

SIMULTANEOUS COD REMOVAL AND PHA PRODUCTION IN AN ACTIVATED SLUDGE SYSTEM UNDER DIFFERENT TEMPERATURES

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ABSTRACT

The purpose of this study was to investigate PHA production by activated sludge biomass under different temperatures. The two-step approach, i.e. utilizing a growth phase followed by a nutrient(s) limitation phase, was applied to stimulate PHA accumulation. Each of three intended temperatures, 10°C, 20°C and 30°C, was investigated with combined N&P limitation. Four liter (L) fully aerobic SBRs were used for all experiments, and operated with a 6 h cycle time, a 10 h HRT and a 10 day SRT. The maximum PHA cellular contents and total concentrations achieved during the N&P limitation periods in the 10 and 20°C systems were very similar, i.e. 45 and 43 % of the TSS and 2133 and 2239 mg/l, respectively, whereas the 30°C results were lower at 33 % of TSS and 1476 mg/l. The biomass temperature clearly had a strong inverse effect upon PHA productivity. It decreased from 427 and 204 to 148 mg/l-day as reactor temperature increased from 10 to 20 and 30°C, respectively. As well, the PHA yields decreased from 0.38 to 0.16 and 0.11 mg PHA/mg COD_u, respectively. The results strongly indicate that activated sludge PHA accumulation stimulated by combined N and P limitation is inversely correlated with temperature.

KEYWORDS

activated sludge, polyhydroxyalkanoate, nutrient limitation, biodegradable plastics, temperature effects

I. Introduction

Polyhydroxyalkanoates (PHAs) are stored by several types of bacteria as a carbon and energy reserve when the cells are subjected to nutritional stress. The conditions that most effectively stimulate bacterial PHA accumulation appears to be those that impose growth limiting conditions on the cells, such as the deprivation of oxygen, nitrogen, phosphate, sulfur, magnesium or potassium in the presence of excess carbon [1], [2].

Unfortunately, the production price of biodegradable PHA plastics is still not competitive with the conventional plastics manufactured by the petrochemical industry. Lee [3] and Braunegg et al.[4] have reported that the price of BIOPOL™, a commercially marketed PHA plastic, is currently about 16-17 times higher than those of conventional plastics. BIOPOL™ is produced using aseptic pure culture conditions and purchased short-chain volatile fatty acids (VFAs), such as acetic, propionic, etc. There have been several efforts to decrease the cost of PHA production, including using mixed cultures such as activated sludge biomass [2], [5], [6] that can store high concentrations of PHA while utilizing an inexpensive carbon source [7] - [10]. These studies have demonstrated that there is considerable potential for the utilization of mixed cultures and industrial (carbon-based) wastewater organics to produce PHAs for the production of fully biodegradable, yet versatile, plastics. Therefore, it is time to consider how wastewater treatment systems can be modified to permit utilization of activated sludge and organic wastewaters for PHA production, so that much less expensive plastics can be produced and reduce the widespread use of disposable, but poorly biodegradable, conventional plastics. If successfully applied, the financial liability of wastewater treatment could be reduced through the production of a commercially marketable by-product. This could prove to be especially attractive to industries that discharge a high strength organic carbon-based wastewater.

A review of the published literature indicates that the knowledge of effects of temperature on PHA storage by activated sludge has been rare. The main objective of this study was to investigate the effects of temperature on PHA production by activated sludge cultures utilizing a synthetic organic industrial wastewater as the growth substrate and subjected to nitrogen and phosphorus limitation.

