0273-2289/1559-0291 (Online)/07/136–140/909–926/$30.00

Functional Stability of a Mixed Microbial Consortium Producing PHA From Waste Carbon Sources

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Abstract

Polyhydroxyalkanoates (PHAs) represent an environmentally effective alternative to synthetic thermoplastics; however, current production practices are not sustainable. In this study, PHA production was accomplished in sequencing batch bioreactors utilizing real wastewaters and mixed microbial consortia from municipal activated sludge as inoculum. Polymer production reached 85, 53, and 10% of the cell dry weight from methanol-enriched pulp and paper mill foul condensate, fermented municipal primary solids, and biodiesel wastewater, respectively. Using denaturing gradient gel electrophoresis of 16S-rDNA from polymerase chain reaction-amplified DNA extracts, distinctly different communities were observed between and within wastewaters following enrichment. Most importantly, functional stability was maintained despite differing and contrasting microbial populations.

Index Entries: Activated sludge; denaturing gradient gel electrophoresis; polyhydroxyalkanoates; wastewater; primary solids fermentate; foul condensate; environmental biotechnology.

Introduction

Engineered biological systems have historically been utilized principally for the remediation and/or treatment of anthropogenic-derived pollution. Only in recent years has this environmental management discipline, appropriately referred to as environmental biotechnology *(1)*, been recognized for its potential to synthesize commodities and provide services beyond waste treatment *(1)*. However, this proposition is not

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without challenges. Foremost, any proposed commodity-producing, biologically based process must be relatively easy to operate, stable, and largely self-correcting (*1*), which demands functional stability within the anticipated diverse microbial community. Although this fundamental requirement is not necessarily congruent with all current biological treatment processes (e.g., biological nitrogen removal and biological phosphorus removal [*2,3*]), recent research applying advanced molecular techniques provides evidence of stable ecological functions within diverse and seemingly different mixed microbial populations (*4,5*). In fact, ecological resilience and maintenance of function is proposed to be predicated on species diversity (*6*).

Within the context of environmental biotechnology and commodity production are biologically derived polyesters known as polyhydroxyalkanoates (PHAs), which represent a potentially sustainable replacement to fossil-fuel based thermoplastics. Synthesis of PHAs, which serve as bacterial carbon and energy storage reserves, is currently estimated to be accomplished by over 300 different bacterial species in the form of cytoplasmic granules (*7*). Biosynthesis is stimulated by either excess soluble carbon with a concurrent macronutrient limitation (typically limited on either nitrogen or phosphorus), a limitation in a terminal electron acceptor (with oxygen as the most common), or a so-called feast/famine environment wherein microorganisms realize a transient excess of soluble carbon without any other nutrient limitations (*8*). Poly-3-hydroxybutyrate (PHB or P3HB) was the first PHA discovered (>75 yr ago), and hence is the most extensively characterized type (*9,10*), although many more forms of hydroxyalkanoic monomer units have since been identified (*9*). Common precursors to PHA synthesis include simple sugars such as glucose and fructose, and organic acids such as acetic and propionic acid. The type of carbon substrate dictates the polymeric structure of the PHA (*9*), with some of the most commonly studied forms including PHB, poly-hydroxyvalerate (PHV), and poly-4-hydroxybutyrate. In turn, each form of PHA yields different polymer properties. PHB exhibits similar properties to polypropylene, including melting temperature and crystallinity, but the polymer is brittle on crystallization and exhibits little stress resistance (*9*). Polymer improvements have been accomplished through copolymerization with PHV to increase ductility and impact resistance and lower processing temperatures (*9*).

Current commercial PHA production practices utilize pure microbial cultures grown on renewable, but refined feedstocks (e.g., glucose) under sterile conditions (*11*), and hence are not necessarily sustainable (*12,13*). However, recognizing the apparent propensity for wild microbial consortia to synthesize the polymer (*14–16*), commercial production of PHA would theoretically appear to be a natural extension of wastewater treatment. In fact PHA synthesis is empirically associated with certain municipal wastewater treatment processes (*17–19*), although biological synthesis of PHA in full-scale wastewater treatment facilities, estimated at upwards

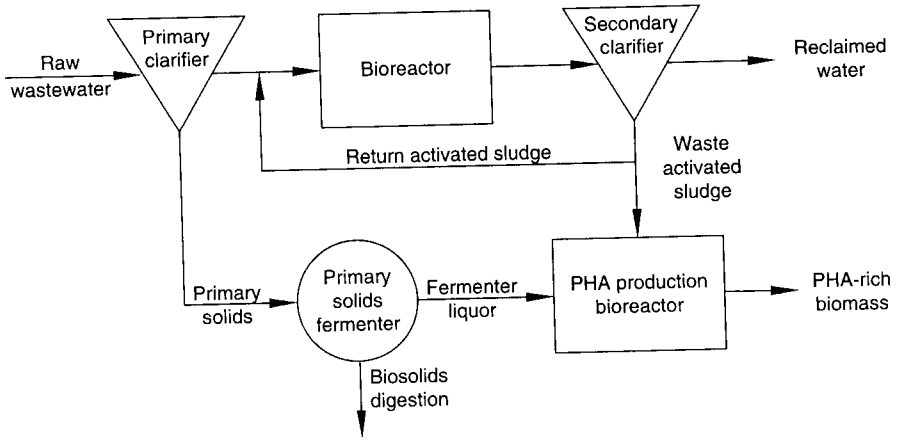

Fig. 1. Schematic diagram of proposed PHA production process integrated within a municipal wastewater treatment scheme.

of 4% (w/w) (data not shown), falls short of quantities necessary for commercial exploitation. Nevertheless, recognizing that many waste streams are rich in PHA precursors, the potential exists for PHA-production concurrent with wastewater treatment.

