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PHA ACCUMULATING BACTERIA SELECTION IN A SBR TREATING FERMENTATION LIQUIDS OF ORGANIC FRACTION OF MUNICIPAL SOLID WASTE

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In appreciation for their help, say thank you to Joan Mata for supervising my work, Joan Dosta for sharing excellent knowledge, my lab-mates for their help and support during difficult moments, Marc for training me into laboratory skills and, finally, Raúl and Ana for teaching me, supporting me, and guide me as a researcher.

Contents

ABBREVIATIONS

Polyhydroxyalkanoates (PHA)
Sequencing Batch Reactor (SBR)
Mixed Microbial Strains (MMS)
Organic Fraction of Municipal Solid Waste (OFMSW)
Fermented Liquid Organic Fraction of Municipal Solid Waste (FL – OFMSW)
Volatile Fatty Acids (VFA)
Dissolved Oxygen (DO)
Hydraulic Retention Time (HRT)
Sludge Retention Time (SRT)
Organic Loading Rate (OLR)
Nitrogen Loading Rate (NLR)
Volatile Solids (VS)
Volatile Suspended Solids (VSS)
Total Solids (TS)
Total Suspended Solids (TSS)
Feast and Famine (F/f)

ABSTRACT

Due to their biodegradability, biocompatibility, chemical-diversity, and being manufactured from renewable carbon resources, PHAs are considered one of the most promising biopolymers to serve as an alternative to synthetic thermoplastics.

In this study is carried out the selection and accumulation of PHA accumulating biomass in two different reactors: a SBR, for selecting bacteria applying a selective pressure based on a carbon excess (feast) and limitation (famine), and an accumulation reactor, operated in batch, for maximizing PHA production. FL-OFMSW was the source of carbon and nutrients which enabled the integrated valorization of waste streams into valuable products. This substrate is composed by a mixture of VFA and N-NH_4^+ ($8 - 9$ g VFA/L and $2 - 4$ g N-NH_4^+/L) and solid fraction. Sludge was collected from a secondary activated sludge system from a local wastewater treatment facility. The objectives for this work were to select PHA accumulating bacteria, to maximize PHA production, to promote nutrient removal from the effluent while PHA production was happening and to study FL-OFMSW affection into PHA accumulation.

The F/f ratio obtained during experimentation was around 0.19 - 0.27 which ensure PHA accumulating bacteria selection. Influent characterization (VFA concentration) is essential for maintaining an optimal ratio for PHA accumulating bacteria selection. Maximum PHA accumulated per gram of TSS was 7.9% in the selection reactor and 37% in the accumulation reactor. Both results were obtained with synthetic mixture of acetic acid which suggested a possible affectation of the FL-OFMSW for PHA accumulation. Among the different possible limiting factors, ammonium concentrations seemed to be the most probable cause. Nutrient removal in the SBR reactor was possible controlling pH during the operation. Basic pH values (over 8.3) could promote high free ammonia concentrations in the rector which inhibits, directly, nitrifying activity.

FL-OFMSW can be used as substrate for the process, nevertheless, due to its nutrient concentration, PHA accumulation and nutrient removal could be limited. For this reason, nutrient removal must be considered for increasing the overall yield of the process.

1. INTRODUCTION

Waste is unwanted or unusable materials formed by any substance which is discarded after primary use, or it is worthless, defective and of no use. Among the different types of wastes, organic waste is one of the most valued materials because of the benefits it gives on waste management. Organic waste is a biodegradable waste that can be broken down into carbon dioxide, water, methane or simple organic molecules by micro-organisms and other living things using composting, aerobic digestion, anaerobic digestion or similar processes. Heat, electricity and fuel are the most important applications obtained from de degradation of organic waste. Nevertheless, the integrated valorisation of organic waste streams, in particular of agro food by-products, effluents and waste, with the production of value added fine chemicals, materials, biofuels and water is a new and challenging development (Fava et al., 2015).

Among the different products that can be obtained from the organic waste stream valorisation, stands out the production of biomaterials from microorganisms with biodegradable properties.

Biopolymers or organic plastics are a form of plastics derived from renewable biomass sources that come, generally, from the integrated valorisation of the organic waste and differ from fossil fuel plastics which are derived from petroleum. Biopolymers provide the dual advantages of conservation of fossil resources and reduction in CO_2 emissions, which make them an important innovation of sustainable development (E. Bugnicourt, 2014). Organic plastic production has been increasing during the past years; in 2013 global biopolymer production capacity for biodegradable plastics was estimated to be 591,000 t and, in 2019, it is expected to increase up to 1,287,000 t (Institute for Bioplastics and Biocomposites).

Due to their biodegradability, biocompatibility, chemical-diversity, and being manufactured from renewable carbon resources, PHA are considered one of the most promising biopolymers to serve as an alternative to synthetic thermoplastics.
PHA is a family of naturally-occurring biopolyesters synthesized by various microorganisms (30% of soil-inhabiting bacteria, Wu et al. 2000) made up of 600 to 35,000 (R)-hydroxy fatty acid monomer units. Each monomer unit harbours a side chain

R group which is usually a saturated alkyl group. Depending on the total number of carbon atoms within a PHA monomer, PHA can be classified as either short-chain length PHA (scl-PHA; 3 to 5 carbon atoms), medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms), or long-chain length PHA (lcl-PHA; 15 or more carbon atoms). PHA are intracellularly stored and serve as energy, carbon and reducing power reserves (Giin-Yu A., 2014).
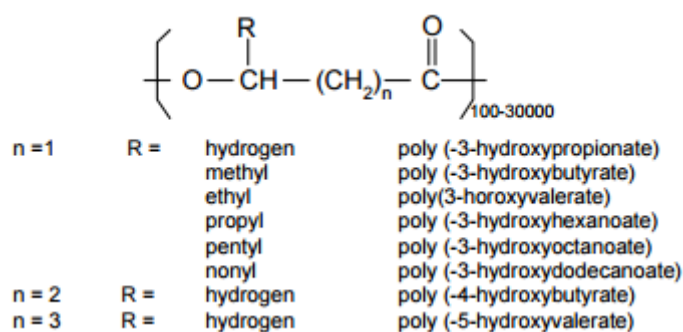

n = 1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figure 1 General structure of a Polyhydroxyalkanoate molecule (G.-Q. Chen, 2014).

Some of the general characteristics of PHAs are: water insoluble and relatively resistant to hydrolytic degradation, good ultra-violet resistance but poor resistance to acids and bases, soluble in chloroform and other chlorinated hydrocarbons. Also, It is biocompatible and hence suitable for medical applications, sinks in water facilitating its anaerobic biodegradation in sediments, nontoxic and less 'sticky' than traditional polymers when melted (G.-Q. Chen, 2014). Some studies revealed that PHAs are very similar to those of polyethylene (PE) and polypropylene (PP). Moreover, copolymers of the PHAs poly(hydroxybutyric acid) (PHB) and poly(hydroxyvaleric acid) (PHV) are far less permeable to oxygen than are polyethylene and polypropylene. This makes PHA copolymers a better material for food packaging because there is a reduced need for antioxidant addition (Salehizadeh & Van Loosdrecht, 2004).

Presently, industrial processes for PHA production are based on the use of pure cultures of selected strains (most of them genetically modified, (Setiadi, Aznury, Trianto, & Pancoro, 2015)). Unfortunately, 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, MMS coming from waste appears to be a promising alternative because it does

not require maintaining sterile conditions and it makes it easier to use low-cost feedstocks (Salehizadeh & Van Loosdrecht, 2004) (see Annex, 7.1).

There are well-known waste substrates for PHA-storing microorganisms such as wastewater from municipal and industrial sources or OFMSW. Nonetheless, when using real wastewater experimental results have indicated that PHA production is primarily associated with the VFA fraction of the wastewater. This correlation is based on the fact that the VFA carbon fraction serves as a PHA precursor and efficiently enriches for a PHA accumulating community (Albuquerque, Torres, & Reis, 2010; Morgan-Sagastume et al., 2015). Other (non-VFA) carbon compounds seem to select for a non-PHA-producing microbial community that solely grows. So, apart from applying the appropriate conditions for enrichment, and thus selecting the microorganism with the highest PHA storage capacity, a key factor for the process productivity is the inflow that is used and what part of this stream can serve as a PHA-precursor (E. Korkakaki, Loosdrecht, M.C.v., Kleerebezem, R.,, 2016).