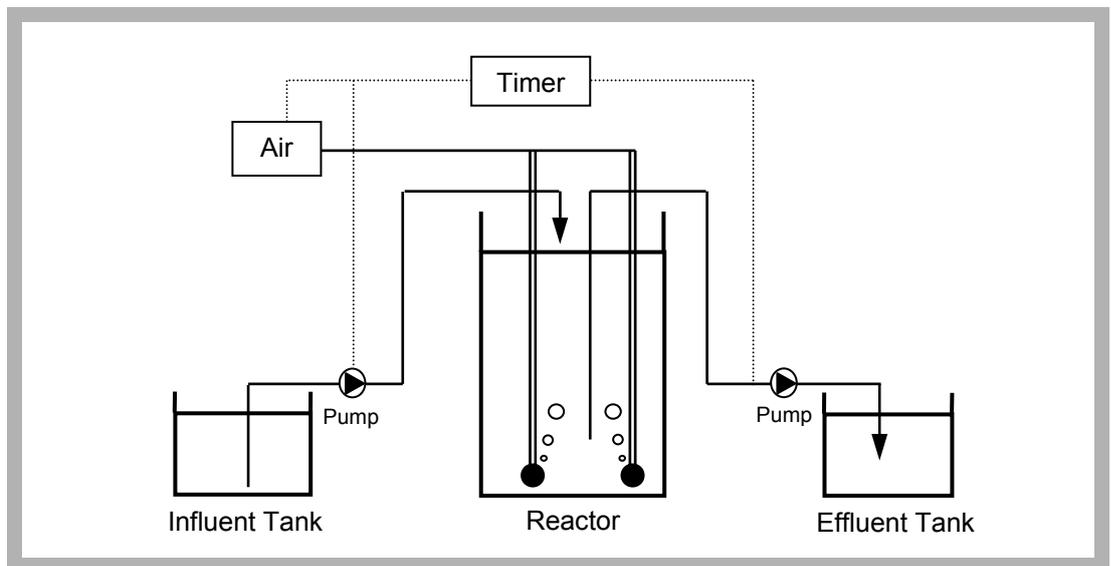
II. Materials and Methods

A fully aerobic SBR system was used for all experiments as Punrattanasin [2] has shown that fully aerobic conditions stimulate higher PHA productivity than typically achieved in Anaerobic/Aerobic (An/Ae) and Microaerophilic/Aerobic (MA/A) cycling systems. Three different temperatures were selected for this study, i.e., 10, 20 and 30°C. Both 10 and 20°C experiments were conducted in temperature control rooms of the laboratory of Civil and Environmental Engineering Department, Virginia Polytechnic Institute and State University in United States of America. While the 30°C reactors were setup in modified water bath equipped with fishing tank heater. Water temperature in every reactor was monitored daily with only $\pm 1.5^{\circ}\text{C}$ fluctuation was allowed. The limitation of both nitrogen (N) and phosphorus (P) were investigated for each temperature. The SBR systems with a working volume of 4 liters as illustrated in Figure 1 were operated with a hydraulic retention time (HRT) of 10 hours, a sludge age of 10 days and a 6-hour operating cycle. Each cycle consisted of 15 min influent feeding time, 4 hr aeration period, and 1.5 hr settling with the last 15 min of effluent withdrawal. The volume in the reactor after effluent discharge was 1.6 l, resulting in a discharge volume of 2.4 l per cycle. For SRT of 10 days, excess sludge was drained approximately 400 ml per day or 100 ml per cycle, which was subjected to be adjusted depending on suspended solid contained in effluent.

The experimental systems were operated using a two-stage approach, i.e. a growth phase followed by a nutrients limitation phase that had been earlier utilized for PHA production with pure cultures [9],[11] and adapted by Punrattanasin [2] for activated sludge. During the growth phase, nutrients were optimized to develop the biomass concentration and cell nutrition. The 10-d SRT was maintained by daily excess sludge withdrawal for a period of 2-4 SRTs in order to allow the biomass in each experiment to adjust and grow similarly on the same influent components. Then, the nutrients limitation phase was introduced by eliminating both N and P from the feed in order to stimulate PHA accumulation. Sludge wasting was terminated during this PHA accumulation phase to maximize biomass concentration in the reactors, with the exception of what was wasted as SS during effluent withdrawal. Each experiment was operated through two consecutive growth & nutrient limitation cycles, in accordance with the conclusion stated by Punrattanasin [2] that the two

consecutive cycles would enrich the PHA accumulating bacteria, reduce the production time, and produce higher PHA content within the cells. PHA accumulation by the sludge was monitored by frequent analysis so that the maximum PHA accumulation and the time required to obtain it could be determined. The systems were inoculated with excessive activated sludge from the UCT/VIP configuration Integrated Fixed Film Activated Sludge (IFAS) pilot plant system operated by Sriwiryarat [12].

Figure 1
Experimental setup



2.1 Synthetic wastewater

The synthetic wastewater was composed of sodium acetate (330 mg/l as COD) and sodium propionate (330 mg/l as COD) as the sole carbon sources. When the system was operated during the growth phase, $(\text{NH}_4)_2\text{SO}_4$ of 33 mg N/l and KH_2PO_4 of 17 mg P/l were added as the N & P nutrients. Other nutrients and micronutrients used in this experiment are shown in Table 1. The synthetic wastewater was prepared daily with tap water.

Table 1
Concentrations
of nutrients and
micronutrients

<u>Nutrients</u>	<u>Concentration (mg/l)</u>	<u>Micronutrients</u>	<u>Concentration ($\mu\text{g/l}$)</u>
CaCl_2	28.3 (10.2 as Ca)	FeCl_3	107 (36.8 as Fe)
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	250 (29.9 as Mg)	HBO_3	18.3 (3.2 as B)
		CuSO_4	3.5 (1.38 as Cu)
		KI	22 (5.2 as K)
		$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	14.7 (4.8 as Mn)
		$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	7.3 (2.9 as Mo)
		$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	31 (7.1 as Zn)
		$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	18.3 (4.5 as Co)

2.2 Analytical methods

MLSS, MLVSS and COD were analyzed according to Standard Methods [13]. PHA, PHB and PHV contents were analyzed using the methanolysis-GC method according to Hart [14], with some modifications described by Punrattanasin [2]. That is, biomass collected from the systems was centrifuged and dried at 100°C for at least 24 hours. Weighed biomass was put into a 5 ml high pressure Wheaton vial. Then 2 ml of benzoic acid solution (50 mg of benzoic acid dissolved in 100 ml of 3% sulfuric acid in methanol (v/v)) was added and followed by 2 ml of chloroform. The vials were sealed by Teflon caps and incubated at 100°C for 3.5 hours. After cooling down, 1 ml of distilled water was added and the vials were then shaken for about 10 minutes. The layers of chloroform and sulfuric-methanol solution were separated from each other

and 1 μ l from the chloroform layer (bottom layer) was injected into a Hewlett-Packard Model 5890 GC equipped with a Stabilwax capillary column (0.25 x 3 mm inner diameter) attached to an FID detector. The oven temperature program used was: 4 min at 90°C, increase from 90 (initially hold to 4 min) to 130°C at the rate of 20°C/min, while the temperatures of injector and detector were 160 and 200°C, respectively. PHA content (%TSS) was defined as the percentage of PHA mass in the dry cell mass. PHA concentration and residual biomass were calculated using equations (1) and (2), respectively.