As proof of this concept, we have previously proposed and implemented an integrated PHA production and wastewater treatment process for municipal wastewaters (Coats et al., in review). In this scheme, wastewater treatment would occur in a biological treatment train designed to create selective environmental pressures necessary to achieve treatment objectives and concurrently enrich for microorganisms capable of producing PHA (Fig. 1). Mass production of PHA would occur in a separate biological reactor (termed a sidestream reactor) receiving biomass routinely wasted from the treatment reactor. Primary solids fermentate, derived from a primary solids fermentation reactor, would be supplied to both the wastewater treatment and PHA production reactors. Implementation of this integrated PHA production–wastewater treatment scheme resulted in a PHA yield of approx 10–22% (w/w) while concurrently realizing soluble carbon removal (Fig. 2); in the sidestream reactor, PHA production peaked at approx 53% (w/w) within 3.5 h (Fig. 3). Similar results were achieved in a sidestream reactor utilizing solids obtained from a wastewater treatment reactor operated under strictly aerobic conditions (data not shown). In all cases PHA production followed a feast-famine pattern, and maximum PHA production consistently occurred at a defined time-point after feeding concurrent with maximum reduction in readily metabolized soluble carbon. Importantly, copolymerization of both PHB and PHV was achieved (Fig. 3). The occurrence of this feast–famine condition is consistent with previous investigations *(8,14,15)* that focused on environmental matrices other than wild microbial consortia and wastewater.

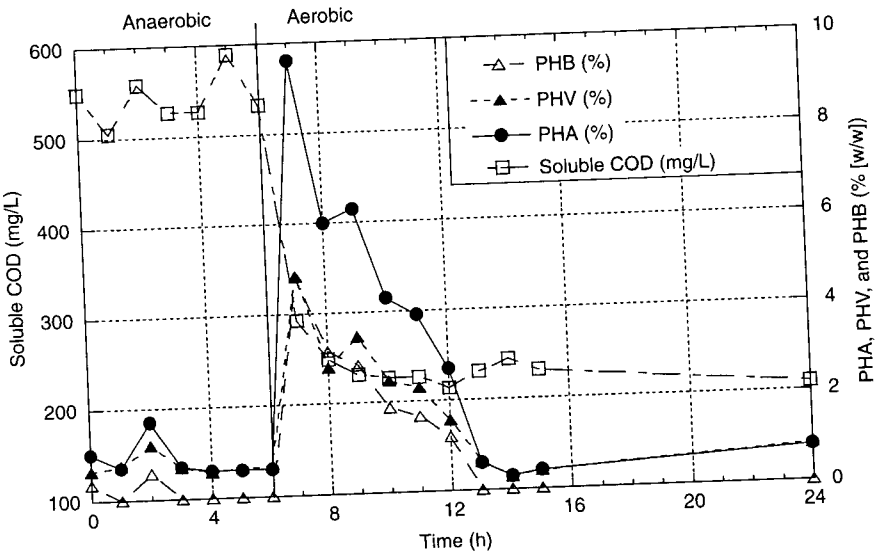

Fig. 2. Transient concentrations of soluble orthophosphate, COD, PHAs, PHB, and PHV in an anaerobic/aerobic SBR seeded with a mixed microbial consortium and fed fermentate (at $t = 0$) derived from municipal primary solids.

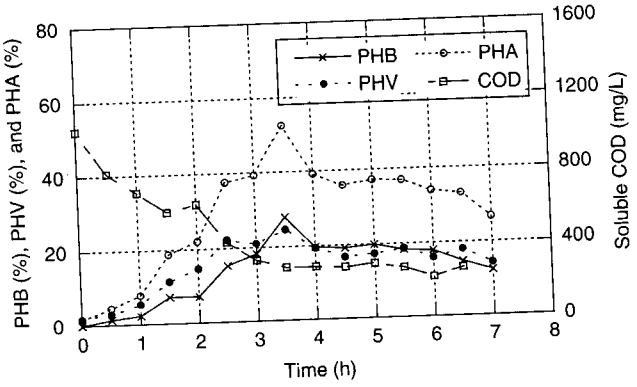

Fig. 3. Transient concentrations of soluble COD, PHAs, PHB, and PHV in a sidestream aerobic batch reactor seeded with cells obtained from the SBR in Figure 2 at $t = 24$ h and fed fermentate (at $t = 0$) derived from municipal primary solids.

The goal of the research presented herein was to extend the proposed PHA-wastewater treatment process to industrial wastewater, and to investigate the functional stability and general diversity of mixed microbial consortia within different PHA-producing wastewater environments. The specific objectives were to:

1. Demonstrate that PHA synthesis can be achieved by a mixed microbial consortium on industrial wastewaters.

2. Demonstrate process and functional stability in a municipal wastewater environment following process upset.
3. Preliminarily characterize the extent of microbial diversity of the PHA-producing consortium within a particular wastewater and among different wastewaters.

