Polyhydroxyalkanoates production using the liquid fraction of hydrolysed municipal organic waste

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Abstract
The production of polyhydroxyalkanoates (PHA) using organic wastes can be a sustainable alternative for the management of the organic fraction of municipal solid waste (OFMSW), providing economic opportunity for waste treatment plants and preserving natural resources. PHA are bacterial polyesters usually produced from volatile fatty acids (VFA) that can be used as biodegradable bioplastics. In this work, the conversion of biodegradable organic waste to PHA was investigated in three phases: VFA production via fermentation of organic waste, use of the VFA stream to select biomass capable of PHA accumulation, and PHA accumulation. Batch test optimization of fermentation parameters revealed that 5.4% solids content, 37°C, and 3.5 d retention time resulted in the greatest VFA production. Up to 14 g L^{-1} VFA was achieved in lab-scale continuous fermentation. The liquid fraction of the fermented organic waste was applied using a feast/famine feed strategy to successfully select PHA accumulating biomass. Finally, the PHA accumulation phase revealed an average maximum of 38% per g VSS was achieved using a low nitrogen feed mixture, with 45% (on VSS basis) being the maximum PHA yield achieved. Overall, this study demonstrates the feasibility of using municipal organic waste for PHA production, although further process optimization and incorporation of nutrient recovery should be investigated to maximize PHA production and process efficiency.

Keywords
Bioplastics, municipal organic waste, PHA, VFA

INTRODUCTION

One of the main sources for production of plastics is crude oil, which is a well-known non-renewable resource. However, plastic materials are often used for only short-term applications, such as plastic bags and packaging. At the same time, the worldwide production of municipal wastes has been continuously increasing in the last years with no signs of significant decrease. Among these municipal wastes, the Organic Fraction of Municipal Solid Waste (OFMSW) is an important part, both because of the amount produced with respect to the total production of municipal waste and because of its potential to be transformed into valuable products, including biopolymers. Biopolymers as an alternative to petroleum based polymers provide the advantage of conserving fossil fuel resources and reducing CO_2 emissions, making them an important innovation of sustainable development (Bugnicourt, 2014). Due to their biodegradability, biocompatibility, chemical-diversity, and being manufactured from renewable carbon resources, polyhydroxyalkanoates (PHAs) are considered one of the most promising biopolymers. Thus, utilizing municipal organic waste for the production of PHA provides a dual advantage: converting a waste into a valuable product and avoiding the production, use, and disposal of petroleum-based plastics.

In general, production of PHA utilizing waste streams such as the OFMSW, in combination with mixture microbial cultures presents many advantages when compared with current conventional PHA production processes. Presently, industrial processes for PHA production are based on the use of pure cultures of selected strains requiring single, pure substrates (Setiadi et al., 2015). This PHA

production is expensive, mostly because of the costs of culture maintenance, substrate formulation, and both substrate and reactor sterilization making the process uncompetitive with synthetic thermoplastics. For this reason, use of mixed microbial strains coming from waste is a promising alternative because it does not require maintaining sterile conditions and it makes it easier to use low-cost feedstocks. However, achieving high PHA content and volumetric productivity with waste streams and mixed cultures is still a significant challenge due to these issues of high solids and nitrogen content (Serafim, et al, 2008; Tamis et al., 2014). Therefore, the objective of this study was to investigate the feasibility of using a complex organic waste stream, specifically non-source-sorted OFMSW referred to as Residual Organic Matter (ROM), as a feedstock for PHA production.

MATERIALS AND METHODS

Substrate and Inoculum

The ROM used in this study was obtained from a Mechanical Biological Treatment (MBT) plant of non-source-sorted municipal waste of the Barcelona Metropolitan area. It was collected in 20 litre carboys and stored at 4°C before use. Anaerobic digester effluent of the same MBT plant served as inoculum for the fermentation phase of this study. It was applied to the reactor directly after collection. The biomass selection reactor was inoculated with waste activated sludge from a municipal wastewater treatment plant of the Barcelona Metropolitan Area.