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

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

III. Results and Discussions

3.1 PHA production

In this study, the duration of the growth phases for the three systems were not the same, depending on how fast steady-state was achieved, e.g. the 10°C and 20°C systems required 2 and 3 sludge ages, respectively, and the 30°C system was operated for 4 sludge ages before starting nutrient limitation conditions because of a sludge bulking problem. Figure 2 shows the profiles of MLSS (mg/l), PHA content (%TSS) and PHA concentration (mg/l) during the experiments at 10°C with combined N and P limitation. The system was operated for 22 days with sufficient N and P added to the influent for biomass growth. The SRT was maintained at 10 days over the 22 days of the growth period by wasting a calculated amount of mixed liquor after accounting for the SS wasted during supernatant withdrawal. After N&P limitation was started, mixed liquor wastage was terminated in order to maximize biomass concentration, and therefore PHA accumulation, in the system.

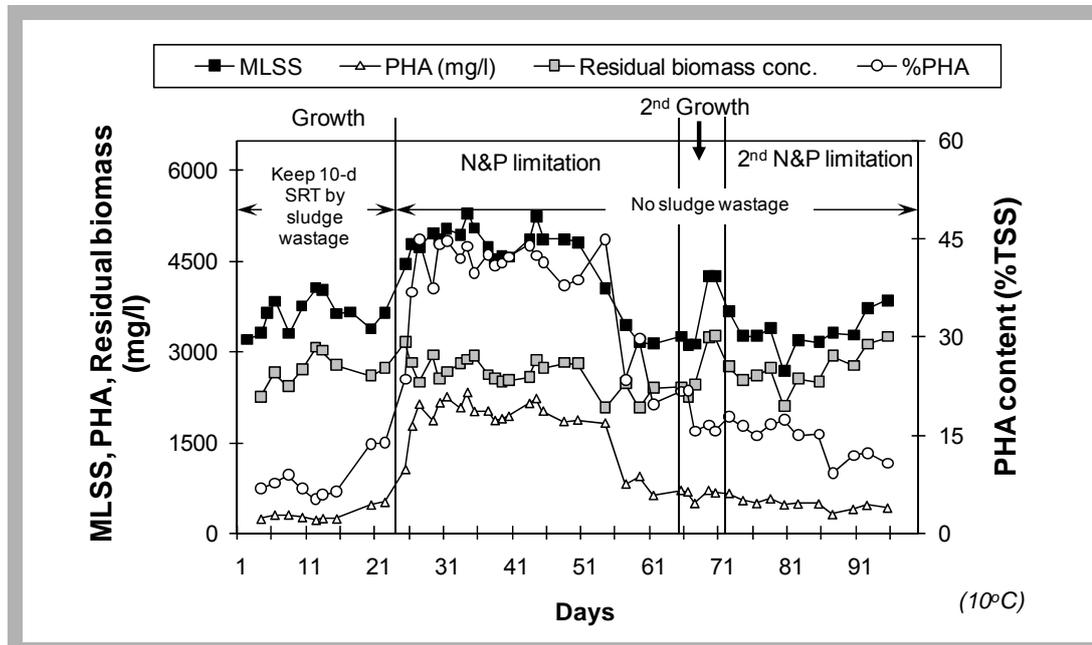


Figure 2
Profiles of PHA content (%TSS), PHA concentration (mg/l), MLSS (mg/l) and residual biomass (mg/l) for the 10°C experiment with N&P limitation

After N&P was eliminated from the influent, PHA accumulation clearly happened as PHA content increased rapidly and reached the maximum content of 45%TSS on day 5 after N&P limitation was started. Surprisingly, the PHA content remained nearly constant above 40%TSS for almost a month after reaching the maximum percentage, before decreasing simultaneously with the biomass concentration.

Next, nutrient addition was resumed and the system was operated under growth conditions again for approximately 5 days, and then both N and P were limited for a second time. During the second N&P limitation period, both the PHA content and the MLSS concentration were nearly the same as during the first growth period. That was inconsistent with the study by Punrattanasin [2], in which the author reported that two consecutive periods of growth and nutrient limitation would typically result in higher PHA content during the second limitation period. However, the operating days during the first N&P limitation of this study totaled 43 days. It is possible the biomass lost its ability to accumulate PHA because it lacked both nitrogen and phosphorus over this long period of time

Figure 3
Profiles of PHA content (%TSS), PHA concentration (mg/l), and residual biomass (mg/l) for the 20°C experiment with N&P limitation

