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New methods for cultivation of mixed microbial cultures towards polyhydroxyalkanoate (PHA) production in aerobic sequential batch reactors.

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“New methods for cultivation of mixed microbial cultures towards polyhydroxyalkanoate (PHA) production in aerobic sequential bacth reactors”

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Dedico esta tesis a mi querido hijo Rafael.

Fuente de toda mi inspiración y deseos de autosuperación.

En todo lo que haga, siempre estaré pensando en darte un mundo cada vez mejor.

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Abbreviations

PHA *polyhydroxyalkanoate*

PHB *polyhydroxybutyrate*

PHV *polyhydroxyvalerate*

MMCs *mixed microbial cultures*

PC *pure cultures*

OLR *organic load rate*

HRT *hydraulic retention time*

SRT *sludge retention time*

VFA *volatile fatty acids*

WAS *waste activated sludge*

COD *chemical oxygen demand*

VSS *volatile suspended solids*

SBR *sequential batch reactor*

Thesis summary

Biodegradable plastics are materials highly required to replace fuel-based plastics, which are getting high environmental impacts nowadays. From bioplastic materials, polyhydroxyalkanoate (PHAs) have a great potential to be produced industrially at low costs. PHAs are polyesters developed by microbes intracellularly as an energy carbon sink, when they are faced to famine conditions. There are strategies to manipulate the production of PHAs by mixed microbial cultures when submitted to stressful feeding conditions in aerobic reactors. These conditions known as "feast and famine" allow the production of PHAs from residues such as wastewaters or industrial effluents, which leads to a decrease in their production costs. On this thesis work, the generation of PHAs and the substrates required for their production were studied. First, volatile fatty acids were produced from olive mill solid waste under acidification reactors. Results showed high acetic acid contents accumulated in biomass under alkaline (pH 9) culture conditions. In a second step, nine treatments for selection of PHA accumulating biomass were applied in a surface response methodology. Two operational conditions were contrasted which were total cycle time of operation in sequential batch reactors and substrate concentration (Acetic acid). Results showed that long cycles with high acetate inputs resulted in a higher conversion of substrate into PHAs. On a third stage, new strategies for control of operation of SBRs under variable feeding conditions were evaluated and contrasted with common feast-famine conditions in a comparative study. The new strategies favored the high accumulation of PHAs when cycles were controlled and forced to auto regulate their feeding cycles. The higher production of polyhydroxybutyrate (PHB) was reached when cycles were regulated in a 0.6 feast to famine ratio.

Thesis outline

This thesis work has been oriented in the studies of the culturing conditions required for biologic production of VFAs and PHAs from mixed microbial cultures. The study was carried out from a macroscopic viewpoint in which the microbial communities were selected in bioreactors submitted to controlled environmental conditions and their metabolic responses were observed trough variations in their growth and compound accumulation responses.

The first chapter of this thesis includes a general overview of the main issues related to research in PHAs. Reader is introduced from the concepts associated to PHAs, understanding their metabolic routes, culture alternatives for PHA accumulating microorganisms, and variables associated to the culture conditions and drawbacks.

In the second chapter, a study for the generation of VFAs, which are substrates necessary for the generation of PHAs, was carried out. This study was developed as an alternative for the revalorization of olive mill solid wastes from olive oil industry, in the form of PHAs. Several aspects about the requirements for VFAs as substrates destined in the generation of PHAs were also detailed.

Consequently, the third chapter entered directly into the study of the culture conditions necessary to stimulate the production of PHAs from mixed cultures, subjecting the microorganisms to diverse conditions of cultivation, which favored a pressure of selection in various magnitudes. In this chapter we directly analyzed the changes in the production costs of PHAs associated with the various cultivation conditions studied.

The fourth chapter utilized the information taken from chapter 3 to develop new strategies for control of operational conditions when variable feed concentrations were applied for the reactors. This strategy was based on the idea of real-scaling the production of PHAs from

MMCs, in which influents likely would tend be supplied in non-stable concentrations when industrial scale production would be applied.

Finally, the fifth chapter of this thesis, gives a general discussion derived from the results obtained in this study and the sixth chapter gives the final conclusions of this thesis and the perspectives reached.

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CHAPTER I

General Introduction

Chapter 1: General Introduction

1.1. PHA and bioplastics.

Polyhydroxyalcanoates (PHAs) are biologically synthesized polymers. They are polyesters of hydroxyalkanoic acids commonly stored inside cytoplasm of many microorganisms (Khanna and Srivastava, 2005). The biologic synthesis of PHAs in nature is performed by some microorganisms, in presence of a high carbon source. They use PHAs as a way to provide carbon and energy when external substrates are unavailable in their environment. Therefore, PHA synthesis in cells represents a biologic evolutionary strategy to enable survival in environments with discontinuous nutrient supply (López et al., 1995). PHAs are molecules condensed in intra-cytoplasmatic cell granules and their chemical structure is based in an esterified polymer with a variable alkanoic secondary aliphatic chain composed by at least 1 carbon (Figure 1). According to the length of the aliphatic chain, two PHA types have been classified. Short-chain length (SCL) PHAs consisting of 3–5 carbon atoms, and medium-chain length (MCL), consisting of 6–14 carbon atoms (Steinbüchel and Valentin, 1995)

The differences in the chain length of these polymers are based on the feeding strategy and substrate used for enrichment. Different monomer composition leads to differences in the PHA physical properties like ductility, brittleness and heat resistance (Daly and Hayward, 2004).

Even though many PHAs have been described in the literature, the ones being produced by bacteria are just few of them. So far, only PHB, copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHB-co-PHV), copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB-co-P4HB), and copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHB-co-PHHx) have been reported to be produced at industrial scales (Chen, 2009). Many other PHA forms have been observed in laboratory scale cultures, but in such lower amounts that no industrial application can be performed with their use (Lee, 1996). The internal production and

degradation of PHAs is a strictly regulated process in cell metabolism. Therefore, the study of different biological pathways into the conversion of energy into PHAs requires detailed attention.

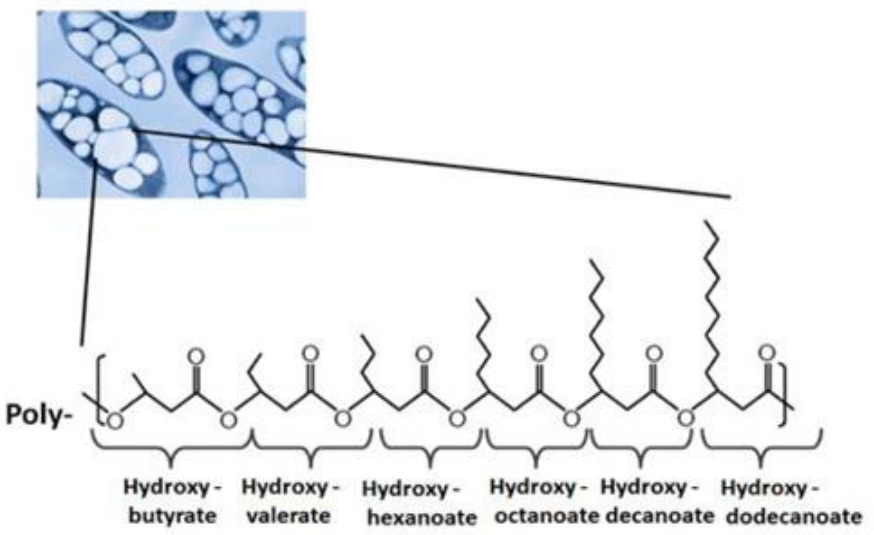

Figure 1. Schematic chemical structures of PHA polyester chains in cell granules.

1.2. Biochemical pathways towards bacterial PHA synthesis.

Principal pathways for the PHA formation in single bacteria have been described and reviewed in the literature (Khanna and Srivastava, 2005; Leong et al., 2014; Luisa S. Serafim et al., 2008a; Taguchi, 2012) Volatile fatty acids (VFAs) such as acetate, propionate, butyrate, valerate and lactate are the most studied compounds which lead to PHA generation. PHA synthesis is mainly based on the tricarboxylic acids cycle (for VFA) and β-oxidation of fatty acids (Khanna and Srivastava, 2005). A general scheme of the PHA formation and degradation route is shown in Figure 2. For PHB formation from acetate, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by the enzyme β-ketothiolase which is then reduced by the enzyme acetoacetyl-CoA reductase into 3-hydroxybutyryl-CoA in expense of NADPH oxidized. Similar behavior has been observed with propionate as carbon source, in which two propionyl-CoA can condense in 3-hydroxy-2-methylvaleryl-CoA (by the enzyme β-ketothiolase) being transformed directly into poly-3-hydroxy-2-methylvalerate (P(3H2MV))

(Luisa S Serafim et al., 2008). When acetate and propionate are present in the substrate (i.e. fermented VFAs), the metabolism can produce poly-3-hydroxyvalerate (PHV) or poly-3-hydroxy-2-methylbutyrate (PHMB) by the condensation of acetyl-CoA + propionyl CoA. In case of other VFAs like butyrate and valerate, direct PHB and PHV can be produced respectively but β-oxidation is previously required. For studies with glucose as substrate, the metabolic product is always glycogen through the tricarboxylic acids cycle based on acetyl-CoA (Dircks et al., 2001).

The polymerization of all mentioned intermediates ((R)-3-hydroxyacyl-CoA substrates) is performed by PHA synthase enzyme. This enzyme releases the Coenzyme A group from the intermediate and add one monomer to the developing polyester chain (Rehm and Steinbüchel, 1999). Rehm, 2003 found that the genetic specificity of PHA synthase is directly related to the specific PHA monomer composition. In this research a wide diversity of 59 genes encoding for the expression these enzymes were found from different PHA accumulating bacteria. This shows a wide variety of synthases in the nature.

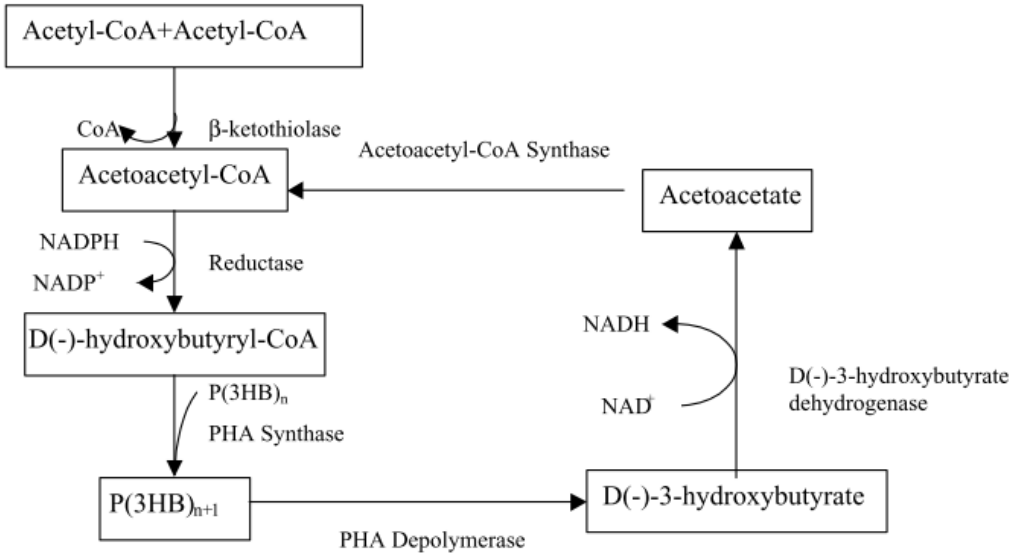

Figure 2. Metabolic pathway involved in the synthesis and breakdown of PHB.

The genetic machinery behind PHA metabolism is being increasingly explored. PHA granules in cells are complexly organized subcellular structures with catabolic, structural and regulatory functions (Jendrossek, 2009). The current knowledge of the biochemical functions of PHA-associated proteins indicates that granules are composed of important proteins as polymerases, phasins and depolymerases, also called as "PhaPs" in their surface layer, which determine their morphology and structure. Authors indicate that PHA granules can be indeed classified as organelles named as "carbonosomes"(Jendrossek and Pfeiffer, 2014).

Respect PHA degradation metabolism, two mechanisms have been proposed: intra and extracellular routes (Jendrossek and Handrick, 2002). In most bacteria, PHA depolymerase that is encoded by phaZ gene performs the depolymerization of PHA to hydroxybutyrate monomers. It has been described that intracellular PHA depolymerases degrade PHA granules in order to mobilize PHA in the absence of a suitable exogenous carbon source or energy source (Rehm, 2003). On the other hand, extracellular PHA depolymerases are secreted in saprophytic organisms in order to utilize PHAs left in the environment after the lysis of cells in which the compounds were previously accumulated (Jendrossek and Handrick, 2002).

1.3 Biological diversity of PHA producers

There is a high diversity of PHA accumulating microorganisms that have been described in literature (Chen et al., 2011). Few bacteria have been well studied for PHA production at industrial scale like *Ralstonia eutropha*. This microorganism is able to accumulate more than 80% of their dry weight as PHA (Brigham et al., 2012). *Escherichia coli* has been extensively studied by genetic manipulation in order to obtain high amounts and diversity of PHA. The recombinant *E. coli* expressing genes from *R. eutropha* resulted in a higher level of poly-3-hydroxybutyrate production compared to the wild-type *R. eutropha* H16 strain (Zhang et al., 2015). There also have been reported copolymers of P(3HB-co-3HV) using genes encoding for PHA synthase from *Salmonella enterica* (Wong et al., 2008) and medium length chain PHAs have been found in recombinant *E. Coli* using genes from *Pseudomonas Aeruginsa* (Lee et al., 2000). Many species of soil bacteria from the genera Pseudomonadaceae and Rhizospheric bacteria like *Azospirillum* and *Azotobacter* have been found to produce PHA naturally under stress conditions (Kadouri et al., 2005). Recently, Moralejo-Gárate et al., 2013 found distinctive G- bacteria tetrads dominating SBR reactors fed with glycerol having a high PHA accumulation capacity under high dissolved oxygen. Moreover, Jiang et al., 2011 found the bacterial species *Plasticicumulans acidivorans* and *Thauera selenatis* predominating cultures fed with acetate and lactate. Other recent studies include PHA production from methane as carbon source when *Methilobacterium organophylum*, a bacteria from a methanogenic consortium, showed up to 57% of cell dry weight on PHB accumulation (Zúñiga et al., 2011). All these results indicate that the PHA internal accumulation is a widely expressed phenomenon in nature, and can be explored from bacteria coming from diverse natural environments. Although PHA biosynthesis is a natural process, their extraction for commercial and industrial purposes has some drawbacks that should be considered and discussed.

1.4 Commercial aspects related to PHA production

When considering PHA production industrially, there have been evaluated several advantages respect to other polymeric substances. The commercial relevance of PHAs, is based on their good properties as plastic material for a broad range of products. Moreover, they are fully biodegradable under aerobic and anaerobic conditions (Jendrossek and Handrick, 2002). PHAs provide then a sustainable alternative, when considering the toxic and hazardous effects of petroleum based polymers (Flieger et al., 2003). During 1980 and 1990s decades, potential increase in petroleum prices derived in industrial interest on bio-based plastic. However, prices didn't increase in those decades, affecting ongoing projects dealing with PHA production for bioplastics. After 2001, petroleum price began a sharp increase leading to the new scientific interest in the development on PHA, resulting in several industrial projects. Nowadays, the top companies in the PHA- business include: Metabolix Inc. (U.S.), Meredian Inc. (U.S.), Biomer (Germany), Tianjin GreenBio Materials Co. Ltd (China), and Shenzhen Ecomann Technology Co. Ltd (China) (Chen, 2009).

According to available reports, 14 Companies in 16 countries will be producing around 0.4 million tons of PHA for 2020. PHAs have been commercialized and used for packaging, food services, bio-medical, and agriculture industries (Sanz et al., 2013). Packaging is the largest consumer of PHA followed by food services. Bio-medical applications are expected to have the highest growth during the next decades (Chen et al., 2011). PHA can be used as biodegradable controlled antibiotic release system, making them ideal for bio-medical applications such as manufacturing implants, sutures, and other medical equipment (Chen and Wu, 2005). Nevertheless, the current market scenario for PHA is still in its infancy. Many research projects are in progress, but until now the demand and market applications are still low. Despite this situation, it is expected that global PHA bio-based polymers will more than quadruple their capacity between 2011 and 2020 Sanz 2013. As the industrialization process of PHA continues

to progress and manufacturing technology remains optimized, the product quality is becoming more stable. All this will drive the cost of PHA to a level that will make it a sustainable and competitive alternative for conventional polymers.

1.5 Strategies to obtain PHAs from bacterial cultures.

Since the discovery of PHA, many strategies have been performed in order to obtain a high storage capacity and high yields in cells. Indeed, culture strategy has been a well- studied topic in PHA production (Kleerebezem and van Loosdrecht, 2007). Currently, two basic strategies have been proposed for PHA production: the use of pure cultures (PC) and the application of mixed microbial cultures (MMC). Pure cultures are focused on a single-strain fast growing and high-accumulating bacteria, commonly grown in sterile medium under batch conditions. Many bacteria have been identified, characterized and genetically manipulated in order to improve PHA yields. Most experiments for PHA production based on PC have used *Pseudomonas, Ralstonia eutropha* and *Escherichia coli* species as accumulating organisms, showing high storage capacity in some cases (Chardron et al., 2010; Lee et al., 2008; Wang et al., 2013; Zhang et al., 2015). Recombinant *Escherichia coli* manipulated with genes from other natural PHA producers, has also been studied in order to obtain PHAs from different sources and wastes (Fidler and Dennis, 1992).

Other widely studied strategy for PHA generation is the use of mixed microbial cultures (MMC). MMCs are a consortium of aerobic microbial organisms, commonly selected from activated sludge systems, applying a selective pressure (Majone et al., 1999). In these systems, accumulating bacteria are naturally selected from any other organism and in a second step, the selected biomass is used to produce PHA in batch systems. Advantages of this culture strategy are well described (Dias et al., 2006a; Luisa S. Serafim et al., 2008a). The open MMC systems become an easily scalable strategy for PHA production by eliminating costs associated with sterility. Moreover, organic wastes can be effectively used as feed for PHA production with

MMC (Kleerebezem and van Loosdrecht, 2007). Therefore, PHA production using MCC has the potential to become a suitable way to recover organics and nutrients into high value products, with potentially low related costs. Biomass residues from the PHA production are a readily degradable organic material, with a relatively low nitrogen content, and can be burned directly for low cost energy generation (Luísa S Serafim et al., 2004).

1.6 Production of PHAs using mixed microbial cultures: Feast/Famine strategy.

One of the main challenges when considering PHA production using MMCs, is the development of a method for culture selection of fast-growing organisms with high PHA storage capacity (Vanloosdrecht et al., 1997). Feast/Famine is a well described strategy for selecting microorganisms in open aerobic biological systems (i.e. activated sludge) (Beun et al., 2002; Luísa S. Serafim et al., 2004a). In Feast/Famine strategy, biomass is submitted to consecutive periods of external substrate accessibility (feast) and unavailability (famine), commonly in the form of sequencing batch reactors (SBR). Bacteria present in biomass consume the substrate in a high rate on the first minutes post feeding. At the same time, PHAs start being generated inside bacteria. When substrate is depleted, microorganisms start consuming the generated PHAs (Dias et al., 2006a). The stress imposed by feast/famine cycles provokes that PHA producers likely become the only remaining species inside the system after a certain period. This selection is performed by cycles which can include feeding, full aeration, settling and withdrawal. Different authors have utilized different reactor configurations and operations to perform Feast/Famine strategies, a summary of some conditions studied is presented in Table 1. The main differences between Feast/Famine strategies are the feeding pattern (i.e. feed time, substrate composition, organic load rate, etc.), the length of the cycles and the type of aeration.

Additionally, some other dynamics have been also explored such as the aerobic/anaerobic PHA generation. This dynamic can be observed in some wastewater treatment plants where true PHA

formation has been observed (Wallen and Rohwedder, 1974). The main groups of bacteria responsible for PHA accumulation selected under these conditions have been denominated as polyphosphate accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (Lemos and Serafim, 2003; Lemos et al., 2007). In both groups of microorganisms, PHA synthesis is performed under stress conditions. In the anaerobic step, carbon substrates are taken up and PHAs are synthesized, with simultaneous consumption of glycogen. In the presence of oxygen (aerobic step), PAOs and GAOs use stored PHA for growth, maintenance and glycogen pool replenishment. PAOs are organisms able to accumulate polyphosphate and release phosphate under anaerobic conditions, gaining energy for PHA accumulation (Lemos and Serafim, 2003). On the contrary, GAOs gain energy only by glycolysis of glycogen under anaerobic conditions; no phosphate is released to the medium or accumulated inside the cells. The amount of PHA accumulated by these groups of microorganisms is generally less than 20% of cell weight which is less efficient than high-accumulating aerobic bacteria mainly found under Feast/Famine conditions (Satoh et al., 1996).

From the past two decades, the scientific knowledge about Feast/Famine strategy has increased significantly and for the purpose of this thesis work, this topic will be discussed with detail in the next pages. For a well understanding of the high potential of MMC under Feast/Famine systems, the different phenomenon behind PHA synthesis and degradation under mixed microbial conditions will be discussed in the next section.

Table 1. Different conditions applied for PHA generation from different substrates.

Substrate	Feed: concentraton	Cycle steps (min)	Reactor volume (L)	Reference
Fermented molasses	3.2 g/L	feeding: 5/ reaction:660/settling:45/ withdrawal:10	0.8	(M G E Albuquerque et al., 2011)
Acetate	0,8g/L	feeding: 13 / reaction: 203 /excess withdrawal:2 /settling:15/ withdrawal:7	21	(Beun et al., 2002)
Acetate + Glucose	0,36 + 0,36g/L	feeding: 13/ reaction:207 / excess withdrawal:2 / settling:15 / withdrawal:7	2	(Carta et al., 2001)
Glutamic acid	0,691g/L	feeding: 3/ anoxic reaction: 117/aerobic reaction: 240	1.5	(Dionisi et al., 2004)
Acetic acid + lactic acid + propionic acid	8,5-31,2 g/L	feeding: 10/ reaction:108 /withdrawal:2	2	(Dionisi et al., 2006a)
Glucose	0,4g/L	feeding: 13/ reaction:207/excess withdrawal:2/settling:15 withdrawal:3	2	(Dircks et al., 2001)
Butyric acid + valeric acid	1,2g/L	feeding: 10 / reaction: 960 / withdrawal:10	3	(Hu et al., 1997)
Acetate	8 g/L	feeding: 13/ reaction:683/ withdrawal:20	2	(Johnson et al., 2009a)
Lactate / acetate + lactate	0.3/0.4 + 1.2 g/L	feeding: 17/ reaction:683/ withdrawal:20	2	(Jiang et al., 2011b)
Acetate/propionate	1.8 g/L	feeding: 15/ reaction:630/settling:60/ withdrawal:30	1	(Lemos et al., 2006)
Glycerol	14,19g/L	feeding: 17/ reaction:1406/ withdrawal:12/ idle: 7	2	(Moralejo-Gárate et al., 2011)
Acetate	3g/L	feeding: 30/ anoxic reaction: 120/aerobic reaction: 240/settling: 60/ withdrawal: 30	4	(Wen et al., 2012)
Acetate	4g/L	feeding: 15/ reaction:615 /settling: 60/ withdrawal:30	0.8	(Luísa S. Serafim et al., 2004b)
Fermented paper mill effluents	8,7 g/L	Continuous	2	(Bengtsson et al., 2008)
Fermented olive mill effluents	8,5 g/L	Feding: 10/ aerobic reaction: 109/ withdrawal: 1	1	(Beccari et al., 2009)

1.7 PHA production with MMCs: Biological and chemical interactions in biomass.

A broad knowledge about the internal bacterial mechanisms for PHA production and degradation is currently available. In this section several elemental topics towards PHA production will be detailed, providing a better understanding of the strategy features.

As the microorganisms involved in mixed microbial cultures and their metabolisms are highly dynamic and complex to study, one of the drawbacks for this strategy is the high variability in the performance of PHA accumulating microorganisms. Therefore, it is possible to improve PHA quality and performance in mixed culture systems by the control of variable parameters, which can define selective operation criterion like the length of feast and famine per each cycle (Dionisi et al., 2001). Some of these parameters are: dissolved oxygen, pH, and ammonium concentration, and are evaluated above in detail.

