Universidade de Aveiro Departamento de Química

Ano 2012/2013

Nuno Miguel Garcez Sardo

Seleção de bactérias produtoras de PHB utilizando efluentes

Selection of PHB-producing bacteria by using waste streams

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau Mestre em Biotecnologia, Ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação científica da Doutora Luísa Serafim, professora auxiliar convidada do Departamento de Química da Universidade de Aveiro e do Doutor Willy Verstraete, professor jubilado da Universidade de Gent e CEO da Avecom NV, Bélgica.

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Keywords

Polyhydroxybutyrate (PHB), activated sludge, waste streams, aerobic dynamic feeding (ADF), volatile fatty acids (VFA), 1,2-propylene carbonate, hydrogen

Abstract

Polyhydroxybutyrate is a type of biodegradable plastic, fully synthesized by bacteria, with similar properties to the ones of conventional plastics. This biopolymer can be produced by mixed cultures (activated sludge form waste water treatment plants) using the volatile fatty acids present in waste streams. Although the huge potential of this process, its application for the industrial production of PHB still lacks development.

Throughout this work, three different strategies to obtain PHB-producing bacteria by using waste streams were tested. In the first one PHB-producing bacteria were first selected by aerobic dynamic feeding conditions, while simultaneously providing hydrogen gas, followed by an accumulation stage. In the second strategy the conventional aerobic dynamic feeding conditions were imposed, followed by an accumulation stage. And a third one, where a mixed culture was straightly submitted to the accumulation stage, without previous selection. Aerobic dynamic feeding was operated in cycles og 8 hours (3 cycles per day). In the first two strategies, feast phase was intended to last 2h30 and the famine 5h30 for a feast/famine ratio of 0.45. While the accumulation stage lasted 22 hours.

High biomass concentration were achieved using strategy 1, in a stable reactor, and it was possible to accumulate PHB up to 59 % of the VSS with a PHB production yield of 0.30 g SLB/g COD fed. The second strategy resulted in less stable rectors, and a PHB content of 40 % of the VSS was achieved, but with PHB production yields as low as 0.09 g PHB/ g COD. Furthermore, it was no always possible to produce PHB as carbon source seemed to be directed to other metabolic pathways. A PHB production yields of 0.31 g PHB/g COD consumed was achieved with the third strategy, although only with a PHB content of 21 % of VSS.

The production of PHB was verified firstly by a thermogravimetric method developed at Avecom previously to this work. This method was replaced by other that comprises the extraction of PHB using 1,2 propylene carbonate as solvent. The development of this method is also addressed in this project.

Palavras-chave

Polihidroxibutirato (PHB), lamas activadas, efluentes, alimentação dinâmica aeróbia (ADF), ácidos orgânicos voláteis (AOV), carbonato de propileno, hidrogénio

Resumo

Polihidroxibutirato é um tipo de plástico biodegradável, completamente sintetizado por bactérias, com propriedades semelhantes aos plásticos convencionais. Este biopolímero pode ser produzito por culturas mistas (lamas ativadas de estações de tratamento de águas) usando os ácidos orgânicos voláteis presentes no efluente. Embora este processo apresente um enorme potencial, ainda é necessário o seu desenvolvimento para a sua aplicação na produção de PHB a escala industrial.

Durante este trabalho, foram testadas três estratégias diferentes para a seleção de bactérias produtoras de PHB forma testadas. A primeira, em que as bactérias produtoras de PHB foram primeiro selecionadas por condições de alimentação dinâmica aeróbia, com a alimentação simultânea de hidrogénio, seguida de uma fase de acumulação. Uma segunda estratégia, onde a alimentação dinâmica aeróbia foi utilizada, seguida de uma etapa de acumulação. E uma terceira, em que uma cultura mista foi imediatamente submetida a uma fase de acumulação, sem seleção prévia. Nas duas primeiras estratégias a alimentação dinâmica aeróbia consistiu em ciclos de 8 horas (3 ciclos por dia), em que se pretendeu-se que a fase de fartura durasse 2h30 A fase de fome por seu lado durou 5h30 para um rácio fome/fartura de 0.45. A fase de acumulação durou 22 horas.

Foram atingidas altas concentrações de biomassa usando a estratégia 1, num reactor estável, em que foi possível atingir um conteúdo em PHB de 59 % dos SSV, com um rendimento de produção de PHB de 0.30 g SLB/g CQO alimentado. A segunda estratégia resultou num reactor menos estável. Um conteúdo em PHB de 40 % dos SSV foi obtido, embora o rendimento de produção de PHB tenho sido só 0.09 g PHB/g CQO. Para além disso, nem sempre foi possível produzir PHB, visto que a fonte de carbono parecia ser direcionada para outras vias metabólicas. Foi atingido um rendimento de produção de PHB de 0.31 PHB/g CQO na terceira estratégia, no entanto o conteúdo em PHB foi só 21 % dos SSV.

A produção de PHB foi inicialmente verificada por um método termogavimétrico desenvolvido na Avecom previamente a este trabalho. Este método foi posteriormente substituído por outro que envolve a extração de PHB usando carbonato de propileno como solvente. O desenvolvimento deste método é abordado no presente trabalho.

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ABBREVIATIONS/ACRONYMS

ADF	Aerobic dynamic feeding
CDW	Cell dry weight
CoA	Coenzyme A
COD	Chemical oxygen demand
DO	Dissolved oxygen
HRT	Hydraulic retention time
LCL	Long chain lenght
MCL	Medium chain length
NAD^+/NADH	Nicotinamide adenine dinucleotide
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NPCB	Non-PHA cellular biomass
OLR	Organic loading rate
P3HB4HB	Poly(R)-3-hydroxybutyrate-co-4-hydroxybutyrate]
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PHBHHx	Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxy-hexanoate]
PHBV	Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate]
q_P	Specific production rate
r_P	Volumetric production rate
SBR	Sequencing batch reactor
SCL	Short Chain Length
SCP	Single Cell Protein
SLB	Stored Lipic Biomass
SLR	Sludge Loading Rate
TSS	Total suspended solids
VFA	Volatile fatty acids
VLR	Volumetric Loading Rate
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

1 Introduction

One of the major challenges that mankind faces today is to achieve harmony between the environment and its own survival and continuous well-being [1] . The current lifestyle is heavily dependent on industry and in the scientific and technological development that supports it [2].

For many years, this development relied on cheap, abundant and easy to use natural resources. In this context, it was simpler and more economical appealing to use resource and dispose them and the end of their "lifetime", rather than reuse them. As a result, a gap between industrial progress and the development of environmental technologies was opened, as the cost associated with environmental protection seemed considerably higher than its return. Therefore, resources were frequently used on the basis of a open cradle-to-grave cycle, instead of a cradle-to-cradle one. This is highly unsustainable, as it generates considerable amounts of waste and leads to the depletion of resources [2].

Nowadays, the paradigm is starting to shift [3]. New sources of raw material have proven to be expensive and difficult to find [2]. In addition, the cost associated with the proper waste management and disposal is increasing [2, 3]. These economical factors are followed up by a significant social and political pressure, as the public grows unease towards its high impact on the environment and legislation is enforced [3].

New processes and techniques need to be developed and implemented in order to comply with the social and environmental laws, while providing economical benefits [2, 3]. This led to the creation of a new area of industrial activities and to the offspring of environmental companies [4], where biotechnology comprises a significant part [3, 4].

1.1 Valorization of waste streams

Waste streams can cause a significant impact on the environment. Currently, industry is obliged by stiff regulation to properly dispose their waste streams [5]. This can be highly expensive and have a hard impact on the economics of their activities [2, 5].

Nevertheless, a lot of industries produce waste streams that have actually the potential to be used in other processes [5]. Waste gases rich in carbon dioxide, an

inorganic carbon source, and waste waters usually rich in organic carbon can be used as substrates in biotechnology processes. At present, these streams are usually treated in wastewater treatment plants (WWTPs) [3]. However, this is mostly faced as an economic burden as the treatment plants are usually designed with the only purpose of treating the stream and not for its valorization [2].

Nowadays, the production of biogas through anaerobic digestion is the most common process for the valorization of waste water. Biogas is composed by 65% of methane and so it can be effectively used as an energy source in the production of green electricity. This "green" electricity is highly funded which allow companies to reduce the cost of waste treatment or even to profit from it. However, this technology requires a significant investment to be installed in a WWTP and funds for the production of electricity from biogas are being cut down. Furthermore, anaerobic digestion does not efficiently remove nitrogen or phosphorus compounds from waste waters. This demands extra treatment steps that can be highly expensive. For these reasons, an alternative process for the valorizations of waste streams is highly desirable.

Polyhydroxyalkanoates (PHA) are a group of "bioplastics" that can be synthesized by bacteria, using waste streams as feedstock. They are fully biodegradable and exhibit some similar properties to the petroleum based ones. Their production at an industrial scale would occur under aerobic conditions, which allows microorganisms to uptake nitrogen and phosphorus with the carbon source [6]. That process could be applied in conventional waste water treatment plants. Thus, the production of these biopolymers would originate added value, while conveniently treating waste streams [7].

1.2 Aims and scopes

Although promising, the industrial production of PHA is not carried out at WWTPs nowadays. This technology still requires development while conventional waste stream treatment technologies are fully developed and successfully applied. As a result, environmental technology companies have their offer of products and services focused on this proven technology. Therefore, expertise in the use of waste streams for the production of PHA can be a differentiating factor for a environmental company to withstand among others.

This work was developed at Avecom NV (Ghent, Belgium), a SME (small and medium sized enterprise) specialized on environmental technology. Its core

activity is to develop and provide services and efficient products for industry and other fields of activity. It has expertise in the field of aquaria and aquaculture, WWTPs and digesters, and soil remediation, offering services and consultation such as feasibility studies, sludge analysis, bio-degrading, nitrification/denitrification and eco-toxicity tests.

The goal was to gain in-house know how on the production of PHA from waste streams and to develop products or services that they could joint to the their current line of offers. In this, the main objective was to develop strategies for the efficient production of PHA by mixed cultures both from gaseous and water waste streams. The development of this process had to have into account the involved economics, so it can be presented as a competitive process to anaerobic digestion.

2 Literature review

2.1 Polyhyroxyalkanoates

Polyhydroxyalkanoates (PHA) are a family of biopolyesters formed by fatty acids. Unlike polylactic acid (PLA) or poly-butylene succinate (PBS) that require chemical polymerization, PHA are the only type of bioplastics known to be completely synthesized by microorganisms [6]. Many bacteria are capable of accumulate these polymers as internal carbon and energy pools. Their synthesis is triggered by growth limited conditions, in the presence of an excess of carbon source [8].

PHA can present diverse structures and be classified into short-chain length (SCL)-PHA, medium-chain-length (MCL)-PHA and long-chain-lenght (LCL)-PHA. This classification is related to the number of carbon atoms in their building blocks. So, while SCL-PHA are composed with monomers having 3 to 5 carbon atoms and properties that are similar to polypropylene, MCL-PHA have 6 to 14 carbon atoms in their monomers and have elastic properties similar to rubber and elastomer [8]. The LCL-PHA are less studied and are uncommon to occur, being constituted by monomers with more than 14 carbon atoms [9].

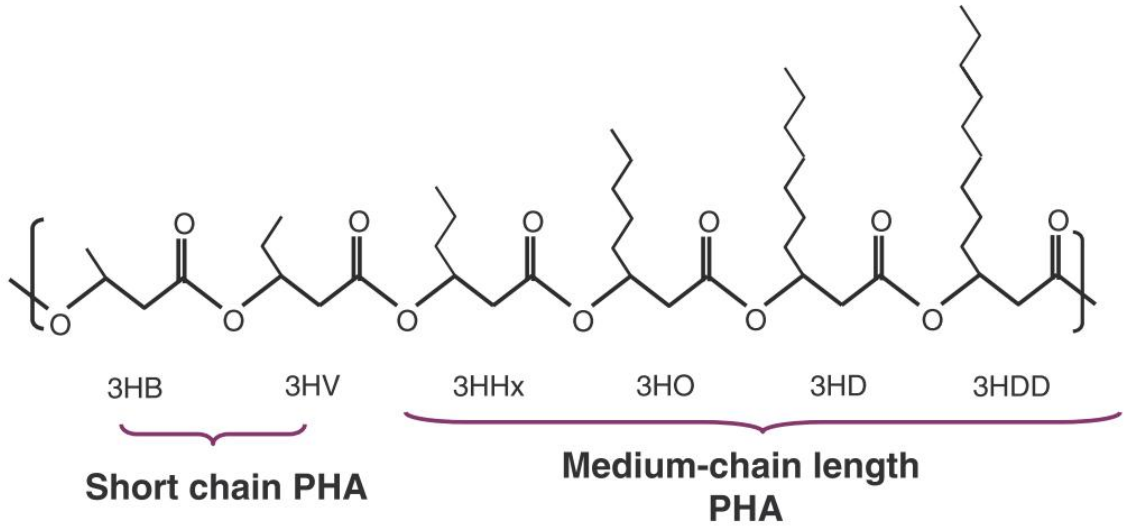

Figure 1 - Common PHA monomers. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV). Medium-chain-length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) [6].

The diverse structural varieties of PHA results in a wide range of different characteristics, namely melting temperatures (T_m) between 60 and 177 ºC, glass

transition temperatures (T_g) between -50 and 4 ºC and thermodegradation temperatures ($T_{d(5\%)}$) between 227 and 256 °C [6]. Regarding their mechanical properties, they can be either brittle or elastic, presenting a very variable Young's modulus, an elongation at break ranging from 2 to 1,000% and a tensile strength of 17 to 104 MPa. Their molecular weight can range between 10,000 and 10,000,000 [6]. Thus these polymers can be molecularly designed to exhibit different properties [6, 10].

PLA is brittle, yet its tensile strength is the highest among these bacterial plastics. Its low glass transition temperature is a handicap for the application of this bioplastic, since it changes shape at 60ºC. In comparison, a PHA with a diversified chain presents a higher glass transition temperature and may more suitable for practical application. PBS is a flexible material, with considerable strength for many applications. Its high thermal decomposition temperature (353 ºC) makes it quite thermostable. Polyethylene (PE) based on bioethanol presents the same characteristics as the petroleum-based PE and so it can be used for the same applications. Poly(trimethylene terephthalate) (PTT) and poly(p-phenylene) (PPP) are two polymers half bio-based, half petroleum-based. While PTT is an elastic, ductile material, PPP is highly brittle, hard to process polymer that exhibits electricity-conducting properties [6].

Despite these different properties, all PHA share similar characteristics. They are biocompatible and biodegradable thermoplastics, formed by chiral monomers and present functional groups [6]. Furthermore they are gas impermeable and hydrophobic, and present non-linear optical activity and piezoelectrical properties [6]. All this makes PHA suitable to be used in a wide range of applications, such as edible packaging material, coating agents, flavor delivery agents and it can be used for making bottles or other containers, cosmetics, films, adhesives, nowoven fabrics and ion-conduction materials. PHA can also be used for the production of laminates with other polymers [11].

As mentioned before, an important characteristic of PHA is their biodegradability. Microorganisms that are able to produce and store polyhydroxyalkanoates (PHA) are usually also capable of degrading and metabolize it when the carbon or energy sources are limited. The decomposition of these polymers into monomers results in (R)-3-hydroxybutyric acid in the case of poly(3-hydroxybutyrate) [12] or in a mixture of (R)-3-hydroxybutyric and (R)-3-hydroxyvalerate in the case of the degradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [13]. These monomers are water soluble and can be easily

metabolized by many bacteria to produce carbon dioxide and water under aerobic conditions [12, 13].

2.1.1 PHB

Polyhydroxybutyrate (PHB) is the most representative PHA. PHB has a very low oxygen permeability, water insolubility and optical purity. Its Young's modulus and tensile strength are similar to polypropylene, but the elongation at break is 6% while for polypropylene is 400%. It has a good UV resistance, but poor resistance to acids and bases. It is also toxicologically safe, being suitable to be used in the food and feed industry, pharmaceutical and medical applications and cosmetics [11]. However, PHB may be to brittle for many application and in terms of PHA processing, the production of copolymers appears to be of a higher commercial interest [14].

2.2 Industrial Production of Polyhydroxyalkanotes

Through the years, several companies, summarized in the Table 2, have invested in research and development (R&D) of PHA production process . It is possible to verify that a representative part of these companies already ceased their PHA related activity. Recent news revealed that ADM and Metabolix, most likely the major players in the PHA market, ended their collaboration for the production of PHA due to the lack of results and huge losses, which may represent a change of direction for Metabolix [15]. This shows that PHA are more a "potential" then a reality.

Table 1 - Worldwide PHA producing and research companies [8]

Company	Types of PHA	Production (tons/year)	Period	Applications
Chemie Linz, Austria	PHB	20 - 100	1980s	Packaging and drug delievery
ICI, UK	PHBV	300	1980-1990	Packaging
P&G, USA	Several PHA	Contract manufacteur	1980 – 2005	Packaging
BTF, Austria	PHB	20 - 100	1990s	Packaging and drug delievery
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Jiangmen Biotech Center, China	PHBHHx	Unknown	1990s	Raw materials
Monsanto, USA	PHB, PHBV	Plant PHA production	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHA	Pilot scale	1990 - 2005	Packaging and medicals
Biocycles, Brazil	PHB	100	1990 to present	Raw materials
Biomers, Germany	PHB	Unknown	1990s to present	Packaging and drug delievery

Company	Types of PHA	Production (tons/year)	Period	Applications
Jiangsu Nan Tian, China	PHB	Pilot scale	1990 to present	Raw materials
Kaneka, Japan (with P&G)	Several PHA	Unknown	1990 to present	Packaging
Zhejiang Tian An, China	PHBV	2000	1990 to present	Raw materials
Shenzen O' Bioer, China	Several PHA	Unknown	2004 to present	Unclear
Tianjin Green Bioscience (+DSM)	P3HB4HB	10000	2004 to present	Raw materials and packaging
ADM, USA (with Metabolix)	Several PHA	50000	2005 to present	Raw materials
Shandong Lukang, China	Several PHA	Pilot scale	2005 to present	Raw materials and packaging
Meredian, USA	Several PHA	10000	2007 to present	Raw materials
Bio-On, Italy	PHA (unclear)	10000	2008 to present	Raw materials
Yikeman, Shandong, China	PHA (unclear)	3000	2008 to present	Raw materials

PHBV poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate], PHB poly[(R)-3-hydroxybutyrate], PHBHHx poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxy-hexanoate], P3HB4HB poly[(R)-3-hydroxybutyrate-co-4-hydroxybutyrate]

Nowadays PHA are mainly produced as raw and packaging material. However in this market they compete directly with cheaper synthetic polymers or with well established bioplastics, like PLA. While synthetic plastics cost around 1.0 €/kg and PLA cost between 2.5 to 5 €/kg, the price of PHA ranges between 6.6 to 10.0 €/kg [16]. This price difference has been a major barrier to the widely acceptance of PHA as a viable alternative to the synthetic plastics.

To achieve the economical success of PHA one of two things must be achieved. The first one would be to find a field of application where PHA could outstand from the competition of synthetic plastics and their cost overlapped by their benefits. While the other one would be to implement new technologies of production and recover in order to decrease the cost of PHA, as their price depends on the substrate cost, process yield, and downstream process chosen [17].

2.3 Microbial culture

PHA can be produced both by pure cultures as well by open mixed cultures. Both offer some advantages and some disadvantages regarding each other. In this section, these two processes are revised using the microorganism *Cupriavidus necator* as an model organism for the production of PHA by pure culture.

2.3.1 Mixed Cultures

Mixed cultures are microbial populations that operate in open biological systems, like those used in biological wastewater treatment processes. The microbial composition of this type of cultures depends directly of the influent fed and of the operational conditions imposed on the bioreactor [7].

The capability of mixed cultures to significantly store PHA was observed in WWTPs were the activated sludge was submitted to periods of excess and lack of external carbon source. This happens in systems were selectors for bulking control are used [7], like for example in WWTPs that are operated in Sequencing Batch Reactor (SBR) system [18].

Although not all the strains present in these mixed cultures are capable of produce PHA, it is possible to enrich the cultures in PHA-storing microorganism by submitting the microbial community to that type of selective pressure [19]. This is usually carried out by submitting the sludge to dynamic conditions, such as aerobic dynamic feeding (ADF), also known as feast and famine regime, or to an anaerobic/aerobic process [20].

It was already demonstrated that mixed cultures acclimated through aerobic dynamic conditions could accumulate PHA up to 77% cell dry weight[21]. Under ADF conditions, the microbial population is submitted to alternating high and low/none carbon substrate concentrations [19], hence being known also by "feast and famine" regime. This transient access to an organic carbon substrate generates a so-called unbalanced growth. When there is abundance of an organic carbon source, the metabolism is directed for the production of PHA. On the other hand, after the exhaustion of the external substrate, the stored polymer is used as an energy and carbon source [14]. Usually this process selects for the *Thauera*, *Azoarcus*, or *Amaricoccus* species, depending of the organic acid used as carbon source. These identifications were reported for synthetic substrates [20].

The anaerobic/aerobic process is based on the capability of polyphosphate accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) to accumulate PHA under anaerobic conditions. In the presence of oxygen, these microorganisms use the stored PHA for growth, maintenance and to stock glycogen, a second storage polymer. These glycogen pools are used simultaneously with the uptake of carbon sources from the medium, during the anaerobic phase. The metabolism of these two groups of microorganism is very similar, with only some differences. PAOs accumulate polyphosphate under

aerobic/anoxic conditions, while the PHA is degraded, in order to store energy and for growth. This polyphosphate is then degraded and phosphate is released to the medium during the anaerobic phase, giving energy to the cell to accumulate PHA. Meanwhile, GAOs only use glycogen as an energy source during anaerobiosis, without the accumulation or release of phosphate [20]. The GAOs microbial community is mainly composed of *Candidatus Competibacter phosphatis*, belonging to the phylum *Gammaprotebacteria*, *Defluviicoccus vanus*-related organism cluster 1, *Defluviicoccus vanus*-related organism cluster 2 and other bacteria belonging to the *Alphaproteobacteria* [20].

However, the amount of PHA accumulated under aerobic/anaerobic process is generally less than 20%, although higher values (between 30% and 57%) were already achieved [20]. Due to this, the anaerobic dynamic feeding is a more efficient strategy for the production of PHA using mixed cultures.

The production of PHA by mixed cultures offer some benefits over their production by pure cultures. The main one is that, since the selection of microorganism occurs on the basis of their capability to store PHA, there is no need to work under sterilized conditions [14]. Furthermore, their PHB specific productivity is 10 times higher than the ones of pure cultures [7].

These types of cultures also allow to use the organic carbon from wastes and industrial influents [19]. It was already demonstrated than mixed cultures are capable of using single VFAs or simple mixtures of them as organic carbon source [22, 23] , but also of using more complex substrates such as olive oil [24, 25] and palm oil [26], paper mill effluents [27] sugarcane molasses [28] and other waste effluents such as food and fruit and municipal wastewaters [19]. However, mixed cultures are not capable of store PHA from sugar-based compounds when submitted to feast and famine conditions. Thus, these more complex substrates have to be submitted to previous fermentation process, to transform carbohydrates into volatile fatty acids (VFAs) and other carboxylic acids, in a process called acidogenesis. These organic acids can be then used by mixed cultures for the production of PHA [20]. Another problem is that, so far, it was not possible to achieve high cellular concentrations associated with high contents in PHA [20].

