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Microbial contribution to biofuels production

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To my husband Pedro
To my daughter Bruna
And to my parents

for their unconditional love and support

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Resumo

A biomassa pode ser convertida em biocombustíveis por dois métodos diferentes: termoquímicos ou bioquímicos. Ambos os processos produzem resíduos que podem ser valorizados, aumentando assim a sustentabilidade do processo de produção de biocombustíveis. A investigação recente sobre a produção de polihidroxialcanoatos (PHA) tem-se centrado na utilização combinada de culturas microbianas mistas (MMC) e substratos de baixo valor comercial. A presente tese teve como intuito estudar e caracterizar MMC capazes de produzir PHA utilizando subprodutos resultantes da produção de biocombustíveis.

A utilização de um bio-óleo resultante da pirólise rápida de camas de galinha como substrato, permitiu selecionar uma cultura com capacidade de produzir um copolímero composto por monómeros de hidroxibutirato e hidroxivalerato (70%:30%). A influência da matriz do bio-óleo na produção de PHA foi investigada sugerindo que existem alguns compostos capazes de inibir/interferir com a capacidade de acumulação. Com o objetivo de maximizar o conteúdo em PHA foram realizadas duas estratégias para modificar o bio-óleo; fermentação anaeróbia e destilação a vácuo. A primeira estratégia permitiu obter melhores resultados uma vez que o aumento de ácidos orgânicos voláteis no bio-óleo fermentado resultou num aumento do rendimento de produção em comparação com os obtidos com o bio-óleo puro (0,63 e 0,31 Cmmol HA/Cmmol S, respetivamente).

Num segundo sistema, utilizando glicerol bruto proveniente da produção de biodiesel como substrato, foi selecionada uma cultura com capacidade de acumular simultaneamente PHA e glicogénio. Embora a fração de metanol presente no subproduto também tenha sido consumida, o glicerol foi a única fonte de carbono que contribuiu para a produção dos biopolímeros. Usando o glicerol bruto em ensaios de acumulação obteve-se 47% de PHA em conteúdo celular.

A comunidade microbiana de ambos os sistemas de produção de PHA foi avaliada através de electroforese em gel com gradiente de desnaturação, hibridação *in situ* de fluorescência e sequenciação. Ambos revelaram uma elevada diversidade microbiana com predominância da classe *Betaproteobacteria* e do género *Amaricoccus* nos sistemas com bio-óleo e glicerol, respetivamente.

Palavras-Chave: Resíduos e subprodutos provenientes da produção dos biocombustíveis; Bio-óleo, glicerol bruto, culturas microbianas mistas (MMC, “mixed microbial cultures”); polihidroxialcanoatos (PHA); ecologia microbiana.

Abstract

Biomass can be converted into biofuels by two different ways: thermochemical or biochemical. Both processes produce waste streams that can be valorised in order to increase the sustainability of the biofuels production process. Recent research on polyhydroxyalkanoates (PHA) production has focused on developing cost-effective processes using low cost substrates combined with mixed microbial cultures (MMC). The intent of this thesis was to study and characterise MMC able to produce PHA using the by-products resulting from the biofuels production.

Bio-oil resulting from the fast-pyrolysis of chicken beds was used as substrate to select cultures under feast/famine conditions with a good PHA storage response. Several operational conditions were investigated and optimized. A copolymer composed by hydroxybutyrate and hydroxyvalerate monomers (70%:30%) was obtained. The impact of the bio-oil matrix on PHA production was also investigated suggesting that some compound may inhibit or interfere with the ability of the enriched culture to accumulate PHA. For further maximization of polymer accumulation two strategies for bio-oil upgrade were performed, anaerobic fermentation and vacuum distillation. The increased of volatile fatty acids on the fermented bio-oil led to an increase on the production yield compared to the ones obtain with pure bio-oil (0.63 and 0.31 Cmmol HA/Cmmol S, respectively).

In another system, MMC selected with crude glycerol from biodiesel production as feedstock had the ability to simultaneously store PHA and glycogen. Although the methanol fraction present in the crude was also consumed, glycerol was the only carbon source that contributed for the biopolymers production. During PHA accumulating assay a content of 47% cell dry weight was achieved.

The dynamics of the microbial community of both PHA production systems was assessed by denaturing gradient gel electrophoresis, fluorescent *in situ* hybridization and sequencing. Both systems had a high microbial diversity with a predominance of *Betaproteobacteria* class and *Amaricoccus* genus in the bio-oil and crude glycerol system, respectively

Keywords: Biofuels wastes/by-products; bio-oil, crude glycerol, mixed microbial cultures (MMC); polyhydroxyalkanoates (PHA); microbial ecology.

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Abbreviations

ADF	Aerobic Dynamic Feeding, also designated as “feast and famine”
AN/A	Anaerobic/aerobic process
BOD_5	Biochemical oxygen demand
C/N/P	Carbon to nitrogen to phosphorus ratio
COD	Chemical oxygen demand
COD/N/P	Chemical oxygen demand to nitrogen to phosphorus ratio
CSTR	Continuous Stirred Tank Reactor
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolve oxygen
E'	Evenness index
EBPR	Enhanced biological phosphorus removal systems
F/F	Feast to Famine ratio
FAA/FAME	Free fatty acids and fatty acids methyl esters ratio
FAAE	Fatty acid alkyl esters
FAME	Fatty acid methyl ester
FISH	Fluorescence in situ hybridization
GB	Glycogen biopolymer
H'	Shannon diversity index
HRT	Hydraulic Retention Time
MCL	Medium chain length (referring to PHA monomers)
MMC	Mixed microbial cultures
OLR	Organic loading rate

OUR	Oxygen Uptake Rate
PCA	Principal component analysis
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
PHAmax	represents PHA at the end of SBR feast phase or batch accumulation test
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
q_{gly}	Specific glycogen storage rate, in Cmol Glucose/Cmol X.h
qMeth	Specific methanol uptake rate, in Cmol Methanol/Cmol X.h
qP	Specific polymer storage rate, in Cmol PHA/Cmol X.h
qS	Specific substrate uptake rate, in Cmol S/Cmol X.h
SCL	Short-chain length (referring to PHA monomers)
SRB	Sequencing Batch Reactor
SRT	Sludge Retention Time
TOC	Total Organic Carbon
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solid
X	Active Biomass
Xi	Initial active biomass concentration
$Y_{O2/S}$	Respiration yield, in Cmol O_2/Cmol S
$Y_{PHA/S}$	Polymer storage yield, in Cmol PHA/Cmol S
$Y_{X/S}$	Growth yield, in Cmol X/Cmol S

CHAPTER 1

THESIS MOTIVATION AND OUTLINE

1.1. THESIS MOTIVATION

Worldwide energy demand increased drastically in recent years as a result of the modern society. The progressive depletion of conventional fossil fuels and increase of the greenhouse gas emission led to a move towards alternative, renewable, sustainable, efficient and cost-effective energy sources with fewer emissions.

Biofuels represent the best renewable alternative to fossil fuels. They are predominantly produced from biomass which is considered as the major world renewable energy source to supplement declining fossil fuel resources. Two main processes can be used to convert biomass into energy/biofuels: thermochemical and biochemical processes. Among the existing thermochemical conversion processes pyrolysis is considered as the best for the conversion of biomass into liquid fuel (bio-oil). Bioethanol and biodiesel are two of the most widely used liquid biofuels and are mainly produced trough biochemical processes.

Nowadays, biofuels can be produced using the existing technologies and be distributed through the available systems. They can be easily applied and are being encouraged by policy measures reaching its global production over 107 thousand million liters per year with a tendency to grow in the next years. As biofuel production increases, the market is being flooded with its waste/by-products and it becomes imperative to investigate alternatives to valorize these surpluses making the overall biofuels production a more sustainable process. The high carbon content in most of these residues makes its use as a substrate in biological conversions to produce biomaterials a viable strategy. Polyhydroxyalkanoates (PHA) are one of the biomaterials with high interest due to the impact of conventional plastics in the environment.

PHA are bio-based, biodegradable and biocompatible plastics with high potential to replace some of the more commonly used conventional plastics. These biopolymers have similar thermoplastic and elastomeric properties to polypropylene (PP) and polyethylene (PE). However they can be synthesized from renewable resources and are fully biodegradable, meeting the criteria of a closed loop life cycle (bio-based to biodegradable) which has a high environmental and economical relevance.

PHA are synthesized and stored intracellularly by a large number of bacteria as carbon and energy sources. The interest to develop and optimize strategies to produce, extract and manipulate these bioplastics has increased significantly since the early 1980s. Nowadays, PHA are already been commercialized, but they are restricted to the use of highly costly synthetic substrates and pure or genetic/metabolic engineered strains. Despite the effort put in the development of pure culture fermentation processes, these have not yet entered bulk materials markets due to high production costs, which is the major drawback in the current PHA production, limiting commercialization to added value applications.

In the last decade, research has focused on the development of alternative and more sustainable production processes aiming to substantially decrease the PHA production costs. The most relevant strategies investigated include the use of low cost substrates (domestic/industrial waste/by-products) and the use of mixed microbial cultures (MMC). It is generally accepted that these strategies will allow the decrease of the global PHA production costs since they requires lower investment and operating costs, due to the use of open systems that do not require sterile conditions. The use of waste-based feedstocks not only permits reducing the PHA production costs but would also make the overall industrial process more sustainable, by valuing an industrial waste/by-product.

Despite the increasing number of references in the literature on the use of several industrial wastes (cheese industries, waste lipids, sugar industries, agriculture crop and other lignocelluloses residues, glycerol and forest and other wood residues) to produce PHA, the majority of them used pure microbial strains. As such the development of further investigation valuing industrial waste/by-products through the production of PHA using MMC can introduce new competitive strategies not only to the PHA market but also to several industries.

The major goals of this thesis can be pointed out as follows:

(I) test the feasibility of MMC to use the liquid fraction resulting from the fast-pyrolysis of a waste biomass to produce PHA without any detoxification process and improve the subsequent PHA production step;

(II) enrich a MMC able to accumulate PHA using the major biodiesel production by-product-crude glycerol and improvement of the production stage;

(III) identify the microbial consortium present in each PHA production systems and correlate different populations with different operation conditions.

1.2. THESIS OUTLINE

This thesis is divided into seven chapters including the current introductory chapter that describes the motivation and outline of the work developed during this PhD project (Chapter 1). The following chapter includes an overview of the state of the art in the biofuels production and respective wastes/by products and an outline of mixed culture PHA production processes as a strategic way to valorized industrial wastes (Chapter 2). Chapter 3 to 6 described the work developed in accordance with the specific objectives laid out above, and a final chapter highlights the main conclusions drawn from this study (Chapter 7). The work performed during this PhD will result in four scientific articles, presented in Chapters 3, 4, 5 and 6, respectively. Chapters 3and 4 are already published in peer reviewed international journal,

Chapter 5 was submitted for publication in peer reviewed international journal and Chapter 6 is being prepared for submission.

Briefly, each chapter includes the following contents:

Chapter 1 (present chapter) provides the motivation and the objectives of this PhD thesis. It also includes the thesis outline with a brief summary of the contents of each chapter.

Chapter 2 includes the state of the art starting to address the current economical and environmental relevance of alternative fuels. It further focuses on the most relevant biofuels, describing their production, economical relevance and applications of the resulting wastes/by-products. PHA production is proposed as an alternative to valorize some wastes/by-products resulting from the biofuels production. A brief overview on PHA structure, proprieties, application and PHA bacterial synthesis is explained followed by the description of the current industrial biotechnology approach to PHA production, using pure culture fermentation. The use of waste-based feedstocks by mixed microbial cultures to produce PHA in a more sustainable form is later introduced. Finally the importance of the study of the microbial communities dynamic is briefly presented.

Chapter 3 considers the development of a process where bio-oil resulting from the fast-pyrolysis of chicken beds was used as substrate to select a mixed microbial culture (MMC) able to produce PHA under feast/famine conditions. During the culture acclimatization to the bio-oil as substrate different conditions were tested, namely the SRT and COD/N/P ratio, in order to optimize the selective pressure imposed to the system.

Chapter 4 describes the different bio-oil upgrading strategies to improve PHA production by the enriched culture. The impact of complex bio-oil matrix was tested in different PHA accumulation batch assays in order to gather information about some possible inhibition problems associated not only with the biomass growth, but also with the substrate uptake and PHA production. Due to the multiplicity of compounds present in bio-oil, the performance on PHA storage capacity of the selected culture using pure bio-oil was tested and compared with the utilization of three defined substrates (acetate, glucose and xylose) known to be present in bio-oil. In addition two strategies for bio-oil upgrade were performed; anaerobic fermentation and vacuum distillation, and the resulting liquid streams were tested for polymer production.

Chapter 5 presents the selection of a mixed microbial community with PHA storage capacity using crude glycerol as substrate and considers also the production step on a 2-stage PHA production process. The influence of the pure synthetic substrates composing crude glycerol (methanol and glycerol, in single or com2010bined mode), on the biopolymers accumulation

was investigated. The storage capacity of the selected culture was study using different feeding strategies of crude glycerol (continuous, pulse feeding) and compared to the use of synthetic glycerol (pulse feeding).

Chapter 6 focuses on the implementation of different strategies to study the bacterial community dynamics in the two different biological PHA production systems. The acclimatization period of the microbial cultures was followed by DGGE analysis. Sequencing of specific DGGE bands allowed to perform bacterial identification and correlate with the PHA storage capacity of the system. Statistical analysis was applied for the presence/absence of DGGE bands for the determination of ecological parameters as well as clustering analysis. FISH technique allowed a direct visualization and quantification of relevant members of the population.

Chapter 7 summarizes the main conclusions achieved in this PhD dissertation. Some possible challenges and suggestions for future research are also presented.

CHAPTER 2

STATE OF THE ART

2.1. BIOFUELS

The energy growing needs of modernized worlds have led to an increased demand of petroleum-based fuels. Today fossil fuels provide up to 80% of the primary energy consumed in the world, of which 58% is consumed by the transport sector (Nigam and Singh 2011). Fossil fuels are non-renewable energy sources and there reserves are estimated to be depleted in less than 50 years, (except coal reserves which should be available until 2112) at the present consumption rate (Shafiee and Topal 2009).

The combustion of fossil fuels is the major contributor to greenhouse gas (GHG) emission, with many negative effects resulting from global warming. Therefore, the progressive depletion of conventional fossil fuels with increasing energy consumption and GHG emission have led to a move towards alternative, renewable, sustainable, efficient and cost-effective energy sources with less emissions. Presently many options are being studied and implemented in practice, with different degrees of success, and in different phases of study and implementation. Examples include solar energy, either thermal or photovoltaic, hydroelectric, geothermal, wind, biofuels, and carbon sequestration. Each one has its own advantages and problems and, depending on the area of application, different options will be better suited.

Fuel demand in the transportation sector is projected to increase by 40% over the period 2010–2040 (ExxonMobil, 2013). One important goal is to take measures for transportation emissions reduction, such as the gradual replacement of fossil fuels by renewable energy sources, where biofuels are seen as real contributors to reach those goals, particularly in the short term. Given that the European transport sector is facing a sustainability issue the European Union has developed objectives to replace fossil fuels by biofuels upon a substitution of 25% by 2030 (Biofuels-A vision for 2030 and beyond). In support of the above, EU has decided to implement an ambitious regional strategy designed to further encourage the development and production of biofuels to set the long term strategy for the development of renewable energy sources (RES) in EU (Directive 2009/28/EC, Directive 98/70/EC). Out of the agreed goal for 20% overall share of RES by 2020, 10 % of all transportation fuels should be derived from biofuels. Recently, due to conflicts caused by the use of edible crop to produce biofuels, an amendments to the directive 2009/28/EC (2012/0288 (COD)) has established that the maximum joint contribution from biofuels and bioliquids produced from cereal and other starch rich crops, sugars and oil crops should be no more than 5%.

2.1.1. Advantages and challenges of biofuels

The term biofuel refers to as solid (biochar), liquid (bioethanol, vegetable oil and biodiesel) or gaseous (biogas, syngas and biohydrogen) fuels that are predominantly produced from biomass. Biomass has been recognized as a major world renewable energy source to supplement declining fossil fuel resources. Besides being a renewable resource that could be sustainably developed in the future, biomass appears to have significant economic potential provided from the increase of fossil fuel prices in the future. Also unlike the combustion of fossil fuels which releases CO_2 that was captured several hundred million years ago, CO_2 released during the utilization of a biomass based fuel is balanced by CO_2 captured in the recent growth of the biomass, resulting in far less net impact on GHG levels (Fig. 2.1). Since biomass utilization can be considered as a closed carbon cycle, the production and usage of biofuels is expected to reduce the net CO_2 emission significantly (Demirbas, 2007).

Fig. 2.1- Biofuels cycle (http://arstechnica.com/)

Biofuels production is expected to offer new opportunities to diversify income and fuel supply sources, to promote employment in rural areas, to develop long term replacement of fossil fuels, and to reduce GHG emissions, boosting the decarbonisation of transportation fuels and increasing the security of energy supply. Large-scale production offers an opportunity for certain developing countries to reduce their dependence on oil imports and in industrialized countries there is a growing trend towards employing modern technologies and efficient bioenergy conversion using a range of biofuels. Biofuels can be produced using existing technologies and be distributed through the available distribution system. For all these reasons biofuels are currently pursued as a fuel alternative that can be easily applied until other options

harder to implement, such as hydrogen, are available. Although biofuels are still more expensive than fossil fuels their production is increasing in countries around the world. Encouraged by policy measures and biofuels targets for transport, its annual global production is estimated to be over 107 thousand million liters (Ren 21, Report 2013).

Besides having several benefits, the production and utilization of biofuels also have several challenges. An improved biomass waste collection network and their storage is the main challenge for establishment of commercial biofuel plant. A strong policy is needed for organic waste collection and blending of biofuels at higher rate. The subsidization for establishment of biofuel plants will accelerate the production of biofuels and tax credits for utilization will create the market for the biofuel. Biofuels production also deals with the same problem as traditional petroleum refining – excess waste. In traditional refining, only about 60 percent of the crude oil becomes gasoline, the rest is used to make other products. Similarly, as biofuel production increases, the market is being flooded with its waste/by-products. Technological improvements could help to increase the system efficiency and provide value added co-products, which will reduce the total production cost

2.1.2. Biofuels classification

Biofuels are broadly classified as primary and secondary biofuels. Primary biofuels are natural and unprocessed biomass such as firewood, wood chips and pellets, and are mainly those where the organic material is utilized essentially in its natural and non-modified chemical form. Primary fuels are directly combusted, usually to supply cooking fuel, heating or electricity production needs in small and large-scale industrial applications. Secondary fuels are modified primary fuels, which have been processed and produced in the form of solids (e.g. charcoal), or liquids (e.g. ethanol, biodiesel and bio-oil), or gases (e.g. biogas, synthesis gas and hydrogen). Secondary fuels can be used for multiple ranges of applications, including transport and high-temperature industrial processes.

The secondary biofuels are further divided into first, second and third-generation biofuels on the basis of raw material and technology used for their production (Fig. 2.2) First generation biofuels refer to biofuels made from sugar, starch, vegetable oils, or animal fats using conventional technology. The basic feedstocks for the production of first generation biofuels are often seeds or grains such as wheat, which yields starch that is fermented into bioethanol, or sunflower seeds, which are pressed to yield vegetable oil that, can be used in biodiesel. Second and third generation biofuels are produce using advanced technology and thus are also called advanced biofuels. Second generation biofuels are made from non-food crops, wastes and

lignocellulosic biomass. Third generation biofuel use algae or sea weeds as feedstock (Demirbas, 2011).

The use of edible feedstocks to produce first generation biofuels creates a direct conflict with food/feed supply. Also, the productions of these biofuels depend on subsidies and in some cases are not cost competitive with existing fossil fuels such as oil. Some of the biofuels allow only limited greenhouse gas emissions savings. When taking emissions from production and transportation into account, life-cycle assessment from first generation biofuels frequently approach those of traditional fossil fuels. As a consequence of first generation manufacture limitations, advanced biofuels technologies have been developed. Second and third generation biofuels are considered to be produced in a more sustainable way and as a truly carbon neutral or even carbon negative in terms of its impact on CO_2 concentrations specially due to the use of non-edible biomass for their production.

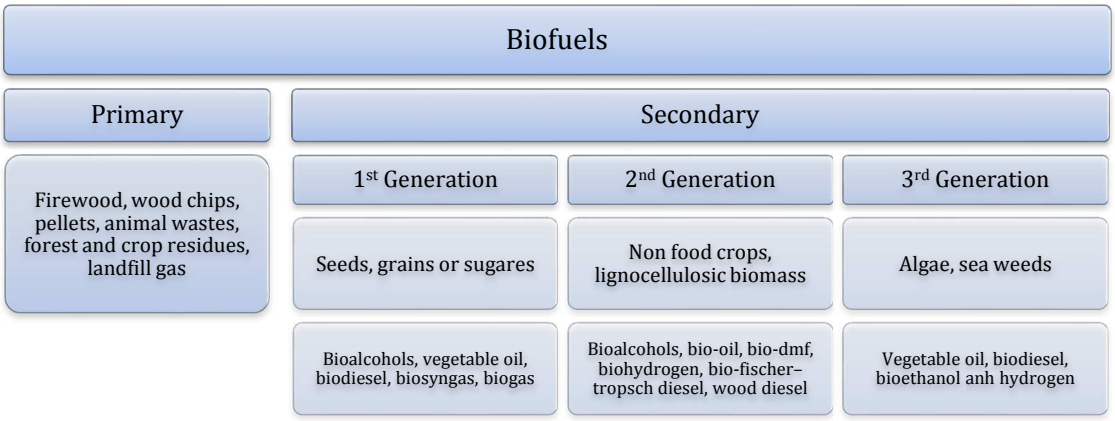


Fig. 2.2- Classification of biofuels (adapted from Nigam & Singh 2011)

2.1.3. Biomass conversion process

Biomass can be converted into energy/biofuels by two main processes: thermochemical and biochemical processes. First generation and a few second-generation biofuels such as ethanol and butanol are produced via biochemical process. However, the main second-generation fuels (i.e methanol, refined Fischer-Tropsch liquids (FTL), and dimethyl ether (DME)) are produced thermochemically. The thermochemical conversion processes include combustion, gasification and pyrolysis. Choice of conversion process depends upon the type and quantity of biomass feedstock, the desired form of the energy, i.e., end use requirements, environmental standards, economic conditions and project specific factors.

Biomass combustion is a worldwide adopted process to obtain a range of outputs like heat, mechanical power or electricity by conversion of the chemical energy stored in biomass. This

process is performed in the presence of air and in order for the combustion to be feasible the biomass moisture content has to be lower than 50%. In most cases biomass rarely arises naturally in an acceptable form of burning, requiring some pretreatment like drying, chopping, grinding, etc., which in turn is associated with financial costs and energy expenditure.

During gasification process the biomass is heated with reduced air supply and converted into a combustible gas mixture by the partial oxidation of biomass at high temperature, in the range 1000-1100K. Methane and hydrogen are also formed simultaneously by thermal splitting of organic material. The low calorific value gas produced can be directly utilized as a fuel for gas turbines and gas engines

Pyrolysis process converts biomass directly into solid, liquid and gaseous products by thermal decomposition of biomass in the absence of oxygen. Although pyrolysis is still under development, this process has received special attention since it offers efficient utilization of biomass with particular importance for countries with vastly available agricultural by-products. (Saxena et al., 2008).

Thermochemical biomass conversion involves processes that require much more extreme temperatures and pressures than those found in biochemical conversion systems. Biochemical conversion uses enzymes to break down structural carbohydrates (for example, the cellulose and hemicellulose found in plant cell walls) into sugars, which are transformed into alcohols, organic acids, or hydrocarbons by microorganisms in fermentation. The conversions typically take place at atmospheric pressure and temperatures ranging from ambient to 70°C.

The two most widely used liquid biofuels are mainly produced as first generation fuels trough biochemical processes: bioethanol and biodiesel. These two biofuels have the ability to replace gasoline and diesel fuels, respectively, in today cars with little or no modifications of vehicle engines. As a result a growing investment on their productions has been observed, especially in the transport sector.

Bioethanol production technology is based on the fermentation of sugar to ethanol. Sugar can be obtained directly from sugarcane (Brazil) and sugar beets (Europe) or indirectly from the hydrolysis of starch-based grains, such as corn (United States) and wheat (Canada and Europe). In the latter case, the starch feedstock needs to be ground to a meal that is hydrolyzed to glucose by enzymes. The resulting pulp is fermented by yeast and bacteria. Finally, the fermented stream is separated into ethanol and residues (for feed production) via distillation and dehydration.

Biodiesel is produced using vegetable oils such as rape seed oil, sunflower seed oil, soybean oil and also used frying oils (UFO) or animal fats mainly by chemical conversion (transesterification).

2.2. PYROLYSIS

In the last decade, increasing efforts have been dedicated to implement biorefinery plants worldwide. Those plants seek for the conversion of lignocellulosic and cellulosic waste into starting materials for the biotechnological production of bioenergy, biopolymers and a range of fine chemicals. From all the thermochemical conversion processes, pyrolysis is considered as the one best suited for conversion of biomass into liquid fuels (Goyal et al., 2008).

Pyrolysis is the thermal degradation of biomass which occurs in the absence of oxygen, resulting in the production of charcoal (solid), bio-oil (liquid) and fuel gaseous products. Depending on the operating condition, pyrolysis can be mainly classified as conventional (slow) or fast-pyrolysis. Conventional pyrolysis occurs under a slow heating rate (0.1–1K/s) and residence time around 45– 550 s. In the first stage (pre-pyrolysis) biomass is thermal decomposed between temperature of 550 and 950K. During this stage, some internal rearrangement such as water elimination, bond breakage, appearance of free radicals, formation of carbonyl, carboxyl and hydroperoxide groups take place. The second stage (main pyrolysis process) proceeds with a high rate and leads to the formation of pyrolysis products. During the third stage, the char decomposes at a very slow rate and forms carbon rich solid residues. Fast pyrolysis occurs at higher temperatures (850–1250K) with fast heating rate (10–200 K/s), short solid residence time (0.5–10 s) and small particle size (<1 mm) (Naik et al., 2010).

Fast pyrolysis processes have been developed for production of food flavors (to replace traditional slow pyrolysis processes which had much lower yields), specialty chemicals and fuels. In fact, fast-pyrolysis of biomass has been shown to be two to three times cheaper than biomass conversion technologies based on gasification and fermentation processes (Vispute et al. 2010). Bio-oil is the main product of fast pyrolysis technology together with the by-product char and gas which can be used within the process to provide the process heat requirements so there are no waste streams other than flue gas and ash. Liquid yield depends on several parameters: biomass type; temperature; hot vapor residence time; char separation and biomass ash content. Research has shown that maximum liquid yields are obtained with high heating rates, at reaction temperatures around 775K and with short vapor residence times (between 30 and 1500ms) to minimize secondary reactions. Both residence time and temperature control is important to ‘freeze’ the intermediates of most chemical interest in conjunction with moderate gas/vapor phase temperatures of 675–775K before recovery of the product to maximize organic liquid yields.

2.2.1. Fast-pyrolysis reactors

In fast pyrolysis technology the reactor is considered as the center of the process. Although the reactor probably represents only about 10–15% of the total capital cost of an integrated system, most research and development has focused on developing and testing different reactor configurations on a variety of feedstocks. The main technologies are bubbling fluid beds; circulating fluid beds and transported beds; the rotating cone which a type of transported bed reactor; and ablative pyrolysis. Fluid bed pyrolysers (Fig. 2.3) give good and consistent performance with high liquid yields of typically 70-75 wt% from wood on a dry feed basis. The key requirements in the design and operation of a fast pyrolysis process are heat transfer and char removal as char and the ash are catalytically active. Recently increasing attention is being paid to control and improvement of the liquid quality and improvement of liquid collection systems (Meier et al. 2013).

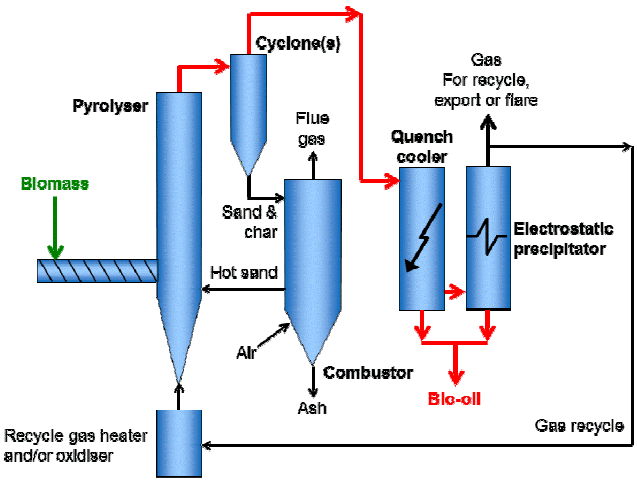


Fig. 2.3- Typical fast-pyrolysis reactor (fluid bed reactor) (http://www.pyne.co.uk/)

2.2.2. Bio-oil characteristics

In fast pyrolysis, biomass decomposes very quickly producing mainly vapors and aerosols and some charcoal and gas. After cooling and condensation, a dark brown homogenous mobile liquid (bio-oil) is formed. Fast pyrolysis produced 60–75% of bio-oil, 15–25% solid char and 10–20% non condensed gases depending upon feedstocks.

Typically, bio-oil is a dark brown, free-flowing liquid. However depending on the feedstock and the fast pyrolysis process the color can be almost black through dark red–brown to dark green,

being influenced by the presence of micro-carbon in the liquid and chemical composition. Bio-oil has a complex chemical composition resulting from three key biomass building blocks: cellulose, hemicellulose, and lignin. Most oligomeric structures are unable to be detected using gas chromatography (GC) or gas chromatography-mass spectroscopy (GC-MS). However, the more than 300 compounds already identified in the bio-oil can be classified into the following five broad categories: (1) hydroxyaldehydes, (2) hydroxyketones, (3) sugars and dehydrosugars, (4) carboxylic acids, and (5) phenolic compounds (Mohan, Pittman,, and Steele 2006).

Particular characteristics of the bio-oil impose some challenges on their future applications. Despite the high water content, pyrolysis liquids (25–45%) can tolerate the addition of some water before phase separation occurs. Water addition reduces viscosity and improves stability while reducing the heating value, meaning that more liquid is required to meet a given duty. Therefore the effect of water is complex and important. Due to the high oxygen content, around 35–40 wt%, the majority of the bio-oils are miscible with polar solvents such as methanol, acetone, etc., but totally immiscible with petroleum-derived fuels. Removal of this oxygen by upgrading requires complex catalytic processes. Bio-oil as a high density compared with the light fuel oils (1.2 Kg/L and 0.85 kg/L, respectively). This means that the liquid has about 42% of the energy content of fuel oil on a weight basis, but 61% on a volumetric basis. This situation has high implications for the design and specification of equipment such as pumps and atomizers in boilers and engines. Viscosity is another important feature in many fuel applications. Bio-oil viscosity can vary from 25 to 1000 m^2s^{-1} (at 40ºC) or more depending on the feedstock, the water content of the bio-oil, the amount of light ends collected and the extent to which the oil has aged. Although bio-oil has been successfully stored for several years (polyolefin plastic drums) without any deterioration that would prevent its use in the applications tested to date, it does change slowly with time (clear gradual increase in viscosity). The recent advances in fast-pyrolysis process design and control as the technology develops have show substantial improvements in consistency and stability of the bio-oils. However, aging is a well known phenomenon caused by continued slow secondary reactions in the liquid which manifests as an increase in viscosity with time and in extreme cases phase separation can occur. The addition of alcohols such as ethanol or methanol can reduce or control the aging process (Meier et al. 2013).

2.2.3. Bio-oil application

Bio-oil can be considered in many applications (Fig. 2.4). The heating value of bio-oil (~17 MJ/ kg.) is lower than that for fossil fuel mainly because of the large number of oxygenated compounds and significant water content. Nevertheless, tested flame combustion showed that

fast pyrolysis oils could be used directly to replace heavy and light fuel oils in standard industrial equipment such as boilers, furnaces, burners, stationary diesel engines, gas turbines and stirling engines (http://www.btgworld.com).

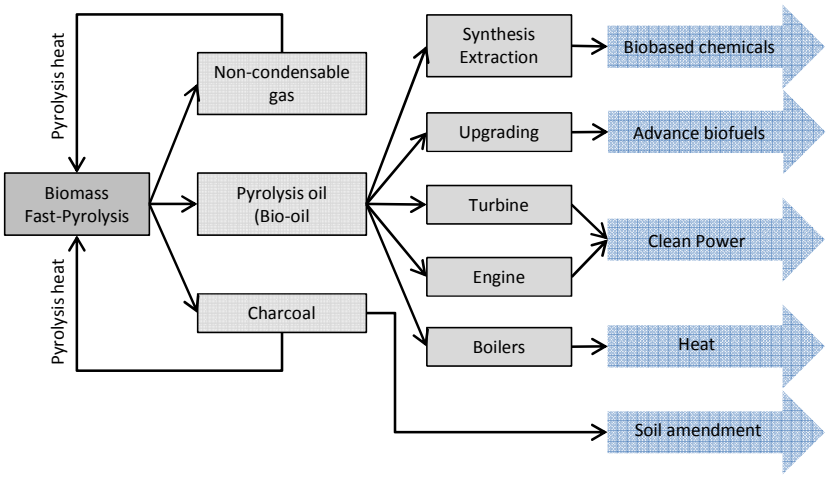


Fig. 2.4- Products from fast-pyrolysis of biomass conversion (adapted from Meier et al. 2013)

Considering an intermediate upgrading step several options for bio-oil utilization can be proposed. Recently there has been considerable research and commercial interest in upgrading bio-oil into synthetic hydrocarbon fuels for transportation applications, however even though is feasible it´s still not yet currently economic profitable. For this type of application, the high oxygen content of bio-oil is reduced through "deoxygenation" processes commonly used in the petrochemical industry: hydrotreating and catalytic cracking. The costs associated with these process increases drastically the price of the final products reducing potential use of those bio-oils as a substitute for petroleum-based fuels.

A different approach to synthesizing transportation fuels from bio-oil is using the pyrolysis liquid as a feedstock for gasification, rather than raw biomass. By gasifying slurry of bio-oil and biochar, it is possible to produce a clean syngas which is then upgraded to transportation fuels using Fischer-Tropsch processing (Henrich et al. 2009). A final upgrading consideration for bio-oil is using steam reforming techniques for the production of hydrogen. Hydrogen is required for many industrial processes, is frequently used in the petrochemical industry and can be used in fuel cells to generate electricity.

Bio-oil contains specific compounds such as acetic acid, levoglucosan, and hydroxyacetaldehyde that have been researched for potential extraction. There are many other "specialty products" originating from bio-oil with commercial potential such as: wood preservatives, insecticides and fungicides, fertilizers, resins, adhesives, numerous food flavorings and additives. In fact, food flavoring from wood pyrolysis products are already

commercially in many countries. All chemicals are attractive possibilities due to their much higher added value compared to fuels and energy products, and lead to the possibility of a bio-refinery concept in which the optimum combinations of fuels and chemicals are produced.

Polar bio-oils resulting from the fast-pyrolyis of lignocellulosic materials usually have high concentrations of alcohols, aldehydes, ketones, carboxylic acids and other polar components. In addition, to the high content in low molecular weight polar components, polar bio-oil has a considerable good water solubility which has motivated the interest in their use as substrate for microbial fermentations. Several studies were focused in the use of sugars present in the bio-oil, especially levoglucosan, to produce ethanol (Chan and Duff 2010; Lian et al. 2010; H. Wang et al. 2012) and some triglycerides (Lian et al. 2010). In all cases, pure single strains (bacterial and yeast) were used and due to the presence of inhibitors compounds (mainly furfural and phenolic compounds) the bio-oil required a detoxification step in order to be metabolized by the organisms.

In recent years it has been reported some developments on technologies to converted petrochemical plastic waste steams into PHA. First, the plastic waste streams are submitted to pyrolysis and then the pyrolysis products are supplied as carbon substrate for microbial fermentation to produce PHA. Ward et al. 2006 converted styrene oil (resulting from the pyrolysis of PS) into mcl-PHA with a yield of 0.1 g PHA/g PS using *Pseudomonas putida* CA-3. Latter, the process was improved through the control of styrene feeding (Nikodinovic-Runic et al. 2011). By changing the mode of liquid feed of styrene by pumping it through the air sparger a 5.4-fold increase in cell dry weight was achieved. Based on the PS to PHA technology the solid fraction –terephthalic acid (TA) - resulting from the pyrolysis of polyethylene terephthalate (PET) was used to produce mcl-PHA (Kenny et al. 2012). Two different *P. putida* strains were able to accumulated PHA at a maximal rate of 8.4 mg PHA/L.h for 12 h before the rate of PHA accumulation decreased dramatically. Mixed plastic pyrolysis oils contain benzene, toluene, ethylbenzene, xylenes, and styrene (BTEXS) in their composition. Nikodinovic et al. 2008 using a synthetic mixture of BTEXS compounds and a defined mixed-culture of *P. putida* strains was able to accumulated 24–36% (cell dry weight) of PHA with a yield of 0.1 g PHA/ g BTEX.

2.3. BIODIESEL

In its main characteristics, biodiesel is quite similar to petroleum-based diesel fuel and can be blended with petroleum diesel to create a stable biodiesel blend. Biodiesel obtained through transesterification is a mono alkyl ester (methyl or ethyl ester) of long chain fatty acids derived from natural, renewable feedstock such as new/used vegetable oils and animal fats. Due to

problems of corrosion, deposits on the engine and warranty issues, a limit on the content of fatty acid methyl ester (FAME) in blended diesel in Europe was established to 7% (v/v) (EN 590). However, this limit is not required for other biofuel production processes, as pure hydrocarbons similar to diesel fuel are obtained from biomass using the Fischer Tropsch process or vegetable oils hydrogenation. Thus, biodiesel is considered as a substitution fuel for traditional diesel in any compression ignition (diesel) engines with little or no modification (Abbaszaadeh et al. 2012).

