Anaerobic Digestion of Polyhydroxybutyrate Accumulated in Excess Activated Sludge

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ABSTRACT
In the present study, to evaluate the conversion of intracellular PHB to methane gas, excess activated sludge with and without the accumulation of polyhydroxybutyrate (PHB) was subjected to anaerobic digestion. Excess activated sludge was collected from a laboratory activated sludge reactor and a part was fed with acetate in aerobic condition to obtain excess activated sludge with PHB accumulation. Then, excess activated sludge with and without PHB accumulation were mixed with anaerobic digested sludge, and the degradation of PHB and formation of methane gas were monitored during anaerobic fermentation at 37°C. For excess sludge with PHB accumulation, more than 75% of PHB was degraded only within the initial 2 days of incubation. Besides, 25% more methane gas was produced from excess sludge with PHB than without PHB accumulation. High production of methane gas indicates that PHB accumulated inside the cells of heterotrophic bacteria can easily be digested in anaerobic digestion process.

Keywords: anaerobic digestion, excess activated sludge, methane, polyhydroxybutyrate

INTRODUCTION
Activated sludge systems are the most commonly used biological processes for the treatment of wastewater. In activated sludge processes, a part of aerobic heterotrophic bacteria can store a part of organic pollutants as temporal carbon storage materials in the form of polyhydroxybutyrate (PHB). The accumulation of PHB in heterotrophic microorganisms is widely recognized as carbon and energy reserve materials (Doi, 1990; Lee, 1996). Takabatake et al. (2002) reported the potential of activated sludge at different wastewater treatment plants. Recent studies on PHB in activated sludge are mostly oriented to the recovery of PHB as a source of biodegradable plastics (Satoh et al., 1998; Chua et al., 2003; Serafim et al., 2004; Verlinden et al., 2007; Johnson et al., 2009; Jiang et al., 2011). But there is another direction to make use of PHB accumulated in activated sludge: a source of bio-energy, if it can be converted to methane gas. Not a few studies have been performed on anaerobic biodegradation of PHB (Budwill et al., 1992; Imam et al., 1995; Reischwitz et al., 1998; Rutkowski et al., 2008; Gutierrez-Wing et al., 2010), but in these studies, anaerobic degradation of industrially processed PHB was studied. On the other hand, PHB in activated sludge microorganisms is mostly located inside cells. And so far, very limited studies have been done on the anaerobic degradation of PHB that accumulated inside the cells of aerobic heterotrophic bacteria in activated sludge.

This study was conducted to evaluate the degradation of PHB accumulated in excess activated sludge and the recovery of methane gas in anaerobic digestion process. Further, a new process to convert organic pollutants in wastewater into methane gas via PHB
accumulation in excess activated sludge is proposed.

MATERIALS AND METHODS
Excess activated sludge was collected from a laboratory-scale sequencing batch reactor. To prepare excess activated sludge with PHB accumulation, a part of it was fed with acetate and aerobically incubated. Excess sludge with or without PHB accumulation was mixed with anaerobically digested sludge, then incubated at 37°C, and the conversion of PHB to methane gas was evaluated.

Inoculum
A laboratory-scale anaerobic digester with a liquid volume of 1.5 L was operated at 37°C under stirring condition. The loading rate of the digester was 1.0 kgVSS/(m³·day) and the sludge retention time (SRT) was 23 days. The anaerobically digested sludge (DS) was used as the inoculum for the batch experiments of anaerobic digestion.

Preparation of excess activated sludge with and without PHB accumulation
Excess activated sludge (2.8 L) was collected from a laboratory-scale sequencing batch activated sludge reactor (SBR) treating synthetic wastewater with acetate and peptone as the main carbon sources. The excess sludge concentration was about 1.9 g/L of MLVSS. Excess activated sludge without PHB accumulation (ES) was concentrated by centrifugation followed by resuspension of the treated water from the SBR.