2.3.2 Pure cultures

Pure cultures are commonly used for the industrial production of PHA. Although sterilized conditions are required for pure cultures, they allow the use of

metabolic engineered strains in order to improve productivity, as well as the use of different substrates or to molecularly design the final product [6].

The species *Pseudomonas aeruginosa* and *Pseudomonas putida* [29], *Aeromonas hydrophila* [30], *Thermus thermophilus* [31], *Methylobacterium organophilum* [32], *Alcaligenes latus* [33], *Cupriavidus necator*, as well as several recombinant strains of *Escherichia coli* [34], among others, have already been used for the successful production of PHA.

Cupriavidus necator, also known in the past as *Hydrogenomonas eutrophus*, *Alcaligenes eutropha*, *Ralstonia eutrophus* and *Wautersia eutrophus* [35], is one extensively studied and used microorganism, due to its capability of producing PHA [36]. This Gram-negative bacteria [37] is a strictly aerobe, facultative chemoautotrophic [36, 38]. It is capable of using hydrogen as a metabolic energy source [39] and can easily grow and accumulate a large amount of PHA (up to 87 % as dry cell mass [40]) in a simple defined medium [38].

This microorganism was used for the industrial production of PHB by the Imperial Chemical Industries (United Kingdom) [38] and for the production of a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Metabolix (USA) using propionic acid and glucose as substrates.

Cupriavidus necator and other pure cultures, unlike mixed cultures, are capable of using complex substrates such as sugar molasses and other sources of carbohydrates for the production and accumulation of PHA and not only organic acids. This way, these cultures do not require the acidogenesis step. Nevertheless it is important to refer that the wild type strain of *Cupriavidus necator* (H16) is not capable of metabolizing glucose, since it does not have the enzymes responsible for glucose transport and its phosphorylation [37]. However, there are natural occurring mutants capable of metabolize it [41] using the Entner-Doudoroff pathway [42], and some strains are commercially available [43]. Notwithstanding, the glucose uptake mechanism by this organism is still not fully understood [35].

Nevertheless, the well known genome and metabolism of *Cupriavidus necator* make it an ideal microorganism for the production of specific PHA, both through natural or recombinant strains [44-46]. The genes of *Cupriavidus necator* are also commonly used to recombine in other species. In these are included bacteria such as *Escherichia coli*, *Lactococcus lacti*, yeasts like *Saccharomyces cerevisiae*, plants like the *Arabidopsis thaliana* or tobacco plants, or even in insect cell lines like *Spodoptera frugiperda* [47].

The great advantage of using pure culture, towards mixed cultures, is the possibility of easily achieve cellular concentrations higher than 80 g/L, with a PHA content that can be more than 80 % of the cell dry weight [20]. This allows a simpler and less expensive downstream process, which may cost of working under the required sterilized conditions [6].

2.3.3 PHA production metabolism

Storage of PHA occurs when the microbial cells uptake the carbon substrate at a higher rate than the one they can grow. This can be due to an external limitation, such as the lack of nitrogen, phosphorus or oxygen, or by an internal limitation regarding the levels or activity of anabolic enzymes [7].

There are some known metabolic pathways leading to the synthesis of PHA. Nevertheless, all of them have in common the production of (R)-3-hydroxyacyl-CoAs using several metabolites and precursors. PHA is then synthesized using these produced (R)-3-hydroxyacyl-CoAs [37]. The different possible metabolic pathways that lead to the synthesis of PHA can be observed in Figure 2.

Most of the PHA-producing bacteria used, like the species *Cupriavidus necator* and *Alcaligenes latus*, are capable of using the Entner-Doudoroff pathway, for the production of acetyl-Coa. This metabolic pathway is responsible for the catabolic degradation of carbohydrates, resulting in the formation of pyruvate, which can be converted into acetyl-CoA. Under conditions favorable to growth, the acetyl-CoA enters the tricarboxylic acid (TCA) cycle where it is oxidized into CO_2, in order to generate anabolic precursors, additional energy, and reducing equivalents. These reducing equivalents are consumed during oxidative phosphorilation, using oxygen as a final electron acceptor, resulting in the production of ATP. On the other hand, under growth-limiting conditions the acetyl-CoA is not oxidized to CO_2. The lack of an external nitrogen source, for example, will cease the production of proteins, causing the accumulation of NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the cell. This results in the inhibition of the TCA cycle enzymes and in the conversion of the acetyl-CoA to PHB [7].

Acetyl-CoA is converted to PHB in a sequence of reactions catalyzed by enzymes. First, two units of acetyl-CoA are condensed by the β-ketothiolase into acetoacetyl-CoA, which is then reduced by the acetoacetyl-CoA reductase to (R)-3-

hydroxybutyryl-CoA, that is incorporated into the PHB chain as 3-hydroxybutyrate by PHA synthase [42]. There are many variants of PHA synthases and they are classified depending on the substrate specificities [7]. For example, *Cupriavidus necator* wild type possesses a class I PHA synthase that accepts short chain-hydroxyacyl-CoAs for polymerization [8]. Nevertheless, since the enzymes involved are stereospecific, all microbially synthesizes hydroxylacyl monomers are in the (R) configuration [7].

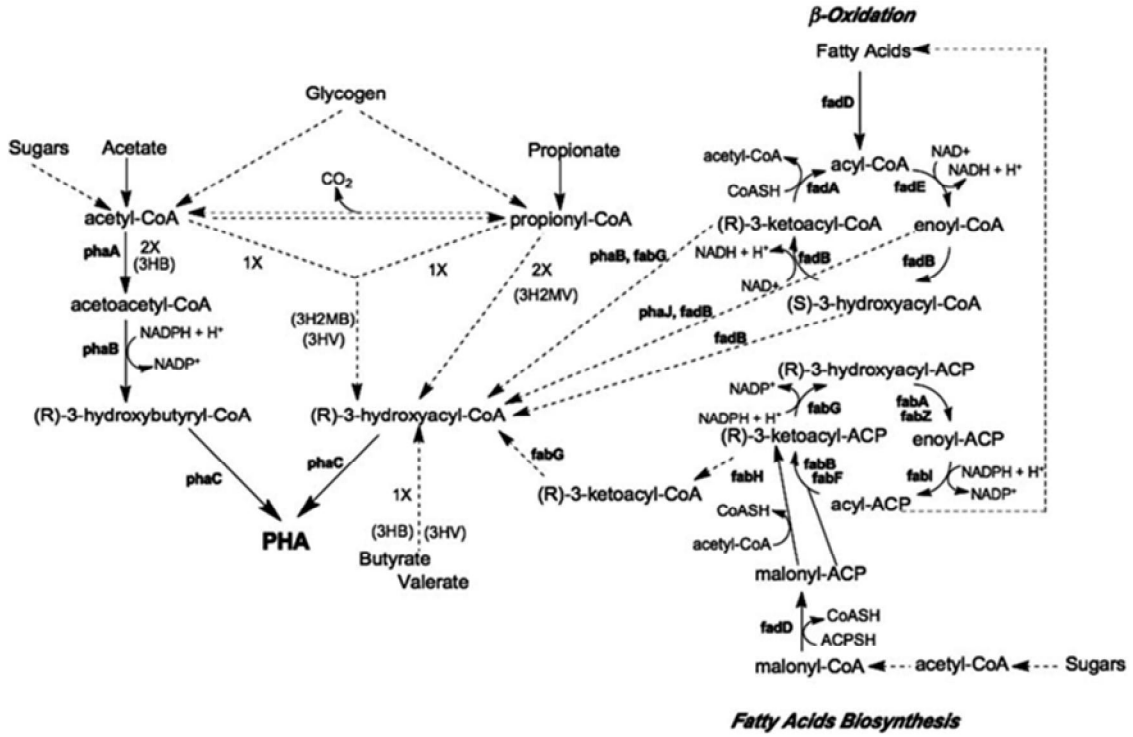

Figure 2 - Methabolic pathways for the synthesis of polyhydroxyalkanoates [20].

Short-chain organic acids, also known as volatile fatty acids (VFAs), can be used for the production of PHA too, as they can be activated to their corresponding acyl-CoA molecule. It is assumed that the metabolic pathways leading to the synthesis of PHA in mixed microbial cultures must be similar to those described for pure cultures using the same substrates [48, 49]. For example, when acetate is used as a carbon source it is converted in to acety-CoA and can be used both for growth or PHB production as described above. Propionate is transformed into propionyl-CoA that can be consumed as such to form a hydroxyacyl unit for the synthesis of PHA or decarboxylated first to acetyl-CoA. A unit of proionyl-CoAs can be combined with another one to form 3-hydroxy-2-methylvalerate (3H2MV) or, instead, with one of acetyl-CoA to form either 3-

hydroxyvalerate (HV) or 3-hydroxy-2-methylbutyrate (3H2MB). Butyrate and valerate can be converted directly to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, thus they do not require a condensation step like it is the case of acetate and propionate, as this will form form 3-hydroxybutyrate (HB) and HV, respectively. The monomers HB, HV, 3H2MV and 3H2MB are the most frequent PHA monomers synthesized by pure cultures [7].

HB, HV and medium- and long- chain lenght fatty acids can be broken down to smaller ones by the β-oxidation pathway to form PHA monomers. Other metabolic pathways, like the novo fatty acid synthesis also can produce intermediates for PHA production and alkanoates, resulting from the oxidtion of the corresponding alkanes, can be also converted to their corresponding acyl-CoA [7].

2.3.3.1 Use of molecular hydrogen in the metabolism

Molecular hydrogen (H_2) can be either oxidized or produced biologically, in a reaction catalyzed by hydrogenases. The most common hydrogenases are the [NiFe] and the [FeFe] enzymes, which are both capable of catalyze the reversible oxidation of molecular hydrogen ($H_2 \leftrightarrow 2H^+ + 2e^-$) [50]. However, while the [NiFe] hydrogenases are O_2-tolerant, the [FeFe] hydrogenases are permanently inactivated by molecular oxygen, as it reacts irreversible with their active site, destroying it [50].

The [NiFe] hydrogenases are the ones present in *Cupriavidus necator* [51], due to is strictly aerobic nature. They present a high affinity to H_2, thus allowing this microrganism oxidize it even when it is present only in trace amounts [52]. The strain *Cupriavidus necator* H16 exhibits a soluble a hydrogen:NAD^+ oxido-reductase [53], which can use H_2 to regenerate NAD^+ to NADH, and a membrane-bound hydrogenase linked to the respiratory chain [53] that oxides NADH back to NAD^+ [54]. This reaction originates potential energy in the form of a pH gradient and electrochemical potential across the membrane, which is used by the membrane enzyme ATP synthase to regenerate ADP to ATP [54]. Thus the oxidation of H_2 is coupled to the reduction of O_2 [52], by the oxidative phosphorylation pathway [54], and is used as a metabolic energy source for this bacterium [52]. This allows *Cupriavidus necator* to grow autotrophically, on minimal salts medium, using carbon dioxide as a carbon source [53].

The autotrophic production of PHA by Cupriavidus necator has already been achieved, with a yield of 5.6 g biomass/ mol H2, a maximum cellular concentration of ≈ 20 g/L and a PHA content of ≈ 85 % [55]. However, no studies have been found on the impact of molecular hydrogen on the cellular growth and PHA production by *Cupriavidus necator* in heterotrophic conditions. The energy source may enhance growth and PHA production, especially in low energy substrates such as acetate [38]. On the other hand, high intracellular concentrations of NADH may completely divert the carbon flux from the citric acid cycle to the PHA synthesis pathway. This may happen since the citrate synthase, the first enzyme from the citric acid cycle, is highly inhibited by NADH, not being capable of accept acetycl-CoA for the cycle [56]. However, the extra NADH may also be used in the synthesis of proteins which could promote growth [57]. It is not clear if Cupriavidus necator is capable of use NADH to reduce $NADP^+$ to NADPH, in a reaction catalyzed by transhydrogenase enzymes. This molecule has a less pronounced inhibitory effect on the citrate synthase, while still promotes the production of PHA [56].

2.4 Industrial waste streams as carbon substrates

The production of PHA usually occurs under aerobic conditions, which results in high losses of the carbon substrate as CO_2 due to cellular respiration. As a result, less than 50% of the carbon source is directed to biomass growth of PHA production. This causes the production costs to be highly determined by the cost of raw materials, which can represent up to 50% of the entire production costs. The use of purified or synthetic substrates significantly increases the production price [37]. Then finding suitable low-cost substrates is a priority for the economically viable production of PHA. The utilization of waste materials for the production of biopolymers is a cost-efficient strategy that could also help to solve the disposable problems of several fields of industrial activity [6, 37].

In Europe, the dairy industry provides huge amounts of surplus whey that can be used as a source of lactose for the production of poly(lactic acid), PHA and bioethanol. Glycerol, a side-product of the biodiesel production, can be used as a substrate for the production of PHA and lactic acid. Meat and bone meal from the slaughtering and rendering industries as well as several grass and silage residues

showed excellent results for the cultivation of microbes capable of producing PHA [6].

In other areas of the world, molasses from the sugar industry, starch, waste lipid and alcohols such as methanol and especially lingo-cellulosic feedstocks may be available in quantities suitable to be used in industrial processes [6]. For example, in Malaysia, palm oil is a potential feedstock for PHA production, since it is produced in very large scales there. The yield from vegetable oils is at least two times the one from sugars [9].

Although all these prospects regarding cheap inexpensive carbon sources, their use usually result in lower growth rates due to the inefficient use of certain nutrients [37].

2.4.1 Volatile Fatty Acids

Volatile fatty acids are the only organic carbon source that can be directly used both by mixed and pure cultures for the production of PHA. As already seen, they are a central part in the PHA synthesis pathway and by feeding of different organic acids to these cultures may result in PHA with different properties.

The complex biomass in wastewater can be pre-fermented in order to convert sugars into volatile fatty acids (VFAs), in an anaerobic process called acidogenesis [14, 37]. This way is possible to form organic acids such as acetic, propionic, butyric and valeric acids, with a high yield [14].

It is important to know how to control the final product of this pre-fermentation, since the VFAs are directly converted by microorganism into their respective acyl-CoA, which are then used in the synthesis of the PHA chain [19]. So, the composition of the used organic carbon source fed stream determines the final PHA structure [14].

However, at high concentrations, these acids can be inhibitory or toxic [37, 58]. This toxicity is attributed to their undissociated lipophilic molecules, which can easily cross the cell membranes, dissociate and acidify the cytoplasm. As a result, the gradient of protons across the membrane is disrupted and the energy production and transport systems associated with this gradient are no longer maintained [37, 58]. In response to the accumulation of fatty acids, free energy is released via ATPase and protons are expelled out of cells in order to maintain the proton gradient. This means, that lower growth rate, acid utilization rate and yield of PHA are usually achieved [11, 37, 58].

The yield and rates are strongly influenced by the pH of the mixed liquor, since at low pHs the short chain fatty acids are predominantly in the undissociated form [37]. It is the pKa value of the organic acid and not its size that influences its inhibitory effect [37, 58]. Since the values for these VFAs are similar (lactic acid pKa = 3.86, acetic acid pKa = 4.76, butyric acid pKa = 4.83 and propionic acid pKa = 4.87), so is the inhibitory effect of all of them at a pH value below 5.0 [37].

At pH level close to the optimal pH for *Cupriavidus necator* (pH 7.0) and for mixed cultures, these organic acids are mainly in the dissociated form. Yet they are not transported so easily across the cell membrane [11, 38], the latter can cause an undesirable increase in osmotic pressure, due to the accumulation of anions [11, 58]. On the other hand, at low initial VFA levels, substrate limitation can occur. The concentration of short chain fatty acids and pH has to be thus carefully regulated, in order for the undissociated fatty acids to enter the cytoplasm, being activated and metabolized into CO_2, cell biomass and/or PHB [37]. For example, it was shown that propionic acid is used more efficiently by *Cupriavidus necator* at a pH of 7.5 [59] and that 5 g.L-1 produce less PHA than 2-3 .L-1 of PHA at pH 7.5 [60].

Lactate is the organic acid preferred acid by *Cupriavidus necator* for growth, followed by acetate, succinate and propionate. Butyrate is only consumed during the growth phase if other organic acids are not present [37], but it is rapidly consumed during stationary phase or in the absence of growth by inhibition [37, 61].

Metabolic simulations showed that acetate is an inefficient source for the production of energy. The ATP required for the uptake of acetate is almost as much as the one synthesized from its metabolism, being the maintenance ATP low for growth. Because of this, growth in acetate is slower than with butyrate or lactate [38]. The utilization of propionate and butyrate are energetically favored, but additional ATP is needed to transport this acid. Lactate is the preferred, since less energy is needed to transport it to the central metabolic pathways [37, 38].

Table 2 - Organic carbon flow and metabolic regulation in Cupriavidus necator grown on various substrates [34]

	Acetate	**Butyrate**	**Lactate**
C in CO2/C in substrate	0.38	0.34	0.56
C in biomass/C in substrate	0.15	0.04	0.07
C in PHB/C in substrate	0.47	0.62	0.37
Mol ATP/mol substrate	0.95	5.97	5.75

The maximum PHB yields achieved with these simulations were of 0.33, 0.33 and 0.67 mol/mol substrate for acetate, lactate and butyrate, respectively [38].

On the other hand, the highest PHA production yields are achieved with butyric acid. Butyrate is a more energetically efficient carbon source, for PHA production that the other organic acids.

2.5 Reactor Operation

Reactor operation conditions for pure cultures are very different from those of mixed microbial cultures. While pure controls require the use of sterile conditions, which greatly increases the production cost, the reactor operation is relatively simple and easier to optimize. On the other hand, mixed microbial cultures do not require sterile conditions, but they require the imposition of selective pressure in order to select for PHA-producing bacteria. Common reactor operation parameters are described below for each case, with emphasis on the microbial mixed cultures.

2.5.1 Pure cultures

The production of PHA by *Cupriavidus necator* mainly occurs during stationary phase and so, their production is usually carried out in two steps. First the cells are fed with enough carbon and nitrogen in order to maximize growth, till a high cellular concentration is achieved [35, 37]. After this, a key nutrient, such as nitrogen, magnesium, molecular oxygen or phosphorus, is limited to trigger PHA synthesis and inhibit growth. This is usually carried out with the suppression of nitrogen [57].

The energy formation and anabolic reactions within the cell are promoted when the Carbon/Nitrogen (C/N) ratio is less than 20, while a C/N ratio between 20 and 200 inhibits cellular growth, but increases the yield and production rate of PHB [62]. This was due to the suppression of the β-oxidative and poly(hydroxyalkanoic) pathways, caused by the lack of nitrogen source [62].

It was also verified that a residual level of phosphorus (0.35 $g.L^{-1}$) and magnesium (10 $mg.L^{-1}$) may enhance PHA production [37]. Since PHB is composed only of carbon, hydrogen and oxygen, the feed of carbon source is of primal significance [38]. The complete exhaustion of phosphorus and magnesium may also

have a negative impact in the polymer molecular weight and increase the polydispersity index and should be maintained at residual levels to enhance PHB production [63].

2.5.2 Mixed Cultures

Studies on production of PHA by mixed microbial cultures are carried out in two separated reactors. The first one, where the microbial population is selected and the second, where PHB is accumulated. This is needed, since anaerobic dynamic feeding conditions are necessary for the selection of PHA-producing bacteria. As a result, the loading rates are limited and are not high enough to achieve a satisfactory PHB content in the growth reactor [7].

The performance of the PHA accumulation stage is highly influenced by the effectiveness of these selective pressure conditions and so the optimization of the selection reactor is of highly importance. Therefore, these conditions must result in the enrichment of the microbial population in bacteria capable of a high and stable PHA storage capacity, as microorganism presenting low storage capacities would have a negative impact on the production and downstream of PHB. As a result, this two stages are usually carried in different reactors, so different optimal conditions can be applied in each one [7].

An ideal selection reactor would allow high organic loading rates and short retention time. Hence, a high growth capacity associated with a high capability to store PHA is desirable. This would allow the operation of the accumulation reactor with higher cellular concentration, so higher volumetric production rates were achievable [7].

The selection reactor is usually operated as a SBR Since they operate per cycles comprising distinct periods. Usually, the cycle starts with the feed of the reactor, followed by the reaction phase that includes both feast and famine, settling phase and discharge of the supernatant. This allows the imposition of a wide range of parameters [64]. Several factor that regulate the use of the carbon source during feast phase either for growth or for the storage of PHA have been studied under different conditions, namely solids retention time (SRT), hydraulic retention time (HRT), pH, temperature, cycle length, feast to famine length ratio (F/F ratio), volumetric loading rate (VLR), influent carbon source and nutrients concentration and the associated limitation/inhibition kinetics [7].

It was demonstrated that for SRTs higher than 2 days, the yield of PHB production under excess nutrients was constant and independent form the specific growth rate. However, at a SRT lower than 2 days, this yield significantly decreases with a decreasing SRT [48].

Table 3 - Usual operational parameters applied on the growh reactor [14]

Operating parameter	*Range*
Related to reactor operating parameters	
Sludge retention time	1–20 day
Hydraulic retention time	1–3 day
Length of SBR cycle	2–12 h
pH	7–9.5
T	20–30 °C
Operation mode	SBR or two continuous reactors sequentially disposed
Related to feedstock	
Organic loading rate	1.8–31.25 g-COD l^{-1} day^{-1}
Substrate concentration	0.9–31.25 g-COD l^{-1}
C/N ratio	9–120 g-C g N^{-1}
Resultant from both feeding and reactor parameters	
F/F ratio	0.1–1.15

The F/F ratio is one of the main parameters regulating if the carbon source is used for growth or for PHA storage. At high PHA storage capacity was observed for low F/F ratios (up to 0.26), while high F/F ratios (higher than 0.90) promoted a growth response in a culture fed with synthetic organic acids and a SRT of 1 day. A similar observation was made in a culture using fermented molasses as feedstock and a SRT of 10 days [7]. This is possible to verify in Figure 3.

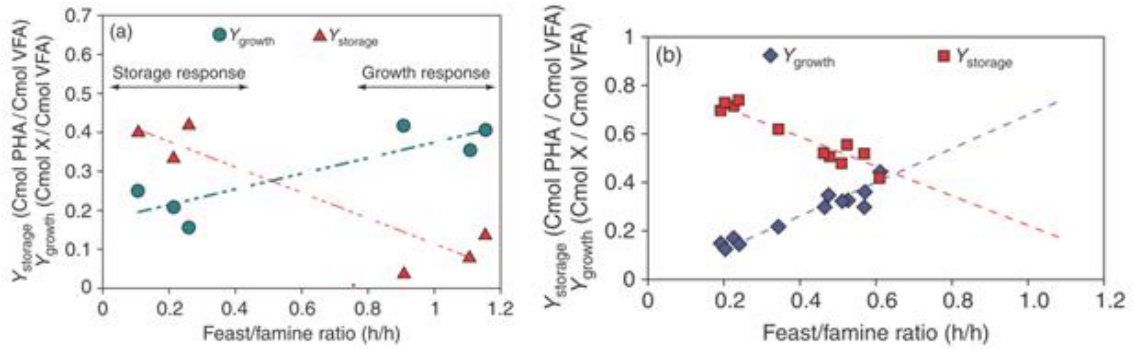

Figure 3 - PHA and active biomass yields on substrate, regarding the feast/famine ratio. SBR operated at a SRT of 1 day (a); and at a SRT of 10 days (b) [7].

