INTRACELLULAR POLYMERS IN AEROBIC SLUDGE OF SEQUENCING BATCH REACTORS

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ABSTRACT: The formation and characteristics of intracellular polymers in aerobic sludge of sequencing batch reactors were investigated at ambient temperature under balanced nutrient conditions. Three substrates of different chemical natures, including fatty acid (acetate), carbohydrate (glucose), and aromatic (benzoate), were fed to individual reactors. When substrates were initially in excess, the sludge in all reactors was capable of converting soluble substrates into intracellular polymers under aerobic conditions. Acetate (up to 27%) and benzoate (up to 51%) were converted to poly-β-hydroxybutyrate, whereas glucose (up to 33%) was converted to intracellular carbohydrates. The initial substrate depletion rates were 208–243 mg-C/g-VSS/h for acetate, 491–590 mg-C/g-VSS/h for benzoate, and 405–558 mg-C/g-VSS/h for glucose. When external substrates were absent in the mixed liquor, the intracellular polymers could be consumed by the sludge for endogenous respiration under aerobic conditions or as a carbon source for denitrification under anoxic conditions. These results suggest a dynamic metabolic mechanism in the sequencing batch reactors.

INTRODUCTION

It has long been recognized that activated sludge can rapidly remove a considerable fraction of organic matter in wastewater during the initial stage of contact (Banerji et al. 1968; Fang et al. 1993; Fang and Ling 1995; Novak et al. 1995). This was often attributed to the adsorption of colloidal/suspended matter by the sludge. A number of processes have been developed based on such characteristics of activated sludge. Among them are the contact stabilization (Ulrich and Smith 1951) and the adsorption/biodegradation (Bohnke 1977) processes.

The development of a biological phosphate removal process in the 1970s (Barnard 1974) led to the discovery that activated sludge could also rapidly remove soluble matter in wastewater under anaerobic conditions. Acetate and other low molecular weight acids in wastewater are sequestered by polyphosphate-accumulating bacteria, forming intracellular polyhydroxyalkanoates (PHAs), the most common of which is poly-β-hydroxybutyrate (PHB) (Fuhs and Chen 1975; Fukase et al. 1982; Wentzel et al. 1986; Nakamura et al. 1991; Satoh et al. 1992, 1994; Mino et al. 1995; Skalsky and Daigger 1995; Szpyrkowicz and Zilio-Grandi 1995). The energy needed for the sequestration is provided by the hydrolysis of polyphosphate. The mixed liquor is aerated subsequently so that bacteria can oxidize PHA and, in the process, produce energy to repolymerize the released phosphate. As a result, phosphate in the wastewater is converted to intracellular polyphosphate and is removed from the system through sludge wasting.

More recently, some bacteria were also found capable of converting carbohydrates, such as glucose, into not just intracellular PHA, but also glycogen (Kohno et al. 1991). At least 28 organic substances could be sequestered by sludge and converted to PHA in batch reactors without the accumulation and/or release of phosphate (Liu et al. 1996); the needed energy was provided by the oxidation of glucose or intracellular glycogen instead.

These recent developments seem to point out that sequestration of soluble substrate may be an important step in the activated sludge process. Yet, little is known about this step of treatment, particularly for the non-polyphosphate-accumulating sludge. Also, there were conflicting results reported on whether the intracellular PHB could be used as the carbon source for denitrification; a report by Wentzel et al. (1989) was negative, but many later reports were positive (Kerrn-Jespersen and Henze 1993; Kuba et al. 1993, 1994, 1996; Bortone et al. 1994; Kerrn-Jespersen et al. 1994).

This study thus was conducted to investigate the sequestration of three soluble substrates of different chemical natures by non-polyphosphate-accumulating sludge and their conversions to intracellular PHB and carbohydrates. The plausibility of bacteria using these intracellular polymers as a carbon source for denitrification was also examined. The substrates included a simple fatty acid (acetate), a key aromatic intermediate (benzoate), and a common carbohydrate (glucose).

MATERIALS AND METHODS

Sludge degrading acetate, benzoate, and glucose, respectively, were developed in three sequencing batch reactors (SBRs), designated correspondingly as Reactors A, B, and C. The cylindrical reactors were made of plexiglass. Each had an internal diameter of 84 mm, with a total volume of 3.33 L and a working volume of 2.27 L. The reactors were operated continuously at a sequencing batch mode at the ambient temperature of 22°–25°C with three 8-h cycles daily. Each cycle consisted of four stages: Fill (30 min), aeration (240 min), settle (180 min), and draw (30 min). The operation was controlled by a personal computer. At the beginning of each cycle, 1 L of wastewater was added to the reactor, and the same volume of supernatant was drawn after settling at the end of the cycle. The sludge retention time was kept at approximately 9 days by discharging one-third of the sludge in each reactor every 3 days.

Activated sludge from a local sewage treatment plant was used to seed all the reactors. Each reactor was fed with a synthetic wastewater containing balanced nutrient, including NH_4Cl (382 mg/L), K_2HPO_4 (20.7 mg/L), KH_2PO_4 (8.25 mg/L), $MgSO_4 \cdot 7H_2O$ (89 mg/L), and $CaCl_2$ (17 mg/L). The COD:N:P ratios in the wastewater were kept at approximately 1100:100:5.5. To prevent the growth of polyphosphate-accumulating bacteria, the phosphorus content was kept at a low level that was just sufficient for the normal bacterial growth (Liu et al. 1996). Sodium bicarbonate was added as the buffering chemical.