Experimental Set-up

Fermentation Phase. For this study, a jacketed fermentation reactor with a working volume of 5L, and mechanical stirring (IKA-Werke, RW16 basic) was operated for the production of VFA from ROM. The fermenter was inoculated with anaerobic digester effluent and the initial HRT established was 2.5 days, with the aim of washing out methanogenic microorganisms present in the inoculum. Operating conditions in the fermentation reactor were determined via initial batch testing to optimize VFA production. Optimal temperature for the fermentation reactor was determined to be 37ºC which was maintained via a water bath (Thermo Electron Corporation, HAAKE DC30). Over the course of the study, HRT in the fermenter was increased from 2.5 to 3.5 days. Manual subtractions and additions of material were made daily to maintain the desired HRT.

Preliminary batch testing was conducted to determine optimal conditions for fermentation of ROM to VFA. Effluent collected during start-up of the fermentation reactor served as inoculum for the batch tests. Three optimisation variables were investigated: solids concentration (3.3, 4.4, 5.6 and 6.1% TS), temperature (33, 35 and 37ºC), and hydraulic retention time (0-5 days). Batch tests were performed in 500 mL bottles. To maintain the desired temperature, bottles were submerged in a thermal bath (Thermo Electron Corporation, HAAKE DC30). The test was carried out for a period of 5 days with regular measurements of VFA concentration.

Biomass Selection Phase. After fermentation, the effluent (hydrolyzed ROM) was filtered via an ultrafiltration membrane (Tami Industries, No. 26110) to achieve separation of the solid and liquid fractions. The liquid fraction of the hydrolysed ROM served as substrate for the biomass selection phase. This fermentation liquid (FL) was preserved at 4ºC to reduce loss of VFA prior to feeding to the biomass selection phase.

The biomass selection phase consisted of a jacketed Sequencing Batch Reactor (SBR) of 3L. The reactor was operated with mechanical stirring (IKA-Werke, RW16 basic) and temperature was maintained at ambient conditions. This reactor was equipped with 3 peristaltic pumps connected to

programmable timers to automatically control: feeding, effluent withdrawal, and biomass purging. The reactor was also equipped with pH, ORP and Dissolved Oxygen (DO) probes all connected to a data collection system (Modules Advantech's ADAM) controlled by a program developed in Advantech ADAMView.

PHA Accumulation Phase. For the accumulation of PHA, a 1L reactor was set-up and operated in semi-batch mode with regular addition of substrate. Each accumulation test was carried out for a period of 24 hours. At the start of each test, biomass collected from the selection reactor was used as inoculum in the accumulation reactor. 150 mL of selection phase biomass purge was collected, rinsed with deionized water, combined with 600 mL deionized water, and added to the reactor. The purpose for rinsing the biomass was to remove the influence of nutrients coming from the selection phase effluent. The accumulation phase reactor was operated with continuous mechanical stirring (IKA-Werke, RW16 basic) and temperature was maintained at ambient conditions.

To assess the viability of the fermentation liquid of hydrolysed ROM as a feedstock for PHA accumulation, three different substrate mixtures were tested: (1) a primarily synthetic feed mixture, which consisted of 10% v/v hydrolysed ROM and 10% v/v acetic acid in deionized water, (2) a mixture of 30% v/v hydrolysed ROM and 10% v/v acetic acid in deionized water, and (3) 100% hydrolysed ROM. Over the course of each 24 hour test period, automatic feedings of 50 mL substrate were made every four hours to the reactor via a peristaltic pump (Spectra, Pericom-1) and programmer. The four hour feed interval was determined via observation of the DO profile and time taken for DO in the accumulation reactor to return to endogenous levels after feeding. VFA, total and volatile suspended solids, and PHA concentration in the biomass were measured at the beginning and the end of each 24 hour tests to compare the effects of the different substrates on PHA accumulation.

Analytical Methods

All analyses were performed according to the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). VFAs were measured using a Shimadzu GC-2010+ gas chromatograph equipped with a capillary column Nukol (0.53 mm ID; 15 m length) and a flame ionization detector (FID). Ammonium nitrogen concentration (NH_4^+-N) was analysed with a specific ammonia electrode (ORION 9512). Inorganic cations and anions were measured by ionic chromatography (Metrohm Advanced Compact IC). PHA extraction and quantification was performed following the method of Lanham et al. (2013).