The reason behind this growth/PHA storage regulation is related with the decrease of the amount of intracellular components required for cell growth, such as RNA and enzymes, during famine phase. Therefore, when the carbon source is

available again cellular growth is internally limited, while the accumulation of PHA is energetically less demanding. This way, the cell can rapidly consume the external carbon source and store it, while the growth metabolism is not reactivated [7]. As a result, after long periods of starvation around 70% (Cmol/Cmol) of the carbon source can be used towards storage, rather than growth and maintenance [48, 49].

The manipulation of the influent substrate concentration and/or the loading rates can be used not only to regulate the F/F ratio, but also to control the substrate uptake rate. This should increase with increasing loading rates, till a maximum value, after which substrate inhibition is verified. Since PHA-producing microorganism use the substrate uptake rate as a competitive advantage towards others, so it does not limit the uptake rate. As a result, if the substrate concentration is kept above a kinetically limiting value and below the substrate inhibition point, this may result in an increased selection efficiency [7].

The selection of cultures with a high capacity to store PHA was already achieved both under nutrient excess and nutrient limiting conditions, as PHA storage occurs due to an internal limitation rather than to an nutrient limitation. Nevertheless, the amount of nutrients available can be used to control the amount of carbon source used for growth, not only during feast, but also during famine. Under excess of nutrients, microorganisms can use the polymer stored during feast in order to grow during famine, giving them an extra competitive advantage as a stable growth rate is possible to maintain. Furthermore, cultures adapted in the presence of nutrients excess are more insensible to them in the accumulation reactor, which may be fundamental if complex feedstocks are used [26].

2.5.2.1 Oxygen

A determinant factor in the achievement of high cell densities and growth rates are the aeration rate and gas stream composition [65]. It seems to be important to maintain oxygen saturation in the mixed liquor of at least 2.0 mg/L. However, during the exponential Phase, the oxygen requirements can be higher than this and the use pure oxygen could be required. This is an expensive process at large scale, that should be compensated with a high volumetric productivity [65].

The dissolved oxygen (DO) is a good way to easily identify the beginning/end of the feast and famine phases. The beginning of the feast phase is associated with a decrease in the dissolved oxygen concentration. This happens

right after the substrate addition, being and indicator that the microbial population is metabolizing the substrate. The concentration of dissolved oxygen remains more or less the same during the feast period, increasing again in the end of it/beginning of famine [14].

2.6 Harvesting and PHA purification

PHA are accumulated in the bacterial cell cytoplasm in the form of granules. This granules have an average size of 0.2 – 0.5 μm. This makes the recovery of PHA a challenging process, with the harvesting of cells and PHB extraction significantly affect the price of the final product [9], which can represent more than 26% [58].

A diagram of the whole PHA production process is possible to observe in Figure 4. First, the cells should be harvested. Usually this is carried out by biomass precipitation, that can promoted by the of 1% Na_2HPO_4, 1% CaCl, and 100 ppm of polyacrylamide to the medium [9]. After that biomass is pressed filter in order to remove water and the cakes are then freeze-dried and ground into powder [6, 9].

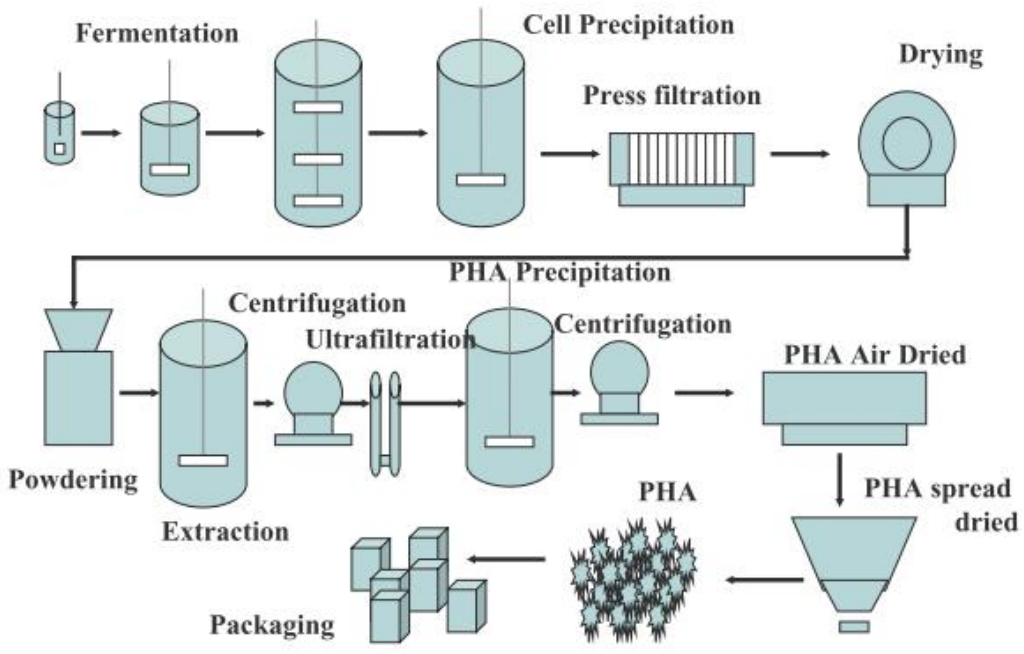

Figure 4 - General PHA production and extraction process [6]

To extract PHA is then necessary to rupture the bacterial cell in order to release the granules. There are several ways to rupture the cells and afterwards

recover the PHA. To disrupt cells, several digestion methods can be applied, as well as mechanical disruption. The recovery of PHA can also be carried out using solvent extraction or two-phase systems [9].

The required PHA purity is determined by the final application it is intended to be used. For example for mulching film or garbage bags, a lower degree of purity may be used. On the other hand, if the PHA is produced to be used in medical applications, that the final product has to be free of any contaminating chemicals, solvents or endotoxins [9].

Regardless the final application of PHA, it must be assured that a sufficiently high molecular weight, so it can be further processed. This may be problematic since, to achieve high purities of the polymer, aggressive extraction methods are usually required, which cause polymer degradation. Thus a compromise between purity, efficiency of recover and the molecular weight of the polymer must be achieved. Furthermore this has to be done in a environmental friendly way, with an associated economical feasibility [9]. Some proposed methods are reviewed in here.

2.6.1 Digestion methods

Digestion methods involve the solubilization of the non-PHA cellular mass. This digestion can be classified into either enzymatic or chemical [6].

Various types of enzymes can be used for the enzymatic digestion of the PHA-producing biomass. The enzymes used can be proteases such as trypsin, chymotrypsin, papain and bromelain, but also β-glycosidases like cellulase and lysozyme [66]. These enzymes are capable of cleaving linkages in the bacteria cell wall [67], which results in the lyses of the cell [68]. Since enzymes are very specific with respect to the reactions that catalyze and since they can operate in mild conditions, it is possible to recover PHA with good quality and without molecular weight degradation.

Chemical digestion is usually carried out using sodium hypochlorite or surfactants. Sodium hypochlorite is a strong, non-selective oxidizer that can be used to disrupt the cells [69]. Among the surfactants, SDS presents a good performance in destabilizing the cell wall and membranes, but other can be used [9]. The problem with this method is while the digestion by hypochlorite can cause severe degradation of the molecular weight of the PHA chains (up to 50%), the

surfactants alone have lower recovery efficiencies [70] and lower degrees of purity are achieved [71].

Nevertheless most of the studies have been directed to the use of chemicals. This happens because of the easy access to various suitable chemicals and to the high cost of enzymes and to the complexity of their recovery from the medium [9].

2.6.2 Mechanical disruption

The mechanical disruption of cell does not involve the use of chemicals, so it minimizes the contamination to the product as well as the environmental pollution. The two main methods used, for large scale cellular disruption in pharmaceutical and biotechnology industries are the bead-milling and the high pressure homogenization [72].

Bead mills cause cellular disruption due to the shearing action and energy transfer from beads to cells in the contact zones. This method is ideal for the extraction of PHA, since it does not require much power supply, is not susceptible to blockages and does not degrade the PHA granules [72]. However, it requires an extensive optimization in order to establish an efficient disruption system [9].

In the high pressures homogenization, cells are obliged to pass through a restricted orifice discharged valve under high pressure. Some possible drawbacks of these method are the possible micronization of PHA, a process that reduces the PHA particles to a micrometric or nanometric scale, and the formation of fine cellular debris that can interfere with the further downstream process [73].

Nevertheless, both technologies have already been successfully tested for the recovery of PHA from biomass [72]. Generally the drawbacks of these methods are the high capital investment required, the long processing time and the difficulty to scale them up [9].

2.6.3 Solvent extraction

Solvent extraction is the most extensively adopted method to recover PHA, especially at laboratory scale, due to its simplicity and rapidness. The solvents used are commonly chlorinated hydrocarbons such as chloroform, 1,2-dichloroethane [74]

First the cell membrane permeability has to be modified in order for the solvent to access the intracellular PHA [75]. After being dissolved by the solvent, PHA are separated from the remaining biomass by filtration or centrifugation. They are then precipitated, usually by addition of a non-solvent such as methanol or ethanol to the solution [74]. This method of extraction is highly efficient, causes negligible polymer degradation and allows for the removal of bacterial endotoxins.

However, chlorinated hydrocarbons are highly pollutant and expensive to be used on high scale. The use of some cyclic carbonates like ethylene carbonate [9] and 1,2-propylene carbonate [76, 77] may overcome this problem.

A problem inherent to all solvent extraction is that they disrupt the amorphous nascent state of the PHA granules [9, 69, 78], which may be useful for some applications. The solubilization of the granules and further precipitation makes PHA to re-crystallize in a more crystalline state [46, 69], becoming more brittle and harder to process [79].

3 Materials and Methods

3.1 Microorganisms

Activated sludge from two different wastewater treatment plants were used as inocula for the bioreactors. The wastewater treatment plants were both from food/feed processing companies and were operated as SBRs.

The activated sludge used in Hydrocell (Chapter 4) came from Solae, a protein processing factory in Ieper, Belgium. On the other hand, the activated sludge for the Production of PHB by ADF (Chapter 5) and for the Production of Fish Feed (Chapter 6) came from Van Steenberge, a brewery in Ertvelde, Belgium.

3.2 Reactor operation

Three different process for the production of PHB were tested: Hydrocell (Chapter 4); Production of PHB by ADF (Chapter 5); and Production of Fish Feed (Chapter 6).

The processes described in Chapter 4 and Chapter 5 share some characteristics and basically comprise two main steps. The first one, the growth reactor, is used for the selection and growth of PHB producing bacteria. While on the second one, the accumulation reactor, the biomass selected in the growth reactor is fed with enough carbon source in order to accumulate a significant amount of PHB. In the process described in Chapter 6, the biomass was not previously acclimated in a growth reactor, but was instead used directly in an accumulation reactor.

The procedures are explained in further detail in each one of their corresponding chapters. Nevertheless, some general methodologies are explained below.

3.2.1 Hydrocell (Chapter 4)

A 3 reactors system was assembled for the Hydrocell process, consisting of a electrolysis, a growth and an accumulation reactor. The electrolysis reactor, as the name implies, was used for the electrolysis of water and the consequent

production of H_2 and O_2. The selection and growth of the PHB producing bacteria from activated sludge was carried out on the growth reactor. The headspace of the electrolysis reactor was connected to this reactor thus allowing the continuous supply of H_2 and O_2 to the growth medium via fine bubbling. Putting things into perspective, these two reactor worked together for the selection of PHB producing bacteria, the first step of a two steps process, as described in Chapter 3.2.

The accumulation reactor comprises the second of those steps. In this step, part of the biomass from the grow reactor is transferred to another reactor where conditions that promote the production of PHB are applied. However, due to the lack of means, this was not done in a regular defined basis. In the cases an accumulation stage was not carried out, the excess sludge was discarded.

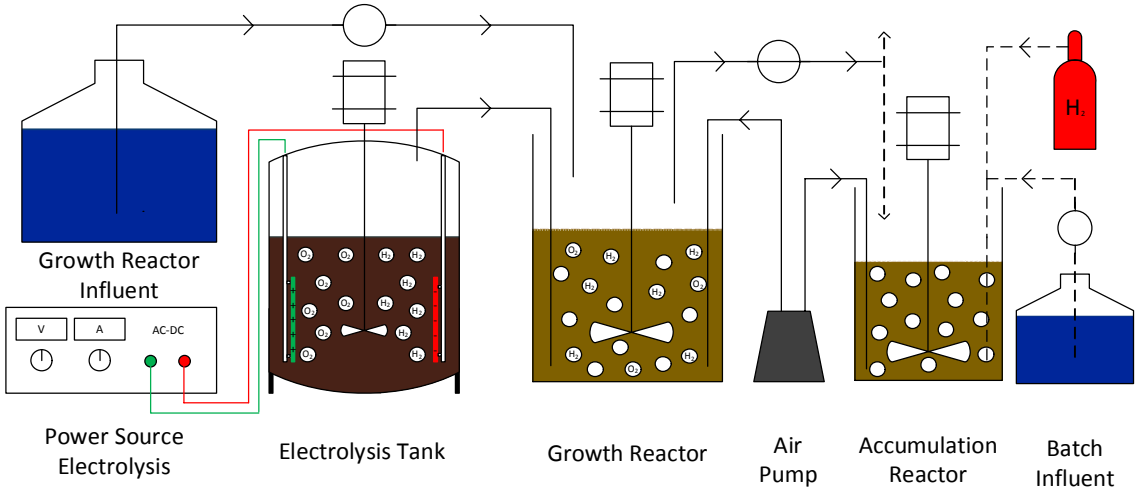

Figure 5 - Diagram of the Hydrocell process with the electrolysis, growth and accumulation reactors

The diagram of the process is described in Figure 5. The methods for each reactor are described further below. Analyses were made as described in chapter 3.3 and the formulas used are described in Appendix B.

3.2.1.1 Electrolysis reactor

An electrolysis system was assembled in a closed reactor for the production of hydrogen (and oxygen) gas. This consisted of two electrodes: one in titanium and the other one in titanium, coated with Iridium oxide. In order to avoid contact

between the electrodes, they were fixed to plastic carriers, at a distance of 15 cm. The area of the electrodes was of 79 cm^2.

The electrodes were connected to an alternating current - direct current (AC-DC) power supply. The positive pole of the power supply was connected to the iridium oxide coated titanium electrode (the electrolysis' anode) and the negative pole was connected to the other titanium electrode (the electrolysis' cathode).

The electrodes were then covered, by adding 4.0 L of tap water containing 1 g/L Na_2SO_4. The salt was added in order to improve the conductivity of the system. This medium was kept under constant stirring. A electric current of 20.2 V (± 0.2) with a intensity of ≈ 0.6 A was applied so the electrolysis of water was achieved.

The headspace of this reactor was coupled to the growth reactor in order to provide H2 and O2 to the growth medium, via fine bubbling.

3.2.1.2 Growth reactor

The reactor was incubated with 4 L of activated sludge from a protein factory waste water treatment plant, as described in Chapter 3.1.

The reactor was operated in the 3 cycles of 8 hours per day. At the beginning of each cycle, 150 mL of fresh influent were added during the 1st minute. The duration of the feast phase was controlled at 2.5 h, while the famine phase lasted for 5.5 h. The compositions of the influents used are described on Appendix A Hydrogen and oxygen gases form the electrolysis were fed continuously during both phases. Aeration was also applied continuously by means of a aeration stone and stirring was kept at 300 rpm. pH in the reactor was kept below 7.8 (± 2%), with diluted sulfuric acid 1 M.

Hydraulic retention time (Equation 10, Appendix B) was not maintained constant over the experiment. It started at 18.1 days, but it was gradually reduced till 8.1 days on on day 5. On day 10 it was verified that the pump responsible for feeding the reactor with fresh influent was not capable of maintaining the calibrated flowrate. As a result, the HRT on this day was of 28 days. After this, the HRT was adjusted to a average value of 14.4 days (±3.4) from day 11 till day 21. For the last ten days, from day 22 till day 32, the HRT was adjusted to an average of 11.2 days (± 0.8). All this gave a global a average retention time of 12.9 (± 3.1) days.

Solid retention time (Equation 11, Appendix B) was the same as HRT as volume was removed from the reactor as mixed liquor. The relatively high retention times were chosen in order to allow the growth of autotrophic bacteria, since this is slower than the heterotrophic one in *C. necator* [80]. This is also evident, in activated sludge where the nitrifying bacteria, that are autotrophic, have slower growth rates than the heterotrophic bacteria [64].

On day 3, 4, 7 and 10 approximately 1/3 of the total volume of mixed liquor was transferred to the accumulation reactor in order to test the sludge for its capability to accumulate PHB, by supplying sufficient carbon source. The volume of the growth reactor was setback to 4 L by adding fresh activated sludge.

On days 14, 17, 21, 24, 28 and 31 the volume in excess in the growth reactor was purged, so the volume was set back to 4L. No fresh activated sludge was added by this times.

3.2.1.3 Accumulation tests

The capability of the biomass selected in the growth reactor to produce and store PHB was tested under both heterotrophic and autotrophic conditions. In order to the this, the mixed liquor withdrawn from the growth reactor on days 3, 4 and 10 was re-inoculated in accumulation reactors.

The accumulation tests in heterotrophic conditions were carried out on days 4 and 10 using acetic acid as carbon and energy source. The accumulation test in autotrophic conditions were carried out on days 3 and 10 and H2 was used as energy source in order to allow the use of inorganic carbon sources. The detailed operation of the two reactors is explained below.

The PHB content and its production was evaluated by means of SLB.

3.2.1.3.1 Accumulation test with acetic acid

1 L of sludge from the growth reactor was incubated on a 2 L vessel. The reactor was fed by the means of pH-stat, in order to dose a influent stream reach in acetic acid. The setpoint was defined to be at pH 7.2 (±3%) and was controlled by PC-Panel μDCU software and a pH meter Easyferm plus K8 425, Hamilton

Bonaduz AG, connected to a Biostat® A plus main system, by Sartorius BBI Systems.

The influent solution was prepared by diluting a solution of acetic acid 80 % (w/w) with water, to a final concentration of 40 g COD/L. No other nutrient sources were added and no pH corrections were done to the influent stream.

The reactor medium was kept under constant stirring by means of an electrical pump, while air was provided through fine bubbling.

3.2.1.3.2 Accumulation test with in autotrophic conditions

1 L of sludge from the growth reactor was incubated on a 2 liter vessel. The reactor was aerated with a hydrogen flow of 50 mL H_2/min via fine bubbling. It was considered that the bicarbonate resultant form the biomass degradation processes in the growth reactor was a sufficient source of inorganic carbon for accumulation. Therefore, no other carbon source was added to the medium, with exception to the normal carbon dioxide content in the air stream.

3.2.2 Production of PHB by ADF (Chapter 5)

Production of PHB by ADF was carried out in a two steps system consisting of a growth reactor and an accumulation reactor. The growth reactor was used for the selection and growth of PHB-producing bacteria, by submitting activated sludge to aerobic dynamic feeding conditions. The volume in excess on this growth reactor was then transferred to an accumulation reactor, where the carbon source (acetic acid) was supplied in excess to the selected microbial community, so PHB could be produced and stored by the PHB-producing bacteria. A diagram of the process is presents in Figure 6.

To achieve this, activated sludge was collected from a brewery wastewater treatment plant, which was operated in a SBR system (described in Chapter 3.1). The collected sludge was left to settle in a cold room overnight, after which the supernatant was discarded (≈50% of the total volume) prior to be incubated in the growth reactor.

This system was tested for a total of 64 days and several operation parameters were tested over time, for both the growth reactor and the

accumulation reactor. However, due to the instability of the reactor it was necessary to restart the growth reactor with fresh sludge on 2 instances, for a total of 3 runs. This implied that the microbial community in the growth reactor was not kept after each restart. In regard to the results, these 3 runs will be referred henceforward as "PHB production A", "PHB production B" and "PHB production C". Each one of these systems will be addressed in a different subchapter comprising the growth reactor and the accumulation reactors.

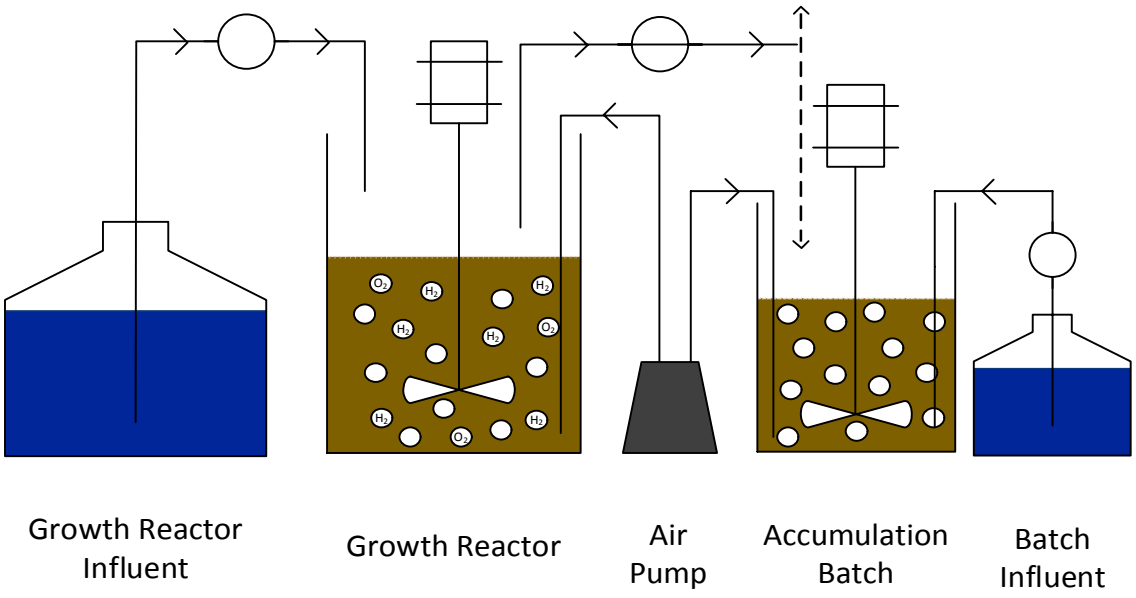

Figure 6 - Diagram of the process of production of PHB by ADF

The operation parameters of the reactors were different from reactor to reactor and varied over time according to the results, the observations made and the knowledge gathered with the previous trials. These are explained in further detail below.

The methods of analysis used are the ones explained in Chapter 3. PHB was quantified by extraction with 1,2-propylene carbonate.

3.2.2.1 PHB prodution A

3.2.2.1.1 Growth reactor - A

A 5 L reactor was incubated with 2 L of the decanted activated sludge. The system was operated in an aerobic dynamic feeding regime (feast & famine), with 3 cycles of 8 hours per day. It was intended that the feast phase occurred during the first 2 h 30 of each cycle, for a maximum feast/famine ratio of 0.45. For that, 200 ML of fresh influent were added to the medium per cycle. The extension of the feast phase was controlled by regulating the COD concentration of the influent, having in consideration the values of the analysis done to the reactor's medium during the cycle. The influent composition can be verified in Appendix A.