During days 135–180 when reactors were operated under steady state conditions, concentrations of substrate and biopolymers (i.e., PHB and carbohydrates) were monitored

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closely in two batches for each reactor. In these batches, wastewater was added manually in just a few seconds. Aeration started immediately after the addition. The PHB was monitored in all the reactors, whereas carbohydrates were only monitored in Reactor C. Sludge was also sampled from each reactor during this period for two additional sets of batch experiments. One was to estimate the degrees of substrate sequestration during the initial stage of each batch. The other was to determine whether the intracellular polymers converted from the sequestrated substrates could serve as the carbon source for denitrification.

Biopolymers for Denitrification

The denitrification experiments were conducted in duplicate in 157-mL serum vials using sludge obtained from the SBR reactors as-is, without acclimation. Each serum vial was filled with 100 mL of feed solution containing substrate, trace metals, vitamins, and nutrient. KNO_3 (60 mg-N/L) was added as a nitrate source, and KH_2PO_4 (182 mg/L) and K_2HPO_4 (230 mg/L) were added as nutrient and buffer chemicals. The mixed solution was purged with nitrogen to strip off dissolved oxygen. After adding a sludge sample and purging the headspace with nitrogen, each vial was sealed with a rubber stopper and was then put on a roller to ensure thorough mixing. Mixed liquor was sampled at various time intervals for measurements of residual nitrate and total organic carbon (TOC).

Analytical Methods

The contents of intracellular PHB and carbohydrates in selected sludge samples were measured after centrifugation and lyophilization. The PHB was digested, methylated, and extracted using the following procedures developed by Brandl et al. (1988) and Comeau et al. (1988). About 10–20 mg of lyophilized sludge was mixed with 2 mL of acidified methanol (20% sulfuric acid) and 2 mL of chloroform in a Pyrex test tube. After sealing with a Teflon-lined screw cap, the tube was heated for 4 h at 105°C. Upon cooling to room temperature, 0.5 mL of 25% aqueous ammonia solution was added, and then the tube was vigorously shaken and then centrifuged at 3,000 rpm for 10 min. The methylated ester was extracted into the dense chloroform phase and was analyzed by a gas chromatograph (Hewlett-Packard 5890 Series II) equipped with a Neutrabond-1 capillary column (GL Science; internal diameter 0.25 mm, film thickness 0.25 μm, and length 30 m) and a flame ionization detector. A 5-μl portion of chloroform solution was injected and analyzed before split injection (split ratio of 1/30). Helium (2 mL/min) was used as the carrier gas. The injector and detector were 230° and 275°C, respectively. The initial temperature of the column was 80°C for 4 min, followed by a ramp of 8°C/min and a final temperature of 160°C for 6 min. The PHB standard was 3-hydroxybutyrate (Sigma-Aldrich Co.).

Glucose and intracellular carbohydrates were measured by the anthrone reaction (Daniels et al. 1994). About 10–20 mg of lyophilized sludge was mixed with 0.6 mL of distilled water in a tube, followed by the addition of 3.0 mL of anthrone reagent. After rapid mixing and heating in boiling water for 10 min, the solution was cooled to room temperature. The carbohydrate content was then measured by the degree of absorption of 625 nm using spectrometry.

Concentrations of nitrate, nitrite, and phosphate were analyzed by an ion chromatograph (Shimadzu HPLC 10A, Japan) equipped with a CDD-6A conductivity detector and a Shim-Pack IC-A3 column. The operation conditions and procedures were the same as those used for sulfate analysis (Fang et al. 1997). The substrate concentration in each sample was measured by the organic content using a TOC analyzer (Shimadzu TOC-5000, Japan). The VSS (volatile suspended solids) were measured according to methods in ''Standard methods'' [American Public Health Association (APHA) 1989].

For transmission electron microscopic (TEM) examinations, sludge samples were first washed three times with 0.1-M phosphate buffer solution (pH 7.2), followed by another washing using a 0.1-M sodium cacodylate buffer solution (pH 7.4). The sludge was then fixed for 12 h at 4°C in a 0.1-M cacodylate buffer solution containing 2.5% glutaraldehyde and 4% paraformaldehyde. After washing three times in the cacodylate buffer, the sludge was heated to 40°C and mixed well with 10% gelatin aqueous solution, also at 40°C. The mixture was then centrifuged for 5 min at 1,500 rpm at 15°C. The gelatin-clotted sludge was cut into pieces. The pieces then were washed with the cacodylate buffer and dehydrated in a graded series of ethanol. Ethanol then was removed by twice washing with propylene oxide; each wash lasted for 15 min. The sample was then soaked in a mixed solution (1:1) of propylene oxide and resin (TAAB 812 Resin, United Kingdom) for 3 h, and then in pure resin for another 12 h, allowing the resin to infiltrate. Finally, the sludge-embedded resin was polymerized at 60°D for 24 h. Ultrathin sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Austria) and stained with uranyl acetate for 20 min and lead citrate for 10 min. The prepared samples finally were examined by the TEM at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

The SBRs were operated for 180 days. During days 135–180 all reactors reached the steady state condition. The average substrate feeding concentrations were 405 mg-C/L (1,080 mg/L of COD) in Reactor A, 400 mg-C/L (1,144 mg/L of COD) in Reactor B, and 421 mg-C/L (1,123 mg/L of COD) in Reactor C. The TOC removal efficiency averaged 99.6%, 98.1%, and 98.7%, respectively. At the end of each cycle the average mixed liquor pH stayed around 8.5 in Reactor A, 7.6 in Reactor B, and 7.8 in Reactor C. The sludge in all reactors settled satisfactorily. The discharged supernatant contained low levels of VSS, averaging 34 mg/L for Reactor A, 41 mg/L for Reactor B, and 47 mg/L for Reactor C. The average VSS contents in Reactors A–C were 3.530, 2.923, and 5.456 g, respectively.

