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

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Abstract The study was designed to investigate the effects of temperature and phosphorus limitation on polyhydroxyalkanoate (PHA) production and storage by activated sludge biomass. The two-stage operation approach, i.e. a growth phase followed by a nutrient limitation phase, was applied to induce PHA accumulation. The pre-selected temperatures of 10, 20 and 30°C were investigated under phosphorus limitation conditions using three four-litre fully aerobic SBR systems operated at an SRT of 10 days with cycle time and HRT of 6 and 10 hours. PHA production was greater in the 10°C system than in the 20°C and 30°C systems but there was little difference between the two higher temperatures. The maximum PHA fractions of the sludge were 52, 45 and 47%TSS for the three temperatures from low to high, and the maximum PHA concentrations in the mixed liquors were 1,491, 1,294 and 1,260 mg/l, respectively. However, it was observed that very low values of PHA yield per unit COD consumed were obtained, i.e., 0.05, 0.03 and 0.04 mgPHA/mgCOD_u, for the 10, 20 and 30°C reactors, respectively. This was because all three systems required several days to reach maximum PHA accumulation in their mixed liquor biomasses. It is probable the bacteria still had some stored poly-P in their cells upon initiation of the phosphorus limited influent, and PHA accumulation was delayed until the stored phosphorus was depleted. Also, PHA productivity was reduced by the large amounts of biomass lost from the systems because of sludge bulking.

Keywords Activated sludge; aerobic SBRs; biodegradable plastics; PHA; phosphorus limitation; polyhydroxyalkanoate accumulation; temperature effects

Introduction

Polyhydroxyalkanoates (PHAs) are intracellular polyesters that can be stored by several types of bacteria. Because PHA is biodegraded to water and CO_2 under aerobic conditions and methane under anaerobic conditions by extracellular enzymes from microorganisms (Lee, 1996), and can be an attractive substitute for common petrochemical thermoplastics because it has similar physical properties, PHA has been used to manufacture completely biodegradable plastics. PHA accumulation in bacteria can be stimulated under conditions of unfavorable growth such as the deprivation of oxygen, nitrogen, phosphate, sulfur, magnesium or potassium in the presence of excess carbon (Lee, 1996). Ryu *et al*. (1997) succeeded in producing PHB, using *Alcaligenes eutrophus* with phosphate limitation, to PHB content and productivity as high as 80% and 3.14 g/l-h, respectively. They also reported that the biomass was in good condition without significant cell lysis when NaOH was added instead of NH_4OH to obtain nitrogen limitation conditions.

There are a lot of attempts reported in the literature to reduce the cost of PHA production so that bio-plastic materials could be produced from it at a price competitive with that of conventional thermoplastics. One approach was the use of excess activated sludge biomass from wastewater treatment plants instead of pure cultures (Chua *et al*., 1997; Chuang *et al*., 1998; Satoh *et al*., 1998). Another has been the use of activated sludge with an industrial wastewater as the source of organic carbon (Punrattanasin, 2001). It is known that the performance of activated sludge during wastewater treatment is significantly affected

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by temperature with better performance at higher temperatures. However, Krishna and Van Loosdrecht (1999) concluded that activated sludge stored less PHB at higher temperatures, and Lishman *et al*. (2000) stated that their aerobic/anoxic SBR system produced more biomass when temperature was decreased.

The purpose of this investigation was to evaluate the effects of phosphorus limitation and temperature on PHA accumulation in activated sludge treating a synthetic wastewater.

Materials and methods

The experimental systems used were fully aerobic sequencing batch reactors (SBRs) that were operated using a two-stage approach that has been utilized for PHA production with pure cultures (Du *et al*., 2001; Wendlandt *et al*., 2001), and adapted by Punrattanasin (2001) for activated sludge. Three fully aerobic SBRs with a working volume of 4 l each were set up and at temperatures of 10, 20 and 30°C. The hydraulic retention time (HRT), sludge age (SRT) and cycle time were 10 h, 10 days and 6 h, respectively. The cycle time consisted of 15 min influent feeding, 4 h aeration, 1.5 h settling and 15 min effluent withdrawal periods. Synthetic wastewater with the composition shown in Table 1 was prepared daily with tap water.

