Biopolymer production through Purple Phototropic Bacteria (PPB) and Activated Sludge (AS) by different engineering processing

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Abstract

The issue of treatment and disposal of sludge produced by wastewater treatment plants (WWTPs), has become relevant worldwide due to the high amounts of residual sludge to be managed, the stringent requirements for agricultural use of sludge, and the cost and disposal constraints associated to landfilling. In Europe, where the availability of fossil fuels is limited, combined treatment of sewage sludge and biowaste, aimed at recovering material and energy, appears to be promising. The present work investigates the feasibility of applying the so-called bio-refinery approach to bio-sludge from WWTP and organic waste, through the analysis of a combined process for converting organic waste (agro-industrial wastes and sewage sludge) into biofuels (bio-H2) and biomaterials (biopolymers).

The management of excess sludge from WWTPs is a critical issue due to the high costs and environmental implications. The conversion of a conventional WWTP into a biorefinery may contribute, while producing a high-quality effluent, to attaining the recovery of valuable elements, materials as well as energy. In particular, a suitable combination of treatment technologies may be directed to producing bio-H2 from mixtures of sewage sludge and agroindustrial biowaste in a first fermentative process stage, and bio-CH4 (under anaerobic conditions) or biopolymers (under aerobic/anaerobic conditions) in a subsequent treatment stage. Among the biopolymers, polyhydroxyalkanoates (PHAs), polyesters synthesized by numerous bacteria, appear to be the most attractive due to their thermoplastic properties similar to that of polypropylene (PP) (Carlozzi et al., 2018). So far, the application of mixed microbial cultures (MMCs) using complex residues for PHA production, through the application of transient carbon availability conditions (the so-called Feast and Famine (FF) strategy), has been widely investigated. However, PHA production with MMCs has been mainly restricted to the utilization of aerobic organisms. In recent years, different studies have proposed the utilization of phototrophic mixed cultures (PMCs). Phototrophic organisms can draw energy from sunlight and by not requiring oxygen to produce ATP, aeration is nonessential, and the high costs associated with system's aeration can be eliminated. (Fradinho et al., 2013a). However, the research on the application of mixed microbial cultures (aerobic and anaerobic) for PHA production, have not yet achieved satisfactory results and needs further investigations applying waste complex feedstock in the process. The experimental campaign of this research project was developed in order to acquire the information necessary to derive the kinetics that describe the production processes of biopolymers, not only to evaluate the yields but also in qualitative terms, by different engineering processing. The specific targets of the research activities were the identification of the effect of different operating conditions on biopolymer production yields and kinetics and identification of the technical and environmental properties of the produced biopolymers. A first set of experiments, carried out at La Sapienza University of Rome with the participation of ACEA SpA, was designed to evaluate the optimal operating

conditions to enrich the PHA-accumulating biomass from the aerobic biological sludge collected at a full-scale WWTP located in Rome. To achieve these targets a synthetic substrate, mimicking the composition of a VFAs-rich effluent from the dark fermentation of mixtures of pre-treated sludge and cheese whey (Akhlaghi et al., 2017), was used as the substrate of an aerobic process for biopolymer accumulation. This study aims to explore the capabilities of the mixed microbial communities (MMCs) to produce PHAs from artificial substrate and complex substrates such as organic waste in a SBR reactor. Five different cycles of SBR were run and 5 different OLR were tested: SBR 1/ SBR 2 (OLR 33 mmolC/ld), SBR 3 (OLR 100 mmolC/ld) SBR 4 (OLR 300 mmolC/ld) and SBR 5 (OLR 150 mmolC/ld). Considering the elements compared, it can be concluded that the operating conditions adopted with SBR3, with an organic load set at 100 mmolC / l × d, were those that led to the best performance of the biomass selection and PHA production process, both in terms of productivity and polymer storage yields. PHA was reflected by a maximum accumulation percentage from 1 % up to 5 % (w/w) in 8 hours and a complete removal of C and N from the reactor. A second set of experiments was carried out at in collaboration with the Advanced Water Management Centre (AWMC) and the School of Chemical Engineering of the University of Queensland. This study was aimed to evaluate the PHA accumulation capacity by the use of another promising mixed culture: The Purple Phototrophic Bacteria (PPB). A continuous 2-L bench scale photo-anaerobic membrane reactor (PAnMBR), was used to test the ability to enrich PHA accumulating microorganisms from sewage sludge, using a synthetic substrate as in the previous experimental set of tests. The operative condition studied in this research had shown a potential capacity of PPB in the PHA accumulation process, reaching more than 15 % (W/W) content of PHA in the microbial cells and 61 % of COD removal and a complete N removal in each cycle. A third series of tests was carried out to investigate an alternative of the conventional biopolymer production setup, trying to minimize the cost of the oxygen supply and the use of expensive substrate. The Study of PHA accumulating capacity of PPBs growing under the natural light/dark cycle evaluating the effect of using molasses as sole carbon source were tested in an out-door 60 l flat plate photobioreactor. This study was carried in collaboration with the School of Chemical Engineering and Advanced Wastewater Management Centre (AWMC) of the University of Queensland (Australia). The operative condition studied in this research had shown a potential capacity of PPB in the PHA accumulation process from fermented molasses, reaching more than 13 % (W/W) content of PHA in the microbial cells and 61 % of COD removal. The results of the batch tests have confirmed the same accumulation capacity of the lab-selected culture so that strengthening the application of this experimental setup could help reaching more than 28 % of PHA content. The outcome of this study identified the PPB as one of the main interesting research challenges for industrial application of biopolymer production from waste.

Acknowledgements

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1 Introduction

1.1 State of the art: critical aspects of wastewater treatment

Nowadays the production and the consequent disposal of wastewater treatment is one of the most critical aspects of the management of the treatment plants, both for the huge increase in the quantities produced and for the evolution of the regulatory framework, which has progressively imposed increasingly restrictive limits for the respect of the environment and the human health. Furthermore, the necessity to identify an alternative domestic wastewater treatment platform seems the more appropriate solution to overcome such a critical issue as described below.

The combination of different strategic actions is required, aimed at developing an innovative wastewater treatment plant, where energy and nutrients are recovered and biomaterials are produced.

The increase in environmental and financial costs of energy production, as well as the availability and cost of minerals and synthetic products have shifted the focus on the management of wastewater treatment aimed at nutrient production and energy recovery (Verstraete et al., 2009). However, as previously mentioned, a paradigm shift in wastewater treatment could lead to rethinking the operation of these plants based on the principles of the green economy and the concepts of biorefinery, directing the treatment process to the production of new materials, to recovery of elements such as nitrogen, and the production of biofuels. The wastewater could be considered as the production chain of innovative biofuels (bio-H2 possibly in combination with bio-CH4), volatile fatty acids and biopolymers. Global petroleum-based plastic production has overcome 320 million tons (Mt) worldwide in 2016 and it is expected to double in the next 20 years (COM, 2018 28. A European Strategy for Plastics in a Circular Economy). The United Nations Environment Program (UNEP) showed that only the 54% of plastic waste was landfilled or recycled, the remaining fraction entering the ocean and accumulating as debris every year, representing a global problem for the marine ecosystems. Biological products are a potential replacement for fossil-fuel-derived feeds in plastic production, attractive for being close to carbon-neutral as the majority of biological carbon is sourced from atmospheric CO_2 rather than

underground petroleum reserves and is both a financial and environmental interest in finding renewable sources of plastic generation.

Bio-plastic production needs to be economically viable to replace current methods, not only for the environmental benefits, but even because the actual worldwide production is only about 1 Mt/y. The demand is continuously growing and, in accordance with the last market data collected by European Bioplastics, the global BP production capacity is expected to increase from around 2 Mt/y in 2017 to around 2.4 Mt/y in 2022.

1.2 Sludge treatment, disposal and recovery

Sludge recovery by spreading in agriculture, has been a long practice due to the simplicity of the application and to the improvement of the chemical-physical characteristics of agricultural soils. However, cause of the presence of contaminants in sludge, such as metals, pathogenic organisms and organic pollutants, the use of this practice has been increasingly limited. The presence of these contaminants in the biological sludge has frequently produced a reduction in the quality of the products of the sludge composting plants, characterized moreover by an increasing cost due to the energy consumption for material turning during the biostabilization process, for supplying oxygen for the maintenance of aerobic condition and for exhaust air collection and treatment. To overcome these problems, aerobic composting has been progressively replaced by the anaerobic digestion processes of the sludge, possibly in combination with other organic substrates (such as agro-food industry treatments, zootechnical waste and urban waste) with the production of biogas to be used for energy purposes. It should be noted that the anaerobic digestion stage must be followed by a stage that completes the biostabilization of the solid fraction and regulates the characteristics of the liquid fraction that could be used, for example, for fertirrigation.

The problem of the treatment and disposal of sludge produced by urban wastewater purification processes has become increasingly important both nationally and internationally in recent years. Since the 90s of the last century, the disposal of excess sludge has been a critical issue for treatment plants due to the high impact on management costs. In fact, the management of sludge is one of the most significant cost items for the wastewater treatment plant. Between 15 and 40% of the total cost of the process, and amounts to values that can vary between 50 and 70 € / t for a recovery plant up to € 140 / t for landfill disposal (Waste Management Costs & Financing and Options for Cost

Recovery, Horizon 2020). The use of sludge in agriculture has long been considered as one of the most efficient solutions, but for several years, it has been at the center of a great debate that has led the current legislation to be much more stringent. European and national legislation has imposed various restrictions, and in particular very strict limits for the concentration of certain metals (such as cadmium and lead), for agronomic and microbiological characteristics and for the maximum amounts of sludge that can be applied to land (Directive Community 86/278 / EEC). The disposal in landfills has also been one of the solutions used for years but even this seems to be always more difficult to apply, because it is considered as the final solution of a more complex treatment cycle and regulated. Moreover, by a regulatory approach that aims not only to limit the quantities to be sent to the landfill but also to improve as much as possible the characteristics of the waste to be dumped (Directive 1999/31 / EC). In the case of excess sludge this implies the need for dehydration and biostabilization systems with increasingly higher performance, however in a context where often the adoption of conventional heat treatment processes is hindered by concerns on the part of the population and the institutions. The importance of identifying innovative ways of managing the wastewater treatment plants and the problems connected to sewage sludge in particular is therefore evident, also taking into account the evolution of the sector regulations. With particular reference to the problems connected with the production of excess sludge, the intervention strategies consist essentially in the reduction of their production during the wastewater treatment process, in the identification of technologies that allow their disposal in compliance with the most restrictive regulatory limits and in the development of material and energy content recovery lines. As for the main technologies for the so-called minimization of sludge production, some of which are currently tested at full scale, they can be classified into biological (enzymatic biolysis, cannibal, enzymatic lysis on MBR reactors ...) and chemical-physical (ozonolysis, biolysis or ultrasound disintegration, mechanical disintegration ...). The technologies for reducing the quantities and increasing the quality of the sludge downstream of their production include thermolysis (thermal hydrolysis), enzymatic digestion, wet oxidation, ultrasound disintegration, mechanical disintegration. However, as previously mentioned, a paradigm shift in the treatment of waste water could lead to rethinking the operation of these plants based on the principles of green economy and biorefinery concepts, directing the treatment process to the production of new materials, to recovery of elements such as nitrogen, and the production of biofuels. The sludge line, in particular, could be the production chain of innovative biofuels (bio-H_2 possibly combined with bio-CH4), volatile fatty acids and

biopolymers. It should be noted that the production of bio-H_2, to be used in combination with fuel cells, would make it possible to obtain energy production with a net negative emission of CO_2, also reducing the "Carbon footprint" associated with the treatment of waste water and use of traditional fuels. *Figure 1.1* shows the main types of disposal arrangements for sludge used in Europe.

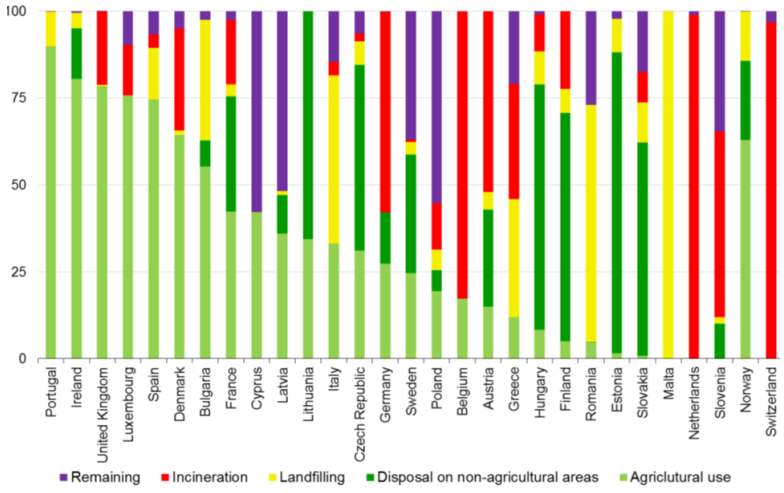


Figure 1.1 European sludge disposal (Vouk et al., 2017 - IWA Specialist Conference On Sludge Management SludgeTech 2017)

1.2.1 Sludge recovery

Rethinking the concepts of waste management, the sludge treatment could be lead into a waste-to-energy conversion or waste-to-byproducts approach through specific recovery strategies. Several solutions have been taken, those which were found relevant are set out below.