Intracellular Polymers in Sludge of SBRs

The formation of biopolymers by aerobic sludge in all reactors was investigated in two series of experiments. Fig. 1 illustrates the metabolic profiles of substrate and intracellular polymers for the series conducted during days 135–140. Content of PHB, which is a common intracellular polymer in many bacteria (Holt et al. 1994), was monitored in all the reactors. On the other hand, intracellular carbohydrates were monitored only in Reactor C, because only carbohydrate-degrading bacteria are capable of converting substrate into intracellular carbohydrates (Liu et al. 1996).

Fig. 1(a) illustrates that acetate concentration decreased rapidly during the initial stage of treatment, reducing from 176 to 33 mg-C/L in 10 min and to 1 mg-C/L within 15 min. The initial substrate-depletion rate (SDR) averaged 271 mg-C/g-VSS/h in the first 10 min. Such an SDR of acetate was two orders of magnitude higher than the rate reported previously (2.1–4.5 mg-C/g-VSS/h) in the anaerobic zone of a system designed for phosphorus removal (Bortone et al. 1994). At the same time, the PHB content increased initially with the depletion of acetate. The PHB content increased from 12 to 50 mg-C/L in 15 min. Based on mass balance, 27% of the depleted acetate was converted to PHB. The PHB concentration began to decrease, thereafter, to 12 mg/L after 45 min. This seems to indicate that bacteria were able to consume intracellular

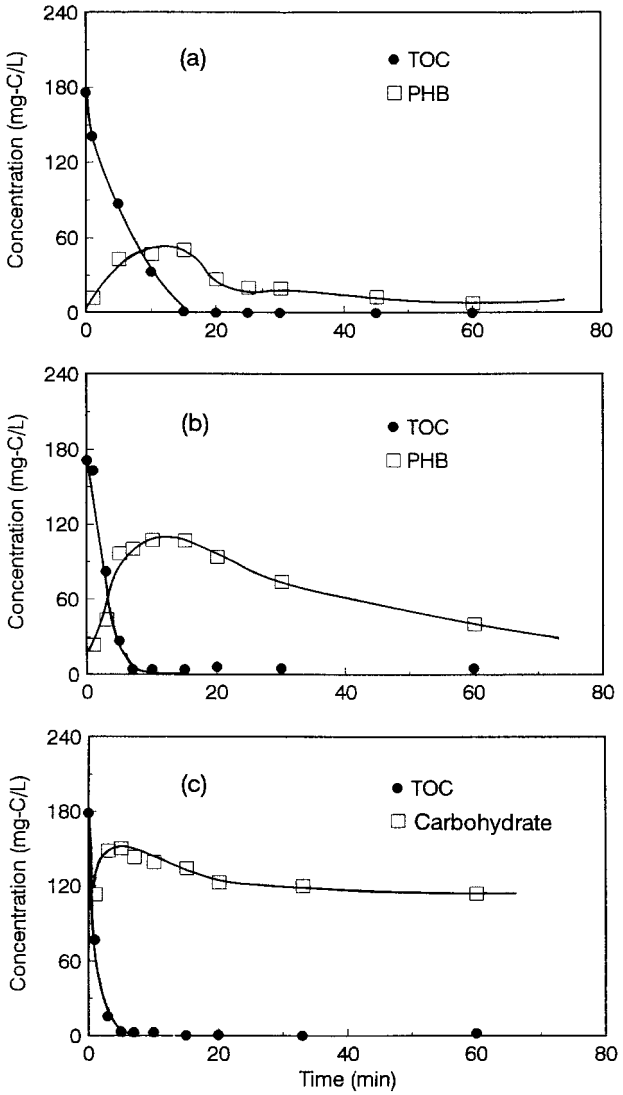

FIG. 1. Interim Formation of Biopolymers in Batches during Days 135–140, and Depletion of: (a) Acetate; (b) Benzoate; (c) Glucose

PHB for endogenous respiration when the substrate became depleted in the mixed liquor.

The microbial population in Reactor B was also capable of converting benzoate, an aromatic substrate, into PHB under aerobic condition. Fig. 1(b) illustrates that benzoate decreased from 171 to 27 mg-C/L within the first 5 min. The observed SDR, 498 mg-C/g-VSS/h, was onefold higher than that of acetate-degrading sludge. During the same period, the PHB concentration increased from 22 to 96 mg-C/L, indicating 51% of the depleted benzoate was converted to PHB. The PHB concentration gradually returned to the steady level of 24 mg-C/L in 180 min, the intracellular PHB was also likely consumed for endogenous respiration in the absence of external substrate.

On the other hand, the accumulation of PHB was not detected in the sludge of Reactor C after the addition of glucose, indicating that the glucose-degrading sludge was incapable of accumulating PHB. This agrees with the observations of other researchers (Matsuzawa and Mino 1991; Goel et al. 1998) that aerobic glucose-degrading bacteria in an environment with balanced nutrient would not produce intracellular PHB. It has been reported that some bacteria, such as *Alcaligenes eutrophus* (Doi 1990) and *Agrobacterium sp. SH-1* and *GW-014* (Lee et al. 1995), can convert glucose into intracellular PHB, but only under nutrient deficient conditions.

Fig. 1(c) illustrates that glucose was reduced from 179 to 3 mg-C/L within the first 5 min. The corresponding SDR was 401 mg-C/g-VSS/h, which was also considerably higher than that of the acetate-degrading sludge. While the glucose concentration was decreasing, intracellular carbohydrates content increased from 101 to 150 mg-C/L in the first 5 min. According to mass balance, 28% of the depleted glucose was converted to intracellular carbohydrates. The accumulated carbohydrates also were used for endogenous respiration in the absence of external glucose, and the concentration gradually returned to the original level after 220 min.

Similar results were observed for the series of experiments conducted during days 175–180. The key results of these two series are summarized in Table 1.