The systems were started with activated sludge collected from the Integrated Fixed Film Activated Sludge (IFAS) pilot plant systems operated by Sriwiriyarat (2002) and initially were operated at growth phase by providing enough nitrogen and phosphorus for cell nutrition and growth. The purpose of the growth phase was to acclimatize the EBPR activated sludge to fully aerobic conditions and obtain steady-state growth. Then the phosphorus limitation phase was initiated by removing all phosphorus from the influent and operating until PHA accumulation was induced. Each experiment was operated through two consecutive growth and nutrient limitation cycles with monitoring of sludge PHA accumulation so that the maximum PHA accumulation and the time to obtain it could be determined, in accordance with the procedure developed by Punrattanasin (2001).

Analytical methods

The following parameters were measured daily throughout the growth and accumulation cycles: MLSS, MLVSS, PHA, SVI and supernatant COD. The PHB and PHV fractions of the PHA were separately determined. MLSS, MLVSS, SVI and COD analyses were performed according to *Standard Methods* (APHA *et al*., 1995). PHA, PHB and PHV contents were determined using the methanolysis-GC method developed by Hart (1994), with modifications described by Punrattanasin (2001). After the extraction step, 1 µl from the chloroform (bottom) layer of each sample was injected into a Hewlett-Packard Model 5890 GC equipped with a Stabilwax capillary column (0.25×3 mm inner diameter) attached to an FID detector. PHA content was defined as %TSS, i.e., the mass of PHA in the total dry weight biomass, expressed as a percent. PHA concentration and residual biomass were calculated using Eqs (1) and (2), respectively.

Table 1 Composition of synthetic wastewater

Substances	Concentration (mg/l)	Micronutrients	Concentration (mg/l)
Sodium acetate	330 as COD	$FeCl_3$	107 (36.8 as Fe)
Sodium propionate	330 as COD	HBO_3	18.3 (3.2 as B)
NH_4SO_4	33 as N	$CuSO_4$	3.5 (1.38 as Cu)
KH_2PO_4	17 as PO_4-P	KI	22 (5.2 as K)
$CaCl_2$	28.3 (10.2 as Ca)	$MnSO_4.H_2O$	14.7 (4.8 as Mn)
$MgCl_2.6H_2O$	250 (29.9 as Mg)	$NaMoO_4.2H_2O$	7.3 (2.9 as Mo)
		$ZnSO_4.7H_2O$	31 (7.1 as Zn)
		$CoCl_2.6H_2O$	18.3 (4.5 as Co)

$$\text{PHA concentration} = \text{PHA content} \times \text{MLSS (mg/l)} \quad (1)$$

$$\text{Residual biomass} = \text{MLSS} - \text{PHA concentration (mg/l)} \quad (2)$$

Results

PHA content and production

The 10°C and 20°C systems were operated under normal growth conditions for 2 to 3 sludge ages, respectively, and then phosphorus, only, was eliminated from the influent feed for the nutrient limitation period or PHA accumulation phase. For the 30°C system, the growth phase was operated for 4 sludge ages because sludge bulking occurred during the early stage of this period. Therefore, this growth phase had to be prolonged. The profiles determined during the experiments at the three temperatures are shown in Figures 1, 2 and 3. Figure 1 shows that the PHA content in the 10°C reactor barely increased during the first two weeks of P limitation, then rapidly increased and reached the maximum content of 52%TSS 22 days after P limitation in the influent was initiated. After a second short growth phase, the PHA content of the sludge increased to the maximum value of 48%TSS within 6 days during the second phase, which was much faster than during the first P limitation phase.

Figure 2 shows that during the 20°C experiment, the PHA content increased gradually and did not reach the maximum content of 45%TSS until 48 days after P limitation was initiated. The PHA content during the second P limitation period at 20°C required 27 days to reach the maximum value of 35%TSS, which was lower than that obtained during the first limitation period. The profile of PHA content during the 30°C experiment as shown in Figure 3 was similar to the results of the other two experiments. The maximum PHA content of 47%TSS was obtained 35 days after initiation of P limitation, and a peak of 41%TSS was obtained 26 days after the second P limitation period.