1.2.1.1 Incineration

The Waste Incineration Directive 2000/76 /EC, which lays down emission limit values for selected heavy metals and chemical compounds, (e.g. NOx, SOx, HCl, particulates, heavy metals and dioxins), regulate waste incineration. The limit values are set in order to prevent and limit as far as practicable negative effects on the environment and the resulting risks to human health. When the biodegradable fraction is incinerated the organic matter is decomposed to carbon dioxide and water. This is short-rotation carbon, thus the energy produced is classified as renewable. However, the

majority of energy gained via the incineration comes from those highly calorific fractions – such as plastics, tyres and synthetic textiles – that are produced from crude oil. The wet fraction of biodegradable waste diminishes the overall energetic efficiency of the incineration process. This means that the combustion of the highly calorific waste fractions is in fact 'helping' the combustion of biodegradable waste. More energy may be gained if biodegradable waste were not to be incinerated along with other wastes. Indeed, refuse-derived fuel (so-called RDF) resulting from the highly calorific fraction can be used in power plants or cement kilns without the need for dedicated incinerators.

1.2.1.2 Cement production

It involves the use of dried sludge as fuel for the production of heat in cement production furnaces. Among the many factors that must be evaluated during the possible disposal of sludge in cement plants, four can be highlighted (Rada et al., 2006). Content of P_2O_5 in the sludge: for the production of clinker, the content of P2O5 must not exceed 0.5% since at high concentrations of this compound the content of C3S in the clinker is reduced. Chlorine content: the chlorine content in the raw mixture should be less than 0.015%; for higher values, the chlorine cycle inside the drum in cement production can cause serious problems for the stability of operations. Quantity of nitrogen: the total nitrogen content in the dried mud can reach levels equal to 8% (with reference to the dry weight); this quantity could make a significant contribution to NOx emissions. Particle size of dried mud: for clinker production it is decisive not only the size of the mud particle (2-10 mm), but also the shape of the same. The sludge consisting of elongated and flat particles is substantially better; "cubic" and "spherical" particles present combustion that is more difficult. In the case of co-incineration of sewage sludge in a cement kiln, the dry content must be very high (at least 80%). This is because with higher water content, the oven temperature would drop below the minimum limit for creating clinker and therefore product quality problems could arise.

1.2.1.3 Land spreading and composting

The sludge recovery by spreading in agriculture, as previously illustrated, has been a practice long used for the simplicity of application and for the consequent improvement of the chemical-physical characteristics of agricultural soils. However, due to the presence of contaminants in the sludge, such as metals, pathogenic organisms and organic pollutants, the use of this practice has been

increasingly limited. Biodegradable waste decomposes in landfills following a long ecological cycle. The decomposition produces landfill gas and highly polluting leachate. However, the major share of the waste remains in the landfill and the nutrients are not available for plant growth. When less organic matter is landfilled, less landfill gas is produced. Landfill gas, which may only be partially captured, contributes considerably to the greenhouse effect. In fact, landfill gas is mainly composed of methane, which is 21 times more powerful than carbon dioxide in terms of climate change effects. It has been calculated that the methane emissions from landfills account for 30% of the global anthropogenic emissions of methane to the atmosphere.

The agronomic reuse of the direct sludge or after composting is a valid solution to the problem of disposal of sewage sludge and requires a considerable importance for agronomic and economic efficiency as it replaces, in whole or in part, chemical fertilization or other types of organic fertilization. To avoid any situation of danger for the environment and for the salutation of the population, it was practiced in full compliance with the regulations regarding the execution of controls on soil. The use of sewage sludge in agriculture is regulated by the Directive 86/278/EEC and was set up to encourage the use of sewage sludge in agriculture and to regulate its use in such a way as to prevent harmful effects on soil, vegetation, animals and man. To this end, it prohibited the use of untreated sludge on agricultural land (Environmental, economic and social impacts of the use of sewage sludge on land, Final report 2008 DG ENV.G.4/ETU/2008/0076r, Milieu Ltd). About 10 million tons' dry matter (DM) of sewage sludge is estimated as produced in Europe every year (Bianchini et al., 2016). The use of sewage sludge in agriculture in Italy is regulated by the Legislative Decree n. 99 of 27 January 1992 which transposes the EU Directive 86/278/EEC. The decree specifically establishes: concentration limit value for some heavy metals that must respected in soil, for toxic, persistent and bio accumulative substance in concentration harmful to soil, crops, animals, humans and environment in general (The precautionary principle in environmental law, Butti 2005).

1.2.1.4 Anaerobic digestion and biogas production

The presence of these contaminants in biological sludge has frequently produced a reduction in the quality of sludge composting plant products. Which are also characterized by high-energy consumption deriving from the need to move material during the biostabilization process and supply of oxygen (and therefore of air) for the maintenance of aerobic and aspiration conditions and treatment of exhausted process air. To overcome these problems, anaerobic digestion processes have progressively replaced the processes based on aerobic composting.

Anaerobic digestion of organic wastes involves methanogenic bacteria and results in the production of biogas, digestate (solid fraction of the residues) and liquor (liquid fraction of the residues). It is rather sensitive to ambient conditions and is difficult to be artificially reproduced because it involves different methanogenic bacteria which work at different temperatures, pH conditions etc. Anaerobic digestion produces biogas, which is typically made up of 65% methane and 35% carbon dioxide with traces of nitrogen, Sulphur compounds, volatile organic compounds and ammonia. This biogas can be combusted directly in modified gas boilers or can be used to run an internal combustion engine. The calorific value of this biogas is typically 17 to 25 MJ/m3. Typically, between 40% and 60% of the organic matter present is converted to biogas.

Thus, anaerobic digestion process is a technique that allows for:

- reduction of the polluting load and stabilization of the waste, since the biodegradable organic substance undergoes a reduction in the volatile fraction, the carbon content and the C / N ratio.
- energy valorization, given that the process involves the production of biogas, consisting mainly of methane (50-80%) and carbon dioxide, which can therefore be used as a fuel.
- recovery of the water contained in the digested biomass for irrigation purposes, through the use of a filter press.

1.2.1.5 Anaerobic co-digestion

A solution to increase biogas production can be the anaerobic co-digestion, which consists of adding other organic co-substrates, including: sewage and manure from livestock (livestock effluents); dedicated vegetable crops (energy crops); organic waste (biowaste); organic waste from agriculture (agro-waste). This last type of substrate is generally characterized by low pH and a low buffer capacity; consequently, often their single anaerobic digestion turns out to be problematic, due to the possible accumulation of VFA. The addition of manure or sludge increases the buffering

capacity of the system, with a better stabilization of the pH. The mixing of two or more substrates also allows to better balance the C: N ratio (Riaño et al., 2011).

1.2.1.6 Dark fermentation

Hydrogen is considered an ideal energy source because it represents a clean fuel that can be easily converted into electricity. The organic production of hydrogen is linked to the production of biogas since they have a similar production process and can be fed with the same substrates. These two gaseous products derive from the same biological process of anaerobic digestion, which activates the production of hydrogen when hydrogenotrophic microorganisms are inhibited. Inhibition is commonly achieved through heat treatment of the inoculum to remove all microorganisms, with the exception of spore-forming fermenting bacteria. The most common bacteria used in the dark fermentation to produce hydrogen are Clostridium and Iermoanaerobacterium. Furthermore, several studies have reported successful hydrogen production from mixed cultures in batches or bioreactors. The advantages of using mixed cultures for the production of biohydrogen are many: no need for sterilization, high adaptive capacity due to microbial diversity, the ability to use a mixture of substrates and the possibility of get a stable and continuous process. The substrate plays an important role in the H2 yield, H2 production rate and the overall economy of the process. These are mainly dependent on the substrate´s carbohydrate content, bioavailability and biodegradation rate (Ausiello et al., 2015; Guo et al., 2010). In more recent dark fermentative studies, complex substrates have been considered, such as the organic fraction of municipal solid waste (OFMSW) (Chen et al., 2012; Nissilä et al., 2011; Tawfik and El-Qelish, 2012; Valdez-vazquez et al., 2005; Zhang et al., 2007). The agricultural residues like lignocellulosic biomasses (e.g. rice straw, wheat straw and corn stalks), agro-industrial wastes like those from food processing industries (e.g. olive mill wastewater and cheese whey).

1.2.1.7 Biopolymer production

As anticipated, an innovative alternative that in recent years is receiving much interest from the scientific community is represented by the production of Polyhydroxyalkanoates (PHA) in a vast number of polymers produced by biochemistry and followed by the metabolism of microorganisms maintained under standard conditions. In this process, the sewage sludge would represent the biomass able to accumulate the biopolymers, using a VFA-rich organic substrate that could derive

from a first stage of sludge fermentation, together with other wastewaters of suitable characteristics, to bio-H_2 .

The use of sludge for the production of PHAs is nowadays studied at laboratory scale, while –to the best of my knowledge - only one pilot scale plant has been put into operation on agro-industrial wastewater. It should also be noted that many of the experimental studies conducted have used synthetic substrates that are intended to simulate the characteristics of real wastewater, obviously characterized by greater complexity.

The use of a mixed microbial consortium such as that found in excess sludge for the production of biopolymers has several advantages. In particular, from the application point of view, a highly differentiated community such as sewage sludge is present that is better able to adapt to complex substrates (and with different characteristics over time) such as real wastewater and effluents from the first stage of hydrogenogenic fermentation, thus guaranteeing greater process stability (in kinetic and qualitative-quantitative product yield terms)

1.3 How Bioplastic can represent an alternative to petroleum-based conventional plastic

1.3.1 Plastic waste pollution and fossil fuel consumption

The increasing production and disposal of sludge is not the sole challenge to be faced as soon as possible regarding waste disposal. Indeed, the amount of urban waste generated in the OECD countries (Organization for Economic Cooperation and Development) has increased since 1980, exceeding the 650 million tons estimated in 2007 (556 kg per person). In most countries for which data are available, economic growth and changes in consumption patterns tend to generate more waste per person. Total waste production in Europe amounts to 88 million tons of food waste, 30-50 million tons of agro industrial waste and 3,5 million tons of sludge produced by sewage treatment plants (FUSION UE Project, March 2016).

However, even more worrying situation, is described by the numbers regarding another waste item, the production of plastics. The world production of plastic waste stands at 322 million tons in 2015, with an increase in 2016 to 335 million tons (PlasticsEurope (PEMRG) / Conversion (Market & Strategy GmbH). In Europe the production amounts stable at 60 million tons (*Figure 1.2*), includes plastic materials (thermoplastics and polyurethanes) and other plastics (thermosets, adhesives, coatings and sealants). 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 (CHEManager International 1-2/2014).

In this panorama, it is necessary to observe how the area with the greatest potential of growth is the Asian developing countries (excluding Japan) where the current per capita plastic consumption is around 20 kg / year. China remains the leading producer in world level with 24% of the market, while Europe reaches 20% of global production, similarly to the countries belonging to the "North American Free Trade Agreement" (NAFTA), the United States, Canada and Mexico.

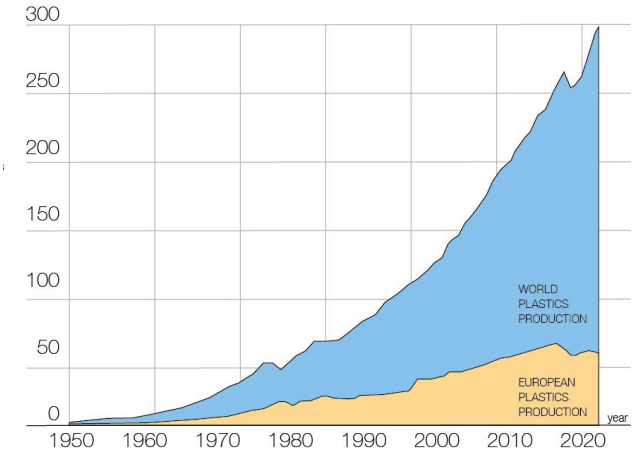


Figure 1.2 World plastic production (1950-2020) (CHEManager International 1-2/2014)

The large quantity of plastic produced globally presents environmental problems linked to the end-of-life options of these materials. The efforts made by the producer countries, and in particular by Europe, in strengthening the forms of recycling, as an alternative, with respect to landfill disposal, also considering the residual market value, represented by plastic materials destined for recycling. In recent years, the increasingly limited availability of fossil fuels, the increase in the price of oil, the ongoing climate changes and the awareness of the environmental awareness of the population are influencing the policies of governments, industries and science, to seek alternatives to oil and its derivatives. To date, 85-90% of the plastics produced are of petrochemical origin, however bio-based plastics and bioplastics are gaining ever greater market shares.