Due to the prospects of replacing fossil fuels, biodiesel production has continuously grown in the last decade. In 2012, the European Biodiesel board estimated that EU biodiesel production totaled 23.54 million metric tons, being in the last years the EU responsible for about half of the world's biodiesel output (http://www.biofuelstp.eu/). Compared with conventional diesel fuels, biodiesel is much less pollutant for the environment and represents a strategic source of energy for the countries that have no oilfields. Therefore, even though the costs of biodiesel are still greater than diesel from petroleum, many governments sustain this production for reducing the environmental impact and the dependence on foreign politically unstable suppliers. For example, the European Directive imposes a 10% volume of biofuels in the transport sector by 2020 (Santacesaria et al. 2012) .

In the production of biodiesel more than 95% of feedstocks come from edible oils since they are produced in many regions of the world and the properties of biodiesel produced from these oils are much suitable to be used as diesel fuel substitute. The fuel potentialities of many vegetable oils (including castor, grapeseed, maize, camelina, pumpkinseed, beechnut, rapeseed, lupin, pea, poppyseed, peanut, hemp, linseed, chestnut, sunflower seed, palm, olive, soybean, cottonseed, shea butter) were considered as early as 1939. Nowadays, the most employed feedstocks in biodiesel production are rapeseed, sunflower, soybean and palm oils.

About 60-80% of the total cost of biodiesel production comes from the cost of raw materials and first generation biofuels are not considered sustainable due to the food/energy competitions which increase both the cost of edible oils and biodiesel. In order to overcome these disadvantages, many researchers, scientists, technologists as well as industrialists are interested in non-edible oil source like waste oils of any sort, oil from *Jathropa curcas* and more recently oils from algae, the later not suitable for human consumption due to the presence of some toxic components in the oils. (Salvi and Panwar 2012; Borugadda and Goud 2012; Santacesaria et al. 2012)

2.3.1. Biodiesel production

Several technologies are accepted and well established for the production of biodiesel fuel. Direct use and blending of raw oils, micro-emulsions, thermal cracking (pyrolysis) and transesterification are considered as the four main procedures to produce biodiesel.

The direct use and blending of raw oils have been considered not satisfactory and unpractical for both direct and indirect diesel engines due to problems such as, high viscosity, acid composition, free fatty acid content, gum formation due to oxidation and polymerization during storage and combustion, carbon deposits and lubricating oil thickening.

Micro-emulsions with solvents such as methanol, ethanol and 1-butanol have been studied for a potential solution for solving the problem of high vegetable oil viscosity. However, micro-emulsion of vegetable oils has resulted in irregular injector needle sticking, heavy carbon deposits and incomplete combustion during 200 h laboratory screening endurance test.

The conversion of vegetable oils and animal fats composed mostly of triglycerides using thermal cracking reactions represents a promising technology since the fuel properties of the liquid product fractions of the thermally decomposed vegetable oil are likely to approach diesel fuels. Although the products are chemically similar to petroleum-derived gasoline and diesel fuel, the removal of oxygen during the thermal processing also removes any environmental benefits of using an oxygenated fuel.

The most common technology of biodiesel production is transesterification with alcohol, most likely methanol, which gives fatty acid alkyl esters (FAAE) as main product and glycerol as by-product. A catalyst is usually involved to improve the reaction rate and yield. Alkalies (sodium hydroxide, potassium hydroxide, carbonates, and corresponding sodium and potassium alkoxides), acids (sulfuric acid, sulfonic acid or hydrochloric acid), or enzymes can be used to catalyze the reaction. Base-catalyzed transesterification is much faster than the acid-catalyzed one and is most often used commercially. The first step of the transesterification reaction is the conversion of triglycerides to diglycerides, which is followed by the conversion of diglycerides to monoglycerides and of monoglycerides to glycerol, yielding one methyl ester molecule from each glyceride at each step (Fig. 2.5; Abbaszaadeh et al. 2012).

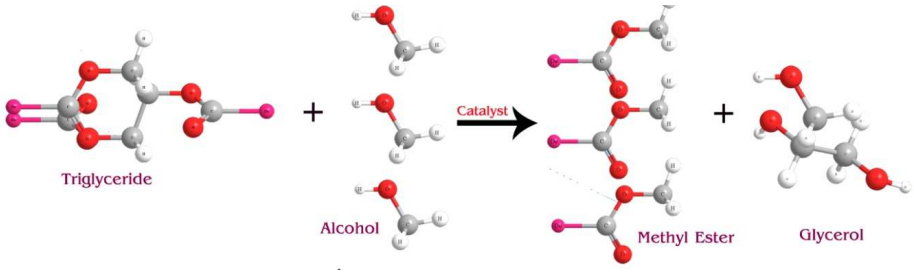


Fig. 2.5- Transesterification reaction (http://share.psu.ac.th)

2.3.2. Crude glycerol composition

The chemical composition of crude glycerol varies mainly with the type of catalyst used to produce biodiesel, the transesterification efficiency, the recovery efficiency of the biodiesel, other impurities in the feedstock, and whether the methanol and catalysts were recovered. The average accepted values are 50 to 70% glycerol, 10 to 20% methanol, 5 to 10% salts, <3 to 10% water, <1 to 5% fatty acids, and 5% non-glycerol organic material (NGOM) by weight.

In most commercial applications the quality of glycerin must be improved until it has an acceptable purity that is completely different from those obtained in biodiesel facilities. There are many actions and processes used to purify biodiesel, recover useful agents for re-cycling, and process the byproduct glycerol. An important post-process of glycerol includes acidification/neutralization to adjust pH and evaporation/distillation to separate water and excess methanol for reuse. Biodiesel manufacturers normally recover methanol by heating and reused in the biodiesel production process. However, because recovery of methanol is less cost effective than using new methanol, this is not always the case (Quispe et al., 2013; Tan et al., 2013).

2.3.3. Crude glycerol market

Biodiesel is considered one of the most promising substitutes for fossil fuels, still its production has increased at a slower rate than expected due to a relatively high production cost. Valorization of biodiesel main by-product, glycerol, is considered as one of the strategies for lowering the production cost.

Biodiesel production generates about 10% (v/v) glycerol as the main byproduct. Supported by governments to increase energy independence and meet the rising energy demand, the biodiesel market is expected to reach 37 billion gallons by 2016, an average growth of 42% per year. This means that around 4 billion gallons of crude glycerol will be produced that year saturating the glycerol market.

From the 1970s until the year 2004 the high-purity glycerin had a stable price between 876 and 1314 €/ton. However, with the arrival of biodiesel this relatively stable market has been drastically altered. In 2006, glycerol price stabilized around 438 €/ton, with a strong falling trend. In fact, in 2011, the price of crude glycerol in the US was so low, 3 to 8 cents/Kg that many biodiesel producers started to store the glycerol waiting for a better market.

The development of sustainable processes for utilizing this organic raw material is imperative. Since purified glycerol is a high-value commercial chemical with thousands of uses, the crude glycerol presents great opportunities for new applications. For that reason, more attention is

being paid to the utilization of crude glycerol from biodiesel production in order to decrease the production cost of biodiesel and to promote biodiesel industrialization on a large scale (Quispe et al., 2013).

2.3.4. Crude glycerol applications

Currently there are more than two thousand uses for glycerol. However, only a few applications use large amounts of glycerol in their composition. The three main uses for refined glycerin are food products, personal hygiene products and oral hygiene products, making up to 64% of total market (Fig. 2.6).

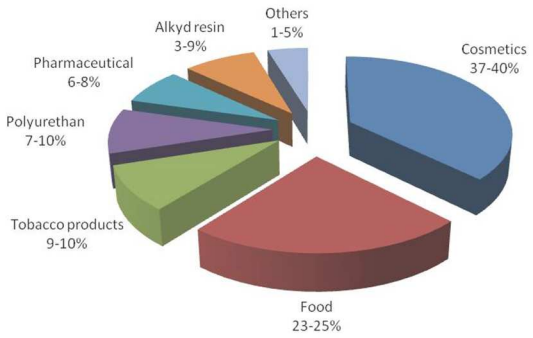


Fig. 2.6- Traditional glycerol applications (adapted from Quispe et al. 2013)

The global market for refined glycerol was estimated to be roughly 900,000 tons in 2005. Considering that by 2016 crude glycerol derived from biodiesel conversion is expected to reach around 4 thousand million gallons it is of great importance for scientists to find new applications for refined and crude glycerol (Yang et al., 2012). Recently, numerous papers have been published on direct utilization of crude glycerol from biodiesel production. Two main applications have been considered: animal feedstock and feedstock for chemicals.

Glycerol as a feed ingredient for animals dates back to the 1970s. This application has been limited by the availability of glycerol. Recently, the possibilities of using crude glycerol have been investigated because of the increase in the price of corn and the surplus of crude glycerol. Glycerol has high absorption rates and once absorbed it can easily be converted to glucose for energy production in the liver of animals by the enzyme glycerol kinase. The addition up to 15% (depending on the animal and the stage of its development) has been proved to have potential for replacing corn in diets. However, one must be aware of the presence of potential hazardous impurities in crude glycerol from biodiesel. For example, residual levels of potassium

may result in wet litter or imbalances in dietary electrolyte balance in broilers. The levels of methanol must be minimized because of its toxicity.

1,3-propanediol **(**1,3-PD) is a simple organic chemical and has a variety of applications in the production of polymers, cosmetics, foods, lubricants and medicines. Currently, the anaerobic fermentative production 1,3-PD is the most promising option for biological conversion of glycerol. Some works have already shown the production of 1,3-PD using crude glycerol as feedstock. Mu et al. 2006 demonstrated that crude glycerol could be directly converted to 1,3-PD without any prior purification by *Klebsiella pneumonia*. The final 1,3-PD concentration on glycerol from lipase-catalyzed methanolysis of soybean oil was comparable to that on glycerol from alkali-catalyzed process (53 and 51.3 g/l, respectively). This fact implied that the crude glycerol composition had little effect on the biological conversion and as such a low fermentation cost could be expected. *Clostridium butyricum* could also be used to produce 1,3-PD from crude glycerol, presenting the same tolerance to raw and commercial glycerol, when both were of similar grade, i.e. above 87% (w/v) (González-Pajuelo et al. 2005).

Ito et al. 2005 demonstrated the production of hydrogen and ethanol using an *Enterobacter aerogenes* strain. Crude glycerol had to be diluted with a synthetic medium to increase the rate of glycerol utilization and observed that the rates of H_2 and ethanol production from biodiesel wastes were much lower than the ones for the same concentration of pure glycerol, partially due to a high salt content in the wastes.

Rhodopseudomonas palustris bacterium was able of photofermentative conversion of glycerol, both pure and crude with nearly equal productions of hydrogen (Sabourin-Provost and Hallenbeck 2009).

Selembo et al. 2009 showed the conversion of glycerol into H_2 and 1-3-PD using anaerobic fermentation with heat-treated mixed cultures. In this study, the highest yields yet reported for both H_2 and 1-3-PD production from pure glycerol and the glycerol byproduct from biodiesel fuel production by fermentation using mixed cultures were achieved.

Pyle 2008 propose to use crude glycerol in the fermentation of the microalga *Schizochytrium limacinum*, which is a prolific producer of docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid with proven beneficial effects on treating human diseases such as cardiovascular diseases, cancers and Alzheimer's. For supporting alga growth and DHA production, 75-100 g/L of crude glycerol were recommended as the optimal range. Further, DHA-containing algae have been developed as replacements for fish oil omega-3 fatty acids (Yang et al., 2012).

Synthetic glycerol can be used by several bacterium to produce PHA (Nikel et al. 2008; Ibrahim and Steinbüchel 2009). Moralejo-Gárate et al. 2011 demonstrated the feasibility of mixed microbial cultures to produce PHA using synthetic glycerol attaining a PHB content of 80% (cell dry weight). The use of crude glycerol as a feedstock has been tested and the available literature is increasing. Ashby et al. 2004 reported a PHB content between 13 to 27% (cell dry weight) using *Pseudomonas oleovorans*. Mothes et al. 2007 attained 70% of PHB (cell dry weight) with *Cupriavidus nector* and Teeka et al., 2012 achieved a PHB content of 45% with a *Novosphingobium* genus. Additionally, Dobroth et al. 2011 explored the use of a mixed microbial consortia that was able to use exclusively the methanol fraction of the crude glycerol to produced PHB.

Beyond the chemicals mentioned above, several other processes for producing useful chemicals from crude glycerol via biotransformations have been developed. Crude glycerol can be used as a raw material for conversion into valued-added products such as: lipids; citric acid, succinic acid; butanol and glycolipid-type biosurfactants. Through conventional catalytic conversions oxygenated chemicals, hydrogen, syngas, monoglycerides propylene glycol and acetol have also been reported to be produced when using glycerol from biodiesel production (Yang et al., 2012).

2.4. POLYHYDROXYALKANOATES (PHA)

PHAs are a unique family of polymers used as intracellular carbon/energy storage for more than 300 species of Gram-positive and Gram-negative bacteria as well as a wide range of *Archaea*. When microbial cell are unable to grow at the same rate at which they can take up the carbon substrate, they store PHA in their cytoplasm as carbon and energy source. Growth restriction can be caused by limited availability of an external factor, such as nitrogen, phosphorus, sulphur or oxygen, or by an internal decline in anabolic enzymatic levels or activity (Q. Wang et al. 2009). Since these polymeric materials do not substantially alter the osmotic state of the cell they can be stored at high concentrations (up to 90% of the dry cell weight). (Laycock et al. 2013)

The mechanical properties of PHA are very similar to the ones of conventional plastics like polypropylene (PP) or polyethylene (PE) and can be extruded, molded, spun into fibers, made into films and used to make heteropolymers with other synthetic polymers. However, PHAs are bio-based polyesters, fully biodegradable and biocompatible which makes them very promising bulk materials for a significant number of industrial applications.

2.4.1. Economical and environmental relevance of PHAs

In the second half of the 20th century, plastics became one of the most universally used and multipurpose materials in the global economy. Today, plastics are utilized in more and more applications and they have become essential to our modern economy. With continuous growth for more than 50 years, global plastic production in 2012 reached 288 million tons - 2.8% increase compared to 2011 (Plastics-The fact 2013). The drawback of plastics is that they are synthetic polymers derived from fossil fuels that can persist in the environment for extended periods of time. As such, sustainable and environmental concerns are important issues that have gradually drawn attention since the last century.

The growing demands for more sustainable solution lead to a growing replacement of petroleum-based plastics by biopolymers. Bioplastics are a family of materials that are bio-based (produced from renewable biological sources), biodegradable, or both. In 2011 bioplastics production capacity achieved approximately 1.2 million tones and is expected to reach 6 million tons in 2016. (EBA, 2013)

Nowadays, some conventional plastics, such as polyethylene (PE) and polyvinyl chloride (PVC) can already be synthesized from renewable resources. However, although they are referred as bioplastics, they are not biodegradable. There is another type of bio-based plastics (e.g. starch plastics, cellulose polymers, polylactide acid (PLA)) that despite being biodegradable require some extra additives to improve their functionality. PHA are a third type of bioplastics made from 100% renewable resources without additives and fully biodegradable, enabling a so-called bio-based-to-biodegradable life cycle (Fig. 2.7). Therefore, PHA can be considered as the only fully bio-based and biodegradable plastic.

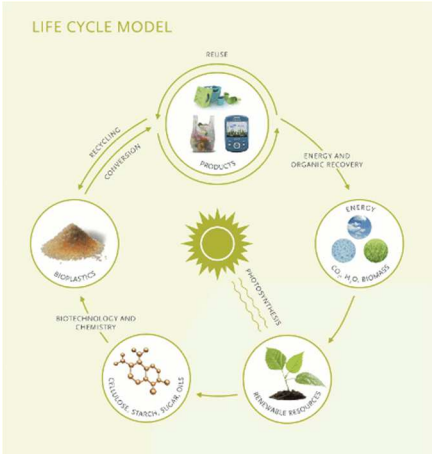


Fig. 2.7- Bioplastics closed loop life cycle (www.european-bioplastics.org)

Life Cycle Assessment (LCA) has become a powerful tool to critically evaluate and direct the overall impact of bioplastics and other bio-based products. Yates and Barlow 2013 review shows that the majority of studies that analyzed the LCA of PHA focused only on the consumption of non-renewable energy and global warming potential. These studies often found that the overall PHA production consumes more non-renewable energy and have higher global warming potential than the petrochemical derived polymers. In contrast, studies which considered other environmental impact categories as well as those which were regional or product specific often found that this conclusion could not be drawn. Despite some unfavorable results for these biopolymers, the immature nature of these technologies needs to be taken into account as future optimization and improvements in process efficiencies are expected.

2.4.2. PHA structure, properties and applications

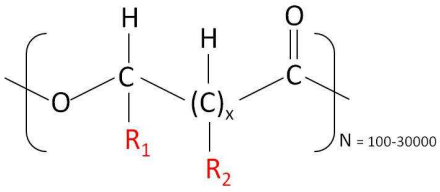
The basic structure of PHA has been identified as primarily linear, head-to-tail polyesters composed of hydroxyfatty acids monomers. To date more than 150 different PHA monomer units have been reported. According to the length of the carbon chains, PHA monomers can be classified into two major groups: (i) Short chain length (SCL) monomers composed by 3-5 carbon atoms. (ii) Medium chain length (MCL) monomers composed of 6-14 carbon atoms. The number of monomers in the polymer ranges from 100 to 30000.

PHA bio-synthetized have a much higher molecular weight than that achieved chemically. Molecular weights (Mw) typically range between 0.2×10^6 and 3×10^6 Da. When Mw is lower than 0.4×10^6 Da the mechanical properties of PHA deteriorate and for thermoplastic applications the value of Mw should be higher than 0.6×10^6 Da. Different bacteria produce P(3HB) with different Mw. Also, substrate type and concentration, nutrient availability and growth conditions such as pH and temperature play an important role and values of Mw as high as 2×10^7 Da have also been reported in mutant strains (Laycock et al. 2013).

PHA polymers properties are influence by several parameters: monomer composition, chain length of the polymer and the microstructure of the polymer (organization of monomers in the polymer chain: randomly or as block co-polymers). PHA are hydrophobic, water-insoluble, non-toxic material, inert and indefinitely stable in air. They also possess thermoplastic and/or elastomeric properties; have very high purity within the cell and a much better resistance to UV degradation than polypropylene. Generally, SCL PHA are more thermoplastic, whereas MCL PHA show more elastomeric properties.

The most common PHA are poly(3-hydroxybutyrate) (P3HB) and poly(3- hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)). The average properties for the homopolymer P(3HB)

are: transition temperature (Tg)≈ 5◦C (by differential scanning calorimetry (DSC)) or 12◦C (by dynamic mechanical thermal analysis (DMTA)); melting temperature (Tm) ≈ 176◦C; tensile modulus 2.9 GPa; tensile strength 37 MPa; maximum elongation to break ~4%. These properties induce a stiff and brittle material with high crystallinity witch has been reported as an obstacle to the practical applications of these materials. Significant research effort has been devoted to manipulating these mechanical properties. One of the solution for improving mechanical properties of PHA is to use copolymeric materials, such as P(3HB-co-3HV) with higher HV contents, or mcl- PHA copolymer. When the 3HV monomers are included in the P(3HB)-type lattice it is observed a "less-perfect" crystals with more defects, smaller crystalline domains and less brittleness. The result is a P(3HB-co-3HV) co-polymer with less stiffness and brittleness and an increased flexibility (higher elongation to break), tensile strength and toughness. (Laycock et al. 2013)



x			R_1	R_2	Name	Abbreviation
1	Poly (3-hydroxy-alkanoates)	SCL (C_3-C_5)	H	H	Poly (3-hydroxypropionate)	P(3HP)
			CH_3	H	Poly (3-hydroxybutyrate)	P(3HB)
			CH_2CH_3	H	Poly (3-hydroxyvalerate)	P(3HV)
			CH_3	CH_3	Poly (3-hydroxy-2-methylbutyrate)	P(3H2MB)
		MCL (C_6-C_{14})	CH_2CH_3	CH_3	Poly (3-hydroxy-2-methylvalerate)	P(3H2MV)
			$(CH_2)_2CH_3$	H	Poly (3-hydroxyhexanoate)	P(3HHx)
2	Poly (4-hydroxy-alkanoates)		H	H	Poly (4-hydroxybutyrate)	P(4HB)

Fig. 2.8- General structure of (PHAs) and examples of the most common monomers

The highly diverse PHA monomers pool (Fig. 2.8) allow for a broad range of application. Initially PHA were used as packing materials, such as shampoo bottles, shopping bags, diapers and cosmetic containers. However, the increasing interest on bio-based, biodegradable and biocompatible polymers PHA has been applied in areas such as industry, medicine and agriculture. The top companies in the PHA business include: Metabolix Inc. (US), Meredian INC. (US), Biomer (Germany), Tianjin GreenBio Materials Co. Ltd (China) and Shenzhen Ecomann Technology Co. Ltd (China). Today's PHA application includes packaging, food-services, agriculture/horticulture, consumer electronics, automotive and consumer goods and

household appliances. But there are a lot more markets starting to use PHA such as building and construction, leisure or fibre applications (clothing, upholstery). Lately, it has been reported an increasing application of PHA on biomedical field as implant materials and drug delivery carriers.

2.4.3. PHA biosynthesis

PHA can be chemical or biological synthesized. The biosynthesis of PHA gives rise to a much higher molecular weight polymer; however this approach does not allow much control over the monomer structures in the PHA (Chen 2010).

Several microorganisms are known to carry metabolic ability to biosynthesize PHA molecules including *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp. and *Methylobacterium* sp.. PHA are stored in the cell cytoplasm as granules. Typically, each cell contains 5-10 discrete granules with diameters ranging from 100 to 500 nm. Each PHA granule is surrounded by membrane coat composed of a phospholipid monolayer with embedded and attached proteins. These proteins include the enzymes involved in PHA synthesis and degradation as well as phasins and regulatory proteins. Phasins are the most abundant protein on the granule surface and their role seems to be related to regulating the size and number of PHA granules as well as stabilizing them (Grage et al 2009)

PHA biosynthesis is controlled by numerous genes encoding a range of enzymes that are directly or indirectly involved in PHA synthesis. Currently it is clear that nature has evolved several different pathways for PHA formation, each suited to the ecological niche of the PHA-producing microorganism. So far, PHA biosynthesis can be summarized in eight pathways (Fig. 2.9).

The first pathway (Pathway I) is the most well known and is represented by the model organism *Cupriavidus necator* (previously known as *Ralstonia eutrophus*). The PHB metabolism involves three key enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase, encoded by genes phaA, phaB and phaC, respectively. Initially the carbohydrates are converted into acetyl-CoA. β-ketothiolase condensate two units of acetyl-CoA into acetoacetly-CoA which is then reduced by the NADPH dependent acetoacetyl-CoA reductase into (*R)*-3-hydroxybutyryl-CoA. Subsequently R*)*-3-hydroxybutyryl-CoA is incorporated into the polymer chain by PHA synthase. Due to the stereo specificity of the enzymes involved, all microbially synthesized HA monomers are in the (*R*) configuration (Sudesh et al., 2000). Biosynthesis and degradation of PHA are a cyclic mechanism. In the depolymerization reaction the accumulated PHA is hydrolyzed into 3-hydroxybutyrate (3HB)

by depolymerase (encoded by phaZ), and can then converted back into acetyl-CoA. The associated pathway was also found in strains of *Aeromonas hydrophila*, *Pseudomonas stutzeri* and *Pseudomonas oleovorans* (Chen 2010).

Pathway II is associated with fatty acid uptake and several microorganisms (*Pseudomonas putida*, *Pesudomonas aeruginosa* and *A. hydrophila*) use this pathway to synthesize mcl-PHA. After fatty acid β-oxidation, acyl-CoA is converted to 3-hydroxyacyl-CoA which follows the PHA monomer synthesis process. Several enzymes are involved in this pathway including 3-ketoacyl-CoA reductase, epimerase and (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I.

In Pathway III substrates are converted into 3-hydroxyacyl-ACP to form PHA monomer 3-hydroxyacyl-CoA, leading to PHA formation under the action of PHA synthase. Enzymes 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonyl CoA-ACP transacylase (FabD) are involved in this pathway.

Pathway IV uses NADPH-dependent acetoacetyl-CoA reductase to oxidize 3-hydroxybutyryl-CoA. All the other pathways (V, VI, VII and VIII) are used to synthesis alternative copolymers. For example, pathways V and VII are used to synthesize P(4HB) by *Clostridium kluyveri* and *A. hydrophila 4AK4*, respectively.(Laycock et al. 2013)

Regulation of PHA metabolism can be performed at several levels. (1) pha gene expression due to specific environmental signals, such as nutrient starvation; (2) PHA synthetic enzymes activation by specific cell components or metabolic intermediates; (3) inhibition of metabolic enzymes of competing pathways and therefore enrichment of required intermediates for PHA synthesis; or (4) a combination of these. For example, during normal bacterial growth, β-ketothiolase from pathway I is inhibited by free coenzyme-A coming out of the Krebs (or TCA) cycle. However, when nutrients other than carbon are limited, acetyl-CoA cannot enter the Krebs cycle and the excess acetyl-CoA is channeled into PHA biosynthesis. If by other reasons growth is limited, protein synthesis stops leading to a high concentrations of NADH and NADPH which inhibits citrate synthase and isocitrate dehydrogenase, slowing down again the Krebs cycle, directing acetyl-CoA towards PHA synthesis (Laycock et al. 2013)

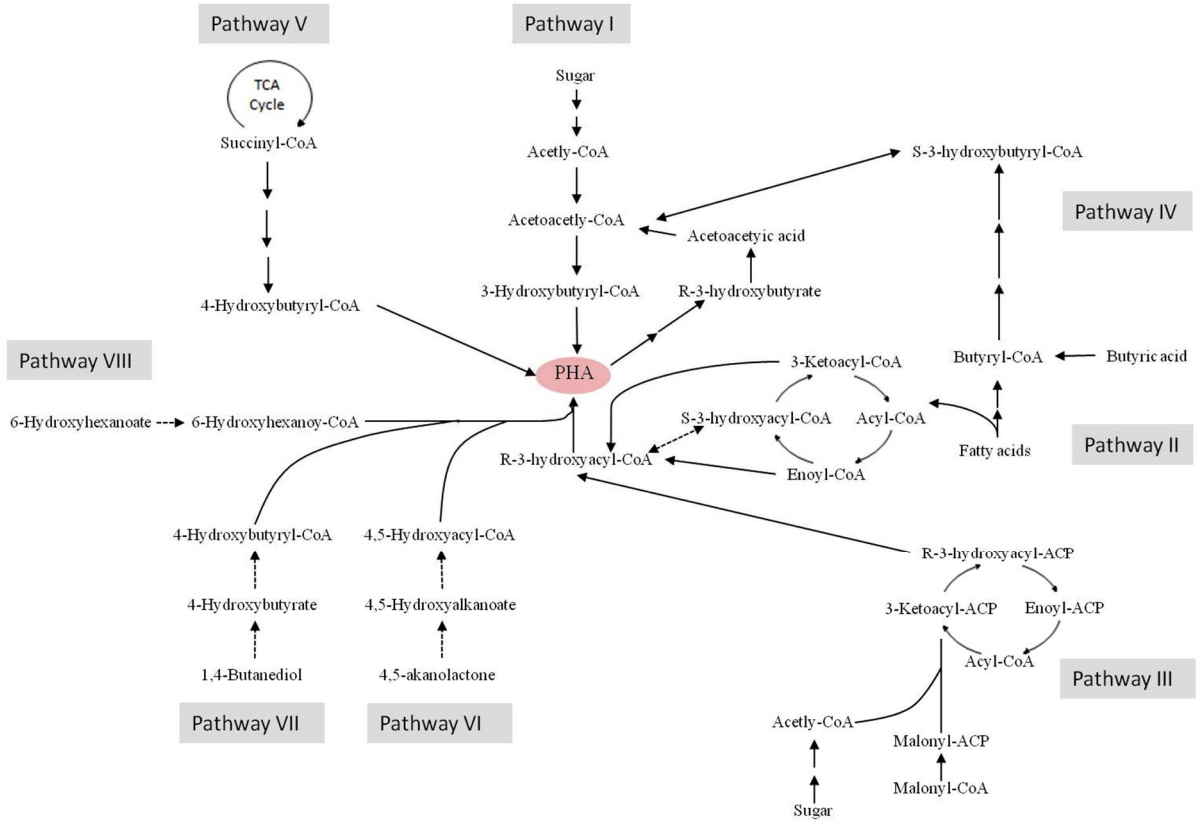


Fig. 2.9- PHA biosynthesis pathways. (adapted from Chen 2010)

2.4.4. Industrial PHA production by pure culture fermentation

Since the early 1980s many industries have made efforts to produce various PHA on pilot or industrial scales. Currently, about 14 companies engage in PHA production using either wild type PHA producers or genetically modified organisms. Despite the vast number of known PHA monomers only the homopolymer PHB, the copolymers P(HB-co-HV), P(HB-co-4HB) and P(HB-co-HHX) and mcl-PHA are produced at large scale.

PHA production involves several important steps: strain development, shake flask optimization, lab and pilot fermenter studies and then industrial scale up. Batch and fed-batch fermentations are typically used in the industrial processes. In fed-batch cultivation, medium composition can be controlled and high initial concentration of substrates fed can be avoided preventing a potential substrate inhibition. With this strategy high products and cell concentration can be achieved. The major limitation of fed-batch cultivation is the long downtime between two batches, which results in high operation costs (Chee et al. 2010).

Initially microorganisms are supplied with an optimized growth media to attain a high cell density. At this stage PHA accumulation is usually very limited. After the depletion of the growth medium, growth limiting conditions are imposed in order to induce PHA storage.

Usually limitation of oxygen or a macroelement, such as ammonia or phosphate, on the presence of carbon excess is the most used strategy to induce and maximize the PHA content of the biomass. When biomass reaches PHA saturation, the cells are recovered and disrupted in order to extract the intracellular biopolymer

The most commonly used wild type strain for the industrial production of scl-PHA is *Cupriavidus necator* due to its ability to accumulate large amount of PHB from simple carbon sources, for example, glucose, fructose and acetic acid (Khanna and Srivastava 2005). Usually this strain is fed with glucose or a mixture of glucose and propionate to produce PHB (Tianjin Northern Food Co. Ltd) or P(HB-co-HV) (Zhejiang Tian An Co. Ltd, China), respectively. The maximum PHA content achieved with this strain is 80% (cell dry weight) of PHB over 60h and 75% (cell dry weight) of P(HB-co-HV) over 48 h (Chen 2009).

Besides *C. necator*, other strains like *Alcaligenes latus*, *Aeromonas hydrophila*, *Pseudomonas oleovorans* and *Pseudomonas putida* are used as natural PHA producers. *A. lactus* was able to accumulate up to 50 wt % PHB on glucose or sucrose in 18h of growth. *A. hydrophila*, *P. oleovorans* and *P. putida* were used to produce mcl-PHA. *A. hydrophila* was employed for large scale production of P(HB-co-HHx) reaching a content of 50 wt%. *P. oleovorans* grown on n-alkanes was reported to produce 63 wt% PHA containing mcl monomers (Chen 2009) .

Genetically modified bacteria have also been employed for PHA production. From all strains recombinant *Escherichia coli* is an obvious choice due to its convenience for genetic manipulation, fast growth, high final cell density and ability to utilize inexpensive carbon sources. *E. coli* is not a natural PHA producer; however recombinant strains are able to produce both scl and mcl-PHA. A recombinant *E. coli* harboring *C. necator* PHA synthase genes reached a PHA content of up to 90% of the cell dry weight (Lee and Choi 1998).

An effective and sustainable microbial PHA production depends on several factors such as the final cell density, bacterial growth rate, percentage of PHA in cell dry weight, time taken to reach high final cell density, substrate to product transformation efficiency, price of substrates and a convenient and cheap method to extract and purify the PHA.

One of the main challenges concerning the replacement of the conventional plastics by PHA is the considerable high cost of the biopolymers. With the increasing financial investments made into production and marketing of bioplastics, PHA prices have been reduced in the last years. However, although the latest market price of Mirel™ (PHB) is quoted at about 1.50€/ kg, the average PHA prices are around 3€/ kg against 1€/ kg for petroleum-based plastic (Chanprateep 2010).

From all the cost associated to the PHA production, raw material accounts for 30-40%. In 2010, food waste were responsible for almost 14% of the total municipal solid waste stream (the second most abundant after paper), <3% of which was recovered and recycled. By the year 2020 it is estimated that annual food waste related emissions would be about 240 Mt. Recent strategies to lowering PHA production cost include the use of a broad range of waste and by-product streams associated with food at different stages of the production/utilization cycle as a sustainable feedstock (Nikodinovic-Runic et al. 2013). Several recombinant strains and wild-type PHA producers have been reported to produce PHA from cheap carbon sources. However, the polymer concentration and content obtained were considerably lower than those obtained using purified carbon substrates. Therefore, there is a need for development of more efficient fermentation strategies for production of these polymers from a cheap carbon source.

2.4.5. Low cost PHA production strategies – Mixed Microbial Cultures

Due to the great potential of PHA, it has been observed in recent years an increasing interest in investigating potential alternative processes that decrease the PHA production costs. These alternatives process include not only the use of low value substrates (waste or surplus feedstocks) but also the use of mixed microbial cultures (MMC).

In opposition to the pure culture process, MMC process operates under non-sterile condition. Since sterilization is not required several operation costs are reduce; less expensive materials can be used for reactor construction and the equipment for control can be minimized. In addition all the contamination and the genetic degeneration of genetically modified strain disadvantages associated to the pure cultures process do not exist in mixed culture process, since mixed culture processes are based on natural/ecological selection. Moreover, mixed culture can use a wide variety of complex substrates, even substrates rich in different compounds. Unlike most pure cultures, PHA storage by MMC is not induced by nutrient limitation (but by an internal growth limitation). This is particularly advantageous if industrial waste feedstocks containing compounds of undefined composition are used. As such, combining the use of low substrate and MMC to produce PHA not only will allowed a significant reduction on the production costs (more than 50%) (Reis et al. 2003) but also to increase the sustainability of PHA production.

In general, mixed cultures are a consortium of microbial population, wherein the total composition is unknown, which are selected in an open biological system by the imposed operational condition. Mixed microbial cultures have been used for decades in wastewater treatment plants (WWTP) to biological remove several nutrient such as phosphorus, nitrogen and sulphate. PHA accumulation in MMC has first observed in enhanced biological phosphorus

removal (EBPR) systems which are operated by alternating anaerobic/aerobic cycles. In these systems phosphate accumulating organisms (PAOs) uptake the external substrate and convert it to PHB with the aid of ATP and reduction equivalents generated by degrading of glycogen and polyphosphate during the anaerobic period. During the subsequent aerobic phase the external substrate is no longer available and PAOs use the stored PHB as carbon source to growth and replenish the polyphosphate and glycogen pool. In EBPR systems PAOs have a natural competitor, glycogen accumulating organisms (GAOs), which have the same metabolism than PAOs with the exception of phosphate removal capacity. In both groups of microorganisms, PHA synthesis plays an important role in their metabolism. Some studies have reported the use of GAOs to produce PHA mainly through the use of synthetic substrate (Dai et al. 2007, 2008; Bengtsson 2009). Through aerobic PHA accumulation, Bengtsson 2009 reported a PHA content of 60% (cell dry weight) using acetate as single carbon source. Studies that used complex fermented waste (paper mill wastewater and molasses) showed lower PHA contents, 42% and 32% (cell dry weight), respectively (Bengtsson et al. 2008a, 2010).

Activated sludge with PHA storage capacity was also observed in aerobic WWTP, where selectors for bulking control were introduced. In this process sludge is submitted to alternate periods of excess of carbon (in the selector reactor) alternated with substrate limitation (in the main reactor) favoring the selection of floc-formers with enhanced PHA storage capacity. This concept of aerobic “feast and famine” process was simulated in lab-reactors and confirmed to enhanced the capacity of MMC to store PHA (Majone et al. 1996). During the feast phase, ammonia and external substrate are consumed by the MMC for simultaneous growth and PHA storage. After external substrate exhaustion (famine phase) the previously stored PHA are consumed along with ammonia, indicating that intracellular biopolymer can be used as carbon and energy source.

It is widely accepted that PHA storage in MMC occurs when growth is restricted. In both strategies, anaerobic/aerobic (AN/A) process and feast and famine (FF) process, PHA storage occurs when growth is prevented. However, the mechanisms by which PAOs and GAOs accumulate PHA under AN/A conditions are different from those observed in aerobic FF process. In the first process, storage is mainly caused by an external growth limitation due to absence of an electron acceptor (oxygen, nitrate). In FF process, it is an internal growth limitation (insufficient intracellular components such as enzymes or RNA) that promotes PHA storage, since both electron donor and acceptor are present in the feast period (Serafim et al. 2008a).

Currently, FF process, also known as ‘‘aerobic dynamic feeding’’ (ADF), is the most well-studied PHA storage process. In these systems, microorganisms obtain enough energy for

storage, growth and substrate uptake from oxidizing a part of the substrate. Therefore, the enriched mixed microbial cultures are independent of poly-phosphate or glycogen synthesis, reducing the tendency to accumulate other storage compounds.

Satoh *et al.* 1999 and Takabatake *et al.,* 2000 have proposed microaerophilic-aerobic processes for selecting PHA producing cultures with low content of other types of storage compounds. In the first phase where the external substrate is still available was operated under microaerophilic conditions in order to limit growth and favor PHA storage. During the second, fully aerobic phase, PHA was used as carbon and energy source for growth and maintenance. Using this condition it was obtained an enriched culture with a maximum PHA content of 62%. However, PHA production was reported to be not stable.