Materials and Methods

Source of Microorganisms

The mixed microbial seed was obtained from the Moscow, Idaho, wastewater treatment facility, which had been determined, through a series of preliminary facility screening studies, to be capable of synthesizing PHA. The microbial seed for the primary solids fermenter was obtained from the anaerobic digester at the Pullman, Washington wastewater treatment plant.

Source of Wastewater

Foul condensate wastewater was obtained from the Lewiston, Idaho, Potlatch Corporation pulp and paper mill. Biodiesel-derived wastewater was provided by the University of Idaho, Department of Biological and Agricultural Engineering (Moscow, Idaho). Two batches of biodiesel wastewater were obtained, one with residual ethanol and one without, and each batch was fed into two reactors, one at 1% (v/v) and the second at 5% (v/v). Thickened primary solids, for operation of the fermentor were obtained from the Pullman, Washington wastewater treatment facility. Raw municipal wastewater was obtained from the Moscow, Idaho wastewater treatment facility.

Culture Conditions and Harvesting Procedures

PHA production on pulp and paper mill foul condensate wastewater was accomplished in three 4-L completely mixed reactors. All reactors were operated as sequencing batch reactors (SBRs) on a 24-h cycle, with a solids retention time (SRT) of 4 d (reactor PO-A and PO-A1) or 6 d (reactor PO-C). Withdrawal and fill cycles of the SBR occurred almost immediately. Given that the mixture under reaction in the SBR was not permitted to settle before withdrawal, the hydraulic retention time (HRT) was equivalent to the SRT. Reactors PO-A and PO-C were continually aerated to maintain fully aerobic conditions; reactor PO-A1 was cycled every 12-h between anaerobic (first 12 h) and aerobic environments. Anaerobic conditions were established by bubbling nitrogen gas continuously into the reactor. Nitrogen gas and air were supplied through a 9-in. diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser (Brown Deer, Wisconsin). PHB production on biodiesel wastewater was accomplished in 500-mL flasks incubated by shaking at 250 rpm for 4 d at 30°C.

Wastewater biosolids fermentate was produced in a 10-L completely mixed primary solids fermenter operated as a SBR, with a 24-h reaction time, and a SRT and HRT of 4 d. The daily decant was centrifuged at approx 10,000*g*, and the supernatant (e.g., fermentate) recovered. The fermentate-fed anaerobic/aerobic reactor (batch fed daily with fermentate) and the raw wastewater methanol fed reactor (batch fed daily raw wastewater and 5 mL of methanol to yield an initial concentration of approx 0.125% [v/v]), consisted of 4-L vessels continuously operated on a 24-h cycle (anaerobically for 6 h following feeding, then aerobically for 18 h) with a SRT and HRT of 5 d. Withdrawal and fill cycles of the SBR occurred almost immediately. Anaerobic conditions were accomplished through the continuous supply of nitrogen gas, and were verified utilizing a dissolved oxygen probe. Nitrogen gas and air were supplied through a 9-in. diameter Sanitaire Silver Series II membrane fine bubble disc diffuser.

Analytical Techniques

Soluble chemical oxygen demand (sCOD or COD) tests were performed in accordance with Standard methods 5220-D *(20)*, with samples filtered through sterilized 0.22 μm filters before analysis (Millipore Corp, Billerica, MA). Hach high-range ampules (Hach Company, Loveland, Colorado) were utilized, with a Hach COD reactor and a Spectronic® 20 Genesys™ spectrophotometer. Biomass PHA content was determined by gas chromatography/mass spectrometry (GC/MS) as previously described *(21)*. Briefly, dried PHA-rich biomass samples were digested at 100°C in 2 mL each of acidified methanol (3% [v/v] sulfuric acid) and chloroform. Benzoic acid was added to the chloroform as an internal standard. Following vigorous vortexing of the mixture with 1-mL deionized water, PHA-rich chloroform was recovered for analysis. The chloroform phase was dehydrated by filtering the PHA-rich solution through sodium sulfate before analysis. GC/MS was performed on a Thermofinnigan PolarisQ iontrap GC/MS instrument (Thermo Electron Corporation, Waltham, MA) in positive electron impact mode. The sample was introduced using split injection. Separation was achieved on a ZB1 (15m, 0.25 mm ID) capillary column (Phenomenex, Torrance, CA) with helium as the carrier gas (1.2 mL/min) using a temperature program of 40°C (2 min) ramped to 200°C at 5°C/min. The Xcalibur software program (Thermo Electron Corporation) was used to analyze the data. The identity of the compounds was confirmed by retention time and mass spectral matching with known standards (Lancaster Synthesis, Ward Hill, MA) as methyl ester derivatives, and quantified based on the internal standard. Total cellular PHA content was determined on a weight basis (e.g., mass PHA : mass of dry biomass, [w/w]).

Molecular Methods

Genomic DNA was extracted from 250 μL liquid samples using MoBio UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA).

