Inês Filipa Cópio Paulo

Licenciada em Bioquímica

Bio-oil anaerobic fermentation as a starting point for the production of PHA by mixed cultures

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Doutor Paulo Costa Lemos,
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Júri:

Presidente: Prof. Dr. Carlos Alberto Gomes Salgueiro
Arguente: Prof. Drª. Luísa Alexandra Seuanes Serafim Martins Leal
Vogal: Dr. Paulo Alexandre da Costa Lemos

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2017

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RESUMO

Nos dias de hoje, a substituição dos produtos derivados do petróleo por carbono renovável é um dos grandes interesses mundiais. Dentro desta estratégia surge a produção de polihidroxialcanoatos (PHA), a partir um resíduo de baixo custo, e com elevado potencial de aplicação. Este facto combinado com uma cultura microbiana mista (MMC) com capacidade de acumulação de polímero permite as condições necessárias para a produção de PHA a baixo custo. O objetivo principal deste estudo foi fornecer um esquema diferente de valorização para resíduos da madeira considerando um novo processo a ser integrado numa abordagem global de biorrefinaria. O bio-óleo de pinheiro foi usado como fonte de carbono para a produção de PHA num processo em três etapas: pré-fermentação, seleção da cultura e produção de PHA, sendo todos os sistemas operados com MMC. Na fermentação acidogénica foram testadas várias condições, das quais se distinguiu o meio contendo 8 g/L de açucares com um rácio C:N:P de 100:3:1 e 10 dias de tempo de retenção, atingindo um máximo de produção de ácidos orgânicos de cadeia curta de 6,3 g/L e um grau de acidificação de 0,14 gCOD/gCOD. O reator de seleção da cultura operou em condições aeróbias e foi alimentado diretamente com bio-óleo não fermentado, atingindo um teor máximo de PHA de 13.93%. Por fim, após a adaptação da cultura foram realizados testes de acumulação em que o teor máximo de PHA produzido foi de 35,60%, utilizando bio-óleo fermentado como substrato. Concluiu-se que o bio-óleo do pinheiro é um possível substrato para a produção de PHA.

PALAVRAS CHAVE: Culturas microbianas mistas; polihidroxialcanoatos; ácidos orgânicos de cadeia curta; valorização de resíduos

ABSTRAT

Nowadays, the replacement of petroleum products by renewable carbon is one of the great concerns in the World. Under this strategy the production of polyhydroxyalkanoates (PHA) starting from a low-cost residue shows with high potential for applications. Combining this with a mixed microbial culture (MMC) with polymer accumulation capacity allows the necessary conditions for the production of PHA at low cost. The main objective of this study was to provide a different valorisation scheme for wood residues considering a new process to be integrated into a global biorefinery approach. Pinewood bio-oil was used as the carbon substrate for the production of PHA in a three-step process: pre-fermentation, culture selection and PHA production, all systems being operated with MMC. In the acidogenic fermentation, several conditions were tested, from which the medium containing 8 g/L of sugars with a C: N: P ratio of 100:3:1 and 10 days of retention time stand out, reaching a maximum short-chain organic acid (SCOA) concentration of 6.3 g/L and an acidification degree of 0.14 gCOD/gCOD. The culture selection reactor operated under aerobic conditions was fed directly with pure bio-oil, reaching a maximum PHA content of 13.93%. Finally, after the adaptation of the culture, accumulation tests were performed in which the maximum PHA content was 35.60%, using fermented bio-oil as a substrate. It was concluded that the pinewood bio-oil is a possible substrate for PHA production.

KEY WORDS: Mixed microbial culture; polyhydroxyalkanoates; short-chain organic acid; waste valorisation

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ABBREVITIONS

3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
3HHX	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
ADF	Aerobic dynamic feeding
AD_{Total}	Acidification degree
AF	Acidogenic fermentation
AMMC	Aerobic mixed microbial culture
AN/AE	Anaerobic/Aerobic process
AnMMC	Anaerobic mixed microbial culture
ATP	Adenosine Triphosphate
CG	Gas Chromatography
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
FF	Feast and Famine
FISH	Fluorescence *in situ* hybridization
GB	Glucose biopolymer B
HA-CoA	Hydroxyacyl-CoA
HB	Hydroxybutyrate
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic retention time
L	Liters
lcl-PHA	Long chain length polyhydroxyalkanoates
MAAS	MicroAerophilic-Aerobic System
mcl-PHA	Medium chain length polyhydroxyalkanoates
mL	Milliliters
MMC	Mixed microbial culture
NADPH	Nicotinamide adenine dinucleotide phosphate
OLR	Organic Loading Rate
OUR	Oxygen Uptake Rate
P(3HB-3HV)	3-hydroxybutyrate-*co*-3-hydroxyvalerate
P(3HB)	Homopolymer of 3-hydroxybutyrate
PA	Polyamide
PBAT	Poly(butylene adipate-*co*-terephthalate)
PE	Polyethylene

PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PLC	Poly(ε-caprolactone)
PolyP	Polyphosphate
PP	Polypropylene
qGB	Glucose biopolymer B specific production rate
qHB	HB specific production rate
RNA	Ribonucleic acid
RT	Retention time
SBR	Sequencing batch reactor
scl-PHA	Short chain length polyhydroxyalkanoates
SCOA	Short-chain organic acid
SRT	Sludge retention time
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WWTP	Wastewater treatment plant
YHB/S	HB storage yield on substrate
YHB/S	GB storage yield on substrate
YO_2/S	Respiration yield on substrate
−qS	Substrate uptake rate

1 INTRODUCTION

1.1 PLASTICS

Plastics are considered the most used daily polymeric material by humankind, in the form of disposable utensils, furniture, household appliances and specially applied in packaging to facilitate our way of life and comfort (Emadian et al., 2016). This material has the ability to be modelled in several different products since it shows versatile qualities of strength, lightness, resistant and durability (Hopewell et al., 2009). Thus, as a result of the high number of uses, the plastic-based annual production is thought to exceed 300 millions of tons in 2015 (Mekonnen et al., 2013). Nowadays, the most sold plastics are mainly derivate from petrochemicals produced from oil and natural gas, engaging almost 4 to 6% of annual consumption of this fossil fuel just in Europe. As its production also requires a high spent of energy, it ends up by consuming an additional quantity similar to the fuel used for production (Hopewell et al., 2009; PlasticsEurope, 2016).

The human population exponential growth took to an excessive production of the plastic polymers and this situation is accompanied by several significant environmental inconvenient. The adverse environmental impacts include carbon dioxide emissions (CO_2) and its accumulation through a long period in the environment due to it is the inability to biodegrade – accumulation of huge quantities of non-degradable waste. As such, they demand sustainable alternatives from renewable resources (Emadian et al., 2016; Jefferson, 2006).

Being it's elimination a strong key aspect, for Europe in 2014 and considering the different plastic applications, packaging represents the biggest part of its use with 39.9%. The packaging also reached the biggest recycle rate with 39,5% (based on large quantities entered in recycling facilities) and they reflect more than 80% of the total recycled quantities (PlasticsEurope, 2016). In 2014, only 69.2% of plastics were processed through recycling and energy recovery, while 30.8% were sent to landfill. These values represent in the last years an increase in recycling and energy recovery, while the landfill as destination had a decrease of 38% (Figure 1.1).

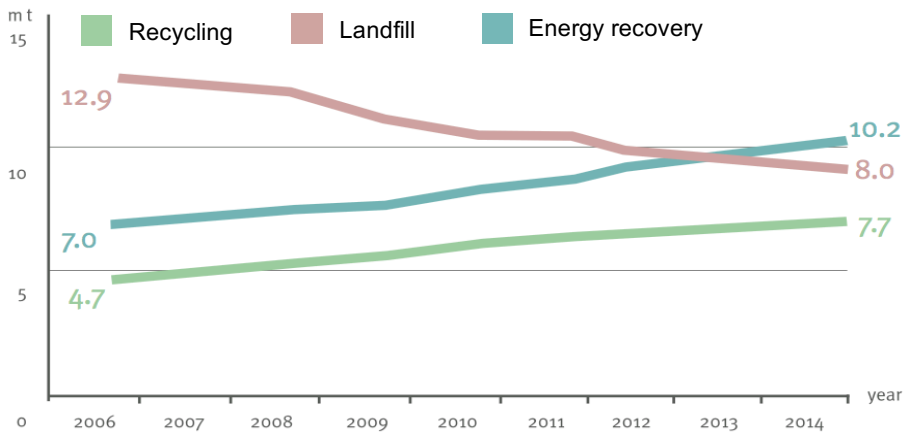

Figure 1.1: Evolution of waste treatment in the period between 2006 and 2014 (PlasticsEurope, 2016).

By minimizing the problem and in response to plastic harmful effects in the environment, there is a huge interest in developing biodegradable materials (Choi and Lee, 1997). Estimates in 2013 indicate that the global production of bioplastics should grow by a high annual rate reaching 30% of the plastic market in the next decade to 3.5 million tons in 2020 (Mekonnen et al., 2013).

1.2 BIOPLASTICS

Synthetic plastics are derivate from limited fossil resources and represent an environmental serious concern due to the absence of biodegradability in nature (Campanari et al., 2014). Considering these obstacles and the oil crises, the search for biodegradable plastics together with the use of renewable resources began to show more interest and experienced a strong development (Kumar et al., 2016; Reddy et al., 2013).

Biomaterials were developed for the first time in 1850, by a British Chemist, using cellulose, a component from wood pulp, as raw material. Since then the research progressed and the polymeric materials have already origin in several vegetable sources and/or biologic resources, such as sweet potato, soya oil, sugar cane, among others (Reddy et al., 2013).

A plastic material is considered a bioplastic when it is bio-based, biodegradable or possesses both properties such as polylactic acid (PLA) and polyhydroxyalkanoates (PHA) (Lunt, 2014). By definition, they are a polymeric material that it is naturally degraded by the action of microorganisms, such as bacteria, fungus, and algae, by chemical products connected to living organisms and/or through abiotic reactions, such as photodegradation, corrosion, and hydrolysis**.** They present several advantages regarding traditional plastics or other synthetic product (Khanna and Srivastava, 2004). Its production promotes less gas emission with the greenhouse effect since for the production of one ton of bioplastic there is a reduction between 0.8 and 3.2 tons of CO_2 than when producing one ton of traditional plastic. They can also help to relieve the energetic crisis, reducing the dependency on fossil fuels of our society. The valuable raw material can be reused and recycled into new products, minimizing the need for virgin material. Their remarkable properties are suitable for several applications (Reddy et al., 2013). So, the PHA besides being biodegradable, assimilated by several microorganisms and so reducing the negative impact of the products once that they are almost reduced if not eliminated, they are also biocompatible since they do not present toxicity for the human body (Luengo et al., 2003; Reddy et al., 2013).

Environmental, economical and safety challenges are surpassed by the replacement of conventional plastics by this material. As a result, several materials are proposed to production through bioplastic. In 2009, European Bioplastics identified packaging, loose fill packaging, and waste collection bags as major applications, in addition to many other applications with reduced impact production (Barker and Safford, 2009).

Despite biodegradable plastics being preferred over the conventional ones, they equally contain some disadvantages. An example considers those materials that are not intended to be recycled in the same way as other plastics (Reddy et al., 2013).

As visible advantages are superior to disadvantages, bioplastic development has awakened an increased interest in a way to approach the request of societies to more ecological biopolymers**.** On the other hand, in competition with large quantities of products, the search for bioplastics is highly influenced by its price. According to the Bioplastic European Association (European Bioplastics, 2016) nowadays the cost is between 1.3 to 4 €/kg. Although being 80% lower than 10 years ago, it still is not competitive with the conventional ones. It is expected that bioplastic global market expands from 7.2 billion Euros – registered value in 2014 – to 39.9 billion Euros in 2020.

Presently, for biodegradable plastics production, the polymeric materials that are used can be classified into three different groups: chemically synthesized polymers, biodegradable materials based on starch and polyhydroxyalkanoates (PHA) (Tan et al., 2014). The last group, PHA, is the one which presents physicochemical characteristics similar to conventional plastics, becoming a viable alternative. PHAs are natural polyesters that are produced by several microorganisms (Chandra and Rustgi, 1998). They can be synthesized from renewable resources and they are completely biodegradable, thus comprising all ecologic requests for the sustainable biodegradable plastics production.

Bioplastics production tend to evolve and it is estimated a three times increasing in production, reaching 17 million tons in 2020. This increase is associated with PLA and PHA production, of which the PHA is the most promising among all, presenting a biological basis and biodegradability. Besides that, they still strongly contribute for big environmental elimination questions (Aeschelmann and Carus, 2015).

1.3 POLYHYDROXYALKANOATES

A high number of microorganisms in specific nutritional and environmental conditions have the ability to synthesise a type of biopolymer, polyhydroxyalkanoates. It's stored internally as a carbon and energy reserve allowing survival in the absence of external substrate (Luengo et al., 2003; L. Serafim et al., 2008). This accumulation occurs in presence of excess carbon, with the imposition of limitation for at least of one nutrient – between phosphorus, nitrogen, potassium and oxygen – resulting in unfavourable conditions for cellular growth and giving rise to a reduction in intracellular granule formation of amorphous PHA or inclusion bodies (Anderson et al., 1989; Luengo et al., 2003; L. Serafim et al., 2008). It's storage can occur in high concentrations inside the cell given that it does not change its osmotic state (Laycock et al., 2013). Bacteria, fungi, and plants are part of the list with more than 250 species of living organisms known to have this ability (Lee, 1996).

PHAs are bioplastics composed by repeat unities of hydroxyalkanoate acids, from which the chemical structure is represented in Figure 1.2. These polyesters consist in a branch of linear carbon with variable size, but usually from 3 to 4 carbons (x=1 and 2, respectively) and alkyl R side-chain, which in case of longer alkyl replacements can incorporate more than 9 carbons (Chandra and Rustgi, 1998).

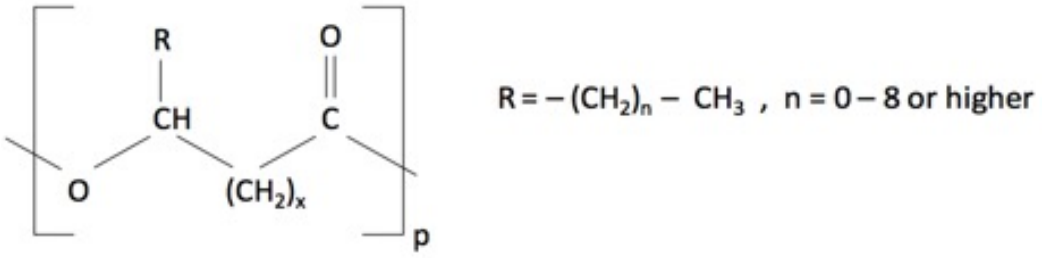

Figure 1.2: Generic structure of PHAs.
p – Number of monomers in the polymer; x – Variable number of carbon atoms in the linear structure; R – alkyl radical; n – Variable number of carbon atoms in the alkyl radical.

The PHAs present different monomeric units and can have different structures depending on the bacterial species and on the carbon feeding source during the synthesis. They are divided into three big groups, based on the monomeric chain length (the number of carbon atoms present in each unit): short chain length PHA (*scl*-PHA) containing 3 to 5 carbon atoms, medium chain length PHA (*mcl*-PHA) containing 6 to 14 carbon atoms, and long chain length PHA (*lcl*-PHA) that present monomers with more than 15 carbon atoms in the constituent chain (Singh and Mallick, 2009).

Different pathways involve the transformation of different molecules and so originating the formation of alternative (co-)polymers, based on the feedstock used in PHAs production that it is normally enriched with SCOA and/or sugar-based compounds (Laycock et al., 2013; L. Serafim et al., 2008). Thus more than 150 different monomeric units in PHAs biological formed were already observed. However, the homopolymer of 3-hydroxybutyrate (P(3HB) and the co-polymer of 3-hydroxybutyrate-*co*-3-hydroxyvalerate (P(3HB-3HV)) are usually the most produced PHAs (Laycock et al., 2013).

The resulting polymers, regardless its composition, share some properties, such as (Laycock et al., 2013; Tsuge, 2002): piezoelectric and optically active since they only present R configuration, high level of purity inside the cell, they present biocompatibility, they are biodegradable since they can be hydrolysed by extracellular PHA depolymerases and the products of cleavage can be used as sources of carbon and energy by many Bacteria and Fungi, zero toxicity, insolubility in water, they are thermoplastics or elastomeric, being inert compounds.

Those characteristics attract industrial attention due to its potential use in several sectors, such as in plastic manufacture industry (Tsuge, 2002). The different monomeric compositions influence the physical and mechanical properties of different PHAs (Khanna and Srivastava, 2004). Their rigidity and fragility vary depending on each monomer content. P(3HB) has high crystallinity, low resistance to rupture and impact, originating very hard and crumbly pellicles and plastics (Chandra and Rustgi, 1998; Laycock et al., 2013; Sudesh and Iwata, 2008). These characteristics have been an obstacle in the practical application of these materials. However, a significant research in a manipulation of these mechanical properties has been performed. And, one of the present solutions is the incorporation of higher contents of 3-hydroxyvalerate monomer (3HV) in order to obtain a more elastic and flexible copolymer (Bengtsson et al., 2008b; Serafim et al., 2004). Some properties of polymers and co-polymers can be seen in Table 1.1.

Table 1.1: Propertie of PHA (adapted from (Laycock et al., 2013)).

	P(3HB)		P(3HB) copolymers		P(3HB-*co*-3HV)		P(3HB-*co*-3HHx)	
	Biomer 240	**Biomer 240**	**Mirel P1001**	**Mirrel P1002**	**ENMA T**	**Biocycle 1000**	**Biocycle 2400-4**	**Kaneka**
	Injection mold	**Injection mold**	**Injection mold**	**Extrusion and injection**	**Injection mold**	**Extrusion and injection**	**Extrusion, injection and fibre**	**Foam mold**
Melt flow rate (g/10min)	5-7	9-13				10-12	15-25	5-10
Density (g/cm^3)	1.17	1.25	1.39	1.3	1.25	1.22	1.2	1.2
Crystallinity (%)	60-70	60-70				50-60		
Tensile strength (MPa)	18-20	24-27	28	26	36	30-40	25-30	10-20
Elongation (%)	10-17	6-9	6	13	5-10	2.5-6	20-30	10-100
Flexural strength (MPa)	17	35	46	35	61			
Flexural modulus (GPa)			3.2	1.9	1.4			
Melting temperature (°C)					147	170-175		0.8-1.8
VICAT softening temperature (°C)	53	96	148	137	143			120-125

PHA degradation may occur in anaerobic and aerobic conditions by quite a few microorganisms in several ecosystems, such as soil, sewers, sea or lake water (Castilho et al., 2009; Lee, 1996). In natural environments, the degradation rate is influenced by factors like the kind of microbial population, temperature, humidity level, pH, nutrients supplying, as well as polymer composition, crystallinity, additives and superficial area of PHA (Khanna and Srivastava, 2004; Sudesh et al., 2000).

1.3.1 PHA APPLICATIONS

Plastic industry uses technologies that can be used in synthetic plastics derivate from oil as in most of PHAs processing since these bioplastics are thermoplastics, which allows being thermally processed, or elastomeric. Its properties can also be adapted according to the final destination, emerging like this an enormous option of possible applications, from hard packaging products until high elastic matters for coating (Sudesh and Iwata, 2008).

PHAs applications have been expanding, dividing into three main areas: Industrial, Agricultural and Medical (Philip et al., 2007; Sudesh and Iwata, 2008).

In an early stage, the main PHA application was in daily items, like shampoo bottles and other packaging materials (Chen, 2009). The range of applications have increased and today they

are already used in the manufacture of pressure sensors, stretch and accelerate measurement instruments, shock wave sensors and lighters, due to its piezoelectric nature. P(3HB-co-3HV) gas barrier properties support its application in food packaging and in beverage plastic bottles production (Philip et al., 2007). PHAs are also used in fabric engineering, printing and photography materials and fine chemical products. In the agricultural field, PHAs are used in the production of devices for the control of fertilizer, herbicide and insecticide release (Philip et al., 2007). Biodegradability and biocompatibility are properties that favour its application in medicine. It made possible the production of medical implants, drugs transporters – matrix for medicine control release –, artificial organ making, surgical sutures and surgical glue. Monomers, especially HB reveal therapeutic effects in Alzheimer and Parkinson, osteoporosis and even memory improvement. Nutritional supplements derived from PHAs is another field of application for these polymers (Chen, 2009; Philip et al., 2007).

Table 1.2: Prices of different types of PHA produced by world manufacturers (adapted from (Możejko-Ciesielska and Kiewisz, 2016)).

Polymer	Trade names	Prices (kg^{-1}) in 2010 (€)
PHB	Biogreen®	2.5-3.0
PHB	Mirel™	1.50
PHB	Biocycle®	n/a
P(HB-*co*-HV) and PHB	Biomer®	3.0-5.0
P(HB-*co*-HV), HBV + *Ecoflex* *blend*	Enmat®	3.26
P(HB-*co*-HV)	Nodax™	2.50
P(HB-*co*-HV)	Nodax™	3.70
P(HB-*co*-HV)	Kaneka PHBH	n/a
P(3HB-*co*-4HB)	Green Bio	n/a
PHA from P&G	Meredian	n/a

The difference between the costs of synthetic plastics to biopolymer causes the major obstacle to the material change (Table 1.2). While PP or PE have a cost of 1,47 and 1,15 €/kg, respectively, the theoretical price of PHA is around the 3,51 €/kg. So, the reduction of production costs is of high interest in order to make the process economically viable (Możejko-Ciesielska and Kiewisz, 2016).

1.4 PHA PRODUCTION

1.4.1 PRODUCING MICROORGANISMS

The industrial production of PHA relies on several factors that contribute significantly to the production costs. In relation to the selection of microorganisms, it should be based on factors as the cell's ability to utilize inexpensive carbon sources, growth and polymer synthesis rates, maximum polymer storage content, and recovery of PHAs (Khanna and Srivastava, 2004).

Table 1.3: Wild type and industrial bacteria strains commonly used for pilot and large scale PHAs production (adapted from (Chen, 2009)).

Strain	DNA manipulation	Final Cell Dry Weight (CDW) (g/L)	Final PHA content (%CDW)	Company
Cupriavidus necator	No	> 200	> 80	Tianjin North. Food, China
Alcaligenes latus	No	> 60	> 75	Chemie Linz, btF, Austria Biomers, Germany
Escherichia coli	phbCAB + vbg	> 150	> 80	Jiang Su Nan Tian, China
Cupriavidus necator	No	> 160	> 75	ICI, UK Zhejiang Tian An, China
Cupriavidus necator	No	> 100	> 75	Metabolix, USA
Escherichia coli	phbCAB			Tianjin Green Biosci, China
Cupriavidus necator	$phaC_{Ac}$	> 100	> 80	P&G, Kaneka, Japan
Aeromonas hydrophila	No	< 50	< 50	P&G, Jiangmen Biotch Ctr, China
Aeromonas hydrophila	phbAB + vgb	~ 50	> 50	Shandong, Lukang
Pseudomonas putida	No	~ 45	> 60	ETH, Switzerland
Bacillus spp.	No	> 90	> 50	Biocycles, Brazil

CDW – cell dry weight; phbCAB – PHB synthesis genes encoding β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase; $phaC_{Ac}$ – PHA synthase gene *phaC* from *Aeromonas caviae*; phbAB – encodes β-ketothiolase and acetoacetyl-CoA reductase; vgb – gene encoding *Vitreoscilla* hemoglobin.

Of over 250 different microorganisms that synthetize PHAs, only several of these are suitable for the production of PHAs with high productivity (Choi and Lee, 1997). In the industrial production, natural or recombinant pure cultures of microorganisms are used since that allow obtaining higher yields particularly applying recombinant strains (Lemos et al., 2006). However, the use of pure cultures still has several disadvantages that can be overcome through the use of microbial mixed cultures (MMC) (L. Serafim et al., 2008). In Table 1.3 some strains commonly used for pilot and large-scale production of PHAs are presented.

1.4.2 Pure cultures versus Mixed microbial cultures

Most researchers that work in this area have preferentially studied pure cultures which are identified as having a high capacity for PHA storage. More recently, mixed microbial cultures appeared as a clear alternative, in which it's possible a reduction of operational costs till 50%. This change combined with re/use of industrial or agricultural subproducts can contribute to a further decrease until 85% of PHA production costs (Tsuge, 2002). MMC can be recalled in wastewater treatment plant (WWTPs) since it was verified that these bacteria have the ability to produce and accumulate PHA (L. Serafim et al., 2008).

Processes with pure cultures, even applying low-cost carbon substrates, such as starch, cheese whey, tapioca hydrolysed or molasses still demand for sterile conditions and consequently the use of more expensive equipment, so it ensures the presence of the bacteria of interest, exhibiting a significant increase in PHA production cost (Albuquerque et al., 2007). The use of MMC do not need sterilization and systems of sterile fermentation, allowing for the use of less sophisticated equipment and less control, and still facilitates the use of complex substrates, being microbial population adapted continuously to the substrate (Serafim et al., 2004). Acids like acetate, propionate, and butyrate found in complex agro-industrial residues are considered precursors to PHA production by MMC, while pure cultures usually use carbohydrates as a carbon source. The absence of SCOA in most of the feedstock, normally rich in carbohydrates, is not an obstacle for MMC processes, once a preliminary acidification performed by MMC under anaerobic conditions can be introduced in the process, so that the organic composts are converted into the desired substrates (L. Serafim et al., 2008).

