Aerobic production of activated sludge polyhydroxyalkanoates from nutrient deficient wastewaters

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Abstract It was found that aerobic strategies combined with multiple nutrient limitations produced greater quantities of polyhydroxyalkanoates (PHAs) than strategies relying on oxygen limitation (either micro-aerophilic or anaerobic/aerobic). This was true both for a synthetic wastewater composed of acetic and propionic acid, and also for a nutrient deficient industrial wastewater. PHA/substrate yields were shown to be comparable to axenic systems for many operating strategies analyzed, and it was found that PHA composition could be affected by process operational conditions. The molecular weight and melting point of the PHA produced were found to be in a desirable range with respect to material properties, which have not been well studied in the previous literature for mixed cultures (Salehizadeh and Van Loodsrecht, 2004). The effects of process staging, multiple treatment cycles, and inocula source were also addressed.

Keywords Activated sludge; biodegradable plastics; nutrient limitation; polyhydroxyalkanoates; wastewater

Introduction

PHAs are an intracellular storage material synthesized by a variety of bacteria, and are materials of interest because they have some properties similar to synthetic plastics. Plastics produced from PHAs have been reported to be truly, fully biodegradable (Page, 1995). The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and the cost of maintaining a pure culture. Lee (1996) reported that the price of biodegradable plastics is \$16/kg, while the price of polypropylene is less than \$1/kg. A good candidate for economical PHA production would be a mixed culture that can store high PHA content while growing on an inexpensive substrate.

Satoh *et al.* (1998) demonstrated the basic feasibility by showing that microaerophilic conditions could be used to increase the PHA in activated sludge to as much as 62% total suspended solids (TSS), using sodium acetate as the primary organic substrate. Serafim *et al.* (2004) achieved the highest PHA content with mixed cultures reported to date with 78.5% achieved with aerobic conditions, acetate pulsing (feast–famine), and nitrogen limitation. Salehizadeh and Van Loosdrecht (2004) reviewed the production of PHA using mixed cultures by both oxygen limitation and feast–famine strategies. They noted that the use of PHA depended on making it more cost effective by finding cheaper substrates and production strategies. Cultivation strategies to achieve high PHA concentration and high productivity in pure cultures are well defined. However, the knowledge is more limited for PHA production using activated sludge cultures. The first objective of this study was to develop an efficient system and suitable operating procedures that

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would maximize PHA production in an activated sludge system using a mixture of acetate and propionate. The second objective was to evaluate a simple aerobic strategy for PHA production from nutrient limited, highly degradable, industrial wastewaters. In addition the properties of PHA produced by the activated sludge were examined to determine suitability in terms of melting point and molecular weight. Salehizadeh and Van Loosdrecht (2004) noted that PHA properties from mixed cultures have been infrequently reported and information is scarce.

Methods

Reactor operation

A 6-hour cycle SBR system with a working volume of 4 litres was used for all experiments. The overall HRT was 10 hours and the SRT was maintained at 10 days during the first experiment. In subsequent experiments, a two-stage or single-stage bioprocess approach was used. The two-stage bioprocess included optimum nutritional conditions during the initial (growth) phase, which was followed by a nutrient limited PHA accumulation phase. For a single-stage bioprocess, nutrient limitation was introduced upon the start up of the systems and no non-limited growth phase was incorporated. The reactor was seeded with fully aerobic sludge obtained from a full-scale wastewater treatment plant unless otherwise stated.

The synthetic wastewater composition of experiment 1 was the same as that used by Satoh *et al.* (1998). In Experiments 2–13, the wastewater components were modified in order to limit the amount of nutrients in the feed, i.e. the system was fed with a 600 mg/LCOD mixture of equal amounts (wt/wt) of acetate and propionate. Experiments 14 to 17 were performed using the high acetic acid industrial wastewater currently discharged and treated by a Cellulose–Acetate fiber manufacturing plant. The wastewater was composed primarily of acetic acid and had a total COD of approximately 1,800 mg/L, but also contained substantial concentrations of isopropyl alcohol and acetone.