600 mL of volume were withdrawn from the reactor each day, in order to maintain the HRT at 4.33 days. However, this was not possible on days 4 and 5, and 11 and 12. As a result, on days 6 and 13 the HRT was off 6.33. This gives an average HRT of 5.13 days. Since there was no settling phase and the volume of sludge in excess was withdrawn as mixed liquor, the SRT was equal to the HRT.

The medium was continuously supplied with oxygen, via aeration through aeration stone, and stirred at ≈ 300 rpm. pH was controlled, fist using HCl 1 M and with H_2SO_4 1 M after day 10, in order to keep the culture medium at pH ≤ 8.5.

3.2.2.1.2 Accumulation reactors - A

No accumulation tests were carried out during this period.

3.2.2.2 PHB Production B

3.2.2.2.1 Growth reactor - B

A 5 L reactor was then incubated with the 2 L of activated sludge. The medium of growth reactor A was neutralized with sodium hydroxide and the biomass was recovered by centrifugation at 3,000 rpm for 10 minutes, while the

supernatant was discarded. The recovered biomass was in the activated sludge inoculated.

The system was operated in an aerobic dynamic feeding regime (feast & famine), with 3 cycles of 8 hours per day. It was intended that the feast phase occurred during the first 2 h 30 of each cycle. For that, 200 ML of fresh influent were added to the medium per cycle, using a peristaltic pump with a flow rate of 6.66 mL/min. Initially, this volume was completely fed in single feed pulse of 30 minuts, at the start of each feast phase. On day 11, the feed volume was split into two equal feed pulses of 15 minutes each, at 0.00 h and 1.00 h. This was changed again on day 16, to 3 equal feed pulses of 10 minutes each, at 0.00 h, 0.75 h and 1.50 h of feast.

The extension of the feast phase was controlled by regulating the COD concentration of the influent, having in consideration the values of the analysis done to the reactor's medium during the cycle. The composition o of the influents used can be observed on Appendix A.

600 mL of volume were withdrawn from the reactor each day, in order to maintain the HRT at 4.33 days. However, this was not possible on days 5 - 6, 12 - 13, 19 - 20 and 26 - 27. As a result, on days 7, 14, 21 and 28 the HRT corresponded to 6.33 days. This gave an average HRT of 5.08 days. Since there was no settling phase and the volume of sludge in excess was withdrawn as mixed liquor, the SRT was design to be equal to the HRT. Nevertheless, this was not verified on days 7 and 14, as the biomass was recovered from the withdrawn mixed liquor, by centrifugation at 3,000 rpm for 10 minutes, and re-inoculated back again in the growth reactor.

On day 28, 2 L of mixed liquor were replaced by 2 L of fresh activated sludge. The biomass from this mixed liquor was also recovered by centrifugation and re-inoculated in the growth reactor.

The medium was continuously supplied with oxygen, via aeration through aeration stone, and stirred at ≈ 300 rpm. pH was controlled, first using HCl 1 M and with H_2SO_4 1 M after day 10, in order to keep the culture medium at pH ≤ 8.5.

3.2.2.2.2 Accumulation reactors - B

Accumulation tests were performed on the activated sludge from growth reactor B on days 2, 7, 10, 15 and 21 of this reactor. In order to carry this out, a

2000 mL reactor was incubated with 400 mL of mixed liquor from the growth reactor, except on day 7 where the accumulation reactor was incubated with 500 mL. The mixed liquor was always taken from the growth reactor at the end of feast phase.

The accumulation reactors were operated in a feed pulse strategy, with the medium being fed every 3 hours with 13.3 mL of synthetic influent, for a total of 8 feed pulses per day. On the accumulation test of day 21 this was changed to 20 feed pulses per day, where 6.7 mL of synthetic influent were added every 72 minutes. The influent solution was composed only by acetic acid/acetate and ammonium chloride, with a COD/N ratio of 100/1 on days 2, 7 and 10 and with a COD/N ratio of 100/2 on days 15 and 21. This solution was prepared so the volumetric loading rate in the fill up reactor would be eight thirds (8/3) of the volumetric loading rate applied on the growth reactor on that day.

The HRT and SRT of the accumulation reactors was kept at 1 day. The medium was continuously supplied with oxygen, via aeration through aeration stone, and constantly stirred.

3.2.2.3 PHB Production C

3.2.2.3.1 Growth reactor - C

A 6 L reactor was incubated with 3 L of the decanted activated sludge. The system was operated in an aerobic dynamic feeding regime (feast & famine), with 3 cycles of 8 hours per day. It was intended that the feast phase occurred during the first 2 h 30 of each cycle, for a maximum feast/famine ratio of 0.45. For that, 400 mL of fresh influent were added to the medium per cycle. This volume was increased to 500 mL on day 14 and forward. These volumes were fed over 6 feed pulses, 1 each 25 minutes, for 10 and 12 minutes respectively. The extension of the feast phase was controlled by the DO values and confirmed by GC analysis. The composition of the influents used can be observed on Appendix A.

The reactor conditions were designed for an active volume of 4.2 L and a HRT of 3.5 days. With the increase of the volume fed per cycle on day 14, these parameters were changed to an active volume of 4.5 L and a HRT of 3.5 days. In order to do so, 1200 mL of mixed liquor were withdrawn from the growth reactor

each day, from day 1 to day 14, being this volume increased to 1.5 L afterwards. Since there was no settling phase and the volume of sludge in excess was withdrawn as mixed liquor, the SRT was equal to the HRT.

The medium was continuously supplied with oxygen, via aeration through aeration stone, and stirred at ≈ 300 rpm. pH was controlled using H_2SO_4 1 M at pH ≤ 8.5, in order to prevent the conversion of ammonium to ammonia.

3.2.2.3.2 Accumulation reactors - C

Accumulation tests were performed in a 2 L reactor. For this, 1200 mL of mixed liquor were withdrawn from growth reactor C at the end of feast phase. The mixed liquor was centrifuged for 10 minutes at 3,000 rpm in order to remove the supernatant, so the nutrients of the growth reactor medium wouldn't interfere with accumulation test. The pellet was used to incubate the accumulation reactor. On days 7, 8 and 9 the pellet was resupended on 1000 mL of tap water, while on days 13 and 14 the volume of water was increased to 1200 mL.

The accumulation reactor was fed every 72 minutes with a 4 minutes feed pulse having a flowrate of 6.66 mL/min. As a result, 0.5 L were fed over the 22 hours of the fed batch. On days 7, 8 and 9 the influent solution was composed by acetic acid/acetate and ammonium chloride in a COD/N ratio of 100/1, while on days 13 and 14 it was composed only of acetic acid/sodium acetate. This solution was prepared so the volumetric loading rate in the fill up reactor would be 3 time higher than the volumetric loading rate of the growth reactor.

The medium was continuously supplied with oxygen, through fine bubbling and constantly stirred.

3.2.3 Production of PHB enriched SCP (Chapter 6)

Activated sludge was collected from the brewery's WWTP operated in SBR described in Chapter 3.1. The sludge was left to settle for an hour, after which it was decanted to 50 % of its initial volume and stored at 4 °C till being used.

The reactor was operated in fed-batch mode. The feed of the reactor was controlled by pH-stat, with a setpoint of 8,2. The influent used had a COD

concentration of 60 g/L, being 50 % of it acetic acid and the other 50 % sodium acetate. The pH of the influent was corrected to 4.75 using sodium hydroxide and no further nutrients were used.

For the test batch, 3 L of decanted sludge were diluted in 3 L of fresh water and inoculated in a 10 L reactor. In the third batch, the reactor was inoculated with 6 L of decanted activated sludge, diluted in 6 L of fresh water.

Every batch was operated overnight and stopped in the next morning. The final volume of the reactor and the volume of influent fed were evaluated. The sludge was left to settle and the water in excess (around 45% of the total volume) was decanted. The sludge was poured into trays and dried overnight, at 60 °C, in order to form flocks.

TSS, VSS and VFA were analyzed at the beginning and end of the batch as described previously. The PHB content was evaluated gravimetrically, after cellular lysis with a hypochlorite solution and recovery using propylene carbonate, as described in more detail in Chapter 7.

3.2.4 Development of a new method for PHB quantification (Chapter 7)

The method develop for the quantification of PHB was based on the gravimetric analysis of PHB. This consists of 3 main steps, namely (1) the lysis of the PHB containing cells; (2) the dissolution on PHB and separation from the cellular debris; (3) and the recovery of the PHB polymer.

3.2.4.1 Cellular lysis

In order to allow a better recovery of the polymer, the cell must be lysed so the solvent can easily access the intracellular PHB. Two methods were used to cause cellular disruption. One based on the mechanical disruption of cells due to the increased volume of water when frozen, and other based on the chemical digestion of cells by hypochlorite.

3.2.4.1.1 Freeze and thaw cycles

Samples of 20 mL of sludge were collected into falcon tubes. They samples were centrifuged at 10.000 rpm for 10 minutes, after which the supernatant was discarded and the pellet resuspended in demineralised water. Then, the samples were immediately placed in a freezer in order to froze. After being completely frozen, the sample was place in a warm bath till it was completely defrosted again. During thaw the sample was shaken several times. The freeze and thaw was repeated more two times before other analysis were carried.

This step was used for PHB quantification throughout Chapter 5 and Chapter 6.

3.2.4.1.2 Hypochlorite digestion

Samples of 20 mL of sludge were collected into falcon tubes and centrifuge it for 10 minutes at 10,000 rpm. The supernatants were then discarded and the pellet was resuspended in 20 mL of a hypochlorite solution 20° (6% of active chlorine).

After 5 minutes, 1 mL of H_2SO_4 1M was added to each sample in order to neutralize the pH of the solution. This would protect the PHB from being hydrolyzed. The addition of acid may originate chlorine gas, so it was done inside a fume hood. The samples were then immediately centrifuged at 10,000. The supernatant was discarded and the pellet rinsed with dematerialized water. The samples were centrifuge once again for 10 minutes at 10,000 rpm and the supernatants were discarded.

3.2.4.2 Extraction with chloroform

The sludge in the falcon tubes was centrifuged at 10,000 rpm for 15 minutes after which the supernatant was discarded and the pellet rinsed with demineralized water. The solution was submitted to another centrifugation, in the same condition, and the supernatant was discarded again.

The pellet was then washed from the falcon tube to a schott bottle with 30 mL of choroform and then incubated at 37 ºC overnight, under constant stirring. In the next day, the chloroform solutions were vacuum filtered using fiber glass filters (0.45 μm of pore size, binding agents free) in order to withdraw the non

dissolved biomass, washing the schott bottles and filters with additional 5 mL of chloroform. The chloroform solution containing the dissolved PHA was then poured into pre-weighted crucibles and the filtration flask was rinsed with methanol.

The chloroform and methanol in the crucibles were let to dry overnight at room temperature in order to evaporate both the chloroform and methanol and further dried at 105 ºC to remove water. After cooling down the crucibles were reweighted.

3.2.4.3 Extraction with 1,2-propylene carbonate

The sludge in the falcon tubes was centrifuged at 10,000 rpm for 15 minutes after which the supernatant was discarded and the pellet rinsed with demineralized water. The solution was submitted to another centrifugation, in the same condition, and the supernatant was discarded again.

The pellet was then washed from the falcon tube to a COD tube with 20 mL of propylene carbonate and incubated at 130 ºC, for at least 15 minutes, with periodic shaking. These solutions were vacuum filtered afterwards with fiber glass filters (0.45 μm of pore size, binding agents free) pre-warmed with propypylene carbonate at 130 ºC. The COD tubes and filters were washed with additional 20 mL of warm propylene carbonate. The filtered solution was stored in schott bottles and the PHA was precipitated with 4 volumes of cold methanol.

After one day, the precipitated PHA was vacuum filtered with pre-weighted fiber glass filters. The cakes were washed with methanol in order to remove traces of propylene carbonate and dried overnight at 105 ºC. After cooling down the filters were weighted again in order to access the amount of material recovered.

3.3 Analythical methods

3.3.1 Biomass Quantification

Biomass was quantified gravimetrically through the determination of Total Suspended Solids (TSS), Volatile Suspended Solids (VSS) and Stored Lipidic Biomass (SLB) / PHB content.

TSS was determined as the remaining residue of a sample after drying at 105 °C, from which the suspended matter was previously separated from the medium, normally through filtration [64].

VSS was determined as the part of TSS that volatizes at 600 °C. This is considered to represent the organic fraction of the sample [64].

Stored lipidic content (SLB) was determined as the part of TSS that volatizes at 250 °C. This method was developed at Avecom previously to this work and is considered to represent the PHB content in the samples. This method was only used throughout Chapter 4 and was then replaced by the method for PHB quantification by extraction with propylene carbonate.

3.3.1.1 TSS

Samples of 20 mL were collected from the biorreactors into 50 mL falcon tubes. The samples were centrifuged at 10,000 rpm, for 10 minutes in a Eppendorf Centrifuge 5810. The supernatants were then discarded and the pellet was resuspended in demineralized water. Next, the resuspended pellets were centrifuged again using the same conditions as before, after which the supernatant was discarded again.

The pellet was then transferred to dry porcelain crucible, pre-weighet using a Sartorius TE64 Analytic Balance. After that, the crucibles with the samples were dried overnight in a oven from Memmert at 105 °C. Next day, the crucibles were let to cool down in an exicator for one hour, before weighting them again. TSS content was given by the difference between the porcelain crucible with the dry matter and the porcelain crucible as such, divided by the volume of sample (Equation 1, Appendix B).

3.3.1.2 VSS

VSS was quantified after the determination of the TSS from the samples. For that, the crucible with the dry matter was placed in a Nabertherm GmbH LE 4/11/R6 incinerator at 600 °C for 2 hours. After cooling down in an exicator, the porcelain crucible was weighted and the PHB content was given by the difference between the weight of the crucible with the dry matter and the weight of the crucible with the ashes, divided by the sample volume (Equation 2, Appendix B)

3.3.1.3 PHB quantification as SLB

When SLB determination was needed, this step was performed between TSS and VSS determination. After determining TSS, the porcelain crucible with the dried biomass was placed in a Nabertherm GmbH LE 4/11/R6 incinerator at 250 °C for 2 hours. After cooling down, the porcelain crucible was weighted and the amount of SLB was given by the difference between the weight of the crucible with the dry matter and the weight of the crucible with the remaining biomass after 250 °C, divided by the sample volume (Equation 3, Appendix B)

VSS could be determined afterwards, using the procedure described previously.

3.3.1.4 PHB quantification by extraction with propylene carbonate

PHB was quantified gravimetrically after extraction with 1,2-propylene carbonate (Equation 4, Appendix B), using a procedure developed at Avecom during this disseration. The method consisted of cellular lyses by freeze and thaw cycles, followed by the extraction of PHB using 1,2-propylene carbonate. This procedure was described above, in Chapter 3.2.4 - Development of a new method for PHB quantification. This method is discussed further in Chapter 7.

3.3.2 pH

The pH of the samples was measured using the pH meter Consort C535.

3.3.3 Dissolved oxygen (DO)

The dissolved oxygen of the reactor was measured using the meter WTW Oxi 315i.

3.3.4 Soluble Chemical oxygen demand (COD)

Chemical oxygen demand was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kits LCK 014 and LCK 514 as

indicated by the manufacturer. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810) in order to remove suspend solids.

3.3.5 Total nitrogen (TKN)

Total nitrogen was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kit LCK 338 as indicated by the manufacturer. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810) in order to remove suspend solids.

3.3.6 Ammonium (NH_4^+-N)

Ammonium (NH_4^+-N) was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kits LCK 302 and LCK 303 as indicated by the manufacturer. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810) in order to remove suspend solids.

3.3.7 Nitrate (NO_3^--N)

Nitrate (NO_3^--N) was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kits LCK 339 and LCK 340 as indicated by the manufacturer. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810) in order to remove suspend solids.

3.3.8 Orthophosphate-phosphorus (PO_4^{-3}-P)

Orthophosphate-phosphorus (PO_4^{-3}-P) was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kit LCK 348 and LCK 350 as indicated by the manufacturer. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810) in order to remove suspend solids.

3.3.9 Volatile fatty acids (VFA)

The concentration of VFA was determined by the standard method of partition of the VFA from samples using diethylether, followed by gas chromatography analysis of the organic phase as described in [81].

For this 2 mL of sample, 0.5 mL of H_2SO_4 solution, 0.4 g of NaCl, 0.4 mL of internal standard solution (2-methyl hexanoic acid) and 2 mL of diethyether were introduced into a centrifuge tube. The tubes were then mixed for 2 min and centrifuged at 3000 rpm for 3 minutes (Eppendorf Centrifuge 5810). Subsequently, the etheric layer was transferred into a GC vial, in which the short chain fatty acids (SCFA) were extracted.

The extracts (1 μL of extract) were analyzed using a GC-2014 gas chromatograph, equipped with an Auto injector AOC-20i and an auto sampler AOC-20s, all from Shimadzu. The temperature profile was set from 110 to 165 ºC, with a temperature increase of 6 ºC per minute. The temperature of the injector was 220 °C, and the temperature of the sampler was 200 ºC. Nitrogen was used as a carrier gas.

4 Hydrocell

4.1 Background

In some european countries is not uncommon that private companies produce and sell electricity from renewable sources, as a complement to their main activity. One example of this is the generation of electricity by the burn of biogas from the anaerobic treatment of wastewaters or by wind turbines.

However, electricity production has to be toughly controlled, since it cannot be stored in a significant way. Its production must always correspond to the amount being consumed. Otherwise the grid would be over or under charged. As a result, sometimes the biogas has to be flared or the wind turbines stopped in order to avoid electric peaks in the grid thus wasting a significant amount of energy, that could be valorised [82]. Furthermore, outside peak hours electricity value is only of 0.05 €/kWh [83].

It would be interesting to valorise these surplus of energy and cheap electricity. One possibility is to use this electricity in the electrolysis of water towards molecular hydrogen and oxygen [84]. Some microorganisms can use molecular hydrogen as an energy source in their metabolism. If these microorganisms are capable of growing and deliver a product with high value, then this process can be economically feasible.

The use of molecular hydrogen as an energy source is widely described for autotrophic microorganisms, who use inorganic carbon, such as carbon dioxide, as a carbon source [50]. CO_2 is a major gaseous waste stream in industry and other human activities and plays a major role in global warming. This gaseous waste streams could be used to feed bacteria in bioreactors. Another source of CO_2 could be biogas, as it is represents roughly 35% of its composition, being the rest mainly methane. If microorganisms could remove CO2 from biogas, its quality would be upgraded.

Electrolysis of water is an economical and environmental friendly process, with an yield of 65 - 80%. At the maximum yield, 1 kWh of electricity generats 9.14 mol of H_2, which correspond to 228 L of this gas at standard ambient temperature and pressure (SATP). This is equivalent to 146 g COD generated per kWh of electricity consumed, since 1 mol of H_2 has COD of 16 g [85].

In electrolysis, the distinction between cathode and anode is not based on the electric charge applied on the electrode, but rather on the electronegativity of the gas formed. Therefore, the cathode is where the hydrogen gas is produced, regardless being negatively charged and the anode is where the oxygen is formed, although the electrode is positively charged. The anode (oxygen formation, positively charged electrode) is typically made of nickel and copper, coated with oxides of metals such as manganese, tungsten and ruthenium. While the cathode (hydrogen formation, negatively charged electrode) is typically made of nickel, coated with small quantities of platinum [86].

The autotrophic production of PHA by pure cultures of *C. necator* was already demonstrated in the literature with fairly good results. It was possible to attain a biomass concentration of 69.3 g/L and a PHA concentrarion of 54.6 g/L, which represents a PHA content of 81.4 % (w/w). This was achieved after 92 hours and the productivity was of 0.61 $g.L^{-1}.h^{-1}$ [87]. In another system, specially designed for high K_La values (a value of 2970 h^{-1}), the biomass concentration was 85.7 g/L after 45 hours. From this biomass 71.8 % (w/w) was PHA, which corresponds to 61.5 g PHA/L [88]. On the other hand, the use of the mixed cultures for the autotrophic production of PHB was already tried at Avecom with poor results (results not published).

In this experiment mixed cultures were submitted to aerobic dynamic feeding conditions in a H_2 enriched media. The purpose of this was to find out if the use of H_2 may act as a selective pressure for the enhanced production of PHB.

4.2 Results and discussion

4.2.1 Growth reactor

The growth reactor was incubated with activated sludge from protein processing factory's WWTP, which was operated in SBR. This represents a nice source of activated sludge for the production of PHB, as the microbial community is adapted to live under the transient conditions of a SBR system [18]

The reactor was maintained over a period of 32 days. The evolution of the biomass of the concentration the growth reactor can be observed in Figure 7.

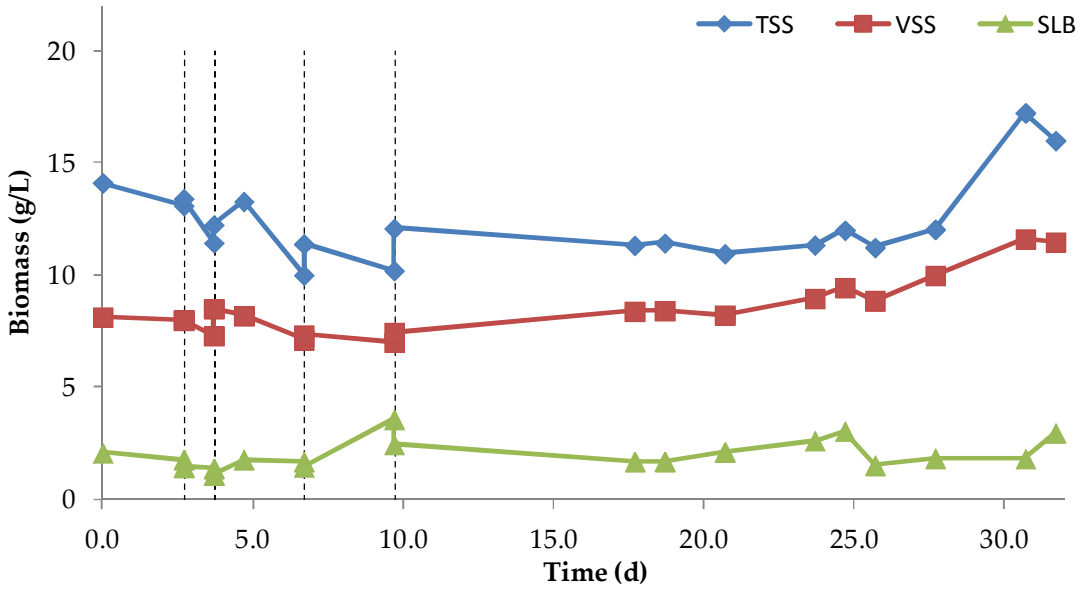

Figure 7 - Biomass concentration as TSS and VSS and SLB content (g SLB/g VSS) in the Hydrocell's growth reactor at the end of feast phase, over a period of 32 days . The dashed vertical lines indicate the times when part of the mixed liquor was replaced by fresh activated sludge.