However, the externalities associated with the use of plastic and plastic packaging are strongly linked to the impact these have on the environment. Especially when they become waste, as they can enter the sewage systems if not properly disposed of or in streams and discharged into the sea (Jambeck et al., 2015) have estimated that between 2010 and the ocean, between 4.8 and 12.7

million tons of plastic came from 192 coastal countries around the world. The study hypothesizes that if waste management strategies remain unchanged, the amount of plastic that will enter the oceans will increase tenfold by 2025. Every year at least 8 million tons of plastic spill into the ocean (Ellen MacArthur Foundation Report, 2016). Globally, plastic materials account for 60-80 percent of all marine litter (Derraik, 2002) and, as shown by some surveys, plastic is as much as 90 percent of the waste on the beach (Pasternak et al., 2017). There is no definitive numerical data on the amount of plastic present in the oceans of the world but referring to quantitative theoretical models it is possible to estimate that there are 5.250 billion pieces of plastic waste weighing 268.940 tons floating in the sea, excluding those present on the seabeds or beaches (Eriksen et al., 2014). According to a study published from environmental engineering of the University of Georgia (Jambeck et al., 2015) 5.3 of the 8.8 million tons of plastic that reach the sea every year come from just five countries. All of the Far East: together with China, with over 3.5 million tons poured into the sea every year, Indonesia, the Philippines, Thailand and Vietnam are in fact responsible for 60% of all oceanic plastics.

Furthermore, in order to protect the health of the sea and the economies that depend on it from the invasion of plastics. It would be necessary to impose prohibitions and limitations on the production of disposable articles and packaging and optimize waste management, investing in infrastructures and services for the collection, in wastewater treatment plants, using the "Zero Waste" approach to avoid dispersion in the marine environment and in particular in coastal areas or near rivers. Where it is not possible to eliminate the use of plastic materials, it is necessary to replace the plastic polymers with biodegradable biopolymers, limiting the maximum consumption of oil for the manufacture of materials and dispersion in the environment.

1.3.2 Bio-based and biodegradable plastic

In 2015, the production capacities for bio-based and biodegradable plastics account for nearly 1% of total global plastics production. The markets for some bio-based and/or biodegradable plastics are expected to grow significantly during the coming years (Bio-PET, PBS and PLA), others are expected to consolidate (CA and Bio-PA) (*Table 1.1*). Overall, it is expected that by 2020 the share of bio-based and biodegradable plastics will increase to 2.5% of fossil plastics production.

For most of the bio-based and biodegradable plastics there are several suppliers and most plastics are readily available (Wageningen Food & Biobased Research 2017). Bio-based plastics are those

materials or products, biodegradable and not, synthesized using bio-derived monomers. It can be obtained through two major routes: using natural polymers (biopolymers) that can be partially modified (for example plastics produced starting from the amid (like as the Mater-Bi®) and by synthesizing bio-based monomers through fermentation processes (as in the case of lactic acid) or chemical synthesis traditional and polymerizing these monomers in a second step. Not all bio-based plastics are biodegradable, as is the case, for example, of bioPET, bioPE and bioPP, which have similar characteristics to those obtained using petrochemical-derived monomers but are made up of monomers of biological origin, the advantage of these materials is undoubtedly that of being able to be obtained from renewable sources.

Table 1.1 Diagram indicating of bio-based versus petrochemical plastics

Type of plastic	Petrochemical	Partly bio-based	Bio-based
Non-biodegradable	PE, PP, PET, PS, PVC	Bio-PET, PTT	Bio-PE
Biodegradable	PBAT, PBS(A), PLC	Starch blends	PLA, PHA, cellophane

According to European Bioplastics, a plastic material is defined as bioplastic if it is bio-based, biodegradable or has both properties. The term bio-based means that the material or product is derived from biomass. The biomass used for bioplastics derives for example from corn, sugar cane or cellulose.

Instead, a biodegradable material is defined as being subject to biodegradation, or to a biochemical process during which microorganisms convert organic matter into inorganic substances. Biodegradability is linked to the chemical structure of a material and does not depend on the nature of the source material: bio-based plastics can be non-biodegradable and plastics derived from fossil fuels can be biodegradable.

The land needed to grow the feedstock for the presently produced bio-based plastics world-wide amounts about 0.02% of the arable area. If we would base all present world-wide fossil plastics production on biomass as feedstock instead, the demand for feedstock would be in the order of 5% of the total amount of biomass produced and harvested each year by mankind. But such scenarios are not likely to happen since it is expected that the industry will develop technologies that can use alternative feedstocks from waste and side streams of agriculture and food production, for example non-edible lingo-cellulosic feedstocks for chemical building blocks.

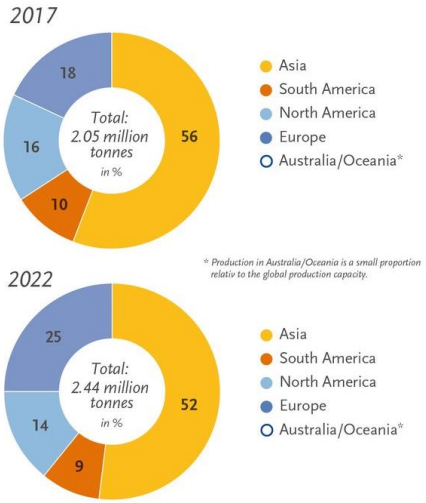


Figure 1.3 Global production capacities of bioplastic (Source: European Bioplastic, Nova-Institute 2017)

The 12th European Bioplastics Conference in Berlin has shown that Europe and packaging are at the epicenter of bioplastics market growth at the moment. The packaging market accounts for (and is set to retain) 58 % of bioplastic production. Meanwhile, Europe is projected to increase its share of global production from 18 to 25 % in the coming five years. Since world production is predicted to increase by 20 % during the same period, this reflects a huge increase in European capacity this fact underlined by the several manufacturers present in Berlin who announced plans to commence or scale up production (results shown in *Figure 1.3*).

Recent research shows that a sustainable co-production of biofuels (and bio-based plastics) and food is possible and that biofuels production may serve as a stabilizer for food prices, providing farmers with more secure markets and thereby leading to more sustainable production.

1.4 Bioplastic production: the "Biorefinery concept"

1.4.1 Industrial biotechnology and sustainable development

Worldwide, problems such as population growth, growing demand for food, energy and water, and climate change highlight the need for economic growth based on the principles of environmental sustainability and eco-friendly processes. In this sense, biotechnologies play a key role since they allow the optimization of the exploitation of resources within a defined production chain and contribute to the improvement of both the overall benefit and the environmental quality of the industrial process. In the past, production strategies have been conceived considering only assessable qualitative parameters in terms of transportability, availability and transformability. Which have led to favoring the use of fossil fuels as primary energy sources, neglecting the environmental impact of these processes and the effects that these could have on the environment in terms of pollution, greenhouse effect, depletion of resources, economic instability and political tensions. Moreover, the availability of increasingly lower fossil fuels has diverted attention from the need to rethink a sustainable energy model aimed at minimizing the use of non-renewable sources, favoring, rather, the use of renewable ones and reducing at the same time waste. The challenge between renewable and non-renewable sources, in terms of competitiveness, is played on the technological level, having to aim for innovative solutions that allow overcoming the critical issues connected to the use of renewable energy sources, both technically and economically. Therefore, a new industrial trend relies on moving from petroleum-based to biomass-based products and sustainable manufacturing processes. The development of a bio-based product industry offers an economical and environmental friendly solution for the surplus agricultural commodities production with low economic income for farmers and for the large amounts of industrial wastes with high disposal (Kamm *et al.* 2006). In this novel bio-based product industry, arose the concept of biorefinery, which was defined by the American National Renewable Energy Laboratory (NREL) as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass".

1.4.2 Integration of PHA production into the biorefinery process

To date, industrial production full-scale applications of PHA are rather limited and fundamentally based on the use of pure cultures, which ensure high biomass productivity (up to 80 g / l, Lee et

al., 1999) and consequently high volumetric productivity of PHA. The processes based on the MMC allow to reach low concentrations of PHA, of which the maximum value of 6.1 g / l has been reported by Dionisi et al. (2006). Since they have a lower production yield than pure crops, however, lower productivity can be compensated by the concrete advantages obtainable from the use and enhancement of waste substrates and microbial cultures that do not require sterile conditions of maintenance and growth, with a considerable reduction in production costs. The technology is therefore very promising but its development requires extensive pilot-scale experimentation, which allows both to acquire technical-economic evaluation data and to obtain a production of polymers in sufficient quantity for workability tests on an industrial scale with a consequent increased competitiveness on the market. According to an article by Jiang et al. (2016), currently the main sources of carbon for the commercial production of PHA are still glucose and food-based vegetable oils. The use of hydrocarbons derived from waste plastic is only exploited on a laboratory scale, but further research is needed to improve the yields and productivity of polymers. The use of waste materials and waste from biorefineries, including glycerol and lignocellulosic sugars, is a promising avenue for sustainable PHA production. So far, comprehensive and cost-effective methods have not been developed to take full advantage of the lignocellulose fermentable sugars. An integration of PHA production into a biorefinery is proposed, which could offset the cost of bioethanol through the co-production of value-added PHAs.

A biorefinery usually uses two paths to convert biomass, from which different products are obtained: the first is a biochemical pathway that transforms the organic substance by enzymatic means. The second is a thermochemical route where biomass is gasified into syngas which is then used to synthesize liquid fuels. So a biorefinery can use biomass and industrial processing residues to produce bioenergy and biomaterials. In a petroleum-based refinery, part of the refined oil is transformed into a chemical substrate, such as ethylene, propylene and terephthalic acid, which is used to synthesize polymers. Biorefineries, through the refining of biomass, obtain biofuels as final products, however polymers have not yet been identified as co-products, as in a traditional refinery. Based on the current developed technology, the PHAs could be a suitable candidate to be integrated into the production process of a biorefinery, since these can be produced by different substrates already present in the system. In fact, in the production of bioethanol with the use of plants containing starch, the waste water contains carbohydrates that can be used to produce PHA. In the

production of biodiesel with vegetable oils or animal fats, large quantities of crude glycerol and methanol are produced, which could be used to produce PHA.

At the end of the process, second-generation industrial biorefineries refine lignocellulose, various agricultural residues, agro-industrial waste, energy crops and forest residues. When these residues are refined, a large amount of hemicellulose hydrolyzate will be produced in the pretreatment process, which contains a high concentration of simple sugars, which can be fermented to obtain PHA. Further optimization of fermentation conditions is needed to improve productivity. Biorefineries are an emerging concept that involves the use of large-scale biomass for the production of biofuels and chemical products based on ecologically compatible raw materials. Biorefinery byproducts can be considered as potential sustainable substrates for PHA production, although further research is needed to identify high-throughput microbial strains and optimal growth conditions to improve PHA yields and productivity in order to achieve an efficient conversion.

The integration of PHA production with biorefineries will open a new path towards bioplastics production and offset the high price of second generation bioethanol and biodiesel. Placing PHA production in a production system based on the use of biomass will allow the biorefinery industry to produce both biofuels and new materials such as biopolymer.

1.5 PHA production through mixed microbial culture (MMC)

1.5.1 PHA production

Among the bioplastics available on the market, the polyhydroxyalkanoates (PHAs), polyesters synthesized by numerous bacteria, are drawing increasing attention, due to their thermoplastic properties similar to that of polypropylene (PP), good mechanical properties and excellent biodegradability in various ecosystems such as fresh water, soil, industrial/domestic compost and sea water (Carlozzi et al., 2018).

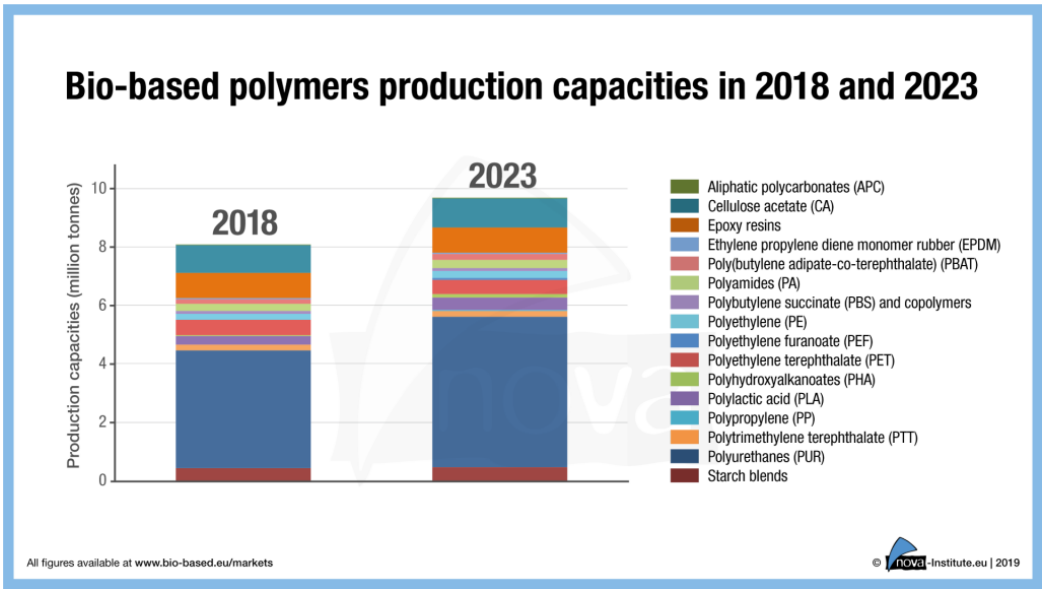


Figure 1.4 Global bioplastic production capacities (Nova-institute GmbH, press release, 2019-02-11.)