Excess activated sludge with PHB accumulation (ESP_HB) was prepared as follows: A part of the excess sludge was placed in a beaker, fed with acetate at a final concentration of 1,000 mgC/L, and incubated for 5 hours under aerobic condition by air bubbling. Then, the sludge was centrifuged at 3,500 rpm for 10 minutes, the supernatant was decanted, and the sludge pellet was re-suspended in treated water from the SBR.

Both the concentrated ES and ESP_HB were stored at 4°C for 2 days before use in batch experiments.

Batch Experiments
Prior to the batch experiments, the volatile solid (VS) concentrations of DS, ES, and ESP_HB were determined. The VS concentrations of DS, ES, and ESP_HB were 9,750 mg/L, 13,600 mg/L, and 19,200 mg/L, respectively. A series of batch experiments were conducted viz. only digested sludge (DS) (control), mixture of digested sludge and excess sludge without PHB accumulation (ES+DS) and mixture of digested sludge and excess sludge with PHB accumulation (ESP_HB+DS). The tests were prepared in 10-mL vials with a liquid volume of 7 mL using 22 vials for each batch experiment. Each vial was added with solids of 68 mgVS except that vials of DS series were added with 34 mgVS. For ES+DS and ESP_HB+DS, the ratio of ES : DS and ESP_HB : DS were 1 : 1 on VS basis. Each vial was added with 1 mL of sodium bicarbonate solution (14 g/L) as a pH buffer and was supplemented with treated water from the SBR to make the total liquid volume to 7 mL. All the vials were then made air-tight with butyl rubber septums and aluminum caps and flushed with nitrogen gas (N₂) for 45 minutes. The contents were mixed thoroughly and incubated at 37°C in a shaker at 200 rpm. Gas and chemical analyses were performed on days 0, 2, 5, 8, 12, 16, 19, 22, 26, 29 and 34 of the
experiment. Two vials were used on each sampling day for chemical and gas composition analyses. The concentration of PHB, the volume of gas and its composition were determined in single measurement, while pH, NH$_3$-N, acetate, total COD, and VS were measured in duplicate.

**Analyses**
Total gas production was measured using a glass syringe equipped with a 20-gauge needle (Owen et al., 1979). Before the release of biogas, the vials were shaken for 15 s in order to equilibrate the gas and liquid phases. Methane was analyzed by a gas chromatograph equipped with a thermal conductivity detector (GC3200, GL Science, Japan) and a SUS column (filled with molecular sieve 5A 30/60, 3 mm i.d. × 2 m) at a flow rate of 20 mL/min using argon as the carrier gas with an injection volume of 0.5 mL and the oven and detector temperatures at 50°C. The volume of methane gas was calculated as the volume under the standard condition (0°C and 1 atm).

Measurement of pH was done with a pH-meter (HM 30G, TOA, Japan). Total COD was measured by the closed-tube colorimetric method using the kit from Hach (USA). Volatile solids (VS) were determined following the Standard Methods (APHA, 2005).

For the analysis of volatile fatty acids (VFA) and NH$_3$-N, 2 mL samples from each vial were centrifuged for 2 minutes at 13,000 rpm, and filtered through a membrane filter (0.2 µm). The concentrations of VFA were analyzed using a liquid chromatograph LaChrome Elite system (Hitachi, Japan) equipped with an SCR 101H column (Shimadzu, Japan), operated at 60°C, with 0.025% sulfuric acid as eluent, with an elution rate of 1 mL/min, and with a UV detector set at 210 nm. For the measurement of NH$_3$-N, the salicylate method was used with the AmVer HR kit (Hach).

Determination of PHB was performed by gas chromatography after methanolytic decomposition as stated by Satoh et al. (1992) and Oshiki et al. (2008). For the detection in gas chromatography, a mass spectrometric detector was used with selective ion monitoring mode at m/z = 74, 103, 105 and 136 using a QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu, Japan). The powder of PHB obtained from Sigma (USA) was used as the standard for quantification.