Polymerase chain reaction (PCR) was used to amplify bacterial 16S rDNA using the 341f-GC and 907r primers described by Ishii et al. (*22*). Amplification was performed in 25 µL reaction mixtures that contained 0.4 pmol of each primer, 0.2 µ*M* of each dNTP, 10.0 µg/mL bovine serum albumin, 1X PCR buffer, 1.5 m*M* Mg^{2+}, 20 units/mL of *Taq* polymerase (buffer, dNTPs, bovine serum albumin, and RED*Taq*, [Sigma-Aldrich, St. Louis, MO]), and approx 100.0 ng target DNA. Reactions began with a 94°C denaturation for 5 min, followed by 30 cycles of 94°C for 1 min, 54°C annealing step for 1 min with a 72°C extension step for 1 min. Final extension was carried out at 72°C for 7 min. Presence of PCR products was confirmed by electrophoresis of 2 µL of the reaction mix on 1.5% agarose gels stained with 0.5 µg/mL ethidium bromide (Bio-Rad Laboratories, Hercules, CA). Four reactions from each sample were pooled to create a single 100 µL composite for denaturing gradient gel electrophoresis (DGGE) analysis.

DGGE (*23*) was performed with the D-Code system (Bio-Rad) at 60°C and 65 V for 900 min. Samples (25 µL) were loaded on a 6% (w/v) polyacrylamide gel (acrylamide : *N,N'*-methylene-bisacrylamide ratio, 37.5 : 1 [Bio-Rad]) in 1X TAE buffer. The denaturing gradient was formed by mixing two stock solutions of 6% acrylamide containing 40% and 80% denaturant (7 *M* urea [Bio-Rad] plus 40% [v/v] formamide [Sigma Chemical Co.]). The DNA was stained with 0.5 µg/mL ethidium bromide and imaged at 302 nm using an AlphaImager (Alpha Innotech Corp., San Leandro, CA).

Results and Discussion

Production of PHA on Industrial Wastewater Treatment

PHA Production in Foul Condensate Wastewater

Pulp and paper mill foul condensate wastewater is typically enriched with methanol, yielding a COD in excess of 10,000.0 mg/L. Methanol can be readily removed from the wastewater through biological processes; however, this wastewater is also nutrient limited, and the addition of nitrogen, phosphorous, and micronutrients is often necessary to achieve adequate removal of COD to meet permitted effluent discharge requirements (*24*). Although these nutrient limitations are often viewed as troublesome from a conventional wastewater treatment perspective, the coupled high-carbon low-macro (micro) nutrient environment is potentially ideal for stimulating PHA synthesis. In addition, methanol is a quality carbon source for PHA synthesis (*25,26*).

Utilizing a PHA-producing mixed microbial seed obtained from the Moscow, Idaho wastewater treatment facility, a foul condensate-fed SBR operated under fully aerobic conditions (reactor PO-A; SRT = 4 d) maintained a microbial consortium capable of producing PHA at 17.2% (w/w). Polymer production was moderately variable, with a 95% confidence interval of 10.4–24.0% (w/w), (Table 1); peak PHA synthesis was 85% (w/w).

Table 1
Summary of PHB Yield on Methanol-Enriched Foul Condensate Wastewater Obtained From a Pulp and Paper Mill, for Samples Collected and Analyzed Over a 4 mo Operational Period

	PHB (% [w/w])		
Reactor	Average	95% Confidence interval	Maximum
PO-A	17.2	6.8	84.6
PO-C	5.0	1.3	7.5
PO-A1	6.6	1.6	16.8

All reactors were operated as SBRs. Reactors PO-A and PO-C were operated under fully aerobic conditions, with a HRT and SRT of 4 and 6 d, respectively. Reactor PO-A1 was operated under alternating anaerobic/aerobic conditions (12 h AN, 12 h AE), with a HRT and SRT of 4 d. PHB yields represent dry weight concentrations (mass PHB : mass dry cell weight).

Reactor PO-C, which was also operated under fully aerobic conditions but with an SRT of 6 d, yielded PHA at 5.0 ± 1.3% (w/w), (95% confidence interval; Table 1); peak PHA synthesis was 7.5% (w/w). The reactors were operated for a period of 4 mo. Analysis of the biomass samples applying GC/MS techniques repeatedly verified both the presence and quantity of PHB (Fig. 4 presents a typical chromatogram). Biomass samples assayed for PHB were collected during routine reactor decant. COD levels in both reactors were consistently reduced by approx 10–20% (data not shown).

The variability in average and peak PHB yield in the fully aerated reactors can be attributed primarily to three factors. First, the sampling time-point may not have corresponded to peak cellular PHA concentration. As evidenced by the temporal distribution of PHA synthesis and degradation on fermentate (Figs. 2 and 3), the sampling time-point is critical, because the microbes will readily and rapidly metabolize the stored carbon. Temporal PHB synthesis within a given operational cycle was not assessed. Second, methanol content in the daily feed of foul condensate may have varied over time; recognizing that methanol is the primary substrate for PHB synthesis in foul condensate wastewater, such variation could significantly affect PHB yield. Conversely, excess methanol has been shown to have an inhibitory effect on overall biomass production and PHB synthesis (*27*). Third, monoterpenes represent the other primary organic carbon constituent in foul condensate (*28*); these carbon forms, which are much more structurally complex than methanol, are biodegraded through different metabolic pathways than methanol. These metabolic processes could have interfered with PHB synthesis.

