Determination of optimum operating conditions for production of polyhydroxybutyrate by activated sludge submitted to dynamic feeding regime

QU Bo, LIU JunXin[†]

State key Laboratory of Environment Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China

Polyhydroxybutyrate (PHB) was produced by activated sludge in a two-stage process. In the first stage, the sludge with high storage potential was enriched in a sequencing batch reactor (SBR) operated by a dynamic feeding regime. The biosynthesis of PHB was sequentially accomplished in a batch reactor in the second stage. This article focused on the optimization of PHB content by evaluating the effects of three critical factors: Dissolved oxygen, pH and food-to-microorganism (F/M) ratio in the batch reactor. The results showed that the rate of substrate uptake, as well as the yield and content of PHB increased with the increase of dissolved oxygen concentration. The control of pH was not necessary under the weak alkaline condition for improvement of PHB content, except for the case in which the pH value was out of rang of 7.0 to 10.0. The enhanced F/M ratio favored PHB accumulation in the range of 1.0 to 4.5 C-mmol/C-mmol and the highest PHB content, 64%, was achieved at 4.5 C-mmol/C-mmol. When F/M ratio was enhanced to 6.0 C-mmol/C-mmol, the phenomenon of utilizing simultaneously intracellular PHB and external substrate by sludge was observed, which resulted in a sharp decrease of PHB content. The microscopic observation clearly confirmed that dynamic feeding regime was effective for selecting and enriching the sludge with a high storage potential.

polyhydroxybutyrate, activated sludge, dynamic feeding regime, optimum operating conditions

Conventional plastics have faced some severe problems, because of their persistency in the environment and non-biodegradability. Therefore, the biodegradable plastic has potentials in applications. Polyhydroxyalkanoates (PHAs) are the polyester of hydroxyalkanoates synthesized by numerous bacteria as intracellular carbon and energy storage compounds, and have similar mechanical properties to conventional plastics like polypropylene or polyethylene. Additionally, PHAs have the attractive features of being completely biodegradable, biocompatible, and produced from renewable sources. Polyhydroxybutyrate (PHB) is the most common and best characterized PHAs. For the economical production of PHAs, various bacterial strains, either wild-type or recombinant, and new fermentation strategies were developed with high content and productivity of PHAs^[1,2].

However, the PHAs production cost is still 8 to 10 times more than that of conventional plastics. The major costs consist in the cost of pure culture fermentation and expensive pure substrates^[3]. Recently, the production of PHB by activated sludge from the wastewater treatment plants has attracted attention^[4–7]. The activated sludge can use cheap substrates (such as organic waste) and avoid the need of sterilization. Thus, the costs may be substantially reduced.

In wastewater treatment process, the microorganisms in activated sludge can often readily transform biodegradable carbon source into PHA before using them for

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[†]Corresponding author (email: jxliu@rcees.ac.cn)

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growth^[8–11]. Hence, it is feasible to transfer activated sludge to PHA producer through appropriate process modification. The dynamic feeding regime (feast-famine regime) has been employed to select and enrich the microorganisms with high PHB accumulation ability in a sequencing batch reactor (SBR), and is believed to be the most promising process for industrial production of PHB by activated sludge^[5,12]. In the “feast” phase, a high organic loading provides an advantage for PHB storage in the microorganisms. In the “famine” phase without external substrate, the PHB in the microorganisms will be degraded for growth and energy production. Based on this consideration, a new two-stage process was proposed for PHB production by activated sludge^[13,14]. In this two-stage process, the SBR and batch reactor are used in the first and second stages respectively. The SBR can enrich sludge with high storage potential by dynamic feeding. The sludge harvested from the SBR, at the end of a cycle, is put into the batch reactor, which is operated at a high organic load to saturate the storage potential. The PHB-rich sludge then flows to the downstream processes for extraction of PHB. The typical PHB content obtained by pure culture and activated sludge in literatures were listed in Table 1. However, by now few works have been focused on the optimization of process parameters for improving PHB production.