MMC for PHA industrial implementation is delayed because of some difficulties that can still be found in the process. The first one is related to biomass concentration since it's too below the values obtained in pure culture process. If the amount of biomass increased, the volumetric productivity also improved and thus it could have a high impact on the downstream process cost, once extraction yield is directly dependent on the amount of collected biomass (L. Serafim et al., 2008). The composition of the selected microbial population is another drawback traditionally attributed to MMC processes. Although a wide range of bacteria was identified in MMC production systems, various microorganisms are related to pure culture systems (Queirós et al., 2015). In addition, the robustness of the MMC systems to support a situation where a periodic variation in the composition of feedstock exist implies a more diverse population (Carvalho et al., 2014; Dias et al., 2006; Pisco et al., 2009). The assumption that diversity may negatively impact on the characteristics of the produced PHA is high, even after some works showed the opposite, where different polymer properties were analysed (molecular weight, polydispersities, melting and glass transition temperatures, melting enthalpies or crystallinity) (Laycock et al., 2013; L. S. Serafim et al., 2008). The decision on the final polymer application requires its characterization. Pure culture responds to the demand of polymer processing industry because they enable better control on the PHA characterization. However, PHA monomeric composition can be controlled by the feed on MMC processes, with defined mixtures of SCOA (Reis et al., 2011; L. S. Serafim et al., 2008). Another pointed disadvantage is that mixed cultures can obtain a very low monomer variety compared to pure cultures since, for pure cultures, monomers of 3 to 14 carbons having a wide variety of side groups have already been obtained (Steinbiichel and Steinbiichel, 1995; Tan et al., 2014).

The downstream processing represents one of the major disadvantages of PHA production by MMC (Madkour et al., 2013). In MMC the cell wall structure and biomass flocculation appear to be more complex and harder to break than in pure cultures, which become fragile when high PHA storage contents are attained. However, researchers are looking for a low-

cost, highly efficient, and environmentally friendly PHA recovery process that allows for high purity without degradation of the polymer.

1.4.3 PHA BIOSYNTHESIS

The chain synthesis of PHA polymer, involving a complex biological and biochemical ways, have undergone a deep study (Laycock et al., 2013; Sudesh et al., 2000; Verlinden et al., 2007). The metabolism for PHA synthesis is well defined for pure cultures, but only a few metabolic studies involving MMCs were reported (Lemos et al., 2006). However, it's assumed that the PHA production in MMCs is similar to the one described for pure cultures (Dias et al., 2006).

PHA synthesis occurs inside the cytoplasm of the bacterial cell, more specifically inside inclusions known as granules. The accumulation is controlled by several genes that code for a group of enzymes that are direct and indirectly involved in PHA synthesis (Laycock et al., 2013). The chain formation and corporation depend on the supply of a suitable substrate and to the conversion in the intended hydroxyacyl-CoA (HA-CoA), through existent metabolic reactions in bacterial cells. Besides that, it's necessary that the cell contains an enzyme capable of incorporating the synthesizes HA-CoA to a polymeric chain, the PHA synthase (Laycock et al., 2013). Initially, the organic acids molecules are transported across the cell membrane and activated to the corresponding acyl-coenzyme A (CoA) or, for glucose, breakdown through glycolysis till acetyl-CoA (L. Serafim et al., 2008). There are several know possible pathways for the production of PHAs that vary according to the substrate used. Figure 1.3 shows metabolic pathways involved in the synthesis of PHAs.

PHA metabolism can be regulated at different levels, such as regulation of enzyme that synthesize PHA (through metabolites or certain cellular components), activation of gene expression caused by specific external conditions (lack of one nutrient), the increase of intermediate metabolites necessary to the synthesis and consequently inhibition of enzyme action by competitor metabolic ways, or even by conjunction of the previously presented (Kessler et al., 1998).

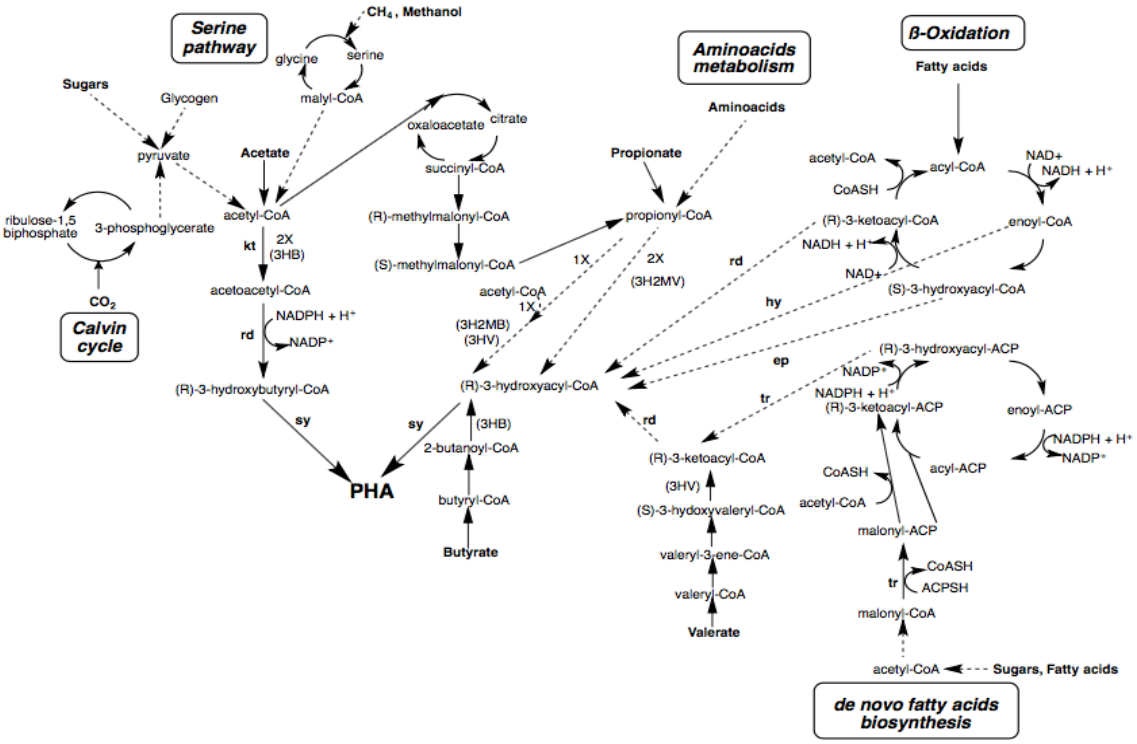

Figure 1.3: Metabolic pathways for production of hydroxyalkanoate monomers for PHA synthesis (adapted from (Serafim et al., 2016).

1.5 STRATEGIES FOR PHA PRODUCTION BY MMC

1.5.1 3-STEP PROCESS

Agroforestry residues, industrial by-products and industrial and urban waste can be reused for PHA production by MMC, in order to become economically competitive with pure culture, since they are low-value substrates. However, these low-value substrates needed to be converted into readily biodegradable carbon sources to be transformed into PHA by microorganisms present in MMC.

Depending on the type of substrate used as feedstock, processes for PHA production involving mixed cultures can be operated in two or three steps. The three-step process was first proposed in 2004, by Dionisi *et al.* for using waste or surplus-based feedstock (Dionisi et al., 2004), as presented in Figure 1.4:

1. Acidification of organic components of the feedstock to obtain readily biodegradable carbon sources, as SCOA
2. Enrichment in PHA-storing microorganisms
3. PHA accumulation

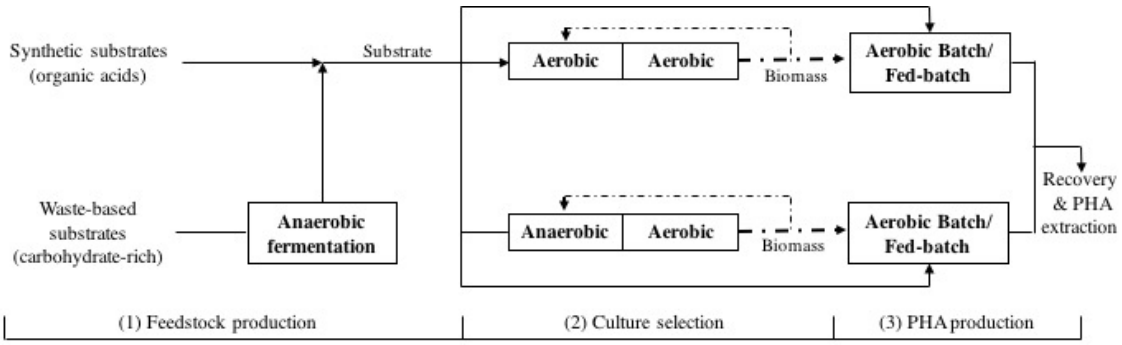

Figure 1.4: Three-step PHA production process by mixed microbial cultures (adapted from (L. Serafim et al., 2008)).

The two-step process (steps 2 and 3 in Figure 1.4) includes the selection of PHA-accumulating organisms (aerobic or anaerobic/aerobic conditions), followed by PHA accumulation, in which PHA storage by the selected culture is maximized. The process optimization is possible from physical separation of the culture enrichment stage from the PHA production phase since different specific conditions for each step are needed (Albuquerque et al., 2007; L. Serafim et al., 2008). This process is applied when feedstock for PHA production contain organic acids, as acetate, propionate, butyrate, valerate or lactate. PHA produced in the accumulation step is then extracted and purified (L. Serafim et al., 2008). For the three-step process an additional preliminary step, acidification, is required whenever the chosen feedstock is poor in SCOA and contains sugar compounds. This is a stage of anaerobic digestion, where carbon-based compounds from waste are transformed into SCOA and alcohols (e.g., ethanol), which can be further used in the selection and accumulation steps (Albuquerque et al., 2007; Dionisi et al., 2005; L. Serafim et al., 2008).

1.5.2 ACIDOGENIC FERMENTATION

Anaerobic digestion is used Worldwide, in full-scale installations for the treatment of industrial and urban wastewater and organic solid waste. Some characteristics, as high organic removal rates, low energy-input requirement, energy production and low sludge production make this process more advantageous than aerobic systems for the same treatment. Anaerobic digestion of organic substrates is a strong alternative for reducing the cost of PHA production since it makes them possible substrates. Whereas, when open culture systems are used with certain substrates, such as amyloid or cellulose hydrolysates, and the end the main result may be the accumulation of glycogen (Reis et al., 2003; Salehizadeh and Van Loosdrecht, 2004).

Anaerobic digestion is a complex process, which can be divided into four individual stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 1.5).

Acidogenic fermentation (AF) step is the most relevant, since soluble organic compounds are fermented into organic acids, such as acetic, propionic, butyric, valeric and lactic acids which are easily converted into other fermentation products such as alcohols and hydrogen. The SCOA produced are also substrates for PHA production, thus making AF a suitable pre-treatment for PHA production (Bengtsson et al., 2008a).

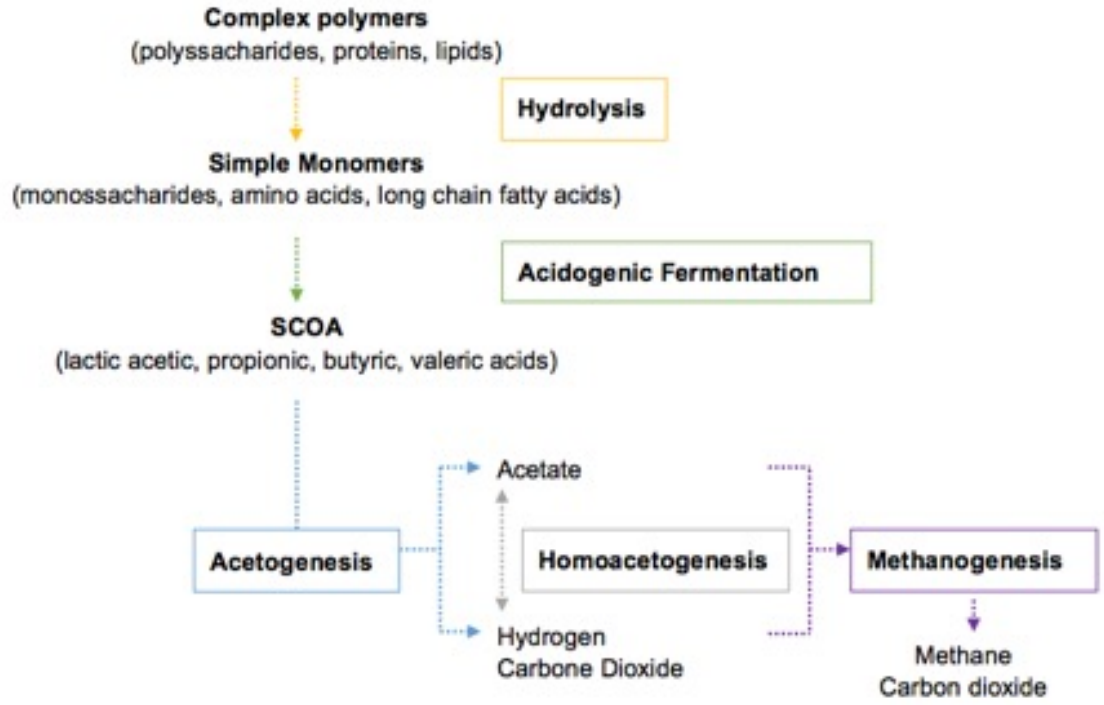

Figure 1.5: Representation of the anaerobic digestion (adapted from (Lee et al., 2014b).

The AF process is very sensitive to operating conditions. So to keep it advantageous, and to ensure the production of SCOA it's essential, for example, to eliminate methanogens, as SCOAs will ultimately be transformed into methane. By manipulating parameters such as hydraulic retention time (HRT), sludge retention time (SRT), the rate of organic load (OLR), temperature and pH the methanogen microbial population can be controlled/eliminated. Consequently, the appropriate reactor type and optimum operating conditions have to be combined, since AF depends significantly on this combination and on the substrate used (Bengtsson et al., 2008a; Jankowska et al., 2015). Longer HRT is recommended for AF in order to allow good culture adaptation and more efficient conversion, since the substrates used in AF can be recalcitrant and difficult to biodegrade, being difficult for the microbial culture to adapt and produce SCOAs in a short time. In addition, a short HRT may lead to the washout of biomass but will have the advantage to decrease the growth of methanogenic microorganisms, since they have slow growth rates. For continuous systems the SRT value equals the HRT, leading to the removal of biomass that did not adapt to the substrate and to becoming excess sludge in the effluent. The SRT may be larger than the HRT when a biomass recirculation system is coupled to the reactor to prevent washout (Jankowska et al., 2015; Lee et al., 2014b; Ozgun et al., 2013).

In relation to the culture selected for AF, MMC has low energy consumption and metabolize complex substrates either aerobically (AMMC) or anaerobically (AnMMC) as inoculum (Wang et al., 2014). Since anaerobic digestion is an anaerobic process, AnMMC is usually used, although AMMC also has a high capacity for the production of SCOA. In addition, AMMC is expected to be more robust than AnMMC as they are derived from aerobic tanks subject to extreme conditions. Therefore, the use of AMMC in AF would have advantages for operational

control of the biological system. Another positive point could be a more effective acidogenic bacterial selection for AMMC since methanogenic microorganisms are strict anaerobes, and AMMCs are composed of aerobic and facultative anaerobes, thus inhibiting the growth of methanogens (Rajeshwari et al., 2000).

The amount and type of PHA produced are dependent on the amount and type of SCOA created by the AF. The type and yield of SCOA produced are intimately related with the pH value of reactor operation. For acidogenic bacteria, a fairly wide range of pH values are known in which their activity and growth are not compromised, although extreme conditions such as pH 3 or pH 12 will present inhibition. (Albuquerque et al., 2010a; Gouveia et al., 2016). Yu and Fang (2002) showed that when using milk wastewater for AF, the production of propionic acid was improved at pH 4 - 4.5, and the production of acetic and butyric acids were favoured at pH 6 - 6.5 (Yu and Fang, 2002). In a study with cheese serum, the production of propionic acid was higher at pH 6, while the production of acetic and butyric acid decreased. Bengtosson et al. (2008) found that using cellulose as the substrate in a pH range of 4.9-6 the production of butyric and propionic acids were favoured, whereas acetic acid production was reduced (Bengtsson et al., 2008a). Therefore, for each substrate and acid production of interest, the study of the optimum pH value is decisive (Tamis et al., 2015).

1.5.3 Culture selection stage for PHA-accumulating microorganisms enrichment

1.5.3.1 Strategies

The enrichment in PHA-storing microorganisms is considered one of the key-points of all PHA production process with MMC. The aim of this step is the selection of a microbial population with a high PHA storage capacity and eliminate, or at least reduce non PHA-storing side populations. Specific conditions are used for eliminating those microorganisms that presented low or no storage capacity since they contribute negatively to the reduction of the average PHA cell content and storage yields by using the carbon source for growth (L. Serafim et al., 2008).

In the processes with MMC, the prevalence of organisms with the ability to store PHA is imposed by operational conditions, which originates a natural selection, without a defined composition (Dias et al., 2006). The most important processes for culture selection are the Anaerobic/Aerobic process (AN/AE), and Aerobic dynamic feeding (ADF).

ADF is the preferred methodology applied for biomass selection. This process is the most promising because of the high sludge PHA content and productivity (Reis et al., 2003). The culture is submitted to transitional feeding conditions in a strategy named Feast and Famine (FF) and is based on the alternation of periods of carbon excess and shortage, allowing only the survival of organisms that store PHAs. In the phase of carbon abundance, the desired bacteria should consume all the available carbon in the substrate in order to truly establish the wanted condition and accumulate it as PHA. In the shortage period, it is intended that non-storing bacteria will be

eliminated and that bacteria that stored PHA use its internal reserves as carbon source and energy (Figure 1.6).

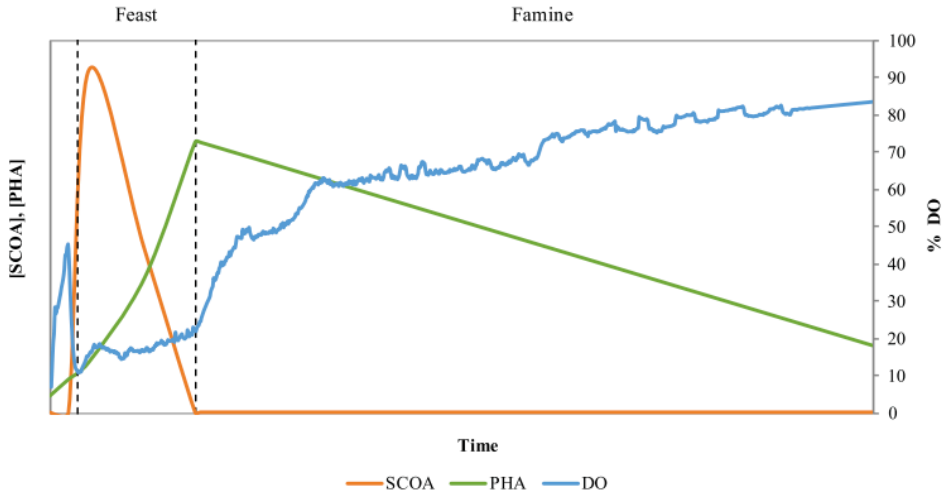

Figure 1.6: Example of an ADF cycle operation by a fully aerobic culture (adapted from (Serafim et al., 2016).

The decrease of reserves will initiate a new storage process when the next period begins and like this the several cycles of FF will favour the growth of producing bacteria, creating a selective pressure for organisms with PHA storage ability (Albuquerque et al., 2010b; Dionisi et al., 2006; Moita et al., 2014; Moita and Lemos, 2012). The ability to store internal reserves gives these microorganisms a competitive advantage over those without this ability when facing transient substrate supply (Salehizadeh and Van Loosdrecht, 2004). The period of famine is needed for competition between the culture, favoring the PHA storage capacity cells (Reis et al., 2003). Several studies validate the importance to establish long phases of famine and short phases of feast in order to obtain low values in the F/F ratio (Albuquerque et al., 2010b; Dionisi et al., 2006). A long period of famine can lead to a decrease of internal growth by the reduction of cellular enzymes needed for growth. During the next phase, cells do not have the ability of immediate growth after the substrate supply, but they can store PHA since this metabolism does not require physiologic adaptation.

1.5.3.2 Factors that influence Feast-Famine microbial selection

The feast and famine strategy for the production of PHAs using MMCs is normally carried out in sequencing batch reactor (SBR), operating in cycles of feeding, reaction, settling and drawn (Dias et al., 2006). SBR is ideal reactor for the selection of robust populations with high PHA-storage ability since biomass grows under transient conditions. Also, these reactors present characteristics as easy control and high flexibility, which allows the rapid change of the process conditions, for example, the duration of feeding and cycle length (Reis et al., 2003).

In relation to microbial cultures, those are sensitive to variations on the growth conditions, and there are several important process parameters in PHA production that must be optimized.

The different factors that can influence MMC response are related to the operating conditions as pH, dissolved oxygen (DO), temperature, OLR influent substrate concentration and other nutrient concentration (as nitrogen and phosphorous), SRT, HRT, cycle length as well as the feedstock (Reis et al., 2011).

pH as a strong influence in PHA accumulation. It was noted that as pH increased from 7.5 to 9.5 the rates and yields of PHA storage decreased. This study was performed using mixed cultures enriched in a SBR starting from activated sludge, with a mixture of acetic (85% on COD basis) and propionic (15%) acid, at an organic load rate of 8,5 g COD/L/day (Villano et al., 2010). In relation to DO concentration in the system, oxygen limitation in PHA production by MMC allows obtaining higher PHA storage yields, since the higher the concentration of DO, the greater will be the proportion of substrate which will be used for cell growth and maintenance, therefore, less substrate will be converted to PHA (Third et al., 2002).

The temperature is another parameter that strongly influences the accumulation of storage polymers. Johnson and Geest (2010) using a culture with a low PHB storage capacity concluded that at higher temperatures (30°C) PHB storage was the predominant process in the feast phase, while at lower temperatures (15°C), growth occurred predominantly, directly on acetate rather than on stored P(3HB), and the length of the feast phase had increased (Johnson et al., 2010).

The OLR is influenced by substrate concentration, which is also an important process parameter. Venkateswar R. and Venkata M. (2012) reported that using activated sludge, from composite wastewater treatment, the higher OLR took more time for maximum PHA accumulation due to the higher available substrate, while, lower OLR showed maximum PHA accumulation more quickly by the minor availability of the substrate (Venkateswar Reddy and Venkata Mohan, 2012).

SRT is an important design and operating parameter for the process since it is the average time that activated-sludge solids are in the system - normally expressed in days -. Chua et al. (2003) observed that a short SRT (3 days) was more profitable than a long SRT (10 days), since in the first condition greater PHA content, up to 10%, could be achieved. Normally, longer SRT means higher biomass concentration in the reactor and for short SRT sludge acclimatization may also be preferable for PHA production purpose. This is due to the fact that sludge production is higher in a short SRT than under a longer SRT (Chua et al., 2003). While, Beun et al. (2002) in their study using acetate also concluded that at a SRT below 2 days, the P(3HB) storage yield and productivity decreased sharply with the decrease of the SRT, while for SRT higher than 2 days, the yield of P(3HB) from acetate under excess nutrients was constant (Beun et al., 2002).

In relation to cycle length and feed frequency, Dionisi et al. (2007) studied the effect of the cycle length on the enrichment and selection of mixed cultures in SBR and found that the selection of microorganisms with storage or growth response was correlated with the ratio of the feast phase/length of the cycle: storage response was observed only when the feast phase was not longer than 20% of the length of the cycle (Dionisi et al., 2007).

Some micronutrients are important for the overall function of the cell. One of the most studied micronutrients is nitrogen that is a component of protein, enzymes and nucleic acids and increasing their use can be beneficial for the cell. Phosphorus has implications in the utilization of carbohydrates and fats for energy production and also in protein synthesis for growth, maintenance, and cell repair. Reddy & Mohan assessed the effect of varying nitrogen concentration on PHA accumulation and reported that lower nitrogen concentration (N1 - 100 mg/ml) showed higher PHA accumulation (45.1%) and vice versa (N2 – 200 mg/ml, 41.5%; N3 – 300 mg/ml, 38%). It was also showed that lower phosphorous concentration favoured PHA accumulation (Venkateswar Reddy and Venkata Mohan, 2012).

The strategy of operation also strongly affects the selection of the microbial culture and consequently the production of PHA. In Feast and Famine processes, the F/F ratio is intended to remain low, since low values ensure physiological adaptation of the microorganisms, and thus the storage of PHA in the feast phase. When this ratio presents higher values, it can cause a partial or complete loss of the physiological adaptation, which will cause the substrate uptake to be driven predominantly toward growth. This condition result from either higher OLR/influent substrate concentration or shorter cycles (Albuquerque et al., 2011). In many of the studies where the strategy of feast and famine has been applied with the ratio lower than 0.2 – 0.3, an efficient enrichment in PHA storing organisms was obtained (Oliveira et al., 2016).

1.6 BIO-OIL

The climate change, international obligations to decrease greenhouse gas emissions, and the scarcity of fossil fuels have driven public awareness for renewable energy. According to the International Energy Agency (2011), biofuel consumption will increase in a sustainable manner, from 2% at present to 27% of the total transport fuel global share, until 2050. Renewable carbon instead of petrol for the production of a variety of products is being implemented in order to change into a bio-based economy. As a result, also wood fuels are becoming increasingly popular sources of heat and power worldwide (Eriksson et al., 2012; Hassan et al., 2016; Koutinas et al., 2014).