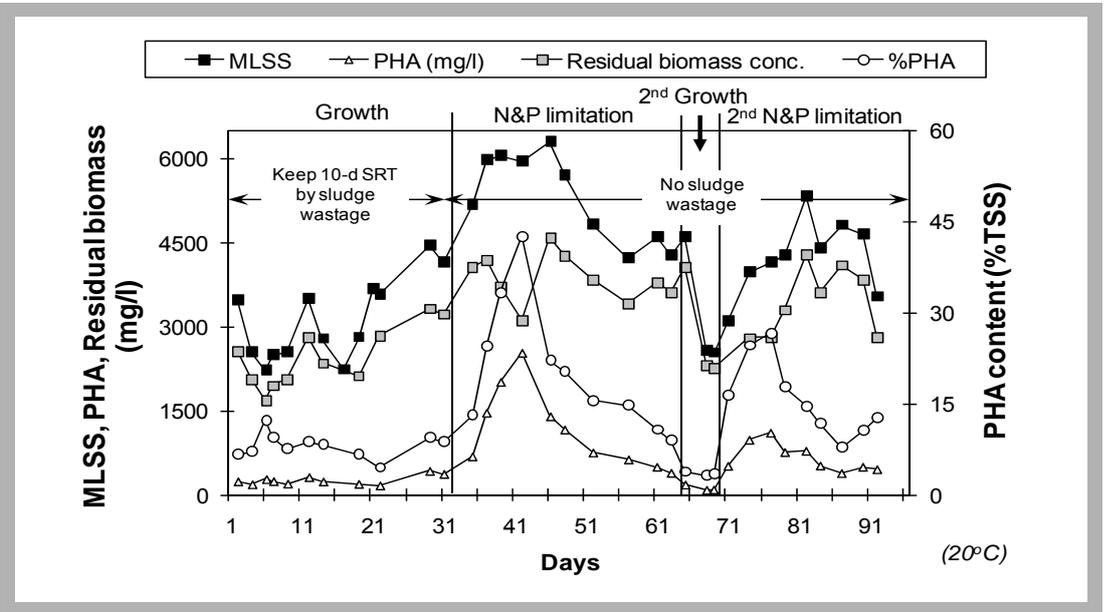
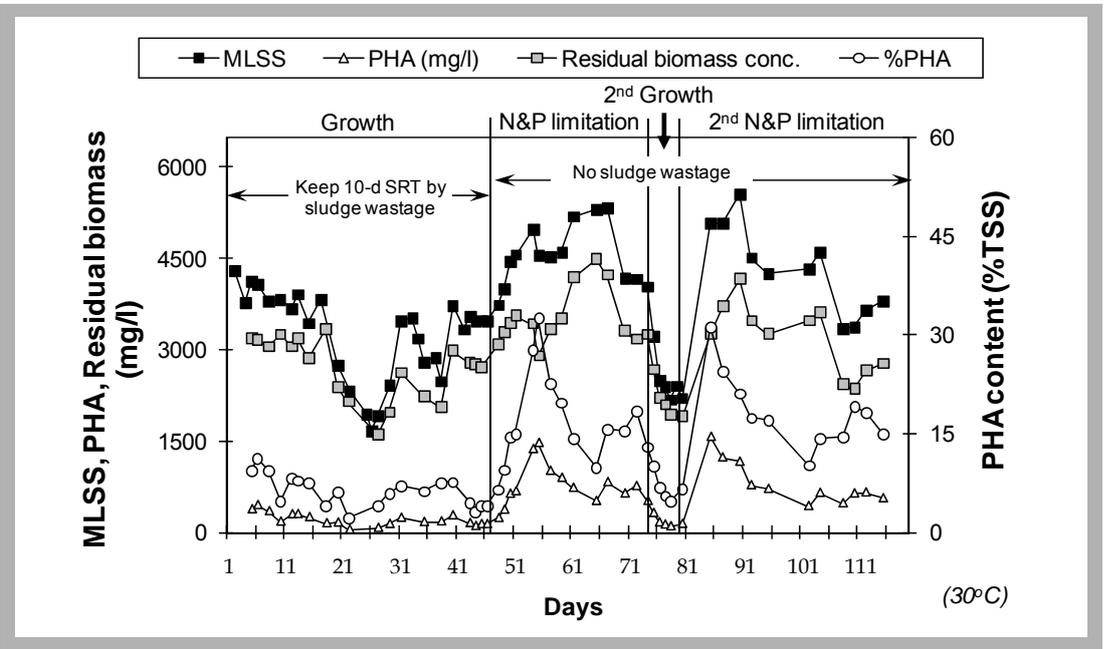


Figure 4
Profiles of PHA content (%TSS), PHA concentration (mg/l), and residual biomass (mg/l) for the 30°C experiment with N&P limitation



Figures 3 and 4 show the profiles of MLSS (mg/l), PHA content (%TSS) and PHA concentration (mg/l) during the experiments at 20 and 30°C, respectively, with combined N&P limitations. Similarly to the 10°C experiment, the systems were operated for 2 SRTs for the normal growth phase. The PHA contents during both experiments increased slower than during the experiment of 10°C. The maximum PHA contents of 43%TSS in the experiment at 20°C and 33%TSS at 30°C were obtained 11 and 10 days after N&P limitations, respectively. Unlike the 10°C experimental results, the PHA contents in both the 20 and 30°C experiments decreased almost immediately after reaching the maximum value. After the second period of N&P limitation conditions were applied, the PHA contents in both experiments reached the maximum values faster than during the first N&P limitations period. The values reached 27%TSS in the 20°C experiment and 31%TSS in the 30°C, after 8 and 6 days, respectively. The peak PHA accumulations occurred during the second nutrient limitation period at a faster rate than the first limitation period similarly to Punrattanasin [2]. The author explained this operating condition (two consecutive cycles of growth & nutrient limitation) would probably enrich the population of PHA accumulating bacteria. But the maximum PHA amounts accumulated were all less than the fractional contents obtained during the first limitations period. The reason of this inconsistency is still unknown.