TABLE 1. SDRs and Percents of Depleted Substrates being Converted to Intracellular Polymers

Parameters (1)	Substrate		
	Acetate (2)	Benzoate (3)	Glucose (4)
SDR (mg-C/g-VSS/h)	243, 208,	491, 590	405, 558
PHB/depleted-substrate (%)	27	51, 32	n/a
Carbohydrates/depleted-substrate (%)	n/a	n/a	28, 33

Note: n/a = not available.

Many researchers reported that some bacteria are capable of converting substrate into intracellular biopolymer under nutrient-deficient conditions (Matsuzawa and Mino 1991; Daniel et al. 1992; van Loosdrecht et al. 1997). It is postulated that bacteria with such a capability would have a distinctive edge over others in competing for food. However, it is interesting to note from results of this study that bacteria also are capable of converting excess substrate as intracellular polymers, even under balanced nutrient conditions.

Aerobic Formation of PHB by Non-Phosphate-Accumulating Sludge

It has been well known that PHB could be formed in phosphate-accumulating sludge during the anaerobic phase of a cyclic anaerobic/aerobic operation. Recently, it was found that PHB could also be formed anaerobically by sludge without accumulating polyphosphate (Liu et al. 1996). Similarly, sludge in this study was non-phosphate-accumulating and did not exhibit any biological phosphorus removal activity; this is due to the high COD:P ratio (1100:5.5) in the wastewater and the lack of an anaerobic phase during the SBR operation. However, unlike the observations of Liu et al. (1996), results of this study show that intracellular PHB could be accumulated by non-phosphate-accumulating sludge under constantly aerobic conditions.

Aerobic Sequestration and Degradation of Benzoate

It is interesting to note from Fig. 1(b) that bacteria could sequester not only acetate, but also benzoate, an aromatic compound, for PHB conversion. Benzoate is a common pollutant in the wastewater from specialty chemical industry. It is also a key intermediate in the degradation of many complex aromatic chemicals. It was detected as the intermediate in the degradation of phenol and chlorophenol (Knoll and Winter 1989; Kobayashi et al. 1989; Zhang et al. 1990). Removal of benzoate from wastewater was studied mostly under anaerobic conditions (Li et al. 1995), the degradation pathway of which has been well understood (Fina et al. 1985). Yet, little information is available on the aerobic degradation of benzoate in wastewater.

Figs. 1(a and b) illustrate that benzoate could be degraded aerobically in SBR reactors at a rate even higher than that of acetate. Furthermore, sludge also was able to convert soluble

benzoate into intracellular PHB under aerobic conditions. The interim production of PHB from benzoate was also higher than that of acetate.

Formation of Intracellular Carbohydrates

Anaerobic conversion of glucose into intracellular polysaccharides, mainly glycogen, has been widely reported (Fukase et al. 1982; Nakamura et al. 1991; Satoh et al. 1992, 1994; Mino et al. 1995; Carucci et al. 1997), but reports of similar conversion under aerobic conditions were very limited. Carbohydrates are part of the normal makeup of a bacterial cell; their content usually remains unchanged during the treatment process. However, some bacteria are capable of sequestering substrate to form intracellular carbohydrates when the mixed liquor contains excess substrate and is deficient of nutrient. Liu et al. (1994) analyzed the intracellular carbohydrate content using an enzymatic method and confirmed that the substrate was converted and stored in a bacterial cell as glycogen. In this study, the intracellular carbohydrate content was analyzed by the anthrone reaction. Although one could speculate that the intracellular carbohydrates were glycogen, the true identity has not been confirmed.

Kohno et al. (1991) reported the glucose could be converted into intracellular glycogen and PHB. The latter however, was not detected in this study. On the other hand, Matsuzawa and Mino (1991) reported that the intracellular glycogen remained stable after 180–240 min of subsequent aeration. Results of this study, however, show that intracellular carbohydrates were not stable. They were gradually consumed by bacteria for endogenous respiration after glucose became depleted in the mixed liquor. Reasons for these discrepancies are not clear. They could be attributed to the difference in substrate concentrations. Substrate concentrations (2–8 g/L) in the two previous studies were considerably higher than those used in this study.

Micrographs of Intracellular Biopolymers

As illustrated in Fig. 1, the interim formation of biopolymers reached the maximum levels in all reactors 10–20 min after the addition of wastewater to the sludge. Sludge samples were taken from each reactor 15 min after addition of wastewater for TEM examinations. Fig. 2 clearly illustrates the presence of biopolymers in the bacterial cells of all sludge samples. The morphology of these polymer-accumulating bacteria ranged from oval to rod.

Biopolymers for Denitrification

Three sets of batch experiments were conducted for sludge sampled from each SBR reactor to evaluate the utilization of intracellular polymers in the denitrification process. As illustrated in Fig. 1, the intracellular biopolymer content in all reactors reached the maximum levels around 15 min after substrate addition and returned to the original levels after extended aeration. Two sludge samples thus were taken from each SBR reactor. A biopolymer-rich sludge was sampled 15 min after substrate addition, and a biopolymer-depleted sludge was sampled after 220 min of aeration. All sludge samples were rinsed with deionized water and centrifuged.

Three denitrification experiments were conducted for sludge from each SBR sludge using KNO_3 (60 mg-N/L) as the nitrate source. The biopolymer-rich sludge was used in the first experiment treating the dilute KNO_3 solution without the addition of any substrate. The biopolymer-depleted sludge was used in the second experiment treating the same solution. The biopolymer-depleted sludge was used again in the third experiment, but substrates used in the corresponding SBR reactors were added to the KNO_3 solution. The substrate concentration was equivalent to 300 mg-COD/L.