2.4.5.1. PHA production process

Since the late 1990s, research related to PHA production under ADF conditions increased markedly. The processes by which MMC produce PHA might be operated in two or three steps, depending on the type of substrate used as a feedstock (reviewed by Dias et al. 2006; Serafim et al. 2008a) (Fig. 2.10). In the two-step process PHA-accumulating organisms are firstly selected using aerobic or anaerobic/aerobic conditions (step 2 in Fig. 2.10) and then PHA storage capacity of the selected culture is maximized in the PHA accumulation step (step 3 in Fig. 2.10). The physical separation of the culture enrichment stage from the PHA production phase allows for process optimization, as different optimal conditions were shown to be required in each step (Serafim et al. 2004; Dionisi et al. 2006; Albuquerque et al. 2007; Johnson et al. 2009). Once PHA storage has reached a saturation stage in the accumulation step, PHA is then extracted and purified. The two-step approach has been mainly applied when organic acids (e.g. acetate, propionate, butyrate, valerate or lactate) were used as feedstock for PHA production.

Recent research has focused on the use of waste-based substrates as feedstock for PHA production using MMC. Waste/surplus materials, such as food scraps (Rhu et al. 2003); municipal wastewaters and municipal activated sludge (Chua et al. 2003; Gurieff 2007; Mengmeng et al. 2009); olive oil mill effluents (OME) (Dionisi et al. 2005; Beccari et al. 2009); industrial wastewaters (Dionisi et al. 2006); palm oil mill effluents (POME) (Din et al. 2006); fruit cannery wastewater (Gurrief, 2007; Liu et al. 2008); sugar cane molasses (Albuquerque et al. 2007); fermented brewery wastewater (Mato et al., 2008); paper mill effluents (Bengtsson et al. 2008a; Jiang et al. 2012), glycerol (Dobroth et al. 2011) and bio-oil (Moita and Lemos 2012) have been tested for PHA production by mixed microbial cultures.

The majority of the waste-based feedstocks are carbohydrate-rich mixtures of compounds (sugar cane and olive oil mill effluents among others). Unlike pure cultures, MMC when submitted to ADF condition tend to store glycogen from carbohydrates instead of PHA (Carta et al. 2001; Dircks et al. 2001). A strategy to overcome this obstacle was to perform a previous acidogenic fermentation step (step 1 of Fig. 2.10) in order to transform the sugars (and other fermentable fractions) of the complex substrates into organic acids, such as VFA, which can be effectively stored as PHAs by mixed microbial cultures. Usually when waste-based substrates are used to produce PHA using MMC the three-step process is applied. Only a few works have reported the two-step approach applied with waste-based substrates (Gurieff et al. 2007 and Liu et al. 2008).

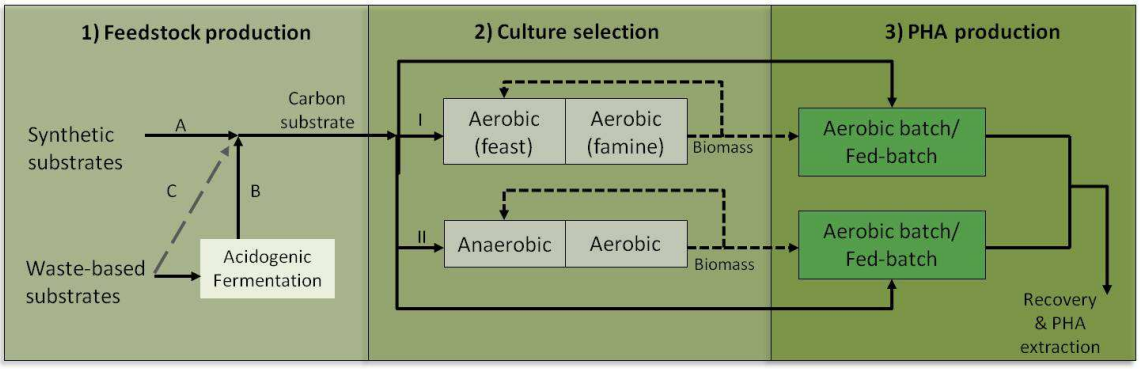


Fig. 2.10- Three-step PHA production process by MMC. Direct used of either synthetic (A) or waste-based substrates (C) or used of fermented waste-based substrates (B) to preformed culture selection using aerobic/aerobic (I) or anaerobic/aerobic (II) dynamic feeding strategies. PHA production (step 3) is carried out in batch/fed-batch mode using the cultures enriched in step 2 and the feedstock produced in step 1 (adapted from Serafim et al. 2008a).

2.4.5.2. Culture selection

The most important aspect for the development of a successful PHA production mixed culture process is culture selection. This stage is responsible for obtaining a culture highly enriched in organisms with high and stable PHA storage capacity. The presence of microorganisms with no PHA storing capacity or with low PHA contents has a negative impact not only on the productivity of the final accumulation stage but also on the downstream processing, increasing the PHA extraction costs. In addition stable highly enriched microorganism community allowed a stable PHA composition which is also highly desirable for future commercialization.

The most frequent reactor configuration used in MMC PHA selection is the sequencing batch reactor (SBR) since it can achieve the dynamic conditions of a feast-famine regime in a continuous way. However, as alternative, continuous reactors were also used in PHA production. Bengtsson et al. 2008a used two sequentially disposed continuous reactors

(mimicking the feast and famine phase) followed by a settler, simulating a WWTP configuration, to produce PHA from fermented paper mill effluent. Latter, Albuquerque et al. 2010a and co-workers compared the performance of culture selection of a SBR and a continuous system, similar to the one used by Bengtsson et al. 2008a, using fermented molasses as carbon source. The results obtained show similar results for PHA content, polymer yield on substrate and specific productivity in both configurations, supporting the possibility of using the existing facilities of WWTP for PHA production from industrial or municipal effluents.

In order to accomplish a good selective pressure, a large number of operational conditions were investigated by several authors (revised by Dias et al. 2006). Among others solid retention time (SRT), hydraulic retention time (HRT), carbon to nitrogen ration in the medium (C/N ratio), organic loading rate (OLR), temperature (T), substrate composition and pH are considered to be the most relevant.

The SRT imposes a selection pressure based on the growth rate of the biomass. Several authors have investigated the optimal SRT, however the results are inconclusive. Long SRT (10 days in average) have been reported for selecting a mixed culture with high storage yield (Serafim et al. 2004; Lemos et al., 2006; Albuquerque et al. 2007; Bengtsson et al. 2008a). However, different results have been observed by Chua et al. 2003 that showed that a mixed culture enriched at a SRT of 3 days accumulated more PHA than that at a SRT of 10 days. Recently, Johnson et al. 2009 and Jiang et al. 2012 have reported selected cultures with high PHA storage capacity (89% and 77% cell dry weight, respectively) using SRTs of 1 and 2 days respectively.

In FF systems the C/N ratio allows to control if the system is operated with carbon limited where growth could occur from PHA stored (famine phase) or under nutrient limited FF cycle where growth only occur in feast phase. Albuquerque et al. 2007 and Johnson et al. 2010a have demonstrated that nitrogen limitation is not favorable for the enrichment and long-term cultivation of PHA producing communities. In fact, in the latter work, it was suggested that the microorganisms with high PHA storage capacity have no advantage over those with low PHA storage capacity under the nitrogen limiting as the stored PHA during the feast phase could only be consumed for maintenance (usually negligible) during the famine phase.

In most studies pH is well controlled during culture selection and PHA accumulation step. Several authors have tested pH range from 6-9.5 (Chua et al. 2003; Serafim et al. 2008b; Villano et al. 2010). Usually higher pH is associated with higher PHA content in accumulation steps. Villano et al. 2010 have reported that PHA composition was strongly affected by the pH; HV content in the PHA composition increased with increasing pH.

Johnson et al. 2010b studied the temperature impact on PHA-producing mixed cultures using non-substrate limiting feeding strategy from 15-30ºC. Results showed that the mixed culture

selected at 30ºC presented a higher storage capacity. In addition, Jiang et al. 2011a demonstrated that the microbial community structure of the PHB-producing enrichments is strongly dependent on temperature: different temperature allowed to selected different dominant PHA producing organism during the culture selection step.

The OLR directly influence the biomass concentration since the higher the amount of carbon substrate supplied, the higher the cellular concentration obtained. Dionisi et al. 2006 and Albuquerque et al. 2010a tested different OLRs in PHA-accumulating culture enrichment SBRs (8.5 – 31.25 gCOD/L.d and 60-120 Cmmol/L.day, respectively). Both works presented the same findings; even though, as expected, the increase in the OLR caused an increase in biomass concentration it also caused a relevant decrease of maximal polymer production rate probably due to a substrate inhibition. The lowest OLRs revealed mixed cultures with the lowest PHA storage capacity probably due to substrate concentration limitation. As such, the best performance of the process was obtained at intermediate OLRs (20 gCOD/L.day and 90 Cmmol/L.day) where both biomass productivity and PHA storage were high enough.

All the operation conditions have a direct or indirect impact on the feast and famine length ratio (F/F ratio) which is considered as a determinant factor on the selection of a culture with good polymer accumulation capacities. Low F/F ratios (≤0.28) have been reported to allow the PHA accumulating organisms to outcompete with non-accumulating bacteria resulting in selected culture with good storage response. F/F ratios higher than 0.55 increase the growth response and the storage mechanisms start to be negligible. (Dionisi et al. 2006; Johnson et al. 2009; Albuquerque et al. 2010a; Jiang et al. 2011b)

2.4.5.3. PHA production

The common strategy used for the PHA accumulation step is the nutrient-limiting conditions during the entire step. Under this condition, the uptake of carbon is mainly driven for PHA storage until it reaches a saturation level inside the cell. As for culture selection, the evaluation of the maximum PHA storage capacity was performed mostly with pure substrates. Only recently real complex wastes were used to evaluate the storage capacity of the cultures selected.

The influence of the initial substrate concentration on the PHA storage has been mostly investigated to optimize the PHA accumulation step. In order to be able to achieve a saturation PHA content it is necessary to apply a high substrate to biomass ration. However, it has been shown that high substrate concentration can be inhibitory and limit the kinetics of substrate uptake and PHA storage (Serafim et al. 2004). Pulse feeding strategy has been widely used to prevent inhibition by the substrate. Alternatively, a continuous feeding strategy has also been

reported (Johnson et al. 2009; Albuquerque et al. 2011) which can only be applied when either a very high feed solution concentration can be used (so as not to affect the reaction volume) or if a membrane separation process can be attached to the bioreactor thereby continuously recirculating the cells back to the reactor.

In general, the results obtain with waste-based substrates are lower than those reports for mixed cultures using synthetic feedstocks. The highest values obtain with MMC and synthetic substrates were reported by Johnson et al. 2009 (89% PHB cell dry weigh with acetate) and Jiang et al. 2011b (92% PHB cell dry weigh with lactate). The majority of works that used waste-based substrates and MMC to produce PHA reported values between 20-48% PHA (cell dry weight). As an exception, Albuquerque et al. 2010b reported a PHA content of 75% (cell dry weight) using fermented molasses to fed MMC in a pulse feeding strategy. Later, using the same substrate but with a continuous feeding strategy, Albuquerque et al. 2011 reported a PHA of 77% (cell dry weight). Recently Jiang et al. 2012 have reported a PHA content of 77% of cell dry weight using MMC and paper mill wastewater as substrate. So far, these two works have reported the highest PHA content using MMC and waste-based substrates. The main different among them are the strategy used to fed the MMC and the feedstock used, resulting in different enriched microbial culture and different type of PHA produced. In the case of Jiang et al. only the homopolymer PHB was produced, on the case of Albuquerque et al. a copolymer (PHB-co-HV) was formed providing a higher broad range of application.

The gap existing between synthetic substrates and waste-based substrates can be justified, on one hand, by the fact real substrate typically contain organic matter other than VFA, even after acidogenic fermentation. This fraction is composed by different types of chemical species with different degrees of biodegradability which may include alcohols, unfermented sugars or compounds not susceptible to fermentation. This non-VFA fraction of the total organic matter present in this type of waste based feedstocks may be consumed by PHA-accumulating organisms but not serve as PHA precursors or eventually may be used for PHA storage but at different rates, or it might also be consumed by non storing organisms, which can have a negative impact on the maximum accumulation capacity of the selected culture. Furthermore, the presence of inhibitory compounds may also negatively affect the process kinetics

The composition of the substrate alone cannot account for the full gap between PHA production using MMC selected with synthetic feedstocks and those selected using fermented waste-based substrates. The impact of the type of feedstock on the enrichment reactor may condition the degree of enrichment obtained from these feedstocks, subsequently limiting batch accumulation performance in the final production step. Considering this, Dionisi et al. 2005 used a synthetic medium (ace+prop+lact) to select a culture with high PHA storage capacity and subsequently

fed with a complex feedstock (fermented olive oil mill effluent (OME)) only in the final accumulation stage achieving a PHA content of 54% (cell dry weight) which is higher than the most cultures selected using fermented feedstocks.

As mention before, VFAs are considered as the main precursors to produce PHAs from MMC. However, a few works have reported the direct used of non-VFA organic matter for PHA storage. Gurieff et al. 2007 obtain a PHA content of 20% (cell dry weight) with primary sludge and 39% with fruit cannery wastewater using a mixed culture enriched with primary sludge. Liu et al. 2008 reported a PHA content of 20% (cell dry weight) using tomato cannery wastewater. Moralejo-Gárate et al. 2011 has able to reach a PHA content of 80% (cell dry weight) using synthetic glycerol as substrate.

Mixed microbial cultures fed with synthetic feedstocks have reported very high specific productivity values (up to 1.97 g PHB/g X.h, Jiang et al. 2011b). This value is about 5 times the highest value reported for pure culture fermentations (0.38 g PHB/g X.h, Lee et al. 1999). These results are one of the major advantages in using MMC to produce PHA production. Since less biomass is necessary to obtain the same amount of biopolymer smaller bioreactors can be used reducing all the adjacent operation costs. However, one of the main drawbacks with using MMC is the low volumetric productivities compared to the ones obtained with pure cultures due to the lower biomass concentrations usually reached in these processes. The maximum cell concentration reported for ADF operated systems was 6.1 g/l (Dionisi et al. 2006), which is much lower than the obtained by pure cultures, usually above 100 g/l (Lee et al., 1999).

2.5. BACTERIAL COMMUNITY DYNAMICS

A stable bioreactor performance is usually achieved with microbial communities that are stable under normal operating conditions, but able to adapt in response to perturbations. As previous discussed, one of the main challenges in the mixed culture PHA production process is culture selection process. Despite the efforts, the consortiums of microorganism used in these processes are enriched in PHA-accumulating organisms but are not strictly composed by them. The selection of a stable culture with a high PHA storage capacity is of major importance for the effectiveness of the process.

During the last two decades, extensive efforts have been made in different research area to better understand all the important features related to the selective pressure imposed on culture enrichment. However, the microbial community responsible for these processes is often considered as a black box. Jiang et al. 2011a study the impact of temperature and cycle length on microbial competition between PHB-producing populations. Recently, two study

investigated a microbial community with high PHA storage capacity selected with fermented molasses; Albuquerque et al. 2013 investigated the substrate preferences of microbial groups in PHA production; and Carvalho et al. 2013 study the relationship between MMC composition and PHA production performance. The scare information on the microbial community found in the literature is not due to an underestimation of the biological component, but is caused by the limitations of methods available for the microbial identification and activity measurements.

Culture-dependent methods are based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays. During several years they have provided extensive information on the biodiversity of microbial communities in natural and engineering systems. However, these conventional methods provide incomplete knowledge about the physiological (nutritional and physical–chemical) needs and the complex syntrophic and symbiotic relations for most microorganisms in natural environments. Another problem is that most culture media tend to favor the growth of certain groups of microorganisms, whereas others that are important in the original sample do not proliferate (Sanz and Köchling 2007). Currently, it is generally accepted that culture-dependent methods are limited for studying natural microbial community composition, because only a small part of bacteria in environmental samples (less than 1%) are culturable under laboratory conditions (Amann et al. 1995; Head et al., 1998)

The possibility of identifying specific populations of microorganisms in their native habitat without the need to isolate them is revolutionizing microbial ecology. Recently, methods based on the 16S ribosomal RNA (rRNA) gene allowed to overcome the problems associated with culture-dependent methods. This gene is universally distributed and is a functionally indispensable part of the core gene set, supporting its used in phylogenetic studies. The presence of highly conserved regions enabled the design of suitable PCR primers or hybridization probes for various taxa at different taxonomic levels ranging from individual strains to whole phyla. However, some specific regions are subjected to variation. The presence of certain variable regions gives the 16S rRNA gene enough diversification to provide a tool for classification (Baldrian 2013). Nowadays, several non-cultured based methods are being used to study microbial communities. Among them denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), fluorescent in situ hybridization (FISH) and restriction fragment length polymorphism (RFLP) are some of the most used. Combining these molecular tools and PHA staining technique (e.g. Nile blue or Sudan black staining), can make the identification of PHA producing bacteria species precisely determined.

The correlation between the PHA storage capacity and culture identification would allow the design of reactor operating conditions favoring the most important PHA-accumulating microorganisms.

2.5.1. DGGE

DDGE is a molecular biology technique that has become a staple of environmental microbiology for characterization of population structure and dynamic. It allows separating highly conserved domains within the 16S rRNA gene but with different nucleotide sequences. Separation is based on the electrophoretic mobility of the PCR fragments in polyacrylamide gels containing a linear gradient of DNA denaturants (mixture of urea and formamide). Once a fragment reaches its melting point, migration will practically stop. Molecules with different sequences will stop migrating at different positions in the gel and correspond to different microbial species (Muyzer et al., 1993). The differential mobility of DNA molecules in the denaturing gradient is generally consistent with guanosine-citosine (G-C) content. In order to prevent a complete denaturation of the PCR products, which would result in a single-stranded fragments migrating out of the gel, a long GC-rich sequence (GC-clamp), generally 40 bases long, is incorporated at the 5' end of the forward primer, becoming incorporated into every amplicon generated during the PCR..

DNA bands in DGGE can be visualized using a nucleic acid stain, such as ethidium bromide, SYBR Green or GelGreen. For microbial species identification, the DGGE bands might be extracted from the gel and sequenced. Moreover, DGGE fingerprinting can be coupled to statistical analysis and calculation of biodiversity indices (e.g. principal component analysis (PCA), Simpson's and Shannon–Weaver indices, cluster analysis, etc.) can be used to compare bacterial communities over time or occurring in different environmental samples (Marzorati et al. 2008).

2.5.2. FISH

FISH, a cultivation-independent method, allowed the *in situ* analysis composition of microbial communities and their dynamics. In the recent years FISH has became widely used for the identification, quantification and characterization of phylogenetically (when combined with other techniques) defined microbial populations in complex environments.

This technique is based on the use of an oligonucleotide probe (binds), labeled with a fluorochrome (fluorescent dye) and whose sequence is complementary to a region in the target

microorganism. Under strictly controlled conditions, probes are allowed to hybridize with the complementary sequence of the target microorganism. Hybridized microorganisms will fluoresce under a fluorescence microscope, whilst microorganisms without a probe will not. Different probes, with different specificities, can be used together, resulting in the simultaneous detection of all bacteria present and of specific bacteria. Information on the identification, morphology, spatial relationship and abundance of different types of microorganisms can be obtained (Amann and Fuchs 2008)

CHAPTER 3

BIOPOLYMERS PRODUCTION FROM MIXED CULTURES AND PYROLYSIS BY-PRODUCTS

ABSTRACT

Polyhydroxyalkanoates (PHAs) production from low value substrates and/or byproducts represents an economical and environmental promising alternative to established industrial manufacture methods. Bio-oil resulting from the fast-pyrolysis of chicken beds was used as substrate to select a mixed microbial culture (MMC) able to produce PHA under feast/famine conditions. In this study a maximum PHA content of 9.2% (g/g cell dry weight) was achieved in a sequencing batch reactor (SBR) operated for culture selection. The PHA obtained with bio-oil as a carbon source was a copolymer composed by 70% of hydroxybutyrate (HB) and 30% of hydroxyvalerate (HV) monomers. Similar results have been reported by other studies that use real complex substrates for culture selection indicating that bio-oil can be a promising feedstock to produce PHAs using MMC. To the best of our knowledge this is the first study that demonstrated the use of bio-oil resulting from fast pyrolysis as a possibly feedstock to produce short chain length polyhydroxyalkanoates.

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3.1. INTRODUCTION

Over the last years, production of energy using renewable resources has gained importance, satisfying more efficiently the environmental concerns than fossil fuel. One of the most promising renewable energy sources able to fulfill the energy needs of the modern society is considered to be biomass. Fast pyrolysis is a good alternative technique to produce energy from biomass, being two-to-three times cheaper than conversion technologies based on gasification or fermentation processes (Vispute et al. 2010). The characteristic features of fast pyrolysis are the very high heating and heat transfer rates, a carefully controlled pyrolysis temperature, a rapid cooling of the products and a very short residence time at the pyrolysis temperature (typically less than 1s). The main product of this process, bio-oil, can be obtained in yields of up to 75 wt% on dry feed basis depending on the pyrolysis temperature. By-product char and gas are used within the process so there are no waste streams other than flue gas and ash. Bio-oil, can be used as a substitute for fossil fuels to generate heat, power and/or chemicals. Boilers and furnaces (including power stations) can be fuelled with bio-oil in the short term, whereas, turbines and diesel engines may become available on the somewhat longer term. Several chemicals can also be extracted or derived from the bio-oil including food flavorings, specialties, resins, agro-chemicals, fertilizers and emissions control agents. Upgrading of the bio-oil to a transportation fuel is technically feasible but needs further development (Bridgwater 2003). Bio-oil costs of production depend mainly on feedstock (pre-treatment) costs, plant scale, type of technology etc. However, because only exist a small number and limited scale of pyrolysis oil production units, the economics of a commercial scale unit can only be estimated. According to the European biomass industry association pyrolysis oils can be produced from 75 to 300 EUR per ton oil, assuming feedstock costs between 0 and 100 euros/t (EUBIA).

Alternatively, fermentation of bio-oil as a post processing biological approach can be applied. Bio-oil has a high carbon content that can be use as substrate for microbial conversions giving rise to high value products, such as bioplastics. Polyhydroxyalkanoates (PHA) are polyesters with similar properties to polypropylene and L,D-polyethylene but completely biodegradable, biocompatible and able to be produced from renewable resources. These biopolymers are stored inside the cells under stress conditions caused by limitation of a nutrient, electron donor or accepter, in the presence of carbon excess. Despite the effort for the development of less costly process with pure culture fermentation, commercialization of PHA is mainly limited to added value applications being their price four to nine times higher than that of the synthetic plastics (Serafim et al. 2008a). In order to develop more cost effective processes for PHA production several different strategies are being applied. One of them involves the use of microbial mixed cultures (MMC) combined with the utilization of low value substrates, as agro-industrial waste and by-products. This approach allows for a lower investment and operating costs for the global

process (Albuquerque et al. 2007; Bengtsson et al. 2008a; Dionisi et al. 2005; Rhu et al., 2003). One of the main problems with those strategies is the low PHA content achieved when compared with the ones reported for pure culture and synthetic substrates. However, recently a similar PHA content (74.6%) using MMC and fermented molasses was obtained (Albuquerque et al., 2010a). Culture selection with a high PHA storage capacity is one of the challenges in the mixed culture PHA production process. By operating the system sequentially under a carbon excess phase followed by substrate exhaustion a selective pressure is imposed to the system. Organisms that were able to store polymers during the feast are selected due to their capacity to use them as an energy and carbon source for cell growth and maintenance during the starvation period. Almost all the studies performed PHA production in two separated steps, first culture selection and then maximization of PHA content (Dionisi et al., 2004; Lemos et al., 2006; Serafim et al., 2004). Experimental conditions are imposed on the first step so that the selected cultures are able to drift almost all carbon for PHA storage. Usually this is achieved by nutrient restriction, being nitrogen limitation one possibility. Mixed cultures are referred as unable to store PHA from sugar-based compounds when submitted to feast and famine conditions (Carta et al., 2001). To overcome this problem some works reported a three-step production process, in which an anaerobic fermentation, with production of short-chain volatile fatty acids, precedes the culture selection and polymer accumulation steps (Albuquerque et al., 2007; Bengtsson et al., 2008; Dionisi et al., 2005).

Some studies report the used of pure cultures with pyrolysis products of petrol-derived residues to produce medium chain length PHA. As an example, from pyrolysis of polystyrene a styrene-enriched oil (82%) was obtained. This oil was later used by *Pseudomonas putida* CA-3 to produce PHA, being the obtained polymer comprised of monomers with 6, 8, and 10 carbons (Ward et al. 2006). Another work used the solid fraction obtained after pyrolysis of polyethylene terephthalate (PET) to generate terephthalate. This substrate was fed to *Pseudomonas sp*. in order to produce a PHA comprised of monomers with 8, 10, and 12 carbons, up to a maximum of 27% PHA content (Kenny et al. 2008). The main goal of this study was to evaluate the possibility of using bio-oil without any pre-treatment as carbon source for selection of PHA-accumulating cultures in an aerobic feast and famine system.

3.2. MATERIAL AND METHODS

3.2.1. Culture medium

The bio-oil used in this study was obtained from the fast pyrolysis of chicken beds. Fast pyrolysis was performed by BTG biomass technology group BV to BIO 73100 which supplied us with the bio-oil. Fast pyrolysis process was performed with reactor temperature around 550ºC, pressure around 2kPa and short residence time. Since bio-oil was obtained from an industrial partner more detailed conditions are protected for the moment.

Preliminary results revealed the presence of volatile fatty acids, different types of sugar and phenolic compounds as the majority of the components of this bio-oil. Phosphate and nitrogen concentration were 0.96g P/L and 38 gN/L, respectively. Chemical and Biochemical oxygen demand of the bio-oil were 742 gO_2/L of COD and 176 gO_2/L of BOD_5, respectively. Of the total BOD_5 present in the bio-oil, 37% (Cmmol/ Cmmol) were sugars.

The value of BOD_5 was used to plan the initial amount of carbon to be fed to the system. In this work we considered as easy biodegradable carbon the amount of BOD_5 measured. In order to achieve an organic loading rate (OLR) of 2 g COD/L.day of biodegradable carbon, bio-oil was initially diluted inside the reactor (1:176) with a mineral solution. Mineral solution was composed by (per liter of tap water): 600mg $MgSO_4 \cdot 7H_2O$, 50mg NH_4Cl, 100mg EDTA, 9mg K_2HPO_4, 20mg KH_2PO_4, 70mg $CaCl_2 \cdot 2H_2O$ and 2ml of trace elements solution. The trace solution consisted of (per liter of distilled water): 1500mg $FeCl_3 \cdot 6H_2O$, 150mg H_3BO_3, 150mg $CoCl_2 \cdot 6H_2O$, 120mg $MnCl_2 \cdot 4H_2O$, 120mg $ZnSO_4 \cdot 7H_2O$, 60mg $Na_2MoO_4 \cdot 2H_2O$, 30mg $CuSO_4 \cdot 5H_2O$ and 30mg of KI. Thiourea (10 mg/l) was added to inhibit nitrification. The pH of the mineral solution was adjusted to 8. Ammonia and phosphate concentrations in the mineral solution were calculated in order to keep the COD/N/P ratios (on a molar basis) at 100:5:1 in the reactor.

3.2.2. Reactor operation

A Sequencing Batch Reactor (SBR) with a working volume of 1500 mL was inoculated with activated sludge from Mutelas's wastewater treatment plant and acclimatized to the bio-oil as feedstock. The SBR 12 h cycles consisted of four periods: fill (10min); aerobiosis (feast and famine) (11h25min); settling (15 min) and withdraw (10min). The hydraulic retention time (HRT) and the sludge retention time (SRT) were kept at 1 and 10 days respectively. The SBR was fed with 1g COD/L per cycle of biodegradable carbon contained in the bio-oil. Air was sparged by a ceramic diffuser and stirring was kept at 250 rpm. pH was controlled to a minimum of 7.2 with NaOH 1M and the reactor stood in a temperature-controlled room (23–

25◦C). At given times, samples were taken periodically from the reactor, centrifuge and the liquid and solid fractions were used for determination of Total organic carbon (TOC) removal, sugar uptake, and PHA and glycogen storage, respectively.

3.2.3. Analytical methods

Biomass concentration was determined using the volatile suspended solid (VSS) procedure described in Standard Methods (APHA, 1995). Total organic carbon (TOC) from clarified samples was analyzed in a Shimadzu TOC automatic analyzer. Total sugars were determined using the Morris method (1948) with modifications. Samples were digested with an anthrone reagent (0.125 g anthrone in 100 ml sulphuric acid) at 100 ◦C for 14 min, and absorbance was measured at 625 nm. Glucose standards (0–100 mg/l) were used to determine total sugars.

Polyhydroxyalkanoate concentrations were determined by gas chromatography using the method adapted from Lemos et al (2006). Lyophilized biomass was incubated for 3h at 100ºC with 1:1 solutions of chloroform with heptadecane as internal standard and a 20% acidic methanol solution. After the digestion step, the organic phase of each sample was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Konik instruments HRGC-3000C). A ZBWax-Plus column was used with hydrogen as the carrier gas (50KPa). Split injection at 280◦C with a split ratio of 1:6 was used. The oven temperature program was as follows: 60◦C; then 20◦C/min until 100◦C; then 3◦C/min until 175◦C; and finally 20◦C/min until 220◦C. The detector temperature was set at 250◦C. Hydroxybutyrate and hydroxyvalerate concentrations were calculated using standards of a commercial P(HB-HV) (88%/12%, Aldrich) and corrected using a heptadecane internal standard.

Glycogen was extracted from lyophilized cells (approximately 2–3 mg) trough an acidic digestion (1 mL HCl 0.6 M, 2 hours, 100ºC). Samples were analyzed by High Pressure Liquid chromatography (HPLC) using an Aminex HPX- 87 H column (Bio-Rad Laboratories, CA, USA) and a Refractive Index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL/min, 60ºC). Phosphate was analyzed by HPLC using an IonPac AS9-HC column (Dionex, CA, USA) (Na_2CO_3 0.9 mM, 30ºC, 1mL/min) coupled with an electrochemical detector (Dionex, CA, USA). Chemical and Biochemical oxygen demand of the bio-oil were analyzed by an external certificated laboratory according to the methods SMEWW 5220-B and SMEWW 5210-B, respectively. Total Nitrogen (Kjedahl) analysis was performed using SMEWW 4500 Norg- A and B method by the same laboratory (APHA, 1995).

3.2.4. Microbial characterization

Biomass samples were fixed in 4% paraformaldehyde and used for fluorescence *in situ* hybridization according to Amman (1995). An estimation of the microbial composition was obtained by observing the cell biovolume of a specific probe in Cy3 against a broad probe covering all bacteria in FITC. The generic probe used was EUBmix containing a mixture of EUB338, EUB338II and EUB338III (Amann et al. 1990; Daims et al. 1999). The specific probes used with Cy3 were: ALF1b, BET42a, GAM42a for the identification of α-, β-and γ-Proteobacteria (Manz et al., 1992) and THAU832 (Loy et al. 2005) AZO644 (Hess et al. 1997), ZRA23a (Rosselló-Mora et al., 1995), AMAR839 (Maszenan et al. 2000) for the identification of *Thauera* spp., *Azoarcus* cluster beta, *Zooglea ramigera* and *Amaricoccus* (except *A. tamworthensis*) respectively, for four known PHA accumulating organism.

With the goal of evaluating the PHA accumulating capacity of the culture, Nile blue staining (Ostle and Holt 1982) was applied to fresh samples taken from the SBR near the end of the feast phase. Both FISH and Nile Blue samples were viewed using an Olympus BX51 epifluorescence microscope coupled to a CCD camera.

3.2.5. Calculations

The sludge PHA content was calculated as a percentage of VSS on a mass basis (% PHA=PHA/VSS*100, in g PHA/g VSS). Glycogen content was calculated as g Glucose/g VSS (%). VSS include active biomass (X), PHA and glycogen. For calculation purposes the value of active biomass was considered constant during all the cycle and was obtained by subtracting the amount of PHA and glycogen produced from the value of VSS. Active biomass was converted into COD according to a conversion factor of 1.42 mg COD/mg biomass (Henze et al 1995). PHA, glycogen and sugar were converted as mg/L of COD using the respective chemical oxidation equation. TOC was converted to COD by a mean ratio value of 2.65 gCOD/gTOC achieved by the analysis of several bio-oil samples .The maximum specific substrate uptake (-qS in g COD/g COD X.h) and PHA storage rates (qP in g COD HA/g COD X.h) were determined by adjusting a function to the experimental data of carbon uptake and PHA concentrations plotted divided by the biomass concentration at that point over time, calculating the first derivative at time zero.

PHA corresponds to the sum of HB and HV monomers. The yields of PHA on substrate (YP/S in g COD HA/g COD) were calculated by dividing the amount of PHA formed by the total amount of carbon consumed during PHA production.

3.3. RESULTS AND DISCUSSION

3.3.1. Reactor performance

According to daily cycles profiles after 167 day of operation a pseudo steady state condition was achieve. Fig. 3.1 shows the results of the SBR daily cycle in this day. At the first hour and half the carbon was consume, at a rate of 0.0934 Cmmol S/Cmmol X.h , along with PHA production. For the rest of the cycle, carbon was still consumed, along with PHA consumption, but with a much lower rate. The feast/famine behavior of the system was establish by the more easily biodegradable fraction of carbon present in the bio-oil since only this fraction was responsible for the PHA production. Although aerobic period of the cycle last about 11h, after 6.5h the carbon uptake can be considered negligible (data not show). Considering the situation, at day 167 the fraction of carbon consumed during the feast phase corresponds to 53% of the carbon consumed during all the cycle. Other carbon fractions were consumed by organisms that didn't have the ability to produce PHA but coexisted in the system. The maximum specific PHA accumulation rate (0.046 Cmmol HA/Cmmol X.h) and the highest storage yield (0.19 Cmmol HA/Cmmol S) obtained with bio-oil fall were on the range of those reported by other works that used MMC and complex substrates (0.00705 to 0.36 Cmmol HA/Cmmol X.h and 0.069 to 0.92 Cmmol HA/Cnnol S), respectively; Serafim et al., 2008). Comparison with literature values was made using parameters reported for batch accumulating assays used for optimization of the PHA storage capacity of the culture, rather than for the selection reactor as in this work since these values are scarcely available. A possible explanation for the specific PHA accumulation rate being in the lower range can be the complexity of the bio-oil matrix. Two effects can be considered: on one hand some of the compounds present in the matrix could inhibit the polymer accumulation and on the other hand a fraction of the carbon present could not be used for polymers synthesis by the selected culture. Another possible limitation is related to the fact that bio-oil contains a considerable amount of slowly biodegradable carbon, making it difficult to accomplish an ideal feast/famine conditions. This situation allows the presence of organisms that don't have the ability to produce PHA as well as decreases the selective pressure imposed to the system. Nevertheless, during culture selection the maximum PHA content observed (9.2% g/g cell dry weight) was is the same range as the ones observed for the selection step of other reported works (Albuquerque et al., 2007; Dionisi et al., 2004). PHA monomers composition was consistent during all the cycles (HB ≅ 70% and HV ≅ 30%). HPLC and GC-MS analysis of the bio-oil showed the presence of several volatile organic acids. Acetate was in majority but propionate was also identified. These organic acids are the main precursors of acetyl-CoA and propionyl-CoA and according to Lemos et al. 2006 these precursors can produce 3HV and 3HB units, one of the possible explanations for the production of a co-polymer P(3HB/3HV) from

bio-oil as a feedstock. Other constituents of the bio-oil that were consumed and used for PHA production had also to be converted either to acetyl or propionyl-CoA since no medium chain length PHAs were detected.

Along with PHA production, glycogen was also produce during the feast phase (Fig. 3.1). The highest glycogen content achieved was 2.9% g Glucose/g cell dry weight showing that the system is more specialized in PHA production. Of the total sugar consumed during the entire cycle 43% (g/g) was consumed over the feast phase. Since 90% (g/g) of this sugar uptake was converted into glycogen there isn't a real competition for this substrate in PHA production. Although 37% of the easily biodegradable carbon present in bio-oil was sugar when considering total carbon consumption of a daily cycle this value decreased to 10%. About one fourth of sugar remained in the systems without being utilized. In order to more efficiently utilize the available carbon sources the introduction of a pre-fermentation step (three step process) would optimize the system. Sugars in this initial step would be converted to volatile fatty acids, one of the preferred substrate for short chain length-PHA by mixed cultures. This strategy would in one end decrease the production of glycogen and on the other hand would increase the availability of substrate for PHA production.