The *Figure 1.4* showed the growing global interest by type of bioplastic and a good increase is expected also for research on PHA. PHAs are intracellular biopolymers synthesized and accumulated by several microorganisms as carbon and energy reserves. The factors that most restrict the application of biopolymers are the competition for the use of renewable materials for the food and energy sectors and their actual high production cost. But these biopolymers have attracted significant interest from the industry and research community due the possibility of a feeding strategy that could be applied to many different feedstocks. Like agro-industrial wastes (e.g. fruit pomaces, animal litter, cheese whey, glycerol) or food and urban wastes (e.g. used cooking oil), where very successful results were obtained in terms of both productivity and accumulation capacity (Kourmentza et al., 2017). This could considerably reduce the impact of costs on production and limit the effect of the contrast food versus energy dilemma. Moreover, in order to decrease PHA production costs and bring this sustainable biopolymer into the market, the utilization of mixed microbial cultures (MMCs) that can use cheap complex residues as feedstock in open conditions has been proposed (Reis et al., 2011). Cost reduction derives mainly from operations being performed under non-sterile conditions and the higher adaptability of MMC to utilize waste streams as substrates. However, PHA production with MMCs has been mainly applied under aerobic conditions, while in fact; the diversity of bacterial species that can produce and accumulate PHA is much wider and includes also anaerobic organisms (Fradinho et al., 2018). In recent years, studies with PHA producing phototrophic systems have proposed the utilization of

phototrophic mixed cultures (PMCs) as means of decreasing operational costs. Phototrophic organisms can draw energy from sunlight and by not requiring oxygen to produce ATP, aeration is non-essential, and the high costs associated with system's aeration can be eliminated. The *Table 1.2* showed the global commercial PHA, the producer company that invested in this industrial production from different substrate.

Table 1.2 global commercial PHA (Kourmentza et al., 2017)

Polymer	Commercial name	Producer	Substrate	Capacity $_{t\,y}$-1	Priece €/kg
PHB	*Biogreen*®	Mitsubishi Gas Chem. Comp. Inc. (Japan)	Methanol	10.000	2.5-3.0
PHB	*Mirel*®	Telles (US)	Sugar derived from corn strach	50.000	1.50
PHB	*Biocycle*®	PHB Industrial Company (Brazil)	Molasses	50	n/a
PHBV e PHB	*Biomer*®	Biomer Inc. (Germany)	Sucrose (idrolyzed cheese whey)	50	3.0-5.0
PHBV PHBV + Ecoflex	*Enmat*®	Tianan Biologic Ningbo (China)	Sugare derived from corn strach	10.000	3.26
PHBH	*Nodax*®	P&G	-	20.000 50.000	2.50
PHBH	*Nodax*®	Lianyi Biotech (China)	-	2.000	3.70
PHBH	*Kaneka PHBH*	Kaneka Corp. (Japan)	waste vegetable oil	1.000	n/a
P(3HB-co-4HB)	*Green Bio*	Tianjin Green Bio-Science Co/DSM	Sugar	10.000	n/a
PHA from P&G	*Meredian*	Meredian (US)	-	272.000	n/a

1.5.2 PHA Synthesis Mechanisms

The structure of PHAs that accumulate within bacterial cells is dependent to a great extent on the carbon sources that are supplied. Thus, the PHA synthesis pathways that supply monomers to the PHA synthase are responsible for the final PHA structures. Carbon sources are divided into PHA structurally related and unrelated to carbon sources. For example, fatty acids are similar to hydroxyalkanoic acids in their structure, and they are termed related substrates, whereas glucose

does not have a structure similar to PHAs and is an unrelated substrate. The biosynthetic pathways of PHA are closely linked to the central metabolic pathways of the bacterium, including glycolysis, the Krebs cycle, β-oxidation, the synthesis of fatty acids, the catabolism of amino acids, the Calvin cycle. Many common intermediates are also shared between PHA and these metabolic pathways, particularly acetyl-CoA (Amy Tan et al., 2014). Path I is the best known of the PHA biosynthetic pathways used by C. necator. In this pathway, 3HB monomers are generated by the condensation of two acetyl-CoA molecules from the tricarboxylic acid cycle (TCA) to form acetoacetyl-CoA from the β-ketothiolase enzyme (Wang et al., 2012). Acetoacetyl CoA reductase acts on acetoacetyl-CoA to form 3-hydroxybutyryl-CoA. Finally, the enzyme PHA synthetase catalyzes the polymerization through the esterification of 3 hydroxybutyryl-CoA in poly (3-hydroxybutyrate) (P (3HB)) (Amy Tan et al., 2014). The pathways involved in fatty acid metabolism generate several hydroxyalkanoate monomers used in PHA biosynthesis. Path II generates substrates by oxidation of fatty acids which can be polymerized by the PHA enzyme synthetase of Pseudomonas aeruginosa (Sudesh et al., 2000). In A. caviae, the β-oxidation intermediate, trans-2-enoyl-CoA is converted into (R) -hydroxyacyl-CoA by a specific enoil-CoA hydratase (R). Path III is of considerable interest because it helps to generate monomers for the synthesis of PHA from cheap and structurally simple and simple carbon sources, such as glucose, sucrose and fructose. The (R) -3-hydroxyacyl intermediates from the biosynthetic pathway of fatty acids are converted from their carrier acyl protein form (ACP) to the CoA form by the enzyme acyl-ACP-CoA transylchylase (encoded by phaG) (Wang et al., 2009) (*Figure 1.5*).

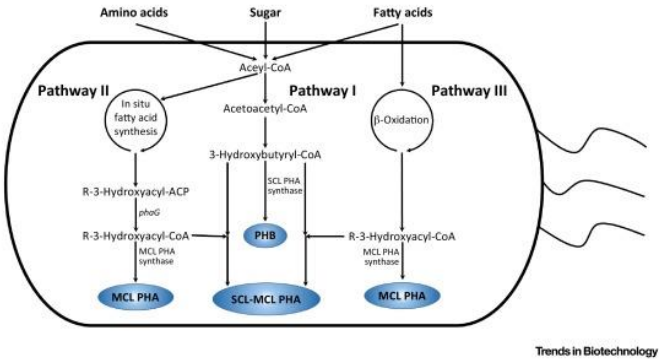


Figure 1.5 Three Major Pathways Related to the Production of Various PHA (Chen et al., 2015)

Whereas such substrates are very expensive, it is also possible to create additional pathways to supply new monomer precursors for non-PHB PHA synthesis, especially using sugars as low-cost substrates. Over time, as multiple pathways leading to different types of PHA monomers have been discovered, a range of microorganisms have been employed to engineer or create new pathways. Increasing the conversion efficiency, tune the features of the PHA, make PHA production more economical, or even to introduce pathways for entirely novel PHAs D.C. (Meng, et al. 2014; Q. Wang, et al. 2013).

1.5.3 Physical and chemical properties of PHA

Thanks to the structural variations of their monomers, PHAs vary considerably in their physical and chemical characteristics. The hydrophobicity of the polymer, the melting point, the glass transition temperature and the degree of crystallinity depend entirely on the composition of the monomer. The PHA family has a wide variety of mechanical properties from hard crystalline to elastic. Short-chain biopolymers are rigid, fragile and possess a high degree of crystallinity (in the range of 60-80%), while medium-chain PHAs are flexible and elastic, with low crystallinity (25%), low tensile strength, high resistance to elongation, low melting temperatures and glass transition temperature below room temperature (Anjum et al., 2016). The PHA family offers a range of properties in polymeric materials from crystalline to elastic. In order to produce PHA materials with the desirable chemical and mechanical properties, many different approaches have been studied, for instance copolymerization, composite production and blending among others (Abe and Doi 2005). PHB is comparable to polypropylene and shows good resistance to moisture and acquires excellent barrier properties against gases. PHAs are insoluble in water, and have good resistance to hydrolytic attacks, are resistant to UV rays, sink into water and facilitate anaerobic biodegradation in sediments. Furthermore, they are biocompatible and biodegradable and behave like piezoelectric materials. Their biodegradation depends mainly on the type and composition of the polymer, the environmental conditions and the type of microorganisms (Raza et al., 2018). PHAs are soluble in chloroform and other chlorinated solvents. Their glass transition temperature ranges from -50 to 4 ° C, melting temperature from 40 to 180 ° C; termodegradation temperature, the tensile strength, the Young's modulus, the transmission speed of water vapor and oxygen vary according to the type of polymer produced and the composition of the monomer unit (Raza et al., 2018). Poly-3-hydroxybutyrate (PHB) is the most common polyester, but it is periodic the regular structure when crystallized makes PHB a rigid and fragile material (55-80% crystallinity), which

also deteriorates over time due to the secondary crystallization (Steinbüchel 2001). On the other hand, the physical and thermal properties of PHA copolymers can be regulated varying their molecular structure and the copolymer compositions. The incorporation of a 3HV fraction (or other monomers with a chain length of 3 to 14 carbon atoms) in the The PHB ridge increases the softness and flexibility of the biopolymer (Noda et to the. 2010), increases thermal stability (Zakaria et al., 2010) and reduces the melting point (McChalicher and Srienc 2007). PHAs polymers are thermoplastic and they differ in their properties depending on their chemical composition (homo- or copolyester, contained hydroxyl fatty acids). Some grades of additivated PHB are similar in their material properties to polypropylene (PP), and offer good resistance to moisture and aroma barrier properties. Polyhydroxybutyric acid synthesized from pure PHB is relatively brittle (i.e. elongation at break typically below 15%) and stiff. PHB is a fragile material due to the re-crystallization with ageing at room temperature, thus mechanical properties change with time and, as reported in the example, samples stored at room temperature for 60 days have lower values for elongation at break than samples stored for 30 days (Bugnicourt et al., 2014). The average PHA's properties are summarized below (Bugnicourt et al., 2014).

Table 1.3 Range of typical properties of PHAs (Bugnicourt et al., 2014)

(* Tg: glass transition temperature, Tm: melting temperature, Xer: crystallinity degree, E: Young's modulus, σ tensile strength, ε: elongation at break, WVTR: water vapour transmission rate, OTR: oxygen transmission rate)

Property [Units]	**Values**
Tg [°C]	2
Tm [°C]	160-175
Xer [%]	46 - 60
E [GPa]	1-2
σ [MPa]	15-40
ε [%]	1-15
WVTR [g.mm/m2.day]	2,36
OTR [cc.mm/m2.day]	55,12

1.5.4 PHA detection method

Various methods are available for the detection and analysis of intracellular microbial PHA. These are useful for identifying new PHA-producing microbes or for routine monitoring of PHA production bioprocesses. Below is a brief description of the various methods most used, referring to a study conducted by Amy Tan et al. (2014).

Methods that detect the presence of PHAs or micro-organisms capable of producing them include cell or cell colony staining, genetic detection of polymerase chain reaction (PCR) and transmission electron microscopy (TEM). Cell or cellular colony staining and PCR genetic detection are often used as first-line methods for high-throughput screening and identification of new microbes with PHA production potential due to the relative ease of sample preparation and in the short analysis time. In the colony / cell staining method, red Nile or Blue Nile A are added as dyes directly in the solid growth medium or in liquid cell cultures. Under UV illumination, the Nile red and the Nile A blue color the PHAs with a pink / red / yellow / orange tint to identify and isolate PHA-producing microorganisms. To confirm this study other publications are reported where the fluorescence spectroscopy was used for the detections of PHA (Khetkorn et al., 2016, Zuriani et al., 2016). In PCR genetic detection, the primer pairs are designed to specifically amplify the phaC gene, which encodes PHA-synthase, an enzyme responsible for PHA synthesis (Solaiman and Ashby 2005). The gene is typically present in bacteria that accumulate PHA and is absent in non-accumulators, serving as a basis for identifying and distinguishing the two types of microorganisms. Although the methods of staining and genetic detection provide an easy way to detect the presence of organisms that produce PHAs efficiently, these methods are also subject to detection errors. False positives may result from the staining of other lipid storage compounds that are not PHAs and non-specific PCR amplification; furthermore, false negatives could derive from unfavorable conditions for the accumulation of PHA accumulating biomass and inadequate detection primers or unsuitable PCR conditions. Thus, these two methods could only be used as a preliminary test to evaluate the PHA production potential.

The Transmission electron microscopy (TEM) allows direct visualization of PHAs, which appear as intracellular granules, providing evidence of polymer bioaccumulation. However, preparing the TEM sample takes a long time and involves the use of radioactive chemicals, making it an

unsuitable method for screening purposes. The detection of the phaC gene and the TEM are effective for verifying PHA production or PHA production capacity, however the disadvantage of these methods is that they are unable to quantify the PHA or provide qualitative information on the monomeric composition of PHA.

1.5.5 Polymer characterization

Traditionally, the analysis of crotonic acid was commonly used as a valid method for the quantitative determination of P3HB: the polymer is dissolved in concentrated sulfuric acid and converted into crotonic acid and measured by UV spectrophotometer, since the latter has a strong absorption to UV wavelength 235 nm. This is a quick and easy way to quantify P3HB, but it tends to overestimate the polymer content (Karr et al., 1983). Currently, the quantification and characterization of various intracellular microbial PHAs can be achieved using modern analytical techniques, including Fourier transform infrared spectroscopy (FTIR).