During this time, there was a small, but rather constant increase in the VSS, from 8.15 g VSS/L on day 0 to 11.49 g VSS/L on day 32. Simultaneously, there was a decrease in the difference between TSS and VSS values, from a VSS/TSS ratio (Equation 6, Appendix B) of 58 % in day 0 to 83 % on day 28 which indicates that non organic material was being removed from the reactor. The TSS value increased abruptly on days 31 and 32 due to the fragmentation in the reactor of one of the aeration stones.

The highest verified specific growth (Equation 14 and 15, Appendix B) rate was of 0.09 h^{-1} on days 18 (0 - 1 h feast) and on day 25 (0 - 2 h feast), while the average specific growth rate was of 0.06 h^{-1}. These values fall far behind 0.19 h^{-1} [14], that was the maximum value found in the literature, but it was close to the value of 0.10 h^{-1} previously reported as the maximum one [89]. This value was achieved while submitting a pure culture of *Paracoccus pantotrophus* (a PHA-producing bacteria commonly present in WWTPs) to ADF conditons, in order to study the effect of the feast and famine cycles [90].

This maximum value of specific growth rate on day 18 was simultaneous with the maximum observed VSS yield of 0.49 g VSS/g COD (Equation 8, Appendix B). Similar yield values were observed on days 3 and 5, but it is not clear if in these days this was a result of the cellular growth or due to the storage of PHB, as nitrogen consumption was not evaluated during these days. These

yield values, as well as the volumetric loading rate (Equation 12, Appendix B) and sludge loading rate (Equation 13, Appendix B) can be observed in Figure 8.

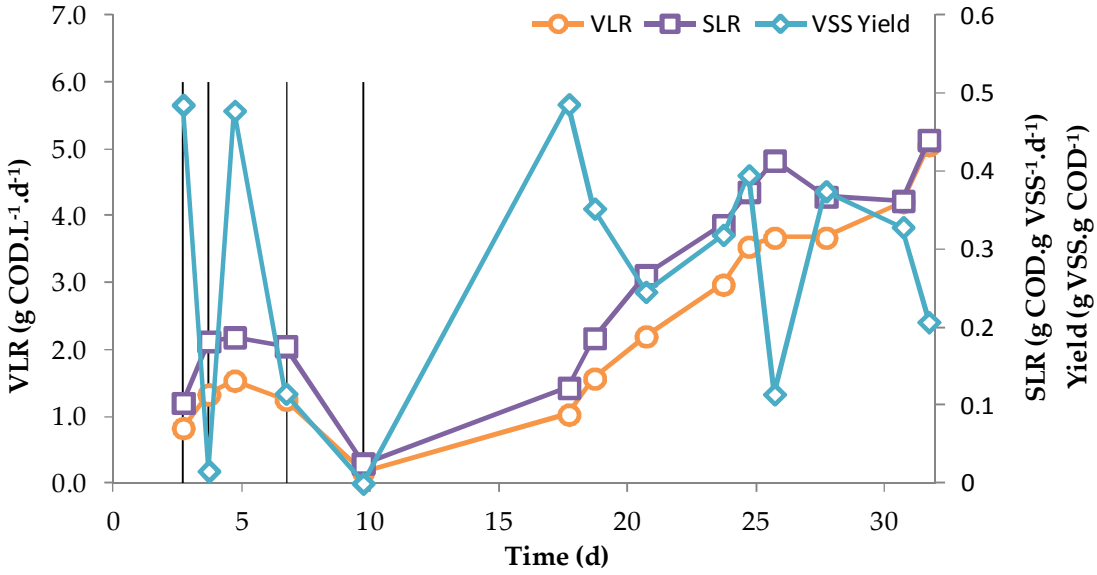

Figure 8 - VLR, SLR and VSS Yields on the Hydrocell's growth reactor. The dashed vertical lines indicate the times when part of the mixed liquor was replaced by fresh activated sludge.

The observed VSS yield was highly inconstant, most likely due to the changes in the hydraulic retention and the replacement of part of the growth reactor mixed liquor with fresh sludge. Actually, the yields seemed to stabilize after day 17, as the HRT and SRT started to be more tightly controlled.

There is not a clear explanation for what cause the low yield values on day 26 and 32. This seems to correspond to the days where the SLR was higher, with 0.41 g COD/g VSS and 0.44 g COD/g VSS respectively. In fact, from day 25 to 26 the influent concentration was increased from 35 g COD/L as acetic acid to 40 g COD/L and on day 32 the influent concentration was already at 50 g COD/L. Nevertheless this can be related either with the higher SLR, since it was already showed that higher loading rates result in lower yields [91].

The loading rates were gradually increased after day 18, maintaining a feast phase duration of 2.5 hours. This explains the increase in VSS already stated previously. In fact the values of VSS are higher than those found in the literature, where a value of 6.8 g VSS/L can be taken as good reference for this type of system [21]. However, these high cellular concentrations are irrelevant if the bacteria are not capable to effectively produce and store PHB.

The evolution of the PHB content, measured as a ratio between SLB and VSS (Equation 7, Appendix B) can be observed in Figure 9. However, the quantification of PHB as SLB revealed to be unreliable, as more extensively described in Chapter 7, so all the observations were made based on values that could be true or not.

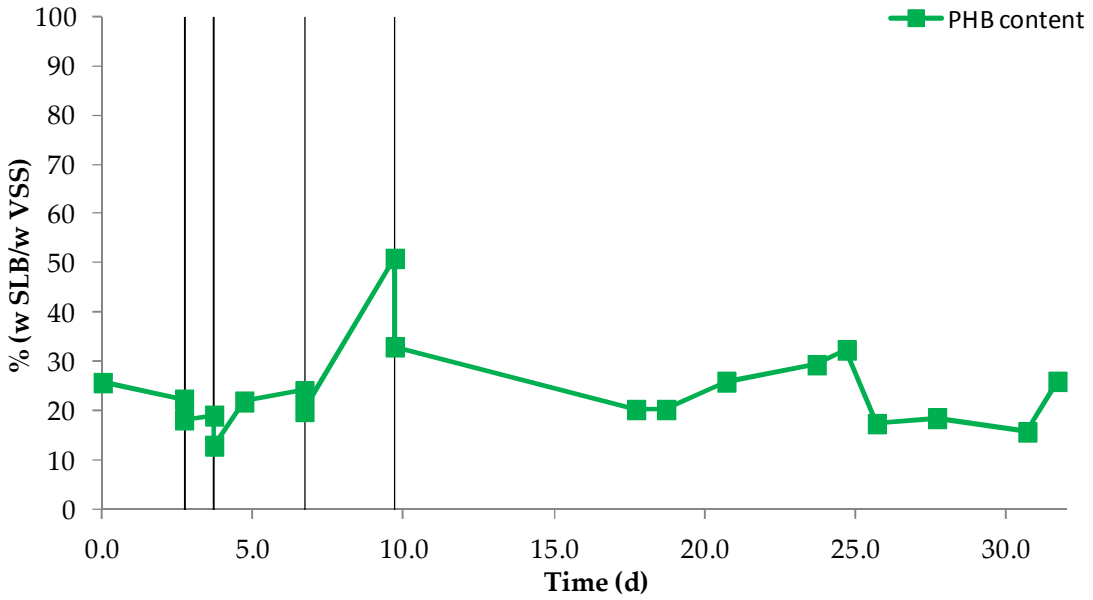

Figure 9 - PHB content (measured as the ratio between SLB and VSS) in Hydrocell's growth reactor at the end of feast phase, during a period of 32 days. The dashed vertical lines indicate the times when part of the mixed liquor was replaced by fresh activated sludge.

The highest SLB contents at the end of feast phase was achieved on day 10 with the SLB accounting for 51 % (w/w) of the VSS. However, this value was observed after a considerably extended famine phase, due to a malfunction with the feeding pump, which resulted in a F/F ratio of 0.04 h/h. The increased famine phase may have led to an effective selection of PHB-producing bacteria, that had the polymer stored and so were capable of surviving without an external carbon source. When the normal reactor operation was resumed, the acetic acid fed must have been rapidly for polymer synthesis in order to restore their PHB reserves, since there was a strong internal limitation to growth [7].

On regular reactor operation, the highest value was verified on day 25 with a SLB content of 31 % (w/w). This value was gradually increasing since day 19, when the SLB content was only 20 % (w/w). This increase in SLB content happened after the reactor's SRT and HRT started to be controlled more judiciously and with the increase of the loading rates, as observed for VSS.

Therefore, the increase on VSS during this time was mainly due to the increased capability of the sludge to store PHB.

However, on the next day, the SLB content decreased to 17 %. This corresponds to the first day of feeding with 40 g COD/L Appendix A. The decrease in the SLB content justifies the low observed VSS yield value, however this does not add extra clues to what might have happened.

The analysis of nitrogen concentration in the medium that day revealed a concentration of 40 mg N/L before the beginning of the feast phase, which indicated some accumulation of nitrogen in the reactor. Since there was a significant increase in the VSS, while the SLB contents stayed low, it is valid to propose that concentration of nitrogen led to a shift in the metabolism, from PHB accumulation to growth.

However, this was against what was observed from day 31 to 32 with an increase in the SLB content from 16 % to 26 %, even with a small decrease in VSS. The nitrogen concentration on day 32 was evaluated again prior to feeding and the it was 100 mg N/L, which clearly shows that nitrogen was indeed accumulating, but does not seem to be related with the decrease in the SLB content.

Looking to the results as a whole, it seems that the capacity of the bacteria to produce and store PHB was not enhanced over time. The average SLB content at the end of feast phase was only of 25 %, while this value was of 26 % on day 0. In fact, this was exactly the same value as the one verified on day 32. This could mean that the conditions applied in the growth reactor may have not been sufficient for an effective selection of PHB-producing bacteria. However, the way that the growth reactor was operated was not designed for a significant accumulation in PHB and so accumulation tests where necessary to determine the capacity of the sludge to store PHB.

4.2.2 Batch accumulation tests

On days 3 and 10 autotrophic accumulation tests were started simultaneously with a heterotrophic ones, so it was possible to compare the results. However, the heterotrophic accumulation test started on day 3 overflowed without being possible to retrieve any values, therefore a new heterotrophic test was started on day 4.

4.2.2.1 Acetic acid

The first accumulations test under heterotrophic conditions was started on day 4. The results of this test are exhibited on Table 4.

Table 4 - Batch accumulation test using acetic acid on day 4 of the growth reactor

Parameter	***Unit***		
Time	h	0	24
Biomass concentration			
- TSS	g/L	11.15	11.48
- VSS	g/L	7.32	8.80
- SLB	g/L	1.40	3.55
- NPCB	g/L	5.92	5.25
Ratio			
- SLB/VSS	%	19	40
- SLB/TSS	%	13	31
- VSS/TSS	%	66	77
Reactor Volume	L	1.00	1.25
COD Fed	g	-	10.0
VLR	g COD/(L.d)	-	8.00
SLR	g COD/(g VSS.d)	-	0.90
Total biomass			
- TSS	g	11.15	14.34
- VSS	g	7.32	11.00
- SLB	g	1.40	4.44
- NPCB	g	5.92	6.56
Yield			
- VSS	g VSS/g COD	-	0.37
- SLB	g SLB/g COD	-	0.30
Productivity			
r_P	g SLB/(L.h)	-	0.10
q_P	g SLB/(g X.h)	-	0.02

The SLB/VSS ratio more than doubled in the first heterotrophic test, from 19% at 0 hour to 40% of SLB content after 24 hours. Although there was some cellular growth, as the total non-PHB cellular mass (Equation 5, Appendix B) increased from 5.92 g to 6.56 g, this only accounted for 17 % of the VSS growth. The PHB production yield of 0.30 g SLB/g COD (Equation 9, Appendix B) was in the range found in the literature for the production of PHB by mixed cultures, 0.25 - 0.56 g PHB/g COD [14], although closer to the lower value. The specific production rate however was of 0.02 g SLB.g $NPCB^{-1}.h^{-1}$ which is way below the values found associated with those yields, that vary between 0.14 and 0.65 h^{-1} [14].

Table 5 - Batch accumulation test using acetic acid on day 10 of the growth reactor

Parameter	***Unit***					
Time	h	0	1	2.5	5.5	22
Biomass concentration						
- TSS	g/L	10.22	9.98	10.02	10.43	11.75
- VSS	g/L	7.04	7.01	7.33	7.84	9.21
- SLB	g/L	3.59	3.41	3.85	4.63	4.01
- NPCB	g/L	3.45	3.60	3.48	3.21	5.2
Ratio						
- SLB/VSS	%	51	49	53	59	44
- SLB/TSS	%	35	35	38	44	34
- VSS/TSS	%	69	70	73	75	78
Reactor Volume	L	1	1.047	1.053	1.077	1.160
COD Fed	g	-	1.88	2.88	4.68	8.80
VLR	g COD/(L.d)	-	1.80	2.74	4.35	7.59
SLR	g COD/(g VSS.d)	-	0.25	0.37	0.55	1.46
Total biomass						
- TSS	g	10.22	10.44	10.55	11.23	13.63
- VSS	g	7.04	7.34	7.72	8.44	10.68
- SLB	g	3.59	3.57	4.05	4.99	4.65
- NPCB	g	3.45	3.77	3.66	3.45	6.03
Yield						
- VSS/COD	g/g	-	0.16	0.24	0.30	0.41
- SLB/COD	g/g	-	0.00	0.16	0.30	0.12
Productivity						
- r_P	g SLB/(L.h)	-	-0.02	0.17	0.24	0.04
- q_P	g SLB/(g VSS.h)	-	-0.01	0.05	0.07	0.01

The activated sludge used on this test already presented a high SLB content prior to the accumulation phase. This happened by coincidence, as this was the day in which a higher SLB content (51% of the VSS) was verified at the end of feast phase in the growth reactor. Probably due to this, there was not a significant increase in the SLB content over time, changing from biomass SLB content of 51 % at 0 hour to a maximum of 59 % after 5.5 hours. Nevertheless, the PHB production yields of 0.30 g SLB/g COD observed at that time was equal to that observed after 24 hours in first accumulation test and so, it is also in range with the values of the literature. There was also a increase in the volumetric productivity and specific production rate regarding the first test, but the values are still lower than the ones on the literature.

It is proposed that these low specific production rates are the result of a poor selection for PHB-producing bacteria in the growth reactor, as 10 days may not be enough to effectively change the microbial community. This may be particularly

true having into account the high HRT and SRT applied and the recurrent addition of fresh unaclimated activated sludge in the growth reactor.

There was no apparent reason for the decay in SLB content after 22 hours. It is possible to verify a decrease in the amount of COD fed per time, but this was probably related with a decrease in the acetate consumption rate, as the PHB-producing bacteria were becoming saturated. Furthermore, the SLR after 22 hours was above 1 g COD.g $VSS^{-1}.d^{-1}$ and the feeding control seemed to be working normally, so it is highly unlikely that the decrease in the SLB content was due to bacteria starvation. It is possible that this unexpected value was caused by the lack of reproducibility of the method used to determine the SLB. This problem was only detected afterwards and willl aborded more extensively in Chapter 7.

4.2.2.2 Autotrophic conditions

Table 6 - Batch accumulation test in autotrophic conditions on day 3 of the growth reactor

Parameter	***Unit***				
Time	h	0	21	24	48
Biomass concentration					
- TSS	g/L	13.11	12.22	12.01	9.26
- VSS	g/L	8.02	7.44	7.62	6.02
- SLB	g/L	1.80	2.42	1.78	1.33
- NPCB	g/L	6.22	5.02	5.84	4.69
Ratio					
- SLB/VSS	%	22	33	23	22
- SLB/TSS	%	14	20	15	14
- VSS/TSS	%	61	61	63	65
Reactor Volume	L	1	1	1	1
COD Fed as H_2	g	-	4.19	5.19	16.76
Yield					
- VSS	g VSS/g COD	-	0.00	0.00	0.00
- SLB	g SLB/g COD	-	0.15	0.00	0.00
Productivity					
- r_P	g SLB/(L.h)	-	0.03	0.00	-0.01
- q_P	g SLB/(g VSS.h)	-	0.01	0.00	0.00

The maximum SLB content of 31 % (g SLB/g VSS) was verified after 21 hours, with a yield of 0.15 g SLB/g COD as H_2. After this, the SLB content decrease to the same verified at 0 hour. Furthermore, the increase in the amount of SLB in the reactor was not followed by an increase in VSS, as it would be expected. In fact, VSS decreases in all the observed times. If the increase in SLB was not due to an

error in its determination, it is only possible to assume that the cells that weren't capable of live in the absence of an organic carbon source died and were lysed by the others. This may imply that the increase in SLB was not due to the autotrophic accumulation of PHB but rather to the used of the cellular debris by the living cells.

Table 7 - Batch accumulation test in autotrophic conditions on day 10 of the growth reactor

Parameter	***Unit***					
Time	h	0	1	2.5	5.5	22
Biomass concentration						
- TSS	g/L	10.22	9.98	10.10	9.92	10.25
- VSS	g/L	7.04	7.01	7.06	6.94	7.21
- SLB	g/L	3.59	3.41	3.66	2.90	1.89
- NPCB	g/L	3.45	3.60	3.40	4.04	5.32
Ratio						
- SLB/VSS	%	51	49	52	42	26
- SLB/TSS	%	35	34	36	29	18
- VSS/TSS	%	69	70	70	70	70
Reactor Volume	L	1.00	1.00	1.00	1.00	1.00
COD Fed as H_2	g	-	2.14	5.63	11.79	47.14
Yield						
- VSS	g VSS/g COD	-	0.00	0.00	0.00	0.00
- SLB	g SLB/g COD	-	0.00	0.01	0.00	0.00
Productivity						
- r_P	g SLB/(L.h)	-	-0.18	0.03	-0.13	-0.08
- q_P	g SLB/(g VSS.h)	-	-0.05	0.01	-0.03	-0.01

The trial carried out on day 10 shows a decreased in SLB with time, while the VSS remains considerably stable. This seems similar to the behaviour verified during a famine phase under excess of nutrients, as the stored PHB seems to be gradually consumed while cellular growth is occurring [7].

5 Production of PHB by ADF

5.1 Background

The production of PHB by submitting mixed cultures to aerobic dynamic feeding conditions had already been researched at Avecom. This research result in the scale up of the process to pilot scale, as part of a project for the profitable treatment of waste water. The objective was to obtain a viable alternative to the production of methane through anaerobic digestion.

Trials revealed a satisfactory capability of the biomass to accumulate PHB (data now shown). However, the system was highly unstable and the biomass concentration decayed overtime. This required the frequent addition of fresh sludge in order to maintain the process. After some months running the pilot and formulating a dried biomass product from the withdrawn mixed liquor, the project was ceased. In this way, other lines of research could be followed. However, the interest of new partner companies in the production of biopolymers led to the restart of the development and production of PHB producing technologies.

The objective of this work was to develop a system for the stable production of PHB by mixed cultures in a two step system. The recovery and extraction of the polymer from the biomass was also of interest, as it was required for other experiments.

5.2 Results and discussion

5.2.1 PHB production A

5.2.1.1 A - Growth reactor

Growth reactor A was operated as SBR, without settling phase, in ADF conditions. A F/F ratio of 0.45 was chosen as previous tests carried out at Avecom showed that this ratio offered a good balance between growth and the capability to store PHB (results not showed). This seems to be supported by the results found in the literature regarding the F/F ratio [7].

The evolution of the biomass concentration in the growth reactor A can be observed in Figure 10, while the VLR, SLR and biomass yield can be observed on Figure 11.

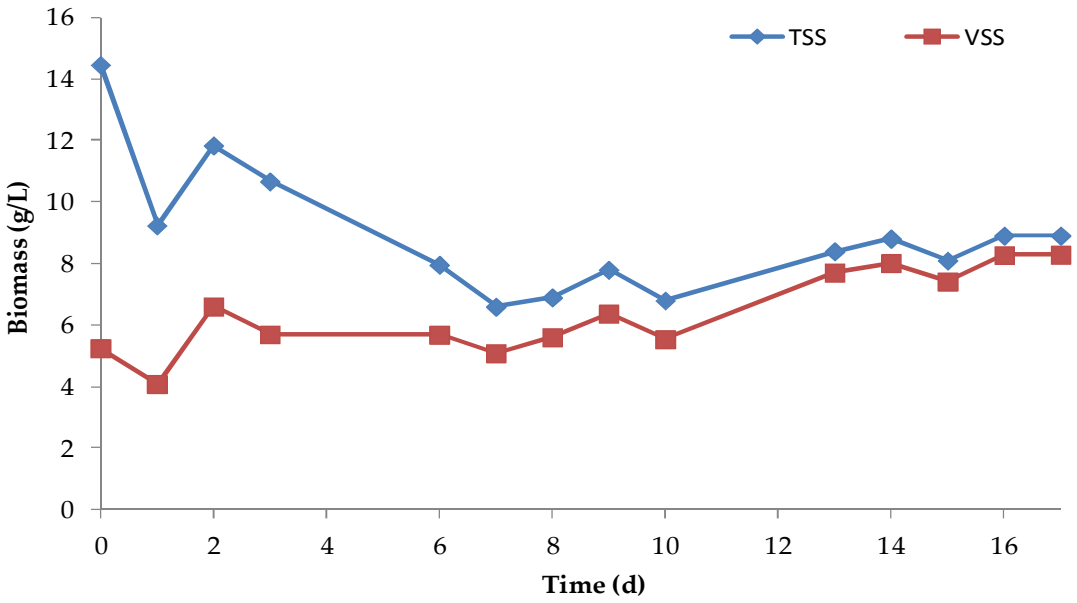

Figure 10 - Evolution of the concentration of TSS and VSS over a period of 17 days.

There were significant fluctuations of the concentration of biomass (measured as VSS) from day 0 to day 3. Upon inoculation, the concentration of biomass in the reactor was of 5.25 g VSS/L. This value was lowered than expected having into account other samples from the brewery WWTP, that usually ranged between 10 and 15 g VSS/L after being decanted. The loading rates had been planned for high cellular concentration and so they were excessive for this concentration of biomass. Probably as a result, the biomass concentration decreased to 4.09 g VSS/L on day 1. With the adjustment of the loading rates, there was a increase in VSS on day 2 to 6.61 g/L, followed by another decrease to 5.7 g VSS/L on day 3.

The concentration of biomass seemed to have stabilized between day 3 and day 6. By this time, it was verified that was possible to increase the VLR from 4.0 g $COD.L^{-1}.d^{-1}$ to 5.8 g $COD.L^{-1}.d^{-1}$, as feast was lasting only 1h30. Initially, this led to a small decrease in the biomass concentration on day 7 (5.09 g VSS/L), but then VSS gradually increased till 6.37 g/L on day 9.

On day 10 the VLR increased again, to 7.4 g $COD.L^{-1}.d^{-1}$. As previously, this led to a decrease in VSS on day 10 to 5.54 g/L. During the next days, till day 13, the

VLR gradually decreased to 5.1 g COD.L^{-1}.d^{-1}, since the volume in excess was not purged from the growth reactor and the same amount of COD was being fed each cycle. However, during this time biomass concentration increased by 44 % to 7.71 g VSS/L. The volume in excess was withdrawn from the reactor on day 14, increasing the VLR back to 7.4 g COD.L^{-1}.d^{-1}. This did not lead to a decrease in VSS this time, in fact it increased to a maximum of 8.29 g VSS/L on the last day.

