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Selective enrichment of a methanol-utilizing consortium using pulp & paper mill waste streams

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ABSTRACT

Efficient utilization of carbon inputs is critical to the economic viability of the current forest products sector. Input carbon losses occur in various locations within a pulp mill, including losses as volatile organics and wastewater . Opportunities exist to capture this carbon in the form of value-added products such as biodegradable polymers. Waste activated sludge from a pulp mill wastewater facility was enriched for 80 days for a methanol-utilizing consortium with the goal of using this consortium to produce biopolymers from methanol-rich pulp mill waste streams. Five enrichment conditions were utilized: three high-methanol streams from the kraft mill foul condensate system, one methanol-amended stream from the mill wastewater plant, and one methanol-only enrichment. Enrichment reactors were operated aerobically in

sequencing batch mode at neutral pH and 25°C with a hydraulic residence time and a solids retention time of four days. Non-enriched waste activated sludge did not consume methanol or reduce chemical oxygen demand. With enrichment, however, the chemical oxygen demand reduction over 24 hour feed/decant cycles ranged from 79 to 89 %, and methanol concentrations dropped below method detection limits. Neither the non-enriched waste activated sludge nor any of the enrichment cultures accumulated polyhydroxyalkanoates (PHAs) under conditions of nitrogen sufficiency. Similarly, the non-enriched waste activated sludge did not accumulate PHAs under nitrogen limited conditions. By contrast, enriched cultures accumulated PHAs to nearly 14% on a dry weight basis under nitrogen limited conditions. This indicates that selectively-enriched pulp mill waste activated sludge can serve as an inoculum for PHA production from methanol-rich pulp mill effluents.

Keywords: foul condensate, waste activated sludge, polyhydroxyalkanoates, pulp mill, natural fiber reinforced thermoplastic composite

INTRODUCTION

The ability to efficiently use waste streams and renewable resources is essential for the development of a sustainable and profitable forest products industry. The pulp and paper industry is continuously evolving to meet the demand for products that are manufactured efficiently, cost-effectively, and in an environmentally friendly manner. In striving to meet increasingly stringent environmental regulations and the socio-political

pressure for sustainability, the pulp & paper industry has made process improvements resulting in an increase in the quality and diversity of its products and a decrease in its energy use and environmental impact [1]. However, opportunities exist to further reduce the environmental footprint of pulp and paper mills. Currently, several input carbon losses occur in various locations within pulp mills, including losses as volatile organic carbon (VOC) compounds such as methanol in kraft mill condensate collection systems. The kraft pulping process uses a hot sodium sulfide-hydroxide solution to digest wood chips and liberate the cellulose fibers, which are subsequently used to manufacture a wide range of paper products [2]. Condensed gases from the digesters are collected to reduce the discharge of volatile organic and reduced sulfur compounds from the mill. These contaminated condensates or "foul condensates" contain such compounds as hydrogen sulfide, methyl mercaptan, ethanol, methanol, acetone, and terpenes. Methanol is the primary hazardous air pollutant of concern and constitutes up to 80% of the organic matter and most of the chemical oxygen demand (COD) from foul condensates [3]. Condensates can either be reused (typically for pulp or chip washing) or can be treated prior to discharge to the wastewater treatment system. Conventional treatment methods rely upon energy-intensive technologies such as incinerators and scrubbers, which ultimately produce VOC, SO_x, NO_x, and particulate emissions [4].

A sustainable alternative treatment of COD from foul condensates can be achieved through a biological conversion of the methanol into commercially useful biopolymers, such as polyhydroxyalkanoates (PHAs). Polyhydroxyalkanoates are polyesters composed of 3-hydroxy fatty acid monomers in which the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer

[5]. Numerous bacteria are able to synthesize and store PHAs as intracellular carbon and energy reserves [6]. Bacteria accumulate PHAs under one or a combination of the following environmental conditions: macronutrient (nitrogen or phosphorous) or micronutrient (potassium, magnesium, or sulfate) limitation in the presence of excess carbon; electron donor/acceptor variability (aerobic/anaerobic cycling); or a feast-famine regime [7].

Polyhydroxyalkanoates exhibit material properties similar, in part, to conventional petroleum-based thermoplastics with the added benefit of being entirely biodegradable [8]. PHAs can serve as an alternative to petroleum-based thermoplastics in selected applications such as natural fiber reinforced thermoplastic composites. These composites are produced by co-extruding mixtures of natural fibers such as wood together with plastics such as high density polyethylene to produce strong materials for durable applications [9]. By replacing the petroleum-derived plastics with thermoplastic bacterial polyesters such as PHAs, the composite products sector could serve as a future market for unpurified PHAs [10]. However, the integration of PHAs with natural fiber reinforced thermoplastic composites faces both technical and economic challenges that need to be met for the end product to be commercially viable. Currently, PHAs are not economically competitive with petroleum-based thermoplastics [11]. The most influential factors driving PHA production costs are extraction, purification, and carbon substrate cost. By minimizing or eliminating extraction and purification steps and by using an inexpensive substrate, the cost of PHAs can potentially be reduced to a competitive level. In applications such as natural fiber composites, PHA extraction from the cell mass may not be required [10]. In addition, the use of pulp mill effluents such as foul condensates as an

inexpensive carbon source can provide a cost-effective means by which PHA is produced using pulp mill effluents. An illustration of this approach can be seen in Figure 1. This approach would also allow for reduced waste production and potentially create an additional profit stream within pulp and paper mills.

Extensive work has been performed using activated sludge from domestic wastewater to produce PHAs from readily degradable carbon sources such as mixed fatty acids and undefined fermentation products from anaerobic sludge digestion [12-14]. In the case of pulp mills, the raw wastewater is less conducive to PHA production because of the recalcitrant nature of the carbon and its low concentration [2]. Therefore, it is necessary to amend the wastewater with an additional carbon source to facilitate PHA production. In this instance, methanol from the foul condensates provides both an available and inexpensive biologically degradable carbon source for PHA production. Since the bacteria in the pulp mill waste activated sludge are not accustomed to methanol as a primary carbon and energy source in the wastewater treatment system, the activated sludge consortium must undergo enrichment to acclimate to the foul condensates. A selective enrichment of the waste activated sludge is required to select for a microbial consortium capable of simultaneously utilizing the methanol in foul condensates while synthesizing PHAs.