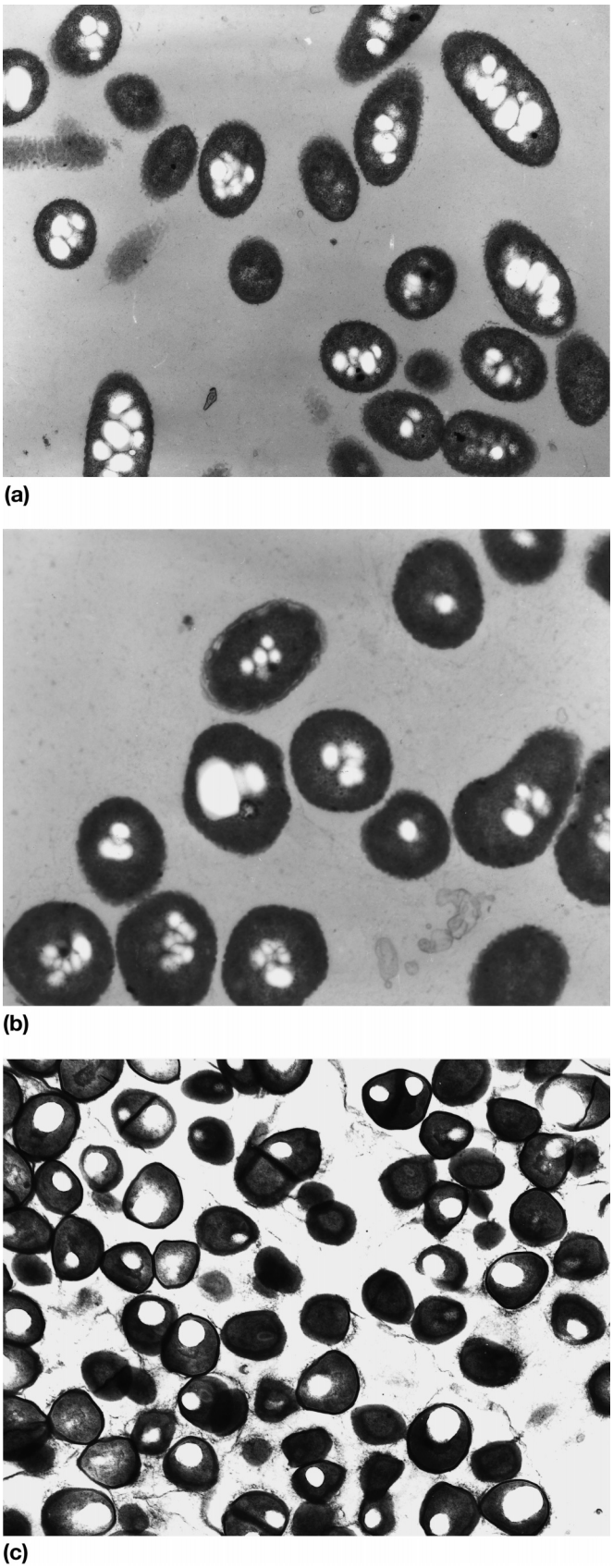

FIG. 2. Intracellular Polymers in Sludges of: (a) Reactor A; (b) Reactor B; (c) Reactor C

Fig. 3(a) illustrates the profiles of nitrate, intracellular PHB, and external substrate during the denitrification process of

those sludge samples taken from the acetate-degrading SBR reactor. It illustrates that the PHB-rich sludge was able to reduce nearly all 60 mg-N/L of nitrate within 30 min in the absence of external substrate. The specific denitrification rate (DNR) was 720 mg-N/g-VSS/d. With the addition of acetate, the PHB-depleted sludge reduced all nitrate in <30 min; the DNR exceeded 742 mg-N/g-VSS/d. On the other hand, in the absence of external substrate the PHB-depleted sludge only was able to reduce nitrate at a considerably lower rate (72 mg-N/g-VSS/d). These results indicate that acetate-degrading bacteria were able to use intracellular PHB as an internal carbon source for denitrification. The rate of denitrification using intracellular PHB was comparable to that using external acetate in the mixed liquor.

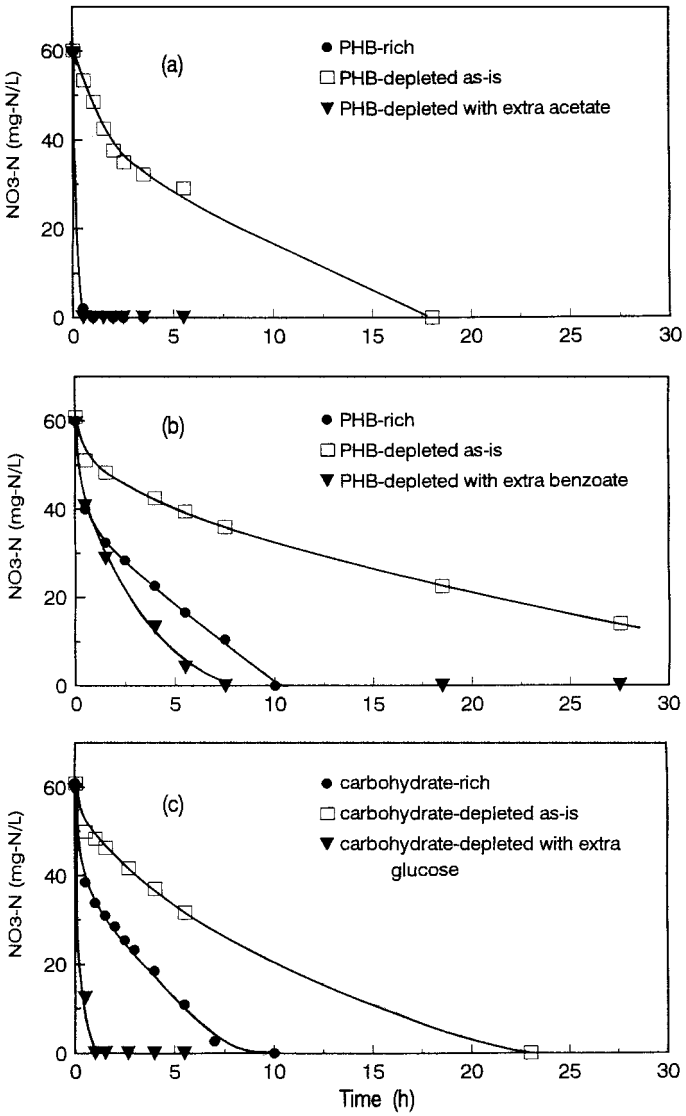

FIG. 3. Denitrification of: (a) Acetate-Sequestered Sludge; (b) Benzoate-Sequestered Sludge; (c) Glucose-Sequestered Sludge