1.7.1 The importance of dissolved oxygen in feast/famine.

Oxygen uptake dynamics is a crucial topic for understanding the biomass behavior inside a feast/famine reactor. As seen in any aerobic system, when biomass becomes active the oxygen uptake rate (OUR) in the reactor is high. In conditions, most of studies have recognized the end of the feast phase coinciding to a drastic decrease in the oxygen uptake rate in the moment in which substrate becomes depleted (Beun et al., 2002). For this reason, in absence of a carbon source, selected biomass stops growing and starts storing polymers as a survival strategy in absence of substrate. However, when microorganisms are storing polymers, they don't stop consuming completely oxygen, but they do it in a lower rate. This phenomenon was proved by Carta et al 2001 when stopped the stirrer in the famine phase and OUR kept constant in the reactor.

In consecutive cycles, the observation of a decrease in OUR to a constant value, is a proper way to establish the initiation of the famine phase in a reactor. OUR depends on many factors, like

substrate type, organic load rate, feeding time and initial ammonium concentration. In studies using SBRs with acetate as substrate and at low organic load rates (OLR) experiments showed a sudden OUR increase after feeding and a sudden OUR decrease after acetate depletion. Meanwhile, in reactors operating at very high OLR, this pattern was lost and OUR never decreased, because acetate did not reach a depletion value in the entire cycle (Dionisi et al., 2001). Using glucose as substrate, OUR increased abruptly and consequently decreased as well in the feeding phase (Dircks et al., 2001). A different response was observed by the use of glutamic acid as carbon source (Dionisi et al., 2004). The consumption of substrate was slow and gradual, so the OUR increased accordingly. When substrate was depleted, OUR decreased quickly. It is worth to notice that PHA production increased sharply at the same degree in which OUR increased. This indicates that in the presence of glutamic acid, accumulating microorganisms were able to grow and store at the same time, which is opposite to observed in other kinetic models (i.e. storage only can occur when growth stops) (Anderson and Dawes, 1990). Variations in aeration intensity and oxygen transference rate (OTR) were also studied by Moralejo-Gárate et al., 2013. At high air flux (60 L/h) and high stirring speed (400 rpm) experiments (high OTR), substrate uptake yield was higher and PHA production was faster than using low OTR (i.e. low air flux (29 L/h)), low stirring speed (128 rpm). In concordance with this work, defining a high KLA value in reactors leads to higher PHA productivities.

1.7.2 Nitrogen use for cell growth in feast/famine reactors.

The principal nutrient for cell growth is nitrogen and several studies have been performed about its dynamics. All studies in which oxygen is the electron acceptor are performed using ammonium as nitrogen source for growth. It is worth to notice that some studies have been performed in anoxic way, in which nitrogen gas (N2) is bubbled meanwhile nitrate is used as electron acceptor. Under those conditions, high yields have been observed, slightly lower than aerobic, but a much lower substrate uptake rate and PHA production rate than common aerobic

systems is showed (Beun et al., 2000a). Ammonium dynamic is described in the same way than OUR when applying feast and famine stages. In feast phase, ammonium is consumed in a higher rate than in famine phase, according to the biomass-needs for growth. Nevertheless, it is unclear the true performance of ammonium utilization because studies show differences according to substrate and OLR used. Studies by Beun et al., 2002 showed that 2 mM ammonium were consumed much slower than acetate (10 mM) in a period of 10.5 hours. Meanwhile, Luísa S. Serafim et al., 2004 showed that acetate and ammonium were totally consumed in a similar moment in almost all experiments (i. e. variations of C:N ratio, pH and cycle length). Even though, in one high carbon-dosage experiment, acetate was consumed almost 2 hours later than ammonium. About the response in PHA production related to the ammonium availability, two theories have been proposed: some authors postulate that high PHA yields are reached with ammonium limitation (Johnson et al., 2010), and other studies showed no changes on PHA production using different ammonium concentrations such as starvation, limitation and excess, proving no effect on PHA yields and production rates (Bengtsson et al., 2010; Jiang et al., 2012; Moralejo-Gárate et al., 2013b).

Anyway, all studies agree that biomass growth is well correlated with the ammonium availability in the system. Higher initial amounts of ammonium lead to a high growth rate in the feast phase and thus, less PHA accumulation. On the other hand, lower initial amounts of ammonium lead to a lower growth rate in the feast phase and more PHA accumulated. These behaviors have been regarded as "growth response" (i.e. high OUR in high ammonium dosage) and as "storage response" (low OUR in low ammonium dosage and consequently all energy used for PHA storage), respectively (Dionisi et al., 2006b). For growth, a constant value of nitrogen/biomass relation (N/X) was determined by subtraction of the ammonium used for growth and ammonium released by glutamic acid in famine phase. This value has been estimated to be 0.11 mg N/ mg DQO of biomass (Dionisi et al., 2004).

1.7.3 Hydrogen ion (pH) influence in biomass

As PHAs are produced using VFAs under aerobic conditions, the influence of pH on these systems is noteworthy. Studies observed the influence of pH in systems showing that hydrogen ion concentration decreases by the uptake of VFAs in the feast phase (Sugimoto et al., 1999). Therefore, as VFAs are progressively being consumed, pH raises. According to this phenomenon, several models based on fixed pH adjustment were proposed (Pratt et al., 2004). In Sugimoto et al. 1999, low yields were observed using a fixed model pH 7 adjustment in batch cultures. Another phenomenon related to pH variations is the ammonium dynamics. The assimilation of ammonium, increases the hydrogen ion concentration because its release during biomass growth and pH drops (Dias et al., 2006a). The chemical equilibrium for hydrogen ion provokes that under no controlled pH conditions, spontaneous raise of pH into values around 8.5 can be observed. The impact of pH is important for PHA yields and composition in cells. Studies with fermented VFAs showed that variations of pH in the fermentation process had a considerable influence in the PHA composition (i.e. HB, HV) during the operation, because different VFAs were formed in the fermenter (Albuquerque et al., 2007). Also, Dionisi et al., 2005 showed that pH had importance in efficiency and productivity of PHA. Extreme pH values (i.e. 4.5 and 10.5) showed a low biomass activity, while higher activity was observed in the range of 6.5-8.5. These results are in accordance with exposed by Chua et al. 2003 who proved that different experiments using pH control at different values (range 7-8) showed no significant differences on storage PHA yields and on the other hand, when no pH control was performed, a spontaneous pH raise into values around 8.5 was observed. Authors concluded that control of this parameter is not necessary when wastewaters are used as substrate. Indeed, studies by Villano et al., 2010 proved that reactors in neutral pH control produced lower PHA yields than reactors without pH control. Therefore, pH has shown to be a parameter of high influence in the fermentation process required to prepare the substrates which will be destined for PHA generation when residual wastewaters are recycled.

1.8 Volatile fatty acids as precursors of PHAs

Feast/famine has demonstrated being an efficient strategy for selection of microorganism adept for PHA accumulation. For the optimal selection procedures under feast/famine conditons, simple carbon sources, such as sugars and volatile fatty acids are commonly used (Rodriguez-Perez et al., 2018). Different proportions on the VFAs composition from substrate can lead to generation of different types of PHAs, commonly expressed as polymers of polyhydroxybutyrate PHB or polyhydroxyvalerate PHV or a mix of them (PHB-co-PHV). On the other hand, changes in OLR would provoke biomass tend to change their metabolisms from storage of PHAs (Storage response) to biomass growth (growth response), which can lead to a decrease in PHA yields and productivities (Dionisi et al., 2006c).

In order to develop feast/famine strategies in more sustainable ways, VFA production from solid and liquid wastes through fermentation pretreatments have been applied. The main studies about fermentation with different substrate and their operational conditions are summarized in Table 2. Sludge, food waste and organic fraction of municipal solid waste (OFMSW) are the three most investigated solid wastes while wastewaters generated from the agricultural, dairy, pulp and paper industries are the liquid wastes most frequently utilized for VFA production. Among them, solid wastes get higher difficulties for hydrolysis than liquid wastes although their higher organic content. As a high variability exists on the VFA production yields through fermentation, it is still unclear which type of waste is more suitable for VFA production due to the use of different operating conditions affect the amounts and composition of VFAs (Table 2). Further research is required to investigate the optimal conditions to transform solid and liquid wastes to VFA substrates.

Considering that sustainable PHA production requires a previous VFA production step, these substrates could be highly variable in terms of concentration and composition. Additionally, considering that the operational conditions for selection of PHA accumulating microorganisms

from MMCs are highly variable, the aim of this thesis work is to implement new strategies to manage microorganisms from mixed microbial cultures to the conversion of AGVs into to PHAs, and to evaluate the variable aspects of the process. On a first step, the conversion from olive mill solid waste was managed to produce VFAs anaerobically under different pH-controlled fermentations. On a second step, changes in operational conditions were evaluated for the conversion of VFAs into PHA observing the influence of these operational conditions on the selection of microorganisms and costs associated to the cultivation steps. Finally, new strategies for DO-based control of feast /famine cycles under variable feeding conditions were evaluated towards a comparison with conventional fixed-cycle feast famine strategies.

Table 2 Different solid and liquid wastes used for the production of VFAs

Type of waste	Organic content (gCOD/L)	Reactor type and operating conditions	VFA production	Reference
Solid waste				
Waste activated sludge	18.6	Batch reactor, pH 9, 35 °C, 5 d	0.298 gCOD/gSV	(Zhang et al., 2009)
	18.6	Batch reactor, pH 8, 55 °C, 9 d	0.368 g COD/g VSS	(Zhang et al., 2009)
	14.8	Batch reactor, 21 °C, 6 d	0.339 g COD/L	(Maharaj and Elefsiniotis, 2001)
	14.8	Batch reactor, 21 °C, 6 d	0.191 g COD/L	(Jiang et al., 2007)
Primary sludge	22.8	Batch reactor, 21 °C, 6 d	0.085 g COD/g VSS	(Ji et al., 2010)
	20.6	Batch reactor, pH 10, room temp., 5 d	0.06 g COD/g VSS/d	(Wu et al., 2009)
Food waste	91.9	Batch reactor, 37 °C, initial pH 5.5	8.950 g COD/L	(Elbeshbishy et al., 2011)
	146.1	Batch reactor, 35 °C, 5 d, enzymatic pretreated food waste	5.610 g COD/L	(Kim et al., 2006)
Kitchen waste	166.1	Batch reactor, pH 7, 35 °C, 4 d	0.036 g/L	(B Zhang et al., 2005)
organic fraction of municipal solid waste	347	Batch reactor, pH 4–5, 14–22 °C, HRT 4–4.5 d	0.040 g/g VS fed	(Bolzonella et al., 2005)
	196.7	Plug flow reactor, pH 5.7–6.1, 37 °C, HRT = SRT 6 d, OLR 38.5 g VS/L/d	23.1 g/L	(Sans et al., 1995)
Liquid waste				
Palm oil mill effluents	88	Semi-continuous reactor (three times feeding per day), pH 6.5, 30 °C, HRT 4 d	15.3 g/L	(Hong et al., 2009)
	30.6	UASB reactor, pH 5.2–5.8, 35 °C, HRT 0.9 d, OLR 16.6 g COD/L/d	4.1 g/L/d (as	(Borja et al., 1996)
Olive mill effluents	70.4	Batch reactor, initial pH 6.5, 25 °C, 45 d	15.6 g COD/L	(D Dionisi et al., 2005)
	37	Packed bed biofilm reactor, pH 5.2–5.5, 25 °C, HRT 1.4 d, OLR 26 g COD/L/d	10.7 g COD/L	(Beccari et al., 2009)
wood mill effluents	11.1	Continuous stirred-tank reactor, pH 5.5, 30 °C, HRT 1.5 d, OLR 2.9 g COD/L/d	42% influent	(Ben et al., 2011)
Paper mill effluents	7.7	Continuous stirred-tank reactor, pH 6, 37 °C, HRT 1 d	0.75 gCOD/gCOD	(Bengtsson et al., 2008)

Continued at next page

Type of waste	Organic content (gCOD/L)	Reactor type and operating conditions	VFA production	Reference
Paper mill effluents	26.3	Batch reactor, 15–25 °C, pH 6, 12 d	60% influent	(Jiang et al., 2012)
Cheese whey	4.5	Continuous stirred-tank reactor, pH 6, 37 °C, HRT 2.1 d	0.84 gCOD/gCOD	(Bengtsson et al., 2008)
Dairy wastewater	4.4	Continuous flow-completely mixed reactor, pH 6.8–7.2, 35 °C, HRT 0.5 d	3.1 g/L/d	(Demirel and Yenigun, 2004)
Mixture of two types of wastes				
	4	UASB reactor, pH 5.5, 55 °C, OLR 6 g COD/L/d	1.032 g/L	(Yu and Fang, 2000)
	12	UASB reactor, pH 5.5, 37 °C, HRT 0.5 d, SRT 15 d	2.07 g/L	(Yu and Fang, 2001)
Primary sludge+ WAS	22.2	Batch reactor, 21 °C, 6 d, mixing ratio 1:1 (on VSS basis)	0.11 g COD/g VSS	(Ji et al., 2010)
	15.4	Semi-continuous reactor, 37 °C, HRT = SRT 5 d	0.11 g COD/g VSS	(Ucisik and Henze, 2008)
Mix food waste + sludge	22.1	Batch reactor, pH 8, 20 °C, 4 d	8.2 g COD/L	(Feng et al., 2011)
	29	Continuous UASB reactor, pH 5.5–5.9, 18 °C, HRT 1 d, 25% food waste + 75% primary sludge	3.6 g/L	(Min et al., 2005)

Adapted from Lee et al., 2014

Hypotheses

1. The operation of acidogenic reactors under high pH conditions can improve the accumulation of volatile fatty acids (VFAs) when solid wastes are used as substrate on the way to polyhydroxyalkanoate (PHA) production.

2. The control of feeding cycles in aerobic mixed microbial reactors, through automatic regulation of feast and famine conditions, can be an effective way to obtain a selected biomass with high polyhydroxyalkanoate (PHA) accumulation capacity.

General objective:

To develop a strategy for polyhydroxyalkanoate production from mixed microbial cultures, based on the control of feeding cycles trough online observation of different parameters in sequential batch reactors (SBRs).

Specific objectives:

1. To determine the effect of pH regulation on the generation of VFAs from wastes (Olive mill solid waste), required in PHA production.

2. To evaluate the influence of operational conditions, such as cycle time and substrate concentration, in SBRs with MMCs towards the elaboration of a feeding strategy for the production of PHA

3. To implement and evaluate an operational strategy for dynamic regulation of feeding cycles in PHA selective reactors.

CHAPTER II

The accumulation of volatile fatty acids and phenols through a pH-controlled fermentation of olive mill solid waste

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Chapter 2: The accumulation of volatile fatty acids and phenols through a pH-controlled fermentation of olive mill solid waste

Abstract

This work aims to compare the use of olive mill solid waste as substrate in pH-controlled fermentation at acid (pH=5), neutral (uncontrolled, pH ≈7) and alkaline (pH=9) operating pH levels. The results obtained in this study indicate that operating pH strongly affected the anaerobic microorganisms and, hence, different target compounds could be obtained by adjusting the operating pH. Fermentation at neutral pH resulted in the conversion of 93.5% of the fed chemical oxygen demand to methane. However, fermentations at pH 5 and 9 resulted in the inhibition of the methanogenic activity. At pH 9, volatile fatty acids reached a maximum concentration of 3.69 g O_2/L, where acetic acid represented up to 79.3% of the total volatile fatty acids. Unlike volatile fatty acid production, an optimal operation of fermentation at pH 5 could allow the recovery of phenols such as vanillin.

Keywords: acidic conditions; alkaline conditions; anaerobic digestion; hydrolysis; methanogenesis.

2.1. Introduction

The population growth and intensification of industrial activity normally imply an increase in the generation of waste, a phenomenon which has created increasing concern in modern society. This creates the need to reuse, recycle and manage waste in order to mitigate the negative impacts of human activity on the environment. As a result, the potential reuse of wastes has attracted the attention of researchers and industry, intensifying the study and application of wastes as feedstocks for different processes, where value-added chemicals, nutrients and energy can be produced. This focus is strengthened when considering the biorefinery concept, in which the maximization of high-value products is not only expected, but also the minimization of final wastes released into the environment. Thus, a double benefit is attained: resource recovery and waste minimization.

Anaerobic digestion is a widely studied and applied technology which promotes waste valorization through biogas production. Anaerobic digestion cannot only produce biogas, but also valuable end-products such as alcohols and VFA (Kleerebezem et al., 2015) VFAs are short-chain fatty acids, produced as intermediate products during the anaerobic digestion of organic matter (Kleerebezem et al., 2015). VFAs have been considered as building-blocks for the production of different compounds in several applications such as the chemical industry (cosmetic, pharmaceutical and petrochemical industry), PHA production (Pagliano et al., 2017; Valentino et al., 2017), chain elongation precursors (Chen et al., 2016), microbial fuel cells (Teng et al., 2010), hydrogen production (Uyar et al., 2009), and biological nutrient removal for wastewaters (S.-J. Lim et al., 2008).

When anaerobic digestion is oriented toward VFA production, it is normally called acid fermentation or dark fermentation. It consists of controlling the operational parameters of traditional anaerobic digestion in order to increase the hydrolysis and acidogenesis steps, while

methanogenesis is inhibited. Thus, VFA formation is increased and VFA consumption for methane formation is diminished or eliminated, resulting in a net VFA accumulation. Relevant factors affecting acid fermentation for VFA production are pH, temperature, hydraulic retention time (HRT), solid retention time (SRT) and ORL (Jiang et al., 2007; Lee et al., 2014a) pH Values which have been reported to be compatible with VFA production are in the range 5.25 – 11, and depend on the type of waste used as the substrate (Lee et al., 2014b). In this sense, it has been reported that favorable conditions for waste activated sludge (WAS) digestion are found under alkaline conditions(Huang et al., 2015; Zhang et al., 2009); whereas for food waste and wastewater, acidic/neutral conditions would increase the VFA production (Oktem et al., 2006; B. Zhang et al., 2005). Different feedstocks for VFA production have been researched and applied, such as sludge (Liu et al., 2018; Yu et al., 2018), wastewaters, food waste (S. J. Lim et al., 2008)and organic matter from municipal solid waste (Bolzonella et al., 2005).

Olive mill solid waste (OMSW) is a residue from the olive industry which is produced during oil extraction from olives. OMSW has been well studied for biogas production as the substrate (Serrano et al., 2017b; Stoyanova et al., 2017) and co-substrate (Fernández-Rodríguez et al., 2014; Fezzani and Cheikh, 2010). However, little research has been focused on VFA production from OMSW through acidogenic fermentation (Rincón and Borja, 2012). OMSW is characterized by high quantities of polysaccharides, proteins, fats, polyphenols and lignocellulosic material, such as cellulose and lignin (Ouazzane et al., 2017). OMSW could be considered as an interesting substrate for obtaining valuable end-products with industrial interest, due to its high initial concentration of acids and phenols.

When considering the recovery of acids and phenols, the hydrolysis of OMSW can be a challenge due to the lignocellulose content. Recently, different pre-treatments such as steam explosion or chemical-thermal treatments have been proposed in order to enhance the

breakdown of fiber structures, which favors the release of valuable compounds for their recovery (Serrano et al., 2017a). However, these methods require high energy consumption and can result in the generation of toxic compounds, such as hydroxymethylfurfural. The control of the operational conditions during acid fermentation could increase the hydrolysis activity to release the target compounds without the mentioned disadvantages. The achievement of a sustainable solubilization method is currently one of the limiting steps for the widespread of biorefineries based on biomass from agricultural waste (Romero-García et al., 2014). Alkaline and acid pre-treatments have been widely proposed in order to facilitate the hydrolysis of lignocellulosic-containing waste (Amin et al., 2017; Hendriks & Zeeman, 2009; Taherzadeh & Karimi, 2008). However, little information can be found about pH-controlled fermentation for lignocellulosic waste and the effect of pH regulation on the accumulation of phenolic compounds.

This work aims to evaluate the obtaining of different products from the fermentation of OMSW by the regulation of the operational pH. Specifically, pH-controlled fermentation was carried out at acidic (pH=5), neutral (uncontrolled, pH≈7) and alkaline (pH=9) operating pH levels. The processes were evaluated in accordance to methane production, the VFA concentration and the accumulation of phenolic compounds. This novel approach could support the transition of the olive oil sector towards a circular economy.

2.2. Materials and Methods

2.2.1. Substrate and inoculum

OMSW was provided by the centralized management plant "Oleícola El Tejar", located in Marchena (Seville), Spain and used as substrate. OMSW was preserved at -18 ºC prior to its characterization and use in order to avoid spontaneous fermentation processes. Anaerobic reactors were inoculated with biomass obtained from a full-scale anaerobic reactor treating waste activated sludge from the "COPERO" urban wastewater treatment plant (Seville, Spain). The characterizations of both substrate and inoculum are summarized in Table 3.

Table 3. Olive mill solid waste (OMSW) and anaerobic inoculum characterization

	OMSW	**Inoculum**
TS (g/kg)	244.4 ± 1.1	41 ± 2.1
VS (g/kg)	229.4 ± 1.0	22.4 ± 1.5
Total COD (g O_2/kg)	401.2 ± 7.2	18.9 ± 1.0
Soluble COD (g O_2/kg)	86.9 ± 0.1	0.2 ± 0.1
pH	4.79 ± 0.01	7.80 ± 0.20

2.2.2. Experimental setup

Batch-fed acid fermentation of the OMSW was evaluated in duplicate under different pH conditions. 2.0 L reactors were used with 1.7 L working volume. Three different pH conditions were studied: neutral, acidic (pH 5) and alkaline (pH 9). pH 5 and pH 9 reactors were controlled by the daily addition of HCl (12 N) and NaOH (6 N), respectively. The neutral pH reactors were operated without pH correction. Figure 3 shows the measured pH during the experimental period. The reactors were continuously stirred, and the temperature was held at 35 ± 1 ºC, by placing them inside a thermostatic chamber. The reactors were equipped with three connections; one to load the feedstock; one to ventilate the biogas; and one to inject inert gas

(nitrogen) and take samples. To remove the CO_2 from the biogas, tightly closed bubblers containing a NaOH solution (3 N) were connected between each reactor and 6-L Boyle-Mariotte flask. The volume measured by the 6-L Boyle-Mariotte flask was assumed to be methane for the neutral and pH 9 reactors. The volume measured in the pH 5 reactors cannot be assumed to be only methane since a significant amount of hydrogen might be also produced at this pH (Kleerebezem et al., 2015).The volume produced at this pH was assumed to be 100% methane and it was compared to the case that 100% were hydrogen.OMSW was batch-fed into each reactor in six successive feeds of 2 g/L VS each, during a total period of 56 days. OMSW feeds were made on days 1, 7, 14, 27, 35 and 48 during the operational time. The last feed, on day 48, was done after the removal of the liquid phase through centrifugation and resuspension of the culture in water and micronutrients on day 45. This part of the experiment was developed to determine whether the accumulation of soluble compounds could limit or interfere with the further acid fermentation of the substrate.

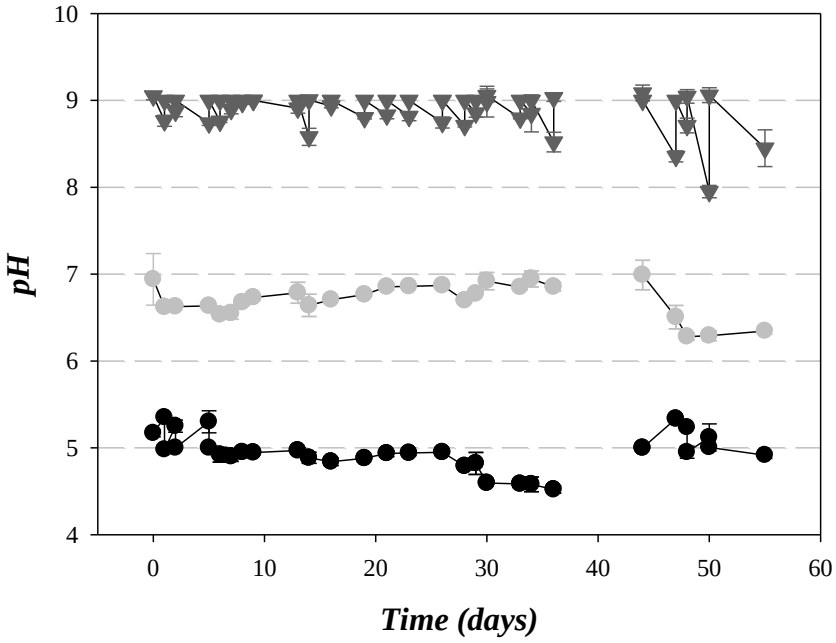


Figure 3. Measured pH over time on (●) neutral pH reactors, (●) pH 5 reactors and (▼) pH 9 reactors.

