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Polyhydroxybutyrate Production from Municipal Wastewater Activated Sludge with Different Carbon Sources

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ABSTRACT: In this study, a sequencing batch reactor was set up and operated for over three months to cultivate polyphosphate-accumulating organisms polyphosphate-accumulating organisms (PAOs) in the enriched activated sludge. Batch studies were then carried out to study the effect of different carbon sources on phosphorus removal as well as polyhydroxybutyrate (PHB) production. The carbon sources investigated were acetate, glucose, wastewater, and beef extract. It was found that enhanced biological phosphorus removal could not be achieved using glucose as substrate. This suggested that glucose was not a good candidate for biological phosphorus removal. In terms of PHB production, using acetate and glucose as substrate resulted in PHB production of 42% and 40%, respectively, of the dry cell weight (DCW). Lower PHB production was obtained from using municipal wastewater and beef extract as a carbon source. This resulted in ~15% and 13% of DCW. It was concluded that municipal wastewater activated sludge can be an economic alternative for PHB production if municipal wastewater is mixed with certain kinds of carbon-enriched industrial wastewater.

KEYWORDS: enhanced biological phosphorus removal, activated sludge, polyhydroxybutyrate, anaerobic phosphorus release, anaerobic/aerobic activated sludge process

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Introduction

Petroleum-based plastic has never been widely used as it is today, and they are manufactured with many desirable properties that can be used in countless applications. However, the fact that petroleum-based plastic is nonbiodegradable causes increasing environmental concern. In addition, the depletion of petroleum resources has significantly increased production costs.[1]

Polyhydroxybutyrate (PHB), a member of polyhydroxyalkonate (PHA) family, is a biodegradable polymer and is used for the production of biodegradable plastic. PHB is produced by microorganisms to store carbon as a response to a stressed environment.[2,3] Unlike conventional plastic, PHB is a renewable and environmental friendly resource for plastic production. It has been documented[4] that more than 300 different microorganisms can synthesize PHB. PHB-based plastic has been considered as an attractive substitute to conventional plastic because of the many similarities in physical properties. However, PHB-based commercial plastic products are rare compared to petroleum-based plastic products. The main obstacle preventing its widespread use is the relatively high cost associated with the production of PHBs, which traditionally require a pure bacterial culture.[5] The cost of commercialization using pure culture fermentation to produce PHB is about four to nine times higher than that of conventional plastic.[6] Intensive studies have been conducted using pure cultures to improve the productivity and to reduce the production cost.[7–9]

Using mixed cultures to produce PHB can be another approach to reduce the production costs. The advantage of using mixed culture is to reduce the cost associated with substrate, which is one of the main cost factors of PHB production.[4] Mixed cultures allow the utilization of the complex substrate.[10] In the wastewater treatment industry, microorganisms employed for phosphorus removal, also called phosphorus-accumulating organisms (PAOs), are capable of synthesizing PHB as their energy pool.[11,12] Research demonstrated that the accumulation of PHBs in PAOs is accomplished through a feast and famine approach of the substrate (carbon source).[13] The reuse of organic matter to produce PHB from carbon-rich industrial wastewater, such as food production waste and brewery waste as well as municipal wastewater, may considerably reduce the cost. This organic matter reuse will also benefit phosphorus removal and may even reduce the excess sludge discharge volume.[14] Combining wastewater treatment with PHB production can be considered as a sustainable approach when dealing with environmental and economic issues.

This study's aim is to investigate the efficiency of biological phosphorus removal and PHB production using four different substrates: municipal wastewater, acetate, glucose, and beef extract. Acetate was selected as a representation of the fermentation industry wastewater. Glucose represented sugar industry wastewater, and beef extract was used to represent a protein-enriched industrial wastewater.