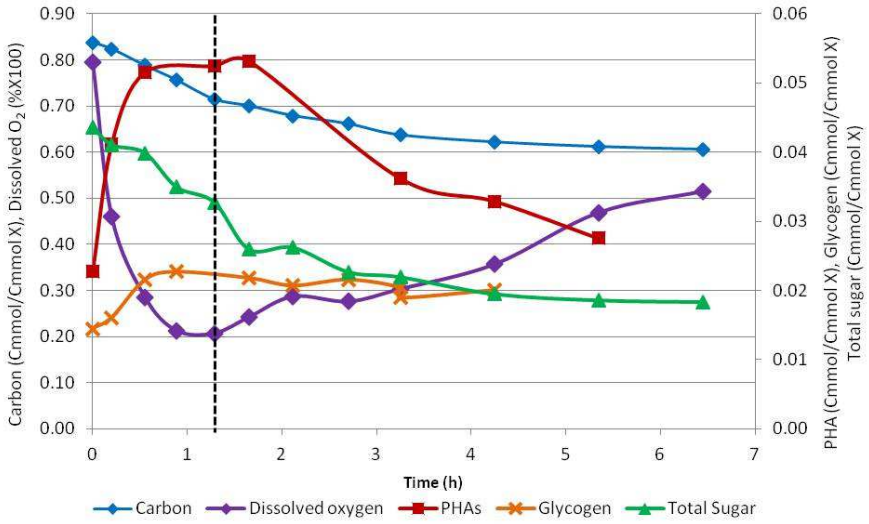

Fig. 3.1-Profiles at day 167 for carbon source, PHA, total sugar and glycogen of a daily SBR cycle

3.3.2. Culture acclimatization

Before reaching a pseudo steady state some changes were performed to the system in order to improve PHA production and culture selection. Fig. 3.2 shows the evolution of the culture performance during the acclimatization period. Improvement of the sludge capacity to use the complex matrix of bio-oil as carbon source to produce PHA can be seen by the increase on

specific substrate uptake rate observed, from 0.032 to 0.087 Cmmol/Cmmol X.h. Also, the increased ability of the sludge to accumulate polymers using bio-oil is denoted both on the enhanced specific PHA storage rate, from 0.0017 to 0.043 Cmmol HA/Cmmol X.h, and also on the augmentation of storage yield, from 0.055 to 0.57 Cmmol HA/Cmmol S.

After 61 days of SBR operation the system reached 6 g/L of biomass. However, no significant improvement in the capacity of the culture to consume the substrate and/or to accumulate PHA was observed along the time. Therefore, from this day on sludge retention time (SRT) was reduced to 5 days, keeping the hydraulic retention time always at 1 day. The expected decrease on the volatile suspended solids (VSS) concentration was observed, together with an enhancement on the performance of the culture to use bio-oil and to produce biopolymers. The decrease on the SRT imposed a selective pressure on the system, favoring organisms with high PHA storage capacity that led to an improvement on substrate uptake/PHA production capacity.

The initial feed was supplemented with nitrogen and phosphorus in a COD/N/P ratio of 100/5/1 molar basis. However, since bio-oil already contains N and P in it composition, after 156 days of operation, tap water started to be use do dilute bio-oil decreasing the COD/N/P ratio to 100/1.7/0.5. This modification can be of major impact in the decrease of production cost since no other nutrients have to be provided. The adjustment led to an increase on the VSS concentration along with an increased on the PHA storage capacity of the culture. As can be seen from the similar profiles obtained for experiments performed at day 167 and 227, five months after the beginning of reactor operation the culture achieved a pseudo steady state (Fig. 3.2)

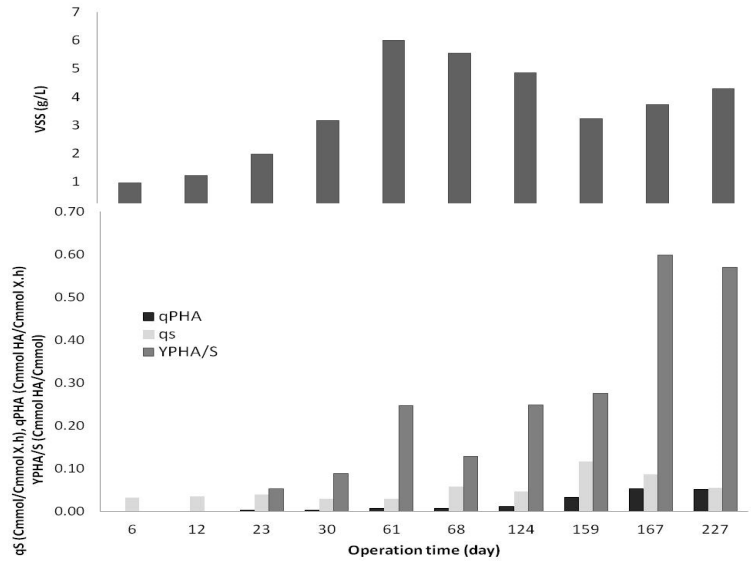

Fig. 3.2- Evolution of the culture performance during the acclimatization period showing volatile suspended solids (VSS) content, specific substrate (qs) and specific PHA (qPHA) rates and polymer yield on substrate (YPHA/S)

3.3.3. Identification of microbial community

Microscopic observation of the sludge was frequently done. Morphologically the bacterial community was mainly composed of cocobacilli together with some less significant cocci and thin filamentous (Fig. 3.3A). Nile Blue staining revealed the presence of PHA granules inside the majority of the bacterial community except for thin filaments.

To get some more information about the microbial composition of the sludge the microbial community was monitored by Fluorescence *in situ* hybridization (FISH) analysis. From the several FISH observation performed, two samples of the SBR bacterial community were characterized in more detail: one at day 117 that corresponds to middle of the acclimatization period and other at day 169 that represents the pseudo steady state (Table 3.1). In both days cells hybridized mainly with probe BET42a (80-90% total *Bacteria*, Fig. 3.3B, C) and with probes ALF969 and GAM42a in small proportions, showing the dominance of *Betaproteobacteria* over the *Alpha* and *Gamaproteobacteria*. The majority of the reported organisms able to produce sort-chain length PHA belong to *Betaproteobacteria* class.

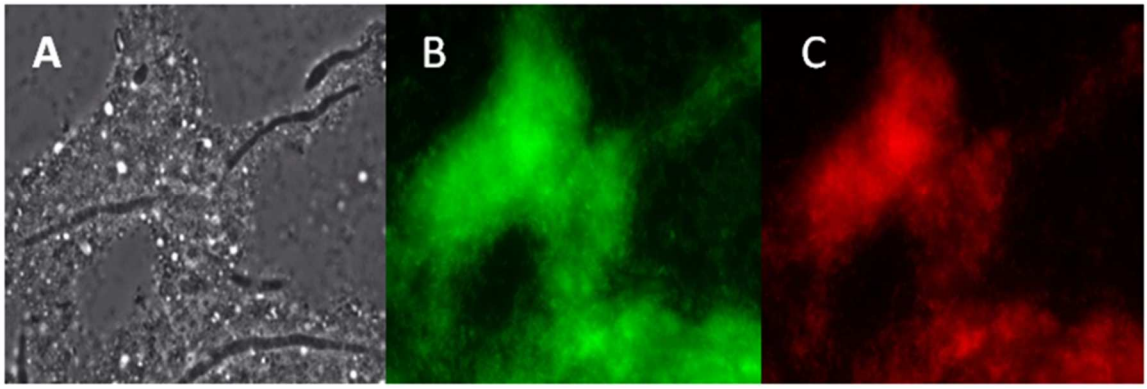

Fig. 3.3-Microscope images of the microbial culture obtained at the end of the acclimatization period. Phase contrast image (A); fluorescence images (B, EUBmix probes; C, specific probe BET42a). Magnification 1000×

Specify probes that hybridize with known PHA accumulating organisms (*Thauera*, *Amaricocus*, *Azoarcus* and *Zooglea* genus) were also tested. At day 117 of the four genus tested only *Thauera* was detected in the system. At day 169 *Amaricocus* and *Zooglea* genus were also detected in the reactor; however *Thauera* genus was still in larger number. This shift on the microbial community composition indicates that the operational condition imposed in the reactor were allowing the selection of specific PHA accumulating organisms able to use bio-oil as feedstock For other mixed culture systems *Azoarcus* genus has been identify as a microbial group able to store high amount of PHA (Serafim et al., 2006) however this genus was not identify in the current system.

Table 3.1- Evolution of the microbial community during acclimatization

FISH Probes	117 day	169 day
ALF969	+[a]	+
BET42a	+++	+++
GAM42a	+	+
THAU832	+	+
AZO644	-	-
AMAR839	-	+
ZRA23a	-	+

[a] +, positive hybridization; -, negative hybridization

3.4. CONCLUSIONS

During culture selection, the system achieved a maximum PHA content of 9.2% g/g cell dry weigh of a co-polymer composed of 70%:30% HB/HV. Glycogen was also produced as a carbon storage compound but in lower amount (2.9 % g glucose/g cell dry weight). Specific carbon uptake rate of 0.0934 Cmmol S/Cmmol X.h, specific PHA accumulation rate of 0.046 Cmmol HA/Cmmol X.h and storage yield of 0.53 Cmmol HA/Cmmol S parameters were in the same range as other studies that also use real complex wastes and MMC indicating that bio-oil can be a promising feedstock to produce short chain length PHAs.

Despite the high carbon content of the bio-oil a good Feast/Famine ratio was accomplished in the selective SBR, allowing a strong selective pressure. During the acclimatization step an enriched on PHA-storing organism was observed by FISH analysis. *Betaproteobacteria* was always the dominant class present in the system being *Thauera* spp. an important representative.

In this work 53% of the biodegradable carbon in bio-oil consumed during a cycle study contributes for the PHA production and 10% for glycogen formation, by the selected mixed culture. If an initial anaerobic step (three-step system) is introduced to the system the fraction used for glycogen synthesis together with 27 % of unused sugars content in bio-oil would be converted into carboxylic acids. With this conversion a higher content of carbon would be available to introduce in the selection or production steps increasing the overall efficiency of the system towards PHA production.

To the best of our knowledge this is the first study that demonstrated the use of bio-oil resulting from fast pyrolysis as a possibly feedstock to produce short chain length polyhydroxyalkanoates, composed of monomers with 4 and 5 carbons (HB and HV), using mixed microbial cultures.

CHAPTER 4

BIO-OIL UPGRADING STRATAGIES TO IMPROVE PHA PRODUCTION FROM SELECTED AEROBIC MIXED CULTURES

ABSTRACT

Recent research on polyhydroxyalkanoates (PHA) has focused on developing cost-effective production processes using low-value or industrial waste/surplus as substrate. One of such substrates is the liquid fraction resulting from pyrolysis processes, bio-oil. In this study, valorization of bio-oil through PHA production was investigated. The impact of the complex bio-oil matrix on PHA production by an enriched mixed culture was examined. The performance of the direct utilization of pure bio-oil was compared with the utilization of three defined substrates contained in this bio-oil: acetate, glucose and xylose. When compared with acetate, bio-oil revealed lower capacity for polymer production as a result of a lower polymer yield on substrate and a lower PHA cell content. Two strategies for bio-oil upgrade were performed, anaerobic fermentation and vacuum distillation, and the resulting liquid streams were tested for polymer production. The first one was enriched in volatile fatty acids and the second one mainly on phenolic and long-chain fatty acids. PHA accumulation assays using the upgraded bio-oils attained polymer yields on substrate similar or higher than the one achieved with acetate, although with a lower PHA content. The capacity to use the enriched fractions for polymer production has yet to be optimized. The anaerobic digestion of bio-oil could also open-up the possibility to use the fermented bio-oil directly in the enrichment process of the mixed culture. This would increase the selective pressure toward an optimized PHA accumulating culture selection.

The contents of this chapter were adapted from the publication: Moita, R., Ortigueira, J., Freches, A, Pelica, J., Gonçalves, M., Mendes, B., & Lemos, P. C. (2013). Bio-oil upgrading strategies to improve PHA production from selected aerobic mixed cultures. New biotechnology *31(4), 297–307.* doi:10.1016/j.nbt.2013.10.009[1]

4.1. INTRODUCTION

Economic, environmental and political concerns driven by the drastic reduction on available petroleum resources and their heterogeneous geographical distribution make it crucial to develop new processes for the production of renewable fuels. Lignocellulosic biomass is considered the most abundant and inexpensive sustainable carbon source (Vispute et al. 2010; Bridgwater 2003; Demirbas 2007). However, enzymatic and fermentative conversions of lignocellulosic feedstock into fuels are, at the moment, not economically feasible procedures. In the last decade biomass thermochemical conversion has been explored as an alternative method for the production of useful forms of energy. From the available thermochemical procedures fast pyrolysis was shown to be two-to-three times cheaper than conversion technologies based on gasification or fermentation processes. The main product of this process, bio-oil, can be obtained in yields of up to 75 wt% on dry feed basis.

Several chemicals, including food flavourings, resins and fertilisers can be extracted or derived from bio-oil. Also it has been accomplished the direct use of bio-oil to substitute fuel oils in many static applications such as boilers, furnaces engines and turbines for electricity generation (Bridgwater 2003). However, bio-oils are considered low-quality fuels that cannot be used in conventional gasoline and diesel fuel engines since they are immiscible with petroleum-derived fuels, primarily on account of their high oxygen content (up to 60 % (wt/wt)). Other challenges with pyrolysis oils are related with their acidity, high water content (25-50 wt %) and the occurrence of phase-separations chemical reactions upon storage. Ideally, pyrolysis oils, in particular those with high-polarity, could be deoxygenated to yield a mixture of organic molecules more stable and more compatible with the current fuels and the chemical manufacturing infrastructure (Vispute et al. 2010). The costs associated with this process increase drastically the price of the final products reducing potential use of those bio-oils as a substitute for petroleum-based fuels.

Polar bio-oils usually have high concentrations of alcohols, aldehydes, ketones, carboxylic acids and other polar components (Oasmaa and Peacocke) which makes them interesting substrates for microbial fermentations due to their good water solubility. Hence, fermentation of bio-oil as a post processing biological approach can be of interest. Bio-oils high content in low molecular weight polar components has motivated the interest in their use as substrate for microbial fermentations due to the good water solubility of those components. Several studies are focused in the use of sugars present in the bio-oil, especially levoglucosan, to produce ethanol (Chan and Duff 2010; Lian et al. 2010; Wang et al. 2012) and some triglycerides (Lian et al. 2010). However, in these studies, pure single strains (bacterial and yeast) were used and the bio-oil required a detoxification step in order to be metabolized by the organisms. More recently, Moita

and Lemos 2012 demonstrated the use of bio-oil, without any detoxification, resulting from fast pyrolysis of chicken beds as a possible feed to produce short chain length polyhydroxyalkanoates (PHAs) by a microbial consortium.

PHAs are a group of bioplastics naturally synthesized by several microorganisms, mostly as intracellular storage compounds for energy and carbon. Their thermoplastic and elastomeric properties are similar to those of a number of conventional commodity fossil based polymers making them very promising bulk material for a significant number of industrial applications. From the emerging bioplastics in the market, PHAs are the only polyesters that are fully biodegradable, biocompatible and able to be produced from renewable resources. With the recent growth of applications, manufacturers and financial investments in the production and marketing of bioplastics, the PHAs prices have been consistently reduced. Nevertheless, commercialization of bacterial PHA is still restricted to the use of pure cultures fermentations and high cost synthetic substrates making their price, in average, two times higher than that of the synthetic plastics (Chanprateep 2010). In the last decade, research has focused on several cost-saving strategies to reduce the PHA production price. Such strategies include the use of microbial mixed cultures (MMC), which avoid sterilization costs and simplify the process control. Also, low value substrates, such as agro-industrial waste and by-products, can be used as carbon sources for PHA production. This strategy allows the reduction of substrate costs and recycles end-of-life materials therefore avoiding the corresponding waste disposal costs. Several authors have already studied such joint strategies at lab-scale showing the potential for lowering PHA production costs (Albuquerque et al. 2007; Bengtsson et al. 2008a; Dionisi et al. 2005; Rhu et al., 2003).

One of the more challenging aspects in mixed culture PHA production is the ability to select a culture with high storage capacity. The most well know strategy for enrichment of MMC with PHA accumulation capacity is the sequential operation of the system under a short carbon excess phase followed by a long substrate depleted phase, known as “feast and famine” or “aerobic dynamic feeding” (ADF). This procedure selects organisms that have the capacity to convert available carbon into storage polymers during the short feast phase and use them during the starvation period as an energy and carbon source for cell growth and maintenance. The use of MMC to produce PHA almost always implies a two-step process. First, a selection step where experimental conditions imposed in the reactor allow the selection of a culture with a good and stable PHA storage capacity. Second, a production step where the maximization of PHA storage efficiency is attempted, usually through nutrient or electron donor restriction so that the selected cultures are able to direct most carbon resources for PHA storage. Carta et al. 2001 reported that mixed cultures are unable to store PHA from sugar-based compounds when submitted to feast and famine conditions. In this case an additional step precedes the culture

selection and polymer accumulation steps, making it a three-step process. This first step is an acidogenic fermentation that allows the production of volatile fatty acids (VFAs), the preferred substrates for PHA production by MMC, from the sugar fraction present in the substrate (Albuquerque et al. 2007; Bengtsson et al. 2008b; Dionisi et al. 2005).

In this study several strategies were investigated to improve the previously reported bio-oil valorisation through PHA production (Moita and Lemos 2012). Since bio-oil is a very complex carbon source the effect of the bio-oil matrix on the bacterial capacity to accumulate PHA was studied. The performance of bio-oil as substrate for PHA production was compared with production attained with defined substrates, namely acetate as representative of carboxylic acids, and glucose and xylose, representatives of C6 and C5 sugars, all of them present in the selection reactor. Two different process of bio-oil upgrading were performed: anaerobic fermentation and vacuum distillation. Fermented and distilled bio-oils were used as substrate in accumulation assays with the goal to maximize carbon utilization towards PHA production.

4.2. MATERIAL AND METHODS

4.2.1. Bio-oil composition

The bio-oil used in this study was obtained from the fast pyrolysis of chicken beds. Fast pyrolysis details were described in Moita and Lemos 2012. Chemical and Biochemical oxygen demand of the bio-oil were 742 g O_2/L of COD and 176 g O_2/L of BOD_5, respectively. The majority of the components detected in this bio-oil were organic acids (acetic, formic, propionic and butyric acid), different types of sugars (ribopyranose, ribofuranose, levoglucosans) as well as phenolic and other aliphatic compounds (unpublished results). Of the total BOD_5 present in the bio-oil, sugar accounts for 37% (Cmmol/ Cmmol). Phosphate and nitrogen concentration were 0.96 g P/L and 38 g N/L, respectively.

4.2.2. Experimental Setup

The set-up consisted of two bench-scale reactors. PHA-accumulating culture selection was carried out in an aerobic sequencing batch reactor (SBR). PHA accumulation assays were performed in a batch reactor under specific conditions.

4.2.3. PHA- accumulating culture selection

A SBR with a working volume of 1500mL was inoculated with activated sludge from the Mutelas's wastewater treatment plant and acclimatized to the bio-oil as feed-stock (Moita & Lemos 2012). Briefly, the daily 12h cycles consisted of: fill (10 min), aeration (11h 25min), settling (15min) and withdraw (10 min). Sludge retention time (SRT) and hydraulic retention time (HRT) were kept at 5 and 1 day, respectively. At the end of the aeration period, a purge of mixed liquor (150 ml) was performed using a peristaltic pump in order to keep the SRT at 5 days. The SBR was fed with 1g COD/L.cycle of biodegradable carbon (based on BOD_5) contained in the bio-oil. Air was sparged by a ceramic diffuser and stirring was kept at 250 rpm. pH was controlled to a minimum of 7.2 with NaOH 1M and the reactor stood in a temperature-controlled room (20–23◦C).

Bio-oil was initially diluted inside the reactor with tap water to a COD/N/P ratio of 100:1.7:0.5 (on a molar basis). The COD contribution was only based on the biodegradable fraction (BOD5). Nitrogen contribution was based on ammonia availability rather than total nitrogen. After 300 days of acclimatization, tap water was replaced by a supplemented tap water composed by (per litre of tap water): 50 mg NH_4Cl, 9 mg K_2HPO_4, 20 mg KH_2PO_4 and 10 mg thiourea was used to dilute the bio-oil, modifying the COD/N/P ratios to 100:5:1 in the reactor. At given times, samples were taken periodically from the reactor in order to determine the total organic carbon (TOC) removal, sugar uptake, nitrogen uptake, PHA and glycogen storage and volatile suspended solids (VSS).

4.2.4. Matrix influence on the accumulation capacity

To study the influence of the bio-oil matrix in the storage capacity of the selected culture, four different assays were performed. For each, the biomass was removed from the main SBR reactor immediately before the end of the daily cycle. In three of the assays, the collected biomass was separated from the liquid phase by decantation (D). In one of these assays half of the supernatant was removed and bio-oil was diluted with tap water (D1). In the second assay (D2) the entire supernatant was removed and bio-oil was diluted with tap water. In the third assay (D3) the entire supernatant was also removed but mineral salt medium was used to dilute the bio-oil. In a fourth assay the entire supernatant was removed by centrifugation(C) and the mineral salt medium was used to dilute the bio-oil. Specific conditions of each assay are summarized in Table 4.1.

The mineral salt medium used was composed of (per litre of tap water): 600 mg $MgSO_4 \cdot 7H_2O$, 100 mg EDTA, 9 mg K_2HPO_4, 20 mg KH_2PO_4, 70 mg $CaCl_2 \cdot 2H_2O$ and 2 ml of trace elements

solution. The trace solution consisted of (per litre of distilled water): 1500 mg $FeCl_3 \cdot 6H_2O$, 150 mg H_3BO_3, 150 mg $CoCl_2 \cdot 6H_2O$, 120 mg $MnCl_2 \cdot 4H_2O$, 120 mg $ZnSO_4 \cdot 7H_2O$, 60 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg $CuSO_4 \cdot 5H_2O$ and 30 mg of KI.

Table 4.1- Experimental conditions used to study the influence of the bio-oil matrix in the PHA storage capacity of the selected culture

Assay	Supernatant removal	Supernatant removal process	Bio-oil dilution
D1(control)	Half volume	Decantation	Tap water
D2	Clear phase	Decantation	Tap water
D3	Clear phase	Decantation	Mineral Solution
C	Totally	Centrifugation	Mineral Solution

4.2.5. Batch accumulation assays

PHA accumulation assays were carried out to determine the maximum PHA accumulation capacity, storage yield on substrate and production rate of the cultures enriched in the SBR. All the assays performed were carried out using sludge from the SBR after the system reached steady-state (stable at least after 3 SRTs). These assays were carried out in a 900 mL working volume reactor. In each assay 400 ml of the SBR content were collected at the end of the famine phase.

All the substrates used (pure bio-oil, acetate, glucose, xylose, distilled bio-oil and fermented bio-oil) were added to the system in a pulse-wise feeding method (30C-mM per pulse) to avoid potential substrate inhibition. In order to maximize storage, batch accumulation assays were carried out under nutrient limitation. A ceramic diffuser supplied air and magnetic stirring provided mixing. pH and oxygen uptake rate (OUR) were monitored over time. The determination of OUR was achieved by recirculation of the mixed liquor through a respirometer (using a peristaltic pump), where mixing was provided by magnetic stirring and an oxygen probe was inserted. Recirculation was stopped at given intervals and the decrease in dissolved oxygen concentration in the respirometer was registered and used to determine the OUR. The accumulation assays were conducted in a temperature controlled room (20–23 ºC).

4.2.6. Bio-oil upgrading

4.2.6.1. Bio-oil distillation

Bio-oil was distilled at reduced pressure in a temperature range from 58ºC to 80ºC. The larger distilled fraction (30% of the initial bio-oil mass) was isolated between 65ºC and 75 ºC. The distilled fractions were characterized by FT-IR to evaluate the functional groups present.

Further characterization was performed by GC-MS, after derivatisation with MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) in order to identify their main components.

4.2.6.2. Acidogenic fermentation

A continuous stirred tank reactor (CSTR; working volume of 1500 ml) was inoculated with sludge from an anaerobic digester from the Beirolas's wastewater treatment plant and acclimatized to the bio-oil feed. The system was fed with 1 g sugars/L present in the bio-oil, diluted inside the reactor with tap water supplemented with ammonia and phosphate concentrations in order to keep the COD/N/P ratio of 100:5:1 (on a molar basis). The bio-oil feed and the mineral nutrients solution flow rates were adjusted to keep the reactor hydraulic/sludge retention time at 2 days. pH was controlled to a minimum of 5.5 with NaOH 2M and the reactor stood at a controlled temperature of 30ºC. Mixing was kept at 250 rpm. The effluent was withdrawn by overflow and collected. Effluent was clarified using a filtration set-up composed of a peristaltic pump and an ultra filtration hollow fibber membrane module (CFP-1-E-5A). The clarified effluent was kept at 4ºC prior to its use in PHA batch accumulation assays.

4.2.7. Analytical Methods

Total suspended solids (TSS) and volatile suspended solid (VSS) were determined as described in standard methods (APHA, 1995). Total organic carbon (TOC) from clarified samples was analyzed in a Shimadzu TOC automatic analyzer. Total sugars were determined using the Morris method (1948) with modifications. Samples were digested with the anthrone reagent (0.125 g anthrone in 100 mL sulphuric acid) at 100ºC for 14 min and absorbance was measured at 600 nm. Glucose standards (0–100 mg/L) were used to determine total sugars.

Polyhydroxyalkanoate concentrations were determined by gas chromatography using the method adapted from. Lemos et al., 2006. Lyophilized biomass was incubated for 3.5 h at 100ºC with 1:1 solutions of chloroform with heptadecane, as internal standard, and a 20% acidic methanol solution. After the digestion step the organic phase of each sample was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Bruker 400-GC). A Bruker BR-SWAX column (60m×0.53mm×1µm) was used with nitrogen as the carrier gas (14.5 Psi). Split injection at 280ºC with a split ratio of 1:10 was used. The oven temperature program was as follows: 40ºC; then 20ºC/min until 100ºC; then 3ºC/min until 155ºC; and finally 20ºC/min until 220ºC. The detector temperature was set at 250◦C.

Hydroxybutyrate and hydroxyvalerate concentrations were obtained using standards of a commercial P(HB-HV) polymer (88/12 %, Aldrich).

Glycogen was extracted from lyophilized cells through acidic digestion (1 mL HCl 0.6M, 2h, 100ºC). Samples were analyzed by high-pressure liquid chromatography (HPLC) using an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA) and a Refractive Index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL/min, 60ºC).

Phosphate was analyzed by HPLC using an IonPacAS9-HC column (Dionex, CA, USA) (Na_2CO_3 0.9 mM, 30ºC, 1 mL/min) coupled with an electrochemical detector (Dionex, CA, USA). Chemical and biochemical oxygen demand of the bio-oil were analyzed by an external certificated laboratory according to the methods SMEWW 5220-B and SMEWW 5210-B, respectively. Initial total nitrogen (Kjedahl) analysis of the crude bio-oil was performed using SMEWW4500 Norg-A and B method by the same laboratory (APHA, 1995). The total nitrogen of the daily samples was analysed using the Laton Kits LCK 338 (Hang Lange).

Gases produced by the CSRT were determined by gas chromatography coupled to a thermal conductivity detector, (GC-TCD, Trace GC ultra, Thermo Electron Corporation). A Supelco Carboxen™ 1010 plot column (30m×0.53mm) was used with helium as the carrier gas (1 ml/min). Split injection at 200ºC with a split ratio of 1:10 was used. The oven temperature was 35ºC. The detector temperature was set at 220ºC.

4.2.8. Calculations

The PHA content was calculated as a percentage of TSS on a mass basis (% PHA=PHA/TSS*100, in g PHA/g TSS). Glycogen content was calculated as g glucose/g TSS (%). Active biomass (X) was obtained by subtracting to the VSS (g/L) the amount of PHAs (g/L) and glycogen (g/L). The complex matrix of the bio-oil made it difficult to directly analyse ammonium. To overcome this situation total nitrogen was determined instead and it was assumed that all the nitrogen consumed was used for growth since using thiurea inhibited nitrification. Active biomass was assumed to be represented by the molecular formula $C_5H_7NO_2$ (Henze et al., 1995). Substrate (S) concentration corresponds to total organic carbon in Cmmol S/L. PHA concentration (in Cmmol PHA /L) corresponds to the sum of HB and HV monomers concentrations (in Cmmol/L).

The maximum specific bio-oil uptake ($-q_S$ in Cmmol S/Cmmol X.h), nitrogen uptake (q_N in Cmmol N/Cmmol X.h), oxygen uptake (q_{O2} in Cmmol O_2/Cmmol X.h), PHA storage rates (q_P in Cmmol PHA/Cmmol X.h) and glycogen storage rates (q_{Glyc} in Cmmol Gluc/Cmmol X.h) were determined by adjusting a function to the experimental data for each variable

concentration divided by the biomass concentration at that point along time, and calculating the first derivative at time zero.

The yields of PHA ($Y_{P/S}$ in Cmmol PHA/Cmmol S), Glycogen ($Y_{Glyc/S}$ in Cmmol Gluc/Cmmol S) and active biomass ($Y_{X/S}$ in Cmmol X/Cmmol S) on substrate consumption were calculated by dividing the amount of each parameter by the total amount of substrate consumed.

The respiration yield on substrate ($Y_{O2/S}$ in Cmmol/Cmmol S) was calculated by integrating the curve of the experimental OUR (in mmol O_2/L h) over time and dividing the value thus obtained by the total amount of substrate consumed (in Cmmol S/L). Oxygen was expressed as carbon assuming that 1 mol CO_2 is formed for 1 mol O_2 consumed.

4.3. RESULTS AND DISCUSSION

4.3.1. Culture selection

Moita and Lemos 2012 described the performance of the culture selection in the first 227 days of operation. During this initial acclimatization period several changes were introduced in the system in order to improve not only the bio-oil consumption but also the PHA production. One of the last changes described was the dilution of the bio-oil only with tap water instead of supplemented mineral solution. With this change, the COD/N/P ratio was decreased from 100:5:1 to 100:1.7:0.5 (molar basis) and an increase on the VSS concentration (3.23 to 4.23 g/L) along with an increased on the PHA storage capacity of the culture was observed. However, after 250 days of operation the system lost the ability to accumulate PHA and the specific substrate uptake decreased from 0.063 to 0.036 Cmmol/Cmmol X.h. This decline led to an increase on the Feast/Famine (F/F) ratio. After the dilution of the bio-oil with tap water the F/F ratio was, on average, 0.2 but when tap water was used this ratio increased gradually reaching 0.4.Beccari et al. 1998 suggested that in F/F processes, cells physiologically adapt to long starvation periods by decreasing their primary metabolism. This physiological adaptation causes an internal growth limitation in the subsequent feast phase, which leads to the enhanced PHA storage response observed. If the F/F ratio increases considerably, physiological adaptation will occur to a lesser extent causing the selective pressure for PHA storage to decrease (Dionisi et al. 2006). Hence, the increase of the F/F ratio observed along with the lower storage capacity of the culture suggest that the storage response of the system started to have a negligible role and that the majority of the carbon was being consumed only for growth. For this reason, after 300 days of operation, the bio-oil feed started to be diluted with tap water supplemented with nitrogen and phosphorus in order to restore the COD/N/P to 100:5:1. After this change, the F/F ratio returned to 0.2 in average and the PHA storage capacity of the system was restored.

According to the daily cycles, the culture achieved a pseudo-steady state after less than 1 month of altering the COD/N/P ratio, with an average biomass value of 3.80 g VSS/L. Fig. 4.1 shows the typical behaviour of the selected culture during a representative daily cycle after 634 days of operation. Since, no significant changes were verified after 6.5 h of the cycle, only this fraction of time was relevant for the daily monitoring. As such, the end of a cycle corresponds to the beginning of the next one. The more easily biodegradable fraction of carbon present in the bio-oil was consumed during the first 2 hours of the cycle along with the PHA production and nitrogen uptake for growth. The remaining carbon fraction, despite being consumed during the entire cycle, had an uptake rate significantly lower and was not involved in the PHA production. The large variety of carbon present in the bio-oil allowed diverse microbial populations to co-exist in the system. Populations without the ability to store polymers were able to grow and persisted in the SBR throughout the consumption of the remaining nitrogen and the less biodegradable carbon fraction. Due to the fact that the carbon was not fully consumed, the F/F ratio of the system was calculated considering only the more easily biodegradable carbon fraction presented in the bio-oil. The sugar fraction existent in the bio-oil was totally consumed after 5 hours of the cycle and did not seem to contribute for the PHA production. Instead, this may be one of the possible substrates used by the microbial population unable to accumulate polymers. After 330 days of operation, under steady-state conditions, the SBR presented, on average, a specific substrate uptake rate and a specific PHA accumulation rate of 0.102 Cmmol/Cmmol X.h and 0.045 Cmmol HA/Cmmol X.h, respectively. The maximum PHA content reached was ≅7% (g/g cell dry weight) and the highest storage yield observed was 0.37 Cmmol HA/Cmmol S.

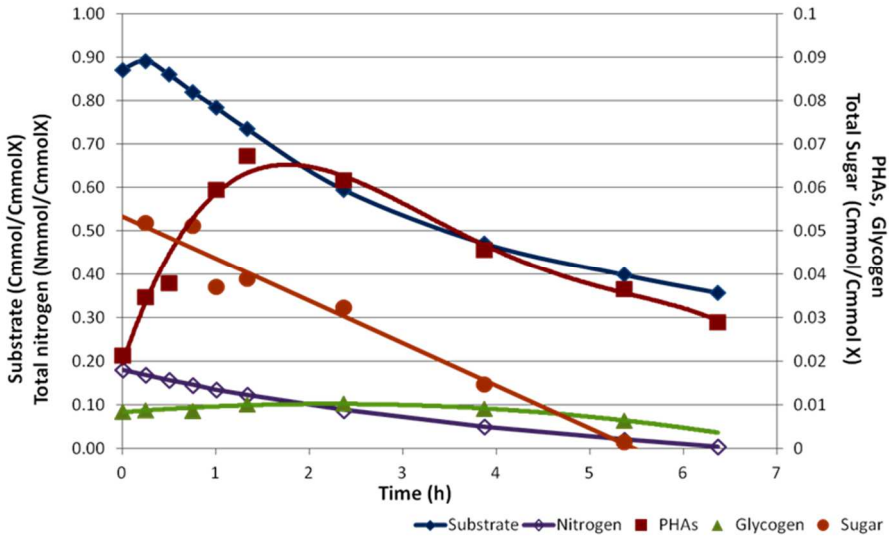

Fig. 4.1- Typical profile of a daily cycle of the reactor SBR operated under ADF conditions and fed with pure bio-oil at 30 Cmmol/L and a COD/N/P ratio of 100:5:1 molar basis (Xi = 124.12 Cmmol/L).

4.3.2. Effect of the bio-oil matrix in the PHA storage response of the enriched culture

Bio-oil is a very complex matrix with very different organic and inorganic substrates. Compounds that were not consumed tended to accumulate during the daily cycles and may have interfered with the maximum PHA storage capacity of the selected culture. Therefore, the effect of the bio-oil matrix was tested in four different PHA accumulation assays using pure bio-oil as substrate. Two different separation methods were used to remove the supernatant: decantation and centrifugation. Tap water or mineral solutions were used to dilute the bio-oil. Table 4.1 summarizes the study conditions for each test.

Fig. 4.2 shows that biomass centrifugation along with complete removal of the supernatant (assay C) increased significantly the specific substrate uptake rate and the specific PHA production rate. However, the storage capacity of the culture was compromised since it generated the lowest PHA production yields of the four assays. The low concentration or even absence of certain compounds, usually present in the residual matrix, could explain that behaviour but that same fact could have influenced the capacity of the biomass to accumulate PHA. Furthermore, centrifugation is a tough separation technique that may have contributed for the weak PHA accumulation capacity observed. All decantation assays had a similar specific substrate uptake rate and specific PHA production rate. D2 assay revealed the highest polymer/substrate yield and PHAs production. D1 assay was considered as the control since the conditions imposed in the batch test mimic the ones imposed in the SBR. The lower PHA storage capacity observed in this assay, when compared with the D2, might result from the fact that some compounds that still remained in the matrix may inhibit or interfere with the production of PHA.

During the selection of the PHA accumulating culture, the use of the tap water supplemented with ammonium and phosphorus to dilute the bio-oil seemed to be absolutely necessary to maintain, as previous discussed, the storage capacity of the microbial populations. When the same condition was applied in D3, it did not achieve comparable results. Hence, the removal of the residual supernatant by decantation and the limitation of nutrients provided by the dilution of the bio-oil only with tap water favoured the storage capacity of the selected culture in batch tests. Further on, in order to achieve maximum storage capacity of the selected culture when bio-oil was used as substrate, the supernatant was almost completely removed by decantation and only tap water was used to dilute the bio-oil in further accumulation assays (D2 conditions).

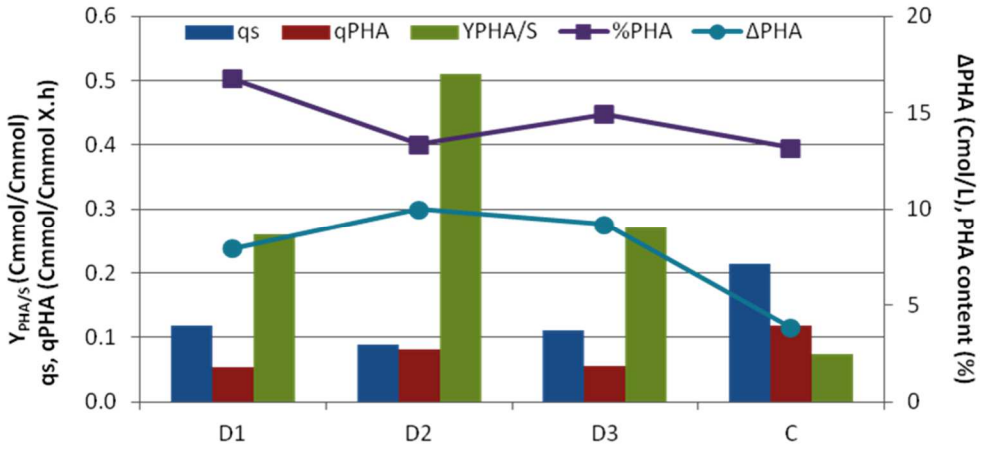

Fig. 4.2- Study of the influence of the bio-oil matrix in the PHA storage. Assays D: Supernatant removal by decantation D1: half of the supernatant removed and bio-oil diluted with tap water; D2: total supernatant removed and bio-oil diluted with tap water; D3: total supernatant removed and bio-oil diluted with mineral solution. Assay C: Total supernatant removed by centrifugation and bio-oil diluted with mineral solution

4.3.3. PHA storage capacity of the selected culture

Several batch production tests under nutrient limiting condition were performed in order to assess PHA accumulating capacity of the SBR enriched culture. Due to the carbon complexity of the bio-oil and to better understand the contributions of different carbon sources present in this substrate, several model compounds were tested. The effect in the PHA storage capacity of the culture was monitored through several kinetic studies comparing pure bio-oil with model substrates as acetate, glucose and xylose.