In this work, PHA-storing microorganisms are fed with fermented OFMSW as a source of carbon and nutrients. The composition of the OFMSW strongly depends on the place and time of collection for a specific municipality or area and, generally, is composed by food rest and some inorganic components.

PHA production from mixed cultures with OFMSW is accomplished by a sequence of operations (see Figure 2), working continuously, which are the following:

(i)   An acidogenic fermentation to produce VFA from organic waste. This inflow is composed by a mixture of VFA, nutrients and solid fraction. After fermentation, OFMSW fermented liquor is filtered and will be the feed for selecting PHA accumulating bacteria.

(ii)   Selection of PHA storing biomass in a sequencing batch reactor (SBR) using VFA as a source of carbon; The most accepted process to select the microbial populations with enhanced capacity to store PHA is the Aerobic Dynamic Feeding (ADF) or Feast and Famine process, in which the MMCs are subjected to an ecological selective pressure caused by continuous shift

of external substrate excess (feast) and limitation (famine). Bacteria with PHA synthesis ability survive famine phases well on their intracellular PHA when external carbon source is limited, outcompeting organisms without this capability. When non-PHA accumulating bacteria diminish, substrate uptake is mainly driven to PHA storage (Z. Chen et al., 2015) (see PHA production under Feast and Famine strategy, Annex).

(iii)   PHA accumulation and maximization in a 24 hours batch process with a "feed on demand" strategy based on high respiration rates of oxygen for substrate uptake.
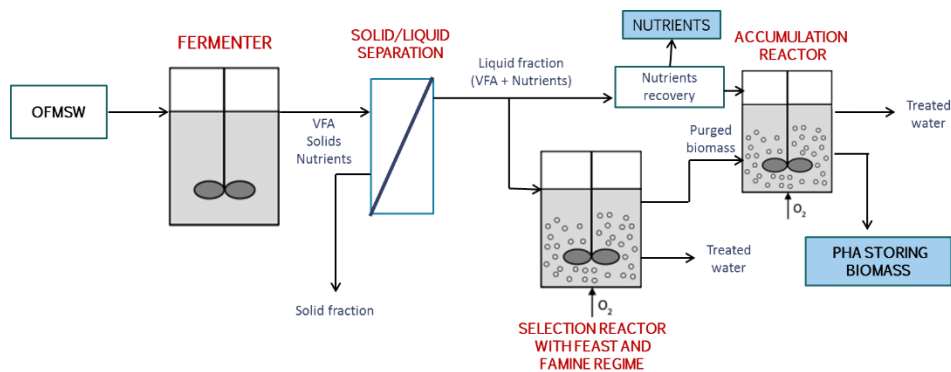

Figure 2 Laboratory operations for PHA production. After the fermenter, a solid/liquid separations gives the liquid fractions composed mainly by VFA and nutrients. This liquid is going to be the feed in the selection reactor where PHA accumulating bacteria will be selected with a selective pressure based on carbon excess and limitation strategy. Finally, selected PHA accumulating biomass will be enriched in an accumulation reactor for obtaining maximum PHA production.

The overall process provides treated water, a residual solids fraction, PHA storing biomass and nutrients. The residual solids fraction could be used as substrate for anaerobic digestion and recovered nutrients (mainly phosphorous and nitrogen) could be employed as fertilizers closing a life cycle started with the integrated valorization of organic waste streams.

1.1 Objectives

The objectives of the present work can be summarized as the following:

- To select PHA accumulating biomass using FL-OFMSW as a source of carbon and nutrients and applying a feast and famine ratio for selecting PHA accumulating bacteria.

- To introduce a strategy for nutrient removal while selecting PHA accumulating biomass.

- To study the involvement of the FL- OFMSW on PHA accumulation in the selection and accumulation reactor, in essence, investigating the effect of: the VFA variability versus synthetic mixtures of VFA and high-ammonium concentrations in the FL-OFMSW.

2. MATERIALS AND METHODS

2.1 Experimental Set Up

The experimental set up consisted in two reactors: selection and accumulation reactor.

The selection of PHA accumulating bacteria was developed in an aerobic reactor with sequential operating cycles consisting of: feeding, reaction, biomass purge, sedimentation and effluent purge. During the process, aerobic and anoxic periods are alternated to promote the degradation of organic matter and, also, nitrification and denitrification processes.

The installation in the selection reactor consists in:

- Reactor (3 Litters)
- Peristaltic pumps:
    - Feed
    - Effluent (hydraulic residence time)
    - Biomass purge (cellular residence time)
- Oxygen diffusers and air compressors (two of each one)
- pH probe
- Dissolved oxygen probe connected to the computer through the program "Adam View"
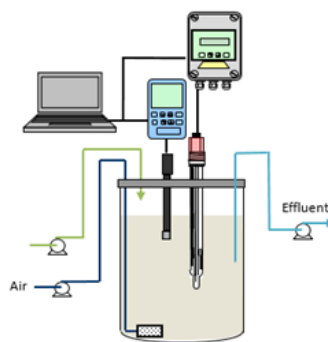- Membrane ultrafiltration (0.3 – 0.45 μm)

Figure 3 Scheme of the SBR reactor.

The accumulation of PHA was developed in an aerobic reactor operating in batch. The installation of the accumulation reactor consisted in:

- Reactor (1 Litter)
- Peristaltic pump
    - Feed
- One oxygen diffusers and one air compressor
- Dissolved oxygen probe

2.1.1 Operational conditions

Operational conditions have been divided into four distinct periods with the characteristics described in Table 1.

The first period, the longest period of experimentation, is characterized by a biomass with very little tendency for PHA production (maximum 14% in the accumulation reactor). Due to the high pH values during the period, influenced by a long hydraulic retention time, nitrogen removal from the influent was not possible due to a complete inhibition of nitrifying and denitrifying bacteria (3.1.3 Nitrification /Denitrification integration). The second period was distinguished by a decrease in reactor biomass concentration at the end of February (Figure 5). In addition to the biomass decline, oxygen profiles showed a gradual shortening of the period of feast which indicated the need for an increase in feedstock and concentration of dissolved oxygen.

During the second period, the volume of food was increased (as a result, HRT and SRT were decreased) and the number of oxygen diffusers was increased from one to two. However, even with these changes, the biomass did not recover, which led to the decision to re-seed the reactor with new biomass on 03/07/2016.

The third period was characterized by a biomass with settling difficulties (resulting in biomass through both the liquid effluent and solids purge) but with high production of PHA (up to 37%). Operational conditions during period three promoted nitrification and denitrification processes. During denitrification nitrogen bubbles were causing bacteria flotation which led to start the fourth period with the same general configuration but

with a different aerobic and anoxic configuration (see Table 3) to promote bacteria sedimentation. Specifically, denitrification was promoted by adding an anoxic stage just after feeding, taking advantage of the volatile fatty acids to transform nitrate to nitrogen gas and avoid occurrence of this process in the final stage of the operation cycle when sedimentation of bacteria is critical.

Table 1 Description of the four operated periods during experimentation

	Period 1	Period 2	Period 3	Period 4
Dates	10/2015 to 01/03/2016	01/03/2016 to 07/03/2016	07/03/2016 to 18/03/2016	18/03/2016 to 10/06/2016
Operational days	98	7	10	84
Aeration configuration (min): aerobic/anoxic	470/10	470/10	470/10	420/60
HRT (days)	10	7.5	7.5	7.5
SRT (days)	22	15	15	15
Ratio F/f	0.20-0.27	0.23-0.27	0.27 – 0.33	0.27 – 0.33
OLR_{AGV} (g/L*d)	-	16 - 28	13 - 31	13 - 31
OLR_{NH4}^{+} (g/(L*d)	-	5 – 5.5	2.2 – 4.6	2.2 – 4-6

Table 2 describes the operational conditions during a cycle in period 1, 2 and 3. During feast conditions, VFA were converted into PHA inside the PHA storing biomass using oxygen as electron acceptor. Therefore, in famine conditions, the PHA storing biomass were able to survive using PHA as a source of carbon.

