Raquel Sofia Corredoura Valente

Licenciada em Química Aplicada

Enrichment of a PHA producing microbial community in a continuous bioreactor setup

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Aknowledgments

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Resumo

Os Polihidroxialcanoatos (PHAs) são poliésteres biosintéticos biodegradáveis e biocompatíveis, propriedades que os tornam de grande interesse para fins industriais. Actualmente, uma das estratégias utilizadas para diminuir os elevados custos de produção dos PHAs é através da utilização de substratos de baixo custo com culturas microbianas mistas. O processo de produção de PHAs requer uma importante etapa de selecção de microorganismos com capacidade para armazenar eficientemente PHAs, a qual é geralmente realizada num reactor descontínuo sequencial.

Esta dissertação teve como objectivo o estudo e optimização da etapa de selecção através de um sistema contínuo de dois reactores, um para simular a fase de fartura e outro a fase de fome. O substrato utilizado foi o acetato e o sistema foi inoculado com biomassa proveniente de lamas activadas enriquecida em *Plasticicumulans acidivorans*. O sistema foi operado sob dois conjuntos diferentes de condições (setup 1 e 2), mantendo um tempo de retenção hidráulico total do sistema de 12 horas e uma carga orgânica de 2,25 Cmmol/L.h^{-1}. No setup 1 obteve-se um conteúdo médio em PHB de 3,3% (peso seco) e de 4,8% (peso seco) no setup 2. Com o intuito de compreender melhor o desempenho do sistema contínuo foram realizadas experiências adicionais em descontínuo, utilizando biomassa do sistema contínuo. Com a experiência de acumulação alcançou-se um máximo de 8,1% PHB armazenado e com a de crescimento, conseguiram-se velocidades específicas de consumo de substrato e de crescimento de 1,15 Cmol $Cmol^{-1}.h^{-1}$ e 0,53 Cmol $Cmol^{-1}.h^{-1}$, próximas às do sistema contínuo (1,12 Cmol $Cmol^{-1}.h^{-1}$ e 0,59 Cmol $Cmol^{-1}.h^{-1}$). A comunidade microbiana foi analisada por visualização microscópica e caracterizada utilizando as técnicas de cromatografia em gel de electroforese com gradiente de desnaturação e hibridização de fluorescencia *in situ*. Por último, foi simulado um sistema contínuo através da aplicação de todas as condições operacionais deste, num reactor descontínuo sequencial com a adição de uma dose extra de substrato durante cada ciclo de 12 horas, mimetizando a passagem de substrato observada entre os reactores de fartura e fome no sistema contínuo. Aferiu-se que, possivelmente, o sistema contínuo era incapaz de seleccionar microorganismos produtores de PHB de uma forma eficiente nas condições utilizadas. Ainda assim, a cultura seleccionada possuía uma boa capacidade de consumo de substrato e subsequente crescimento. Dois factores mostraram ter um possível impacto no desempenho do sistema contínuo: a concentração de azoto presente no reactor de fartura e a quantidade de substrato não consumido no reactor de fartura que passa para o reactor de fome.

Palavras-chave: Polihidroxialcanoatos; Fase de Fartura; Fase de Fome; Reactor descontínuo sequencial; Reactor contínuo

Abstract

Polyhydroxyalkanoates (PHAs) are biosynthetic polyesters, biodegradable and biocompatible making them of great interest for industrial purposes. The use of low value substrates with mixed microbial communities (MMC) is a strategy currently used to decrease the elevated PHA production costs. PHA production process requires an important step for selection and enrichment of PHA-storing microorganisms which is usually carried out in a Sequencing Batch Reactor (SBR).

The aim of this study was to optimize the PHA accumulating culture selection stage using a 2-stage Continuous Stirrer Tank Reactor (CSTR) system. The system was composed by two separate feast and famine bioreactors operated continuously, mimicking the feast and famine phases in a SBR system. Acetate was used as carbon source and biomass seed was highly enriched in *Plasticicumulans acidivorans* obtained from activated sludge. The system was operated under two different sets of conditions (setup 1 and 2), maintaining a system total retention time of 12 hours and an OLR of 2.25 Cmmol/L.h^{-1}. An average PHB-content of 3.3 % wt was obtained in setup 1 and 4.8% wt in setup 2. Several other experiments were performed in order to better understand the continuous system behaviour, using biomass from the continuous system. With the fed-batch experiment a maximum of 8.1% PHB was stored and the maximum substrate uptake and specific growth rates obtained in the growth experiment (1.15 Cmol $Cmol^{-1}.h^{-1}$ and 0.53 Cmol $Cmol^{-1}.h^{-1}$) were close to the ones from continuous system (1.12 Cmol $Cmol^{-1}.h^{-1}$ and 0.59 Cmol $Cmol^{-1}.h^{-1}$). The microbial community was characterized trough microscopic visualization, Denaturing Gradient Gel Electrophoresis (DGGE) analysis and Fluorescent in situ hybridization (FISH). The last studied performed mimicked the continuous system by building up a SBR system with all the same operational conditions while adding an extra acetate dosage during the 12 h cycle, simulating the substrate passing from the feast to the famine reactors under continuous operation. It was shown that possibly the continuous system was not able to efficiently select for PHB storing organisms under the operational conditions imposed, although the selected culture was capable of consuming the substrate and grow fast. This main conclusion might have resulted from two major factors affecting the system performance: the ammonium concentration in the Feast reactor and the amount of substrate leaching from the Feast to the Famine reactor.

Keywords: Polyhydroxyalkanoates (PHAs); Feast reactor; Famine reactor; Sequencing Batch Reactor (SBR); Continuous Stirred Tank Reactor (CSTR)

List of abbreviations

Ac – Acetate

ADF - Aerobic Dynamic Feeding

C/N/P - Carbon/nitrogen/phosphorus ratio, in C-mol /N-mol/ P-mol

C_X - Biomass concentration

CSTR - Continuous stirred tank reactor

C_S - Substrate concentration

C_N – Nitrogen concentration

C_P – Product concentration

DGGE – Denaturing gradient gel electrophoresis

DO - Dissolved oxygen

EtOH - Ethanol

FF - Feast and famine regime

F/F- Feast and famine ratio

FISH – Fluorescent *in situ* hybridization

GC - Gas chromatography

HAc - Acetic acid

HB - Hydroxybutyrate

HPLC - High performance liquid chromatography

HRT - Hydraulic retention time, in days

HV - hydroxyvalerate

mcl-PHA - Medium chain length PHA

MMC - Mixed microbial culture

OLR - Organic loading rate, in C g $L^{-1}d^{-1}$

P - Product

PB - polybutene

PBAT - Poly(butylene adipate-co-terphthalate)

PCR - Polymerase Chain Reaction

PE-LD – Low density polyethylene

PE-LLD - Linear low density polyethylene

PE-HD - High density polyethylene

PHA - Polyhydroxyalkanoate

PHB - Polyhydroxybutyrate

PLA - Polylactic acid

PP - Polypropylene

PS - Polystyrene

PVC - Polyvinyl chloride

qCO_2 – Specific carbon dioxide evolution rate

qN - Specific nitrogen consumption rate

qO_2 - Specific oxygen uptake rate

qP - Specific product production rate

qS - Specific substrate uptake rate

qX - Specific biomass growth rate

S - Substrate

SBR - Sequencing batch reactor

scl-PHA - Short chain length PHA

SRT - Sludge retention time, in days

TCA - Tricarboxylic acid cycle

VFA - Volatile fatty acids

V_{Famine} - Volume of Famine reactor

V_{Feast} - Volume of Feast reactor

VSS - Volatile suspended solids, in g L^{-1}

X - Active biomass

$Y_{PHB/Ac}$ - Yield of PHB storage on acetate

$Y_{X/Ac}$ – Yield of biomass on acetate

$Y_{CO2/Ac}$ – Yield of carbon dioxide on acetate

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1.Introduction

After the Stone Age, the Bronze Age, and the Iron Age, nowadays society seems to live in the Plastic age, with plastics being a crucial part of our modern lifestyle. In current industrial society it is almost unimaginable to have an activity without plastics playing a role. Their improved mechanical and thermal properties as well as stability in severe conditions made them durable and strongly resistant allowing them to continuously replace other materials in a wide range of applications (Mulder 1998; Lange 2007). Yet, plastics long life period in the ambient lead to great wastes accumulation which became a major environmental concern. Besides the decades taken to degrade in nature, plastics also originate toxins during the degradation process. Furthermore, production of structural materials like plastics is highly dependent on fossil fuels. Worldwide there is currently a consumption of roughly 140 million tons of plastics per year, a production that requires approximately 150 million tons of fossil fuels which are quite hard to replace (Suriyamongkol *et al*., 2007). As fossil fuels are a finite resource, the environmental issues allied with the wish for a secure and independent energy supply have gradually induce the replacement of petroleum-based products for materials derived from renewable sources. Consequently, there is a lusty trend to a more bio-based economy rather than an oil-based economy (Lange 2007; Ojumu *et al*., 2004 ; Johnson 2010a). An increasingly pursuit has taken place to discover new commonly used materials that can be produced by sustainable renewable sources and easily eliminated from the environment. This way, biomaterials have been under research and development focusing by industry. Biomaterials are natural products synthesised and catabolised by different organisms. They can be biodegradable, as they can be assimilated by many species and be biocompatible since they do not provoke toxic effects which positively stand them out from the conventional synthetic products. Bioplastics are a special type of biomaterials, as they are polyesters produced in cells as storage materials by a range of microbes, cultured under different nutrient and environmental conditions. Depending on the source material and the microorganism used, polyesters can be obtained with different properties which concede them broad biotechnological applications. The most widely produced microbial bioplastics are the polyhydroxyalkanoates (PHAs) and their derivatives (Luengo *et al*., 2003; Steinbüchel & Füchtenbusch, 1998). Nowadays, there are already companies like Biocycle™ and Metabolix Inc. commercializing bioplastics as polyhydroxyalkanoates (PHAs) aiming to compete with conventional polymers. However, PHAs price in the market is still more than three times higher than traditional plastics (Digregorio, 2009). PHA industrial production requires pricy feedstock and the use of pure microorganisms cultures that need aseptic conditions, which are two of main factors sharply augmenting the production process.

Therefore, current research is focused on developing PHA production processes able to use low cost subtracts as industrial wastes and mixed microbial cultures (MMC) that do not require sterile conditions allowing to significantly decrease the overall process price. While none of the bioplastic available on the market today is able to compete with the price and meet the need for every petroleum-based product made, the race is on. For this reason, the aim of this work is to develop and improve a 2-stage continuous system capable of selecting PHA-storing microorganisms by using mixed microbial culture (MMC).

This thesis is organized in 5 sections. The present section is the first one, composed by the motivation that led to this project realization and by this paragraph outlining the main structure of the dissertation. Section 2 is the background supporting this research, starting by framing the need for PHA development, followed by elucidation of PHA structure, properties, different applications and biosynthesis. Then it is explained the concept of eco-biotechnology and the PHA production process from low value substrates using mixed microbial cultures is described and compared for SBR and CSTR systems. In section 3 are described all the practical methods and procedures used during this research work. Section 4 is dedicated to show all the results obtained and discuss them. In section 5 the final considerations and recommendations are presented.

2. Background

2.1. Polyhydroxyalkanoates as a step forward in bioplastics industry

2.1.1. Plastics and bioplastics

Plastics became one of the most multipurpose and universally used materials in the worldwide economy along the last half of the 20th century. The per capita plastics consumption in Western Europe and North America is evaluated at about 100 kg annually, with aproximally 260 million tons of plastics being manufactured worldwide each year (Jonhson *et al*., 2010a). They have become an essential part of our daily lives, from medical and pharmaceutical industry to simple household use. In areas like medicine they made possible massive progresses such as the development of techniques that enable unblocking blood vessels, develop artificial corneas or hearing devices (Plastics Europe, 2014). Plastics allowed human race pushing forward the boundaries so we could go further in a faster and safer way than we have dared to go before.

Different types of plastics with specific properties were developed in order to reach most of industry demands, among them are: polyethylene (low density PE-LD, linear low density PE-LLD and high density PE-HD), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET). To produce these kinds of materials more than 140 million tons of petroleum-based polymers are manufactured worldwide. Essentially using oil, coal and natural gas as raw materials for their production these products withstand too well to our hasty throwaway society remaining in the environment for 2.000 years or more (Digregorio, 2009).

The utilization of fossil resources for the production of polymers strongly contributed to severe problems in actuality, like the greenhouse effect and the global warming. These materials are used for a reasonably brief period of time, after which they are often incinerated leading to a rising in the atmospheric CO_2 concentration. This way, carbon that was not part of the natural carbon cycle is converted to CO_2 and often accumulated in the atmosphere resulting in climacteric issues (Koller *et al*., 2010b). Incineration of plastics also releases toxic compounds and when not burned they are simply accumulated in the environment year after year. Due to plastics floatability they are also easily spread in the open ocean, which allied with its ubiquity and recalcitrance, has led to serious side effects in marine organisms (Morét-Ferguson *et al*., 2010). Although recycling has become more and more popular along the past years, 38% of

plastics waste is still going to landfill (Plastics Europe 2013). Plus, crude's price is unexpectedly changing which builds up an intense unpredictably for plastics industry along with the fact that fossil feedstocks will eventually be completely exhausted. Consequently, a consciousness concerning the promotion of manufacturing techniques based on renewable recourse has been sharply growing (Braunegg *et al*., 2004).

Nature is filled with polymers, from wood, leaves, fruits and seed till animals, all containing natural polymers. For thousands of years bio-based polymers have been applied for food, clothing and furniture. In 1926, the French chemist and bacteriologist Maurice Lemoigne discovered for the first time that a Gram-positive bacterium *Bacillus megaterium* was able to produce an intracellular biopolyester, polyhydroxybutyrate (PHB), which, nowadays, is one of the most ordinary derived forms of polyhydroxyalkanoates (PHAs). More than 100 different PHAs have been discovered from a broad range of microorganisms. Yet, most of the discoveries and inventions involving bio-based polymers done in the 1930s and 1940s never passed from the laboratory stage to the commercial production mainly due to the discovery of crude oil reserves in the 1950s. Only around the 1980s, when chemists and biologists started realising the severe negative impact of petroleum-based plastics in the planet´s health that bio-based plastics have began a gaining strength (Mulder 1998 ; Digregorio, 2009 ; Shen *et al*., 2009).

Currently, bioplastics still represent merely 1% of the million tonnes of plastics produced annually. However, with more sophisticated and improved bioplastic materials showing up in the global market an increasingly demand is visibly.

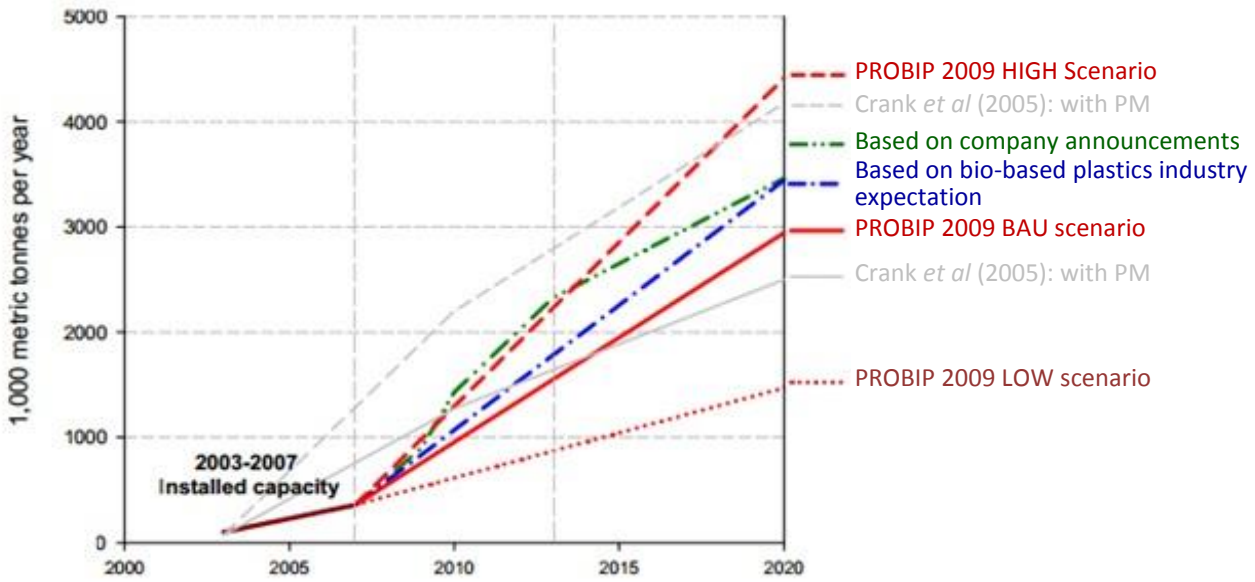


Figure 2.1. Projection of the worldwide production capacity of bio-based plastics until 2020. Adapted from Shen *et al*.. 2009.

According with Shen *et al*., 2009, the worldwide capacity of bio-based plastics in line with company announcements increased from 0.36 Mt to 2.33 Mt from 2007 to 2013 and will probably rise to 3.45 Mt in 2020. These values resulted in an average annual growth rate of 37% between 2007 and 2013 and possibly 6% between 2013 and 2020, with more and more sophisticated bioplastic materials and products entering the market. The PROBIP study conducted by the University of Utrecht in 2009 demonstrated that bioplastics could be able to replace around 85% of conventional plastics. Consumers interested in buying products with the minimum impact to the environment are continuously growing, particularly in Europe constituting about 80% of the consumers (Eurobarometer survey EC 2013). There are several market segments where bioplastics are becoming tightly applicable, such as: packaging, food services, agriculture, consumer goods, household appliances and electronics. They are also starting to stand out in automotive, sports equipment and toys sectors.

European Bioplastics association describes the term bio-based as the part of a material or product that drifts from biomass. The chemical process of biodegradation occurs when materials degrade back into water, CO_2 and biomass with the help of microorganisms. This process is dependent on the material itself, on the circulating environmental conditions (such as temperature or location) and on the material application. It is meaningful to highlight that the biodegradation property of a material is not related with the kind of source used but is rather directly connected with its chemical structure. In a general way, bioplastics are partially or completely based on natural resources like cellulose or biomass that mainly drifts from corn grain, sugar cane, potatoes or castor oil. Therefore, bioplastics are materials with a wide range of properties and characteristics but all related to each other. They are pooled in three groups with its own characteristics:

- ✓ **Biobased and partially biobased (non-biodegradable) –** In this group are included the bio version of the commodity plastics like PE, PP and PVC which are already currently produced from renewable resources such as bioethanol biofuel. Included in this group is also the partially biobased polyester PET. These bioplastics are used for a wide diversity of applications, from packaging to automotive.
- ✓ **Biobased and biodegradable plastics –** here are included the bioplastics manufactured from starch blends and other biodegradable polymers and polyesters such as (PLA) polylactic acid and (PHA) polyhydroxyalcanoate. Contrary to cellulose materials they have become industrially available just since a few years ago.
- ✓ **Biodegradable fossil-based plastics –** Materials from this relatively smaller group are still produced using petrochemical production processes but has the future possibility of being partially made from renewable resources. They are mostly applied to combine with starch and other bioplastics. Among them are Poly(butylene adipate-co-terphthalate) (PBAT) and polybutene (PB). (European Bioplastics, 2014).