RESULTS AND DISCUSSION

Proposed PHA Production System

The initial aim of this research was to show the feasibility of producing PHA from the complex substrate that is non-source-sorted OFMSW (ROM) using a mixed biomass. Figure 1 shows a schematic outlining of the three-part process (Reis et al., 2011; Katsou et al., 2015; Frison et al., 2015; Basset et al., 2016) which includes (1) Fermentation of the ROM to produce a fermentation liquid rich in VFA, (2) Selection of PHA accumulating microorganisms using a feast/famine strategy, (3) Accumulation of PHA in the selected biomass. With this process there is also potential for energy production via anaerobic digestion of the solids fraction remaining after fermentation, and nutrient recovery from the liquid fraction prior to use for PHA production.

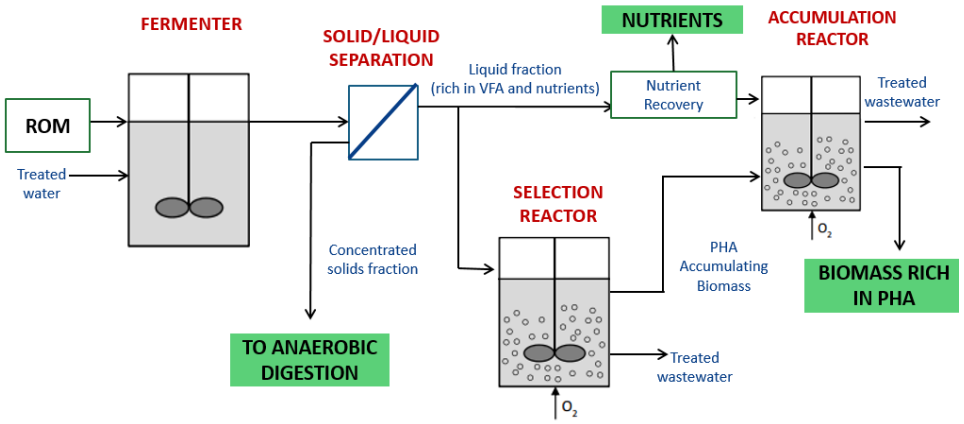

Figure 1. Schematic of three-part process for production of PHA from ROM including potential for nutrient and energy recovery.

Fermentation of ROM

Several batch tests were carried out to assess proper operational conditions at short term in order to maximize VFA production from ROM. The influence of three operation parameters was studied: solids concentration (from 3.3 to 6.1% TS), temperature (33-37ºC) and retention time (batch tests duration was set at 5 days). Batch fermentation tests were performed and analysed using surface response curve methodology to determine optimal operating parameters. Higher VFA production was registered at the highest temperature tested, namely 37ºC. Figure 2 shows the response curve resulting from batch fermentation of ROM at 37°C. When comparing the resulting VFA concentrations at various retention times and solids concentrations, it was concluded that maximum VFA production at short-term conditions was achieved at 5.4% TS and 3.4 days of retention time, under 37ºC.

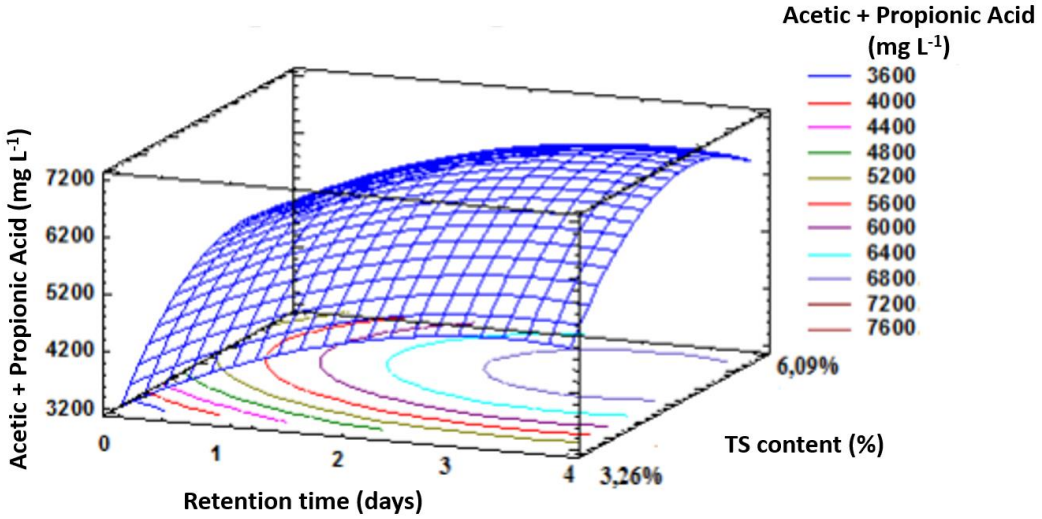

Figure 2. Surface response curve from batch test fermentation of ROM at 37ºC comparing resulting VFA concentrations at various retention times and solids concentrations.