The results indicated that peak PHA content can typically be obtained faster during the second phosphorus limitation period, but the maximum PHA content obtained will probably be less than during the first accumulation phase. It was noted that the P limitation experiments of this study required significantly longer times to obtain maximum PHA content than was required during nitrogen limitation experiments (Chinwetkitvanich *et al*., 2003). Based on the results obtained by Punrattanasin (2001), it is probable that, even though the influent contained no phosphorus, the biomass still had phosphorus stored within it that had to be depleted before PHA accumulation would begin.

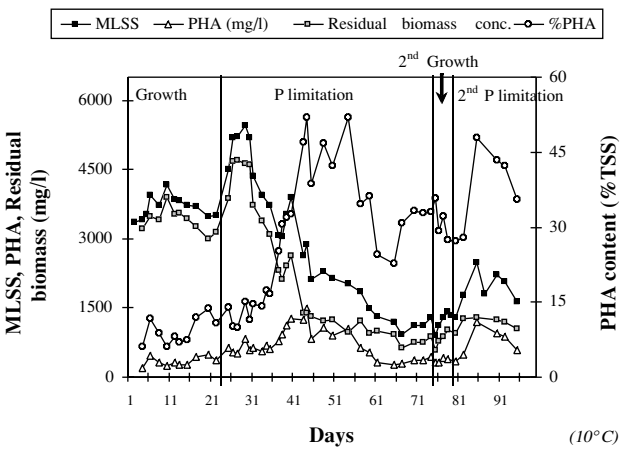

Figure 1 Profiles of PHA content (%TSS), PHA concentration (mg/l), MLSS (mg/l) and residual biomass (mg/l) for the 10°C experiment

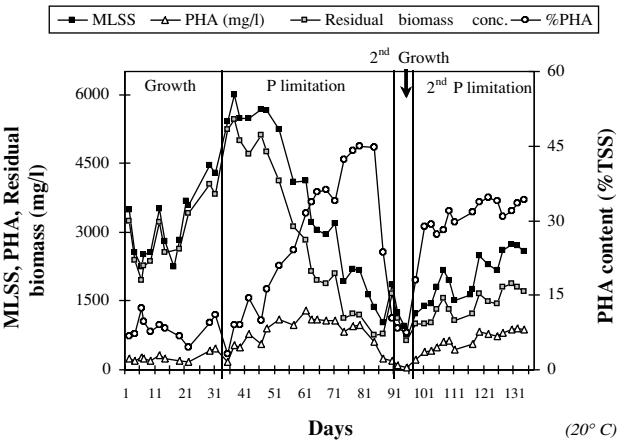

Figure 2 Profiles of PHA content (%TSS), PHA concentration (mg/l), MLSS (mg/l) and residual biomass (mg/l) for the 20°C experiment

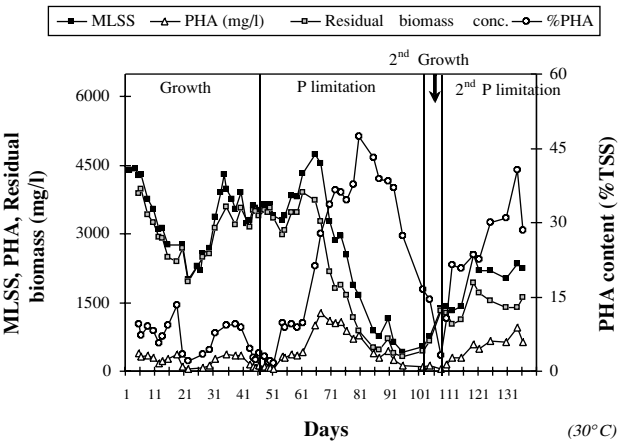

Figure 3 Profiles of PHA content (%TSS), PHA concentration (mg/l), MLSS (mg/l) and residual biomass (mg/l) for the 30°C experiment

Table 2 Summary of PHA production and biomass concentration of experiments with P limitation