Methodology

Operation of anaerobic/aerobic (A/O) activated sludge process. A laboratory-scale sequencing batch reactor (SBR) with A/O configuration was set up to achieve biological phosphorus removal and was operated for over 90 days before the start of this study. The reactor was seeded with activated sludge from the local wastewater treatment plant that is a non-BNR plant (Winnipeg South End Water Pollution Control Center) and had a working volume of 3 L. The SBR was operated with three cycles per day, and solid retention time was controlled for 10 days. Temperature was constant at ~20–22°C, and pH was controlled between 7.0 and 7.5 by adding 0.5 N H_2SO_4 solution. The reactor was fed with synthetic wastewater using acetate, yeast, and beef extract as the carbon sources. The composition of synthetic wastewater is listed in Table 1, and the reactor operational configuration is shown in Figure 1.

Batch test for phosphate uptake and release. The enriched PAO sludge was taken from the A/O SBR at the end of the aeration phase and was subjected to batch tests. The batch experiment, consisting of an anaerobic phosphorus release test and an aerobic phosphorus uptake test, was conducted using 1 L of activated sludge mixed liquor with a solid concentration of 1.5–1.8 g/L. Glucose, sodium acetate, beef extract, and municipal wastewater were used as the sole carbon source in each batch test. For the anaerobic phosphorus release test (150 minutes), nitrogen gas was purged to the fermenter. For the aerobic phosphorus uptake test (210 minutes), compressed air was bubbled into the system. Throughout the batch experiments, the pH was controlled between 7.0 and 7.5 by adding 0.5 N H_2SO_4 solution.

Batch experiment test for PHB production test.

- For the acetate, glucose, and beef extract test, the sludge was taken at the end of aeration phase of the SBR and was placed into a 500 mL flask. One gram of each substrate was added to obtain an approximate concentration of 2 g/L.
- For the wastewater test, approximately the same amount of sludge was taken at the end of the aeration phase of the SBR. However, in order to provide enough substrate, 3 L of wastewater was used.

The fermenters were operated under complete mixing (moderate speed) conditions for 24 hours in a closed system. For these experiments, the pH was not monitored.

Analytical procedure. Carbon content of the substrates was measured as dissolved organic carbon (DOC). A Phoenix 8000 TOC Analyzer (Tekmar Dohrmann) was used for the determination of DOC. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) measurements were performed according to Standard Methods.[15] Dissolved phosphate was measured by Lachat Instruments QuikChem 8500, following the QuikChem® Method for orthophosphate 10-115-01-1-O.

PHB analysis. Extraction and estimation of the PHB were performed according to the procedures from the Manual of Methods for General Bacteriology.[16] The biomass was pelletized by centrifugation (10,000 × *g*, 15 minutes) followed by lysis of biomass by incubation at 37°C with sodium hypochlorite. The lipid granules were deposited by centrifugation, followed by washing with water, acetone, and ethanol. The PHB was extracted with hot chloroform and obtained by evaporating the chloroform. Finally, sulfuric acid was added to convert PHB polymer into crotonic acid. The absorbance of the sample was measured by Ultrospec 2100 pro UV/visible spectrophotometer (Biochrom Ltd.) at 235 nm against a sulfuric acid blank. The standard curve was prepared with 3-hydroxybutyric acid (Sigma-Aldrich), and the concentration of each sample was obtained from this standard graph.

Table 1. Synthetic wastewater composition.[24]

SYNTHETIC WASTEWATER		MINERAL SOLUTION	
INGREDIENTS	CONCENTRATION (mg/L)	INGREDIENTS	CONCENTRATION (g/L)
NaAc	355	$FeCl_3 \cdot 6H_2O$	1.5
Beef extract	65	H_3BO_3	0.15
Yeast extract	65	$CuSO_4 \cdot 5H_2O$	0.03
$MgSO_4 \cdot 7H_2O$	170	KI	0.03
$CaCl_2 \cdot 2H_2O$	14	$MnCl_2 \cdot 4H_2O$	0.12
P (K_2HPO_4)	9	$Na_2MoO_4 \cdot 2H_2O$	0.06
TN (organic)	14–15	$ZnSO_4 \cdot 7H_2O$	0.12
		$CoCl_2 \cdot 2H_2O$	0.15
Mineral solution	0.3 mL	EDTA	10

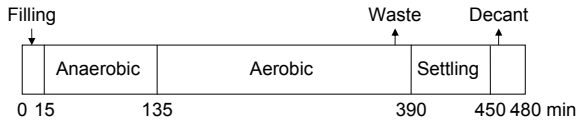

Figure 1. Schematics of A/O process configuration.

