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Licenciada em Biologia

Investigation of the Regulation Mechanisms for Bioplastics Production from Industrial Residues

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Sumário

A actual elevada procura de plásticos tornou-se insustentável. Polihidroxialcanoatos são biopolímeros que podem, potencialmente, substituir os plásticos devido a: variedade de aplicações; biodegradabilidade; utilização de recursos renováveis como substrato. Os custos elevados de produção de polihidroxialcanoatos actuais podem ser reduzidos com a aplicação de culturas mistas de organismos. Lamas activadas de plantas de tratamento de águas residuais são seleccionadas para produção de polihidroxialcanoatos pela imposição de ciclos de alimentação intermitente.

Neste estudo, a aclimatização de lamas activadas usando ácidos gordos voláteis (VFAs) sintéticos como substrato resultou numa cultura rica em *Paracoccus* spp. e bactéria filamentosas não identificadas.

Podem utilizar-se substratos de baixo custo como melaço de cana-de-açúcar (SM) ou soro de leite (CW) para uma maior redução de custos. Isto requer um passo adicional antes da selecção microbiana para fermentação dos resíduos em VFAs.

Neste trabalho, mudou-se o substrato de SM para CW. A população alimentada com SM era rica em Actinomycetaceae, enquanto a população alimentada com CW era rica em Streptococcaceae, afectando a composição em VFAs. Consequentemente a população acumuladora e o polímero foram afectados. Na fase com SM fermentado (FSM) a população era rica em Azaorcus (41.5 - 64.6%) e na fase com fCW era mais diversa.

Alterar o pH no reactor de fermentação também afectou o passo da selecção com o aumento em *Thauera* e *Azoarcus* e diminuição de *Paracoccus*. Observou-se uma população não identificada significativa de bactérias formadoras de colónias espalmadas.

Em último lugar, investigou-se a ocorrência de comunicação célula-a-célula (QS). Possivelmente, moléculas de QS foram detectadas aquando da depleção da fonte de carbono.

Todos os passos da produção de polihidroxialcanoatos estão interligados e para a sua optimização todos as fases devem ser estudadas e melhoradas. Ainda, se QS estiver envolvido na produção de polihidroxialcanoatos, a aplicação de moléculas de QS no processo pode ser explorada.

Palavras-chave: Polihidroxialcanoatos; culturas mistas de organismos; dinâmica de populações; Fluorescence *in situ* Hybridisation; Quorum sensing

Abstract

The current high demand for plastics has become unsustainable. Polyhydroxyalkanoates are biopolymers stored by bacteria that can potentially replace modern plastics due to: wide range of applications; biodegradability; use of renewable resources as feedstock. High costs of current Polyhydroxyalkanoates production can be reduced using mixed cultures of organisms. Activated sludge from wastewater treatment plants is selected for Polyhydroxyalkanoates production through the imposition of cycles of intermittent feeding.

In this study, the acclimation of activated sludge using synthetic volatile fatty acids (VFAs) as substrate resulted in a culture rich in *Paracoccus* spp. and unidentified filamentous bacteria.

Low cost substrates such as sugarcane molasses (SM) or cheese whey (CW) can be employed as feedstock for further cost reduction. This requires an additional step before the microbial selection to ferment the feedstock into VFAs.

In this work, the feedstock was changed from SM to CW. The population fed with SM was rich in Actinomycetaceae, while the population fed with CW was rich in Streptococcaceae, affecting the VFA composition. Consequently, the PHA-storing population and the polymer were affected. In the fermented SM (fSM) phase, the population was rich in *Azoarcus* (41.5 - 64.6%) and in the fCW phase the population was more diverse.

Changing the pH in the fermentation reactor also affected the selection stage with an increase in *Thauera* and *Azoarcus* and a decrease in *Paracoccus*. A significant unidentified population of one layer sheet- forming bacteria was observed.

Lastly, the occurrence of cell-to-cell communication (QS) in the selection stage was investigated. Possibly, QS molecules were detected when the carbon source was depleted.

All steps of polyhydroxyalkanoate production are interconnected and for optimization, all stages must be studied and improved. Moreover, if QS proves to be involved in polyhydroxyalkanoate storage, the addition of QS molecules to the process may be explored for further optimization.

Keywords: Polyhydroxyalkanoates; mixed microbial cultures; population dynamics; Fluorescence *in situ* Hybridisation; Quorum sensing

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List of Abbreviations

3-oxo-C8-HSL - 3-oxo-octanoyl-L-homoserine lactone

ADF – Aerobic dynamic feeding

AHLs - Acylated homoserine lactones

AHT - N-acylhomocysteine thiolactone

CO_2 - Carbon dioxide

CSTR - Continuous stirred tank reactor

CW – Cheese whey

DGGE - Denaturing gradient gel electrophoresis

EBPR - Enhanced biological phosphorus removal

fCW – Fermented cheese whey

FF – Feast and famine

FISH – Fluorescence *in situ* hybridisation

fSM – Fermented sugarcane molasses

GAOs – Glycogen accumulating organisms

HHL - N-hexanoyl-L-Homoserine lactone

HPLC - High performance liquid chromatography

HRT – Hydraulic retention time

LB - Lysogeny broth

MBR – Membrane bioreactor

MCL - Medium-chain-length

MMC – Mixed microbial cultures

MS - Mass spectrometry

OLR – Organic loading rate

OTUs - Operational taxonomic units

P(3HB) – Poly(3-hydroxybutyrate)

P(3HB-co-HHx) - poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

P(3HB-co-HV) – Poly(3-hydroxybutyrate-co-hydroxyvalerate)

PHB – Polyhydroxybutyrate

PAOs – Phosphorus accumulating organisms

PBS - Phosphate buffered saline

PE – Polyethylene

PFA - Paraformaldehyde

PHA – Polyhydroxyalkanoates

poly-P – Polyphosphate

PP – Polypropylene

PS – Polystyrene

QS – Quorum sensing

SBR – Sequencing batch reactor

SCL - Short-chain-length

SM – Sugarcane molasses

SRT – Sludge retention time

VFAs – Volatile fatty acids

WWTP – Wastewater treatment plant

Chapter 1

General Introduction

1.1. Motivation

The wide usage of polymers is recent and it has been increasing heavily in our society during the last century (see Figure 1.1)[1], its production becoming the fastest growing one among bulk materials. They are also slowly becoming substitutes for other bulk materials, such as glass and, to a lesser extent, steel.[2] Plastics have a very wide range of applications due to their desirable characteristics namely durability and resistance to degradation. Nowadays, they are immensely involved in our daily lives in packages, technological accessories such as computers and mobile phones, and other appliances. Most of these applications are of the disposable kind.

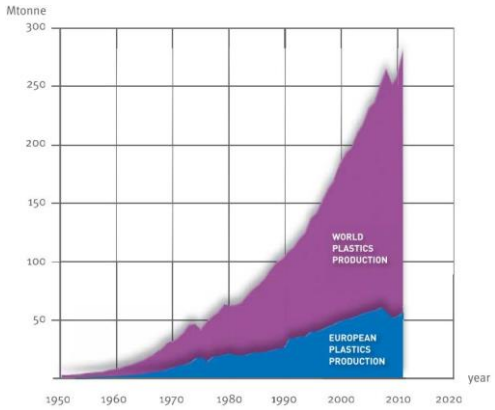


Figure 1.1 – World plastic production since 1950. Adapted from PlasticsEurope report, 2012[1]

But, like most things in our modern society, plastics depend on fossil fuels as a resource. These non-renewable resources are diminishing and will soon be depleted.[3] It is estimated that plastic production growth rate slows down by 2020 unless better alternatives for these polymers are found.[2] Together with this depletion, the rising dependence on fossil fuels as a resource has also raised environmental concerns regarding high carbon dioxide (CO_2) emissions that led to climate changes.

On a short-term scale, we have the resource depletion that affects us directly, during this life-time. On the other hand, and on a longer-term scale, there are the environmental issues derived from the plastic production industry. It may not affect us directly in this life-time, but will affect near-future generations. The pollution resultant from this industry comes both from the manufacturing process (greenhouse gas emissions)[4] and the waste resultant post-usage. As a result from the very large amounts of plastic used for short-term or disposable applications plastic waste can occupy a high volume fraction in municipal landfills[5]. Plastic waste is either incinerated or recycled or is disposed of into landfills or the marine environment[6]. Incineration generates toxic waste[7–9] and sorting is difficult due to the large variety of plastics used and the consequent variety of treatment processes[7,8]. Despite this, in Europe the recovery rate from recycling and energy recovery reached almost 60% in 2011.[1] But it is in the marine environment where they cause most damage because although they aren't degradable, they break into small polymers with very high recalcitrance to biological degradation[10] and while doing so they release hazardous chemical substances. They can also be ingested by marine life and eventually end up in our organisms.

To tackle this issue, it is essential to think of an alternative that covers both the diminishing resources and the non-biodegradability of plastics. We need biodegradable polymers synthesised from renewable feedstock.

1.2. Biopolymers - A New Hope

"Biobased polymers" are all naturally occurring polymeric materials that have been chemically and/or biologically polymerized into high molecular weight materials[3], meaning polymers derived from renewable resources.[10] All naturally occurring carbon-based polymers are biodegradable, but the same does not apply to all bioplastics based on naturally occurring monomers because their biodegradability can be lost due to chemical modification and polymerization.[2]

Based on the processes involved and the type of polymer, the production methods of biobased and biodegradable polymers can be divided into three main groups, one being the modification of natural polymers, as happens with starch and cellulose, another being the chemical polymerization of monomers derived from biological processes, as is the case of poly(lactic acid) (PLA)[11], and the last being the direct biosynthesis of polymers in microorganisms, such as in polyhydroxyalkanoates (PHAs)[11]. The last two have been the most studied materials.[2,3]

Starch is a major polysaccharide in higher plants and is available in abundance in nature. Starch polymers are biodegradable and incinerable and can be fabricated into finished products through existing technology. And even though the technologies for the processing of native starch are already well established and successfully commercialized[3], they still involve major physical and chemical reactions, using solvents and high temperature and pressure[12].

PLA is a crystalline, thermoplastic polyester with potential areas of application that include packaging and containers, agricultural and civil engineering materials as well as composting materials.[3] A patent has been developed for a low-cost continuous process for the production of lactic acid-based polymers through lactide formation[13] allowing for PLA production for bulk applications.[2,3]

Both PLA and PHA have mechanical properties that resemble those of commodity plastics such as polyethylene (PE), polystyrene (PS) and polypropylene (PP) and both can be produced from renewable biobased resources.[8,14,15] Since PLA has been available in large quantity, its application research is ahead of PHA's.[14] But while PHA is completely degradable under a wide range of conditions,[16,17] PLA hydrolytic degradation needs to be initiated by relatively high temperatures, at around 608°C.[3] Besides, biological processes produce less or no residues, originate less CO_2 emissions, less water consumption and less energy consumption which lead to lower costs and environmental impact.

1.3. Polyhydroxyalkanoates

Polyhydroxyalkanoates started receiving serious attention as a natural substitute for synthetic plastics during the petroleum crisis in the 1970s which served as a wake-up call to the fact that petroleum is a non-renewable resource that will one day be exhausted[2,18]. With the increasing reliance on petroleum, research on PHAs has also continued with an encouraging rythm.[3]

1.3.1. PHA Structure

PHAs are naturally occurring biodegradable polymers that are synthesized by bacteria under unbalanced growth conditions[15,19], usually when exhaustion of a single nutrient such as nitrogen limits growth in the presence of excess of carbon source.[20,21] Deposition occurs in the form of granules[22] (shown in Figure 1.2) of materials that serve as specialized bacterial reserves of carbon and/or energy. These granules are subsequently consumed by the cells when necessary.[21]

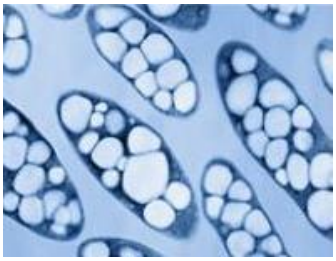


Figure 1.2 – PHA granules inside bacterial cells.[23]

Poly(3-hydroxybutyrate) (P(3HB)) is the most common PHA and was first described in 1926 in *Bacillus megaterium*[24]. Since then the ability to store PHAs has been proved to be a widely spread phylogenetic characteristic, being present in Archaebacteria[25], Gram positive[16,24,26] and Gram negative bacteria[26–28] and photosynthetic bacteria[29]. Most of the known polyhydroxyalkanoates are polymers of 3-hydroxyacids[22] and their general formula is presented below in Figure 1.3. The composition of the side chain or atom R and value of x determine together the identity of a monomer unit.[30] Currently, over 150 different monomer constituents of PHA are known.[31]

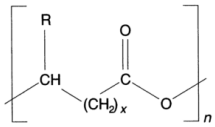


Figure 1.3 - General formula of polyhydroxyalkanoates.[30]

According to the constitution of the monomers, PHAs can be divided into two major groups: short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs.[15,16] SCL-PHAs are comprised of five or less carbon atoms in a monomer and have a high degree of crystallinity. MCL-PHA's have Monomers with 6 to 14 carbon atoms and are elastic materials with a low degree of crystallinity and a low melting temperature.[20]

1.3.2. PHA Metabolism

There are four major pathways that supply the hydroxyalkanoate monomers for PHA synthesis as shown in Figure 1.4.[18] The pathway for the biosynthesis of PHB is pathway I[32] and is considered the simplest.[33] It consists of three enzymatic reactions catalyzed by three distinct enzymes:[16,33,34]

- In the first reaction, two acetyl coenzyme A (acetyl-CoA) molecules are condensed to form acetoacetyl-CoA by β-ketothiolase (encoded by the *phaA* gene).

- Then follows the reduction of the previously formed acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by the *phaB* gene).
- Lastly, the polymerization of the (R)-3-hydroxybutyryl-CoA monomers occurs, catalyzed by PHA synthase (encoded by the *phaC* gene).

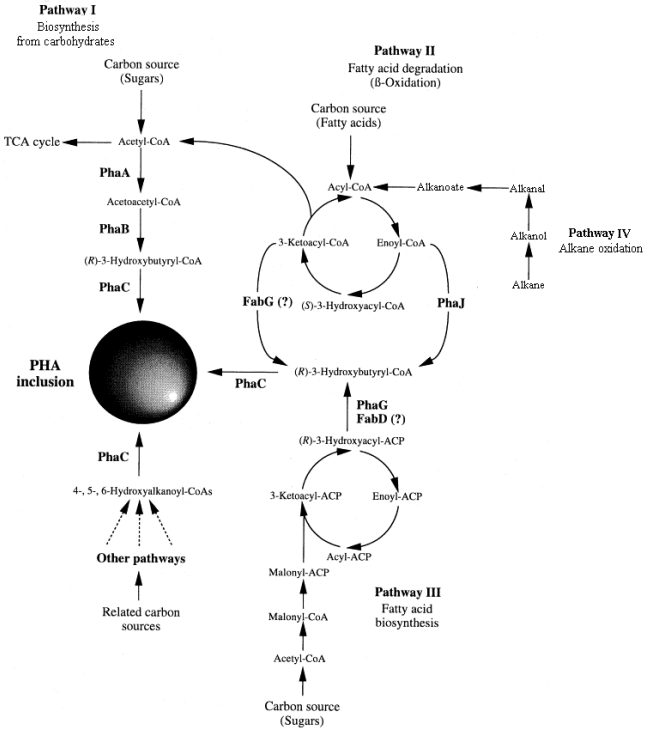


Figure 1.4 – Four major metabolic pathways that lead to the formation of the PHA monomers. Adapted from Sudesh et al., 2000[32] and Luengo et al., 2003[18].

This pathway has, until now, been shown to create only SCL-PHAs. Pathway II produces MCL-PHA from the intermediates of fatty-acid β-oxidation pathway, as pathway IV does from alkane oxidation[7]. The monomers formed through pathway III derive from the de novo fatty acid biosynthesis pathway.[32] Other enzymes involved in PHA accumulation include phasins (PhaP), which are part of the PHA inclusions and are thought to be involved in their formation, and intracellular PHA depolymerase (PhaZ), which are used to recover the stored carbon and, in some cases, an extracellular PHA depolymerase exists to degrade crystalline PHA material in the environment[32].[18,32]

The *pha* loci are considerably diverse.[16] The loci encoding the genes for PHA synthesis have been characterized from several species. Genes specifying enzymes for SCL-PHA formation are designated *phb*, and those specifying enzymes for MCL-PHA formation are designated *pha*. The genes encoding for the enzymes involved in PHA synthesis are not necessarily clustered and are organized differently in the genome depending on the organism.[16] The substrates that react with the PHA enzymes also depend on the organism (substrate specificity) and dictate the PHA synthesis pathway and consequently the resulting monomer.[15,16,33] PHA synthase enzyme shows broad substrate specificity and therefore a wide variety of monomers can be polymerized.[32] Currently, three different classes of PHA synthases (I, II and III) are distinguished, according to their subunit composition and their substrate specificity.[34]

1.3.3. State of the Art

Several PHAs have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics.[16] SCL-PHAs are the most studied for commercial application.[15] This is due to their physical and mechanical properties that resemble the properties of common commodity thermoplastics.[33] MCL-PHAs are elastomers and rubbers[33] and have a much lower melting point and glass transition temperature. Their molecular structure is analogous to soft PP. This is due to chain defects which cause crystal disruption and enhanced molecular entanglement, resulting in a highly amorphous material.[2]

P(3HB) is, as already mentioned, the most common PHA, and is the most extensively studied, triggering its commercial interest[16].P(3HB) has good thermoplastic properties (melting point of 180°C)[11,16] and can be processed as classic thermoplastics.[2] Its mechanical properties are comparable to those of PP,[32] and it has a wide temperature resistance range, retaining its original shape from -30°C to 120°C. P(3HB) is water insoluble and relatively resistant to hydrolytic degradation, differentiating P(3HB) from most other currently available bio-based plastics, such as starch-based plastics which need chemical modification to reduce its hydrophilicity[3]. Due to P(3HB)'s high crystallinity[16] (60 to 70%) it has excellent resistance to solvents. Resistance to fats and oils is fair to good. It has good UV resistance, but poor resistance to acids and bases. The oxygen permeability is very low, making P(3HB) a suitable material for use in packaging oxygen-sensitive products. The monomer and the polymer are natural components and metabolites of animals,[33] making it toxicologically safe. Thus, P(3HB) has potential in application for articles which come into contact with skin or food.[2] Commercialisation of this polymer, the trial product of the PHA family, was first attempted in the 1950s by W. R. Grace Co., although production inefficiencies, poor thermal stabilities and a lack of available extraction technologies limited application.[35]

Besides the obstacles in the production process, the elongation required to break for P(3HB) is poor compared to that of PP, which means that P(3HB) is stiffer and more brittle[35], somewhat limiting its applications[2]. Also, because of its high crystallinity, it is difficult to dye.[2]