The goal of this study was to enrich a culture from pulp and paper secondary waste activated sludge that can grow aerobically using methanol-rich pulp and paper mill foul condensate streams originating from a kraft chemical recovery process. The enrichments will be used in future experiments to explore the range of environmental conditions necessary to produce and accumulate PHAs using pulp and paper effluents

with the ultimate goal of incorporating the PHA-rich biomass into natural fiber reinforced thermoplastic composite materials.

MATERIALS AND METHODS

Source of Microorganisms. A mixed microbial consortium was obtained from the return waste activated sludge line within the wastewater treatment facility at the P.H. Glatfelter pulp & paper mill in Chillicothe, OH. Activated sludge was shipped overnight at 4°C and used within 48 hours of receipt. The activated sludge had a mixed liquor volatile suspended solids (MLVSS) concentration of 3200 mg L^{-1}, a pH of 7.43, and a COD of 180 mg L^{-1}. The activated sludge was used to inoculate sequencing batch reactors used in enrichment experiments at one-fourth of the total volume of the reactors.

Pulp & Paper Mill Waste Streams. Five conditions were chosen for the enrichment of the original waste activated sludge. Three foul condensate effluents from the pulp mill kraft chemical recovery process were selected based on their high COD: 1) the combined evaporator condensates (3200 tank foul condensate or FC 3200), 2) blow heat accumulator overflow foul condensate (FC BHAO), and 3) evaporator foul condensate (FC EVAP). The remaining two media used for enrichment consisted of primary clarifier effluent (primary out abbreviated as "1° OUT") supplemented with methanol, and waste activated sludge supplemented with methanol (WAS-only).

The COD of the foul condensates were highly variable (see Table 1). In the FC BHAO the COD ranged from 4600 to 46300 mg L^{-1}. In the FC 3200 the COD ranged from 810 mg L^{-1} to 47400 mg L^{-1}. In the FC EVAP the COD ranged from 4800 mg L^{-1} to

17400 mg L^{-1}. On the other hand, the 1° OUT received had a reasonably consistent COD between 300 and 400 mg L^{-1}. The primary carbon source in the foul condensates was methanol, which accounted for 51-72% of the COD, depending on the date sampled. This large variability demonstrates the changing compositions of the materials received, and is assumed to be a result of the variability of the wood species used as feedstocks for the pulp mill. The primary carbon source in the 1° OUT was not identified.

The foul condensates were not a significant source of total suspended solids (TSS). The average values for TSS were consistently an order of magnitude less than the TSS of the waste activated sludge that was used as the source of inoculum. The average values for foul condensate COD, pH, and TSS are in Table 1. When samples were received from the pulp mill, pH, COD, and TSS were immediately measured. Foul condensates were adjusted to neutral pH using 1 N HCl (Fisher Scientific, Fair Lawn, NJ) and the materials were stored at 4°C to minimize biological activity and COD degradation. In some cases, the foul condensates contained a non-aqueous phase liquid that was removed by decanting and disposed prior to using the foul condensates.

Nutrient Media. To ensure the presence of the necessary macronutrients and micronutrients to select for a community capable of consuming methanol as the main carbon source, Methanol-Utilizing Bacteria Medium B [15] was utilized as a nutrient addition. The nutrient media was supplied to all enrichment reactors to ensure balanced growth conditions. The nutrient medium was used without methanol addition for the FC 3200, FC BHAO, and FC EVAP enrichments, since the foul condensates contained sufficient concentrations of methanol. Methanol (99.9%; Fisher Scientific, Fair Lawn,

NJ) was added to the nutrient media supplied to the 1° OUT and WAS-only enrichment reactors.

System Design and Operation. Fifteen sequencing batch reactors, each with a working volume 160 mL, were run at a hydraulic retention time (HRT) equal to the solids retention time (SRT) of four days. Air was supplied (200 mL min^{-1} or 1.24 vvm) to provide oxygen and mixing. Each enrichment condition was prepared in triplicate. The reactors were operated on a 24 hour feed/decant cycle with one quarter of the reactor volume (40mL) being replaced with fresh feed. In the FC 3200, FC BHAO, and FC EVAP reactors the feed consisted of a mixture of the nutrient media and the respective foul condensate. The 1° OUT reactors were fed a mixture of the nutrient medium supplemented with methanol and the 1° OUT from the pulp mill. Finally, the WAS-only reactors were simply fed the nutrient media supplemented with methanol. The ratio of nutrient media to the waste being fed was determined by reaching a final total COD in the feed of 2500 mg L^{-1}. This value was chosen as the ceiling for COD/methanol concentration as it is within the range of methanol concentrations demonstrated to be optimal for PHA production in pure culture [16-18] yet minimize any potential toxic effects on the microbial community [19]. The reactors were supplied continuously with oil-free instrument air, which was humidified to reduce evaporation from the reactors. Thereafter, the airflow was separated using a gang valve and then regulated using a flow meter (Cole-Parmer Vernon Hills, IL).

The reactors were constructed using 2" × 6" (*d* × *h*) glass process pipe, closed at both ends with Teflon® end caps, and sealed with rubber gaskets and stainless steel clamps (Ace Glass, Vineland, NJ). Reactors were sealed to contain the hydrogen sulfide,

methyl mercaptan, and other malodorous and hazardous compounds present in the foul condensates. Reactors were vented through a series of two granular activated carbon beds (CC601 and Midas OCM; USFilter, Los Angeles, CA) that effectively removed hydrogen sulfide and methyl mercaptan from the effluent gases. The experimental process flow diagram is shown in Figure 2.

Prior to initiating the enrichments, a nonsteady-state mass balance was performed to account for methanol stripping due to aeration. The amount of methanol stripped over a 24 hour period was calculated according to Equations 1 and 2:

$$\% \text{ Methanol Stripped} = \frac{C_i - C_f}{C_i} \text{ x } 100\% \quad (1)$$

$$C_f = C_i \exp\left[-\left(\frac{Q_{air}\Delta t H_{methanol}}{V_{reactor}}\right)\right] \quad (2)$$

where C_i is the methanol concentration at time zero, C_f is the methanol concentration in reactor after 24 hours, Q_{air} is the air flow rate, Δt is the time interval, $V_{reactor}$ is the reactor volume, and $H_{methanol}$ is the dimensionless Henry's constant for methanol ($1.858*10^{-4}$ for methanol at 25°C [20]).