Table 2 Operation configuration in Period 1, 2 and 3

Operation	Start (min)	Final (min)	Duration (min)
Feed	0	2	2
Aerobic reaction	2	470	468
Cellular purge	360	361	1
Sedimentation	470	480	10
Effluent withdrawal	479	480	1

Table 3 shows the operation during period 4. It was carried out in the same SBR reactor, this time with nitrogen removal through nitritation/denitritation and the PHA selection process by applying a feast and famine regime. Specifically, during aerobic conditions, ammonium was oxidized to nitrate and the VFAs converted to PHA, while during the anoxic conditions, nitrate was reduced to nitrogen gas (N_2) using VFA as a carbon source.

Table 3 Operation configuration during period 4.

Operation	Start (min)	Final (min)	Duration (min)
Feed	0	2	2
Anoxic reaction	0	20	20
Aerobic reaction	20	440	420
Cellular purge	360	361	1
Anoxic reaction	440	480	40
Sedimentation	470	480	10
Effluent withdrawal	479	480	1

2.2 Analytical Methods

The analytical methods used in this work were performed according to the *Standard Methods for the examination of Water and Wastewater (American Public Health Association (APHA), 2005).*

2.2.1   Total suspended solids

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined following the reference methods 2540D and 2540E, respectively. A known volume of sample (V) was filtered through a 1.2 µm Millipore standard filter, previously weighted (P_1). Then, the filter with the TSS was placed at 105°C during 24h, afterwards in a desiccator for 10 minutes and it was weighted (P_2). TSS concentration was calculated according to Equation 1. Finally, the filter with TSS was introduced at 550°C for 150 minutes, after that in a desiccator for 10 minutes and was weighted (P_3). The VSS were calculated as shown in Equation 2.

$$TSS\ (g/L)\ = \frac{P_2(g) - P_1(g)}{V\ (L)} \tag{1}$$

$$VSS\ (g/L)\ = \frac{P_2(g) - P_3(g)}{V\ (L)} \tag{2}$$

2.2.2   Total ammonium nitrogen

Total ammonium nitrogen concentration (N-NH_4^+) was analysed with a specific ammonia electrode (pH/mV Crison MicropH 2002) following the standard method 4500-NH_3D. The method is based on the conversion of dissolved ammonium NH_3(aq) and NH_4^+-N(aq) to NH_3(g) by raising the pH above 11 with a strong base (NaOH) and a subsequent NH_3(g) diffusion through the electrode membrane. Immediately, the electrode was submerged into the sample, and provided a potential (ΔV) in mV. This potential was related to the NH_4^+-N concentration by means of a semilogarithmic expression (Equation 3), obtained from 4 standards of 5, 25, 50 and 100 mg NH_4^+-N L^{-1}.

$$Ln(NH_4^+ - N) = a \times \Delta V + b \tag{3}$$

2.2.3   Nitrates, nitrites and ammonium

When nitrogen was in an ion form as nitrate, nitrite or ammonium, it could be measured by ionic chromatography. Ionic chromatograph Metrohm Advanced Compact IC can measure the concentration of both anionic and cationic species in a liquid sample. The sample, previously centrifuged and filtered, was automatically injected into the anionic column in which anions were separated giving a response in terms of peaks. Each peak corresponded to different anionic specie, and its area was related to its concentration in the sample.

2.2.4   Gas chromatography

Individual VFAs (acetic, propionic, butyric, valeric, hexanoic and heptanoic acids) were analysed by 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). Specifically, the chromatograph oven temperature program was as follows: increase from 85°C to 110°C at 10°C min^{-1}; increase to 145°C at 15°C min^{-1}; increase to 190°C at 20°C min^{-1}, and hold 0.10 min. Injector and detector temperature was set at 280°C and 300°C, respectively. Carrier gas was helium at a rate of 36.9 mL min^{-1} and 17.6 kPa.

2.2.5   Polyhydroxyalkanoates extraction and quantification

PHA extraction was performed following the method of Lanham et al. (2013) The samples collected from the reactor were centrifuged and immediately frozen in order to stop the biological activity. Before the PHA extraction, all the collected samples were lyophilized (LyoQuest - 50 Telsar). Around 10 mg of sample was placed in a closed digestion vial together with 1 ml of chloroform.
The mixture was digested at 70°C for 3 h, thus the PHA was dissolved in the chloroform. Once the samples reached room temperature, the remaining solids were filtered and the organic phase was collected in 3 ml of methanol. PHA species dissolved in chloroform tend to precipitate in contact with methanol. Finally, the solid phase obtained was filtered through Whatman 0.45 μm membranes and weighted to calculate the percentage of PHA (%, gPHA·gSS^{-1}) in the biomass

2.3 Substrate and Inoculum

2.3.1 Substrate: OFMSW-FL

Influent in this study came from residual organic matter fermented with a low HRT (approximately 3 days), enough to promote only acidogenic fermentation. In this way, the resulting fermentation liquid is rich in volatile fatty acids and high in nitrogen. Table 4 summarizes the characteristics of the input feed to the reactor 2. The concentration of volatile fatty acids remained constant around 8-9 AGV g/L during the four periods of operation. The concentration of ammonium remained high around 4000 – 2500 ppm NH_4^+ which can be a limiting factor for PHA production as other studies revealed ([E. Korkakaki et al., 2016](#)). The VS to TS ratio provides information on the amount of organic matter in the total solids (a higher ratio VS/TS means more volatile rather than inert solids). The optimum ratio has a value close to 1, which means that the proportion of organic matter to inert material is very high. Two batches of residual organic material were collected from Ecoparc to use in this study. Because the characteristics of these two batches were not identical the proportion of organic matter in the first period was higher than in the period 4.

Table 4 Reported characteristics of the feed introduced in reactor 2.

	Period 1	Period 2	Period 3	Period 4
VFA (g VFA/L)	8.2 ± 1.7	8.7 ± 0.4	9 ± 0.45	8.9 ± 2
NH_4^+ -N (mg NH_4^+/L)	4000 - 4500	4000 - 4500	3000 - 4000	2500 - 3000
TS	5.10 ± 1.6	-	-	4.8 ± 1.2
VS	3.6 ± 1.25	-	-	3.2 ± 1
VS/TS	0.7 ± 0.02	-	-	0.66 ± 0.04
pH	5.2 – 7.2	5.2 – 7.2	5.2 – 7.2	5.2 – 7.2

2.3.2 Active Sludge

Active sludge, which was used as the inoculum for PHA selection reactor, was collected from a secondary activated sludge system from a local wastewater treatment facility.

3. RESULTS AND DISCUSSION

3.1 Operation in the selection reactor

In the following section, the most important effects during the operation in the selection reactor, will be shown and discussed focusing attention on the results obtained specifically during period 1 and period 4 which allow us to compare specifically between the selection of PHA accumulating biomass with or without a strategy for removing nutrients present in the influent.

3.1.1 Effect of feast/famine ratio and OLR_{VFA} on the selection and accumulation of PHA accumulating biomass in the selection reactor

The ratio F/f is essential for selecting only PHA accumulating biomass and, the length the feast phase, depends directly on the VFA concentration in the FL-OFMSW or the OLR due to VFA. An optimal ratio must be achieved in order to insure selection of PHA accumulating bacteria and accumulation of some amount of PHA in their biomass so that only PHA accumulating biomass will survive during the famine period.