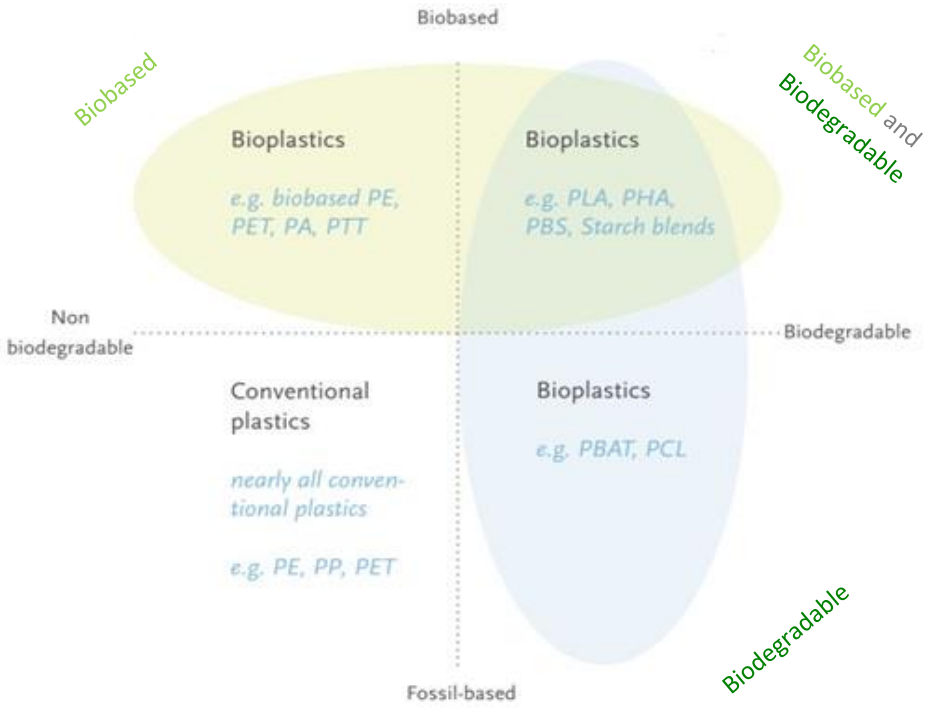


Figure 2.2. Relation between biobased, biodegradable, non biodegradable and fossil-based. Adapted from European Bioplastics 2014.

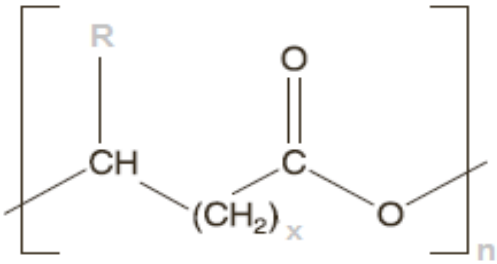
Together with the diversity growth of bioplastics materials, also properties like the durability, printability, transparency, barrier, heat resistance, flexibility and a lot more have been significantly studied and improved. Biobased and partially biobased plastics as bio-PE, bio-PET and bio-PVC can only be distinguish from conventional commodity plastics by scientific analysis due to the high similarity of properties. Also resembling the conventional plastics, the new bio-based plastics and potentially biodegradable are produced from bacteria able to consume renewable resources (such as starch, cellulose or fatty acids) in order to grow and produce building blocks which have been already successfully used for polymerization of bacterial plastics. Among those building blocks are hydroxyalkanoic acids with many structural variations (where PHAs are included), lactic acid, succinic acid, (*R*)-3-hydroxypropionic acid, bioethylene produced from dehydration of bioethanol, 1,3-propanediol and *cis*-3,5-cyclohexadiene-1,2-diols from microbial transformation of benzene and other chemicals. These fresh biodegradable materials have unlocked new ways of organic recycling and recovery providing a green alternative to crude plastics, from where PHAs stood out due to their particular chemical structure. (European Bioplastics, 2014; Chen, 2010a).

2.1.2. Polyhydroxyalkanoates

2.1.2.1. Structure / Properties / Applications

Properties of Polyhydroxyalkanoates are dependent on the starting carbon feedstock, the metabolic pathways in which take place the conversion of that feedstock into precursors for PHAs and the activities and substrate specificities of the enzymes entering in the process (Lu *et al*., 2009).

Polyhydroxyalkanoates are polyesters of hydroxyalkanoates (HAs) that can be synthesized by more than 300 different bacteria, encompassing Gram-negative and Gram-positive species. Polyhydroxyalkanoates (PHAs) are the only bioplastics fully synthesized and polymerized by microorganisms making part of a family of biopolyesters with several structures. Since 1926, when the first PHA was identified, the poly(3-hydroxybutyrate) [P(3HB)], over 80 distinct monomer units have been found as constituents of PHAs in diverse bacteria. (Chen, 2010b ; Lee, 1996). Among them are 3 HAs of 3 to 14 carbon atoms that can have a wide variety of straight or branched and saturated or unsaturated chains containing aliphatic or aromatic side groups (Figure 2.3).



			Polymer
x = 1	**R** =	hydrogen	Poly(3-hydroxypropionate)
		methyl	Poly(3-hydroxybutyrate)
		ethyl	Poly(3-hydroxyvalerate)
		propyl	Poly(3-hydroxyhexanoate)
		pentyl	Poly(3-hydroxyoctanoate)
		nonyl	Poly(3-hydroxydodecanoate)
2		hydrogen	Poly(4-hydroxybutyrate)
3		hydrogen	Poly(5-hydroxyvalerate)

n = 100 to 30000

Figure 2.3. General structure of polyhydroxyalkanoates. With "R" as the variable side chain; "x" as the number of carbon atoms in the linear polyester structure and "n" as the molecular weight. Adapted from Lee 1996.

PHAs can be formed with a wide range of molecular weights, from 100 to 30000 Da. Depending on the number of carbon atoms in the monomer units, PHAs can be split in two groups:

- ✓ **Short-chain-length (scl)-PHAs** – monomer units have 3 to 5 carbon atoms. These PHAs are stiff and brittle with a high degree of crystallinity. The most known elements of this group are poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)), the most common PHAs.
- ✓ **Medium-chain-length (mcl)-PHAs** – monomer units consist of 6 to 14 carbon atoms. PHAs are flexible, with low crystallinity, tensile strength and melting point. Among them are 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) and (Philip *et al*., 2007).

PHAs can be formed with many different physical characteristics in accordance with the co-polyester formed (Chen 2010a). These polymers are piezoelectric and perfectly optically active with only the (R)-configuration. They are thermoplastic and/or elastomeric, non toxic and possess a quite high purity in the cell and are also hydrophobic, insoluble in water, inert and indefinitely stable in air. PHAs have reached commercial interest as bioplastic materials due to their notable resemblance in physical properties with synthetic polymers such as polypropylene. PHA is less solvent resistant comparing to propylene; yet, it holds a greater resistance to UV degradation. Most importantly, PHAs are fully biodegradable (Braunegg *et al*., 2004 ; Laycock *et al*., 2013). One of the main PHAs benefit is the fact that their physical properties and rate of degradation can be changed just by using different bacterial species and corresponding fermentation conditions (pH or substrate concentration). Blending the PHAs with other polymers, altering the surface or combining them with other inorganic materials allows improving the mechanical properties and biocompatibility of PHAs (Akaraonye *et al*., 2010). The variety of structures concedes rich properties to PHAs making them quite attractive for applications in fields like commodity packaging, agriculture, medicine and pharmaceutics, textile industry, fine chemical industry, food industry, and biofuels (Chen 2009). In 1996, PHAs received European approval for food contact opening opportunities in food service and packaging industry, which became the current leading market segment (European Bioplastics 2013). This area of applications includes packaging films (for food packages), bags, containers, paper coatings, feminine hygiene products, cosmetics containers, shampoo bottles, cups, as well as disposable items such as razors, utensils, diapers, etc (Jain *et al*., 2010). PHAs biodegradability in soil is what makes them very appealing for agriculture purposes. They can be used as supply for small molecules that can be used as biodegradable solvents and as carriers for long-term slow release of insecticides, herbicides or fertilizers (Akaraonye *et al*., 2010). Medical and pharmaceutical are the main areas of research and improvement for PHA use, in which biodegradability and biocompatibility are the more meaningful characteristics. In the last

years, PHAs have been used to develop many medical devices, such as bone plates, cardiovascular patches, orthopaedic pins and also in drug delivery (Dai *et al*., 2009).

2.1.2.2. Biosynthesis as microbial storage reserves

For PHA-producing microbial cells, PHAs serve as carbon and energy storage material in times of unbalanced nutrient availability. Under conditions of starvation, these reserve materials can be mobilized, giving a survival advantage to the cells (Koller *et al*., 2010a). They are produced intracellularly as insoluble spherical inclusions named PHA granules (Figure 2.4). Usually, under maximum PHA-conditions, around 5 to 10 granules can fill almost the entire cell, having diameters ranging between 100 and 500 nm (Grage *et al*., 2009). PHA inclusions can be observed by Transmission Electron Microscopy (TEM). They also can be stained with Sudan black B which indicates that they are of a lipidic nature. PHA is even more specifically stained using oxazine dye Nile Blue and its fluorescent oxazone form, Nile Red that can already be used to detect PHA directly in growing bacterial colonies. Staining methods are utilized to indicate the presence of PHA, while chemical analysis are often required to determine their monomeric compositions, like Gas chromatography (GC) and Nuclear Magnetic Resonance (NMR) spectroscopy analysis (Spiekermann *et al*., 1999; Williamson *et al*., 1958).

The PHA granules consist of a polyester core surrounded by a phospholipid layer with proteins attached. PHA synthase is one of these proteins, which is the key enzyme of PHA biosynthesis by catalysing polyester formation from different (R) – 3-hydroxyacyl-CoA precursors. PHA synthase stands covalently attached to the polyester and, consequently, to the PHA granule. There are also other proteins associated to the PHA granule that are related to functions like depolymerisation, regulation or structural stabilization (Fuller, 1999 ; Grage *et al*., 2009).

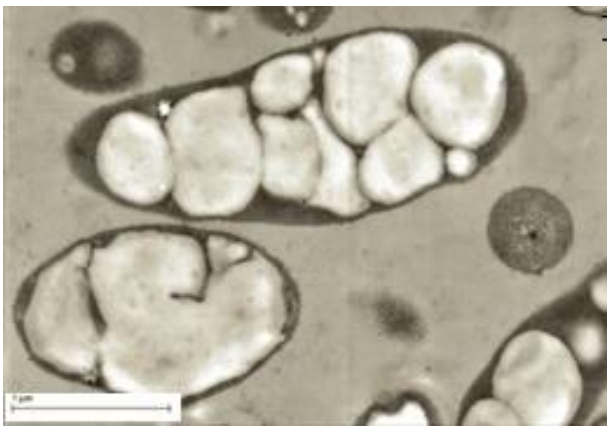


Figure 2.4. Electron-microscopic picture of polyhydroxyalkanoate (PHA)-rich *Cupriavidus necator* DSM 545 cells cultivated in a continuous fermentation process on glucose. Magnification 1/72 000 . Scale of 1µm. Adapted from Koller *et al*., 2010b.

Microorganisms able to produce and store PHA under nutrient-limited conditions can usually degrade and metabolize it when the carbon or energy source is limited. The degradable PHA monomers are soluble in water and they can also be metabolized by ß-oxidation and the tricarboxylic acid cycle of many organisms producing carbon dioxide and water under aerobic conditions (Chen 2010). Along with the identification of new PHA-synthesizing microorganisms and how PHAs influences bacterial fitness, it became evident that this polymer is more than just an intracellular carbon-storage material used only when carbon becomes a limiting resource. It was found that PHAs magnifies the survival capacity of several bacteria under environmental stress conditions (Kadouri *et al*., 2005). Free-living bacterial cells with high-PHA content may be skilled to survive longer than cells with low PHA content or lacking it. This skill may be because they are protected from adverse factors or due to their reserve material that they can use longer and more efficiently comparing with bacteria with low PHA-producing capacity or lacking this ability at all (Matin *et al*., 1979). Therefore, the exact mechanism of PHA granule formation and production is still under tough investigation. Knowing so far that, *in vivo*, under favourable PHA accumulation conditions, PHA biosynthesis begins as soon as the substrate (R) – 3-hydroxyacyl-CoA is available (Grage *et al*., 2009).

2.1.2.3. Metabolic pathways for synthesis by bacteria

Since PHA accumulation is controlled by many genes that encode a range of enzymes, which are directly or indirectly involved in PHA synthesis the biology of PHAs is quite complex (Rehm 2010). Most of the PHA synthesis studied so far is specific for the production of scl-PHA or mcl-PHA, mainly due to the substrate specificity of the PHA synthesis that only accepts 3-hydroxyalkanoates of a certain range of carbon length (Ojumu *et al*., 2004). Since identification and characterization of the enzymes involved in PHA synthesis, hundreds of genes have been cloned or identified as putative PHA biosynthesis genes, which make microorganisms able to use different pathways for PHA biosynthesis. Poly-(*R*)-3-hydroxybutyrate (PHB) is one of the best-studied PHA. In PHB synthesis mechanism three main enzymes are involved. Both ß-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) are involved in general lipid metabolism while PHA synthase (PhaC) is uniquely involved in the biosynthesis part, by performing the polymerization of ß-hydroxyalkanoyl-CoA monomers into poly(ß-hydroxyalkanoate) (Castro-Sowinski *et al*., 2010 ; Philip *et al*., 2007). At least 88 PHA synthases have been sequenced, with four major classes being identified depending on the number of carbon atoms of the substrate used (Peoples *et al*., 1989; Sudesh *et al*., 2000; Laycock *et al*., 2013). So far, eight pathways for PHA biosynthesis have been summarized, being three of them already quite well studied (Figure 2.5).

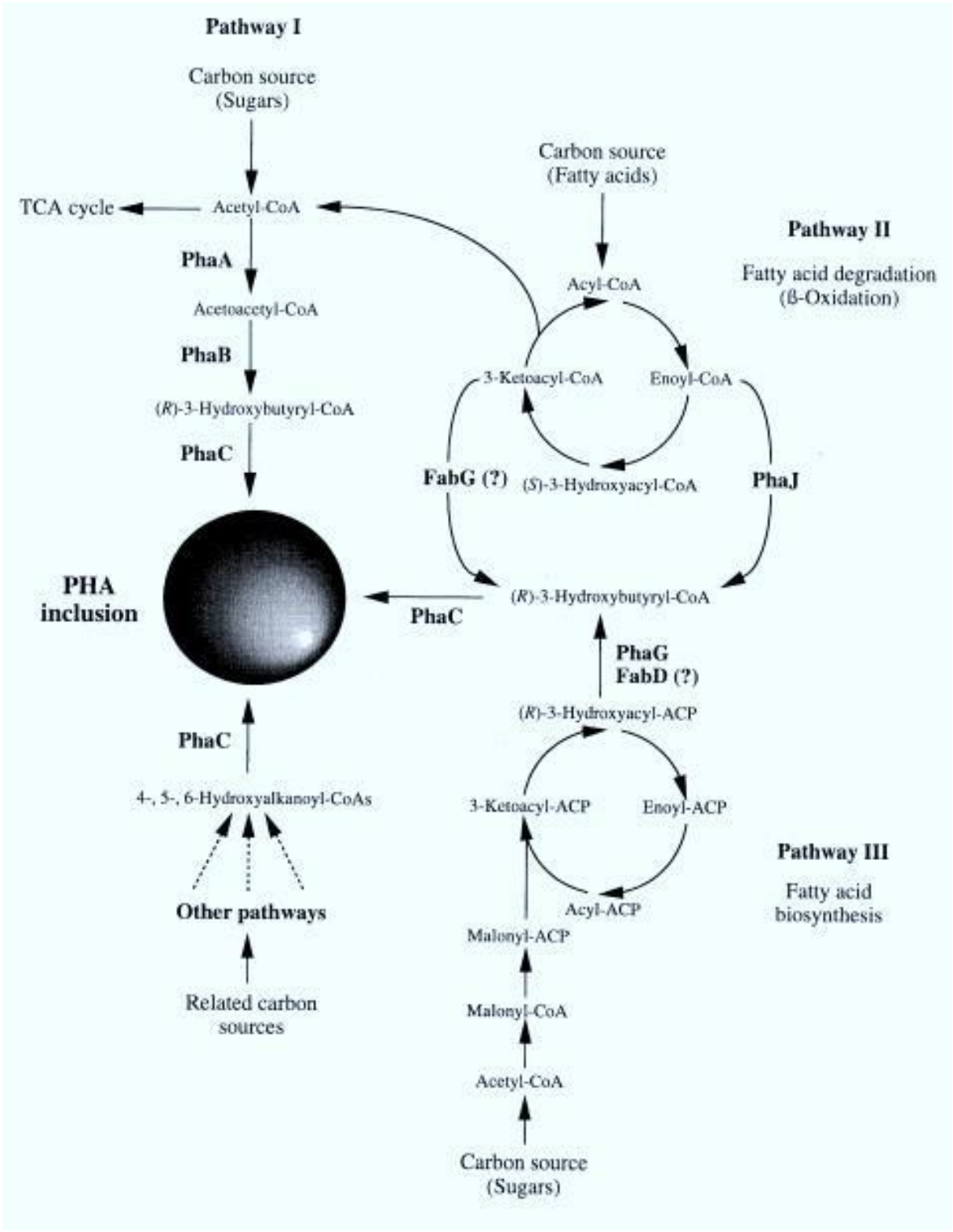


Figure 2.5. Metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis. PhaA, ß-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (R)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA reductase. Adapted from Sudesh *et al*., 2000.

Pathway I is the pathway used by the model organism *Cupriavidus necator,* formerly *Rahlstonia eutropha,* for PHA biosynthesis, which is one of the more extensively studied Bacteria for this purpose. The carbon source (as acetate) is initially converted into (R)-hydroxyacetyl-CoA by ß-ketothiolase (PhaA). The product is then reduced by an NADPH-dependent reductase (PhaB) producing (R)- 3-hydroxybutyryl-CoA which is finally converted by PHA synthase (PhaC) into Poly- 3-hydroxybutyrate (PHB) (Laycock *et al.*, 2013; Chen 2010b). Various carbon sources can be utilized by *C. necator* for growth or PHA production. It is capable of producing the P(3HB) homopolymer from even carbon numbered n-alkanoates, while odd-carbon numbered n-alkanoates resulted in the accumulation of copolymers of 3HB and 3HV (Akiyama *et al*., 1992).

PHA biosynthesis can also take place by microorganisms that can synthesize mcl-PHA from diverse alkanes, alkanols or alkanoates. These kinds of Bacteria use the 3-hydroxyacyl-CoA substrates of C6 to C14 from the intermediates of fatty acid β-oxidation pathway (Figure 2.5 - Pathway II). In this pathway fatty acids are used as substrate trough β-oxidation where they start being oxidized originating acyl-CoA that is converted to 3-hydroxyacyl-CoA which can then form PHA under synthase catalysis. Specific enzymes such as the enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-CoA reductase (FabG) are presumably involved in the conversion of fatty acid β-oxidation intermediates into suitable monomers that can then be polymerized by the PHA synthase.

Many bacteria are also capable of synthetize mcl-PHA from unrelated carbon sources such as carbohydrates, like fructose and lactate or volatile fatty acids (VFAs), like acetate, propionate, butyrate and valerate. Through this route, the 3-hydroxyacyl monomers can be derived from the *de novo* fatty acid biosynthesis pathway (Figure 2.5 - Pathway III). An extra biosynthetic step is necessary in order to convert the intermediate that is in the form of (R)-3-hydroxyacyl-ACP into the (R)-3-hydroxyacyl-CoA form. An enzyme known as 3-hydroxyacyl-ACP-CoA transferase (PhaG) was found to be capable of channelling the intermediates from the *de novo* fatty acid biosynthesis pathway to PHA biosynthesis (Rehm *et al*., 1998 ; Anderson *et al*., 1990 ; Sudesh *et al*., 2000).