2.2.3. Analytical methods

The following parameters were determined in the OMSW and/or in the effluent of the reactors: total Chemical Oxygen Demand (CODt), soluble Chemical Oxygen Demand (CODs), Total Solid (TS), total Volatile Solids (VS), pH, and ammoniacal nitrogen ($N\text{-}NH_4^+$) concentration. All determinations were made in accordance to Standard Methods (American Public Health et al., 2005).

Individual VFA were quantified using a gas chromatograph equipped with a 15m × 4mm Nukol-silica capillary column and a flame ionization detector. The oven temperature was gradually increased from 100 to 150 ºC at a rate of 4 ºC/min. Helium (28.6 kPa), Nitrogen (28.6 kPa), Hydrogen (14.3 kPa) and synthetic air (28.6 kPa) were used as carrier gas at a flow rate of 50 ml/min.

The concentrations of individual phenolic compounds were also measured in the reactors during the experimental period. The individual phenolic compounds quantified were simple phenols, such as Hydroxytyrosol (HT), tyrosol (Ty), 3,4-dihydroxyphenylglycol (DHPG), 4-ethylphenol (4-EP), Vanillin (Va) and vanillic acid (VA). HT, Ty or DHPG are formed by the hydrolysis of complex phenolic compounds (Bartella et al., 2018; Lama-Muñoz et al., 2013). 4-EP is formed by the degradation of benzoic structures present in phenols (Serrano et al., 2017).Va and VA are formed from lignin degradation (Wang et al., 2018). Individual phenols were quantified using a Hewlett-Packard 1100 liquid chromatography system with a C-18 column (Mediterranea SEA 18, Teknokroma, 250 mm x 4.6 mm, i.d. 5 μm) and diode array detector (DAD, the wavelengths used for quantification were 254, 280, and 340 nm) with Rheodyne injection valves (20 μL loop). The mobile phase was Milli-Q water acidified with 0.01 % trichloroacetic acid and acetonitrile (A) with the following gradient over a total run time of 55 min: 95% A initially, 75% A in 30 min, 50% A in 45 min, 0% A in 47 min, 75% A in 95 min, and 95% A in 52 min until completion of the run. Quantification was carried out by integration of the peaks at different wavelengths in function of the compounds with reference to calibrations made using external standards. 3,4-dihydroxyphenylglycol, vanillic acid, vanillin and 4-ethylphenol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Hydroxytyrosol was obtained from Extrasynthese (Lyon Nord, Geney, France). Tyrosol was obtained from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany) and ultrapure water was obtained using a Milli-Q water system (Millipore, Milford, MA, USA).

2.3. Results and discussion

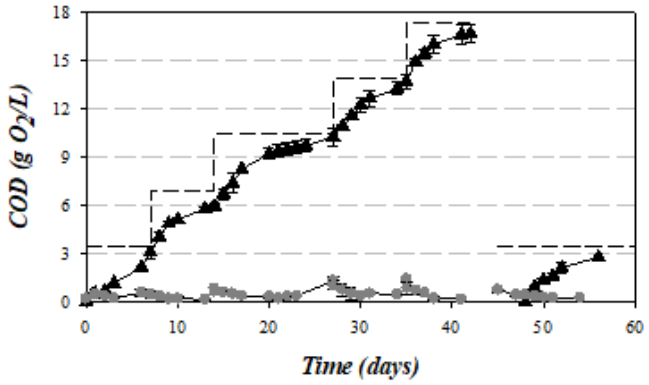
2.3.1 Methane production and COD accumulation at different pH levels

At neutral pH, most of the fed CODt from the OMSW was transformed into methane after the different OMSW feeds (Figure 4A). Methane production at the end of each feed reached an average production of 93.5% of fed CODt. During the 56 days of operation, little accumulation of CODs was observed (Figure 4A). Almost all of the measured CODs were consumed in less than four days after every feed. The biodegradability of the OMSW at neutral pH, measured as percentage of CODt converted into methane, was markedly higher than the one observed by Serrano et al. (2017), who reported a biodegradability of 57.3% for the same substrate under mesophilic conditions. The difference could be the result of the higher inoculum to substrate ratio and the low OLR applied in the present study (Borja et al., 2004).

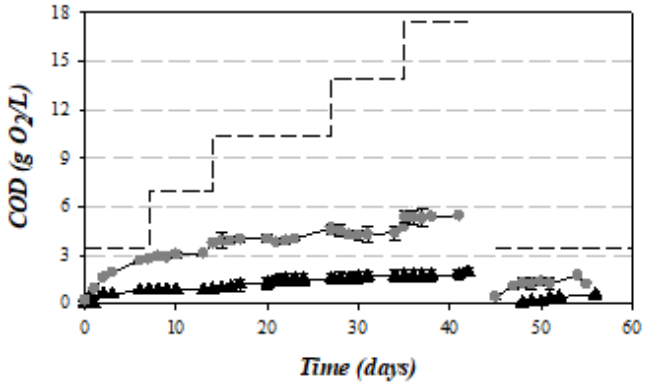
At pH 5, in the case that all the measured volume were methane, methane production at the end of each feed corresponded to an average of 29% of fed CODt (Figure 4B); whereas if all the measured volume were hydrogen, the hydrogen production at the end of each feed corresponded to an average of 14% of fed CODt. The accumulation of CODs at the end of each feed corresponded to an average of 20% of the fed CODt, and was 18.6% on day 42 of operation (Figure 4B). By the end of each feed, the accumulation of soluble COD as well as methane production corresponded to an average transformation of 50% of the fed CODt (35% of the fed CODt in the case that all the generated volume was hydrogen). That is around half of the transformation of fed CODt at neutral pH. Therefore, both hydrolysis and biogas productionwere inhibited at pH 5, when compared to neutral pH. The lower biogas production at pH 5 indicates the inhibition of methanogenic activity compared to neutral pH. The optimal pH range for methanogens is reported to vary from 6.8 to 7.8 (Wheatley, 1990). It should be taken into account that recent studies have indicated that the reduction in biogas production is

closely related to the increase in free acetic acid, rather than to the pH of operation (Zhang et al., 2018). Although Xiao et al. (2016) observed methane production even at high concentrations of acetate in acidified reactors (pH 4.8 and 8.2 g acetate/L). The observed reduced biogas production at pH 5 could explain the low, but existing, biogas production at acid conditions. As indicated, inhibition of biogas production under acid conditions increases the production of VFAs in comparison to neutral pH conditions (Feng et al., 2009). However, at pH 5 the inhibition of hydrolysis minimized the accumulation of CODs (Figure 4B), and therefore the possible production of VFAs due to a minor transformation of CODt compared to neutral conditions.

At pH 9, very low methane production was observed, corresponding to less than 15% of the fed CODt (Figure 4C). The accumulation of CODs at the end of each feed corresponded to an average of 40% of the fed CODt, and was 30.1% on day 42 of operation. Both the accumulation of soluble COD and methane production at the end of each feed reached an average transformation of 56.3% of the fed CODt. As was the case of pH 5, both hydrolysis and biogas production were inhibited at pH 9, when compared to neutral pH. The transformation of CODt at pH 9 was higher than the one observed at pH 5. The higher transformation of CODt compared to pH 5 could be related to the chemical effects of the alkaline environment, which might facilitate the breakdown of molecular bonds (Chen et al., 2007). pH 9 showed the highest accumulation of CODs during the operation, i.e. over 20% higher than at pH 5. The low methane production observed indicates that alkaline conditions strongly affected the methanogenic stage. Chen et al. (2007) observed a significant increase in CODs under alkaline pH (9, 10 and 11), with respect to pH 5 and 7, mainly due to a decrease in methanogenesis. Similar to pH 5, the inhibition of hydrolysis minimized the accumulation



A)



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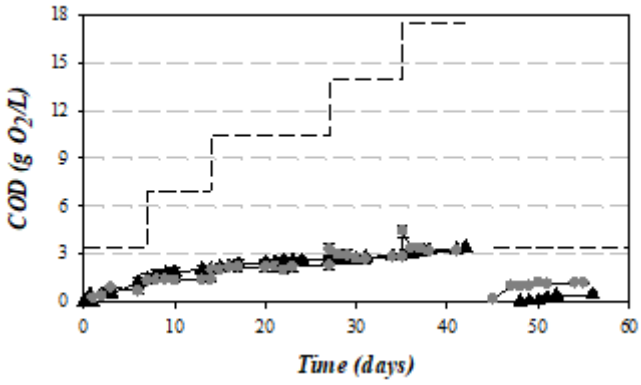


C)

Figure 4. Transformation of compounds among time in the form of COD. A) Neutral (without pH control) reactors. B) Acidified (pH 5) reactors. C) Alkaline (pH 9) reactors. Black triangles (▲), produced methane over time. Grey circles (●), CODs over time. Line (– – –), indicates accumulated CODt input over time of CODs (Figure 4C), and therefore the possible VFA production, due to a minor transformation of CODt compared to neutral conditions.

In order to study whether the accumulated CODs inside the reactors might influence the OMSW transformation process, the last feed on day 45 was performed after the replacement of the liquid phase with water and micronutrients. This was done by centrifugation and re-suspension of the culture in water and micronutrients. Methane and COD production followed the same trend as before this procedure under all pH conditions tested (Figure 4A, 4B, and 4C). At pH 9 and 5, the results indicate that the previously accumulated CODs did not affect the transformation processes. Specifically, the reactors at pH 5 transformed 50.5% of the fed CODt during this final load; whereas the reactors at pH 9 transformed 50.6% of the fed CODt during this final load.

Significant differences in residual ammoniacal nitrogen ($N\text{-}NH_4^+$) were observed at different pHs. By the end of the operation on day 56, the $N\text{-}NH_4^+$ concentrations were 115 mg/L, 216 mg/L and 653 mg/L at neutral pH, pH 5 and pH 9, respectively. The release of $N\text{-}NH_4^+$ observed at pH 9 can be related to an increase in protein degradation. These results are in agreement with previous studies, which report an enhancement in the hydrolysis of soluble compounds under alkaline pH conditions, mainly carbohydrates and proteins (Zhang et al., 2009). The $N\text{-}NH_4^+$ concentration is especially relevant due to its relation with toxic free ammonia (NH_3), which is freely membrane-permeable (Chen et al., 2008). In fact, the concentration of free NH_3 at pH 9 was 440 mg/L according to the NH_4^+/NH_3 equilibrium; whereas at neutral and pH 5, it was almost negligible. Despite the observed difference, free NH_3 concentration remained below the inhibition thresholds for mesophilic anaerobic digestion, i.e. 470-1450 mg/L (Hadj et al., 2009; Nakakubo et al., 2008). However, the high free NH_3 advises against anaerobic digestion under alkaline conditions of other substrates with higher nitrogen contents due to the risk of inhibition.

2.3.2 Volatile fatty acid accumulation in the pH 5 and pH 9 reactors.

Total VFA accumulation at pH 5 showed a constant and gradual increase, reaching a concentration of 1.69 g COD-VFA/L on day 42 (Figure 5). This total VFA content on day 42 accounted for 52% of CODs (Figure 5). A higher increase in total VFAs was observed at pH 9 compared to pH 5. VFA accumulation reached a maximum total of 3.69 g COD-VFA/L on day 42 at pH 9 (Figure 5). At this pH, total VFAs accounted for 68% of measured CODs (Figure 5). The total VFA concentration at pH 9 was twice as much as that for pH 5 (9.7% and 21.2 % of fed CODt at pH 5 and pH 9, respectively). These results are in agreement with other studies, which show how alkaline conditions can favor VFA accumulation in contrast with acidic conditions when using waste-activated sludge or food waste as substrates (Stein et al., 2017; Zhang et al., 2009). A higher production of total VFAs under alkaline conditions compared to acidic conditions was also reported by Stein et al. (2017) for the production of butyric acid from food waste. These authors reported average butyric acid concentrations of 2.06 and 8.52 g/L, for fermentations carried out at pH 5.5 and 9.0, respectively.

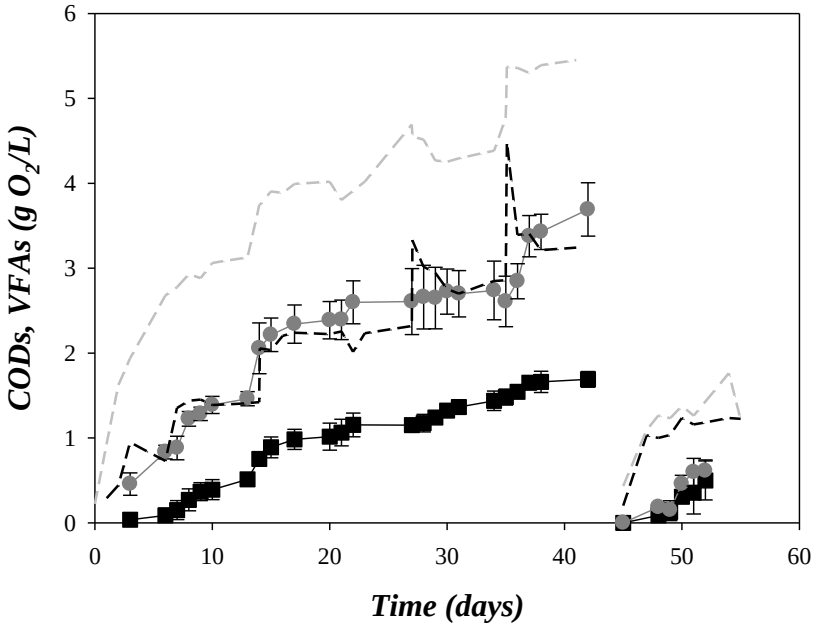
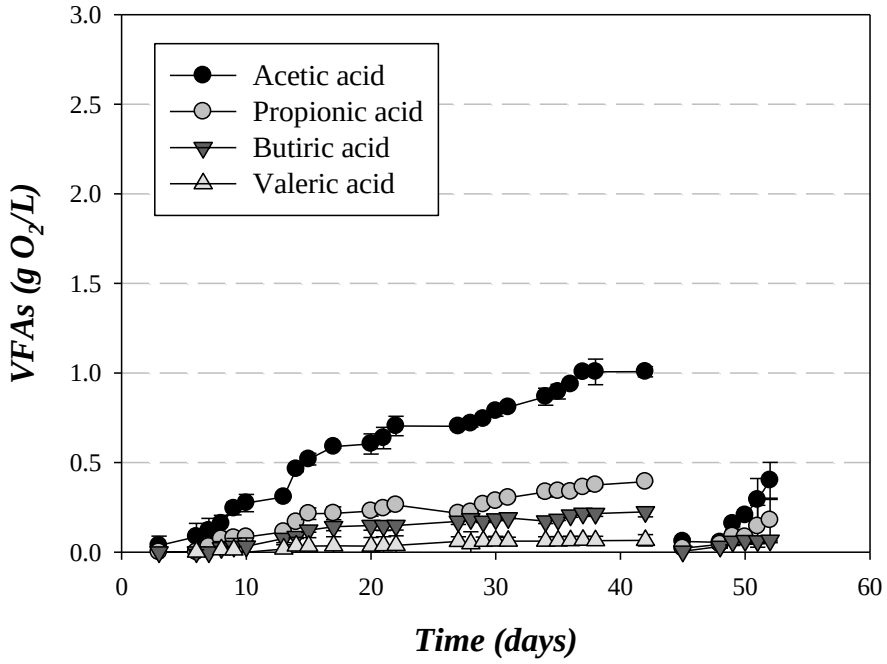


Figure 5. CODs and total VFAs accumulation at pH 5 and 9. Black squares (■) indicate total VFAs at pH 5 and gray circles (●) indicate total VFAs at pH 9. Discontinuous black line (- - -) indicate CODs in pH 5 and discontinuous gray line (- - -) indicate CODs in pH 9.

Individual VFA showed a gradual increase at both pH 5 and pH 9 (Figure 6). Acetic acid was the most relevant VFA under both conditions, reaching a maximum on day 42 of 1.01 g COD-VFA/L and 2.93 g COD-VFA/L at pH 5 and pH 9, respectively. In fact, acetic acid represented up to 79.3% of the total VFA under pH 9 conditions (Figure 6B). The marked preponderance of the acetic acid in the VFA composition at pH 9 can be related to a strong inhibition of the acetoclastic methanogens, such as *Methanoculleus* or *Methanocorpusculum*genera (Pagliano et al., 2018), but not of the acetogenesis stage, since most of the VFA were actually degraded up to acetic acid. The VFA profile at pH 5 was more heterogeneous than at pH 9 (Figure 6), with propionic and butyric acids accounting for up to 23.2% and 13.2% of the total VFA, respectively. Thus, in the case of VFA for PHA production, it would be expected that under the VFA profile obtained at pH 9, PHB production would be the main product; whereas a PHB/PHV mixture would be produced from a VFA profile at pH 5(Muhammadi et al., 2015;

Rodriguez-Perez et al., 2018). The increase in the diversity of VFAs at pH 5 can be explained by a decreased activity not only of acetoclastic methanogens but also of propionic oxidant bacteria and/or butyric oxidant bacteria, which work syntrophically during the degradation of these compounds. The metabolic pathways for the degradation of propionic and butyric acids are different, and depend on the microorganisms involved in the degradative process of the substrate (Zhou et al., 2018). Several bacteria from the order of *Syntropobacterales* degrade propionate in syntrophic association with methanogens (Müller et al., 2010). In the case of butyrate degradation, two groups of bacteria are involved belonging to the *Syntrophomonadaceae* family and *Syntrophobacterales* order. Butyrate oxidizing bacteria uses the beta-oxidation pathway (McInerney et al., 2008), a pathway also used for long-chain fatty acid degradations like valerate and caproic acid.



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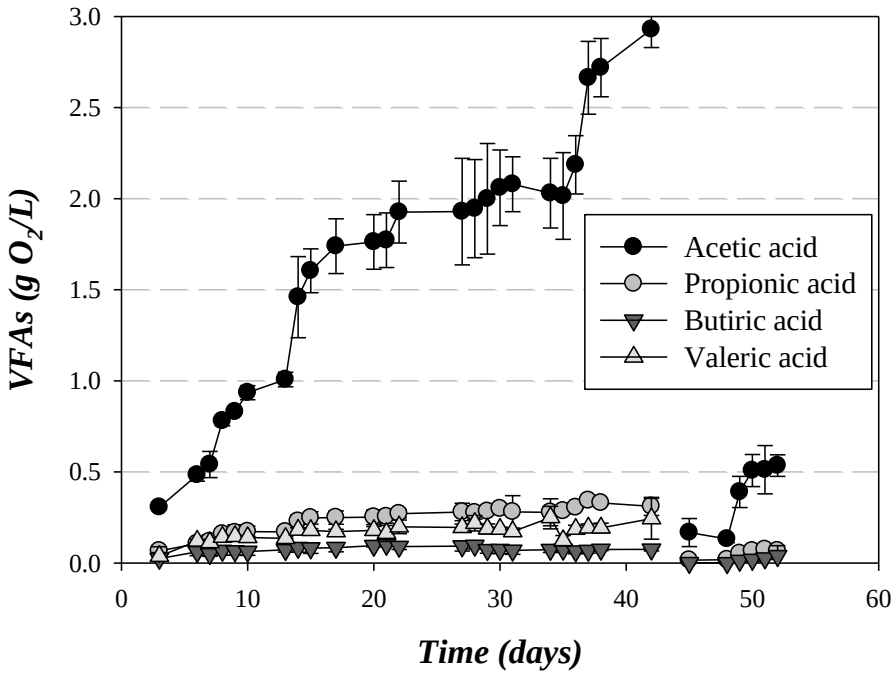


B)

Figure 6. Characterization of Volatile fatty acids produced among pH-controlled rectors. A) VFAs profile and amounts at pH 5 B) VFAs profile and amounts at pH 9.

Acetic acid accumulation at pH 5 was continuous; whereas the accumulation seems to be stepped at pH 9 (Figure 6). This difference could be related to the slightly higher hydrolytic activity at pH 9, where the marked increases in VFA production coincided with the OMSW feeds. At pH 9, the concentration of other VFA, apart from acetic acid, was almost constant from day 15 (Figure 6B). This indicates that the acidogenic microorganisms present in the system were able to survive and did not lose their acidogenic activity at pH 9. This fact contradicted the results of Ma et al. (2016), who reported that microbial populations tend to decrease under alkaline conditions, since alkaline pH would not be suitable for the growth and metabolism of acidogenic microorganisms.

2.3.3 Phenols accumulation at pH 5 and pH 9 reactors.

The measured values for each individual phenol in the reactors are shown in Table 4, in addition to the added ones, indicating the sum of each phenol in the OMSW used in each feed. The balance of individual phenols after 42 days of operation showed an effective degradation of most of them. Measured 3,4-dihydroxyphenylglycol (DHPG) disappeared completely at pH 9; while at pH 5 it did not. At pH 5 the measured concentration of DHPG varied in the range of 0 to 5.49±1.68 mg/L, while always remaining lower than the added values. The measured concentration of hydroxytyrosol (HT) and tyrosol (ty) increased markedly after 14 days at both pH 5 and 9, most likely due to the hydrolysis of the complex phenols present in the OMSW. From day 27 the balance between the formation and degradation of HT and ty started to be negative, with higher decrements at pH 9 than at pH 5. No vanillic acid (VA) was detected after each OMSW addition at both pH levels under study. A similar behavior at pH 9 was observed for the other metabolite of lignin, vanillin (Va). At pH 5 Va concentration increased by up to 48 mg/L on day 27, went down on day 35, and significantly increased once again on day 42. This means that the lignin present in the OMSW was not equally accessible by the microorganisms at pH 5. The concentration of measured 4-ethilphenol (4-EP) increased

Table 4. Individual phenolic compounds concentration in mg per liter of reactor. Measured and added (3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol (HT), tyrosol (Ty), vanillic acid (VA), vanillin (Va) and 4-ethylphenol (4-EP)) in the reactors at pH 5 and pH 9, at days 0, 14, 27, 35 and 42 of operation.

Time (days)	**Values**	**mg/L at pH 5**						**mg/L at pH 9**					
		DHPG	HT	Ty	VA	Va	4-EP	DHPG	HT	Ty	VA	Va	4-EP
0	**Added**	2.74	5.85	1.55	0.20	0.74	n.d.	2.74	5.85	1.55	0.20	0.74	n.d.
	SD	0.11	0.06	0.08	0.03	0.13	-	0.11	0.06	0.08	0.03	0.13	-
14	**Measured**	n.d.	18.00	4.00	n.d.	21.00	16.50	n.d.	18.75	4.50	n.d.	n.d.	12.75
	SD	-	5.66	0.00	-	0.00	6.36	-	0.35	3.54	-	-	5.30
	Added	5.49	11.70	3.09	0.39	1.49	n.d.	5.49	11.70	3.09	0.39	1.49	n.d.
	SD	0.23	0.12	0.15	0.06	0.26	-	0.23	0.12	0.15	0.06	0.26	-
27	**Measured**	5.52	16.50	4.67	n.d.	47.95	18.67	n.d.	9.50	3.20	n.d.	n.d.	22.90
	SD	1.68	3.96	1.46	-	3.61	4.14	-	0.99	4.24	-	-	4.67
	Added	8.23	17.55	4.64	0.59	2.23	n.d.	8.23	17.55	4.64	0.59	2.23	n.d.
	SD	0.34	0.18	0.23	0.09	0.38	-	0.34	0.18	0.23	0.09	0.38	-
35	**Measured**	1.95	7.85	4.25	n.d.	4.10	51.55	n.d.	4.55	4.00	n.d.	n.d.	17.80
	SD	2.76	1.48	1.48	-	7.71	4.31	-	0.07	0.85	-	-	5.37
	Added	10.97	23.40	6.18	0.78	2.97	n.d.	10.97	23.40	6.18	0.78	2.97	n.d.
	SD	0.46	0.24	0.30	0.12	0.51	-	0.46	0.24	0.30	0.12	0.51	-
42	**Measured**	4.57	10.73	2.00	n.d.	23.35	4.60	n.d.	5.25	1.80	n.d.	n.d.	7.87
	SD	1.46	0.38	0.00	-	0.49	1.56	-	0.64	0.85	-	-	2.17
	Added	13.71	29.25	7.73	0.98	3.71	n.d.	13.71	29.25	7.73	0.98	3.71	n.d.
	SD	0.57	0.30	0.38	0.15	0.64	-	0.57	0.30	0.38	0.15	0.64	-

n.d., non detected. SD, standard deviation.

from day 0 to 35 at both pHs, reaching a maximum of over 50 mg/L at pH 5 on day 35. On day 35, the measured concentration of 4-EP at pH 9 was three times lower than at pH 5. It is worth notiing that at pH 5 on day 35 the concentrations of other phenolic compounds were lower than 4-EP (Table 4). This observation could be due to the additional formation of 4-EP from other benzoic structures derived from condensed phenols (Wöhlbrand et al., 2008). On day 27, at pH 5, the sum of the concentrations of all the measured phenolic compounds was 180% higher than the phenols added to the reactor. This increase is higher than the one reported by Serrano et al. (2017), who reached an increment in the phenolic compounds from OMSW of up to 60.4% by carrying out a thermal treatment (170 ^{o}C, 60 min). Therefore, the fermentation of OMSW at pH 5 could be a promising amendment alternative prior to phenol purification since it allowed a high solubilization increment without costly energy requirements. On another hand, the sum of the concentrations of the phenolic compounds was markedly lower than the inhibition limits described in the literature for anaerobic digestion, i.e. 2000 mg/L (Calabrò et al., 2018). Hence, the low methane productions at pH 5 and 9 cannot be related to an inhibition phenomenon by the accumulation of phenolic compounds.