4.3.3.1. PHA accumulating assay using pure bio-oil as substrate

To determine the maximal PHA storage capacity of the selected culture when pure bio-oil was used as substrate, 3 consecutive pulses were added (≈ 3x25 Cmmol/L) in a total period of 6 hours. In this accumulation assay the pure-bio-oil was diluted only with tap water. Although ammonia was not added, complete nitrogen absence could not be achieved since bio-oil contains nitrogen in its composition. In each bio-oil pulse ≈18 Nmmol of total nitrogen per litre was added to the system, preventing a complete growth inhibition.

Fig. 4.3 shows the result of a typical batch study using pure bio-oil as substrate for PHA production. PHAs were mainly produced during the first two pulses, reaching a maximum PHA content of 9.8 % (g/g cell dry weight). A final PHA monomer composition of 75 % of HB and 25% of HV was achieved. Despite the drastic decrease of the specific PHAs accumulation rate in the second pulse (Table 4.2), the storage yield was maintained almost constant (0.31-0.32

Cmmol HA/Cmmol S). The PHA accumulation rate and the storage yield obtained with pure bio-oil (1º pulse) as a substrate were between the values reported by other studies that use MMC and real complex substrates, such as fermented olive oil mill effluents, fermented paper mill effluent and fermented molasses (Albuquerque et al., 2010a; Bengtsson et al. 2008a; Liu et al. 2008; Beccari et al. 2009). The maximum PHA content achieved with pure bio-oil was much lower than the ones reported in the previously mentioned studies. Unlike the other real complex substrates tested to produce PHA, pure bio-oil contains a lower VFAs content, the main precursors to produce PHA from MMC, which could explain the low PHA content obtained.

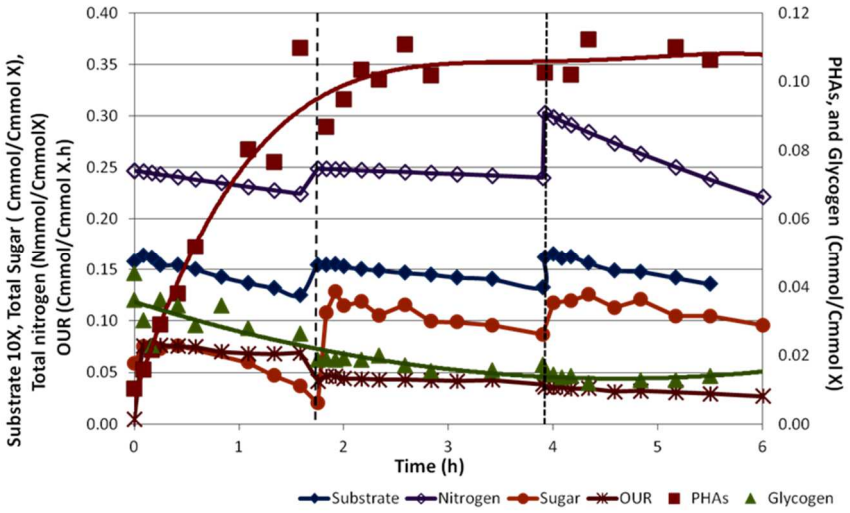

Fig. 4.3- PHA accumulation assay using the selected culture in the SBR and pure bio-oil as a substrate (three consecutive pulses of 30 Cmmol/

During the third pulse of pure bio-oil PHA storage was considered negligible. However, the substrate was consumed with a higher rate than the one observed in the preceding pulse (Table 4.2). Specific nitrogen uptake rate along with respiration yield also increased drastically in this last pulse. Actually, the amount of nitrogen consumed in this last pulse was at least ten-fold higher than the nitrogen consumed in the remaining pulses. These results suggest that all the carbon consumed was drifted towards growth and respiration.

Table 4.2- Stoichiometric and kinetic parameters of the accumulation assays

Substrate	$-q_s$	q_p	$-q_N$	PHA_{max}	X_i	$Y_{PHA/S}$	$Y_{O2/S}$	$Y_{X/S}$	PHA comp. (%HB :%HV)
Pure Bio-oil									
1º Pulse	0.25 (0.035)	0.058 (0.012)	0.0143 (0.0002)	8.87	72.06	0.31	0.35	0.16	
2º Pulse	0.10 (0.015)	0.027 (0.006)	0.0041 (<0.0001)	9.79		0.32	0.48	0.10	75:25
3º Pulse	0.18 (0.041)	-	0.0395 (0.0007)	7.64		-	0.65	nd	
Acetate									
1º Pulse	0.21 (0.017)	0.073 (0.016)	-	15.74	87.38	0.42	0.24	-	
2º Pulse	0.27 (0.073)	0.068 (0.025)	-	25.85		0.43	0.16	-	100:0
3º Pulse	0.20 (0.033)	0.070 (0.033)	-	32.47		0.31	0.27	-	
Distilled Bio-oil									
1º Pulse	0.14 (0.027)	0.051 (0.007)	0.0059 (<0.0001)	9.35	81.21	0.47	0.41	0.21	
2º Pulse	0.19 (0.039)	0.065 (0.012)	0.0229 (0.0007)	15.23		0.46	0.45	0.41	83:17
3º Pulse	0.27 (0.056)	0.047 (0.027)	0.0350 (0.0001)	16.76		0.16	0.49	0.52	
Fermented Bio-oil									
1º Pulse	0.23 (0.019)	0.137 (0.025)	0.0286 (0.0005)	10.58	144.67	0.63	0.12	0.27	73:23
2º Pulse	0.17 (0.026)	0.052 (0.012)	0.0323 (0.0007)	16.83		0.53	0.21	0.48	

(st desviation); nd: not determined;
$-q_S$ (C-mmol S/C-mmol X.h); q_p (C-mmol HA/C-mmol X.h); $-q_N$ (N-mmol /C-mmol X.h); PHA_{max} (% g/g cell dry weight);
X_i active biomass at the beginning of the assay (Cmmol/L); $Y_{PHA/S}$ (Cmmol HA/Cmmol S); $Y_{O2/S}$ (Cmmol/Cmmol S) and $Y_{X/S}$ (Cmmol X/Cmmol S)
PHA composition (%HB:%HV): Overall monomer produce during the entire assay

The presence of nitrogen in the bio-oil may have led to growth stimulation during the entire accumulation assays. In fact, the drastic increase of the specific nitrogen uptake rate on the third pulse supports this hypothesis, allowing the culture enough time to drift their metabolism, preferably, to growth in detriment of PHA storage. Hence, it is unclear if, after the consumption of 50 Cmmol/L of biodegradable carbon present in the bio-oil, the maximum PHA storage capacity has been reached.

Carta et al. 2001 demonstrated that mixed cultures tended to accumulate glycogen from sugar-based compounds instead of PHAs when submitted to feast and famine conditions. In this study we verified that the sugar fraction present in the bio-oil was consumed along with glycogen uptake and PHA production. The glycogen fraction present in the culture was slowly consumed, essentially in the first hours of the assay (q_{gly}=0.007 Cmmol/Cmmol X.h). It was unclear if glycogen consumption contributed to the PHA production. Still, as discussed previously,

populations unable to accumulate PHAs can co-exist in the system and may be responsible for the consumption of the sugar-based compounds registered during the entire assay.

During the accumulation assay all the biodegradable carbon present in the bio-oil (≅ 23%) was consumed. However, an important carbon fraction remains in the effluent along with nitrogen and other organic and inorganic compounds. Chemical extraction from this effluent (ex. phenolic compounds) could be a strategy to add value to the overall PHA production process by extending the overall degree of substrate valorisation.

4.3.3.2. PHA accumulating assay using acetate as substrate

Volatile fatty acids (VFAs) are considered to be the best substrates for PHA accumulation by mixed cultures. Serafim et al. 2004 showed that mixed microbial cultures subjected to dynamic feeding conditions using acetate as the carbon source may accumulate PHB up to 65% cell dry weight. From all the organic acids already identified in the bio-oil, acetate was the most abundant. Fig. 4 shows the accumulation capacity of the selected culture using acetate as the only substrate. Three pulses of 30 Cmmol/L were added, during 8 hours of assay, where a maximum PHA content of 32% was achieved. Only the HB monomer was produced in this assay.

The three pulses of acetate achieved very similar specific acetate uptake rates and specific PHA production rates, on average 0.23 Cmmol S/ Cmmol X.h and 0.071 C mmol HA/C mmol.h, respectively. The overall yield of PHB on acetate was 0.40 Cmmol HB/Cmmol. (Johnson et al. 2009; Jiang et al. 2011c) reported higher kinetic and stoichiometric parameters using acetate to produce PHA with mixed cultures. In both studies an overall storage yield of 0.60 Cmmol HB/Cmmol S and PHA contents higher than 87% were reported. The culture used in the referred studies was acclimatized in the selection reactor with acetate as carbon source, which may have contributed to the higher rates and yields observed in their accumulating assays. Fig. 4.4 shows that, despite the slight decrease on PHB storage yield verified on the third pulse (Table 4.2), the maximal PHA storage capacity of the culture was not reached in this assay after the consumption of 90 Cmmol/L of acetate.

The specific substrate uptake rates of the first pulse in both acetate and pure bio-oil assays were similar. However, in the bio-oil assay, a significant decrease of those rates was observed in the following pulses. This observation supports the idea that some of the bio-oil components may negatively influence the substrate uptake. The amount of carbon used in the pure bio-oil experiment to produce both biomass and polymer was in the same range of the total carbon used for PHA production when acetate was used as substrate (Table 4.2).

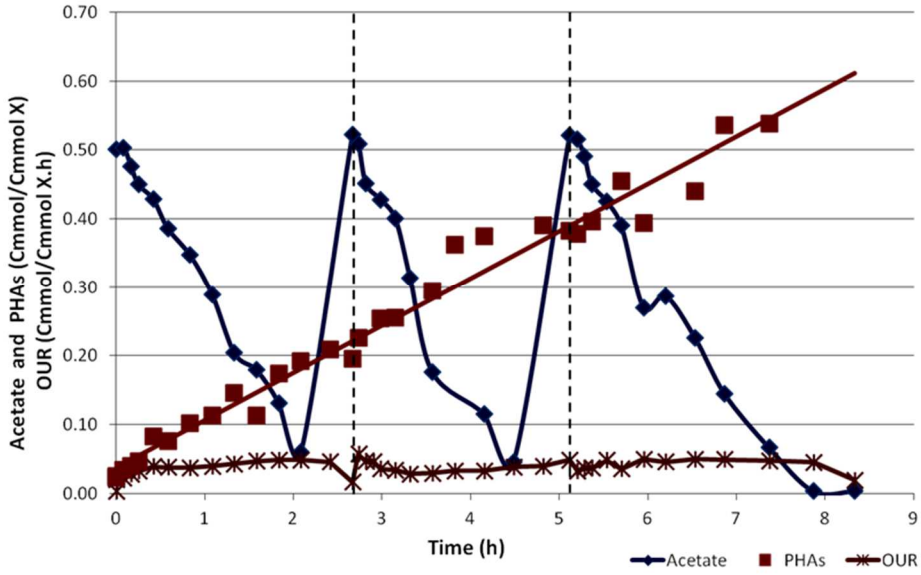

Fig. 4.4- PHA accumulation assay using the selected culture in the SBR and acetate as a substrate (three consecutive pulses of 30 Cmmol/L, each).

4.3.3.3. PHA accumulating assay using C5 and C6 sugars as substrate

Thirty seven percent of the biodegradable carbon present in bio-oil corresponded to sugar-based compounds. During the daily cycles this sugar fraction was totally consumed. Bio-oil characterization showed the existence of glucose (C6 sugar) and xylose (C5 sugar) as part of the sugar-based compounds detected (unpublished results). In order to understand their individual contribution to the overall system performance these two substrates were independently tested. In each assay 30 Cmmol/L of sugar was used as substrate but, in both cases, the selected culture did not reveal the ability to uptake either glucose or xylose as substrates. To confirm that the microbial population was active a pulse-fed of acetate was given after several hours without sugar consumption and the culture responded immediately. It seems that the two sugars, glucose and xylose, which are consumed in the daily cycles with pure bio-oil, could probably only be used as carbon sources when other co-substrates were present. This interpretation of the observation requires further investigation.

4.3.4. Bio-oil upgrade: Effect on the PHA accumulation capacity of the culture selected

Accumulation assays with pure bio-oil only reached a maximum PHA content of 10% and a production yield of 0.32 Cmmol HA/Cmmol S. The low PHA production capacity of the system may be associated to the high complexity of the pure bio-oil as a substrate. The presence of the sugar-based compounds that were usually involved in the glycogen production instead of PHA

in mixed cultures under ADF condition (Carta et al. 2001) may contribute for an inefficient selection of organisms with high PHA accumulating capacity. Also, the high amount of nitrogen present in the bio-oil may also have contributed for the low production yields observed during the accumulation assays, since more than 10% of the substrate consumed was used for biomass growth. To overcome these problems two bio-oil upgrading strategies were performed: bio-oil distillation and acidogenic fermentation. The use of these two upgraded bio-oils as substrate for PHA production with the selected mixed culture was studied in accumulation assays.

4.3.4.1. PHA accumulating capacity of the selected culture using distilled bio-oil

Bio-oil was distilled under reduced pressure, at temperatures between 58ºC and 80ºC, with a global yield of 43,8%. The resulting liquid fraction, from now on referred to as distillate, was characterized using the techniques FT-IR, GC-MS to identify its main components and to compare it with the crude bio-oil (unpublished results). The main products obtained after distillation were aromatic compounds (phenols, xylenes, pyrazines and pyrimidines) and long chain fatty-acids (C20-C23). The sugar fraction initially present in the bio-oil was no longer detected in the distillate, as expected given the low volatility of those functional groups. The distillation process concentrated volatile aromatic compounds with oxygen functional groups, mainly phenol derivatives and oxygen heterocycles such as 2,4-dimethylfuran or 2-metoxy-cresol. Nitrogen containing compounds were also concentrated in the distilled bio-oil with predominance of pyrazine derivatives such as 2,5-dimehylpyrazine or trimethylpyrazine. The TOC of the distillate was 68 g C/L. During the distillation process only 30% of the carbon was recovered on the liquid fraction. BOD_5 of the distilled was not measured, however previous analysis of this bio-oil revealed that 25% of the total carbon was biodegradable and sustainable for fermentation uses (Moita and Lemos 2012). As such, for this experiment, it was assumed that the majority of the non-biodegradable carbon remained in the thick residue obtained after the distillation.

An accumulation assay was performed to test the capacity of the mixed culture to use the distillate as substrate to produce PHA. The substrate was added in 3 pulses with an average of 30 Cmmol/L each, over the duration of the experiment (4.5 h) (Fig. 4.5). In the first 15 min of the assay a lag phase on the substrate uptake was observed. Since pure bio-oil was used to select the culture, this lag phase may represent an adaptation of the system to the distillate as substrate. A shorter lag phase was also verified in the second pulse. The initial specific substrate uptake rate observed with the distillate was significantly lower than the ones observed with pure bio-oil (Table 4.2) but, as opposed to the bio-oil accumulation test, this rate increased in the consecutive pulses reaching, by the third pulse, a value of -0.27 Cmmol S/Cmmol X.h. This rate

was slightly higher than the one obtained in the first pulse with pure bio-oil and those attained when synthetic acetate was used as substrate. Although the culture had the necessity to adapt to the bio-oil distillate, the increase on the substrate uptake rate indicated a good adaptation of the culture to this substrate. In each pulse about a total of about 14 Cmmol/L were consumed, showing that at least 50% of the carbon that remains in this distillate fraction was biodegradable or able to be used by the mixed culture.

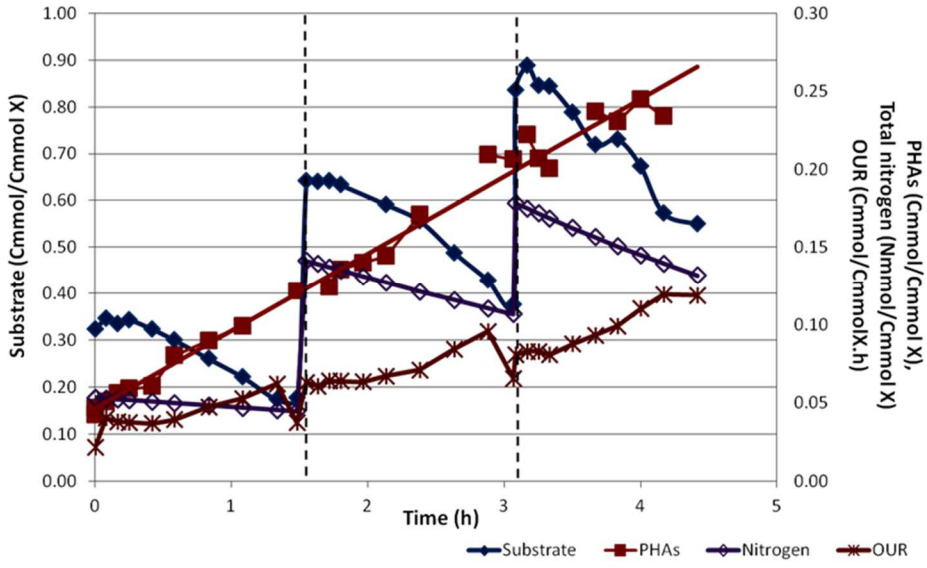

Fig. 4.5- PHA accumulation assay using the selected culture in the SBR and distilled bio-oil as a substrate (three consecutive pulses of 30 Cmmol/L, each).

The specific PHA production rate ranged from 0.047 to 0.065 Cmmol HA/Cmmol X.h, with an average value of 0.054 Cmmol HA/C mmol X.h for the three pulses. Even though the culture needed a period of adaptation to the bio-oil distillate as substrate, the ability to produce PHA did not seem to be very affected. The PHA production yields for the first two pulses was identical, decreasing significantly in the third one, 0.16 Cmmol HA/ Cmmol S. This decrease was accompanied by an increased in the biomass production yield. The profile for PHA production seems to indicate that the maximum accumulation capacity of the culture has not been reached after the consumption of 45 Cmmol/L of this substrate. However, in this last pulse, the culture appeared to start favouring growth over PHA production. The co-polymer produced with the bio-oil distillate had a monomer composition of 83% of HB/ 17% of HV.

As it has been observed with the accumulation assays with pure bio-oil, the presence of nitrogen allowed the biomass to grow, despite the fact that the distilled bio-oil only contains one-fourth of the nitrogen present in the pure bio-oil. In order to achieve the maximum storage capacity with the selected culture, an attempt to remove the nitrogen or inhibit growth has to be promoted. The PHA/S yield reported for the first two pulses with distilled bio-oil were in the

same range as those observed when synthetic acetate was used as substrate. Therefore, the replacement of the pure bio-oil by this distilled fraction in the selection reactor could possibly allow an increase on the F/F pressure behaviour, increasing the selection of populations with higher PHA storage capacity.

4.3.4.2. PHA accumulating capacity of the selected culture using fermented bio-oil

VFAs are considered the preferred substrate for PHA accumulation by mixed cultures. Several studies that use real wastes with high sugar content usually apply anaerobic fermentation as pre-treatment to convert several organic compounds to VFAs and by so doing increase the potential to produce PHA by mixed cultures. This same approach was applied to bio-oil, as a pre-fermentation step. A CSTR was fed with 1 g sugars/L present in the bio-oil, diluted inside the reactor with supplemented tap water in order to keep the COD/N/P ratio of 100:5:1 (on a molar basis). The effluent was clarified with an ultrafiltration membrane and kept at 4ºC prior to its use in PHA batch accumulation assays.

After the CSTR reached a steady-state the resulting fermented bio-oil was collected and the TOC, sugar content and organic acids present were analysed. Under the condition imposed to the CSRT, the selected culture had the capacity to consume 40% of the total sugar content, being this sugar-fraction responsible for the production of only 12% of the total VFAs produced (in C molar basis). The remaining sugar fraction possibly corresponded to molecules with higher complexity, unable to be metabolized by the selected culture. Future work will involve optimization of the conditions imposed to the CSTR in order to study the possible increase of the sugar fraction conversion by the selected culture. Table 4.3 summarizes the different organic acids quantified in the pure and the fermented bio-oil. The residual formic acid present in the pure bio-oil was undetected in the CSRT. Three of the major relevant organic acids involved in the PHA production were produced in the anaerobic fermentation. Those were acetic, propionic and butyric acids, presenting a five-fold increase in VFAs concentration, and showing the feasibility of the pre-fermentation step towards a better PHA production.

Table 4.3- VFAs identified and quantified in the pure and fermented bio-oil

	Formic acid (Cmmol/L)	Acetic acid (Cmmol/L)	Propionic acid (Cmmol/L)	Butyric acid (Cmmol/L)
Bio-oil (feed)	0.75	19.89	5.83	8.12
Fermented bio-oil	nd	62.84	32.15	73.50

nd: Not detected

Again, an accumulation assay was performed to test the ability of the enriched culture to use the fermented bio-oil as substrate for PHA production. The substrate was added in 2 pulses, on average 30 Cmmol/L each, over the duration of the experiment (3.5h) (Fig. 4.6). Due to the higher content in VFAs, the fermented bio-oil was immediately consumed by the selected culture without any lag phase, as it was observed for the other experiments except for the distilled bio-oil. The maximum specific substrate uptake rate was achieved in the first pulse (Table 4.2) being of the same order of magnitude as the ones verified for the assays where pure bio-oil (1ª pulse) or acetate were used as substrates. All the organic acids identified and produced during the anaerobic fermentation were consumed in the accumulation assay. Butyric acid showed the highest maximum specific uptake rate (-0.100 Cmmol But/Cmmol X.h) while for acetic acid and propionic acid the values were -0.075 Cmmol Ac/CmmolX.h and -0.040 Cmmol Prop/CmmolX.h, respectively.

Although the fermented bio-oil was consumed with similar rates as the ones verified for the other tested substrates, the maximum specific PHA production rate (0.134 Cmmol HA/CmmolX.h) and the PHA production/substrate yield (0.63 Cmmol HA/Cmmol S) were the highest. The storage yield obtained with the fermented bio-oil was very similar to the one reported by Albuquerque et al. 2010a (0.66 Cmmol HA/Cmmol S) which, to the best of our knowledge, has reported the highest storage yield using MMC and fermented streams (molasses) to produce PHAs. Despite the complex matrix, the increase of VFAs on the fermented bio-oil allowed the microbial population to have more available carbon for PHA accumulation. The maximum PHA content achieved with the fermented bio-oil (≅17%) was in the lower range of the ones reported by other studies (20% - 75% PHA content) (Liu et al. 2008; Bengtsson et al. 2008a; Albuquerque et al., 2010a). Results in Fig. 4.6 suggest that even though the specific production rate decreased in the second pulse, the maximum accumulation capacity of the culture has not been reached with this substrate, after the consumption of 60 Cmmol/L.

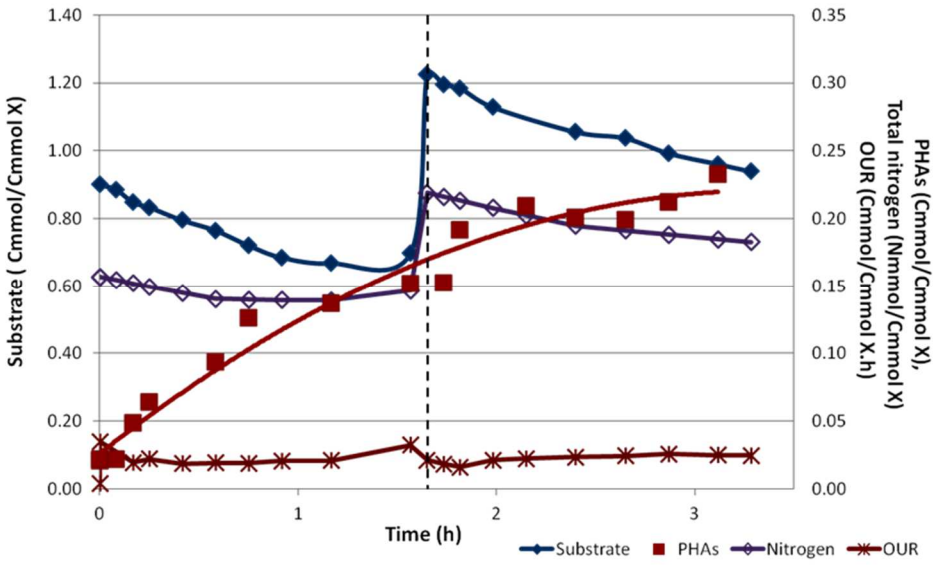

Fig. 4.6- PHA accumulation assay using the selected culture in the SBR and fermented bio-oil as a substrate (two consecutive pulses of 30 Cmmol/L, each).

In order to keep the COD/N/P ratio of 100:5:1 (on a molar basis) during the acidogenic fermentation, ammonium was added to the supplemented tap water used to dilute the bio-oil. As a result, the nitrogen content of the fermented bio-oil when added to the assay was higher than when pure bio-oil was used. Consequently, the initial specific nitrogen uptake with fermented bio-oil achieved the highest values. Two different nitrogen uptake rates could be observed for each fermented bio-oil pulse. The more available nitrogen source, ammonium, remaining from the pre-fermentation step, could be responsible for the faster initial nitrogen uptake rate observed, being the more complex fraction of nitrogen consumed afterwards at a lower rate. An attempt to overcome this problem may be possible by controlling the ammonium fed to the CSTR. If a residual level of ammonia in the fermented bio-oil is maintained, biomass growth in accumulation assays can be kept to minimum levels and thus increase the PHA storage capacity. Further comparison between the two upgraded bio-oils tested showed that the fermented bio-oil is the most promising substitute for the pure bio-oil in the selection reactor. The higher amount on VFAs of this substrate will possibly allow an improved selection of organisms with high PHA storage capacity. Even though some sugar content was still detected in the fermented bio-oil, no significant consumption was observed in the accumulation assay and the initial glycogen present in the biomass remained constant during the entire assay. Consequentially, in further future work, the use of the fermented bio-oil in the SBR will be investigated to assess how this pre-treatment could help to improve the performance of the enrichment biomass step for PHA accumulation.

4.4. CONCLUSION

In this study, it was demonstrated that even though the bio-oil contains some compounds that may inhibit or interfere with the production of PHA, there was no necessity to detoxify the bio-oil in order for it to be metabolized by the selected organisms. To the best of our knowledge this is the first study that used the entire bio-oil, resulting from a fast pyrolysis process, as feedstock.

Specific PHA accumulation rate and storage yield with pure bio-oil (1º pulse) as substrate were similar to the values reported in other studies that use MMC and real complex substrates, indicating that bio-oil can be used as a feedstock to produce short chain length PHA. The presence of nitrogen in the bio-oil led to growth stimulation during the entire accumulation assays, allowing the culture enough time to drift their metabolism preferably to growth in detriment of PHA storage. In order to be able to study the maximum PHA storage capacity of the selected culture, nitrogen removal or growth inhibition would be necessary.

Since VFAs are considered as the best substrates from PHA accumulation by mixed cultures and acetate is the most abundant organic acid identified in the bio-oil, the higher PHA content (32%) achieved with synthetic acetate as substrate in the accumulation assay was expected. The higher PHA production yield observed with acetate versus bio-oil seems to reflect the easier conversion of this substrate into PHA and the biomass growth inhibition accomplished by imposing a nitrogen limitation.

Distillation of the bio-oil reduced the nitrogen content by 75%. However, biomass growth was still observed and, in the last pulse, the culture appeared to start favouring growth instead of PHA production. The distilled bio-oil revealed similar production yield to acetate, although at least 50% of the carbon that remained in this fraction was biodegradable or able to be used by the mixed culture. As such, only a small fraction of the total carbon present in the bio-oil was metabolized and converted to PHA.

From all the real complex substrates tested, the fermented bio-oil appears to be the best choice to produce PHA from the selected culture. The higher amount of VFAs in this substrate suggests that this fermented stream is a promising substitute for the pure bio-oil in the selection reactor, allowing for a more effective selection of organisms with high PHA storage capacity.

CHAPTER 5

CRUDE GLYCEROL AS FEEDSTOCK FOR POLYHYDROXYALKANOATES PRODUCTION BY MIXED MICROBIAL CULTURES

ABSTRACT

The increase in global biodiesel production makes imperative the development of sustainable processes for the use of its main by-product, crude glycerol. In this study the feasibility of polyhydroxyalkanoates (PHA) production by a mixed microbial community using crude glycerol as feedstock was investigated. The selected culture had the ability to consume both glycerol and methanol fraction present in the crude. However, glycerol seemed to be the only carbon source contributing for the two biopolymers stored: poly-3-hydroxybutyrate (PHB) and glucose biopolymer (GB). In this work the culture reached a maximum PHB content of 47% (cdw) and a productivity of 0.27 g X/L.d, with an aerobic mixed cultures and a real waste substrate with non-volatile fatty acids (VFA) organic matter. The overall PHA yield on total substrate obtained was in the middle range of those reported in literature. The fact that crude glycerol can be used to produce PHA without any pre-treatment step, makes the overall production process economically more competitive, reducing polymer final cost.

The contents of this chapter were adapted from the publication: Moita, R. Freches, A, Lemos, P. C. "Crude glycerol *as feedstock for polyhydroxyalkanoates production by mixed microbial cultures". Water Research, 58, 9–20. doi: 10.1016/j.watres.2014.03.066.*[1]

5.1. INTRODUCTION

Due to the prospects of replacing fossil fuels, biodiesel production has continuously grown in the last decade. As a consequence, biodiesel industries are facing a surplus of its main byproduct, glycerol, which represents 10% (v/v) of the final ester. Supported by governments to increase energy independence and meet the rising energy demand, the biodiesel market is expected to reach 37 billion gallons by 2016, an average growth of 42% per year. This will result in a production of 4 billion gallons of crude glycerol that year, saturating the glycerol market (Quispe et al. 2013).

Industrial application of crude glycerol in food, pharmaceutical and cosmetics industries, its main markets, requires a costly refining process in order to achieve a necessary high purity. In the last years many research projects have been conducted with the aim of finding a new utilization for raw glycerol. In addition to new applications in the food industry, polymer industry, glycerol has also been considered as a feedstock for new industrial fermentations (Yang, et al.2012). Particularly attractive is the microbial conversion of raw glycerol into 1,3-propanediol (Hiremath et al. 2011), H_2 and ethanol (Ito et al. 2005) and citric acid (Papanikolaou and Aggelis 2003). Equally interesting could be the conversion of the glycerol into polyhydroxyalkanoates (PHA).

PHA are biodegradable polyesters with market capacity to replace some of the more commonly used elastomeric/thermo plastics. These biopolymers are naturally synthesized and stored inside the cells by several microbial species. With the rising financial investments made into production and marketing of bioplastics, PHA prices have been reduced in the last years. However, commercialization of bacterial PHA is still restricted to the use of pure cultures fermentations and high cost synthetic substrates making their price, in average, two times higher than conventional plastics (i.e PVC) (Chanprateep 2010). In recent years, research has focused on the development of alternative PHA production processes, including the use waste/surplus based feedstocks and mixed microbial cultures (MMC). This approach permits for a lower investment and operating costs for the global process (Albuquerque et al. 2007; Bengtsson et al. 2008b). The main problems associated with those strategies are the lower PHA content and the lower volumetric productivities achieved when compared with the ones reported for pure culture and synthetic substrates. A critical step in this strategy is the selection of a stable culture with a high PHA storage capacity. This can be achieved by subjecting microbial cultures to alternate periods of short carbon availability followed by a long unavailability, designated as aerobic dynamic feeding (ADF, also known as feast/famine). Using this approach Jiang et al. (2012) obtained a PHA content of 77% (cdw) with MMC and fermented paper mill wastewater.

These results make the gap between the PHA production using pure cultures/synthetic substrates (88% of cdw) (Lee et al. 1999) and MMC/complex feedstock considerably narrowed.

Most of the study that reported the use glycerol to produce PHAs used pure cultures and observed that only the PHA homopolymer, poly-3-hydroxybutyrate (PHB) was stored. Recent studies (Moralejo-Gárate et al. 2011; Dobroth et al. 2011) have explored the use of MMC to produce PHB from glycerol. This strategy represents an opportunity to further decrease the process environmental footprint, primarily due to reduced energy usage associated with the absence of aseptic conditions. Moralejo-Gárate et al. (2011) proved the feasibility of glycerol-based PHA production by a MMC where the enriched mixed community achieved a PHA content up to 80 % of cdw (0.40 g PHB/g glycerol). Dobroth et al. (2011) was the only study until now that enriched a stable mixed culture using crude glycerol. However, although the authors reported the enrichment of a MMC with an intrinsic high PHB content (62% cdw) the selected culture uses exclusively the methanol fraction of the crude glycerol to produced PHB with a low polymer yield on substrate (0.10g PHB/g methanol).

The aim of this work was to investigate and demonstrate the feasibility of PHB production by a mixed microbial community using crude glycerol as substrate. A two-step process was used, comprising (1) selection of a PHA-accumulating culture under ADF conditions, and (2) batch PHA accumulation using the selected culture. The impact of the synthetic substrates versus crude glycerol on the PHB storage was study. Also the storage capacity of the selected culture using crude and synthetic glycerol under different feeding strategies was investigated. To the best of our knowledge this was the first study that shows the valorisation of crude glycerol into PHAs using an aerobic mixed microbial consortium.

5.2. MATERIAL AND METHODS

5.2.1. Crude glycerol composition

The crude glycerol used in this study was obtained from an industrial biodiesel manufacturing plant (Sovena) in Portugal. Multiple vegetable oil sources are used by this industry to produce the biodiesel. The crude glycerol was removed after the bio-diesel production and before any purification step. This fraction was mainly composed by glycerol (71.66%, g C/g TOC) and methanol (25.69%, g C/g TOC). Crude glycerol also contained a small fraction (2.58% w/w) of free fatty acids and fatty acids methyl esters (FFA/FAME).

5.2.2. PHA-accumulation culture enrichment

The PHA-accumulating culture enrichment on crude glycerol was conducted in a sequencing batch reactor (SBR) with a working volume of 1500 ml. The reactor was inoculated with a PHA-accumulating mixed culture acclimatized to bio-oil as feedstock (Moita el al., 2013). The SBR was operated under ADF conditions. Each SBR cycle (24h) consisted of four periods: fill (15min); aerobiosis (23h); settling (20 min) and withdraw (15min). HRT was kept at 2 days. A peristaltic pump was calibrated to purge mixed liquor (300 ml) at the end of the aeration period in order to keep SRT at 5 days.

At the beginning of each cycle the reactor was fed with 30 CmM of crude glycerol. A mineral nutrients solution was added separately to the reactor that included nitrogen and phosphorus source (NH_4Cl and KH_2PO_4/Na_2HPO_4) to keep the C/N/P ratio (on a molar basis) at 100:8:1. The solution was prepared in tap water and thiourea (10 mg/l) was added to inhibit nitrification. Air was sparged (± 1L/min) through a ceramic diffuser and stirring was kept at 400 rpm. pH was controlled between 7.2 and 8.2 with NaOH 1M and HCl 1M and the reactor stood in a temperature-controlled room (20 - 23◦C).

5.2.3. Batch accumulation assays

Two different sets of batch accumulation assays were performed. In the first one the influence of the single synthetic substrates composing crude glycerol (methanol and/or glycerol), on the biopolymers production was investigated. In the second one, the storage capacity of the selected culture was studied using different feeding strategies. All the batch experiments were carried out using sludge from the SBR (400 ml), collected at the end of the famine phase, after the system reached steady-state. The collected biomass was washed twice with mineral solution (without any carbon and nitrogen source) before the beginning of the accumulation assays. The accumulation assays were carried out in a 600 mL working volume reactor. Due to the long duration of the assays where the storage capacity of the culture was tested using crude and synthetic glycerol a 900ml working volume reactor was used instead.

With the exception of one batch assay where crude glycerol continuous feeding was tested, all substrates (crude glycerol, synthetic glycerol, synthetic methanol and a synthetic mixture of glycerol and methanol) were added to the system in a pulse-wise manner to avoid potential substrate inhibition. The decision of adding a new pulse was based on the DO profile. Once the carbon was depleted the DO increased abruptly and a new pulse of carbon was immediately added. In order to maximize storage, the accumulation assays were carried out under ammonia limitation. When crude glycerol was used as substrate, the mineral medium was prepared with

tap water and included only a phosphorus source to keep the C/P ratio equal to the condition imposed in the SBR. When synthetic substrates were used, 2 ml per litre of a trace micronutrients solution (Moita et al. 2013) was added to the mineral medium. Thiourea (10 mg/l) was added in all the assays to inhibit nitrification. All the other condition used during accumulation assays were as described in Moita et al. 2013

5.2.3.1. Crude glycerol versus pure substrate: influence on the biopolymers production

In addition to glycerol and methanol, crude glycerol contains FFA/FAME, salts and other impurities. To study the influence of synthetic substrates in the storage capacity of the selected culture, four different assays were performed with 3 pulses (3X30CmM) of each tested substrate: Crude glycerol (GM1), synthetic glycerol (GM2), synthetic methanol (GM3) and a synthetic mixture of glycerol and methanol (GM4) at the same proportion existing in the crude glycerol.