There are more then 250 different natural PHA-producers, yet, just some bacteria have been used for PHA biosynthesis. Among them are *Alcaligenes latus, B. megaterium, C. necator* and *P. oleovorans* (Chee *et al*., 2010). Nowadays, companies like Biomer™ (PHB) and Biocycle™ (PHB and P(HB-co-HV)) are producing PHA using pure cultures in their natural state.

Over the past decades, there has been a notable gain of knowledge about the native biosynthetic pathways for the production of PHA monomers in various microorganisms. This understanding of the global physiological process of the PHA accumulating cells has lead to new strategies like the introduction of synthetic pathways into organisms for the biosynthetic

production of various PHA polymers. The strains built can then be further metabolically engineered in order to produce PHAs in a sufficiently high concentration resulting in a high productivity and yield using quite inexpensive carbon source through fine-controlled fermentation. Metabolic engineering of PHA producers has become one of the main strategies to allow PHA to efficiently and economically compete with the petroleum-derived polymers, being made by companies like Biopol™ (P(HB-co-HV) and Nodax™ (P(HB-co-HHx) (Jung *et al*., 2010; Lu *et al*., 2009; Khanna *et al*., 2005; Lemos *et al*., 2006). Some of the genetic engineering PHA producers efficiently able to use inexpensive renewable substrates like genetically modified *Escherichia coli* and *Alkaligenes* species are presented in Table 2.1.

Table 2.1. PHA-producing bacteria using plant oils or wastes.

Strain	**PHA**	**Substrate used**	**PHA content (wt%)**	**References**
Recombinant *Escherichia coli*	P(3HB-coHHx-co-3HO)	Soybean oil	6	Fonseca *et al*., 2006
Alkaligenes latus DSM 1124	P(3HB)	Soya waste ; malt waste	33.71	Yu *et al*., 1999
Comamonas testosteroni	mcl-PHA	Castor, coconut, mustard, cottonseed, groundnut, olive and sesame oils	79-88	Thakor *et al*., 2005
Cupriavidus necator H16	P(3HB-co-3HV)	Crude palm kernel, palme kernel, olive, sunflower, cooking, palm and crude palm oils	65-90	Lee *et al*., 2008
Cupriavidus necator DSM 545	P(3HB)	Waste glycerol	50	Cavalheiro *et al*.,2009
Pseudomonas guezennei biovar.tikehau	mcl-PHA	Coprah oil	63	Simon-Colin *et al*., 2008

Nonetheless, pure culture production of PHA entails disadvantages resulting from the sterilization costs associated to the process, since the sterile pre-cultivation of the bacteria utilized till the sterile operation of the final production process (Kleerebezem & van Loosdrecht, 2007).

2.2. Eco-Biotechnology

The eco-biotechnology principle relies on microorganism's natural selection and competition rather than on genetic or metabolic engineering them, that is, the ecosystem is engineered instead of the organisms (Jonhson *et al*., 2009a). This biotechnological research field has been developed by allying the traditional elements from environmental biotechnology concerning the cleaning of waste streams with the industrial biotechnology that is focused on product maximization. In this approach, pure microbial cultures are replaced by mixed microbial cultures, allowing the production process to be only based on natural selection by manipulation of either the bioprocess operation or the source of the natural inoculum. Since PHA was for first time detected in MMC in a wastewater treatment plant by Wallen and Rohwedder (1974) much effort have been done to develop and optimise production processes using MMC. The maximum MMC PHA cell content (77 %) was reported by Jiang *et al*. (2012) using paper mill wastewater.

The use of mixed cultures in industrial biotechnology instead of pure cultures offers specific advantages, namely: no sterilization requirements, adaptive capacity due to the microbial diversity, possibility of using mixed substrates and the opportunity to develop a continuous process. Polyhydroxybutyrate (PHB), the dominant PHA produced by *Bacteria* is a great example of a process combining the objectives of elimination of undesirable compounds from a waste stream by generating a valuable product (Kleerebezem & van Loosdrecht, 2007).

The major obstacle to the replacement of synthetic plastics by biopolymers is their great cost contrast, while polypropylene is produced at €1/kg, PHA production costs are over three times more varying from €3.5 to 5.0/kg. The economics of PHA depends mainly on the substrate price, on the PHA yield on substrate and on the extraction efficiency of the polymer from the cells (Lemos *et al*., 2006). Although the replacement of mixed cultures by pure cultures helps decrease the production expenses, the substrate costs is a determinant step, as almost 50% of the entire production costs arises from there. These results from the fact that PHA accumulation takes place under aerobic conditions, resulting in high losses of the carbon substrate by intracellular respiration thereby only around 50% of the carbon source is driven towards biomass and PHA formation. Worldwide, substantial amount of excess agro-industrial waste effluents or activated sludge is generated daily. About 40 to 60% of the total operational

costs of an activated sludge treatment plant consist in handling, treatment and ultimate disposal of the excess sludge. Hence, the replacement of raw materials by waste materials, like activated sludge, as starting materials for PHA biosynthesis constitutes a viable strategy for cost-efficient biopolymer production and a strong help for industry to fight disposal problems (Koller *et al*., 2010a; Zhang *et al*., 2009). The utilization of activated sludge for PHA production has the handicap that storage rates of activated sludge are usually quite low since only a fraction of the microbial biomass can be stored. Also, at high sludge age and low organic load rates biomass becomes less active. Aiming to overcome this limitation various strategies have been proposed, among them the application of the regime named Aerobic Dynamic Feeding (ADF) or Feast and Famine (FF) for PHA producing processes (Beccari *et al*., 2002; Majone *et al*., 2006).

2.2.1. PHA production process from low value substrates using mixed microbial cultures

The maximum PHA cell content reported in activated sludge has been rising along the past years, from modest values like 50% of the cell dry weight to a latter value quite close to the reported for pure cultures (~90%) (Lemos *et al*., 2006; Serafim *et al*., 2008) The PHA production process from low value substrates using mixed microbial cultures requires different stages, which are strictly interconnected. Wastes or fermented stream are fed into two successive stages, the first one with the purpose of selecting and enrichment of PHA-storing microorganisms and the second one for subsequent polymer maximum accumulation (Figure 2.6). Culture selection can be reached under conditions of feast and famine (FF) regime, usually established in a sequencing batch reactor (SBR) (Villano *et al*., 2014; Majone *et al*., 2006). When the need for the fermentation of the waste steam exists, we are in the presence of a three-step process instead of the most commonly two-stage process.

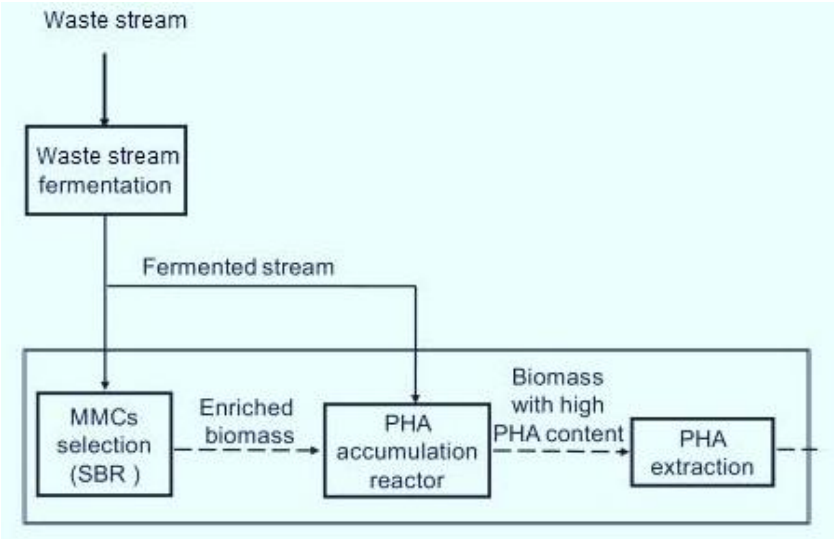


Figure 2.6. Multi-stage process for PHA production by food industry by-products with mixed microbial cultures. Adapted from Villano *et al*., 2014.

2.2.1.1. Culture selection stage using Feast and Famine principle

Among the different processes studied for industrial PHA production by mixed cultures, the feast and famine process is the most promising due to the high sludge PHA content and productivity obtained (Reis *et al*., 2003).

In a system with a feast and famine regime the substrate is fed during a short period of time (feast), followed by a longer period of substrate lack (famine). Under these dynamic conditions, during the period of excess of external substrate, carbon uptake is lead to cell growth while PHA storage is occurring simultaneously. Once all the external substrate is exhausted, the stored polymer is used as energy and carbon sources (Figure 2.7). The storage phenomenon is usually dominant relative to growth since the biochemical route of substrate conversion to PHA is quite shorter and requires less energy than the one for biomass production (Majone *et al*., 1999; Serafim *et al*., 2004; Kleerebezem & van Loosdrecht, 2007). Under conditions where substrate is continuously present during a long period of time, physiological adaptation occurs and growth can overcome. Still, when facing brief substrate supplies, the microorganisms having the ability to store internal reserves own a competitive advantage over those without this ability (Salehizadeh & van Loosdrecht, 2004).

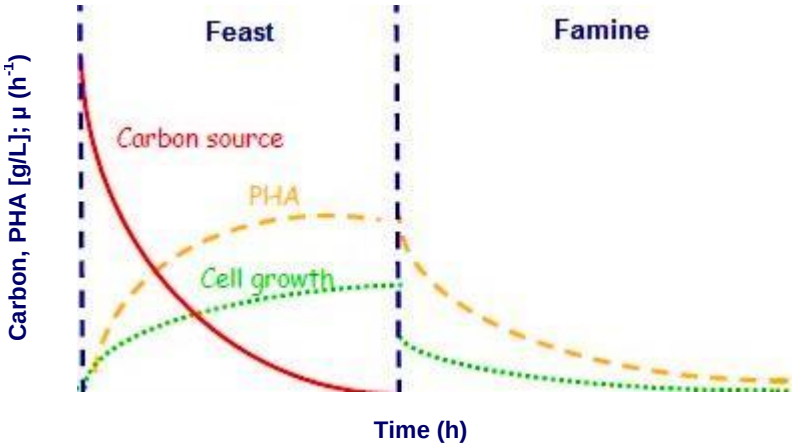


Figure 2.7. Feast and Famine cycling. Feast corresponds to an excess of external carbon substrate and Famine to a long period of starvation and Famine. "μ" is the specific growth rate. Adapted from BIND 2011/12 DQ-FCT-UNL RO.

In a system with a feast and famine regime, the synthesis of cell enzymes and RNA needs to be induced in *Bacteria* for a level of average growth rate in the system instead of maximal growth rate, enhancing the efficiency of the overall growth process. Consequently, the amount of PHA that can be accumulated is strongly dependable on the history of the cells. In systems with short sludge age the growth process becomes more dominant which drives to lower maximal PHA contents. In turn, PHA degradation rate depends on the cells polymer content (Reis *et al*., 2003 ; Salehizadeh & van Loosdrecht, 2004). Besides sludge age other

parameters significantly affect the performance of the selection stage, namely, length of the feast and famine phase (F/F ratio). In general, low F/F ratio ensures selection of microorganisms more capable of storing PHA and with an enhanced physiological adaptation towards PHA synthesis in the feast phase. The aim of the selection stage is mainly to produce the PHA-storing culture at the highest productivity possible, which means, produce large amounts of biomass per unit of volume of reactor and per unit of time. In order to reach high biomass volumetric productivities, the reactor should be operated at elevated organic load rates (OLR). This is possible by supplying high concentrations of substrate and/or imposing short hydraulic retention time (HRT). However, too high OLR can result in unstable or poor performance, therefore, a balance between F/F ration, OLR and HRT is the key for a successful selection stage (Villano *et al* 2014; Reis *et al*., 2011).

In an industrial PHA-production process, following the selection stage is the accumulation phase. During this step the microorganisms are saturated with PHA by supplying an excess of substrate, thereby, 70 to 80% of the dry matter formed in the process is recovered as PHA. This value of PHA content is quite close to the one obtained with modified *E. coli* showing the potential of this process to be economically feasible (Kleerebezem & van Loosdrecht, 2007).

2.2.2. Downstream process

A good strategy to efficiently recover PHA should minimise polymer degradation, and maximise extraction yield and final product purity, taking into account two different principles: the polymer solubility in appropriate solvents; and the disruption of the cell membrane (Dias *et al*., 2006). The most used methods to extract PHA from cells involve solvents, such as chloroform, methylene chloride, propylene carbonate and dichloroethane, methods that can lead to very pure PHA. However, the solvents used not only increase the final costs due to their prices, as also result in adverse environmental consequences concerning their toxicity (Reis *et al*., 2011). There is an alternative method using hypochlorite in which the PHA granules are isolated from the cell by centrifugation and then biomass is treated with a sodium hypochlorite solution that degrades the cellular material other than PHA (Berger *et al*., 1989). The main disadvantage of this method is the possible degradation of PHA, resulting in PHA with a lower molecular weight (Chee et al., 2010). Enzymatic digestion is another method to extract PHA, during which the broth culture is submitted to a short period of heat shock treatment in order to break the cells. Enzymes like protease, lysozyme and phospholipase can be used being very specific, with no polymer degradation, and resulting in the efficient recovery of high purity

polymers (Dias *et al*., 2006; Reis *et al*., 2011). To ensure a polymer with high purity, it has to be additionally subjected to several purification procedures, which can decrease product recovery or have a negative impact on polymer properties. Thereby, a balance between product recovery and purity, with its impact on the polymer properties, must be taken into account during the selection of the most suitable procedure to each specific PHA production process. As a crucial point concerning the high costs, further research is required for both improvement and development of new approaches for downstream processes (Freitas *et al*., 2011).

2.3. Two-stage CSTR system *vs* SBR for PHA accumulating culture selection

PHA production processes by microbial mixed cultures are based in a minimal of two steps operated separately, the culture selection stage and the subsequent PHA accumulation stage. This way, each stage can be operated independently in two reactors at different optimized conditions, like distinct nutrient concentrations. Usually, a Sequencing Batch Reactor (SBR) is used to perform the step for selection of PHA-accumulating organisms while the PHA accumulation of the enriched culture takes place in batch mode (Dionisi *et al*., 2004; Serafim *et al*., 2004). One of the possible approaches to improve the efficiency of MMC PHA production is trough the maximization of PHA storage efficiency in the final production stage by optimization of the culture selection stage. This strategy is based on the principle of eco-biotechnology that applies different operation conditions to the culture enrichment system. The efficacy of this strategy was already proven by Alburqueque *et al*. (2010) and Johnson *et al*. (2010b). By optimizing the selection stage they were able to reach in the final batch production step, a PHA content of 74% and 89% using fermented molasses and acetate, respectively.

The culture selection stage is more commonly performed in an SBR which is basically a suspended growth biological reactor where all the metabolic reactions take place in one tank and in a well-defined and continuously repeated time sequence (SBR cycle). In this kind of reactor, microorganisms' exposure time, frequency of exposure and amplitude of the respective concentration can be defined independent of the inflow pattern. This way, periodic exposure of the microorganisms to defined process conditions is effectively achieved. The transient conditions imposed to the biomass growers makes this kind of reactors quite effective for selection of populations with a raised PHA-storage capability. Besides, it's very easy and flexible to modify and control the process conditions like the time for feeding the substrate or the cycle duration. So, several important variables influences the SBR operation like the HRT, OLR and total cycle time employed which are some of the parameters currently in study to

improve PHA-producing processes trough this system (Singh & Srivastava, 2011; Mahvi, 2008; Reis *et al*., 2003).

The SBR system has some drawbacks concerning the scale up of the PHA process to an industrial level. Since both phases are performed in a unique reactor and as the equipment designed is based on the maximum oxygen consumption, the equipment is over dimensioned for famine phase needs. Also, during the effluent and feeding phase large volumes have to be transferred during short periods of time, which implies the utilization of large pumps. Both factors, the oversized equipment and the large pumps, lead to an increment in the process operational costs. On the other hand, a continuous system (CSTR) offers some advantages for scale-up comparing with the SBR. In this kind of systems the hydraulic retention times (HRT) of the first and second reactors correspond to the Feast and Famine phases on a SBR cycle. Instead of a feast and famine phase in a single SBR reactor, substrate uptake and microbial growth are uncoupled in space rather than in time. Different cultivation conditions can so be applied, allowing the separate design of each reactor which can trigger the cultivation conditions in each stage seeking for its individual highest performance (Atlic *et al*., 2011; Jung *et al*., 2001). Considering a desired amount of product per unit of time and choosing a continuous culture over a batch process, the bioreactor volume can be sharply reduced. Using lower volumes allows achieving the desirable mixing conditions with less energy expenditure. Therefore, multi-staged CSTRs can be operated providing a more rigorous control of the PHA produced in the cells in each reactor individually (Braunegg *et al*., 2004). Lately, continuous reactors to select for PHA-accumulating organisms under Feast and Famine conditions have been raising its commercial interest especially for strains with a high maximum specific growth rate. As an example of this strategy a process was studied including two sequentially disposed continuous reactors followed by a settler mimicking a wastewater treatment plant configuration and using fermented paper mill wastewater as feedstock. The selected culture achieved a maximum PHA content of 48 wt% PHA (Bengtsson *et al*., 2008). Other study about the production of PHA with enrichment cultures using a 2-staged system was carried by Albuquerque *et al*. (2010) obtaining a maximum PHA content of 61 wt% using fermented sugar molasses as feedstock. Other systems with more stages were studied as well, namely, Atlic *et al*. (2011) that reached 77 wt% PHA in a five-stage system with *Cupriavidus necator* and using glucose as carbon source.

3.Materials and Methods

3.1. Systems for culture enrichment

3.1.1. Two-reactor CSTR system

3.1.1.1. Setup 1

A continuously fed two-stage CSTR system consisting of a Feast and a Famine reactor was installed for enrichment of a PHA-producing microbial community and operated for 29 days. The enrichment reactor system was designed to impose Feast and Famine conditions by feeding the Feast reactor with carbon substrate and passing part of the resulting effluent to the Famine reactor trough a continuous flow. The system was composed of a double-jacket glass bioreactor with 1L working volume (Applikon, The Netherlands), representing the Feast reactor, and a single-wall bioreactor with internal heat exchanger and 11.5 L working volume (Applikon, The Netherlands) representing the Famine reactor. The retention time of the Feast and the Famine was, respectively, 0.5h and 11.5h. Therefore, the sum of the retention time of the Feast and Famine reactor was 12 hours and the effluent flow was set as half of the Feast outflow in order to simulate a sequential batch reactor (SBR) system with a 12 hour cycle and an SRT of 1 day. The flow rate of the feeding entering in the Feast reactor (Nutrients + acetate), the recycle between Feast and Famine reactors and the effluent going out was 1 L/h. There was no settling phase, so, the hydraulic retention time (HRT) equalled the SRT of 1 day.

The reactors were equipped with two standard geometry six-blade turbines and the stirring speed was programmed to 900 rpm for the Feast and 500 rpm for the Famine reactor. The airflow of the reactors was controlled at 0.4 L_N/min and 1.0 L_N/min in the Feast and Famine reactor, respectively, with a mass flow controller (Brooks Instruments, USA). The dissolved oxygen (DO) was determined with a DO electrode (Applikon, The Netherlands). The partial pressures of oxygen and carbon dioxide entering and leaving the reactors were quantified using a NGA 2000 gas analyser (Rosemount, USA). The reactors were temperature controlled at 30±1°C by means of a thermostat bath (Lauda, Germany) connected to the water jacket of the Feast reactor and the internal heat exchanger of the Famine reactor. The pH of the reactors was monitored with a pH electrode (Mettler Toledo, USA) and maintained at 7.0 ± 0.1 using 1 M HCl and 1 M NaOH.