The overall degradation of phenolic compounds at pH 9 was higher than at pH 5. The results show that the accumulation of some phenolic compounds through pH regulation might be feasible at pH 5. However, further research would be necessary to maintain sufficiently stressful conditions inside the reactors to avoid further phenolic degradation. The evaluation of the community composition at long-term operation would provide a deeper understanding of how a community would evolve according to the different operationing conditions.

2.4. Conclusions

The results obtained in this study indicate that pH choice must be based on value-added compounds. Fermentation at neutral pH resulted in the conversion of 93.5% of fed CODt to methane. Fermentations at pH 5 and 9 resulted in the inhibition of the methanogenic activity. At pH 9, VFA reached its maximum concentration of 3.69 g COD-VFA/L, where acetic acid represented up to 79.3% of the total VFA. Unlike VFA production, an optimal operation of fermentation at pH 5 could allow the recovery of phenols such as vanillin. At pH 5 a 180% increase was achieved in the sum of the concentrations of all the measured phenolic compounds with respect to the phenols added to the reactor.

CHAPTER III

Effect of operational conditions on the behavior of a mixed microbial culture for PHA production

Chapter 3: Effect of operational conditions on the behavior of a mixed microbial culture for PHA production

Abstract

Massive production and disposal of petrochemical derived plastics represent a relevant environmental problem. Polyhydroxyalkanoates (PHA) are a renewable alternate, that can even be produced form wastes. The production of PHA from acetate using mixed microbial cultures was studied. The effect of 2 key operational conditions was studied, i.e. substrate concentration and cycle length. The effect of these factors on several responses was studied sung surface response methodology. Several reactors were operated under selected conditions for at least 10 solids retention times, to ensure stable operation. Results shows that conditions providing higher PHB content involve lower biomass productivities. This has a great impact of biomass production costs. Results suggests then that PHB content alone may not be a reasonable criterion for determining optimal conditions for PHB production. If costs need to be reduced, conditions providing a lower PHB content in the selection reactor, but a higher biomass productivity may be of interest.

3.1. Introduction

Plastics represent a serious environmental problem. They are usually non-biodegradable, are produced from non-renewable resources and have low densities, meaning that they occupy large volume in municipal landfills. Moreover, marine plastics pollution is a growing source of concern. It is mainly caused by single used plastics, which is rapidly changing policies and legislation in many countries around the world (Xanthos and Walker, 2017). Polyhydroxyalkanoates (PHA) have been proposed as a potential replacement for traditional petrochemical based plastics, since they can be used in a wide range of industrial applications

(Hong et al., 2009). PHA are polyoxoesters of hydroxyalkanoic acids, which are synthesized by some bacteria and as intracellular storage compounds (Koller et al., 2011). They are biodegradable and can be produced from renewable resources (Alvarez-Chavez et al., 2012).

The production of PHAs has shown to be technically feasible when using known high-PHA accumulating bacterial cultures like *Ralstonia Eutropha* and *Cupravidus Necatoror* (Lee et al., 2008; Zhang et al., 2015), or modified bacteria like *Escherichia coli.* These microorganisms have reached internal PHA contents up to 90% dry weight, when working with batch reactors (Tan et al., 2014). The costs associated to inoculum preservation, raw materials and downstream in PHA production, makes these polymers nowadays 5 to 10 times more expensive than their fuel-based counterparts (Raza et al., 2018). Moreover, when well defined substrates are used, this item can contribute with 1/3 of the operational costs (Choi and Lee, 1997). In order to decrease costs of inoculum preservation in axenic conditions, reducing costs of raw materials and increasing production efficiencies of PHAs, a lot of research has been conducted during the last 20 years in the use mixed microbial cultures (MMCs). Application of MMCs enables the use of volatile fatty acids (VFA) as substrate, which in turn can be derived from organic wastes by dark fermentation, further reducing potential costs and revalorizing waste organic compounds (Kleerebezem and van Loosdrecht, 2007).

PHAs production using MMCs requires a first stage of culture enrichment of PHA accumulating microorganisms (Albuquerque et al., 2010a). Transient conditions of carbon supply are normally used for this purpose: consecutive phases of carbon source availability (feast) and scarcity (famine), which induces a selective pressure favouring the development of PHA accumulating microorganisms. This feast-famine operation strategy has also been regarded as aerobic dynamic feeding (ADF) (Dias et al., 2006). Starvation for certain period can cause a decrease in the amount of intracellular components needed for growth. After starvation, when substrate is

available again, storage occurs instead of growth since the amount of enzymes required for storage are lower than RNA and enzymes required for growth (Daigger and Grady, 1982).

Several parameters have been identified as relevant for the selection of MMC with improved PHA accumulation capacity. Some of them are organic load rate (OLR), influent substrate concentration, type of substrate, sludge retention time, temperature, pH, oxygen supply, carbon to nitrogen ratio and cycle length (Dias et al., 2006; Serafim et al., 2008; Wen et al., 2010). In general, it has been observed that very high OLR in selective reactors is related with a decrease in the polyhydroxybutyrate (PHB) storage capacity, due to prevalence of cellular growth (Dionisi et al., 2006). Furthermore, differences on the feeding pattern and substrate concentration have shown considerable differences in the PHA content and yields (Valentino et al., 2014). On the other hand, the length of the cycle (total cycle time) has shown to influence the performance of PHA production as well, increasing the substrate uptake rate when decreasing the cycle length (Beun and Dircks, 2002).

When considering PHA production using VFA from waste, significant variations in substrate concentration, composition and availability may be expected. Changes in conditions will most likely require adjusting operational parameters to ensure efficient selection of an active PHA producing population. This problem could be addressed by adjusting feast and famine intervals of each operation cycle, according to the influent availability and characteristics. In order to do so, information is needed about the influence of conditions such as feed concentration (which determines OLR) and cycle length on PHB accumulation. At the same time, different conditions will also affect costs associated with the production of PHA-enriched biomass. Then, conditions favouring maximal PHA accumulation may not coincide with those providing lower costs. The

aim of this work is to evaluate the influence of cycle length and carbon concentration on the dynamics of the feast-famine process. The influence of both parameters was determined using surface response methodology. By means of the kinetics of acetate and oxygen consumption, feast and famine phases were identified and studied. Moreover, costs estimations were performed, in order to evaluate the effect of the same parameters on the first stages of PHA production: selection, enrichment and biomass separation. Studding costs associated with PHA production is relevant, since price of PHA would be mainly influenced by process costs (Lettner et al., 2017)

3.2. Materials and methods

3.2.1. Reactor operation

Several sequential batch bioreactor (SBR) runs were performed. 4 SBRs with working volume of 2 L were implemented for that purpose. All SBRs were operated under ADF conditions, which consisted of a feed period, a variable period of aerobic reaction, and a final withdrawal of the mixed liquor from the mixed vessel. SBRs were operated using acetate as sole carbon source. Feed and withdrawal periods were 6 and 10 minutes. Feed flow was 2.5 L/h. Withdraw was set to remove the same volume added during feeding (0.25 L). No settling phase was performed. Then, sludge retention time (SRT) was equal to hydraulic retention time (HRT).

Conditions imposed during SBR runs were organized using surface response methology, applying a face-centred central composite design (Montgomery, 2009). Studied factors were feed concentration and total cycle length (feast + famine). Each factor was studied at 3 levels: 30, 75, 120 mM and 4, 8, 12 h for acetate concentration and total cycle length, respectively. Central point was replicated 3 times. Conditions applied are shown in Table 5 (11 reactor runs). Different responses were studied, such as Feast/Famine (F/F) length ratio, biomass productivity, maximum

PHB content and PHB productivity. ANOVA analyses were made for each response. In all cases, model significance and lack of fit tests were performed to check that models were relevant and fitted the experimental data. All statistical analyses were made considering $\alpha = 0.05$.

A complementary SBR was run to provide a fairly stable inoculum for each of the reactor runs described in Table 5. This SBR was in turn inoculated with activated sludge from the sewage treatment plant of the city of Temuco (Chile). Feed concentration was 120 mM, and cycle length was 6 h. During the operation of all SBRs, aeration was provided at an airflow rate of 3-5 L/min. All reactors were also mechanically stirred at 60 rpm.

Influent was composed by sodium acetate and a mineral medium. Mineral medium composition was 600 mg/L $MgSO_4$-$7H_2O$, 160 mg/L NH_4Cl, 100/L mg EDTA, 92/L mg K_2HPO_4, 45 mg/L KH_2PO_4, 70 mg/L $CaCl_2$-$2H_2O$, thiourea (10 mg/L) and 2 mL/L of trace elements solution. The trace solution composition was 1500 mg/L $FeCl_3$-$6H_2O$, 150 mg/L H_3BO_3, 150 mg/L $CoCl_2$.$6H_2O$, 120 mg/L $MnCl_2$-$4H_2O$, 120 mg/L $ZnSO_4$-$7H_2O$, 60 mg/L Na_2MoO_4-$2H_2O$, 30 mg/L $CuSO_4$-$5H_2O$ and 30 mg/L of KI.

Dissolved oxygen (DO) and pH were acquired online by means of electrodes. DO was measured using an optic industrial probe (WQ401, Global Water, USA). Signals from sensors and pumps control were handled using a CompactDAQ system (cDAQ-9178 chasis, National Instruments) and a routine specially programmed for this purpose using LabView software (National Instruments).

3.2.2. Analytical Methods

Acetate was determined by gas-chromatography using a Flame Ionization Detector (Clarus 400, Perkin Elmer), using a Nukol™ capillary column (Sigma-Aldrich). Cell dry weight was quantified using the volatile suspended solids (VSS) technique according to Standard Methods (APHA, 2011).

PHB determination was performed according to Serafim et al. (2004). 5 mL of homogenized culture were collected, and 5 drops of formaldehyde were added to stop biological activity: Samples were then freezed and lyophilizated for storage. The biomass samples were later resuspended in 1 mL acidic methanol (20% H_2SO_4) with 0.65 mg/mL of benzoic acid as internal standard (Sigma Aldrich™). The chloroform phase was collected and molecular sieves (0.3 nm) were added for water adsorption. One mL of the chloroform phase obtained was injected on-column in the same gas chromatograph used for acetate determination. A calibration curve was prepared by injecting standard concentrations of hydroxybutyric acid sodium salt (Sigma Aldrich) previously submitted to the procedure described for reactor samples.

3.2.3. Estimation of costs for PHA-enriched biomass production

An estimation of the costs associated with the production of PHA-enriched biomass was performed, for each of the conditions described in Table 5. Capital and operating costs were considered, for the process described in Figure 7. It consisted of 3 stages: biomass selection, PHA accumulation and biomass harvest (by centrifugation). This study included only biomass production (and not PHA extraction or purification) since that stage is the one that will be most affected by changes of the operational parameters studied in this research.

Analysis was made considering the following conditions or assumptions:

- An annual production of 500 ton of PHA was considered as calculation basis.

- Total volume needed for enrichment and accumulation stages were calculated according the production rate of PHB of each stage. To calculate the number of reactors required, reactors with a useful volume of 8 m^3 were considered.
- Enrichment reactor produced biomass with a PHA content equals to that observed by the end of each operation cycle tested experimentally. Accumulation reactor produces biomass with a PHA content equals to that observed by the end of each feast cycle tested experimentally.
- Pumping capacity needed for each reactor was calculated taking into account the inlet flowrate supplied during the feeding time. Pumping energy consumption was calculated basis on the inlet flowrate supplied and considering a reactor height of 4.6 m and an energy efficiency of the pump of 0.7.
- Oxygen requirements for enrichment and accumulation reactors were determined by means of chemical oxygen demand (COD) mass balances. Air flowrate supplied was calculated using a mass transfer efficiency factor of 20%. Fans with a capacity of 3 m^3 air/s were considered.
- The total volume required for feeding and buffer tanks was determined basis on the flowrates of each stream and considering a storage period of 12 h. Tanks with a volume of 60 m^3 were chosen. Each tank was provided with one agitator whose specific power and energy efficiency were of 0.01 $kW \cdot h/m^3$ and 0.7, respectively.
- To separate the biomass generated during the accumulation stage, centrifuges with a capacity of 30 m^3/h were considered. A solid separation efficiency of 100% was supposed
- Energy costs were calculated taking into account energy consumed by agitators, pumps and fans, considering a price of 15.8 cents USD/kW·h.

- Capital cost were calculated according (Couper et al., 2009). Prices were updated taking into an annual increase of costs of 3%. A lifetime of 20 years was considered.

All equations used for costs estimations are reproduced in the Appendix of this thesis.

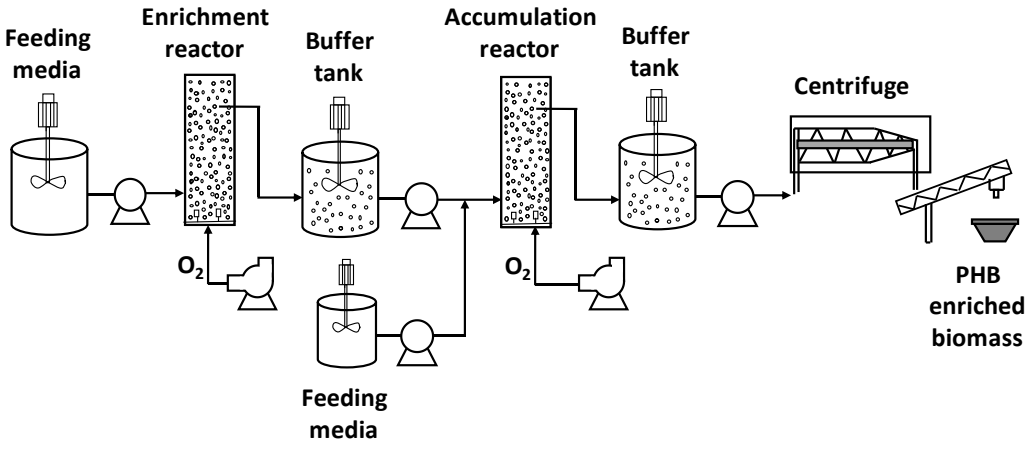


Figure 7. Process considered for costs estimation of biomass-enriched PHB.

3.3. Results and discussion

3.3.1 SBRs operation

Figure 8A shows a typical operation cycle (data from SBR #2, see Table 5). After feeding, oxygen uptake increases, producing a sudden drop in DO. Oxygen concentration remains almost null, while acetate is present in the reactor, as a result of a high oxygen uptake rate. During this period biomass PHB content increases. After acetate is depleted, oxygen rapidly increases as oxygen consumption rate is reduced. As described by previous authors (Villano et al., 2014), these changes in the patterns of OD are well correlated with the limits of feast and famine phases. Then, oxygen profile can be easily used to determine feast/famine boundary. Figure 8A also shows how PHB is produced during feast and consumed during famine. As a result, similar PHA concentrations were observed at the beginning and end of the cycle.

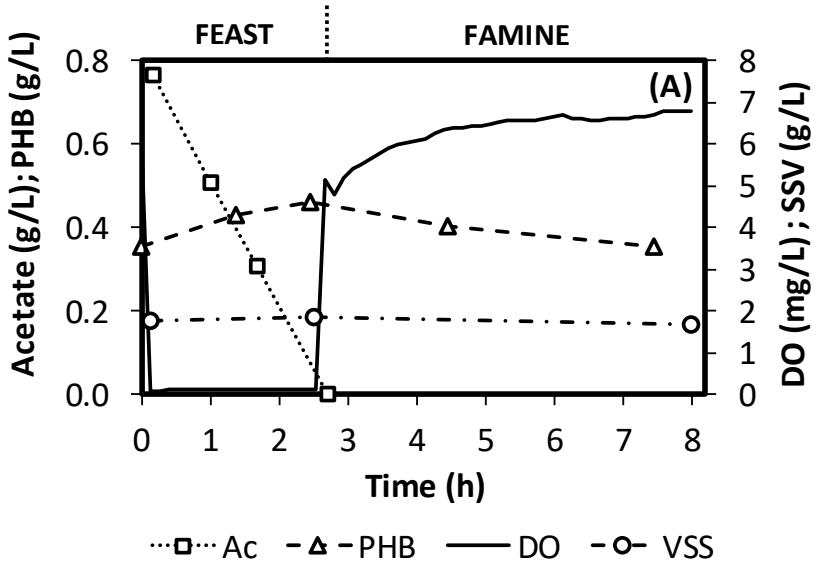
Obviously, application of the conditions described in Table 5 induced changes in the biomass developed in the reactors. Figure 8B presents the evolution of the feast phase length of SBR #10. It can be observed that feast length decreases rapidly during the first 30 days of operation. No changes are observed after day 60, indicating that SBR is in a stable state of operation. Time required to reach steady state depended on the applied operational conditions. To ensure that the responses measured in the design of experiments were representative of the applied conditions, operation was kept until steady state conditions were achieved, which was identified by a constant feast length. Only then analyses were performed, and responses were evaluated. As a result, all SBRs were operated for a period exceeding 10 SRT. As expected, SBR stabilization took longer in those cases when applied conditions were far from those applied in the SBR operated to provide the inoculum. Table 6 presents feast and famine phases lengths, for each SBR. Values by the end of the operation are presented, when stable operation was identified, based on the criteria described above.

Table 5. Operational conditions in all enrichment SBRs from the experimental design.

Run	Cycle length (h)	Acetate in the feed (mM)	Organic loading rate (g/L·d)	SRT* (d)
SBR #1	4	30	1.4	1.3
SBR #2	4	75	3.4	1.3
SBR #3	4	120	5.4	1.3
SBR #4	8	30	0.7	2.7
SBR #5	8	75	1.7	2.7
SBR #6	8	75	1.7	2.7
SBR #7	8	75	1.7	2.7
SBR #8	8	120	2.7	2.7
SBR #9	12	30	0.5	4.0
SBR #10	12	75	1.1	4.0
SBR #11	12	120	1.8	4.0

* Since no biomass retention was applied, SRT is equal to HRT.

A)



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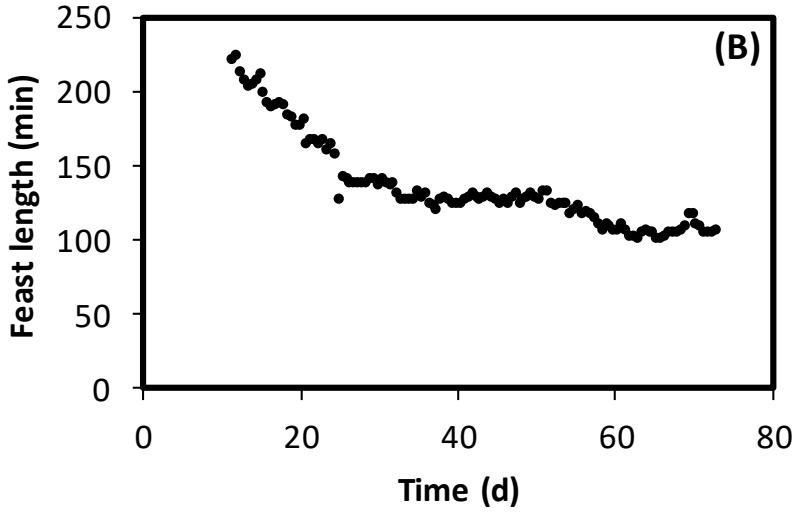


Figure 8. (A) Typical behaviour of an operation cycle (SBR #2). (B) Evolution of feast length during operation of SBR #10

3.3.2. Effect of substrate concentration and cycle length on SBR operation.

Figure 9 presents the effect of the studied factors (cycle length and acetate concentration in the feed) over the studied responses. Figure 9A shows the OLR. Since fed volume per cycle remained constant, changes in influent concentration resulted in different applied OLRs. On the other hand, increases in cycle time reduced applied loading. As a result, OLRs were in the range 0.5-5.5 g acetic acid/L/d. Figure 9B presents F/F ratios, which is the relation between times presented in Table 6. Figure 9B shows a direct relation between F/F ratio and acetate concentration, and an inverse relation with cycle length. Such result is expected, since a higher feed concertation results in a higher loading rate, requiring the culture more time to consume substrate, increasing feast time. On the other hand, a higher total cycle will result in a higher famine time. In general, F/F ratios observed in this study are high when compared with the ones reported by other authors (Albuquerque et al., 2010b; Freches and Lemos, 2017; Jiang et al., 2011) who normally applied values below 0.5. Famine lengths observed in this study were in the range 1-11 h (see Table 6). Figure 9C presents the maximum biomass PHB content, which is attained by the end of feast period (beginning of famine), as can be seen in Figure 9A. Maximum PHB content ranged between 10 and 90%, revealing how determinant the studied parameters are in terms of selecting microorganisms with high PHB storage capacity. An increase in cycle time induced an increment on maximum PHB content. This is most likely the result of the consequent increment of famine length. Under tested conditions, feast is mainly a function of applied acetate concentration, so increases of cycle time at constant acetate concentration will increase famine length. Decreasing F/F enhances growth limitation, favouring PHA storage (Albuquerque et al., 2010b). On the other hand, a clear increase in maximum PHB content was observed when increasing acetate concentration (and therefore organic loading rate), behaviour that has been already observed in previous studies (Dionisi et al., 2006; Jiang et al., 2013).

Table 6. Feast and famine phases length (by the end of operation), for the SBR runs described in Table 1.