The FTIR method was applied to detect and distinguish the different types of PHA (ie scl-PHA, mcl-PHA and scl-mcl-PHA), present inside intact cells or as purified polymers. The carbonyl ester band characteristic for intracellular scl-PHA, mcl-PHA and scl-mcl-PHA was observed at 1732 cm-1, 1744 cm-1 and 1739 cm-1 respectively, while the same band for scl- purified polymer PHA, mcl-PHA and scl-mcl-PHA were observed at 1728 cm-1, 1740 cm-1 and 1732 cm-1 respectively. Arcos-Hernandez et al. showed that the scl-PHA content of the biomass, between 0.03 and 0.58 w / w, could be coupled to the FTIR spectra using a partial least-squares model, allowing the determination of the scl-PHA content within a standard error of the prediction value of 0.023 w / w. The solvent-free nature of the FTIR technique and the short analysis time eliminate risk exposure to hazardous chemicals, while providing rapid data delivery. However, methods based on the FTIR technique have a lower detection sensitivity than others, are not suitable for describing or detecting changes in the PHA monomer composition and cannot discriminate mixtures of PHAs and copolymers. Thus, FTIR spectroscopy-based methods are more suitable for routine monitoring of PHA production for standard processes with well-characterized PHA products (Akdogan and Celik 2018).

The methods reported on LC and GC are much more accurate as regards the control and accuracy of polymers and qualitative information on their monomeric composition. Compared to the FTIR methods, the methods on chromatography have a higher detection rate of 0.014 to 14 μg for HPLC

and 0.05 pg to 15 mg for GC depending on the detectors and chemical derivatization methods used. The automation of the LC platforms has improved the analysis of the scl-PHA. The improvement in the measurement accuracy of the P3HB-derived crotonic acid was possible by applying the HPLC to an ionic coupled with a UV detection. Simultaneous analysis and quantification of 3HB and 3HV monomers can also be obtained by ion chromatography (IC) with an anion column and a conductivity detector. Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique on the use of liquid chromatography together with mass spectrometry: the chromatograph separates the compounds present in the sample while the mass spectrometer acts as a detector. This technique, compared to other gas chromatography, uses a pressure control window fast and robust enough for the routine analysis of PHA monomers, however, to date, LC-MS has been applied only to a limited extent as a complementary technique for analyzing PHA monomers. At present, GC remains the preferred method for the qualitative and quantitative analysis of PHA monomers due to its high separation power and detection sensitivity. One of the first works on the determination of PHA by GC was reported by Braunegg et al., (1998) that developed a method for the accurate and reproducible determination of the P3HB content in bacterial biomass using the GC with flamed ionization detector (GC- FID). They showed that after subjecting the bacterial biomass containing P3HB to methanolysis, P3HB can be completely recovered as its methyl esters derivatives and quantified at levels as low as 10-5 g / l. The GC-FID analysis was subsequently extended to P3HV and mcl-PHA. The robustness of the GC-FID determination, however, depends on the inclusion of the appropriate PHA analytical standards. Conversely, the GC coupling to the mass spectrometry detector (GC-MS) guarantees a more reliable detection, confirms the identity and quantification of PHA monomers, as well as allowing the provisional identification of new PHA monomers in the absence of standards analytical. A recent study by Tan et al. showed that PHA monomers with carbon numbers between 4 and 16 have strong linear correlations with their respective retention times and response factors ($R2 \geq 0.987$) in the GC-MS, which allowed to predict the retention time and the response factor of other PHA monomers. This method allowed to detect a wide range of PHAs, ranging from scl-PHA to lcl-PHA, and to reliably quantify them in the absence of reference standards. It is also expected that the coupling of GC to tandem mass spectrometry (GC-MS / MS) could offer improvements in terms of sensitivity and specificity for the determination of PHA monomers. Despite their advantages, chromatography-based methods require that PHAs be depolymerized and chemically transformed into acids, diols or methyl ester

derivatives before analysis. This means that methods based on chromatography are not able to distinguish whether different PHA monomers are part of different homogeneous PHA polymers or whether they are part of a single PHA copolymer.

1.5.6 PHA Extractions

PHAs are a product of an intracellular nature, therefore at the end of the production process it is necessary to extract them from the cells of the accumulating microorganisms. Being stored in the form of insoluble intracellular granules, the PHA recovery methods typically involve lysis of the cell wall / cell membrane, solubilization and purification and precipitation of the polymers. The extraction process involves two steps: cell lysis, which is the break of the membrane and the cell, with the consequent dissolution of the cell itself. Common methods for recovering PHAs from microbial biomass are solvent extraction methods or digestion methods based on the use of enzymes and chemicals. In the solvent extraction process, the choice of the latter is linked to the type of PHA to be extracted: in the case of short side chains such as for PHB and PHBHV, in the literature, the use of chlorinated organic solvents such as chloroform is preferred, while in the case of long side chains, the most used solvent is acetone. In most cases, before the extraction process a biomass pre-treatment is necessary, necessary to remove any contaminants (such as residual nutrients from the PHA accumulation phase) and to favor the subsequent cell lysis phase. In addition to these pre-treatments, polymer purification processes are usually required after the extraction. The most commonly applied extraction methods are illustrated below, referring to various studies carried out on this topic, including those of Amy Tan et al. (2014) and Raza et al. (2018). Solvent extraction is the most commonly used technique for PHA extraction due to its simplicity and ease of use. The solvent has a double action, as it acts on the permeability of the cell membrane, so that the polymers become accessible, and solubilizes the polymer granules.

The solvent extraction is most used for the PHA characterization. The first solvents used were the chlorinated organic solvents such as chloroform, 1,2-dichloroethane, chloromethane, which are used for the extraction of short-chain polymers. While for medium-long chain polymers, the extraction with acetone; in these cases, the recovery of the polymer can take place through the evaporation of the solvent or by precipitation by adding a non-solvent, such as methanol or ethanol in refrigerated form. Generally, the recovery of the polymer through a direct solvent extraction if on the one hand it allows to reach high purities (> 97%), on the other hand it shows a recovery

yield deficit (≤ 90%). A new method for short chain PHA extraction was developed using acetone at elevated temperature and pressure as an anti-solvent in a closed system that combines components for product extraction, filtration and processing. The quality of the polyesters extracted with acetone does not show significant differences with respect to those extracted from chloroform, providing a promising substitute in terms of greater solvent recyclability, without negatively affecting the structural characteristics of the biopolymers. After extraction with solvent, the cellular residues are removed by filtration and the solution is concentrated by rotary evaporation; then the P3HB polymer is precipitated by adding frozen ethanol. Chloroform and 1,2-dichloroethane have been used for the extraction of polymers, obtaining a high recovery of P3HB (68%) with high purity (from 96% to 98%), while through methylene chloride a recovery was obtained of 25% and a purity of 98%. 1,2-propylene carbonate is the solvent that is preferred because it has a lower degree of toxicity than others. The preponderance of solvent extraction is based on the high purity of PHA recovery, however it has several disadvantages related to high operating costs and the negative environmental impact caused by the production of hazardous waste.

1.5.7 PHA applications

The main applications of biopolymers are in the agricultural and medical field. Due to their diverse structures and properties, PHAs have penetrated different markets sectors as bioplastics, fibers, biomedical implants and drug delivery carriers. Bioplastics, nowadays, are being used for the manufacture of short-lived materials and products like films, catering products, food packing, packing and waste bags, paper coating (Bioplastics 2011; Coats et al. 2011). Based on their characteristics, PHAs have been used mostly in drug delivery, tissue engineering, wound dressing in surgery, wound management, bioimaging, and biosensors with great success (Yao et al. 2008; Atwood and Rehm 2009; Wu et al. 2009). The principal PHA application are summarized in *Table 1.4*

Table 1.4 Application of PHA in various fields (Chen 2009).

Applications	Examples
Packing industries	All packing materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances, etc.
Printing & photographic industry	PHAs are polyesters that can be easily stained.
Other bulk chemicals	Heat sensitive adhesives, latex, and smart gels. PHA nonwoven matrices can be used to remove facial oils.
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers.
Plastic Processing	PHA can be used as processing aids for plastic processing.
Textile Industries	Like nylons, PHA can be processed into fibers.
Fine chemical industries	PHA monomers are all chiral *R-forms*, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals.
Medical implant biomaterials	PHAs have biodegradable and biocompatibility, and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices.
Medical	PHA monomers especially R3HB, have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement.
Healthy food additives	PHA oligomers can be used as a food supplements for obtaining ketone bodies
Industrial microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performances of industrial microbial strains
Biofuels or fuel additives	PHA can be hydrolyzed from hydroxyl-alkanoate methyl esters that are combustible.
Protein purification	PHA granules binding proteins phasin or PhaP are used to purify recombinant proteins.
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting diseased tissues.

1.5.8 Substrates used for PHA production

As already mentioned, the composition of PHAs produced by the different bacterial strains varies according to the type of current substrate as a carbon source. Among the substrates used sugars (eg. Fructose, maltose, lactose, xylose, arabinose, etc.), N-alkanes (hexane, octane, dodecane, etc.), n-alkanoic acids (eg. acetic acid, propionic acid, butyric acid, valeric acid, lauric acid, oleic acid, etc.), n-alcohols (eg. methanol, ethanol, octanol, glycerol, etc.) and gas (eg. methane and carbon dioxide). Furthermore, it is a PHA production process. These products used oils, waste fats, food waste, agricultural waste, domestic waste water, vegetable oil effluents, raw glycerol from biodiesel production, plastic waste, landfill gas, etc. (Amy Tan, 2016). Since the raw material is the main source of carbon for the synthesis of PHA and the growth of bacteria, the use of organic waste as a source of organic substance is presented as a promising alternative for the reduction of PHA production costs. As already described, there are three main paths for the synthesis of PHA: two use sugars as a carbon source (Route I, from Acetyl-CoA to 3-Hydroxybutyryl-CoA and Path III, with the biosynthesis of fatty acids) and a third via uses fatty acids (Route II, with the degradation of fatty acids). Therefore, the sugars and / or fatty acids contained in the waste could be the best candidates to use in the production of PHA. For substrates containing fatty acids, yields of around 0.6-0.8 g PHA / g ΔS were recorded, which are higher than those for sugars 0.3-0.4 g PHA / g ΔS. Different types of waste have been reported as a carbon source for PHA production.

Depending on their origin, waste streams have been classified as agro-food (solid plant waste, livestock waste, waste from the agro-food industry), non-agro-food industrial (generated from the processing of biofuels), foodstuffs (solid waste) or liquids), residues from urban waste treatment plant (both urban solid waste and wastewater) and synthetic substrates (glucose solutions, VFA). In particular, the study carried out by Raza et al. shows some examples of waste materials used as a substrate for PHA production.

First the whey is mentioned, which is a by-product of the dairy industry that represents up to 90% of the volume of processed milk; contains about 4.5% (weight / volume) of lactose, 0.8% of proteins, 1% of salts and 0.1-0.8% of lactic acid and its high biological oxygen demand (40 gBODl-1) makes it difficult to dispose of. Half of the whey produced is converted into products usable for humans and animals and the rest is usually thrown away and released into the environment. The discharge of large quantities of whey in the environment can damage the chemical and physical structure of the soil, pollute groundwater and can also affect the health of the air. Several attempts to use whey, as a carbon source for bacterial growth, as an economic substrate for the PHA production strategy have been studied. A PHA yield of 1.27 g / l was reported with a biomass yield of 5.0 g / l using a pure P. idrogenovora culture. C. necator is one of the best known bacteria among PHA-producing microorganisms, but it is not able to hydrolyse lactose or metabolize galactose. Therefore, E is used for the production of PHB from glucose. Recombinant coli, with which concentrations of 5.2 g / l of PHB were obtained, corresponding to 81.3% (w / w) of PHB, with a concentration of 30 g / l of whey solution. Another study looked at the production of PHB from recombinant E. coli strains under conditions of O2 deficiency and without O2 limitation. The accumulation of higher PHB (80%) was observed under limiting conditions, with a PHB concentration of 25 g / l. Instead, without O2 limitation, 57% of PHB was reached with a concentration of 32 g / l.