The mass balance model predicted that 28% of the methanol would be stripped over a 24 hour period (one feed/decant cycle). This was verified by an average methanol decrease of 26% over a 24 hour period in five replicate stripping columns constructed and operated identically to the enrichment reactors (data not shown). Based on these results, an additional 36 μL of 99.99% methanol was added daily to the reactors along with the feed to compensate for methanol loss due to stripping.

Sampling and Analysis. Samples were collected from the daily decants immediately before feeding and analyzed for MLVSS, COD, methanol, and ammonium (NH_4^+) concentration. A 40 mL sample was recovered from each reactor every 3rd and 4th day of an HRT period. The unfiltered samples from the 3rd day were stored at -80°C for future microbial community analysis. The unfiltered samples conserved on the 4th day were initially used to measure MLVSS according to ASTM Standard Method 2540 E [21] using Millipore TCLP AP40 glass fiber filters. The samples were then filtered through a Millex GP 0.22 μm Express PES Membrane filter unit (Millipore Corporation, Billerica, MA) and analyzed for soluble COD, methanol, and NH_4^+.

COD.

COD was measured according to ASTM Standard Method 5220 D [21] using Hach high–range ampoules with a Hach DRB 200 digestion block and Hach DR 2010 portable data logging spectrophotometer (Hach Company, Loveland, CO) set at a 620 nm wavelength.

Methanol.

Methanol was analyzed by high performance liquid chromatography (HPLC) using a Hitachi HPLC D-6000 Series HPLC system (Tokyo, Japan) consisting of a Hewlett Packard 1047A RI Detector (Agilent Technologies, Palo Alto, CA), a Hitachi L6200A Gradient Pump (Tokyo, Japan), and a Hitachi AS-4000 autosampler (Tokyo, Japan). The mobile phase consisted of 0.01% H_2SO_4 at 0.6 ml min^{-1}. Twenty microliter samples were prepared at 1:1 dilution and injected into a Bio Rad Aminex HPX-87H (300mm x 7.8mm ID) column (Hercules, CA) with a Bio Rad Micro-Guard cartridge (Cat. No. 125-0131). Methanol standards were prepared at 0.01 – 0.1% by volume using

99.99% methanol. Samples were run at 60°C, with the detector set at 50°C. The method detection limit (MDL) for the methanol analysis was 100 mg L^{-1}.

Ammonium.

Soluble ammonium ion concentrations were measured by ion chromatography using a system consisting of a Dionex ED40 conductivity detector with a GP50 gradient pump, an AS50 autosampler, an AD20 absorbance detector, and an EG40 eluent generator (Dionex Corporation, Sunnyvale, CA). Samples were analyzed using 5 µL injections at a flow rate of 1 mL min^{-1}. Samples were injected into a Dionex IonPac CS12A (250mm x 4mm ID) for cations with the Dionex ECG-MSA cartridge in the eluent generator system. Data were collected using Peaknet v. 5.21 (Dionex Corporation, Sunnyvale, CA). Cation standards were prepared using the Dionex 6 Cation Standard II #46070. A seven level cation standardization was performed (DI water, 5 standards, and the non-dilute Cation Standard II solution). The MDL for the NH_4^+ analysis was 0.5 mg L^{-1}.

Polyhydroxyalkanoates.

PHA analysis was conducted as described by Braunegg [22], with the following modifications. Upon completion of the 20th HRT, the reactors were fed their respective substrate media and four 40 mL samples were taken for PHA analysis per reactor over a 24 hour period. The unfiltered 40 mL biomass samples were bleached by adding 2 mL of commercial grade bleach (5% NaClO) (Clorox, Oakland, CA) and centrifuged at 4300 x g for 15 minutes. The pellet was then dried at 60°C for 24 hours. Between 20-40 mg of the dried biomass was weighed and suspended in a mixture of 2 mL acidified methanol (3% H_2SO_4, v/v) and 2 mL chloroform. The chloroform contained 0.5 mg mL^{-1} benzoic

acid as an internal standard. The mixture was then digested at 100°C for 4 hours in a Hach DRB 200 digestion block (Hach Company, Loveland, CO).

Once the digestion was completed, 1 mL of deionized water was added and the mixture was vortexed for 30 seconds. After allowing the organic phase to separate from the aqueous phase, Pasteur pipettes were used to remove the organic phase. The organic phase was then filtered through another Pasteur pipette packed with a cotton plug and 1 g of sodium sulfate (Fisher Scientific, Fair Lawn, NJ) to remove any remaining water. The organic phase, which contained the methyl ester monomers of the PHA molecules, was analyzed by gas chromatography using an Agilent Technologies 6850 Network GC System with a Thermal Couple Detector system (Agilent Technologies, Palo Alto, CA). The samples were run at 60°C and at 1 ml min^{-1} with 1 μL injections using a Zebron ZB-624 column (250mm x 1.4mm ID) (Phenomenex Inc., Torrance, CA). The GC Chemstation 2001 software (Agilent Technologies, Palo Alto, CA) was utilized for data analysis. The concentration of PHA was quantified against standards of PHB and PHV (Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) standard) (Sigma-Aldrich, St. Louis, MO) as derivatized methyl esters and extraction efficiency was calculated based on the benzoic acid internal standard.

RESULTS

The enrichments were run for a total of 20 HRTs, or 80 days, to allow the selective enrichment of a consortium capable of utilizing methanol in the various media as their main carbon source. The data shown in Figures 3 – 6 are the averages of the three

replicate reactors run for each medium, and represent the end point values for each HRT (40 mL 4th day samples; see above). Error bars shown indicate one standard deviation.

MLVSS. MLVSS in all reactors declined as a result of washout during enrichment of the culture for methanol-utilizing bacteria. The MLVSS, which is representative of the total microbial biomass in the cultures, stabilized in all reactors between approximately HRT 8 and 12. Because stabilization of MLVSS is not necessarily indicative of stability of the microbial community, it was decided to continue the enrichment feedings until either rebound of the MLVSS was observed (indicating growth of the selected community), or until the MLVSS was stable for an extended period (indicating steady state cell concentration for the carbon loads supplied). The FC 3200 and FC BHAO reactors required the longest amount of time to stabilize the MLVSS (Figs 3A and 3B), whereas, the WAS-only enrichments required the least amount of time (Fig. 3E). In the FC EVAP and 1°OUT reactors, the MLVSS rebounded before stabilizing (Figs. 3C and 3D).