Run	Feast length (h)	Famine length (h)
SBR #1	0.88	3.12
SBR #2	1.90	2.10
SBR #3	2.97	1.03
SBR #4	0.81	7.19
SBR #5	2.92	5.08
SBR #6	3.40	4.60
SBR #7	2.86	5.14
SBR #8	4.79	3.21
SBR #9	1.15	10.85
SBR #10	2.85	9.15
SBR #11	4.38	7.63

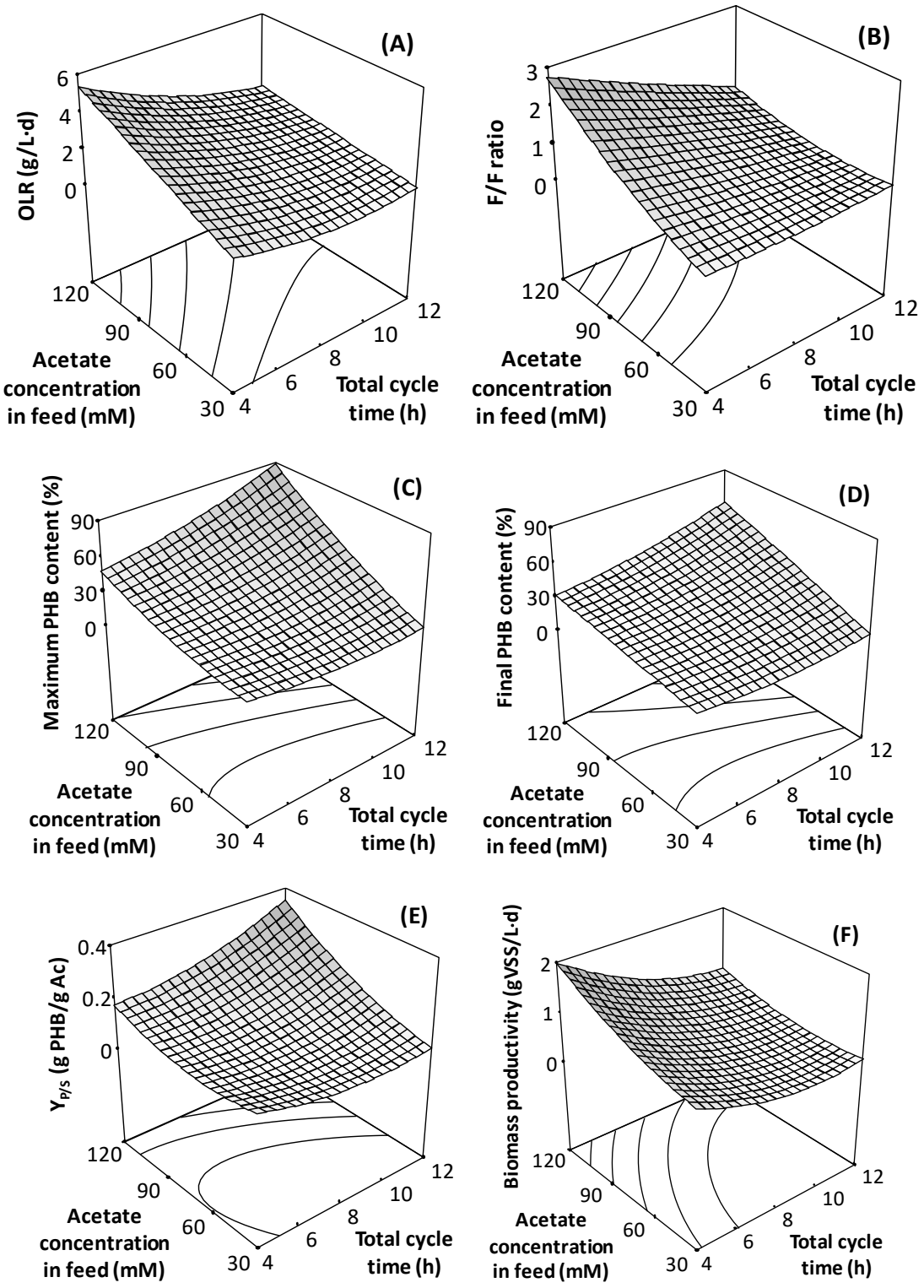


Figure 9. Selected responses observed during surface response methodology. Factors tested: acetate concentration in the feed and total cycle time. Responses: (A) OLR, (B) F/F ratio, (C) maximum PHB content of biomass, (D) PHB content of biomass by the end of the cycle, (E) yield PHA/acetic acid, (F) biomass productivity.

Considering that draw of biomass takes place by the end of each cycle, final PHB content i.e. the one by the end of famine, is probably a more relevant information for reactor operation, than maximum content (see Figure 9D). As expected, values are lower than those shown in Figure 9C, because of PHB consumption during famine. Nevertheless, a constant relation between maximum and final PHB contents was observed, irrespective of the conditions tested, as can be seen in Figure 10. It is clear that during famine about 30% of the PHB was consumed. Proportionality between maximum and final PHB content indicates that second value can be good indicator of the first one. Then, decisions may be taken based on PHB content at the end of the cycle, which may be easier to determine under full-scale operational conditions, or when detailed control or follow up of reactor may be not feasible.

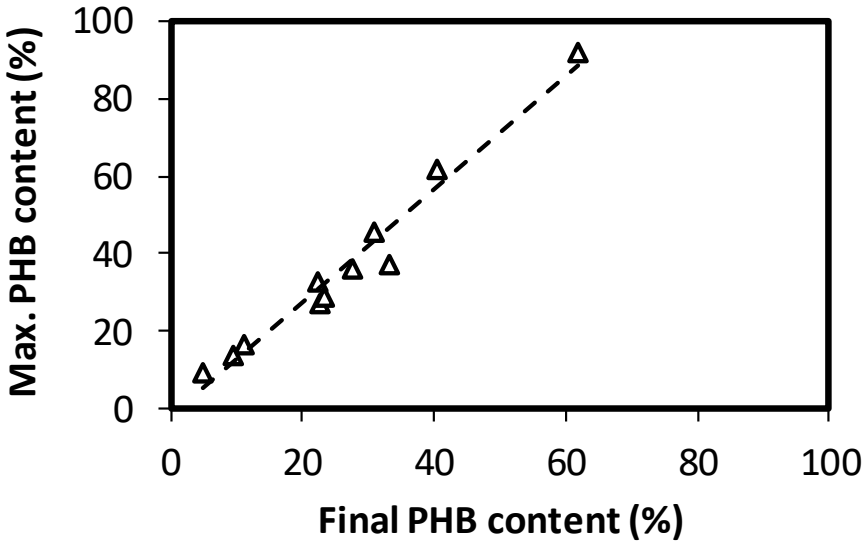


Figure 10. Relation between maximum and final PHB contents for the operation of SBR reactors.

A similar trend as that described for biomass PHB content can be observed for $Y_{P/S}$, the product/substrate yield (Figure 9E). This value has been computed considering maximum PHB biomass content. As expected, conditions providing a higher $Y_{P/S}$ are those generating a higher PHB biomass content, since more substrate is oriented towards polymers accumulation, as already described.

Results correspond to selection reactors, that will produce biomass for an accumulation step, where further conditions may be imposed to enhance PHB accumulation (such as nitrogen limitation). Then, biomass productivity is also relevant for full-scale application, since a higher productivity will produce more biomass to potentially feed such accumulating reactor. Biomass productivity, in terms of VSS, is presented in Figure 9F. Average productivity is presented, i.e. the mass of biomass exiting the reactor after each cycle, per volume of reactor divided by cycle length. Values presented correspond to total biomass, including intracellular accumulated PHB. As expected, biomass productivity follows the same pattern as applied OLR (Figure 9A), since complete substrate consumption is obtained under all conditions. Comparison of Figures 9D and 3F shows a compromise between biomass production and observed PHA accumulation, phenomenon that has been previously descried (Dionisi et al., 2006; Villano et al., 2014). Depending on the eventual performance of an accumulation step, a lower content of PHB may be accepted, if a higher amount of biomass can be produced, in the selection step. A parameter that may provide useful criteria for determination of operational conditions could be PHB productivity during selection reactors operation. Unfortunately, PHB productivity cannot be presented in the form of a surface respond, since ANOVA analysis showed a significant lack of fit ($\alpha = 5\%$), meaning that the second order model used does not correctly represent observed response variation. Then, observed PHB productivity is presented in Table 7. Two productivities can be evaluated, one in terms of maximum PHB content (observed by the end of feast) and one based on the amount of PHB at the end of cycle. First productivity would be the one observed if biomass would be harvested by the end of feast. Both productivities are anyway proportional, as is the case of both biomass PHB contents (Figure 10).

Table 7. PHB average productivity for the operation of SBR

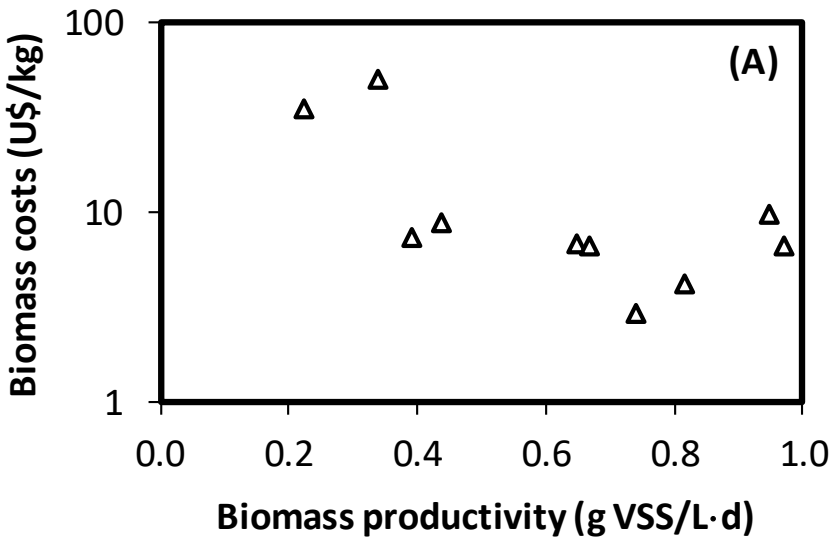
Run	**PHB productivity based on maximum PHB (g/L·d)**	**PHB productivity based on final PHB (g/L·d)**
SBR #1	0.16	0.10
SBR #2	0.32	0.22
SBR #3	1.01	0.68
SBR #4	0.03	0.02
SBR #5	0.18	0.15
SBR #6	0.19	0.16
SBR #7	0.16	0.12
SBR #8	0.51	0.33
SBR #9	0.03	0.02
SBR #10	0.15	0.13
SBR #11	0.68	0.46

3.3.3. Estimation of costs for PHA-enriched biomass production

Figure 11 shows the costs estimation for PHA-enriched biomass, as a function of biomass productivity and PHA content by the end of each cycle. To construct these charts the data coming from the experiments described in Table 7 were used. Few reports are available dealing with costs of PHA production in literature, such as (Fernandez-Dacosta et al., 2015; Gurieff and Lant, 2007). These studies are very useful to determine industrial applicability of mixed culture based PHB production. However, depend greatly on the particular conditions and process selected for the analyses. This study has certainly limitations, such as the definition of a sole reactor volume, so replication of reactors was considered when higher fermentation volumes were required. Then, economy of scale is not taken into consideration. However, it provides an opportunity to visualize the potential impact of operational conditions on potential associated costs. Numbers are expected to serve as a way to relatively compare costs and not necessary to provide absolute values. Observation of Figures 11A and 11B clearly shows the already identified compromise between biomass productivity and PHA content. Conditions providing a high level of PHB accumulation in the selection process involve low biomass productivity, which in turns affect overall costs. Conditions tested in this research provided a wide range of PHB contents and biomass productivities, producing great differences in associated costs for biomass production. Results provided by this research confirms the great relevance that selection appropriate conditions for biomass selection can produce on PHB-enriched biomass quality and production costs. Results also suggests that PHB content alone may not be a reasonable criterion for determining optimal conditions for

PHB production. If costs need to be reduced, conditions providing a lower PHB content in the selection reactor, but a higher biomass productivity may be of interest. Of course, as long as the

reduction of PHB content does not involve significant increases in PHB extraction and purification costs.



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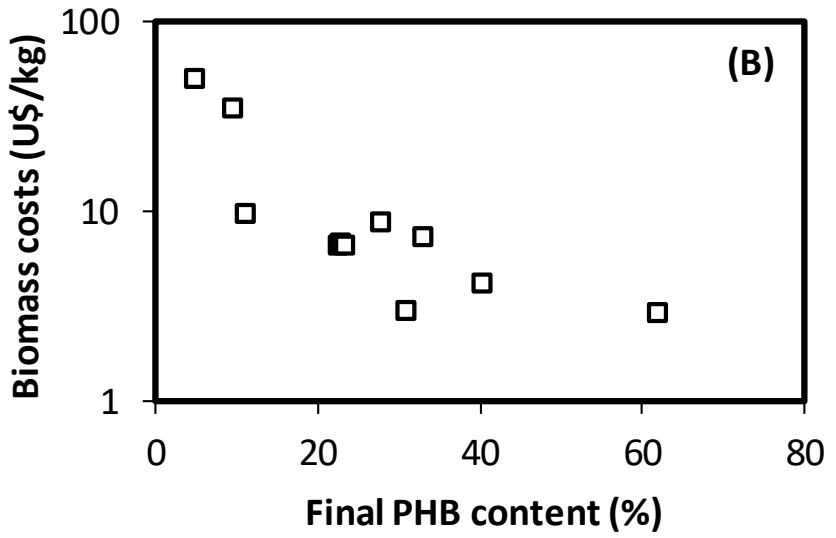


B)

Figure 11. Cost estimation for production of PHA-enriched biomass as a function of (A) total biomass productivity and (B) PHB content by the end of the cycle.

3.4. Conclusions

Substrate concentration and cycle length proved to have a deep impact on SBR operation for the selection of PHB-accumulating biomass. Both factors showed to have statistically significant effect over biomass productivity, PHB content and product/substrate yield. PHB content by the end of feast and famine stages showed to have a constant relation, irrespective of the conditions tested. In all cases 30% of existing PHB was consumed during famine. Biomass productivity was expected to have a relevant effect of the costs associated with the production of PHA-enriched biomass. Since results showed a negative relation between biomass productivity and PHA content, costs for biomass production are higher for those conditions providing higher PHA contents in the biomass. Then, maximising PHB content in the selection reactor may provide excessive costs for biomass production.

CHAPTER IV

On-line control of feast/famine cycles to improve PHB accumulation during cultivation of mixed populations in sequential batch reactors

Chapter 4: On-line control of feast/famine cycles to improve PHB accumulation during cultivation of mixed microbial cultures in sequential batch reactors

Abstract

Nowadays, the production of Polyhydroxyalkanoate (PHAs) has gained much interest so that these compounds can be used for bioplastic production. In order to reduce production costs of PHAs, many researches has been directed for producing these bioplastics from residues and using mixed microbial cultures (MMC). Moreover, the operational strategy "feast/famine" is the most used for PHAs selecting bacteria in sequential batch reactors (SBRs). In this sense, the typical operation under feast/famine strategy maintain an organic substrate load and a "feast/famine" ratio (f/f ratio) is reached in a stablished and constant total cycle time. However, the possibility of operating these systems in a full-scale process implies that a fluctuating organic load enters to the SBRs system, which will provoke constant changes in f/f ratio, defaulting the global operation in this step. Thus, on the present study, a strategy for control and regulation of feeding cycles in sequential batch reactors (SBRs) destined for PHA production was applied. The strategy was imposed to biomass and compared to the conventional "feast/famine" strategy to evaluate the contents, and productivities reached in both conditions. Our results indicated that higher PHB productivity up to 0.16 g/L/d and internal PHB contents up to 45% w/w were observed when controlling feeding cycles compared to normal feast/famine cycles through evaluation of SBR performance and batch accumulation tests. Thus, control of f/f ratio will increase the performance of PHA selecting step.

4.1. Introduction

During the last decades, an increased interest for the production of polyhydroxyalkanoate (PHAs) has been evidenced, as a result of the search for biodegradable thermoplastic materials,

as alternative to petroleum-based plastics. The increased use of plastics in packing and many other applications, has triggered the plastic accumulation in lands and waters, which has generated diverse ecological and environmental problems (Możejko-Ciesielska and Kiewisz, 2016).

PHAs are bacterial polymers destined to energy storage. They are only produced by microorganisms that possess the enzymatic pool to transform the carbon to polyester chains and store them in intracytoplasmic granules (Jendrossek and Pfeiffer, 2014). Traditionally, the industrial production of PHAs has been linked to the selection of microbial strains that allocate a large extent of their cellular content into storage(Khanna and Srivastava, 2005). In this way, high PHA contents from the biomass have been achieved (Chen et al., 2011; Shang et al., 2003; Zhang et al., 2015). Nevertheless, these PHA production routes have turned out to be, so far, more expensive than petroleum-based polyesters production because of several aspects such as substrate, culture sterilization and extraction costs (Chen, 2009). PHA production using mixed microbial cultures (MMCs) has been proposed as an alternative way to produce PHA, and researches dealing this alternative have steadily increase since 1990s decade. The MMCs have the versatility that come from the operation under non-axenic environments, therefore not sterilization process is needed, and tend to be more robust to changes in operating conditions, which makes them a suitable alternative for large-scale production of PHAs (Dias et al., 2006b; Reis et al., 2003; Luisa S. Serafim et al., 2008b). To produce PHAs from MMCs, a strategy of consecutive intervals of availability and scarcity of substrate is developed under sequential batch reactors, which forces selection of microorganisms that accumulate PHAs. This strategy has been called as feast/famine (Beun et al., 2000b).

Recent findings related to costs of PHA production from MMCs indicate that the substrate, selection of culture to obtain a highly productive inoculum and extraction of PHAs from biomass are the main operational costs are associated with this process (Raza et al., 2018;

Rodriguez-Perez et al., 2018). Therefore, for a sustainable production of PHAs, the use of substrates such as solid wastes and wastewaters is a suitable way to decrease substrate-associated costs (Albuquerque et al., 2010; Ntaikou et al., 2014) Besides the reduction of costs, this process allows the transformation of waste into valuable compounds, generating a double benefit. However, it has to be considered that in situations of real scaling, substrates from wastewaters can be variable in terms of concentration and composition, since pre-fermentation processes are required to obtain them, and substrate containing effluents are commonly variable, in terms of flow and organic load (Tan et al., 2014). This could be problematic for PHA generation with Feast/Famine strategy, due to feeding cycles on SBRs are programmed at fixed time intervals (Luisa S. Serafim et al., 2008b). In consequence, a lower PHA productivity could be obtained because of over-exposition to famine intervals. Therefore, is necessary to find operational methods that can regulate the feeding cycles when operating under variable feeding conditions.

On the present study, an alternative strategy for PHB-accumulating biomass selection, based on the traditional feast/Famine strategy has been tested. The strategy consisted in the implementation of a control platform for sequential batch reactors (SBRs), which based on dissolved oxygen (DO) monitoring, controlled the feast and famine intervals in constant and defined feast to famine ratios (F/F ratio), for variable feed of acetate as substrate were entered to reactor. Using this control platform, the auto-regulation of feeding cycles was achieved by maintaining a f/f ratio constant, which provoked variations in total cycle lengths when different loads were applied. The results were evaluated and compared to a fixed cycle length operation in terms of storage yields, internal cell contents and PHB productivity on SBRs.

4.2. Materials and methods

4.2.1. SBR reactors

Three SBR reactors with 2L useful volume were operated to conduct this study. Operation cycles in SBRs included feeding, aerobic reaction phase and mixed liquor withdrawal. In all cases feeding time was 6 min in which 250 mL of media were fed. Reaction time varied among conditions and withdrawal of 250 ml was performed during 10 minutes. No settling was applied, so hydraulic and sludge retention times were the same. Aeration and mechanical mixing were supplied during the whole cycle, at 4 L/min and 60 rpm, respectively.

PHA-producing biomass was acclimated from an activated sludge plant treating sewage, in a SBR operated for 30 days (6 hours cycle length, 60 mM acetate). This biomass was used as inoculum to start-up the three studied SBRs. Two reactors were operated with the proposed strategy (described below), adjusting automatically cycle length to provide feast/famine ratios of 0.2 (R0.2) and 0.6 (R0.6). Last reactor was operated as a control at a constant 12 hours cycle time. Table 8 describes the main operational characteristics of each reactor.

Several SBR reactors were operated to conduct this study. All were made of glass and had 2L of useful volume. They were operated using cycles including feeding, an aerobic reaction phase and mixed liquor withdrawal. In all cases feeding time was 6 min during which 250 mL of media were fed. No settling was applied, so hydraulic and biomass retention times were the same. Aeration and mechanical mixing were supplied during the whole cycle, at 4 L/min and 60 rpm, respectively. During withdrawal period (10 min), 250 mL were removed from the reactor.

Table 8: Operation conditions of SBR reactors used in this study

	R0.2	R0.6	Control
F/F ratio	0.2	0.6	Variable
Cycle length	variable	variable	12 h
Influent acetate concentration	alternating 30 and 120 mM	alternating 30 and 120 mM	alternating 30 and 120 mM
Media fed per cycle	250 mL	250 mL	250 mL

All SBRs were fed with the same synthetic media, containing sodium acetate as the carbon source. In order to test the effect of fixed f/f ratio, acetate feed was applied alternating two concentrations: 30 and 120mM, in successive cycles. Mineral medium was composed of 600 mg/L MgSO4-7H2O, 160 mg/L NH4Cl, 100 mg/L EDTA, 92 mg/L K2HPO4, 45 mg/L KH2PO4, 70 mg/L CaCl2-2H2O, 10 mg/L Thiourea and 2 mL/L of trace element solution. The trace solution consisted of 1500 mg/L FeCl3·6H2O, 150 mg/L H3BO3, 150/L mg CoCl2·6H2O, 120 mg/L MnCl2·4H2O, 120/L mg ZnSO4·7H2O, 60 mg/L Na2MoO4·2H2O, 30 mg/L CuSO4·5H2O, and 30 mg/L of KI.

Peristaltic pumps (Cole Parmer, USA) were used for feed and withdrawal. Dissolved oxygen (DO) was acquired online by means of an optic industrial probe (WQ401, Global Water, USA). Signals from sensors and pumps control were handled using a CompactDAQ system (cDAQ-9178 chasis, National Instruments), connected to a PC running a routine specially programmed for this purpose using LabView software (National Instruments).

Reactors were monitored until reaching pseudo-stationary phase (i.e, period of operation in which biomass concentration remained stable ad feast/ famine dynamic showed constant PHB production and degradation during every cycle) and evaluated through PHB accumulation kinetics for storage yield ($Y_{P/S\ (feast)}$),PHB productivity ($q_{PHB\ (feast)}$), substrate uptake rate ($-q_{Acet.}$

$_{(feast)}$) and PHB content (as part of the biomass) at the beginning of cycle and the end of the feast phase.

4.2.2. DO-based Feast/Famine ratio control for PHB-accumulating SBRs

During this research, feast-famine boundary was determined on the basis of the fast DO concentration increase that characterizes the end of feast phase. Then, by determining when DO exceeds a previously determined threshold concentration, the length of feast can be determined by a simple routine. In the case of this research threshold concentration was fixed at 5 mg/L. The alternation of acetate feed in two concentrations was achieved by preparing two feeds, and using a control valve to switch automatically the substrate injection.

4.2.3. Batch accumulation tests

Biomass from reactors was extracted and mixed with culture medium in order to perform batch PHB accumulation experiments. 125 mL of biomass was mixed with a 30 mM acetate solution in a 1:1 proportion (250 mL final volume). Then, samples were incubated at 150 RPM and 25°C until all acetate was depleted from the medium. The culture medium was composed identically as described for SBRs cultures, with exception of the nitrogen source (NH4Cl) in order to enhance the PHB accumulation through nitrogen depravation. Batch tests were evaluated in terms of PHB and biomass accumulation, and acetate consumption.

4.2.4. Analytical Methods

The substrate (acetate) was measured on filtered samples (0.45 μm pore size) by gas-chromatography (Nukol™ capillary column 25 m, 0.25 mm), using a Flame Ionization Detector (FID) (Clarus 400, Perkin Elmer™). Polyhydroxyalkanoate determination was performed according to Serafim et al 2004. Five ml of homogenized culture were collected from culture. Five drops of formaldehyde were added to stop all biological activity in cells and samples were

freezed for lyophilization. Biomass was re-suspended in 1 mL acidic methanol (20% H2SO4) with 0.65 mg/ml benzoic acid as internal standard (Sigma aldrich™). The chloroform phase was collected and molecular sieves (0.3 nm) were added for water adsorption. Hydroxybutyric acid was measured then by gas chromatography (FID detector). A calibration curve was done by injecting standard concentrations of hydroxybutyric acid sodium salt (Sigma Aldrich)

4.2.5. Calculations.

Kinetic and stoichiometric parameters were calculated through kinetics of acetate consumption and PHA production. Theses kinetics were applied to SBRs on different cycles when reached stationary phase. PHB productivity was calculated as the overall amount of PHB produced per day. It consisted on the observed increase in PHB per cycle associated to the withdrawed volume per day. Initial and maximum PHB concentration refers to the volumetric amounts of PHB (g/L PHB) observed at the beginning of the cycle and at the end of feast phase respectively. Storage yield (YP/S (feast)) was computed dividing variation of PHB at initial and final time by variation of acetate at initial and final time (g PHB/g acetate). Substrate (acetate) uptake rate ($-q_{Acet.\ (feast)}$) was computed as the slope of linear regression between acetate concentration and time in feast cycle (g Acetate/L- hour).