In this study, the effects of three critical parameters, dissolved oxygen (DO) concentration, pH value and food-to-microorganism (F/M) ratio, were evaluated to improve the PHB content by optimizing the process operating conditions. Moreover, the nutrient elements, such as nitrogen and phosphorous, were sufficient in synthetic medium in this experiment. It is significant for PHB production by activated sludge with recoverable organic waste.

1 Materials and methods

1.1 SBR set-up and operation

The experimental setup of a SBR with a working volume of 2.0 L was used for selection and enrichment of sludge with a high storage potential. The SBR was operated with a cycle of 6 h consisting of 300 min of aerobic reaction, 10 min of settling, 3 min of effluent withdrawal and 47 min of idle. During the initial 10 min of aerobic reaction, 1.0 L fresh medium was pumped into the reactor by a peristaltic pump. The sludge retention time (SRT) was maintained in 6 days by mixed liquor withdrawal from the reactor once every cycle. The effluent was withdrawn in the middle of the reactor, leaving a working volume of 1 L at the beginning of the next cycle, which resulted in a hydraulic retention time (HRT) of 12 h. Oxygen was supplied by an air compressor, and the DO concentration was always above 40% of the saturation value. The temperature was maintained at $(25 \pm 0.5)^\circ\text{C}$ by using a water jacket and a thermostat bath. DO and pH were continuously monitored on-line. The SBR was operated without pH control.

The SBR was inoculated with activated sludge from a municipal wastewater treatment plant in Beijing, and the initial biomass concentration in the reactor was 2000 mg MLSS /L. After the experimental startup, the SBR was monitored and sampled at regular intervals. The batch tests for PHB storage were performed after the steady-state in the SBR was reached. The criterion of the steady state was that a constant biomass concentration in the SBR was maintained over 7 days, and the dynamic pattern in DO and pH during one cycle did not change.

1.2 Batch experiments

Batch experiments were performed to investigate the effects of DO, pH and F/M ratio on the PHB storage.

Table 1 The results of PHB production in literatures

Microorganisms	Carbon source	Process	PHB (%)	References
<i>A. latus</i>	Sucrose	Nitrogen limitation	50	[15]
<i>A. eutrophus</i>	Glucose	Phosphorus limitation	82	[16]
<i>R. eutropha</i>	VFA	Nitrogen limitation	60	[17]
Recombinant <i>R. eutropha</i>	Soybean oil	no	57	[18]
Recombinant <i>E. coli</i>	Glucose	no	77	[19]
Activated sludge	Acetate	Nitrogen limitation	78	[12]
Activated sludge	VFA	no	50	[14]

a) On chemical oxygen demand (COD) basis.

For each test, the batch reactor with a volume of 400 mL was inoculated with biomass from the SBR collected at the end of cycle, to the chosen concentration (1000 mg MLVSS/L) by using the same medium of the SBR (without acetate and ammonia). At the beginning of tests, a concentrated solution including acetate and ammonia was fed with a pulse in the batch reactor to provide carbon source and nitrogen with pre-selected concentration. Aerobic reaction was followed to the end of storage reaction indicated by DO concentration increase suddenly.

Oxygen was supplied by an air compressor through a ceramic disperser inside the batch reactor. The airflow rate was measured and controlled by an airflow meter to maintain pre-selected DO concentration in reactor. The pH value in the reactor was not controlled, except for the experiments where the effect of pH was investigated. A pH auto-controller was used to keep pH at a pre-selected value by addition of 1 M HCl or 1 M NaOH. Oxygen uptake rates (OUR) were determined on line by a respirometer as described by Smolders et al.^[20]. DO and pH were continuously on-line monitored. The temperature and stirring rate were the same to those in SBR. The biomass in the batch reactor was sampled at regular intervals for analytical determination of acetate, PHB, and ammonia.