Soluble COD. Soluble COD present in the reactors at the end of the 24 hour feeding cycle initially varied widely for both the FC 3200 and FC BHAO reactors (Figs 4A and 4B). This variability mirrored the larger than expected variability in the COD of these foul condensates. The large increase in COD between HRT 2 and 6 for the FC BHAO reactors was likely due to the unexpected increase in the FC BHAO foul condensate COD from 4600 to 46300 mg L^{-1} (Fig. 4B). The other enrichments began COD degradation immediately (Figs. 4C-E). In all reactors the COD was degraded an average of 79-89% after enrichment for 20 HRTs. In the enrichments that were fed foul condensates, 11-21% of the input COD was found to be non-biodegradable.

Methanol. Methanol was the main contributor to COD in the enrichment reactors. Methanol comprised 51-72% of the COD in the foul condensate wastes. This wide range was due to the varying nature of the foul condensates, and ultimately, to the composition of the wood feedstocks entering the mill. The enriched consortia in all of the reactors consumed methanol to levels below the 100 mg L^{-1} methanol (HPLC) detection limit by HRT 8 (Fig. 5A-E). This indicates functional stability in the system by HRT 8 with respect to methanol consumption. To ensure community stability in the reactors, as well as to assess the long-term functional stability of the consortia, the reactors were further run to HRT 20. The resulting communities were capable of degrading added methanol and methanol present in the pulp mill effluents.

Ammonium (NH_4^+). All reactors were fed a balanced growth medium containing 300 mg L^{-1} NH_4^+ as the primary source of nitrogen. Nutrient conditions were chosen to foster balanced microbial growth—no nutrient limitations were anticipated or observed. Figure 6A-E clearly demonstrates that the organisms in each enrichment scenario were not lacking in nitrogen. The concentration in the balanced growth medium was asymptotically reached after the community had stabilized in both the 1°OUT and WAS-only enrichments. Notably, the foul condensates from the Chillicothe mill proved to be a source of additional NH_4^+ (Figs. 6A-C), which resulted in nitrogen sufficiency reflected as C:N ratios as low as 3 in the FC 3200 enrichments. Peak COD and NH_4^+ concentrations occurred simultaneously in the FC 3200 and FC BHAO reactors (Figs. 6A-B and 4A-B).

DISCUSSION

The objective of this work was to selectively enrich methanol-utilizing organisms from the original pulp mill waste activated sludge. Secondary waste activated sludge from the P. H. Glatfelter wastewater treatment plant was tested for its ability to degrade methanol and produce PHA. It was found that without enrichment, the bacteria within the waste activated sludge from the pulp mill wastewater facility did not degrade methanol or accumulate PHA over a 24 hour period (data not shown). Thus, the selective enrichment of the activated sludge microbial community on methanol was necessary to obtain a culture that could aerobically degrade methanol and accumulate PHA derived from methanol carbon. After a period of 20 HRTs, the pulp mill waste activated sludge was enriched for consortia capable of using the methanol from foul condensates and the methanol added to the WAS-only and 1°OUT enrichments.

As a consequence of the selection process, the MLVSS of the activated sludge initially decreased in all of the enrichment reactors due to washout of members of the microbial community. This was expected since the organisms present in the pulp mill activated sludge were not accustomed to methanol as a primary carbon source since significant methanol does not reach the wastewater treatment facility but is accumulated in the foul condensates, which are combusted during disposal. After initially decreasing, the MLVSS in all reactors stabilized—FC EVAP and 1° OUT eventually showed a rebound in MLVSS, indicative of the selection for an altered microbial community. The decrease in MLVSS was evidence of the washout of non-methanol-utilizing bacteria during the enrichments, as well as washout of methanol-utilizing bacteria having doubling times longer than the SRT (4 days). Nonetheless, the underlying microbial

community structure, dynamics, and functional stability and how they are related in mixed consortia are poorly understood [23]. Despite the potential structural and functional changes during the enrichment process, the mixed microbial communities were continuously capable of degrading methanol, which was their intended function. It is believed that functional stability observed in engineered systems such as sequencing batch reactors is a result of the functional redundancy within the system [24], especially with respect to microbial carbon assimilation pathways. Therefore, despite process upsets or influent changes, the dynamic changes within a biological reactor utilizing a diverse microbial community are overshadowed by functional stability when the microbial community is faced with a consistent set of operating conditions over a long period of time [25]. Based on this knowledge, the enrichment reactors were run for 20 HRTs to attempt to assess long-term functional stability and attain microbial community stability. Future work will characterize the microbial population throughout the enrichment process using t-RFLP.

The COD, pH, and TSS of the foul condensates used in the enrichments were highly variable resulting at least in part from the transient nature of the wood species utilized at the pulp mill. However, while COD variability was expected in the foul condensates because of varying throughput at the facility, seasonal variations, and quality of wood being processed, the magnitude of COD variation observed was not expected. The pulp mill effluents used were taken directly from pre-existing sample points within the facility. It is believed that the sampling lines may not have been allowed to fully purge before sample collection due to the extensive presence of toxic and/or odorous compounds including hydrogen sulfide, methyl mercaptan, ethanol, methanol, acetone,

terpenes, and various other VOCs in the foul condensates. This may have contributed to added variability in the foul condensates. This is supported by the case of the 1°OUT, as it was sampled from a well-purged sampling line and had consistent COD, pH, and TSS throughout the enrichment process. Unless improved foul condensate collection procedures are employed, the variability of the foul condensates will present a challenge in the design of a biological treatment system to synthesize PHA from pulp mill waste effluents. Such fluctuations in waste quality can negatively impact biological treatment systems if the fluctuations are not attenuated prior to treatment. A potential improvement to the collection procedure would be the use of feedstock mixing and dilution tank in which the condensates would be diluted to a pre-determined COD using another lower carbon concentration effluent prior to biological treatment. Monitoring and controlling effluent carbon and nitrogen concentrations will be vital for the successful operation of a biological process that degrades methanol and produces biopolymers from pulp mill effluents.