4.2.6 Statistical Analysis

A one-way ANOVA analysis was computed to investigate significant differences in PHB productivity, storage yield, substrate uptake rate and PHB content between reactors RC, R0.2 and R0.6. When a significant difference was found, post-hoc Tukey tests were computed in order to compare the means of the analyzed values. The change in initial and final PHB content for batch assays in accumulation step was analyzed through independent sample t-student tests. All these analyses were carried out using the statistical software SPSS19. A significance level of 5% ($\alpha = 0.05$) and N = 3 were used in all cases.

4.3. Results and discussion

4.3.1. Setup performance of SBRs

Figure 12 presents biomass concentration during operation time of reactors R0.6, R0.2 and Control. Since biomass from all SBRs was obtained from previously feast-famine managed biomass (reactor RI), little changes in biomass concentration (SSV) were observed during operational period. In pseudo-stationary stage R0.6 and R0.2 showed an average biomass content of 1.8 and 1.5 gSSV/L respectively, while control showed a slightly higher content of 2.0 g SSV/L. In general, SBRs showed stable amounts of biomass and stable feast/famine dynamics from third week until the end of the operation. Therefore, kinetic and stoichiometric parameters of PHA production were evaluated after one month of continuous operation.

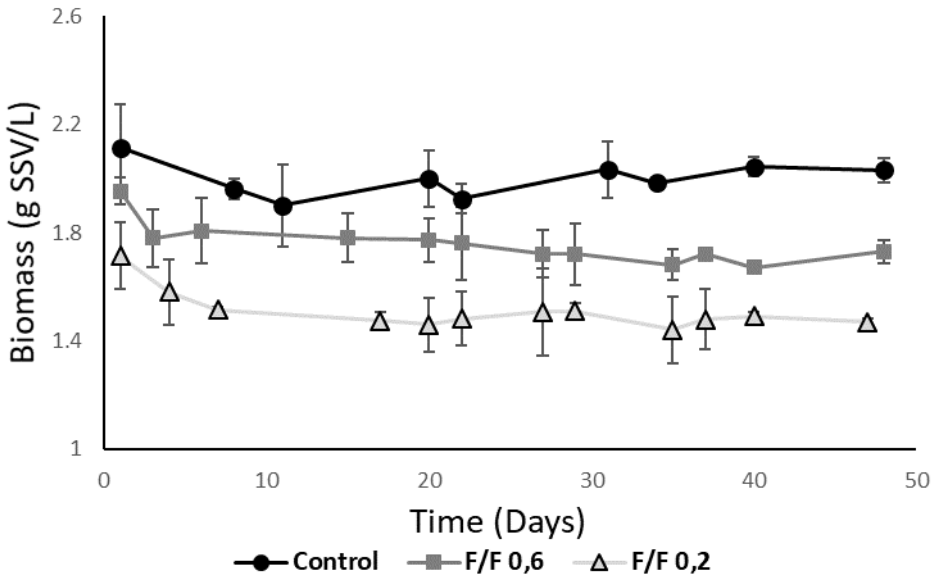
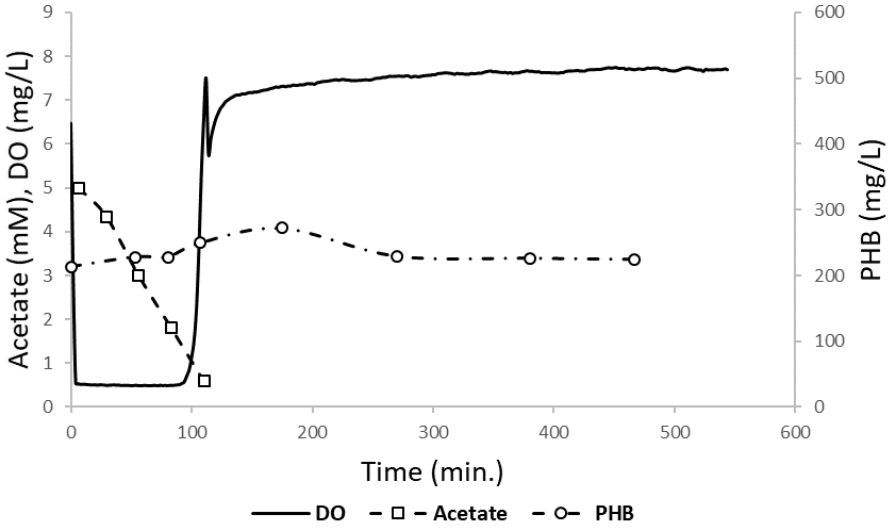


Figure 12. Biomass content of different SBRs during the operation time.

Figure 13 presents typical operation cycles for R0.2 and R0.6. The dynamics of oxygen, acetate and PHA can be observed on a cycle with a feed of 30 mM acetate. Both cycles are quite

different in total length, and observation of oxygen profiles indicates that the DO-based Feast/Famine ratio control worked as expected.



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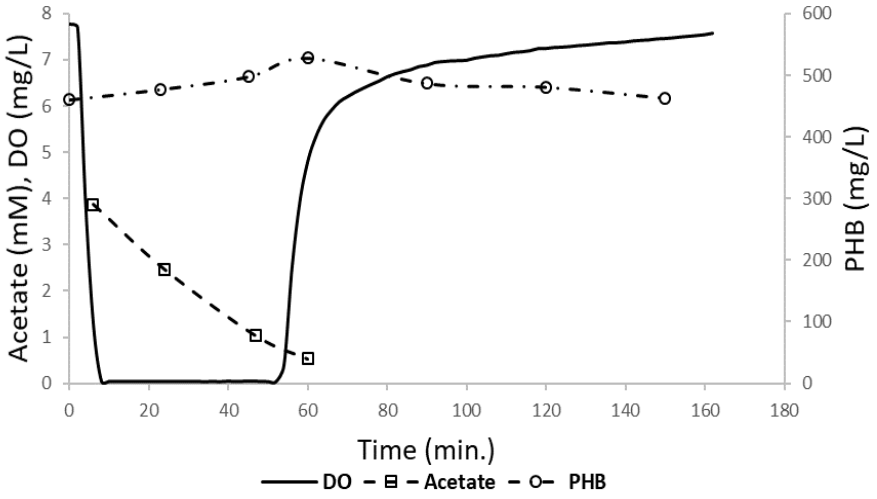
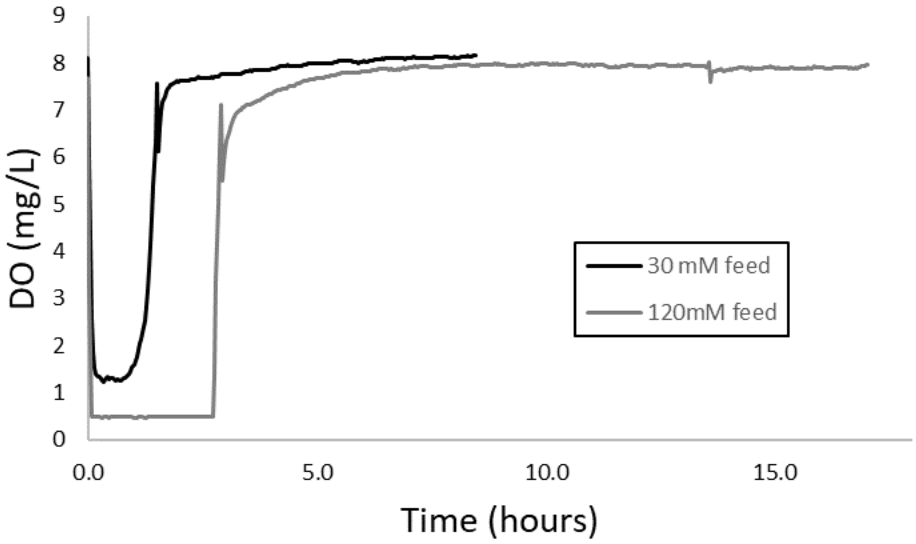


B)

Figure 13. Scheme of acetate consumption, DO variations and PHB generation dynamics in one stationary-phase cycle of operation. A) R0.2 SBR; 120mM Feed B) R0.6 SBR; 30 mM Feed.

It is noteworthy that the operation worked under fully automatic regulation of cycle time, and this caused that both reactors self-regulated their feast and famine intervals. The behavior of the F/F control can be further checked observing Figure 14. It represents typical OD evolutions during SBR cycles operated with 30- and 120-mM feeds, for both reactors R0.2 and R0.6. As expected, longest cycles were observed for R0.2, ranging from 9 to 18 hours, depending on acetate concentration in the feed. In case of R0.6, cycles were shorter, ranging from 2,5 to 5 hours. Shorter cycles mean more cycles per day, indicating a more frequent feed and therefore higher organic load rates. As a result, estimated OLR were (in g/L/d) 2,5 – 2,71 in R0.2 and 9.55-11.1 in R0.6.

This operation strategy also provoked changes in the length of the feast phase between different conditions. When increasing the f/f ratio from 0.2 to 0.6, feast phase length became shorter Also, both f/f ratio controlled SBRs turned out to have a shorter feast phase, compared to the control SBR (Figure 15). This phenomenon occurred likely to an increment on substrate uptake rate ($-q_{Acet.\ (feast)}$), when cycles became shorter by reducing the famine length in R0.6, which can be related to the increment in OLR observed in R0.6. These results are in agreement with Albuquerque et al 2010 experiments, where an increase in $-q_{Acet.\ (feast)}$ was observed when increasing OLR in different PHA production SBRs due to a stronger competition of microorganisms for the substrate.



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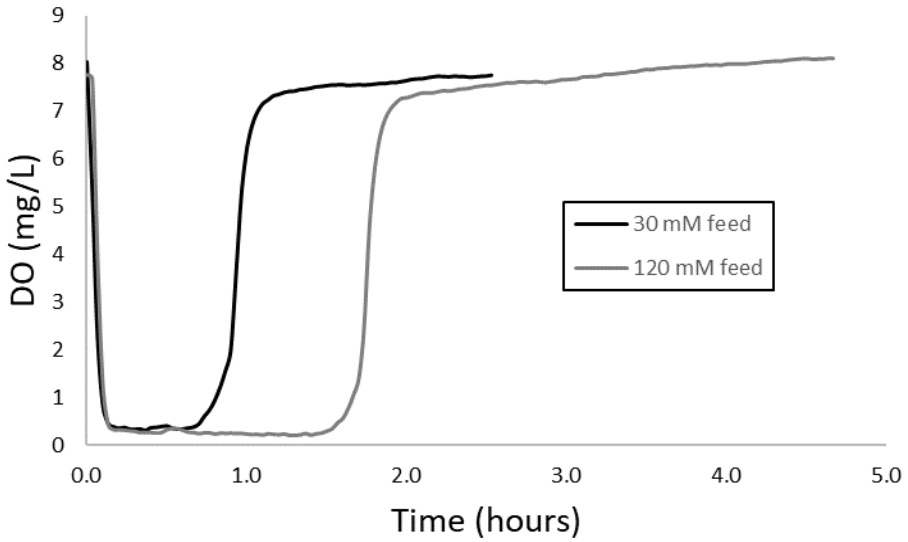

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Figure 14. Scheme of the DO signals obtained during both f/f ratio controlled SBRs. A) f/f 0.2 SBR. B) f/f 0.6 SBR

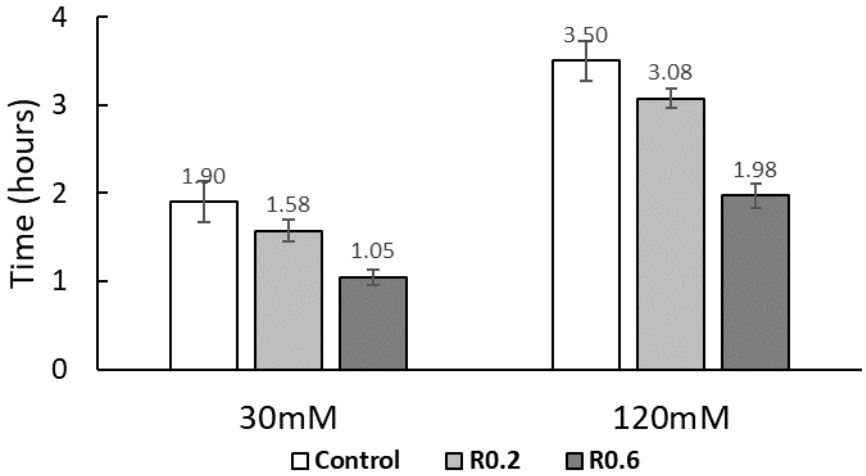


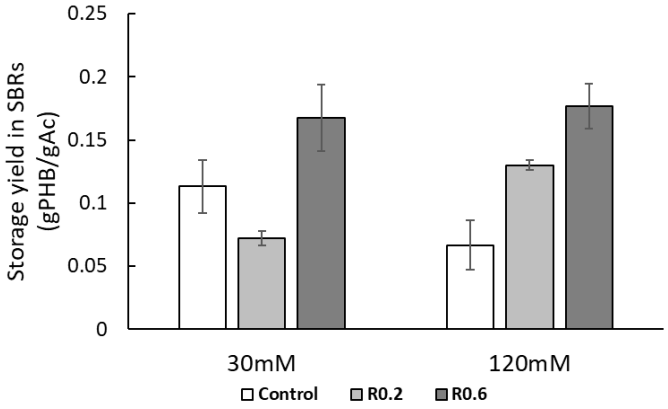
Figure 15. Observed feast time in different SBRs and variations respect to the feed concentration.

4.3.2. PHB generation and accumulation in F/F ratio controlled SBRs.

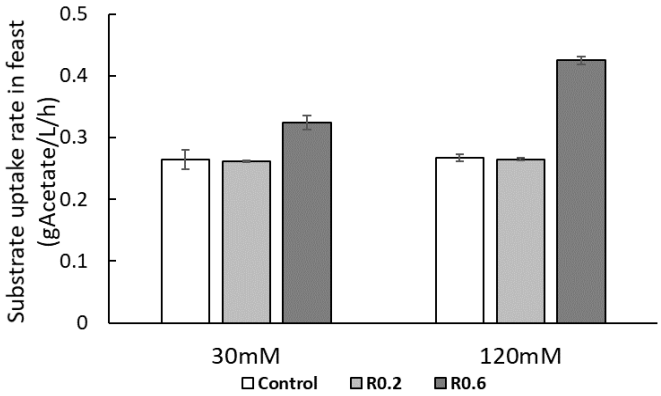
In order to determine the effect of control of feeding cycles through f/f ratio on PHB generation, the accumulated PHB in biomass was evaluated during the operation of SBRs when reached stationary phase (Figure 16). Kinetics of PHB storage yield ($Y_{P/S\ (feast)}$) showed different storage capacities among conditions. R0.6 showed the higher $Y_{P/S\ (feast)}$ reaching a value of 0.18 gPHB/gAc in cycles fed with 120mM acetate (Figure 16A). $Y_{P/S\ (feast)}$ on cycles fed with 30mM acetate was slightly lower (0.17 gPHB/gAc.) showing no significant differences on this parameter respect to the imposed feed concentration. For R0.2, $Y_{P/S\ (feast)}$ decreased from 0.13 to 0.07 gPHB/gAc. When decreasing feed concentration during the cycle. only R0.6 showed statistically significant differences among this parameter compared to control. On a first instance, these results indicated that a major fraction of acetate was derived for PHB storage when feast/famine control forced to reduce famine time. From the scarce information that exist about the influence of the length of cycles and frequency on storage yields, it can be observed that when feed frequency favors higher OLRs and enough famine time exists to create stressful growth conditions, the storage response increases (Dionisi et al., 2006b; Freches and Lemos, 2017; Jiang et al., 2011c; Valentino et al., 2014). These conditions were applied more favorably in R0.6, where a high OLR existed and enough famine time was set. Nevertheless, it has to be considered that our $Y_{P/S\ (feast)}$ results are quite lower than observed in that literature. This can indicate that under all evaluated conditions, a large extent of the substrate could be destined for maintaining the biomass population stable, instead of accumulating PHB. On the other hand, when comparing the PHB productivity ($q_{PHB\ (feast)}$) per day in different operations, it is observed that R0.6 increased considerably respect R0.2 and control in all substrate inputs (Figure 16B). Same as $Y_{P/S\ (feast)}$ calculations, $q_{PHB\ (feast)}$ had no significative differences among R0.2 and control reactors. These results can indicate that the productivity increase under R0.6 strategy is related to an increase on the number of cycles per day, and in consequence, a higher OLR

respect to the other feast/famine strategies. According to Valentino et al., 2014, when cycle length is reduced because of an increase in OLR in reactors with sludge low residence time (SRT), it exists an increase in the polymer production yield and rate. It has to be considered that under non-regulated feast/famine intervals, low substrate inputs could provoke time loss which could affect the productive capacity for biologic PHB obtention (Freches and Lemos, 2017).

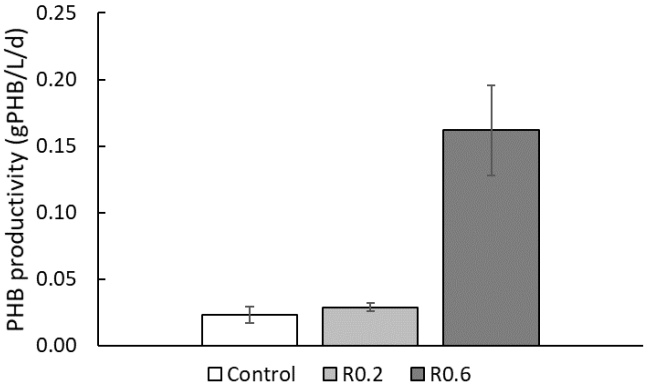
On the present study, R0.6 condition had short cycle lengths which provoked also a short SRT. This condition allowed both, enough famine time to stimulate PHB production, and short SRTs which increased OLR respect to R0.2 and control. In concordance with described by Chen 2016, our results indicated that the OLR increase in R0.6 was related to a higher PHB storage capacity. In a common feast-famine dynamic, very high OLR is related to a decrease in PHB storage capacity due to a prevalence of growth metabolism under high organic loads operation (Dionisi et al., 2006b). Thus, compared to a common fixed cycle operation, the auto-regulation of feeding cycles through f/f ratio could control excessive carbon supply, favoring PHB metabolism over biomass growth.



A)



B)



C)

Figure 16. Kinetic and stoichiometric parameters evaluated under different SBRs. A) Storage yields from SBRs. B) PHB productivity from SBRs. C) A) Substrate uptake rates from SBRs.

Otherwise, the contents of PHB at the beginning of the cycles and accumulated during the feast phases were also compared among conditions (Figure 17). As a consequence of the storage capacity evaluated previously in the different reactors, the higher PHB contents were observed in f/f 0.6 SBR. The PHB contents increased during feast phase from 28.2 to 35.8 %w/w and from 25.1 to 30.8 %w/w at 30mM and 120mM feed, respectively. This variation represented an average increase of 7.6 %w/w and 5,7 %w/w for both conditions. The condition imposed in f/f 0.6 SBR derived into the higher accumulation of PHB in biomass in our study, but anyway this content is not as high as observed by other studies (Johnson et al., 2009b; Moralejo-Gárate et al., 2013b). In relation to f/f 0.2 SBR condition, the initial PHB contents evaluated were just slightly higher than Control SBR. Statistical differences respect to the control contents were obtained only at f/f 0.2 with 120mM acetate, which showed a maximum content of 19.6 %w/w.

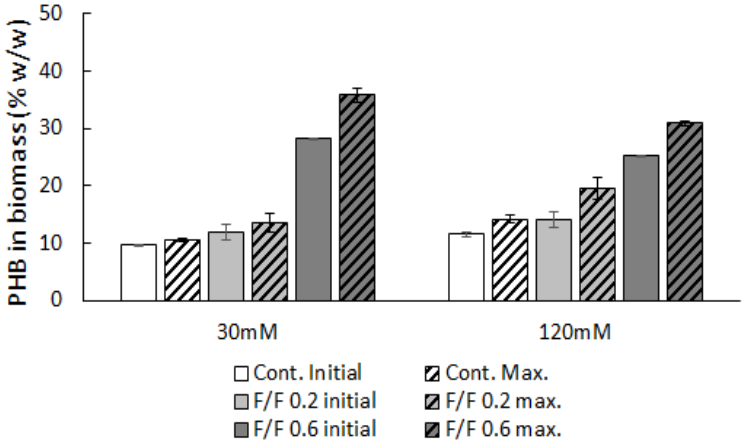


Figure 17. Comparison of initial and maximum PHB contents per cycle of operation among different conditions, when reached stationary phase.

4.3.3. Batch PHB accumulation experiments

The different operations under fixed control of f/f ratio were validated through batch accumulation tests where the source of nitrogen was eliminated as part of the feeding with the aim of increasing the intracellular content of PHB and inhibiting cell growth. The results of the PHB accumulation in batch tests showed the same trend compared to the operation in SBRs, and parameters are summarized in Table 9. The results indicated that R0.6 had the highest concentrations of PHB both in the selection reactor and in the accumulation reactor. Although the tendency suggests that contents observed in batch experiments had similar behaviors than SBRs, the maximum observed contents were low compared to observed in other studies in which batch experiment contents range from 50 to 80% (Johnson et al., 2009a; Montiel-Jarillo et al., 2017). During batch experiments from this research, time required for consumption of acetate was very high compared to other studies, which could have provoked difficulties in this accumulation experiments. On the other hand, it is considered that differences in the microorganism's composition respect to other studies could have provoked these changes. Further studies about bacterial communities present in culture are required to evaluate this topic in detail. Respect of storage kinetics in batch experiments, results of $Y_{P/S}$ were 0.026, 0.04 and 0.086 gPHB/gAcetate in control, R0.2 and R0.6, respectively. These results can be used to reaffirm our evidence that the reactor operated at a f/f 0.6 ratio possessed higher capacities for the generation and accumulation of PHB than SBR operated at fixed cycle length.

Table 9. Kinetics of PHB accumulation experiments

Unit	Substrate uptake rate	Specific PHB productivity	Storage yield	Initial PHB content	Maximum PHB content
	(g/L-h)	(g/L-h)	(gPHB/gAc.)	(% w/w)	(% w/w)
control SBR	0.04	0.002	0.043	7.01	11.61
f/f 0.2 SBR	0.09	0.005	0.052	16.58	25.00
f/f 0.6 SBR	0.09	0.007	0.086	29.82	44.49

4.4 Conclusion

The results obtained in this study indicate that the control of feeding cycles trough regulation of f/f ratio increased contents, storage yields and productivities of PHB in biomass, compared to conditions without f/f ratio control. Fixed cycle operation resulted in a low conversion of 11% of biomass in batch tests, as the form of PHB which indicated that this fixed cycle was not an optimal condition for polymer storage when feeding biomass with variable acetate concentrations.

When automatic regulation was applied, f/f ratio 0.6 had the best results of the experiment, indicating that a shorter famine interval guided biomass to produce more PHB (45% w/w of PHB). As the contents obtained at this research were not enough high compared to other studies, it is concluded that more experiments have to be developed in order to find the optimal conditions for automatic control of the feeding cycles in SBRs

Chapter 5

General discussion and concluding remarks

Chapter 5: General discussion and concluding remarks

5.1. General Discussion

In order to achieve a sustainable production of polymers from PHAs, it is essential to advance in the use of mixed microbial cultures adapted to transform substrates coming from residual effluents. Given that under the current costs of PHA production, polymers from axenic cultures are not competitive against the petroleum-based polymer costs, this thesis analyzed both the obtaining of a suitable substrate for production, as well as alternatives for obtaining inoculums from mixed microbial cultures. Drawbacks of both topics are discussed in this section:

About the generation of VFAs which come from residual effluents, it is observed that the operational conditions for acidogenic reactors could generate strong variations of the accumulation of these compounds depending on the culture conditions. pH variations imposed to acidogenic reactors affected microorganisms and both VFAs and phenols were progressively accumulated when methanogenics were inhibited. According to results from Chapter II, Alkaline conditions affected strongly methanogenics and acetogenic bacteria, favoring a high accumulation of acetic acid. These results are in concordance with most recent reports about this topic (Huang et al., 2018). Alkaline conditions favored hydrolysis making available higher amounts of organic matter to the reactor. Total amounts of VFAs accumulated had a value of 3,6 g VFA/L. These generated amounts of VFAs are in a higher range compared to other studies (See table 2) making this process, a valid alternative on the PHA production, considering the higher amounts of feedstock required for continuous operation of selective SBRs. Due to this fact, new researches about optimal substrates and pH conditions for VFA generation are required to advance on the implementation of this process at industrial scale.