In addition to the aforementioned factors, reactor operating conditions adversely influenced PHB synthesis. Increased HRT/SRT (e.g., reactor PO-C), which corresponded to an "older" microbial consortium, appeared to result in more carbon utilized for cell maintenance and growth and less for PHB

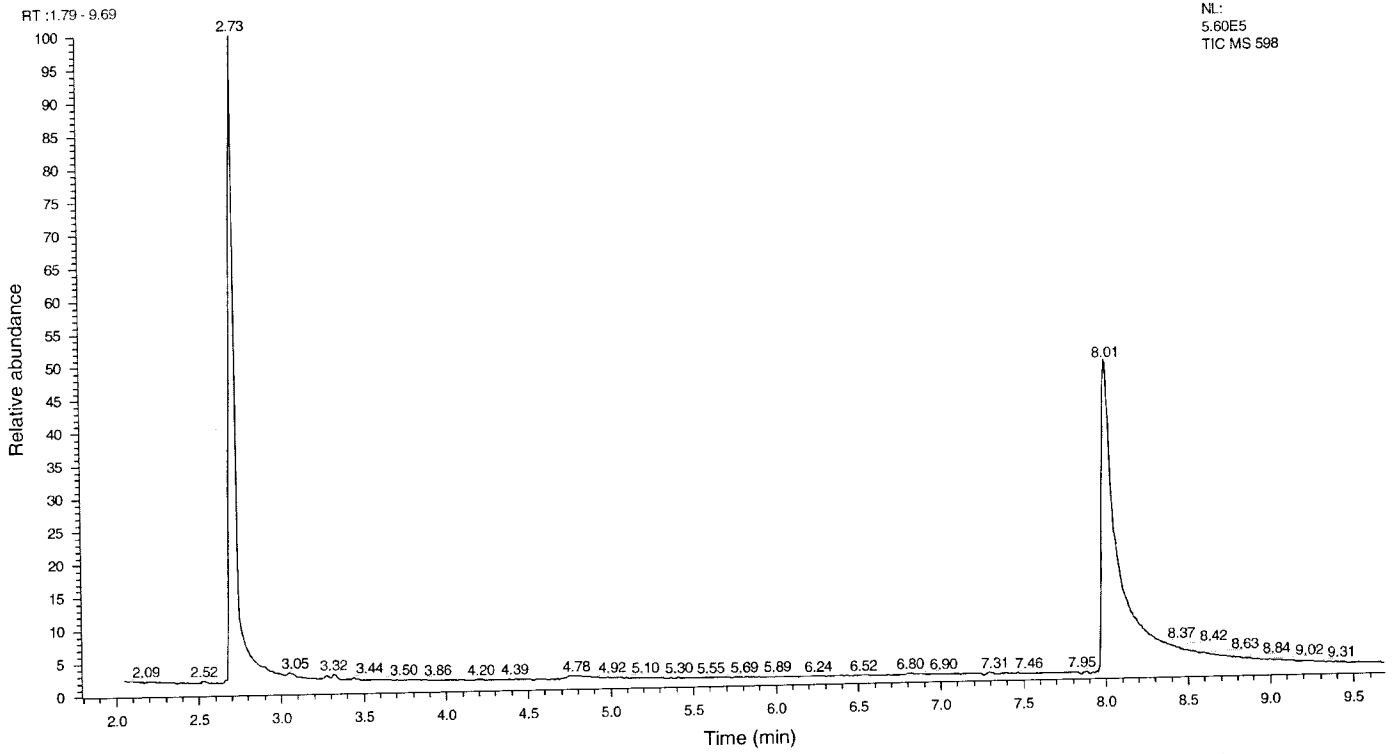

Fig. 4. Gas chromatograph plot of PHB-rich biomass produced on pulp and paper mill foul condensate. PHB methyl esters eluted at 2.73 min, whereas benzoic acid methyl esters (e.g., internal standard) eluted at 8.06 min. No other significant peaks occurred. PHB yield was estimated at approx 85% (w/w).

synthesis (Table 1). Further, as contrasted with reactor PO-A, PHB yield was significantly less under alternating anaerobic/aerobic reactor conditions (e.g., reactor PO-A1; average 6.9% [w/w], Table 1). The removal of oxygen appears to have created antagonistic conditions for PHB synthesis associated with nearly complete inhibition of microbial activity consistent with aerobic methanol oxidation by obligate methylotrophs *(29)*.

Methylotrophic bacteria are the principal microbial species associated with PHB synthesis on methanol *(30,31)*, and certain species are capable of producing upwards of 80% PHB *(30)*, which is consistent with our peak yield. The mechanism for stimulating polymer synthesis appears to be one of macronutrient limitation *(27,30)*, which is also consistent with our operations. However, previous research suggests carbon-to-macronutrient ratios may need to be optimized to maximize PHB synthesis *(27,30)*. In terms of process scale-up, site-specific investigations will be needed to optimize nutrient conditions. However, clearly there is potential to integrate polymer production into this industry as a value-added commodity generated during wastewater treatment.