TABLE 2. Maximum DNR of Sludge

Denitrification condition (1)	Substrate (mg-N/g-VSS/d)		
	Acetate (2)	Benzoate (3)	Glucose (4)
PHB-rich sludge	720	122	n/a
PHB-depleted sludge as-is	72	67	n/a
PHB-depleted sludge with addition of extra substrate	742	125	n/a
Carbohydrate-rich sludge	n/a	n/a	236
Carbohydrate-depleted sludge as-is	n/a	n/a	116
Carbohydrate-depleted sludge with addition of extra substrate	n/a	n/a	311

Note: n/a = not available.

The benzoate- and glucose-degrading sludge also exhibited similar denitrification characteristics to the acetate-degrading sludge. Figs. 3(b and c) illustrate that, in the absence of external substrate, the sludge accumulated with intracellular polymer was able to rapidly reduce nitrate. The DNR using PHB was comparable to that using external supplies of benzoate, but the DNR using intracellular carbohydrates was lower than that using glucose. On the other hand, the PHB- and carbohydrates-depleted sludges reduced nitrate at much lower rates in the absence of external carbon supplies.

Table 2 summarizes the maximum specific DNR of those sludge samples under different conditions. Little data were available in literature for comparison for sludge incapable of accumulating polyphosphate. For the PHB- and polyphosphate-accumulating sludge, the reported DNR values varied widely, ranging from 13 to 28 mg-N/g-VSS/d (Kerrn-Jespersen and Henze 1993), 440 mg-N/g-VSS/d (Kuba et al. 1993), and 60 mg-N/g-VSS/d (Bortone et al. 1994).

Filamentous Acetate-Degrading Sludge

Prior to day 180 when the reactor was operated under steady state conditions, the acetate-degrading sludge in Reactor A was dominated by microorganisms with good flocculent and settling properties. After experiments were terminated on day 180, Reactor A continued to operate for three more months. During this period, all conditions remained unchanged except sludge was not wasted regularly. Sludge concentration increased drastically from 3.5 to >6 g/L in about 10 days. The sludge settleability deteriorated quickly; the sludge volume index increased from 70 to >250 mL/g. The proliferation of the filamentous bacteria was confirmed by microscopic observations, as illustrated in Fig. 4. These bent and irregularly coiled filamentous bacteria were >100 μm in length and 1.2–1.6 μm in diameter. They are positive to Gram staining but negative to Neisser and PHB staining. In addition, they had clear cell septa but no branching. Judging from these characteristics, they are likely to be *Nostocoida limicola II* (Jenkins et al. 1993). Its dominance was probably due to the lowering of the food/microorganism ratio. When the sludge discharge resumed to a regular basis, these *N. limicola II*-like bacteria gradually disappeared in 2 months.

During this period, the *N. limicola II*-like bacteria were ex-

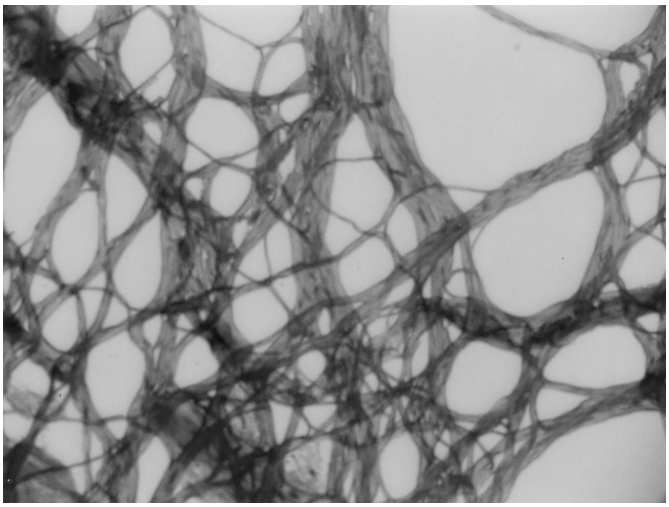

FIG. 4. Micrographs of Acetate-Degrading Filamentous Sludge

amined for acetate sequestration and PHB formation. Results show that the acetate was reduced from 191 to 22 mg-C/L within 120 min. The SDR of these bacteria was 29 mg-C/g-VSS/h, about one-seventh of that observed for the flocculent sludge. Further, these bacteria were incapable of converting acetate to PHB; the PHB content was only 0.5–1.9 mg-C/g-VSS. These results agreed with a number of previous studies (Cech and Chudoba 1983; Van den Eijnde et al. 1984; Matsuzawa and Mino 1991; Rensink and Donker 1991) that floc sludge had higher initial biosorption and COD removal abilities than filamentous sludge.