Considering these operational parameters at short-time effect, a lab-scale continuous fermentation reactor (working temperature 37ºC) was set-up and investigated to assess the production of VFA at long-term conditions. The fermenter was inoculated with anaerobic digester effluent of the same MBT plant and the initial HRT established was 2.5 days, with the aim of washing out methanogenic microorganisms present in the inoculum. Figure 3 shows VFA production in the continuous fermenter over a range of HRTs from 2.5 to 3.5 days, where the average TS concentration of the

influent MOR was in the range of 45.7 ± 10.2 g L^{-1}. In this Figure, it can be observed that VFA concentrations increased with increasing HRT, although it was also dependant on the feeding substrate (see initial VFA concentration). Average VFA concentrations at 2.5, 3.0, and 3.5 day HRT were 8,880 ±765; 9,359 ±1,506; 11,886 ±1,360 mg L^{-1}, respectively. Moreover, longer retention time resulted in a greater proportion of acetic and propionic acid within the total mixture of VFAs produced. Acetic acid represented an average percentage of 35.2, 37.5 and 45.3% of the total VFA generated in the fermentation reactor for HRT 2.5, 3.0 and 3.5 days, respectively. However, it should be highlighted that the ROM fed in the last period (HRT 3.5 days) contained more VFA than that used in the previous periods. Considering the mass balance of VS, the effluent VS represented approximately a 90% of the VS fed to the reactor, which could be related to VS degradation, some accumulation of solids in the bottom of the fermentation reactor or biogas production.

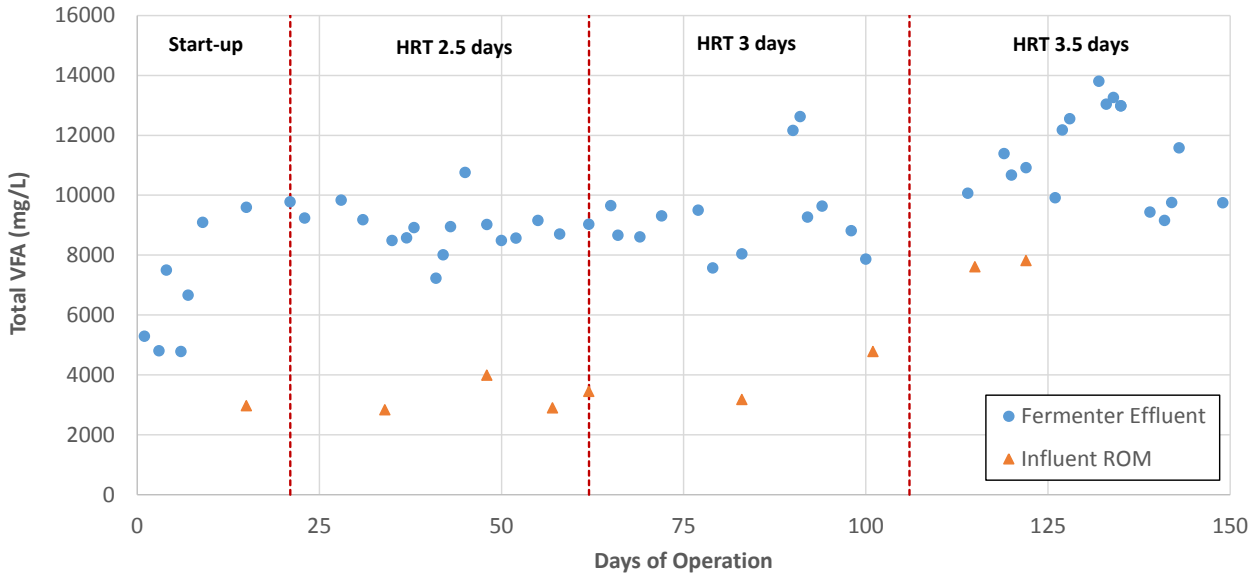

Figure 3. Concentration of total VFA (influent and effluent) in the lab-scale continuous fermenter over time.