	P limitation					
	10°C		20°C		30°C	
	1st	2nd	1st	2nd	1st	2nd
Max. PHA (%TSS)	52	48	45	35	47	41
Days to accumulate max. %PHA (days)	22	6	48	27	35	26
Max. PHA concentration (mg/l)	1,491	1,184	1,294	832	1260	954
Corresponding PHA (%TSS)	52	48	32	34	28	41
Corresponding MLSS (mg/l)	2,867	2,477	4,108	2,477	4,533	2,343
Days to accumulate max. PHA concentration (days)	22	6	30	24	22	26
PHA productivity (mg/l-d)	68	197	43	35	57	37
PHV/PHA on day of max. PHA concentration (%)	45	56	14	26	37	46
PHA yield/substrate utilized ($mgPHA/mgCOD_u$)	0.05	0.13	0.03	0.03	0.04	0.03

Information regarding the PHA accumulation data is tabulated in Table 2. The primary conclusion from the data is that PHA accumulation decreased with an increase in temperature. The maximum PHA concentrations were 1,491, 1,294 and 1,260 mg/l for the 10°C, 20°C and 30°C experiments, respectively, and the corresponding PHA productivity rates were 68/197, 43/35 and 57/37 mg/l-d for the two runs at each temperature. The PHA accumulation at the temperature of 10°C was significantly higher than the amounts and rates observed at 20 and 30°C, but the difference was small between 20 and 30°C. The actual difference between PHA accumulation at 10°C was even greater than the PHA numbers indicate because the MLSS concentration maintained at the lowest temperature was substantially less than those at the other temperatures were. Table 2 shows that the respective MLSS concentrations were 2,867, 4,108 and 4,533 mg/l at the 10, 20 and 30°C temperatures when the first PHA concentration peak was observed. The lowest temperature would have been considerably better for PHA accumulation if the biomass solids had been retained better in the reactor. Note, however, that all three reactors had lost a lot of biomass by the time of the second % PHA peak.

The PHA yields per unit substrate at 10°C, 20°C and 30°C were 0.05, 0.03 and 0.04 mg PHA/mg COD_u, respectively. These PHA yields were significantly lower than those which happened in the experiment with nitrogen limitation (Chinwetkitvanich *et al.*, 2003) as these experiments required longer PHA accumulation periods (P limitation phase) before accomplishing the maximum PHA concentration. In addition, the utilization of COD still functioned quite well till the maximum PHA content was reached (Figures 4–6); therefore the COD utilization was totally high and consequently gave the low values of PHA yield. In the circumstances, it can be therefore concluded that the P limitation step shall not be employed in the PHA production process using activated sludge biomass.

Biomass concentration and sludge quality

Sludge bulking problems caused considerable variation in the MLSS concentrations in the reactors. The MLSS concentration in the 10°C system was 3,514 mg/l when the P limitation phase began, then increased to 5,433 mg/l when sludge wasting was discontinued to achieve PHA accumulation. Thereafter, it decreased drastically throughout the first P limitation phase to a value of 930 mg/l because of sludge bulking problems. The biomass concentration changes in the 20 and 30°C systems had similar patterns to the 10°C system, and the 30°C MLSS decreased from 3,573 to 422 mg/l because of sludge bulking. Figures 7–9 show that the SVI changes correlated with the MLSS changes. Krishna and Van Loosdrecht

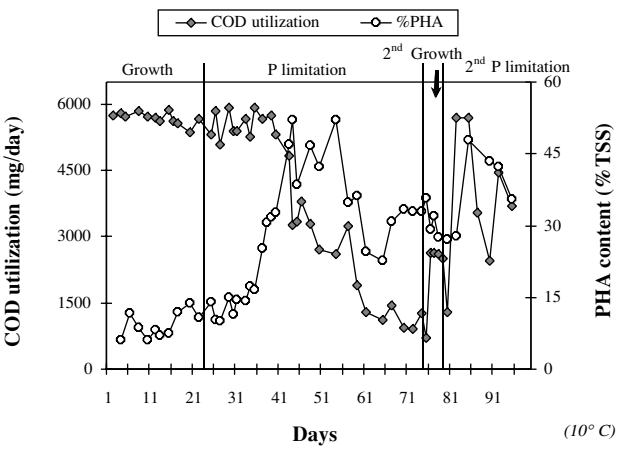

Figure 4 Profiles of COD utilization (mg/day) and PHA content (%TSS) for the 10°C experiment.