The process occurs in bio-refineries that convert biomass into fuels, chemicals, polymers, materials, food, feed and value-added products maximizing both profit and raw material usage. The biomass, initially, was of vegetable origin and more recently wastes and by-products from difference sources, industrial, domestic or agroforestry, are being considered for use as renewable resources (Koutinas et al., 2014). Its abundance, being renewable, readily available and emit relatively low CO_2 content were the characteristics that roused the attention of biomass as a clean and alternative energy source. Furthermore, their use for energy production can mitigate CO_2 build up in the atmosphere, reducing global warming (Hassan et al., 2016).

Industrial roundwood is the wood that enters the forest processing sector, not accounting for energy production. Sawing and wood furniture manufacturing industry produced most of the wood residues (Figure 1.7). Despite these residues be mainly rejects, they have several applications such as the production of particleboards and fibreboards, feedstock in the pulp

industry and used in the wood pellet industry. With the possibility of biomass to be converted into bio-oils or intermediate compounds for the chemical industry, the wood waste has gained new applications (Hassan et al., 2016; Peksa-Blanchard et al., 2007). This conversion can be through a thermochemical process, such as pyrolysis, gasification, liquefaction, and high-pressure supercritical extraction (Hassan et al., 2016).

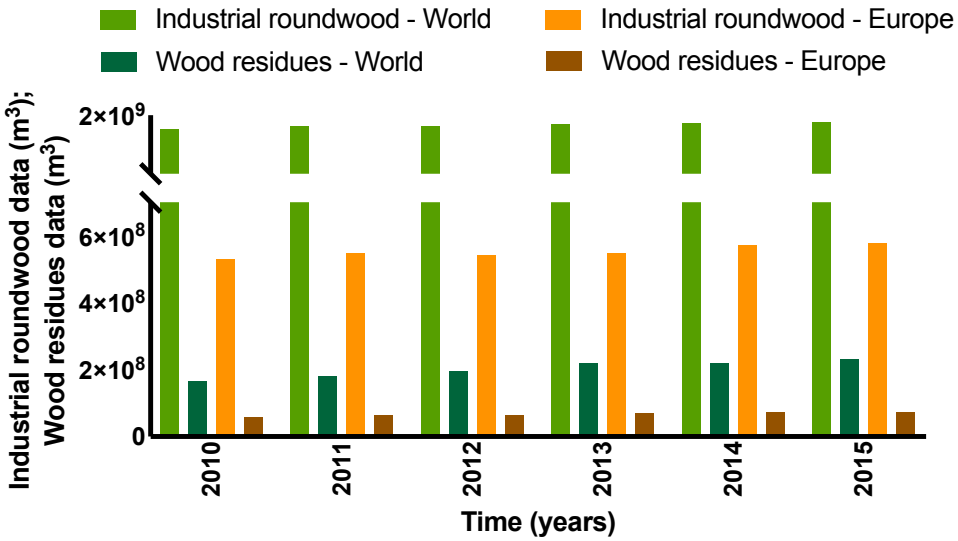

Figure 1.7: Production of wood residues and industrial roundwood (adapted from (FAO, 2017)).

Pyrolysis is a low-cost thermochemical process, defined as thermal degradation of biomass in the absence of air/oxygen. This process occurs at atmospheric pressure and moderate temperatures (usually 450 - 550 °C), converting biomass into three types of products, namely, solid (biochar), liquid (bio-oil) and volatile gaseous components. The advantage of pyrolysis process is the flexibility of feedstock use also allowing for the utilization of low-quality high-ash feedstocks (Hassan et al., 2016; Stefanidis et al., 2015). Bio-oils produced from lignocellulosic material results from the breakdown of cellulose, hemicellulose, and lignin, existing in its composition. These oils are dark brown organic liquids, that present potential as fuels for numerous applications and are environment-friendly. Despite its advantages, bio-oil also faces numerous technical challenges for commercialization because of its high-water content (15–30 wt.%) and high composition of oxygenated compounds (35–60 wt.%) such as acids, ketones, ethers, aldehydes, and alcohols. The yield and composition of pyrolysis products may vary depending on feedstock (Aboagye et al., 2016; Hassan et al., 2016; Stefanidis et al., 2015).

Wood residues are used to produce bio-oil since they are composed mainly of cellulose (33-51%, db), hemicelluloses (20-30%, db) and lignin (21-32%, db). These wastes from the forest industry have favourable physical properties as they have low moisture content (less than 20%) and they are, generally, a clean solid uniform material without impurities, making them as an excellent substrate for thermochemical transformation (Koutinas et al., 2014). Table 1.4, shows the properties of two types of bio-oil and heavy fuel oil.

Table 1.4: Properties of bio-oil from pine, other biomasses and heavy fuel oil (adapted from *(Aboagye et al., 2016; Stefanidis et al., 2015)*).

Property	Typical properties of bio-oil from biomass	Pine	Heavy fuel oil
Carbon (% wt.)	32 – 49	52,60	85
Hydrogen (% wt.)	6,9 – 8,9	5,94	11
Oxygen (% wt.)	44 – 60	40,55	1
Nitrogen (% wt.)	0,0 – 0,2		0,3
Sulphur (% wt.)	0,0 – 0,05	0,05	
Volatile %, wt.			
Moisture Content % wt.	15 – 30	5,89	0,1
HHV (kJ/kg)	13 – 18		40
Fixed Carbon (% wt.)			
Ash (%, wt.)	0,004 – 0,3	0,86	0,1
Density (kg/m^3)	1200 – 1300		
Viscosity (at 50 °C), cP	13 – 80		40

HHV – Heat of combustion.

As an alternative to pyrolysis as a treatment, cellulose and hemicelluloses contained in wood residues can be converted to C5 and C6 sugar monomers by hydrolysis either in acidic conditions (concentrated or diluted acid hydrolysis) or by combined chemical and enzymatic treatment. The sugars obtained can be used as carbon sources for chemical and biopolymer production via fermentation (Koutinas et al., 2014).

1.7 MOTIVATION

The replacement of fossil-based products with the ones derived from renewable resources is of increasing global interest. Currently, petroleum-based plastics are among the most worrisome materials in the world. In addition to being widely used in a wide range of applications, such as food packaging, clothing, and healthcare, it also has slow decomposition kinetics, which means that a high volume is required in landfills once these are the main deposition system. In order to circumvent this worldwide problem, bio-based and biodegradable polymeric materials are increasingly sought as alternatives for most applications of petrochemical plastics having similar improved characteristics. With this, a more in-depth study of the development and production of bioplastics is made every day to make the process sustainable. Ideally with the use of less valuable raw materials for the production of something with added value in order to minimize the use of other resources. The treatment and transformation of waste for the production of PHAs, such as lignocellulosic biomass and industrial or urban waste, has a great advantage in reducing the price of the substrate and thus the cost of the final product (Queirós et al., 2014). The properties of PHAs make it attractive for the production of biodegradable and biocompatible thermoplastics with a wide range of applications capable of replacing various petroleum-based plastics.

Fast pyrolysis is the most useful technique for producing energy from biomass. It consists of heating at a controlled temperature for a short time, followed by a rapid cooling, showing as main product bio-oil, with a low process cost. Bio-oil presents a wide range of applications, one of them being its fermentation in a biological way. Due to its high carbon content, it's possible to use it as a substrate for microbial conversions, and thus produce high-value products such as bioplastics. In this study, the biomass to be used as substrate has agroforestry origin. Interest in this substrate arised since millions of tons of woodwastes are produced annually, with a composition that makes it an excellent substrate for thermochemical transformation (Moita and Lemos, 2012).

Acidogenic fermentation has high importance in the sense of rendering the substrate profitable. In this process, the production of SCOA occurs from the total sugar present in the bio-oil. The use of a MMC also favours the process since the sterilization step of the system is not necessary, reducing costs (Bengtsson et al., 2008a; Serafim et al., 2004).

In this work, it is proposed to valorise pinewood residues by its conversion into bio-oil that will be further used for PHA production by MMC, after acidogenic fermentation for SCOA production. The main objectives of this research were:

- Operation of an acidogenic fermentation reactor for substrate enrichment in SCOA and conversion of other compounds present in the bio-oil, particularly sugars and phenolics;
- Selection of a PHA accumulating culture by operating a sequencing batch reactor (SBR) under dynamic aerobic feeding conditions (ADF);
- To test the accumulation capacity of the selected culture in the SBR through several batch tests using as substrate acetic acid or a SCOA-rich effluent collected from the acidogenic fermentation;
- To identify the major groups composing the microbial community using fluorescence *in situ* hybridization (FISH) and to monitor the evolution of the culture considering the PHA producers.

2 MATERIALS & METHODS

2.1 ACIDOGENIC FERMENTATION

2.1.1 CULTURES

In the first phase of the reactor operation, the microorganisms used in this work were obtained from a full-scale aerobic digester receiving mixed domestic-industrial wastewater (Beirolas, Lisbon). The inoculum was later changed to MMC collected from an anaerobic digester of the same local. The sludge underwent a pre-treatment during 30 min at 93°C, in an oven (Loading Modell 100-800, Memmert) in such a way of eliminating methanogenic bacteria.

2.1.2 FEED PREPARATION

The bio-oil used in this study was obtained through the thermochemical conversion of pine trees residues from the wood industry.

After the bio-oil is weighed according to the desired concentration and dissolved in 1 liter (L) deionized water, the preparation of the feed medium consisted of pH adjustment at 5.5, centrifugation at 5000 rpm for 10 min and filtration of the supernatant for discard of the particles that precipitate. Glass microfibers filter (0.45 µm of internal diameter) was used for filtration. The filtrated bio-oil volume was adjusted with deionized water to make 5 L.

The feed introduced into the reactor was an enriched medium composed of filtrated bio-oil amended with macro, micro- and trace-nutrients. The micronutrient solution was composed (per L of distilled water): 0.07 g $CaCl_2 \bullet 2H_2O$, 0.6 g $MgSO_4 \bullet 7H_2O$, 0.025 g $C_{10}H_{16}N_2O_8$, 0.01 g $C_4H_8N_2S$, and 2 mL of trace solution – (per L of distilled water): 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.15 g $CoCl_2.6H_2O$, 0.12 g $MnCl_2.4H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.03 g $CuSO_4.5H_2O$ and 0.03 g of KI –. NH_4Cl, KH_2PO_4, K_2HPO_4 vary their concentration according to the C:N:P ratio tested during reactor operation. Since phosphate and ammonium salts precipitate irreversibly during sterilization when together with filtrated bio-oil, all salts and trace solution were added to the filtrated bio-oil except for phosphates and nitrogen that were prepared separately. After sterilization in the autoclave (120ºC, 20 min) the filtrated bio-oil, phosphates, and ammonium solutions were mixed under sterile conditions.

Initially, the medium was prepared using 16 g sugars/L, with the C:N:P ratio (on a molar basis) of 100:3:1, having been changed to 100:6:1. Subsequently, the feed was changed to 8 g sugars/L and C:N:P ratio for 100:3:1. A medium with 16 g/L was tested with a C:N:P ratio of 100:1.5:1. Finally, a medium with 4 g/L was tested with a C: N: P ratio of 100:1.5:1.

2.1.3 BATCH MODE AND CONTINUOUS FLOW STIRRED-TANK REACTOR (CSTR)

In this work, the reactor had two modes of operation and was initially inoculated with aerobic sludge and then with anaerobic sludge (Figure 2.1). During its operation, several conditions were kept constant: agitation by a magnetic stirrer at 50 rpm, temperature controlled (35°C) and pH=5.5. Redox and pH were monitored and acquired using in-house developed

BioCTR software running on LabView with Data Acquisition Control (National Instruments, NI USB-6009)). This reactor had a working volume of 1.5 L and working in fed-batch mode. In continuous – CSTR – mode, a HRT of 10 days was imposed with the flow rate of the feeding solution at 0.15 L/d (ISMATEC ISM831C). The reactor had no system for retaining the biomass; therefore, the SRT was the same as the HRT. The effluent was collected at the outlet of the reactor by overflow.

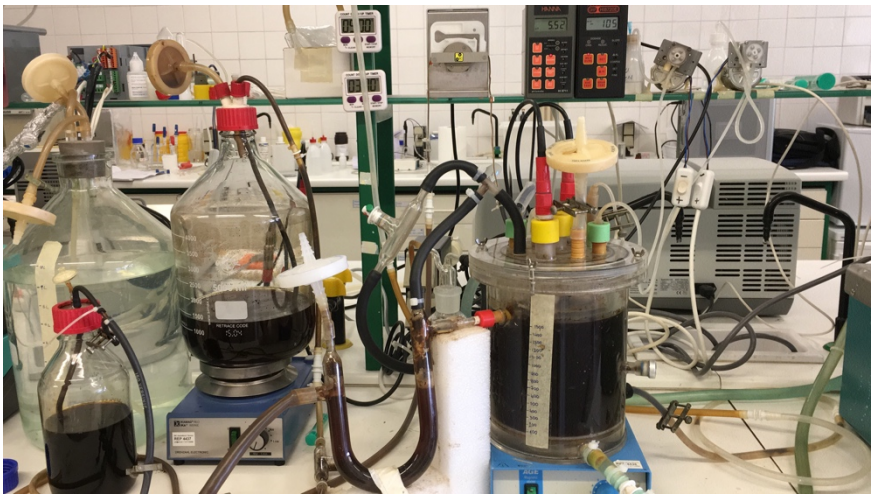

Figure 2.1: Anaerobic fermentation, bio-oil with mineral medium, feed pump, redOx and pH electrode.

Several conditions were tested for this reactor along time and so the operating period was divided into phases according to reactor operation (Figure 2.2, Table 2.1).

Phase 1. The acidogenic fermenter was inoculated with facultative aerobic MMC. The reactor was operated during 79 days. The initial volume was comprised of 1.125 L of biomass and 0.375 L of feed, and it worked in fed-batch. Initially, the retention time was expected to be 4 days, with 0.375 L of purge and addition of the same volume in new medium, but after the first 4 pulses it was found that a large amount of sugars and phenolic compounds, essentially, remained in the medium. Then, new pulses were given when the sugar concentration reached 0.5 g/L. The concentration of sugars in each pulse was 2 g/L, which translates into an organic load of 7.8 g COD/L.

Phase 2. The reactor was inoculated with anaerobic MMC. The concentration of each pulse was maintained, and the interest continued to be the selection of anaerobic bacteria, in fed-batch operation.

Phase 3. In this phase, the reactor was inoculated with a new anaerobic MMC and the concentration of sugars at each pulse was also 2 g/L. The operation started in fed-batch and at day 42 of incubation, it started operating in continuous. In continuous, 16 and 8 g/L of sugar concentration were tested and the ammonia concentration was 20 and 5 N-

mM, respectively. The C:N:P ratios then tested were 100:6:1 and 100:3:1. Lastly, it was fed again with 16 g/L but one with C:N:P of 100:1.5:1.

Phase 4. New anaerobic sludge was added. The reactor was working in continuous and two trials were carried out with 16 g/L and 4 g/L of sugars, respectively. The ammonia concentration was always the same with a C:N:P ratio of 100:1.5:1.

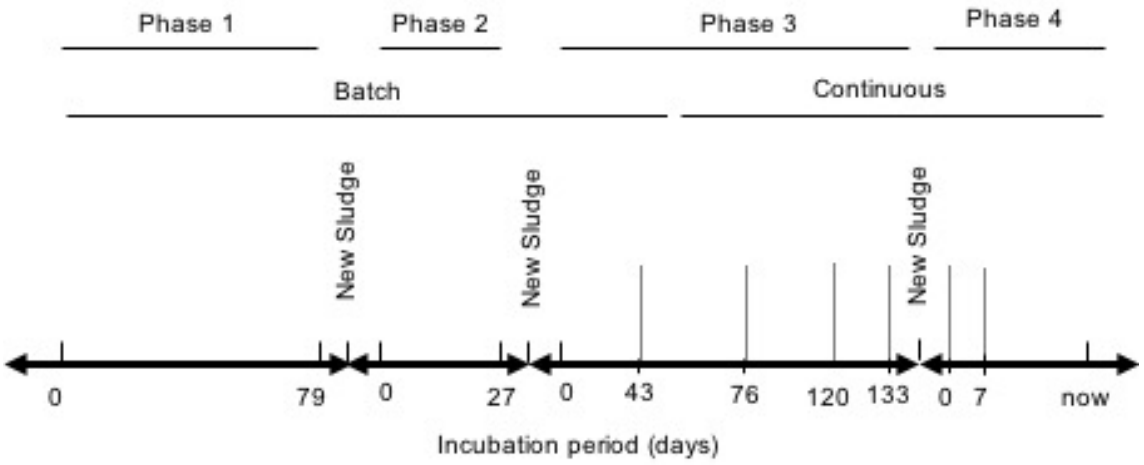

Figure 2.2: Phases of acidogenic fermentation.

Table 2.1: Conditions of acidogenic fermentation in different phases.

Phase	Incubation period (days)	Operation	Total sugars (g/L)	C:N:P ratio	RT (days)
1	0 – 79	Pulse-feed	Variable	100:6:1	Undefined
2	0 – 27		2		
3	0 – 42		2		
	43 – 75	Continuous	16	100:6:1	10
	76 – 119		8	100:3:1	
	120 – 133		16	100:1.5:1	
4	0 - 6		16		
	7 - now		4		

During the motorization of reactor operation, samples were daily collected, registering at that time, pH, temperature, and redox potential. Each sample was centrifuged at 10 000 rpm during 5 min (Centrifuge 5430 R, Eppendorf), and the supernatant was separated from the pellet. The supernatant was stored in the freezer at – 16°C and the pellet was discharged. The supernatant was used to determine the production of acids (acetic, propionic, valeric, lactic, formic and butyric acids) and consumption of sugars, chemical oxygen demand (COD), total phenolic compounds and ammonia. Samples for determination of total suspended solids (TSS) and volatile suspended solids (VSS) were collected weekly.

2.2 SBR FOR PHA CULTURE SELECTION

2.2.1 CULTURE

The microorganisms used in this work were obtained from a full-scale aerobic digester of a domestic-industrial wastewater treatment plant (Beirolas, Lisbon).

2.2.2 FEED PREPARATION

Using the same substrate of the wood industry, the prepared feed contained 2 g/L COD from pine bio-oil, which corresponds to a concentration of 60 CmM of OLR.

The procedure for preparation of the medium was identical to the one for the anaerobic reactor except that it did not need pH adjustment prior to centrifugation. Thus, after weighing the bio-oil, it was dissolved in water, centrifuged at 5000 rpm for 10 min and subsequently filtered with the same previous filters. In the dilution of the filtrated medium tap water was used, while for the dissolution of the salts distilled water was used.

The enriched medium was only composed of NH_4Cl, KH_2PO_4, K_2HPO_4 and $C_4H_8N_2S$ and these micronutrients were added at a concentration dependent on the desired C:N:P ratio, being always added 0.005 g $C_4H_8N_2S$ (per L of water) to inhibit nitrification.

2.2.3 SEQUENCING BATCH REACTOR

The reactor had a working volume of 1.5 L, operated under feast and famine conditions. The SBR was operated with aerobic dynamic substrate feeding (Figure 2.3).

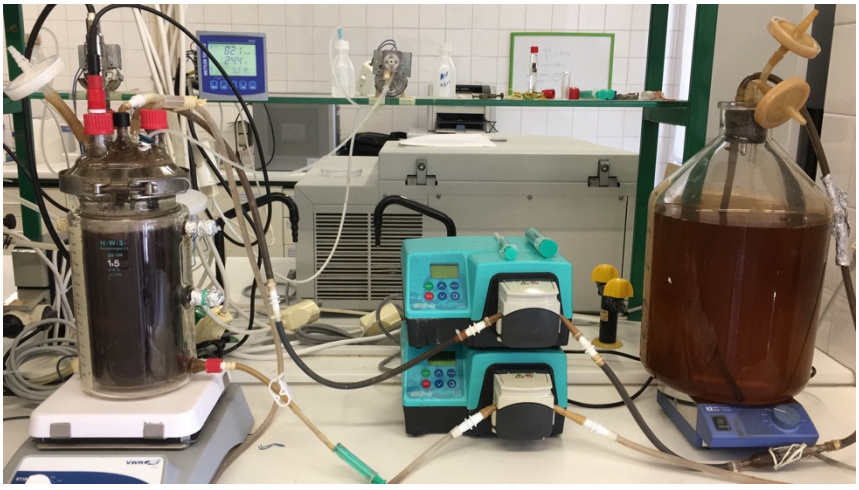

Figure 2.3: SBR, bio-oil with mineral medium, air flux, feeding and withdrawing pumps, dissolved oxygen/temperature and pH electrodes.

Each SBR cycle consisted of four periods: fill, aerobiosis (feast and famine), settling and withdraw. Several different operational conditions were tested. The aerobiosis phase was never lower than 98.3% of the daily cycle, and the time of the other parameters were adapted. A peristaltic pump was calibrated to purge the mixed liquid in the reactor at the end of the aeration period in order to maintain the desired SRT and after the settling phase to maintain the desired

HRT. At the beginning of each cycle, the reactor was fed to maintain the organic loading rate that corresponded to 1 g/COD (30 CmM of OLR) per day.

The mineral nutrients medium included nitrogen and phosphorus source (NH_4Cl and KH_2PO_4 / K_2HPO_4) to keep different C:N:P ratios (on a molar basis). As so, the reactor went under five stages of different operating conditions.

Stage 1. It was operated under 30 CmM/day of OLR, with 12h cycle, SRT of 5 and HRT of 2 days. The C:N:P ratio was 100:5:1.

Stage 2. It was operated under 30 CmM/day, with 24h cycle, SRT of 8 days and HRT of 2 days, with the same C:N:P ratio that the previous stage.

Stage 3. It was maintained the organic load and the duration of the cycle, but the SRT went to 10 days and HRT to 2 days. This stage had a C:N:P of 100:3:1.

Stage 4. All conditions were equal to Stage 3, except the C:N:P ratio that was changed to 100:4.5:1.

Stage 5. In this stage returned to the conditions of Stage 3.

Table 2.2: Different stages of SBR operation.

Stage	Incubation period (days)	Organic loading rate (CmM/day)	Cycle (h)	SRT	HRT	C:N:P
1	0 – 19	30	12	5	2	100:5:1
2	20 – 49		24	8		100:5:1
3	50 – 84			10		100:3:1
4	85 – 114					100:4.5:1
5	115 - now					100:3:1

The reactor worked at a temperature of approximately 22°C. pH was controlled between 7.95 and 8.40 with NaOH 1 M and HCL 1 M, through the use of probes inside the reactor. Air was sparged through a diffuser and stirring was kept at 400 rpm. Pumping (fill and draw), aeration and mixing were automatically controlled by BioCTR and LabView with Data Acquisition Control (National Instruments, NI USB-6009)). In addition, the software was also used to acquire pH and DO data. At given times, samples were taken periodically from the reactor in order to determine ammonia and carbon consumption, PHA, glucose biopolymer B (GB), and biomass production.

During the motorization of SBR cycles, samples were collected after the feeding stopped (t = 0 h) and every 10 min periods during the first half hour of the cycle, followed by every 15 min during an hour. After this, samples were collected every hour during the first 8h of the cycle. Samples for determination of TSS and VSS were collected after the 8^{th} h of the cycle motorization

period. When each sample was collected, pH, temperature and DO percentage of the reactor were registered. After sample collection, pH was measured again. Each sample was centrifuged at 10 000 rpm during 5 min (Centrifuge 5430 R, Eppendorf) and the supernatant was separated from the pellet. The pellet and supernatant were stored in the freezer at – 16°C. The supernatant was used to determine the consumption of acids (acetic, propionic, valeric, lactic, formic and butyric acids) and total sugar, COD, and ammonia. Initially, total sugar and total phenolic compounds were analysed, but neither the initial concentration nor their variation over the cycle represented significant values, therefore this analysis was discontinued. The lyophilized pellet was used to determine the PHA concentration by Gas Chromatography (GC) and GB concentration by High Performance Liquid Chromatography (HPLC).

2.3 ACCUMULATION TESTS

Accumulation tests were performed with several pulses of feeding either with acetic acid or fermented medium obtained from the CSTR. All the batch experiments were carried out using sludge from the SBR (0.5 to 0.9 L), collected at the end of the famine phase after the system reached steady-state, and at all pulses phosphorus was added, being the only micronutrient present in the assay. It was added in a concentration so as to maintain the C:P ratio equal to the condition imposed in the SBR.

The kinetic tests were performed in a 0.9 L working volume reactor operated in pulse-wise feeding method, Figure 2.4. Pulse feeding tends to avoid potential substrate inhibition and to maximize storage, the accumulation assays were performed under ammonia limitation.

Air was supplied by a ceramic diffuser and mixing was provided by magnetic stirring in the same condition as the SBR. With the aid of a respirometer (and using a peristaltic pump) the reactor was recirculated, where there was also magnetic stirring and where the oxygen probe was inserted. Thus, it was possible to determine the oxygen uptake rate (OUR), with recirculation being interrupted at certain intervals, leading to a decrease in dissolved oxygen concentration in the respirometer, which was recorded and used to determine OUR. pH and DO were monitored over time. The bioreactor was operated in a room controlled temperature (20-23°C).