Respect to the strategies evaluated on the selection of adapted biomass inoculums for PHA generation, in chapter III it was observed that cycle length and substrate concentration affected strongly the amounts and yields of PHB and biomass generated.

Despite a large increase in storage yield and PHB content with a 12-hour cycle and 120 mM acetate, the biomass content was low compared to other conditions evaluated and some other reports (Jiang et al., 2011a; Rodriguez-Perez et al., 2018). Our analysis of costs associated to the biomass selection step in SBRs (Chapter III) indicated that, for optimal PHB production at industrial scale, high amounts of biomass are required from the inoculum, in order to decrease associated costs (Figure 9). As it was previously indicated, there is an inverse proportional relation between PHB accumulated and biomass increase as growth (Dionisi et al., 2006b). These dynamics indicate that conditions which favor the biomass growth are not suitable for PHB accumulation in that biomass, leading to low storage yields of polymers. Further studies have to be taken, in order to accomplish a high biomass production with enough internal PHB accumulation which would be sustainable at industrial scales.

Otherwise, when evaluating wastewaters as feedstock for PHA generation, scarce research about variations in influents concentration has been developed to date. The importance of evaluate these variations lies in a hardly stability on OLRs when using VFAs from wastes in real-scale production. In this sense, the control strategy evaluated in Chapter IV worked well regulating the cycle time when different feed concentrations were applied alternately. Both controlled SBRs showed a higher performance on PHB storage, in comparison to control reactor. Nevertheless, all conditions showed low accumulation of PHB, in comparison to other studies when were evaluated with batch tests (M. G E Albuquerque et al., 2011; Montiel-Jarillo et al., 2017; Tamis et al., 2014). These results seem to be related to characteristics of the selected biomass in the previous stage acclimation reactor. Biomass selections is a highly important

issue when working with MMCs, and little variations in culture conditions can affect strongly the structure of microbial community selected(Albuquerque et al., 2013). Despite these drawbacks, studies carried out on Chapter IV of the present thesis work could effectively approximate to the developments of new technics towards the management of residues for highly value-added compounds such as PHAs, at high scale.

5.2. Concluding remarks

Chapter II:

As pH regulation played an important role in the destiny of accumulated compounds inside acidification reactors, it is concluded that alkaline conditions are highly stressfull conditions for anaerobic microbial communities which result in favour of VFAs accumulation. More research is required towards the optimization of VFAs obtention at alkaline conditions. As PHB production require constant and high VFAs influent concentration, a system which could produce high contents of short chain VFAs continuously must be developed.

Chapter III:

Operational conditions such as cycle length and substrate concentration in the influent of SBRs destined for PHA production, can affect significatively the productivities of biomass and PHB. According to results obtained from associated costs of all evaluated conditions, it is necessary to increase the biomass productivity, as equal to maintain enough sorage yield of PHAs in order to reduce costs for PHA production. As storage of PHB becomes low when high biomass is present in SBRs, there is necessary to find strategies for cultivation PHA accumulating microorganisms in conditions of high biomass contents

Chapter IV:

As real-scaling production of PHB is assumed to have difficulties in control of the influent concentrations when developed using waste streams, the strategies elaborated through this thesis can be an effective alternative to manage and regulate cycles under variable

concentrations. Through the DO monitoring, control platforms of reactors are able to regulate feeding cycles under variable feed conditions. Respect the control of cycle time through feast and famine intervals, short famine cycles showed to be an interesting alternative to obtain high storages of PHB with enough biomass contents.

References

Albuquerque, M.G.E., Carvalho, G., Kragelund, C., Silva, A.F., Barreto Crespo, M.T., Reis, M.A.M., Nielsen, P.H., 2013. Link between microbial composition and carbon substrate-uptake preferences in a PHA-storing community. ISME J. 7, 1–12. https://doi.org/10.1038/ismej.2012.74

Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R., Reis, M.A.M., 2007. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J. Biotechnol. 130, 411–421. https://doi.org/10.1016/j.jbiotec.2007.05.011

Albuquerque, M.G.E., Martino, V., Pollet, E., Avérous, L., Reis, M. a M., 2011. Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: effect of substrate composition and feeding regime on PHA productivity, composition and properties. J. Biotechnol. 151, 66–76. https://doi.org/10.1016/j.jbiotec.2010.10.070

Albuquerque, M.G.E., Martino, V., Pollet, E., Avérous, L., Reis, M. a M., 2011. Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. J. Biotechnol. 151, 66–76. https://doi.org/10.1016/j.jbiotec.2010.10.070

Albuquerque, M.G.E., Torres, C.A. V, Reis, M. a M., 2010. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection. Water Res. 44, 3419–3433. https://doi.org/10.1016/j.watres.2010.03.021

Anderson, A., Dawes, E., 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 450–472.

APHA, 2011. American Public Health Association. Standard methods for the examination of water and wastewater. 20^{o}ed. https://doi.org/10.2307/3071946

Beccari, M., Bertin, L., Dionisi, D., Fava, F., Lampis, S., Majone, M., Valentino, F., Vallini, G., Villano, M., 2009. Exploiting olive oil mill effluents as a renewable resource for production of biodegradable polymers through a combined anaerobic-aerobic process. J. Chem. Technol. Biotechnol. 84, 901–908. https://doi.org/10.1002/jctb.2173

Ben, M., Mato, T., Lopez, A., Vila, M., Kennes, C., Veiga, M.C., 2011. Bioplastic production using wood mill effluents as feedstock. Water Sci. Technol. 63, 1196–1202.

Bengtsson, S., Pisco, A.R., Reis, M. a M., Lemos, P.C., 2010. Production of polyhydroxyalkanoates from fermented sugar cane molasses by a mixed culture enriched in glycogen accumulating organisms. J. Biotechnol. 145, 253–63. https://doi.org/10.1016/j.jbiotec.2009.11.016

Bengtsson, S., Werker, A., Welander, T., 2008. Production of polyhydroxyalkanoates by glycogen accumulating organisms treating a paper mill wastewater. Water Sci. Technol. 58, 323–30. https://doi.org/10.2166/wst.2008.381

Beun, J.J., Dircks, K., Van Loosdrecht, M.C.M., Heijnen, J.J., 2002. Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. Water Res. 36, 1167–1180. https://doi.org/10.1016/S0043-1354(01)00317-7

Beun, J.J., Paletta, F., Van Loosdrecht, M.C.M., Heijnen, J.J., 2000a. Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures. Biotechnol. Bioeng. https://doi.org/10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2

Beun, J.J., Verhoef, E. V, Van Loosdrecht, M.C.M., Heijnen, J.J., 2000b. Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism under denitrifying conditions in

activated sludge cultures. Biotechnol. Bioeng. 68, 496–507. https://doi.org/10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2

Bolzonella, D., Fatone, F., Pavan, P., Cecchi, F., 2005. Anaerobic Fermentation of Organic Municipal Solid Wastes for the Production of Soluble Organic Compounds. Ind. Eng. Chem. Res. 44, 3412–3418. https://doi.org/10.1021/ie048937m

Borja, R., Banks, C.J., Sánchez, E., 1996. Anaerobic treatment of palm oil mill effluent in a two-stage up-flow anaerobic sludge blanket (UASB) system. J. Biotechnol. 45, 125–135. https://doi.org/10.1016/0168-1656(95)00154-9

Brigham, C.J., Zhila, N., Shishatskaya, E., Volova, T.G., Sinskey, A.J., 2012. Reprogramming Microbial Metabolic Pathways. Subcellular Biochemistry 64, 343–366. https://doi.org/10.1007/978-94-007-5055-5

Carta, F., Beun, J.J., Loosdrecht, M.C.M.V.A.N., Heijnen, J.J., 2001. Simultaneous Storage and Degradation of Phb and Glycogen in Activated Sludge Cultures 35, 2693–2701.

Chardron, S., Bruzaud, S., Lignot, B., Elain, A., Sire, O., 2010. Characterization of bionanocomposites based on medium chain length polyhydroxyalkanoates synthesized by Pseudomonas oleovorans. Polym. Test. 29, 966–971. https://doi.org/10.1016/j.polymertesting.2010.08.009

Chen, G.-Q., 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem. Soc. Rev. 38, 2434–2446. https://doi.org/10.1039/b812677c

Chen, G.-Q., Wu, Q., Jung, Y.K., Lee, S.Y., 2011. Pha/Phb. Compr. Biotechnol. 217–227. https://doi.org/10.1016/B978-0-08-088504-9.00179-3

Chen, G.Q., Jiang, X.R., Guo, Y., 2016. Synthetic biology of microbes synthesizing polyhydroxyalkanoates (PHA). Synth. Syst. Biotechnol. 1, 236–242.

https://doi.org/10.1016/j.synbio.2016.09.006

Chen, G.Q., Wu, Q., 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26, 6565–6578. https://doi.org/10.1016/j.biomaterials.2005.04.036

Daly, J., Hayward, D., 2004. Ageing and rejuvenation of Biopol™, copolymers: A dielectric study. J. Mater. … 9, 925–931.

Demirel, B., Yenigun, O., 2004. Anaerobic acidogenesis of dairy wastewater: the effects of variations in hydraulic retention time with no pH control. J. Chem. Technol. Biotechnol. 79, 755–760. https://doi.org/10.1002/jctb.1052

Dias, J.M.L., Lemos, P.C., Serafim, L.S., Oliveira, C., Eiroa, M., Albuquerque, M.G.E., Ramos, A.M., Oliveira, R., Reis, M. a M., 2006a. Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. Macromol. Biosci. 6, 885–906. https://doi.org/10.1002/mabi.200600112

Dias, J.M.L., Lemos, P.C., Serafim, L.S., Oliveira, C., Eiroa, M., Albuquerque, M.G.E., Ramos, A.M., Oliveira, R., Reis, M. a M., 2006b. Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. Macromol. Biosci. 6, 885–906. https://doi.org/10.1002/mabi.200600112

Dionisi, D., Carucci, G., Papini, M.P., Riccardi, C., Majone, M., Carrasco, F., 2005. Olive oil mill effluents as a feedstock for production of biodegradable polymers. Water Res. 39, 2076–84. https://doi.org/10.1016/j.watres.2005.03.011

Dionisi, D., Carucci, G., Papini, M.P., Riccardi, C., Majone, M., Carrasco, F., Petrangeli Papini, M., Riccardi, C., Majone, M., Carrasco, F., 2005. Olive oil mill effluents as a feedstock for production of biodegradable polymers. Water Res. 39, 2076–2084. https://doi.org/10.1016/j.watres.2005.03.011

Dionisi, D., Majone, M., Miccheli, A., Puccetti, C., Sinisi, C., 2004. Glutamic acid removal and PHB storage in the activated sludge process under dynamic conditions. Biotechnol. Bioeng. 86, 842–51. https://doi.org/10.1002/bit.20091

Dionisi, D., Majone, M., Tandoi, V., Beccari, M., 2001. Sequencing Batch Reactor: Influence of Periodic Operation on Performance of Activated Sludges in Biological Wastewater Treatment. Ind. Eng. Chem. Res. 40, 5110–5119. https://doi.org/10.1021/ie001008k

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S., Beccari, M., 2006a. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. Biotechnol. Bioeng. 93, 76–88. https://doi.org/10.1002/bit.20683

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S., Beccari, M., 2006b. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. Biotechnol. Bioeng. 93, 76–88. https://doi.org/10.1002/bit.20683

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S., Beccari, M., 2006c. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. Biotechnol. Bioeng. 93, 76–88. https://doi.org/10.1002/bit.20683

Dircks, K., Beun, J.J., Loosdrecht, M. Van, Heijnen, J.J., Henze, M., 2001. Glycogen Metabolism in Aerobic Mixed Cultures Experimental Set-Up.

Elbeshbishy, E., Hafez, H., Dhar, B.R., Nakhla, G., 2011. Single and combined effect of various pretreatment methods for biohydrogen production from food waste. Int. J. Hydrogen Energy 36, 11379–11387. https://doi.org/10.1016/j.ijhydene.2011.02.067

Feng, L., Yan, Y., Chen, Y., 2011. Co-fermentation of waste activated sludge with food waste

for short-chain fatty acids production: effect of pH at ambient temperature. Front. Environ. Sci. Eng. China 5, 623–632. https://doi.org/10.1007/s11783-011-0334-2

Fernández-Rodríguez, M.J., Rincón, B., Fermoso, F.G., Jiménez, A.M., Borja, R., 2014. Assessment of two-phase olive mill solid waste and microalgae co-digestion to improve methane production and process kinetics. Bioresour. Technol. 157, 263–269. https://doi.org/10.1016/j.biortech.2014.01.096

Fezzani, B., Cheikh, R. Ben, 2010. Two-phase anaerobic co-digestion of olive mill wastes in semi-continuous digesters at mesophilic temperature. Bioresour. Technol. 101, 1628–1634. https://doi.org/10.1016/j.biortech.2009.09.067

Fidler, S., Dennis, D., 1992. Polyhydroxyalkanoate production in recombinant Escherichia coli. FEMS Microbiol. Lett. 103, 231–235.

Flieger, M., Kantorová, M., Prell, a, Rezanka, T., Votruba, J., 2003. Biodegradable plastics from renewable sources. Folia Microbiol. (Praha). 48, 27–44. https://doi.org/10.1007/BF02931273

Freches, A., Lemos, P.C., 2017. Microbial selection strategies for polyhydroxyalkanoates production from crude glycerol: Effect of OLR and cycle length. N. Biotechnol. 39, 22–28. https://doi.org/10.1016/j.nbt.2017.05.011

Hong, C., Hao, H., Haiyun, W., 2009. Process optimization for PHA production by activated sludge using response surface methodology. Biomass and Bioenergy 33, 721–727. https://doi.org/10.1016/j.biombioe.2008.11.004

Hu, W., Chua, H., Yu, P., 1997. Synthesis of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from activated sludge. Biotechnol. Lett. 7–8.

Huang, C., Liu, C., Sun, X., Sun, Y., Li, R., Li, J., Shen, J., Han, W., Liu, X., Wang, L., 2015.

Hydrolysis and volatile fatty acids accumulation of waste activated sludge enhanced by the combined use of nitrite and alkaline pH. Environ. Sci. Pollut. Res. 22, 18793–18800. https://doi.org/10.1007/s11356-015-4822-y

Huang, X., Dong, W., Wang, H., Feng, Y., 2018. Role of acid/alkali-treatment in primary sludge anaerobic fermentation: Insights into microbial community structure, functional shifts and metabolic output by high-throughput sequencing. Bioresour. Technol. 249, 943–952. https://doi.org/10.1016/j.biortech.2017.10.104

Jendrossek, D., 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). J. Bacteriol. 191, 3195–3202. https://doi.org/10.1128/JB.01723-08

Jendrossek, D., Handrick, R., 2002. Microbial degradation of polyhydroxyalkanoates. Annu. Rev. Microbiol. 56, 403–432. https://doi.org/10.1146/annurev.micro.56.012302.160838

Jendrossek, D., Pfeiffer, D., 2014. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ. Microbiol. 16, 2357–2373. https://doi.org/10.1111/1462-2920.12356

Ji, Z., Chen, G., Chen, Y., 2010. Effects of waste activated sludge and surfactant addition on primary sludge hydrolysis and short-chain fatty acids accumulation. Bioresour. Technol. 101, 3457–3462. https://doi.org/10.1016/j.biortech.2009.12.117

Jiang, S., Chen, Y., Zhou, Q., Gu, G., 2007. Biological short-chain fatty acids (SCFAs) production from waste-activated sludge affected by surfactant. Water Res. 41, 3112–3120. https://doi.org/10.1016/j.watres.2007.03.039

Jiang, Y., Hebly, M., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2011a. Metabolic modeling of mixed substrate uptake for polyhydroxyalkanoate (PHA) production. Water Res. 45, 1309–1321. https://doi.org/10.1016/j.watres.2010.10.009

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2011b. Polyhydroxybutyrate production from lactate using a mixed microbial culture. Biotechnol. Bioeng. 108, 2022–35. https://doi.org/10.1002/bit.23148

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2011c. Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor. ISME J. 5, 896–907. https://doi.org/10.1038/ismej.2010.174

Jiang, Y., Marang, L., Tamis, J., van Loosdrecht, M.C.M., Dijkman, H., Kleerebezem, R., 2012. Waste to resource: Converting paper mill wastewater to bioplastic. Water Res. 46, 5517–30. https://doi.org/10.1016/j.watres.2012.07.028

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., Van Loosdrecht, M.C.M., 2009a. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10, 670–676. https://doi.org/10.1021/bm8013796

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., Van Loosdrecht, M.C.M., 2009b. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity, in: Biomacromolecules. pp. 670–676. https://doi.org/10.1021/bm8013796

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. J. Biotechnol. 147, 73–9. https://doi.org/10.1016/j.jbiotec.2010.02.003

Kadouri, D., Jurkevitch, E., Okon, Y., Castro-Sowinski, S., 2005. Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Crit. Rev. Microbiol. 31, 55–67. https://doi.org/10.1080/10408410590899228

Khanna, S., Srivastava, A.K., 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem. 40, 607–619. https://doi.org/10.1016/j.procbio.2004.01.053

Kim, H.J., Kim, S.H., Choi, Y.G., Kim, G.D., Chung, H., 2006. Effect of enzymatic pretreatment on acid fermentation of food waste. J. Chem. Technol. Biotechnol. https://doi.org/10.1002/jctb.1484

Kleerebezem, R., Joosse, B., Rozendal, R., Van Loosdrecht, M.C.M., 2015. Anaerobic digestion without biogas? Rev. Environ. Sci. Biotechnol. 14, 787–801. https://doi.org/10.1007/s11157-015-9374-6

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Curr. Opin. Biotechnol. 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001

Lee, S.Y., 1996. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 14, 431–438. https://doi.org/10.1016/0167-7799(96)10061-5

Lee, S.Y., Wong, H.H., Choi, J. Il, Lee, S.H., Lee, S.C., Han, C.S., 2000. Production of medium-chain-length polyhydroxyalkanoates by high-cell- density cultivation Pseudomonas putida under phosphorus limitation. Biotechnol. Bioeng. https://doi.org/10.1002/(SICI)1097-0290(20000520)68:4<466::AID-BIT12>3.0.CO;2-T

Lee, W.-H., Loo, C.-Y., Nomura, C.T., Sudesh, K., 2008. Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. Bioresour. Technol. 99, 6844–51. https://doi.org/10.1016/j.biortech.2008.01.051

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014a. A review of the production and applications of waste-derived volatile fatty acids. Chem. Eng. J. 235, 83–99. https://doi.org/10.1016/j.cej.2013.09.002

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014b. A review of the production and

applications of waste-derived volatile fatty acids. Chem. Eng. J. 235, 83–99. https://doi.org/10.1016/j.cej.2013.09.002

Lemos, P., Serafim, L., 2003. Metabolic pathway for propionate utilization by phosphorus-accumulating organisms in activated sludge: 13C labeling and in vivo nuclear magnetic resonance. Appl. … 241–251. https://doi.org/10.1128/AEM.69.1.241

Lemos, P.C., Dai, Y., Yuan, Z., Keller, J., Santos, H., Reis, M. a M., 2007. Elucidation of metabolic pathways in glycogen-accumulating organisms with in vivo 13C nuclear magnetic resonance. Environ. Microbiol. 9, 2694–706. https://doi.org/10.1111/j.1462-2920.2007.01382.x

Lemos, P.C., Serafim, L.S., Reis, M. a M., 2006. Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. J. Biotechnol. 122, 226–238. https://doi.org/10.1016/j.jbiotec.2005.09.006

Leong, Y.K., Show, P.L., Ooi, C.W., Ling, T.C., Lan, J.C.W., 2014. Current trends in polyhydroxyalkanoates (PHAs) biosynthesis: Insights from the recombinant Escherichia coli. J. Biotechnol. 180, 52–65. https://doi.org/10.1016/j.jbiotec.2014.03.020

Lim, S.-J., Kim, E.-Y., Ahn, Y.-H., Chang, H.-N., 2008. Biological nutrient removal with volatile fatty acids from food wastes in sequencing batch reactor. Korean J. Chem. Eng. 25, 129–133. https://doi.org/10.1007/s11814-008-0023-4

Lim, S.J., Kim, B.J., Jeong, C.M., Choi, J. dal rae, Ahn, Y.H., Chang, H.N., 2008. Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor. Bioresour. Technol. 99, 7866–7874. https://doi.org/10.1016/j.biortech.2007.06.028

Liu, H., Han, P., Liu, H., Zhou, G., Fu, B., Zheng, Z., 2018. Full-scale production of VFAs from sewage sludge by anaerobic alkaline fermentation to improve biological nutrients removal in domestic wastewater. Bioresour. Technol. 260, 105–114.

https://doi.org/10.1016/j.biortech.2018.03.105

López, N.I., Floccari, M.., Steinbüchel, a., García, a. F., Méndez, B.., 1995. Effect of poly(3-hydroxybutyrate) (PHB) content on the starvation-survival of bacteria in natural waters. FEMS Microbiol. Ecol. 16, 95–101. https://doi.org/10.1111/j.1574-6941.1995.tb00273.x

Maharaj, I., Elefsiniotis, P., 2001. The role of HRT and low temperature on the acid-phase anaerobic digestion of municipal and industrial wastewaters. Bioresour. Technol. 76, 191–197. https://doi.org/10.1016/S0960-8524(00)00128-0

Majone, M., Dircks, K., Beun, J., 1999. Aerobic storage under dynamic conditions in activated sludge processes. The state of the art. Water Sci. Technol. 39, 61–73. https://doi.org/10.1016/S0273-1223(98)00776-8

Martins, A.M.P., Heijnen, J.J., van Loosdrecht, M.C.M., 2003. Effect of feeding pattern and storage on the sludge settleability under aerobic conditions. Water Res. 37, 2555–70. https://doi.org/10.1016/S0043-1354(03)00070-8

Min, K.S., Khan, A.R., Kwon, M.K., Jung, Y.J., Yun, Z., Kiso, Y., 2005. Acidogenic fermentation of blended food-waste in combination with primary sludge for the production of volatile fatty acids. J. Chem. Technol. Biotechnol. 80, 909–915. https://doi.org/10.1002/jctb.1261

Montiel-Jarillo, G., Carrera, J., Suárez-Ojeda, M.E., 2017. Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: Effect of pH and N and P concentrations. Sci. Total Environ. 583, 300–307. https://doi.org/10.1016/j.scitotenv.2017.01.069

Moralejo-Gárate, H., Kleerebezem, R., Mosquera-Corral, A., van Loosdrecht, M.C.M., 2013a. Impact of oxygen limitation on glycerol-based biopolymer production by bacterial enrichments. Water Res. 47, 1209–17. https://doi.org/10.1016/j.watres.2012.11.039

Moralejo-Gárate, H., Mar'atusalihat, E., Kleerebezem, R., van Loosdrecht, M.C.M., 2011. Microbial community engineering for biopolymer production from glycerol. Appl. Microbiol. Biotechnol. 92, 631–9. https://doi.org/10.1007/s00253-011-3359-3

Moralejo-Gárate, H., Palmeiro-Sánchez, T., Kleerebezem, R., Mosquera-Corral, A., Campos, J.L., van Loosdrecht, M.C.M., 2013b. Influence of the cycle length on the production of PHA and polyglucose from glycerol by bacterial enrichments in sequencing batch reactors. Biotechnol. Bioeng. 110, 3148–55. https://doi.org/10.1002/bit.24991

Możejko-Ciesielska, J., Kiewisz, R., 2016. Bacterial polyhydroxyalkanoates: Still fabulous? Microbiol. Res. 192, 271–282. https://doi.org/10.1016/j.micres.2016.07.010

Ntaikou, I., Valencia Peroni, C., Kourmentza, C., Ilieva, V.I., Morelli, A., Chiellini, E., Lyberatos, G., 2014. Microbial bio-based plastics from olive-mill wastewater: Generation and properties of polyhydroxyalkanoates from mixed cultures in a two-stage pilot scale system. J. Biotechnol. 188, 138–147. https://doi.org/10.1016/j.jbiotec.2014.08.015

Oktem, Y.A., Ince, O., Donnelly, T., Sallis, P., Ince, B.K., 2006. Determination of optimum operating conditions of an acidification reactor treating a chemical synthesis-based pharmaceutical wastewater. Process Biochem. 41, 2258–2263. https://doi.org/10.1016/j.procbio.2006.05.016

Ouazzane, H., Laajine, F., El Yamani, M., El Hilaly, J., Rharrabti, Y., Amarouch, M.Y., Mazouzi, D., 2017. Olive mill solid waste characterization and recycling opportunities: A review. J. Mater. Environ. Sci. 8, 2632–2650.