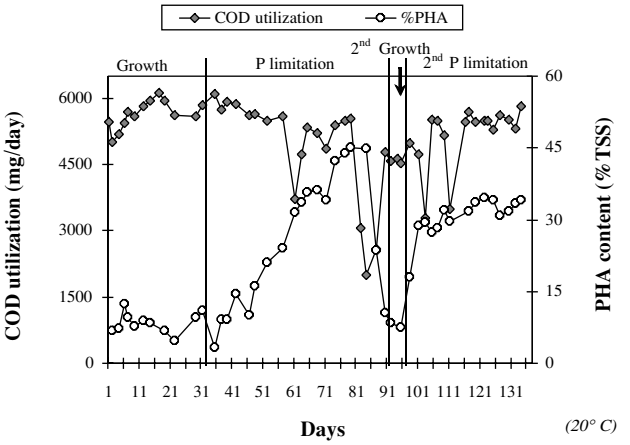

Figure 5 Profiles of COD utilization (mg/day) and PHA content (%TSS) for the 20°C experiment

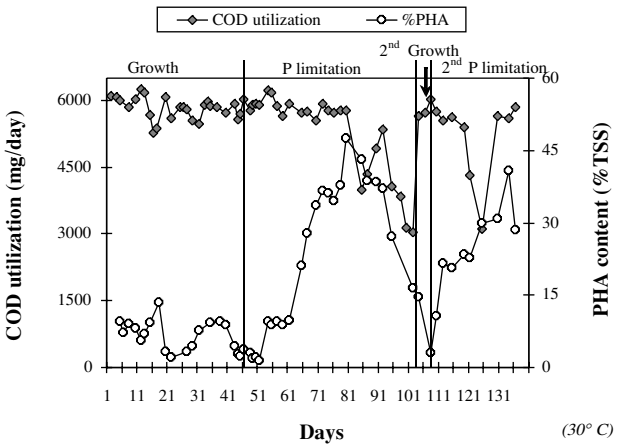

Figure 6 Profiles of COD utilization (mg/day) and PHA content (%TSS) for the 30°C experiment

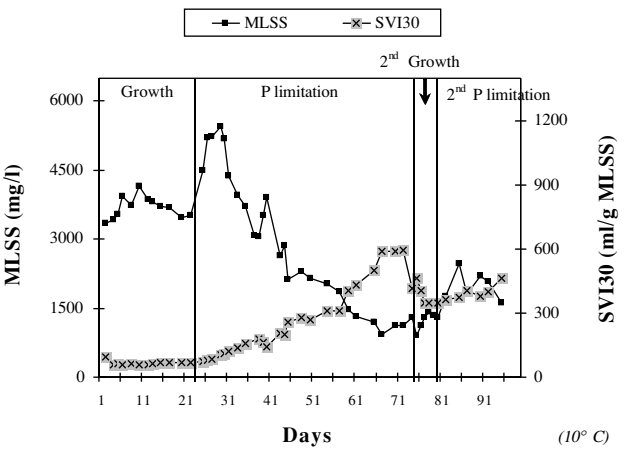

Figure 7 Profiles of MLSS (mg/l) and SVI (ml/g MLSS) for the 10°C experiment

(1999) have stated that sludge bulking is likely to happen when the temperature exceeds 30°C. It is of interest that the sludge bulking problems were worse during the phosphorus limitation experiments than during the preceding nitrogen limitation experiments (Chinwetkitvanich *et al.*, 2003).