**RESULTS AND DISCUSSION**
The pH and NH$_3$-N concentrations during anaerobic incubation of DS, ES+DS, and ESPHB+DS are shown in Figs. 1 and 2 respectively. At the startup of the experiment, pH was 8.1 in ES+DS and 8.7 in ESPHB+DS; NH$_3$-N was 384 mg/L in ES+DS and 380 mg/L in ESPHB+DS. Within two days, pH dropped and then stayed at 7.7 – 7.9 (Fig. 1), and NH$_3$-N increased to around 730 mg/L in both groups (Fig. 2). In the control series (DS only), pH was 8.1 (Fig. 1) and NH$_3$-N was 480 mg/L (Fig. 2). The desired pH for anaerobic treatment is between 6.6 and 7.6 (Rittmann and McCarty, 2001), and the inhibitory concentration of NH$_3$-N is reported to be 1,500 – 3,000 mg/L (Metcalf and Eddy, 2004). The measured values for pH were found higher than the desired range but the concentrations of NH$_3$-N were within the desirable range for anaerobic digestion.

During the anaerobic incubation of DS, ES+DS, and ESPHB+DS, measurements of PHB concentration, acetate concentration, total COD concentration, and methane production
were done and the values are shown in Figures 3(a) through 3(e).

In the present study, PHB was reduced rapidly by 77% during the initial 2 days of incubation in ESPHB+DS series, then the degradation rate was slowed down, and even after day 34, 10% of PHB remained undegraded. In DS+ES series also, initial PHB degradation was fast (56% reduction in 2 days), and after that, degradation of PHB almost stopped (Fig. 3a). A little amount of PHB (30 mg/L) was also initially found in DS (control) which was also degraded and then stabilized.

At the beginning of the experiment, the acetate concentrations (Fig. 3b) were almost the same in ES+DS and ESPHB+DS and they increased rapidly in both series as the experiment progressed. Acetate concentration increased from about 130 mg/L to around 700 mg/L for ES+DS, and 900 mg/L for ESPHB+DS until day 26, and then reduced. In the control series also, acetate was increased (70 mg/L) within the initial 2 days of incubation and later decreased.

Total COD concentrations decreased gradually and linearly from day 0 to the end in all batch experiments (Fig. 3c). Total COD in DS, ES+DS and ESPHB+DS were 7,400 mg/L, 14,300 mg/L, and 14,600 mg/L, respectively, on day 0 which decreased to 6,000 mg/L,
10,100 mg/L, and 10,100 mg/L, respectively, on the last day (Fig. 3c). Total COD in the vials linearly decreased by 29% and 31% in excess sludge with and without PHB, respectively, while in the control set, the reduction was around 20%. The reduction rates were 0.006 mg/(L·d) ($r^2 = 0.87$) in DS, 0.011 mg/(L·d) ($r^2 = 0.99$) in ES+DS and 0.012 mg/(L·d) ($r^2 = 0.93$) in ESPHB+DS. The reduction of VS was found almost similar in both ES+DS (21%) and ESPHB+DS (22%), whereas in control series, it was 15%, though VS measurement was more susceptible to error (Fig. 3d).

As shown in Fig. 3(e), the highest production of methane was recorded in ESPHB+DS (188 mL-CH₄/gVS) followed by ES+DS (150 mL-CH₄/gVS) and DS (control) (27 mL-CH₄/gVS). The methane gas production in ESPHB+DS was increased by 25% compared to ES+DS. As the initial amounts of biomass in the vials were the same for ES+DS and ESPHB+DS, it is apparent that ESPHB was more readily converted to methane gas than ES.
Figure 4 shows the mass balance of COD in ES+DS and ES\textsubscript{PHB}+DS experiments between days 0 and 34. Figure 4 shows the masses of COD from excess sludge (with or without PHB) as positive bars and those from digested sludge as negative bars. Masses of COD in Fig. 4 were obtained by calculations as follows:

\[ \text{Biomass}_{[DS]} = \text{Total COD}_{\text{ctrl}} - \text{CH}_4_{\text{ctrl}} \]
\[ \text{CH}_4_{[DS]} = \text{CH}_4_{\text{ctrl}} \]
\[ \text{Biomass}_{[EXP]} = \text{Total COD}_{\text{exp}} - \text{Biomass}_{[DS]} - \text{PHB}_{\text{exp}} - \text{VFA}_{\text{exp}} - \text{CH}_4_{\text{exp}} \]
\[ \text{PHB}_{[EXP]} = \text{PHB}_{\text{exp}} - \text{PHB}_{[DS]} \]
\[ \text{VFA}_{[EXP]} = \text{VFA}_{\text{exp}} - \text{VFA}_{[DS]} \]
\[ \text{CH}_4_{[EXP]} = \text{CH}_4_{\text{exp}} - \text{CH}_4_{[DS]} \]

where the following parameters were directly measured and converted to values per vial,

\text{COD}_{\text{ctrl}}: \text{the amount of total COD in DS (control) experiment}
\text{CH}_4_{\text{ctrl}}: \text{the amount of generated methane in DS (control) experiment}
\text{COD}_{\text{exp}}: \text{the amount of total COD in DS+ES or DS+ES\textsubscript{PHB} experiment}
\text{PHB}_{\text{exp}}: \text{the amount of PHB in DS+ES or DS+ES\textsubscript{PHB} experiment}
\text{VFA}_{\text{exp}}: \text{the amount of VFA in DS+ES or DS+ES\textsubscript{PHB} experiment}
\text{CH}_4_{\text{exp}}: \text{the amount of generated methane in DS+ES or DS+ES\textsubscript{PHB} experiment}

and the terms on the left side of the equations are defined as follows:

\text{Biomass}_{[DS]}: \text{the amount of remaining digested sludge biomass excluding PHB}
\text{CH}_4_{[DS]}: \text{the amount of methane generated from biomass and PHB in digested sludge}
\text{Biomass}_{[EXP]}: \text{the amount of remaining excess sludge biomass excluding PHB}
\text{PHB}_{[EXP]}: \text{the amount of PHB in excess sludge biomass}
\text{VFA}_{[EXP]}: \text{the amount of VFA from biomass and PHB in excess sludge}
\text{CH}_4_{[EXP]}: \text{the amount of methane generated from biomass and PHB in excess sludge}

After 34 days of incubation, around 90% of COD was recovered, as can be seen in Fig. 4. The digestion of anaerobic sludge was small in comparison to those of excess sludge with and without PHB accumulation, while in control experiment Biomass\textsubscript{[DS]} was only reduced by around 20%, and Biomass\textsubscript{[EXP]} was reduced by 39% and 42%, respectively, in ES+DS and ES\textsubscript{PHB}+DS experiments. In Fig. 4, the differences of mass balance between ES+DS and ES\textsubscript{PHB}+DS experiments are not very clear, but a small difference can be found in the amount of initial PHB\textsubscript{[EXP]} and the methane gas formed, CH\textsubscript{4}[EXP]: the difference in the amount of PHB caused the difference in the amount of methane generated.

The PHB granules in the ES\textsubscript{PHB}+DS experiment were thought to have been inside the cells of aerobic heterotrophs (intracellular) in excess activated sludge. As shown in Fig. 3(a), a rapid degradation of PHB was observed during the initial 2 days of incubation. There is a possibility that not only the enzymes of anaerobic microorganisms but also the enzymes of aerobic microorganisms contributed to the rapid degradation of PHB. If the enzymes from anaerobic microorganisms facilitated rapid degradation of the intracellular PHB, the penetration of the enzymes from anaerobic microorganisms...
would take more time. On the other hand, aerobic PHB-accumulating bacteria have their own PHB-degrading enzymes. Aerobic heterotrophs will be able to tolerate anaerobic condition for a while and will not easily allow the enzymes from anaerobic microorganisms to go into their cells. While further studies are needed to confirm, it is suggested that the enzymes from aerobic bacteria can help to degrade the PHB granules initially in anaerobic condition.