Figure 4 shows the evolution of the ratio F/f and the measured OLR_{VFA} during period 1 and their correlation to % PHA quantification in the TSS. The OLR_{VFA} remained constant during period 1 unlike the ratio F/f that was observed to be quite variable. The first peak recorded could be related to an increase in biomass that resulted in a doubling of the biomass concentration in the reactor (see Figure 5, day 11-2). The increase in biomass directly impacted the oxygen requirements in the reactor (due to the biomass increase there was low DO), that limited substrate degradation, resulting in a significant lengthening of the feast period. After biomass concentration stabilized, the ratio F/f decreased by reducing the OLR_{VFA}. The second peak can be directly related to a increase, again, in normal values of the OLR_{VFA} which affected the bacteria's population capacity for degrading VFA. The ratio F/f was finally stabilized when the oxygen requirements of the reactor were met with the addition of an additional oxygen diffuser. These two oxygen diffusers

promote stabilization of the F/f ratio until the final day of the period 1. PHA was measured just as the F/f ratio had stabilized, insuring selection of PHA accumulating bacteria (0.19 – 0.25, ratio F/f suggested through bibliographic data (Albuquerque et al., 2010); (E. Korkakaki et al., 2016)). Maximum PHA quantification obtained during period 1 was 1.02% of PHA per gram of TSS which was lower than expected result for a selection reactor ((Albuquerque et al., 2010) obtained a maximum PHA production of 25% in the selection reactor with sugar molasses).

During the final days of period 1 the ratio of F/f decreased (famine phase increased too much) promoted by an uncontrolled death of microbial population that started on day 26-2 (see Figure 5). To overcome that problem, the OLR_{VFA} was increased from 115 mL to 168 mL in order to increase the feast phase (period 2). However, even with that change, the microbial population did not recover (see day 7-3, Figure 5) which led to the decision of re-starting the reactor with new biomass capable of accumulating more PHA in the selection reactor (that may directly affect to the PHA accumulating capacity in reactor 3) and, at the same time, having the capacity to remove nutrients from the main influent.
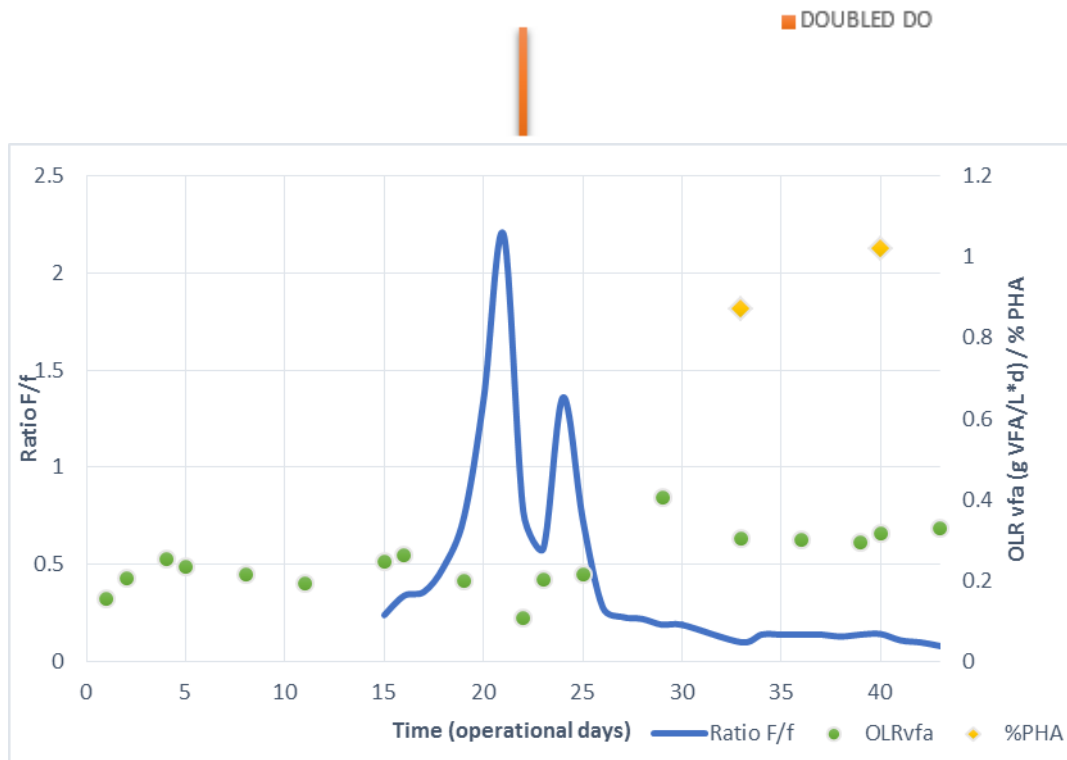

Figure 4 Evolution of the feast and famine ratio and OLR_{VFA} during period 1. In the upper graphic the day the oxygen supply was increased with two diffusers is noted.
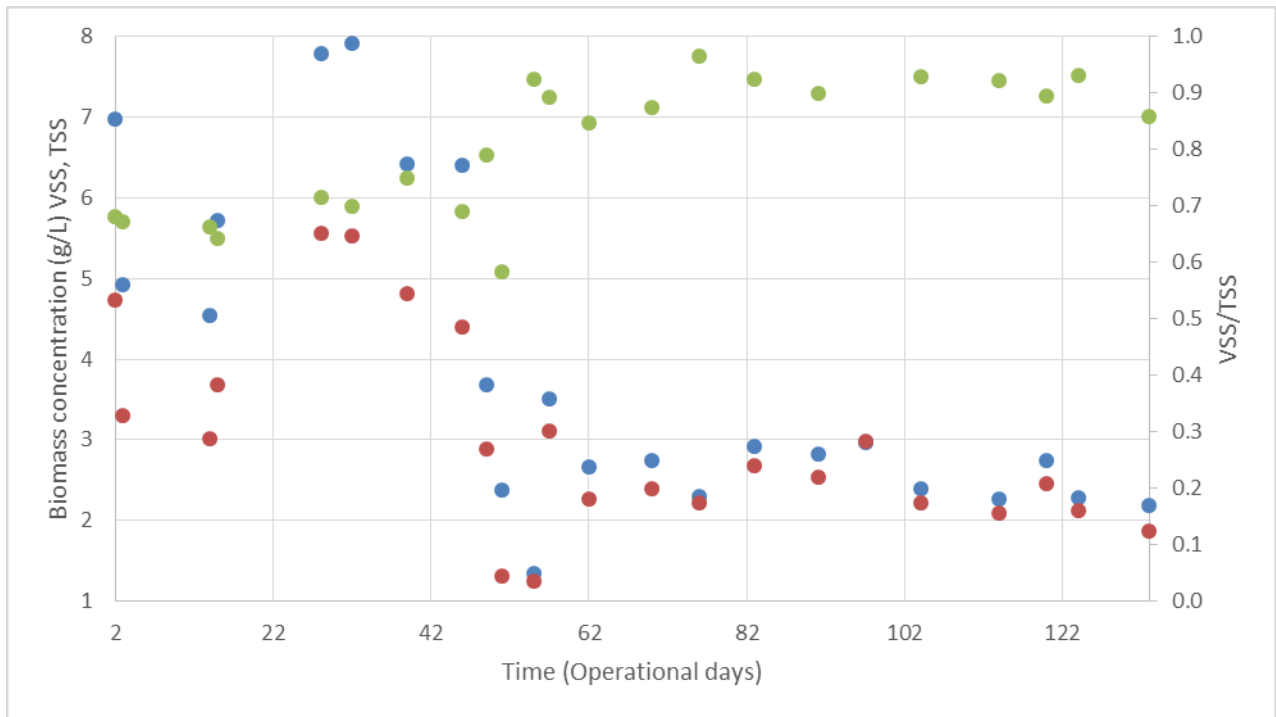
Figure 5 Biomass evolution during the four periods. TSS (Total suspended solids), VSS (Volatile Suspended Solids). VSS/TSS ratio must be relatively closer to 1 which means that majority part of the biomass is VSS which can be related to biomass and mainly to PHA.