1.3 Medium

The culture medium used in this study was composed of (per liter of distilled water) 2460 mg CH₃COONa (60 C-mmol), 321 mg NH₄Cl (6 N-mmol), 100 mg MgSO₄, 20 mg EDTA, 160 mg K₂HPO₄, 38 mg KH₂PO₄, 70 mg CaCl₂·2H₂O, 20 mg yeast extract and 2 ml of trace elements solution. The trace elements solution consisted of (per liter of distilled water) 1500 mg FeCl₃·6H₂O, 150 mg H₃BO₃, 150 mg CoCl₂·6H₂O, 120 mg MnCl₂·4H₂O, 120 mg ZnSO₄·7H₂O, 120 mg KI, 60 mg Na₂MoO₄·2H₂O, 60 mg NiCl, 30 mg CuSO₄·5H₂O. Thiourea (20 mg/L) was also added to inhibit nitrification, and then ammonia consumption was related to active biomass formation. In batch experiments, the mineral medium was the same to that used in SBR except for acetate and ammonia.

1.4 Analytical procedure

Mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), specific oxygen uptake rates (SOUR) and ammonia were determined by using standard methods^[21]. PHB and acetate were also

determined during the experimental operation. 5 ml of the mixture liquor was treated immediately after sampling with 6 M HCl solution, and the mixture was centrifuged at 10000 rpm for 15 min. The pellet obtained was lyophilized for PHB determination. The supernatant was filtered with 0.22 μm membrane for acetate and ammonia determination. PHB was extracted, hydrolyzed, and esterified to 3-hydroxyacyl methyl esters, determined by gas chromatography (Angilent, 6890 NT with autoinjector 7683B), equipped with a 30 m×0.32 mm × 0.25 μm capillary column (HP-5, Angilent)^[22,23]. The acetate was determined by using the same gas chromatography with FID detector and a 30 m × 0.32 mm × 0.50 μm capillary column (DB-FFAP, Angilent). The temperatures of the injector and detector were 240°C and 280°C, respectively. The initial oven temperature was 70°C for 3min followed by a ramp of 20°C per min for 5.5 min and a final temperature of 180°C for 3min. Nitrogen was used as the carrier gas with a flow rate of 2.6 mL/min. The detectable level was 1 mg/L for acetate.

1.5 Microscopic observation

Mixed cultures (sludge) from batch reactor were investigated by cell staining procedures and by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Dual staining with DAPI and Nile Blue was performed for the detection of PHB. The sample was observed with a Carl Zeiss Axioplan epifluorescence microscope. The detail was described by Kawaharasaki et al.^[24] and Serafim et al.^[25]. SEM (HITACHI S-3000N) and TEM (HITACHI H-7500) were performed according to protocols described by Deneus-Mustin et al.^[26].

1.6 Calculations

The PHB content in the sludge was determined by:

$$\text{PHB(\%)} = \frac{\text{PHB}}{\text{MLSS}} \times 100 \text{ (mg/mg)}. \quad (1)$$

The specific acetate uptake rate (Ac per X·h, $-q_s$ in C-mmol/C-mmol·h), specific PHB storage rate (PHB per X·h, q_P in C-mmol/C-mmol·h), the specific ammonia uptake rate (N per X·h, $-q_N$ in N-mmol/C-mmol·h) and the yield of PHB (PHB per Ac, $Y_{P/S}$ in C-mmol/C-mmol) were calculated according to Serafim et al.^[11]. The yield of active biomass (X per Ac, $Y_{X/S}$ in C-mmol/C-mmol) was calculated from the ammonia uptake considering the molecular formula for biomass C₅H₇NO₂: 8 mg of bio-

mass contains 1 mg of N^[27]. In experiments, it was assumed that all the ammonia was used for growth because nitrification was inhibited by thiourea.

2 Results and discussion

2.1 SBR performance

The objective of the SBR operation was to enrich the sludge with a high PHB storage potential. The PHB storage capacity of the inoculating sludge could be gradually increased under a dynamic feeding condition. Figures 1(a) and (b) showed a typical cycle behavior of acetate, PHB, SOUR, DO, pH, and ammonia in the SBR. The observations in Figure 1(a) indicate that the cycle is clearly divided into two phases, i.e. the feast period (initial 40 min of the operation) and the famine period (after 40 min). During the feast period, acetate was quickly removed and PHB content increased accordingly (Figure 1(a)). At the end of feast period, the PHB content of 21% in the sludge was obtained. In the same period, the DO decreased immediately to a minimum due to acetate consumption. At the same time, the SOUR increased to a very high level and lasted for initial 40 min.