After fermentation of the ROM, the liquid and solid fractions of the fermentation liquid were separated via membrane filtration. The resulting liquid fraction, rich in VFAs, was applied as substrate in the biomass selection phase of the process. Table 1 describes pH, VFA content and NH_4^+-N concentration of the ROM before fermentation, the resulting fermentation effluent before filtration, and the final fermentation liquid fraction after filtration. From Table 1 it can be seen that there is a minor loss (8%) of VFA after filtration.

Table 1. Characteristics of ROM, fermentation effluent, and filtered fermentation liquid

Parameter	Units	Residual Organic Matter (ROM)	Fermentation Effluent (before filtration)	Liquid Fraction of Fermentation Effluent
pH	-	6.3 ±0.3	6.0 ±0.4	6.2 ±1.4
Total VFA	mg L^{-1}	4,388 ±1,982	9,492 ±1,931	8,700 ±356
NH_4^+-N	mg L^{-1}	1,794 ±631	2,087 ±779	2,079±725

Selection of PHA Accumulating Biomass

To select PHA-accumulating microorganisms, an SBR was started-up in 8 hour cycles under a regime of feast-famine, achieving an average feast/famine time ratio ranging from 0.15 to 0.21, which falls near the range of 0.20-0.25 reported by Albuquerque et al. (2010). Total VFA concentration in the clarified effluent was always below 30 mg VFA L^{-1}, so VFA removal

efficiency was above 99%. Figure 4 shows the evolution of TSS and VSS in the mixed liquor and the percentage of PHA in the purged biomass. Several strategies were followed during the operation of the selection reactor: In a first stage (0-15 days) the SBR was started up with secondary sludge from a municipal WWTP and fed with diluted fermentation liquid (50%) spiked with Acetic Acid in order to obtain 6 g VFA L^{-1} in the feed. In this stage, HRT and SRT were set at 7.5 and 17 days, respectively. After start-up in the selection reactor, average VSS and TSS stabilized to around 2.5 and 2.7 g/L, respectively. PHA content in the sludge purge was in the range of 1.5-2.5% (on VSS basis). In this stage, air supply was performed during 7 hours of the SBR cycle, and the 30 minutes before biomass settling and effluent withdrawal were dedicated to mixing without air supply. Nitrification activity was registered and episodes of sludge flotation during the settling step of the SBR occurred. Therefore, in a second stage, a 30 min anoxic stage was set after feeding in every SBR cycle in order to avoid uncontrolled denitrification during the settling step. This strategy leads to a decrease in the PHA content of the purged biomass (namely 1.2-1.7% on VSS basis) which the authors related to a lower initial VFA concentration in the feast phase under aerobic conditions (part of the VFAs were consumed for denitrification) and to the proliferation of heterotrophic denitrifying biomass and autotrophic nitrifying biomass without PHA storing capacity. For this reason, in a third stage (days 64-105), the anoxic period after feeding was suppressed, so the PHA content of purged biomass also increased. Finally, from day 105 on (Stage 4), undiluted fermentation liquid was fed to the SBR, HRT and SRT were set at 6 and 20 days, respectively, and an increase in both the volatile suspended solids and the PHA content of purged biomass (13.8-17.6% on VSS basis) were registered.