A new start-up of the selection reactor to allowed study of the process and the effect of the FL-OFMSW as substrate in more detail. During the first days of the start-up, with a stable OLR_{VFA}, PHA was extracted and quantified in order to see the effect of the FL-OFMSW with fresh biomass. Figure 6 shows % PHA obtained during period 3. As the fresh biomass worked with FL-OFMSW, the measured % PHA decreased which could mean that FL-OFMSW as influent for the process may have a negative effect on PHA accumulation in the selection reactor and, perhaps, it could be limiting PHA accumulation in reactor 3, the accumulation reactor.
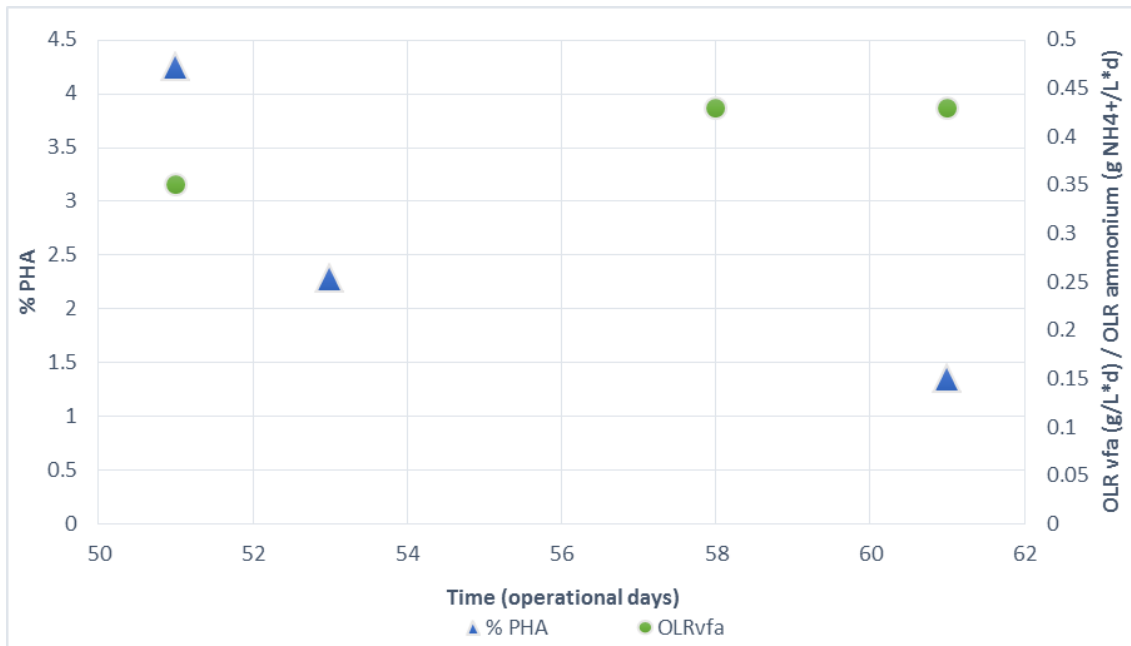
Figure 6 % PHA and OLR$_{VFA}$ during period 3 or start up with new biomass.

Figure 7 shows the evolution of the F/f ratio and OLR$_{VFA}$ during period 4. The F/f ratio was variable during the 84 days of experimentation and has been mainly changing influenced by OLR$_{VFA}$ (see day 102 or day 128). During this period the influent substrate for the selection reactor was alternated between FL-OFMSW and a synthetic VFA mixture which allowed study the effect of the FL-OFMSW to PHA accumulation on the selection reactor. Between days 62 to 81, 85 to 107 and 122 to 144 the reactor was fed with FL-OFMSW. Maximum PHA accumulated during these periods was 2.1% PHA of TSS, obtained on day 72 of operation. Between days 108 to 122 the reactor was fed with a synthetic mixture of VFA (acetic acid and water) and the maximum PHA obtained during all experimentation in the selection reactor, 7.9%, was achieved during this period. This is supporting the hypothesis that FL-OFMSW could be affecting PHA accumulation in the selection reactor.

Korkakaki et al. 2016ab, in their paper called "*PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix*" presented experimental data that confirmed that specific substrate uptake rates were significantly reduced when the biomass selection was conducted with OFMSW leachate compared to an equivalent artificial VFA mixture. This could be directly affecting to the storage capacity of the biomass making PHA production uncompetitive. Testing the different possible inhibitors, such as salt, ammonium or VFA concentration suggested

that the main inhibition most probably was caused by the high ammonium concentration of the OFMSW leachate.
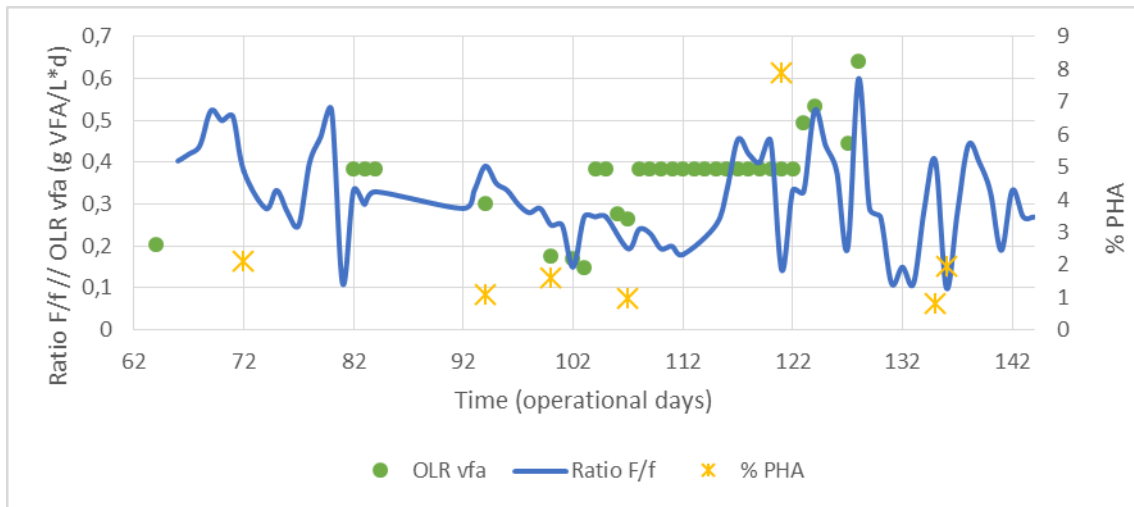

Figure 7 Evolution of feast/famine ratio depending on OLR_{VFA} and %PHA obtained per gram TSS during period 4.

Another possible explanation for the limited PHA production could be related to the biomass inability to remove ammonium from the influent. Frison et al. 2016 suggested that nitrite limitation under famine conditions could be affecting PHA production rates. Experiments confirmed that, after nitrite addition during anoxic conditions, PHA degradation rates where enhanced which could be directly affecting PHA accumulation during the next step (PHA accumulation).

Furthermore, is shown how nitrification and denitrification processes are affected, and mainly inhibited, by pH in the selection reactor which, also, could be affecting PHA accumulating capacities in the selection reactor.