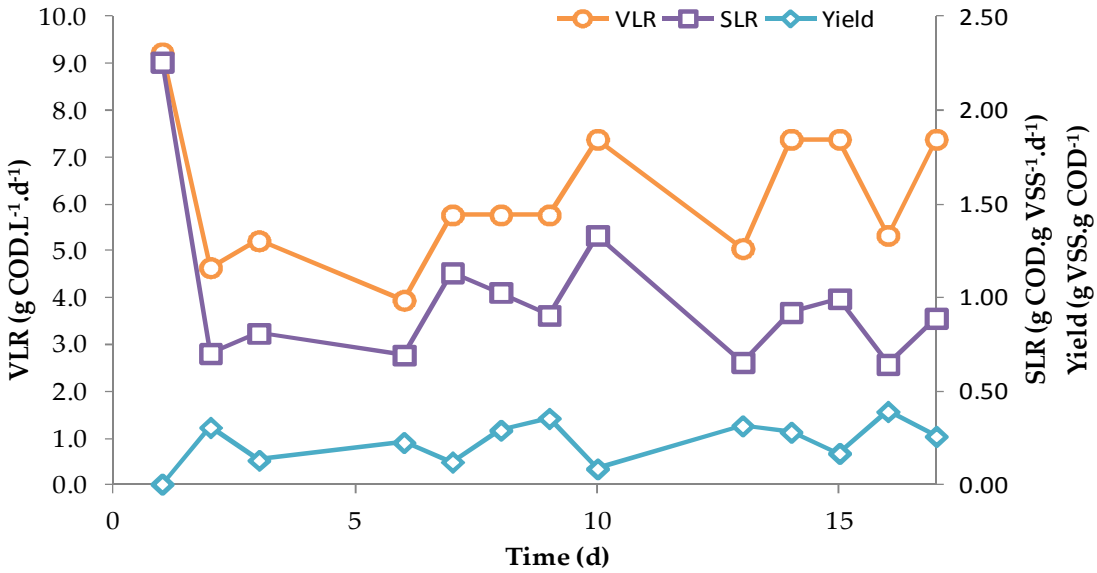

Figure 11 - Evolution of the Volumetric and Sludge Loading Rate over a period of 17 days

The decreases in the biomass concentration on days 7 and 10 may appear to be caused by some of required microbial adaptation to the increased VLR. However, this is probably not a direct consequence, but a result of the increased SLR. On day 7 and day 10, the SLR was of 1.13 g COD.g VSS^{-1}.d^{-1} and 1.33 g COD.g VSS^{-1}.d^{-1} respectively. This high SLR seemed to have caused metabolic inhibition thus slowing down growth and PHB accumulation. This would explain the decrease in VSS as a result of the dilution rate.

The observed yields seemed to support this idea, as they seem to vary in a opposite way regarding the SLR. This was already verified in the literature where higher loading rates, result in smaller yields [7]. During the time of the experiment the minimal VSS yield value was of 0.01 g VSS/g COD, on day 1 for a SLR of 2.26 g COD.g VSS^{-1}.d^{-1}, but this was before the loading rates where in fact regulated for the initial concentration of biomass. After this was carried out, the minimum VSS yield was 0.09 g VSS/ g COD (day 10, 1.33 g COD.g VSS^{-1}.d^{-1}) and the maximum VSS yield was 0.36 g VSS/ g COD (day 9, 0.91 g COD.g VSS^{-1}.d^{-1}). The average VSS

yield value was of 0.22 g VSS/ g COD. This observed VSS yield value falls short of the yield value of 0.43 g VSS/g COD, using fermented olive oil mill effluents as feedstock [25].

PHB was not quantified during this time. PHB quantification as SLB revealed to be inaccurate and an alternative method was still being developed (Chapter 7). So, to have a notion of how much carbon source was being used for growth or for the production of PHB, non-PHA cellular biomass (NPCB) growth was quantified stoichiometrically considering a chemical composition of $C_7H_{12}O_4N$ [3].

On day 13 a concentration of 2240 mg N/L as total soluble nitrogen was verified on the reactor, revealing that nitrogen was pilling up. Influent without a nitrogen source was used in the next 6 cycles and it was possible to observe that, regarding the consumption of nitrogen, approximately almost all carbon source had been converted to NPCB by the end of each cycle. This reveals that if PHB was been produced during feast, than it was being successfully used for growth during famine. On day 17, the concentration of nitrogen in the reactor by the end of famine was 0 g N/L, so it was possible to observe this in more detail in the next cycle. After the initial feed pulse, nitrogen was almost completely consumed after 90 minutes, while there was still 1044 mg acetate/L in the medium, tanking another 60 minutes to being consumed. This carbon source was most likely mainly converted into PHB. On the overall, during this cycle 43 % of the carbon source fed seemed to have been directed towards the production of NPCB, while the remaining 57% was used for cellular maintenance and the production of PHB.

5.2.1.2 A - Accumulation reactors

No accumulation tests were carried out with the sludge of the growth reactor A, due to limitation in the available material, as most of it was being used for the run of tests in parallel in others Avecom's R&D projects.

5.2.2 PHB production B

5.2.2.1 B - Growth reactor

Growth reactor B had similar operation conditions to those of growth reactor A. However, in this case nutrient excess conditions were imposed, by increasing the N/COD ratio. This was expected to act as a selective pressure in order to enhance growth and to select for bacteria capable of produce PHB efficiently, in a accumulation reactor, even in the presence of nutrients [7].

The reactor was maintained over a period of 31 days, after which it was stopped. The evolution of the biomass concentration in the reactor can be observed in Figure 12.

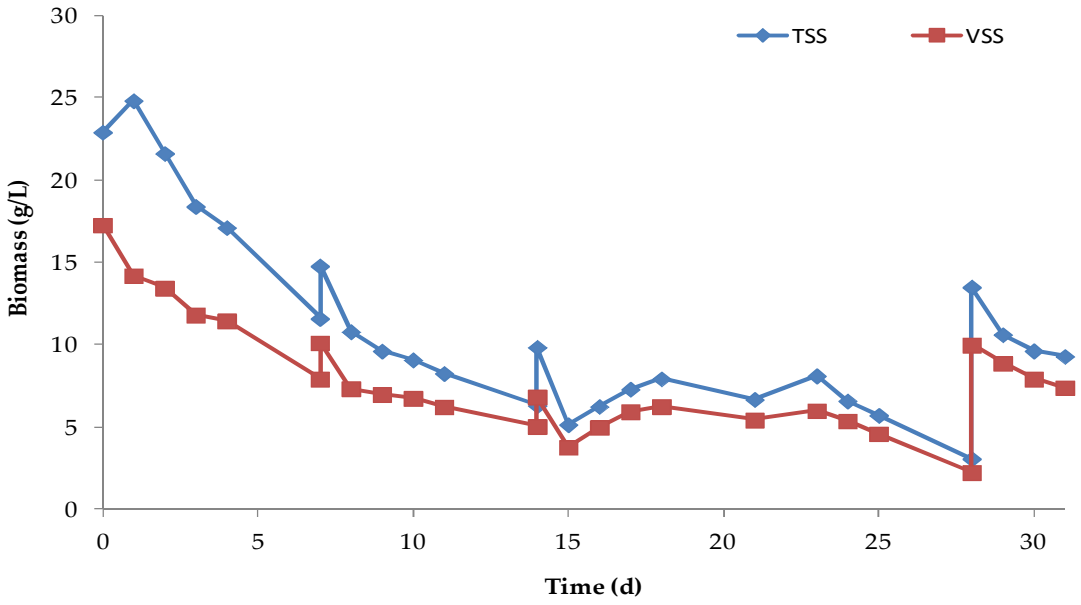

Figure 12 – TSS and VSS in the growth reactor over a period of 31 days

The concentration of TSS and VSS decreased considerably from 17.28 g VSS/L on day 0 to 5.16 g VSS/L on day 15. It was expected that the biomass recovered from growth reactor A would be dead and so it would be washed away with time. Thus, its concentration should be meaningless after day 5, as a result of the SRT of 4.33 days. Nevertheless, VSS continued to decrease at a similar rate even after this point. It was not predicted that the applied parameters would result in wash conditions, since a similar SRT had been used in growth reactor A. Hence, it was expected that the biomass concentration in the reactor would eventually stabilize, when the VSS concentration reached normal value for this type of systems.

On day 7 and 14 the biomass was recovered from the withdrawn mixed liquor by centrifugation and reinocculated back again in the growth reactor. These were attempts to maintain high cellular concentrations in the reactor. Yet, they proven to be useless as the biomass continued to decrease.

The VLR was regulated along the time in order to approach the SLR of values close to 1 g COD.g $VSS^{-1}.d^{-1}$. Their evolution during the experiment, as well as the yields, can be observed in Figure 13. The low SLR form day 0 to day 3 could help explain the decrease in biomass. Nevertheless, after this day the SLR value was high enough, so it was possible to prevent the biomass concentration from decreasing, which only happened after day 15. Thus, the decrease in biomass concentration appears to be caused by growth inhibition due to a limitation in oxygen, since the reactor was inoculated with a high concentration of biomass an high loading rates were applied. In fact, during this period, a DO of 0 mg O_2/L was measured during the entire time of feast. In oxygen-limiting conditions, the carbon source is diverted from the TCA cycle to other metabolic pathways, like those leading to the production of PHB, as described in Chapter 2.5.

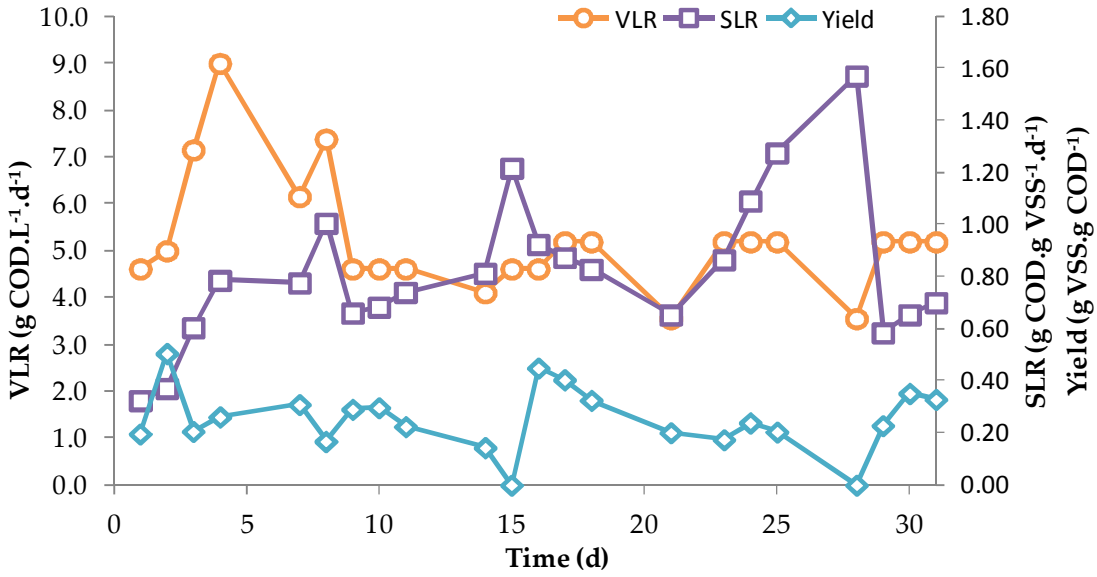

Figure 13 - Volumetric and sludge loading rates over a period of 32 days

Nevertheless, this might have had other consequences as unidentified white layer started to appear on the samples on day 9. Unlike the cells, this layer did not sediment when centrifuged for 10 minutes at 3,000 rpm. Nutrient analysis ere carried and it was verified that the total soluble nitrogen in the reactor was above 2 g N/L. In the impossibility of microorganisms to grow, nitrogen could not be

uptake and so it was pilling up. The white layer may be in fact exopolymer produced by bacteria as a stress mechanism, in order to protect them from this high concentrations of ammonium and other nitrogen compounds. This exopolymer most likely required a carbon substrate to synthesize and thus it may have hindered the capacity of the reactor to select for PHB-producing bacteria.

Several measures were taken in order to correct this situation. Firstly the COD concentration in the influent was reduced from 40 g COD/L to 25 g COD/L without neither a nitrogen nor a phosphorus source. This would diminish the concentration of phosphorus and nitrogen on the reactor, while the lower VLR would require a lower amount of oxygen for the acetate to be consumed. Furthermore, it was verified on that day that the feed with 40 g COD/L was tanking already taken 3h30 to be consumed. On day 11 the influent volume fed per cycle was split into 2 equal feed pulses instead of a only one. This way a lower amount of COD was fed per pulse, resulting in lower concentration of acetate in the medium, and requiring less oxygen for that given time.

The number of feed pulses was rearranged again on day 15, dividing the feed volume in 3 equal feed pulses, which reduced the feast phase for less than 2 hours. Thus, it was possible to gradually increase the loading rates over the next days. This was followed for an increase in the concentration of biomass from 3.79 g VSS/L on day 15 to 6.26 g VSS/L on day 18. This concentration was more or less maintained till day 23 (6.00 g VSS/L), decreasing almost linearly afterwards till a biomass concentration of 2.26 g VSS/L was achieved on day 28. During the period where the biomass concentration was relatively stable, the average observed yield was of 0.23 g VSS/g COD which is similar value to that observed for growth reactor. Nevertheless, during the phase where the concentration of VSS increased, this yield rises to an average value of 0.39 g VSS/g COD which is close to the yield value of 0.43 g VSS/g COD, using fermented olive oil mill effluents as feedstock, found in the literature [25].

On day 28 the biomass was recovered from the mixed liquor by centrifugation at 3.000 rpm for 10 minutes and re-incubated in the reactor with 2 L of fresh activated sludge. This was carried out in order to increase the biomass concentration and basically do a reset in the selection process for PHB-producing bacteria. A decrease in VSS was observed in the next few days, but it was not possible to verify if it would stabilize after sometime or duplicate the previous behaviour, as the reactor overflowed on day 31.

5.2.2.2 B - Accumulation reactors

Five different accumulation tests were carried out in fed-batch reactors. This time a time controlled feed pulse strategy was preferred over the pH-stat method. This way it is possible to define the amount of carbon source fed and when should it be fed, allowing a tighter control of the accumulation reactors.

The influents used were composed of acetic acid / sodium acetate and ammonium chloride in a COD/N ratio of 100/1 in days 2, 7 and 10 and of 100/2 in days 15 and 21 in order to test the capability of the sludge to accumulate PHB in the presence of this nutrient.

Table 8 - Batch accumulation test using acetic acid on day 2 of the growth reactor B

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	21.62	25.32
- VSS	g/L	13.45	16.38
- PHB	g/L	0.27	1.08
- NPCB	g/L	13.18	15.3
Ratio			
- PHB/VSS	%	2	7
- PHB/TSS	%	1	4
- VSS/TSS	%	62	65
Reactor Volume	mL	400	500
COD Fed	g	-	7.69
VLR	g COD/(L.d)	-	15.38
SLR	g COD/(g VSS.d)	-	0.94
Total biomass			
- TSS	g	8.65	12.66
- VSS	g	5.38	8.19
- PHB	g	0.11	0.54
- NPCB	g	5.27	7.65
Yield			
- VSS	g VSS/g COD	-	0.37
- PHB	g PHB/g COD	-	0.06
Productivity			
- r_P	g PHB/(L.h)	-	0.04
- q_P	g PHB/(g VSS.h)	-	0.00

There was a significant increase in the VSS concentration, with a yield of 0.37 g VSS/g COD. Hover, only 0.06 g PHB were produced per gram of COD. The initial PHB content of 2% (PHB/VSS) was quiet low for a sludge sample taken

from the growth reactor at the end of feast and its increase during the accumulation fed batch was meaningless.

As a result, another accumulation test was carried on day 7 and day 10.

Table 9 - Batch accumulation test using acetic acid on day 7 of the growth reactor B

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	14.79	14.76
- VSS	g/L	10.12	10.40
- PHB	g/L	0.72	0.96
- NPCB	g/L	9.40	9.44
Ratio			
- PHB/VSS	%	7	9
- PHB/TSS	%	5	7
- VSS/TSS	%	68	70
Reactor Volume	mL	500	600
COD Fed	G	-	4.94
VLR	g COD/(L.d)	-	8.23
SLR	g COD/(g VSS.d)	-	0.79
Total biomass			
- TSS	g	7.40	8.88
- VSS	g	5.06	6.24
- PHB	g	0.36	0.58
- NPCB	g	4.70	5.66
Yield			
- VSS	g VSS/g COD	-	0.24
- PHB	g PHB/g COD	-	0.04
Productivity			
- r_P	g PHB/(L.h)	-	0.02
- q_P	g PHB/(g VSS.h)	-	0.00

Table 10 - Batch accumulation test using acetic acid on day 10 of the growth reactor B

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	8.27	14.73
- VSS	g/L	6.75	9.95
- PHB	g/L	0.22	1.07
- NPCB	g/L	6.53	8.88
Ratio			
- PHB/VSS	%	3	11
- PHB/TSS	%	3	7
- VSS/TSS	%	82	68
Reactor Volume	mL	400	500
COD Fed	g	-	9.23

VLR	g COD/(L.d)	-	18.46
SLR	g COD/(g VSS.d)	-	1.85
Total biomass			
- TSS	g	3.31	7.37
- VSS	g	2.70	4.98
- PHB	g	0.09	0.54
- NPCB	g	2.61	4.44
Yield			
- VSS	g VSS/g COD	-	0.25
- PHB	g PHB/g COD	-	0.05
Productivity			
- r_P	g PHB/(L.h)	-	0.04
- q_P	g PHB/(g VSS.h)	-	0.00

The results of these test were similar to the first one, with the VSS increasing considerably, but not as a result of PHB storage. In the accumulation test started on day 10 it was possible to verify an increase in the "white layer" described in the growth reactor section. At that time the presumable exopolymer had not yet been associated with the high concentrations of nitrogen in the medium. The "exopolymer" and its amount before and after the accumulation test can be verified on Figure 14.

Figure 14 - Pellet of the mixed liquor sample of the growth reactor (left) and from the accumulation reactor (right) after centrifugation at 10,000 rpm for tem minutes.

This image shows that the increase of VSS is mostly due to the production of this "exopolymer". The results of the accumulation tests on days 15 and 21 were very similar to the ones above, since the cause of this stress mechanism was not corrected. The results of this accumulation tests can be verified on Appendix C.

5.2.3 PHB production C

5.2.3.1 C - Growth reactor

The evolution of the biomass concentration in the growth reactor A can be observed in Figure 15 while the VLR, SLR and biomass yield can be observed on Figure 16.

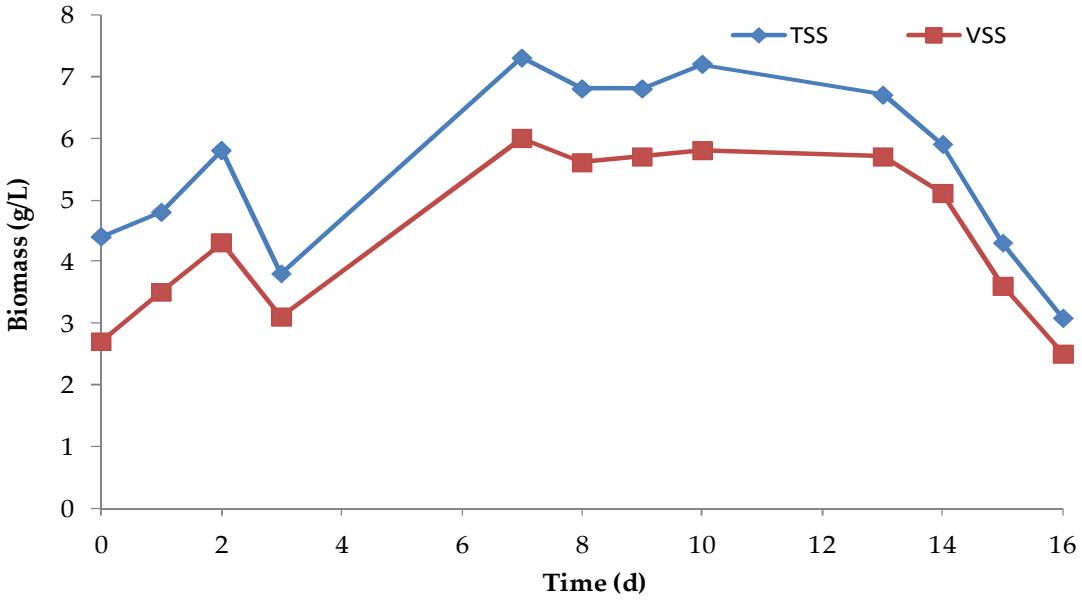

Figure 15 - Evolution of the concentration of TSS and VSS over a period of 15 days.

The reactor was inoculated with activated sludge having a biomass concentration of 2.7 g VSS/L. This value increased linearly over the first two days till a VSS concentration of 4.3 g/L on day 2. On day 1 it was verified that the feast phase was lasting for 3h30 and so the volumetric loading rate was lowered from 6 g $COD.L^{-1}.d^{-1}$ to 4.3 g $COD.L^{-1}.d^{-1}$.

VFA analysis on day 2 showed that the concentration of acetic acid on the medium at the end of each feed pulse was minimal, allowing the increase of the VLR to 6.3 g $COD.L^{-1}.d^{-1}$ for day 3. This resulted in a decrease in the biomass concentration, to 3.1 g VSS/L. Nevertheless, the VFA analysis clearly showed that the acetic acid was almost completely deployed at the end of each feed pulse. This behaviour, both on day 2 and day, 3 indicated that the acetate uptake rate was being equal to the feeding rate. Therefore, if the decrease in the VSS concentration on day 3 was caused by substrate inhibition in the first two cycles of the day, it

seemed that the microbial population managed to adapt to the imposed loading rates and the VLR was maintained.