The pH, DO, and acid and base dosage were managed by an ADI 1030 Bio Controller (Applikon, The Netherlands) and, including the off-gas composition, recorded by a PC using the software MFCs/win (Sartorius Stedim System, USA).

Both online – pH, DO, acid and base dosage, and off-gas O_2 and CO_2 – and offline measurements – acetate, ammonium, biomass and PHB – were performed along the entire experimental period.

The inoculum of the Feast and Famine reactor was biomass highly enriched in *Plasticicumulans acidivorans*, from activated sludge contained in an acetate-fed SBR with 18-hour cycles. Together with an inoculum of 3.5 L, 70 mL ammonium 3.4 mM and 280 mL nutrients solution plus 7.6 L of water was dosed in the Famine reactor. In the Feast reactor, a pulse of 17.5 mL nutrients solution plus 0.3 L of biomass and 0.5 L of water was dosed.

The Feast reactor was cleaned about twice a week in order to remove biofilms from the walls, metal parts and electrodes.

3.1.1.2. Setup 2

After analysing the results obtained from setup1 a new set of conditions was determined for the experimental setup (setup 2).In setup 2, the residence time in the Feast reactor was increased to 0.8 hours and in the Famine reactor decreased to 11.2 hours, keeping the total retention time of the system of 12 hours. The feed flow and concentration were adjusted in accordance with the new retention times, leading to a carbon dosage of 48.6 CmM and an ammonium dosage of 3.0 mM. The reactors were re-inoculated with biomass highly enriched in *Plasticicumulans acidivorans*, from activated sludge contained in the acetate-fed SBR with 18-hour cycles as in setup 1.

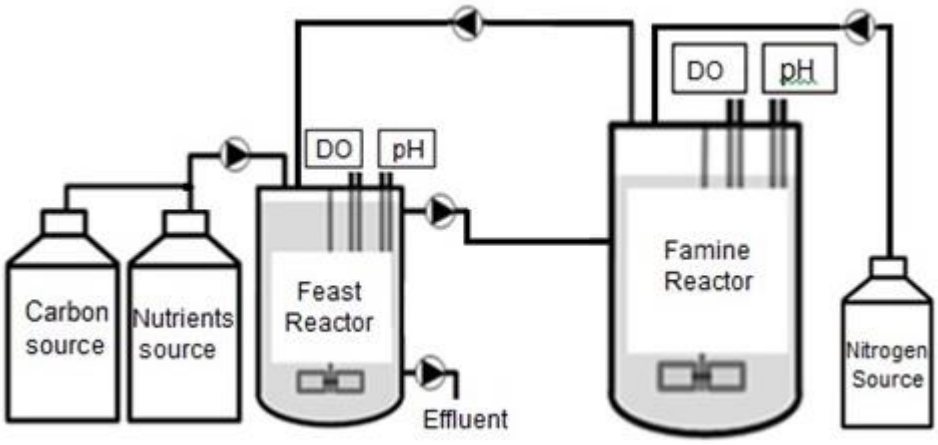


Figure 3.1. Two-reactor CSTR system experimental setup.

Figure 3.2. Two-reactor CSTR system.

3.1.2. SBR system

A sequencing batch reactor (SBR) was performed using a double-jacket glass bioreactor with a working volume of 2 L (Applikon, The Netherlands). For a period of 3 weeks the reactor was operated as a non-sterile SBR with 12 h batch cycles. Due to the lack of a settling phase, the SRT equalled the HRT of 1 day. Each cycle consisted of a feeding phase (10 min), reaction phase (695 min) and effluent phase (15 min). The reactor was running continuously sequences of batch reactions; once a batch was finished it was immediately followed by the feeding phase of the next batch.

The stirring speed was programmed to 750 rpm. The airflow rate was kept at 0.15 L_N/min with a recirculation flow of 0.14 L_N/min using a mass flow controller (Brooks Instruments, USA). The temperature of the reactor was maintained at 30±1 ºC using a thermostat bath (Lauda, Germany) connected to the water jacket. The pH was maintained at 7.0 ± 0.1 using 1 M HCl and 1 M NaOH and determined with a pH electrode (Mettler, Toledo, USA).

The reactor was considered to be in steady state when, after around seven days, the length of the Feast phase, as indicated by the DO profile, and the concentration of total suspended solids (TSS) at the end of the cycle were constant. When the reactor was in steady state, in addition to the online measurements, a cycle of the SBR was monitored by offline samples (acetate, TSS, PHB, biomass and ammonium).

The reactor was initially inoculated with 1 L volume comprising 600 mL of biomass highly enriched in *Plasticicumulans acidivorans*, from activated sludge contained in the acetate-fed SBR with 18-hour cycles (as in CSTR), 150 mL of nutrients and 250 mL of water. The MMC was supplied, in each batch cycle, with 54 Cmmol of NaAc (carbon source), a nutrient source (same composition as nutrient solution of CSTR system) with 6.75 mmol ammonia (NH_4Cl) and 5 mg L-1 allylthiourea to avoid nitrification. In the influent phase of each batch cycle, 100 mL of carbon source, 100 mL of nutrient source, and 800 mL of dilution water were mixed and pumped into the reactor.

After the system was running for 7 days extra acetate (18 Cmmol) was dosed besides the initial feeding (54 Cmmol) at the beginning of each cycle. This extra amount of acetate was dosed continuously along every Feast and Famine phase (705 min) and it was 1/3 of the initial feeding. Briefly, every 12 hours cycle began by feeding 54 Cmmol of acetate and once the feast phase started an extra amount of 18 Cmmol of acetate was continuously pumped till the end of the cycle. After 3 days, in order to avoid a possible ammonium limitation due to the extra acetate dosage, 15 mL NH_4Cl 2.25 mmol were added to the nutrient supply bottle.

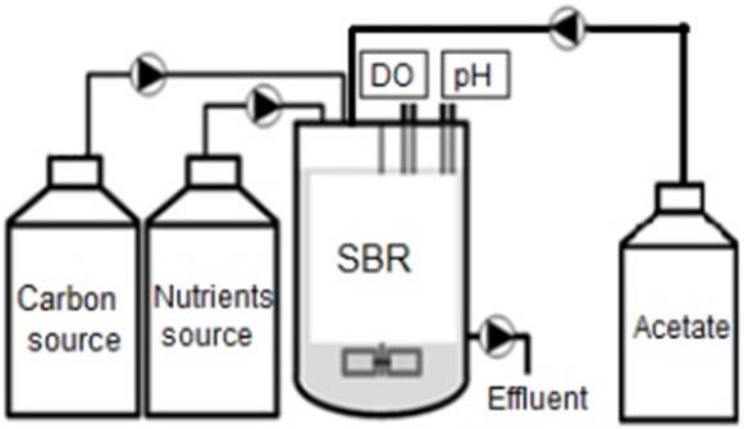


Figure 3.3. Experimental setup of Sequencing Batch Reactor experiment.

3.2. Medium composition

The carbon source used to feed the system was sodium acetate ($CH_3COONa \cdot 3H_2O$) at a concentration of 54 CmM and the concentrations of the other nutrients composing the feed solution were: 2.49 mM KH_2PO_4, 0.55 mM $MgSO_4 \cdot 7H_2O$, 0.72 mM KCl, 1.5 mL/L trace elements and 5 mg/L allylthiourea (to prevent nitrification). The composition of the trace element solution (Vishniac and Santer, 1957) was: 63.69 g/L EDTA triplex III, 22 g/L $ZnSO_4 \cdot 7H_2O$, 1.61 g $CoCl_2 \cdot 6H_2O$, 5.06 g/L $MnCl_2 \cdot 4H_2O$, 1.51 g/L $CuSO_4 \cdot 5H_2O$, 2.99 g/L $FeSO_4 \cdot 7H_2O$, 1.10 g/L $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ and 7.34 g/L $CaCl_2 \cdot H_2O$. By addition of potassium hydroxide (KOH) the pH of the trace elements solution was increased to 6.0. The nitrogen source, 3.4 mM ammonium chloride (NH_4Cl), was fed to the Famine reactor.

3.3. Side experiments

3.3.1. Accumulation Experiment (Fed-batch)

The PHA accumulation performance of the culture selected in the CSTR system (setup 2) was assessed by conducting an accumulation experiment under ammonia limiting conditions. For this fed-batch experiment a double-jacket glass bioreactor with a working volume of 2 L (Applikon, The Netherlands) was used. Since in this system the effluent is after the Feast reactor, a volume of 2 L was collected from the effluent flow of the Feast reactor and used as inoculum for the experiment. The production of PHA was initiated by feeding a pulse of 12 mL

sodium acetate (4 M). Acetic acid (1.5 M) was used for pH control at 7.0 ± 1.0 and simultaneously used as carbon source (fed-batch).

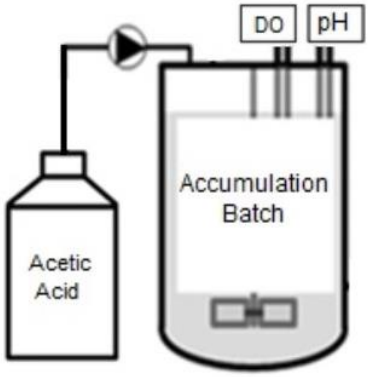


Figure 3.4. Fed-Batch experimental setup.

The stirring speed was programmed to 750 rpm. The airflow rate was kept at 0.4 L_N/min with a recirculation flow of 0.96 L_N/min using a mass flow controller (Brooks Instruments, USA). The temperature of the reactor was maintained at 30±1 ºC using a thermostat bath (Lauda, Germany) connected to the water jacket. The pH was determined with a pH electrode (Mettler, Toledo, USA) and the dissolved oxygen concentration (DO) with a DO electrode (Applikon, The Netherlands). The pH, acid and base dosage were controlled by a Biostat B plus (Sartorious Systems, USA) and all the data recorded by a computer running the MFCs/win software (Sartorius Stedim System, USA). The DO, pH, acid and base dosage were monitored online. Substrate, PHB and biomass concentrations were measured offline.

The experiment was performed during 7 hours, the second sample was taken 30 min after the beginning and then one every hour. A last sample was taken the following day. All the samples were treated and analysed as explained below in analytical procedures (3.4).

3.3.2. Growth Experiment

A growth experiment in the presence of ammonium was done in order to evaluate the growth rate of the culture selected in the CSTR (setup 2). For this experiment a double- jacket glass bioreactor with a working volume of 2 L (Applikon, The Netherlands) was used. A volume of 2 L was collected from the effluent flow of the Feast reactor. A pulse of 15 mL sodium acetate 4 M and 22 mL NH_4Cl 0.8 M (stock solution) was dosed at the beginning.

The stirring speed was programmed to 750 rpm. The airflow rate was kept at 0.4 L_N/min with a recirculation flow of 0.96 L_N/min using a mass flow controller (Brooks Instruments, USA). The temperature of the reactor was maintained at 30±1 ºC using a thermostat bath (Lauda, Germany) connected to the water jacket. The pH was maintained at 7.0 ± 0.1 using 1 M

HCl and 1 M NaOH. Acid and base dosages were controlled the same way in the side experiments.

The DO, pH, acid and base dosage were monitored online. Substrate, PHB and biomass concentrations were measured offline.

Samples were taken each 15 minutes during 3 hours. All the samples were treated and analysed as explained below in analytical procedures (3.4).

3.4. Analytical procedures

Samples taken from the reactors for analysis of acetate and ammonium levels were immediately filtered with a 0.45 µm pore size filter (PVDF membrane, Millipore, Ireland). The acetate concentration of the filtered samples was measured by High Performance Liquid Chromatography (HPLC) using a UV detector (Waters 484, 210 nm) and an automatic injector (Waters 717plus, 10 µl). The mobile phase, 1.5 mM H_3PO_4 in Milli-Q water, moved at a flow rate of 0.6 ml/min and at 59°C through the BioRad Animex HPX-87H column (300 by 7.8 mm). The ammonium concentration in the supernatant was determined spectrophotometrically, using a commercial cuvette test kit (LCK302/303/304, Hach Lange, Germany).

Nitrogen biomass content was assessed by taking 3 samples from the Feast reactor and 3 samples from the Famine reactor with a dilution of 1:2. The samples were then submitted to a Koroleff Digestion (Peroxodisulphate) and photometric detection using a commercial cuvette test kit (LCK 338, Hach Lange, Germany).

Biomass and PHB quantification was performed by taking 15 mL samples from the Feast and Famine reactor into previously weighed Greiner tubes with five drops of formaldehyde (37%), in order to stop all biological activity. The samples were then centrifuged for 10 minutes at 4500 rpm (3850 x g). The supernatant was discarded and the samples subsequently frozen (-20°C) and lastly freeze dried (Labconco, USA) in around 24 hours (-50°C, 10^{-1} atm). The freeze-dried biomass was weighed as total suspended solids (TSS).

For PHB quantification, the freeze-dried samples were used. Pure PHB (Sigma-Aldrich CAS 26063-00-3) was used as a standard in the analysis and treated alongside with the samples. The freeze-dried biomass and three standards were weighed on an analytical balance (Mettler Toledo, The Netherlands) and transferred to glass tubes. Firstly, 50 µL of 1 mg benzoic acid in 50 mL 1-propanol was added to each tube. Secondly, 1.5 mL solvent, dichloroethane, and 1.5 mL HCl in 1-propanol (1:4) were added. The HCl was used to break the cell walls and

hydrolyse the PHB and the propanol to produce the linear ester of PHB. The tubes were then heated for two hours in a block heater (100°C). Addition of 3 mL Milli-Q water, after cooling, promoted the extraction of free acids from the organic phase. The separation of the aqueous and organic phase was speeded up by centrifugation (5 min, 2500 rpm/1188 x g). Afterwards, 1 mL of the organic phase was filtered over water-free sodium sulfate into GC vials. The propylesters formed were measured using a gas chromatograph (Agilent 6890N, USA) equipped with a flame ionization detector (FID) and an HP-INNOWax colum. The injection volume was 1 µL with an inlet at 230ºC and 240.3 kPa and the carrier gas was He. The detector was operating at 250ºC. The temperature profile of the oven was, starting at 110ºC, hold 3 min, increase to 170ºC at 15ºC/min, increase to 240ºC at 40ºC/min and hold for 6.25 min, adding up a total run time of 15 min. The flow through the column was 1.7 mL/min. Results were expressed as the weight percentage PHB of the total biomass.

The amount of PHB was subtracted from TSS to determine the concentration of active biomass. A biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ was assumed with a molecular weight of 25.1 g/Cmol, including ash (Beun J.J., 2002).

3.5. Microbial community analysis

3.5.1. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

Biomass samples from the Famine reactor of the CSTR system (setup 2) were collected after 66 days of experiment to analyse the microbial composition of the enrichment culture and washed with TE (Tris-EDTA) buffer.

Genomic DNA was extracted using the Ultra Clean Soil DNA extraction kit (MoBio Laboratories, California). Subsequently, the DNA extracted was used as template DNA for PCR. A "touchdown" PCR program (Appendix A; Table 7.1) was done using primers 341-GC and 907R (Appendix A; Table 7.2) for DGGE analysis. (Schäfer, H. *et al*., 2001). The fragment length obtained was proximately 550bp.

16S rRNA gene amplicons were applied onto 8% polyacrylamide gels with denaturing gradient from 20% to 70% DNA denaturants (100% denaturants is a mixture of 5.6 M urea and 32% formamide) for 16 hours (960 min) at 100V and 60ºC (Schäfer, H.*et al*., 2001). DNA was visualized by UV illumination and photographed with a digital camera after staining with SYBR® Safe. A sterile razor blade was used to excise individual bands from the gel. The bands were incubated overnight in 50 µL water at 4 °C. Re-amplification was performed using the

same primer pair (341) without the GC clamp and the PCR products were sequenced by a commercial company (Macrogen, South Korea).

3.5.2. Fluorescent *in situ* hybridization (FISH)

Biomass samples collected from the CSTR system (setup 2) after 75 days were fixed in paraformaldehyde, immobilized on microscopic slides and hybridized. Probes were commercially synthesized and 5′-labeled with either FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). A probe mixture for all bacterial species in the sample was used (EUB338mix) and a Cy5-labeled 16S rRNA probe specific for *P. acidivorans* (UCB823) was used to indicate its presence in the sample.

For the two samples, one from the Feast and one from the Famine reactor, were used 4 wells in order to stain both samples in two different ways (Appendix A; Table 7.3):

1. Using "α mix" (Cy3) and "β/γ" (Cy5 and FLUOS respectively) being α-Proteobacteria stained red, β-Proteobacteria blue and γ-Proteobacteria green.
2. Using "EUB338mix" (Cy5), "γ/β-" (Cy3 and unlabeled, respectively) and "UCB823" (FLUOS) being all eubacteria stained blue, γ-Proteobacteria red and *Plasticicumulans* green.

The unlabelled beta probe in case 2 is there in order to minimize erroneous hybridizations of the gamma probe. In case 1, the same strategy was used in beta and gamma mix, except that both were labelled.

For the fixation and immobilization the samples were centrifuged and the supernatant removed. The cells were washed with 500 µL PBS, centrifuged and resuspended in 100 µL PBS. To one volume (300 µL) of suspension were added tree volumes of fixative (PBS(3x); 390 mM NaCI in 30 mM phosphate buffer pH 7.2, paraformaldehyde, 1M NaOH,, 1M HCl) and kept in ice for 3 h. Cells were washed, re-suspended in 200 µL PBS(1x) and 250 µL of 98 % ethanol (-20°C) were added to one volume of cell suspension.

A microscopic slide was used to fix the previously fixated biomass samples. A dehydration step was performed by putting the samples for 3 minutes in each solution of 50% (v/v), 80%(v/v) and 96%(v/v) ethanol. The samples were hybridized with probes in a mixture composed by 1 µL of each probe stock solution (Appendix A; Table 7.3) (final concentration 5 pmol/µL for CY3 and CY5-labeled probes and 8.3 pmol/µL for FLUOS labelled probes) and 10 µL hybridization buffer containing 5M NaCl, 1M Tris-HCl pH 8 and 30% (v/v) formamide. Hybridizations were carried out for 1.5 h at 46 °C in a sealed moisture chamber and stopped rinsing the non-bound probes from the slides with washing buffer (Tris-HCl pH 8, 5M NaCl,

0.5M EDTA pH 8, 10% (w/v) sodium dodecyl sulfate (SDS). The slides were incubated for 20 minutes at 48ºC in the rest of the washing buffer, then washed shortly with distilled water and dried at room temperature. The wells were embedded with Vectashield and the coverslip fixated with nail polish. The samples were then analysed under the fluorescence microscope.

3.6. Data treatment

3.6.1. Two-reactor CSTR system for culture enrichment

3.6.1.1. Calculations

In order to calculate yields, specific rates, carbon balances and electron balances average values of acetate consumed, PHB produced, biomass produced, nitrogen consumed, carbon dioxide produced and oxygen consumed (all in carbon-mole base) were considered. These values were determined using the measurements made along the experiment together with the system mass balances described in the following section 3.6.1.2 (Table 3.1 and 3.2). Since these were average values the error associated was determinate according with, $E = \sqrt{\sigma_{\bar{a}}{}^2 + \sigma_{\bar{b}}{}^2}$, being σ the standard deviation and $\bar{a}\ and\ \bar{b}$ the average values.