Another substrate that is mentioned is wheat and rice bran, which is the outer layer of the grain that contains proteins, carbohydrates and other minerals. Trials using wheat bran waste as a carbon source for bacterial growth and PHA production were carried out by growing Halomonas boliviensis LC1 resulting in biomass production of 3.19 g / l and 1.08 g production / l of PHB. A high biomass content of up to 140 g / l and the PHA accumulation of 55.6% (w / w) of CDW were reported using rice bran together with cornstarch using Mediterranean haloferax. Therefore, the

use of this waste can be a credible alternative to the production of PHA and to the reduction of problems of disposal in the environment. As a source of carbon, starch is mentioned, which is a cheap and easily available material on the market; however, it has a rather complex nature which limits the ability of many bacterial strains to hydrolyze and produce α-amylase. This enzyme must be introduced from an external source, so that these microorganisms can hydrolyse the starch and use it as a carbon source. The H. mediterranei bacterium together with the enzymatic extruded starch gave a biomass yield of 1.14 g / l with a PHA accumulation of 43% (weight / weight) of CDW. Potato starch was used as the sole carbon source in high cell density cultures using Ralstonia eutropha NCIM 5149 as a bacterial strain, obtaining a total cellular biomass of 179 g / l with PHA content up to 55% (p / p) of CDW. Other studies have been conducted using sulphide and non-sulfur molasses, or viscous residues of sugar cane; if for the extraction is used sulfur dioxide as stabilizer, we speak of sulfur molasses, otherwise of non-sulfur molasses. Sugar cane molasses contains sucrose, glucose, fructose in addition to iron, magnesium, calcium, potassium and vitamins including B7, all of which are suitable for the growth of bacterial cells. Up to 6.0 g / l of PHA were reported when sugar cane molasses was used as a carbon source. The accumulation of PHA using sugar cane molasses is favored by a lower pH during fermentation, since the production of butyric and valeric acid is favored, while, at higher pH, the main products were acetic and propionic acids; however, the high concentration of ammonia has a negative impact on PHA accumulation. P. fluorescents A2a5 was grown on a low-cost liquid sugar cane medium as a carbon source and produced PHA up to 70% (w / w) of CDW, thus significantly reducing the cost of PHA production.

Finally, wastewater is mentioned, which can be used to produce PHA with a mixed culture method. Using the UWD strain of Azotobacter vinelandii on wastewater, a PHA accumulation of 58% (w / w) of CDW was recorded; a PHA yield of 43% (w / w) of CDW was reported using acetate as a source of carbon added to the wastewater from the paper industry. Textile wastewater has also been used successfully for the production of PHA and for the biodegradation of dyes. To reduce the cost of the production the research is focused on the utilization of MMC fed with complex substrate as waste. in terms of bio refinery, the use of low-cost waste and the environmental advantage are the main reasons for these studies to continue investing to obtain polymers with a high commercial value. The numerous possibilities of using industrial and agricultural waste are summarized *Table 1.5* below.

Table 1.5 List of the most common renewable sources investigated for PHA production (Valentino et al., 2014)

Reusable substrate	Reference
Olive mill wastewater	Dionisi et al., Wat Res 2005 Beccari et al., J Chem Technol Biotechnol 2009 Ntaikou et al., Biores Technol 2009
Olive pomace	Waller et al., Biores Technol 2012
Sugare cane molasses	Albuquerque et al., J Biotechnol 2007
Paper mill wastewater	Bengtsson et al., Biores Technol 2008
Palm oil wastewater	Marsudi et al., Appl Microb Biotechnol 2008 Md Din et al., Wat Sci Technol 2006
Tomato	Liu et al., Wat Environ Res 2008
Sugar beet syrup	Wang et al., Industrial Crops and Product 2013
Cheese whey	Duque et al., New Biotechnol 2013
Cellulose hydrolyzed	Nduko et al., J Biosci Bioeng 2012
Agro-industrial waste and amid	Shamala et al., Brazilian J Microb 2012
Amid hydrolyzed	Chakraborty et al., J Biomed Biotechnol 2012
Tapioca starch	Setyawaty et al., World Appl Sci J 2011
Municipal wastewater	Morgan-Sagastume et al., Wat Res 2010 Mengmeng et al., Biores Technol 2008

1.6 PHA production through Mixed Photosynthetic Consortia

However, PHA production with MMCs has been mainly restricted to the utilization of aerobic organisms. In recent years, different studies have proposed the utilization of phototrophic mixed cultures (PMCs). A new approach relying on the photosynthetic activity of mixed consortia has been explored recently (Fradinho et al., 2013). The results analyzed above has shown the higher costs for the production of PHA are associated with the supply of oxygen. An alternative without the use of oxygen has been proposed eliminating the costly need for aeration during ADF enrichments. In such a system, photosynthetic bacteria uptake an external carbon source, in the form of acetate, during the feast phase using light as an energy source PHB was produced at the same time as a sink of NADH, given that no electron acceptor was present. During the famine

phase PHB was consumed using oxygen as an electron acceptor, which was not provided though aeration but produced by algae also present in the SBR (Kourmentza et al., 2017). This was based on the fact that photosynthetic accumulating bacteria out-compete other bacteria and algae

without the need of oxygen. However, this process is energy saving, since sterilization and aeration are not sufficient, which has an impact on the final price of the polymer.

1.7 PHA production through Purple Phototrophic Bacteria (PPB)

The PPB show a high potential to accumulate intracellular PHB granules as energy and carbon reserves and are active H2 producers when they are grown under stress conditions (Suzuki et al., 1995). These bacteria preferably grow by a photoheterotrophic metabolism with organic substances as electron donors during their photosynthetic activity (Koku et al., 2002). Compared to other phototrophic organisms, their key advantages include high growth rates, versatile metabolic modes (Tabita, 1988) and the utilisation of IR light, which lowers the power required per photon emission and facilitates the specific selection and enrichment over algae (Bertling et al., 2006). Initial studies with PMCs also applied the FF strategies to obtain PHA storing phototropic bacteria, and by using acetate as a carbon source. The PHA accumulation values, reported by Fradinho et al., 2013, were 20 and 30 % g PHA/ g VSS obtained under continuous illumination.

2 Research objectives

The experimental campaign of this project was developed in order to investigate the kinetics governing the production processes of biopolymers, as well as the yields – even qualitatively - of different engineering processing. Two different mixed culture were compared: MMC (AS) taken from a WWTP and the PPB enriched from wastewater. Two different set up were tested: an aerobic SBR for the selection of the biomass and an anaerobic flat plate photobioreactor. The project was divided into three main phases to achieve the overall objective of the thesis, which will be listed below.

2.1 Research objective 1 (RO1)- Preliminary test for the production of polyhydroxyalkanoates (PHAs) by Mixed Microbial Culture (MMC)

In the above-described context, the main general goal of the proposed project is to develop an innovative approach for biowaste/FW valorization aimed at integrating the recovery of both energy (thorough bio-H2 production) and material (via bio-polymer production) from different kinds of organic residues. The research is thus devoted to highlighting the mechanisms at the base of bio-H2 and biopolymers production, the influence of operating conditions on each stage (as well as on the whole process) yields and kinetics. The biomass supply to the metabolic stage is selected from the biological sludge of a municipal wastewater treatment plant, so as to attaining a further overall target of the proposed research, namely the valorization of an additional problematic waste stream.

The specific objectives of the research can be summarized as follows:

1) Development of an innovative treatment based on a staged process. During the first stage, the conversion of sewage sludge, possibly in combination with other wastewater of agro-industrial origin, into bio-H2 is attained; the second stage is aimed at producing biopolymers from volatiles fat acids (VFA) exiting the biohydrogenogenesis stage, using the excess sludge as biopolymers producing organisms;

2) understanding of the effect of the operating conditions of each stage on the kinetics and production yields of bio-H2 and biopolymers, as well as on their qualitative characteristics;

3) evaluation of the full-scale transferability of the process through the realization of mass and energy balances and through the preliminary estimation of the avoided impacts

2.2 Research objective (RO2) - Photofermentative biopolymer production by mixed culture of Purple phototrophic bacteria

The results obtained so far, have not solved the problem of the high cost of producing biopolymers through the application of mixed bacterial cultures. The PHA production with MMCs has been mainly restricted to the utilization of aerobic organisms. For this reason, the capacity of anaerobic growth in photobioreactor of PPB it is studied to allows the decrease of

production costs, by removing the oxygen supplying and by the use of the sun light during the process.

The specific objectives of the research are summarized below:

- enrich PHA-accumulating PPB
- Study how the VFA feed affects polymer composition
- Optimize the operative condition and apply to a large-scale reactor
- Polymer characterization:
  - Qualitative/quantitative
  - Mechanical and thermal property
  - Molecular weight and chemical structure

2.3 Research objectives (RO3) - Photofermentative biopolymer production by mixed culture of Purple phototrophic bacteria from fermented molasses

The need to find a solution to the high cost of bioplastics production, introduced the importance of finding economically viable alternatives. The results obtained so far on the application of PPB for the production of PHA, have not yet solved the problem of market competitiveness with plastics. For this reason, in this thesis the use of complex waste from agricultural industries for the production of lower cost PHA has been studied. The feasibility of application during the production phases of PHA of sugar cane molasses has been studied.

The specific objectives of the research are summarized below:

- Study of a single-stage photo fermentation process from sugar-rich waste
- Study the production processes by comparing the use of artificial and complex feedstock
- Optimize the operative condition and apply to an out-door large scale reactor
- enrich PHA-accumulating PPB
- Accumulation batch test to study the effect of the nutrient limitation on the PPB-PHA accumulation capacity
- Polymer characterization:

- Qualitative/quantitative
- Mechanical and thermal property
- Molecular weight and chemical structure

3 Research activities for reaching RO1: Preliminary tests for the production of polyhydroxyalkanoates (PHAs) by Mixed Microbial Culture (MMC)

3.1 Introduction

The management of excess sludge from WWTPs is a critical issue due to the high costs and environmental implications. The conversion of a conventional WWTP into a biorefinery may contribute, while producing a high-quality effluent, to attaining the recovery of valuable elements, materials as well as energy. In particular, a suitable combination of treatment technologies may be directed to producing bio-H_2 from mixtures of sewage sludge and agroindustrial biowaste in a first fermentative process stage, and bio-CH_4 (under anaerobic conditions) or biopolymers (under aerobic/anaerobic conditions) in a subsequent treatment stage. Among the biopolymers, polyhydroxyalkanoates (PHAs), polyesters synthesized by numerous bacteria, appear to be the most attractive due to their thermoplastic properties similar to that of polypropylene (PP) (Carlozzi et al., 2018). So far, the application of mixed microbial cultures (MMCs) using complex residues for PHA production, through the application of transient carbon availability conditions the Feast and Famine (FF) strategy, has been widely investigated. However, PHA production with MMCs has been mainly restricted to the utilization of aerobic organisms.

3.1.1 PHA production from pure culture

To obtain high productivity in terms of PHA, pure cultures of microorganisms are collected. Within the mixed crops deposition of certain PHA-producing microbial strains may occur; these are well known in terms of genetics and biochemical processes that lie at the base of their strategies of carbon assimilation and PHA accumulation. Personal information is available based on evaluation criteria (Amy Tan et al., 2014). The research carried out by Amy Tan et al. focuses on the different

microbial strains that accumulate PHA and their productivity. To date, most of the PHA-producing bacteria have been found to be Gram-negative bacteria, which synthesized short-chain PHAs in considerable quantities (between 24 and 88% in dry weight), producing both poly (3-hydroxybutyrate) (P3HB) and poly (3-hydroxyvalerate) (P3HV) from different sources, including various sugars (glucose, fructose and sucrose), fatty acids, methanol, n-pentanol. The production of PHA with a medium chain reaches 30 or 70% in dry weight, depending on the species used (especially Pseudomonas); the monomer present is 3-hydroxybutyrate (3HB). Pure culture is used for their storage capacity and high PHA high density.

PHA accumulation has also been observed in gram-negative extremophilic bacteria, which resist extreme cultivation conditions, with high salinity or high temperatures (up to 70 ° C) (Bertrand et al., 1990). Compared to other Gram-negative bacteria, extremophiles are advantageous in terms of lower sterility demand and their potential for direct application with originally high discharge effluents in salt concentrations or temperatures, eliminating the need and cost required for effluent pretreatment of discharge.

The main concern for Gram-negative bacteria, however, is the presence of lipopolysaccharide endotoxins (LPS) in the outer cell membrane of bacteria; LPS is a pyrogen that can cause a strong inflammatory response, making the PHA polymer unsuitable for biomedical applications (Koller et al., 2013). The removal of the LPS endotoxin can be obtained by treating the PHA polymer with oxidizing agents (sodium hypochlorite and NaOH, ozone, hydrogen peroxide and benzoyl peroxide), with repeated solvent extraction or with solvent extraction followed from purification with activated carbon. However, these methods increase the overall cost of PHA production and lead to changes in the properties of the PHA polymer (ie reduction in molecular mass and polydispersion).

Compared to Gram-negative bacteria, Gram-positive bacteria produce mainly short-chain PHAs, in lower contents (about 2 to 50% of dry weight), which is why Gram-positive bacteria have not yet been adopted for commercial production of PHA. Despite generally accumulating lower amounts of PHA, Gram-positive bacteria are advantageous compared to Gram-negative bacteria due to their lack of LPS, which can make them a better source of PHA for biomedical applications (Luef et al., 2015).

However, the cultivation of microbial species also requires particular environmental conditions, which must be maintained during the polymer production process. This implies significant burdens

both in terms of management and in terms of costs, which is why pure cultures of microorganisms are not often used in industrial processes.