Figure 1 The typical SBR cycle during a SBR cycle under dynamic feeding regime. (a) The profile of acetate (▲), PHB (■) and ammonia (○); (b) the profile of pH (■), SOUR (○) and DO (▲).

During the famine period, the PHB stored in the cell was consumed as energy and carbon resource, because of acetate exhaustion. The DO immediately increased

and the SOUR decreased simultaneously. Therefore, the inflections in the profiles of DO and SOUR were a direct observation to define the transition of the feast to the famine period. The pH in the SBR was not controlled and fluctuated in the range of 8.8 to 9.5 in one cycle, as shown in Figure 1(b). Though ammonia concentration gradually decreased, it was still over 5 mg/L at the end of cycle. Therefore, ammonia was a no limiting factor in this test based on the result of the previous research^[28].

These results indicate that the microorganisms with high PHB capacity storage can be enriched through the dynamic feeding regime in the SBR. In the following batch experiments, the process parameters in batch reactor were investigated for optimization of PHB production.

2.2 Optimization of operating conditions effect of DO

Figure 2 showed the results of acetate removal and PHB production under different DO concentrations in the batch reactors. The acetate removal rate and PHB storage rate obviously increased with the increase of DO concentration. Moreover, the maximum PHB production increased with the DO concentration, and was positive correlative with the removal rate of acetate, though the initial concentration of acetate was the same. For example, at the end of storage reaction, the PHB productions were 25 C-mmol/L and 17 C-mmol/L, respectively, under the conditions of 70% and 10% of DO saturation (Figure 2).

Figure 2 The profile of acetate consumption and PHB accumulation in the batch reactors with different DO ● 10%; △ 20%; ■ 40%; ○ 70% of DO saturation.

A previous literature reported that an oversupply of oxygen would result in a decrease of PHB yield because a higher proportion of acetate was converted into a biomass growth at higher DO concentration^[29]. However, our research found that high DO concentration favored to enhance PHB content. As a result of the increased DO concentration, the increase of acetate uptake rate out-classed the increase of growth rate. Thus, the excess acetate had to be converted to PHB to maintain a balanced metabolism, which resulted in the enhanced PHB yield and content.

Enhanced DO concentration means that more electron acceptors are available for the production of energy by microorganisms in sludge. This consequently means that substrate uptake activity is enhanced due to the fact that the microorganisms cultivated in chemostat-like reactor always induce the maximal level of substrate uptake enzymes^[30]. However, the growth rate is determined by the enzyme and RNA content for growth processes. Induction of these enzymes strongly depends on the previous growth condition of microorganisms^[4]. On the other hand, the transport of acetate over the cell membrane is an energy required process^[31]. Based on this condition, we postulate that enhanced DO concentration accelerates the uptake of acetate and then improves PHB storage.

(1) Effect of pH

According to some related literatures, the experiments of PHB storage were usually carried out at pH 7.0 or pH 7.5^[14,32,33]. In order to evaluate the effect of pH on PHB production, a set of experiments were performed with pH control and compared with the one without pH control. The results were shown in Figure 3.

In the pH range of 7.0 to 10, the PHB storage had no significant change regardless of pH control or un-control, and the similar PHB contents were obtained. This result indicated that the PHB metabolism was not sensitive to pH under the weak alkaline condition. However, the acetate uptake and PHB storage stopped when pH was controlled at 6.0 or 11 (the date was not shown). At pH of 6.0, the majority of acetate was in the form of undissociated weak acid, which would cross cell membranes and acidify the cytoplasm^[34]. Thus, the PHB was not synthesized as a result of the acetate uptake inhibited. A similar phenomenon was observed by Bond et al.^[35]. In their study, intracellular acidification of sludge inhibited PHB formation under a low pH condition in the enhanced biological phosphorus removal process. When

Figure 3 The profile of acetate consumption and PHB accumulation in the batch reactors with different pH ◆ 7.0; ○ 8.0; ▲ 9.0; * 10; △ un-control.

pH was controlled at 11, it was reasonable to consider that the biological activity was inhibited completely by so high alkalinity.