3.1.2   Effect of high-ammonium streams on PHA accumulation in the selection reactor

As seen before, Korakaki et al. 2016ab, confirmed that high ammonium concentrations in OFMSW can affect the bacteria's capacity for accumulating PHA. Figure 8 shows the effect of high-strength ammonium inflows to new biomass during period 3. The graphic did not give conclusive data, but it can be seen that when ammonium

concentration increases and stabilizes, PHA production diminished to the minimum levels recorded during period 3.
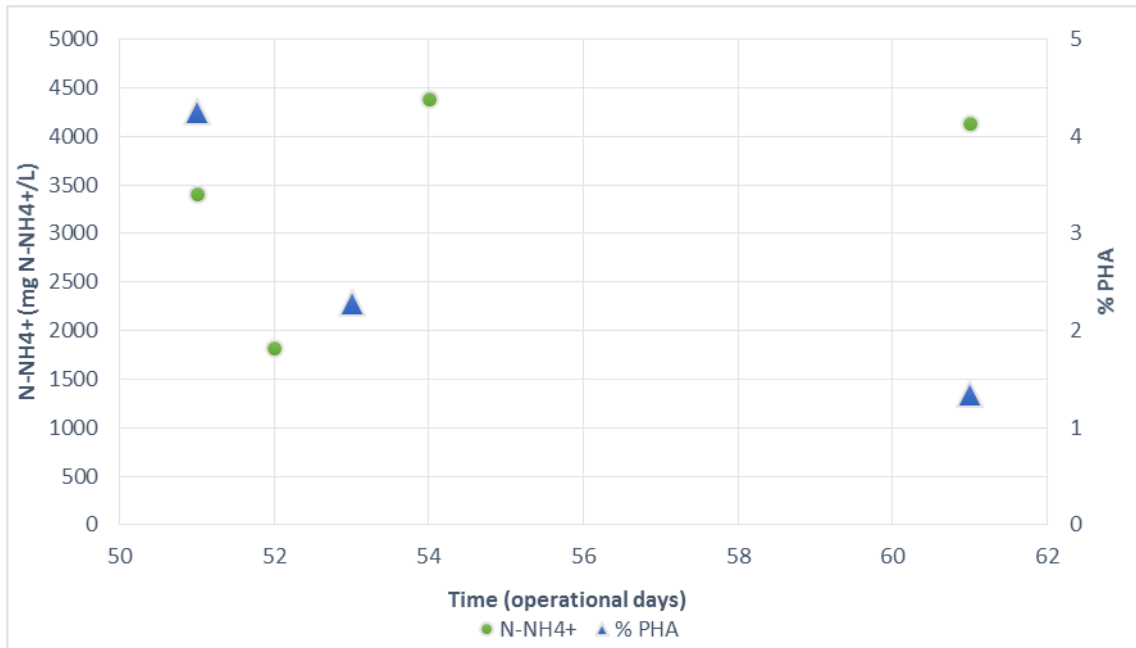

Figure 8 Effect of the ammonium concentration to the % PHA over TSS during period 3 or start-up

3.1.3 Nitrification /Denitrification integration during PHA production

Table 5 shows the ammonium concentration during different stages of an entire feed cycle in Period 1 and 3. In Period 1, only 56.4% of the NLR was removed which means that nutrient elimination was not performed. On day 11, during Period 2, operation with the nutrient removal configuration resulted in removal of nearly all the ammonium inside the reactor. Nevertheless, these positive results could be linked to the dilution of the influent during the first days due to a shortage of FL-OFMSW.

Table 5 Ammonium concentrations in period 1 and 3 during different stages of the cycle

	Period 1	Period 3
FL-OFMSW (mg N-NH$_4^+$/L)	4408	2572.50*
Inside reactor (After feast) (mg N-NH$_4^+$/L)	2093.75	737.40
Liquid effluent (mg N-NH$_4^+$/L)	1821.2	296.4
% Removal	**56.4**	**92.8**

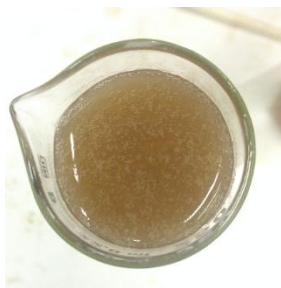*Concentration achieved with dilution

Figure 9 Nitrogen gas bubbles in the effluent tube after one 8 hours cycle. This means denitrification was happening in anoxic conditions during period 3.

Anthonsien et al. presented in 1976 a model that described the way the nitrifying bacteria could be inhibited by free ammonium in the reactor. Between 1 mg $N-NH_3$/L and 150 mg $N-NH_3$/L, Nitrite Oxidizing Bacteria (NOB) became inhibited by this compound. Over 150 mg $N-NH_3$/L, Ammonium Oxidizing Bacteria (AOB) and NOB became fully inhibited and no nitrifying activity could be possible (see Figure 10).
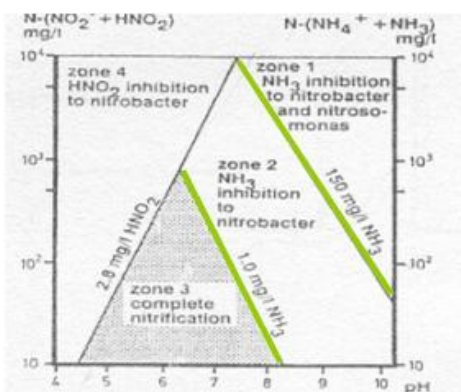

Figure 10 Anthonsien et Al. Model for nitrifying inhibition due to free ammonia

For this reason, free ammonia was calculated (see Calculations, Annex 7.2) to demonstrate that nutrient removal was not possible in the selection reactor during conditions of high pH and high ammonia concentrations in the OFMSW:

Table 6 Calculated free ammonia inside the reactor during period 1 and 4

	Free NH$_3$-N (mg/L)	
	Period 1	**Period 4**
Selection reactor	1068.4 ± 547.5	129.75 ± 10.2

During periods 1 and 2, nitrogen removing bacteria were inhibited due to the free ammonia effect. During period 1, both AOB and NOB were inhibited, were as in period 2 only NOB were inhibited. During all periods, as shown in Figure 10, it appeared that denitrification was happening in the effluent during period 3 after having exited the reactor. However, this denitrification would have only been possible via nitrite because no significant levels of nitrate were ever detected in the reactor or effluent.

3.2 Operation in the accumulation reactor

Operation in the accumulation reactor aims to maximize PHA accumulation inside the selected PHA accumulating bacteria under a strategy called "feed on demand". The biomass is collected from the selection reactor, under famine conditions during the aerobic phase and is concentrated gravimetrically by applying 30 min of settling. The objective of settling the biomass is to reduce the level of nutrients coming with the biomass from the selection SBR.

The "feed on demand" technique is based on high biomass respiration rates driving the demand for substrate. Thus the addition of substrate to the accumulation reactor is controlled via monitoring of biomass respiration. When DO levels in the reactor rise, indicating that the biomass has consumed all the available substrate, another addition of substrate is added. With this control strategy of periodically and repeatedly subjecting the biomass to conditions of "feast", the respiration and activity of PHA storing bacteria can be sustained such that they accumulate large quantities of PHA. (Valentino, 2015).

In this study, knowing the possible limitation that FL-OFMSW can have on PHA accumulation due to nutrient inhibition, two experiments were performed: PHA accumulation was investigated using first synthetic wastewater substrates and second, using real fermentation liquid as the substrate in the accumulation reactor.

3.2.1   Synthetic wastewater

The first accumulation reactor experiments were performed using a synthetic wastewater feed which consisted of 10% acetic acid and (10% filtered fermentation liquid from the acidogenic reactor). Several accumulation batch tests were performed using this synthetic feed in order to determine a baseline PHA yield that could be achieved with a low nutrient, high VFA content substrate. Table 7 summarizes the

conditions for each batch that was performed. During the experiments, the maximum % PHA in the TSS achieved was 37%.

Table 7 Operational conditions during batch experimentations with synthetic (10% real food) water

Operation Time (hrs)	Feed Interval (hrs)	OLR_{vfa} (kg COD/m³-d)	Inital Food:Microb (g VFA/g TSS)	VFAs Consumed (% of total fed)	Avg. VFA Consumption Rate (g/g VSS-d)	TSS (g/L)		VSS (g/L)		VSS/TSS		PHA %	Inoculum Age (days)	Feed Type
						Initial	Final	Initial	Final	Initial	Final			
24	4	7.30	0.88	96.7	1816.2	0.62	1.36	0.54	1.36	0.87	1.00	31	3	Synthetic
24	4	4.39	0.87	85.5	2344.8	0.42	0.40	0.60	0.56	1.43	1.40	32	1	Synthetic
24	4	4.71	--	77.3	1633.7	--	0.79	--	0.78	--	0.99	--	2	Synthetic
24	4	5.12	--	24.2	943.7	--	0.50	--	0.46	--	0.92	31	1	Synthetic
24	4	5.41	0.38	51.2	889.5	1.18	1.19	1.07	1.09	0.91	0.92	37	3	Synthetic
24	4	--	--	--	--	0.39	0.53	0.38	0.45	0.99	0.85	16	1	Synthetic
24	4	--	--	--	--	0.58	1.06	0.51	0.92	0.88	0.87	20	3	Synthetic

3.2.2 Real wastewater

The next phase of experimentation in the PHA accumulation reactor was performed using real filtered fermentation liquid as the substrate. Different dilutions of FL-OFMSW were tested, ranging from 17% to 100%, in order to assess the possible effects on PHA accumulation. Maximum % PHA accumulated per gram of TSS was 24%. This means that, as observed during experimentation in reactor 2, FL-OFMSW is limiting biomass capacity for accumulating PHA.