CONCLUSIONS

The following conclusions could be drawn based on results of this study:

- Under balanced nutrient conditions, aerobic SBR sludge was capable of converting excess soluble substrates—including acetate, glucose and benzoate, an aromatic intermediate—into intracellular polymers.
- The initial substrate depletion rates were 208–243 mg-C/g-VSS/h for acetate, 491–590 mg-C/g-VSS/h for benzoate, and 405–558 mg-C/g-VSS/h for glucose. Acetate (up to 27%) and benzoate (up to 51%) were converted to PHB, whereas glucose (up to 33%) was converted to intracellular carbohydrates.
- When substrates became depleted in the mixed liquor, intracellular PHB and carbohydrates could be consumed by bacteria for endogenous respiration under aerobic conditions or as the carbon source for denitrification under anoxic conditions.
- The DNRs using intracellular PHB as the carbon source were comparable to those using external substrates in the mixed liquor, but for the sludge using intracellular carbohydrates as the carbon source the rate was lower.
- Filamentous acetate-degrading bacteria resembling *N. limicola II* were incapable of converting acetate into PHB.

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APPENDIX. REFERENCES

American Public Health Association (APHA). (1989). ''Standard methods for the examination of water and wastewater.'' 17th Ed., Washington, D.C., 2–77.

Banerji, S. K., Ewing, B. B., Engelbrecht, R. S., and Speece, R. E. (1968). ''Kinetics of removal of starch in activated sludge systems.'' *J. Water Pollution Control Fed.*, 40, 161–173.

Barnard, J. L. (1974). ''Cut P and N without chemicals.'' *Water and Wastes Engrg.*, Great Britain, 11(7), 33–36.

Bohnke, B. (1977). ''Das adsorption-belebungsverfahren.'' *Korrespondenz Abwasser*, Germany, 24, 121–127.

Bortone, G., Malaspina, F., Stante, L., and Tilche, A. (1994). ''Biological nitrogen and phosphorus removal in an anaerobic/anoxic sequencing batch reactor with separated biofilm nitrification.'' *Water Sci. and Technol.*, 30(6), 303–313.

Brandl, H., Gross, R. A., Lenz, R. W., and Fuller, R. C. (1988). ''*Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters.'' *Appl. Envir. Microbiology*, 54(8), 1977–1982.

Carucci, A., Majone, M., Ramadori, R., and Rossetti, A. (1997). ''Biological phosphorus removal with different organic substrates in an anaerobic/aerobic sequencing batch reactor.'' *Water Sci. and Technol.*, 35(1), 161–168.

Cech, J. S., and Chudoba, J. (1983). ''Influence of accumulation capacity of activated sludge micro-organisms on kinetics of glucose removal.'' *Water Res.*, 17, 659–666.

Comeau, Y., Hall, K. J., and Oldham, W. K. (1988). ''Determination of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate in activated sludge by gas-liquid chromatography.'' *Appl. Envir. Microbiology*, 54(9), 2325–2527.

Daniel, M., Choi, J. H., Kim, J. H., and Lebeault, J. M. (1992). ''Effect of nutrient deficiency on accumulation and relative molecular weight of poly-β-hydroxybutyric acid by methylotrophic bacterium, *Pseudomonas* 135.'' *Appl. Microbiology Biotechnology*, 37, 702–706.

Daniels, L., Hanson, R. S., and Phillips, J. A. (1994). ''Chemical analysis.'' *Methods for general and molecular bacteriology*, P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, eds., American Society for Microbiology, 518.

Doi, Y. (1990). *Microbial polyesters*, VCH, New York, 1–9.

Fang, H. H. P., and Ling, J. W. C. (1995). ''Removal of nitrogen and phosphorus in batch reactor with fibrous packing.'' *Biores. Technol.*, Great Britain, 53, 141–145.

Fang, H. H. P., Liu, Y., and Chen, T. (1997). ''Effect of sulfate on anaerobic degradation of benzoate in UASB reactors.'' *J. Envir. Engrg.*, ASCE, 123(4), 320–328.

Fang, H. H. P., Yeong, C. L. Y., Book, K. M., and Chiu, C. M. (1993). ''Removal of COD and nitrogen in wastewater using sequencing batch reactor with fibrous packing.'' *Water Sci. and Technol.*, 28(7), 125–131.

Fina, L. R., Bridges, R. L., Coblentz, T. H., and Roberts, F. F. (1978). ''The anaerobic decomposition of benzoic acid during methane fermentation. III: The fate of carbon four and the identification of propanoic acid.'' *Arch. Microbiol.*, 118, 169–172.

Fuhs, G. W., and Chen, M. (1975). ''Phosphate removal in the activated sludge process.'' *Microbial Ecology*, 2(2), 119–138.

Fukase, T., Shibata, M., and Miyaji, Y. (1982). ''Studies on the mechanism of biological phosphorus removal.'' *Japan J. Water Pollution Res.*, 5, 309–317.

Geol, R., Mino, T., Satoh, H., and Matsuo, T. (1998). ''Intracellular storage compounds, oxygen uptake rates and biomass yield with readily and slowly degradable substrates.'' *Water Sci. and Technol.*, 38(8/9), 85–93.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994). *Bergey's manual of determinative bacteriology*, 9th Ed., Williams and Wilkins, Baltimore, 39–165, 428–632, 739–746.

Jenkins, D., Richard, M. G., and Daigger, G. T. (1993). *Manual on the causes and control of activated sludge bulking and foaming*, Lewis, Ann Arbor, Mich.

Kerrn-Jespersen, J. P., and Henze, M. (1993). ''Biological phosphorus uptake under anoxic and aerobic conditions.'' *Water Res.*, 27(4), 617–624.

Kerrn-Jespersen, J. P., Henze, M., and Strube, R. (1994). ''Biological phosphorus release and uptake under alternating anaerobic and anoxic conditions in a fixed-film reactor.'' *Water Res.*, 28(5), 1253–1255.

Knoll, G., and Winter, J. (1989). ''Degradation of phenol via carboxylation to benzoate by a defined, obligate syntrophic consortium of anaerobic bacteria.'' *Appl. Envir. Microbiology*, 30, 318–324.