After day 2 of incubation, the degradation of PHB was significantly slowed down and even after 34 days about 10% of initial PHB still remained. The cause is not clear but some of the aerobic bacteria might have gone into a dormant status where they can tolerate anaerobic stress and PHB is maintained without degradation. The residual PHB granules led to the increase in the amount of solids that have to be treated and disposed. Further investigation would be needed on the following points: 1) the mechanisms of the stabilization of PHB during anaerobic digestion, 2) the estimation of the solids volume which will finally remain, and 3) the development of methods to reduce the residual PHB.

The accumulation of acetate as shown in Fig. 3(b) implies that acetate was the intermediate product in the conversion of PHB to methane. Typical degradation pathway of PHB to acetate is via D(−)-3-hydroxybutyrate, acetoacetate, acetoacetyl-CoA (Gottschalk, 1985; Doi, 1990). Acetate formed can easily be converted to methane gas.

As shown in Fig. 3(e), excess sludge with PHB accumulation generated more methane gas. The degradation rate of excess sludge with PHB accumulation was found higher than that of excess sludge without PHB accumulation. It is revealed that temporarily stored carbon can be effectively and easily converted to methane gas. Yet, it is also worth to note that a small fraction of PHB remained undegraded at the end of anaerobic
digestion. From practical point of view, this part of residual PHB directly contributes to the increase of sludge which has to be treated for final disposal. It is worth to pursue the mechanisms of the formation of stable PHB granules.

A NEW PROCESS
The results presented above demonstrate that the digestibility of PHB that accumulated in excess sludge biomass is better than that of excess sludge itself, and can be a good source of methane gas. In order to take advantage of the findings, the authors are proposing a modified activated sludge process as shown in Fig. 5. Excess sludge is usually withdrawn from the secondary settling tank. Microorganisms in activated sludge come to the secondary settling tank after being sufficiently aerated in the aeration tank, and can accumulate PHB when they are contacted with organic materials in wastewater. Thus, the excess sludge is fed with wastewater, shortly aerated to allow microorganisms to accumulate PHB, settled, and then sent to sludge treatment processes. Since the process as a whole is not to oxidize organic pollutants but rather to recover it in the form of PHB, oxygen consumption for aeration will be less. Further, as was demonstrated in the present study, accumulated PHB can be used as the source of methane. The authors named the reaction as Final AeRation of Excess sludge With Excess Loading (FAREWEL). The efficiency, applicability, and the design and operational criteria of the FAREWEL process should be studied next.

Fig. 5 - A new process to improve energy efficiency in activated sludge process.
CONCLUSIONS
Conversion of PHB that accumulated in excess activated sludge to methane during anaerobic incubation at 37°C was studied. More than 75% of PHB was degraded only within the initial 2 days of incubation. The degradation of PHB was followed by the accumulation of acetate and the formation of methane gas. Within 34 days of incubation, 25% more methane gas was produced from excess sludge with PHB accumulation in comparison to that of excess sludge without PHB accumulation. It was demonstrated that PHB which accumulated in excess activated sludge can be converted to methane gas than excess sludge itself.

In order to take advantage of the finding, the authors proposed the “FAREWEL” process. The “FAREWEL” process is a modified activated sludge process in which excess activated sludge is subjected to aeration with an excess loading to fix organic pollutants in wastewater to excess activated sludge biomass in the form of PHB. Tentatively accumulated PHB can then be utilized as the source of methane gas.

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REFERENCES