PHA Production in Biodiesel Wastewater

Biodiesel is a potential replacement or supplement to petroleum-based diesel fuels *(32)*. However, within the context of green engineering *(33)*, the "green" label is arguably a misnomer because the high strength coproduct wastewater stream *(32)* has simply shifted the environmental impacts within the overall life-cycle of the product. Biodiesel wastewater, which exhibits a COD in excess of 10,000,000 mg/L, principally consists of residual ethanol, glycerol, fatty acid ethyl (or methyl) esters, and residual fatty acids *(32)*. Glycerol, ethanol, and fatty acids are direct precursors to PHA synthesis *(25,26)*. Production of PHA on biodiesel wastewater is not presented here as the exclusive method of making this product "green", but rather as an example of how the production of commodities within the context of wastewater treatment (viewed here simply as raw materials) can be used to mitigate the shift in environmental impacts within the overall life-cycle of a product.

Utilizing biodiesel wastewater and a PHA-producing microbial seed derived from the Moscow, Idaho wastewater treatment facility, PHB yield ranged from approx 6% (w/w) on the ethanol-enriched biodiesel to approx 10% (w/w) on wastewater that contained no ethanol. Somewhat surprisingly, the yield on the ethanol-enriched biodiesel wastewater, which represents a diverse carbon substrate for PHA synthesis, was lower than the waste stream that contained no ethanol. Concurrent COD reduction was approx 67 and 60%, respectively. Considering the COD strength of biodiesel, this level of treatment is quite significant. Whereas PHB yield was low, reactor optimization would likely result in improved PHA yield concurrent with additional COD reduction. In fact, previous research with pure microbial cultures grown on biodiesel wastewater has yielded upwards of 42% PHA (w/w) *(32)*.

Process and Functional Stability following Process Upset

A fermentate-fed SBR (identified as reactor FE-1) operated in an alternating anaerobic/aerobic scheme consistently maintained a microbial consortium capable of producing PHA (Fig. 2). The described conditions were replicated in three discrete reactors operated under steady-state conditions at different times over a 9 mo period. Comparable treatment efficiency and PHA production patterns were achieved each time. Each of these reactors was established with a new microbial seed obtained from the Moscow, Idaho wastewater treatment facility, with the primary solids fermenter operated with similarly new (e.g., fresh) material. Moreover, the reactor microbial seed and primary solids were obtained under different seasonal conditions (e.g., fall, winter, and spring) and under varying City wastewater conditions (e.g., with and without the contributions of the seasonally large university student populations in Pullman and Moscow). As further validation of this proposed process, the results presented herein were replicated at the University of California-Davis utilizing a mixed microbial seed derived from the Lincoln, CA wastewater treatment facility and primary solids derived from the Davis, CA wastewater treatment facility (data not shown).

As a contrast to the fermentate-fed wastewater treatment reactor, a mixed microbial seed derived from the Moscow, Idaho wastewater treatment facility was cultured on raw wastewater augmented with methanol (identified as reactor RW-1). The consortium generally utilized carbon at a constant rate throughout both the anaerobic and aerobic periods, and no appreciable PHA synthesis occurred (Fig. 5). A limited quantity of PHA was produced in the form of PHB. Clearly, this form of augmentation and reactor operation does not yield conditions suitable for concurrent PHA production and wastewater treatment. In fact, the results were comparable with those obtained on foul condensate wastewater when the reactor was operated under alternating anaerobic/aerobic conditions.

To evaluate how each established microbial consortium would function under dynamic nutrient feed conditions (e.g., process upset), recognizing that wastewater constituents can vary over time within a municipal wastewater treatment environment, the substrate to the respective reactors was switched. Specifically, the feedstock to reactor RW-1 was converted to fermentate (reactor identified as FE-2), and vice versa (FE-1 renamed as RW-2). As the reactors were batch fed daily, the conversion was effectively instantaneous. The induced "upset" conditions were associated with (a) a significant increase in COD (e.g., FE-1 to RW-2) and (b) a significant change in the carbon : nitrogen : phosphorus ratios in the substrate (e.g., RW-1 to FE-2 and FE-1 to RW-2). All other operating parameters remained the same. Two interesting mechanistic responses were observed. First, each microbial consortium ultimately switched metabolic responses. For example, the methanol-amended raw wastewater-fed reactor that was switched to fermentate, ultimately stabilized to cycle carbon consistent with the results

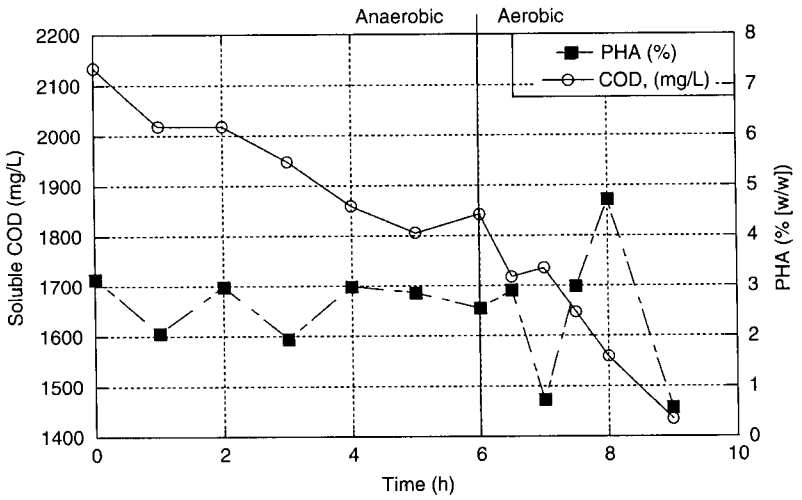

Fig. 5. Transient concentrations of soluble COD and PHAs in an anaerobic/aerobic SBR seeded with a mixed microbial consortium and fed raw wastewater and methanol (at $t = 0$).

shown in Fig. 2. Second, the microbial consortium in reactor FE-2 adapted to synthesize both PHB and PHV in copious amounts, whereas the consortium was previously producing only minimal amounts of PHB. Conversely, reactor RW-2 yielded negligible quantities of PHB, similar to RW-1.