Foul condensates were a significant source of ammonium nitrogen. Ammonia is produced in the pulping process due to the caustic digestion of proteins in the wood fed to the mill. The feedstock wood species has a significant impact on the concentration of ammonia present in the pulping process streams since it dictates the amount of wood nitrogen present [26]. Once the wood and pulp are digested, the primary exit point for ammonia in a pulp mill within a kraft chemical recovery process is in the foul condensates [27]. Foul condensates are a condensed caustic waste stream in which the ammonia either remains in the gas phase or is dissolved in the foul condensate as aqueous ammonia. In the present work, once the foul condensates were received for the

enrichment experiment, they were neutralized and ammonia was converted into the soluble ammonium ion. The combination of the nitrogen in the foul condensates and ammonium present in the nutrient media provided conditions of nitrogen sufficiency for all of the enrichments (C:N of 3).

The three main environmental conditions that stimulate PHA storage in a mixed consortium are nutrient limitation, variation in electron donor/acceptor availability, or the presence of a feast-famine environment [28]. In this study, the enrichment reactors were operated with the intent of enriching the original pulp mill activated sludge for consortia capable of utilizing methanol in pulp mill effluents while synthesizing PHA in a nutrient-sufficient feast-famine environment. Therefore, it was expected that PHA production could be stimulated solely by a feast-famine regime in the presence of excess nutrients [29]. However, the enrichment reactors did not produce PHA under the nutrient-sufficient enrichment conditions, even though feast-famine conditions were maintained. Although a low C:N ratio condition was more advantageous for cell growth, it did not favor PHA accumulation. Based on these observations, it was hypothesized that it would be necessary to combine a feast-famine regime with nutrient limitation (by increasing the C:N) to stimulate PHA storage using the enriched cultures. With a lower nitrogen concentration with respect to available carbon, it has been shown that a higher proportion of the carbon can be directed toward PHA synthesis [30-32]. Cultures from the WAS-only enrichments were subsequently tested for PHA production under a combined feast-famine and N-limited environment, resulting in the production of up to 13.9% PHA on a dry cell weight basis. By contrast, the original non-enriched waste activated sludge did not yield any PHA under N-limited conditions.

The results of this study indicate that the enrichment process altered the original activated sludge community – which was originally unable to degrade methanol or produce PHA – and produced a consortium capable of degrading methanol under both N-sufficient and N-limited conditions. Alterations to the community will be studied in future experiments using molecular methods. While we expected the enriched community to produce PHA from methanol under feast-famine conditions, both N-limitation and feast famine conditions were ultimately required to stimulate PHA production. This indicates that nutrient limitation, variation in electron donor/acceptor availability, and feast-famine may not each be singly sufficient in all cases for stimulating PHA accumulation by bacteria.

CONCLUSIONS AND FUTURE WORK

Activated sludge obtained from the pulp mill's wastewater treatment plant was successfully enriched for consortia capable of aerobically degrading methanol from high-strength carbon pulp mill waste streams. The COD in all of the enrichments was reduced by 79 to 89 %. Foul condensates were found to be a suitable feedstock, although their variability must be mitigated to avoid process design challenges. Besides being a source of high strength COD, the foul condensates were also a significant source of ammonium nitrogen. This makes it unnecessary to add nitrogen during either growth (N-sufficient) or PHA production (N-limited) phases when using the foul condensates as the carbon source.

The enrichments were intentionally run under N-sufficient conditions to assure balanced growth, but this condition did not stimulate PHA production. The enriched cultures were later tested for PHA production under N-limited conditions and produced PHA. The non-enriched waste activated sludge did not produce PHA under N-limited conditions. Therefore, in order to stimulate PHA production from pulp mill effluents it is necessary to not only provide a feast-famine regime, but also a nutrient-limited condition.

Future work will focus on optimizing the PHA yield from pulp mill effluents because successful integration of PHAs with natural fiber composites requires higher biomass PHA concentrations [10]. As part of the effort to optimize PHA production using pulp mill effluents, future work will involve characterization of the microbial community throughout the enrichment process, investigation of the influence of C:N, F:M (food to microorganism ratio), HRT, and SRT on PHA production, and a pilot test of PHA production from FC 3200 at the mill using activated sludge enriched onsite.

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Figure 1

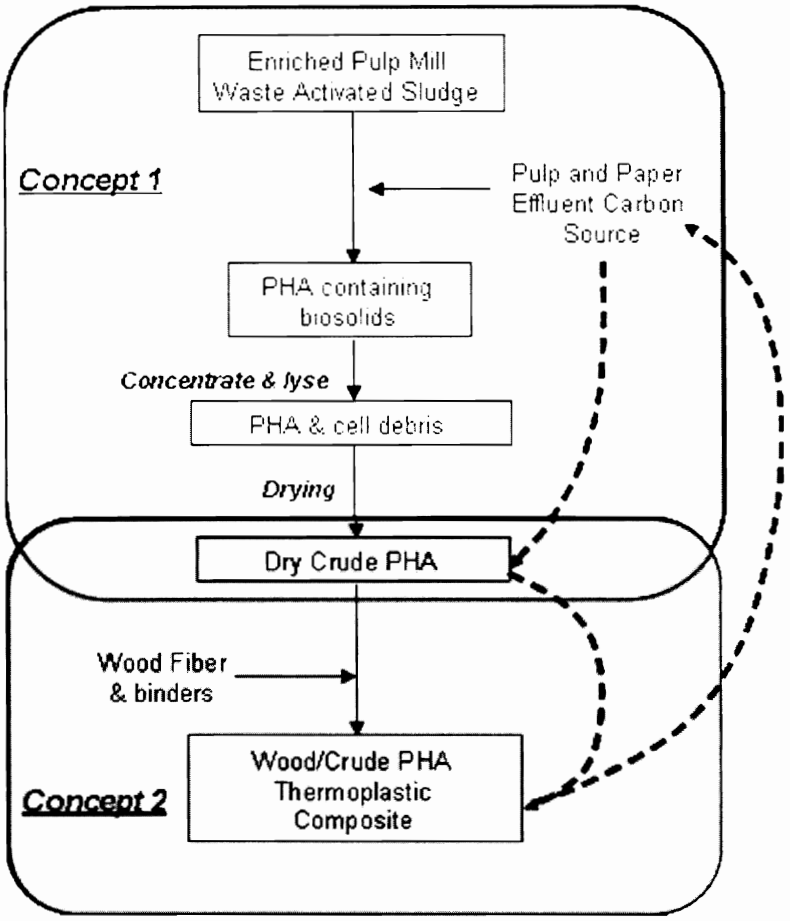

Figure 1. Approach for minimizing costs associated with the manufacture of natural fiber reinforced thermoplastic composites using unpurified PHAs