Experimental design

Three variable parameters were selected for study, i.e. oxygen, nitrogen (N), and phosphorus (P). Three types of oxygen operating conditions were investigated, i.e. the absence of oxygen (anaerobic, AN), oxygen limitation (microaerophilic, MAA), and without oxygen limitation (fully aerobic, AE). Three sets of conditions were selected for the N and P experiments, i.e. limitation of either N or P, or limitation of both N and P. Experiment 13 was focused on multiple cycles with single inocula, while all other experiments were for a single cycle. The complete set of experimental conditions investigated is listed below.

Experiment 1: MAA/AE cycling without nutrient limitation
Experiment 2: MAA/AE cycling with P limitation
Experiment 3: MAA/AE cycling with combined N&P limitations
Experiment 4: AN/AE cycling with P limitation
Experiment 5: AN/AE cycling with combined N&P limitations
Experiment 6: fully AE conditions with P limitation
Experiment 7: fully AE conditions with N limitation
Experiment 8: fully AE conditions with N&P limitations
Experiment 9: fully AE conditions with partial N&P limitations
Experiment 10: fully AE conditions with P limitation (single-stage)
Experiment 11: fully AE conditions with N&P limitations (single-stage fully aerobic inocula)
Experiment 12: fully AE conditions with N&P limitations (single-stage EBPR inocula)

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Experiment 13: fully AE conditions with multiple periods of N&P limitations
Experiment 14: Effects of fully AE conditions with N,P&K limitations
Experiment 15: Effects of fully AE conditions with N,P&K limitations (single-stage)
Experiment 16: Effects of fully AE conditions with N&P limitations (single-stage)
Experiment 17: Effects of fully AE conditions with P limitations (single-stage)

Parameters measured

Each experiment was monitored for mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), PHA, PHB, PHV, chemical oxygen demand (COD), anions (nitrite, nitrate, phosphate, and sulfate), cations (ammonia-N, potassium, magnesium, and calcium), and intracellular carbohydrate content. MLSS, MLVSS, and COD were analyzed according to *Standard Methods for the Examination of Water and Wastewater* (1998). Intracellular carbohydrate content was measured according to the Manual of Methods for General Bacteriology (Gerhardt, 1981). Anions and cations were analyzed by a Dionex 2010I and Dionex 120 ion chromatograph, respectively (Dionex Corp., Sunnyvale, CA).

PHA (PHB and PHV) was measured by the methanolysis-GC method described by Hart (1994) and Liu *et al.* (2002), with some modifications. Firstly, the MLSS was centrifuged for at least 10 minutes. Solids were dried for 24 hours at 100 °C and then weighed as they were placed into a 5 mL high pressure vial. At least 5 PHA external standards were used to make a standard curve for each analysis run. Benzoic acid was used as an internal standard. Benzoic acid solution was prepared by mixing 50 mg of benzoic acid into 100 mL of 3% sulfuric acid in methanol solution (v/v). Two millilitres each of the benzoic acid solution and chloroform were added into each vial. The vials were then incubated at 100 °C for 3.5 hours. PHA was extracted by adding 1 mL of distilled water into each vial. 1 mL of the chloroform phase was injected into a GC with a 3 metre 2% Reoplex 400 Chromosorb GAW column attached to an FID detector. The oven temperature was 130 °C, while injector and detector temperatures were 160 and 200 °C, respectively.

Chloroform extraction was used to purify PHA. The extraction process was obtained by personal communication with Satoh *et al.* (1998). Firstly, activated sludge biomass was dried under vacuum conditions using a freeze-drying unit. Freeze dried sludge was extracted for PHA using hot chloroform in a Soxhlet extractor. Approximately 1–2 grams of dried sludge pellets and 100 mL of chloroform were used per batch of extraction. Sludge pellets were extracted for 4–6 hours. Then, the chloroform mixture was condensed to approximately 5 mL and PHA was extracted by adding 100 mL of methanol. Pure PHA was obtained as the resulting white precipitate. Molecular weight was determined using a Water model 2690 gel permeation chromatograph. Approximately 3 mg/mL of the sample was eluted by chloroform at a flow rate of 1 mL/min. Melting point was measured using a differential scanning calorimeter. Approximately 10 mg of the purified PHA sample was encapsulated in an aluminum pan and heated at the rate of 20 °C/min from 30 to 195 °C.