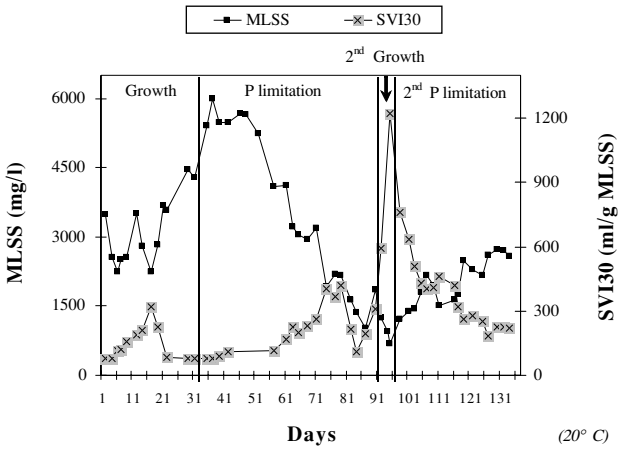

Figure 8 Profiles of MLSS (mg/l) and SVI (ml/g MLSS) for the 20°C experiment

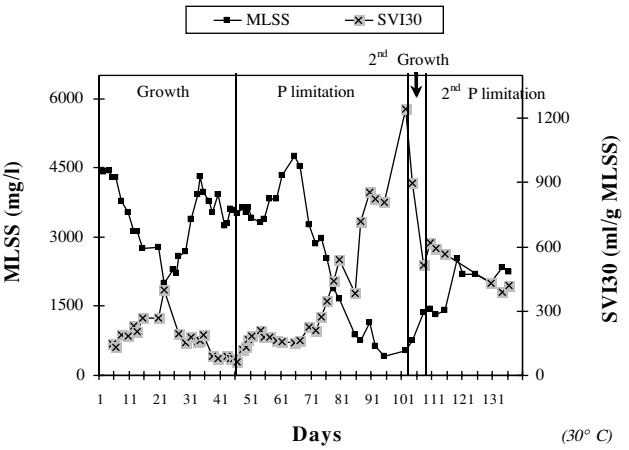

Figure 9 Profiles of MLSS (mg/l) and SVI (ml/g MLSS) for the 30°C experiment

As shown earlier in Figures 1–3, the residual biomass of all three experimental temperatures increased for some time during the early period of P limitation. That means biomass in that period still had the ability to utilize substrate for cell growth although phosphorus was not added to the feed anymore. Then, the residual biomass decreased drastically before the PHA fractions reached the maximum. This decreasing of residual biomass should not be interpreted that cell growth was terminated, but the major reason should be granted to the occurrence of a sludge bulking problem that caused severe loss of biomass from the reactor with the effluent withdrawal.

Discussion

Although all experiments with P limitation resulted in large accumulations of PHA, expressed as % of TSS in the biomass, the PHA yields (mg PHA/mg COD_u) and productivities (mg/l-d) were significantly lower than those obtained during the nitrogen limitation experiments (Chinwetkitvanich *et al.*, 2003). Based on results previously reported by Punrattanasin (2001), it is probable that the biomass cells contained substantial amounts of stored P when P limitation was initiated, and PHA accumulation was delayed until the stored P had been depleted. In addition, the feed contained nitrogen throughout the P limitation experiments, which enabled the biomass to continue cell growth and anabolic

metabolism. Several researchers (Steinbuchel and Schlegel, 1991; Kessler and Witholt, 2001; Du *et al.*, 2001) have explained that under growth conditions, intracellular concentrations of acetyl-CoA (precursor for PHB synthesis) are presumably low as it mostly enters the TCA cycle. This liberates large amounts of free coenzyme A (CoASH), which could inhibit the condensation of acetyl-CoA to acetoacetyl-CoA by the enzyme 3-ketothiolase and, thus, inhibit PHB synthesis. Some researchers (Ryu *et al.*, 1997; Wendlandt *et al.*, 2001) have reported that they could accumulate PHB under P limitation better than limitation of nitrogen or other essential nutrients. Similar results might have been obtained during these studies if much of the biomass had not been lost from the systems because of sludge bulking. It is recommended that future P limitation experiments utilize a method to reduce the loss of biomass, such as the use of membrane separation or the use of batch or fed-batch systems applied to pure culture instead of a continuously fed (like wastewater treatment) system.

Conclusions

From the experimental results, P limitation is not a good strategy for PHA production by activated sludge biomass because they produced low PHA productivities and yields, although the maximum PHA contents (% of TSS) were substantially high at every experimental temperature. Nonetheless, the results strongly indicate that PHA accumulation in activated sludge under P limitation conditions requires less time and increases to a higher fraction of the TSS when the mixed liquor temperature is 10°C, as compared to 20 or 30°C. They also indicate that PHA accumulation will peak in a much shorter time period during the second P limitation period than the first, but probably not to as high a fraction of the TSS. The probable cause of the prolonged period of PHA accumulation during the first limitation period is the presence of stored P in the cells when the feed without P is initiated. However, P limitation might be a considerable strategy for PHA production if the local wastewater already contained a nitrogen source.

Acknowledgement

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