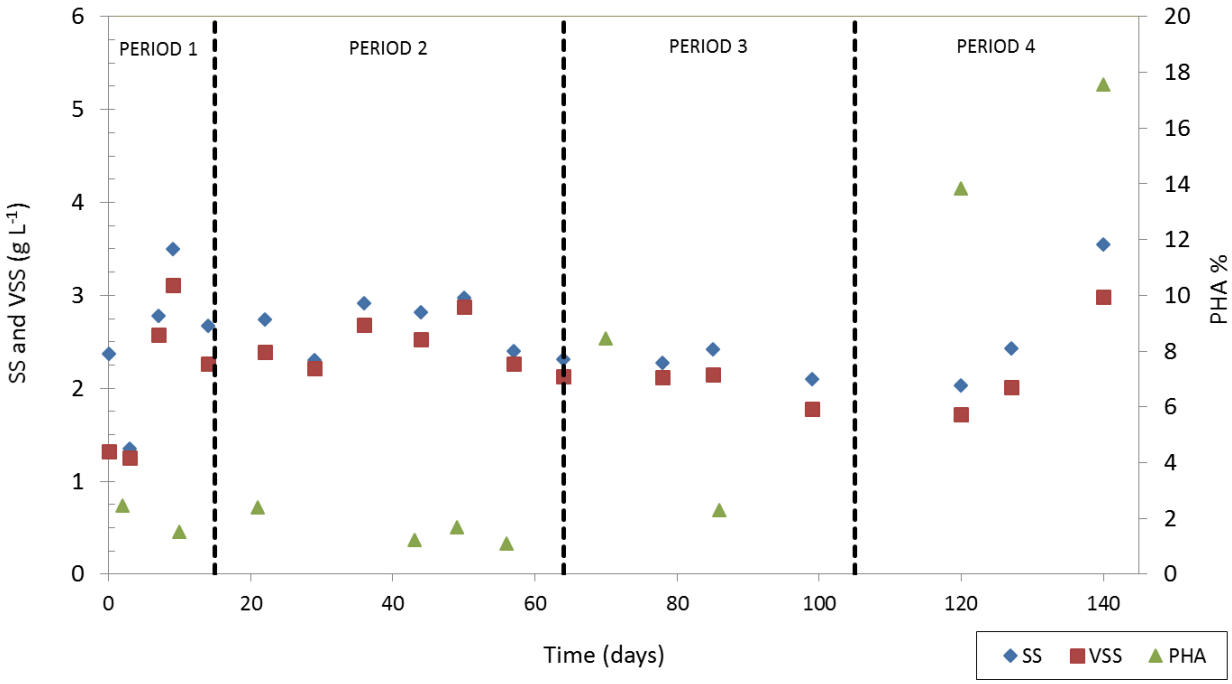

Figure 4. TSS, VSS, and PHA% per gram VSS obtained in the selection phase reactor over time.

Table 2 shows the operational conditions during Stage 4, when undiluted fermentation liquid was fed to the reactor. It is important to highlight that during this stage high pH values (in the range of 9.4) were achieved which promoted high free ammonia concentration and the consequent nitrification inhibition. Total Ammonia Nitrogen was in the range of 1.1-1.3 g NH_4^+-N L^{-1}, so NH_3 stripping was registered. Therefore, ammonia recovery prior to the selection reactor treatment will be performed in future work. Figure 3 shows a representative dissolved oxygen (DO) profile in the selection reactor over time of 4 consecutive SBR cycles (HRT of 6 days). This profile reflects the feast and famine periods achieved in the reactor, with low DO reflecting the period of VFA

consumption (feast), and increasing/high levels of DO reflecting the period of famine. A rise in the ORP profile was also recorded at the end of feast conditions, which could also be used to identify the feast and famine periods. During the famine period, bacteria that are able to store organic carbon as PHA have an advantage, as they can use their stored PHA for energy (Lee, 1996; Van Loosdrecht et al., 1997). Thus, with multiple cycles of feast and famine (in this case, with a feast to famine time ratio of 0.15) selection of PHA accumulating microorganism was achieved. Biomass samples were collected after feast periods to determine the PHA concentration. The maximum concentration achieved was 17.6% of PHA (on VSS basis), confirming successful selection of PHA accumulators in the biomass.

Table 2. Operation conditions of the selector reactor treating undiluted fermentation liquid of the ROM

Parameter	Average Value	Range Value	Units
Cycle duration	8	-	h
Feeding (with mixing)	2	-	min
Aeration + mixing	432	-	min
Mixing (without aeration)	30	-	min
Settling	15	-	min
Effluent withdrawal	1	-	min
OLR	1.29	0.90-1.67	g VFA (L day)$^{-1}$
% VFA removal	>99	-	%
HRT	6	-	days
SRT	20	-	days
TSS	3.02	2.03-3.54	g SS L^{-1}
VSS	2.47	1.72-2.99	g VSS L^{-1}
Feast/Famine time ratio	0.15	0.14-0.15	-
% PHA in the purged biomass	15.7	13.8-17.6	% (on VSS basis)