The yields of PHB ($Y_{PHB/Ac}$) and active biomass ($Y_{X/Ac}$) on acetate were determined by dividing the amount of PHB and biomass produced by the total amount of acetate consumed, respectively. The respiration yield on substrate ($Y_{CO2/Ac}$) was calculated by dividing the cumulative amount of CO_2 produced by respiration by the amount of substrate consumed.

The average biomass specific substrate uptake rate (qS), PHB production rate (qP), growth rate (qX) and nitrogen consumption rate (qN) were calculated trough the mass balances described below (Table 3.1 and 3.2, equations 1 - 8). The average specific carbon dioxide evolution rate (qCO_2) and oxygen uptake rate (qO_2) were calculated from the difference between the air entering the system and the off-gas leaving the system divided by the biomass concentration. The relative errors associated with the yields and rates calculated were determinate trough the standard deviations of the average values referred in the first paragraph according with, $E = x.\sqrt{\left(\frac{\sigma_{\bar{a}}}{\bar{a}}\right)^2 + \left(\frac{\sigma_{\bar{b}}}{\bar{b}}\right)^2}$, being x the yield or rate value, σ the standard deviation and $\bar{a}\ and\ \bar{b}$ the average values.

Electron balances were calculated relative to the reference oxidation states of carbon dioxide and water for organic carbon and oxygen. The oxidation state of the remaining reactants

can be calculated (in carbon-mole) trough the growth reference system: 4.0 for acetate, 4.5 for PHB, 4.2 for biomass, and -4.0 for oxygen. According with this system in all chemotrophic growth systems H_2O, HCO_3^-, H^+ and N sources occur as chemical compounds. To each chemical compound three numbers are designated: γ (degree of reduction that represents the electron content per C-mol); ΔG_e (Gibbs energy per electron present in the compound); ΔH_e (enthalpy per electron present in the compound). The three numbers are calculated from the reference redox half reaction where 1 C-mol of organic or 1 mol of inorganic compound is converted into the reference chemicals and a number of electrons which is, by definition, equal to γ. As example, for biomass with NH_4^+ as N source, the half reaction is, $-1CH_{1.8}O_{0.8}N_{0.2} - 2.5H_2O + HCO_3^- + 0.2NH4^+ + 5H^+ + 4.2e^-$ (Heijnen *et al*., 1999).

For carbon balances, the total amount of acetate consumed, PHB produced or consumed, biomass produced, and carbon dioxide produced was considered.

3.6.1.2. System mass balances

The system mass balances were determinate according with the equations in Table 3.1. and Table 3.2.

Table 3.1. Mass balances of Feast reactor

1. <u>C-Source mass balance</u> $\frac{\mathrm{ds}}{\mathrm{dt}} = \mathrm{q_s} \times \mathrm{C_x} \times \mathrm{V_{Feast}} + \mathrm{C_{sFeed}} \times \mathrm{Inflow_{Feed}} + \mathrm{C_{sFamine}} \times \mathrm{Outflow_{Famine}} - \mathrm{C_{sOut}} \times \mathrm{Outflow_{Feast}} = 0$
2. <u>Biomass mass balance</u> $\frac{\mathrm{dx}}{\mathrm{dt}} = \mu \times \mathrm{C_{xFeast}} \times \mathrm{V_{Feast}} + \mathrm{C_{xFamine}} \times \mathrm{Outflow_{Famine}} - \mathrm{C_{xOut}} \times \mathrm{Outflow_{Feast}} = 0$
3. <u>Product mass balance</u> $\frac{dP}{dt} = q_p \times C_{xfeast} \times V_{Feast} + C_{pFamine} \times Outflow_{Famine} C_{pOut} \times Outflow_{Feast} = 0$
4. <u>Ammonium mass balance</u> $\frac{dN}{dt} = q_N \times C_x \times V_{Feast} + C_{NFeed} \times Inflow_{NFeed} + C_{NFamine} \times Outflow_{Famine} - C_{NOut} \times Outflow_{Feast} = 0$

Table 3.2. Mass balances of Famine reactor

5. C-Source mass balance $\frac{ds}{dt} = q_s \times C_x \times V_{Famine} + C_{sFeast} \times Inflow_{Famine} - C_{sOut} \times Outflow_{Famine} = 0$
6. Biomass mass balance $\frac{dx}{dt} = \mu \times C_{xFamine} \times V_{Famine} + C_{xFeast} \times Inflow_{Famine} - C_{xOut} \times Outflow_{Famine} = 0$
7. Product mass balance $\frac{dP}{dt} = q_p \times C_{xFamine} \times V_{Famine} + C_{pFeast} \times Inflow_{Famine} - C_{pOut} \times Outflow_{Famine} = 0$
8. Ammonium mass balance $\frac{dN}{dt} = q_N \times C_x \times V_{Famine} + C_{NFeast} \times Inflow_{Famine} + C_{NFeed} \times Inflow_{Famine} - C_{NOut} \times Outflow_{Famine} = 0$

3.6.2. Side experiments

Online and offline data collected during the accumulation and growth experiments were corrected for effects of sampling, addition of liquids and inorganic carbon dissolution according with Johnson *et al*. (2009b) (Table 7.6 and 7.7 – Appendix B). The data was corrected using Microsoft Excel and carbon and electron balances were determined to assess the accuracy of the measurements.

Jonhson *et al*. (2009b) adapted the reactions 1 till 6 (Table 3.3) from van Aalst-van Leeuwen *et al*. (1997), for the development of a metabolic model for the production of PHA from acetate. All the equations are in carbon-mole base where applicable. The metabolic model was used to evaluate the corrected measurement data and determine kinetic parameters (q_s^{max}, μ^{max}, q_{PHB}^{max}). Balances of the conserved moieties (NADH, ATP, and acetyl-CoA) produced and consumed can be used to calculate the theoretical yields for the production of PHB on acetate, biomass on acetate, biomass on PHB and biomass on PHB and acetate.

Table 3.3. Reactions considered in the metabolic model on a carbon-mole base adapted from Johnson *et al*., 2009b. The efficiency of the oxidative phosphorylation (δ) was considered to be 2.0.

1. Acetate uptake, activation	1 HAc+1 ATP→1 Ac-CoA
2. PHB production	1 Ac-CoA+0.25 NADH→1 PHB
3. PHB consumption	1 PHB+0.25 ATP→1 Ac-CoA+0.25 NADH
4. Catabolism	1 Ac-CoA→2 NADH+1 CO_2
5. Oxid. phosphorylation	1 NADH+0.5 O_2→δATP (with δ=2)
6. Anabolism	1.267 -CoA+0.2 NH_3+2.16 ATP→$CH_{1.8}O_{0.5}$ $N_{0.2}$+0.267 CO_2+0.434 NADH

4. Results and discussion

4.1. Two-reactor CSTR system for culture enrichment

The main goal of this project was to develop and study a continuous process for enrichment of PHA producing bacteria. With that purpose, the 2-reactor CSTR system was operated under two different set of conditions (application of two different resident times in the Feast reactor) resulting in setup 1 and setup 2. For both setups, the carbon source was supplied in the Feast reactor and the ammonium was dosed in the Famine reactor in a way that bacteria were exposed to residual carbon concentrations in the Feast and carbon limiting concentrations in the Famine reactor.

4.1.1. Reactor operation

In a 2-reactor continuous system is essential to maintain both Feast and Famine reactors with a stable volume, with that purpose, all the pumps needed to be adjusted with a high accuracy in a way that a constant flow between the effluent going out, the Feast and Famine reactors was always kept.

Setup 1, with a resident time of 0.5 hours in the Feast reactor and 11.5 hours in the Famine reactor was operated during 29 days. Along the 29 days, the performance of the selected culture, in terms of PHB accumulation and cell growth was monitored. Both reactors were characterised on a daily basis by monitoring acetate and ammonium uptake as well as PHB and biomass production (Figure 4.1 A, A1, B and B1).

Following concentration profiles in Figure 4.1 A, A1, B and B1 it is possible to describe a system with residual carbon and ammonium limitation in the Feast and residual ammonium and carbon limitation in the Famine, in agreement with the desired conditions.

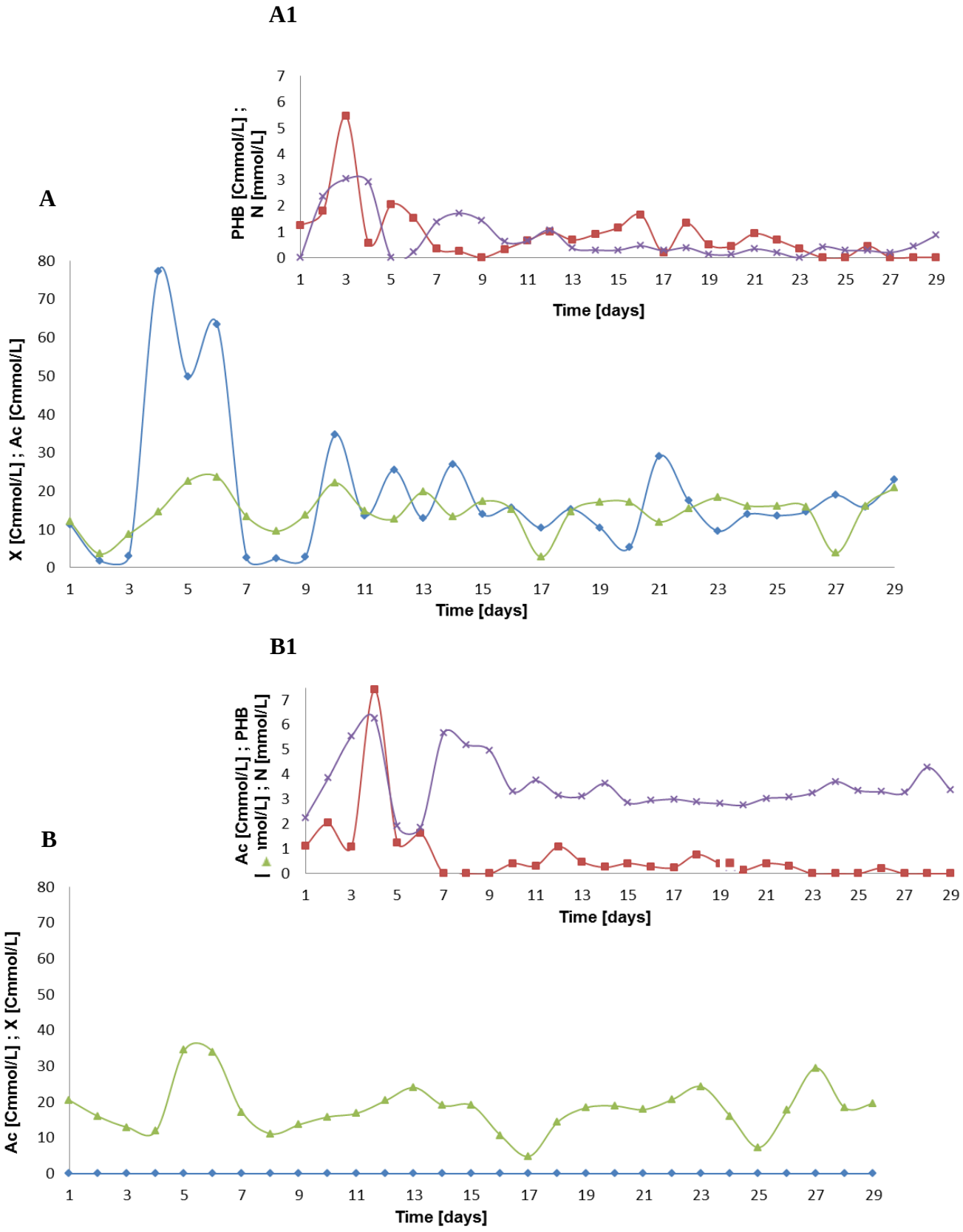


Figure 4.1. 2-reactor CSTR system for culture enrichment (setup 1). (A) and (B) amount of acetate (♦) and amount of active biomass (▲) present in the reactor were represented, (A1) and (B1) amount of ammonium (×) and amount of PHB (■) present in the reactor were represented.

Table 4.1 shows the average performance of PHA-accumulating culture in the 2-reactor CSTR system. After 10 days of operation setup 1 reached a stable state. Since the system was instable during the first 11 days only the last 18 days were considered to determine an accurate performance. As a continuous system, even when running stable, the reactors volume slightly fluctuated from one day to the other. Consequently, the pondered standard deviations associated with the average values determined were high in some cases.

Table 4.1. PHB content, average yields and rates from two-reactor CSTR system (setup 1). These kinetic parameters were calculated using the average values of acetate, biomass, ammonium and PHB consumed/produced (which were determined trough the measurements made along the experiment, Table 7.4 and 7.5 – Appendix B).

		Feast	Standard deviation	**Famine**	Standard deviation
[Cmmol/h]	Ac consumed	21.60	±6.3	16.20	±6.3
	N consumed	2.62	±0.4	0.44	±0.4
	X produced	12.84	±4.8	3.25	±4.8
	PHB produced	0.84	±0.6	-0.28	±0.6
PHB content [wt%]		3.3	±2.2	1.5	±1.5
Yields [Cmol Cmol^{-1}]	$Y_{x/Ac}$	0.60	±0.3	-	-
	$Y_{PHB/Ac}$	0.04	±0.0	-	-
	$Y_{x/PHB+Ac}$	-	-	0.20	±0.6
	$Y_{N/x}$	0.20	±0.1	0.14	±0.2
Rates [Cmol Cmol^{-1}.h^{-1}]	q_S	1.34	±0.4	0.07	±0.0
	q_P	0.05	±0.0	0.00	±0.0
	μ	0.80	±0.3	0.01	±0.0
	q_N	0.16	±0.0	0.01	±0.0

The yield of biomass on acetate, 0.60 Cmol Cmol^{-1}, reached a much higher value then the yield of PHB on acetate, 0.04 Cmol Cmol^{-1}. Although there was ammonium limitation in the Feast reactor, 3.21 mmol.h^{-1} of ammonium was leaching from the Famine reactor. Once reached the Feast reactor, 82% of the ammonium was consumed with an average rate of 0.16 mol Cmol^{-1}.h^{-1}. Thus, besides PHB storage there was also direct growth on acetate.

From the carbon available in the Feast reactor, 40% was consumed and 2% of this consumed carbon was PHB. The average substrate consumption rate was 1.34 Cmol Cmol^{-1}.h^{-1}

with an average growth rate of 0.8 Cmol $Cmol^{-1}.h^{-1}$ and an average PHB production rate of only 0.05 Cmol $Cmol^{-1}.h^{-1}$. The low PHB production rate is in concordance with the PHB concentration profile described in Figure 4.1.A1. Average growth rate and ammonium consumption rate also revealed that most of the substrate consumption resulted in growth. However, ammonium was never completely depleted in the Feast reactor. In the continuous system being studied half of the flow going out from the Feast reactor was discarded trough the effluent (effluent was after the Feast reactor) and the other half was recirculated to the Famine reactor. This way, among the 60% of carbon that went out of the Feast reactor, 30% was discarded trough the effluent and the other 30% leached to the Famine reactor.

In the Famine reactor all the carbon was consumed with an average substrate uptake rate of 0.07 Cmol $Cmol^{-1}.h^{-1}$. With acetate and ammonium available bacteria started first growing on acetate and once it was depleted began consuming the PHB. This is the reason why the PHB content in the cells barely varied between Feast (3.3 wt%) and Famine reactors (1.5 wt%). Most of the carbon consumed was probably used to grow at a growth rate of 0.01 Cmol/Cmol.h^{-1}.

Using enrichment cultures from substrate mixtures like wastewater for PHA production unavoidably leads to the settlement of non-PHA-storing populations aside from the PHA-producing bacteria. The maximum PHB content that can be reached is then reduced and the downstream-processing costs increased (Marang *et al*., 2014). Therefore, after some weeks running the continuous system and achieving a low PHB content it was hypothesized that it could be due to the fact that only 40% of the acetate was consumed in the Feast reactor with the rest (30%) leaching to the Famine reactor. This acetate together with the ammonium available in the Famine reactor was probably giving plenty of time for microorganisms to grow directly on acetate. With the intuit of raising the selective pressure for PHA producers the residence time of 30 minutes in the Feast reactor was extended to 50 minutes. This way, more time would be provided to the bacteria to consume the acetate at qS maximum under ammonium limited conditions and, therefore, less acetate would leach to the Famine reactor. The system operating conditions were then modified in order to increase the Feast residence time to 50 minutes (increase Feast/Famine ratio from 0.04 to 0.07) while keeping the system total retention time of 12 hours and the OLR of 2.25 Cmol.$L^{-1}h^{-1}$.

To start operating under setup 2 conditions only few adjustments on setup 1 were needed, so, a stable state was easily achieved. Figures 4.2 A, A1, B and B1 describe the 2-reactor CSTR system performance along the 79 days of operation as setup 2 showing a quite similar behaviour to setup 1. The desired conditions of residual carbon and ammonium limitation in the Feast and residual ammonium and carbon limitation in the Famine were also achieved.

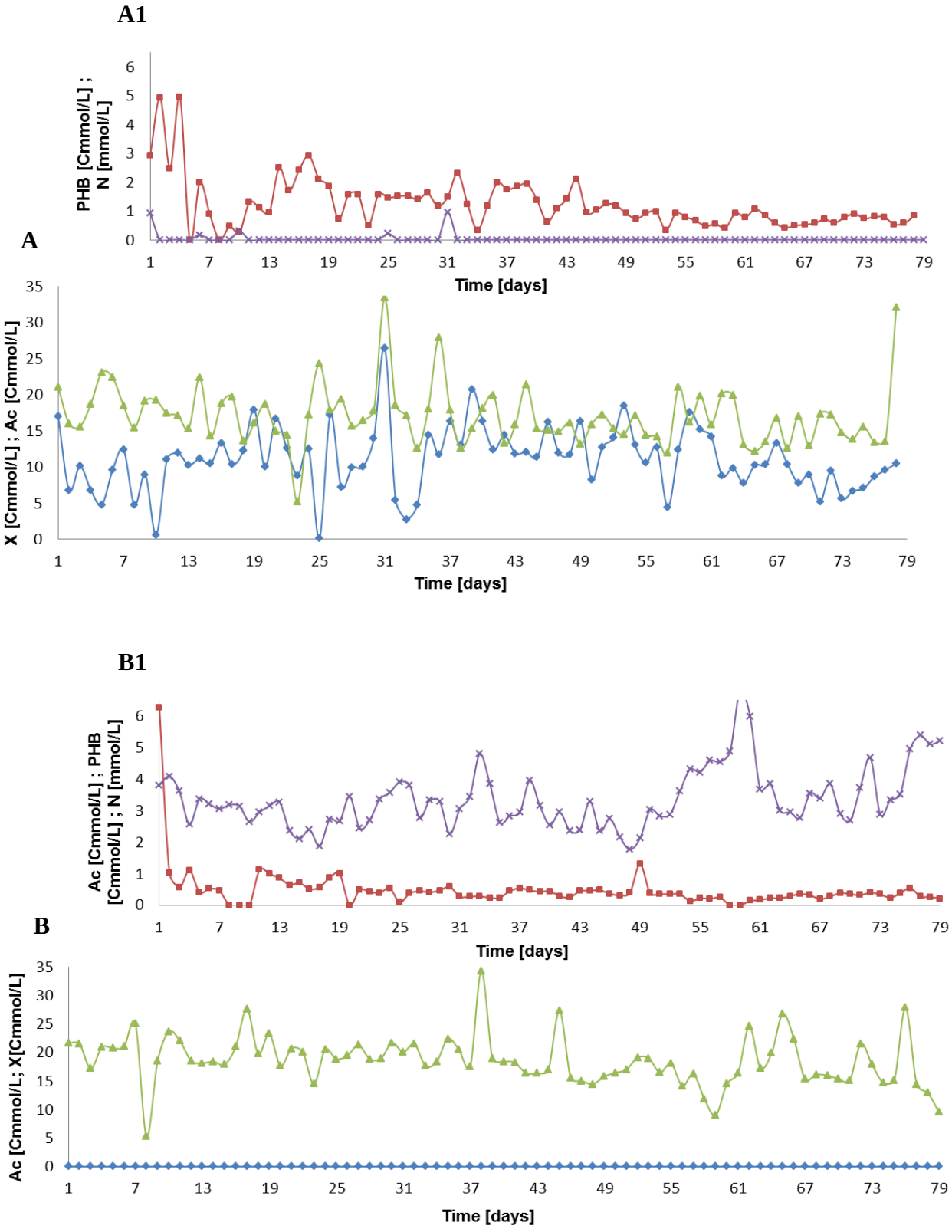


Figure 4.2. 2-reactor CSTR system for culture enrichment (setup 2). (A) and (B) amount of acetate (◆) and amount of active biomass (▲) present in the reactor were represented, (A1) and (B1) amount of ammonium (×) and amount of PHB (■) present in the reactor were represented.