On the other hand, the copolymer P(3HB-co-3HV) has lower crystallinity and improved mechanical properties including decreased stiffness and brittleness, increased tensile strength and toughness, compared to P(3HB).[2] Its mechanical properties depend on the molar ratio of 3HV and in general, the tensile strength decreases gradually as the 3HV molar ratio increases and the copolymers become more flexible compared to P(3HB).[35] In the early 1990s, Imperial Chemical Industries (ICI), was the pioneer in developing a biosynthesis process that is capable of producing P(3HB-co-3HV).[14] The process involved bacterial fermentation using a mixture of glucose and propionic acid. At the time, the polymer was offered at US $30 per kg, projected to go down to US $8-10 per kg, still a prohibitive price for bulk applications. Later on, the business was sold to Monsanto, who commercially produced small volumes of Biopol® P(3HB-co-3HV). When Monsanto ceased its PHA operations sold its Biopol® assets to the U.S. biotechnology company Metabolix who is producing PHAs through fermentation of commercial-grade corn. In this process, *R. Eutropha* is fed with a combination of glucose and propionate produces.[2]

Nevertheless, P(3HB-co-3HV) copolymers have almost the same degree of crystallinity throughout a wide range of 3HV compositions because of isodimorphism.[35] Contrarily, the

copolymer P(3HB-co-3HHx) has variable crystallinity, dependent on its monomer ratio,[3] which decreases with an increase of the 3HHx composition, since 3HHx is an elastomer[10]. The 3HHx units cannot crystallize in the sequence of 3HB units and act as defects in the P(3HB) lattice.[35] This effect produces a broader range of physical, thermal and mechanical properties for P(3HB-co-3HHx) when compared with P(3HB-co-3HV) copolymer, resulting in much interest in the development of P(3HB-co-3HHx) for bulk applications.[3] Procter and Gamble (P&G), in partnership with Kaneka Corporation, has engaged in R&D efforts to develop and commercialise P(3HB-co-3HHx). For its production, a range of oils (lipids, saccharides etc.) is used to feed genetically modified *Escherichia Coli*, obtaining a range of different compositions of Nodax®.[2]

Due to the hydrophobic nature of the surface of these polymers, its colonization is an arduous task for the degrading organisms. Therefore, the shelf life of a PHA product is virtually unlimited, requiring a high biological activity for biodegradation to occur.[36] Total degradation of these polymers can take from a few months (sewage)[37] to a few years (fresh water)[38], depending on polymer constitution, environmental conditions such as temperature and the microbiota responsible for the degradation.[37,39]

1.3.3.1. Production Strategies

The culture conditions required for PHA biosynthesis are important criteria to be taken into consideration for the development of cultivation techniques used in the large scale production of PHA.[15]

Currently, high value substrates are used as feedstock in PHA production. In the EU beet sugar predominates while in the US, the raw material source is chiefly corn steep liquor. Other popular sources include palm kernel or soybean oil which are also used with some microorganisms.[2]

It is important to produce PHA with high productivity and high yield to reduce the overall cost. Fed-batch and continuous cultivations have been used to improve productivity.[17] Fed-batch cultivation is employed for bacteria that require the limitation of an essential nutrient, such as nitrogen, phosphorous or magnesium, together with excess carbon source for the efficient synthesis of PHA.[7,15] A two-step cultivation method (not necessarily with two fermentation vessels) is most often employed. First, cells are grown to a desired concentration without nutrient limitation. Then, an essential nutrient is limited, allowing for efficient PHA synthesis.[7,40] But it is necessary to calculate the limitation because a premature limitation of nutrients will result in low final cell and PHA concentrations, resulting in low PHA productivity, even though high intracellular PHA contents may be obtained, whereas a delay in the nutrient limitation leads to the cells not being able to accumulate much polymer, resulting in a low PHA content and a low PHA productivity even though high cell concentrations can be achieved.[40] The representative bacteria that require this strategy for proper PHA accumulation include *Cupriavidus necator*, *Alcaligenes eutrophus*, *Protomonas extorquens* and *Protomonas oleovorans*.[7,15]

Another group of bacteria does not require nutrient limitation for PHA synthesis and is capable of storing polymer during the exponential growth phase. For these bacteria cell growth

and PHA accumulation need to be balanced to avoid incomplete accumulation of PHA or premature termination of fermentation at low cell concentration, becoming crucial the development of a nutrient feeding strategy. Complex nitrogen sources such as corn steep liquor, yeast extract or fish peptone can be supplemented to enhance cell growth as well as polymer accumulation since PHA synthesis is not dependent on nutrient limitation in these bacteria. [7,40] The bacteria in this group include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, and recombinant *E. Coli* harbouring the PHA biosynthetic operon of *C. Necator*.[7,15]

Fed-batch cultivation allows achieving higher product and cell concentration in comparison to batch cultivation because the medium composition can be controlled by substrate inhibition. As a result, high initial concentration of substrates can be avoided, which could be potentially inhibitory.[15]

After maximum storage is achieved, the PHA must be retrieved. Since PHA is an intracellular product, the method used in the effective separation of PHA from other biomass components can be complex and expensive.[15] To isolate and purify PHA, the cells are concentrated, dried and extracted with hot solvent.[2] The most commonly utilised is chloroform[15] which allows for the attainment of high purity without degrading the polymer.[5] Even so, it presents major disadvantages concerning the hazards associated with the solvents and the high price/low recovery correlation.[41] Another simple and effective extraction method that has been employed in PHA recovery involves cell digestion with sodium hypochlorite. In this method, the cell biomass is treated with sodium hypochlorite solution before the PHA granules are isolated from the cell debris by centrifugation. However, the use of this method results in severe PHA degradation, which leads to PHA with lower molecular weight. To obtain higher purity, higher degradation occurs.[42] Conversely, by combining hypochlorite digestion with surfactant pre-treatment, a higher molecular weight can be obtained (730,000–790,000 g/mol vs. 680,000 g/mol obtained with hypochlorite control) and also higher purity (97–98% vs. 87% obtained with hypochlorite control).[43] In general, different extraction and pre-treatment methods will affect the recovery of the PHA.[15]

1.3.3.1.1. Pure Cultures

In these processes the protagonists are the bacteria. Even though more than 250 different natural PHA-producers have been recognized, only a few bacteria have been employed for the biosynthesis of PHA.[15] Today, industrial production of PHAs is based on the use of pure cultures of microorganisms in their wild form, such as *Ralstonia eutropha,* which can store up to 90% of its dry weight in PHA granules,[33] *Alcaligenes latus*, and *Burkholderia sacchari*,[44] using expensive pure substrates, as already mentioned. Cost of substrate is the highest expense in PHA production,[40,45] followed by the polymer extraction. This leads to a much higher selling price of PHAs compared to petroleum-based plastics.[46]

Furthermore, natural PHA producers have become accustomed to accumulating PHA during evolution, often leading to long generation time, which implies lower growth rates. Also, many have relatively low optimal growth temperature, which requires cooling during operation. Additionally, they are often hard to lyse, and contain native machinery for polymer degradation for PHA degradation, making the recovery of PHA difficult.[16,33] This makes these PHA producers unsuitable for industrial production of bulk biomaterials.[33,47]

Accordingly, the improvement of PHA production has been attempted in recent years through the combination of genetic engineering and molecular microbiology techniques. [15,33] Various attempts at recombinant strains have striven to grant them rapid growth, high cell density, ability to use several inexpensive substrates, and simple polymer purification for cost-effective PHA production.[16] These strains have been developed by cloning the PHA synthase genes from many microorganisms, including *C. necator*. *E. coli* does not naturally possess any part of the PHA production metabolism, but it has the ability to grow fast and is easy to lyse. The faster growth enables a shorter cycle time for the production process, while the easier lysis of the cells provides cost savings during the purification of the PHA granules.[16] That, together with the existing extensive study of several aspects of *E. coli*, including genome, make it the appropriate host for generating higher yields of the biopolymer.[32,48] The production of PHA by recombinant *E. coli* harbouring *C. necator* can reach 80–90% of the cell dry weight. However, the high cellular concentration obtained implies a high oxygen demand during fermentation which, along with the need for feed and equipment sterilization, has a negative impact on the process costs.[5,47]

Additionally recombinant strains present other barriers to their commercial application. One of the major obstacles in producing PHB in recombinant organisms is associated with the instability of the introduced *pha* genes. Even after vast attempts at maximizing PHB production in non-PHB producing microorganisms, the PHB accumulation level is not as high as what could be obtained with the natural producers of the biopolymer.[33]

PHA properties can be tailored to suit numerous applications[3] and these polymers show promise in novel applications which require non-toxicity, biodegradability and the use of renewable feedstock, qualities that conventional synthetic thermoplastic polymers cannot meet[2]. Furthermore, most PHAs are thermoplastics and can be thermally processed using existing technologies in the plastics industry. Despite these numerous advantages, the commercialization of PHA has been ongoing since 1980s with limited success.[15] The high production cost of these polymers has been a major drawback to their application to replace petrochemical plastics.[5,6,33] While petrochemical plastics such as PP and PE can cost less than US$1/Kg, PHAs can cost up to US$16/Kg.[40,49,50]

Although commercialisation efforts are underway, bulk volume applications appear to be still many years off.[2] If PHA production is to attain bulk commercial viability, it is necessary to reduce substrate costs, relying on cheap renewable resources, reduce fermentation costs and improve productivity, and optimize separation and reduce its cost. Furthermore, the price of these biopolymers must be reduced by increasing the volumetric production capacity of fermenter systems and improving process technology, especially the downstream processing.[46]

1.3.3.1.2. Mixed Cultures

In theory, any carbon source can be utilised as feedstock for PHA production, including agricultural lignocellulosic by-products. In recent years, a variety of low-cost carbon substrates (e.g., starch, whey, and molasses) have been tested for PHA production by pure cultures. Nevertheless, PHA production costs remain high, mainly due to substrate and operating costs.[45]

To make possible the use of more complex carbon sources and simultaneously reduce operating costs, mixed cultures of organisms, known as Mixed Microbial Cultures (MMC), can be employed. These types of cultures are able to make use of a wide variety of more complex substrates as opposed to many pure strains[26], and also they allow for considerable production cost reduction due to not requiring high-cost sterile and strictly controlled conditions[9,10,46].

Differing feedstock leads to different PHA compositions.[32] MMC can easily produce a broad range of PHA compositions based on a variety of feedstock which may be due to the diversity of organisms in MMC that are likely to make use of a range of PHA synthetic pathways.[35] One barrier to the application of MMC for PHA production was the low PHA cellular contents typically obtained, but cellular PHA contents and production rates comparable to those of pure cultures have already been achieved in some studies, achieving a maximum P(3HB) content of 89 wt% of the dried biomass in less than 8 hours[47]. Volatile Fatty Acids (VFAs) are energetically advantageous substrates because their complete β-oxidation generates more chemical energy equivalents in the form of ATP molecules than the complete oxidation of a molar equivalent of glucose does,[51] hence the broad utilization of organic acids as feedstock in studies on PHA production by MMC[35].

PHA storage in MMC occurs in systems where electron donor and acceptor availability are separate, like anaerobic/aerobic dynamics, or where there is imbalance in substrate availability.[10,46] These transient conditions lead to unbalanced growth and the storage of substrates as internal polymers becomes an important mechanism of response.[46,52] Consequently, ecological selective pressures that favour organisms with elevated PHA storage capacity are applied to MMC, to engineer the microbial consortium.[53] For this population selection, different methods can be exploited, depending on the instability that leads to the unbalanced growth.[27]

In systems where electron donor and acceptor availability are separate, PHA is crucial for certain bacteria, the most well-known being the polyphosphate accumulating organisms (PAOs) and the glycogen accumulating organisms (GAOs)[10], which are relevant in wastewater treatment processes with biological P-removal. These two types of microorganisms are capable of storing the carbon source during the anaerobic stage while consuming glycogen, a second storage polymer. PHA is then used in the aerobic phase for cell growth, maintenance, and glycogen pool replenishment.[10] PAOs derive energy from the hydrolysis of stored polyphosphate (poly-P) with the simultaneous release of phosphorus to the external medium. That energy is utilized to store exogenous substrate in the form of PHA when there is no electron acceptor (oxygen or nitrate) available for energy generation, giving them a competitive advantage. The electrons (NADPH) necessary for PHA formation derive from glycogen conversion to PHA.[46,53] On the other hand, GAOs have been recognized as competitors of PAOs.[53] For GAOs, energy comes from the glycolysis and is subsequently used to accumulate VFAs in the form of PHA.[46,53] The presence and relative proportion of different PHAs is dependent on the type of carbon source available.[53]

Despite the existence of such systems, recently, much research has concentrated on the production of PHAs by MMC that respond to transient external substrate supply.[53] The imposition of these transient conditions is currently known as ‘aerobic dynamic feeding’ (ADF) or

Feast and Famine (FF) regime.[5,10] This regime comprises two phases, a short one of abundance (feast) and a long one of lack (famine) of carbon source,[46] represented in Figure 1.5. In these systems, microbial competition is based on substrate uptake rate rather than on growth rate: bacteria that can take up the most substrate during the short feast phase are more capable to proliferate in the system, using the substrate for PHA production and growth, simultaneously.[46,47]

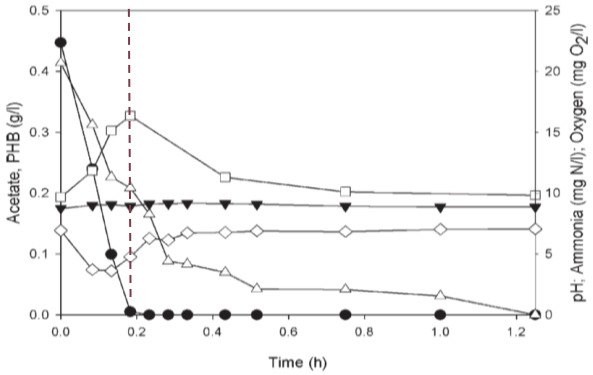


Figure 1.5 – Illustration of a typical FF cycle carried out in a SBR, using acetate (●) as substrate for PHB (□) production. The progress of pH (▼), ammonia (Δ) and oxygen (◇) is also represented. The traced line marks the transition from the feast to the famine phase.[5]

This way, during the initial feast phase organisms with high and stable capacity of PHA production are selected.[10] With the external substrate depletion, the famine phase commences. In this phase, the stored polymer can be used as internal carbon and energy source for cell growth and maintenance.[10,47,53] In order to survive the famine phase without having stored polymer in the feast phase, the bacteria need to take up the substrate as fast as the PHA producers and at least double their biomass during the feast phase in order to remain in the system, in addition to having to endure starvation during the famine phase.[47] The famine period is needed to stimulate the PHA storage capacity of cells.[46]

Before the new cycle begins, a portion of the consortium is removed, selectively enriching the remaining biomass.[35] Thus, under these dynamic conditions PHA storing bacteria have a competitive advantage over other bacteria in this process, being able to grow continuously throughout the cycle, albeit at a faster rate during the feast phase.[5,10,46,47,53]

For industrial production of PHAs, the feast and famine approach is the most promising because of the high sludge PHA content and productivity. It promotes the conversion of the carbon substrate to PHA rather than to glycogen or other intracellular materials. A sequencing batch reactor (SBR) is commonly used for FF processes. This type of reactor is easy to control and highly flexible, allowing for a quick modification of the defined process conditions (i.e., the length of feeding and the cycle time).[5,46,53] Usually, the SBR is operated with cycles of feeding, reaction, settling, and withdrawal.[5]

However, exactly how do the cells “know” they have to store PHAs rather than using the entire carbon source for growth? How do they “predict” the following famine period? It is possible that cell-to-cell communication takes place. Indeed, PHA synthesis has been shown to be highly dependent on cell density,[54] leading to the hypothesis that storage is a quorum sensing-induced process associated with a response to famine.

1.3.3.1.2.1. Quorum Sensing

Quorum sensing is a communication phenomenon, mostly observed in bacteria, that depends on population density. The term “quorum sensing” was introduced to describe an environmental sensing system that allows bacteria to monitor their own population density.[55] It was first discovered and described in bioluminescent marine bacteria as “autoinduction”.[56] The researchers observed that synthesis of the luciferase enzyme by *Vibrio fischeri* was controlled at the transcriptional level[56] by an autoinducer, a molecule that accumulates in the growth medium,[57] activating the synthesis during exponential growth.[56,57] That being so, microorganisms perceive population density and respond when a certain threshold is reached, relying on the production and subsequent response to diffusible signalling molecules.[58,59] The minimum unit required to trigger a response is a quorum of bacteria.[55]

The quorum sensing phenomenon requires the possession of certain characteristic features which are: (i) production of the signalling molecules occurs during specific stages of growth, under certain physiological conditions, or in response to environmental changes; (ii) the signalling molecules accumulate extracellularly and are recognized by a specific bacterial receptor; (iii) the signalling molecules accumulate up to a critical concentration threshold that generates a concerted response; (iv) the cellular response extends beyond physiological changes required to metabolize or detoxify the molecule. All four criteria must be met because the first three criteria on their own are also met by many other molecules.[60]

Acylated Homoserine Lactones (AHLs) are the most studied signalling molecules involved in quorum sensing and are exclusive to gram-negative bacteria.[59] They are produced for the purpose of communication, and have specific receptors. Today, more than 70 species are recognized as AHL producers,[61] and these signals have been found to regulate a diverse range of bacterial processes including bioluminescence,[62] virulence,[63] antibiotic biosynthesis[64] and formation and dispersal of biofilms[65]. Although AHL structures have the homoserine lactone ring moiety in common, the acyl side chain can vary in length, degree of substitution, and saturation (see Figure 1.6).[59] All currently known AHLs have side chains usually with an even number of carbons that range from 4 to 18 carbons in length.[61] The overall length of the side chain and chemical modification provide specificity to quorum-sensing systems.[59]

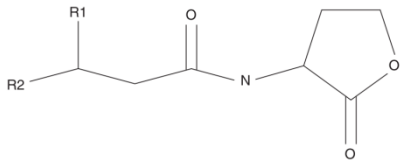


Figure 1.6 - Basic structure of the AHL signal molecule. R1 represents H, OH, or O, and R2 represents C1–C18.[66]

AHLs are synthesized at a low basal level by AHL synthases (LuxI proteins), and are immediately removed from the cell by diffusion down their concentration gradient. The AHLs accumulate slowly in the growth medium in proportion to the increasing bacterial population and so, an increase in population density elevates the local AHL signal concentration both intra- and extracellularly. When a threshold level is reached, the binding of the AHL signal to receptor molecules (LuxR protein) is promoted, and the activated LuxR–AHL complex forms dimers or multimers which in turn act as a transcriptional regulator that modulates expression of the quorum sensing–regulated genes.[55,59] This regulatory circuit in shown in Figure 1.7.