Mixed Microbial Consortia for the Production of PHA in Wastewaters

Whereas the fact that PHA could be produced by a mixed microbial consortium at a commercial level on various wastewaters certainly is significant, and that the consortia demonstrated robust functional capabilities, another observation carries similar weight. Quantitative PHA analysis on the original microbial seed indicated approx 0.2% (w/w) PHB and insignificant amounts of PHV, suggesting either limited numbers of PHA-producing organisms, or a limited production capacity. In either case, when exposed to more optimum PHA-producing conditions, this same PHA-producing consortium flourished.

Diversity of the PHA-Producing Microbial Population

DGGE was performed to provide preliminary qualitative information regarding microbial community composition in the different reactors as a result of both the contrasting and altered feedstock conditions. The utilized primers amplified a bacterial 16S rDNA region corresponding to *Escherichia coli* positions 356–906. DGGE profiles indicate the number and relative abundance of the bacterial 16S rDNA amplicons in the reaction mixture. Each discrete band represents a single amplicon that corresponds

to a different bacterial strain present in the original sample. Despite biases of this method described in the literature *(34)*, this method nonetheless provides a "fingerprint" that can be used to describe similarities between and changes within microbial communities without the need for cultivation or cloning and sequencing.

DGGE analysis was performed on samples from: (a) reactors FE-1, FE-2, and RW-2, (b) two foul condensate reactors (PO-A and PO-C), (c) the original microbial seed from the Moscow, Idaho EBPR facility, and (d) the primary solids fermenter liquor (e.g., fermentate). The resulting fingerprints revealed starkly different microbial populations, with few common bands (Fig. 6). Patterns from FE-1 indicated the presence of at least eight different strains with two dominant strains relative to others within the lane. RW-2 contained only two distinct bands, which interestingly were not present in FE-1; this is of significance given that RW-2 was initially FE-1 before switching feedstock. The absence of common bands between these two samples indicates a complete change in the dominant communities present. FE-2 contained 5 faint bands; contrasting FE-1 and FE-2 revealed little apparent similarity between populations despite receiving the same substrate. The microbial seed from the Moscow, Idaho wastewater facility (lane D) exhibited eight bands of approximately equivalent intensity; additional faint bands were observed in replicate electrophoresis gels (data not shown), suggesting a rich, diverse community of bacteria present in the seed material. Fermenter liquor (lane E) contained five bands, two of which were dominant. Throughout all lanes, smears are interpreted as a large number of incompletely resolved bands likely resulting in an underestimate of diversity.

Potential explanations for the appearance and disappearance of bands between related reactors (e.g., FE-1 vs RW-2; FE-1 vs FE-2; all reactors contrasted with the microbial seed) include enrichment of strains that were originally beyond detection, addition of microorganisms in the feedstock, or microbial contamination. Feedstock effects are possible, because the clarified fermenter liquor feedstock contained low quantities of microorganisms. However, the DGGE profiles of FE-1 and FE-2 (Fig. 6) showed minimal-to-no influence of the dominant communities from the feedstock, as indicated by the predominant absence of common bands. The environmental pressure in these reactors appears to have selected a microbial consortium exclusive of not only the fermenter liquor feedstock but also the dominant microorganisms in the original seed. Raw wastewater from the City of Moscow (no DGGE sample collected) contained less than 200.0 mg/L total suspended solids (of which only a small portion would represent microbes). Thus, whereas this feedstock is potentially a source of inoculum, the cell density is low relative to the cell density established within the operating reactor. However, in the absence of DGGE profiles for the raw wastewater there is no way to characterize its influence on the resulting communities in the raw wastewater fed reactors.