Results and discussion

Effect of nutrient limitations

Experiment 1 was unsuccessful due to sludge bulking. Table 1 illustrates that nutrient deficiency conditions stimulate massive PHA production. PHA contents of all experiments were greater than 42%TSS. The highest PHA content was 62%TSS under AN conditions with P limitation and MAA conditions with P limitation. However, process productivity and PHA yield were dependent on the aeration strategy used,

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Table 1 Results of experiments using synthetic wastewater

Experiment	2	3	4	5	6	7	8	9
Aeration condition	MAA	MAA	AN	AN	AE	AE	AE	AE
Limiting nutrient	P	N&P	P	N&P	P	N	N&P	Partial N&P
No. of stage	2	2	2	2	2	2	2	2
PHA content (%TSS)	62	48	62	47	53	42	57	43
Process productivity (mg/L/day)	58	37	34	79	379	156	497	78
PHA yield (mgPHA/mgCOD)	0.04	0.05	0.03	0.07	0.27	0.34	0.39	0.05
PHV/PHA ratio	52	13	36	38	66	37	45	56

i.e. performance under fully AE conditions was much greater than that of AN or MAA conditions. The fully AE conditions resulted in process productivity of 156 to 497 mg/L/day while the oxygen limited strategies ranged from 34 to 79 mg/L/day. PHA yield under fully AE conditions with N&P limitations ranged from 0.27 to 0.39 mgPHA/mgCOD in comparison to the range of 0.03 to 0.07 mgPHA/mgCOD under an oxygen limited strategy. However, when the limiting nutrients were increased in the influent wastewater (experiment 9) the PHA productivity for fully AE conditions fell to a level in the upper range obtained using oxygen limited strategies. The major finding is that fully AE conditions for nutrient limited wastewater were much more effective for PHA accumulation than oxygen limitation. Oxygen limitation is a prerequisite for enhanced biological phosphorus removal (EBPR) but was not optimal for the different objective of maximizing PHA accumulation using wastewater organics.

It is notable that even the PHA production in experiment 8 was an order of magnitude or more lower than productivities in axenic production. The highest MLSS concentration achieved in this study was 4,567 mg/L while concentrations in axenic systems can be well over 10 g/L (Preusting *et al.*, 1993). These experiments have shown, however, the yield of PHA per unit substrate was similar to those obtained with axenic culture. Salehizadeh and Van Loosdrecht (2004) noted that pure culture yields ranged from 0.185 to 0.50 g/g and that the maximum observed process productivity was 2,000 mg/L/day. The PHA yield under fully AE experiments in this study ranged from 0.27 to 0.39 gPHA/gCOD. Again taking into account the much more dilute nature of the biomass in these mixed culture reactors the yield and productivity results demonstrated that activated sludge biomass used the substrates at an efficiency at least in the same region as, if not comparable to, the pure cultures for the most successful experiment (experiment 8). Perhaps the cost of PHA production could be further reduced if a higher strength wastewater could be used and the activated sludge culture could be further concentrated, such as with a membrane separation system, or if concentrated biomass from the secondary clarifier underflow was used.

Effect of reactor operation strategy

From an economical point of view, PHA production cost could be minimized if the production time could be reduced. A possible way to reduce PHA production time would be by eliminating the growth phase of the two-stage operation. However, the single-stage operation, where no growth phase was incorporated, had lower PHA content and process productivities than corresponding two-stage strategy as summarized in Table 2 (compare experiment 10 with 6, and experiment 11 with 8). We will see later that this was not always the case when we look at the results obtained using a real industrial wastewater. This split result may indicate that success with operation strategy is wastewater specific, but the definitive reason for this is not known. It is also possible that the inocula used

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Table 2 Results of reactor operation strategy experiments

Experiment	6	10	8	11
Aeration condition	AE	AE	AE	AE
Limiting nutrient	P	P	N&P	N&P
No. of stage	2	1	2	1
PHA content (%TSS)	53	22	57	33
Process productivity (mg/L/day)	379	59	497	215
PHV/PHA ratio	66	77	45	51

in single-stage experiments had some polyphosphate in the biomass and therefore full nutrient limitation was not achieved.