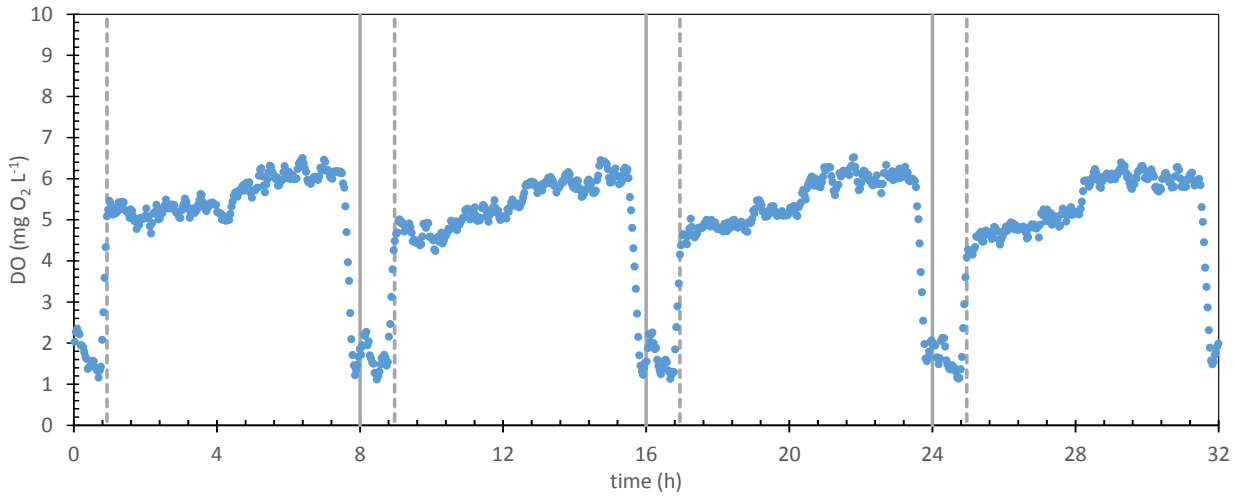

Figure 4. Dissolved oxygen profile in the selection reactor over time during stage 4 of the selection reactor, reflecting the feast/famine feed strategy. (End of SBR cycle: ——; End of Feast period in each SBR cycle: – – –)

PHA Accumulation

In the final phase of the process, PHA accumulation within the selected biomass was investigated. PHA accumulation in select microorganisms occurs when they are subjected to stress conditions such as limitation of a nutrient, electron donor or acceptor. In this study, nutrient limitation was the chosen strategy, and the impact of ammonia nitrogen concentrations on PHA accumulation was investigated. Biomass collected from the selection reactor (mainly during Period 2 and 3) was used to seed the PHA accumulation reactor. Prior to seeding the reactor, the biomass was rinsed to

remove any nutrients that were present in the liquid effluent of the selection reactor. Three concentrations of filtered fermentation liquid (10%, 33%, and 100%) were applied as substrate in the accumulation reactor to achieve a comparison of low, medium, and high ammonia nitrogen addition. In the 10% and 33% conditions, acetic acid was added to the dilution so that the level of VFA in all three substrate mixtures was similar. The majority of the fermentation liquid that was used for PHA accumulation testing was collected during Period 2 of the fermentation phase, when average acetic acid represents approximately 34% of total VFA. The liquid was stored in the refrigerator prior to use in an attempt to reduce the loss of VFA, however, some loss of VFA did occur. Table 3 provides a description of the three substrate mixtures.

Table 3. Characterization of substrate applied to the PHA accumulation reactor.

Fermentation Liquid Concentration	**10%**	**33%**	**100%**	
Total VFA	6050 ±705	5839 ±1345	5722 ±1512	mg L^{-1}
Acetic	99.2	64.6	32.0	
Propionic	0.2	14.8	39.1	
Isobutyric	0.0	5.3	11.4	
Butyric	0.2	5.3	13.2	
Isovaleric	0.0	4.5	11.7	% of Total
Valeric	0.1	3.3	10.3	
Isocaproic	0.0	1.0	2.6	
Caproic	0.1	0.5	3.1	
Heptanoic	0.3	0.8	0.8	
NH_4^+-N	6.9 ±4	725.3*	2,198 ±716	mg N L^{-1}
N/COD	0.46	49.8	114.3	mg g^{-1}
pH	5.7 ±0.3	5.7 ±0.5	6.2 ±1.4	-

*Calculated based on NH_4^+-N concentration in 100% fermentation liquid

A series of accumulation batch tests were performed using the different substrate mixtures. Results are shown in Table 4. The maximum PHA yield achieved was 45% on VSS basis (37% on TSS basis) using the 10% fermentation liquid feed mixture. Overall, PHA yield was highest in the 10% fermentation liquid feed mixture, and decreased with increasing concentration of fermentation liquid. Average PHA yield in 10, 33, and 100% fermentation liquid were 38, 27, and 19% per g VSS respectively.