In the first acetic acid test, only one carbon pulse was given, while in the second assay three feed pulses were given. For the fermented stream five pulses were supplied to the sludge in the first assay and in the second three pulses were supplied. Since each fermented medium was administered the feed volume was adjusted relative to the composition of each, always maintaining a concentration of 30 CmM of SCOA. Soon after the feed pulses samples were collected every 5 min, then passed to 10, 20 and 30 min intervals. The pH and DO percentage were recorded for each collected sample. Each sample was centrifuged at 10 000 rpm during 5 min (Centrifuge 5430 R, Eppendorf) and the supernatant was separated from the pellet. The pellet and supernatant were stored in the freezer at – 16°C. The supernatant was used to determine the consumption of SCOA and COD. The lyophilized pellet was used to determine PHA concentration by GC and GB concentration by HPLC.

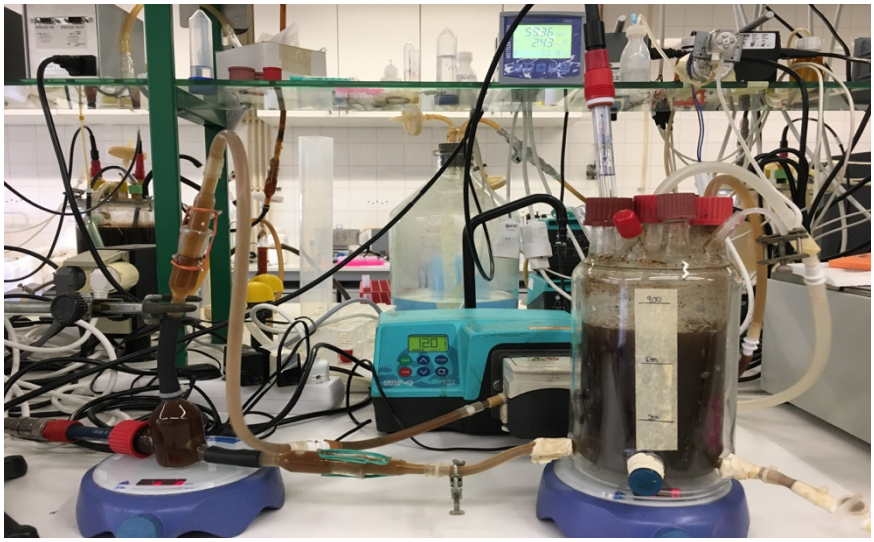

Figure 2.4: Bioreactor used in kinetic test.

2.4 ANALYTIC METHODS

Several different analytical methods were used for the characterization of bio-oil, clarified samples from the reactors and the feed solution. Among them, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), COD, ammonia quantification, Folin-Ciocalteu method, Anthrone method and Phenol-Sulfuric Acid method were performed. GC and HPLC were used too.

2.4.1 TOTAL SUSPENDED SOLIDS AND VOLATILE SUSPENDED SOLIDS

Biomass concentration was determined using TSS and VSS procedure described in Standard Methods (Clesceri et al., 1998). In this work, 10 mL of sample were filtered by vacuum filtration using filters previously dehydrated and weighed (Glass microfiber filters, 0.45 µm of internal diameter, 47 mm, FILTER-LAB). The membranes were placed in an oven (Loading Modell 100-800, memmert) at 100°C for 24h. After cooling in a desiccator, the membranes were weighed and the TSS was determined in g/L (equation 2.1). For the determination of the concentration of VSS in g/L (equation 2.2), the membranes were further placed in a muffle (Lenton Thermal Designs LTD, Lenton Furnaces) at 550°C for 2h, being again weighed after cooling.

$$TSS = \frac{(mass\ of\ filter + sample\ dried\ in\ the\ oven\ at\ 105°C) - (mass\ of\ filter)}{volume\ of\ the\ filtered\ sample\ (mL)} . 1000 \quad (2.1)$$

$$VSS = \frac{(sample\ dried\ in\ the\ oven\ at\ 105°C) - (volatilized\ sample\ in\ the\ muffle\ at\ 550°C)}{volume\ of\ the\ filtered\ sample\ (mL)} . 1000 \quad (2.2)$$

2.4.2 CHEMICAL OXYGEN DEMAND

From the samples of both reactors, the COD has measured accordingly to *Standard Methods* (Clesceri et al., 1998). In this protocol, after some proportional reductions of volumes, to each tube, it was added 2.5 mL of sample, 1.5 mL of digestion solution and 3.5 mL of acidic solution. Then, the tubes were shaken and placed in a block digester (QBH2 Grant) pre-heated to 150°C for 2h. A calibration curve was prepared with standards of glucose, and followed the same protocol as the samples, in the range of 0 g/L to 1.6 g/L. After cooling down to room temperature, the absorbance was measured at 610 nm in a spectrophotometer Helios Omega UV-VIS, Thermo Scientific. The blank made with water.

The digestion solution was composed by 10.2 g/L of $K_2Cr_2O_7$, 33.3 g/L of $HgSO_4$, 167 mL H_2SO_4 and diluted with H_2O to a total volume of 1 L. The acidogenic solution was prepared using 5.5 g $AgSO_4$/kg H_2SO_4.

2.4.3 AMMONIA QUANTIFICATION

The ammonia concentrations present in all reactors were determined through the use of a Thermo Scientific Ion Selective Electrode - ammonia gas sensing electrode - (Thermo Orion 9512). The analysis was performed with 1 mL of sample, to which 20 μL of Ionic Strength Adjuster (ISA) solution were added, in order to adjust the ionic strength. The calibration curve was prepared using standard solutions of NH_4Cl, with a range of 0 mM to 4.73 mM. The concentration values of the calibration curve and each sample were measured in mV, registered after 3 min of measurement.

2.4.4 FOLIN-CIOCALTEU METHOD

Total phenol compounds were determined using the Folin-Ciocalteu method, reading absorbance at 765 nm with a UV/visible spectrophotometer, Helios Omega UV-VIS, Thermo Scientific. The samples were compost by 0.02 mL of reactor sample, 1.5 mL of H_2O, 0.1 mL of Folin-Ciocalteu solution and 0.3 mL of the sodium carbonate solution, added after 30 sec until 8 min. The calibration curve had phenol concentration between 0 to 0.5 g gallic acid/L, the effective range of the assay. The solutions were kept at 20°C for 2h and then absorbance was determined.

The gallic acid solution had a concentration of 5 g/L and the sodium carbonate solution of 200 g/L.

2.4.5 PHENOL-SULFURIC ACID METHOD

Quantification of total sugar was achieved using the phenol-sulfuric acid method (Nielsen, 2010). This method consisted in phenol-sulfuric acid digestion, where to 2.5 mL of sample, 20 μL of phenol (80%) and 2.5 mL of sulfuric acid (96%) were added. The calibration curve was prepared with glucose in the range between 0 and 0.05 g/L. After cooling down to room temperature, some check reading the absorbance at 490 nm with a UV/visible spectrophotometer (Helios Omega UV-VIS, Thermo Scientific).

2.4.6 Gas Chromatography

PHA quantification and monomeric composition were determined using GC following the procedure adapted from Lemos (2006). Lyophilized biomass was incubated for 3.5h at 100°C with 1:1 solutions of chloroform with heptadecane, as the internal standard, and a 20% acidic methanol solution. After the digestion step, the organic phase of each sample was extracted with half a volume of water and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Bruker 400-GC). A Bruker BR-SWAX column (30 m × 0.25 m × 0.25 μm) with splitless injection at 240°C was used. The oven temperature program was as follows: 40°C, then 20°C/min until 100°C, then 3°C/min until 155°C, and finally 20°C/min until 220°C. The detector temperature was set at 230°C. HB and HV concentrations were obtained using standards of a commercial P(3HB-*co*-3HV) polymer (88%/12%, Aldrich) and corrected using the heptadecane internal standard.

2.4.7 High Performance Liquid Chromatography

For the anaerobic reactor, the production of SCOA (acetic, propionic, butyric, valeric, lactic and formic) was measured by HPLC. While that, the samples of the aerobic reactor were analysed by HPLC for determination of glucose biopolymer. The samples of the aerobic reactor were initially lyophilized and then underwent acid digestion – 1 mL HCL 0.6 N, 2h, 100°C –. All samples were filtered and the liquid fraction was analysed by HPLC.

In both analyses, a refractive index detector (Merck) and an Aminex HPX-87H column (Bio-Rad Laboratories) were used. Sulphuric acid 0.01 N was used as the eluent at a flow rate of 0.5 mL/min and an operating temperature 60°C (Freches and Lemos, 2016). A calibration curve using standard curves for glucose, acetic, propionic, butyric, lactic, valeric and formic acids was applied, in a concentration range of 0 to 1.0 g/L for glucose, propionic, butyric, valeric and formic acids, 0 to 0.5 g/L for acetic and lactic acids.

2.5 Activated carbon: Phenolics extraction

The activated carbons method was used for the pre-treatment of bio-oil.

In this method, adsorption experiments were performed adapted from Jung (2000). In all tests a fixed amount of activated carbons and sample solution was applied, always maintaining an L/S ratio equal to 10 – 0.1 g of charcoal in 10 mL of sample –. They were placed in a glass vial and shaken at 150 rpm at 23°C for 24h. After mixing, the solutions were filtered through microfiber filters > 1.2 μm. The supernatant was subsequently analysed. The activated carbons used were: commercial activated carbon (CAF and GAC), and carbon produced in the laboratory from biomass residues typical of the Azores archipelago (RCB_2).

The percentage of total phenolic compounds removed (%Removal) was calculated by following equation (2.3):

$$\%Removal = \frac{(Control - Sample) \times 100}{Control} \quad (2.3)$$

2.6 MICROBIAL COMMUNITY ANALYSIS

The characterization of the biomass present in reactors was based on traditional staining techniques as Nile Blue staining and molecular methods as fluorescence *in situ* hybridization (FISH).

2.6.1 NILE BLUE STAINING

About 1 mL of sample was withdrawn from the reactor, put in an Eppendorf, 3 drops of Nile Blue solution (1% w/v) were added, and incubated at 55°C for 10 min. The sample was removed from the water bath and washed with 8% acetic acid solution followed by brief centrifugation. Finally, the pellet was resuspended in phosphate buffered saline (1xPBS) (Rees et al., 1992). To the microscope slides 1 drop of prepared solution was added and observed under oil immersion at 1000X magnification using an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software). The presence of PHA granules in bacteria were visualized in such a way.

2.6.2 FLUORESCENCE *IN SITU* HYBRIDIZATION

FISH is a molecular technique used to detect and localize the presence or absence of certain DNA sequences according to Nielsen *et al.* (2009). In this case, it was used to identify communities of bacteria.

After the sample was collected, a fixation step follows in which the sample was centrifuged at 4000 rpm for 5 min. Then, the pellet was washed with 300 μL of 1x PBS, centrifuged at 4000 rpm again and the supernatant discarded, being this procedure repeated three times. Pellets were resuspended in 300 μL of 1x PBS, 900 μL of paraformaldehyde solution (4%) added, incubated for 3h or overnight at 4°C. Posteriorly, the sample was centrifuged at 5000 rpm for 5 min and the supernatant was discarded. The pellet was washed with 1000 μL of 1x PBS and centrifuged again. As the samples were not immediately analysed, the pellet was again resuspended in 500 μL of 1x PBS to which 500 μL of ice-cold ethanol was added, and store at −20°C.

At this moment, the sample was already prepared for the hybridization on slides. The specimen was prepared on a teflon coated slide, spot fixed cells and dried at 46°C for about 10 min. In the meantime, the hybridization buffer was prepared and kept at room temperature. The dehydrated specimen on the slide was exposed to an increasing ethanol series – 50, 80 and 100% – for 3 min each. Meanwhile, the oligonucleotide probes were thawed. 10 μL of

hybridization buffer was dropped onto each well and 0.5 μL of each probe was added without scratching the teflon surface. The hybridization tube (50 mL falcon tube) was prepared by folding a piece of tissue, putting it into the tube and pouring the rest of the hybridization buffer onto the tissue. The slide was immediately transferred into the hybridization tube and incubate in the hybridization oven (46°C) for 1.5h. Different hybridization cameras were required according to the stringency of each probe since different probes have different stringency. In the meantime, the washing buffer was prepared and preheated at 48°C (water bath). After incubation, the slides were rinsed with washing buffer and incubated in the washing buffer for 10 min in a preheated water bath (48°C). The excess of washing buffer was removed with distilled water (do not detach the cells) and the slide dried quickly (compressed air). The slide was embedded with embedding liquid (Vectashield) and a cover slip put onto the slide.

The wash buffer has a final volume of 50 mL. It consists of a 5M NaCl – specific amount for the each probe –, 1 mL of 1M Tris/HCL, 500 μL of 0,5M EDTA, 50 μL of 10% SDS and make up to volume with milli-Q water. While, hybridization buffer had 360 μL of 0.9 M NaCl, 40 μL of 1 mM Tris/HCL, 2 μL of 0.01% SDS, the amount of formamide which was according to the percentage of formamide of each probe, and milli-Q water to make up to 2 mL.

For each slide sample EUBmix (generic) probe was always applied with another specific probe (Table 2.3). For all probes, the fluorophore was cyanine 3 (Cy3), excepted in EUB338mix that was 6 fluorescein amidite (6-FAM). A blank for each sample was prepared without addition of probe to the well in order to detect auto fluorescence.

Table 2.3: FISH probes used.

Probe	% FA	Specificity	Sequence (5' – 3')	References
EUB338[1]	0 – 50	*Bacteria*	GCT GCC TCC CGT AGG AGT	(Amann *et al* 1995)
EUB338 II[1]	0 – 50	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	(Daims *et al* 1999)
EUB338 III[1]	0 – 50	*Verrucomicrobiales*	GCT GCC ACC CGT AGG TGT	(Daims *et al* 1999)
EUB338 IV[1]	0 – 50	*Bacterial lineages not covered by the previous one*	GCA GCC TCC CGT AGG AGT	(Shimit *et al* 2005)
EUB338 V[1]	0 – 50	*Bacterial lineages not covered by the previous one*	GCT GCC CCC CGT AGG AGT	(Vannini *et al* 2010)
ALF969	20	*Alphaproteobacteria, except of Rickettsiales*	TGG TAA GGT TCT GCG CGT	(Oehmen *et al* 2006)
BET42a	35	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	(Manz *et al* 1992)
DELTA495a[2]	35	*Deltaproteobacteria and Gemmatimonadetes*	AGT TAG CCG GTG CTT CCT	(Loy *et al* 2002; Lücker *et al* 2007)
DELTA495b[2]	35	*Deltaproteobacteria*	AGT TAG CCG GCG CTT CCT	(Loy *et al* 2002; Lücker *et al* 2007)
DELTA495c[2]	35	*Deltaproteobacteria*	AAT TAG CCG GTG CTT CCT	(Loy *et al* 2002; Lücker *et al* 2007)
GAM42a	35	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	(Manz *et al* 1992)
DECL585	35	*Actinobacteria PAO*	ACG CCT GTC TTA CAA AAC CGC	(Kong *et al* 2002)
Actino-221a[3]	35	*Actinobacteria—potential PAOs*	CGC AGG TCC ATC CCA GAC	(Kong *et al* 2005)
Actino-658a[3]	40	*Actinobacteria—potential PAOs*	TCC GGT CTC CCC TAC CAT	(Kong *et al* 2005)
Lgc354a[4]	35	*Firmicutes (Gram+ bacteria with low GC content)*	TGG AAG ATT CCC TAC TGC	(Meier *et al* 1999)
Lgc354b[4]	35	*Firmicutes (Gram+ bacteria with low GC content)*	CGG AAG ATT CCC TAC TGC	(Meier *et al* 1999)
Lgc354c[4]	35	*Firmicutes (Gram+ bacteria with low GC content)*	CCG AAG ATT CCC TAC TGC	(Meier *et al* 1999)
Gnsb941	20	*Chloroflexi (green nonsulfur bacteria)*	AAA CCA CAC GCT CCG CT	(Gich *et al* 2001)
TM7905	20	*Candidate division TM7*	CCG TCA ATT CCT TTA TGT TTT A	(Hugenholz *et al* 2000)
Pla46	30	*Planctomycetales*	GAC TTG CAT GCC TAA TCC	(Neef *et al* 1998)
Cf319a	35	*Flavobacteria, Bacteroidetes, Sphingobacteria*	TGG TCC GTG TCT CAG TAC	(Manz *et al* 1996)
ARC915	20	*Archaea domain*	GTG CTC CCC CGC CAA TTC CT	(Stahl and Amman 1991)

* FA – Formamide; 1,2,3,4 – Probes in mix.

2.7 Calculation of Kinetic and Stoichiometric Parameters

The sludge hydroxybutyrate (HB) and glucose biopolymer B (GB) content were calculated as a percentage of TSS on a mass basis (%HB = g HB/g TSS∗100, and %GB = g glucose/g TSS∗100).

Active biomass (X) was obtained by subtracting the storage products from the VSS as X = VSS - PHA - GB (in g/L). It was assumed that all the ammonia consumed was used for growth and it was the only possible source of nitrogen. Active biomass elemental composition was represented by the molecular formula $C_5H_7NO_2$ (Henze et al., 1995; Serafim et al., 2004).

The maximum specific HB production rate (qHB, in Cmmol HB/ Cmmol X.h), specific GB production rate (qGB, in Cmmol Glu/ Cmmol X.h), and maximum specific substrate uptake rate (−qS, in Cmmol S/ Cmmol X.h) were determined by adjusting a linear function to the experimental data (PHA, GB, substrate) concentrations plotted over time, calculating the first derivative at time zero (taking the slope of the fitting) and dividing by the corresponding biomass concentration at that point. For this rates, the associated error was also calculated.

The yields of HB (YHB/S, in Cmmol HB/ Cmmol S), GB (YGB/S, in Cmmol GB/ Cmmol S) and active biomass (YX/S, in Cmmol X/ Cmmol S) on substrate consumed were calculated by dividing the amount of HB formed, GB formed or the active biomass formed, respectively, by the total amount of substrate consumed (S). The respiration yield on the substrate (YO_2/S, in Cmmol/ Cmmol S) was calculated by integrating the curve of the experimental OUR (in mmol O_2/L.h) over time, dividing the resulting value by the amount of substrate consumed and with the subtraction of endogenous OUR (in Cmmol S/L).

For the acidogenic fermentation process the total acidification degree (AD_{Total}) was calculated by the expression:

$$AD_{Total} = \frac{[SCOA]}{COD_{in}} * 100 \quad (2.4)$$

and represents the percentage of substrate consumed for production of SCOA, considering all the organic matter entering the reactor (COD/L).

3 RESULTS & DISCUSSION

3.1 BIO-OIL AND PREPARATION OF THE FEEDING MEDIUM

The bio-oil used as the substrate in the present work would be ultimately used for the production of PHAs. Two different approaches were studied, in which the first consisted on feeding bio-oil to an anaerobic fermentation reactor for the conversion of sugars into SCOA and the second one in an aerobic production reactor feed directly with bio-oil. The first to be tested in the laboratory was the anaerobic system – acidogenic fermentation –.

Based on the data obtained from the characterization of the pure substrate to be used in the reactor, such as COD, total sugars, total phenolic compounds, total ammonia and total phosphorus (Table 3.1), a protocol was developed for preparing the medium desired to feed the reactors.

Table 3.1: Bio-oil composition.

COD (gO_2/L)	Total sugars (g/L)	Phenolic compounds (g/L)	Total ammonia (g/L)	Total phosphorus (g/L)
1106	222.12 (sd=22.57; n=3)	201.31 (sd=8.99; n=4)	1.105	0.07 – 0.087

The bio-oil has low viscosity at low temperatures, while at room temperature the substrate becomes more viscous. When in contact with water, forms an insoluble paste. This effect also occurred after titration to pH 5.5. The formation of the insoluble paste, which was discarded, causes that the expected concentration of sugar that was to be supplied to the anaerobic reactor did not coincident with the actual one. For determining the total sugars concentration in the bio-oil, the phenol-sulfuric method was chosen, where the reproducibility of the analyses was verified since the substrate in question interfere with the anthrone method and being the analyses not reproducible with the last method.

In order to achieve the desired sugar concentration and as the substrate is not readily accessible, its handling has been tested under various conditions to obtain the least wasteful medium, making it also more profitable. In total, 12 procedures were tested for protocol optimization (Table 3.2). In the control protocol – procedure 1 –, water was added to the substrate and the titration was performed at pH 5.5, followed by centrifugation and filtration. The mineral salts were added and the total volume was completed. It was later autoclaved. The proportion of substrate volume (bio-oil) to total volume was 28 in 1000 mL, which corresponds to na expected sugar concentration of 2 g/L. In the other procedures tested different conditions were varied such as the addition of a centrifugation and filtration step before titration – procedure 2 – with the objective of avoiding the formation of more precipitates. Reduction of substrate volume maintaining the same total volume – procedures 3 and 4 – in order to minimize the amount of insoluble paste when in addition to water. Addition of acetone and antifoam – procedures 5 to 11 –, since these reagents had good dissolution results of the insoluble paste in water. When added

before the titration it was intended to dissolve the paste formed with the substrate and water junction, whereas prior to centrifugation it was for dissolution of the precipitate formed in the titration.

Table 3.2: Different preparation procedures for the feed medium of the fermentation reactor.

Procedure	Total sugars $_{theoretical}$ (g/L)	Total sugars $_{end}$ (g/L)	Error (%)	Variable
1	8	7.01	12	Control
2	8	8.86	17	Centrifugation and filtration prior to titration
3	4	2.96	26	Reduction of substrate volume
4	1	0.93	7	Reduction of substrate volume
5	2	1.19	40	100 μL of acetone prior to titration
6	2	1.24	38	500 μL of acetone prior to titration
7	2	1.97	2	1000 μL of anti-foam prior to titration, without centrifugation
8	2	1.31	35	1000 μL of anti-foam prior to titration
9	16	13.38	16	500 μL of anti-foam prior to titration
10	16	11.95	25	2000 μL of anti-foam prior to titration
11	16	11.02	31	1000 μL of anti-foam prior to centrifugation

In general, the procedures tested showed no improvement compared to the control, with a considerable error. The procedures 4 and 7 presented better results in the sense that there was a lower loss of sugar. However, the protocols were not suitable for the needs, since the sugar concentration in procedure 4 was not sufficient to feed the reactor and in procedure 7 filtration became a non-viable step due to the insoluble paste present.

Another condition to be tested was the addition of the mineral salts after the autoclave in order to avoid the formation of precipitates when exposed to high pressure and temperature. This variable had a great impact on the clarity of the medium obtained at the end of the process because when autoclaving the minerals separately and only mixed when both were at room temperature, it made the medium lighter and without precipitation.

Thus, it was decided to opt for the control protocol with two new conditions: addition of the mineral salts after the autoclave and adjustment of the substrate volume/total volume ratio. The change in substrate volume consisted of determining the amount of sugar that was lost during the process and correcting it accordingly. Then, the bio-oil that was previously added by volume

began to be added by weight, after calculating its density, for greater accuracy and less waste due to its physical characteristics.

3.2 ACIDOGENIC FERMENTATION

Given the characteristics of the bio-oil used, acidogenic fermentation was an important step to develop. The bio-oil, although rich in sugars, also presented several elements in its composition that made it a very complex substrate. With this, the adaptation of microbial cultures was a difficult stage, requiring several phases (Figure 2.2, section 2.1.3). After adaptation of the culture several conditions were tested (Table 2.1, section 2.1.3).

3.2.1 PHASE 1

The bacterial culture inoculated into the fermentation reactor had previously been directly enriched with bio-oil and the biomass originated from a full-scale aerobic digester receiving mixed domestic-industrial wastewater. The choice of an aerobic inoculum was based on the fact that most of the acidogenic bacteria are facultative anaerobes, eliminating methanogens that are strict anaerobes (Fernández-Morales et al., 2010). At this stage, the reactor started operating with an initial total sugars concentration of 1.5 g/L (Figure 3.1), 0.7 g/L of total phenolic compounds (Figure 3.2) and 0.2 g/L of ammonia (data not shown). The reactor was operating without an initial defined RT. The system was started with the addition of a bio-oil pulse, and then before the addition of new pulses medium with biomass purge was collected under stirring conditions until the 55th day. From that day on the medium was collected without stirring.

At the beginning, the reactor had a VSS of 4.17 g/L, which allowed the total consumption of sugars in the first pulse in a period of 10 days. However, 10 days of incubation between pulses was not desirable, is a very extensive period which will not lead to a higher production of SCOA, reaching a plateau phase. A shorter SRT also favours the growth of acidogenic organisms in relation to methanogens (Fang and Yu, 2000; Lee et al., 2014a). The ammonia concentration was still not exhausted, accumulating 0.1 g/L in the system. In an attempt to circumvent this situation, the bio-oil feed was reduced to 0.5 g/L of total sugars making the concentration of phenolic compounds within the system also reduced to values lower than 0.5 g/L, and ammonia concentration was reduced for 0.1 g/L. This would be favourable to the system since these last compounds were not consumed in the same proportion as the total sugars and can act as inhibitors of SCOA production (Koutinas et al., 2014). Two consecutive pulses – at days 13 and 16 of incubation – were applied with these conditions. The new strategy proved to be inefficient, since the low substrate concentration within the reactor resulted in a wash-out of the sludge, with the biomass concentration drastically reduced in a short time – 4.17 g VSS/L at day 1 to 1.54 g VSS/L on day 18 –. The analysis of SCOA production confirmed the previously mentioned (Figure 3.3) since in the three bio-oil pulses SCOA were not produced. The presence of SCOA in the system represented a residual value supplied by the feed.