Microscopic analysis. PHB granule staining was prepared following the Manual of Methods for General Bacteriology,[16] and was observed under a phase contrast microscope (Leitz Wetzlar Germany).

Results and Discussion

Performance of the SBR. The phosphorus removal from the SBR was gradually achieved as the sludge was taken from a non-BNR plant. The phosphate release and uptake rates were increased over time. At day 16, ~95% phosphate removal was achieved; however, the phosphate release and uptake rates continued to increase until they were stabilized at day 72. The continuing increase of P release and uptake rates indicated the increase of the PAO population. Figure 2 shows the DOC and phosphate profiles in a typical cycle of the SBR at day 85. It was noticed that rapid DOC removal (substrate uptake) occurred during the first 60 minutes, and this resulted in a rapid phosphate release. The phosphate release rate and uptake rate were measured as 31 mg P/(g VSS hour) and 26 mg P/(g VSS hour), respectively. The SBR was continuously operated for over 90 days before starting the batch tests.

Effects of carbon source on anaerobic P release. The phosphate release and uptake tests using acetate, glucose, beef extract, and municipal wastewater as the carbon sources were monitored in the batch reactors with an initial phosphate concentration of around 6 mg/L. As can been seen from Figure 2, the highest DOC uptake under the anaerobic phase was achieved by glucose. This was followed by acetate, municipal wastewater, and beef extract, whereas, phosphate release was most rapid using acetate as substrate, followed by glucose, wastewater, and beef extract (Fig. 3).

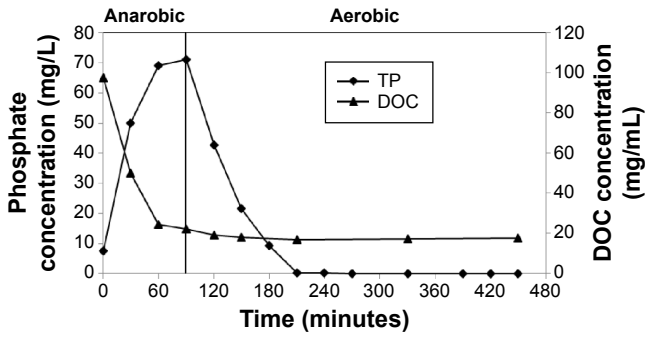

Figure 2. Typical cycle of DOC and phosphorus profiles of SBR with biological phosphorus removal.

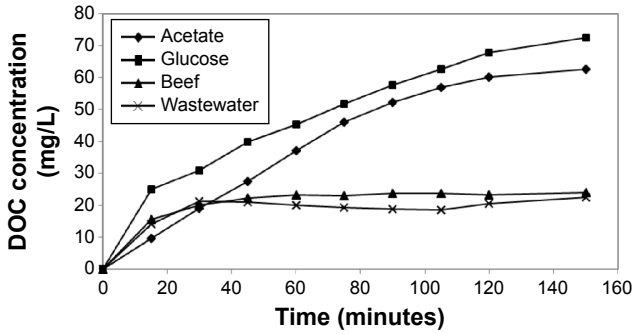

Figure 3. Utilization of different carbon source during anaerobic phosphorus release batch test.

Under anaerobic conditions, when the microorganisms in the reactor were exposed to acetate and glucose, a considerably higher carbon uptake and phosphate release was observed in comparison with wastewater and beef extract. It is well documented that acetate is the most-preferred and -effective substrate for PAOs, while the effect of glucose on the selection and growth of PAO is quite controversial. Researches have showed that glucose is detrimental to the biological phosphorus removal system, as glucose promotes the growth of glycogen accumulation bacteria (G-bacteria). G-bacteria compete with PAOs for acetate, and unlike PAOs, they can uptake acetate anaerobically without phosphorus release, ie, no performance of phosphorus removal. On the other hand, researches have demonstrated that glucose can be used for PAOs to achieve phosphorus removal.[17,18] In this batch test, the result of high phosphorus release when using glucose as substrate was consistent with their observation. The phosphorus release to carbon uptake ratio can be used as an indicator for the effectiveness of substrate. As can be seen in Table 2, acetate, with the lowest ratio, is the preferred substrate for PAOs.