Under aerobic conditions, the theoretical oxidation of acetate is as follows:

Eq. (2) shows that the oxidation of acetate consumes hydrogen ions in a ratio of 1 mole to 1 mole, i.e. an increase in the solution pH is inevitable as acetate is oxidized. In this study, the pH in batch reactor increased from the initial value of 7.6–8.3 after acetate addition, and then maintained at a high level (9.3–9.6) to the end of experiment under the condition without pH control. Moreover, the yield of PHB was slightly higher under pH un-control condition than those under pH-control conditions. It was obvious that pH control was not necessary to improve PHB production, except for the case in which pH value was out of the range of 7.0 to 10.

(2) Effect of F/M ratio

The F/M ratio is an important parameter affecting the biological reaction process. In the following experiments, the initial concentration of biomass was fixed and a different amount of acetate was added to obtain a different F/M ratio. Thus, the different F/M ratio was represented as the different initial concentrations of acetate in the batch reactors. Figure 4 showed the experimental results of the acetate removal and PHB production in the batch

reactors with different F/M ratios. The time used for acetate uptake increased with the initial concentration of acetate (Figure 4(a)), and the maximum PHB production increased correspondingly, excepting the F/M ratio of 6.0 (Figure 4 (b)). The highest amount of PHB production and PHB content were 85C-mmol/L and 64%, respectively, at the F/M ratio of 4.5 C-mmol/C-mmol.

Figure 4 The profile of acetate consumption and PHB production in the batch reactors with different F/M ratios • 1.0; △ 1.5; ■ 2.0; ○ 3.0; ▲ 4.5; ◇ 6.0 C-mmol/C-mmol.

When F/M ratio was 6.0C-mmol/C-mmol, the rates of acetate uptake and PHB storage decreased sharply. Serafim et al.^[13] speculated that high acetate concentration inhibited the metabolism of PHB storage. The PHB

concentration began to decrease at 540 min after acetate addition, though acetate was not depleted at that time (Figure 4). This implied that the utilization of intracellular PHB and external substrate occurred simultaneously when the storage reaction proceeded for a long time. The phenomenon was not reported in previous literatures. Therefore, the selection of appropriate F/M ratio is important for maximizing PHB content. If the substrate is oversupplied, e.g. F/M ratio above 4.5C-mmol/C-mmol, two negative effects occur. Firstly, too high substrate concentration inhibits the uptake of substrate, resulting in decreased PHB content. On the other hand, high F/M ratio results in the need of a longer time for acetate uptake (Figure 4).

(3) Analyse of operating conditions for high PHB production

The foregoing experimental data and calculated kinetic parameters were summarized in Table 2. Some tendencies can be found from these data. At a fixed F/M ratio of 1.0C-mmol/C-mmol, when the DO concentrations were increased to 70% from 10% of saturation, the specific uptake rate of acetate ($-q_s$), specific storage rate of PHB (q_p) and yield of PHB (Y_p) all had an obvious increase, but the yield of active biomass (Y_x) presented a little of decrease. Moreover, the PHB content increased clearly with DO concentration. When the DO concentration was fixed at 70% and the F/M ratio was increased to 6.0 from 1.5C-mmol/C-mmol, an opposite result was obtained, which the q_s , q_p and Y_p had all decrease, whereas Y_x increases. According to the experience of the effect of DO, the PHB content should be decreased here. However, the PHB content stored in the sludge in-

Table 2 Typical kinetic and stoichiometric parameters for the experiments with different operational conditions