5.2.3.2. Maximizing storage capacity of the selected culture

To maximize the storage capacity of the selected culture crude glycerol and synthetic glycerol were used and compared. The effect of feeding regimen (pulse-wise and continuous) was assessed in assays GA1 and GA2 with crude glycerol as carbon source. The accumulation assay with synthetic glycerol (GA3) was performed in pulse wise feeding mode and served as a control. In the pulse regime accumulation assays (GA1 and GA3) the substrate was added in pulses of 30 CmM until the carbon consumption had ceased. In the accumulation assay (GA2) with continuous feeding a peristaltic pump added crude glycerol with the same rate to which it was consumed (determined in previous assays). The accumulation assays were stopped when the OUR achieved a similar value to the endogenous OUR measured in the beginning of the assay.

5.2.4. Analytical Methods

Biomass concentration was determined using the volatile suspended solid (VSS) procedure described in Standard Methods (APHA, 1995). Glycerol and methanol concentrations were determined by high performance liquid chromatography (HPLC) using a Refractive Index detector (Merck, Germany) and Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA).

Sulphuric acid 0.01M was used as the eluent at a flow rate of 0.6 ml/min and 50ºC operating temperature.

Polyhydroxyalkanoate was determined according to Moita et al. 2013. Glucose biopolymer (GB) was determined as total glucose and it was extracted from lyophilized cells trough an acidic digestion (1 mL HCl 0.6 M, 2 hours, 100ºC). Samples were analyzed by HPLC at the same condition of glycerol and methanol.

Ammonia concentrations were determined using an ammonia gas sensing combination electrode (ThermoOrion 9512). Calibration was conducted with NH_4Cl standards (0.01–10 Nmmol/L). Total nitrogen was analyzed using a Vario TOC select (Elementar) and a mixture of ammonium chloride and sodium nitrate as standard for calibration following the equipment instruction.

FFA/FAME fraction present in the crude glycerol (10 g) was extracted three times with hexane (50/100/150 ml). The hexane extracts (300 mL) were collected together and dried in a rotavapor at 40ºC. The residues remaining corresponded to the FFA/FAME extracted from the crude glycerol.

5.2.5. Calculations

The sludge HB and GB content was calculated as a percentage of TSS on a mass basis (% HB= g HB/g TSS*100, and % GB= g glucose/g TSS*100). Active biomass (X) was obtained by subtracting the storage products from VSS as: X = VSS - PHA - GB (in g/L). It was assumed that all the ammonia consumed was used for growth since it was the only possible source of nitrogen. Active biomass elemental composition was represented by the molecular formula $C_5H_7NO_2$ (Henze et al., 1995).

The maximum specific substrates uptakes rates (−qGly, −qMeth, qN, qO2) and biopolymers production rates (qP, qGB) were determined by adjusting a linear function to the experimental data for each variable concentration divided by the biomass concentration at that point along time, and calculating the first derivative at time zero.

The yields of HB ($Y_{HB/S}$), GB ($Y_{GB/S}$), oxygen ($Y_{O2/S}$) and active biomass ($Y_{X/S}$) on substrate were calculated by dividing the amount of each parameter by the total amount of substrate consumed (S). When only glycerol was consumed the substrate contribution was defined as S_g.

5.3. RESULTS AND DISCUSSION

5.3.1. PHA-accumulating culture enrichment

A sequencing batch reactor (SBR) under ADF condition was started to select for a culture able to accumulate PHA using crude glycerol as feedstock. Since glycerol is not a preferred substrate to produce PHA by MMC, the SBR was inoculated with a PHA-accumulating culture selected with a complex substrate, a by-product (bio-oil) resulting from the fast-pyrolysis of chicken beds (Moita et al., 2014). Due to the vast carbon mixture present in this bio-oil (organic acids, sugars, phenolic and other aliphatic compounds), it allowed for the selection of a heterogeneous microbial consortium able to store PHA, possibly from different carbon sources.

The adaptation of the inoculum to the crude glycerol was initially followed by the variation on the feast/famine ratio duration. Following the DO concentration along time inside the reactor the feast phase can be monitored. At the beginning of a cycle the DO decreases due to substrate consumption and as the carbon source depletes, a sudden increase on DO occurs, indicating the transition between feast and famine phases. Fig. 5.1A shows the variation of the feast and famine ratio (F/F) during the first 60 days of SBR operation. In the first 5 days no significant changes in the DO were observed, as a result of the very low substrate consumption. After this initial period a clear feast and famine pattern was established. Analysis of daily cycles demonstrated a clear tendency on the preferential substrate storage, as shown by the increase in the biopolymers production yield during the culture enrichment, followed by a decrease in biomass production yield (Fig. 5.1B). These results confirm that the conditions imposed to the SBR developed a community specialized on PHB and GB storage through the consumption of crude glycerol. The F/F ratio together with other parameters such as pH profile, VSS, consumption/storage rates and production yields can be used to assess the stability of a SBR. On this basis, and considering that after 43 days no significant changes in the kinetic and stoichiometric parameters were observed, it was considered that the system reached a steady-state (≅ 8.5 SRT). Moralejo-Gárate et al. (2013b) reported a longer stabilization period of 19 SRT and 20 SRT for two identical SBRs, both fed with synthetic glycerol and SRT of 48h but operated with different cycle times (6 and 24h, respectively). The shorter stabilization period verified in this study may be due to the fact that an inoculum already enriched in PHA-accumulating organism was used, being the system acclimatized to the crude glycerol as feedstock with some minor changes on operational conditions (cycle length 24h).

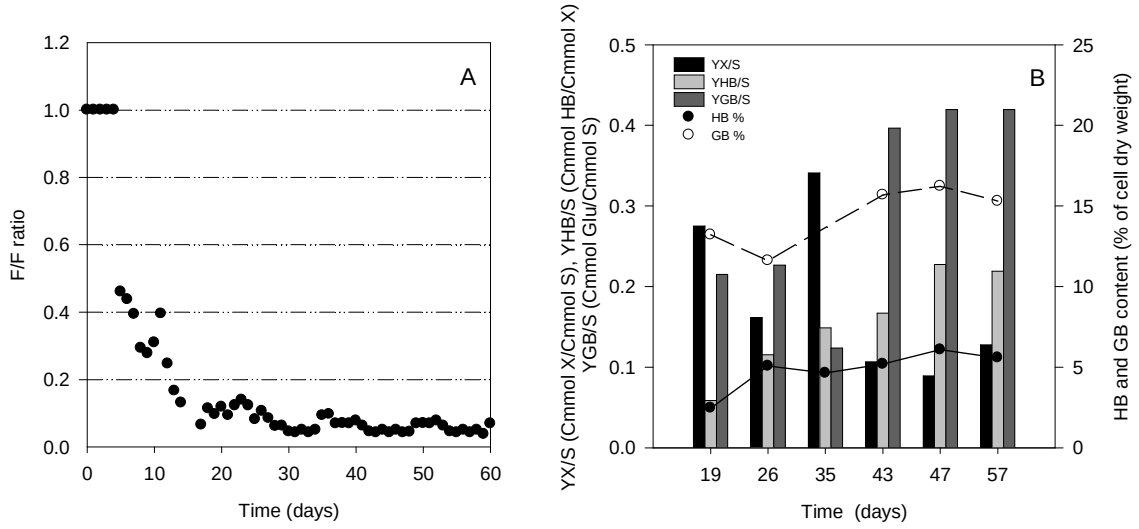

Fig. 5.1- Evolution of the bacterial enrichment **A**: F/F ratio during the first 60 days. **B**: Stoichiometric parameters and biopolymers content for selected days.

Several authors demonstrated that the F/F ratio imposed to the SBR is a determinant factor on the selection of a culture with good polymer accumulation capacities (Dionisi et al. 2006; Johnson et al. 2009; Albuquerque et al. 2010a; Jiang et al. 2011b). All these studies reported that low F/F ratios (≤0.28) allow the PHA accumulating organisms to outcompete with non-accumulating bacteria and that the selected culture shows a good storage response. F/F ratios higher than 0.55 increase the growth response and the storage mechanisms start to be negligible. In this work, after the culture has been acclimatized to the crude glycerol the F/F ratio was maintained in the range of 0.04-0.12 (Fig .5.1A), indicating that the SBR was operated under appropriate condition to select organisms with a preferential storage capacity.

A typical daily cycle under steady operational conditions is shown in Fig 5.2. Since no significant changes were verified after 5 h of the cycle, only this fraction of time was relevant for the daily monitoring. The end of a cycle corresponds to the beginning of the next one being both biopolymers produced during the feast phase consumed during the long famine phase. The SBR were characterized on a weekly basis by monitoring substrate and ammonia uptake as well as biopolymers and biomass production. Stoichiometric and kinetics parameters were calculated for the feast phase of each cycle monitored, average values are presented in Table 5.1. The crude glycerol used mainly contains two different carbon sources: glycerol and methanol. In the first hour of the cycle, glycerol was totally consumed at a specific rate of -0.27 Cmmol S/Cmmol X.h and it was accompanied by the production of PHA (only PHB) and a glucose biopolymer (referred to as GB). Comparing the specific production rates of both biopolymers, GB synthesis was almost three times faster than PHB (0.11 Cmmol GB/Cmmol X.h and 0.04

Cmmol HB/Cmmol.X.h, respectively). Also GB storage yield (0.41 Cmmol GB/Cmmol S_g) was higher than the PHB storage yield (0.20 Cmmol HB/Cmmol S_g). These results were consistent with the findings by Dircks et al. (2001), which showed that glycogen storage was faster than PHB production. Dircks demonstrated that not only glycogen storage was more efficient in terms of ATP than PHB but also that less oxygen was necessary to convert glucose into glycogen than acetate into PHA. Also the maintenance based on glycogen consumption was 10% to 15% smaller than maintenance based on PHB consumption.

Moralejo-Gárate et al. (2011) also reported the production of PHB and GB using synthetic glycerol and MMC. However, in this last case specific PHB storage rate and PHB storage yield were higher than the specific GB storage rate and GB storage yield. In recent studies Moralejo-Gárate et al. (2013a and 2013b) investigated the influence of oxygen concentration and cycle length on the PHB and GB production by the bacterial enrichments. The presence of oxygen limitation during the community enrichment step was show to favored GB storage over PHB (Moralejo-Gárate et al. 2013a). When comparing two SBRs operated under similar conditions but with different cycle lengths, 24h and 6h, for the second one the selected culture preferred GB over PHB storage (Moralejo-Gárate et al. 2013b). The authors suggested that the reduction on the food to microorganism (F/M) ratio verified in the 6h cycle, from 1.94 Cmol S/Cmol X to 0.25 Cmol S/Cmol X, led the selective pressure to favor the fastest storage polymer, GB. In the present study, the lowest oxygen concentration observed was at the end of the feast phase (Fig. 5.2B) with an average value of ±1.7 mg O_2/L. Under those conditions the preference of GB over HB could not be driven by oxygen limitation. Considering the F/M ratio, although the OLR (≅25 Cmmol/L.d) was very similar to the one reported in Moralejo-Gárate et al. (2011) their low biomass concentration (0.57 g/L at the end of the cycle) lead to a higher F/M comparing to present results (0.26 Cmol S/Cmol X). Since the F/M observed in this work was the same as the one reported for the 6h cycle reactor of Moralejo-Garáte et al. (2013b) the observed favoured GB storage over PHB verified in this study should be due to the low F/M ratio imposed to the SBR.

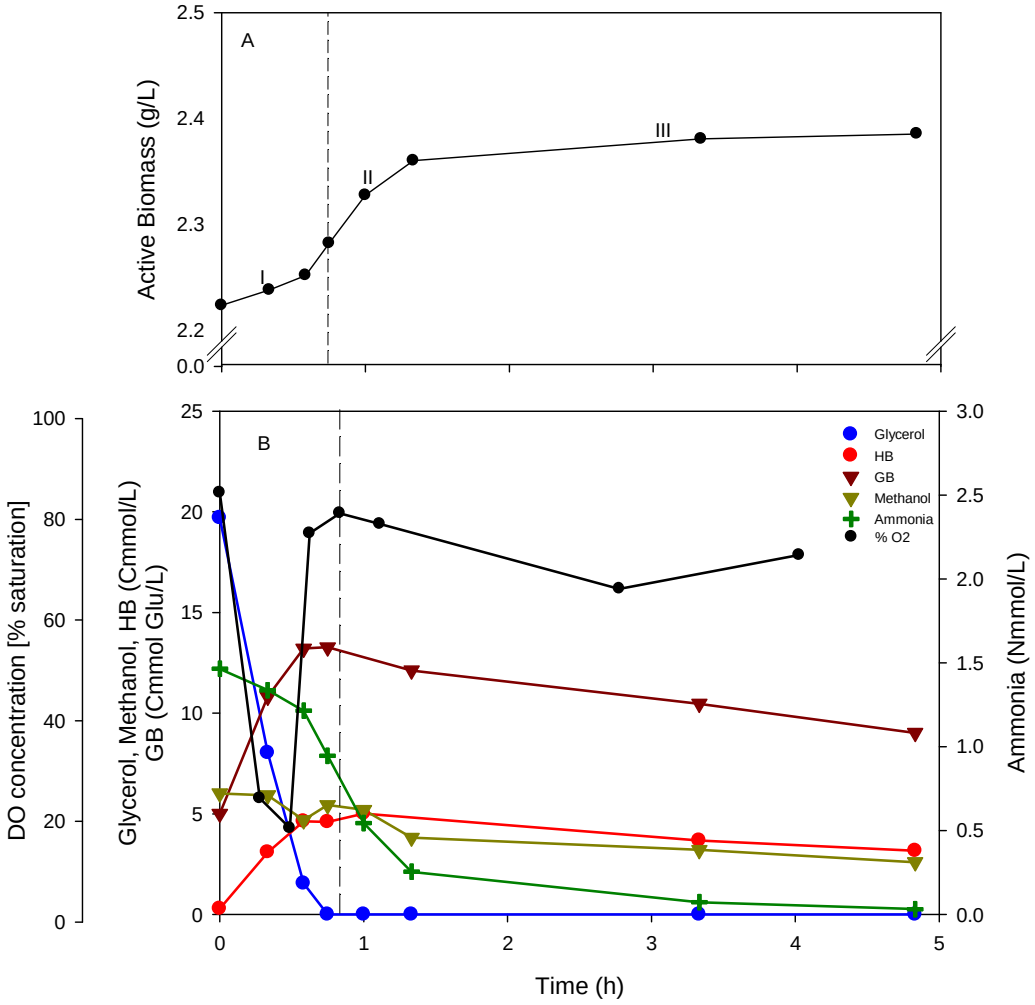

Fig. 5.2- Typical profile of a daily SBR cycle during steady-condition operated under ADF conditions and fed with crude glycerol (30Cmmol/L). **A:** Active biomass increase. **B:** Glycerol, methanol and ammonia consumption and biopolymers (HB and GB) production.

Methanol was also consumed in the SBR, but at a much lower rate compared to glycerol (Fig 5.2B). However, accurate determination of methanol consumption rates was not possible. The analysis of methanol present in crude glycerol showed some inconsistencies that were not observed when using pure methanol, suggesting the presence of interfering compounds in the crude glycerol. Contrary to what happened with glycerol, after 1.33h methanol consumption was considered negligible, being not totally exhausted at the end of the cycle. Once glycerol was depleted both stored polymers began to be consumed, regardless of methanol being present. This fact suggests that polymers production were mainly associated to glycerol consumption and the biopolymers yields were calculated only based on glycerol consumption.

In order to understand methanol disappearance pattern, either being biological consumed or being stripped by the aeration of the system, an assay mimicking the SBR conditions but with

no biomass, was performed. Samples were taken during the first 4 hours of operation and no methanol stripping was detected (data not show). The observed methanol consumption can be tentatively attributed to a second microbial community that, despite not having the ability to accumulate polymers, was able to grow and persist in the SBR.

Fig 5.2A shows the active biomass growth profile during the represented daily cycle (TSS_i = 2.58 g/L) where three different steps (I, II and III) were clearly established. During the biopolymers production (I) the specific biomass growth rate was 0.02 h^{-1} and 0.07 Cmmol X/L of biomass was synthesized. However, once the biopolymers began to be consumed (II), the specific growth rate increases to a maximum of 0.06 h^{-1} and 0.23 Cmmol X/L of biomass was produced. Comparing the growth yield ($Y_{X/S}$ =0.11 Cmmol X/Cmmol S) during the feast using glycerol, with the growth yield ($Y_{X/Polymers}$ =0.75 Cmmol X/Cmmol S), during the famine from both polymers we can observe that the glycerol uptake was essentially drift for polymers storage and that their later consumption, in the famine phase, allowed a good growth response. Also the fact that 76% of the biomass synthesized occurred during the famine phase indicates that the biopolymers produced during the feast were the main carbon source responsible for the biomass production in the SBR. When ammonia reached a limiting concentration (III) the growth rate decreased being ammonia consumed slowly until exhaustion. Albuquerque et al. (2010) also observed the difficulty of MMC to grow in the beginning of the cycle suggesting that it was due to the physiological adaptation of the cells after a starvation period.

Johnson et al. (2010) investigated the influence of the C/N ratio on the performance of PHB producing SBR at short SRTs. It was reported that biomass in strongly nitrogen-limited SBRs (medium C/N ratios 15–24 Cmol/Nmol) had higher baseline PHA contents in the SBR, but carbon-limited SBRs (medium C/N ratios 6–13.2 Cmol/Nmol) usually resulted in biomass with higher maximal PHA storage capacities. In this study it was chosen to start the system with a C/N of 12.5, which was in the upper limited of carbon-limited SBRs considered by Johnson. Although the ammonia was almost depleted after 5h, being the system N-limited, the faster glycerol uptake rate compared with the ammonia uptake during the feast phase indicates that the system can be considered as a carbon-limited SBR.

5.3.2. Crude glycerol *versus* pure substrates

To study the influence of each substrate present in crude glycerol in the biopolymers storage capacity by the MMC four batch accumulation assays were performed: crude glycerol (GM1); synthetic glycerol (GM2), synthetic methanol (GM3) and a synthetic mixture of glycerol and methanol (GM4) in the same proportions to those in crude glycerol. Table 5.1 summarizes the stoichiometric and kinetic parameter of these assays.

Table 5.1- Biopolymers storage performance of the microbial consortium during a daily cycle and in batch tests performed with crude glycerol and synthetic substrates

Assay	Substrate		$-q_s$	q_{HB}	q_{GB}	% HB $_{max}$	% GB $_{max}$	ΔHB	ΔGB	X_i	$Y_{HB/S}$	$Y_{GB/S}$	$Y_{O2/S}$	$Y_{X/S}$
Daily cycle	Crude Glycerol	-	0.27 (0.044)	0.039 (0.006)	0.111 (0.005)	5.72 (0.47)	15.44 (0.92)	4.11 (0.47)	8.31 (0.14)	80.50 (15.53)	0.20 (0.03)	0.41 (0.01)	0.15*	0.11 (0.02)
GM1	Crude Glycerol	1º Pulse	0.35 (0.024)	0.092 (0.022)	0.122 (0.018)	6.25	11.82	6.25	7.24		0.29	0.34	0.20	0.10
		2º Pulse	0.33 (0.029)	0.127 (0.019)	0.106 (0.040)	11.26	18.33	8.91	7.23	83.12	0.39	0.32	0.23	0.09
		3º Pulse	0.32 (0.055)	0.118 (0.040)	0.062 (0.022)	17.46	19.51	9.49	5.13		0.41	0.22	0.23	0.09
GM2	Synthetic Glycerol	1º Pulse	0.36 (0.023)	0.116 (0.021)	0.078 (0.008)	10.20	11.53	8.40	7.22		0.37	0.22	0.20	nd
		2º Pulse	0.32 (0.023)	0.0950 (0.018)	nd	15.91	12.84	9.74	3.07	83.12	0.35	0.13	0.22	nd
		3º Pulse	0.22 (0.012)	0.066 (0.026)	-	20.53	10.41	10.13	-		0.36	-	0.36	0.10
GM4	Synthetic mixture of Glycerol and Methanol	1º Pulse	0.21 (0.016)	0.064 (0.006)	0.071 (0.013)	9.62	12.97	10.71	6.24		0.31	0.27	0.33	nd
		2º Pulse	0.17 (0.017)	0.104 (0.015)	0.051 (0.016)	18.13	14.00	9.14	3.36	79.05	0.39	0.12	0.44	0.05
		3º Pulse	0.17 (0.005)	0.061 (0.017)	-	23.43	14.78	9.41	-		0.37	-	0.45	0.16

*only measured in one daily cycle

(st desviation); (nd)- not determined

$-q_S$ (Cmmol S/Cmmol X.h); q_{HB} (Cmmol HB/Cmmol X.h); q_{GB} (Cmmol Glu/Cmmol X.h);

$\%PHA_{max}$ (% g/g cell dry weight); $\%GB_{max}$ (% g/g cell dry weight); ΔHB (Cmmol HB/L) and ΔGB (Cmmol Glu/L)

X_i (Cmmol/L); $Y_{HB/S}$ (Cmmol HB/Cmmol S_g); $Y_{GB/S}$ (Cmmol Glu/Cmmol S_g), $Y_{O2/S}$ (mmol O_2/Cmmol S) and $Y_{X/S}$ (Cmmol X/Cmmol S)

Glycerol consumption rate in GM1 was somewhat similar along the three pulses and as it was observed during the daily cycles, methanol was also consumed but at a lower rate than glycerol in all pulses resulting in a buildup of methanol along the assay (≅8 Cmmol/L at the end of the assay, average pulse concentration of ≅ 5 Cmmol/L).

Concerning the biopolymers production, HB production rate increased from the first pulse to the second and them remained relatively constant in the third pulse. However the GB production rate decreased during the entire assay (Table 5.1). After 90 CmM of crude glycerol the selected culture was able to store 17.46% of HB (cdw) and the HB storage yield significantly increased from 0.29 to 0.41 Cmmol HB/ Cmmol S_g in the third pulse. Even though a higher GB content was achieved (19.51% of cell dry weight) with the 90CmM of substrate, the culture seems to lose GB storage capacity in the third pulse since the GB storage yield decreased from 0.34 to 0.22 Cmmol GB/ Cmmol S_g. Thus, although GB was store at a faster rate than PHB in the beginning of the assay the selected culture seems to have a lower GB storage capacity.

In spite of biomass growth has been limited by the lack of ammonia in the feed, it was observed an increase of the active biomass considering the difference between VSS and both produced biopolymers. The methodology used to determine the GB can include glycogen and other forms of sugar as exopolymeric substances (EPS). During the accumulation assays no significant viscosity was detected, which normally indicates EPS synthesis. Ammonia determinations in the soluble fraction confirmed that no residual concentration was transiting from the SBR. A concentration of 2.30 N-mmol/L of total nitrogen was detected in the sample before the addition of the substrate and it remained constant during the entire assay.

In GM1 the first pulse of the assay mimics the feast condition of the SBR but in this experiment no ammonia was added to the feed. When comparing the kinetic and stoichiometric parameters of both assays (Table 5.1) it can be verified that the biopolymers production was slightly affected. In GM1 a higher HB storage rate along with a higher HB production yield was observed. Concerning the GB production although a similar storage rate was verified in both assays, GM1 presented a lower GB production yield. The lack of ammonia in the feed (and by so a lower nitrogen availability) seemed to favor the HB production over the GB storage by the selected culture.

In GM2 synthetic glycerol was used as single substrate. The specific glycerol uptake rates were relatively constant (≅ 0.34 Cmmol S/CmmolX.h) during the first two pulses and then decreased significantly (0.22 Cmmol S/Cmmol X.h) along with the increase on the respiration yield (Table 5.1). PHB storage yield was maintained constant along the three pulses with an average value of 0.36 Cmmol HB/Cmmol S_g. Comparing the GM2 with GM1 it can be observed that similar specific glycerol uptake rate were obtained with the first two pulses of GM2 and the entire GM1

assay (≅ 0.34 Cmmol S/CmmolX.h and ≅ 0.33 Cmmol S/CmmolX.h, respectively on average). Also, the PHB storage yield obtained with synthetic glycerol was very similar to the ones in the second and third pulses of crude glycerol (0.39-0.41 Cmmol HB/Cmmol S_g). Having the enriched culture being selected with crude glycerol these results suggest that the culture was fully adapted to this carbon source and that the crude glycerol composition did not had a negative influence on the biopolymers production (mainly PHB). In this assay, GB biopolymer production had lower production rates and yields than the HB biopolymer (Table 5.1). In fact GB production ceased during the second pulse of synthetic glycerol. The decrease on the GB storage of the culture had already been observed with crude glycerol in GM1, being this capacity more affected when synthetic glycerol was used as carbon source, probably by the lack of some compound present in the crude glycerol.

The response of the selected culture to the synthetic methanol as the only carbon source available was study in GM3 assay. After the pulse of 30 CmM of synthetic methanol the accumulation batch was followed during 2h and no methanol consumption was observed (data not show). High methanol concentration can negatively impact many bacterial growth and such, the 30 CmM of methanol used in this assay may have had a toxic effect on the microbial community selected in the SBR blocking its consumption.

A synthetic mixture of glycerol (25 Cmmol/L) and methanol (5 Cmmol/L) in the same proportions to those in real substrate was used in GM4. As it was observed in GM3, synthetic methanol was not consumed during the entire assay (data not show). Since in GM4 the synthetic mixture of glycerol and methanol mimics the crude glycerol it can be ensure that the initial concentration of methanol used did not inhibit the selected culture, at least in the first pulse. Available literature reported the consumption of methanol to produce PHB from different organisms (Khosravi-Darani et al. 2013). In most cases, a single strain was used to produce PHB from synthetic methanol. Mockos et al. 2008 and Dobroth et al. 2011 reported the production of PHB using MMC and methanol present in real wastes (pulp and paper mill wastes and crude glycerol, respectively). Therefore, synthetic methanol and methanol as an integral part of complex waste can indeed be used to produce PHB. Although in this study methanol consumption did not seemed to be involved in PHB production, the fact that this culture was only able to consume the substrate when it was present in the crude glycerol suggested that a specific compound present in the crude composition can acts as a co-factor for the methanol consumption. In GM4 the specific glycerol consumption rate was higher in the first pulse (-0.21 Cmmol S/Cmmol X.h) and then decreases (-0.17 Cmmol S/Cmmol X.h) in the other pulses. Comparing with the other assays GM4 presented the lowest specific glycerol consumption rate, which can be explained by a possible inhibition effect of cumulative synthetic methanol concentration (≅12Cmmol/L at the end of the assay, average pulse concentration of ≅ 4

Cmmol/L). The high respiration yield observed during this assay also supports these findings, mainly in the last two pulses where the $Y_{O2/S}$ was ≅0.44 mmol O_2/Cmmol S. The synthetic mixture allowed reaching a PHB content of 23% (cdw) after 3 pulses and a HB production yield on the higher range during the entire assay (Table 5.1). Comparing the GM4 assay with the others accumulation assays preformed (Fig 5.3) it can be observed that although it shows the lowest glycerol uptake rate also presents the highest PHB content and similar production yields. Regarding the GB production the same effect as the one reported in GM2 was observed. The maximum specific GB production rate was obtained in the first pulse followed by a drastic decrease on the production in the following pulses, reaching a step were no GB production was observed.

5.3.3. Study of the maximum storage capacity of the selected culture

In order to overcome potential substrate inhibition (Albuquerque et al. 2007) a multiple pulse addition of crude glycerol was used to investigate the maximum storage capacity of the selected culture (GA1). A second feeding strategy (continuous feeding) was assessed with crude glycerol (GA2) and compared with pulse feeding. Synthetic glycerol was also used as substrate in a pulse feeding strategy (GA3) to evaluate the maximum storage capacity of the selected culture using a pure and defined carbon source.

Fig 5.3 shows the results from the crude glycerol pulse feeding assay GA1. As it was reported before, glycerol and methanol were simultaneously consumed by the selected culture and PHB was produced with a maximum storage rate of 0.03 Cmmol HB/Cmmol X.h and a production yield of 0.46 Cmmol HB/Cmmol Sg from glycerol. After almost 30h, the accumulation assay was stopped due to the lack of available reactor volume and a maximum PHB content of 46.91% (cdw) was achieved (ΔPHB = 43%).

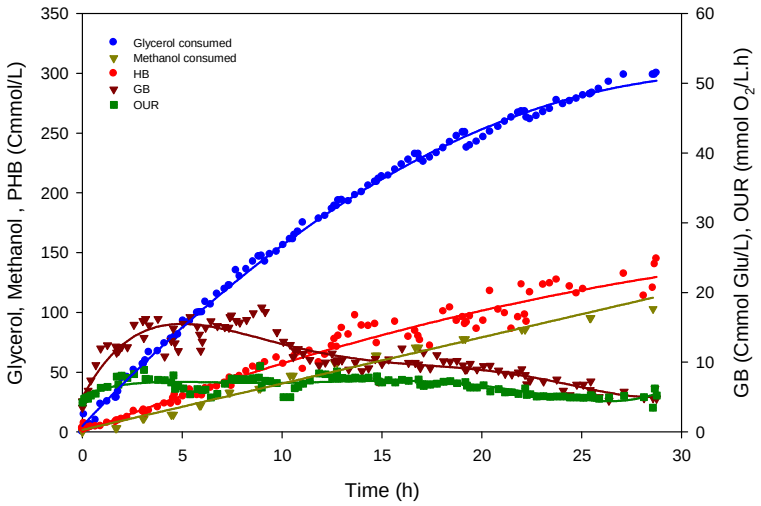

Fig. 5.3- PHB accumulation assay (GA1) using crude glycerol in a pulse-feed strategy (14X30C-mM). The amount of glycerol, methanol, HB and GB were represented in a cumulative mode.

In the accumulation assay with continuous feeding of crude glycerol (GA2) the maximum glycerol uptake rate was 0.16 Cmmol S/Cmmol X.h. The rate at which the substrate was added to the system was previous determined by the glycerol uptake rate measured in other assays (GM1). However, the culture showed a much lower rate and the substrate accumulated over time (data not show). After 9h of assay, PHB stopped being produced (Fig. 5.4) and a maximum PHB content of 32.08% (cdw) with a HB production yield of 0.28 Cmmol HB/Cmmol Sg were achieved. Since higher PHB content and storage yield were obtained in GA1 (pulse feeding), the low productivity observed in GA2 was associated to a potential substrate inhibition.

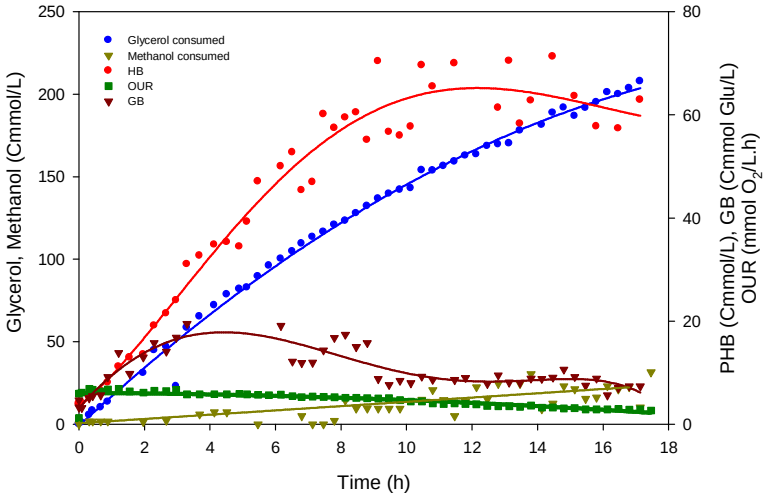

Fig. 5.4- : PHB accumulation assay (GA2) using crude glycerol in a continuous feeding strategy (0.55 CmM/min). The amount of glycerol, methanol, HB and GB were represented in a cumulative mode.

In GA3 synthetic glycerol was feed in a pulse feeding strategy and it was consumed at maximum rate of 0.20 Cmmol S/Cmmol X.h (Fig 5.5). The selected culture was able to accumulate PHB with a production yield of 0.51 Cmmol HB/Cmmol Sg, reaching a maximum PHB content of 53.31% (cdw). Moralejo-Gárate et al. (2011) reported that the maximum theoretical yield that can be obtained in the conversion of glycerol to PHB based on the PHB production pathway via acetyl-CoA was 0.67 Cmmol HB/Cmmol glycerol and that the simultaneous occurrence of a polyglucose polymer and growth, could explain the gap between the theoretical and the observed yield of PHB over glycerol. Considering these assumptions, the same findings were observed in this study since that, taking into account the simultaneous GB storage ($Y_{GB/S}$= 0.02 Cmmol GB/ CmmolS) and growth ($Y_{X/S}$= 0.13 Cmmol X/ CmmolS), the gap between the theoretical and the observed yield of PHB from glycerol was totally fulfilled. As it has been observed in previous accumulation assays, GB biopolymer was initially produced in the entire accumulation batch performed but rapidly started to be consumed, remaining low during the rest of the assays. The reason why the selected microbial community stops the GB storage while continuing accumulating PHB through glycerol consumption is still unclear and future work will be necessary to better understand the PHB/GB metabolism of this culture.

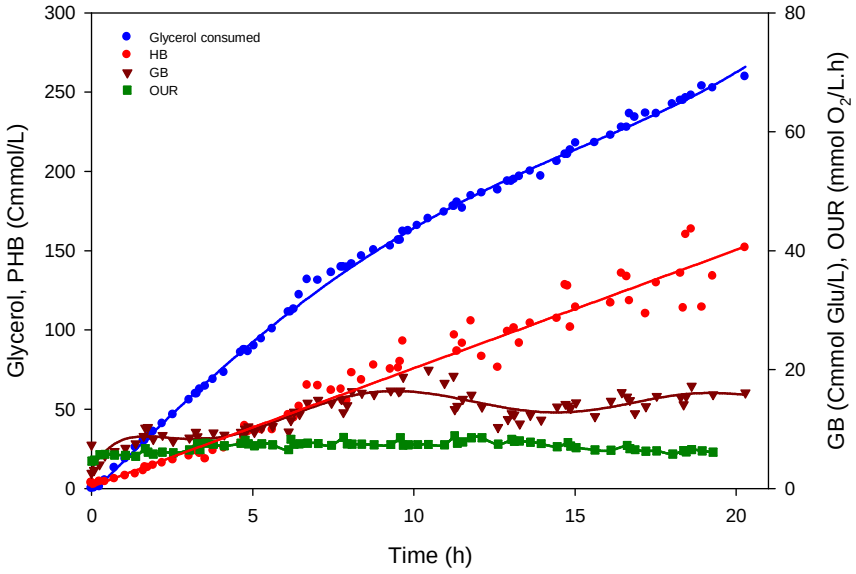

Fig. 5.5- PHB accumulation assay (GA3) using synthetic glycerol in a pulse-feed strategy (12X30C-mM). The amount of glycerol, HB and GB were represented in a cumulative mode.

Comparing the GA1 with the GA3 it can be observed that the crude glycerol matrix did not had a major impact on PHA production, as already stated for the GM1 and GM2 assays. Not only the maximum glycerol uptake rate was equal in both assays, as the PHB production yield and PHB content were only slightly lower with crude glycerol (Table 5.2). The major difference

between these two assays was the specific PHB storage rate which was two times higher with synthetic glycerol (0.07 Cmmol HB/CmmolX.h). The accumulated methanol verified in GA1 (≅100 Cmmol/L at the end of the assay) can induce an inhibitory effect resulting in a lower specific PHB storage rate. However, most of biodiesel manufactures recover the methanol present in the crude and recycle it, making in this way the biodiesel production more efficient from both economical and environmental point of view. Therefore the potential toxic effect of the methanol verified in this study can be devalued, since the majority of the industrial crude glycerol can have low methanol concentration.

Since crude glycerol matrix does not seems to greatly influence the PHA production, the higher PHB storage yield (0.57 Cmmol HB/ Cmmol S) and PHB content (67% cdw after 6h and a slowly increase to a maximum storage of 80% after 28h) reported by Moralejo-Gárate et al. 2011 seems to reflect a better culture selection efficiency. In this study the culture enrichment was performed with synthetic glycerol, an operational temperature of 30ºC and with a high F/M ratio (1.94 Cmmol S/Cmmol X), which has already been discussed as an important parameter favouring PHB over GB simultaneous storage using glycerol as a carbon source. These operational conditions allowed achieving biomass productivity of 0.29 g X/L.d and to select a microbial community with a high PHB storage capacity resulting in a PHB productivity of 1.15 g PHB/L.d. In the present study the same biomass productivity was achieved (0.27 g X/L.d), however due to the selection of a population with a lower PHB storages capacity a 4 time lower PHB productivity was achieved (0.30 g PHB/L.d) when synthetic glycerol was tested (GA3). Culture selection efficiency using crude glycerol could be further improved by increasing F/M ratio, OLR and C/N ratio in the SBR system.

Although Moralejo-Gárate et al. (2011) achieved a higher PHB productivity the use of temperature and synthetic substrates increases drastically the HB production costs. In addition in this study the accumulation test stopped after 20h, the maximum storage capacity of the enriched culture had not been achieved and the PHB still seemed to be produced linearly (Fig. 5.5). As such, a higher PHB content could be anticipated using the selected culture and synthetic glycerol as carbon source increasing the PHB productivity. Future work is necessary to confirm this hypothesis.