Table 8 Operational conditions during batch experimentations with real food (17%, 33% and 100% real food)

Operation Time (hrs)	Feed Interval (hrs)	OLR_{vfa} (kg COD/m³-d)	Inital Food:Microb (g VFA/g TSS)	VFAs Consumed (% of total fed)	Avg. VFA Consumption Rate (g/g VSS-d)	TSS (g/L)		VSS (g/L)		VSS/TSS		PHA %	Inoculum Age (days)	Feed Type
						Initial	Final	Initial	Final	Initial	Final			
24	4	9.19	3.5	80.3	2084.5	0.25	1.35	0.24	1.24	0.96	0.92	24	1	17% RF
24	4	2.39	--	--	--	--	0.87	--	0.83	--	0.95	22	1	33% RF
24	4	3.41	0.75	32.3	406.2	0.38	1.01	0.37	0.95	0.97	0.94	12	1	100% RF
24	4	--	--	--	--	0.54	1.04	0.52	0.94	0.96	0.90	22	1	100% RF
24	4	--	--	--	--	0.54	1.08	0.52	1.01	0.96	0.93	18	1	100% RF

Figure 11 shows the final results obtained from different batch experiments. Maximum % PHA per gram of TSS obtained was with a wastewater substrate mixed with only 10% of fermentation liquid (the synthetic feed mixture). Contrarily, 100% real fermentation liquid resulted in the lowest results for PHA accumulation during all experimentation.
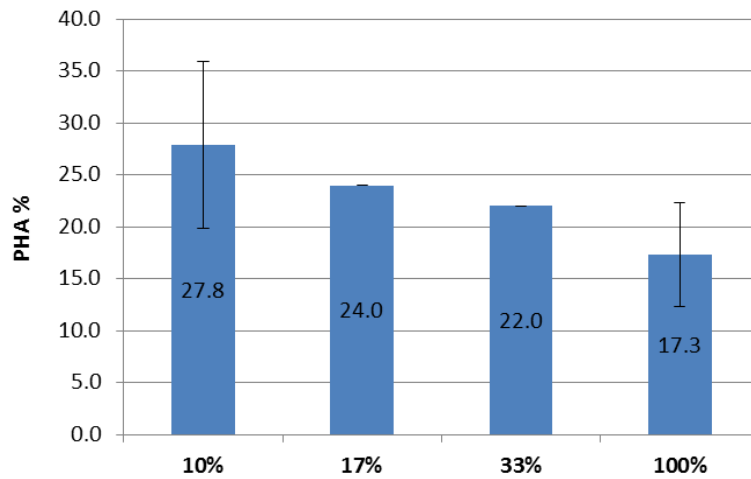

Figure 11 % PHA obtained over TSS with different percentages of real food (10, 17, 33 and 100%)

4. CONCLUSIONS

Conclusions of this work can be summarized as the following:

- Due to FL-OFMSW variability, ratio feast to famine has been changing during all experimentation. Influent characterization and control must be considered in order to maintain a stationary state while selecting PHA accumulating bacteria.

- Nutrient removal from the influent cannot be possible with high pH values. Free ammonia can inhibit nitrifying activity preventing nutrient removal. Further studies must prove if this has some implications into PHA accumulation in the selection reactor.

- Experiments with real and synthetic wastewater revealed that FL-OFMSW is limiting PHA accumulation in the selection and accumulation reactor probably due to high ammonium concentrations. Maximum % PHA per gram TSS in the selection and accumulation reactor were:

    o Selection reactor: 7% using synthetic wastewater
    o Accumulation reactor: 37% using synthetic wastewater

5. RECOMMENDATIONS

- Most of the research on ADF mode has focused on how to improve the PHA accumulating capacity through operation optimization but not on how to minimize the time necessary to achieve such an enrichment, which will significantly influence the PHA production cost.

- Selective retention of PHA-producers can be achieved by implementing a settling step at the end of the feast phase (Korkakai et al 2016).

- Recover nutrients from the influent for further uses as fertilizers. Chemical precipitation must be considered.

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7. ANNEX

7.1 Effect of substrate cost and PHB yield on the production cost of PHB (Salehizadeh & Van Loosdrecht, 2004)

| Substrate	Price (US $ kg^{-1})	Yield (g PHB/g substrate)	Substrate cost (US $ kg^{-1} PHB)
Glucose	0.493	0.38	1.35
Sucrose	0.295	0.40	0.72
Methanol	0.180	0.43	0.42
Acetic acid	0.595	0.38	1.56
Ethanol	0.502	0.50	1.00
Cane molasses	0.220	0.42	0.52
Cheese whey	0.071	0.33	0.22
Hydrolyzed corn starch	0.220	0.185	0.58
Hemicellulose hydrolyzate	0.069	0.20	0.34

7.2 PHA production pathways in feast/famine conditions (Salehizadeh & Van Loosdrecht, 2004)
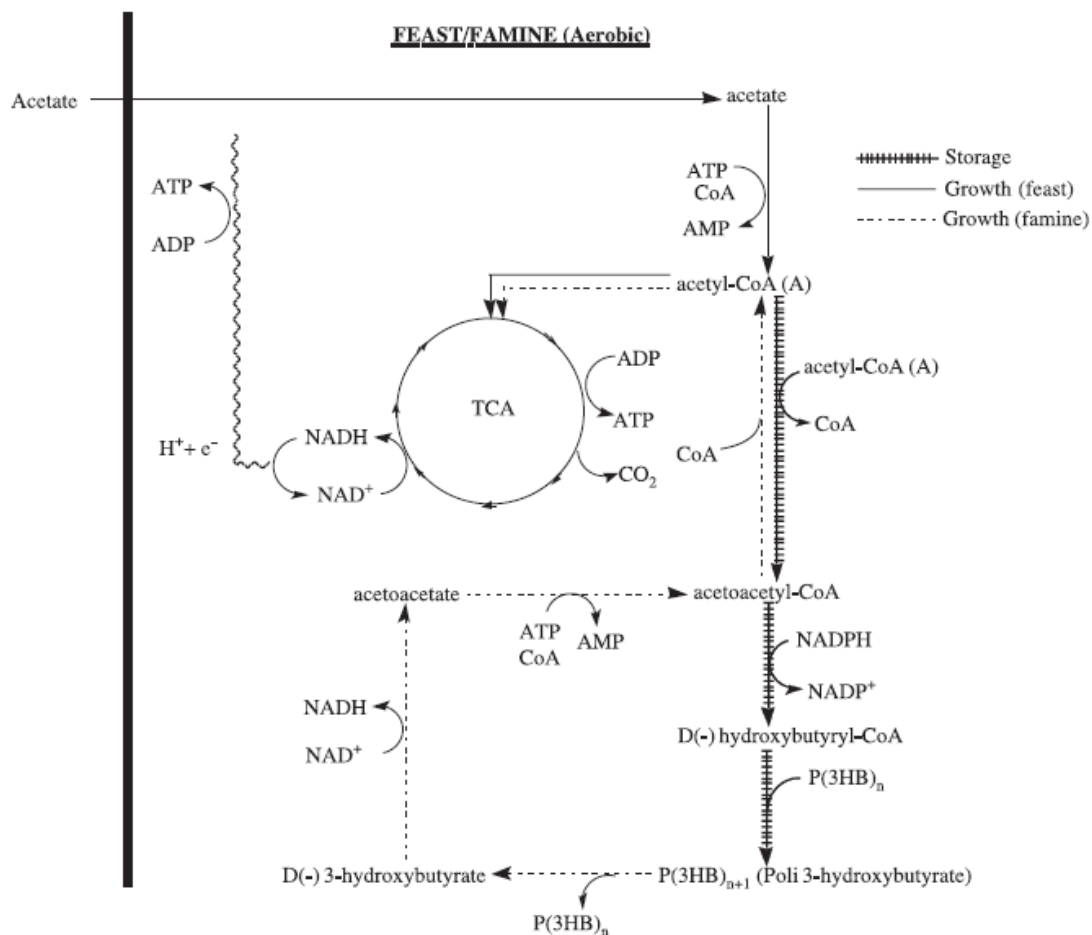
7.3 Calculations