In the case of municipal wastewater and beef extract, carbon uptake shows a similar trend (Fig. 2). In both reactors, the carbon uptake reached a plateau at 30 minutes. However, their phosphate release was quite different. For the beef extract reactor, the phosphate release gradually increased with time, while in the municipal wastewater reactor, the phosphate release occurred rapidly and reached a maximum after 30 minutes. One possible reason for low carbon uptake and gradual phosphate release in the reactor with beef extract was that amino acids are the main component of beef extract. Deamination had to occur before the microorganisms could utilize this carbon source. Therefore, the slow phosphorus release is a result of the slow amino acid degradation and subsequent conversion to PHB.

As shown in Table 2, wastewater and acetate had the same phosphate release to carbon uptake ratio. This similarity suggested that the main carbon source in the wastewater that facilitated the PAO's phosphate release was volatile fatty acids. These fatty acids, which are mainly acetic acid, were

Table 2. Effect of substrate on phosphate release and uptake.

	ACETATE	GLUCOSE	MUNICIPAL WASTEWATER	BEEF EXTRACT
Initial TP (mg/L)	5.7	5.2	6.3	6.0
ΔP release (mg/L)	78.3	72.6	26.9	17.2
ΔP uptake (mg/L)	83.0	54.4	33.2	23.2
$\Delta DOC_{removal}$ (mg/L)	62.9	72.5	22.4	23.1
MLVSS (g/L)	1.65	1.62	1.64	1.65
$\Delta Prelease:\Delta C_{DOC}$	1:0.8	1:1.0	1:0.8	1:1.3
$\Delta Prelease:\Delta C_{DOC}$ (molar ratio)	1:1.0	1:0.4	–	–

produced by fermentative microorganisms in the raw wastewater during storage.

Effects of carbon source on aerobic P uptake. During 210-minute aeration period, the highest phosphate uptake was caused by acetate, followed by glucose, municipal wastewater, and beef extract (Fig. 4). Although using beef extract as a substrate caused the lowest phosphate release under anaerobic conditions, it took up a net of 6 mg/L of phosphate in only 120 minutes. Municipal wastewater and acetate took 150 minutes and 180 minutes, respectively, to uptake this net 6 mg/L of phosphate. Using glucose as substrate, phosphate released under anaerobic conditions was not fully taken up, even after 210 minutes of aeration. This result was probably because of the presence of residual glucose in the aerobic phase. The initial carbon load in the form of glucose in this batch test was twice as high as the other substrates, and only half the amount of glucose was taken up at the end of anaerobic phase. Therefore, not all the glucose was utilized, resulting in some residual glucose present in the aerobic phase. This existence of substrate in the aerobic phase would have hindered phosphate uptake because of the competition for oxygen between the PAOs and the other heterotrophic bacteria in the system.

Production of PHB using different carbon substrates. After 24 hours of fermentation, samples were taken to determine the PHB content in the biomass. PHB as high as 42% dry cell weight (DCW) was measured from the acetate substrate, and ~40% of DCW was measured from the glucose substrate. For wastewater and beef extract, PHB content of DCW was 15% and 13%, respectively (Fig. 5). This suggests that PHB production was related to the type of substrate. Results from this experiment showed that acetate was an effective carbon source for PHB production. Samples from the biomass supplied with the acetate substrate were taken for microscopic examination. Large PHB granules were observed inside the cell.

Table 3 lists the PHB production from different types of wastewater using different microorganisms from literature reviews. The PHB production obtained from this test using acetate as substrate is comparable with other reported results.