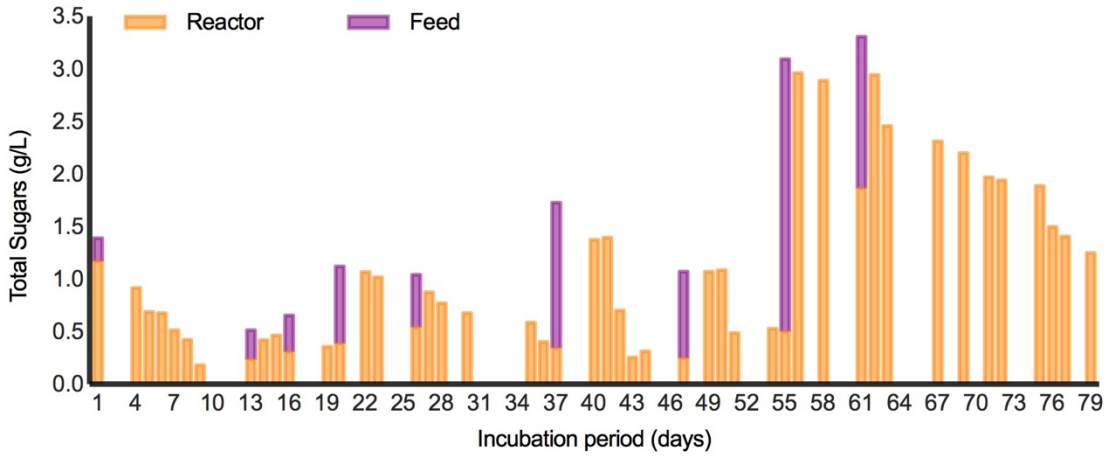

Figure 3.1: Total sugars profile for AF in Phase 1.

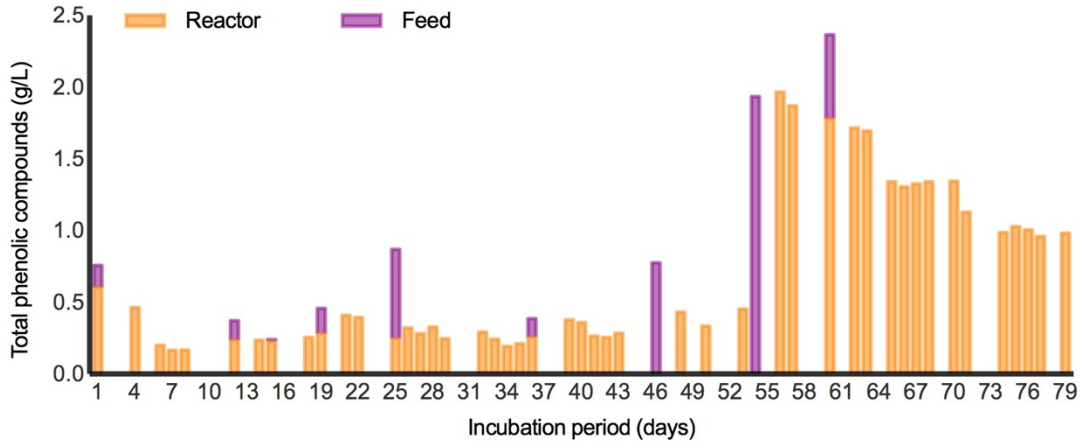

Figure 3.2: Total phenolic compounds profile for AF in Phase 1.

Sugar concentration, phenolic compound concentration, days between pulses (which influence the RT) and the SCOA production profile are all parameters that when related are important for the study. So, to perfect the compromise between these, various changes in organic load were tested. In the pulse of day 20, and in the three consecutive pulses, it was verified that after the feeding in the reactor the concentration of total sugars present was 1 g/L and the total phenolic compounds remained with low values (Figures 3.1 and 3.2). While the ammonia was totally consumed. Although good sugar consumption was observed in all the pulses, the production profile of the SCOA revealed lactic acid consumption and a small production of acetic acid (concentration <0.3 g/L) (Figure 3.3).

With these results, on the next two pulses (days 55 and 61) the total sugars available in the reactor were increased to 3 g/L in order to give a final competitive advantage to the SCOA-producing fraction of the mixed microbial culture. The total phenolic compounds also increased, reaching an average concentration of 1.5 g/L, and ammonia increased for 0.2 g/L. Although sugar

and ammonia were being consumed, the total phenolic compounds remained in high concentration and an acceptable production of SCOA was not observed, maintaining the same scheme of lactic consumption and small acetic acid production. During this phase of the reactor, acetic acid was the main SCOA produced but remained at low concentrations (<0.4 g/L) and it started to be consumed by the end of reactor operation. Lactic acid was present at high concentrations (1.4 g/L) after the bio-oil pulse (Figure 3.3), but consumption was observed during the following days of operation. Fernández-Morales *et al.* (2010) reported that the production of acetic acid was related to the evolution of acidogenic organisms, whereas the decrease of lactic acid was associated with the reduction of facultative microorganisms, which favoured the conversion of glucose into SCOA.

Several strategies concerning the change in total sugar/total phenolic compounds available and the time between the pulses of bio-oil did not produce consistent and satisfactory results, which means that the selected facultative anaerobic bacteria were not a suitable slurry for the fermentation of pine bio-oil. So, a new reactor was designed.

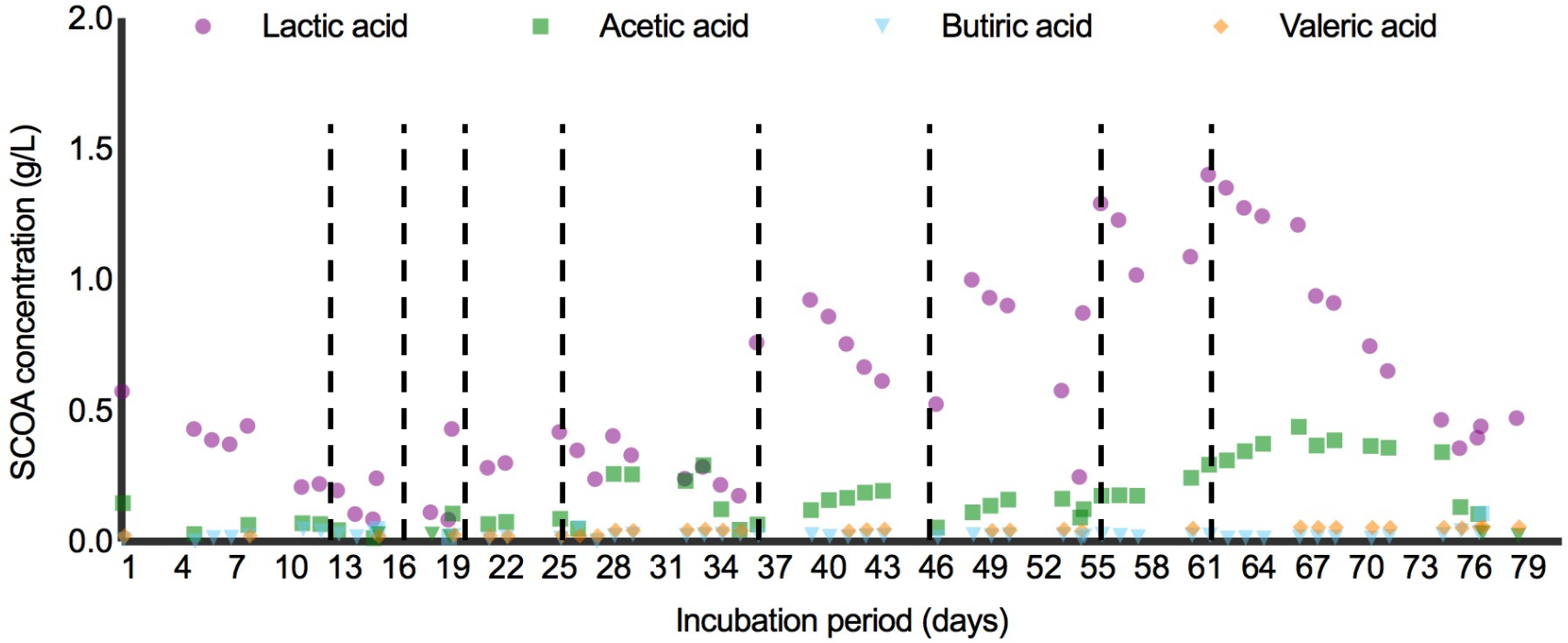

Figure 3.3: SCOA profile for AF in Phase 1.
Dashed lines represents bio-oil pulses.

3.2.2 PHASE 2

Due to the results of the previous reactor, aerobic sludge was replaced by anaerobic sludge as the seed sludge, as this has advantages as a more robust capacity for the substrate consumption, which could potentially help to increase the accumulation of SCOA (Rajeshwari et al., 2000). The second fermentation reactor was inoculated with sludge from the anaerobic stream of the Beirolas wastewater treatment plant. In the anaerobic sludge, the presence of methanogenic bacteria should be accounted, and for this reason, a pre-treatment of the sludge was performed, consisting of 30 min at 93°C.

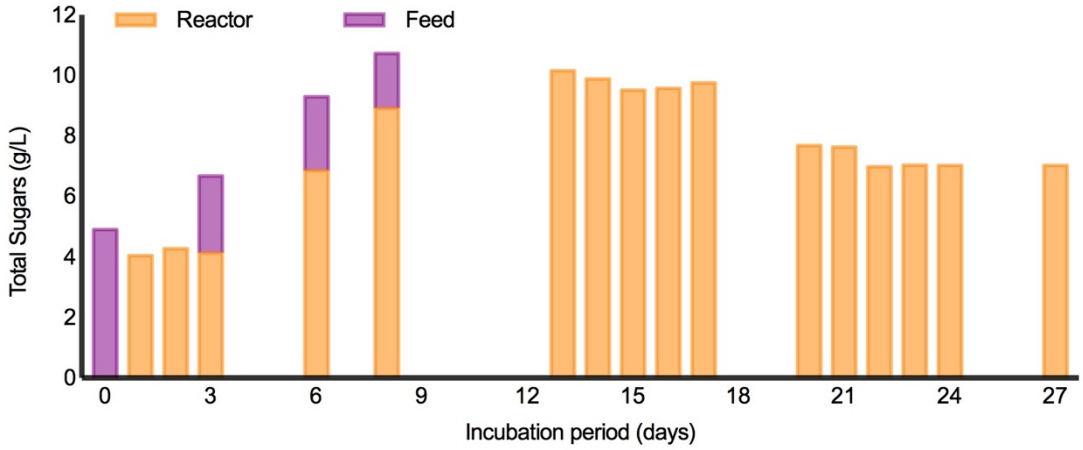

Figure 3.4: Total sugars profile for AF in Phase 2.

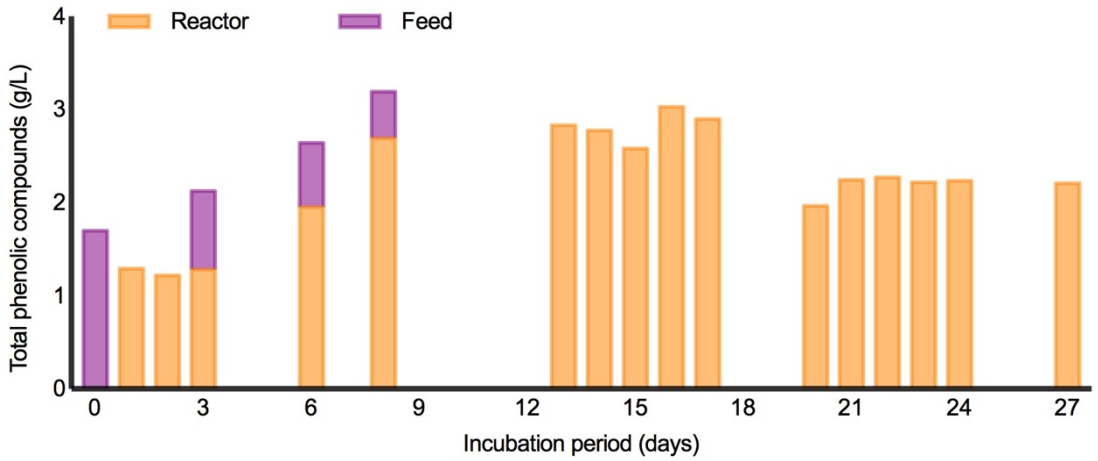

Figure 3.5: Total phenolic compounds profile for AF in Phase 2.

The Phase 2 started and the pH profile of system was the only parameter possible to follow during the initial 10 days, showing systematic additions of NaOH 1M to the reactor, revealing a significant volume of spent NaOH. This indicated that the medium acidified probably by the production of SCOA and sugar was being consumed. It was assumed that reactor started working in a promising way. The RT was undefined and before each feed pulse medium was collected with biomass purge. The feed concentration in total sugars was 2 g/L in all pulses.

Subsequent analysis of the available carbon substrate showed the accumulation of total sugars and phenolic compounds to 10 g/L and 3.2 g/L, respectively at the fourth pulse (8 days of operation; Figures 3.4 and 3.5). Inhibition of the system was evident as the substrates were no longer consumed. In view of these results, it was decided to start a new reactor with the same bacteria, now with a lower feed concentration.

3.2.3 PHASE 3

In Phase 3, the reactor was inoculated with anaerobic sludge as previous and it was started as pulse-feed operation up to day 32. Subsequently, it began to be operated in continuous mode being several conditions tested. In total, this phase was composed of four different periods.

3.2.3.1 Phase 3, pulse-feed operation

For this stage, a target concentration of 1.5 g/L of total sugar after each pulse was defined (Figure 3.6), and consequently, as expected, a concentration of total phenolic compounds of 0,6 g/L (Figure 3.7). With the ammonia concentration of 0.2 g/L (data not shown). For each new pulse, the same volume was removed from inside the reactor while stirring. In Figure 3.6 it's possible to see that the microbial culture consumed 1 g/L of total sugar in 10 days but instead of increasing, the acetic acid concentration fell from 0.45 to 0.21 g/L, while the other acids had a residual value (<0.1 g/L). Only from day 7 on a slight growth in the production of acetic and propionic acids occurred reaching 0.34 and 0.20 g/L, respectively, to the next pulse. Since the system was at the beginning of a new adaptation period, SCOA production may have been unstable during the first pulse due to acclimatization with new anaerobic sludge from the Beirolas wastewater treatment plant.

After stabilizing the consumption of sugars from the first pulse, the second feeding pulse was given. At this time – from day 10 to day 21 –, it can be seen that the SCOA profile was similar to the previous pulse. There was a slight consumption of acetic acid followed by production up to 0.60 g/L. The remaining acids were neither consumed nor produced, having a stable concentration around 0.2 g/L (Figure 3.8). The concentration of the total phenolic compounds did not decrease (Figure 3.7), which represented a negative impact on acclimatization of MMC since this compound becomes inhibitory when present in high concentrations (Koutinas *et al.* (2014)).

On the third pulse of bio-oil, the initial 2 g/L of total sugars decreased by 1 g/L, while the concentration of total phenolic compounds did not change significantly (0.2 g/L, approximately) till day 42. Lactic acid showed higher production (0.85 g/L) than acetic acid at 0.79 g/L followed by significant consumption of both. The butyric and propionic acids were stable soon after the pulse, and after 15 days increased the concentration by about 0.2 g/L. The shift from production to consumption of lactic and acetic acids, the beginning of increase on butyric and propionic acids concentrations and the decrease of total sugars all occurred at the same time, aiming at a promising adaptation of the acidogenic microorganisms with respect to the methanogenic ones (Fernández-Morales et al., 2010). During the first two pulses, there was no ammonia consumption, only starting from the third pulse. Still, there was an accumulation of about 0.33 g/L.

The best total acid concentration was the 36th day with 2.3 g/L and the following composition: 26% lactic, 37% acetic, 19% butyric and 18% propionic, and an acidogenic degree of the 0.07 gCOD/gCOD. Acetic acid as the main SCOA produced followed by propionic and butyric acids as also found in the study by Chang *et al.* (2010) where several substrates such as food residues, rice straw, and corn flour were tested.

Although SCOA production in Phase 3 with feed pulse was already higher than in every system/strategy tried before in this study, in order to prevent phenolic compounds accumulation and to maximize SCOA production the only viable strategy to pursue seemed to be to operate the reactor under continuous feeding.

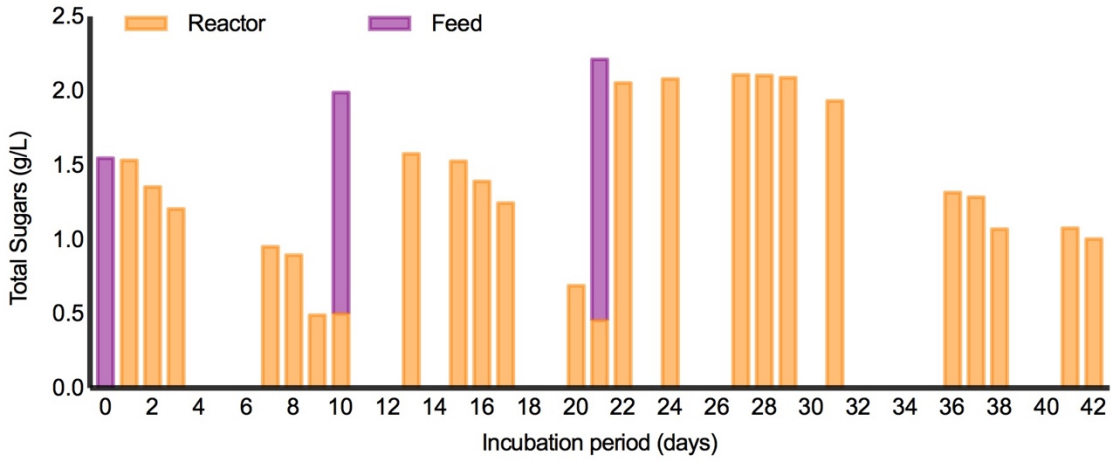

Figure 3.6: Total sugars profile for AF in Phase 3, 0 to 42 days.

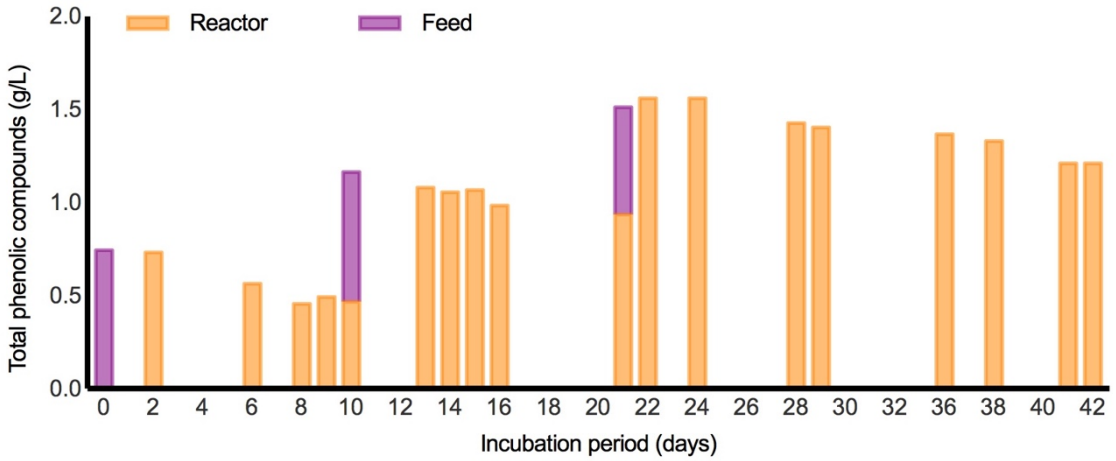

Figure 3.7: Total phenolic compounds profile for AF in Phase 3, 0 to 42 days.

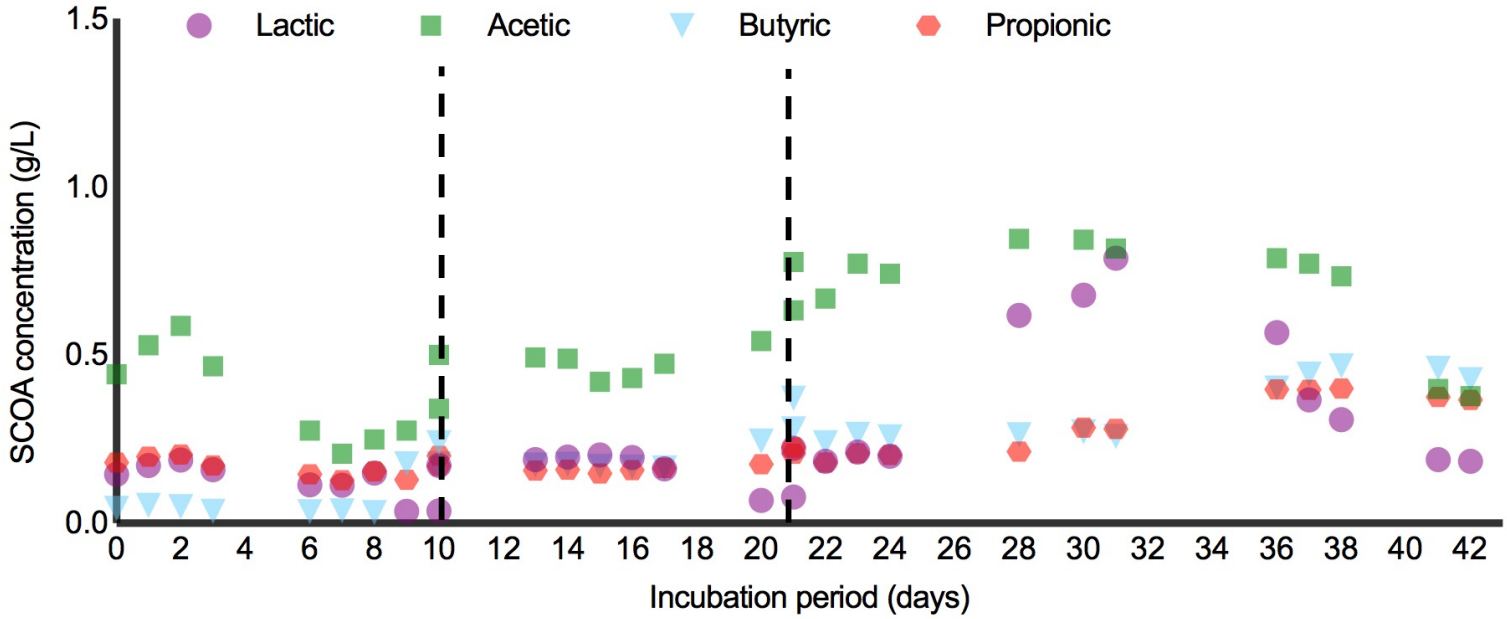

Figure 3.8: SCOA produced profile for AF in Phase 3, 0 to 42 days.
Dashed lines represents bio-oil pulses.

3.2.3.2 Phase 3, continuous operation

At this stage, the reactor started to be operated in continuous mode. With 16 g/L.day of feed concentration, containing 5 g/L.day of total phenolic compounds (Figure 3.9), a C:N:P ratio of 100:6:1 and with a RT of 10 days.

As expected, the sugar concentration inside reactor has been increasing over the incubation period. After 30 days of operation, the concentration was equal to, approximately, 14 g/L, which means that only 2 g/L.day were being consumed. At the same time, total phenolic compounds concentration increased, approximately, for 5 g/L. After the three retention times, there was a stabilization at both concentrations by inhibition of the system. Koutinas *et al.* (2014) describe total phenolic compounds as inhibitors in AF systems.

The concentration of ammonia is also an important parameter to take into account. Based on the C: N: P ratio used, 1.7 g/L.day of ammonia was supplied to the system, and only 0.11 g/L.day were consumed, with a high accumulation of this micronutrient.

In view of the results, it was decided a reduction of sugar concentration for 8 g/L.day, thus passing the organic load to half, and consequently, a decrease of total phenolic compounds. The C:N:P ratio also switched to 100:3:1. These conditions started on the 76th day and as the last feed medium had a higher concentration, it was necessary to wait for the first RT so that the system re-established the inside concentration. Between days 76 and 86 it was observed an exponential decrease in the concentration of sugar of 14 g/L for 11 g/L, total phenolic compounds of 5 g/L for 4 g/L and ammonia reduced from 0.91 g/L to 0.55 g/L. Right after – from day 87 to 107 – the results did not show a good functioning of the reactor since the concentration of sugar in system stabilized around 8 g/L, which means there was no consumption. From day 107 the sugar content reduced to values close to 6 g/L. Total phenolic compounds showed the same profile as sugar, stable over a period of 10 days with 3 g/L, followed by a decrease to 2 g/L. Ammonia now at a lower concentration, was not completely consumed, being accumulated in the system at a concentration of approximately 0.21 g/L.

Figure 10 shows the production profile of SCOA during the entire Phase 3 in continuous operation mode. Acetic acid increased throughout the period, reaching a concentration of 0.73 g/L. Propionic acid had the same behaviour, ending with a higher concentration (1.23 g/L), while lactic acid increases preferentially from day 63 and reached 3.9 g/L. The butyric acid always presented a decreasing behaviour, going from 1.7 g/L to 0.9 g/L.

With the reduction of sugar in the feed, SCOA production was shown to reach maximum values at day 92 with 56% of lactic, 11% of acetic, 16% of propionic and 17% of butyric, which translates into a concentration of SCOA of 6.3 g/L. On this day, the degree of acidification also reached the maximum value in this system with 0.14 gCOD/gCOD. Lactic acid increased exponentially, being the main acid present. The reactor in this period was possibly in a phase of instability, which favoured the production of this acid (Gouveia et al., 2016; Itoh et al., 2012). Concentrations of butyric and propionic acids appeared somewhat inversely related since their composition varied symmetrically. The literature suggests the existence of different types of

populations for the production of the two acids, which compete for the carbon source (Bengtsson et al., 2008a; Horiuchi et al., 2002). After day 92, there was a biomass wash-out where the VSS went from 1.10 to 0.80 g/L and consequently, the production of all acids also fell, where the lactic acid changed to 1 g/L, the acetic acid to 0.7 g/L, the propionic acid to 0.3 g/L, excepted the butyric acid that maintained at 0.7 g/L (Figure 3.9 and 3.10).