As setup 2 resulted from modifications in setup 1 parameters but remained with the same operation characteristics, the results from setup 2 were analysed together with the ones from setup 1 in order to better understand the impact of the changes in the overall continuous system performance.

Table 4.2 shows the average performance of PHA-accumulating culture in the 2-reactor CSTR system from setup 1 and setup 2. Although the system operated under setup 2 conditions was stable along almost all the operating time, due to some sampling and analysis problems, only the last 42 days were considered to settle an accurate system performance.

Table 4.2. . PHB content, average yields and rates from two-reactor CSTR system (setup 1 and setup 2). These kinetic parameters were calculated using the average values of acetate, biomass, ammonium and PHB consumed/produced (which were determined trough the measurements made along the experiment, Table 7.4 and 7.5 – Appendix B).

		Setup 1				**Setup 2**			
		Feast	Standard deviation	**Famine**	Standard deviation	**Feast**	Standard deviation	**Famine**	Standard deviation
[Cmmol/h]	Ac consumed	21.60	±6.3	16.20	±6.3	27.30	±3.2	10.50	±3.2
	N consumed	2.62	±0.4	0.44	±0.4	2.88	±0.8	0.03	±0.8
	X produced	12.84	±4.8	3.25	±4.8	14.28	±4.4	0.33	±4.4
	PHB produced	0.84	±0.6	-0.28	±0.6	1.38	±0.5	-0.54	±0.5
PHB content [wt%]		3.3	±2.2	1.5	±1.5	4.8	±2.5	1.6	±0.9
Yields [Cmol Cmol^{-1}]	$Y_{x/Ac}$	0.60	±0.3	-	-	0.52	±0.2	-	-
	$Y_{PHB/Ac}$	0.04	±0.0	-	-	0.05	±0.0	-	-
	$Y_{x/PHB+Ac}$	-	-	0.20	±0.6	-	-	0.03	±
	$Y_{N/x}$	0.20	±0.1	0.14	±0.2	0.20	±0.1	0.08	±
Rates [Cmol Cmol^{-1}.h^{-1}]	q_S	1.34	±0.4	0.07	±0.0	1.12	±0.3	0.06	±0.0
	q_P	0.05	±0.0	0.00	±0.0	0.06	±0.0	0.00	±0.0
	μ	0.80	±0.3	0.01	±0.0	0.59	±0.2	0.00	±0.0
	q_N	0.16	±0.0	0.01	±0.0	0.12	±0.0	0.00	±0.0

Analysing the values from table 4.2 it was noticed that the yield of biomass in acetate slightly decreased and the yield of PHB in acetate lightly rose, from setup 1 to setup 2. These differences were not very significant and the average growth rate was still quite higher than the average PHB producing rate. Thus, the PHB content in the cells continued low, rising only from 3.3 wt% (setup 1) to 4.8 wt% (setup 2) in the Feast reactor. Although there was still 2.88 mmol/h of ammonium leaching from the Famine to the Feast reactor, all was then consumed here at a quite similar average rate of 0.12 mol $Cmol^{-1}.h^{-1}$.

The carbon available in the Feast reactor was similar to setup 1 and the consumption heightened to 56%, with 5% of that consumed carbon being PHB. Yet, the average substrate consumption rate decreased, showing that the raise in carbon consumption was mainly due to the higher retention time in the Feast reactor that gave more time for bacteria to consume the substrate. Along with a slight decrement of the average substrate consumption rate and increment of the PHB production rate from setup 1 to setup 2, TSS samples indicated a light increase in active biomass concentrations. There was still 22% of carbon leaching from the Feast to the Famine reactor.

Once in the Famine reactor, all the carbon was consumed at an average rate of 0.06 Cmol $Cmol^{-1}.h^{-1}$. From the PHB accumulated in the Feast reactor 65% was consumed in the Famine reactor, slightly more than what was consumed in setup 1 (50%). The average substrate consumption rate barely varied comparing with the significant reduction from the yield of biomass in acetate and PHB. The average growth rate and ammonium consumption rate had an accentuated diminution, as well, being the ammonium consumption close to 0%.This could suggest that while the substrate kept being consumed with the same low speed there was almost no growth or ammonium consumption and, therefore, the substrate consumed was mostly used to be convert into CO_2.

The very low ammonium consumption resulted in a high amount of ammonium being available in the Famine reactor. With the amount of acetate also present it would be expected that bacteria would consume the acetate, the PHB and grow. The reason why the ammonium consumption was so low could be justified by the presence of protozoa that decomposed the organic compounds from grazed bacteria releasing nitrogen as ammonium. It is known that predator-prey relationships between protozoa and bacteria are expected in activated sludge due to the permanent presence of protozoa in this habitat (Güde *et al*., 1979). Grazing can stimulate mineralization of nutrients, diminish bacterial numbers and probably affect the structures of the bacterial communities. There are several studies demonstrating that grazing by protozoa is an important factor in shaping the morphological and taxonomical compositions of bacterioplankton communities in activated sludge (Rønn *et al*., 2002). This way, either bacteria were using the substrate only for maintenance or they did actually grow but were being eaten at a similar rate.

It is known that PHB-storing bacteria are able to outcompete non-storing microorganisms due to their fast substrate uptake rate. If a high amount of substrate is available for a short period of time then the microorganisms capable to store it as PHB and use the stored PHB in the Famine phase to grow have a competitive advantage over the microorganisms just able to grow (Dionisi *et al*., 2005). With the low Feast/Famine ratio (0.04 in setup 1 and 0.07 in setup 2) it would be expected that the storing bacteria would be capable to overcome the non-storing ones. Raising slightly the F/F ratio, in setup 2, while still keeping it at a low value allowed to decrease the average growth rate from 0.8 Cmol $Cmol^{-1}.h^{-1}$ to 0.59 Cmol $Cmol^{-1}.h^{-1}$, which still was not low enough for storing populations to outcompete the non-storing ones. The ammonium consumption augmented from 82% (setup 1) to 100% (setup 2) in the Feast reactor. Aiming to understand this total consumption of ammonium, biomass samples were taken from the system and analysed using a Total Nitrogen test to assess the ratio of nitrogen to carbon in the medium. An average nitrogen biomass content of 17% was found for both Feast and Famine reactors. This balance lies within the expected range (14 to 26%, Nagata, 1986). Thus, there was a total exhaustion of the ammonium available in the Feast reactor, a condition that was not predicted for this continuous system, which might have led to a negative impact in the system behaviour.

Residual substrate concentration in both Feast and Famine reactors have been proven to influence the selective pressure for PHB storage along with the length of Feast and Famine phases, as demonstrated in Albuquerque *et al*., 2010. Yet, in this study, the ammonium concentration in Feast reactor also revealed to possible play an important role in the system performance. Once the culture was submitted to a slightly higher period to consume the substrate during the Feast phase and the residual substrate decreased (from 16.2 Cmmol/L in setup 1 to 11.7 Cmmol/L in setup 2) a change in ammonium consumption and growth was noticed. Although the growth rate decreased significantly, the ammonium was completely depleted, which led to an ammonium exhaustion in the Feast reactor. Accordingly with the initial conditions settled for the continuous system it was suppose to be imposed an ammonium limitation in the Feast reactor, however, ammonium ended up being completely consumed. Wen *et al*., (2010) investigated the effects of nitrogen limitation on PHA production by activated sludge biomass with acetate as carbon source in a SBR system and it was shown that indeed high percentage of PHA (59%) was accumulated under nitrogen limitation. Still, the influence of the complete ammonium exhaustion in a continuous system performance has not been analysed yet. So far, it is only possible to interrogate if this total depletion might or might not influence the culture's behaviour, hereupon, it is a question that deserves further investigation.

The 2-stage CSTR system for selection of PHA-accumulating bacteria with enrichment cultures is not a quite well studied system, nevertheless, some studies have been already

performed with different feed stocks and operating conditions. Bengtsson *et al*. (2008) achieved a PHA content of 48 wt% by using fermented paper mill wastewater. Albuquerque *et al*. (2010), using sugar molasses, obtained 32 wt% of PHA content with a residual carbon concentration of 6 Cmmol VFA/L in the Feast reactor, and 61 wt% maximum PHA content in an accumulation batch experience (no saturation was reached). Multiple-stage systems were also studied, like the five-stage system studied by Atlic *et al*. (2011) where a 77 wt% of PHA content was reached. Although relevant parameters like the OLR and F/F ratio were different among the several studies it would be expected that a higher PHA content would be achieved in this study. Comparing also with SBR systems, which are the more commonly used for selection of PHA-accumulating bacteria, quite lower contents were achieved. An example of an effective study was Jiang *et al*. (2011), that with mainly the same operational conditions as the continuous system in study, at 30ºC with a 12h cycle, 1 day SRT and inoculated with a microbial community highly enriched in *P. acidivorans*, a 70 wt% PHA content would be expected. With the 2-stage CSTR studied a much lower content of 4.8 wt% average PHA content in the Feast reactor with a residual substrate concentration of 11.7 Cmmol/L was achieved.

The two different reactor strategies utilized, while with the same operational parameters gave distinct results (this work, Jiang *et al* 2011). In order to better study the system behaviour and the selected culture some kinetic assays to investigate the real PHB-accumulating capacity and growth propensity of the microbial community were performed.

4.1.2. Kinetics studies

In order to assess the PHB accumulation performance of the culture, a fed-batch PHA production assay under growth limiting conditions (ammonium limitation) and using a pH controlled acetic acid feed was conducted.

This fed-batch experiment was performed using biomass collected from the Feast reactor from the continuous system (setup 2) after 64 days of operation. With an initial sodium acetate (Ac) concentration of 272.5 Cmmol/L the selected culture PHB storage capacity and production rate were characterised during 7 hours (~400 min) of experiment (Figure 4.3).

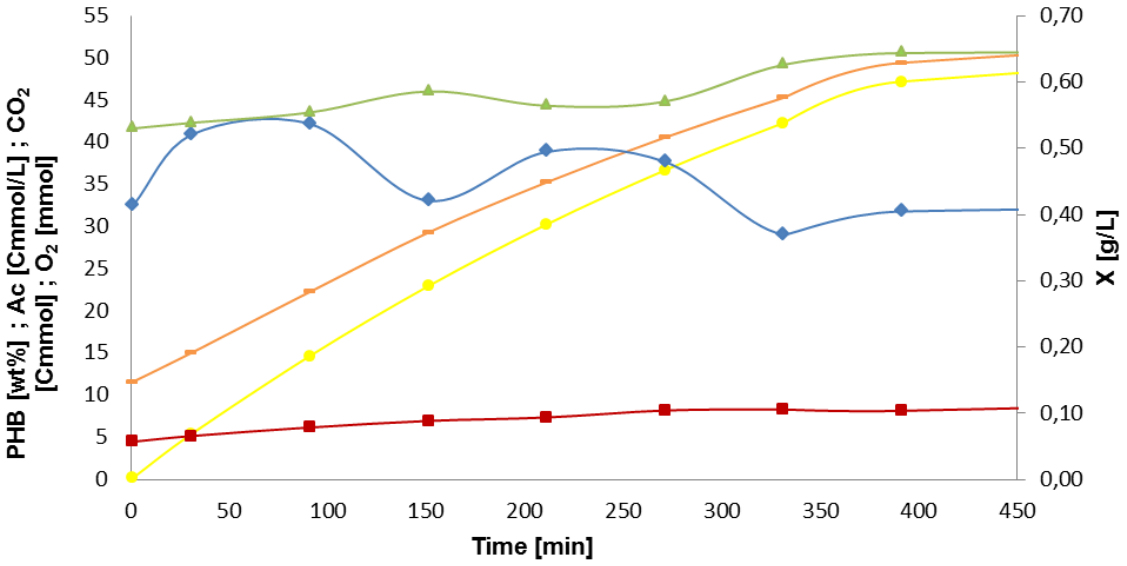


Figure 4.3. Fed-batch experiment with microbial enrichment community from the CSTR system. Evolution of amount of acetate taken up (Ac)(◆), weight percentage of PHB in TSS (■), amount of active biomass (TSS excluding PHB) present in the reactor (X) (▲), cumulative carbon dioxide (–) and cumulative oxygen uptake (●) was followed along the experiment.

Table 4.3 shows the kinetic performance of the accumulation experiment and also some of the relevant values from the continuous system setup 2 in order to better compare and analyse the culture behaviour.

Table 4.3. PHB content, average yields and rates from the accumulation experiment and the two-reactor CSTR system setup 2.

Yields [Cmol/Cmol]/ **Rates** [Cmol $Cmol^{-1}.h^{-1}$]	**Accumulation experiment**	**Setup 2**
$Y_{PHB/Ac}$	0.06	0.05
q_S	0.11	1.12
q_P	0.05	0.06
PHB content [wt%]	8.1	4.8

The PHB production rate (0.05 Cmol $Cmol^{-1}.h^{-1}$) was initially quite similar with the average PHB production rate determined in the continuous system (0.06 Cmol $Cmol^{-1}.h^{-1}$). However, it significantly decreased along the experiment till it stopped accumulating after proximally 270 min (~ 4.5 h) of experiment. The overall yield of PHB on acetate (Ac + HAc) hit the same value as the one measured in the continuous system (0.06 Cmol $Cmol^{-1}$). The overall substrate uptake rate (0.11 Cmol $Cmol^{-1}.h^{-1}$) was a lower value compared with the one determine in the continuous system (1.12 Cmol $Cmol^{-1}.h^{-1}$). A 4.5% PHB content was achieved only after 1 minute of experiment which was really close to the 4.8% average PHB content found in the continuous system setup 2 as it would be expected. The evolution of PHB accumulation was quite insignificant and a maximum modest value of 8.1 % stored PHB was achieved.

This batch experiment allowed to build the possibility that the continuous system operational conditions did not afforded to efficiently select for PHB storing organisms. Consequently, aiming to keep gaining a better understanding of the mechanisms governing the efficiency of the culture selection stage performed in the continuous system other experiments and analysis were made. Thus, the next step was to evaluate the growth rate of the selected culture in presence of ammonium by performing a growth experiment.

The growth experiment was performed using biomass collected from the Feast reactor of the continuous system after 67 days of operation. And it began by giving a pulse of acetate (4 M) and ammonium (0.8 M). Once the acetate and ammonium were almost depleted, after approximately 3 hours, the experiment was stopped. Using the values measured along the 3 hours, the growth experiment was modelled (Table 7.6 and 7.7 – Appendix B) in order to predict the maximum growth specific rate and maximum substrate uptake rate. The culture behaviour as well as the performance resulted from the modelling are represented in Figure 4.4.

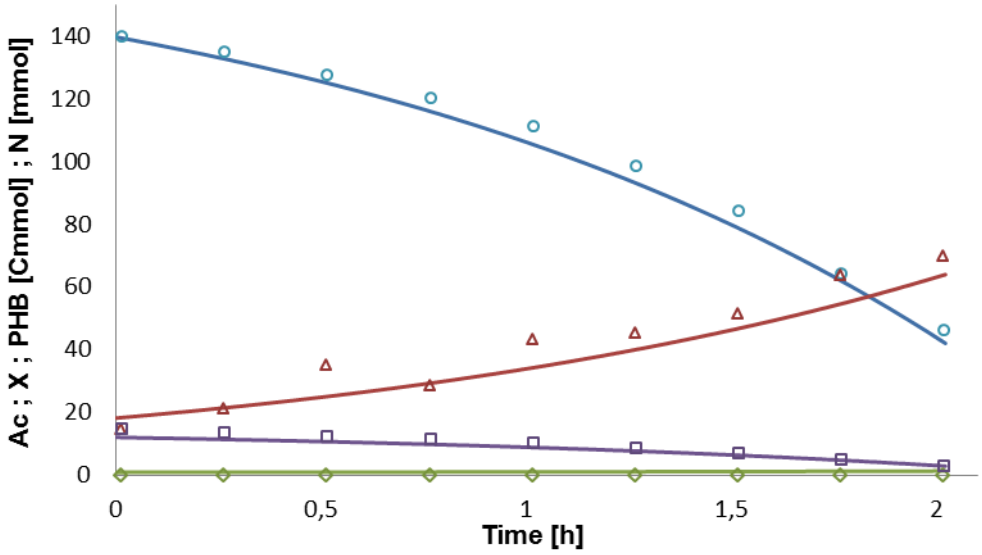


Figure 4.4. Growth experiment with microbial enrichment community from the CSTR system. Evolution of acetate (○), biomass (△), ammonium (□) and PHB (◇) was followed along the experiment. The modelled values are represented by the lines (same colours).

Table 4.4 exhibits the kinetic performance of the growth experiment (measured and modelled) as well as the rates from setup 2 that help the interpretation of the results obtained.

Table 4.4. Rates from the growth experiment, the modelling of the growth experiment and setup2.

Rates [Cmol Cmol^{-1}.h^{-1}]	**Growth Experiment**	**Growth Experiment (modelled)**	**Setup 2**
q_S	1.15	1.10	1.12
μ	0.53	0.50	0.59

The values of maximum substrate uptake rate and maximum specific growth rate predicted by the model (1.10 Cmol/Cmol.h^{-1} and 0.50 Cmol/Cmol.h^{-1}, respectively) were very alike with the ones calculated trough the measurements made along the experiment (1.15 Cmol/Cmol.h^{-1} and 0.53 Cmol/Cmol.h^{-1}). The former results were also quite similar with the ones obtained for the continuous system (1.12 Cmol/Cmol.h^{-1} and 0.59 Cmol/Cmol.h^{-1}), which showed that the microbial community selected was actually able to consume the substrate and grow quite fast.

Putting together the results obtained from both kinetic essays a more supported assumption could be made about the continuous system performance. Although the selected

culture was capable to consume the substrate available and grow it was not able to store high PHB-contents. Hence, at this point of the investigation, a closer look into the community microbial diversity could help to better analyse and understand the overall results obtained so far.

4.1.3. Microbial community analysis

The microbial community populating the continuous system was analysed under the microscope by taking biomass samples from Feast and Famine reactors as shown in Figure 4.5. These essays under the microscope were made frequently along the entire experiment (setup 1 and 2) and the pictures presented here are the ones that better represented the overall taken.