The PHA concentrations (mg/l), PHA productivities (mg/l-day) and PHA yields (mg PHA/mg COD_u) obtained during the N&P limitation periods are summarized in Table 2. The PHA productivities and yields clearly decreased as temperature increased, i.e., declining from 427 to 204 to 148 mg/l-day, and from 0.38 to 0.16 to 0.11 mg PHA/mg COD_u at the temperatures of 10, 20 and 30°C, respectively. The PHA productivities in this study were significantly less than those reported in several papers using pure cultures. This is because the concentrations of activated sludge biomass and substrate used in these experiments were much lower, based on the intention to use wastewaters as carbon source in subsequent research. However, the above PHA yields were quite promising, especially at 10°C (0.38 mg PHA/mg COD_u) when compared to the theoretical yield of 0.48 mg PHA/mg acetic acid, (equivalent to 0.45 mg PHA/mg COD_u), calculated by Yamane et al. [15], or the yields obtained using pure cultures, e.g., a yield of 0.46 g PHB/g glucose with *Alcaligenes eutrophus* DSM 545 [16] and a yield of 0.36 g PHB/g glucose with *Ralstonia eutropha* [11].

Items	10°C		20°C		30°C	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
Max. PHA (%TSS)	45	18	43	27	33	31
Max. PHA concentration (mg/l)	2133	654	2239	1107	1476	1577
Corresponding PHA (%TSS)*	45	18	43	27	33	31
Corresponding MLSS (mg/l)**	4740	3673	5255	4147	4542	5070
Days to Accumulate Max. %PHA (days)	5	2	11	8	10	6
PHA Productivity (mg/l-day)	427	327	204	138	148	263
PHV/PHA on day of Max. PHA concentration (%)	43	48	21	31	25	42
Yield of PHA on Substrates (mgPHA/mgCOD _u)	0.38	0.22	0.16	0.10	0.11	0.17

Remark: *Corresponding PHA (%TSS) is PHA content on the day that PHA concentration is maximum, not always the same day of maximum PHA content
 **Corresponding MLSS (mg/l) is MLSS on the day of maximum PHA concentration.

Table 2
 Summary of PHA production and biomass concentration data from experiments with combined N&P limitations

3.2 Substrate utilization

If wastewaters are used as the organic carbon provider, high COD or substrate utilization is preferred. Figures 5 to 7 show the profiles of COD utilization, COD removal efficiency and PHA content during the experiments. The results indicate that there is a relationship between these two parameters during N&P limitation conditions, and a similar relationship was observed when only nitrogen was limited, as reported by Chinwetkitvanich et al. [17]. The data plots of the experiments at 10, 20 and 30°C show that COD utilization and COD removal efficiency decreased rapidly while the PHA fraction increased to peak values.

During the second N&P limitation periods, the decrease of COD utilization and increase of PHA content at 10°C and 20°C occurred concurrently, while COD utilization in the 30°C system was maintained at a surprisingly high level. Regarding the residual biomass curve of the 30°C system, shown in Figures 4, there was cell growth during the second PHA accumulation. It was observed that the 30°C biomass utilized COD for both PHA storage and cell growth, while the biomass maintained at the two lower temperatures utilized COD mostly for PHA accumulation. However, this study had not yet considered another intracellular storage such as glycogen. It is possible that the residual biomass would not represent only cell growth, but also glycogen storage. If glycogen storage significantly involved with COD utilization, the explanation of temperature effect on PHA accumulation would be more complicated. For COD removal efficiencies, they were mostly lower than 50% during PHA accumulation, especially in the 10°C system (only 20% left).

Figure 5
Profiles of COD utilization and PHA content (% of TSS) for the 10°C experiment with N&P limitation