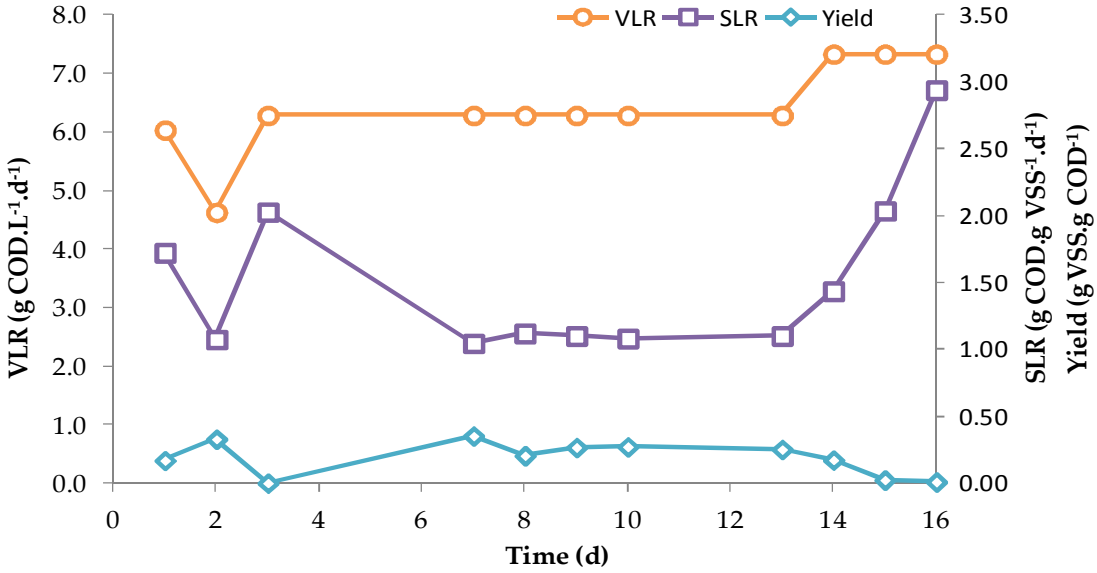

Figure 16 - Volumetric and sludge loading rates over a period of 15 days

There was a significant increase in VSS from day 3 to 7 with the biomass concentration almost doubling, from 3.1 g VSS/L to 6.0 g VSS/L. By maintaining the VLR it was possible to maintain a stable concentration of biomass in the reactor till day 13. Hence, stabilizing the SLR between 1.05 and 1.12 g COD.g $VSS^{-1}.d^{-1}$ and the yields at an average of 0.27 ± 0.05 g VSS/g COD. The uptake rates continued equal to the feed rate during this time.

From day 13 to day 14 the HRT and SRT was changed from 3.5 to 3.0 days, by increasing the volume of influent fed to reactor and consequently withdrawn from in, for 1.2 L to 1.5. No alterations were made to the influent concentration as so, as a result, this led to an increase in the VLR to 7.3 g $COD.L^{-1}.d^{-1}$. It was expected that by reducing the HRT and SRT growth would be estimulated, but the opposite happened. All this together led to a significant decrease in the biomass concentration, reaching a minimum value of 2.50 g VSS/L on the last day.

5.2.3.2 C - Accumulation reactors

The accumulation tests started on day 7, 8 and 9 used and influent with a COD/N/P ratio of 100/1/0 similar to the accumulation tests performed on growth

reactor. However, in this case there was not production of the "exopolymer" in the growth reactor and the concentration of nitrogen in the medium was more controlled. The importance of these tests was to assess the influence of a nitrogen source in the accumulation of PHB. Some articles state that presence of a small concentration of nitrogen, for example in a COD/N ratio of 100/1 enhances the production of PHB in pure cultures of *Cupriavidus necator*. This happened as nitrogen is required for the enzimes responsible for the polymerization of PHB [92]. Since mixed microbial cultures produce PHB mainly by an internal limitation mechanism [7], this should not compromise the results.

Table 11 - Batch accumulation test using acetic acid on day 7 of the growth reactor C - feed pulse

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	5.07	5.08
- VSS	g/L	4.31	4.67
- PHB	g/L	0.32	1.32
- NPCB	g/L	3.99	3.35
Ratio			
- PHB/VSS	%	7	28
- PHB/TSS	%	6	26
- VSS/TSS	%	85	92
Reactor Volume	mL	1000	1355
COD Fed	g	-	20.2
VLR	g COD/(L.d)	-	14.90
SLR	g COD/(g VSS.d)	-	3.19
Total biomass			
- TSS	g	5.07	6.88
- VSS	g	4.31	6.33
- PHB	g	0.32	1.79
- NPCB	g	3.99	4.54
Yield			
- VSS	g VSS/g COD	-	0.10
- PHB	g PHB/g COD	-	0.07
Productivity			
- r_P	g PHB/(L.h)	-	0.05
- q_P	g PHB/(g VSS.h)	-	0.01

The accumulation test was started with the biomass having a PHB content of 7 % (g PHB/g PHB) which is quiet low having into account the sludge was at the end of feast. This value increased to 28 % (g PHB/ g VSS) after 22 hours of accumulation which is a rather low value for a microbial population acclimated to ADF conditions. Both the yields and the productivity rates were also considerably

low. Another test was run in the next day exhibiting a similar behaviour, with even lower results Appendix C.

A final test still using an influent with a COD/N ratio of 100/1 was ran on day 9 (Table 12).

Table 12 - Batch accumulation test using acetic acid on day 9 of the growth reactor C - feed pulse

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	6.50	9.40
- VSS	g/L	5.50	8.46
- PHB	g/L	0.37	1.20
- NPCB	g/L	5.13	7.26
Ratio			
- PHB/VSS	%	7	14
- PHB/TSS	%	6	13
- VSS/TSS	%	85	90
Reactor Volume	mL	1000	1360
COD Fed	g	-	21.6
VLR	g COD/(L.d)	-	15.88
SLR	g COD/(g VSS.d)	-	1.88
Total biomass			
- TSS	g	6.50	12.78
- VSS	g	5.50	11.51
- PHB	g	0.37	1.63
- NPCB	g	5.13	9.87
Yield			
- VSS	g VSS/g COD	-	0.28
- PHB	g PHB/g COD	-	0.06
Productivity			
- r_P	g PHB/(L.h)	-	0.04
- q_P	g PHB/(g VSS.h)	-	0.01

The biggest difference in this test was the high VSS yield. Nevertheless, the production of "exopolymer" was not verified in any of these accumulation tests. The increase in VSS on this last test seemed to be a consequence of cellular growth, since nitrogen was consumed in a stoichiometric proportion.

These reveals that the selected microbial population has little tolerance for the production of PHB under nutrient excess conditions. This is may be caused by the short SRT, that may act as a strong selective pressure to growth, instead of a PHB accumulation. This may result in a big handicap in a industrial process using complex fermented stream, where there may be abundance of nutrients and short SRT are desired.

Due to this observation, on the following fill ups no nitrogen source was used on the influent, having acetic acid/acetate as the only nutrient. Two feeding strategies were applied during the several fill ups in order to compare their results. The first one was pH-stat method that was used to run a accumulation test on day 13 and other on day 14, where the influent was fed automatically to the reactor, every time the pH exceeded 8.2. This value was chosen as it was reported that the use of pH-stat at higher pH values resulted in a high PHB content [93]. While the other strategy consisted of time controlled feed pulse feeding, where the reactor was fed every 72 minutes (20 times per day) for 3 minutes, with a influent flow rate of 6.66 mL.

The results of the accumulation test using the pH-stat method on day 13 are present on Table 13.

Table 13 - Batch accumulation test using acetic acid on day 13 of the growth reactor C - pH stat strategy

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	6.4	4.07
- VSS	g/L	5.5	3.70
- PHB	g/L	0.40	0.97
- NPCB	g/L	5.10	2.73
Ratio			
- PHB/VSS	%	7	26
- PHB/TSS	%	6	24
- VSS/TSS	%	86	91
Reactor Volume	mL	1200	1300
COD Fed	g	-	6
VLR	g COD/(L.d)	-	4.62
SLR	g COD/(g VSS.d)	-	1.25
Total biomass			
- TSS	g	7.68	5.29
- VSS	g	6.60	4.81
- PHB	g	0.48	1.26
- NPCB	g	6.12	3.55
Yield			
- VSS	g VSS/g COD	-	0.00
- PHB	g PHB/g COD	-	0.13
Productivity			
- r_P	g PHB/(L.h)	-	0.03
- q_P	g PHB/(g VSS.h)	-	0.01

There was not a significant production of PHB nor a significant increase in the PHB content. This may be a consequence of the low VLR, since there was a

decrease in the total VSS, which may indicate cellular starvation, although the SLR was of 1.25 g COD.g $VSS^{-1}.d^{-1}$. This is one problem of this method, as the COD fed to the system cannot be imposed. As a result it may take a lot of time for the pH to reach the value where more influent will be added, as this is influenced by the pH of the influent and the buffer capacity of the reactor's medium.

Because of this on day 9, to fill ups were started in order to compare the feed pulse strategy with the ph-stat. In this day, the pH of the influent for the feed pulse strategy was set to 5.5, while for the pH-stat the influent was set at pH 5.0.

Table 14 - Batch accumulation test using acetic acid on day 14 of the growth reactor C - feed pulse strategy

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	5.73	4.92
- VSS	g/L	4.91	4.73
- PHB	g/L	0.17	1.87
- NPCB	g/L	4.74	2.86
Ratio			
- PHB/VSS	%	3	40
- PHB/TSS	%	3	38
- VSS/TSS	%	86	96
Reactor Volume	mL	600	1010
COD Fed	g	-	24.6
VLR	g COD/(L.d)	-	24.36
SLR	g COD/(g VSS.d)	-	5.15
Total biomass			
- TSS	g	3.44	4.97
- VSS	g	2.95	4.78
- PHB	g	0.10	1.89
- NPCB	g	2.844	2.89
Yield			
- VSS	g VSS/g COD	-	0.07
- PHB	g PHB/g COD	-	0.07
Productivity			
- r_P	g PHB/(L.h)	-	0.08
- q_P	g PHB/(g VSS.h)	-	0.03

Table 15 - Batch accumulation test using acetic acid on day 14 of the growth reactor C - pH stat strategy

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	5.73	6.18
- VSS	g/L	4.91	5.99
- PHB	g/L	0.17	2.27

- NPCB	g/L	4.74	3.72
Ratio			
- PHB/VSS	%	3	38
- PHB/TSS	%	3	37
- VSS/TSS	%	86	97
Reactor Volume	mL	600	975
COD Fed	g	-	22.5
VLR	g COD/(L.d)	-	23.08
SLR	g COD/(g VSS.d)	-	3.85
Total biomass			
- TSS	g	3.44	6.03
- VSS	g	2.95	5.84
- PHB	g	0.10	2.21
- NPCB	g	2.84	3.63
Yield			
- VSS	g VSS/g COD	-	0.13
- PHB	g PHB/g COD	-	0.09
Productivity			
- r_P	g PHB/(L.h)	-	0.10
- q_P	g PHB/(g VSS.h)	-	0.03

It is possible to observe that both methods exhibited similar results and very decent PHB contents were achieved. This proves that the pH of the fresh influent may have a tremendous impact on the performance of the accumulation reactor if a pH-stat strategy is used. Nevertheless, both accumulation tests revealed low yields and low productivity rates. This means that a lower amount of COD could have been used to feed the reactors and probably in a shorter fed batch run.

6 Production of PHB enriched SCP

6.1 Background

Single cell protein (SCP) is the use of microorganisms as a feed/food source of protein. This concept started to become popular in the 60s and 70s. However, changes in the economical landscape and mainly the resistance of the public to the product led to a significant decrease in the development and investments related to this technology.

Nowadays, there is a renewed interest in the production of microbial protein. SCP can be produced in a economical feasible way, by using waste materials as substrates. This can be used in animal feed or even in products for human consumption [94]. This way, costly conventional protein sources like soy- or fishmeal can be replaced as protein supplements. The use of waste as a negative cost substrate also benefits the economy of the process [95].

PHB enriched mixed cultures could be used as a protein source. This may seen contradictory, since the PHB is considered a lipid [4] and would therefore decrease the value of the biomass [96]. However, PHB has been proven to have a highly positive influence on the gastrointestinal health [97-99].

There are bacteria present in the intestine that can efficiently degrade PHB into butyrate [97, 98]. This can be effectively absorbed by the intestinal bacteria or by the host to be used as carbon source in their metabolism [98]. Studies performed with juvenile European seabass showed that partially feeding them with PHB enhances their growth [99]. Furthermore, other studies reveal that these compounds and the enriched intestinal bacterial community result in an increased resistance off the host to pathogenic agents [97, 98]. These factors are considerably relevant for the industry of aquaculture as it allows better productivities and to minimize the use of antibiotics for the control of diseases.

Given those reasons, it was considered of economical interest to develop a method to produce PHB enriched SCP for being used as fish feed.

6.2 Results and discussion

A method for the quick production of a large quantity of PHB enriched single cell protein was necessary in order to produce enough fish feed so it could be used in zootechnical trials. For that, it was defined that the PHB content should be at least 20 % of the VSS.

It was demonstrated in the literature that unacclimated activated sludge from WWTPs operated in SBR could store PHA if submitted to feed pulses of organic acids, even without previous selection [100]. This way it is not possible to achieve high PHB contents, which would endear significantly the downstream process if a pure product was desired, but allows high concentrations of biomass slightly increased in PHB. This process was therefore considered suitable for the desired goal, as it consisted in a simple way of producing PHB enriched single cell protein.

The results of a first fed-batch test are presented in Table 6.

Table 16 - Excessive feeding, fed-batch number 1

Parameter	Unit	0 h	22 h	Delta
Reactor volume	mL	6000	7650	1650
Biomass				
-TSS	g/L	14.03	13.22	-0.81
-VSS	g/L	8.91	8.84	-0.07
-PHB	g/L	1.27	1.88	+0.61
Ratio				
-VSS/TSS	%	63	67	+4
-PHB/TSS	%	9	14	+5
-PHB/VSS	%	14	21	+7
Acetic acid fed	g		99.0	
Acetic acid consumed	g		45.3	
Total biomass in the system				
-g TSS	g	84.2	101.1	+16.9
-g VSS	g	53.5	67.6	+14.1
-g PHB	g	7.7	14.4	+6.7
Yield (COD fed)				
-TSS/COD	g/g	-	0.17	-
-VSS/COD	g/g	-	0.14	-

-PHB/COD	g/g	-	0.07	-
Yield (COD consumed)				
-TSS/COD	g/g	-	0.37	-
-VSS/COD	g/g	-	0.31	-
-PHB/COD	g/g	-	0.15	-

There was an increase in the PHB content from 14 % to 21 % (PHB/VSS) which fulfills the main objective. This is value considerably lower than the PHB contents achieved with acclimated sludge, that can represent 50 % of the VSS or even higher. Nevertheless, this method allows that high biomass concentrations are easily obtained, which is not the case when activated sludge is submitted do ADF conditions, were a stable reactor operation is hard to maintain for high VSS values. On the other hand, this process has the disadvantage of having a low VSS/TSS ratio, while in the selection reactor this could easily achieve a value of 0.85. This high ash content (the difference between TSS and VSS) can be a barrier to the commercialization of this product as feed, both in terms of legislation and quality control.

The observed yield of 0.15 g PHB/g COD consumed is below the ones found in the literatures for the production of PHB by unacclimated activated sludge using volatile fatty acids, that range from 0.20 g PHB/ g COD to 0.28 g PHB / g COD. However, around 50 % of the acetic acid/acetate fed was not consumed. Excessive feeding of a carbon source was already discussed in the previous chapters as having a negative effect on the yields and even leading to inhibition of metabolic pathways. Therefore, it is rather probable that this could be increased with a better optimization of the process.

7 Development of a new method for PHB quantification

7.1 Background

The thermal instability of PHB during melt is a considerable drawback for its use as substitute to nonbiodegradable polymeric materials in commercial products [101]. PHB starts to degrade and volatilize at temperatures above 200 °C and more significantly after 230 °C [101, 102].

The procedure initially used at Avecom for the determination of the PHB content in the biomass was based in this characteristic. Dried biomass samples, in which the TSS was already determined, were submitted to a temperature of 250 °C for 2 hours in an incinerator. It was considered that this was sufficient to volatilize all PHB in the samples and that the weight loss was mainly due to this. This was a quick, practical and economic method to estimate the PHB content of the activated sludge samples. Nevertheless, this method was never carefully validated and with time it was possible to observe a huge standard deviation between replicas (results not shown). Different incineration times were tried in order to refine the method, but the increased reproducibility was followed by an increased inaccuracy of the determination, most likely due to the volatilization of the NPCB. Therefore, there was the need to implement another PHB quantification protocol at Avecom.

The quantification of PHB is a challenging procedure, since it is hampered by the complexity of the matrix and the relatively low content of the polymer, especially in activated sludge [103]. Currently, it is possible to quantify PHB by NMR [104, 105], mass-spectometry [106] or, more commonly, by gas chromatography [96, 107, 108].

The GC quantification is based on the acidic transesterification of PHB in the presence of a chlorinated solvent (simultaneous extraction and derivatization) followed by the analysis of the hydroxybutyrate alkyl-ester by GC [96, 107, 108]. The most common differences in this method are regarding the solvent used (chloroform, dichloroethane or dichloromethane), the transesterification alcohol (methanol or propanol) or in the reaction catalyst (sulfuric acid or HCl).

Although the high sensitivity and reproducibility of the method [109], its drawbacks meant a technically difficult implementation at Avecom. The method

is lengthy and requires the handling and disposal of harmful and highly pollutant solvents [103]. Furthermore, in some variations of the method PHB recovery is inefficient in the presence of water, while the acids and cellular debris in the sample cause premature GC column degradation. This can be avoided, but only by introducing sample lyophilisation and an additional purification step to the method [107], which increases the cost and complexity of the method.

Other quantification methods relying on HPLC or flow cytometry was not possible due to the lack of the required devices. Because of this there was a need to develop a quantification method to be used at Avecom. This method should be relatively cheap, not recur to chlorinated or other high pollutant solvents and preferably allow the quantification of PHB without its destruction.

The development of a method based on the extraction of PHB by propylene carbonate, fitted these three conditions. Unlike the chlorinate hydrocarbons, 1,2-propylene carbonate presents a low toxicity and a high boiling point (240 ºC). This way, it has a low vapor pressure at environmental temperatures and its evaporation to the environment is avoided. PHB is soluble in 1,2-propylene carbonate at temperatures higher than 100 °C even for short contact times, allowing its separation from the biomass. After this step, methanol or ethanol could be used in order to precipitate the dissolved PHB , that could then be recovered by filtration or centrifugation. The methanol or ethanol could be easily recovered afterwards by distillation, give the different boiling points. A PHA recover yield of 95% and a purity of 84% was reported for the extraction from *Cupriavidus necator* cells, without any pre-treatment. The extraction procedure was carried out at 130 ºC for 30 min [76, 77]. These results are comparable to the values obtained from chloroform extraction (94% yield and 98% purity) [9].

7.2 Results and discussion

The values of PHB quantificaiton by chloroform extraction and 1,2-propylene carbonate (PC) for two different samples and different replicas are compared in Table 17.

Table 17 - Comparison between the amount of PHA obtained wiht chloroform and propylene carbonate extraction

	Sample volume (mL)	Polyhydroxyalkanoates (g/L)	
		Chlorofom extraction	PC extraction
PHB Enriched Sludge	20	4.71 (±0.27)	6.50 (±0.34)
	10	5.82 (±1.66)	6.66 (±0.49)
	Average	5.27 (±1.17)	6.58 (±0.36)

	Sample volume (mL)	Polyhydroxyalkanoates (g/L)	
		Chlorofom extraction	PC extraction
Activated Sludge	20	0.53 (±0.10)	0.08 (±0.01)
	10	0.53 (±0.16)	0.14*
	Average	0.53 (±0.11)	0.10 (±0.04)

*Only one sample taken into account

These values were then converted to PHA content as part of TSS and as part of VSS and were compared with the values obtained by SLB quantification.

Table 18 - Comparison between the PHA/TSS and PHA/VSS ratios determined by chloroform and propylene carbonate extraction and by thermogravimetric analysis of SLB

	Ratio	Chloroform	Propylene Carbonate	SLB
PHB Enriched Sludge	PHA/TSS (±Δ)	28 %	35 % (+7%)	50 % (+ 22%)
	PHA/VSS (±Δ)	36 %	45 % (+ 9%)	64 % (+ 28%)
Activated sludge	PHA/TSS (±Δ)	2,2 %	0,4 % (-1.8 %)	26 % (+24 %)
	PHA/VSS (±Δ)	5,2 %	1.0 % (-4,2 %)	59 % (+54 %)

Chlorofom extraction is currently used as a standard method for PHA recovery in lab scale, so the values obtained with it were used as reference.

It is possible to verify in table 1 and 2 that the propylene carbonate extraction allowed to obtain a higher amount of PHA form the pilot fill up sludge than the

ones with chloroform. Furthermore, the method does not decrease its sensibility and reproducibility when different volumes of sample are used. Finally, but not least, the methanol used in this method allows the removal of other oils from the extracted PHA, so a higher purity is expected. This seems to be proven by the values obtained from the brewery sludge, where more biomass was extracted is chloroform.

8 Conclusions

The use of mixed microbial cultures for the production of PHB in reactors operated in ADF conditions was possible. However, relatively low biomass concentrations were achieved, even when it was possible to obtain values higher than the ones found in the literature.

The low biomass concentration is a limitation of the ADF conditions, since the loading rates are limited by the need of a famine phase. Excessively high loading rates seemed to have a negative impact on the performance of the reactor inhibiting growth and the production of PHB. This is clearly the main limitation regarding this type of system, for its appliance at an industrial scale. It was possible to achieve better values using the Hydrocell system than by conventional ADF conditions. However, the increased operation costs might not overcome the advantages.

The control of the feed by the levels of DO may play a key role in the development of new feeding patterns and in the control of this type of systems at an industrial scale.

The problems regarding the production of PHB under nitrogen excess conditions revealed that this process cannot be used with any type of wastewaters. As a result, their carefully selection must be carried out before the implementation of a full scale process.

8.1 Hydrocell

A unusual high biomass concentration for mixed microbial cultures submitted to ADF conditions was achieved. Furthermore, the biomass concentration of the reactor was stable during the time of the experiment, without major fluctuations in VSS, although the unstable HRT, SRT, VLR and SLR applied.

The specific growth rate of 0.09 h^{-1}, the maximum one observed in the Hydrocell's growth reactor, is in range with the data found in the literature. The maximum observed VSS yield was of 0.49 g VSS/g COD which was a very good result, although the average VSS yield was only of 0.28 g VSS/COD.

A good capability to store PHB using acetic acid as a carbon source was observed, with a maximum PHB content of 59 % of the VSS. Although the low productivity rates, this was achieved with PHB production yield of 0.30 g SLB/g

COD which was a very acceptable value. On the other hand, it was not possible to significantly produce PHB under autotrophic conditions on the accumulation tests.

8.2 Production of PHB by ADF

The growth reactors on this system were less stable than the one in the Hydrocell system, as the microbial population tended to decay with time.. Nevertheless it was possible to achieve a stable growth reactor during PHB production C, while stable conditions were imposed. This sensibility to the change of conditions may due to the different system used, to the different source of activated sludge or to both.

The biomass concentration of the growth reactor was similar to the values found on the literature. In fact, the concentration of biomass in growth reactor A was higher than the usual values, but the capability of microbial population to accumulate PHB was not verified. The maximum observed VSS yields were in range with the literature.

High biomass concentrations are technically difficult to obtain or to apply in growth reactors operated in ADF conditions, due to the limitations in the loading rates that can be applied. VLR must be regulated regarding the capability to provide oxygen to the medium, as a limitation in O2 result in lower uptake rates and in an increased feast phase. While the SLR must be high enough to maintain the concentration of biomass in the reactor, while higher values may inhibit growth and PHB production. A SLR value close to 1 g COD.g VSS^{-1}. d^{-1} seemed to be appropriated for the growth reactor. The observed VSS yield usually decrease when the SLR is increased, while they to increase when the SLR is decreased.