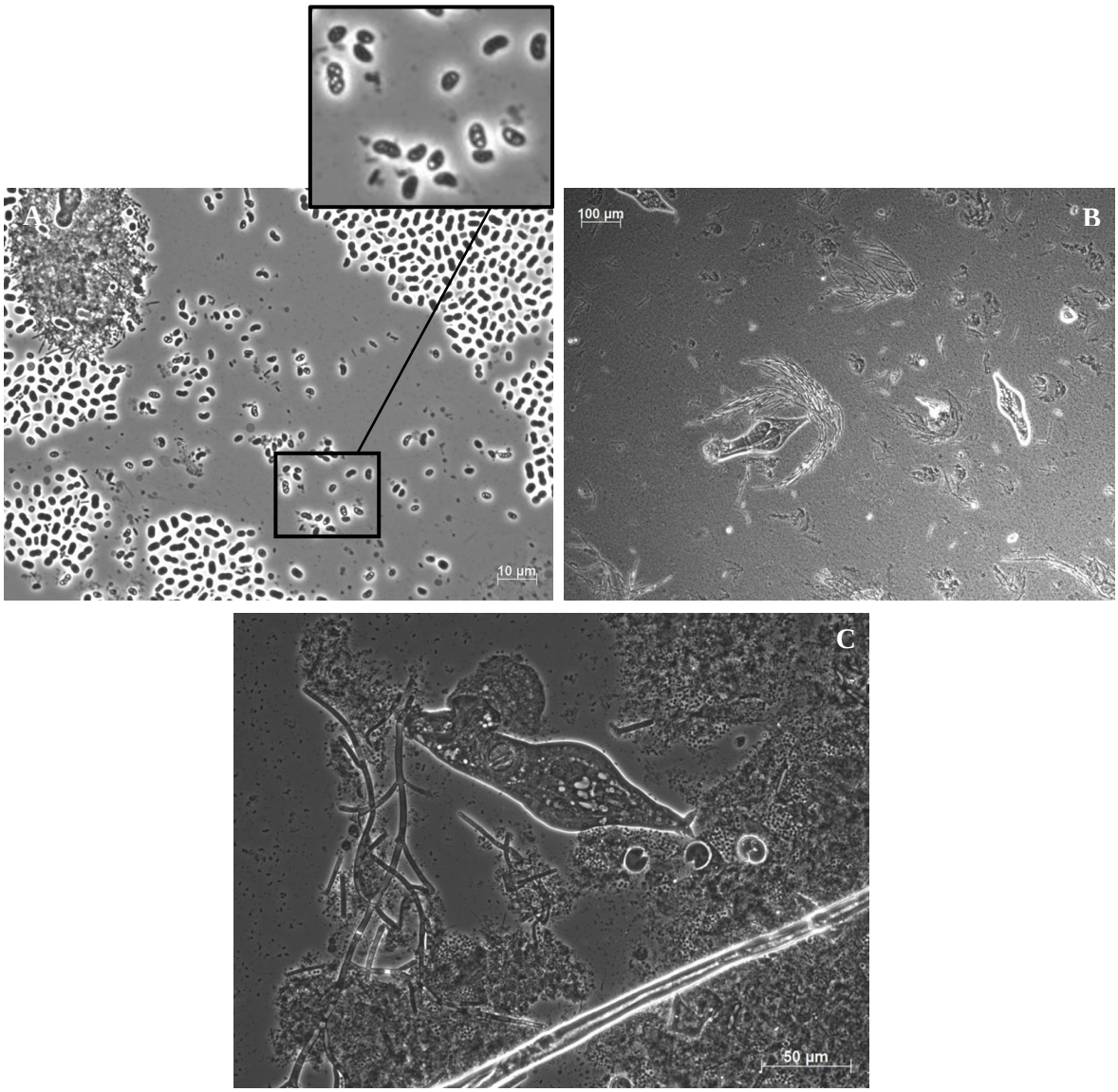


Figure 4.5. Diversity of the microbial enrichment culture in setup 2 of the CSTR system, at the last day of operation. (A) Single cells with very low PHB content present in the Feast reactor (magnification 100x) (B) Presence of protozoa in the Feast reactor (magnification 10x) (C) Bacteria and protozoa present in the Famine reactor (magnification 40x).

From Figure 4.5 C it is possible to verify the presence of protozoa and rotifers in the Famine reactor. Figures 4.5 A and B describe the Feast reactor community which is also composed by protozoa. The bacteria present had a quite low PHB content as demonstrated by the few inclusion bodies found (Figure 4.5 A) which is in concordance with the results presented previously. Thus, along the several months of operation, the system microbial community was highly constituted by protozoa. The microbial community populating the reactor of the fed-batch experiment was also analysed under the microscope by taking a biomass sample after 7 hours running. It was observed small populations with some inclusion bodies similar to Figure 4.5 B, yet not in a big quantity. There were plentiful of filaments present as well as quite a lot of protozoa. These protozoa were not very active.

So far, some hypotheses could be disposed about the 2-reactor continuous system studied. It was a possibility that the system was not able to efficiently select for PHA-accumulating organisms and that protozoa could be one of the kind of microorganisms populating it.

Seeking for a clear understanding of the system conduct, the microbial community from the continuous system was also examined by performing Denaturing Gradient Gel Electrophoresis (DGGE) analysis and Fluorescent in situ hybridization (FISH).

A biomass sample taken from the Famine reactor (setup 2) after 66 days of operation allowed the analysis of the selected culture by DGGE showing the presence of tree main bacterial species (Table 4.5 and Figure 4.6): *Acinetobacter* sp. (Gammaproteobacteria) from a sequence with 98% similarity, *Acidovorax* sp. (Betaproteobacteria) with 99% similarity and two sequences with 95-96% and 96-97% of similarity with *Rhodobacteraceae/Paracoccus* (Alphaproteobacterium). In taxonomy the γ-Proteobacteria *Acinetobacter* belongs to the Pseudomonadales Order and Moraxellaceae Family, the β-Proteobacterium *Acidovorax* pertains to the Burkholderiales Order and Comamonadaceae Family and the α-Proteobacteria Rhodobacteraceae is included in the Rhodobacterales Order and Rhodobacteraceae Family. Both *Rhodobacteraceae* and *Acinetobacter* are included in the bacteria that are able to produce PHA.

Table 4.5. Hits/similarity with database after BLAST of sequences from the DGGE gel band excised.

Band	Sequence	Similarity
1	M_SEQ_Bac_E10_BAC907rM (cut at bp 65-543)	-
2	M_SEQ_Bac_F10_BAC907rM	99% identical to *Acidovorax sp.* (Betaproteobacteria)
3	M_SEQ_Bac_G10_BAC907rM (cut at bp 88-544)	Rhodobacte/Paracoccus (Alphaproteobacteria) (94% query, 95-96% identical)
4	M_SEQ_Bac_A10_BAC907rM	Artificial *Acinetobacter sp.*
5	M_SEQ_Bac_B10_BAC907rM (cut at bp 38-548)	98% identical to *Acinetobacter sp.* (Gammaproteobacteria)
6	M_SEQ_Bac_C10_BAC907rM	Rhodobacter/Paracoccus (100% query, 96-97% identical)

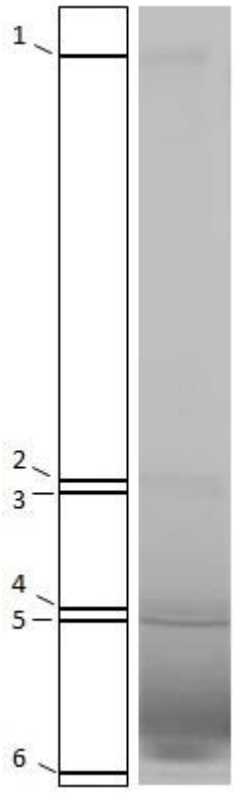


Figure 4.6. DGGE gel of PCR-amplified 16S rRNA gene fragments from the enrichment culture of the continuous system (setup 2). The band was excised and re-amplified for microbial identification.

With the aim to check the relative abundance of these different kinds of Bacteria and better comprehend the population dynamics, FISH was performed (Figure 4.7). For FISH analysis, one sample from the Feast and one from the Famine reactor were taken after 75 days of system operation.

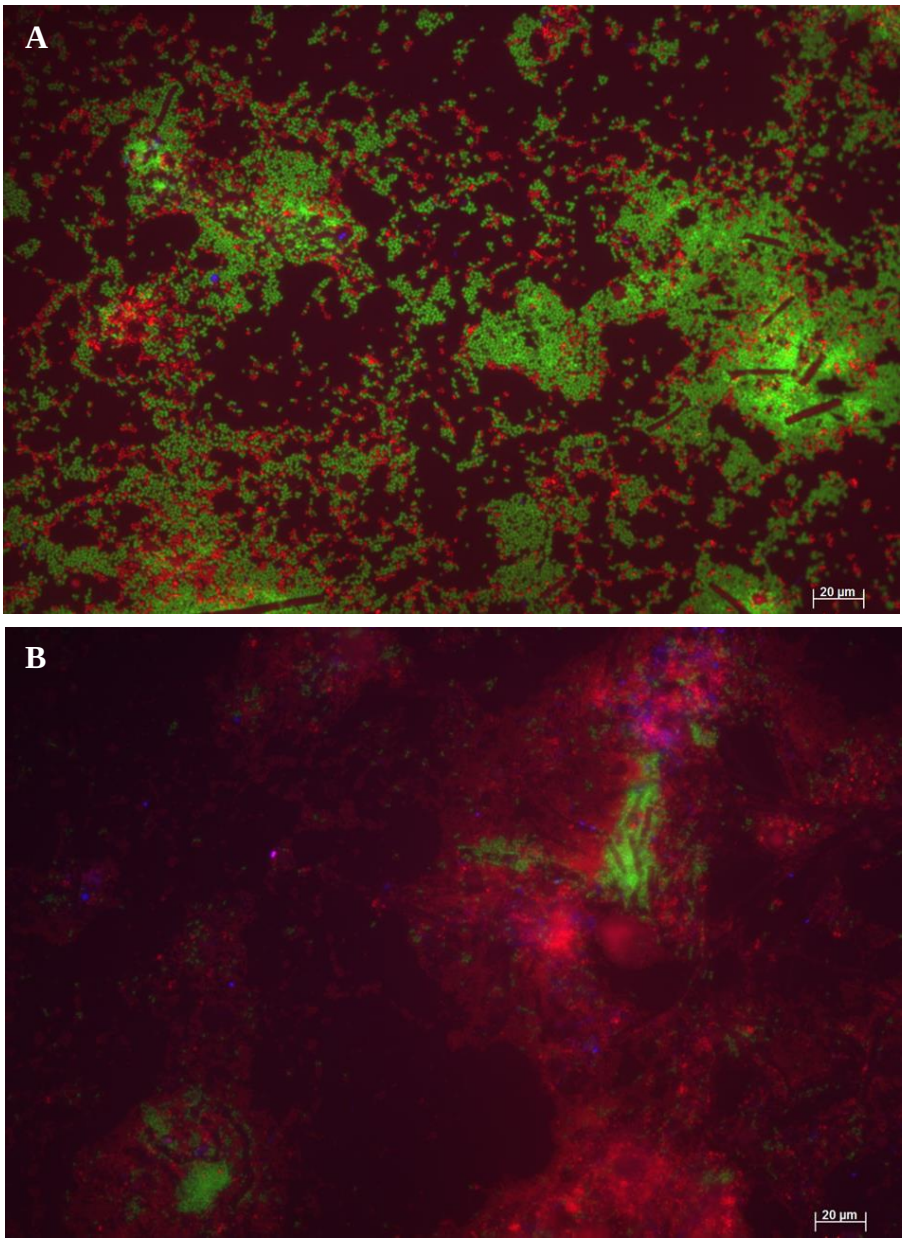


Figure 4.7. Fish images from CSTR system using the probes: "α mix" (Cy3) and "β/γ" (Cy5 and FLUOS respectively) being α-proteobacteria stained red, β-proteobacteria blue and γ-proteobacteria green. (**A**) Feast reactor (magnification 40x). (B) Famine reactor (magnification 40x).

From Figure 4.7 A it is visible that Feast reactor´s microbial community was predominated by γ-Proteobacteria (green) and just very few α-Proteobacteria (red). On the other hand, it showed that most of the microbial community in Famine reactor was dominated by α-Proteobacteria (red). This quite significant change between Feast and Famine reactors in the relative abundance of the two different Proteobacteria wasn't expected since in the continuous system the biomass was permanently recirculated between both reactors. Due to this ambiguous result another FISH analysis was performed. Both analyses made, with samples taken after 75 and 79 days of system operation, showed the same result: a clear distinction between the two Proteobacteria classes abundance in Feast and Famine reactors.

By the microbial community analysis and characterization (DGGE and FISH analysis) it was shown that the Feast reactor's microbial community was dominated by γ-Proteobacteria in contrast with the Famine reactor, where most of the microbial community was dominated by α-Proteobacteria. This accentuated change between the bacteria populating in the two reactors could be a possible justification for the results showed earlier concerning the almost absent ammonium consumption in the Famine reactor. If the bacteria were simply being eaten and giving place for new species to grow, at the same rate then the ammonium consumption would be quite insignificant. Yet, the reason why such a significant microbial change was happening in the selected culture and the dominant *P. acidivorans* in the inoculum was washed out of the system remains unknown. For this particular question, before any other kind of experiment, it would be proposed to perform a new DGGE analysis with the samples previously used in order to corroborate the results obtained. And then perform a closer analysis into the different species found in the system.

At this point, both kinetic studies and microbial community analysis made were not enough to fully understand the behaviour of this continuous system. With the intention of finding more possible explanations or hypothesis instead of keep looking further into the microbial community itself, a different kind of experiment was made. As it was previously discussed, it is common in this kind of 2-reactor CSTR system that an amount of substrate leaches from the Feast to the Famine reactor. In order to further explore how the acetate leaching to the Famine reactor could influence the continuous system performance a Sequencing Batch Reactor experiment reproducing the continuous system was conduct.

4.2. Sequencing Batch Reactor Experiment

An SBR setup was built mimicking the 2-reactor continuous system by applying the same operational conditions, same carbon and nutrients concentration with a 12 hours cycle and 1 day SRT. Passing 7 days from the inoculation, once the typical Feast-Famine profile of dissolved oxygen (DO) was established an extra acetate dosage started being dosed. Besides the amount of 54 Cmmol of acetate fed at the beginning of each 12h cycle (same feeding as the continuous system) an extra acetate dosage, corresponding to 1/3 of initial amount dosed (18 Cmmol), was continuously dosed along the cycle (at 0.07 mL/min for 695 min). This third of acetate concentration roughly corresponded to the amount reaching the Famine reactor in the continuous system. Figure 4.8 describes a Feast/Famine cycle from the SBR system while operating with the extra acetate dosage.

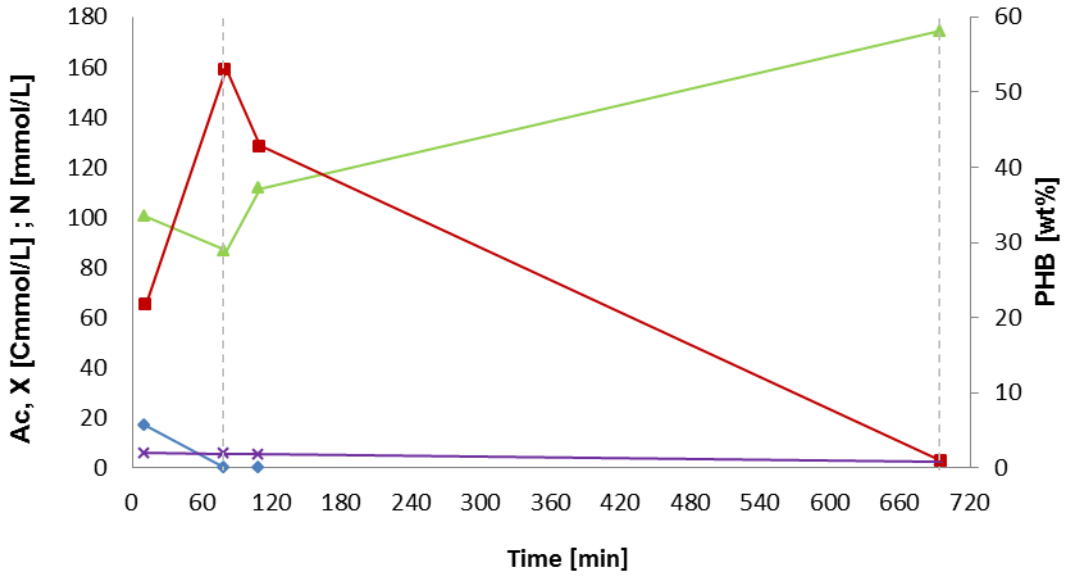


Figure 4.8. Feast/Famine cycle of the SBR experiment during which it was operated with an extra acetate dosage (6th day after beginning the acetate dosage). The amount of acetate present in the reactor (◆), amount of active biomass (TSS excl. PHB) (▲), amount of ammonium (×) and the weight percentage of PHB in TSS (■) were represented.

From Figure 4.8 it was possible to find that the Feast phase length was around 80 minutes which comparing with the Feast resident time (50 min) of the continuous system it was a little bit longer. Thus, after 6 days with extra acetate dosage the F/F ratio raised from 0.07 (typical continuous system F/F ratio) to aproximally 0.13.

DO evolution was followed along the entire experiment including the first 7 days without the extra acetate dosage in order to assure that stable cycles were achieved as well as to keep track of the Feast and Famine lengths. The PHB content and biomass at the end of each

Feast and Famine phases was also monitored along the entire experiment. Figure 4.9 shows the biomass and maximum PHB-content at the end of the Feast phases along the total 15 days of experiment and the F/F ratio evolution along the 9 days with the extra acetate dosage.

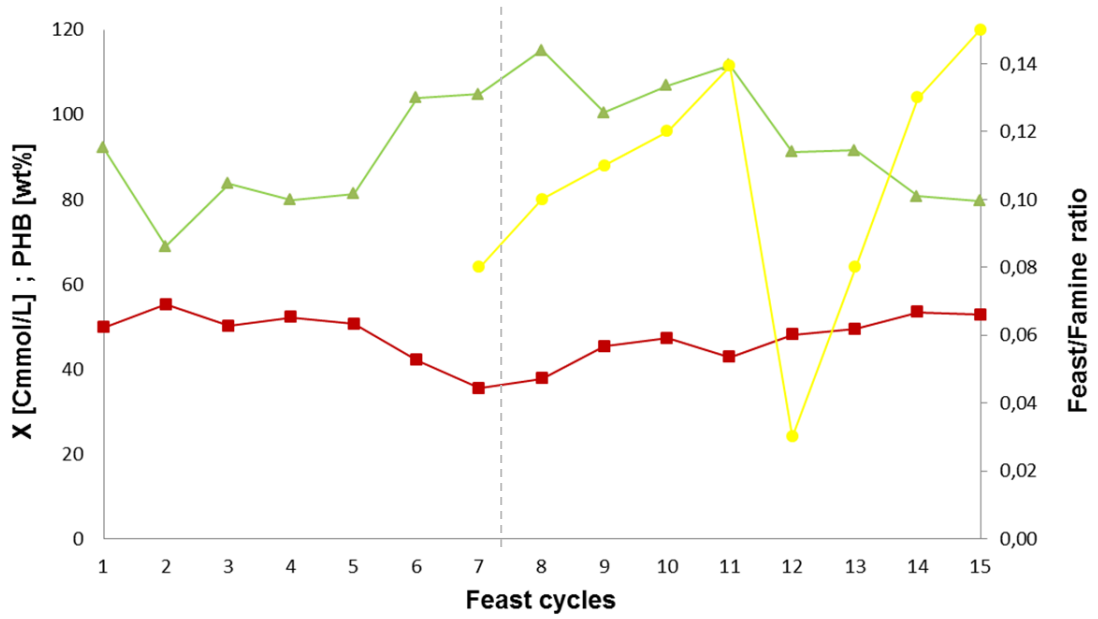


Figure 4.9. Active biomass (TSS excluding PHB) (▲) and PHB content (wt%) (■) at the end of each Feast cycle during the total 15 days of experiment were represented. Feast/Famine ratio (●) during the 9 days while the SBR experiment was operated with an extra acetate dosage was followed.