DO (%)	pH	F/M	C	N	$-q_s$	q_p	Y_x	Y_p	PHB (%)
10	uncontrol	1.0	40	1.5	0.19 (0.021)	0.07 (0.010)	0.14	0.36	26
20	uncontrol	1.0	40	1.5	0.26 (0.036)	0.10 (0.007)	0.13	0.41	30
40	uncontrol	1.0	40	1.5	0.34 (0.020)	0.16 (0.024)	0.11	0.48	33
70	uncontrol	1.0	40	1.5	0.56 (0.031)	0.30 (0.006)	0.11	0.56	37
40	7	1.0	40	1.5	0.40 (0.010)	0.16 (0.030)	0.11	0.40	32
40	8	1.0	40	1.5	0.41 (0.021)	0.14 (0.017)	0.10	0.37	31
40	9	1.0	40	1.5	0.40 (0.020)	0.17 (0.028)	0.15	0.41	32
40	10	1.0	40	1.5	0.42 (0.008)	0.17 (0.034)	0.10	0.43	32
70	uncontrol	1.5	60	2.25	0.68 (0.006)	0.34 (0.007)	0.09	0.51	44
70	uncontrol	2.0	80	3.0	0.67 (0.001)	0.34 (0.019)	0.10	0.51	53
70	uncontrol	3.0	120	4.5	0.63 (0.031)	0.29 (0.029)	0.13	0.46	57
70	uncontrol	4.5	180	6.75	0.61 (0.029)	0.28 (0.039)	0.13	0.46	64
70	uncontrol	6.0	240	9.0	0.46 (0.017)	0.14 (0.041)	0.16	0.30	40

The values in parentheses are the standard deviation; DO in % of air saturation; F/M ratio in C-mmol C-mmol⁻¹; C in C-mmol L⁻¹; N in N-mmol L⁻¹; $-q_s$ in C-mmol C-mmol⁻¹ h⁻¹; q_p in C-mmol C-mmol⁻¹ h⁻¹; Y_x in C-mmol C-mmol⁻¹; Y_p in C-mmol C-mmol⁻¹.

Figure 5 SEM image of the coccus-shap morphology of the cells, Bar is 5 μm .

creased significantly with the increase of F/M ratio from 1.5 to 4.5C-mmol/C-mmol, the highest PHB content of 64% was obtained at the F/M ratio of 4.5C-mmol/C-mmol, but it decreased at 6.0C-mmol/C-mmol. The reasons why the PHB content was lower at F/M ratio of 1.5-3.0C-mmol/C-mmol than 4.5C-mmol/C-mmol was likely to substrate limitation. It can be confirmed by the experimental results in Figure 5.

2.3 Microscopic observation

Microscopic observation was performed, and the sludge collected from the batch reactor with the F/M ratio of 4.5C-mmol/C-mmol. The SEM images in Figure 5 showed that the cells in sludge had a considerable homogeneous morphology. Almost all of the cells were cocci that occur singly or in packed. Few short-rod shape cells were observed. The typical length of a single cell varied from 1 to 2 μm . Abundant intracellular granules were observed (Figure 6). These accumulated cellular granules stained positively with PHB dye (Figure

Figure 6 TEM image the present of intracellular PHB granules, Bar is 2 μm .

Figure 7 Fluorescence micrographs of dual staining with DAPI and Nile Blue. (a) The sludge stained with DAPI. Blue fluorescence means all cells; (b) the sludge stained with Nile Blue at identical microscopic field as in (a). Red fluorescence means the cells with PHB granules. Bar is 2 μm for (a) and (b).

7(b)), and further confirmed to be PHB by gas chromatography. Dual staining with DAPI and Nile Blue revealed the presence of PHB inclusions in almost all of the cells (Figure 7). The electron micrographs clearly confirmed that the microorganisms with storage ability were effectively selected and enriched in SBR reactor, and a large amount of PHB was stored in the batch reactor.

The microscopic observation confirmedly supported the experimental data. Though the evolution of microbial population structure was not included in this study, it can be concluded that the microorganisms with the ability to store PHB were the dominant population in system.

3 Conclusion

The DO concentration and F/M ratio have a strong effect to PHB content stored in sludge. However, the pH value is not an important factor on PHB production in the range of 7 to 10. The PHB content increases with DO concentration. An opportune F/M ratio is crucial to obtain the highest PHB content. The DO of 70% and F/M ratio of 4.5C-mmol/C-mmol are suggested as optimum parameters of PHB production by the activated sludge.

The PHB content of 64% achieved in this study was close to those of pure culture microorganisms and was considerably high comparing with those of activated sludge (Table 1). Especially, it should be pointed out that the PHB production by the activated sludge in this study without growth limitation is caused by nutrient element insufficiency, which was substantially significant for PHB production by reusing the organic waste.

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