The PHB production of 40% DCW was achieved using glucose as substrate in this test. Singh et al[9] and Bhuwal et al[19] obtained much higher PHB production (ie, 51.8% and 70%–80% DCW, respectively) by using pure cultures from carbon-enriched industrial wastewater. In the current tests, lower PHB productions were expected because of the lower efficiency of mixed cultures when compared to the pure culture. The comparable PHB production from using glucose and acetate as substrate in this test suggested that glucose is also a good substrate for PHB production. It was reported[23] that when glucose was used as the sole substrate, the majority of the glucose was converted by the succinate–propionate pathway to propionyl-CoA, ending up as PHV with very low PHB production. However, this experiment revealed that the microorganisms in this operating system probably could convert glucose in the same manner as acetate. In other words, the TCA cycle was not fully carried out. It was assumed that

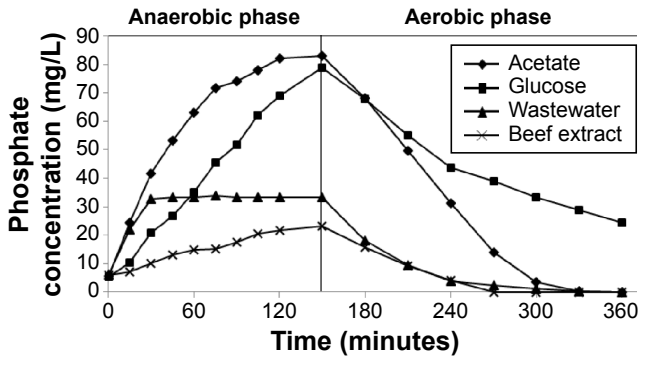

Figure 4. Phosphorus profile by using different carbon sources during batch test.

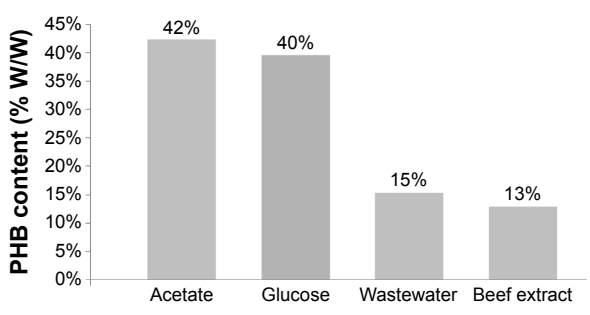

Figure 5. PHB content of biomass from different substrates.

Table 3. PHB production from different types of wastewater and cultures.

MICROORGANISMS	SUBSTRATE	PHB CONTENT (W/W) OF DCW	REFERENCES
Bacillus subtilis NG220	Sugar industry wastewater	51.8%	9
NAP11, NAC1 Isolates	Pulp wastewater	77–80%	19
Enterobacter aerogenes	Domestic wastewater	96.25%	12
Mixed cultures	Synthetic wastewater (acetate)	58.2–78.5%	20
Mixed cultures	Synthetic wastewater (acetate)	16–55%	21
Mixed cultures	Synthetic wastewater (acetate, yeast exact)	28.8–50%	22

in this experiment, glucose was converted to Acetyl-CoA through the embden-meyerhof-parnas (EMP) pathway and subsequently condensed to PHB (Figs. 6 and 7). However, further research is necessary to validate this hypothesis.

The PHB content of biomass produced by municipal wastewater was only 15% of DCW. This low production was probably because of the low carbon content in the wastewater. Ceyhan and Ozdemir[12] reported much higher PHB production from domestic wastewater. Their high production could be the result of (1) using pure culture and (2) using a mixture of high carbon-enriched wastewater. From this current test, it was suggested that if municipal wastewater is mixed with certain carbon-rich industrial waste, it can be a suitable substrate for PHB production.

The lowest PHB production from beef extract (13% DCW) suggested that the PAOs in this system could not efficiently utilize beef extract. Therefore, substrate high in amino acid was not ideal for PHB production.