Effect of inocula origins

This study also isolated inocula source as an experimental variable. The inocula for experiment 12 were obtained from a pilot-scale biological nutrient removal (BNR) system. The inocula were taken from the last aerobic zone of the BNR system when polyphosphate content was at a maximum. The BNR biomass had much lower PHA content (20.7%TSS) and process productivity (46 mg/L/day) than those of an aerobic system (experiment 11). Intracellular carbohydrate contents were in the range of 6–15%TSS when the seeded sludge was obtained from the aerobic system, even when the systems were operated using the single-stage approach. In contrast, when the seeded sludge was obtained from the BNR system, intracellular carbohydrate content ranged from 20 to 38%TSS. It clearly illustrates that the source of the biomass used for PHA production can in some cases have a significant impact upon production. The best PHA production system would be the one that promotes the conversion of organic substrates to PHA, not to intracellular carbohydrates, and it has been anecdotally observed that oxygen limited system biomasses have much higher carbohydrate contents than corresponding aerobic systems. It is also notable that most axenic systems are fully aerobic. Another possible factor contributing to the observed results may have been that the internal polyphosphate prevented phosphorus limitation in experiment 12. It should be noted that Serafim *et al.* (2004) had successful PHA accumulation using EBPR inocula but in that case their experiment ran for almost two years meaning that any EBPR characteristics were almost certainly lost in their system.

Effect of multiple-cycles operation

Experiment 13 was designed to investigate the effect of multiple operational cycles on mixed culture PHA production. The system was operated under the same conditions as experiment 8, which had the highest process productivity. Figure 1 emphasizes the importance of nutrient limitation for inducing PHA accumulation under fully AE conditions. Note that even higher and more rapid PHA accumulation was realized during the 2nd cycle than in the 1st cycle. During the 3rd cycle of N&P limitation the system began to fail due to sludge bulking. The highest PHA content of 70% and PHA yield of 0.34 gPHA/gCOD were obtained using the multiple-cycles operation strategy. The results during this phase of study indicated that multiple cycles may enrich the population of PHA producing bacteria resulting in a reduced production time.

Effect of nutrient limitation on polymer composition

The PHV/PHB ratios of the copolymers produced by the activated sludge mixed culture were different under the different operating conditions, even though the organic substrates used were the same. This was surprising because it is considered to be well known that

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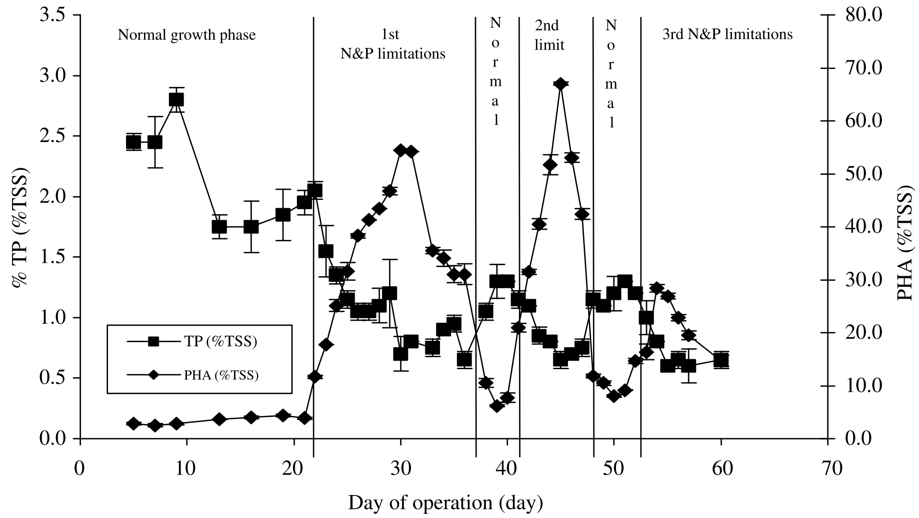