Kobayashi, T., Hashinaga, T., Mikami, E., and Suzuki, T. (1989). ''Methanogenic degradation of phenol and benzoate in acclimated sludges.'' *Water Sci. and Technol.*, 21(4/5), 55–56.

Kohno, T., Yoshina, K., and Satoh, S. (1991). ''The roles of intracellular organic storage materials in the selection of microorganisms in activated sludge.'' *Water Sci. and Technol.*, 23(4–6), 889–898.

Kuba, T., Smolders, G., van Loosdrecht, M. C. M., and Heijnen, J. J. (1993). ''Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor.'' *Water Sci. and Technol.*, 27(5/6), 241–252.

Kuba, T., van Loosdrecht, M. C. M., and Heijnen, J. J. (1996). ''Phosphorus and nitrogen removal with minimal COD requirement by integration of denitrifying dephosphatation and nitrification in a two-sludge system.'' *Water Res.*, 30(7), 1702–1710.

Kuba, T., Wachtmeister, A., van Loosdrecht, M. C. M., and Heijnen, J. J. (1994). ''Effect of nitrate on phosphorus release in biological phosphorus removal systems.'' *Water Sci. and Technol.*, 30(6), 263–269.

Lee, E. Y., Kang, S. H., and Choi, C. Y. (1995). ''Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by newly isolated *Agrobacterium sp. SH-1* and *GW-014* from structurally unrelated single carbon substrates.'' *J. Ferment. Bioengrg.*, Japan, 79(4), 328–334.

Li, Y. Y., Fang, H. H. P., Chen, T., and Chui, H. K. (1995). ''UASB treatment of wastewater containing concentrated benzoate.'' *J. Envir. Engrg.*, ASCE, 121(10), 748–751.

Liu, W. T., Mino, T., Nakamura, K., and Matsuo, T. (1994). ''Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic-aerobic activated sludge with a minimized polyphosphate content.'' *J. Ferment. Bioengrg.*, Japan, 77(5), 535–540.

Liu, W. T., Mino, T., Nakamura, K., and Matsuo, T. (1996). ''Glycogen accumulating population and its anaerobic substrate uptake in anaero-

bic-aerobic activated sludge without biological phosphorus removal.'' *Water Res.*, 30(1), 75–82.
Matsuzawa, Y., and Mino, T. (1991). ''Role of glycogen as an intracellular carbon reserve of activated sludge in the competitive growth of filamentous and non-filamentous bacteria.'' *Water Sci. and Technol.*, 23(4–6), 899–905.
Mino, T., Liu, W. T., Kurisu, F., and Matsuo, T. (1995). ''Modelling glycogen storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes.'' *Water Sci. and Technol.*, 31(2), 25–34.
Nakamura, K., Masuda, K., and Mikami, E. (1991). ''Isolation of a new type of polyphosphate accumulating bacterium and its phosphate removal characteristics.'' *J. Ferment. Bioengrg.*, Japan, 71, 258–263.
Novak, L., Larrea, L., and Wanner, J. (1995). ''Mathematical model for soluble carbonaceous substrate biosorption.'' *Water Sci. and Technol.*, 31(2), 67–77.
Rensink, J. H., and Donker, H. J. G. W. (1991). ''The effect of contact tank operation of bulking sludge and biosorption processes.'' *Water Sci. and Technol.*, 23(4–6), 857–866.
Satoh, H., Mino, T., and Matsuo, T. (1992). ''Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intercellular carbohydrates under anaerobic conditions in the biological excess phosphorus removal process.'' *Water Sci. and Technol.*, 26, 933–942.
Satoh, T., Mino, T., and Matsuo, T. (1994). ''Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation.'' *Water Sci. and Technol.*, 30(6), 203–211.
Skalsky, D. S., and Daigger, G. T. (1995). ''Wastewater solids fermentation for volatile acid production and enhanced biological phosphorus removal.'' *Water Envir. Res.*, 67(2), 230–237.
Szpyrkowicz, L., and Zilio-Grandi, F. (1995). ''Seasonal phosphorus removal in a Phostrip process-II phosphorus fractionation and sludge microbiology during start-up.'' *Water Res.*, 29(10), 2327–2338.
Ulrich, A. H., and Smith, M. W. (1951). ''The biosorption process of sewage and waste treatment.'' *Sewage and Industrial Wastes*, 23, 1248–1255.
Van den Eijnde, E., Vriens, Z., Wynants, M., and Verachtert, H. (1984). ''Transient behaviour and time aspects of intermittently and continuously fed bacterial cultures with regard to filamentous bulking of activated sludge.'' *Appl. Microbiology Biotechnology*, 19, 44–52.
van Loosdrectht, M. C. M., Pot, M., and Heijnen, J. J. (1997). ''Importance of bacterial storage polymers in activated sludge processes.'' *Water Sci. and Technol.*, 35(1), 41–47.
Wentzel, M. C., Ekama, G. A., Loewenthal, R. E., Dold, P. L., and Marias, G. V. R. (1989). ''Enhanced polyphosphate organism cultures in activated sludge systems. Part II: Experimental behaviour.'' *Water SA*, 15(2), 71–88.
Wentzel, M. C., Lotter, L., Loewenthal, R. E., and Marais, G. V. R. (1986). ''Metabolic behaviour of *Acinetobacter spp*. enhanced biological phosphorus removal—A biochemical model.'' *Water SA*, 12, 209–224.
Zhang, X., Morgan, T. U., and Wiegel, J. (1990). ''Conversion of ^{13}C-1 phenol to ^{13}C-4 benzoate, an intermediate step in the anaerobic degradation of chlorophenols.'' *FEMS Microbiology Letter*, 87, 63–66.