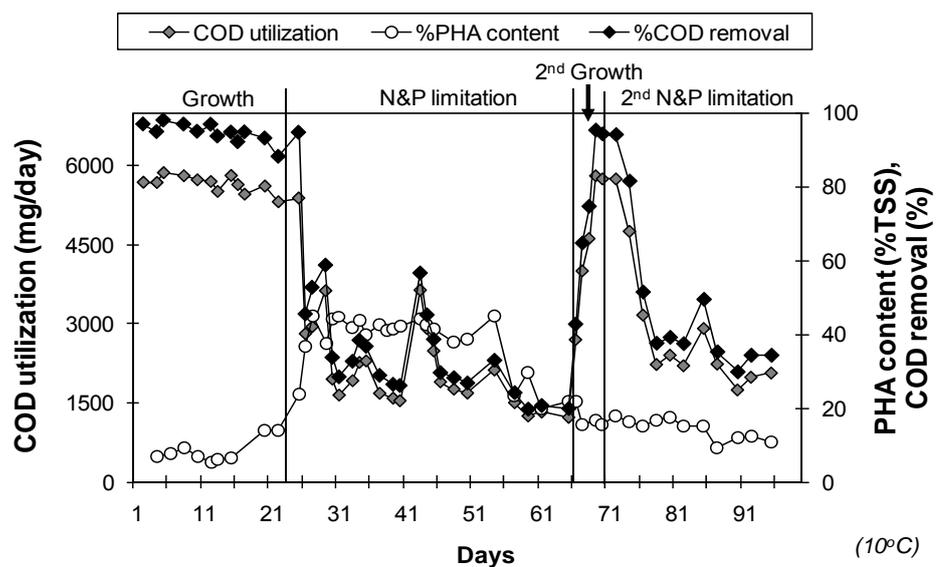
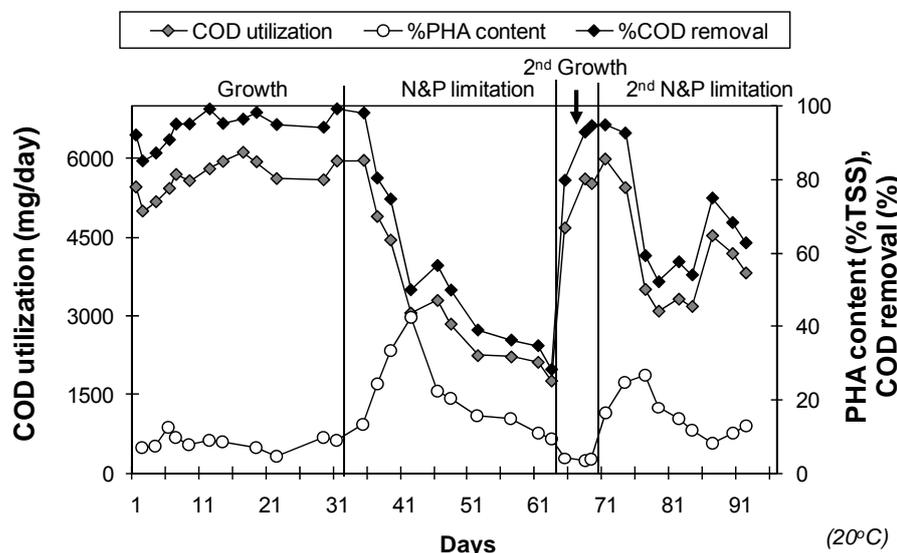


Figure 6
Profiles of COD utilization and PHA content (% of TSS) for the 20°C experiment with N&P limitation



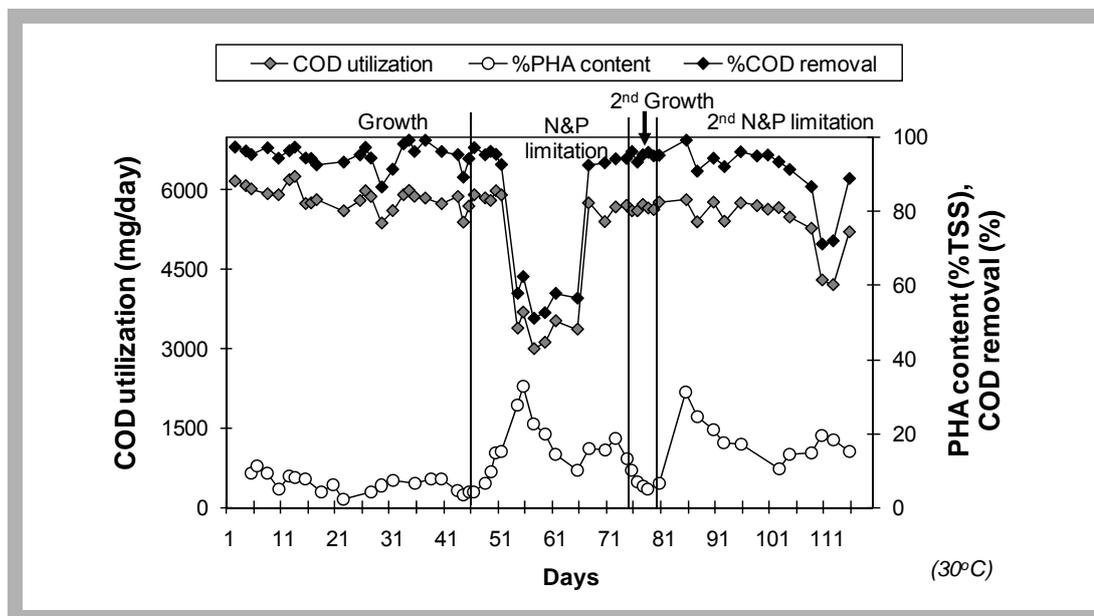


Figure 7
Profiles of COD utilization and PHA content (% of TSS) for the 30°C experiment with N&P limitation

3.3 Comparison of PHA productions with different nutrient limitation scenarios

The production of PHA with nitrogen limitation [17], phosphorus limitation [18] and combined N&P limitation from this study were comparatively summarized in Figure 8. The best PHA yield of 0.38 mg PHA/mg COD_u (Figure 8a) was obtained in the experiment at 10°C with N&P limitation. This yield was quite close to the theoretical yield of 0.45 mg PHA/mg COD_u [15], and the observed yield of 0.42 mg PHA/mg sucrose (equivalent to 0.37 mg PHA/mg COD_u) accumulated by *Alcaligenes latus* [19]. In the case of P limitation, PHA yields ranged from 0.03 to 0.05 mg PHA/mg COD, which are very much lower due to the longer time of PHA accumulation required. Therefore, P limitation is not recommended for PHA accumulation with activated sludge biomass. However, not only nutrient limitation, but also temperature influences the observed PHA yield, i.e., higher temperatures result in lower PHA yields.