Due to the reduction of 8 g/L.day of total sugars and being the specific content of each type of sugar unknown, it was possible that the system was limited by the lack of accessible sugars. Low SCOA production and constant biomass loss encouraged the return of 16 g/L.day of sugar in the feed medium, keeping now a low ammonia concentration. With this decision, the maintenance of the biomass inside the reactor was also expected. At the 120th day, the new condition began but the system did not respond. Sugar, total phenolic compounds, ammonia and SCOA concentration did not change over time (results not shown). Thus, with only 13 days of operation, it was decided to give a spike with new biomass (Phase 4).

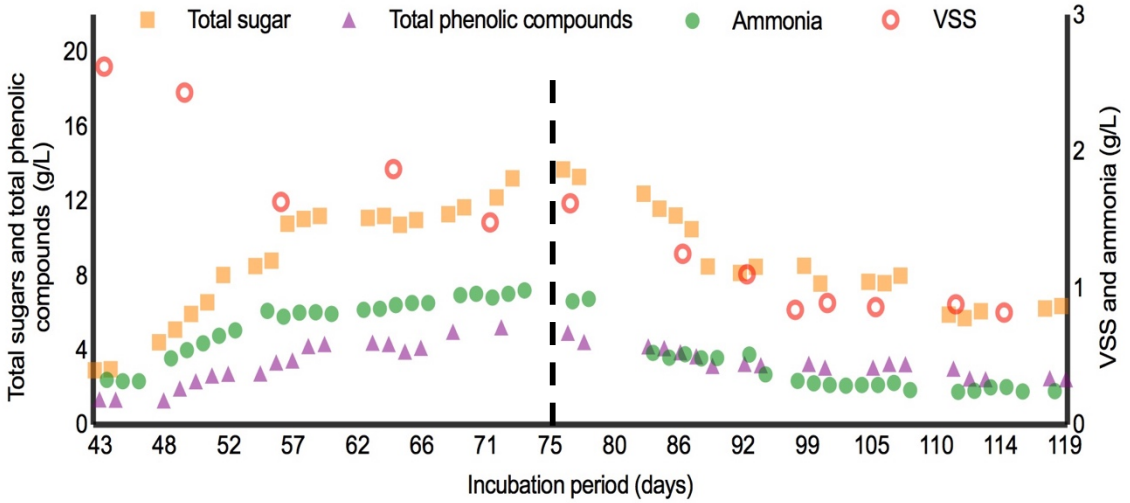

Figure 3.9: Total sugars, total phenolic compounds, ammonia and VSS for AF in Phase 3 in continuous mode.
The dashed line represents the division of the conditions tested during Phase 3, continuous operation**.**

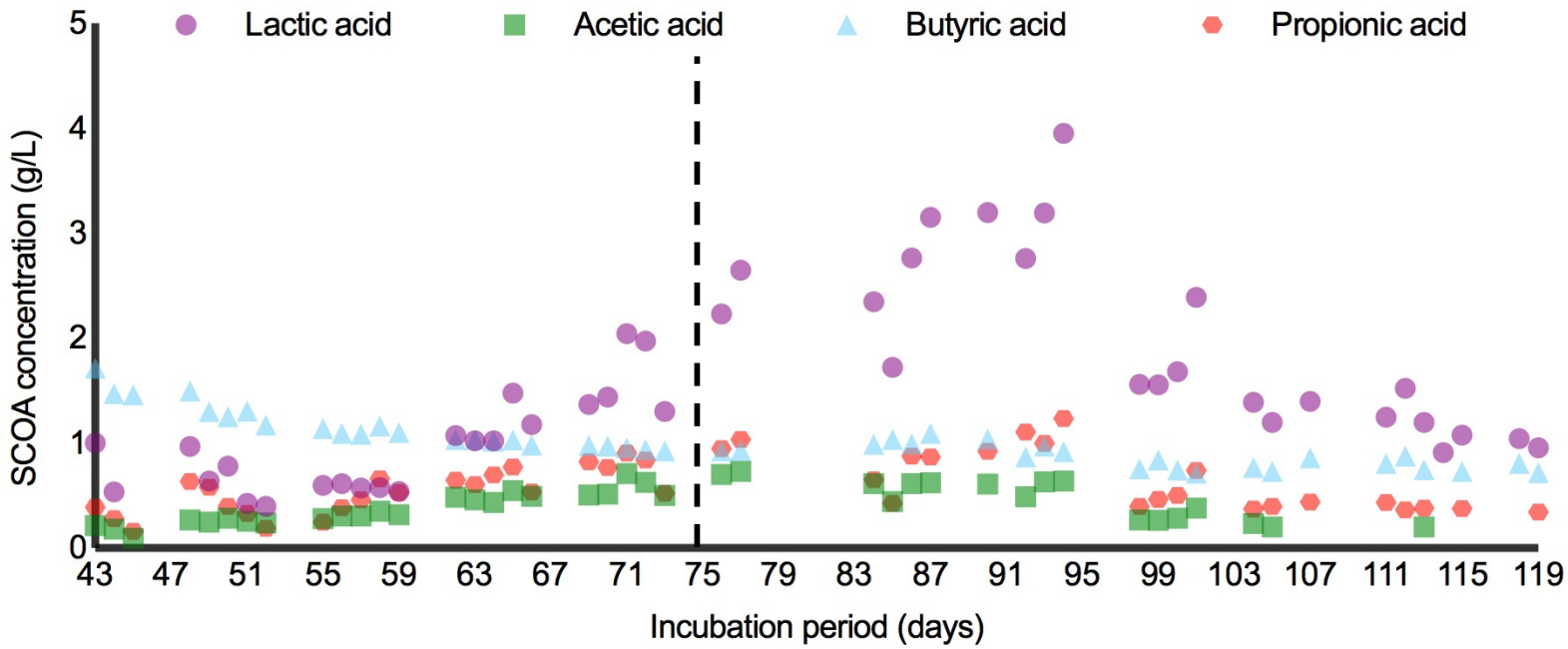

Figure 3.10: SCOA produced profile for AF in Phase 3 in continuous mode.
Dashed line represents the division of the conditions from days 43 to 75 and from days 76 to 199.

3.2.4 PHASE 4

After biomass almost wash-out, new sludge was added. In Phase 4, the reactor was working in continuous and it was two different conditions tested. The RT of 10 days and C:N:P ratio of 100:1.5:1 was kept.

The feed conditions were maintained as previously for a short time - 6 days -, the sugar concentration of 16 g/L.day, and the ammonia concentration of 0.6 g/L.day, to favour the adaptation of the added culture. During this period, there was only the increase in the concentration of sugar, total phenolic compounds, and ammonia up to 11.3 g/L, 4.2 g/L, and 0.5 g/L, respectively (data not shown). Given the results, reducing the concentrations that were being accumulated was the goal as to minimize undigested sugar in the reactor and also to reduce total phenolic compounds in order to avoid the inhibiting effect. The accumulation of ammonia in the reactor was also a point to consider. The concentration of sugar in the feed medium was reduced to 4 g/L.day and, consequently, the total phenolic compounds were present at a concentration of 2 g/L.day and only 0.12 g/L.day of ammonia.

In first RT of the 2nd condition, the sugar concentration reduced from 12 g/L to 3.5 g/L. The same happened with total phenolic compounds and ammonia that reduced significantly their concentration reaching a concentration of 1.8 g/L and 0.1 g/L, respectively (Figure 3.11). In the following days, the sugar and total phenolic compounds concentration stabilized at 2 g/L and 1.5 g/L, respectively. This result shows that a consumption of about 2 g/L of sugar was occurring, and the low phenolic concentration reached did not interfere with the good functioning of the reactor. The ammonia concentration reached zero, being the whole ammonia consumed. It can be also verified that VSS at this stage was stabilized at about 1.40 g/L, after decreasing from 3.68 g/L (value after the spiking with new biomass). So, it may mean that the concentration of ammonia is not enough to produce more, and in that sense, it becomes limiting.

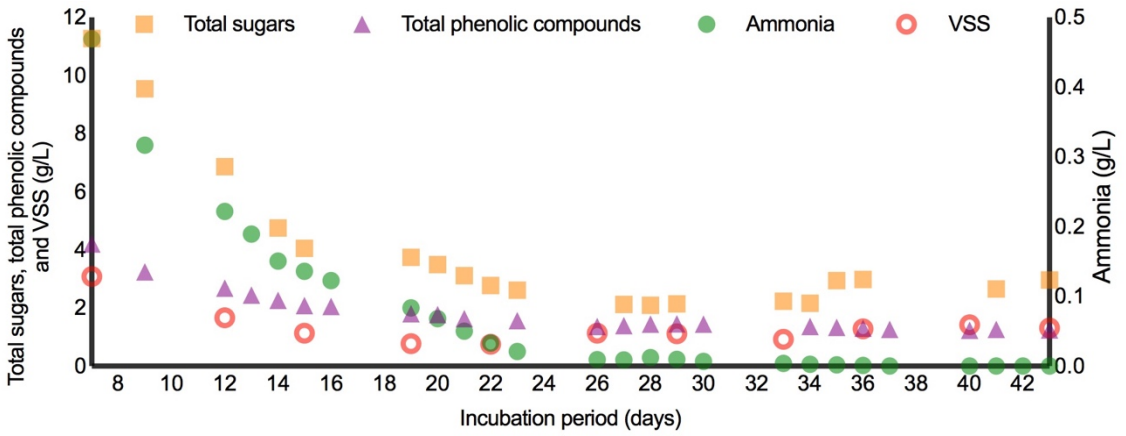

Figure 3.11: Total sugar, total phenolic compounds, ammonia and VSS concentration for AF in Phase 4 in continuous mode.

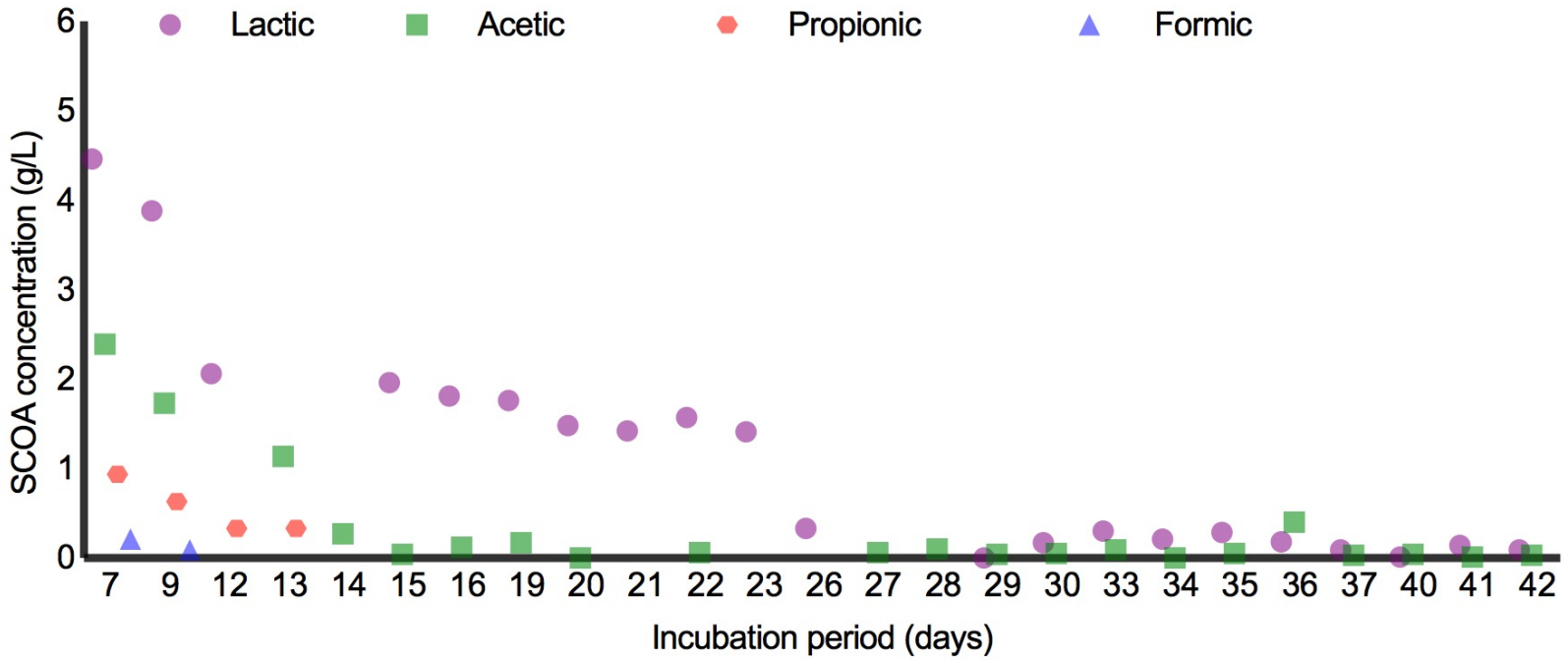

Figure 3.12: SCOA produced profile for AF in Phase 4 in continuous mode.

In contrast to the sugar consumption profile, Figure 3.12 shows that the reactor was not functioning well. The production of all the acids was decreasing during the operation until it was almost completely extinguished after day 26. The lactic acid is still the one with the highest concentration, which indicates the instability of the system (Gouveia et al., 2016). A minimum production of formic with a maximum of 0.2 g/L was verified, which is associated with the spike of new biomass. This profile indicates that at the time of the condition change the system was not yet adapted to the substrate and the low feed concentration did not favor the selection of the desired culture.

3.3 SEQUENCING BATCH REACTOR

After analysis of crude pinewood bio-oil, it was found that several SCOA were present in its composition. With the objective of feeding the second reactor with the SCOA-rich stream from acidogenic fermentation, a sequential batch reactor (SBR) was designed in order to start to acclimatize a PHA-accumulating microbial culture able to use the unrefined bio-oil.

The reactor was operated in feast and famine conditions, being the microbial selection based on periods of carbon availability (feast phase) and long starvation periods (famine phase). This selective pressure ensures that the microbial fraction with an ability to store biopolymers during the feast phase could be dominant in the system. During the operating time, several conditions were tested in order to optimize the selection of PHAs-accumulating cultures, and such conditions are described in Table 2.2, section 2.2.3. Several parameters were monitored such as consumption of COD, ammonium, and production of PHAs, glucose biopolymer B and biomass. The F/F ratio was also followed.

The reactor started (1 Stage) with 12h cycles and 5 day SRT, but under these conditions, it was found that there was a huge loss of biomass. The cycles were increased to 24 hours and the SRT to 8 days after 20 days of operation (2 Stage). With this change, it was expected that the biomass would remain in the reactor, which did not happen. A biomass wash-out occurred again and it was also found that there was a high accumulation of ammonia. The Stage 3 was started at 50^{th} day, where the SRT changed to 10 days and the C:N:P ratio decreased from 100:5:1 to 100:3:1. During this period, the biomass appeared to be more stable within the reactor, but by analysing ammonia, it was observed that it reached zero values and there was no increase in the growth of the culture. So, at 85 days ammonium limitation may be occurring and it was increased from 1 mM to 1.5 mM (4 Stage). This change caused ammonia accumulation for the next cycles and PHA production declined. Due to this fact the conditions applied in 3 Stage were restored at 115 days, since the most satisfactory results were obtained in this stage (5 Stage).

Figure 3.13 shows the evolution of F/F ratio and biomass along the SBR operational time. The concentration of biomass inside the reactor was very unstable, being necessary the addition of biomass collected in the previous cycles, corresponding to the points out of trend. The F/F ratio is an important parameter in the culture selection, indicative of the adaptation of the inoculum to the bio-oil as new substrate (Albuquerque et al., 2010b). This ratio can be obtained by monitoring the duration of the feast phase, following the dissolved oxygen (DO) concentration and/or the pH

along time inside the reactor. In relation to the values, high F/F ratios reveal that growth is favoured rather than polymer accumulation, while low F/F values ensure PHA storage. The best response of given culture with a good storage capacity is observed when the feast phase is no longer than 20% of the length of all cycle (Albuquerque et al., 2010b; Dionisi et al., 2007; Reis et al., 2011). As shown in Figure 3.13, after day 120 the trend of F/F ratio was below 0.2 and decreasing towards 0.1, meaning that the SBR operated under the desired conditions for the selection of the culture presented preferential storage capacity.

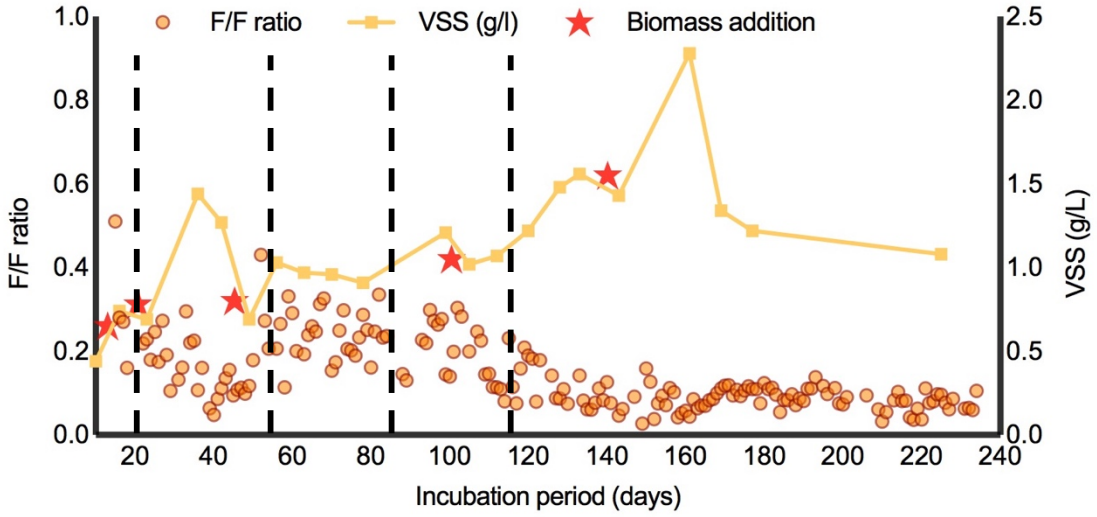

Figure 3.13: F/F ratio and VSS for the selection reactor.
Dashed lines represents a new stage.

3.3.1 A TYPICAL DAILY CYCLE

Figure 3.14 shows a typical daily cycle under steady operational conditions of the SBR. Typically, the start of the feast phase coincides with an abrupt reduction in DO value, and it ends when increasing DO and pH to higher values (Serafim et al., 2004). Through the DO profile, it could be considered three phases. The first increase in DO corresponds to the consumption of the preferred carbon sources – approximately 3h –, followed by a slower increase that revealed the consumption of other less accessible sources (feast phase). There were no true feast/famine conditions since some COD was still consumed during the rest of the cycle (famine phase).

The beginning of the cycle corresponded to the moment when the reactor was fed with the medium, starting the feast phase. In this system, the pH was controlled between 7.95 and 8.40 with the addition of 1M NaOH, with pH control switched off during the feed. After completing the feed, it was possible to verify that pH decreases to 6.5 and at the same time started the addition of the base, in order to stabilize the pH in the desired values. During this phase pH always tend to decrease and when the feast ends the pH increases rapidly until approximately 8.40. This pH range goes according to the findings of Serafim et al. (2004) where it was determined that the polymer yield on substrate and the intracellular content in P(3HB) was higher at pH 8 than at pH

7 and that those were still higher when pH was uncontrolled (8 to 9.5) (Serafim et al., 2004). pH control has some advantages, as described by Oehmen *et al.* (2011), where a higher volumetric productivity of PHA production was observed when pH was controlled at 8 than without pH control (pH 8-9). It also has drawbacks, such as increased costs, when is the need for chemicals for this purpose in large-scale applications, and the complexity of the process (Oehmen et al., 2014).

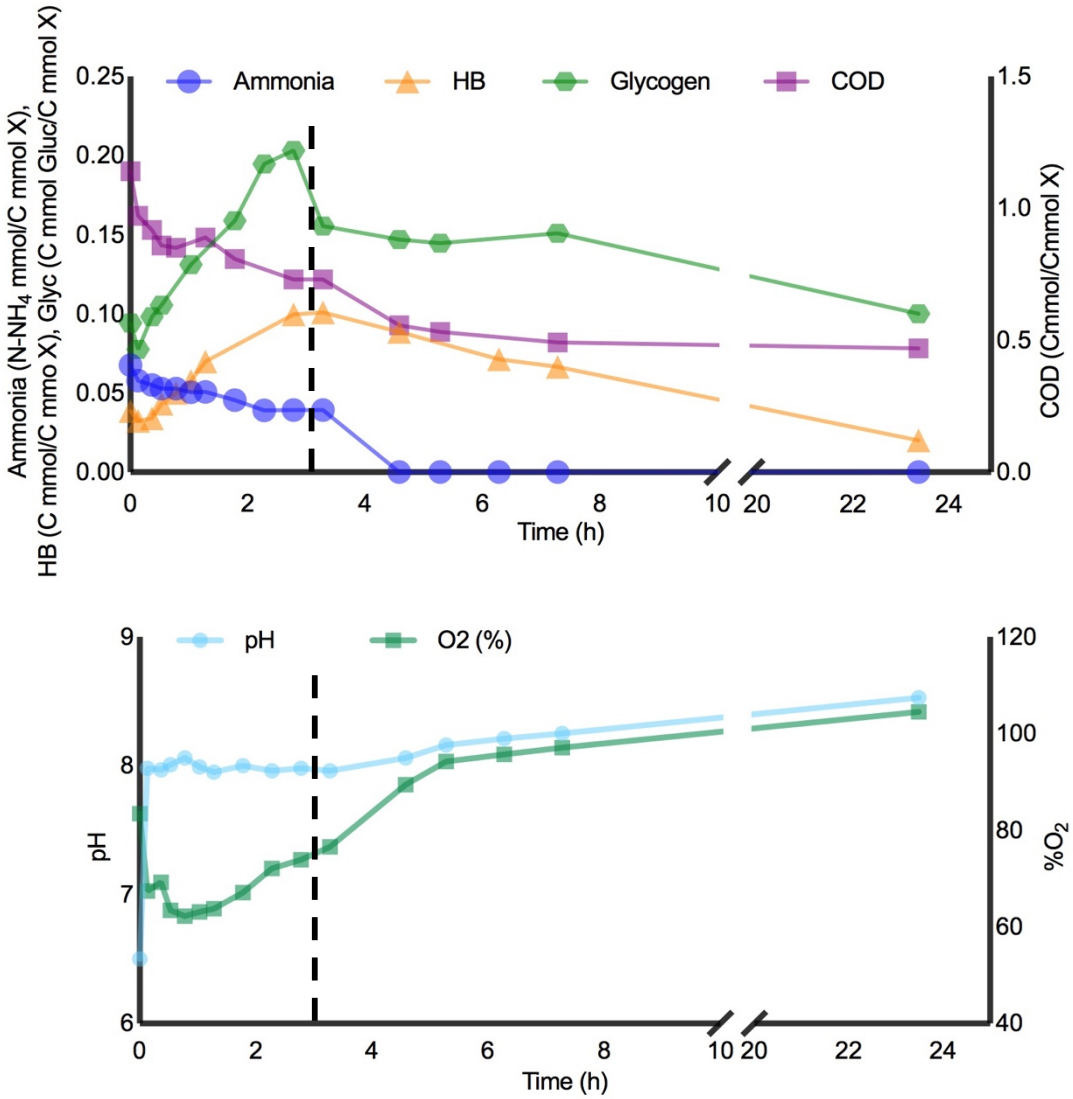

Figure 3.14: SBR cycle from the 112th day of operation, with the evolution of pH, oxygen, ammonia, HB, glycogen and COD.

It can be seen in Figure 3.14 that the substrate consumption was directly related to the accumulation of two different internal carbon reserves in the microbial culture, in this case, PHA (mainly HB) and glucose biopolymer B (GB). It can be observed that 0.25 Cmmol/Cmmol X of COD was consumed until 3h for the production of HB homopolymer (0.11 Cmmol HB/Cmmol X) and GB (0.10 Cmmol Glu/Cmmol X). Near the 3h, COD consumption stabilizes and even though micronutrients limitation was not observed, there was 0.62 Cmmol/Cmmol X of COD still not consumed by the microbial culture. For this reason, feast phase duration was estimated at 3

hours. The biopolymers accumulated during this phase are important during the famine phase, in order to maintain the microbial culture. Total sugar consumption can be related to GB accumulation, since the conversion of sugars to glucose biopolymer B is a common metabolic pathway for aerobic microbial cultures (data not shown). Comparing both polymers yields on the substrate, YHB/S (0.23 Cmmol HB/Cmmol S) was lower than the YGB/S (0.43 Cmmol GB/Cmmol S). The specific production rates of the biopolymers presented the same behaviour, being HB synthesis slower than GB (0.03 Cmmol HB/Cmmol X.h and 0.05 Cmmol GB/Cmmol X.h, respectively). In the adaptation phase of the PHA accumulating culture, it was expected that the SCOA present in the feed medium would be preferentially consumed by bacteria producing HB, which had not yet occurred at this time of operation since the largest production was of GB. Besides that, COD consumption was remarkable, accompanied by the consumption of ammonia used for cell maintenance during the cycle. Ammonia was predominantly consumed during the feast phase, at a rate of 0.009 N-mmol/C-mmol X.h. After the feast phase and consequent exhaustion of the preferred carbon sources, the cells began to consume the polymers stored previously, which justifies the decrease visible in Figure 3.14, of HB and GB. The slight consumption of external COD during this phase makes the selection process less efficient. In this cycle, the feast phase had a duration of 1/10 of the total cycle time, thus having the famine phase a duration of 21h. The F/F ratio was 0.1.