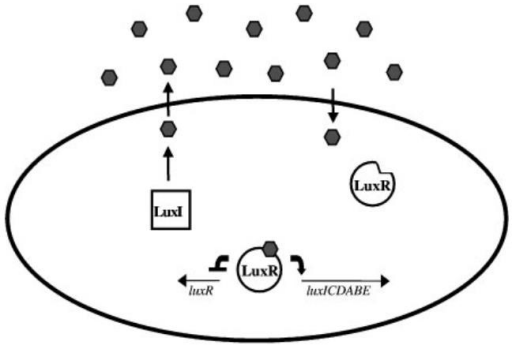


Figure 1.7 - The most extensively studied quorum sensing system, the LuxI/LuxR quorum sensing circuit present in *Vibrio fischeri*. There are five luciferase structural genes (luxCDABE) and two regulatory genes (luxR and luxI) involved in quorum sensing. The LuxI protein (square) synthesises AHLs (hexagons). When the AHLs reach the concentration threshold, they bind to the LuxR protein (circle). The LuxR-autoinducer complex binds at the luxICDABE promoter activating transcription. This results in an exponential increase of AHL synthesis due to the increase in luxI transcription and an exponential increase in light production due to the increase in luxCDABE transcription. The LuxR-autoinducer complex also binds at the luxR promoter, but to repress luxR transcription. This negative action compensates for the positive action at the luxICDABE promoter.[67]

Quorum sensing can be affected by several external factors, such as pH, temperature or even other interfering molecules. Under alkaline conditions most AHLs are chemically unstable due to the hydrolysis of the lactone ring, resulting in accelerated signal degradation. The resistance to ring opening increases with the length of the side chain.[68] Concerning other molecules, extracellular reactants may interact with AHL, destabilizing or sequestering the signal and could affect accumulation.[59] The combination of all these factors dictates the success of the communication between the cells.

Homoserine lactones have been shown to be involved in the synthesis of PHB. Sun et al.[54] and Miyamoto et al.[69] observed that the *lux* autoinducer N-(3-hydroxybutanoyl)homoserine lactone controlled the synthesis of PHB in *Vibrio harveyi*, showing that the *lux* autoinducer acts as a general signal transductant. On the other hand, the presence of AHLs has also been found in activated sludge from an industrial WWTP.[70,71] Valle et al.[70] showed that the addition of AHLs to reactor sludge affected community function and the community itself.

Isolating these signalling compounds will possibly allow for PHA production enhancement, with production of a fair amount of PHA even in low population concentrations, lowering PHA production costs.[14] Understanding if our PHA producing systems make use of quorum sensing to store biopolymer is one step closer to process optimisation.

1.3.3.1.3. Low-cost Substrates

Agricultural feedstock, e. g., starch, sugars, and oils, are currently the most desired raw materials for the industrial production of biobased and biodegradable plastics. Vegetable oils such as soybean oil and palm oil seem to be the most efficient fermentation substrate for the production of PHAs for bulk applications. However, the same raw materials are also in high demand for the production of biofuels such as bioethanol and biodiesel. Using such substrates would be tapping into valuable food supplies for the growing global human population as well as for livestock feed, making the use of plant biomass as a feedstock in the industrial production of biodegradable plastics not commercially viable.[3]

The exploitation of agricultural or industrial waste or by-products as feedstock for PHA production allows for the substantial decrease in production costs. Furthermore, utilizing waste saves the cost of waste disposal.[8,51] Many waste streams and co-products from agriculture and its associated industries are potentially useful substrates for microbial production of PHAs.[51,53] Several have been employed successfully using MMC, such as sugarcane molasses[45], paper mill wastewater[72], oil mill effluent[73] and cheese whey[74].

1.4. Thesis Objectives and Work Outline

For the improvement of PHA production processes, it is important to study the system and understand the dynamics of the population responsible for the process, how the population reacts to changes in the system and the regulation mechanisms responsible for PHA production. Accordingly, this work was aimed at studying the communities involved in the process of PHA production rather than the PHA production itself.

This work sets out to study the microbial population and regulation mechanisms in PHA producing systems. First and foremost comes the identification of the most prevalent groups of organisms in PHA producing cultures enriched from activated sludge and observation of their evolution throughout the selection period. Also, an investigation of how those groups of organisms react to different feedstock (cheese whey and sugarcane molasses) and how altering reactor conditions and feedstock composition affects the biomass which in turn affects the PHA outcome was carried out. Another chief goal was to understand if quorum-sensing takes any part in the PHA accumulation process in these systems, and if occurring, where it intervenes.

This thesis is comprised of 7 chapters, including the present one. In Chapter 2, the Materials and Methods used in the analyses of the communities are described. Then, the four studies conducted are divided in different Chapters (3-6). The first study (Chapter 3) regards the acclimation of activated sludge from a WWTP to the production of PHA using synthetic VFAs as substrate. Next, in Chapter 4, different renewable resources were employed as substrate and the effect of the change from one feedstock to another in the communities of the fermentation and the selection reactors was studied. Then (Chapter 5), the pH of the fermentation reactor was modified and the effect of that change in the SBR community was investigated. The last study regards the occurrence of quorum sensing in the PHA-producing system previously studied. Finally, Chapter 7 is a general conclusion of this work.

Chapter 2

Materials and Methods

2.1. Nile Blue Staining

Samples were taken at the end of the feast phase for PHA granules observation using Nile Blue staining, which was performed according to Bengtsson et al., 2008[72]. After staining, the slides were observed under an Olympus BX51 epifluorescence microscope using the 100x objective with immersion oil. The samples were also observed in phase contrast and brightfield. The images were collected with the cell-F soft imaging system GmbH.

2.2. FISH Analysis[75]

To carry out FISH analysis, samples were taken from the SBR at the end of the feast phase and fixed for gram negative bacteria as follows: three volumes of Paraformaldehyde (PFA) 4% were added to one volume of sample and then stored at 4°C for one to three hours; subsequently, the samples were centrifuged at 10,000 rpm for three minutes; the supernatant was disposed of, 1mL of Phosphate buffered saline (PBS) 1x was added to the pellet of cells and the pellet was then resuspended to wash off the matrix and the PFA; this step was repeated one more time; after washing, the cells were resuspended with one volume of PBS 1x and absolute ethanol at -20˚C was added in the same proportion; the samples were mixed and then they were stored at -20˚C.

For the FISH analysis, 10µl of sample were used in each well. After the sample application, the slides were air dried and then put through a series of ethanol dehydration steps: the slides were inserted in Falcon tubes with increasing ethanol concentrations (50%, 80% and 98%) for three minutes each.

For the probe hybridization, a hybridization buffer was prepared in final concentrations of 0.9M NaCl, 0.01% SDS, 20mM Tris/HCl and pH7.2. Formamide concentration varied according to probe requirements (see Table 2.1). 8µl of this solution was added to the wells, followed by 1µl of each probe. After the probe application, the slides are kept at 46˚C for 1.5 to three hours.

Table 2.1 - Probes used in the identification of the bacteria present in the SBRs.

Probe Name	Specificity	Sequence	Formamide %	Reference
ALF969	Some Alphaproteobacteria	5'- TGG TAA GGT TCT GCG CGT -3'	35	Oehmen et al., 2006[76]
AMAR839	*Amaricoccus* spp.	5'- CTG CGA CAC CGA ACG GCA AGC C -3'	20	Maszenan et al., 2000[77]
AZO644	Most members of the *Azoarcus* cluster	5'- GCC GTA CTC TAG CCG TGC -3'	30	Hess et al., 1997[78]
BET42a	Betaproteobacteria	5'- GCC TTC CCA CTT CGT TT -3'	35	Manz et al., 1992[79]
CF319a	Most *Flavobacteria*, some Bacteroidetes, some Sphingobacteria	5'- TGG TCC GTG TCT CAG TAC -3'	35	Manz et al., 1996[80]
DF988	"*Defluvicoccus vanus*"-cluster D2 related organisms	5'- GAT ACG ACG CCC ATG TCA AGG G -3'	35	Meyer et al., 2006[81]
DF1020	"*Defluvicoccus vanus*"-cluster D2 related organisms	5'- CCG GCC GAA CCG ACT CCC -3'	35	Meyer et al., 2006[81]
H966	Helper probe	5'- CTG GTA AGG TTC TGC GCG TTG C -3'	-	Meyer et al., 2006[81]
H1038	Helper probe	5'- AGC AGC CAT GCA GCA CCT GTG TGG CGT -3'	-	Meyer et al., 2006[81]
EUB338	Most bacteria	5'- GCT GCC TCC CGT AGG AGT -3'	0-50	Amann et al.,1990[82]
EUB338 II	Planctomycetales	5'- GCA GCC ACC CGT AGG TGT -3'	0-50	Daims et al., 1999[83]
EUB338 III	Verrucomicrobiales	5'- GCT GCC ACC CGT AGG TGT -3'	0-50	Daims et al., 1999[83]
GAM42a	Gammaproteobacteria	5'- GCC TTC CCA CAT CGT TT -3'	35	Manz et al., 1992[79]
HGC69a	Actinobacteria (high G+C Gram-positive bacteria)	5'- TAT AGT TAC CAC CGC CGT -3'	25	Roller et al., 1994[84]
PAR651	*Paracoccus* genus	5'- ACC TCT CTC GAA CTC CAG -3'	40	Neef et al., 1996[85]
TFO-DF218	"*Defluvicoccus*"-related TFO in Alphaproteobacteria	5´- GAA GCC TTT GCC CCT CAG -3'	25-35	Wong et al., 2004[86]
TFO-DF618	"*Defluvicoccus*"-related TFO in Alphaproteobacteria	5'- GCC TCA CTT GTC TAA CCG -3'	25-35	Wong et al., 2004[86]
THAU832	*Thauera* genus	5'- TGC ATT GCT GCT CCG AAC -3'	30	Loy et al., 2005[87]
ZRA23a	Most members of the *Zoogloea* lineage, not *Z. resiniphila*	5'- CTG CCG TAC TCT AGT TAT -3'	35	Rosselló-Mora et al., 1995[88]

The slides were subsequently washed in a washing buffer at 48°C with varying amounts of NaCl and EDTA depending on formamide concentration (see Table 2.2). The slides were observed under an Olympus BX51 epifluorescence microscope and the images were collected using the cell-F soft imaging system. Group specific probes were Cy3-labeled, while the probe for all bacteria EUBmix was FitC labeled.

Table 2.2 – NaCl and EDTA amounts used in the washing buffer, depending on the amount of formamide used.

Formamide (%)	NaCl amount(µl)	EDTA amount (µl)
20	2150	500
25	1490	500
30	1020	500
35	700	500
40	460	500

2.2.1. Quantitative FISH Analysis

For quantitative FISH analysis, the probes AZO644, PAR651, THAU832 and EUBmix were used. Hybridized samples were viewed with a Zeiss LSM 510 Meta confocal laser scanning microscope. FISH quantification of Cy3-labeled *Azoarcus*, *Paracoccus* and *Thauera* in respect to all Bacteria (Cy5-labeled) was done by image analysis (20 - 25 images of each sample) with the Daime software[89], which determines the biovolume fraction of the specifically labelled target population relative to the biovolume of the total bacteria. The standard error of the mean (s.e.m.) was calculated as the standard deviation divided by the square root of the number of images.

2.3. Next Generation High Throughput Sequencing

High throughput sequencing of 16S rRNA gene PCR amplicons was carried out by Søren M. Karst at the Aalborg University, Denmark using Illumina technology.

Chapter 3

Acclimation of PHA Storing Sludge from a WWTP to Synthetic Volatile Fatty Acids Feeding

3.1. Introduction

In this chapter the acclimation of activated sludge from a WWTP was studied. The sludge was exposed to a feast and famine regime to select the PHA producing biomass, while using synthetic VFA's as substrate. Instead of renewable resources, synthetic VFAs were employed in order to have less variables and better study the competition among organisms from an activated sludge inoculum.

Throughout time, samples were taken to observe the biomass microscopically and Nile Blue was used to assess if the biomass was being able to store PHA and how much of the biomass was involved in production. Nile Blue is a stain used in the detection of PHA granules that is quite specific, with cell membranes or other lipid-containing cell components not being able to absorb enough dye to give detectable fluorescence. It exhibits a strong orange fluorescence at an excitation wavelength of 460nm.[90]

The microbial community selected was characterized through semi-quantitative fluorescence *in situ* hybridization (FISH) and next generation high throughput sequencing in order to identify the dominant microbial groups, particularly those with PHA-storage properties. FISH analysis allows for the identification in the biomass of known microorganisms through specific probes that target specific DNA sequences[75] and can provide semi-quantitative results. On the other hand, next generation high throughput sequencing can be used to determine the most abundant microbial groups present without knowing what to look for, and is a broader approach than cloning or denaturing gradient gel electrophoresis (DGGE) followed by sequencing. It was employed to try to identify an unknown group of bacteria present in the system.

THAU832, AZO644, PAR651, AMAR and Zra23a were the probes used for the study of the evolution of the biomass through FISH analysis. The genera targeted by these probes have all been previously found in activated sludge systems and have been reported to store PHAs.

Thauera, *Azoarcus* and *Zoogloea* belong to the Betaproteobacteria class, which is known to play a role in organic material degradation, nutrient removal and floc formation[91], and its dominance in activated sludge communities has been observed by many researchers[9,88,92]. In plants treating mainly domestic wastewater with biological nitrogen and/or phosphorus removal *Azoarcus* and *Thauera* are usually abundant, representing 3–16% of the biovolume.[93] They are known denitrifiers, as is *Zoogloea*[9,88,92].

From the genera presently studied, *Zoogloea* was the one reported the earliest. By 1964[94] it was already considered of great importance for the wastewater treatment process, and a year later it was described the accumulation of PHAs by organisms of the genera isolated from activated sludge[95]. For a long time it was considered responsible for floc formation[96], but other groups of organisms have been shown to be involved in this phenomenon[97]. It has been described both as abundant[9,88,92] and as present in small numbers[93].

The *Thauera* genus was first described as a PHA producer by Dionisi et al.[98]. The genus had already been found in activated sludge,[92] but it had never been associated with PHA production[5,98]. The *Azoarcus* genus was reported to accumulate PHA[99] after having been identified in abundance in activated sludge[92].

Amaricoccus were first identified as such from activated sludge isolates by Maszenan et al.[77]. Later, the genus was reported to produce PHA[100]. These organisms have been associated with poor performance of enhanced biological phosphorus removal (EBPR) plants, by out-competing the polyphosphate accumulating bacteria.[77]

The *Paracoccus* genus has been found in activated sludge[28,101], but its abundance is seldom studied[102]. Organisms belonging to the *Paracoccus* genus were among the first genera presently studied to be identified as PHA producers[103].

3.2. Experimental Set-up

A 30L SBR was inoculated with activated sludge obtained from the WWTP in Beirolas, Lisbon. For four days, the reactor went through prolonged famine, for a "pre-selection" of PHA accumulating organisms. After the "pre-selection", the reactor was set up with SBR cycles of 12h. The sludge retention time (SRT) was of 4 days and the hydraulic retention time (HRT) was of 1 day. The reactor was fed with a solution of synthetic VFAs, with the composition shown in Table 3.1. Besides the synthetic VFAs, the reactor was supplemented with a nutrient solution containing NH_4Cl and KH_2PO_4.

Table 3.1 – Constitution of the feeding solution

VFAs	Concentration (% Cmolar basis)
Acetate	65
Propionate	15
Butyrate	15
Valerate	5

3.3. Results and Discussion

For 152 days, samples were taken regularly for microscopic observation of the biomass at the end of the feast phase in order to observe the cells with their maximum PHA content. The biomass was characterized using FISH analysis,

3.3.1. Morphological Characterization of the Biomass

The microscopic observation of the biomass with brightfield and phase contrast was used to examine the biomass and detect obvious changes in the biomass at the morphological level. Nile Blue was used to follow the evolution of PHA-storage by the population and evaluate if the biomass was being selected for that purpose.

The first major change observed was the reduction of extracellular material detectable in the flocs. Also, in the first 60 days, there was a considerable increase in the PHA producing community as well as the cells' storage capacity. The bacteria with storing ability became mostly organized in microcolonies within the flocs. As the selection progressed, the biomass became more homogeneous with larger cells and many tetrad-forming organisms (see Figure 3.1).

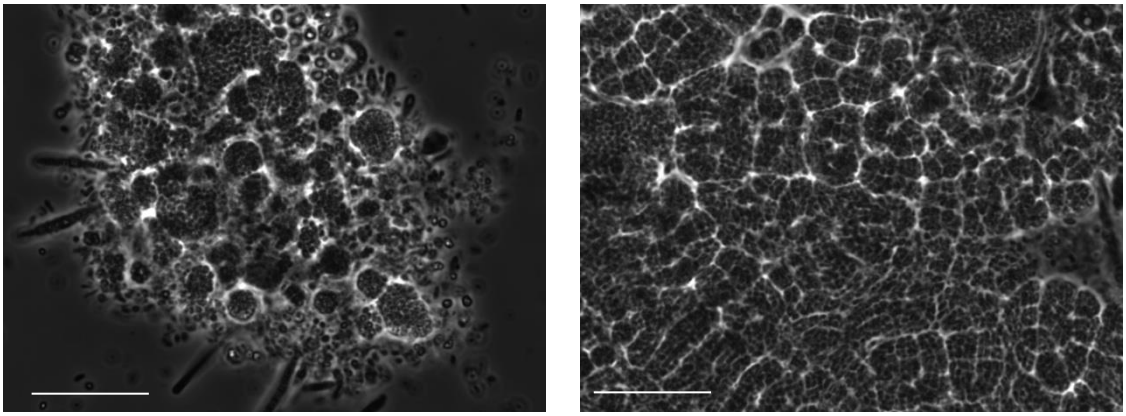


Figure 3.1 – Phase contrast images: (a) day 49 of reactor operation and (b) day 150 have different biomass compositions. Bar=20µm

Filamentous bacteria were present in the system since inoculation. After around 40 days some very long filaments comprised of ovoid to discoid cells were present in large amount and their amount continued to increase considerably and these filaments became dominant after 60 days of reactor operation. The increase in filamentous bacteria accompanied an increase in the total biomass. The abundance of these large filaments lasted throughout the rest of the study. The variety and abundance of filaments can be observed in Figure 3.2.

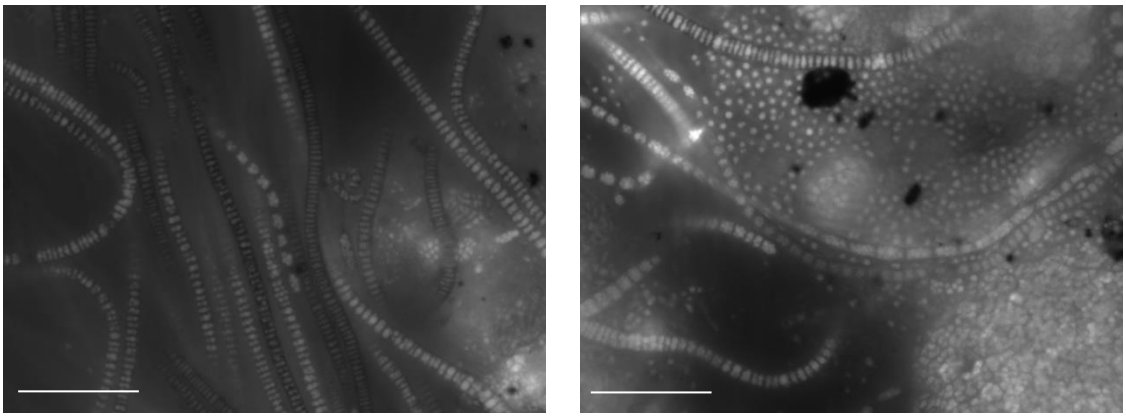


Figure 3.2 – Nile Blue staining shows the PHA granules (in white) inside the large filaments that dominated the community throughout reactor operation. Bar=20µm

This regular investigation helped understanding certain events, such as the difficulty in settling (bulking) and the subsequent cell washout that accompanied the establishment of the filamentous bacteria in the system.