Krishna and Van Loosdrecht [20] have explained that under famine phase (or unbalanced growth conditions), cell maintenance played a more important role than cell growth. As well, the authors stated that cell maintenance and decay rates increased with higher temperature, resulting in decreased biomass formation. Therefore, at higher temperatures, some of accumulated PHA may also be oxidized to obtain enough energy for anabolic metabolism, resulting in lower observed PHA yield.

Phosphorus-accumulating organisms (PAOs), the important microorganisms in anaerobic-aerobic activated sludge processing, have been widely recognized for their ability to accumulate PHA. Panswad et al. [21] concluded that PAOs are lower-range mesophiles or psychrophiles. Thus, at higher temperatures there would be an increased requirement for cell maintenance energy, and less energy availability for cell production; hence, the presence of PAOs in the biomass would be reduced as the system temperature decreases. Therefore, though N&P limitation seems to stimulate PHA production better than other nutrient limitations, PHA production with N&P limitation at high temperature is not likely to be economical.

The PHA productivities obtained in this study (Figure 8b) were significantly lower than those reported by Wang and Lee [19] and Choi and Lee [22], whose studies were based on using pure cultures and high concentration carbon sources, because of a lower biomass concentration in this study. In commercial production studies with pure cultures, the biomass concentrations used for cultivation were substantially higher, e.g., a final biomass concentration of 164 g/l was obtained by Kim et al. [23] and 143 g/l by Yamane et al. [24], whereas the biomass concentrations in the activated sludge systems operated during these experiments were always lower than 5 g/l during the growth phase and never exceeded 8 g/l. Also, the concentrations of carbon substrate fed during this study were only about 0.66 g/l COD while others [23] - [25] fed glucose concentrations of 10-20 g/l. Nevertheless, the experiment at 10°C with N&P limitation

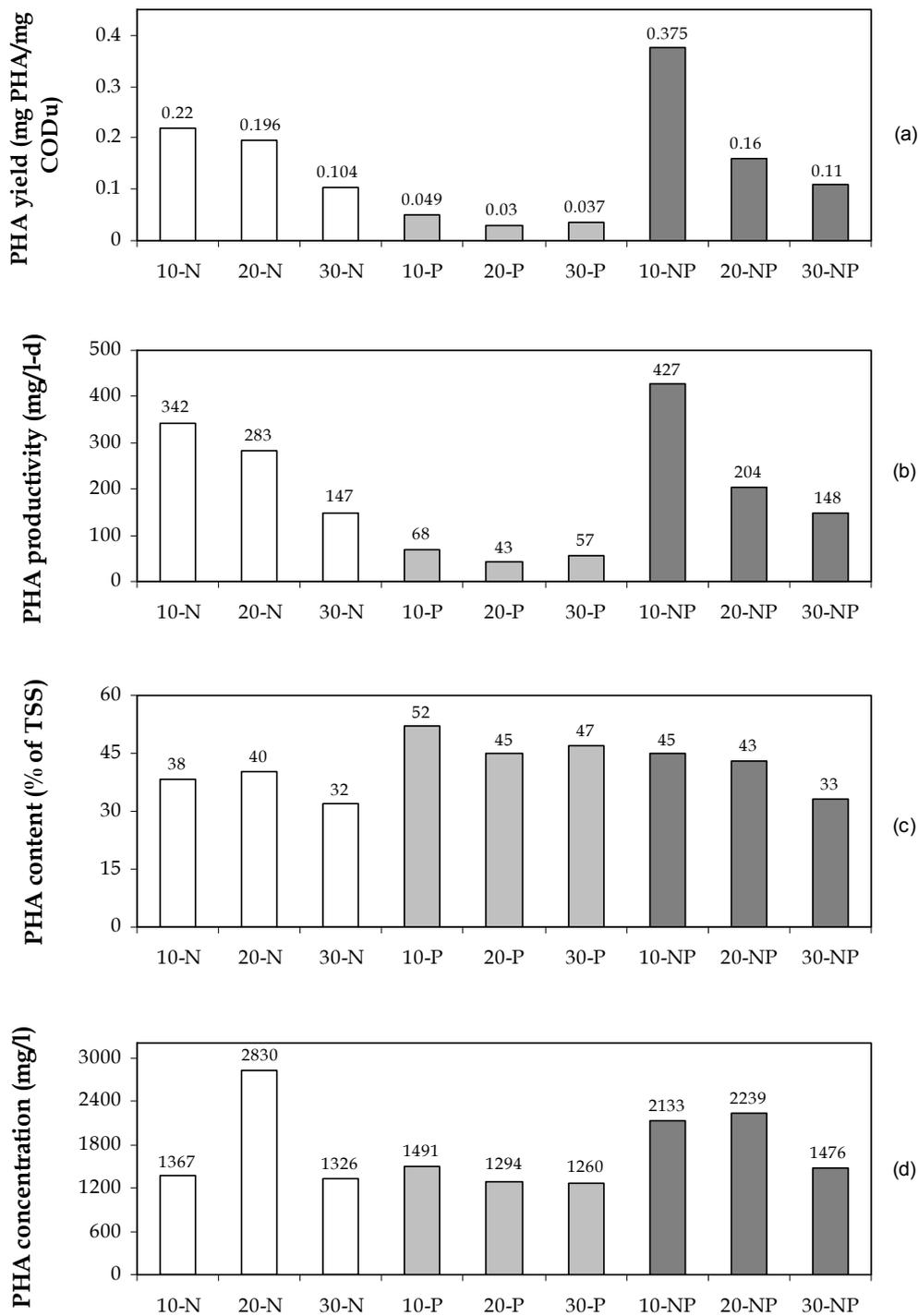
provided the best PHA productivity of 427 mg/l-d and PHA yields of 0.38 mg PHA/mg COD_u. This PHA yield was only 15% less than those theorized by Yamane [15] and slightly greater than the 0.37 mg PHA/mgCOD_u obtained by Wang and Lee [19]. However, the low PHA productivities obtained from the experiments with P limitation confirmed that P limitation was not suitable for PHA accumulation utilizing activated sludge biomass. Of course, increases in temperature above 10°C reduced both PHA yield and the resulting PHA concentration.