3.3.2 SBR: EVOLUTION

To compare the evolution of HB, GB and biomass yields on the substrate, all yields were calculated for the total available carbon. The analysis of several daily cycles (Figure 3.15) showed yields of biopolymer production (YHB/S and YGB/S) with satisfactory values, but with some variations throughout the incubation period, with a good adaptation of the culture towards the production of the desired polymer – PHB –.

In the initial operation phase of the reactor – day 16 to 63 – acceptable values were verified for HB and GB production yields (both higher than 0.10 Cmmol/Cmmol S) and stable maximum HB and GB levels (4-6 and 6-10 in %dw, respectively), except in 56th day where the YHB/S on substrate presented a practically null value (0.06 Cmmol/Cmmol S) with a high GB yield. These values although with low reach allowed to observe that the mixed culture specializes in the accumulation of biopolymers. During this period GB was the dominant polymer with yields between 0.15-0.30 Cmmol Glu/Cmmol S reaching a content of 10% of dw. The reason for this may be the availability of sugar in unfermented bio-oil (data not shown). At this stage, the biomass yield remained stable with high values and connected with the biomass spikes that were needed. Between days 70 and 128 there was a marked decrease in the GB content produced from 17.38 to 4.71% of dw, accompanied by the reduction in yield values. While HB content did not significantly change, maintained at approximately 6%, HB yield showed a tendency to decrease until day 128, reaching 0.05 Cmmol HB/Cmmol S. The biomass yield on substrate in that period increased due to a spike of biomass, but soon went down again to 0.15 Cmmol X/Cmmol S. After the 128th days of incubation, the production of PHB showed a marked increase in yield (0.18

Cmmol HB/Cmmol S), accompanied by an increase in the PHB content, reaching 13.93% on 224th day. The yield of GB production on substrate increased slightly but rapidly decreased to 0.001 Cmmol GB/Cmmol S, with a content of 2.43%. These results reveal that, contrary to what had happened in the initial phase of the reactor, the accumulation of HB was preferential, selecting the best PHA-accumulating microorganisms.

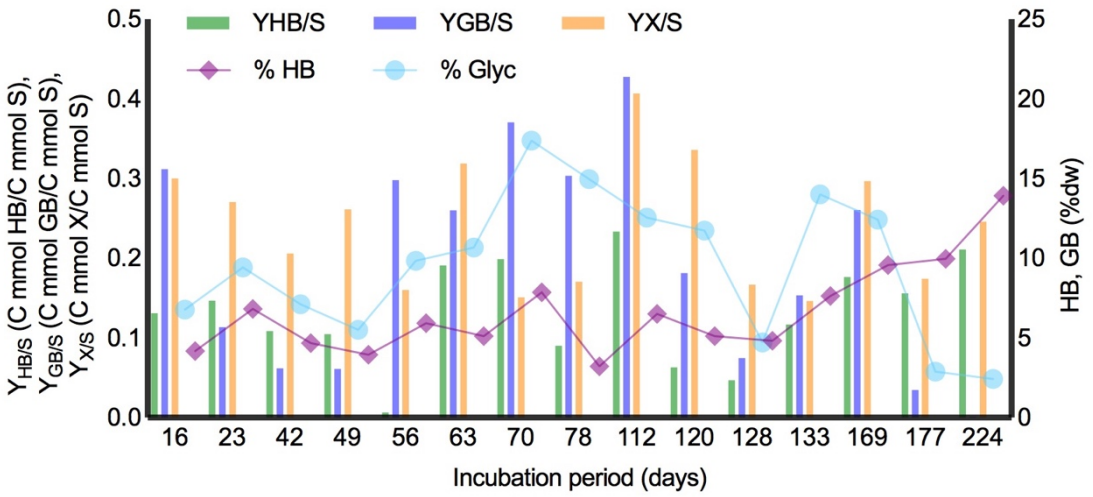

Figure 3.15: Polymer (HB and GB) yields and contents.

The results confirm that the reactor operated under optimal conditions to develop a microbial culture specialized in the production and storage of PHB with the consumption of unrefined bio-oil. Good acclimatization under these conditions shows that even the pine bio-oil can be used directly for PHA accumulation. Besides that, the acidogenic fermentation step is important for the enrichment of this substrate in SCOA since it contains a great part of organic compounds neglected by the microbial culture. The pre-treatment could also be a solution to the results of the first days of operation of the reactor in which the GB presented higher yields than HB since the contents in sugar of the fermented bio-oil are practically zero.

3.3.3 Accumulation tests

After adapting the SBR microbial culture under optimum conditions for selection of the PHA-producing organisms, several accumulation tests were performed under batch conditions in order to analyse the storage response capacity of the culture. The inoculum for the accumulation tests consisted of six SBR purges from the previous days. The three trials were performed at the 212th, 234th and 239th days of SBR operation, respectively. For all assays, the SBR was already in a stable phase where the culture showed promising results for good adaptation/selection. The process of the following tests consisted in subjecting the MMC to consecutive feeding pulses. In the first test, the pulses were only of acetic acid. Subsequently, the accumulation reactor was fed with the fermented bio-oil obtained through the acidogenic fermentation. In all assays, phosphorus was added and there was ammonia limitation.

3.3.3.1 Accumulation test with acetic acid

Both kinetic tests performed with acetic acid were carried out with three feed pulses with the same molar concentration as that used in SBR – 30 CmM –, only with the addition of phosphorus in a concentration of 0.86 mM. One of the tests is described in Figure 3.16 and the kinetic and stoichiometric parameters are shown in Table 3.3. After the first pulse, the following pulses were provided when there was an increase in DO, that variation being associated with the termination of SCOA. The increase in acetic acid, and the decrease on OUR and pH were verified after each supplied pulse.

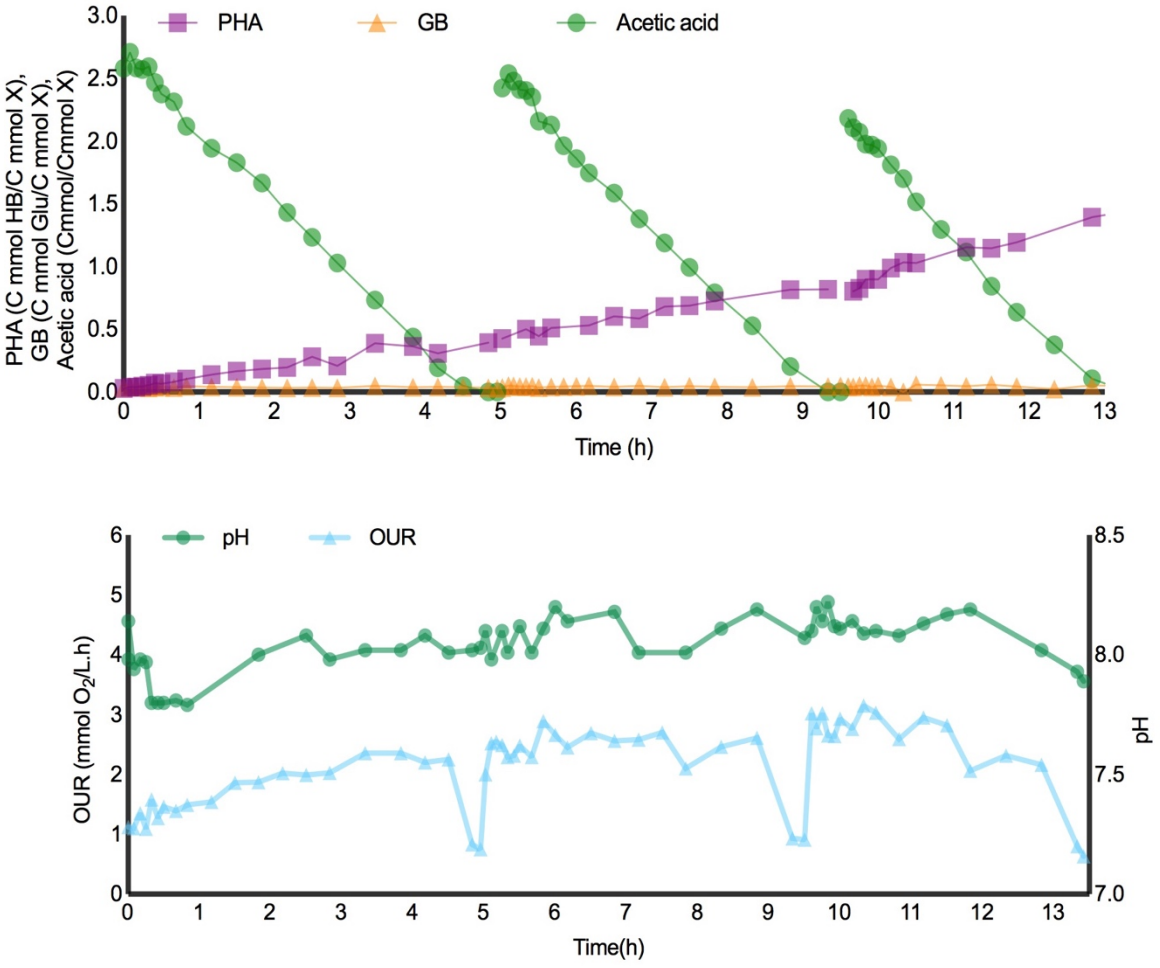

Figure 3.16: Evolution of pH, OUR, PHA, GB and acetic acid in a kinetic test. Test performed on the 212th day of operation.

Acetic acid was consumed entirety on each pulse. The time of exhaustion was identified with a rise in DO concentration and a decrease in OUR. The rapid identification of this event allowed that the accumulated PHA was not consumed for cell growth and maintenance between pulses since at these points there were no external carbon sources. Thus, continuous accumulation was achieved reaching a maximum PHA content of 46.14% of dw at the end of the

third pulse. In these tests, the GB concentration was always kept at residual values. Since the feed consisted only of acetic acid, with no presence of sugars, it was assumed that glycogen was already present in the biomass at the beginning of the accumulation reactor, having been produced in the SBR from which the sludge was collected. The reduced time between exhaustion and the new carbon pulse also made with that GB was never consumed by the culture.

The HB production rate increased over the pulses, ranging from 0.080 to 0.166 Cmmol HB/C mmol X.h, while there was no GB production during the incubation period. Substrate consumption started at 0.571 and increased slightly to 0.623 Cmmol S/Cmmol X.h. In relation to yields, YHB/S in the first two pulses was around 0.150 Cmmol HB/Cmmol S, and in the third pulse increased exponentially to approximately 0.300 Cmmol HB/Cmmol S. The YO2/S maintained at approximately 0.250 Cmmol O_2/Cmmol S, during the three pulses.

As described in Table 3.5, Johnson *et al.* (2009) used a community culture operated during years, using acetate as carbon source, reaching 89% of PHA content. While that, Moita *et al.* (2013) presented more identical results with a culture adapted with bio-oil in which the accumulation was 32.47% of PHA, qPHA around 0.071 Cmmol HA/Cmmol X.h, -qS around 0.230 Cmmol S/Cmmol X.h. The yields of YPHA/S and YO2/s were 0.400 Cmmol HA/Cmmol S and 0.200 Cmmol O_2/Cmmol S, respectively. Colombo *et al.* (2016) using acetate as carbon source got a qPHA of 0.290 Cmmol PHA/Cmmol X.h, -qS of 0.440 Cmmol S/Cmmol X.h, and YPHA/S of 0.680 Cmmol PHA/Cmmol S. In this test, the content of PHA was an intermediate value compared to the literature. The HB specific production rate was identical with Moita *et al.* (2013) in which a complex bio-oil was also used to adapt the culture, but YHB/S was lower. The difference between the VSS of each assay may lie behind this low YHB/S value since all other parameters were identical.

With these results, it can be affirmed that a promising culture was selected for PHA production. In this assay, it would still be possible to give more pulses so as to obtain a higher accumulation since the accumulated polymer concentration continued to increase linearly.

Table 3.3: Relevant kinetic and stoichiometric parameters analysis of the PHA accumulation tests.

Substrate	Pulse	$-q_s$	q_{HB}	q_{GB}	%HB (max)	%GB (max)	ΔHB	ΔGB	$Y_{HB/S1}$	$Y_{GB/S1}$	$Y_{O2/S1}$	$Y_{HB/S2}$	$Y_{GB/S2}$	$Y_{O2/S2}$
Acetic acid	1st	0.571	0.080	nd	19.09	2.40	6.06	nd	0.136	nd	0.206			
	2nd	0.588	0.096	nd	35.75	2.60	6.50	nd	0.155	nd	0.253			
	3rd	0.623	0.166	nd	46.14	2.29	10.67	nd	0.296	nd	0.257			
Fermented bio-oil I	1st	0.366	0.027	0.020	5.44	9.31	3.37	2.73	0.498	0.404	0.695	0.200	0.162	0.279
	2nd	0.532	0.035	0.011	10.66	9.62	4.66	1.19	0.396	0.146	0.786	0.259	0.096	0.514
	3rd	0.444	0.038	nd	18.48	12.75	5.72	3.49	0.509	0.311	0.579	0.282	0.172	0.320
	4th	0.327	0.022	0.004	20.59	10.44	3.77	nd	0.396	nd	0.942	0.153	nd	0.363
	5th	0.293	0.052	nd	26.57	10.31	9.37	2.23	0.481	0.279	0.999	0.246	0.142	0.604
Fermented bio-oil II	1st	0.078	0.082	nd	15.32	9.27	16.19	0.02	0.722	0.099	0.384	0.308	0.042	0.164
	2nd	0.072	0.086	nd	28.26	10.60	22.04	0.02	0.879	0.061	0.527	0.546	0.043	0.369
	3rd	0.022	0.068	nd	35.60	11.32	11.60	0.06	0.417	0.130	0.449	0.329	0.165	0.355

nd – not determined; $-q_S$ (Cmmol S/Cmmol X.h); q_{HB} (Cmmol HB/Cmmol X.h); q_{GB} (Cmmol Glu/Cmmol X.h); %HBmax and %GBmax (%g/g cell dry weight); ΔHB (Cmmol HB/L); ΔGB (Cmmol Glu/L); $Y_{HB/S}$ (Cmmol HB/Cmmol S); $Y_{GB/S}$ (Cmmol Glu/Cmmol S); $Y_{O2/S}$ (Cmmol O_2/Cmmol S); $Y_{/S1}$ (in relation of the SCOA); $Y_{/S2}$ (in relation of total COD).

3.3.3.2 Accumulation test with fermented bio-oil

During the Phase 3 of the acidogenic fermentation, fermented bio-oil was collected. Table 3.4 shows the composition of two fermented streams that were used in accumulation tests with SBR sludge. In both assays, each pulse of feed medium had an organic load of 30 CmM of SCOA.

Table 3.4: Fermented bio-oil composition.

Fermented bio-oil	Total sugar	Phenolic compounds	Total ammonia	Acetic acid	Lactic acid	Propionic acid	Formic acid
	g/L	g/L	g/L	Cmmol/L			
I	2.54	2.22	0.04	24.98	326.04	74.49	2.17
II	9.42	4.28	0.14	39.30	107.57	8.13	1.74

In the first assay the medium I was used as the carbon source. The results of this test are shown in Figure 3.17 and the kinetic and stoichiometric parameters in Table 3.3. Five pulses were administered, and the only added micronutrient was phosphorus in a concentration of 0.86 mM, with ammonia limitation. The end of each pulse was again identified by increased DO.

The acetic, propionic and formic acids were consumed totally in each pulse, whereas the lactic acid was never exhausted since the initial concentration of lactic acid was also much higher than that of the other acids. Propionic, acetic and formic acid were consumed tendentially according to their concentration. Thus, the end of each pulse was associated with an increase in DO and to the depletion of acids that were present in less concentration in the feed medium. A very low concentration of ammonia was present in the fermented medium composition. This value as soon as inside the reactor became residual and was undetectable (data not shown). The maximum content of PHA was at 13,53h with 26.57% dw, containing only HB. The profile tended to increase, but before the second and third pulses, a slight decrease occurred although lactic acid was always available. In this test, the low sugar concentration in the feed medium did not favor GB production, with only a residual value (10% dw of maximum content). There was a slight increased along the pulses, but production and yield were always lower than for HB. The qHB maintained a value close to 0.030 Cmmol HB/Cmmol X.h during the first three pulses. At the 4th pulse, there was a decrease followed by an increase up to 0.052 Cmmol HB/Cmmol X.h. The -qS increased from the first to the second pulse, reaching a maximum value of -0.532 Cmmol S/Cmmol X.h, and then decreased (-0.293 Cmmol S/Cmmol X.h) in the other pulses, possibly due to an inhibition effect of cumulative of total phenolic compounds (data not shown). The YPHA/S obtained in this accumulation test had a variable behavior, reaching maximum values at 1 and 3 pulses (0.498 and 0.509 Cmmol HB/Cmmol S (based on SCOA), respectively).

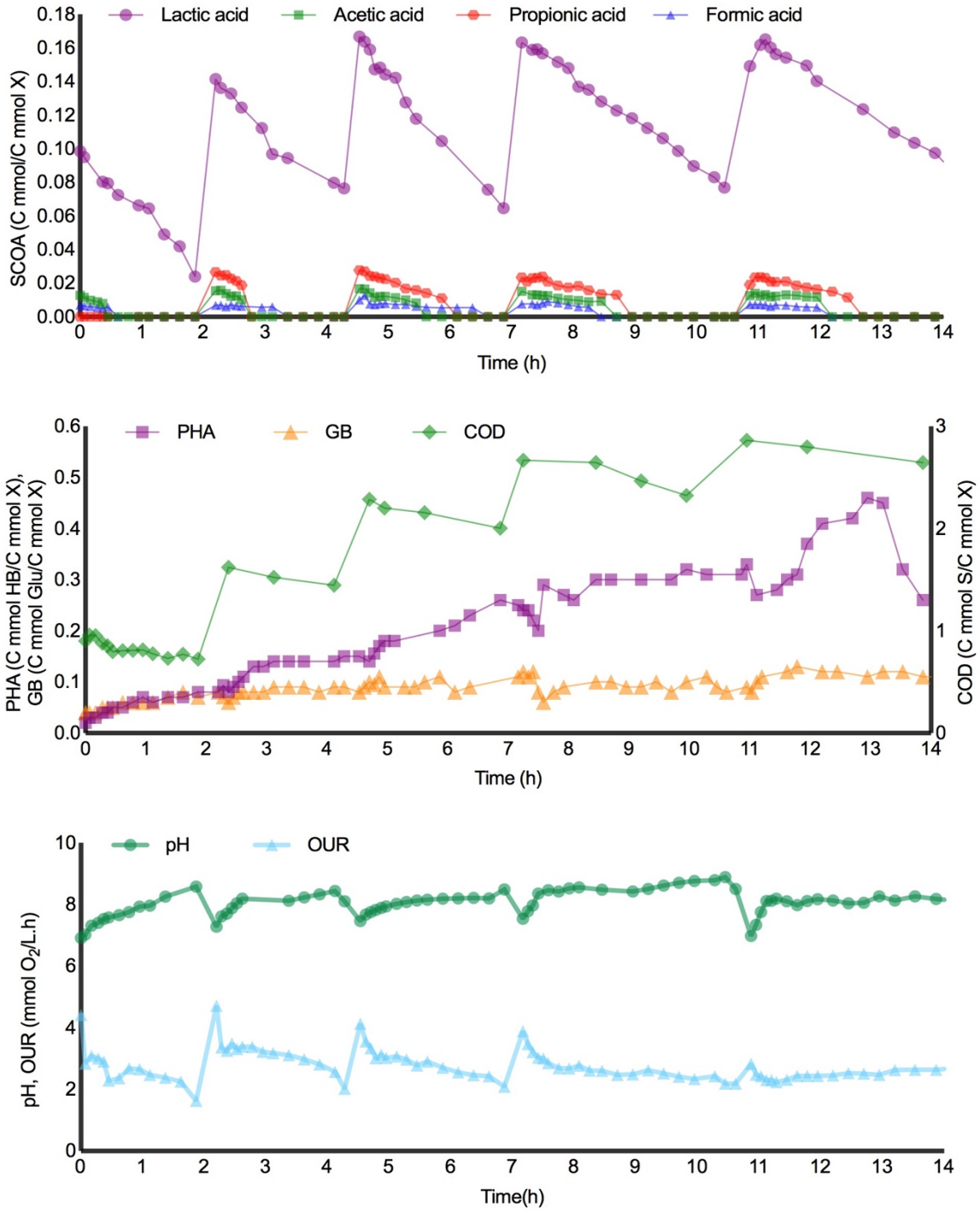

Figure 3.17: Evolution of SCOA, PHA, GB, COD, pH and OUR concentrations in kinetic test performed with médium I. Test performed on the 234th day of operation.

Based on the SCOA consumed, the yields considering only HB and O_2 were much higher than 1, which reveals the consumption of other carbon sources present in the feed. Considering, the first pulse, the SCOA COD consumed was 0.20 gCOD, whereas in relation to the total COD 0.41 gCOD was consumed. Also since respiration yields on SCOA presented high values, all yields were calculated in terms of total COD too. As expected the yields in order of total COD were lower. The YO2/S revealed a random profile, in which an initial increase was observed,

followed by a decrease up to 0.350 Cmmol O_2/Cmmol S. On the last pulse, it increased markedly to 0.600 Cmmol O_2/Cmmol S. The YHB/S kept the same profile that the one when calculating with SCOA COD, only lower.

This assay allowed the production of a homopolymer of HB, achieving a promising polymer content on biomass (26.57 %), relative to the complexity of the feed medium. However, the YHB/S obtained was much lower than those presented in the literature (Table 3.5) for substrates of identical complexity.

In the second assay with fermented bio-oil medium II was used. Figure 3.18 shows the results and Table 3.3 the kinetic and stoichiometric parameters are described. In this test, only 3 pulses were given and in each one, the phosphorus was added at 0.86 mM. Again, the increase in DO allowed to identify the end of the carbon in each pulse, indicating when it was necessary to give the following.

As in the previous test, lactic acid was not completely consumed between pulses. In this case, there was a small passage of acetic acid from the second to the third pulse. The acids with lower concentration in the feed medium – propionic and formic acids – were consumed completely. The accumulation of lactic and acetic acids may be due to the high initial concentration. Also for this feed medium originated from the pre-fermentation reactor, a small amount of ammonia was present (data not shown). The concentration of ammonia was very low, extinguishing almost immediately after the pulse be administered to the reactor, being undetectable on all pulses. Despite an acidogenic fermentation was performed to enriched the feed medium in SCOA it still contained a considerable amount of sugars, that was reflected as a slight production of GB, though unstable. As HB was the preferential polymer produced, after the three pulses it reached the maximum value of 35.60 in %dw, showing a linear increase throughout the test. Both qHB and -qS presented similar values for the first and the second pulse, decreasing in the third one, from 0.082 to 0.068 Cmmol HB/Cmmol X.h and from 0.078 to 0.022 Cmmol S/Cmmol X.h, respectively. The YHB/S had a slight increase from the first to the second pulse (0.722 to 0.879 Cmmol HB/Cmmol S), followed by a marked decrease in the third pulse (0.417 Cmmol HB/Cmmol S). This decrease is due to possible inhibition by the substrate.

Both SCOA COD and total COD were used to calculate yields since the sum of all yields was greater than 1 when using SCOA COD. The first pulse was explanatory of that, as the consumed SCOA COD was 0.73 gCOD while 1.58 gCOD were consumed in relation to the total COD. Based on total COD, the polymer and respiration yields decreased. The YO2/S had an increasing behavior along the pulses, reaching a maximum value of 0.355 Cmmol O_2/Cmmol S, while the YHB/S declined, with a maximum of 0.546 Cmmol HB/Cmmol S on the second pulse.

More promising results were obtained. Although the substrate consumption rate was lower than the previous assay, the accumulation rate improved which provided a high conversion into the desired polymer, once the culture was selected for the production of PHA. HB was the only polymer produced, at a PHB content of 35.60%. YHB/S also increased and approached more of the yields reported in other trials.