Table 5.2- Average performance of the PHB accumulation assays preformed to assess the maximum storage capacity of the selected culture

Batch	Substrate	Feeding Regime	$-q_s$	q_{HB}	q_{GB}	% HB $_{max}$	% GB $_{max}$	X_i	ΔHB	ΔGB	$Y_{HB/S}$	$Y_{GB/S}$	$Y_{O2/S}$	$Y_{X/S}$
GA1	Crude Glycerol (14X 30CmM)	Pulse feeding	0.20 (0.046)	0.034 (0.008)	0.131 (0.346)	46.91	16.69	67.64	137.27	12.71	0.46	0.04	0.64	0.11
GA2	Crude Glycerol (0.55CmM/min)	Continuous feeding	0.16 (0.025)	0.056 (0.011)	0.047 (0.014)	32.08	14.17	82.08	59.16	9.72	0.28	0.05	0.36	0.19
GA3	Synthetic Glycerol (12X 30CmM)	Pulse feeding	0.20 (0.035)	0.070 (0.017)	0.059 (0.021)	53.31	12.88	60.95	160.34	7.68	0.51	0.02	0.42	0.13

(st desviation)

($-q_S$ (Cmmol S/Cmmol X.h); q_{HB} (Cmmol HB/Cmmol X.h); q_{GB} (Cmmol Glu/Cmmol X.h);

$\%PHA_{max}$ (% g/g cell dry weight); $\%GB_{max}$ (% g/g cell dry weight); ΔHB (Cmmol HB/L) and ΔGB (Cmmol Glu/L)

X_i (Cmmol/L); $Y_{HB/S}$ (Cmmol HB/Cmmol S_g); $Y_{GB/S}$ (Cmmol Glu/Cmmol S_g), $Y_{O2/S}$ (mmol O_2/Cmmol S) and $Y_{X/S}$ (Cmmol X/Cmmol S)

According to the available literature Dobroth et al. (2011) has the only study that used crude glycerol to enrich MMCs able to produce PHB. In this study several optimal condition (SRT, HRT and cycle length) were tested and it was showed that PHB synthesis was driven by a macronutrient deficiency, possibly phosphorus. In all the assays performed, it was verified that the glycerol fraction of the crude remained constant during the entire cycle and that only the methanol was contributing for the PHB synthesis. In general the authors have reported the selection of a culture with an intrinsic high PHB content (20-62% of cell dry weight). However, although it can be considered as a good exploratory work no true PHA producing cycle was observed and the maximum PHB production in a cycle (ΔPHB) was never above ≅ 9% of cdw (≅0.27 g PHB/L), which was achieved in a 5 days cycle SBR with a SRT of 20 days. In addition to the low PHB production, the enriched MMC has a low production yield on the substrate (0.10 g PHA/g methanol). Recently Cavaillé et al. (2013) also investigated phosphorus limitation to induce PHB production directly in waste activated sludge by using fed-batch mode. In this study a high PHB content was achieved (67% cdw) with acetate as substrate in a 60 days assay. The authors stated that the use of P-limitation usually generates low PHA yields (0.21 Cmol PHB/ Cmol S) due to the loss of carbon required for new catalytic cell growth.

Several other studies have reported the use of real complex wastes to produce PHAs. Table 5.3 presents the most relevant of these studies concerning the PHA productivity. With the exception of the present work, all the other studies have in common the fact that the complex waste undergoes a pre-fermentation step in order to increase the volatile fatty acids (VFAs) content of the feedstock. VFAs are considered as the main precursors to produce PHAs from MMC and hence, feedstocks with high VFA content are more suitable to achieve high PHA content. Jiang et al. (2012), Albuquerque et al. (2010), Bengtsson et al. (2008) and Dionisi et al. (2005) have reported not only PHA contents higher than those obtain in this study with crude glycerol, but also higher PHA production yield during the PHA production step. However, for the industrial scale-up of any process it is important to consider the overall efficiency and determine the PHA yield over the entire process accounting for all the carbon added, being consumed or not. Thus, it can be verified that the PHA yield (0.32 g COD HB/g COD crude glycerol) was within the overall PHA yields reported in those works (0.08-0.58 g COD PHA/g COD real waste). In a three-step process, the PHA production includes a pre-treatment stage, usually anaerobic fermentation applied to convert several organic compounds into VFAs. In such processes, not only it has to be considered the conversion yield but the overall acidogenic fermentor performance since it will have a great impact on the quality and quantity of the final produced biopolymer (Bengtsson et al. 2008b). This initial step deals with several additional costs. They involve not only an additional reactor (with all the operation costs associated) but also a system

(usually ultrafiltration) to separate biomass from the effluent. The fermented effluent can then be used on the PHA production process (second and third stages). The separation procedure usually allows the recovery of 90-95% of the effluent (in optimized condition), having high maintenance with significant cost. Although the overall PHA yield was not the highest reported so far, the fact that crude glycerol can be used to produce PHA without any pre-treatment step, makes the overall production process economically more interesting, reducing PHB final cost.

Table 5.3- Summary on the PHA production from MMC and real complex wastes

Reference	Complex waste	Max PHA content	YPHA/S (g COD/g COD)	PHA productivity (gPHA/L.d)	Overall productivity (gCOD HB/gCOD waste)
Albuquerque et al. 2010**b**	Sugar molasses	75%	0.84	1.57	0.58
Ben et al. 2011	Wood mill	29%	0.59	0.08	0.23
Bengtsson et al. 2008 a,b	Paper mill	48%	0.66	nd	0.49
Bengtsson et al. 2010	Sugar molasses	37%	0.73*	0.15	nd
Dionisi et al. 2005	Olive oil mill	54%	1	nd	nd
Jiang et al. 2012	Paper mill	77%	0.80	2	0.55
Mato et al. 2010	Wood mill	25%	0.24	nd	0.08
This study	Crude Glycerol	47%	0.44	0.24	0.32

*Cmmol PHA/Cmmol VFA

Only few works reported PHA production using MMC and real waste substrate with non-VFA organic matter. Gurieff et al (2007) enriched a mixed culture using primary sludge as the feedstock and in the accumulation step obtain a PHA content of 20% (cdw) with primary sludge and 39% with fruit cannery wastewater. Liu et al. (2008) reported a PHA content of 20% (cdw) using tomato cannery wastewater and recently Dobroth et al. (2011) which have been already extensively discussed. With the exception of the latter study that, as mentioned, had a very low PHB productivity the PHB content of 47% (cdw) obtained with crude glycerol in this work was the highest PHB content reported with aerobic mixed cultures and a real waste substrate with non-VFA organic matter.

5.4. CONCLUSION

Crude glycerol can be efficiently used as a feedstock to produce PHB by aerobic mixed cultures. The obtained results suggest that the culture selection can be further improved by increasing F/M ratio, OLR and C/N ratio of the SBR system. Nevertheless the selected culture allowed achieving a high PHB content (47% cdw) using real waste substrate with non-VFA fraction. The fact that crude glycerol does not need a pre-fermentation step to be converted into PHB makes the overall production process economically more sustainable were compared with the majority of the three-step process.

CHAPTER 6

BIOREACTORS USING BIOFUELS BY-PRODUCTS FOR POLYMER PRODUCTION: MICROBIAL COMMUNITY ANALYSIS

ABSTRACT

PHA production from industrial wastewater or surplus by mixed microbial cultures (MMC) has been extensively studied in recent years. The microbial community dynamics and composition of two PHA producing systems, each one fed with an industrial biofuel byproduct (bio-oil or crude glycerol), was investigated through denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH) of 16S rRNA. Principal component analysis (PCA) and clustering analysis of the system fed with bio-oil resulting from fast-pyrolysis of lignocellulosic biomass showed that different operational condition induce an adaptation of the microbial community that was reflected in PHA storage capacity of the system.. The bio-oil enriched culture was composed mainly by *Betaproteobacteria* (73.4% at the end of the operation) and the *Pseudomonas*, *Brachymonas*, *Burkholderia* and *Alcaligenes* were identified as the most relevant genera responsible for the reported PHA storage capacity. This microbial community was later adapted for the use of crude glycerol. The microbial community attained at steady state after 60 days of operation had a 55% similarity with the inoculum demonstrating the microbial adaptation to the new feedstock. Nile Blue staining and FISH analysis identified *Amaricocus*, *Azoarcus* and *Zoogloea* genera in this last enriched culture, all these organisms being well known PHA accumulators.

6.1. INTRODUCTION

A wide variety of petroleum-based synthetic polymers were produced worldwide, to an extent of 280 million tons in 2011(www.plasticseurope.org), and remarkable amounts of them were introduced in the ecosystem as industrial waste products. Polyhydroxyalkanoates (PHAs) are polyesters intracellularly stored by several microorganisms that have gained increasing attention as an alternative to the conventional petroleum-based plastics. These bioplastics display a wide range of elastomeric/thermoplastic properties and are both bio-based and biodegradable, allowing for a closed loop carbon cycle. Commercial PHA production is based on the utilization of pure cultures and synthetic substrate, which implies high production costs due to high substrate prices and the need for sterile operation. Even thought their market price has been reduced in recent year's (Chanprateep 2010), it is still not competitive for the replacement of traditional petroleum-based plastics. Several research studies have been directed towards the reduction of PHA production costs. The main strategies focus on the use of mixed microbial cultures (MMC) and real complex wastes as feedstock. Combining these two approaches fermented olive oil mill effluents (D Dionisi et al. 2005; Beccari et al. 2009); fermented paper mill effluents (Bengtsson et al. 2008a; Jiang et al. 2012); fermented molasses (M G E Albuquerque et al. 2007); pyrolysis by-products (Moita and Lemos 2012) and crude glycerol (Dobroth et al. 2011) among others have been considered for PHA production.

One of the most important steps in optimization of PHA production process using MMC is the maximization of the selective pressure imposed on the culture for enrichment. Extensive research has been carried out on the impact of different SBR-operating conditions (Dias et al. 2006; Serafim et al. 2008a), but scarce information can be found in the literature on the microbial community composition. Culture-dependent methods are restrictive to study natural microbial community composition since only a small fraction of bacteria present in environmental samples (less than 1%) are culturable under laboratory conditions (Amann et al., 1995a; Head et al., 1998; Moyer et al., 1994). On the contrary, DNA-based molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridisation (FISH), provide a more comprehensive, rapid and concise characterization of the bacterial population diversity in biological systems.

Lemos et al. 2008 identified the genera *Amaricoccus*, *Azoarcus*, *Thauera* and *Paraccoccus* in a sequencing batch reactor fed with propionate (SBR P) as PHA accumulating organisms using reverse transcriptase–polymerase chain reaction (RT-PCR) on micromanipulated cells. Using quantitative FISH the amount of each genus was determined in the SBR P and in two other SBRs fed with acetate (SBR A and A1). SBR A and P had the same sludge retention time (SRT, 10 days) while A1 was operated with lower SRT (1 day) and the double organic loading

rate (OLR, 120 C mmol $L^{-1}day^{-1}$). The systems fed with acetate became enriched in *Thauera* while only present in minor amount of the system with propionate. *Azoarcus* cells were found in all the analyzed systems with the same trend as *Thauera*. Both SBR A and P present *Amaricoccus,* being this organism favored in the latter, while *Paracoccus* was scarcely present in any system.

Beccari et al. 2009 enriched two different microbial communities, both with high PHA storage response, using fermented olive mill effluent (OME) and a synthetic VFA mixture. Using DGGE technique two different bacterial strains were identified: *Lampropedia hyalina* and *Candidatus Meganema perideroedes*, with synthetic feed or fermented OMEs, respectively. *Lampropedia* spp was described as a polyphosphate and polyhydroxybutyrate (PHB) storing bacterial strain in activated sludge and *Candidatus Meganema perideroedes* shown to have a remarkably high storage capacity, forming PHA from a wide variety of substrates. Jiang et al. 2011a studied the impact of temperature and cycle length on microbial competition in polyhydroxybutyrate (PHB)-producing populations enriched in feast-famine SBRs. DGGE analysis revealed that the microbial community structure was strongly dependent on temperature, but not on cycle length. FISH was performed to estimate the relative abundance of the *Plasticicumulans acidivorans* in the reactors and it was observed that *Zoogloea* and *P. acidivorans* dominated the SBRs operated at 20ºC and 30ºC, respectively.

Recently, two studies investigated microbial communities with high PHA storage capacity selected with fermented molasses. Albuquerque et al. 2013 investigated the substrate preferences of microbial groups in PHA production. PHA-storing populations were identified and quantified through a 16S rRNA gene clone library and FISH. The community was composed by the genera *Azoarcus*, *Thauera* and *Paracoccus*. Microautoradiography-FISH showed that *Azoarcus* and *Thauera* primarily consumed acetate and butyrate, respectively,. *Paracoccus* consumed a broader range of substrates, having a higher specific substrate uptake. Carvalho et al. 2013 study the relationship between the MMC composition reported in Albuquerque et al. 2013 and PHA production performance. FISH quantification combined with DGGE analysis showed that the dominance of either *Thauera* or *Azoarcus* seemed to be determined by the organic loading rate imposed in the SBR. *Azoarcus and Paracoccus* abundance were related with higher and lower PHA production capacity, respectively. *Thauera* was strongly linked to higher hydroxyvalerate (HV) fractions and *Paracoccus* with lower HV fractions.

The purpose of this study was to characterize the bacterial diversity of two different PHA-accumulating communities and monitor changes in those populations during the reactors operation. This was achieve using a 16S PCR-DGGE approach that allowed identify the phylogenetic affiliation of community members by UPGMA analysis. DNA sequencing of bands excised from DGGE gels identified predominant bacterial phylotypes and the results were confirmed by FISH.

6.2. MATERIAL AND METHODS

6.2.1. PHA–accumulating organisms enrichment: Experimental setup

Two sequencing batch reactors (SBRs) were used to select PHA accumulating organisms from two different industrial biofuels by-product: bio-oil, resulting from the fast pyrolysis of chicken beds; and crude glycerol from biodiesel production.

In order to select a stable PHA accumulation culture fed with bio-oil (SBR-B) several operational modifications were performed to the system (Moita and Lemos 2012 and Moita et al. 2013). The experimental setup and operation of the SBR fed with bio-oil was described in Chapters 3 and 4. Briefly, in the initial condition (phase I) sludge retention time (SRT) and hydraulic retention time (HRT) were kept at 10 and 1 day, respectively in a 12h cycle. Tap water supplemented with phosphorus (P) and nitrogen (N) was used to dilute the bio-oil maintaining the COD/N/P ratios to 100:5:1 inside the reactor. After 61 days of the start up of the system (phase II) the SRT was reduced to 5 days. Since bio-oil already contains N and P in it composition, after 156 days of operation (phase III), tap water with no supplements started to be use do dilute bio-oil, decreasing the COD/N/P ratio to 100:1.7:0.5 Due to a decrease on the PHA storage capacity of the selected culture, after 300 days (phase IV), the COD/N/P ratios were restored to 100:5:1 using the supplemented tap water to dilute the bio-oil.

The experimental setup of the SBR fed with crude glycerol (SBR-G) was described in Chapter 5. Briefly, an SBR was inoculated with a PHA-accumulating mixed culture acclimatized to bio-oil (SBR-B) and fed with 30 CmM of crude glycerol in aerobic dynamic feeding condition. Tap water supplemented with phosphorus and nitrogen was used to dilute the crude glycerol inside the SBR maintaining the C/N/P ratios to 100:8:1. The SBR was operated with a 24h cycle and the HRT and SRT were kept at 2 and 5 days respectively

6.2.2. PCR-DGGE of the microbial community

The genomic DNA extraction of the samples was performed using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions. The extracted DNA was kept at -20 °C until use.

Primers 968F-GC and 1401R were used for the amplification of the hypervariable region V6 to V8 region of bacterial 16S rRNA gene fragments (Nübel et al. 1996). Each 50 µl reaction mixture contained 10 µl of 5X PCR buffer, 0.5 µl of each primer (100µM), 1 µl of RANGER DNA polymrase (BioLine) and 2 µl DNA template. Thermo cycling consisted of an initial denaturation at 95ºC for 1 minutes followed by 35 cycles of 95ºC for 30 s, 58ºC for 30 s, and 68ºC for 1min. The final extension was at 68ºC for 7 minutes. After PCR amplification, the size of the PCR products was verified on a 1% agarose gel.

DGGE was performed with Dcode™ Universal Mutation Detection System (Bio-Rad, USA). Aliquots of PCR samples (40 µl) were loaded on to 8% polyacrylamide gel in 0.5X TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM disodium EDTA, pH 8.3). The polyacrylamide gel was made with a denaturing gradient ranging from 45% to 55% (where 100% denaturant contains 7M urea and 40% formamide). The gel was run for 16 hours at 60 V and 60 ºC. After electrophoresis, the gel was stained with SYBERsafe (1:10000 dilution, Invitrogen, USA) for 30min. The DGGE images were acquired using a Safe Imager™ Blue-Light Transilluminator (Invitrogen™, USA).

DGGE profiles were analyzed using Totallab software (GE Helthcare). Every gel contained 2 or 3 lanes with a standard DNA ladder for normalization and as an indication of the quality of the analysis.

6.2.2.1. Analysis of DGGE profiles

The structural and functional diversity of the microbial community was assessed using the Shannon diversity index,, H' (Shannon and Weaver, 1963) and the evenness index, E' (Pielou, 1966):

$$H' = \sum \left(\frac{ni}{N}\right) \log\left(\frac{ni}{N}\right)$$

$$E' = H / \log S$$

where "ni" is the relative surface intensity of each DGGE band, "S" is the number of DGGE bands (used to indicate the number of species) and "N" is the sum of all the surfaces for all bands in a given sample (used as estimates of species abundance) (Formin et al 2002).

DGGE bands identified in the fingerprinting of each SBR were classified in different band types. A binary data matrix was created, considering the presence (1) or absence (0) of the individual bands. A dissimilarity matrix based on the Jaccard coefficient (Sj) was then calculated, and a dendrogram built using the UPGMA method (unweighted pair group average linkage method). The dissimilarity matrix was also used to perform a principal components analysis (PCA). All of the statistical analyses were done using the MVSP 3.1 software (http://www.kovcomp.co.uk/Mvsp/)

6.2.2.2. DNA Sequencing of selected DGGE bands

Selected bands from the DGGE gel of the SBR-B system were excised with a sterile scalpel and eluted in 100 µl of sterile Tris-HCl buffer (10 mM Tris-HCl, pH 8.00). After 5 days at 4 °C, 2 µl of the supernatant was used for re-amplification with the original primer set, but without the GC clamp attached to the forward primer (968F). The reaction mixture for this PCR was the same used in the first PCR reaction. Thermo cycling consisted of an initial denaturation at 95ºC for 1 minutes followed by 30 cycles of 95ºC for 10 s, 48ºC for 30 s and 68ºC for 30s. The final extension was at 68ºC for 7 minutes. After PCR amplification, the size of the PCR products (≅ 430bp) was confirmed on a 1% agarose gel.

For sequencing analysis, PCR products were purified using GeneJET PCR Purification Kit (ThermoScientific, USA), according to the manufacturers' instructions. DNA sequencing was performed by Eurofins MWG Operon (Germany). Band sequences were compared using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for identification and phylogenetic classification.

6.2.3. FISH

Biomass samples were fixed in 4% paraformaldehyde and used for fluorescence *in situ* hybridization according to Amann et al. 1995. The FISH probes used in this study are listened in Table 6.1. EUBmix probes were labeled with 6-FAM while every other probe was labeled with CY3. Visualization was carried out using an Olympus BX51 epifluorescence microscope coupled to a CCD camera.

FISH quantification (Q-FISH) of specific samples was preformed to quantify the *Betaproteobacteria* class in the SBR-B system. A LEICA TCS SPE confocal laser scanning microscope (CLSM) was used for observation of the hybridized samples and image acquisition. FISH quantification of Cy3-labeled *Betaproteobacteria* class with respect to all *Bacteria* (Cy5-labeled) was done by image analysis (30 images of each sample) using the Daime software (Holger Daims, Lücker, and Wagner 2006). The determination of the biovolume fraction of the specifically labeled target population was done relatively to the biovolume of total *Bacteria*.

6.2.4. Nile Blue Staining

With the goal of evaluating the PHA accumulating capacity of the culture, Nile blue staining (Ostle and Holt 1982) was applied to fresh samples taken from the SBR near the end of the feast phase. Visualization was carried out using an Olympus BX51 epifluorescence microscope coupled to a CCD camera.

Table 6.1- Information relevant to the FISH oligonucleotides used in this study

Probe	Target organisms	Reference
Actino-221a	*Actinobacteria*—potential PAOs	Kong et al (2005)
Actino-658a	*Actinobacteria*—potential PAOs	Kong et al (2005)
ALF969	*Alphaproteobacteria*, except of *Rickettsiales*	Neef A. (1997)
AMAR839	*Amaricoccus* (except *A. tamworthensis*)	Maszenan et al., 2000
ARC915	*Archea* domain	Stahl and Amman, 1991
AZA483	*Azoarcus* cluster	Hess et al(1997) and Loy et al (2005)
Bet42a	*Betaproteobacteria*	Manz et al (1992)
Cf319a	*Flavobacteria, Bacteroidetes, Sphingobacteria*	Manz et al. (1996)
DELTA495a	Most *Deltaproteobacteria* and most *Gemmatimonadetes*	Loy et al (2002) and Lückeret al (2007)
DELTA495b	Some *Deltaproteobacteria*	Loy et al (2002) and Lückeret al (2007)
DELTA495c	Some *Deltaproteobacteria*	Loy et al (2002) and Lückeret al (2007)
EPSY549	*Epsilonproteobacteria*	Lin X et al (2006)
EUB338	Most *Bacteria*	Amann et al (1995)
EUB338-II	*Planctomycetales*	Daims et al (1999)
EUB338-III	*Verrucomicrobiales*	Daims et al (1999)
EUB338- IV	Bacterial lineages not covered by probes EUB338,EUB338-II e III	Shimit et al (2005)
EUB338- V	Bacterial lineages not covered by probes EUB338,EUB338-II ,III and IV	Vannini et al (2010)
GAM42a	*Gammaproteobacteria*	Manz et al (1992)
GB742	*Candidatus Competibacter phosphatis* (Competibacter) subgroups 1to 8	Kim et al (2011)
Gnsb941	*Chloroflexi* (green nonsulfur bacteria)	Gich et al. (2001)
G_Rb	*Rhodobacter, Roseobacter*	Eilers et al (2000) and Giuliano et al (1999)
Lgc354a	Firmicutes (Gram+ bacteria with low GC content)	Meier et al. (1999)
Lgc354b	Firmicutes (Gram+ bacteria with low GC content)	Meier et al. (1999)
Lgc354c	Firmicutes (Gram+ bacteria with low GC content)	Meier et al. (1999)
Pla46	Planctomycetales	Neef et al. (1998)
THAU832	Thauera spp.	Loy et al 2005
TM7905	candidate division TM7	Hugenholz et al. 2000)
UCB-823	Plasticicumulans acidivorans	Johnson et al. (2009)
ZRA23a	Most members of the Zooglea lineage, not *Z.resiniphila*	Rosselló-Mora et al., 1995

Actino-221a and Actino-658a used together as Actino mix

DELTA495a, DELTA495b, DELTA495c used together as DELTA mix

EUB338, EUB338-II, EUB338-III, EUB338-IV, EUB338-V used together as EUB mix

Lgc354a, Lgc354b and Lgc354c used together as Lgc mix

6.3. RESULTS

6.3.1. PHA accumulating organism selected using fast pyrolysis by-product as feedstock

6.3.1.1. Reactor performance

The evolution of the microbial consortium in the bio-oil SBR reactor was followed for almost 2 years (Fig. 6.1) and it was described in detail in Moita and Lemos 2012 and Moita et al. 2013. Briefly, during the initial condition (phase I) the system showed a good response of the bacterial community to the bio-oil. Substrate (S) was consumed at an average rate (qs) of 0.074 Cmmol S/Cmmol X.h (X, active biomass), the polymer production yield on substrate ($Y_{P/S}$) increased to an average value of 0.19 Cmmol HA/Cmmol S and the Feast to Famine ratio (F/F) decreased below 0.2. F/F ratios lower than 0.28 are considered to allow PHA accumulating organisms to outcompete non-accumulating bacteria improving the good storage response by the selected culture (Dionisi et al. 2006; Johnson et al. 2009; Albuquerque et al. 2010a; Jiang et al. 2011b). During this phase, biomass increased drastically reaching a maximum of 6 g/L of volatile suspended solids (VSS). With this increase the food to microorganism ratio (F/M) would have the tendency to decrease to values where the carbon source could became limiting. In order to maintain a good F/M, two strategies could be applied: increase of the organic loading rate (OLR) or decrease of the SRT. Since bio-oil is a very complex feedstock with compounds that may inhibit or interfere with the production of PHA the strategy to decrease the SRT to 5 days was preferred and applied after 68 days of operation. After altering the SRT from 10 to 5 days (phase II) the VSS decreased as expected to an average value of 4 g/L and the selective pressure imposed to the system led to an improvement on substrate uptake rate.

Bio-oil contains nitrogen and phosphorus in its composition. With the intent to decrease the production cost of PHA production process, tap water started to be use do dilute bio-oil after 156 days of operation. The COD/N/P ratio decreased from 100:5:1 to 100:1.7:0.5 molar basis (phase III). Initially an apparent increased on the PHA storage capacity of the culture was observed (Fig. 6.1). However, after 250 days of operation the specific substrate uptake rate decreased significantly resulting in an increase of the F/F ratio (0.4). As a consequence of the increased F/F ratio the storage mechanisms of the microbial community began to be neglected and the storage yield of the system decreased. The shorter famine phase lead to the inability of the PHA-accumulating organisms to fully consume the polymer previously accumulated, during the feast phase. As such while the maximum PHA content showed a tendency to increase during this period of time the volumetric PHA production decreased from 5.34 to 2.36 Cmmol HA/L (Fig. 6.1). The N limitation imposed to the system seemed to be responsible for the selection of

a population with a lower substrate uptake rate and therefore endangering the F/F pressure imposed to the system.

After 300 days of operation, the COD/N/P of 100:5:1 was restored with tap water for the dilution of the bio-oil, supplemented with nitrogen and phosphorus (phase IV). After this change, the F/F ratio returned to 0.2 in average. As a consequence of the increased substrate uptake rate (0.0420 to 0.1410 Cmmol S/Cmmol X.h) the PHA storage capacity of the system was restored, with a PHA production yield reaching ≅ 0.40 Cmmol HA/Cmmol S and a PHA content of ≅ 7% cell dry weight, on average.

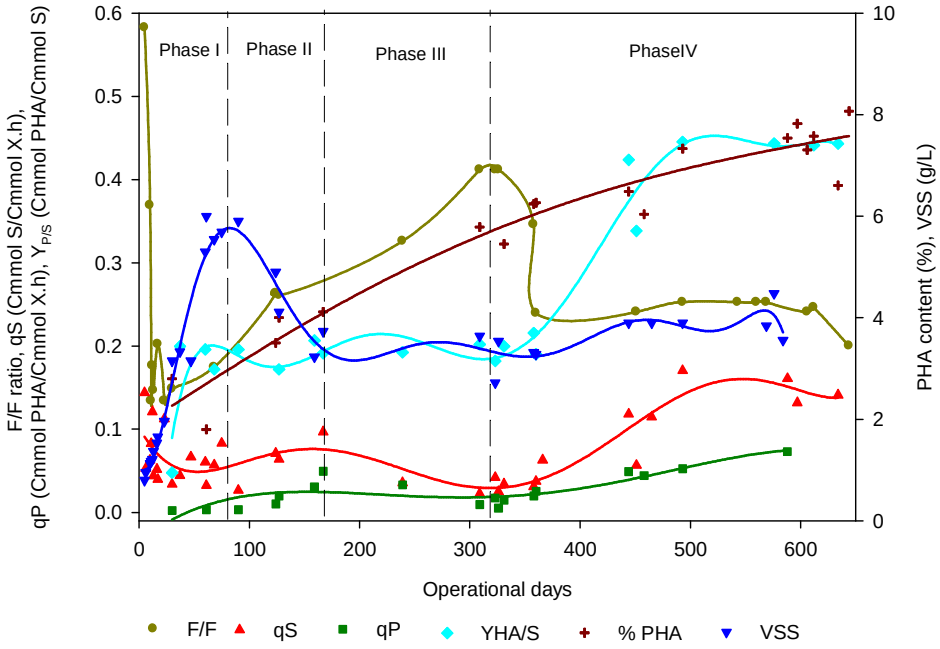

Fig. 6.1- Evolution of the SBR-B performance.

6.3.1.2. DGGE analysis of bacterial community

DGGE analysis of 16S rRNA gene was performed to investigate bacterial community changes in the SBR fed with bio-oil (SBR-B). Representative samples of each phase of acclimatization period were selected and the DGGE profiles obtained for each sampling day are shown in Fig. 6.2. Phase I was represented by day 17 and 68; phase II was represented by day 124; phase III was represented by days 182 and 212 and finally phase IV was represented by days 309, 339, 416, 458, 569, 634. The number of DGGE bands per lane varied between 8 and 17. DGGE fingerprints revealed the microbial changes during the SBR operation period with some shared

bands in all samples (i.e bands 9 and 13), while others were only present in some sampling days (i.e bands 4 and 8).

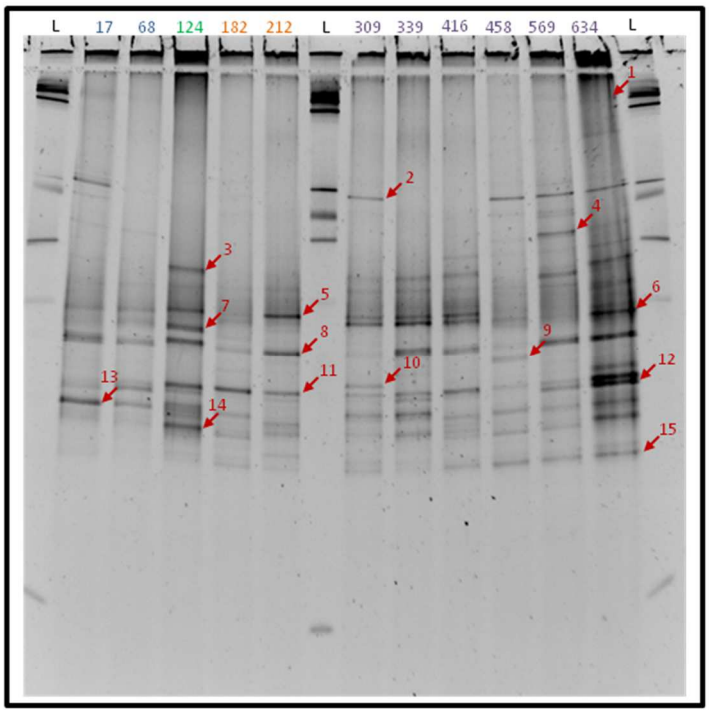

Fig. 6.2- DGGE community fingerprints of the bio-oil enriched biomass along time ("L" corresponds to ladder; top numbers indicate the acclimatization days of the sample; arrows and numbers relative to excised bands for sequencing identification)

Cluster analysis was performed using the Jaccard's coefficient aiming to investigate the similarity between the samples (Fig. 6.3). The dendogram showed significant variations in bacterial communities during operation of the SBR-B. The largest shift in bacterial assemblage was identified between samples from phase I (17 and 68) to the rest of the samples (39.9% of similarity). The similarity of the bacterial community during phase II, III and start of phase IV varies between 63.4% and 91.7%, demonstrating a large shift of population occurring during the parameters adjustments imposed to the SBR-B. This group of samples has only a similarity of 49.2% with the days 458, 569 and 634. These last three days represent a period where a new pseudo-steady state of the system was achieved. However a significant shift of population was still observed between day 458 and the last two days (67.2% of similarity) and the two month period that separates day 569 and day 634 have also allowed a considerable shift of population (81.3% of similarity).

Despite the high shift in populations observed during the last period of the SBR-B system no significant differences on stoichiometric and kinetic parameters of the referred days were

observed. Moita et al. 2013 reported that the diversity of carbon present in bio-oil allowed diverse microbial populations to co-exist in the system. The more easily biodegradable fraction of carbon present in the bio-oil was show to be consumed at a higher rate by PHA accumulating organisms. Populations without the ability to store polymers were able to grow and persisted in the system throughout the consumption of the less biodegradable carbon fraction that was consumed during the entire SBR cycle. Hence, since the PHA storage capacity of the system was maintained constant during the pseudo-steady state, the high shift of population verified in this period of time could be explained by shifts on the non-PHA storing organism present in the microbial consortium.

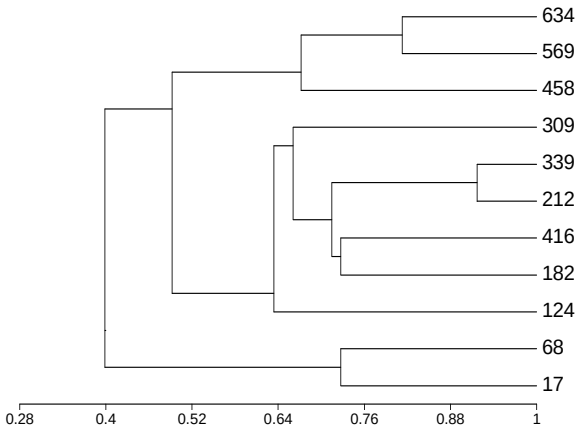

Fig. 6.3- Cluster analysis of the microbial community present in the SBR-B based upon DGGE profiles. Similarities were calculated using Jaccard's coefficient.

To better visualize the relationships among samples a binary matrix was constructed based on the presence or absence of bands. The resulting matrix was used to conduct a PCA analysis allowing identifying different clusters. In PCA analysis, PC1 captured 31.7% of variance and PC2 captured 28.3%, totalizing 60% of variance. The subsequent PCs captured progressively lower variance percentages, thus only the loadings of the first 2 principal components were analyzed. The loadings of PC1 and PC2 (Fig. 6.4) showed three main clusters which corresponded to the three main operation periods verified in the system. Cluster1 represented the initial operational conditions (phase I). Day 124 as the only representative sample from phase II was not inserted into any of the three defined clusters. Cluster 2 corresponded to the operation period were the PHA production yield of the system was low (≅ 0.2 Cmmol HA/Cmmol S) and the system performance was not stable. This cluster could be divided in two clusters that were positively correlated, 2A and 2B, and represented phase III and the start of

phase IV of the SBR-B, respectively. Cluster 3 stands for the pseudo-steady state period of the SBR, which included the final phase of operation (IV, i.e samples 458, 569, 634).

In agreement with results obtained during cluster analysis of the DGGE fingerprinting, the PCA analysis clearly showed that all samples were grouped according with the selective pressure imposed. This demonstrated the effect that changes in operation condition had on the microbial community and how it affected the selection of organisms with a good PHA storage capacity.

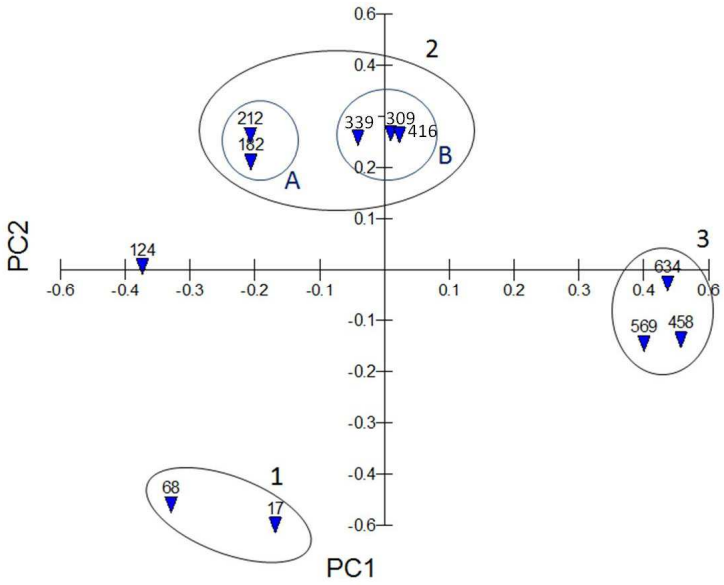

Fig. 6.4- PCA analysis using the presence/absence matrix of the DGGE profiles of the operation of MMC with bio-oil as carbon source (SBR-B). PC1 and PC2 captured 60% of variance (31.7 and 28.3 respectively).

The use of indexes based on relative band intensities provides information about community composition other than the number of species (Moura et al. 2009). Densiometric curves of DGGE patterns were used to calculate the relative surface intensity of each DGGE band present. A numerical analysis of the DGGE patterns was performed using two indexes (H' and E'), each one describing a different aspect of community diversity. No significant differences could be observed for all the operations periods when the Shannon diversity index was considered, being all the values on the low range. The microbial diversity (H', Table 6.2) was higher at the end of phase IV reaching a maximum of 1.14 ± 0.03 (day 634). Phase II and Phase IV presented the same operational conditions, however the H' was higher in the latter (H'= 0.89± 0.01, day 124 and H'= 1.14± 0.03, day 634). The difference on the time of operation that separates these days (510 days) seems to have allowed an acclimatization of the culture to different carbon sources present in the bio-oil. In fact, the higher and increased microbial diversity observed during Phase IV together with a higher substrate uptake rate raises the hypothesis that along the

operation time the selection of organisms able to better utilize the available carbon sources occurred.

The community evenness index (E) of all samples were close to one and no significant different were again obtain between samples. These results indicated that the distribution of microbial groups at different operational phases was uneven, probably due to the high diversity of carbon sources present in the bio-oil which could be metabolized by different microbial populations (PHA storing and non-PHA storing organisms).