From Figure 4.9 it was noticed that after the extra acetate started being dosed the F/F ratio was slightly augmenting, fact that goes in accordance with the SBR cycle profile described in Figure 4.8.

During the first 7 days while no extra acetate was being given to the SBR, the average PHB content at the end of Feast and Famine phases was, 52% and 2%, respectively. After adding the continuous acetate dosage the average PHB content at the end of Feast and Famine phases just lightly decreased, being 45% and 1%, correspondingly. Yet, Figure 4.9 shows that along the days with the extra acetate dosage the maximum PHB accumulated at the end of the Feast phase lightly increased evidencing that the PHB-accumulating capacity of the culture barely varied due to the extra acetate dosage.

The sharp decrement of the F/F ratio at the 12th feast cycle (6 days after starting the acetate dosage) resulted from an unexpected problem in the pump administrating the extra acetate dosage to the system which stopped working. Once the problem was detected and the extra acetate dosage started being dosed again the F/F ratio quickly returned to its previous values. However, the PHB content remained unaffected by the extra acetate shutdown. This

accident just helped reinforce the idea that the influence of the extra acetate in the PHB-storage capacity of the community wasn't very significant.

The microbial population was also analysed under the microscope upon 10 days of operation under acetate dosage. Some filaments were present resembling the ones detected in the continuous system, although in a lower amount, and the selected culture was still dominated by *P. acidivorans* with a high PHB-content (Figure 4.10). Thus, the presence of acetate during the Famine phase did not seem to have a significant impact in the PHB-accumulating capacity of the culture.

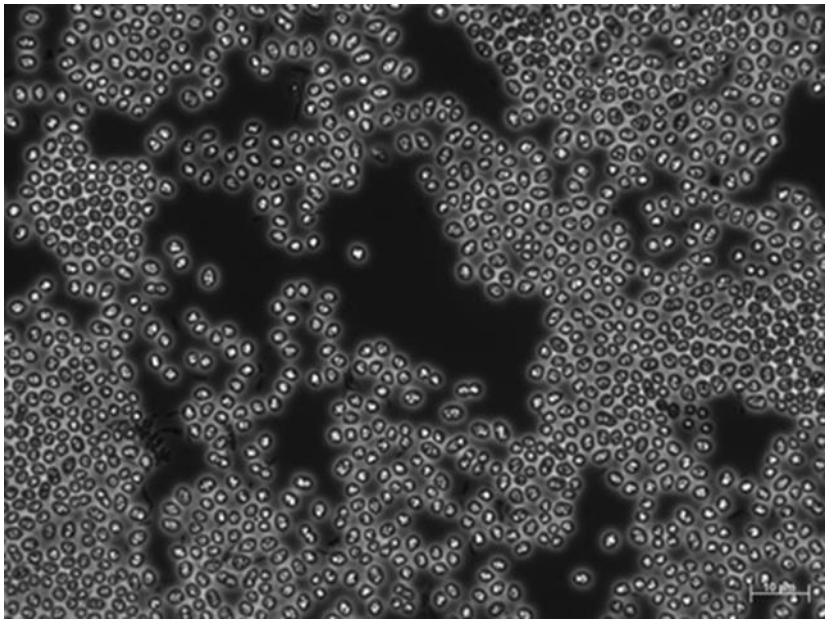


Figure 4.10. Cells with high PHB content after one week operation of the SBR with continuous acetate dosage. Phase contrast was used to obtain the picture.

The performance of SBR system studied could then be connected with the analysis of the continuous system behaviour. Substrate uptake rate in the Famine reactor was quite low in setup 1 (0.07 Cmol $Cmol^{-1}.h^{-1}$) and setup 2 (0.06 Cmol $Cmol^{-1}.h^{-1}$), around 20 times smaller than the substrate uptake rate in the Feast reactor (1.34 Cmol $Cmol^{-1}.h^{-1}$and 1.12 Cmol $Cmol^{-1}.h^{-1}$ in setup 1 and setup 2, respectively). Other studies (Beccari *et al*., 1998) showed that in Feast and Famine processes cultures adapt to long starvation periods (high Famine/HRT) by decreasing their primary metabolism resulting in an internal growth limitation in the next Feast phase inducing an enhanced PHB storage answer. Accordingly, if the Famine substrate uptake rate is at a low value, even with a significant substrate presence, the physiologic adaptation allowing enhancing the PHB-storage capacity once cells are recirculated to the Feast can still occur. It was already hypothesised by Albuquerque *et al*. (2010) that the Famine phase should be related to an amount of substrate that only let the culture grow till a certain rate (growth limitation)

rather than a complete absence of substrate. The SBR setup built in this study allowed assessing that if proximally 1/3 of the substrate fed in the Feast phase (18 Cmmol) is present during the Famine phase it does not affect significantly the culture PHB-storage ability, yet, it revealed to have an impact in the Feast phase length. As explained above, short periods of time in the presence of substrate enhance the selective pressure for storing bacteria, but what happened in this SBR system was an increment of the Feast periods. Therefore, it could be presumed that the presence of an extra dosage of acetate during the Famine phase was lowering the selective pressure. The same way, the acetate leaching to the Famine reactor in the continuous system could be a reason for the low system efficiency. However, the fact that the selected culture kept being capable of storing high PHB-content while the extra acetate was being dosed goes against the previous hypothesis. So, more studies concerning till which point residual substrate reaching to the Famine reactor compromises or not the culture performance should be performed. And if so, the maximum residual substrate allowed reaching the Famine reactor should also be assessed .Once known this limit it would be possible to increase the substrate concentration in the system till a maximum substrate uptake rate is reached which would probably result in a raised selective pressure for PHB storage.

Linking the SBR system behaviour with the side experiments and the microbial community analysis from the continuous system also allowed furthering hypothesise about the selected culture. With the analysis made to the microbial community protozoa was shown as one of the possible microorganisms populating the continuous system. In the accumulation batch experiment performed the Protozoa present were barely active. This lack of activity might have been due to the fact that the experiment was performed in a SBR system instead of in a CSTR system. Ni *et al*. (2010) developed a model to predict the impact of predators on biomass in these two kinds of systems according to which the predators have a stronger effect in the continuous system then in the SBR system. SBR systems exhibit two clearly defined phases, in contrast with continuous systems, that have almost permanent near-Famine conditions due to the low residual substrate concentration. This nearly permanent Famine conditions favours predators increasing the predator biomass fraction which has impact in the OUR (oxygen uptake rate) and active biomass distribution (active bacteria and extracellular polymeric substances). Therefore, Ni *et al*. (2010) model hypothesis agrees with the CSTR and SBR system behaviours analysed in the present study.

A key difference between SBR and CSTR for PHB-enrichment processes is the less rigid division between Feast and Famine conditions in the CSTR. At the beginning of a SBR cycle the culture is submitted to a high substrate concentration which then decreases over time, contrary to CSTR, where a constant substrate concentration is available in the Feast reactor. Knowing that substrate concentration influences the substrate uptake rate and consequently

PHB production rate, in the 2-stage CSTR, the substrate concentration is constant leading to an also constant substrate uptake rate. Whereas, in the SBR system, as the substrate is being depleted towards the end of Feast phase the substrate uptake rate is also decreasing till the culture is under substrate limiting conditions. On one hand, having a residual substrate concentration can be an advantage for the selective pressure since a faster substrate uptake rate of storing bacteria will let them out-compete the non-storing microorganisms. On the other hand, lacking the strict separation between Feast and Famine phases has not been fully clarified as a drawback or not in the 2-stage CSTR system.

5.Final considerations and recommendations

The 2-stage continuous system built allowed the settlement of specific Feast and Famine conditions in two different reactors. Even though a stable culture performance was reached accomplishing to cycle PHB between the Feast and Famine reactors the percentage of PHB stored was quite modest, accumulating an average of 4.8% in the Feast and 1.6% in the Famine reactor. The microbial community selected trough the 2-stage continuous system was not able to efficiently store a high PHB content, although, it proved capable of consuming the substrate and grow.

Multiple-stage CSTR systems have proven before to be a potential step forward in the valorisation of waste materials by PHA production. This was the reason why several assays were made in order to clear up the behaviour of the culture selected under the system studied. Two major final considerations to which some recommendations for further research can be presented.

1. The not fully exhaustion of the ammonium leaching into the Feast reactor has not been reported as a significant parameter in the continuous system performance, but it showed in this study that it might have an impact in the culture's behaviour. Therefore, a closer look into the impact of the ammonium concentration in the Feast reactor should be investigated. With that concern, it is proposed to operate the continuous system build up in this study (keeping all the same operating conditions) but feeding the ammonium in the Feast reactor instead of in the Famine reactor in order to assess how this change may or may not influence the culture selection in the continuous process.

2. During the continuous system operation a certain amount of substrate leaching to the Famine reactor was detected but the respective impact in the system behaviour was not clearly understood. The knowledge of the maximum residual substrate concentration in the Famine reactor that will not affect the PHA-storage capacity of the culture seems to be a key point to improve the 2-stage CSTR system viability. In this study it was shown that 1/3 of the substrate amount dosed in the Feast phase reactor reaches to the Famine phase reactor. When this same situation was applied for the SBR operated mode the PHA-accumulation capacity of the microbial population was not affected. Thereafter, it is suggested to perform a SBR experiment with all the same operating conditions and only raising the extra substrate dosage to 1/2 of the substrate amount fed at the beginning of each cycle. Also different concentrations of acetate leaking to the Famine reactor (lower) should be tested in the continuous system. It would be

interesting as well to analyse the impact of the substrate leaching to the Famine reactor by setting up a 3-reactor CSTR system. The substrate would be dosed in the first reactor, the total depletion of the substrate would be ensured in the second reactor and the third reactor would be the Famine reactor, where no residual subtract would be present.

6.References

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7.Appendices

Appendix A

Table 7.1. PCR.cycling conditions for PCR-DGGE assay.

Primer combination	Specificity	PCR program	Reference
341F-GC/907R	Bacteria	5 min at 94ºC, followed by 20 cycles of 1 min at 94ºC, 1 min at 65º ..55ºC (touchdown – 0.5ºC cycle^{-1}), and 3 min at 72ºC, followed by 15 cycles of 1 min at 94ºC, 1 min at 55ºC and 3 min at 72ºC, followed by 7 min final extension at 72º	Muyzer *et al*., 1996

Table 7.2. Primers for PCR and DGGE analysis.

Primer	Target site	Sequence (5' to 3')	Specificity	Reference
341F-GC	341-357	CCT ACG GGA GGC AGC AG*	Bacteria	Muyzer *et al*., 1996
907R	907-926	CCG TCA ATT CMT TTG AGT TT	Bacteria	Muyzer *et al*., 1996

***** Contains GC-clamp (5´-cgcccgccgcgccccgcgcccgtcccgccgcccccgcccg-3´) at the 5´ end of the primer.

Table 7.3. Oligonucleotide probes for FISH analysis used in this study

Probe code	Sequence (5´-3´)	Specificity	Formamide (%)	Label	Reference
EUB338	GCTGCCTCCCG TAGGAGT	Bacteria	0-50	Cy5	Amann *et al*. (1990)
EUB338II	GCAGCCACCC GTAGGTGT	Bacteria	0-50	Cy5	Daims *et al*. (1999)
EUB338III	GCTGCCACCC GTAGGTGT	Bacteria	0-50	Cy5	Daims *et al*. (1999)
UCB-823	CCTCCCCACCG TCCAGTT	*P. acidivorans*	35	Cy3	Jonhson *et al*. (2009a)
BET42a	GCCTTCCCACT TCGTTT	Betaproteobacteria	35	Cy5 / unlabeled	Manz *et al*. (1992)
GAM42a	GCCTTCCCACA TCGTTT	Gammaproteobacteri a	35	FLUOS / Cy3	Manz *et al*. (1992)
ALF968	GGTAAGGTTC TGCGCGTT	Alphaproteobacteria	20	Cy3	Loy *et al*. (2007)
ALF1B	CGTTCGYTCTG AGCCAG	Alphaproteobacteria	20	Cy3	Loy *et al*. (2007)

Appendix B – Results and discussion

Table 7.4. Average and standard deviation error of the measurements made along the 2-reactor CSTR system experiment (setup 1). The first 11 days of experiment were not considered to estimate the average values due to the instability of the system (last 18 days were considered).

	Feast		Famine	
	Average	**Standard Deviation**	**Average**	**Standard Deviation**
Acetate [Cmmol/L]	16.2	6.3	0.0	0.0
Biomass [Cmmol/L]	16.1	2.3	19.3	4.2
Ammonium[mmol/L]	0.3	0.2	3.2	0.4
PHB [Cmmol/L]	0.6	0.5	0.3	0.3

Table 7.5. Average and standard deviation of the measurements made along the 2-reactor CSTR system experiment (setup 2). The 42 days in which the system was stable (and no problems in the analytical analysis were detected) were considered to determine the average values.

	Feast		Famine	
	Average	**Standard Deviation**	**Average**	**Standard Deviation**
Acetate [Cmmol/L]	16.2	6.3	0.0	0.0
Biomass [Cmmol/L]	16.1	2.3	19.3	4.2
Ammonium[mmol/L]	0.3	0.2	3.2	0.4
PHB [Cmmol/L]	0.6	0.5	0.3	0.3
CO2 [Cmmol/L]	0.3	0.1	0.2	0.0
O2 [Cmmol/L]	8.2	0.2	8.5	0.1

Table 7.6. Model kinetics used for the growth experiment (Johnson *et al*., 2009b).

Feast phase	Acetate uptake	$\tilde{q}_{Ac,1}(t) = \tilde{q}_{Ac}^{max}\frac{\tilde{c}_{Ac}(t)}{K_{Ac}+\tilde{c}_{Ac}(t)}\ If\ \tilde{q}_{PHB,1}^{feast} \le \tilde{q}_{PHB,2}^{feast}$
	With PHB inhibition	$\tilde{q}_{Ac,2}(t) = \tilde{\mu}^{feast}(t)\frac{1}{Y_{X/Ac}^{feast}} + \tilde{q}_{PHB}^{feast}\frac{1}{Y_{PHB/Ac}^{feast}} + m_{Ac}\ If\ \tilde{q}_{PHB,1}^{feast} > \tilde{q}_{PHB,2}^{feast}$
	Growth	$\tilde{\mu}^{feast}(t) = \tilde{\mu}^{max}\frac{\tilde{c}_{NH_3}(t)}{K_{NH_3}+\tilde{c}_{NH_3}(t)}\frac{\tilde{c}_{Ac}(t)}{K_{Ac}+\tilde{c}_{Ac}(t)}$
	Maintenance	$m_{Ac=\frac{m_{ATP}}{Y_{ATP/Ac}^{feast}}}$
	PHB production	$\tilde{q}_{PHB,1}^{feast}(t) = \left(\tilde{q}_{Ac}(t) - \mu^{feast}(t)\frac{1}{Y_{X/Ac}^{feast}} - m_{Ac}\right)Y_{PHB/Ac}^{feast}$
	With PHB inhibition	$\tilde{q}_{PHB,2}^{feast}(t) = \tilde{q}_{PHB}^{max}\frac{\tilde{c}_{Ac}(t)}{K_{Ac}+\tilde{c}_{Ac}(t)}\left[1-\left(\frac{\tilde{f}_{PHB}(t)}{\tilde{f}_{PHB}^{max}(t)}\right)^{\alpha}\ If\ \tilde{q}_{PHB,1}^{feast} \le \tilde{q}_{PHB,2}^{feast}\right]$
	CO2 evolution	$\tilde{q}_{CO_2}^{feast}(ti) = \tilde{\mu}^{feast}(ti)Y_{CO_2/X}^{feast} + \tilde{q}_{PHB}^{feast}(ti)Y_{CO_2/PHB}^{feast} + m_{Ac}Y_{CO_2/Ac}^{feast}$
	O2 uptake	$\tilde{q}_{O_2}^{feast}(ti) = \tilde{\mu}^{feast}(ti)Y_{O_2/X}^{feast} + \tilde{q}_{PHB}^{feast}(ti)Y_{O_2/PHB}^{feast} + m_{Ac}Y_{O_2/Ac}^{feast}$
	NH3 uptake	$\tilde{q}_{NH_3}^{feast}(ti) = \tilde{\mu}^{feast}(ti)Y_{HN_3/X}^{feast}$
Famine phase	PHB degradation	$\tilde{q}_{PHB,1}^{fam}(t) = k\tilde{f}_{PHB}(t)^{2/3}$
	Maintenance	$m_{PHB} = \frac{m_{ATP}}{Y_{ATP/PHB}^{fam}}$
	Growth	$\tilde{\mu}^{fam}(t) = Y_{X/PHB}^{fam}\left(\tilde{q}_{PHB}^{fam}(t) - m_{PHB}\right)$
	CO2 evolution	$\tilde{q}_{CO_2}^{fam}(t) = \tilde{\mu}^{fam}(t)Y_{CO_2/X}^{fam} + m_{PHB}\,Y_{CO_2/PHB}^{fam}$
	O2 uptake	$\tilde{q}_{O_2}^{fam}(ti) = \tilde{\mu}^{fam}(ti)Y_{O_2/X}^{fam} + m_{PHB}\,Y_{O_2/PHB}^{fam}$
	NH3 uptake	$\tilde{q}_{NH_3}^{fam}(ti) = \tilde{\mu}^{fam}(ti)Y_{NH_3/X}^{fam}$

Table 7.7. Model parameters used for modelling the growth experiment (Johnson *et al*., 2009b).

Parameter/initial conditions	Value	Constant or estimated
Half-saturation constant for acetate	K_{Ac} = 0.2 Cmmol/L	Constant
Half-saturation constant for ammonia	K_{NH3} = 0.0001 mmol/L	Constant
Efficiency of oxid. Phosphorylation	δ = 2mmolATP/mmolNADH	Constant
Maintenance ATP requirement	m_{ATP}	Estimated
Max. acetate uptake rate	$\tilde{q}_{Ac}^{max}$	Estimated
Max. growth rate feast	$\tilde{u}^{max}$	Estimated
Max. PHB production rate	$\tilde{q}_{PHB}^{max}$ = 2 Cmmol/Cmmol/h	Estimated in fed-batch, constant in SBR experiments
Exponent of PHB inhibition term	α = 1.24	Estimated Estimated in fed-batch, constant in SBR experiments
Max. fraction of PHB	$\tilde{f}_{PHB}^{max}$	Estimated Estimated in fed-batch, constant in SBR experiments (Value of fed-batch experiment)
Rate constant PHB degradation	K	Estimated for SBR experiments
Initial concentration of acetate	$\tilde{c}_{Ac}(t=0)$	Estimated
Initial concentration of PHB	$\tilde{c}_{PHB}(t=0)$	Estimated
Initial concentration of biomass	$\tilde{c}_{X}(t=0)$	Estimated
Initial concentration of ammonia	$\tilde{c}_{NH_3}(t=0)$	Estimated
Initial carbon dioxide evolution	$cumC\tilde{E}(t=0)=0\ mmol$	Constant
Initial oxygen uptake	$cumO\tilde{U}(t=0)=0\ mmol$	Constant