Conclusions

The effects of different carbon sources, namely, acetate, glucose, beef extract, and municipal wastewater, on biological phosphorus removal and PHB production were investigated in this study. In light of the key results from this experiment, the following conclusions can be made:

- Using acetate as substrate resulted in the lowest phosphate release to carbon uptake ratio, which indicates that acetate is the preferred substrate for PAOs.
- Biological phosphor can be achieved by using acetate, beef extract, and municipal wastewater as carbon source.
- The presence of substrate in the aerobic phase hinders the aerobic phosphate uptake.
- Acetate and glucose produce comparable amounts of PHB.
- Municipal wastewater can be used to produce PHB if it is mixed with certain carbon-rich industrial waste. However, beef extract is not an ideal substrate for PHB production.

Author Contributions

Conceived and designed the experiments: QY. Analyzed the data: QY, RS. Wrote the first draft of the manuscript: QY.

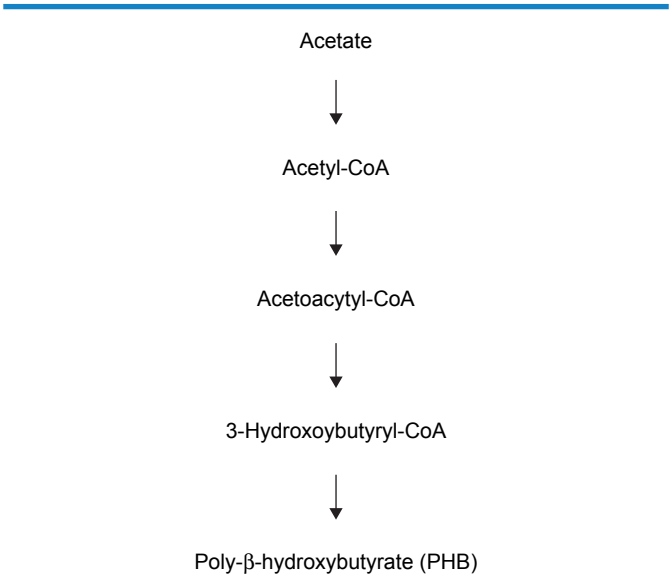

Figure 6. Pathway to produce PHB from acetate.

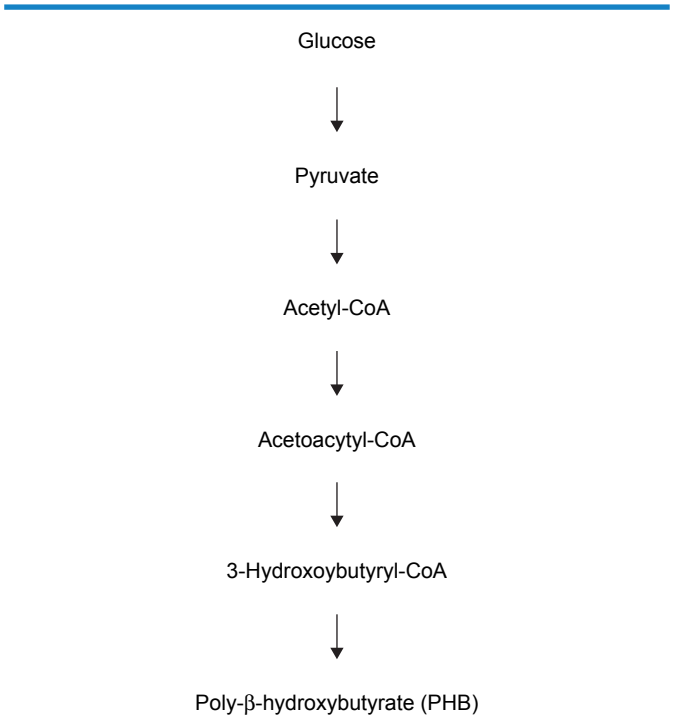

Figure 7. Proposed pathway to produce PHB from glucose.

Contributed to the writing of the manuscript: QY, RS, JO. Agree with manuscript results and conclusions: QY, RS, JO. Jointly developed the structure and arguments for the paper: QY, RS, JO. Made critical revisions and approved final version: QY, RS, JO. All authors reviewed and approved of the final manuscript.

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