Despite the occurrence of bulking, the filamentous bacteria showed great storage capacity, as seen in Figure 3.2, leading to an interest in their identification which was attempted through next generation high throughput sequencing and FISH analyses. High throughput sequencing was employed to see the dominant groups of organisms present in the biomass. The high throughput sequencing results showed that Proteobacteria comprised around 97% of the biomass, with Alphaproteobacteria making up more than 80% of the Proteobacteria and Betaproteobacteria constituting 17% of Proteobacteria (see Table 3.2). Both these classes of bacteria are commonly found in activated sludge systems with PHA production[76,98,104,105]. Bacteroidetes followed the Proteobacteria phylum in abundance, comprising 2% of the total amplicons. This group has been found in low amount in other PHA producing systems[106].

Table 3.2 – Most abundant OTUs found in the SBR at the end of the study and their relative abundances.

Phylum	Class	Order	*Genus*
Bacteroidetes (2%)			
	Flavobacteria (1%)		
	Sphingobacteria (1%)		
Proteobacteria (97%)			
	Alphaproteobacteria (81%)		
		Rhizobiales (66%)	
		Rhodobacterales (12%)	
			Paracoccus (10%)
	Betaproteobacteria (16%)		
		Burkholderiales (3%)	
			Hydrogenophaga (1%)

Moreover, sequencing identified the most abundant order as Rhizobiales, which is commonly found in activated sludge systems[107], but no family could be identified. While on one hand, next generation high-throughput sequencing allows for a broader investigation of the community than FISH, on the other hand it does not allow for a very thorough phylogenetic identification due to the employment of relatively short amplicons and is not as quantitatively accurate. The Rhodobacterales order of the Alphaproteobacteria class was the second most abundant order in that class, with the genus *Paracoccus* being the most abundant member of the order. This abundance was confirmed with FISH analysis.

Hydrogenophaga was shown to be the most abundant genus of the Betaproteobacteria family. This genus has been found to be the most abundant group belonging to the Betaproteobacteria class in a system enriched for PHA production from activated sludge[106].

To make an identification attempt through FISH analysis, the ALF969, BET42a and GAM42a probes were employed. Even though the high throughput sequencing results showed the Flavobacteria class and the Actinobacteria phylum to be present in very low amount, the CF319a (for most Flavobacteria) and HGC69a (for Actinobacteria) probes were also used, since filamentous bacteria belonging to these groups have been observed in activated sludge[108,109], All the long filamentous bacteria were positively identified as Alphaproteobacteria. The FISH analysis also revealed Alphaproteobacteria to be the most abundant class, followed by Betaproteobacteria and Gammaproteobacteria.

Filamentous bacteria can be found in all types of WWTP and are often responsible for bulking, or foaming (transportation of biosolids to the surface of the tank). Several factors are known to promote the growth of filamentous bacteria, including presence of sulphides in the influent, lack of nutrients and low oxygen concentration.[102]

Contrarily to what occurred in the present study, FF conditions are known to select for floc-forming bacteria over filamentous bacteria.[10] However, the same phenomenon has been reported in other systems selected under FF conditions. Beccari et al.[110] reported the selection of bulking sludge under intermittent feeding dominated by PHB accumulating filamentous bacteria.

The filamentous bacteria found in this study resembled Eikelboom Type 021N and "*Nostocoida limicola*" morphotypes[111]. The typical morphology of the first morphotype is of bent filaments, composed of disk to rod shaped cells with a diameter of 1.2-1.5 µm. They are mainly seen in plants treating industrial effluents[112] in the bulk liquid between the flocs. "*Nostocoida limicola*" filaments commonly occur in domestic wastewater systems[111], but resembling filaments also appear to be widespread in industrial systems as bent/twisted filaments consisting of disc shaped to spherical cells with a diameter variable from 0.8-1.4 µm.[113]

Most Eikelboom Type 021N filaments have been found to belong to the *Thiothrix* genus of the Gammaproteobacteria class, but some have to failed to hybridise with the probe for the genus[112]. The "*Nostocoida limicola*" morphotype contains a wide group of phylogenetically unrelated bacteria,[113] but filaments belonging to the Alphaproteobacteria class have only rarely been reported in domestic treatment plants[114].

Filamentous Alphaproteobacteria have been shown to be very important in industrial WWTPs where they are often associated with bulking incidents or deteriorating settling properties of the sludge.[113] 7 phylogenetic clusters of filamentous Alphaproteobacteria have been described: '*Candidatus Combothrix italica*', '*Candidatus Catenimonas italica*', '*Candidatus Sphaeronema italicum*', '*Candidatus Alysiosphaera europaea*', '*Candidatus Monilibacter batavus*', '*Candidatus Alysiomicrobium bavaricum*' [113], the last four of which have been shown to produce PHAs[104] and *Meganema perideroedes* that also produces PHAs[115].

Both high storage response and resistance to starvation can contribute to competitive advantage of these filamentous bacteria. Their dominance confirms that the filaments are able to remove the substrate and store it as fast as floc-formers and are able to successfully compete against them under intermittent feeding. This demonstrates that FF conditions always select for PHA-storing organisms rather than against filamentous organisms.[110]

3.3.2. FISH Analysis

In the inoculum sludge, it was possible to observe that all the groups investigated were present in low amount, with *Thauera* being the most abundant of the five genera.

Thauera responded well to the four days of "pre-selection" with its presence in the reactor increasing substantially. In the inoculum sludge, *Thauera* cells were small and round, and organized in small clusters. After the pre-selection, besides the increase in amount, the morphology also changed, appearing larger cells and much larger clusters. These alterations can be observed in Figure 3.3.

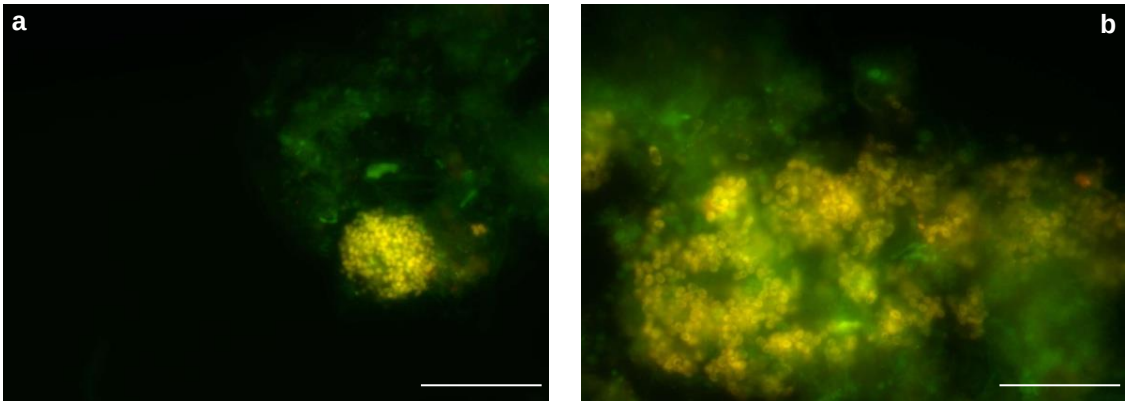


Figure 3.3 – FISH images show (a) the *Thauera* population just before inoculation and (b) after the pre-selection. THAU832 targeted cells appear in yellow and other bacteria appear in green. Bar=20µm

Paracoccus also responded to the initial four days of famine, but not as noticeably. Its amount increased slightly but the biggest difference between the two dates was the morphology. Initially, *Paracoccus* cells were either large and round, or small and rod-shaped, with the latter morphology being the majority, and formed small loose clusters. After the "pre-selection", the large round morphology dominated, forming mostly tight clusters. This evolution can be observed in Figure 3.4.

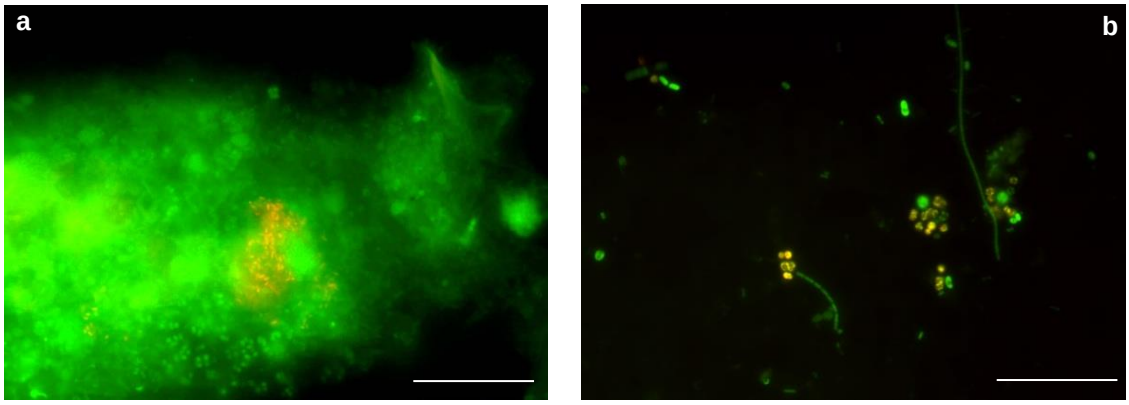


Figure 3.4 – FISH images show (a) the *Paracoccus* population just before inoculation and (b) after the pre-selection. The PAR651 targeted population appears in yellow while other bacteria appear in green. Bar=20µm

Contrarily to the two previously described genera, *Azoarcus* didn't seem to react to the "pre-selection". In fact, the amount of *Azoarcus* cells present in the reactor appeared to slightly decrease in the reactor, and no morphological differences were detected. The cells were round and relatively large forming microcolonies (data not shown).

The amount of cells belonging to the genera *Amaricoccus* and *Zoogloea* seemed to decrease with the "pre-selection" as well. The *Amaricoccus* cells were small cocci organized in small and somewhat loose clusters, as seen in Figure 3.5 (a). The *Zoogloea* genus was represented by different morphologies: small cocci or coccobacilli organized in tight clusters (see Figure 3.5 (c)) or scattered in the flocs. The clusters occurred after the pre-selection as well, as shown in Figure 3.5 (d).

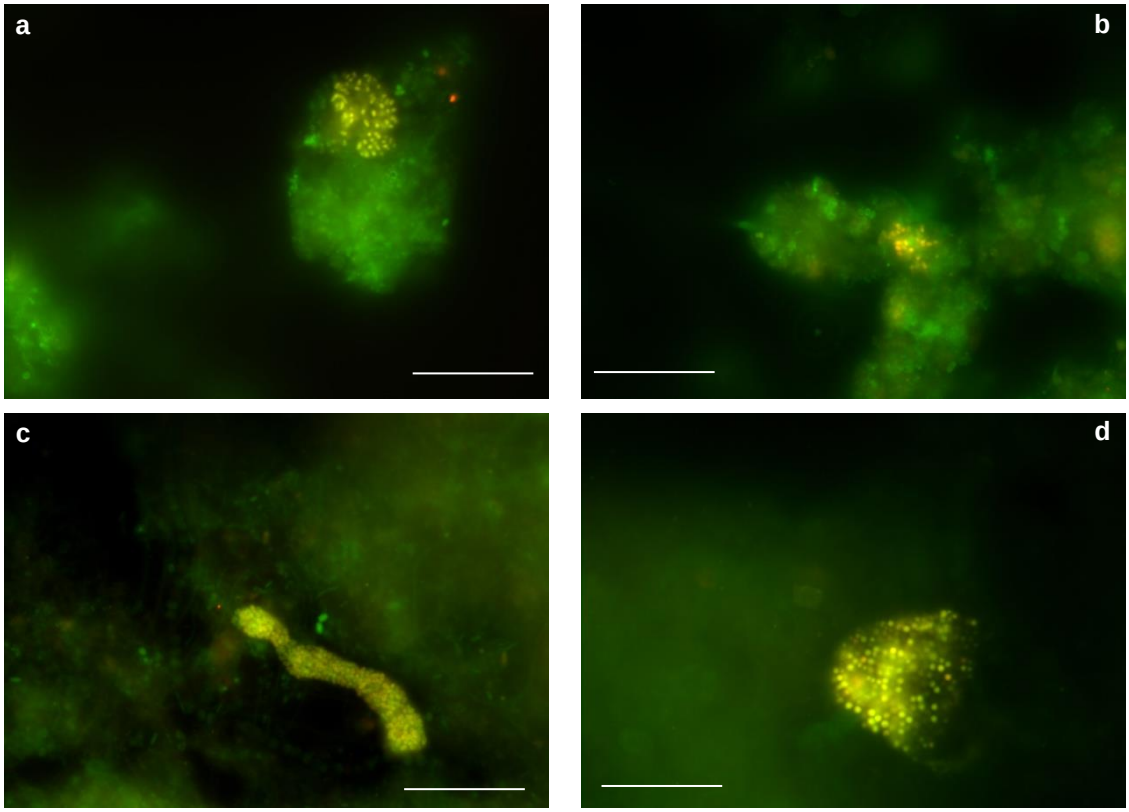


Figure 3.5 – FISH images show (a) & (b) *Amaricoccus* and (c) & (d) *Zoogloea* (a) & (c) before and (b) & (d) after the initial four days of famine. In image (c) a characteristic "finger-like" *Zoogloea* cluster is shown. The targeted populations (AMAR839 and ZRA23a) appear in yellow and all other bacteria appear in green. Bar=20µm

Most *Zoogloea* cells are rod-like,[93] and are typically described as forming characteristic colonies in branched gelatinous matrices, sometime referred to as finger-like,[88,102] as the one seen in Figure 3.5 c).

The presence of *Thauera* in the system continued to increase and after 19 days, *Thauera* represented a large majority of the biomass (see Figure 3.6 (a)). The cells were mostly cocci and coccobacilli. Also, some small filaments were observed, as can be seen in Figure 3.6 (b).

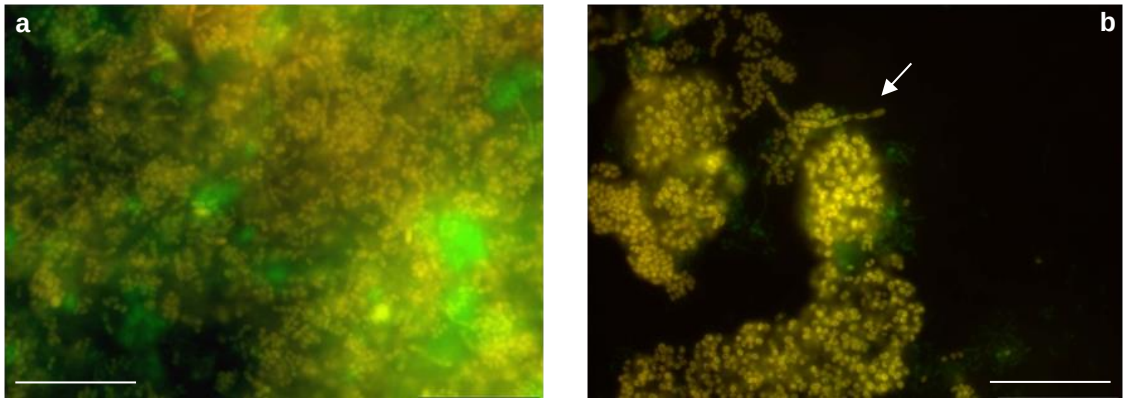


Figure 3.6 – FISH images: (a) *Thauera* cells are abundant at 19 days of reactor operation and (b) show different morphologies and even form small filaments at 22 days of reactor operation. THAU832 targeted cells appear in yellow and other bacteria appear in green. Bar=20µm

A population abundant in *Thauera* was also obtained by Dionisi et al.[98], in a system fed with acetate (40%), lactate (40%) and propionate (20%), and by Lemos et al.[28] when using acetate as carbon source. In the present study, acetate was also the most abundant VFA.

However this pattern did not last, and *Thauera* cells started to disappear from the reactor. By day 47, the presence of the *Thauera* genus had decreased notably and lost its bulk presence, and many cells were smaller (Figure 3.7 (a)). The decreasing trend continued, until the cells belonging to this genus were almost absent by the end of the study (Figure 3.7 (b)).

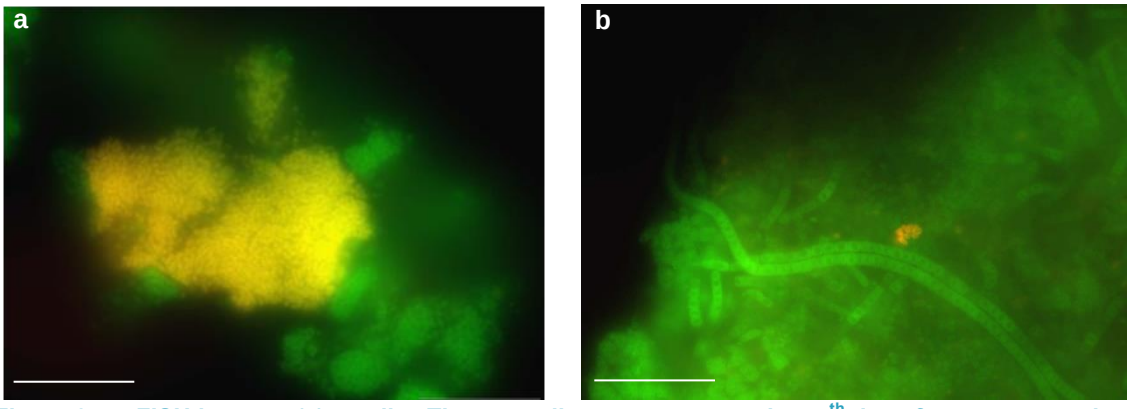


Figure 3.7 – FISH images: (a) smaller *Thauera* cells are present on the 47th day of reactor operation and (b) the amount of organisms belonging to the genus have decreased substantially by day 152. THAU832 targeted cells appear in yellow and other bacteria appear in green. Bar=20µm

After the beginning of the FF cycles, the amount of *Paracoccus* cells decreased and the genus seemed to be almost absent from the reactor by day 19 and, at least, until day 22. The cells were arranged in very small groups (see Figure 3.8 (a)), or as single cells. However, after 47 days of reactor operation, the amount of *Paracoccus* cells in the reactor increased noticeably and the genus seemed to constitute a considerable amount of the floc-forming biomass. The cocci were organized in small regular clusters (see Figure 3.8 (b)).