It is possible to increase the loading rates by splitting the feed volume through several feed pulses during feast phase, instead of a single one at the beginning of feast. This results in the decrease of the amount of COD present in the reactor at a given moment, thus requiring less oxygen to incorporate it. This can be controlled by measuring the levels of DO in the reactor's medium.

The use of time controlled feed pulses during accumulation tests revealed to be preferred over the pH-stat method, as it allows a direct control of the reactor and the imposition of defined conditions. On the other hand, the pH-stat method efficiency seemed to be highly influenced by the pH of the influent used, and most likely by the buffer capability of the accumulation reactor's medium also.

The presence of high amounts of ammonium seem to select for bacteria other than PHB-producing ones or to activate a stress response where an exopolymer seemed to be produced. This stress mechanism most likely diverted the consumed carbon source for other metabolic pathways rather than for PHB production

8.3 Production of PHB enriched SCP

It was possible to produce PHB enriched SCP using non acclimated activated sludge, by feeding a relatively high biomass concentration with acetic acid. This may constitute a simple and quick method to originate added value from wastewaters, as this PHB enriched biomass may have an interesting nutritional value.

8.4 Development of a new method for PHB quantification

A method for the quantification of PHB using 1,2-propylene carbonate was developed. This method was reproducibly and exhibited to be at least as accurate as the one using chloroform for the extraction of PHB from biomass, followed by gravimetric quantification.

9 Further work

The economics of the process must be determined in order to see if this process could in fact present advantages over the anaerobic treatment and in which circumstantiates. For this, distinct types of wastewater must be evaluate for the production of PHB.

It is necessary to develop a strategy that can be applied in production of PHB, as the control and optimization of this type of systems can be highly difficult. More studies focused on the use of DO in order to control the process must be carried out in the future.

New applications for PHB must be searched, as it can be more useful in other areas rather than competing with other types of plastic. In these other areas the price of PHB or PHB enriched sludge may be an advantage rather than a limitation.

A stronger proof of concept regarding the Hydrocell system and the production of PHB enriched SCP must be developed in order for these systems to become economically atractive.

The development of a quicker method for the quantification of PHB using non chlorinated solvents, preferably by GG or HPLC would be of great interest.

9.1 Hydrocell

The obtained results should be confirmed using a similar system. However, in that study thiourea should be used in order to avoid nitrification. This is necessary, as nitrifying bacteria are autotrophic [64] and the high biomass concentrations may result of that.

The characterization of the microbial population selected using this conditions would be of great interest in order to really understand the effect of having an electrolysis reactor coupled to the growth reactor and to confirm if hydrogen gas can indeed be used as a selective pressure for PHB production by mixed cultures.

Since no accumulation tests were carried out after day 10, it would be important to verify if the capacity of the bacteria to accumulate PHB would be affected by increasing the loading rates in the hydrocell's growth reactor.

9.2 Production of PHB by ADF

Tests using real wastewater efluents would be of great importance for the activities of Avecom. This can change greatly in compositions and so a strategy for the selection of PHB-producing bacteria capable of operate under different nutrient conditions would be desirable.

It is necessary to develop a strategy that can be applied in production of PHB, as the control and optimization of this type of systems can be highly difficult. More studies focused on the use of DO in order to control the process must be carried out in the future.

9.3 Production of PHB enriched SCP

The production process must be optimized, in order to achieve yields and productivity rates. The downstream of the process also need to be developed so a stable, sterilized product can be delivered.

This product must be more extensively characterized regarding the amount of protein and amino acid composition, the ribonucleic an deoxyribonucleic acids content, as well as carbohydrate and lipid content. Toxicity and contamination tests must be also carried out in order to access for the safety of this product.

The introduction of extra steps that could lead to the increase of the VSS/TSS ratio, maintaining the process simples and allowing high biomass concentrations and yields would be highly desirable.

9.4 Development of a new method for PHB quantification

The method should be further validated, as the use of other sources of activated sludge or the presence of certain nutrients could affect the reproducibility and accuracy of the method. The results should also be compared to those of more widely accepted methods like the protocols used for quantification using GC or HPLC.

Economical and feasibility studies regarding the use of 1,2-propylene carbonate as a solvent for the extraction and purification of PHB at industrial scale should be carried out in order to verify if they can be indeed used as a viable alternative to chlorinated solvents.

The development of GC or HPLC analysis protocol for the PHB quantification using 1,2-propylene carbonate, or other non-chlorinated solvent, in replacement of the chlorinated solvents used nowadays on those protocols would be very useful.

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APPENDIX

CONTENTS

Appendix A: Influents

A.1 Hydrocell

Table A.1 - Composition of the influents used on the Hydrocell's growth reactor

Day		1 - 16	17 - 20	21 - 25	26-30	31 - 32
COD	g/L	15	25	35	40	50
COD/N/P		100/5/1	100/5/1	100/6/1	100/5/1	100/5/1
CH_3COOH (80 %)	mL/L	18.75	18.75	18.75	18.75	18.75
$NaCH_3COO{\cdot}3H_2O$	g/L	-	23.05	46.10	56.83	79.33
Urea (40%)	ml/L	3.15	5.25	7.87	8.4	10.5
K_2HPO_4	g/L	3.44	3.44	3.44	3.44	11.46
Pepton	g/L	0.75	1.25	1.75	2.50	2.50
$MgSO_4{\cdot}7H_2O$	g/L	0.2685	0.4475	0.6265	0.7000	0.8750
Nutriflok	g/L	0.15	0.25	0.35	0.40	0.50

A.2 PHB production A

Table A.2 - Composition of the influents used on the growth reactor A of the PHB production by ADF

Day		1	2	3	4 - 9	10 - 17
COD	g/L	40	10	20	25	32
COD/N/P		100/6/2	100/0/1	100/6/2	100/6/2	100/6/2
CH_3COOH (80 %)	mL/L	23.36	12.5	18.7	14.6	18.69
$NaCH_3COO{\cdot}3H_2O$	g/L	27.80	-	9.2	17.375	22.24
NH_4Cl	g/L	9.325	-	3.82	5.87	7.51
Pepton	g/L	0.65	0.5	1	0.25	0.32
Nutriflok	g/L	0.39	0.1	0.2	0.25	0.32
$MgSO_4{\cdot}7H_2O$	g/L	0.6992	0.1750	0.3580	0.4370	0.5594
K_2HPO_4	g/L	2.2	0.229	1.124	1.375	1.76

A.3 PHB production B

Table A.3 - Composition of the influents used on the growth reactor B of the PHB production by ADF

Day		1	2	3	4	5 - 7
COD	g/L	20	25	31	39	39
COD/N/P		100/5.8/1	100/5.8/1	100/5.8/1	100/6.3/1	100/6.5/1
CH_3COOH (80 %)	mL/L	18.7	23.38	29.22	36.53	36.53
$NaCH_3COO{\cdot}3H_2O$	g/L	9.20	11.5	14.36	17.97	17.97
NH_4Cl	g/L	3.82	4.76	5.97	9.325	9.325
Pepton	g/L	1.00	1.25	1.56	-	0.65
Nutriflok	g/L	0.20	0.25	0.31	0.39	0.39
$MgSO_4{\cdot}7H_2O$	g/L	0.36	0.45	0.56	0.70	0.70
K_2HPO_4	g/L	1.12	1.41	1.76	2.2	2.2

Day		8	9	10 - 11	12 - 14
COD	g/L	32	20	20	26
COD/N/P		100/8/1.2	100/7.7/1.2	100/7.9/1.2	100/7.8/1.2
CH_3COOH (80 %)	mL/L	23.36	14.6	14.6	18.69
$NaCH_3COO{\cdot}3H_2O$	g/L	27.80	17.36	17.36	22.24
NH_4Cl	g/L	9.33	5.87	5.87	7.51
Pepton	g/L	0.65	-	0.25	0.32
Nutriflok	g/L	0.39	0.24	0.24	0.31
$MgSO_4{\cdot}7H_2O$	g/L	0.70	0.44	0.44	0.56
K_2HPO_4	g/L	2.2	1.38	1.38	1.76

Day		15	16	17 - 32	33 - 34
COD	g/L	20	20	22.5	22.5
COD/N/P		100/6.2/1.2	100/6.2/1.2	100/5/1	100/6.2/1.2
CH_3COOH (80 %)	mL/L	14.6	16.36	16.36	14.6
$NaCH_3COO{\cdot}3H_2O$	g/L	17.36	19.46	19.46	17.36
NH_4Cl	g/L	4.62	5.18	4.26	4.62
Pepton	g/L	0.25	0.28	0.23	0.25
Nutriflok	g/L	0.24	0.28	0.23	0.24
$MgSO_4{\cdot}7H_2O$	g/L	0.44	0.46	0.40	0.44

K_2HPO_4	g/L	1.38	1.54	1.27	1.38

A.4 PHB production C

Table A.4 - Composition of the influents used on the growth reactor C of the PHB production by ADF

Day		1	2 - 9	8	9 - 16
COD	g/L	60.4	22	22	22
COD/N/P		100/5/1	100/5/1	100/5/1	100/5/1
CH_3COOH (80 %)	mL/L	35.28	12.85	12.85	12.85
$NaCH_3COO{\cdot}3H_2O$	g/L	68.45	24.93	24.93	24.93
NH_4Cl	g/L	11.17	4.07	-	4.07
Pepton	g/L	0.60	0.22	-	0.22
Nutriflok	g/L	0.60	0.22	0.22	0.22
$MgSO_4{\cdot}7H_2O$	g/L	1.07	0.39	0.39	0.39
K_2HPO_4	g/L	3.40	1.24	-	1.24

Appendix B : Formulas

B.1 TSS concentration

TSS is the quotient between the weight difference of the crucibles, after being overnight at 105 °C and before being used, and the volume of the sample.

$$TSS(g/L) = \frac{Crucible_{105} - Crucible_{unused}}{SampleVolume} \quad \text{(Eq. 1)}$$

B.2 VSS concentration

VSS is the quotient between the weight difference of the crucibles after being overnight at 105 °C and after being for 2 hours at 250 °C, and the volume of the sample.

$$VSS(g/L) = \frac{Crucible_{105} - Crucible_{600}}{SampleVolume} \quad \text{(Eq. 2)}$$

B.3 SLB concentration

Stored lipidic content (SLB) is the quotient between the weight difference of the crucibles after being overnight at 105 °C and after being for 2 hours at 600 °C, and the volume of the sample.

$$SLB(g/L) = \frac{Crucible_{105} - Crucible_{250}}{SampleVolume} \quad \text{(Eq. 3)}$$

B.4 PHB concentration

PHB concentration is the amount (weight) of PHB extracted from a sample using 1,2-propylene carbonate, divided by the sample volume

$$SLB(g/L) = \frac{Extracted\ PHB}{SampleVolume} \quad \text{(Eq. 4)}$$

B.5 NPCB concentration

NPCB is calculated as the part of VSS that is not PHB

$$NPCB(g/L) = VSS - SLB = VSS - PHB \quad \text{(Eq. 5)}$$

B.6 VSS/TSS ratio

The VSS/TSS ratio was given by the quotient between the volatile suspended solids concentration and the total suspended solids concentration.

$$VSS/TSS\,(\%) = \frac{VSS}{TSS} * 100 \quad \text{(Eq. 6)}$$

B.7 PHB content

The lipid content was given by the quotient between the lipid concentration (SCO) and the volatile suspended solids concentration.

$$PHB\ Content\ (\%) = \frac{SLB}{VSS} * 100 = \frac{PHB}{VSS} * 100 \quad \text{(Eq. 7)}$$

B.8 Observed VSS yield

The sludge yield was given by the quotient between the volatile suspended solids concentration formed and the total amount added of COD.

$$Observed\ VSS\ yield = \frac{(VSS \times V)_t - (VSS \times V)_{t-1}}{COD\ consumed}(\%) \quad \text{(Eq. 8)}$$

B.9 SLB/PHB production yield

The SCO production was given by the quotient between the lipid concentration formed and the total amount added of COD.

$$PHB\ prodction\ yield = \frac{(PHB \times V)_t - (PHB \times V)_{t-1}}{COD\ consumed}(\%) \quad \text{(Eq. 9)}$$

B.10 Hydraulic retention time

The Hydraulic Retention Time (HRT) was given by the quotient between the volume of the reactor and the flow rate of the influent used.

$$HRT\ (t) = \frac{Active Volume}{Influent\ Flow\ Rate} \qquad \text{(Eq. 10)}$$

B.11 Sludge retention time

The sludge retention time (SRT) is given by equation . In cases where there is not a settling phase before the purging volume from the reactor or there in not recirculation of sludge the SRT is equal to the HRT.

$$SRT\ (t) = \frac{V \times X}{(Q - Q_W)X_e + Q_w \times X_r} \qquad \text{(Eq. 11)}$$

B.12 Volumetric loading rate

The Volumetric Loading Rate (VLR) was given by the product between the organic concentration of the influent and the flow rate of the influent used divided by the volume used in the reactor.

$$VLR\left(\frac{w\ COD}{V \times t}\right) = \frac{[Influent] \times Flow\ rate}{Volume} \qquad \text{(Eq. 12)}$$

B.13 Sludge loading rate

The Sludge Loading Rate was given by the quotient between organic loading rate and the and the volatile suspended solids concentration present in the reactor. Specific growth rate

$$SLR\left(\frac{w\ COD}{w\ VSS \times t}\right) = \frac{VLR}{VSS} \qquad \text{(Eq. 13)}$$

B.14 Specific growth rate

Specific growth rate (μ) is given by the growth as nitrogen consumption during time, divided by the NPCB

$$\mu\,(t^{-1}) = \frac{growth\ as\ nitrogen\ consumption}{NPCB \times t} \qquad \text{(Eq. 14)}$$

B.15 Growth as nitrogen consumption

Growth as nitrogen consumption is calculated regarding the consumption of nitrogen during two given times and using a considering the following chemical formula for NPCB [3].

$$NPCB = C_7H_{12}O_4N \qquad \text{(Eq. 15)}$$

Appendix C: Accumulation reactors

On the fill up of day 15 to day 16 the COD/N ratio was changed from 100/1 to 100/2. The reactor was operated in the hot room, since is described in the literature that a higher production of PHB is achieved with a temperature of 30 °C instead of 20 °C.

C.1 Accumulation reactor B

Table C1 - Batch accumulation test using acetic acid on day 15 of the growth reactor B

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	5.16	4.88
- VSS	g/L	3.79	3.87
- SLB	g/L	0.00	1.04
- NPCB	g/L	3.79	2.83
Ratio			
- SLB/VSS	%	0	27
- SLB/TSS	%	0	21
- VSS/TSS	%	73	79
Reactor Volume	mL	400	400
COD Fed	g	-	5.38
VLR	g COD/(L.d)	-	13.45
SLR	g COD/(g VSS.d)	-	3.47
Total biomass			
- TSS	g	2.06	1.95
- VSS	g	1.52	1.55
- SLB	g	0.00	0.42
- NPCB	g	1.52	1.13
Yield			
- VSS	g VSS/g COD	-	0.00
- SLB	g SLB/g COD	-	0.08
Productivity			
- r_P	g SLB/(L.h)	-	0.05
- q_P	g SLB/(g VSS.h)	-	0.02

The results obtained with this fill up were even worse than the ones of previous week. The heat inside the warm room, causes an excessive evaporation in the reactor, that may negatively affect its performance and the difference in temperature between the growth and the fill up reactor may be excessive for the bacteria.

The fill up of day 21 was carried outside the warm room. For this feel up, the reactor every 72 minutes (it was intended to feed the reactor every 60 seconds, but the timer used to control the feed pump doesn't allow more than 20 programs). For the second approach it was necessary to increase the volume fed per day from 100 mL to 125 mL.

The fed solution was composed only by acetic acid/acetate and ammonium chloride in a COD/N ratio of 100/2. This solution was prepared so the volumetric loading rate in the fill up reactor would be eight thirds (8/3) of the volumetric loading rate of the growth reactor.

Table C2 - Batch accumulation test using acetic acid on day 21 of the growth reactor (inoc 2)

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	7.14	7.65
- VSS	g/L	5.78	6.43
- SLB	g/L	0.04	0.13
- NPCB	g/L	5.74	6.30
Ratio			
- SLB/VSS	%	1	2
- SLB/TSS	%	1	2
- VSS/TSS	%	81	84
Reactor Volume	L	400	525
COD Fed	g	-	10.34
VLR	g COD/(L.d)	-	19.70
SLR	g COD/(g VSS.d)	-	3.06
Total biomass			
- TSS	g	2.86	4.02
- VSS	g	2.31	3.38
- SLB	g	0.02	0.07
- NPCB	g/	2.30	3.30
Yield			
- VSS	g VSS/g COD	-	0.10
- SLB	g SLB/g COD	-	0.00
Productivity			
- r_P	g SLB/(L.h)	-	0.00
- q_P	g SLB/(g VSS.h)	-	0.00

C.2 Accumulation reactor C

An additional accumulation tests to the ones shown on Chapter 5.3.2.3.2 C - Accumulation reactors. The results of that accumulation test are exhibited on Table 14.

Table 19 - Batch accumulation test using acetic acid on day 8 of the growth reactor (inoc 3) - feed pulse

Parameter	***Unit***		
Time	h	0	22
Biomass concentration			
- TSS	g/L	7.55	7.01
- VSS	g/L	7.48	6.83
- PHB	g/L	0.42	0.95
- NPCB	g/L	7.06	5.88
Ratio			
- SLB/VSS	%	6	14
- SLB/TSS	%	6	14
- VSS/TSS	%	99	97
Reactor Volume	mL	1000	1340
COD Fed	g	-	20.4
VLR	g COD/(L.d)	-	15.22
SLR	g COD/(g VSS.d)	-	2.23
Total biomass			
- TSS	g	7.55	9.39
- VSS	g	7.48	9.15
- SLB	g	0.42	1.27
- NPCB	g	7.06	7.88
Yield			
- VSS	g VSS/g COD	-	0.08
- SLB	g SLB/g COD	-	0.04
Productivity			
- r_P	g SLB/(L.h)	-	0.03
- q_P	g SLB/(g VSS.h)	-	0.00

The results were very similar to those already showed, without further comments to add.

Appendix D: PHB Quantification - Standard Operation Protocol

D.1 Background

Polyhydroxybutyrate (PHB) is a type of endo-polymer synthesized by bacteria. It quantification is usually a complicated process that requires highly acidic conditions and temperature in order to hydrolyze the polymer chain. After that, the monomers are further derivate to a compound possible to quantify through gas or high pressure liquid chromatography.

Alternatively, PHB can be extracted and purified from the remaining biomass. However, this polymer is not soluble in most solvents and its susceptible to hydrolysis, both on acid and alkaline conditions, makes its separation from the cellular debris a difficult task. Chlorinated solvents are commonly used in lab scale to extract and purify PHB. Nevertheless these solvents are highly pollutant, toxic and even carcinogenic, so their use is highly undesirable.

In this protocol 1,2-propylene carbonate is used as an effective way to extract and purify PHB, without the drawbacks of chlorinated compounds. These solvent requires a temperature higher than 100 °C in order to achieve an efficient solubilization of the PHB. However, its high boiling point (242 °C), low vapor pressure at room temperature and non toxicity makes it a very easy solvent to work with.

This protocol consists of three steps, namely 1) cellular lyses and release of the PHB chain; 2) solubilization of PHB and separation from the cellular debris; 3) recover and quantification of the PHB. The complete procedure is described below:

D.2 Cellular lysis

D.2.1 Material needed:

a) Hypochlorite solution (bleach) with 6% of active chlorine (20°);
b) Falcon tubes (one per sample)

c) 20 mL sampling pipettes and pipette filler
d) Centrifuge

D.2.2 Method:

a) Withdrawn 20 mL of sample and centrifuge it for 10 minutes at 10,000 rpm.
b) Discard the supernatant and resuspend the biomass in 20 mL of a hypochlorite solution 20° (6% of active chlorine).
c) After 5 minutes, add 1 mL of H_2SO_4 1M in order to neutralize the pH of the solution. This will protect the PHB from being hydrolyzed. The addition of acid may originate chlorine gas and thus should be done inside a fume hood.
d) Immediately centrifuge for 10 minutes at 10,000 rpm. Discard the supernatant and rinse it with demineralized water. Centrifuge one more time for 10 minutes at 10,000 rpm and discard the supernatant.

D.3 Dissolution of PHB and separation from the cellular debris

D.3.1 Material needed:

a) 1,2-propylene carbonate (40 mL per sample)
b) Destruction tubes (1 per sample + 2 extra ones)
c) Heating plaque for destruction tubes at 130 °C
d) Glass vacuum filtration system
e) Fiber glass filters without binding agents, pore size of 0.47 μm
f) Cold methanol (~ 170 mL per sample)
g) 100 mL cylinder
h) 500 mL Schott bottles with stopper (1 per sample)
i) Tweezers

D.3.2 Method:

a) Resuspend the lysed biomass in 20 mL of 1,2-propylene carbonate and pour it to a destruction tube.

b) In a clean tube, add 20 mL of 1,2-propylene carbonate. This tube, with solvent only, will be used to warm the filtering system before filtering the samples. This will avoid PHB precipitation due to the cool down of the solvent.
c) In another clean tube without biomass, add 10 mL of propylene-carbonate per each one of the samples. This will be used to wash the destruction tube and filter after each filtration.
d) Warm all the tubes at 130 °C for at least 15 minutes, shaking the samples every 5 minutes. The samples should remain at 130 °C till they are filtrated, in order to avoid PHB precipitation.
e) In the meanwhile, prepare the vacuum filtration system.
f) After warming the samples for 15 minutes, use the extra tube with solvent (prepared on step b) to warm up the filtering system, in order to avoid PHB precipitation and clogging of the filter.
g) Take the solvent from the filtering erlenmeyer flask back to the destruction tube and put it back in the heating plaque. This may be useful later on if more hot solvent is necessary.
h) Filter one sample and let it be filtered completely. Use 10 mL of hot solvent to wash the sample tube and pour it on the top of the filter.
i) Recover the liquid in the filtration erlenmeyer to a Schott bottle and rinse the flask with cold methanol (-20 °C). Pour that methanol to the Schott bottle as well, till a final volume of ~ 200 mL. Methanol is added as an anti-solvent, thus precipitating the PHB in the Schott bottles.
j) Repeat steps “h” and ”i” for the other samples still in the heating plaque. Replace the fiber glass filter every time that necessary.
k) Let the content in Schott bottles to rest overnight.

D.4 PHB recovery

D.4.1 Material:

a) Vacuum filtration system
b) Drying oven at 105 °C
c) Freezer

d) (Pre- weighted, unused) fiber glass filters without binding agents, pore size of 0.47 μm (1 per sample)
e) Petri dishes (1 per sample)
f) Methanol (-20 °C)

D.4.2 Method:

a) Put unused fiber glass filters in Petri dishes and dry them overnight at 105 °C in order to remove any water.
b) Weight the filters after cooling down and register the value.
c) Use these filters to recover the precipitated PHB in the Schott bottles.
d) The bottle and filter should be washed with an additional methanol in order not to lose any PHB and to remove any trace of propylene carbonate.
e) Dry the filters on their respective Petri dish in the oven. After cooling down, weight them again.
f) Calculate the concentration of PHB in the sludge sample.