Figure 2

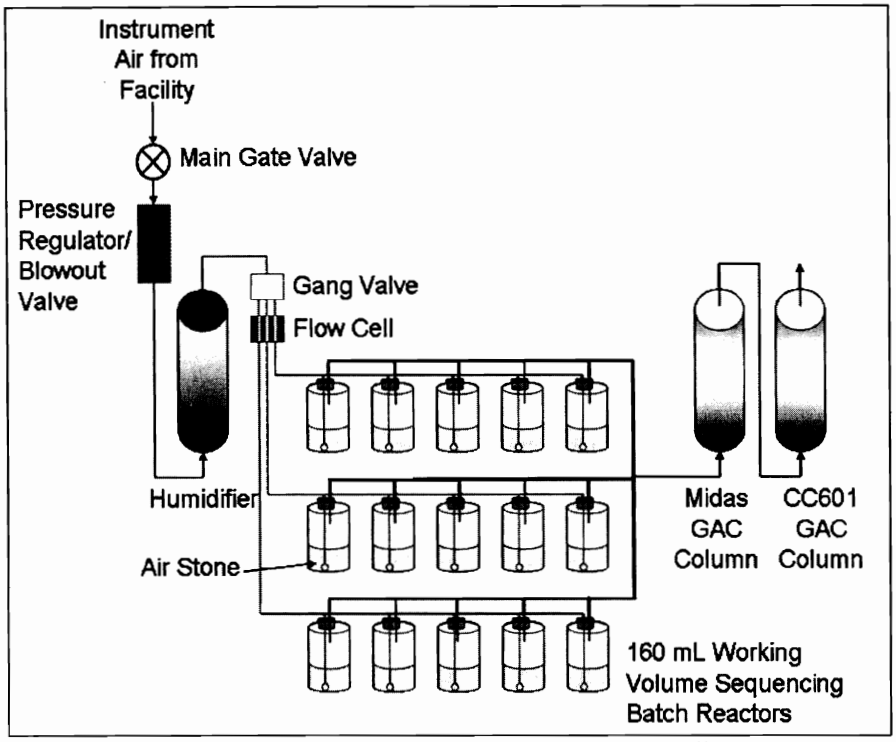

Figure 2. Enrichment experiment process flow diagram

Table 1

Table 1. Average COD, TSS, and pH for the foul condensates and 1°OUT as received

Waste Stream ID	COD[1] (mg/L)	TSS[2] (mg/L)	pH
FC 3200	30200 ± 13545	47 ± 23	9.66 ± 0.84
FC BHAO	13314 ± 16433	976 ± 1537	9.39 ± 0.99
FC EVAP	5487 ± 4247	116 ± 29	9.48 ± 1.11
1° Out	480 ± 286	600 ± 303	7.48 ± 0.45

[1]Chemical Oxygen Demand ± one standard deviation

[2]Total Suspended Solids ± one standard deviation

Figure 3A-E

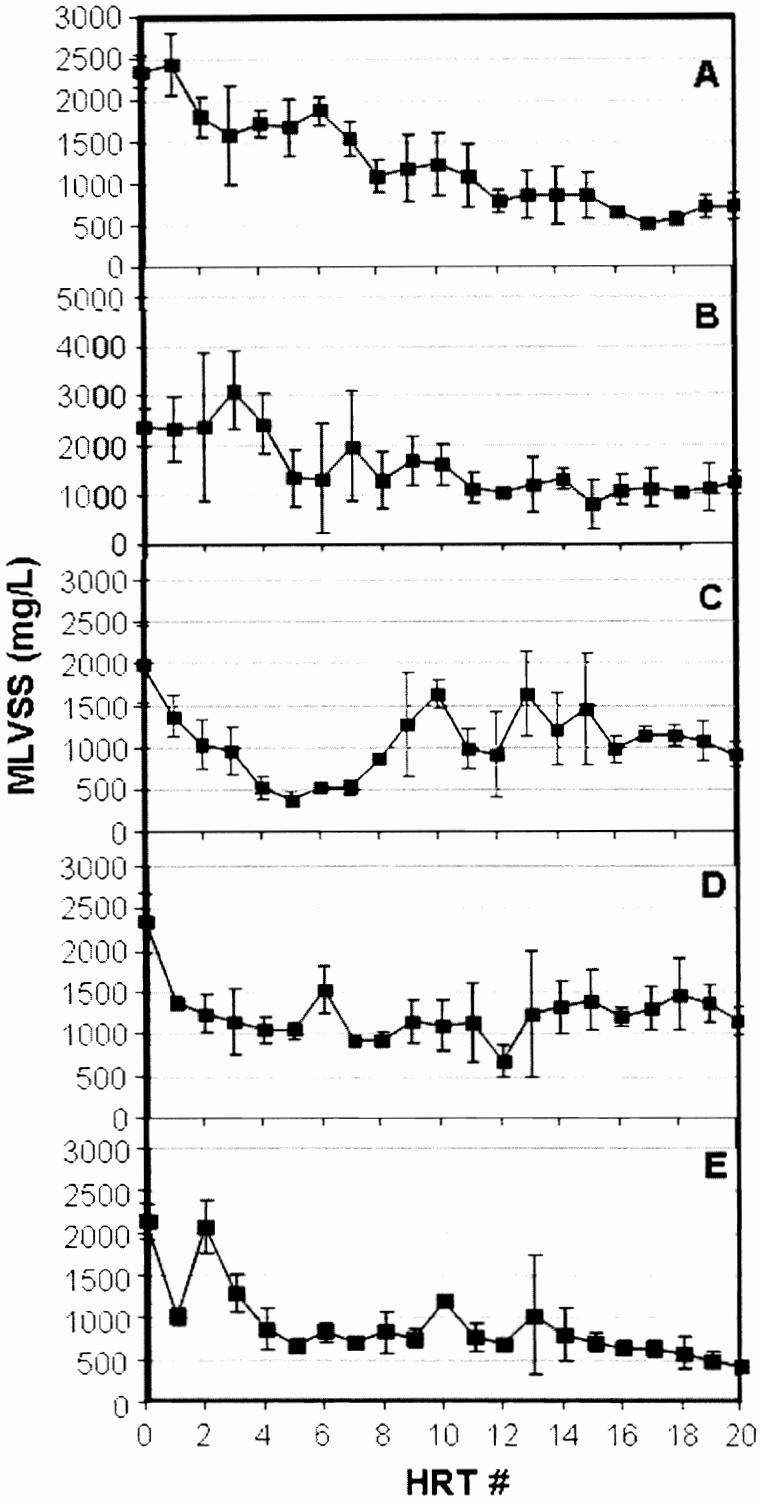

Figure 3A-E. Variation of MLVSS with HRT. Data shown are from samples taken at the end of the 24 hour feed period, and are the average of three replicate reactors. **A** FC 3200. **B** FC BHAO. **C** FC EVAP. **D** 1°OUT. **E** WAS-only.