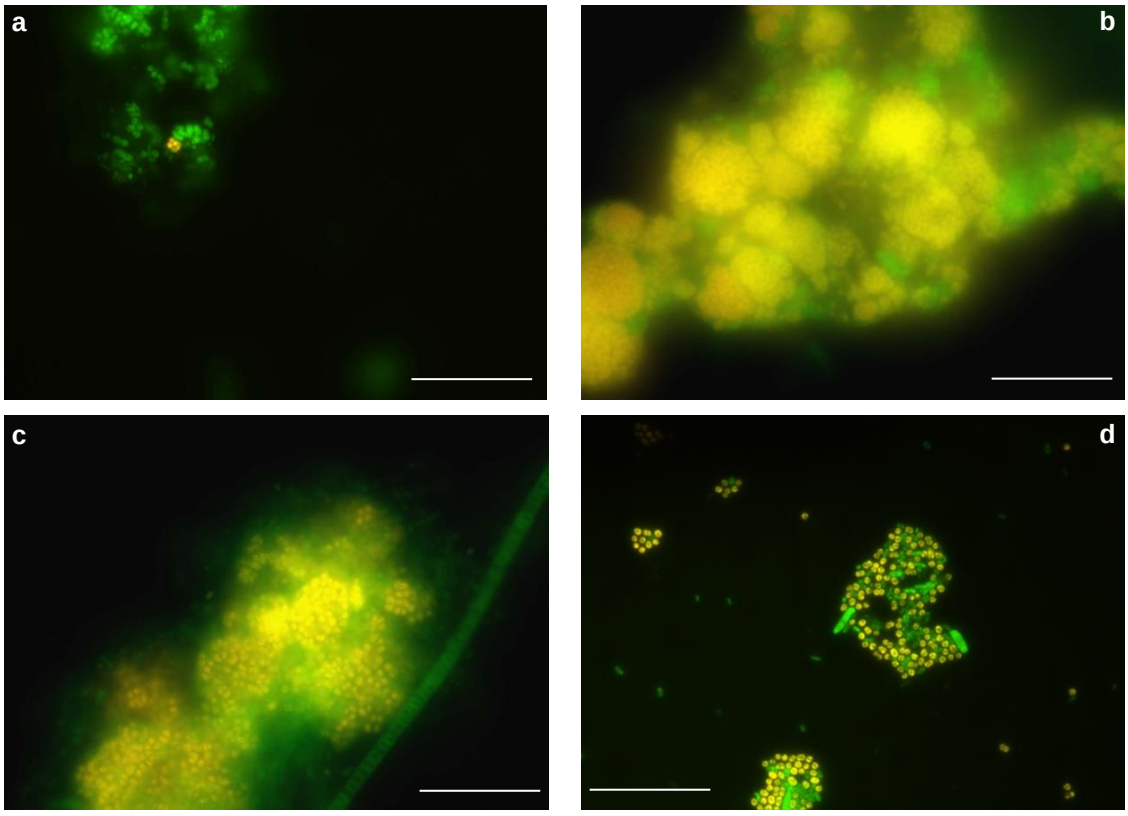


Figure 3.8 – FISH images: the *Paracoccus* population increased substantially between days (a) 22 and (b) 47 of reactor operation. The *Paracoccus* population (c) 70 days after inoculation and (d) after 152 days of reactor operation. PAR651 targeted cells appear in yellow and other bacteria appear in green. Bar=20µm

Between days 22 and 47 of reactor operation, the most dominant group seemed to shift from *Thauera* to *Paracoccus*. During this time, the reactor was reinoculated twice with previous purges and the aeration was reduced. These factors may have contributed to the shift in the population.

The predominance of PAR651 targeted cells among floc-forming bacteria persisted throughout the rest of the study, and with similar morphology, as seen in Figure 3.8 (c) and (d). Sequencing of samples from the end of the study showed that *Paracoccus* comprised about 10% of the population. These results are comparable to those obtained by Albuquerque et al.[101], who found that *Paracoccus* consumed a broader range of substrates as compared with *Azoarcus* and *Thauera*, which seemed to be more specialized in acetate and butyrate, respectively, and had a higher cell-specific substrate uptake. In that study, the community obtained was also rich in *Paracoccus* with a feeding with a similar VFA profile to the present study, with acetate comprising the majority of the VFAs, valerate being in the lowest amount and propionate and butyrate being in similar small amounts.

After the FF cycles began, the amount of *Azoarcus* cells increased. The cocci were mainly gathered in very small groups, while the bacilli were scattered, as showed in Figure 3.9 a). The amount of *Azoarcus* cells continued to increase more visibly and reached its peak by day 53, shown in Figure 3.9 b). This predominance was shared with the *Paracoccus* genus. This abundance can be due to the amount of acetate in the feed, as *Azoarcus* have been shown to prefer acetate to the other VFAs presently employed[101]. Even so, these bacteria took longer to become abundant, which can mean they have a lower growth rate than *Thauera* and *Paracoccus*.

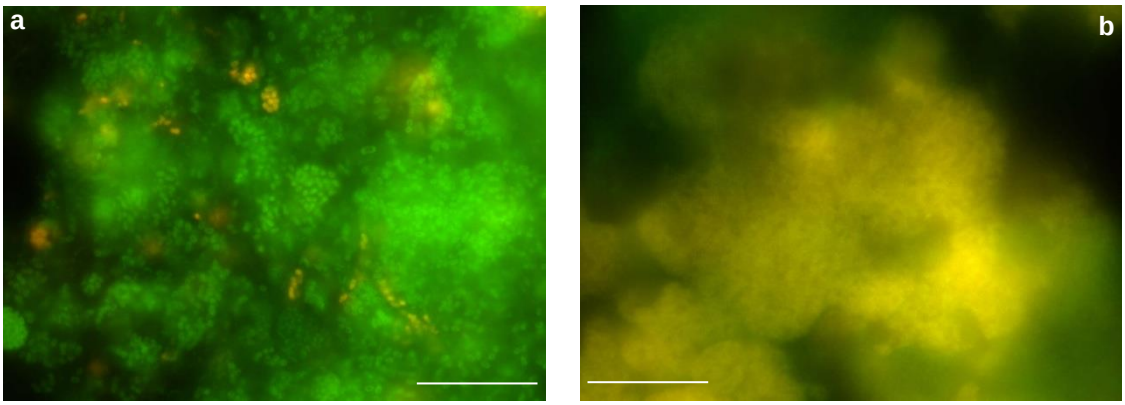


Figure 3.9 – FISH images: Evolution of *Azoarcus* from (a) day 22 to (b) day 53. AZO644 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

Azoarcus cells were mostly round and formed large and regular microcolonies, or, very rarely, were as single cells. *Azoarcus* cells remained in large amount for some time, but were not as organized as before, rather were scattered throughout the flocs (see Figure 3.10 a)). The cells were mostly bacilli. The presence of *Azoarcus* started decreasing in the reactor by day 89, but not as much as *Thauera*. The cells were also smaller, as Figure 3.10 b) illustrates.

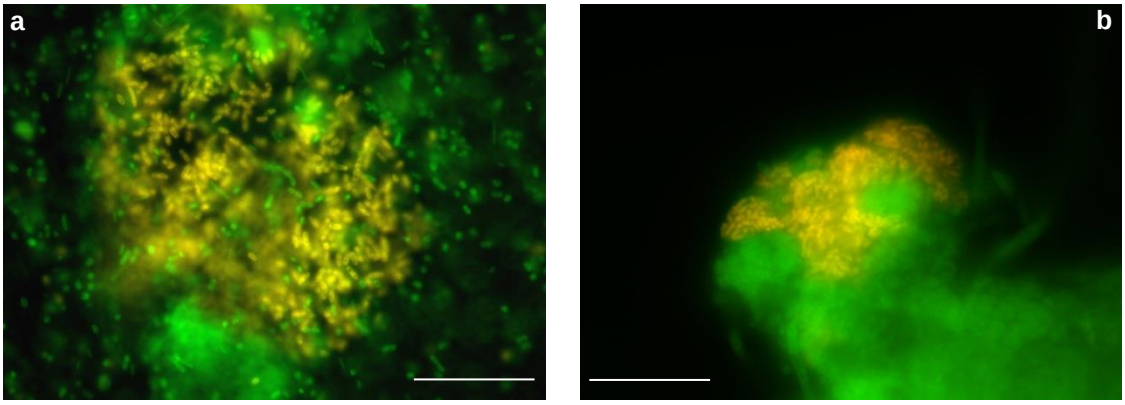


Figure 3.10 – FISH images: *Azoarcus* cells (a) at 71days of reactor operation and (b) after 152 days of reactor operation. AZO644 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

The amount of *Azoarcus* decreased between days 71 and 89 of reactor operation. During this time, the reactor was left without feed for a weekend. Possibly, the *Azoarcus* population is less resistant to long starvation than *Paracoccus*, causing their decline during this period.

The *Amaricoccus* genus remained in the reactor in low amount, organized in small clusters or scattered tetrads. Its amount decreased and *Amaricoccus* cells were almost absent from the reactor until the end of the study. Sequencing of samples from day 152 showed the presence of *Amaricoccus* being close to 0%. This decrease can be observed in Figure 3.11. *Amaricoccus* have distinctive morphology of cocci in tetrads[100], but their presence in single cells is also common. In the present study, both tetrads and single cells were identified with the AMAR839 probe.

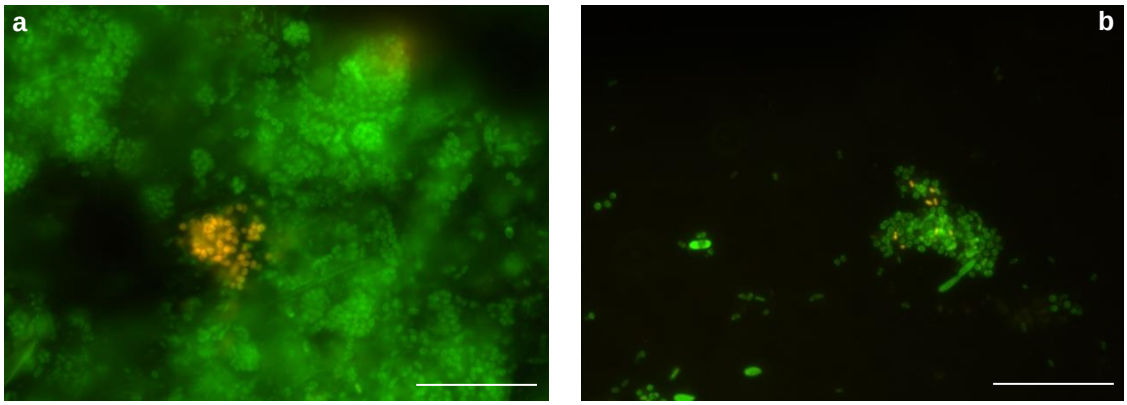


Figure 3.11 – FISH images: *Amaricoccus* cells (a) present in small amount after 22 days of reactor operation and (b) are almost absent at the end of the study. AMAR839 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

Amaricoccus has been observed in low amount in PHA-producing systems fed with acetate, which is the most abundant VFA in the present study[28]. In the same study, a population rich in *Amaricoccus* (around 61%) was obtained using propionate as substrate, which was presently employed in low amount.

The *Zoogloea* genus continued decreasing and had disappeared from the reactor by day 88. This was confirmed by the sequencing results. These two genera were present in fewer amounts than the other genera throughout the entire study.

3.4. Conclusions and Future Prospects

To improve the organism selection, it is important to know the organisms in the community. Identification of the filamentous bacteria can help control the problems created by their presence because different filaments are caused by different factors. Due to the morphological variability of the filaments and the likeness of different species makes morphological features unreliable for identifying these different bacteria.[113] Knowing that the filaments belong to the Alphaproteobacteria class, further FISH probes can be used to identify the filaments, namely the ones described by Levantesi et al.[113], Thomsen et al.[115] and Kragelund et al.[104]. Also, isolation can be attempted using micromanipulation, for a phylogenetic study. Furthermore, these filamentous bacteria can be exploited for their PHA-storage potential. In order to do so it is important to confirm their PHA-storage capacity by assessing the PHA productivity in a system enriched with these bacteria.

To better understand the shifts in the population presently described, quantitative FISH should be applied. It would permit assessing the quantity of organisms belonging to each genus relatively to the biovolume and detect smaller differences in amount. This would help comprehend how the population changes throughout time, contributing to the understanding of how the bacteria behave in PHA-producing systems.

Detecting which of the identified organisms actually store PHA can be done through post-FISH Nile Blue staining, which has been accomplished successfully[101].

Chapter 4

Population Dynamics in PHA-storing Systems Alternating Between Cheese Whey and Molasses as Feedstock

4.1. Introduction

In this chapter, renewable feedstock was used for the production of PHAs. Unlike with pure cultures, the use of carbohydrates by MMC results in the accumulation of glycogen instead of PHAs.[10] This can be overcome by the addition of an acidification step, where the sugars from industrial or agricultural sub-products are converted into VFAs. These acids are then used as precursors in PHA biosynthesis,[5,53] that takes place in an SBR, where selection of storing organisms occurs.

Accordingly, in this study a step was added to the system and the microbial consortium of a three-step process for PHA production were studied. In the first stage, acidogenesis occurred in a membrane bioreactor (MBR); the cleared permeate resultant from the first stage was used to feed the selection SBR; the selected biomass was used in a fed-batch reactor to achieve maximum storage. The selection of suitable raw materials for PHA production is very much dependent on their ready availability and current cost. There is also the dependency on product seasonality and other variations that may occur in the industry.[116] Consequently shifting feedstock to accommodate those variations may be necessary and it is desirable to do so without suffering alterations in productivity and product composition. Therefore, different agro-industrial substrates were used to feed the MBR in this study.

Sugarcane molasses is a by-product of the sugar refinery industry with very high sugar content, with total sugars making up 54 % w/w of the molasses, composed mainly of sucrose (62 %) and fructose (38 %).[45,51] Sugar beet and sugar cane refining plants are the major sources for sugar molasses that contains high sucrose content. Depending on the grades and sources, sugar molasses may not be appropriate for further used in foods or feeds.[51] On the other hand, the dairy industry is an important part of the agricultural sector and cheese whey is a large-volume sub-product of the industry. It is rich in fermentable nutrients such as lactose, lipids and soluble proteins. The direct disposal of cheese whey represents an environmental concern because of the high volumes produced and its high organic matter content, making its application more valuable.[51,117] Both these wastes are readily available in Portugal.

The MBR was initially fed with cheese whey. Then, the substrate was changed to sugarcane molasses, and back to the cheese whey. High throughput sequencing was employed in the study of the community of both reactors, while quantitative FISH was only employed in the study of the SBR. FISH was not employed in the study of the anaerobic reactor due to the lack of probes available that target these communities. The microbial population profiles of the two reactors and their dynamics throughout the study were correlated to the shifts imposed on the feedstock and VFA profile, respectively. The FISH probes selected target well-known PHA producers commonly found in activated sludge: AZO644 for the *Azoarcus* genus, PAR651 for the *Paracoccus* genus and THAU832 for the *Thauera* genus, as well as EUBmix for all bacteria.

4.2. Experimental Set-up

The system studied is represented in Figure 4.1. The operation of both reactors was carried out by someone else.

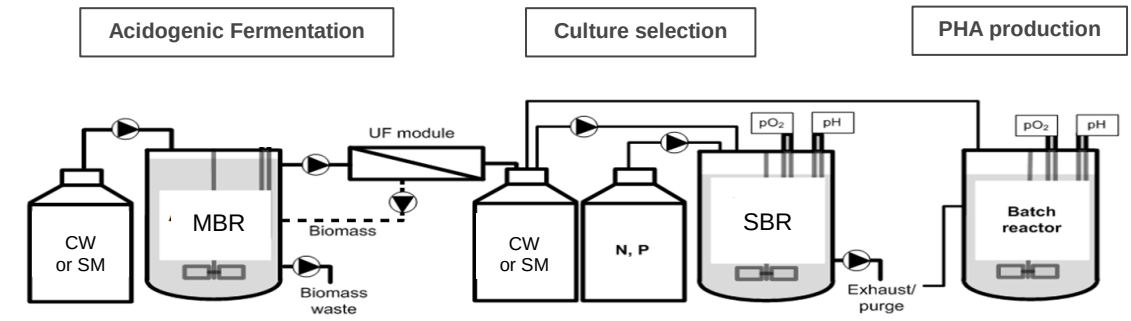


Figure 4.1 – Schematic representation of the three-step process presently studied.

4.2.1. Fermentation Reactor

For 16 days, the MBR reactor was fed with cheese whey (CW), with SRT of 2.5 days. Then, the feedstock was changed from the CW to sugarcane molasses (SM). This phase lasted 75 days, with SRT of 3.4 days. Lastly, the feedstock was changed back to the CW, with SRT of 2.9 days. The HRT was of 1 day. A time-line of these changes is represented in Figure 4.. The phases were long enough for reactor stability to be obtained (more than 3x SRT). Several samples were taken throughout the three phases for sequencing.

4.2.2. Selection Reactor

Firstly, the SBR was fed with a solution of synthetic VFAs containing acetate, butyrate, propionate and valerate, for 10 days. Then, the fermented sugarcane molasses (fSM) resultant from the second stage was fed to the reactor for a period of 28 days. After this period, the SBR was fed with the fermented cheese whey (fCW) for 49 days. A time-line of these changes is represented in Figure 4.2. The SRT was of 4 days, and so the phases were long enough for reactor stability to be obtained (more than 3x SRT), and the HRT was of 1 day. Several samples were taken throughout the three phases for FISH analysis and sequencing.

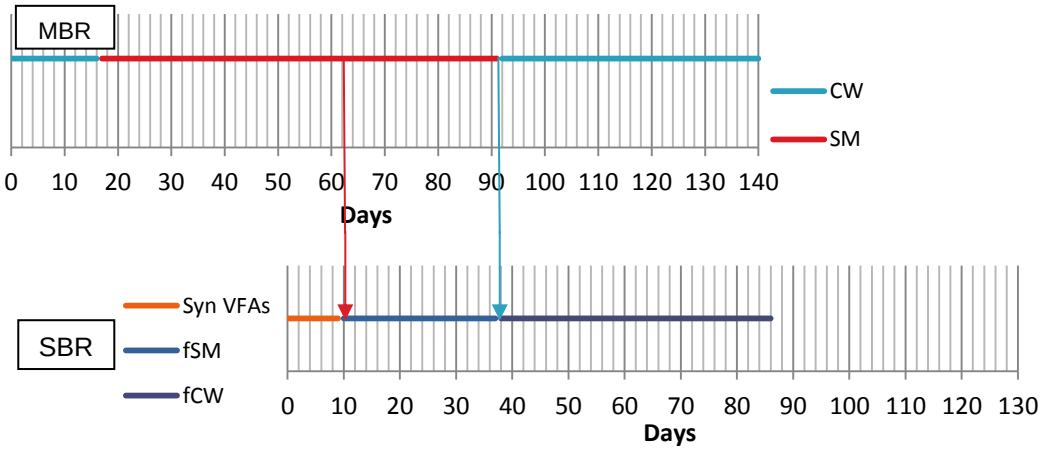


Figure 4.2 – Time-line of the feedstock changes imposed on the reactors.