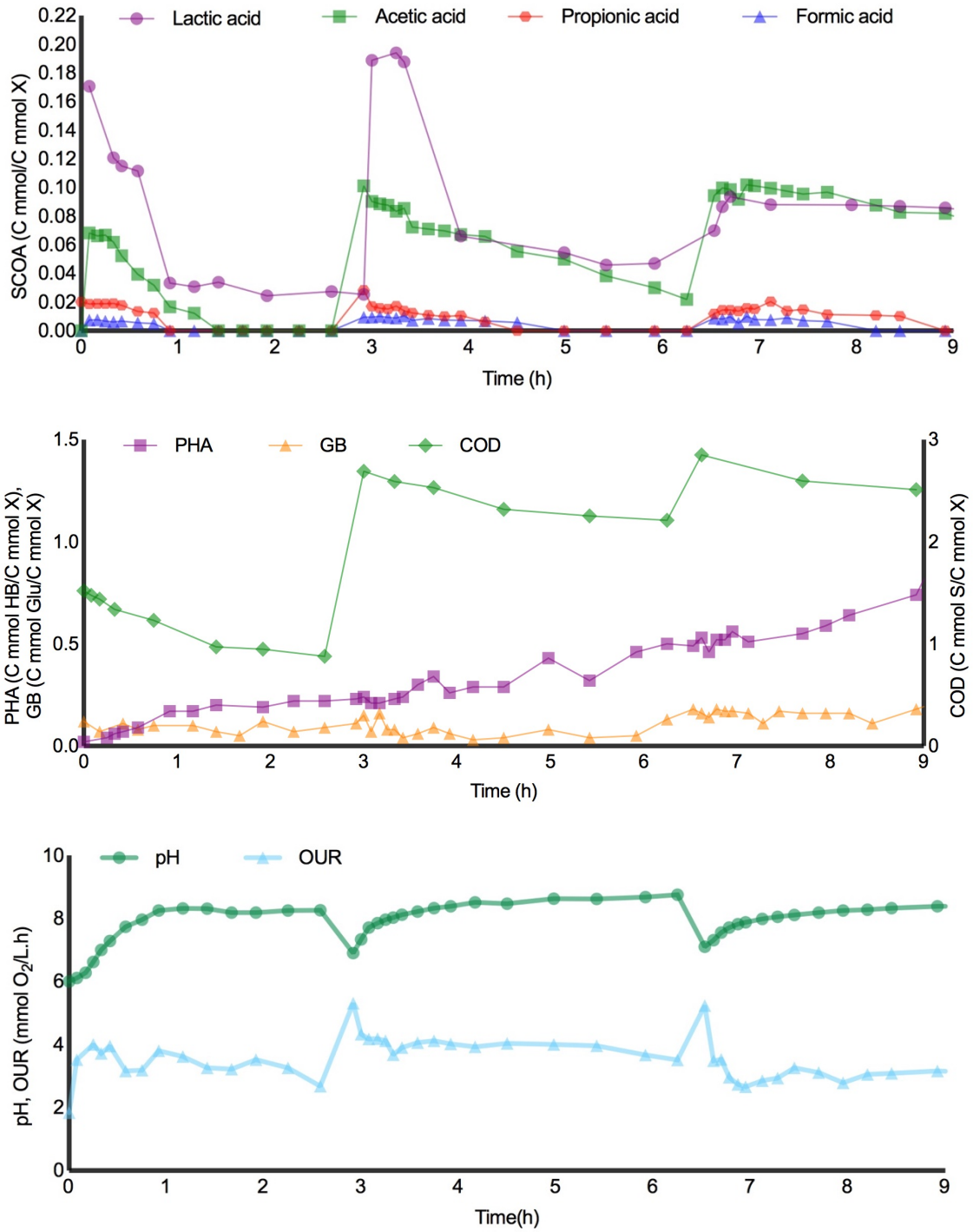

Figure 3.18: Evolution of SCOA, PHA, GB, COD, pH and OUR concentrations in kinetic test performed with médium II. Test performed on the 239th day of operation.

Comparing the two accumulation tests with the different fermented media, the observed profiles were also quite different. In the first assay, it could be verified that the culture after the five pulses administered should be able to continue the accumulation test, presenting high rates of substrate consumption. On the assay with medium II, the maximum HB content (35.60% dw) was reached, in a shorter period of time. In this last assay for the third pulse, inhibition could be

observed since most yields decreased during the last pulse. The inhibitory effect could be caused by the presence of as sugars and phenolics present in the feed medium. The amount of SCOA per biomass unit (Cmmol / Cmmol X) was much higher in the first test, which may have influenced the higher substrate consumption rate compared to the second assay. Meanwhile, the lower production of HB and lower HB production rate was verified in the first assay. The lactic acid consumption was also higher in this assay, which justifies the higher substrate consumption with the lower production of HB since there is no metabolic pathway of lactic acid consumption that leads to the production of HB.

Compared with the study of Albuquerque *et al.* (2010) that used fermented sugar cane molasses in accumulation test and reached a maximum PHA content of 74,6%, the results of these tests are not the best. Whereas comparing with the study by Moita *et al.* (2013) although production and consumption rates were significantly lower and yields were slightly lower, the maximum PHA obtained was much higher. In the assay with the medium I, the substrate consumption rate was close to the study of Colombo *et al*. (2016) with fermented cheese whey as substrate obtaining 0.300 Cmmol S/Cmmol X.h of –qS. The other parameters (qPHA and YPHB/S) were much lower (Colombo et al., 2016). The assay with the medium II obtained similar results to the Bengtsson *et al.* (2008b) study with fermented paper mill effluents (Table 3.5).

Table 3.5: Acetate and fermented streams used on PHA accumulation.

Substrate	Pulse	$-q_S$	q_{PHA}	PHA_{max}	$Y_{PHA/S}$	$Y_{O2/S}$	Reference
Acetate	1	–	1.400	89.00	0.600	–	Johnson *et al.* (2009)
Acetate	1	0.210	0.073	15.74	0.420	0.240	Moita *et al.* (2014)
	2	0.270	0.068	25.85	0.430	0.160	
	3	0.200	0.070	32.47	0.310	0.270	
Acetate	1	0.500	0.200	42.00	0.400	–	Colombo *et al.* (2016)
Fermented paper mill effluents	1	0.061	0.104	44.00	0.550	–	Bengtsson *et al.* (2008b)
Fermented molasses	1	0.540	0.430	74.60	0.660	–	Albuquerque *et al.* (2010)
Fermented bio-oil	1	0.230	0.137	10.58	0.630	0.120	Moita *et al.* (2013)
	2	0.170	0.052	16.83	0.530	0.210	
Fermented cheese	1	0.300	0.200	65.90	0.700	–	Colombo *et al.* (2016)

q_S (C-mmol S/C-mmol X h); q_{PHA} (C-mmol HA/C-mmol X h); PHA_{max} (%g/g cell dry weight); $Y_{PHA/S}$ (Cmmol HA/Cmmol S); $Y_{O2/S}$ (Cmmol/Cmmol S).

In relation to the literature, the maximum content obtained in this study was in the middle range. While that, the consumption and production rates presented promising results. The results obtained from the tests showed that the fermented stream could be promising. However, solving problems such as the passage of sugars from AF into the SBR feed medium, and the presence of total phenolic compounds in the substrate is very relevant for the improvement in PHA production.

3.4 MICROBIAL COMMUNITY CHARACTERIZATION

3.4.1 NILE BLUE STAINING

During the SBR operation time, several samples were collected for analysis with Nile Blue staining. The presence of a microbial culture of PHA accumulation can be confirmed with the use of this technique. The results are shown in Figure 3.19.

From the beginning of the SBR operation, the presence of PHA inclusion bodies was easily perceptible by the existence of bright spots on the cells stained under epifluorescence. In the several observations, it was possible to identify three types of present arrangements: dense agglomerates – A – , circular agglomerates – C – and dispersed cocci – E and G –. Figures are in order of incubation time, corresponding to day 58 (A), 108 (C, E) and 157 (G), and in all arrangements, from the start of the reactor, a good intracellular accumulation of biopolymers of different morphologies have been observed. In dense clumps, the fluorescence was clearer and the biopolymer occupied most of the cell space, whereas in the circular agglomerates there was a high cell density, making the fluorescence less clear and the biopolymer did not occupy all the cellular space. Finally, although in a smaller presence than in the other morphologies reported, in the dispersed clusters the biopolymer was also visible within the cells. The density of bright spots on stained cells increased in the samples collected over the incubation time which also confirmed the selection of the microbial culture for a PHA storage culture.

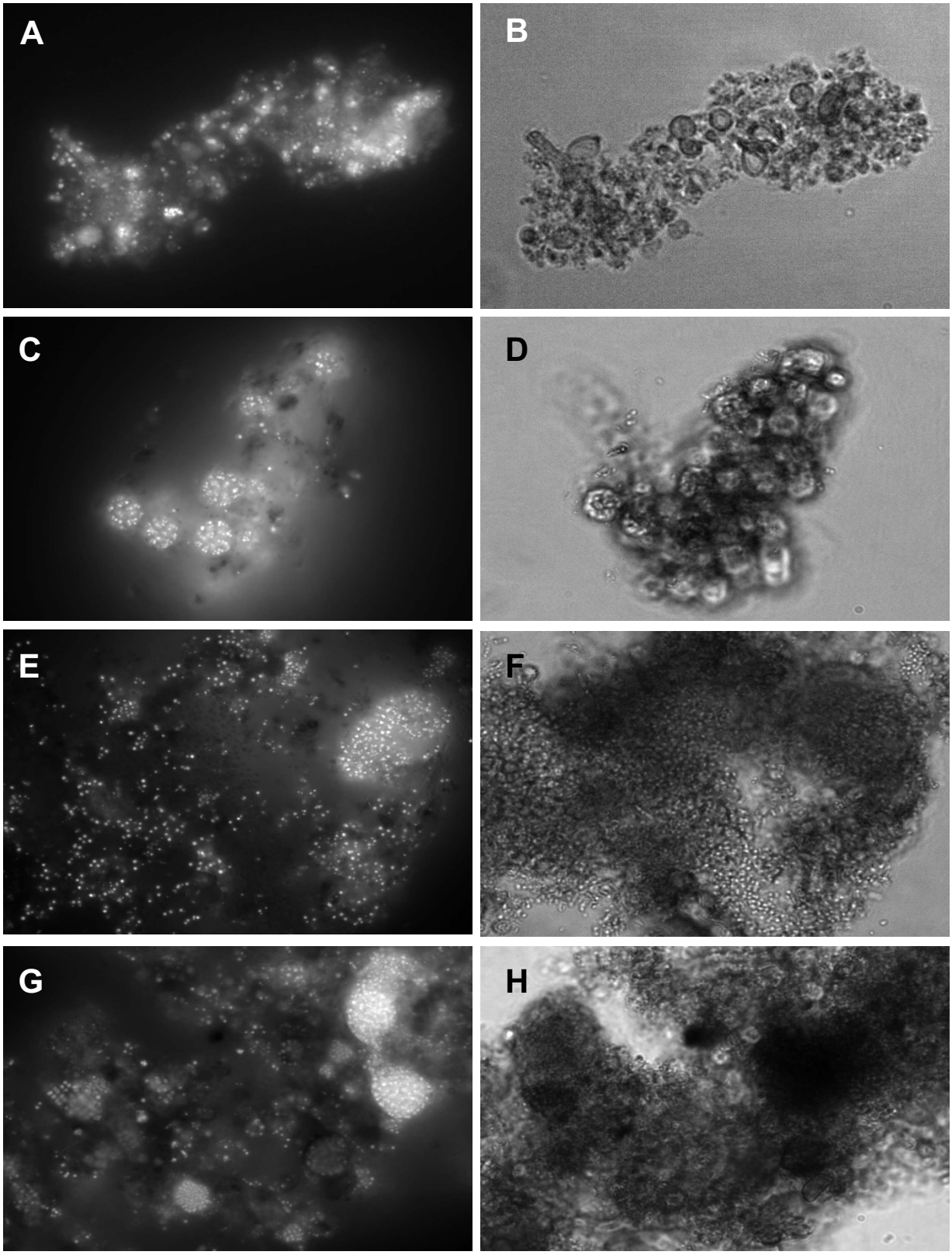

Figure 3.19: Microscopic observations (1000x) of SBR. A, C, E, G – Nile Blue staining; B, D, F, H – Phase contrast.

3.4.2 FISH

FISH was the technique used to monitor the microbial population over the SBR operation period. This analysis is essential to identify the different groups of microorganisms that are present in the population and relate them to the storage and kinetic capacities (Queirós et al., 2015). The FISH analysis was performed on samples collected from the SBR on days 23, 78 and 169 of operation.

In the first assay, it was found that there was a large cell aggregation, which may have a negative impact on the visualization of FISH hybridized cells. In order to circumvent the problem, a pre-treatment with ultrasound was performed, which reduced the sizes of the cellular aggregates of MMC. Yet during the analysis, identification of FISH for some probes was not possible due to the high auto fluorescence of the sample. This indicates that the ultrasound pre-treatment was not completely effective in disrupting the aggregates.

For the first day analysed, it was verified that the microbial community was composed of *Bacteria* since no *Archaea* was detected. For identification of the MMC, specific probes within the *Bacteria* domain were applied also taking into account bacteria previously reported as PHA accumulating organisms. In these specific probe assays the major groups in the *Bacteria* domain – Table 2.3, section 2.5.2. – that showed positive results to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria*, *Bacteroidetes*, *Sphingobacteria*, *Actinobacteria*.

Betaproteobacteria was the only group that remained during the entire time of SBR operation, which reveals that the operational conditions favoured the maintenance of this group within the MMC, a group reported to present PHA production capacity. At the beginning of the reactor, *Gammaproteobacteria* was also present. This group during the operation time was extinguished and then reappeared in the last FISH analysis. This reappearance may be related to a biomass spike that was necessary to administrate about 20 days prior to analysis. After some time of operation *Alphaproteobacteria*, *Flavobacteria*, *Bacteroidetes*, *Sphingobacteria* and *Actinobacteria* were identified in the MMC. On the 169th day of operation only *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* were present, with the *Betaproteobacteria* dominance in the bacterial community. A qualitative evolution of the different groups of the bacterial community is represented in Table 3.6.

Between days 78 and 169, despite being a large space of time, the results show that the dominant bacterial community remained stable during this period. *Betaproteobacteria* remained the dominant group and *Gammaproteobacteria* were the second most abundant group in the bacterial community during this time.

Table 3.6: Qualitative results from FISH analysis.
(–) Non-present (0%); (•) Almost non-existent (1-5%); (+) Present (5-20%); (++) Abundant (20-50%); (+++) Extremely Abundant (>50%).

Incubation time (days)	23	78	169
Alphaproteobacteria	**(–)**	**(•)**	**(–)**
Betaproteobacteria	**(+)**	**(+ +)**	**(+ + +)**
Gammaproteobacteria	**(+ +)**	**(–)**	**(+)**
Flavobacteria*, *Bacteroidetes*, *Sphingobacteria	**(–)**	**(•)**	**(–)**
Actinobacteria	**(–)**	**(•)**	**(•)**

FISH allowed to conclude that the composition of the bacterial community has undergone significant changes during the operational period, with the exchange of the dominant group. The two groups that during the operation were in majority are referred in the literature as PHA accumulators selected under aerobic dynamic feeding conditions (Lemos et al., 2008; Moita and Lemos, 2012; Queirós et al., 2014). The main morphotypes present in the different groups of the Bacteria domain identified in this analysis are shown in Figure 3.20.

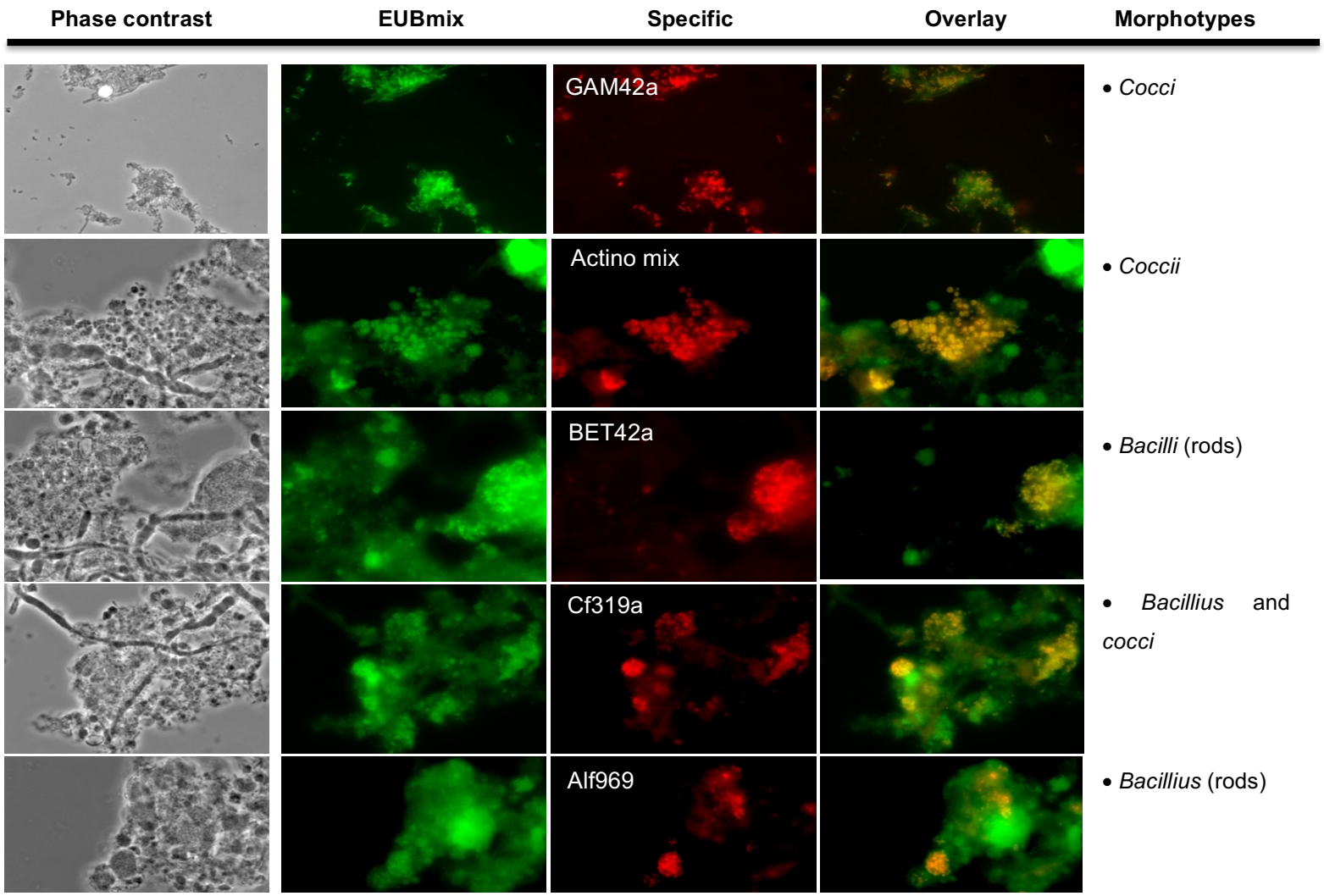

Figure 3.20: Microscopic observations (1000x) of different groups of the *Bacteria* domain. Fluorophores used: Cy3 (specific) and 6-FAM (EUBmix).

3.5 ENHANCEMENT OF BIOPROCESSING OF BIO-OIL: ADSORPTION OF MICROBIAL INHIBITORS

Koutinas *et al.* (2014) observed that microbial growth was often inhibited in the presence of various compounds, of which the total phenolic compounds are part. This represents a disadvantage for the low-value substrates, since in their composition usually, these compounds are present, which cause low yields and productivities of the products in AF. To reduce the amount of phenolic in the substrates a pre-treatment is then suggested. Nam *et al.* (2014) present the activated carbon as a possible material due to its characteristics as large surface areas, micro nature, large adsorption capacity, high purity and good accessibility.

The high concentration of total phenolic compounds in the bio-oil was a condition expected to be circumvented since these have a great negative influence when inside the reactor. Table 3.7 shows the results before and after the pre-treatment of the bio-oil with different activated carbons. The pH of 5.5 and 2.7 were chosen for the control, the first pH being used inside the reactor and the second the original pH of the bio-oil diluted in water. In relation to activated carbons, GAC is basic and CAF is neutral, and both are commercial activated carbons with physical adsorption. While RCB2 is a laboratory-produced carbon with acid character, and with the chemical adsorption.

From the outset, it was found that raising the pH provided a reduction of the total phenolic compounds. This reduction is related to the precipitate formed with increasing pH.

Table 3.7: Influence of pre-treatment with activated carbons in the removal of total phenolic compounds in bio-oil.

Activated carbon	$pH_{initial}$	pH_{end}	Phenolic compounds (g/L)	%Removal
None	5.5	5.5	6.0	
GAC		5.2	3.4	43
CAF		5.2	3.7	38
RCB_2		5.1	2.6	57
None	2.9	2.9	7.1	
GAC		2.7	4.7	33
CAF		2.8	4.6	35
RCB_2		2.6	4.7	34

In this analysis, the concentration of activated carbon was the same in all tests, varying the acidity. For pH 2.9, the variation had no impact on the removal of total phenolic compounds, around 34% of any of the assays. The %removal was higher at pH 5.5 for all types of carbons studied, with the best result when used the activated carbon with acid pH – RBC2 – reaching 57% of removal, followed by activated carbon with basic pH – GAC – with 43% and then neutral pH – CAF – with 38% removal. This fact is in accordance with the literature since previous studies affirm that there is a higher adsorption with acidic carbons (Gupta et al., 2014).

Samples at pH 5.5 revealed a better removal of the compounds than at pH 2.9, with the mean removal being 46% and 34%, respectively. The difference in the results at pH 5.5 and 2.9 was related to the oily composition of the samples at low pH, which causes a competition effect during treatment with activated carbon. The viscosity of the sample eventually deactivates the carbon by clogging the pores. While the formation of the precipitate with the pH adjustment promotes the removal of several compounds making the sample more accessible to pre-treatment.

Therefore, pre-treatment of the substrate after titration and use of an acid activated carbon is the most promising method for the removal of the total phenolic compounds in the bio-oil. In this study, the bio-oil subjected to the pre-treatment was not used in the preparation of the medium, and the should be tested for future application.

4 CONCLUSION

Agroforestry wastes are known as a cheap and abundant source, capable of replacing several substrates in biotechnological productions, which together with an AF process allows the production of value-added compounds such as SCOA. The three-step process used in this work allows the use of the produced SCOA as a precursor on the PHA production in the acummulation step by a MMC.

Regarding the AF step, several tests were performed under different conditions, in order to optimize the fermentation method for a specific substrate – bio-oil from pine –. Initially, it was verified that for the pulse-feed system the MMC needed a long period of adaptation to the bio-oil, with a low efficiency in the conversion of sugars into SCOA, reaching a maximum concentration of total SCOA of 2.3 g/L. This behaviour could be due to the high concentration of total phenolic compounds present during the pulse-feed system, which seems to inhibit the system. Subsequently, the continuous-feed system was tested. The best results achieved in the AF stage were registered during the condition initiated on the 76th day that consisted in 8 g/L.day of total sugar, C:N:P ratio of 100:3:1 and RT of 10 days. The maximum SCOA concentration was 6.3 g/L (56 lactic, 11 acetic, 17 butyric, 16 propionic, in %) which corresponded to a degree of acidification of 0.14 gCOD/gCOD. The main SCOA produced was lactic acid, and in small amounts, acetic, butyric, and propionic acid. The presence of lactic acid was associated with phases of instability and adaptation of the system. Overall, the production of SCOA through the fermentation leads to a valorisation of fermented bio-oil rich in organic acids. During the continuous operation, it was also noticed a biomass wash-out several times, which makes the addition of a decanter to the reactor in a future perspective an important step for the maintenance of the culture. In order to improve this system, an in-depth study of the bio-oil composition in terms of total sugars and total phenolic compounds would also be important to understand how and each carbon compounds of the bio-oil are used. These changes are expected to maximize SCOA production.

Simultaneously to the previous AF process mentioned, an ADF system was operated for 240 days in order to select a PHA accumulating MMC. Considering the complexity of the system there was a reasonable acclimatization time, in which after day 120, the trend of F/F ratio was below 0.2 and decreasing towards 0.1. In the initial phase of SBR, GB was the biopolymer preferably produced with a content of 10% of dw, being exceeded by the production of HB which achieved a yield of 0.18 Cmmol HB/Cmmol S, and 13.93% of dw. These results confirm that the reactor was operated under ideal conditions for the selection of a PHA accumulating culture. With the Nile Blue technique, it was possible to monitor the high abundance of PHA inclusion bodies that was growing during the operational feast period of the reactor. On the other hand, the FISH analysis showed that *Betaproteobacteria* was the most abundant phylogenetic group in the microbial culture in the pseudo-stationary phase of the SBR. These results are according to the literature that describes the *Betaproteobacteria* as PHA accumulating microorganisms. The remaining bacteria belong mainly to the groups *Gammaproteobacteria*, and *Actinobacteria* in a smaller amount.

After the stabilization period of the culture, accumulation tests were carried out under fermented bio-oil feeding, obtaining a higher polymer content (35.60%). In this context, with this work, it can be concluded that the production process of PHA from pinewood bio-oil, using a mixed culture is promising, since it presented good rates, yield, and productivity. The yields of polymer were higher in the medium II assay, reaching values of 0.546 Cmmol HB/Cmmol S, considering the total COD. The HB specific production rate also obtained the maximum values in this assay, with 0.086 Cmmol HB/Cmmol X.h. An accumulation test using acetate as a substrate was also performed. The PHA content was higher (46.15%), but the yield values were not promising.

This work revealed the high potential of the three-step process for producing PHA from MMC using residues as a substrate, which can have a large impact on reducing polymer costs. In a future perspective, all the operational parameters that influence the production of SCOA should have an in-depth study to optimize the process of valorisation of bio-oil as carbon source, where the conditions that influence the production of SCOA must be well-defined, which consequently will influence the composition of PHA and the HB:HV ratio. In the SBR, new conditions such as cycle duration, HRT and SRT should be tested, as well as the use of the fermented stream from the anaerobic reactor as feed for biomass enrichment.

In view of the composition of the medium, it will be crucial to carry out a pre-treatment to the bio-oil with activated carbon for phenolic compounds removal and potential valorisation. With this, new studies should be carried out in the future and a new characterization of the detoxified feed must be done to better know the types of sugars available.

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