A valid alternative is to use mixed microbial cultures (MMC), which are less demanding in terms of management and less expensive, as they do not require the maintenance of sterile conditions; they are also more easily integrated into the waste and waste treatment process than the use of pure crops (Huang et al., 2017). To enrich the mixed biomass of PHA accumulating microorganisms, particular conditions are imposed on the process (which will be described in the following chapters), in order to select the biomass and ensure that cell growth is not the prevalent metabolic process

3.1.2 PHA production from mixed microbial culture

Two different strategies have been studied so far for the PHA production by mixed microbial culture: anaerobic-aerobic cycling and a feast-famine (Aerobic Dynamic Feeding (ADF) approach. In the first process two different bacteria strains are involved. Glycogen-accumulating organisms (GAO) have the potential to directly compete with polyphosphate-accumulating organisms (PAO) in EBPR systems as both are able to take up VFA anaerobically and grow on the intracellular storage products aerobically (Zeng et al., 2002). Using MMC cultured on waste substrate is predicated on maximizing the enrichment of a microbial consortium capable of feast-famine PHA synthesis. Feast-famine PHA synthesis is a metabolic response to aerobic dynamic feeding (ADF) conditions applied to an MMC fed an organic carbon and nutrient-rich substrate. ADF-induced PHA synthesis on VFA-rich substrate is a well-established phenomenon. By combining short periods of exogenous substrate availability with long periods of exogenous substrate deficiency, PHA-producing bacteria are enriched over non-PHA producing bacteria (Reis et al., 2003). Bacteria that are able to convert carbon to PHA during the feast phase have a competitive advantage towards the rest of the microbial population, as they utilize PHA as a carbon and energy reserve during the famine phase, allowing them to grow over non-PHA storing microorganisms (Albuquerque et al., 2007). In both strategies, anaerobic/aerobic cycles and the feast/famine approach, PHA storage occurs when cellular growth is not favored. In the anaerobic/aerobic process, an external limitation of an electron acceptor (such as oxygen or nitrate) limits cellular growth and enhances PHA storage. By contrast, in the ADF process, an internal limitation

(insufficient intracellular components necessary for growth) prevents growth while both an electron acceptor (oxygen) and its corresponding electron donor (PHA) are present during the substrate uptake.

3.1.3 Three-stage process for PHA production by mixed microbial cultures

PHA production further valorizes wastewater treatment by recycling and channeling carbon towards products and reducing sludge production. PHA production with wastewater treatment implies selectively growing/enriching PHA-storing bacteria in activated sludge based on engineered environments exerting dynamic conditions with respect to substrate feeding (i.e., alternating conditions of carbon availability and unavailability) (Dionisi et al. 2014). The most consolidated process based on the selection / enrichment of mixed crops from activated sludge, comprises three stages. A first anaerobic stage in which the wastewater is fermented (to obtain VFAs, easily convertible to PHAs); a subsequent aerobic biomass selection phase in a SBR reactor, under the Feast-Famine (FF) regimen, and a last aerobic phase for PHA accumulation; finally, the polymer is extracted from the cells and purified. The analysis of the results obtained in laboratory-scale experiments conducted up to now and available in the literature, suggests the feasibility of the process of producing biopolymers from sludge, possibly in combination with other substrates, although further studies are needed to allow a theoretical interpretation of aspects that are not yet fully understood. Since many industrial wastes/by-products feedstocks used so far for PHA production are rich in carbon compounds but poor in nutrients (sugarcane molasses, paper mill wastewater, olive oil mill wastewater), nutrient supplementation is required during culture selection (Albuquerque et al., 2010). In another study it was studied the selection efficiency and stability of the reactor operated under feast and famine regime and fed with fermented cheese whey at high OLRs might be achieved by imposing an additional selective pressure: uncoupling the availability of nitrogen and external carbon (Oliveira et al., 2016). Not only agro-industrial waste is studied for the PHA production. The feasibility of producing a functional biomass with PHA-storing ability is demonstrated, at laboratory- and pilot-scales while providing some level of municipal wastewater treatment, in a work carried out by Morgan-Sagastume et al., 2014. Furthermore, PHA production from VFAs obtained from waste activated sludge (WAS) acidogenic fermentation is benchmarked with biomass with PHA storage capacity. The piloting system reported herein constitutes, to the best knowledge of the authors, the first pilot-scale system treating

municipal wastewater and producing biomass with enhanced PHA storage capacity (Morgan-Sagastume et al., 2014). PHA production together with municipal wastewater and sludge treatment is investigated as a four-stage process is shown in *Figure 3.1*

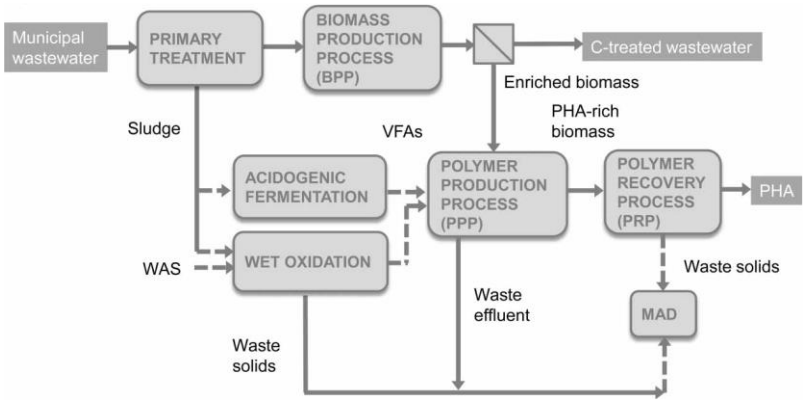


Figure 3.1 Schematic process flow diagram of municipal wastewater and sludge treatment in conjunction with PHA production (F. Morgan-Sagastume et al., 2014)

3.1.4 Methods for enhanced production of mixed culture PHAs

As previously stated, the economic feasibility of mixed culture PHAs can be increased by improving the volumetric process productivity and for this purpose is necessary to understand the effects of process parameters on PHA yield.

3.1.5 Parameters affecting the process

The PHA production process can be summarized in three phases: pre-treatment (phase I, fermentation), growth / acclimatization of the culture or enrichment of the culture (phase II, SBR phase) and production / accumulation of PHA (phase III, batch phase). The stage of fermentation is a process of production of waste materials from wastewater. During the phase I, is performed an anaerobic acidogenic fermentation of the substrate, in order to obtain the organic content in volatile fatty acids (VFA), carbohydrates and proteins, which precede the PHA precursors and are readily available for biomass (Mengmeng et al., 2009). The conversion of the complex organic substrate takes place in four phases:

- Hydrolysis, where the complex organic substrate is broken down into simpler compounds, thanks to hydrolytic bacteria that split proteins, fats and carbohydrates into, amino acids, fatty acids and monosaccharides.

• Acidogenesis, where fermentative bacteria carry out a further split into even simpler molecules, namely volatile fatty acids (for example acetic, propionic, butyric and valeric acid), alcohols and ketones.

• Acetogenesis, where the simple molecules produced in the previous stage are further digested by acetogenic bacteria and transformed into carbon dioxide, hydrogen and acetic acid.

• Methanogenesis, with production of methane, CO2 and water.

Phase II aims to reach a sufficient amount of active biomass able to produce and accumulate the maximum PHA content. To achieve this goal two different strategies are applied based on the type of crop used; as already mentioned, pure crops or mixed crops can be used. The main difference between these strategies lies in the fact that processes with pure culture are performed with strains of PHA-producing microorganisms, already known from the bibliography; therefore, these processes require phase II only to reach the sufficient amount of active biomass. While the mixed culture processes require the selection and enrichment of the strains capable of producing PHA. In order to cultivate pure culture, many authors have used a synthetic medium with an easily degradable carbon source. Furthermore, some pure cultures have been acclimatized before phase III with a medium characterized by a nutrient limitation, made possible through the regulation of the C / N or C / N / P ratio, since the restriction of nitrogen or phosphorus could improve the PHA accumulation. In the PHA production processes with mixed crops, cultivation enrichment, carried out in phase II (phase SBR), is mostly performed with the imposition of the feast / famine regime. In this regime, the feeding is added intermittently: the carbon source is added at the beginning of the test, in the feast phase, in which thanks to the abundance of nourishment the microorganisms grow; subsequently the substrate is no longer added, therefore in the famine phase there is a lack of nourishment. The microorganisms that accumulated polymers during the feast phase, use them, during the famine phase, as a carbon source once the external substrate is exhausted. This generates a selective pressure able to select the microorganisms that can produce and accumulate PHA (Rodriguez-Perez et al., 2018). PHA production and accumulation occur in phase III, or Batch phase, for which generally (for 75% of laboratory studies) a different reactor is used compared to phase II. The studies carried out the two phases in the same reactor used a pure culture, or a photosynthetic consortium of bacteria and algae. The reactor is fed with the substrate enriched with

VFA and as an inoculum the accumulating biomass selected in the previous phase is used, so as to obtain the maximum polymer content and high productivity.

3.1.5.1 Fermentation Phase

The acidogenic fermentation phase is influenced by various parameters, including the type of reactor used and the operating conditions adopted (including HRT and SRT), the composition of the substrate supplied and the feeding frequency, pH and temperature. These factors influence the VFA composition of the mixture leaving the process and consequently the composition and properties of the PHAs produced in the subsequent phases. A study carried out by Gouveia et al. it is proposed to evaluate the effect of pH on the fermentation phase, evaluating the possibility, through the variation of the pH in a range between 4 and 7, to customize the composition of the polymer in a PHA production process, using a substrate as an industrial by-product, whey. The concentration of fermentation products (FP) obtained at each tested pH value was fairly constant (about 13 gCOD / l), except at pH 4, where the production of FP fell to around 4 gCOD / l. There was also a decrease in the production of VFA at a pH below 5. On the other hand, the removal of proteins reached its highest value at pH 4 (about 59%); since no nitrogen or phosphorus was added, the proteins were necessarily hydrolyzed to release N and P in order to support the observed cell growth. However, the concentration of FP was lower at pH 4 and the biomass concentration decreased, suggesting some chemical hydrolysis. The pH change can also affect the composition of the VFA products. In the same study by Gouveia et al. it was found that, regardless of the pH value, lactate was the dominant product acid. A linear relationship was found between the production of acetate and lactate and the pH, which occurs independently of the structure of the microbial community: there is a predominance of acetate at a higher pH and lactate at a lower pH during the fermentation of dairy products. The linear correlation may be due to the activation or inactivation of different metabolic pathways, since the pH influences the rate of bacterial growth which in turn influences the structure of the microbial community and the production of VFA. Furthermore, in this study the pH changes did not have important effects on the yields and rates of fermentation and growth of biomass, on the degree of fermentation and on the removal of proteins.

PH has two effects on biomass: on the one hand, it selects the microbial community, on the other it regulates metabolic pathways. The changes in pH imposed have led to various changes in the

microbial community, but this did not significantly affect fermentation products, a sign that biomass can vary while maintaining similar performance.

As already mentioned, the pH influences the composition of the polymers produced in the subsequent phases. At high pH (> 6) more propionate (HV precursor) is produced; on the contrary, with low pH (<6) more acetate is produced, which is a precursor of HB. It is therefore possible to obtain a manipulation of the polymer composition maintaining the same composition of the substrate, but varying the operating conditions of the acidogenic reactor.

3.1.5.2 SBR Phase

SBR operation is influenced by various parameters that are set at the beginning of the process. Below is a brief description of the influence these have on the system. The hydraulic retention time (HRT) and the sludge retention time (SRT) are decisive for checking the correct operation of the plant, as they directly affect the biomass, both in terms of concentration and selection, and the f / f ratio, varying the length of the feast and famine phases. Low SRT values lead to a reduction in the biomass concentration present at the beginning of the feast phase, which can be a restrictive factor for the substrate consumption rate (Chen, 2017), as the biomass present in low concentrations fails to consume nourishment in a short time. This leads to an increase in the duration of the feast phase, to the detriment of the famine phase, with an increase in the f / f ratio and a negative impact on the enrichment of the accumulating biomass. Furthermore, the SRT has a direct effect on the selection of bacterial strains: it is widely attested that, during the feast phase, the micro-organisms that store PHA possess a kinetic advantage compared to non-accumulating heterotrophs, therefore low SRT values increase this advantage and leading to a higher specific substrate consumption rate (-qs). On the other hand, higher SRT values favor the accumulation of non-accumulating biomass, which leads to a reduction of –qs. As already mentioned, the feast / famine (f / f) ratio assumes a fundamental importance in the biomass selection process and is derived from the OD trend within the reactor: the feast phase, characterized by low OD values, it ends when the concentration rises, or when the biomass ceases its metabolic activity, having consumed the available nourishment. Several studies have shown that a ratio lower than 0.28 provides a competitive advantage to PHA accumulating microorganisms compared to non-accumulating ones, while a ratio greater than 0.55 favors bacterial growth to the detriment of polymer storage (Freches, 2017). This derives from the need to impose an effective internal limitation during the famine phase to induce PHA storage. If