4.2.3. Accumulation Fed-Batch

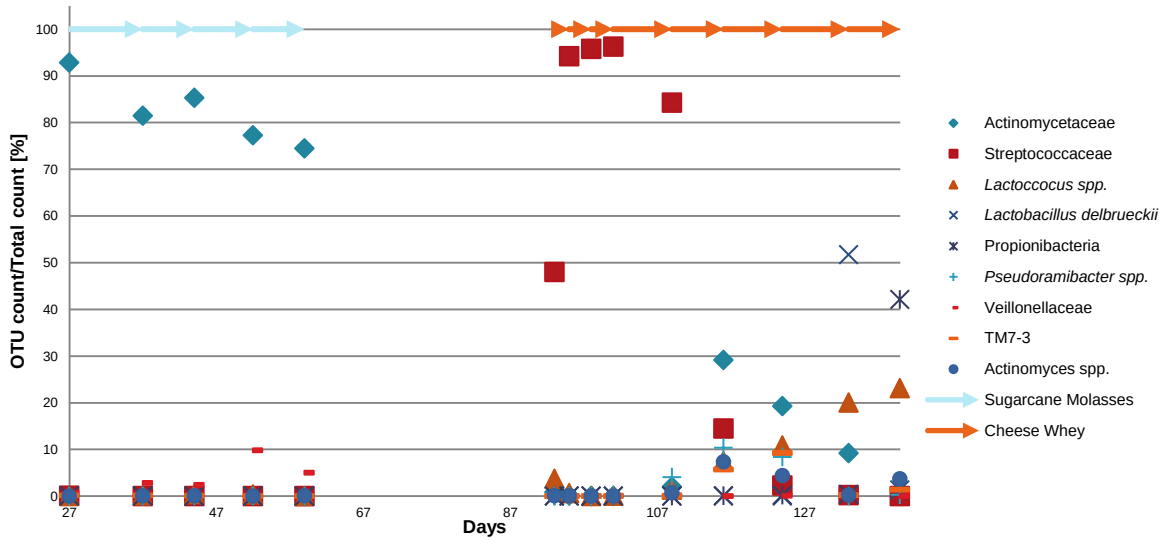
For the evaluation of maximum PHA storage, a Fed-Batch reactor was set-up, fed with fCW or the fSM.

4.3. Results and Discussion

To observe how the feedstock shift affected the biomass, samples were taken throughout reactor operation. Sequencing was performed for both the fermentation stage and the selection stage and quantitative FISH analysis was performed to support the results obtained through sequencing.

4.3.1. Fermentation Reactor Community

High throughput sequencing was performed to identify the major groups of organisms that populated the reactor in each phase and evaluate the occurrence of changes in the population to relate them to the change in feedstock. In the fermentation reactor, a clear shift in the population accompanied the substrate shift, with different families dominating in each phase, as can be seen in the sequencing results represented in Figure 4.3.



Phylum	Class	Order	Family	Genus	Species
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae		
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	*Actinomyces*	
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae		
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*delbrueckii*
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae		
Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	*Pseudoramibacter*	
Firmicutes	Clostridia	Clostridiales	Veillonellaceae		
TM7	TM7-3				

Figure 4.3 – Most abundant operational taxonomic units (OTUs) in the membrane bioreactor throughout operation with sugarcane molasses and cheese whey as substrate, and respective phylogeny.

During the sugar molasses phase, the Actinomycetaceae family constituted 75-93% of the total amplicon count. In the beginning of the phase, it represented 93% of the total count, and its amount slowly decreased to 75%, reached at the end of the sugar molasses phase. The Actinomycetaceae family disappeared with the introduction of cheese whey as substrate. After 25 days of operation with cheese whey, there was a peak of the OTU related to Actinomycetaceae of 29% but it decreased until it disappeared again after 24 days. Most

members of the Actinomycetaceae family are capable of fermenting glucose and many have acetate as a major fermentation product and some produce lactate as well.[118,119] Accordingly, there was a lactate peak in the permeate solution (see Figure 4.4) during the cheese whey phase which coincides with the peak in the population of Actinomycetaceae.

Another group of bacteria seemed to be present in the reactor during the sugar molasses phase, the Veillonellaceae family, in <10%. No organisms belonging to this family were detected in the cheese whey phase. Other OTUs were established but their count was close to 0% of total OTUs.

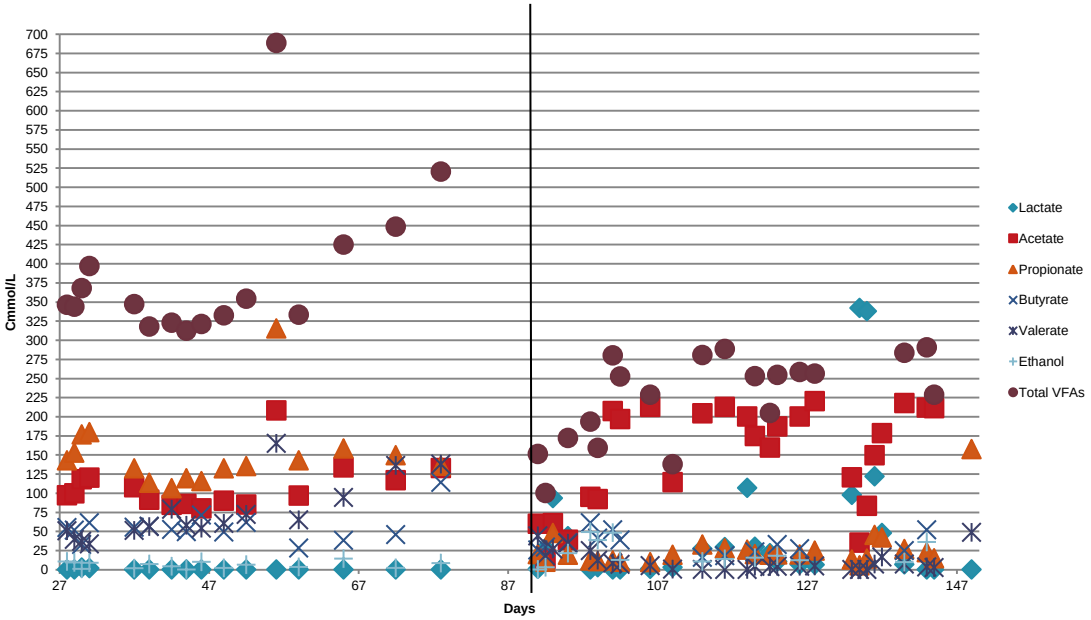


Figure 4.4 – VFA concentration of the permeate solution throughout reactor operation with sugarcane molasses and cheese whey as substrates.

The bacterial community during the cheese whey phase was less stable and more groups of organisms were detected. The Streptococcaceae family was non-existent with sugar molasses as the carbon source; however it was dominant during the first 18 days of the cheese whey phase. It reached 96% of total amplicon count after 10 days of operation with cheese whey, and then it started to decrease abruptly (in 9 days decreased from 84% to 14%). *Streptococcus* is one of the representative genera of the Streptococcaceae family. *Streptococci* are well recognized lactic acid bacteria that grow well under high nutrient conditions and produce lactate.[120] Davilla-Vasquez et al.[121] investigated a fermentative biohydrogen-producing continuous stirred tank reactor (CSTR) with cheese whey as substrate and occasionally detected *Streptococcus* sp. during operations. Several studies[122,123] on the use of granular sludge to enhance dark fermentation biohydrogen production have identified the existence of *Streptococcus* sp. in the bacterial community, using starch, xylose, glucose, and sucrose as substrates. The presence of *Streptococcus* sp. is possibly the most important factor in granular sludge formation in these efficient dark fermentation granular sludge systems.[124] Streptococcaceae are facultative anaerobic bacteria and produce mostly lactate by fermentation,[125] and members of this family are able to produce ethanol[126]. In the beginning of

the cheese whey phase, when the Streptococcaceae family dominated, the amount of ethanol produced increased as did the amount of lactate.

The abrupt decrease of the Streptococcaceae -related OTU coincided with the increase of the Actinomycetaceae family. With this decrease, the *Lactococcus* genus of the Streptococcaceae family appeared and increased slowly to make up 23% of the total amplicon count. The organisms belonging to this genus are characteristic facultative anaerobes that are able to ferment sucrose.[127] It is known that small amounts of *Lactococcus* can have an inhibiting effect to hydrogen production and can produce ethanol.[126] Ethanol production was overall greater during the cheese whey phase than in the molasses phase, and was slightly superior in the end of the phase than in the middle, when the *Lactococcus* population reaches its highest amount.

Bacteria of the *Actinomyces* genus were detected at the same time as the abrupt decrease of the Streptococcaceae family occurred, and remained in low amount until the end of the study. This genus is usually associated with milk and milk products.[128]

A similar behaviour was shown by the *Pseudoramibacter* genus and the candidate phylum TM7, which seemed to reach a higher amount. *Pseudoramibacter* is a genus of strictly anaerobic bacteria. The growth of these bacteria is stimulated by fermentable carbohydrates and the fermentation end products include acetate and butyrate.[129] Acetate was the VFA produced in highest amount during the CW phase, and butyrate was produced in a slightly larger amount when this OTU appeared. Organisms belonging to the TM7 candidate division have been detected in a range of chemically and geographically diverse habitats[130] including WWTPs[109]. Filaments of this division have been shown to take up glucose.

After 42 days of the cheese whey phase, OTUs related to *Lactobacillus delbrueckii* appeared in the reactor with a total count of 52% of total OTUs, but 7 days later it was just over 1%. *Lactobacillus delbrueckii* seemed to emerge in the end of the cheese whey phase. A subspecies, *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), is used in the dairy industry to transform milk into yogurt.[131] *L. delbrueckii* has also been employed at the start of a fed-batch system so as to utilize glucose and produce lactate to then serve as substrate for PHB production.[132] The last lactate production peak coincides with the peak in the OTU related to *L. delbrueckii*.

When the amount of *Lactobacillus delbrueckii* decreased, the family Propionibacteria appeared in considerable amount, with an OTU count of 42%. This family is usually associated with milk and milk products[128] and has been found in anaerobic sludge[127]. This family is a known propionate producer[118] and the emergence of the OTU related to this family matches a slight increase in propionate production in the reactor.

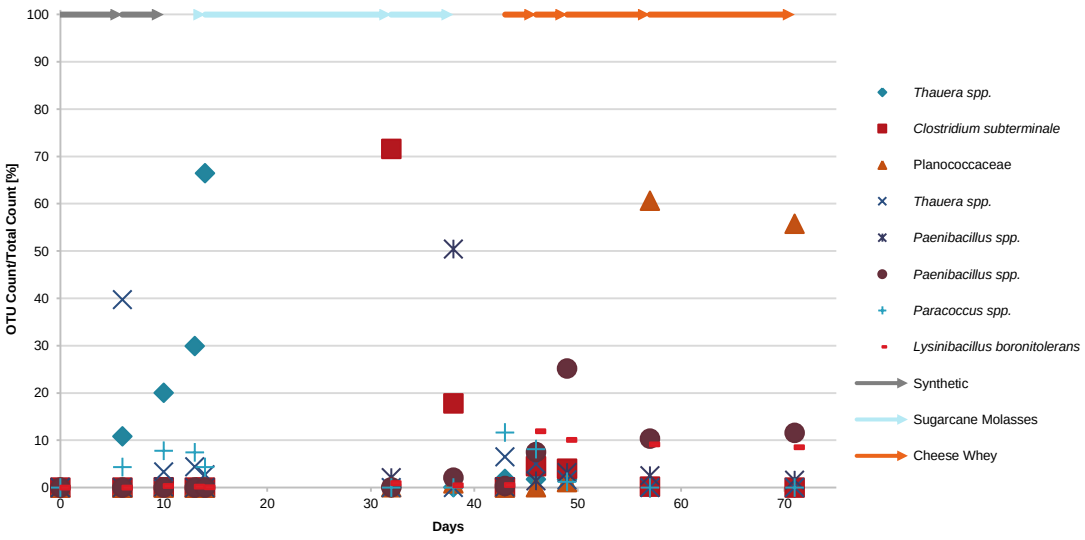
4.3.2. Overall Volatile Fatty Acids Profile

In the sugar molasses phase the permeate solution contained a higher concentration of VFAs, and propionate and valerate, HV precursors, constituted around 58% of the total VFAs. With the beginning of the cheese whey phase, the amount of VFAs in the permeate solution decreased, especially of the HV precursors. Nevertheless, the acetate concentration increased, and HB precursors (acetate and butyrate) comprised around 89% of the total VFAs. Despite the

apparent unstable population during the cheese whey phase, the VFA profile was relatively stable.

4.3.3. Selection Reactor Community

Despite the clear change in the population accompanying the feedstock shift as happened in the MBR, there was not a dominant group for each phase, as can be seen in Figure 4.5. Furthermore, FISH quantification of the AZO644, PAR651 and THAU832 targeted cells was performed to compare with the sequencing results, since high throughput sequencing is not as quantitatively accurate.



Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	*Thauera*	
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*subterminale*
Firmicutes	Bacilli	Bacillales	Planococcaceae		
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	*Thauera*	
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Paracoccus*	
Firmicutes	Bacilli	Bacillales	Planococcaceae	*Lysinibacillus*	*boronitolerans*

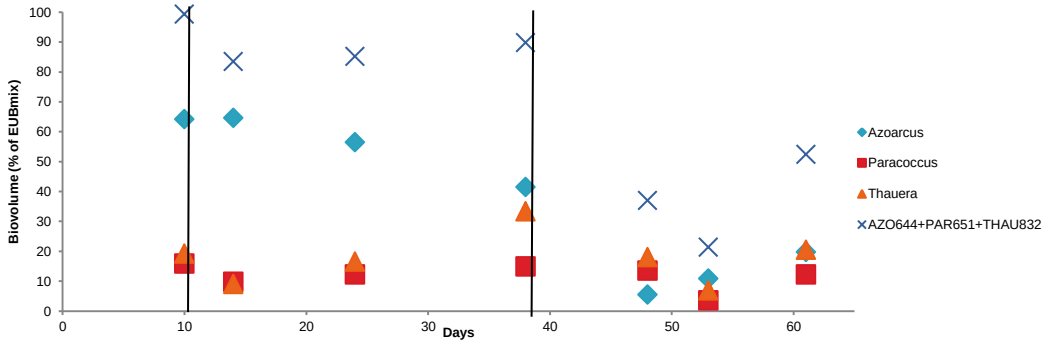
Figure 4.5 - Most abundant OTUs in the sequencing batch reactor throughout operation with sugarcane molasses and cheese whey as substrate, and their respective phylogeny.

Sequencing results show the presence of two separate OTUs related to *Thauera* during the "synthetic" phase, one comprising 40% of total amplicon count and the other, 11%. *Paracoccus* was also present. The quantitative FISH analysis revealed that these two genera together with *Azoarcus* virtually covered the entire biovolume (see Figure 4.6). *Azoarcus* wasn't detected through sequencing, but due to the phylogenetic proximity between *Azoarcus* and *Thauera*, it is possible that they were gathered in the same OTU, which could explain the high count of a *Thauera* related OTU when FISH did not show *Thauera* to be present in a very high amount.

After the feeding with fSM started, one of the OTUs related to *Thauera* decreased in four days and remained in low amount. In the end of operation with sugar molasses the same population was not detected. Similarly, a slight decrease in *Thauera* after the feeding with fSM

was initiated was observed through the quantitative FISH analysis. The high throughput sequencing results also showed the presence of another *Thauera*-related OTU that increased considerably with the beginning of the feeding with fermented molasses, reaching 66% of total amplicon count. Similarly, with the quantitative FISH, *Azoarcus* was shown to reach 65% in the beginning of the molasses phase. Quantitative FISH also showed a decrease in the *Azoarcus* and *Thauera* populations with the beginning of the fCW phase, reaching 5.5% and 6.9% of the biovolume, respectively. However, sequencing did not show either OTU related to the *Thauera* genus to be present in the acclimated biomass fed with fCW.

The OTU related to *Paracoccus* was detected in low amount throughout the study, as confirmed by quantitative FISH analysis. Although no OTU belonging to the genus was found in the fSM acclimated biomass, FISH analysis showed that the *Paracoccus* population remained somewhat constant throughout the entire study.



	AZO644			PAR651			THAU832		
	Abundance [a] (% of EUBmix)	s.e.m. (% of EUBmix)	Congruency [a] (%)	Abundance [a] (% of EUBmix)	s.e.m. (% of EUBmix)	Congruency [a] (%)	Abundance [a] (% of EUBmix)	s.e.m. (% of EUBmix)	Congruency [a] (%)
D 10	64.2	2	99	15.9	2.3	100	19.2	2	100
D 14	64.6	1.1	99	9.8	0.9	100	14.9	1.5	99
D 24	56.4	2.1	100	12.2	0.3	100	16.5	0.9	97
D 38	41.5	2.4	100	14.9	1.5	100	33.3	2.1	100
D 48	5.5	1.1	100	13.5	0.8	100	18	2.4	99
D 53	10.8	1.6	100	3.6	0.3	100	6.9	0.8	99
D 61	19.7	1.4	99	12.2	1.4	100	20.5	1.7	100

[a] Abundance determined as biovolume fraction of total bacteria and congruency between the population and general probes, as per the Daime software

Figure 4.6 - Illustration of the biovolume of each genus studied relatively to all bacteria (EUBmix).

The VFA profile of the permeate solution remained constant throughout much of reactor operation with sugarcane molasses. However after 20 days of feeding the SBR with the fermented molasses, the VFA profile changed and the four VFAs were present in the reactor in similar amount. In the acclimated biomass of the fSM phase, new groups of organisms appeared, some in high amount, namely, *Clostridium subterminale* (72%) and *Paenibacillus* sp. (50%). *Clostridium subterminale* is a PHA producer,[133] and organisms related to the genus *Paenibacillus*, have been shown to produce PHA as well[134], showing that the conditions applied were efficient in selecting PHA producing organisms. However, after the feeding changed to fermented cheese whey, both groups stayed in the reactor in very low quantity.

Another OTU related to the *Paenibacillus* genus emerged in the fCW phase, reaching 25% of total amplicon count, and stayed in the system at around 10%. Another group of organisms was found in the community in high amount well into the fCW phase, the Planococcaceae family, which made up more than half of the total amplicon count (55-60%). Also *Lysinibacillus boronitolerans*, which belongs to the Planococcaceae family, appeared in the system with the

fermented cheese whey as feedstock and remained at around 10%. All three new OTUs are related to the order Bacillales which has been shown to possess PHA producers[135].

The morphology of the targeted organisms changed slightly with the feedstock shift, reflecting the changes observed in microbial population structure described above. In the acclimatised biomass of the "synthetic" phase, *Azoarcus*, the most abundant group, was represented by bacilli and coccobacilli. With the introduction of fermented molasses as substrate, the cells became bigger and most became rounder as well. This trend continued with the introduction of cheese whey, when *Azoarcus* cells were mostly cocci. These changes can be observed in Figure 4.7.