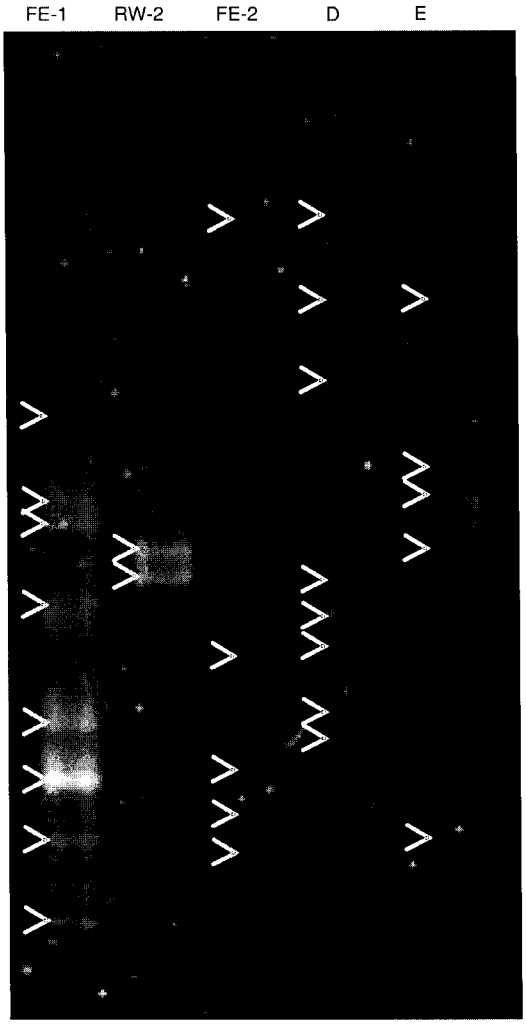

Fig. 6. DGGE gel of bacterial 16S rDNA PCR products amplified from mixed microbial consortium PHA-producing reactors, seed material from the Moscow, Idaho wastewater treatment facility (lane D), and the primary solids fermenter liquor (lane E). Lane FE-1 was inoculated with a microbial seed obtained from the Moscow, Idaho wastewater treatment facility and fed fermentate-rich wastewater. Lane RW-2 was previously run as FE-1 but was switched to a feedstock of raw wastewater and methanol. Lane FE-2 was previously run as RW-1 but was switched to a feedstock of fermentate-rich wastewater.

In contrast to the appearance and disappearance of multiple bands noted above, results from DGGE analysis on samples from reactors PO-A and PO-C (Fig. 7) indicated a loss of only one band and a subsequent change in the intensities of the other bands. The lower dilution rate of PO-C resulted in an increased sludge age (e.g., older microbial population) that appears to

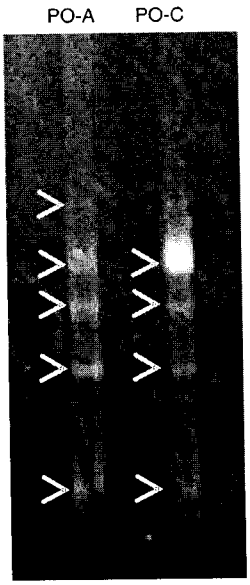

Fig. 7. DGGE gel of bacterial 16S rDNA PCR products amplified from mixed microbial consortium PHA-producing reactors. Lane PO-A represents a reactor operated at a HRT/SRT of 4 d, and lane PO-C represents a reactor operated at a HRT/SRT of 6 d. Both reactors were inoculated with a microbial seed obtained from the Moscow, Idaho wastewater treatment facility, fed pulp and paper mill foul condensate wastewater, and aerated continuously.

have enriched for one member of the community at the expense of at least one other, as well as decreased net PHA accumulation. Further, recognizing that PO-C did not receive as much substrate as PO-A, the reduced "feast" environment may have also facilitated the enhanced selection of non-PHA producing microbes.

A common approach to understanding the interplay between microbial community structure and function in biological wastewater treatment has relied on the identification of the microorganism present in either a single sample or a few samples over time *(35)*. Although there arguably is value in identifying which microorganisms are performing the critical functions associated with PHA synthesis, the task is certainly daunting, even with the many molecular tools available today. Conversely, an argument can also be made that phylogenetic specificity is not necessary to accomplish phenotypic stability; in fact, Rittman et al. *(1)* suggest that the field of environmental biotechnology within the context of full-scale engineered biological systems should focus on managing microbial communities rather than focusing on a "solves-all problems superbug."

The results presented herein demonstrate that a diverse microbial consortium can achieve a stable function under dynamic conditions, thereby

validating an application of this "function over structure" approach. Recognizing the diversity of municipal and industrial wastewater streams, and the associated diversity of bioreactor operating conditions necessary to treat these wastewaters, this apparently transcendent functional stability for PHA synthesis is viewed as a prerequisite condition for ultimate commercial development of the proposed PHA process. Taken together, these results suggest that nutrient conditions more significantly affect microbial function, rather than dominance of certain genotypes, and furthermore, that "functional redundancy" *(36)* results in retention of major or dominant community functions despite changes in community structure. These results further imply that if given a suitable carbon and energy source, reactor operational conditions such as induction of a feast/famine regime, the HRT, and/or the SRT, rather than starting inoculum composition, drive the microbial functions of PHA accumulation in a mixed microbial consortium.

Conclusions

Based on the results presented herein, the following conclusions can be drawn:

1. The genetic capability to synthesize PHA appears to be common in mixed microbial consortiums present in conventional wastewater treatment bioreactors, as demonstrated through process success in two geographically distinct regions and on different waste carbon substrates.
2. The proposed process can recover from a process upset associated with instantaneous changes in either substrate carbon concentration or carbon : nitrogen : phosphorus ratios.
3. The genetic capability to synthesize PHA (e.g., function) is a critical factor for process success, rather than the presence of a specific microbial structure.
4. Successful integration of PHA production with wastewater treatment will demand optimizing bioreactor operations (e.g., SRT, HRT, and operating environment) with the substrate.
5. Additional investigations are necessary to develop appropriate design and operational criteria such that the proposed process can be successfully scaled up. Within this context, investigations should be conducted to identify the specific microbes-producing PHA, which could generate mechanisms to monitor and maintain process success.

Acknowledgments

This material is based on work supported by the National Science Foundation under Grant Number DMI-0400337 and by the U.S. Department of Energy, Industrial Technologies Program, Forest Products Industries of the Future, under DOE-NE Idaho Operations Office Contract

DE-AC07-05ID14517. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding agency.

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