Figure 1 Fully aerobic PHA production under transient N&P limiting conditions

copolymer composition is primarily influenced by the substrate used (Doi, 1990). Also, the compositional change with experimental conditions was not predicted by the hypothesis proposed by Liu *et al.* (2002). This difference was probably due to the fact that PHA carbon could be recycled multiple times in these continuous experiments unlike the batch experiment of Liu *et al.* (2002). For example it is well known that acetic acid results in PHB but in multiple cycle experiments or because of glycogen carbon, PHV is also formed to varying degrees, e.g. Pereira *et al.* (1996). Renner *et al.* (1996) found that different bacteria were able to produce PHAs with different compositions when growing on the same substrate. Therefore, it is possible that the different operating conditions in this study selected for different groups of PHA producing bacteria. According to Doi (1990), PHA production and degradation could occur simultaneously under nutrient limiting conditions, a phenomenon known as cyclic metabolism. Lafferty *et al.* (1988) stated that PHA is degraded more rapidly in an anaerobic environment than in an aerobic one. Therefore, it is also possible that the degradation rate of PHA under AN or MAA conditions is somewhat more rapid than that under fully AE conditions. The rapid rate of PHA degradation under these conditions could result in more available acetyl-CoA that can be used to form more HB units in the polymer chains as observed in higher PHB fractions produced under MAA and AN conditions.

Physical properties of the PHA

Activated sludge PHAs under the best production conditions (experiment 8) had physical properties comparable to those obtained from pure cultures, e.g. molecular weight of 1,051,000 and melting point of 62 °C. Polymer with high molecular weight (>250,000) is more desirable because it can be processed for a larger variety of applications. In addition, a copolymer has a lower melting point than that of PHB homopolymer (approximately 179 °C). Major advantages of the PHB–PHV copolymer over the PHB monopolymer are that the copolymer can be processed over a larger range of temperature conditions and it has a lower level of crystallinity, which makes it tougher and more flexible.

Experiments with a nutrient deficient industrial wastewater

Experiments 14 to 17 were performed using a high acetic acid industrial wastewater that was discharged and treated by a cellulose-acetate fiber manufacturing plant.

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Table 3 Results of experiments using real industrial wastewater

Experiment	14	15	16	17
Aeration condition	AE	AE	AE	AE
Limiting nutrient	N, P&K	N, P&K	N&P	P
No. of stage	2	1	1	1
PHA content (%TSS)	18	40	29	26
Process productivity (mg/L/day)	379	577	365	392
PHA yield (mgPHA/mgCOD)	0.25	0.5	0.18	0.21
PHV/PHA ratio	1.8	2.1	0.9	1.4

The concentrations of ammonia-nitrogen, phosphorus, and potassium were minimal, i.e. NH_4-N = 0.7 mg/L, PO_4-P not detectable, $K^+ = 0.2$ mg/L. It was determined that the single-stage strategy was a better approach than the two-stage strategy for this wastewater. Fully aerobic reactor conditions containing minimal concentrations of N, P, and K were determined to be better conditions for PHA production than fully aerobic conditions with only N&P limitations or just P limitation. The results obtained during the industrial wastewater experiments are summarized in Table 3. It is notable that the highest process productivity and PHA yield observed in the study were in experiment 15. The results imply there is considerable potential in a simple, single-stage, aerobic process to produce PHAs from nutrient deficient, highly degradable, industrial wastewaters.

Conclusions

Based upon the results of this study, the major findings are summarized as follows.

- Activated sludge was used as a source of organisms that overproduce PHA. Fully aerobic conditions, in combination with N&P limitations, resulted in higher PHA accumulation than oxygen limitation or single nutrient limitation.
- A simple aerobic reactor resulted in the highest process PHA productivity of the study for a N, P&K deficient industrial wastewater. This process also only required a single stage. This result showed the potential of aerobic PHA accumulation using nutrient deficient, highly degradable, industrial wastewaters as the organic substrate.
- For synthetic (acetic/propionic) wastewater a 2-stage bioprocess (growth → nutrient limitation) obtained greater PHA accumulation than a single-stage continuous SBR process. In addition, PHA accumulations were increased by operating the system for two consecutive cycles of N&P limitations under fully aerobic conditions.
- The activated sludge biomass under the conditions of this study obtained a PHA to substrate yield as high as that of pure cultures used in commercial production.
- Conventional activated sludge inocula resulted in higher PHA production than EBPR biomass.
- The results implied that mixed cultures of activated sludge have the potential to be used to produce polymers for specific applications by operating under different nutrient limiting conditions since these were observed to affect PHA composition.
- The physical properties of the PHAs produced by mixed culture of activated sludge were comparable to PHAs produced by axenic cultures.

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