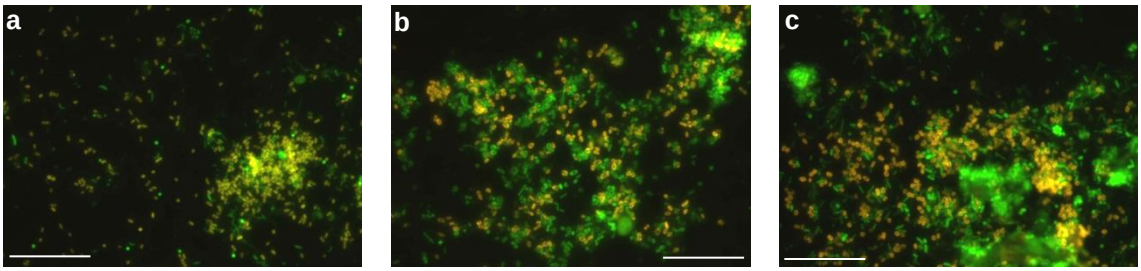


Figure 4.7 – FISH images show the morphology of the *Azoarcus* population during a) the synthetic phase, b) the fermented molasses phase, and c) the fermented cheese whey phase. AZO644 targeted cells appear in yellow while all other bacteria appear in green. Bar=20µm.

THAU832 targeted organisms showed a similar response to that of *Azoarcus* cells and can be seen in Figure 4.8. In the "synthetic phase", *Thauera* cells were mainly small bacilli, mostly present in the flocs, not in suspension. The introduction of molasses permeate gave rise to bigger and round cells, but the small bacilli were also present. In the last phase, the cells of the *Thauera* population were overall rounder, with many cocci.

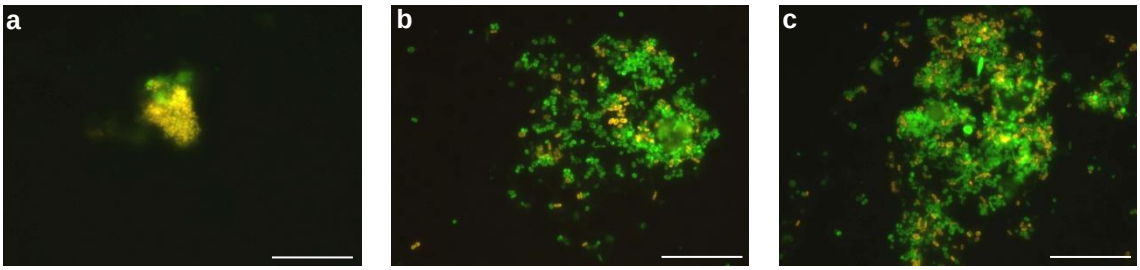


Figure 4.8 - FISH images show the morphology of the *Thauera* population during a) the synthetic phase, b) the fermented molasses phase, and c) the fermented cheese whey phase. THAU832 targeted cells appear in yellow while all other bacteria appear in green. Bar=20µm.

The *Paracoccus* population was less evidently affected by the feedstock shift (see Figure 4.9). Firstly, they were small and round, and organized in small microcolonies. With the introduction of fermented molasses as carbon source, the *Paracoccus* cells became bigger, but the difference is not as noticeable as in the other two genera. No changes were observed in the last phase.

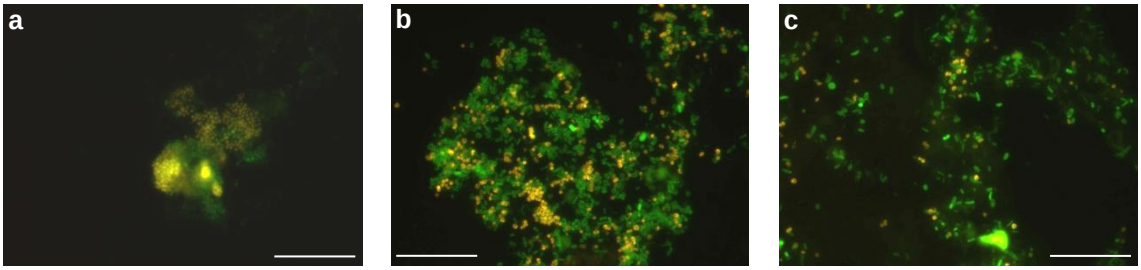


Figure 4.9 - FISH images show the morphology of the *Paracoccus* population during a) the synthetic phase, b) the fermented molasses phase, and c) the fermented cheese whey phase. PAR651 targeted cells appear in yellow while all other bacteria appear in green. Bar=20µm.

4.3.4. Polymer Production

With the shift in the VFA profile, the composition of the produced polymer also changed. During the sugar molasses phase, the polymer HB:HV ratio was of 47:53 while with the cheese whey permeate it was of 83:17. Maximum accumulation determined in accumulation batch tests was superior with the cheese whey, reaching 65% w/w, while with sugarcane molasses only 56% w/w was obtained.

4.4. Conclusions and Future Prospects

Changing the feedstock used for PHA production can be necessary to accommodate changes that can occur in the industry that provides the substrate. Here, two readily available substrates in Portugal were used, cheese whey and sugarcane molasses. The communities of the fermentation and the selection reactors were studied.

It was possible to observe that the feedstock affected both communities and their respective products. In the fermentation MBR, the dominant microbial community, according to high throughput sequencing data, shifted from the Actinomycetaceae to the Streptococcaceae family. As a result of changing the feedstock, the produced VFA profile also changed, which affected the microbial population in the selection SBR. While during operation with fSM the population was clearly dominated by members of the *Azoarcus*, *Thauera* and *Paracoccus* genera, operation with fCW selected other organisms, where *Lysinibacillus* and *Paenibacillus* were possibly relevant groups. However, it is unclear if these genera are involved in the observed PHA storage.

For a feedstock shift to be viable, it is necessary that there are no major shifts in the resulting polymer. Therefore, it is important to explore different operation conditions and different substrate combinations that can result in the most similar VFA profiles possible.

Moreover, as the accumulating population, and consequently the polymer, are directly affected by the feedstock, the identification of the carbon sources preferred by the different organisms in the community is relevant for the development of segregated metabolic models that incorporate the fraction of each PHA-producing population and their corresponding substrate preferences, which would allow more specific metabolic flux analysis.

Chapter 5

Effect of a pH Change in the Fermentation Stage on the PHA-producing Population

5.1. Introduction

Apart from the substrate, the operation parameters also affect the microbial community of the fermentation reactor and consequently the resulting VFAs and the VFA conversion yield.[10] The products from the acidogenic reactor depend on conditions such as pH[45], organic loading rate (OLR), hydraulic retention time (HRT) and temperature[136]. Also, as different VFAs lead to different polymer compositions, the conditions in the fermentation reactor can be changed to accommodate our requirements and attain higher productivity.

Moreover, fermentation conditions can be altered to avoid the formation of side products. In an acidogenic reactor, it is intended to maximize acidogenic fermentation while minimizing methanogenic fermentation. Methanogenic activity can be prevented by employing conditions which favour acidogenic bacteria such as low HRT, low temperature,[73] low pH,[45] or a combination of the different approaches. Accordingly, in this chapter the pH of the fermentation reactor was altered. With this change, it is expected for changes to occur in the VFA profile and consequently in the SBR community. The effect on the SBR community was studied.

FISH analysis and sequencing were performed to identify key elements of the microbial community, particularly those with PHA-storage properties, and evaluate the effect of the pH change on the producing population. Also, samples were taken regularly for light microscopic observation to detect major changes in the biomass, and for Nile Blue staining to assess the occurrence of changes in the storing population.

5.2. Experimental Set-up

Operation of both reactors was carried out by someone else.

Cheese whey was used as feedstock. The MBR was acclimatised with (i) pH6, HRT of 0.5d and OLR of 40g/L/d; (ii) and then the pH was changed to 5.

The selection reactor was fed with the fermented cheese whey and supplemented with a nutrient solution containing NH_4Cl and KH_2PO_4. It worked in F/F 12h cycles. Samples from the SBR were taken every few days for microscopic observation of the biomass. The reactor was fed with fCW from phase (i) for 42 days, and with fCW from phase (ii) for 30 days.

5.3. Results and Discussion

For 73 days, samples were taken regularly for microscopic observation of the biomass. The biomass was characterized using FISH analysis, but the samples were also observed with brightfield and phase contrast to detect immediate changes in the biomass morphology and composition, and Nile Blue helped assess if the biomass was being selected for PHA storage.

Despite the pH change in the MBR, the pH of the SBR was not affected.

5.3.1. Population Dynamics

FISH analysis showed *Thauera* to be the most prevalent genus of the three genera studied throughout the entire study despite the occurrence of fluctuations. In the biomass acclimated with fCW from the pH6 phase, the *Thauera* population was present in substantial amount, and was comprised of bacilli in loose clusters, small and, mostly, large. After permeate from the pH5 phase was introduced, the amount of *Thauera* organisms in the reactor increased considerably

and seemed to constitute most of the bacterial community. This trend continued throughout the study, together with the appearance of cocci. This evolution can be seen in Figure 5.1.

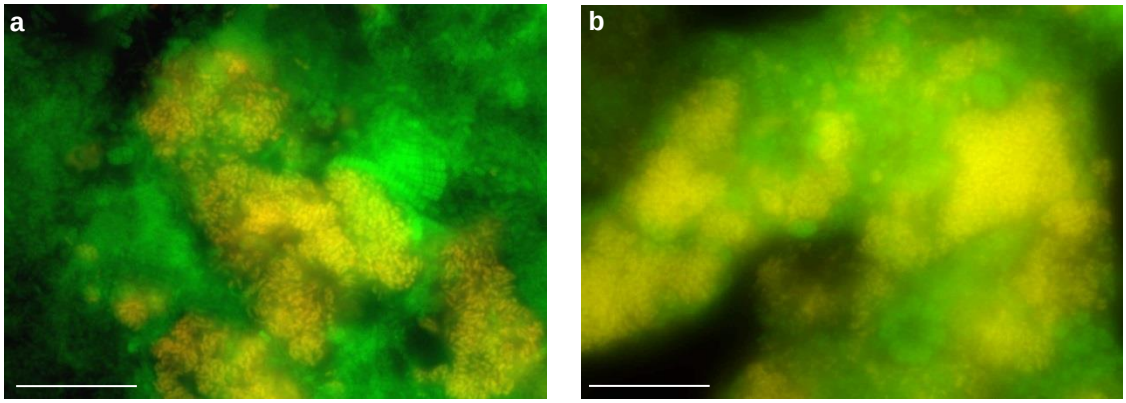


Figure 5.1 – FISH images: the *Thauera* population (a) before and (b) after the shift from the fCW of the pH6 phase to the fCW of the pH5 phase. THAU832 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

The *Azoarcus* population also seemed to react positively to the change in the MBR, albeit not as evidently. Before changing the permeate solution, *Azoarcus* cells were present in low amount, and the cells were mostly scattered in the flocs. The genus was represented by bacilli and some coccobacilli. With the introduction of the fermented cheese whey from the pH5 phase, the amount of organisms belonging to *Azoarcus* increased. In addition, the observed cells belonging to this genus were rounder and were more organized in tight clusters. This change is shown in Figure 5.2. As the pH5 phase progressed, the *Azoarcus* population continued to increase slightly and the clusters became looser.

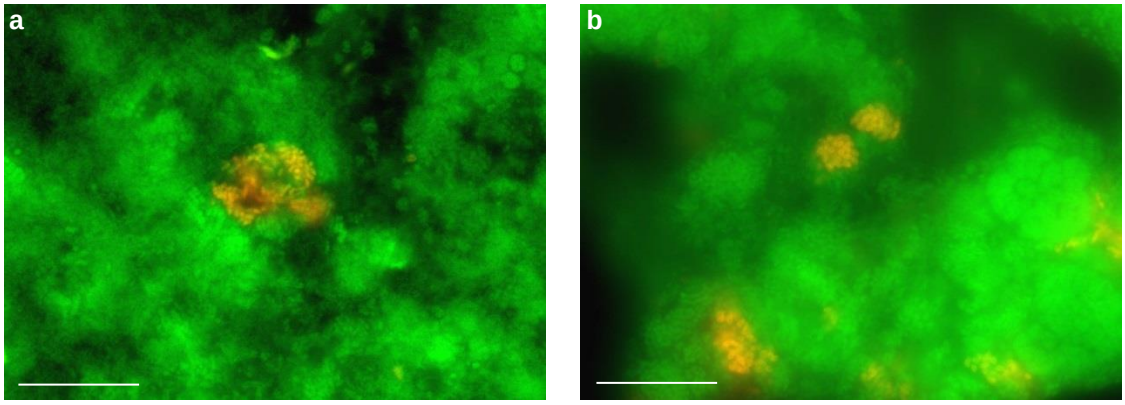


Figure 5.2 - FISH images: the *Azoarcus* population (a) before and (b) 8 days after the shift from the fCW of the pH6 phase to the fCW of the pH5 phase. AZO644 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

Paracoccus seemed to be the least affected population of the three groups monitored through FISH. *Paracoccus* organisms had a contrary response to the alteration than *Azoarcus* and *Thauera* organisms (see Figure 5.3). The amount of *Paracoccus* cells decreased substantially and was almost non-existent by the end of the study, although it was already low in the beginning. The cells were round and mostly organized in small clusters. Some bigger clusters could be found.

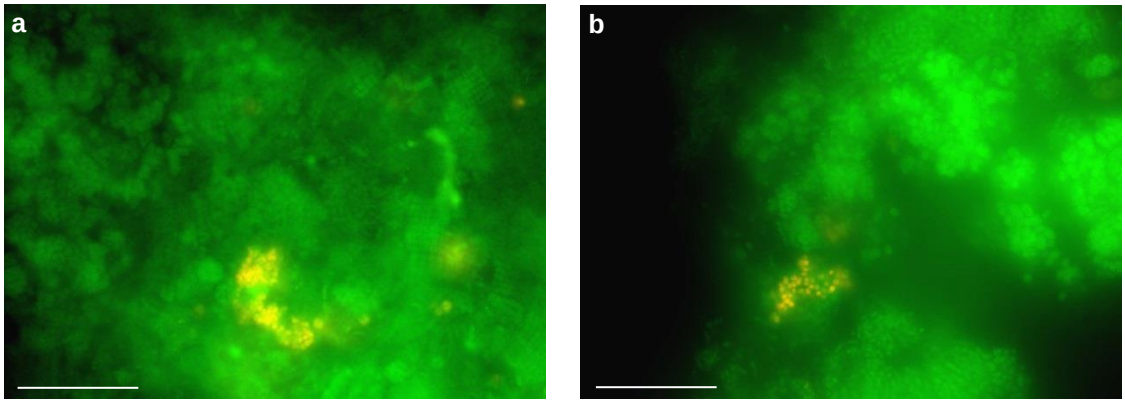


Figure 5.3 - FISH images: the *Paracoccus* population (a) before and (b) 8 days after the shift from the fCW of the pH6 phase to the fCW of the pH5 phase. PAR651 targeted cells appear in yellow and all other bacteria appear in green. Bar=20µm

The change in pH in the fermentation reactor impacted on the selection reactor. It led to an obvious change in the VFA profile (see Figure 5.4). Even though butyrate and acetate made up the majority of VFAs produced in both phases, the change in pH led to a considerable decrease in acetate production and a substantial increase in the production of butyrate (around 60% of total VFAs) and lactate. Both propionate and valerate were produced in low amount in both phases.

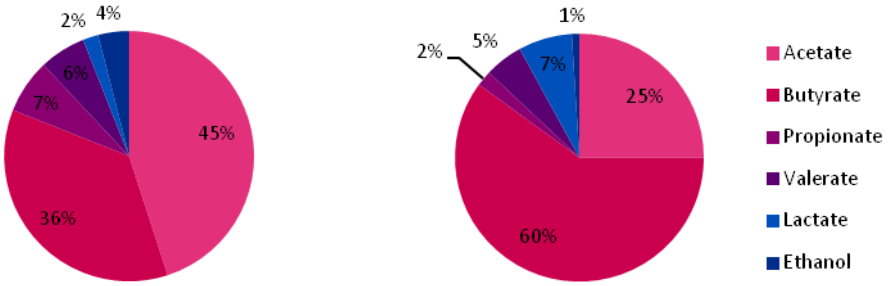


Figure 5.4 – VFA profile during the (a) pH6 phase and the (b) pH5 phase.

Azoarcus, *Thauera* and *Paracoccus* can consume acetate efficiently.[28,101] Albuquerque et al.[101], using fermented sugarcane molasses as feedstock, showed *Thauera* to efficiently consume butyrate while *Azoarcus* did not take up butyrate at all. However, many studies have shown that *Azoarcus* cells are able to use butyrate as carbon source for growth[137]. Albuquerque et al.[101] also reported that *Paracoccus* preferred butyrate over the other VFAs, but in this study the amount of *Paracoccus* in the system decreased with the increase in butyrate, although the size of the *Paracoccus* population was very small to begin with. Possibly, the *Paracoccus* population present in this study is comprised of different species than the ones found by Albuquerque et al.[101]. In that study, the SRT was of 10 days, which could have led to the selection of a different population because longer SRTs allow for slower growing organisms to thrive in the reactor.

5.3.2. Promising Unknown Storing Population

Throughout the entire study, interesting clusters with high storage potential were observed (see Figure 5.5). They formed rectangular sheets of various sizes, and could be highly organized or as if falling apart.

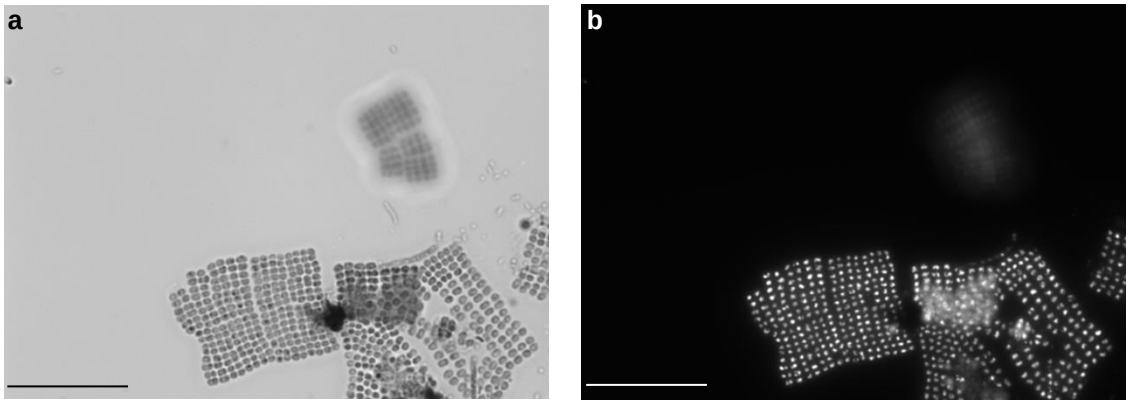


Figure 5.5 – (a) bright field microscopic image of the sheets; (b) Nile Blue image shows PHA storage capacity of the clusters. Bar=20µm

Next generation high throughput sequencing was performed to detect the major bacterial groups present in the biomass acclimated with fCW from phase (i). The results showed the Proteobacteria phylum to be the most abundant group, comprising around 93% of total amplicon count, followed by Actinobacteria (4%) and Bacteroidetes (2%) (see Table 5.1).