Table 4. PHA accumulation batch test parameters and results, comparing different concentrations of fermentation liquid in the substrate (10%, 33% and 100%).

Fermentation Liquid Concentration	**10%**	**33%**	**100%**	
OLR	1.9 ±0.35	1.86 ±0.56	1.86 ±0.58	kg VFA $(m^3day)^{-1}$
Initial F:M	0.63 ±0.18	0.98 ±0.11	0.93 ±0.16	g VFA g^{-1} TSS
VFA removal	58 ±25	50	44 ±13.24	%
TSS				g L^{-1}
Initial	0.74 ±0.29	0.46 ±0.10	0.49 ±0.09	
Final	1.29 ±0.62	1.15 ±0.13	1.54 ±0.09	
VSS				g L^{-1}
Initial	0.62 ±0.30	0.42 ±0.07	0.47 ±0.09	
Final	1.18 ±0.68	1.07 ±0.16	1.42 ±0.08	
PHA				% (on VSS basis)
Initial	2.3 ± 0.2	7.5 ± 9.0	6.2 ± 3.9	
Final	**37.5 ±6.3**	**27.1 ±5.8**	**18.8 ± 5.8**	

In general, PHA yields obtained in this study were near the range of previously reported PHA accumulation values. Serafim et al. (2008) reported a range of 20-54% PHA per g biomass for PHA

accumulation using aerobic dynamic feeding strategies with mixed cultures and complex substrates. In this study, the lower PHA yield observed with increasing concentration of fermentation liquid is likely due to the increase of nutrients. Previous research has found that high levels of nutrients can cause inhibition of PHA accumulation. Korkakaki et al. (2016) presented experimental data that confirmed that specific substrate uptake rates were significantly reduced when OFMSW leachate was used as the substrate as compared to an equivalent artificial VFA mixture. Testing the different possible inhibitors, such as salt, ammonium or VFA concentration suggested that the main inhibition most likely was caused by the high ammonium concentration of the OFMSW leachate. Optimal N/COD of 2-15 mg g^{-1} was reported by Valentino et al. (2015). In this study, the 33% and 100% fermentation liquid condition had N/COD ratios of 49.8 and 114.3 mg/g respectively, well beyond the suggested optimal range. Therefore, inhibition of PHA accumulation due to high ammonia concentrations is highly likely, and in order to improve PHA production from ROM, a means for removing and ideally recovering nitrogen from the fermentation liquid prior to use is recommended.

Overall, PHA accumulation using the liquid fraction of fermented ROM was successful under conditions of low nitrogen concentration, achieving an average maximum PHA yield of 38% on VSS (33% on TSS basis). Increasing the concentration of fermentation liquid in the feed stream inherently increased ammonia nitrogen concentrations beyond those optimal for PHA accumulation, and a reduction in PHA yield was observed. Further studies will investigate options for recovery of ammonia nitrogen from the fermented ROM in order to achieve greater PHA yields. Recovery of ammonia will also provide a valuable by-product from the process, advancing the progress of shifting waste treatment processes towards a circular economy.

CONCLUSIONS

The production of bio-plastics from municipal organic waste can shift the paradigm of municipal waste management towards a more circular economy. In this study, the feasibility of PHA production from Residual Organic Matter (ROM) was investigated and achieved in a three-phase process. Firstly, the production of VFA from fermentation of ROM was determined to be optimal under the following conditions: 5.4% solids, 37ºC, and 3.4 day HRT. Secondly, a biomass selection reactor, fed with undiluted liquid fraction of fermented ROM, was operated at a feast/famine ratio of 0.15, SRT of 20 days, and HRT of 6 days, achieving PHA-accumulating enriched biomass (up to 17.6% PHA on VSS basis). Finally, biomass from the selection reactor was inoculated in an accumulation reactor where a maximum PHA content of 45% on VSS basis (37% on TSS basis) was obtained using a low nitrogen feed mixture. Using the liquid fraction of fermented ROM as substrate in the PHA-accumulation phase resulted in reduced PHA production likely due to inhibition from high ammonia concentrations. Overall, this study demonstrates the feasibility of using ROM as a substrate for PHA production, although further process optimization and incorporation of nutrient recovery should be investigated in order to maximize PHA production and improve process efficiency.

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