<u>Free ammonia concentration</u>

The amount of ammonia present in the reactor can be calculated from the pH and temperature using the following equilibrium equations:

$$NH_4^+ \xleftrightarrow{K_e^{NH}} NH_3 + H^+$$

$$HNO_2 \xleftrightarrow{K_e^{NO}} NO_2^- + H^+$$
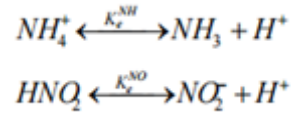
With TAN= NH3 + NH4+ and KeNH= (NH3*H+)/ NH4+ the fraction of total ammonium present as uncharged ammonia (NH3) is calculated as:

$$C_{NH_3} = \frac{C_{TAN}}{1 + \frac{10^{-pH}}{K_e^{NH}}}$$

<u>Hydraulic Retention Time (HRT)</u>

$$HRT = \frac{Reactor\ capacity\ (litre)}{Fresh\ substrate\ added\ dialy\ (\frac{litre}{day})} = days$$

<u>Sludge Retention Tiem (HRT)</u>

$$SRT = \frac{Reactor\ capacity\ (litre)}{Dialy\ cell\ purge\ (\frac{litre}{day})} = days$$

<u>Organic Loading Rate (OLR)</u>

$$OLR = \frac{Organic\ solids\ added\ dialy\ (kg\ \frac{OS}{day})}{Reactor\ capacity\ (litre)}$$
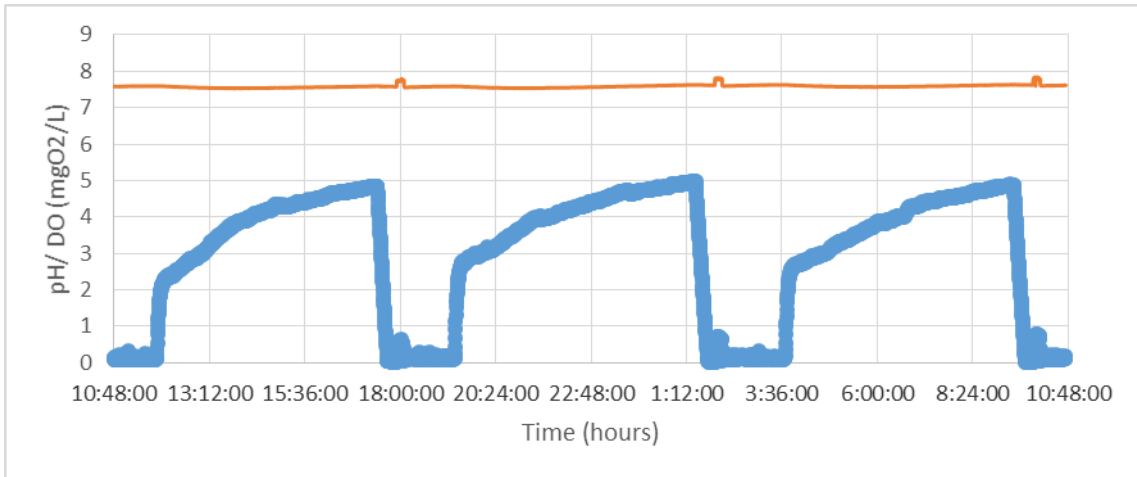
7.4 Dissolved Oxigen and pH profiles from the four representative periods

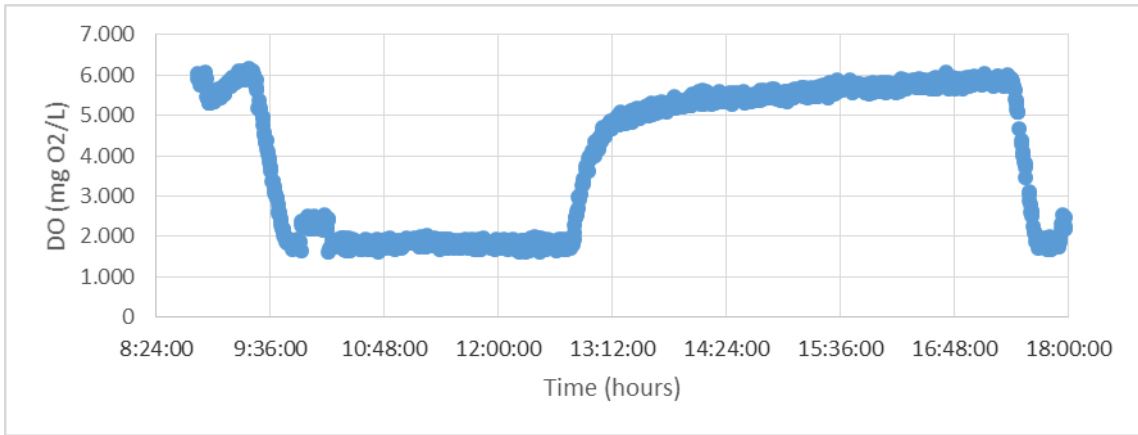Dissolved oxigen and pH profiles are an important tool for

Period 1
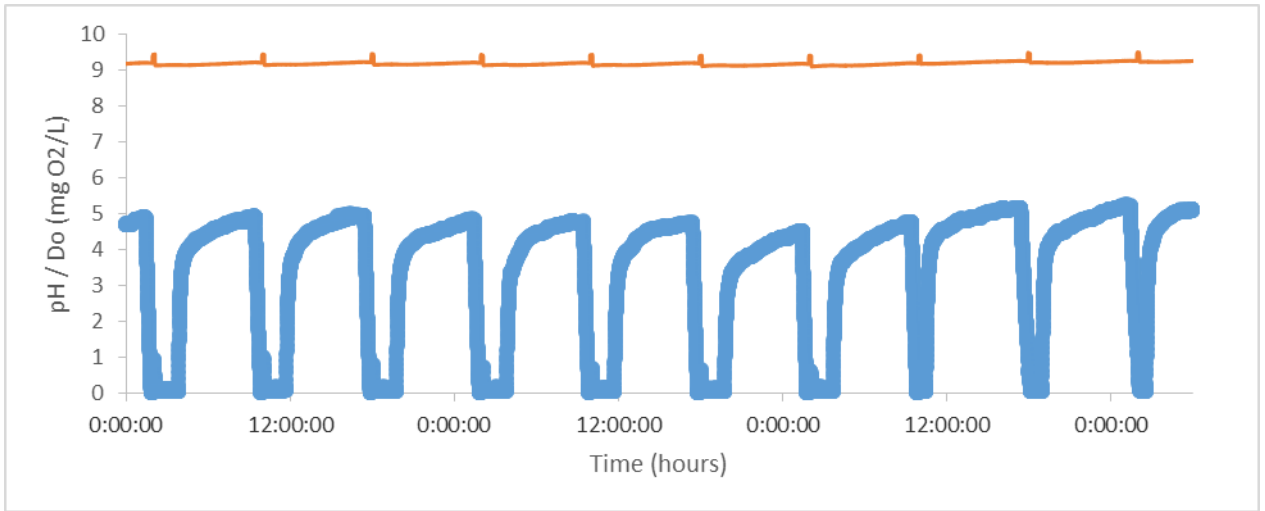
Period 2

Period 4

7.5 Laboratory Installation