Table 5.1 - Most abundant OTUs found in the SBR at the end of the study and their relative abundances

Phylum	**Class**	**Order**	***Genus***
Actinobacteria (4%)			
Bacteroidetes (2%)			
Proteobacteria (93%)			
	Alphaproteobacteria (12%)		
		Rhizobiales (4%)	
		Rhodobacterales (6%)	
			Paracoccus (2%)
	Betaproteobacteria (74%)		
		Burkholderiales (54%)	
			Lampropedia (35%)
			Rubrivivax (10%)
		Rhodocyclales (17%)	
			Thauera (15%)
	Gammaproteobacteria (6%)		
		Xanthomonadales (6%)	

FISH was performed to try to identify the group the organisms belonged to, using probes for the three major Proteobacteria (ALF969, BET42a and GAM42a) and of known tetrad forming organisms[86]: TFOmix (TFO-DF618 + TFO-DF218), HGC69a, and DEFmix (DF988 + DF1020 + helper probes). None of these probes seemed to hybridise with the clusters.

Sequencing results show the *Lampropedia* genus of the Betaproteobacteria class to constitute around 35% of total amplicon count. Although no results were obtained with the

BET42a probe, the description of the genus is consistent with the organisms found in this study (see Figure 5.6). Organisms belonging to the *Lampropedia* genus are gram-negative cocci found in enhanced biological phosphorus removal (EBPR) plants.[138] They form sheets of cells arranged in square tablets of 16–64 cells, occasionally separated into pairs or tetrads; the cells of a tablet are enclosed within a complex, structured envelope; are obligate aerobic, having a strictly respiratory type of metabolism with oxygen serving as the terminal electron acceptor. Intracellular granules of PHB are prominent.[139]

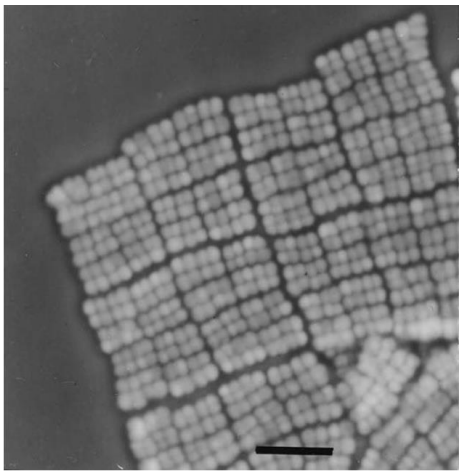


Figure 5.6 - Light micrograph of *Lampropedia hyalina* showing a corner of a sheet of actively growing tablets of cells. Bar=5µm.[139]

Bergey's Manual of Systematic Bacteriology[139] reports an optimal temperature for growth of 30°C and optimal pH of 7. The reactor in the present study was at room temperature (20-22°C) and the pH was close to 8. The genus is described as being able to consume butyrate and acetate,[140] and a *Lampropedia* population has been found in activated sludge selected for PHA production and fed with VFAs.[141]

5.4. Conclusions and Future Prospects

The pH is an important parameter in acidogenic fermentation as it can be used to minimise methanogenic fermentation and so, avoid that the substrate be used in other pathways aside from VFA production. This study revealed that changing the pH of the MBR affects the SBR community. The VFA profile shifted and became rich in butyrate. This led to an increase in *Thauera* organisms, mostly, but also *Azoarcus*.

Also, the population contained a substantial population of sheet-forming organisms throughout the entire study, which is consistent with the description of *Lampropedia*, despite the negative result in FISH analysis using a Betaproteobacteria-specific probe (BET42a). For an accurate identification of these bacteria, the BET42a probe could be used in a *Lampropedia* spp. pure culture and in the culture presently studied concurrently, under the same conditions to compare the results. Also, FISH probes have been designed for the identification of *Lampropedia* organisms,[140] which could be employed to validate their identity. Furthermore, isolation of the organisms from the system can be attempted through micromanipulation and subsequent sequencing can be performed for identification. If the organisms observed in the study are confirmed to belong to the *Lampropedia* genus, this is the first report of the ability of these organisms to store PHA and their occurrence in high numbers in PHA-production

systems. The operating conditions known to be optimal for these organisms could be imposed on the system as a strategy to further increase their abundance, and evaluate their impact on the overall community stability and process productivity.

Chapter 6

Occurrence of Quorum Sensing in PHA Producing Systems

6.1. Introduction

Establishing the occurrence of quorum sensing (QS) in PHA production can be a step further towards industrial optimisation. QS has been shown to determine PHB production at late stages of growth in *V. harveyi*, where polymer production does not take place in the absence or insufficiency of LuxR which is the protein receptor that binds to the autoinducer and acts as a transcriptional factor.[69] Nevertheless, not much more has been investigated concerning the relationship between PHA production and quorum sensing, especially in mixed culture systems, although quorum sensing has been shown to occur in activated sludge and to affect the community's performance[70].

The first measure to confirm the occurrence of quorum sensing and its implication in biopolymer production is to investigate the presence of signalling molecules involved in this regulatory mechanism in the system.

In this chapter, the presence of AHLs in a PHA producing system using activated sludge and fermented cheese whey as carbon source was investigated. In order to do so, a probe bacterial strain was used to detect the presence of such molecules. *Chromobacterium violaceum* is a bacterium that produces violacein, a water-insoluble purple pigment with antibacterial activity.[142] The *Chromobacterium violaceum* mutant CV026 is a mini-Tn5 mutant which involves the insertion of a kanamycin resistance cassette in violacein production gene. Consequently, violacein is produced only in the presence of exogenous short-chain AHLs. Also, Violacein production by *C. violaceum* CV026 is inhibited by AHLs with N-acyl chains from C10 to C14. This makes the CV026 mutant a convenient biosensor for the detection of a broad range of AHL and N-acylhomocysteine thiolactone (AHT) analogues[143] and it has been successfully employed to investigate the presence of AHLs in mixed cultures such as activated sludge[70,71,144]. It is more sensitive to 3-oxo-C6-HSL and C6-HSL.[143]

The reactor tested here was the one studied is the previous chapter. Samples were taken throughout the cycle to assess the presence of AHL's in different points of a FF cycle to possibly relate the quorum sensing phenomenon to certain occurrences.

6.2. Quorum Sensing Experimental Set-up

6.2.1. CV026 Growth Conditions

Chromobacterium violaceum mutant CV026 (kindly supplied by the Centre for Biomolecular Sciences at the University of Nottingham) was grown overnight in Lysogeny Broth (LB) supplemented with Kanamycin (25µg/ml), in a 100ml Erlenmeyer flask at 30°C with 200rpm shaking. The LB was prepared as follows: for 1L of solution, it was used 10g of Bacto-Tryptone, 5g of Yeast Extract and 10g of Sodium Chloride, and the pH was adjusted to 7 with Sodium Hydroxide 5M. For LB agar, 20g of agar were added, apart from when otherwise indicated.

6.2.2. Reactor Supernatant Bioassays

Presence of AHLs in the SBR's supernatant was tested by evaluating both stimulation and inhibition of violacein production by CV026. The SBR tested was fed with fermented cheese whey.

6.2.2.1. Violacein Stimulation

15ml of LB agar supplied with Kanamycin (25µg/ml) were poured into Petri dishes. After they dried, 5ml of LB agar (0.3 %, w/v) seeded with 50µl of the CV026 culture grown overnight were poured onto the LB agar plates. When dry, wells with approximately 6mm in diameter were punched in the agar with a sterile plastic tip.[143] Also, besides using wells, the supernatant was evenly spread over the surface together with the CV026 culture grown overnight.

Several samples were taken throughout the 12h SBR cycle. Samples from the feeding solution were also tested to establish that it did not interfere with the process. The samples were centrifuged at 8000rpm for three minutes and the resulting supernatant was sterilized with a 0.20µm filter. The wells previously punched in the agar plates were filled with 50µl of the treated supernatant. The plates were incubated overnight in the upright position, and were analysed for violacein production the following day.[143]

Both positive and negative controls were performed, leaving empty wells in the plates and wells filled with synthetic AHLs, and in the plates where the supernatant was spread, plates were left with no supernatant and in other synthetic AHLs were poured.

6.2.2.2. Violacein Inhibition

The inhibition bioassay set-up was similar to the violacein stimulation one, except a synthetic N-hexanoyl-L-Homoserine lactone (purchased from Sigma-Aldrich, Belgium) solution (5mM in Dimethyl sulfoxide) was added to the LB agar (0.3 %, w/v) seeded with 50µl of the CV026 culture with a final concentration of 5µM. The supernatant was treated similarly and the plates were incubated the same way.[143]

Negative controls were performed, leaving empty wells in the plates.

6.2.3. Detection Limit Assay

The detection limit of the assay was established using synthetic HHL. The assay was prepared similarly to the ones employing supernatant from the reactor, but different concentrations of filter sterilised HHL solution (5µM – 0.02µM) were poured into the wells. The HHL solution was prepared by diluting the stock solution with autoclaved distilled water.

6.3. Results and Discussion

Several samples were taken throughout a FF cycle and tested for the presence of short-chain AHLs and Long-chain AHLs up to 14 C long.

6.3.1. Violacein Stimulation Bioassay

Violacein production is determined by the appearance of violet halos around the wells against the colour of the agar. No pigment production was observed in the violacein stimulation bioassay plates (results not shown).

6.3.2. Violacein Inhibition Bioassay

Violacein inhibition occurs when white halos arise around the wells against the violet background of the seeded LB. There has to be microbial growth in the white halos, otherwise the occurring inhibition is of growth and not of violacein production.

In the inhibition bioassays, most samples showed no results, including the feeding solution. The absence of inhibition is shown in Figure 6.1 a) & b). However, very thin white halos with microbial growth were observed around the wells with supernatant from the end of the feast phase and shortly after the beginning of the famine phase (Figure 6. c) and d)), suggesting the presence of long-chain AHLs in the beginning of the famine phase.

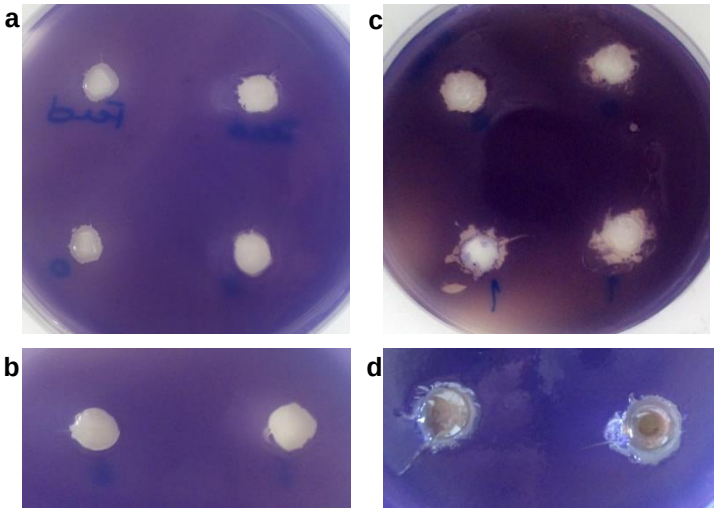


Figure 6.1 – Results from the violacein production bioassays. a) & b) show absence of inhibition of violacein. The top wells contain feed solution treated similarly to the supernatants; the bottom wells had supernatant from the end of the famine phase, when PHA concentration is the lowest. c) & d) show white halos around wells with supernatant from the end of the feast phase and the beginning of the famine phase, which suggests the occurrence of inhibition of violacein production.

When setting out to study the presence of AHLs in this system, if QS was detected, it was expected to be at the end of the famine phase to make the organisms store the carbon source instead of using it for growth, so they could survive the subsequent famine phase. Instead, in this study, AHLs were possibly detected in the end of the feast phase. At this point of the cycle the population concentration is the highest achieved during the entire cycle due to the simultaneous growth and accumulation of PHA that take place during the previous feast phase[46,47]. As the presence of QS molecules in the medium is dependent on population concentration, this can justify the AHLs detected in the end of the feast phase. Also, perhaps, at this point of the operation, the cell-to-cell communication that was observed is related to the slow consumption of the stored polymer for maintenance rather than a more rapid growth.

Nevertheless, these results are preliminary because the halo is thin and therefore it is not easy to evaluate the existence of microbial growth on the medium. It is difficult to ascertain that the halo is in fact inhibition of violacein production rather than simple inhibition of CV026 growth.

The detection limit observed was of 0.5μM. The detection limit assay showed that the halo gets thinner with the decrease in HHL concentration due to weaker activation. Furthermore, McClean et al.[143] observed that even if a compound is able to weakly activate violacein production there may not be observable pigment and attributed it to low concentration in the wells. Accordingly, the uncertainty of the results obtained in this study may be due to low concentration of autoinducers in the supernatant and so these results do not exclude the presence of AHLs at the end of the feast phase as expected. Further treatment to the supernatant may be required to carry on these bioassays successfully. Other studies have

employed extraction methods before applying the supernatant to the wells,[144] cleaning the supernatant of possible interferents.

6.4. Conclusions and Future Prospects

Identification of the autoinducers produced by the community in study is advantageous because it offers the possibility of catering more specifically to a certain system while identification of the organisms with AHL activity can prove useful there being the possibility of enriching the community accordingly. This can facilitate the enhancement of PHA production or other quorum sensing mediated phenomena desired.

Alternative bioassays employing the CV026 mutant have been used with positive results. In these studies, the reporter strain is streaked across the agar plate and cross-feeding[71] or cross-streaking[70] were performed, followed by isolation of the colonies showing AHL activity.

Another biosensor used for AHL detection is *Agrobacterium tumefaciens* mutant NTL4. *A. tumefaciens* responds to 29 homoserine lactones as autoinducer analogues of 3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) with great sensitivity.[145] The mutant *A. tumefaciens* NTL4[146] overexpresses TraR which binds exogenous AHLs and then activates the expression of the traI-lacZ gene fusion. β-Galactosidase, encoded by lacZ, hydrolyzes the non-inducing substrate X-Gal, producing a blue precipitate.[147] This strain has also been employed successfully in the study of quorum sensing in activated sludge.[70,71] These pigment induction methods have greatly simplified and facilitated screening for AHL production.

Nonetheless, the R protein is highly specific and bacteria often produce more than one type of autoinducer, making these detection systems limited in the range of AHLs to which they respond. Thus, a thorough screening requires the use of several reporter systems, each responding to different signaling molecules with their own structural features. To facilitate this screening, Shaw et al.[148] designed a simple, rapid, and sensitive thin-layer chromatography (TLC) assay useful for analysing bacterial cultures for the presence of autoinducers.

However, these assays only provide tentative identification. After a screening for the presence of autoinducers has been performed, it is important to identify them. For unequivocal identification of the autoinducers other analysis are necessary such as liquid chromatography and mass spectrometry (MS).[59,149] High performance liquid chromatography (HPLC) analysis makes possible the identification of AHLs by comparing the results of the samples with known standards. In this study, after detecting the presence of long-chain AHLs, HPLC could be carried out using standards for long-chain AHL to determine if there were in fact long-chain AHLs present in the system. But HPLC is not enough to unequivocally identify autoinducers. The final structural confirmation needs to be obtained by chemical synthesis to demonstrate that the properties of both natural and synthetic materials are identical. In order to do so, MS can be performed. This method has been shown to be the most valuable tool for the identification and characterisation of AHLs.[149]

After the occurrence of quorum sensing has been established, it is necessary to ascertain if there is a connection between this phenomenon and PHA accumulation. The determination of interference of autoinducers in PHA production can be accomplished making use of either signal augmentation or signal extinction. Signal augmentation is achieved with the increase of

signalling molecules in the medium, supplementing exogenous autoinducers to the medium, as experimented by Valle et al.[70]. If quorum sensing is involved in PHA production, an increase in PHA production is expected. Nevertheless, in a not-so-stable system with varying PHA production and in the event that not enough exogenous autoinducer is supplemented, it may be difficult to evaluate a slight increase in production. To more easily asses the existence of a link between cell-to-cell communication and PHA storage, quorum quenching can be explored. Quorum quenching is the occurrence of interference in quorum sensing, diminishing the communication. Recently, Kim et al.[150] used acylase, a quorum quenching enzyme, to study the formation of biofilms. The researchers found that the introduction of acylase in the reactor led to the decrease in quorum sensing bacteria. If employed in PHA systems, a decrease in PHA production would indicate a link between quorum sensing and PHA production.

Chapter 7

Conclusion

We live in the age of plastic. To continue to be that way, replacements must be found, for petrochemical plastics have become unsustainable due to resource depletion and the pollution derived from the plastics industry. PHAs have potential to replace modern plastics but before that is possible PHAs have to be made easily available and at similar price. Ongoing research leans towards the cost reduction through the employment of low cost – or even free – feedstock and the use of MMC.

For this work, the microbial dynamics of PHA producing systems was studied to better understand the connection between reactor operation and the PHA-producing community. Central to this system is the selection stage that took place in a SBR. In this stage FF cycles were used to enrich the activated sludge population with PHA-storing organisms.

Firstly, the acclimation of activated sludge from a WWTP to this regime was studied, using a solution of synthetic VFAs. The result was a population very rich in large filamentous bacteria belonging to the Alphaproteobacteria class, as revealed by FISH analysis, and in *Paracoccus* spp, known PHA-producers and more generalist substrate-wise than *Azoarcus* and *Thauera*, other abundant groups present in the system. Although the abundance of the filamentous bacteria impacted negatively on the process causing bulking, they could still be an asset in moderate amount due to their high storage capacity and help in structuring floccs, and so equilibrium must be established in the amount of filamentous bacteria.

Then, the impact on the population of alternating the feedstock between SM and CW was studied, as exploring different carbon sources can be useful to accommodate changes in the industries that originate the substrate and to obtain different polymers for different applications. For the employment of such substrates, a step of fermentation is added before the selection to generate VFAs. The change in feedstock affected both the fermentation stage and the selection stage. Overall, the population was more homogeneous and stable during the SM phase than the CW phase, for both reactors. With the CW as substrate, more polymer was produced by the cells, up to 65% w/w vs. 56% w/w from the SM phase. The population in the SM phase was rich in *Azoarcus* (up to 64.6%) and together with the *Paracoccus* and *Azoarcus* populations constituted up to 83.4% ±3.5 – 99.3% ±6.3 of the total biovolume. In the CW phase, the three groups comprised 21.3% ±2.7 – 52.4% ±4.5, indicating the presence of a more varied community.

Furthermore, in a system already acclimatised using CW as substrate, the pH of the fermentation reactor was changed and the impact on the SBR population was investigated. With an increase in butyrate in the fermented CW (from around 50% to more than 60%), the *Thauera* population increased significantly, the *Azoarcus* population increased slightly and the *Paracoccus* population was reduced to near non-existence. With the change, the selection reactor had a poor performance and PHA production. Studying the impact of different operating conditions is crucial to the optimization of the process, to obtain higher productivities or different co-polymers with different characteristics.

Lastly, one step more can be taken towards production optimization. The awareness of the importance of quorum sensing in PHA production can lead to application of quorum sensing enhancing strategies to improve PHA production. With that in mind, the existence of QS molecules in the system was investigated. The results were not conclusive, but other strategies

can be employed, namely taking advantage of quorum quenching to evaluate the effect of the absence of QS in PHA production.

Knowing the microbial population structure, dynamics and regulation mechanisms contributes to a better understanding of how PHA production works and what affects it, for a more efficient system set-up.

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