Figure 4A-E

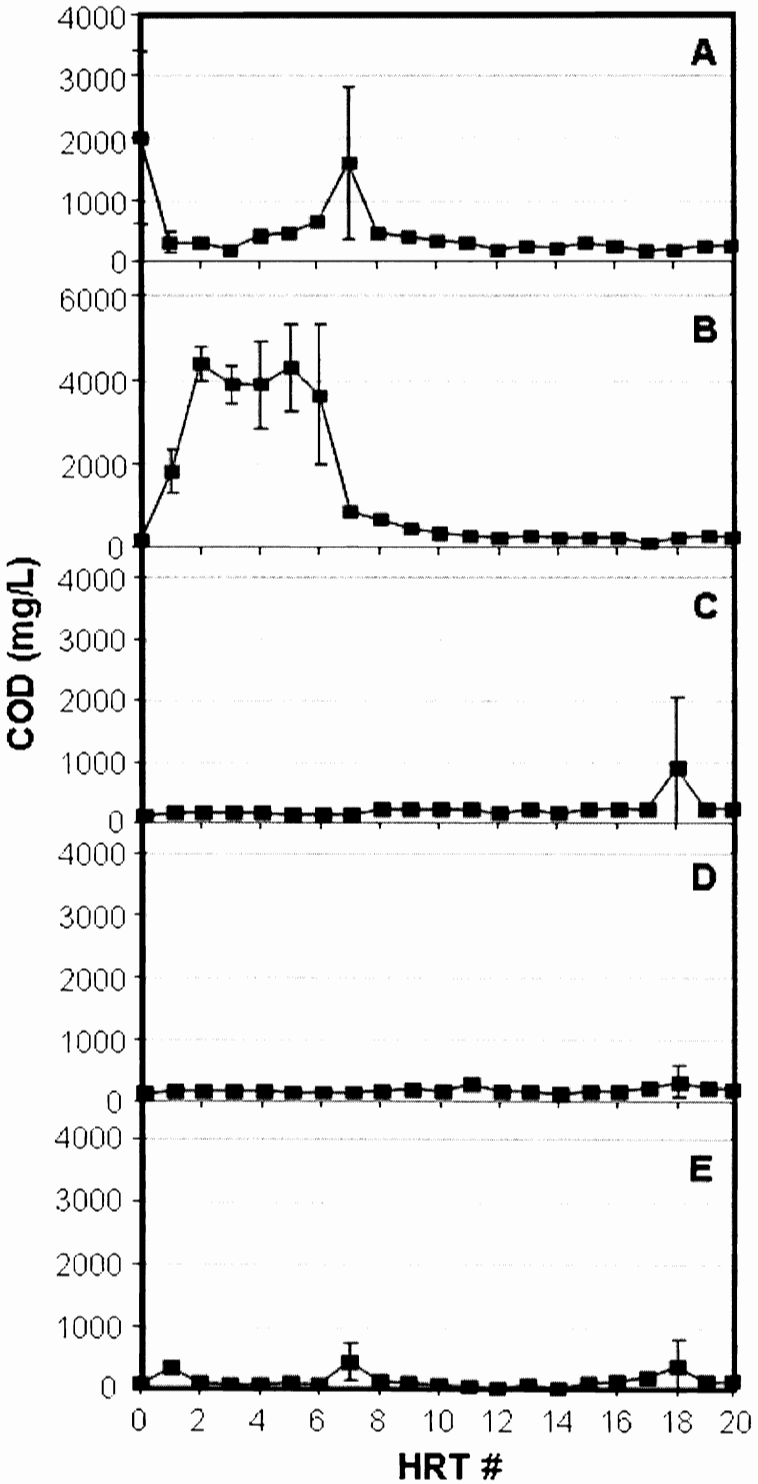

Figure 4A-E. Variation of COD with HRT. Data shown are from samples taken at the end of the 24 hour feed period, and are the average of three replicate reactors. **A** FC 3200. **B** FC BHAO. **C** FC EVAP. **D** 1°OUT. **E** WAS-only.