Table 6.2- Shannon diversity index (H) and evenness index (E) of each sample analyzed trough DGGE

Acc. Days	**17**	**68**	**124**	**182**	**212**	**309**	**339**	**416**	**458**	**569**	**634**
H'	0.94	0.86	0.89	0.87	0.93	1.00	0.95	0.92	1.01	1.03	1.14
E'	0.90	0.95	0.99	0.91	0.86	0.90	0.91	0.92	0.90	0.93	0.93

6.3.1.3. *Sequencing of DGGE bands*

The identification of the microorganisms represented by bands in the DGGE profiles was determined by excision and sequencing. It was possible to obtain 15 sequences (corresponding bands are indicated in Fig. 6.2). Each partial 16S rRNA gene sequence was submitted to a BLAST search and the results of their closest relative are shown in Table 6.3. Many of the sequences were similar to 16S rDNA sequences reported for uncultured organisms obtained from environmental samples, such as activated sludge, wastewater and soil, reinforcing the importance of culture-independent methods for the study of microbial communities

Sequencing results show that β and γ-*proteobacteria* were the predominant classes present in the microbial consortium. For the γ-*proteobacteria* six different bands (2, 3, 7, 8, 11 and 12) were identified with the genus *Pseudomonas*. This genus contains several species with the ability to accumulate PHA (C. S. K. Reddy et al. 2003) and it was present in all the samples analysed. Other genera known to have PHA-accumulating organism were also identified. Considering the ß-*proteobacteria,* genera *Comamonas* (band 5), *Brachymonas* (band 6), *Burkholderia* (band 14) and *Alcaligenes* (band 15) were identified. The three latter genera were only present after phase III of the acclimatization period. *Comamonas* genus was identified in most samples (exception days 458, 569 and 634). Despite the fact that *Comamonas* is an

important genus among the PHA accumulators, it did not contributed for the high PHA storage capacity observed in the system during that period of time, the end of Phase IV.

Genera *Bordetella*, *Achromobacter* and *Herminiimonas* can be found in several environmental samples (wastewater, soil etc). They were detected in only a few days without any perceived correlation between their presence and the operational conditions. *Arthrobacter* genus is commonly found in the soil, being present in all the samples. All species of this genus are obligate aerobes and some strains have been show to grow on a variety of aromatic compounds (O'Loughlin, Sims, and Traina 1999), in which bio-oil is rich.

The bands excised did not allow identifying a specific genus (or genera) responsible for the high storage capacity of system represented by cluster 3 in the PCA analysis. The most relevant genera in the microbial community selected with bio-oil resulting from the fast pyrolysis of chicken bed were *Pseudomonas, Brachymonas, Burkholderia* and *Alcaligenes,* possibly responsible for the reported PHA storage capacity of the system. Since these organism were present during all the operation period the conditions imposed to the SBR-B in phase IV did not allowed for the selection of new PHA accumulating organism, but rather to increase the amount of pre-existing ones.

DGGE technique is considered has a qualitative rather than a quantitative technique having some general and specific limitations that need to be considered. Some general potential biases are linked to sample type, handling and storage as well as biases in the PCR technique and inefficiencies in DNA extraction methods. Also DGGE has limited detection sensitivity for some rare community members (only predominant species in a community are displayed). Moreover, the choice of bands to excise can be highly subjective and due to the possible co-migration of DNA fragments with different sequences, excising and sequencing bands can miss hidden diversity (Muyzer 1999).

Table 6.3- Phylogenetic sequence affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from DGGE gels band

	Phylogenetic affiliation			
Band Number	Class	Genus	Similarity (%)	Accession number
1	*α-proteobacteria*	*unknown*	87	JN679095
2	*γ-proteobacteria*	*Pseudomonas*	76	C255076
3	*β-proteobacteria*	*Bordetella*	92	JQ965641
4	*γ-proteobacteria*	*Pseudomonas*	87	KC255152
5	*β-proteobacteria*	*Comamonas*	100	JQ912536
6	*β-proteobacteria*	*Brachymonas*	99	EU434401
7	*γ-proteobacteria*	*Pseudomonas*	94	JF94771
8	*γ-proteobacteria*	*Pseudomonas*	95	KC255152
9	*β-proteobacteria*	*Achromobacter*	91	JX512462
10	*β-proteobacteria*	*Herminiimonas*	100	KF556700
11	*γ-proteobacteria*	*Pseudomonas*	100	KC963965
12	*γ-proteobacteria*	*Pseudomonas*	99	HG416957
13	*Actinobacteridae*	*Arthrobacter*	99	KC683723
14	*β-proteobacteria*	*Burkholderia*	100	JQ023738
15	*β-proteobacteria*	*Alcaligenes*	96	DQ152012

6.3.1.4. Microbial community analysis by FISH

During the acclimatization period microscopic observation of the sludge was frequently preformed and no significant changes in the organism's morphology were observed. The microbial community had the tendency to aggregate in dense flocs which difficult morphological observation. Regardless this situation, morphologically the bacterial community was mainly composed of cocobacilli. Nile Blue staining revealed the presence of PHA granules.

Initially, several generic FISH probes (Table 6.1) were tested in order to identify relevant bacterial phylum/classes and also specific genus of known PHA accumulating organisms like *Thauera, Zooglea, Azoarcus* and *Amaricocus*. From all the tested probes only probes ALF969, ARC915, BET42a, EUB338mix, GAM42a, G_Rb, GB-742 and THAU832 hybridized. Table 6.4 summarizes the semi-quantitative hybridization of these probes for samples characterizing phase I, II, III and IV of SBR-B.

Table 6.4- Hybridization of FISH probes during the operation of the SBR feed with bio-oil

FISH probes	Operation days								
	Phase I		Phase II		Phase III		Phase IV		
	0	17	92	127	182	267	458	569	639
ARC915	+[a]	+	+	+	+	+	+	+	-
ALF969	+	+	+	+	-	-	+	+	+
G_Rb	+	+	+	+	-	-	+	+	+
BET42a	+	+	++	++	++	++	+++	+++	+++
THAU832	-	-	+	+	-	-	-	-	-
GAM42a	+	+	+	-	+	+	+	+	+
GB-742	+	-	-	-	+	-	-	-	-

[a] + positive hybridization; −, negative hybridization.

The majority of organisms present during all the operation time belong to the *Bacteria* domain being *Archeae* domain also present (except sample on day 639) but at trace amounts. The three classes of Proteobacteria, *Alpha, Beta* and *Gama*, were present in almost all samples. Except for Phase III of the SBR-B (days 182 and 267), all the samples belonging to the other operation periods hybridized with the G-Rb probe (*Alphaproteobacteria*). This probe hybridizes with *Rhodobacter* genus which contains several known species able to accumulate PHAs (Philip et al., 2007). Probe GB-742 reacted with samples at time 0 and 182. *Gammaproteobacterial GAO* (GB) known as '*Candidatus Competibacter phosphatis'* have been intensively studied and are widely present in lab- and full-scale enhanced biological phosphorus removal (EBPR) processes. *Betaproteobacteria* class reveled to be the most dominant class. For the known PHA accumulating organism tested only *Thauera* was detected in Phase II (days 92 and 127) of the system.

Fig. 6.5 shows the positive hybridization of probes ALF969, BET42a, G_Rb and GAM42a at the end of phase IV (day 639), demonstrating not only the high diversity microbial population present but also that the dominance of the *Betaproteobacteria* class at this period (Fig 5C).

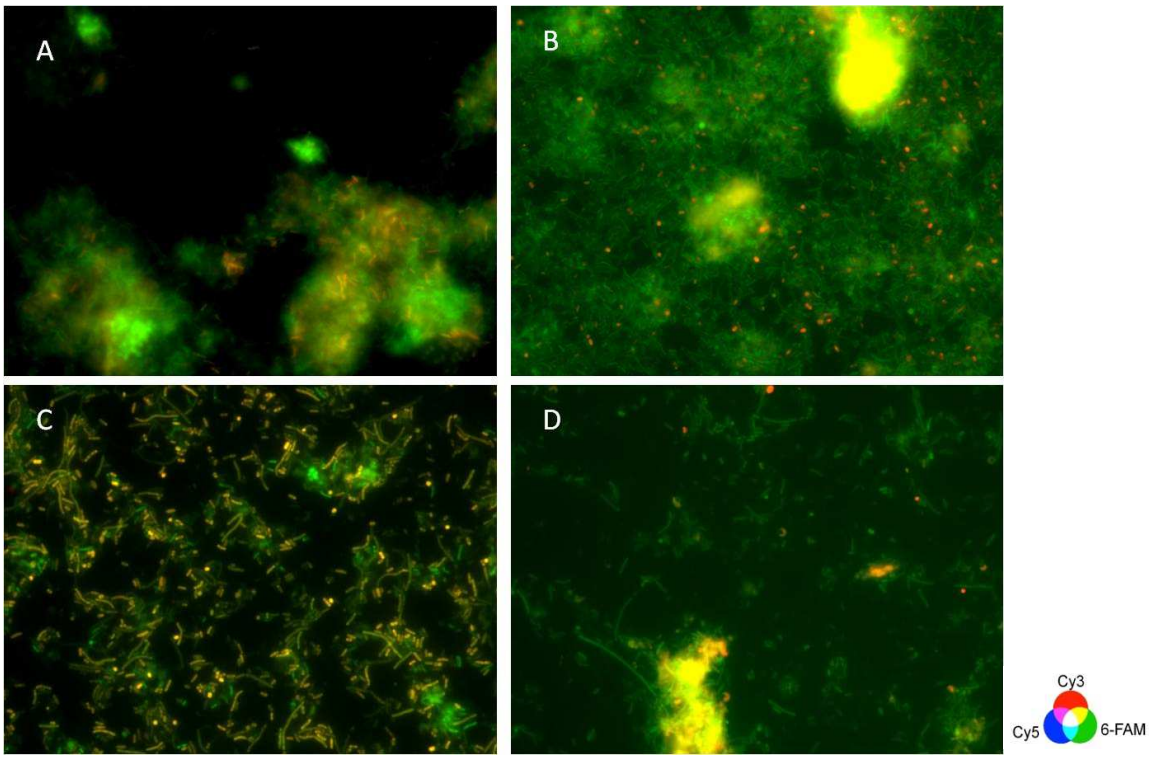

Fig. 6.5-FISH images at day 639. Combined hybridization of: **(A)** EUBmix probes (6-FAM) with Alpha969 probe (CY3); **(B**) EUBmix probes (6-FAM) with G-Rb probe (CY3); **(C)** EUBmix probes (6-FAM) with Bet42_a probes (CY3); **(D)** EUBmix probes (6-FAM) with Gama42_a probe (CY3); 1000x

Q-FISH using confocal laser microscopy was performed in one representative sample of each acclimatization phase: day 17 (phase I), day 127 (phase II), day 182 (phase III) and day 639 (phase IV). An increase of the *Betaproteobacteria* class along the operational period from 4.7% at Phase I to 73.4% at Phase IV was observed (Fig. 6.6A). Fig. 6.6B shows a Q-FISH image clearly demonstrating the dominance of this genus at the end of the phase IV. Three of the four genera identified during the DGGE band sequencing as PHA accumulators belong to the *Betaproteobacteria* class (*Brachymona*, *Burkholderia* and *Alcaligenes*). The increase on the *Betaproteobacteria* class along with the higher PHA storage capacity of the SBR-B system reported during Phase IV may be explain by an increase of these three genera.

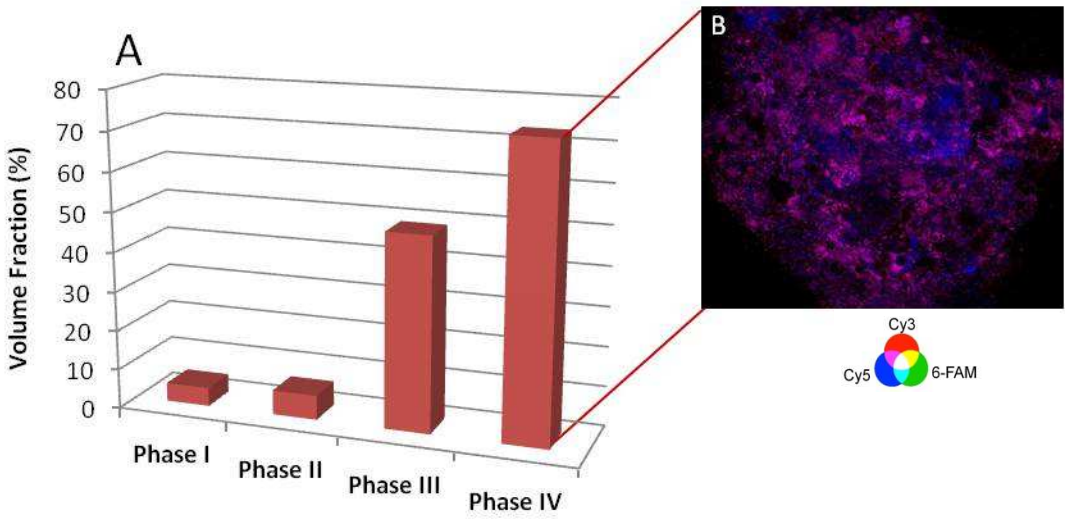

Fig. 6.6-(A) Evolution of the *Betaproteobacteria* class during the SBR-B operation (Biovolume relative to total *Bacteria*). **(B)** Q-FISH image at day 639 (Phase IV). Combined hidridization of EUBmix probes (Cy5) with BET42a (Cy3); 400X

6.3.2. PHA accumulating organism selected using crude glycerol as a feedstock

6.3.2.1. Reactor performance

The SBR-G was inoculated with a PHA-accumulating culture selected with bio-oil from the final Phase IV of SBR-B. The operation period of the microbial consortium adapted to crude glycerol utilization was described in detail in Chapter 5 and lasted for 2 months. Briefly, both main crude glycerol carbon sources, glycerol and methanol, were consumed by the selected culture having the latter a significant lower rate. Glycerol was totally consumed during the first hour of the cycle (-0.32 Cmmol S/Cmmol X.h) while methanol stop being consumed after 1.33h and it was not totally exhausted at the end of the cycle. Glycerol was the only carbon source contributing for both biopolymers production: PHB and glucose biopolymer (GB). GB had a specific production rate almost two times faster than PHB (0.11 Cmmol GB/Cmmol X.h and 0.06 Cmmol HB/Cmmol.X.h, respectively). Also GB storage yield (0.42 Cmmol GB/Cmmol S) was higher than the PHB storage yield (0.22 Cmmol HB/Cmmol S). Although the system seemed to be more specialized for glycogen production, accumulation assays performed with crude glycerol (Chapter 5) demonstrated that along the duration of the assay the selected culture rapidly losses the ability to accumulate glycogen and maintains the PHB production. A maximum PHB content of 47% cell dry weight and a storage yield of 0.46 Cmmol HB/Cmmol S was obtained.

6.3.2.2. DGGE analysis of bacterial community

The operation time of the SBR-G was short and the bacterial community changes were only investigated between day 0 (inoculation time) and at the end of the second month of operation (day 61). DGGE fingerprinting (Fig. 6.7) revealed that the majority of the bands were maintained and some of them present a higher intensity at the end of the analyzed period (i.e bands 11 and 13). Band excision and sequencing was performed in the same way as for the SBR-B system: However some unidentified problems, probably in the DNA quality made it impossible to obtain quality sequences for a good identification.

Cluster analysis performed using the Jaccard's coeficient establish a similarity of 55% between samples. This significant verified shift in the population clearly demonstrates the adaptation of the selected culture to the crude glycerol as the new feedstock.

Although the microbial culture was totally acclimatized to the crude glycerol as the new feedstock no significant changes in the microbial diversity were observed. Densiometric curves of the DGGE patterns reported that the Shannon diversity index (H') between the beginning and the end of the acclimatization period of the SBR-G was very similar (H'= 1.09 and 1.02, respectively). The community evenness indexes (E') was close to one in both samples and again no significant different were observed between samples (E'=0.90 and 0.87, respectively).

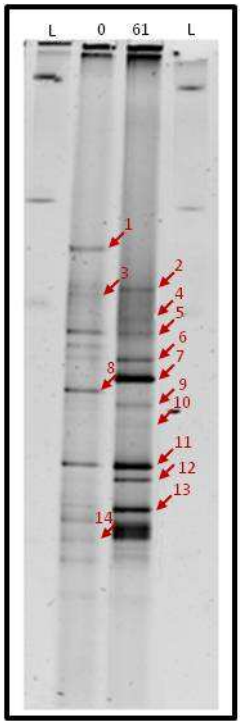

Fig. 6.7- DGGE community fingerprints of the crude glycerol enriched biomass at the beginning and end of the acclimatization period ("L" corresponds to ladder; top numbers indicate the operation days of the sample; arrows and numbers relative to excised bands for sequencing identification)

6.3.2.3. Microbial community analysis by FISH

The bacterial community selected with crude glycerol (day 61) was mainly composed by three distinguish morphotypyes: tetrad-forming organism (TFO), cocci and coccobacilli. Nile Blue staining revealed the presence of PHA granules inside the TFO and cocci bacteria (Fig. 6.8). Both populations appeared in an aggregated form. In opposite, the cocobacilli population was wide dispersed and did not present any PHA granules.

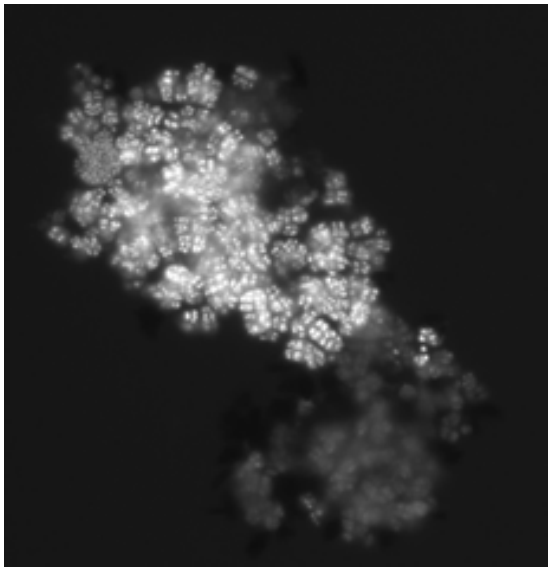

Fig. 6.8- PHA staining by Nile Blue A of the mixed community (SBR-G); 1000X

FISH analysis of the microbial community from day 61 of SBR-G was performed using the probes identified in Table 6.1. The generic *Proteobacteria* FISH probes showed the presence of *Alpha*, *Beta* and *Deltaproteobacteria*. The coccobacilli population that hybridized with the DELTAmix probes (Fig. 6.9A) did not present the capacity to accumulate PHA.

The TFO morphotype hybridized with ALF969, G-Rb and AMAR839 probes (Fig. 6.9 B and C) and it was identified as *Amaricocus*. Falvo et al. 2001 reported that *Amaricoccus kaplicensis*, a Gram-negative with a distinctive morphology of cocci arranged in clusters or tetrads found in samples of biomass from activated sludge plants all over the world, had a high storage capacity of accumulate PHB from acetate at high rates. *Azoarcus* and *Zoogloea* genus were also detected (*Betaproteobacteria*). All the other probes tested did not show a positive signal.

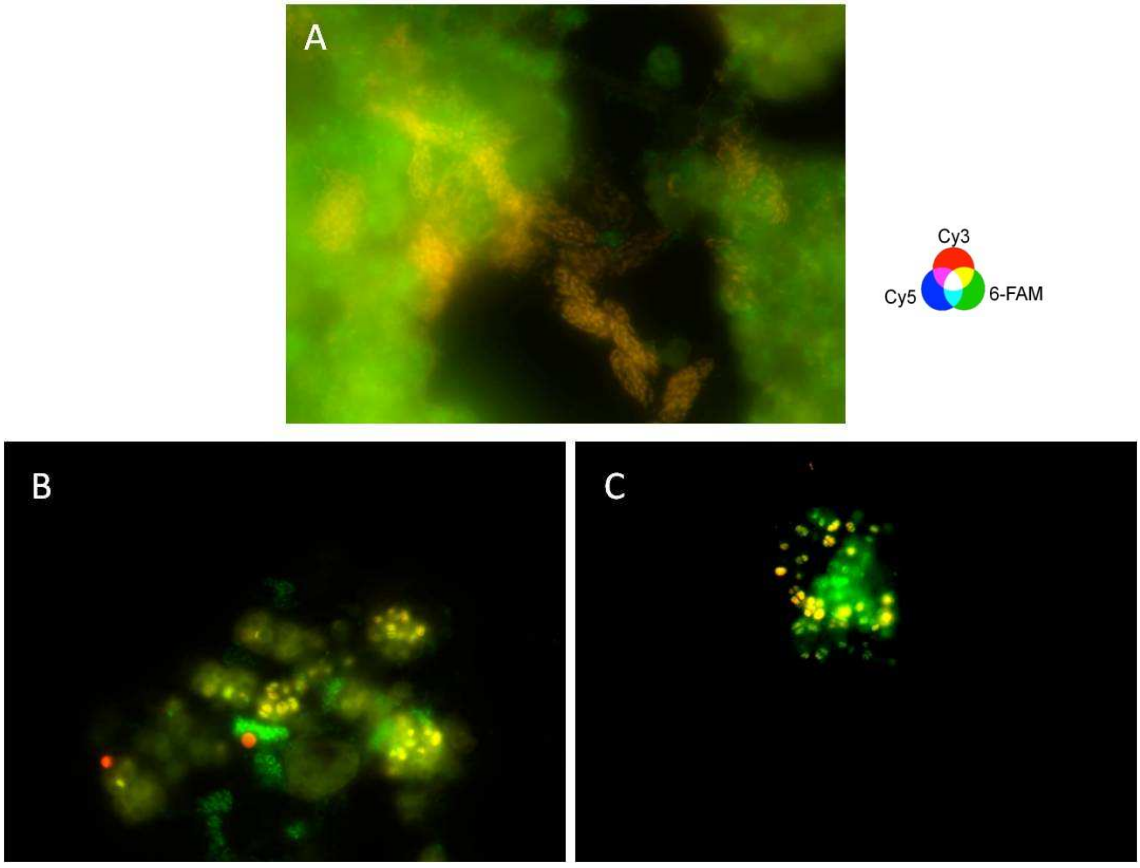

Fig. 6.9- FISH image at day 61. Combined hybridization of:.(**A**) EUBmix probes (6-FAM) with DELTAmix (Cy3); (**B**) EUBmix probes (6-FAM) with G-Rb (Cy3); (**C**) EUBmix probes (6-FAM) with AMAR839 (Cy3); 1000X

6.4. CONCLUSIONS

This study investigated the dynamics and composition of two established PHA-producing bioreactors fed with biofuels by-products. The evolution of the microbial community present in the system fed with bio-oil was accompanied during the entire operation period were different operational condition were imposed. The statistical methods used to interpret the PCR-DGGE fingerprinting, (PCA and clustering analysis), demonstrated that different operational conditions induced in the SBR a strong selective pressure on the microbial community. Although it was observed a significant change in the microbial community during the reactor operation period, the diversity indexes (H' and E') were very similar along time. These observations could be a consequence of the high variety of carbon sources present in the bio-oil which allowed the selection of PHA-producing and non-PHA producing organisms. Sequencing of excised bands from the DGGE gel and FISH analysis identified *Pseudomonas*, *Brachymonas*, *Burkholderia* and *Alcaligenes* as some of the genera responsible for the reported PHA storage capacity of the SBR-B system. FISH quantification confirmed that *Betaproteobacteria* has the most dominant class in this system reaching 73.4% of the bacterial population at the end of the operation time.

The observed increase in the *Betaproteobacteria* class was directly related with the reported growth of the PHA storage capacity of the SBR-B system.

The microbial analysis of the system fed with crude glycerol demonstrated the versatility of an enriched culture to adapt to a new substrate. Clustering analysis establish a similarity of 55% between the beginning and two months later of reactor operation, clearly indicating the adaptation of the selected culture to the crude glycerol as the new feedstock. Two morphotypyes (TFO and cocci) identified in the bacterial community selected with crude glycerol revealed the presence of PHA granules inside the cells. A third morphotype (coccobacilli) without the ability to accumulate PHA appears in lightly higher numbers and FISH analysis identified them as belonging to the *Deltaproteobacteri*a class. The TFO population was identified has *Amaricocus sp.*, which has been reported has a PHA accumulator genus. The high amount of the TFO observed at the end of the acclimatization time, suggests that the *Amaricocus* was one of the most relevant genus responsible for the high PHA accumulation reported during an accumulation assay with crude glycerol (47% cdw).

The results of this study demonstrate that the statistical analyses combined with molecular techniques are good strategies to follow the microbial community evolution during the enrichment period in SBR PHA-producing systems.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1. GENERAL CONCLUSIONS AND FINAL OVERVIEW

Investigating new and improved ways to valorized biofuels waste and by-products will allow reducing the total production cost, making the overall biofuels production a more sustainable process. In this thesis two different by-products resulting from distinguish biofuels production processes were successfully used as feedstock to produce PHA using aerobic mixed cultures.

Biological conversion processes have been widely used for biotechnology research, but relatively unexplored for the conversion of pyrolytic products to biomaterials. The main drawback in using bio-oil resulting from the fast-pyrolysis of lignocellulosic material as feedstock to produce transportation fuels is its characteristic as corrosive, thermally unstable and the fact that it contains large amounts of small oxygenated species (and water) with little economic value. The high water content in addition with the high concentrations of alcohols, aldehydes, ketones, carboxylic acids and other polar components present in some bio-oils has recently motivated the interest in their use as substrate for microbial fermentations. To the data only ethanol and some triglycerides were produced by pure single strains using the sugars fraction present in the bio-oil, after a detoxification step.

One of the main contributions of this work was to demonstrate the possible valorization of a waste lignocellulosic material (chicken beds), with no other valorisation rather than combustion, through the direct used of the resulting fast-pyrolysis bio-oil to produce PHA.

A two-step process for PHA production by mixed cultures using bio-oil resulting from the fast-pyrolysis of chicken beds was successfully established. Although bio-oil was directly used without any pre-detoxification step results suggest that bio-oil contained some compounds that may have inhibited or interfered with the polymer production. To the best of our knowledge this was the first study that used the entire bio-oil, resulting from a fast pyrolysis process, as feedstock.

Despite the high carbon content of bio-oil it was possible to achieve a good feast/famine ratio (≅0.2) on the selection reactor, considering only the consumption of the more easily biodegradable fraction of bio-oil. The imposed selective pressure allowed selecting a microbial culture able to produce a co-polymer composed of 70%:30% HB/HV with an average PHA content of ≅7% (g HA/g cell dry weight) at the end of feast phase of the SBR and a storage yield of 0.37 Cmmol HA/Cmmol S.

Bio-oil contains a high number of carbon sources able to be metabolized by microbial cultures. From all available substrates, sugar-based compounds were determined to be important (37% of

CBO_5) and their consumption by the selected culture was investigated. Bio-oil characterization revealed the presence of glucose and xylose. However, when these two sugars were tested independently as carbon source no consumption was observed. As such, these results suggest that both sugars could only be used as carbon source by the selected culture when other co-substrates were present. Sugar consumption seems to be responsible for the accumulation of another storage material by the selected culture: glycogen. Although two different biopolymers were stored by the culture, the system seems be more specialized in PHA production since glycogen was store to a lower extent (≅1 % g glucose/g cell dry weight).

The high amount of nitrogen naturally present in this bio-oil revealed to be an obstacle to study the maximum PHA storage capacity of the selected culture. For the accumulation assays results showed that growth was present along the entire process, allowing the culture to drift their metabolism preferably to growth in detriment of PHA storage overtime. However, the specific PHA accumulation rate and storage yield with pure bio-oil for the 1st pulse in a multi pulse-feed strategy were between the values reported in other studies that use MMC and real complex substrates suggesting that bio-oil can be used as feedstock to produce short chain length PHA.

With regard to the maximum PHA content obtained with bio-oil (9.8% cell dry weight), this was below the vast majority of studies using real complex substrates and MMC. Several bio-oil features could explain these results. First the bio-oil nitrogen content that drift the metabolism preferably to growth in detriment of PHA storage during accumulation assays. Secondly, unlike the other real complex substrates tested to produce PHA, pure bio-oil contains a lower VFAs content, the main precursors to produce PHAs from MMC. Finally, the large variety of carbon present in the bio-oil allowed diverse microbial populations to co-exist in the system. Populations without the ability to store polymers were able to grow and persisted in the SBR throughout the consumption of the remaining nitrogen and the less biodegradable carbon fraction.

When synthetic acetate was used as the only carbon source to produce PHA from the enriched culture with bio-oil, a significantly higher PHA content (≅32 % cell dry weight) along with a higher storage yield were obtained. Acetate was identified as one the most relevant VFAs present in the bio-oil. As such, these results suggest that the lower PHA storage capacity reported with pure bio-oil does not result from a low storage capacity of the selected culture but from the complexity of the bio-oil as substrate.

The two strategies used to upgrade bio-oil in order to maximize the PHA accumulation, exhibited completely different results. The main products of the distillate bio-oil were aromatic compounds (phenols, xylenes, pyrazines and pyrimidines) and long chain fatty acids. Results

suggested that at least 50% of the carbon that remained in distillate was not biodegradable or not able to be used by the mixed culture. Overall, considering the total carbon present in the bio-oil only a small fraction was metabolized and converted to PHA. Bio-oil distillation allowed to reduce the nitrogen content of the distillate by 75%. However, despite the lower content of nitrogen in the distillate bio-oil when it was used as a carbon source in an accumulation assay, biomass growth was still observed and, in the last pulse, the culture appeared to favor growth in detriment of PHA production. This situation highlights the importance of nitrogen removal in order to study the maximum PHA storage capacity of the selected culture. Acidogenic fermentation of the bio-oil was responsible for the conversion of 42% of the sugar-based compounds into VFAs. The amount of acetic, propionic and butyric acid present in the fermented bio-oil increased three, five and nine times respectively. Results demonstrated that other carbon sources rather than sugar were converted into VFAs since only 12% of the produced VFAs came from the sugar fraction consumed. The increased of VFAs in the fermented bio-oil resulted in a significant increase on PHA production yield (0.63 Cmmol HA/Cmmol S, 1st pulse). Also, due to the lower sugar content of the fermented bio-oil, no glycogen production was reported using this feedstock.

In summary, microbial cultures could use the bio-oil without any detoxification process. The direct used of the bio-oil to produce scl-PHA using MMC showed to be a feasibly process. However, results using the fermented bio-oil suggested that by using a three-step process for PHA production by mixed cultures from bio-oil would probably allow for a more effective selection of organisms with high PHA storage capacity in the selection reactor step. The selection of a culture with a higher PHA storage capacity would eventually result in higher PHA content and storage yield in the accumulation step.

With the biodiesel industry booming all over the world, an excess of crude glycerol is being created. Because it is prohibitively expensive to purify this glycerol into material that can be used in the pharmaceutical, food, or cosmetics industries, new value-added uses for this glycerol need to be investigated. Several pure cultures showed the ability to produce PHA using crude glycerol.

A two-step process for PHA production using crude glycerol resulting from the biodiesel production and aerobic MMC was successfully established. The selected culture had the ability to consume both glycerol and methanol fraction present in crude. However, glycerol seemed to be the only carbon source contributing for the two biopolymers stored: PHB and glycogen. Glycogen storage yield (0.42 Cmmol GB/Cmmol S_g) showed to be higher than the PHB storage yield (0.22 Cmmol HB/Cmmol S_g) which is consisted with Dircks et al. (2001) findings that

demonstrated that glycogen storage is faster than the PHB production. Studies using synthetic glycerol to produce PHA from MMC (Moralejo-Gárate et al., 2011, 2013) also reported the production of this two biopolymers. The results obtained in these last works compared with the ones reported in this thesis suggest that the preference of glycogen storage over PHB was influenced by the low F/M ratio imposed to the selection SBR .

In all the accumlation assays preformed, the selected culture consumed the crude glycerol at the same rate as the synthetic glycerol indicanting that the other compounds present in crude did not interfere with the crude glycerol consumption. The main difference on using synthetic and crude glycerol relie on the biopolymers production. When a synthetic mixture of glycerol and methanol in the same proportions to those in real substrate was used as a substrate, the synthetic methanol was not consumed but the results suggest that the cumulative methanol may exhibit an inhibition effect. As such, although the methanol fraction in the crude does not interfere with the glycerol consumption it seems to have an adverse effect on the PHB production.

In accumulation assays the selected culture was able to achieve a maximum PHB content of 47% cell dry weight with a production yield of 0.46 Cmmol HB/Cmmol Sg using crude glycerol. The overall PHA yield on substrate was in the same range as the ones reported for others studies with MMC and real wastes. Since VFAs are the preferred substrates for PHA production by MMC many complex waste substrates need a pre-fermentation step for their production. The fact that crude glycerol did not need this step to be converted into PHB makes the overall production process economically more attractive. This PHA content with crude glycerol was the highest polymer content using real waste substrate with non-VFA fraction reported to data. In addition, to the best of our knowledge this is the first study that shows the valorisation of crude glycerol into PHAs using an aerobic mixed microbial consortium.

Molecular techniques (DGGE, FISH and sequencing of specific DGGE bands associate with statistic analysis (PCA) showed to be promising techniques to investigate the composition and dynamic of the microbial culture during the acclimatization of PHA production systems. Results from PCA analysis and cluster analyses resulting from the DGGE fingerprinting of the bio-oil system showed that all samples are clearly grouped according with the selective pressure imposed. Furthermore, sequencing of excised bands from the DGGE gel and FISH analysis identified several genera responsible for the reported PHA production of the SBR-B system at the end of the operation time: *Pseudomonas*, *Brachymonas*, *Burkholderia and Alcaligenes*. FISH quantification confirmed that at this period of time *Betaproteobacteria* has the most dominant class in this system reaching 73.4% of the bacterial population. DGGE fingerprinting of the stable glycerol system shows the adaptation of the microbial culture to the crude glycerol

as the new feedstock. Three morphotypyes, TFO, cocci and coccobacilli, were identified in bacterial community selected with crude glycerol. The latter was the only morphotype that did not revealed the ability to accumulate PHA and FISH analysis identified as belonging to the *Deltaproteobacteri*a class The TFO population was identified as *Amaricocus* and the high number of this population indicates as one of the main genus responsible for the high PHA accumulation reported during an accumulation assay with crude glycerol (47% cdw).

Two different aspects can be considered as the main contributions of this thesis for the optimization of polyhydroxyalkanoates production. On one hand it showed the valorisation of two waste streams (bio-oil and glycerol) resulting from different ways for biomass conversion into biofuels (thermochemical and biochemical, respectively). On the other hand the results presented in this thesis, especially concerning the conversion of crude glycerol into PHA, illustrate the potential in using low cost substrates with non-VFA fraction to produce PHA using MMC.

7.2. FUTURE WORK

The outlined future work considers four distinct areas:

Three-step process for PHA production using bio-oil

Acidogenic fermentation of the bio-oil is currently ongoing. Further work will involve investigating strategies to improve the productivity of this stage, not only as a way to improve the overall process productivity but also as a means of producing a clarified fermented bio-oil effluent with a high VFA concentration. One possible way is to test different pH and COD/N/P ratio during the fermentation step and evaluate its effect on the organic acids profile and productivity. Under consideration is also to study the maximum organic loading rate that the microbial community can metabolized. These studies may additionally be coupled to the investigation of the use of a membrane bioreactor for the acidogenic fermentation stage in order to reach higher cell concentrations, thus improving productivity and allowing higher organic loadings.

Use the fermented bio-oil effluent to select, in an aerobic SBR system, a microbial population with a higher PHA storage capacity. Strategies to optimize the culture selection stage will be evaluated. Different parameters may be tested, such as OLR, SRT, temperature, pH and COD/N/P and their impact on the selective pressure for PHA storage and/or impact on cell's growth capacity determined by monitoring reactor performance.

Concerning the toxicity of the bio-oil, if necessary some solvent extraction can be tested in order to remove inhibitors compounds (mainly furfural and phenolic compounds) as a strategy to enable the increase of the organic loading rate in the fermentation step.

Regarding the accumulation step using the fermented bio-oil, concerns about the amount of nitrogen (especially ammonia) present in the feedstock will be investigated. The COD/N/P ratio used during the fermentation step should allow a residual level of ammonia in the fermented bio-oil in order to maintained the biomass growth in accumulation assays to minimum levels and thus increase the PHA storage capacity. In addition, analytical determination of the nitrogen fraction naturally present in the bio-oil will be investigated in order to better understand it consumption. If necessary, strategies to remove the nitrogen or inhibit the biomass growth during the PHA accumulation step must be applied. One hypothesis to control bacterial growth is to induce phosphorus limitation during the accumulation step

Moreover, studies on a possible chemical extraction (e.g. phenolic compounds) from the effluent resulting after the culture enrichment and PHA accumulation step will be evaluated. This approach could be a strategy to add value to the overall PHA production process by extending the overall degree of substrate valorisation.

Improving the PHA productivity using crude glycerol

Concerning the optimization of the culture selection reactor using crude glycerol, different operational conditions can be tested in order to maximize the selection of PHA accumulating organism with a high storage capacity, such as OLR, SRT, temperature, pH. Studies on the influence of the F/M ratio on the scale production of glycogen and PHA will be preformed. One hypothesis is to increase the organic loading rate without compromised the F/F ratio necessary to maintain a good selective pressure.

Moreover, 13C-NMR studies will be performed using 13C-labelled glycerol. This study will give inside information about the metabolic pathways used by the selected culture aiming to better understand the contribution of the glycerol on the production of both biopolymers: PHA and glycogen.

Regarding the batch production stage, strategies to improve productivity will be tested. On one hand by studying the maximum crude glycerol uptake due to potential substrate inhibition, specially induced by cumulative methanol. Secondly, optimization of continuous feeding strategy during the production stage will be performed in order to evaluate this feeding strategy on the PHA production.

PHA characterization

Characterization of the PHA produced in both aerobic systems is currently ongoing. Techniques like thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), size exclusion chromatography (SEC), and nuclear magnetic resonance (NMR), will allowed to better understand the biopolymer produced by the bacterial communities in terms of quality, polymer properties. Correlation between polymers produced and operational conditions imposed will be explored as a way for producing of tailor-made polymers.

Microbial community analysis

Microbial characterization of both SBR systems will continue, pondering any operational change performed and allow linking the PHA-storing community with several operational conditions. Moreover, specific FISH probes will be used to quantify specific genus and potential other organism in both microbial consortia, especially the ones identified by sequencing of DGGE bands, aiming at relating the results of the microbial analysis to the performance assessed already.

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