Figure 8c illustrated that the PHA contents obtained from the experiment with P limitation were somewhat higher than others, especially, from the experiment at 10°C, 52% of TSS, which was quite similar to the value of 53% of TSS obtained from activated sludge biomass with P limitation [2]. However, the PHA accumulation, till the highest PHA content (52% of TSS) was obtained, took quite longer time than other experiments. During the PHA accumulation, the problem of sludge bulking severely occurred and caused the massive loss of biomass, resulting in the lowest yield and the lowest productivity of PHA in this experiment (P limitation). Still, these PHA contents were very much lower in comparison with PHA content of 80% of cell dry weight obtained by pure culture, *Alcaligenes eutrophus*, with P limitation [26].

The experiments with N&P limitation produced even lower PHA contents (the highest content was 45% of TSS in the experiment at 10°C). On the other hand, in the case of PHA content, effects due to different nutrient limitations and temperature were not so distinct.

The PHA concentrations obtained during the P limitation experiments, i.e., 1491, 1294 and 1260 mg PHA/l at 10, 20 and 30°C, respectively, were somewhat lower than those obtained during the N&P limitation or nitrogen limitation experiments (Figure 8d). The biomass concentrations were declining throughout the P limitation experiments. The best PHA concentration of 2830 mg PHA/l was obtained from the experiment at 20°C with nitrogen limitation due to its high biomass concentration of 7548 mg/l. Regarding to this PHA content parameter, the setup with N&P limitation and low temperature was still the best among the others.

Choi and Lee [22] concluded that PHA content was the most important factor affecting the performance and economics of PHA production because of its effects on PHA yield and recovery efficiency. However, the results gained from this study were a little different; for example, the experiments with P limitation had higher PHA contents, but much lower PHA yields and productivities than others. This occurred because the biomass required a much longer time to reach high PHA content and prolonged cultivation with P limitation caused instability of biomass and consequently the loss of biomass with supernatant withdrawal. Hence, in this study, we have to conclude that P limitation was not suitable for PHA production using activated sludge biomass even though these conditions produced higher PHA contents. Consider the experiments at 30°C; PHA productions with combined N&P limitation or only N limitation were much alike. This will be an advantage for future study because finding the ideal real wastewater (high carbon with low nitrogen and phosphorus) for using in PHA production would be more flexible. The preceding inquiry needs explained better.



Note: 10-N (at 10°C with N limitation), 20-N (at 20°C with N limitation), 30-N (at 30°C with N limitation), 10-P (at 10°C with P limitation), 20-P (at 20°C with P limitation), 30-P (at 30°C with P limitation), 10-NP (at 10°C with N&P limitation), 20-NP (at 20°C with N&P limitation), 30-NP (at 30°C with N&P limitation).

Figure 8
Comparison of PHA production with various types of nutrient limitation

IV. Conclusions

It is evident from the N&P limitation experimental data that PHA accumulation was better in the 10°C system compared to the two systems operated at the higher temperatures of 20 and 30°C. Although the 20°C system produced the highest MLSS concentration during PHA accumulation, the 10°C biomass stored a higher fraction of PHA content with less production time, resulting in the highest PHA yield (mg PHA/mg COD_u). It was also observed that the PHA content during the second N&P limitation period reached the maximum value in shorter time than required during the first limitation period, but greater productions were obtained during the first limitation periods. The experiment with N&P limitation at 10°C produced the higher PHA productivity and yield in comparison with the experiments with either N or P limitations at the same temperature. The P limitation condition provided the lowest PHA productivities and yields at all experimental temperatures and, as a result, is not recommended for further study. Based on the results of these experiments, it is recommended that low temperatures, i.e. 10 to 20°C, be used for PHA production and harvesting for biodegradable plastics production. Anyway, COD removal efficiency (or COD utilization) was reduced during a peak of PHA accumulation and may not satisfy wastewater treatment purpose. The additional process line for biodegradable plastics production (using the same wastewater) may be required in parallel with a conventional process line of wastewater treatment in order to accomplish both benefits.

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