Pagliano, G., Ventorino, V., Panico, A., Pepe, O., 2017. Integrated systems for biopolymers and bioenergy production from organic waste and by-products: a review of microbial processes. Biotechnol. Biofuels 10, 113.

Pratt, S., Yuan, Z., Keller, J., 2004. Modeling aerobic carbon oxidation and storage by

integrating respirometric, titrimetric, and off-gas CO2 measurements. Biotechnol. Bioeng. 88, 135–47. https://doi.org/10.1002/bit.20199

Raza, Z.A., Abid, S., Banat, I.M., 2018. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. Int. Biodeterior. Biodegrad. 126, 45–56. https://doi.org/10.1016/j.ibiod.2017.10.001

Rehm, B.H.A., 2003. Polyester synthases: natural catalysts for plastics. Biochem. J. 376, 15–33. https://doi.org/10.1042/BJ20031254

Rehm, B.H.A., Steinbüchel, A., 1999. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis, in: International Journal of Biological Macromolecules. pp. 3–19. https://doi.org/10.1016/S0141-8130(99)00010-0

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., Van Loosdrecht, M.C.M., 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosyst. Eng. 25, 377–385. https://doi.org/10.1007/s00449-003-0322-4

Rincón, B., Borja, R., 2012. Assessment of the anaerobic acidogenesis of wet olive cake from a two-phase olive oil mill. J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 47, 1439–1445. https://doi.org/10.1080/10934529.2012.672389

Rodriguez-Perez, S., Serrano, A., Pantión, A.A., Alonso-Fariñas, B., 2018. Challenges of scaling-up PHA production from waste streams. A review. J. Environ. Manage. 205, 215–230. https://doi.org/10.1016/j.jenvman.2017.09.083

Sans, C., Mata-Alvarez, J., Cecchi, F., Pavan, P., Bassetti, A., 1995. Acidogenic fermentation of organic urban wastes in a plug-flow reactor under thermophilic conditions. Bioresour. Technol. 54, 105–110. https://doi.org/10.1016/0960-8524(95)00098-4

Sanz, A., Scholz, L., Carus, M., 2013. Market study on Bio-based polymers in the world,

capacities, production and applications: Status Quo and trends towars 2020. nova-Institute GmbH.

Satoh, H., Ramey, W.D., Koch, F. a., Oldham, W.K., Mino, T., Matsuo, T., 1996. Anaerobic substrate uptake by the enhanced biological phosphorus removal activated sludge treating real sewage. Water Sci. Technol. 34, 9–16. https://doi.org/10.1016/0273-1223(96)00489-1

Serafim, L.S., Lemos, P.C., Albuquerque, M.G.E., Reis, M. a M., 2008. Strategies for PHA production by mixed cultures and renewable waste materials. Appl. Microbiol. Biotechnol. 81, 615–28. https://doi.org/10.1007/s00253-008-1757-y

Serafim, L.S., Lemos, P.C., Albuquerque, M.G.E., Reis, M.A.M., 2008a. Strategies for PHA production by mixed cultures and renewable waste materials. Appl. Microbiol. Biotechnol. 81, 615–628. https://doi.org/10.1007/s00253-008-1757-y

Serafim, L.S., Lemos, P.C., Albuquerque, M.G.E., Reis, M.A.M., 2008b. Strategies for PHA production by mixed cultures and renewable waste materials. Appl. Microbiol. Biotechnol. 81, 615–628. https://doi.org/10.1007/s00253-008-1757-y

Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M. a M., 2004. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–60. https://doi.org/10.1002/bit.20085

Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M., 2004a. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–160. https://doi.org/10.1002/bit.20085

Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M., 2004b. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–160. https://doi.org/10.1002/bit.20085

Serrano, A., Fermoso, F.G., Alonso-Fariñas, B., Rodríguez-Gutierrez, G., Fernandez-Bolaños, J., Borja, R., 2017a. Olive mill solid waste biorefinery: High-temperature thermal pre-treatment for phenol recovery and biomethanization. J. Clean. Prod. 148, 314–323. https://doi.org/10.1016/j.jclepro.2017.01.152

Serrano, A., Fermoso, F.G., Rodríguez-Gutierrez, G., Fernandez-Bolaños, J., Borja, R., 2017b. Biomethanization of olive mill solid waste after phenols recovery through low-temperature thermal pre-treatment. Waste Manag. 61, 229–235. https://doi.org/10.1016/j.wasman.2016.12.033

Shang, L., Jiang, M., Chang, H.N., 2003. Poly(3-hydroxybutyrate) synthesis in fed-batch culture of Ralstonia eutropha with phosphate limitation under different glucose concentrations. Biotechnol. Lett. 25, 1415–1419. https://doi.org/10.1023/A:1025047410699

Steinbüchel, A., Valentin, H., 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128, 219–228.

Stoyanova, E., Lundaa, T., Bochmann, G., Fuchs, W., 2017. Overcoming the bottlenecks of anaerobic digestion of olive mill solid waste by two-stage fermentation. Environ. Technol. (United Kingdom) 38, 394–405. https://doi.org/10.1080/09593330.2016.1196736

Sugimoto, T., Tsuge, T., Tanaka, K., Ishizaki, A., 1999. Control of acetic acid concentration by pH-Stat continuous substrate feeding in heterotrophic culture phase of two-stage cultivation of Alcaligenes eutrophus for production of P(3HB) from CO2, H2, and O2 under non-explosive conditions. Biotechnol. Bioeng. https://doi.org/10.1002/(SICI)1097-0290(19990320)62:6<625::AID-BIT1>3.0.CO;2-D

Taguchi, S., 2012. Poly (hydroxyalkanoate) s, Polymer Science:A Comprehensive Reference. Elsevier B.V. https://doi.org/10.1016/B978-0-444-53349-4.00223-5

Tamis, J., Lužkov, K., Jiang, Y., Loosdrecht, M.C.M. van, Kleerebezem, R., 2014. Enrichment of Plasticicumulans acidivorans at pilot-scale for PHA production on industrial wastewater. J. Biotechnol. 192, 161–169. https://doi.org/10.1016/j.jbiotec.2014.10.022

Tan, D., Wu, Q., Chen, J.-C., Chen, G.-Q., 2014. Engineering Halomonas TD01 for the low-cost production of polyhydroxyalkanoates. Metab. Eng. 26C, 34–47. https://doi.org/10.1016/j.ymben.2014.09.001

Teng, S.-X., Tong, Z.-H., Li, W.-W., Wang, S.-G., Sheng, G.-P., Shi, X.-Y., Liu, X.-W., Yu, H.-Q., 2010. Electricity generation from mixed volatile fatty acids using microbial fuel cells. Appl. Microbiol. Biotechnol. 87, 2365–2372. https://doi.org/10.1007/s00253-010-2746-5

Ucisik, A.S., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. Water Res. 42, 3729–3738. https://doi.org/10.1016/J.WATRES.2008.06.010

Uyar, B., Eroglu, I., Yücel, M., Gündüz, U., 2009. Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. Int. J. Hydrogen Energy 34, 4517–4523. https://doi.org/10.1016/J.IJHYDENE.2008.07.057

Valentino, F., Beccari, M., Fraraccio, S., Zanaroli, G., Majone, M., 2014. Feed frequency in a Sequencing Batch Reactor strongly affects the production of polyhydroxyalkanoates (PHAs) from volatile fatty acids. N. Biotechnol. 31, 264–275. https://doi.org/10.1016/j.nbt.2013.10.006

Valentino, F., Morgan-Sagastume, F., Campanari, S., Werker, A., 2017. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23. https://doi.org/10.1016/J.NBT.2016.05.007

Vanloosdrecht, M., Pot, M., Heijnen, J., 1997. Importance of bacterial storage polymers in bioprocesses. Water Sci. Technol. 35, 41–47. https://doi.org/10.1016/S0273-1223(96)00877-3

Villano, M., Beccari, M., Dionisi, D., Lampis, S., Miccheli, A., Vallini, G., Majone, M., 2010. Effect of pH on the production of bacterial polyhydroxyalkanoates by mixed cultures enriched under periodic feeding. Process Biochem. 45, 714–723. https://doi.org/10.1016/j.procbio.2010.01.008

Wallen, L.L., Rohwedder, W.K., 1974. Poly-B-hydroxyalkanoate from Activated Sludge. Environ. Sci. Technol. 8, 576–579. https://doi.org/\url{10.1021/es60091a007}

Wang, B., Li, Y., Ren, N., 2013. Biohydrogen from molasses with ethanol-type fermentation: Effect of hydraulic retention time. Int. J. Hydrogen Energy 38, 4361–4367. https://doi.org/10.1016/j.ijhydene.2013.01.120

Wen, Q., Chen, Z., Wang, C., Ren, N., 2012. Bulking sludge for PHA production: Energy saving and comparative storage capacity with well-settled sludge. J. Environ. Sci. (China) 24, 1744–1752. https://doi.org/10.1016/S1001-0742(11)61005-X

Wong, M.S., Causey, T.B., Mantzaris, N., Bennett, G.N., San, K., 2008. Engineering Poly (3-hydroxybutyrate- co -3- hydroxyvalerate) Copolymer Composition in E . coli 919–928. https://doi.org/10.1002/bit

Wu, H., Yang, D., Zhou, Q., Song, Z., 2009. The effect of pH on anaerobic fermentation of primary sludge at room temperature. J. Hazard. Mater. 172, 196–201. https://doi.org/10.1016/j.jhazmat.2009.06.146

Yu, H.Q., Fang, H.H.., 2001. Acidification of mid- and high-strength dairy wastewaters. Water Res. 35, 3697–3705. https://doi.org/10.1016/S0043-1354(01)00077-X

Yu, H.Q., Fang, H.H.P., 2000. Thermophilic acidification of dairy wastewater. Appl. Microbiol. Biotechnol. 54, 439–444. https://doi.org/10.1007/s002530000390

Yu, L., Zhang, W., Liu, H., Wang, G., Liu, H., 2018. Evaluation of volatile fatty acids production and dewaterability of waste activated sludge with different thermo-chemical pretreatments. Int. Biodeterior. Biodegradation 129, 170–178. https://doi.org/https://doi.org/10.1016/j.ibiod.2018.02.008

Zhang, B., Zhang, L.-L., Zhang, S.-C., Shi, H.-Z., Cai, W.-M., 2005. The Influence of pH on Hydrolysis and Acidogenesis of Kitchen Wastes in Two-phase Anaerobic Digestion. Environ. Technol. 26, 329–340. https://doi.org/10.1080/09593332608618563

Zhang, B., Zhang, L.-L., Zhang, S.-C., Shi, H.-Z., Cai, W.-M., 2005. The Influence of pH on Hydrolysis and Acidogenesis of Kitchen Wastes in Two-phase Anaerobic Digestion. Environ. Technol. 26, 329–340. https://doi.org/10.1080/09593332608618563

Zhang, P., Chen, Y., Zhou, Q., 2009. Waste activated sludge hydrolysis and short-chain fatty acids accumulation under mesophilic and thermophilic conditions: Effect of pH. Water Res. 43, 3735–3742. https://doi.org/10.1016/j.watres.2009.05.036

Zhang, Y.-Z., Liu, G.-M., Weng, W.-Q., Ding, J.-Y., Liu, S.-J., 2015. Engineering of Ralstonia eutropha for the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from glucose. J. Biotechnol. 195, 82–8. https://doi.org/10.1016/j.jbiotec.2014.12.014

Zúñiga, C., Morales, M., Le Borgne, S., Revah, S., 2011. Production of poly-β-hydroxybutyrate (PHB) by Methylobacterium organophilum isolated from a methanotrophic consortium in a two-phase partition bioreactor. J. Hazard. Mater. 190, 876–82. https://doi.org/10.1016/j.jhazmat.2011.04.011

Appendix

Equations for costs estimation of PHA-enriched biomass

In order to determine the operating conditions that minimize the bioplastic production costs, capital and operating costs were calculated for a annual production of PHB of 500 Tons.

Calculations:

Number of SBRs for PHB accumulation:

$$N_{SBR_{Accum}} = round.up\left(\frac{PHB\ production\ \left(\frac{Ton\ PHB}{year}\right)\cdot 1000\left(\frac{kg\ PHB}{Ton\ PHB}\right)\cdot\frac{1}{365}\left(\frac{year}{d}\right)}{\left[(r_{PHB})_{feast}\cdot\left(\frac{kg\ PHB}{m^3\cdot d}\right)\cdot\frac{t_{feast}}{t_{fed}+t_{feast}+t_{withdrawal}}+[VSS]_{end\ of\ enrichment\ cycle}\left(\frac{kg\ VSS}{m^3}\right)\cdot PHB_{fraction\ end\ of\ enrichment\ cycle}\left(\frac{kg\ PHB}{kg\ VSS}\right)\cdot(1-VF_{fed})\cdot\frac{24\ \left(\frac{h}{d}\right)}{(t_{fed}+t_{feast}+t_{withdrawal})\ (h)}\right]\cdot V_r\ (\frac{m^3}{reactor})}\right) + 1$$

VF_{fed}= Volumetric fraction of feeding media supplied per cycle (1/8)

V_r= Useful volume of the reactor (8.0 m^3; Total volume: 10.5 m^3; H/D: 4)

Number of SBRs needed to carry out biomass enrichment:

$$N_{SBR_{Enrichment}} = round.up\left(\frac{PHB\ production\ \left(\frac{Ton\ PHB}{year}\right)\cdot 1000\left(\frac{kg\ PHB}{Ton\ PHB}\right)\cdot\frac{1}{365}\left(\frac{year}{d}\right)-(r_{PHB})_{feast}\cdot\left(\frac{kg\ PHB}{m^3\cdot d}\right)\cdot\frac{t_{feast}}{t_{fed}+t_{feast}+t_{withdrawal}}\cdot(N_{SBR_{Accum}}-1)\ (reactor)\cdot V_r\ (\frac{m^3}{reactor})}{\left[[VSS]_{end\ of\ enrichment\ cycle}\left(\frac{kg\ VSS}{m^3}\right)\cdot PHB_{fraction\ end\ of\ enrichment\ cycle}\left(\frac{kg\ PHB}{kg\ VSS}\right)\cdot VER_{Enrichment}\cdot\frac{24\ \left(\frac{h}{d}\right)}{t_{cycle\ Enrichment}\ (h)}\right]\cdot V_r\ (\frac{m^3}{reactor})}\right)+1$$

$VER_{Enrichment}$= Volumetric exchange ratio of the enrichment reactor (1/8)

V_r= Useful volume of the reactor (8.0 m^3; Total volume: 10.5 m^3; H/D: 4)

Flow rate fed of SBRs for PHB accumulation:

$$Flowrate_{Fed\ Acum}\left(\frac{m^3}{d}\right)$$

$$= VF_{fed}\ \cdot V_r\ (\frac{m^3}{reactor}) \cdot (N_{SBR_{Accum}} - 1)\ (reactor) \cdot \frac{24\ (\frac{h}{d})}{(t_{fed} + t_{feast} + t_{withdrawal})\ (h)}$$

Flow rate from enrichment SBRs to SBRs for PHB accumulation:

$$Flowrate_{Enrich\ Accum}\left(\frac{m^3}{d}\right)$$

$$= (1 - VF_{fed})\ \cdot V_r\ (\frac{m^3}{reactor}) \cdot (N_{SBR_{Accum}} - 1)\ (reactor) \cdot \frac{24\ (\frac{h}{d})}{(t_{fed} + t_{feast} + t_{withdrawal})\ (h)}$$

Flow rate fed of SBRs for PHB accumulation:

$$Flowrate_{Fed\ Enrich}\left(\frac{m^3}{d}\right) = VER_{Enrichment}\ \cdot V_r\ (\frac{m^3}{reactor}) \cdot (N_{SBR_{Enrich}} - 1)\ (reactor) \cdot \frac{24\ (\frac{h}{d})}{t_{cycle\ Enrichment}\ (h)}$$

Pumping capacity:

$$Flowrate\ Pump_{Fed\ Accum}\left(\frac{m^3}{s}\right) = \frac{VF_{fed}\ \cdot V_{r\ Accum}(m^3)}{t_{fed}(\min) \cdot \frac{1}{60}\ (\frac{min}{s})}$$

$$Flowrate\ Pump_{Enrich-Accum}\left(\frac{m^3}{s}\right) = \frac{(1 - VF_{fed}) \cdot V_{r\ Accum}(m^3)}{t_{fed}(\min) \cdot \frac{1}{60}\ (\frac{min}{s})}$$

$$Flowrate\ Pump_{Fed\ Enrichment}\left(\frac{m^3}{s}\right) = \frac{VER_{Enrichment}\ \cdot V_{r\ Enrichment}(m^3)}{t_{fed}(\min) \cdot \frac{1}{60}\left(\frac{min}{s}\right)}$$

Number of pumps:

$$N_{Pump\ fed\ Accum} = round.up\left(\frac{Flowrate_{Fed\ Accum}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Fed\ Accum}\left(\frac{m^3}{s}\right)\cdot 86400\ \left(\frac{s}{d}\right)}\right)+1$$

$$N_{Pump\ Enrich-Accum} = round.up\left(\frac{Flowrate_{Enrich-Accum}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Enrich-Accum}\left(\frac{m^3}{s}\right)\cdot 86400\ \left(\frac{s}{d}\right)}\right)+1$$

$$N_{Pump\ fed\ Enrichment} = round.up\left(\frac{Flowrate_{Fed\ Enrich}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Fed\ Enrichment}\left(\frac{m^3}{s}\right)\cdot 86400\ \left(\frac{s}{d}\right)}\right)+1$$

Pumping energy consumption:

$$Pump\ Power\ (W) = \frac{\rho_{H_2O}\left(\frac{kg}{m^3}\right)\cdot Flowrate\ Pump\left(\frac{m^3}{s}\right)\cdot H_r(m)}{\eta_{pump}}$$

η_{pump}= Energy efficiency of the pump (0.7)

H_r= height of reactor (4.6 m)

$$Pumping\ energy\ consumption_{\ Fed\ Accum}\left(\frac{kW\cdot h}{d}\right) = \frac{Pump\ Power_{\ Fed\ Accum}\ (W)\cdot Flowrate_{Fed\ Accum}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Fed\ Accum}\left(\frac{m^3}{s}\right)\cdot 3.6\cdot 10^6\ (W/(\frac{kW\cdot h}{d}))}$$

$$Pumping\ energy\ consumption_{Fed\ Enrichment}\left(\frac{kW\cdot h}{d}\right) = \frac{Pump\ Power_{\ fed\ Enrichment}\ (W)\cdot Flowrate_{Fed\ Enrichment}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Fed\ Enrichment}\left(\frac{m^3}{s}\right)\cdot 3.6\cdot 10^6\ (W/(\frac{kW\cdot h}{d}))}$$

$$Pumping\ energy\ consumption_{\ Enrich-Accum}\left(\frac{kW\cdot h}{d}\right) = \frac{Pump\ Power_{\ Enrich-Accum}\ (W)\cdot Flowrate_{\ Enrich-Accum}\left(\frac{m^3}{d}\right)}{Flowrate\ Pump_{Enrich-Accum}\left(\frac{m^3}{s}\right)\cdot 3.6\cdot 10^6\ (W/(\frac{kW\cdot h}{d}))}$$

Sodium acetate consumption:

$$Inlet\ AcNa_{Enrichment}\left(\frac{kg\ AcNa}{d}\right) = [AcNa]_{fed}\left(\frac{kg\ AcNa}{m^3}\right) \cdot Flowrate_{Fed\ Enrichment}\left(\frac{m^3}{d}\right)$$

$$Inlet\ AcNa_{Accum}\left(\frac{kg\ AcNa}{d}\right) = [AcNa]_{fed}\left(\frac{kg\ AcNa}{m^3}\right) \cdot Flowrate_{Fed\ Acum}\left(\frac{m^3}{d}\right)$$

Oxygen consumption: Oxygen consumed during the operation of both enrichment and accumulation systems was calculated based on a COD balance:

$$m_{O_2}\left(\frac{kg\ O_2}{d}\right) = m_{AcNa_i}\left(\frac{kg\ AcNa}{d}\right) \cdot \left[0.78\left(\frac{kg\ COD}{kg\ AcNa}\right) - Y_{\frac{PHB}{AcNa}}\left(\frac{kg\ PHB}{kg\ AcNa}\right) \cdot 1{,}67\left(\frac{kg\ COD}{kg\ PHB}\right) - Y_{\frac{X}{AcNa}}\left(\frac{kg\ Biomass}{kg\ AcNa}\right) \cdot 1{,}42\left(\frac{kg\ COD}{kg\ Biomass}\right)\right]$$

Fan flow rate calculation:

$$Q_{O_2}\left(\frac{m^3{}_{air}}{s}\right) = \frac{m_{O_2}\left(\frac{kg\ O_2}{d}\right) \cdot 22.4\left(\frac{m^3{}_{O_2}}{kmol\ O_2}\right)}{32\left(\frac{kg\ O_2}{kmol\ O_2}\right) \cdot 0.21\left(\frac{m^3{}_{O_2}}{m^3{}_{air}}\right) \cdot 86400\left(\frac{s}{d}\right) \cdot 0.2\left(\frac{kg\ O_2 transfered}{kg\ O_2 supplied}\right)}$$

Number of fan:

$$Number\ of\ fan_{Accum} = round.up\left(\frac{Q_{O_2}\left(\frac{m^3{}_{air}}{s}\right)}{Fan\ Flowrate\left(\frac{m^3{}_{air}}{s \cdot fan}\right) \cdot \frac{t_{feast}}{t_{fed} + t_{feast} + t_{withdrawal}}}\right) + 1$$

$$Number\ of\ fan_{Enrichment} = round.up\left(\frac{Q_{O_2}\left(\frac{m^3{}_{air}}{s}\right)}{Fan\ Flowrate\left(\frac{m^3{}_{air}}{s \cdot fan}\right) \cdot \frac{t_{feast} + t_{famine}}{t_{fed} + t_{feast} + t_{famine} + t_{withdrawal}}}\right) + 1$$

Fan flowrate: 3 m^3 air/s

$$Fan\ energy\ consumption\ \left(\frac{kW \cdot h}{d}\right) = 1\ \frac{kW \cdot h}{kg\ O_2} \cdot \left(m_{O_2 Enrich}\ + m_{O_2\ Acum}\right)\left(\frac{kg\ O_2}{d}\right)$$

Storage and buffer tanks:

To determine the total volume of tanks required in each stage, a storage period of 12 h was considered.

$$Number\ of\ storage\ tanks = round.up\left(\frac{Flowrate\ \left(\frac{m^3}{d}\right) \cdot 0.5\ d}{Volume\ of\ tank\ (m^3)}\right) + 1$$

Volume of tank: 60 m^3

Agitators:

One agitator per tank was selected and the required power was calculated taking into account a requirement of 0.01 kW·h/m^3 and an energy efficiency of 0.7.

Solids generated:

$$VSSgenerated\ \left(\frac{kg\ VSS}{d}\right) = \frac{PHB\ production\ \left(\frac{Ton\ PHB}{year}\right) \cdot 1000\left(\frac{kg\ PHB}{Ton\ PHB}\right) \cdot \frac{1}{365}\left(\frac{year}{d}\right)}{PHB_{fraction\ feast}\left(\frac{kg\ PHB}{kg\ VSS}\right)}$$

Centrifuge energy consumption:

$$Centrifuge\ energy\ consumption\ \left(\frac{kW \cdot h}{d}\right) = 0.3\ \frac{kW \cdot h}{kg\ VSS} \cdot VSSgenerated\ \left(\frac{kg\ VSS}{d}\right)$$

Number of centrifuges:

$$N_{centrifuge} = round.up\left(\frac{(Flowrate_{Enrichment-Accum} + Flowrate_{Fed-Accum})\left(\frac{m^3}{d}\right)\cdot\frac{1}{24}\left(\frac{h}{d}\right)}{Centrifuge\ capacity\ \left(\frac{m^3}{h}\right)}\right) + 1$$

Centrifuge capacity: 30 m^3/h