Figure 5A-E

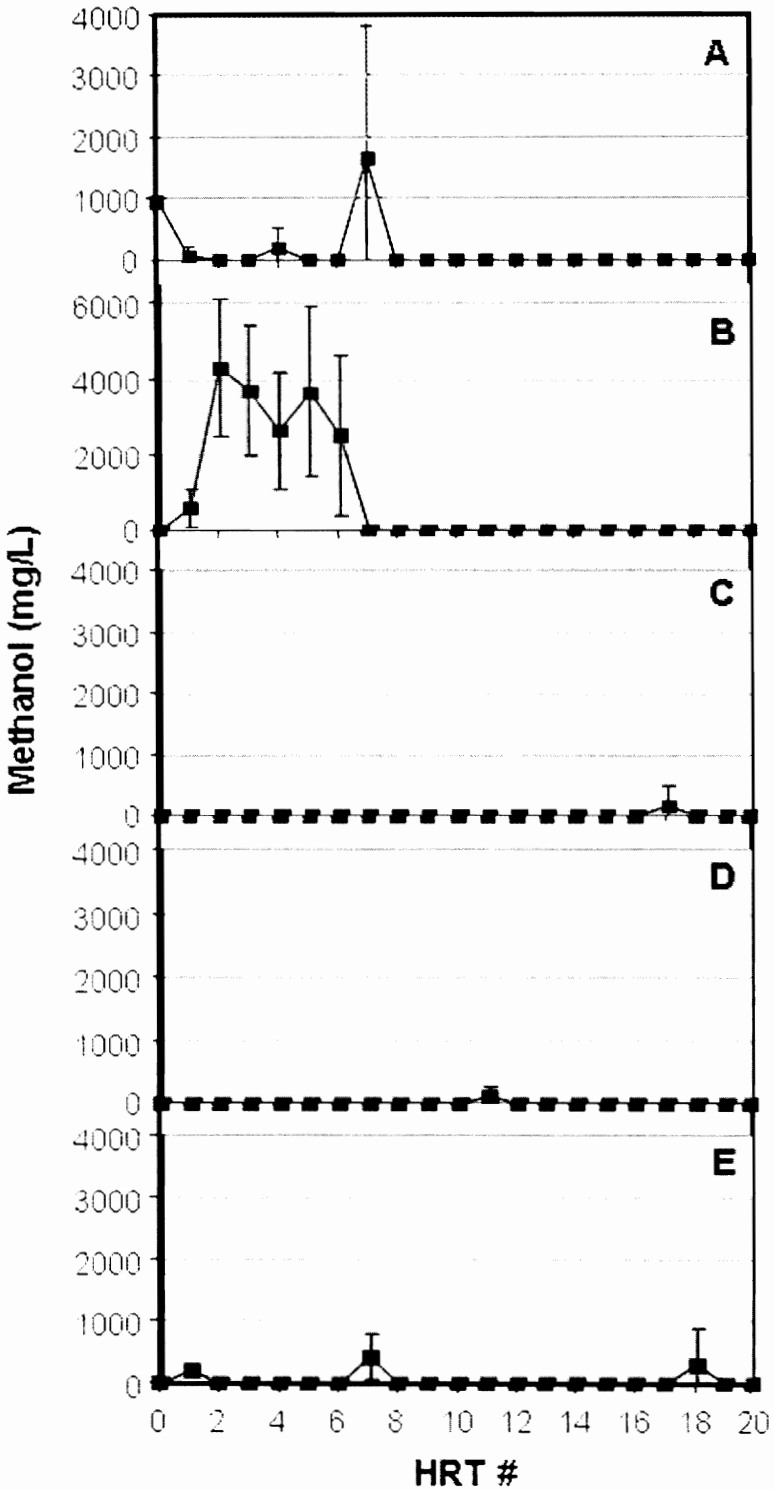

Figure 5A-E. Variation of Methanol with HRT. Data shown are from samples taken at the end of the 24 hour feed period, and are the average of three replicate reactors. **A** FC 3200. **B** FC BHAO. **C** FC EVAP. **D** 1°OUT. **E** WAS-only.

Figure 6A-E

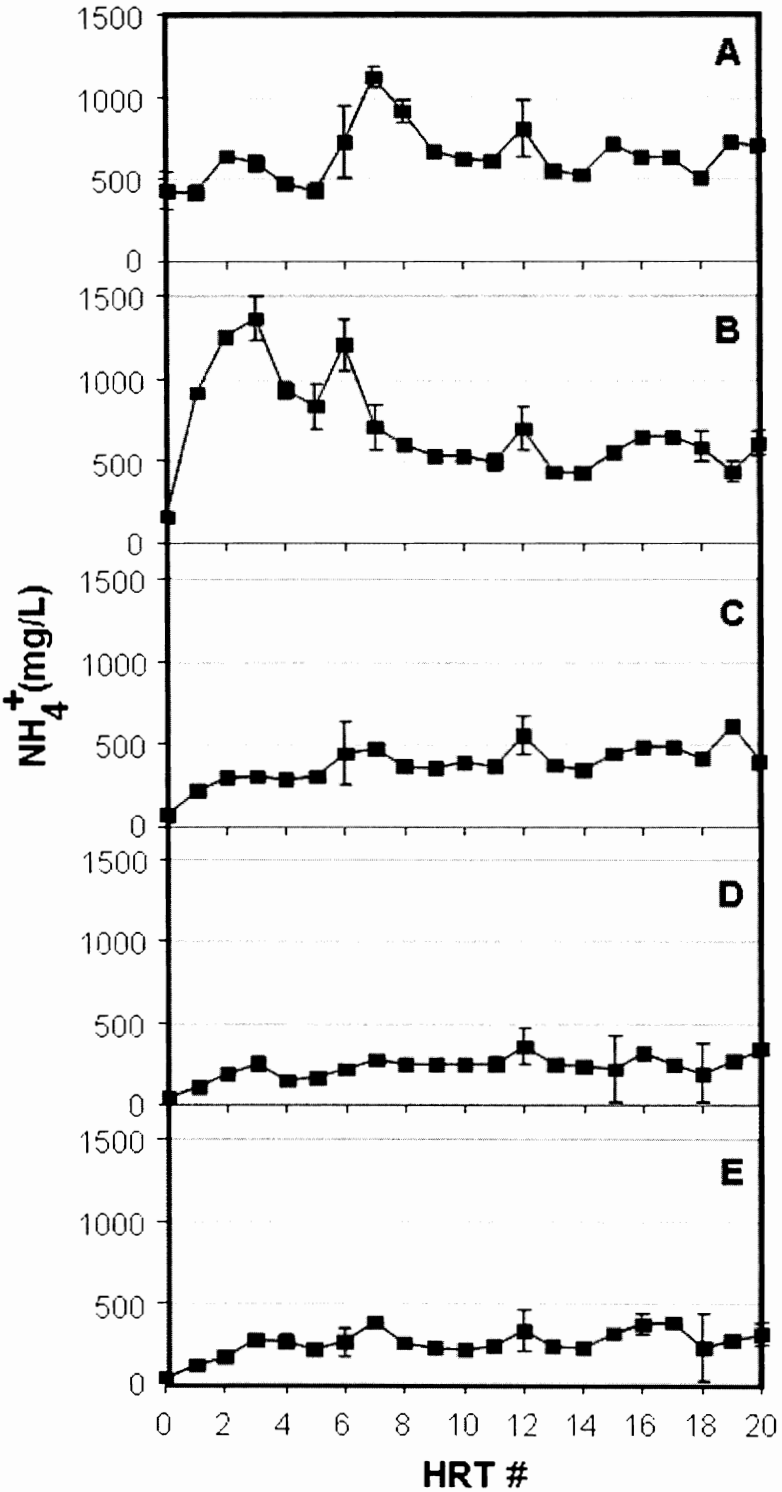

Figure 6A-E. Variation of NH_4^+ with HRT. Data shown are from samples taken at the end of the 24 hour feed period, and are the average of three replicate reactors. **A** FC 3200. **B** FC BHAO. **C** FC EVAP. **D** 1°OUT. **E** WAS-only.