the f / f ratio assumes high values, the famine phase is short and the limitation of internal growth factors is low, so yes will have a predominant bacterial growth compared to the storage of polymers, with a decrease in the specific production rate of PHA (Oliveira et al., 2017). Dissolved oxygen (OD) represents a further parameter that influences the process, for this reason it has been the subject of several studies. Third et al. (2003) observe that in the feast phase of an SBR fed with acetate, the growth of biomass is favored by high OD values, while the yield of PHB conversion is higher with low DO levels, since more ATP is required for growth cellular compared to polymer synthesis. Moralejo-Garate et al. (2013), using glycerol as a substrate, showed that with low OD levels, microorganisms consume the substrate through metabolic pathways with lower energy requirements, favoring the production of polyglucose at the expense of PHB accumulation. However, these works concentrated only single substrates. With the use of fermented industrial wastewater containing different VFAs (acetate, propionate, butyrate and valerate), the composition of the substrate becomes a crucial factor for the enrichment of microbial communities and the performance of PHA production. Wang et al. (2017) have carried out trials at different OD levels, concluding that this has a lower impact than the SRT on biomass selection, while it influences the abundance of selected microbial communities. The length of the cycle also affects the performance of the system, as it can vary the length of the feast and famine phases (and therefore the f / f ratio, with the consequences already described) and the response of the biomass in terms of polymer storage. It has been shown that cycles of between 2 and 4 hours have led to higher values in terms of storage rate and yield and PHA productivity, with the same OLR and HRT (Dionisi et al., 2007); moreover, the selection of the accumulating biomass was better when the feast phase did not exceed 20% of the entire cycle. According to the study by Freches et al., The length of the cycle also influences the composition of the polymers produced, based on the metabolic preferences of the selected biomass. The Organic Loading Rate (OLR), or organic load, influences the biomass selection and the F/F ratio. With low OLR values a low F/F ratio and a good selection of the accumulating biomass is obtained, while higher values of OLR lead a cell growth and an increase in biomass concentration (Dionisi et al., 2007). At the start of the system, the non-selected biomass will contain a small part of rapidly storing microorganisms. If the organic load is low, these microorganisms are able to quickly remove the substrate, establishing the feast / famine regime. On the other hand, if the OLR is very high, the low percentage of accumulating biomass will not be able to quickly remove all the substrate, which will be available to non-accumulating

microorganisms for a longer time. Less strong kinetic pressure will not be sufficient to create a stable predominance of accumulating biomass. Therefore, the different organic load during start-up will push the system towards a "steady state" where the accumulating biomass will not dominate the microbial consortium (Dionisi et al., 2006). Therefore, the optimal starting procedure of the SBR system would be to maintain low OLR values for the first cycle, in order to select the accumulating biomass, and then gradually increase the organic load. According to a study carried out by Silva et al. (2017), the C / N ratio influences the process from different points of view. Increasing the C / N ratio increases the length of the feast phase and decreases the production of PHA and the polymer storage rate, since the nutrients are used by the biomass for cell growth (increases the concentration of active biomass). The composition of the P (HB / HV) copolymers produced also changes: increasing the C / N ratio decreases the HV content. Finally, on the most influence factor is the pH. Villano et al., 2010 conducted tests by varying the pH values (from 7.5 to 9.5). The results showed that the production of biomass (SSV) is not influenced by pH variations in the range of values investigated. As far as the production of polymers is concerned, under the most alkaline conditions the substrate removal rate has suffered a slight decrease, as the PHA storage rate and yield. Moreover, the pH also influences the composition of the polymers: higher HV content was obtained using the biomass enriched in SBR at pH 8.5 and accumulated in the batch tests at pH 9.5, and in this case the highest PHA content was reached (Villano et al., 2010).

3.1.5.3 Batch test phase

The Batch phase is also influenced by various parameters. First of all, it is important to consider the beginning of the new phase, as this will affect the biomass capacities under the new operating conditions. Several studies show that, to saturate the sludge storage capacity, the process must be conducted at high organic load and in aerobic conditions; furthermore, the feeding of the substrates must be intermittent and high frequency, in order to avoid possible inhibitory effects for the biomass at high concentrations of VFA and to avoid the exhaustion of the nourishment during the test.

This is the level of hydroxivalerate (HV) present in PHAs. In this regard, a study carried out by Montiel-Jarillo et al. (2017) the influence of nutrient concentrations on PHA accumulation. The results that the limitation of a nutrient, N or P, increase the PHA content within the biomass. Under

N limit a PHA content almost three times higher (51% gPHA / gVSS) was obtained compared to 21% gPHA / gVSS obtained when nitrogen was supplied in excess. A similar trend in terms of PHA content was observed by changing the conditions of phosphorus availability. In the absence of P, a 42% gPHA / gVSS of PHA was obtained, while a lower accumulation of PHA (13% gPHA / gVSS) was reached when P was in excess, showing that the concentration of P could also be a key factor for maximizing the PHA. Therefore, the PHA contents in the biomass were evaluated when they are not present in limited concentrations, since they are metabolic pathways towards the accumulation of PHA rather than towards the growth of biomass. The conversion rates and yields of the substrate in PHA are higher in non-limiting conditions, as well as the specific rate and PHA storage yield.

Other studies have shown that the pH influences the composition of the polymers produced: in fact, for low pH (around 5) the content of hydroxivalerate (HV) present in PHA is lower (10%) compared to that obtained at higher pH (30 % at pH 9.5). In the same study by Montiel-Jarillo et al. (2017) the influence of pH on the PHA accumulation process is also analyzed. The lowest PHA accumulations were found at acid pH values of 4.0, 5.5 and 6.5, where no significant accumulation was observed with respect to the PHA content of the enriched biomass (Chua et al., 2003; Dias et al., 2006). These trends could be explained because at low pH conditions, acetate remains as a non-dissociated form (acetic acid); this non-dissociated acetic acid spreads rapidly in bacterial cells, then dissociates and imposes a load of intracellular protons that lowers the intracellular pH. This decrease in internal pH could be the cause of the decrease in PHA production. Furthermore, the PHA content of the biomass at pH 8.8-9.2 could be related to the higher acetate absorption rate (0.57 Cmol Ac Cmol-1 X h- 1) compared to the rest of the pH values evaluated. In the pH range> 7.0 the PHA production rates were significantly higher than those of acid pH values. Moreover, the yield of the polymeric substrate at pH 8.8-9.2 was higher (0.33 Cmol PHA Cmol-1 Ac) compared to the rest of the pH values, demonstrating that the carbon source is used for PHA accumulation (Serafim et al., 2014). Therefore, the amount of PHA stored on the biomass enriched during the batch tests is influenced by the system pH: for pH $\geq$ 7.5 there is a high PHA accumulation, demonstrating the importance of this parameter to improve biomass performance enriched (Chua et al., 2003).

Another parameter that influences the accumulation of PHAs during the Batch phase is dissolved oxygen (DO), the effect of which was studied by Wang et al. (2017), which during the SBR phase carried out the biomass selection process once at high DO concentrations (3.47 ± 1.12 mg / L in the feast phase and 3.78 ± 1.00 mg / L in the famine phase) and a second time at lower concentrations (0.86 ± 0.50 mg / L in the feast phase and 2.42 ± 1.26 mg / L in the phase). The DO had a significant impact on the accumulation of PHA by the culture selected in high DO conditions during the feast phase: the substrate absorption rate, the PHA accumulation rate, the PHA conversion yield and the PHA storage capacity decreased with decreasing DO concentration. While, when using the selected culture with insufficient DO concentrations for PHA accumulation, the DO had a limited impact on PHA yield and storage capacity, however, the substrate absorption rate and the rate of PHA accumulation were much lower at the low DO level. In Table 1.2 below were summarized the principal operating parameter and the range of the most influent factors in the PHA chain production.

3.1.6 Operating parameters

As previously stated, to optimize the production of biopolymer is necessary to understand the effects of the process parameters on the PHA yield. As outcome of bibliographic research, described above, the following operational parameters were analyzed and established:

- HRT (Hydraulic Retention Time) is given by the ratio between the reactor volume and the inlet flow and represents the residence time of the fluid inside the reactor. It has been set to 1 day.

$$HRT = \frac{V}{Q}$$

- SRT (Sludge Retention Time) is the cellular residence time, or the average stay time of the activated sludge within the system. Also, this parameter gives an information about the age of the sludge; is given by the ratio between the mass of solids present inside the biological reactor and the quantity of solids that is removed daily through the effluent current or the purging. It was set at 4 days.

$$SRT = \frac{VX}{nV_W X}$$

- The OLR (Organic Loading Rate) is the organic load factor that expresses the amount of input substrate referred to the unit of volume of the reactor itself and to the time. For the first tests it was decided to maintain low OLR values (30 mmolC / ld) to evaluate the response of the biomass; then the organic load was increased to simulate the concentrations of a real wastewater, such as those of whey digestate, which will have to be used as a substrate once the process has been stabilized and optimized. The C / N / P ratio is given by the ratio between the three main nutrients (carbon, nitrogen and phosphorus), whose presence in different concentrations influences the metabolic processes that occur during the reaction phase.
- The length of the cycle, set at 8 hours, influences the duration of the feast and famine phases and, consequently, the selection of the biomass.
- Length of feed phase and discharge cycles influences system performance, as it can vary the f / f ratio and the response of the biomass in terms of polymer storage. It has been shown that the selection of the accumulating biomass was increased when the feast phase does not exceed the 20% of the entire cycle; consequently, the length of the phases also influences the composition of the polymers produced, based on the metabolic preferences of the selected biomass.
- Dissolved oxygen (DO) is another parameter that influences the process. It has been shown that the growth of biomass is favored by high DO values, while the yield of PHA conversion is higher with low DO levels, since more ATP is required for cell growth than polymer synthesis; however, the OD has a lower impact than the SRT on biomass selection, while it influences the abundance of selected microbial communities.
- The inoculum / substrate ratio influences the removal of the substrate: the lower it is, the higher the concentration of microorganisms and the degree of oxidation of organic substances. It also influences the metabolic pathways favored by biomass, since when an excess of nutrients is present the microorganisms use the substrate for cell growth.

Table 3.1 principal operating parameter and the range, which influence the production chain

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

3.2 Experimental approach

3.2.1 Methodology for achieving the objectives

The achievement of the specific objectives of the project was obtained thanks to the execution of an experimental campaign on a laboratory scale, carried out using the reactors designed, realized and made operational at the Sanitary-Environmental Engineering Laboratory of the University of Rome "La Sapienza", which will be described later in the text. The experimental campaign was organized in order to acquire the information necessary to derive the kinetics that describe the production processes of bio-H_2 and biopolymers, and to evaluate the yields also in qualitative terms. The necessary information was acquired to understand the metabolic pathways prevalent in the course of the different processes studied, and to understand the influence that the different process operating conditions that will be analyzed have on them. The innovative element of the project proposal consists in the study of real matrices (sewage sludge in possible combination with wastewater of agro-industrial origin) since a large part of the literature studies use synthetic substrates or selected biomasses. Furthermore, the execution method of the experimental campaign allowed the acquisition of detailed information about the evolution of the processes, also allowing

the characteristics of the final products (biopolymers) to be correlated with the operating conditions in which the biochemical process is conducted. This results are used for the evaluation of the feasibility and sustainability of the process. This element certainly represents an element of particular innovation in the proposed project. Below is reported a description of the process configurations to be analyzed on a laboratory scale, in view of a possible transfer to pilot scale and full scale. In *Figure 3.2* is shown the process scheme with co-digestion of activated sludge (inoculum) and whey (substrate) for the production of bio-H_2 and PHAs.

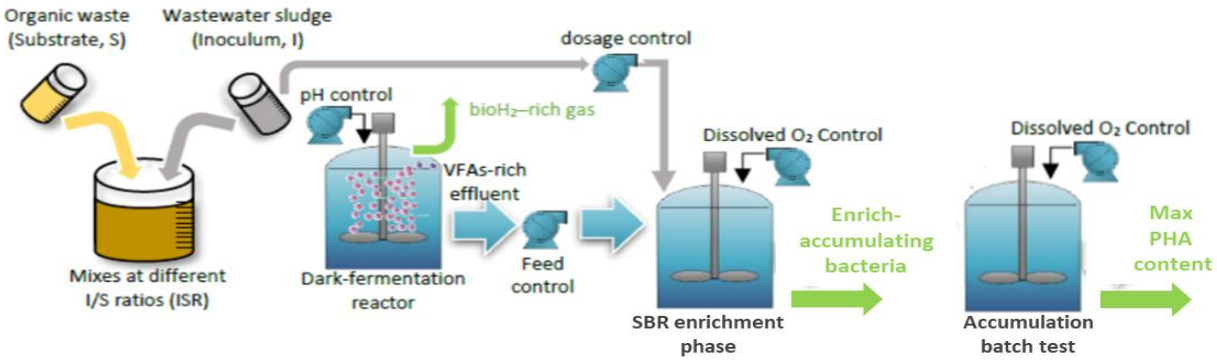


Figure 3.2 the process scheme with co-digestion of activated sludge (inoculum) and whey (substrate) for the production of bio-H2 and PHAs

3.3 Material and method

The PHA production process is based on a first phase aimed at selecting the appropriate biomass; this phase involved the design, construction and commissioning of a laboratory-scale SBR reactor at the Sanitary and Environmental Engineering Laboratory, in order to analyze the effect of the alternation of specific nutritional stress conditions during the reactor operating cycles. The alternation of feast and famine phases is managed in order to apply pressure on the biomass, allowing the selection of only one capable of accumulating PHAs. Starting from a mixed microbial culture, those bacterial strains able to produce and store biopolymers inside them are favored in the selection. To begin implementing the system, a bibliographic study was conducted on scientific articles in the literature, in order to understand the principal operating conditions to start the process. Once the operating conditions to be applied to the process were chosen and fixed,

preliminary, non-automated tests were carried out to evaluate the actual operation and optimization of the plant.

3.3.1 Characterization of input matrices

Activated sludge (AS) from the aerobic unit of a municipal wastewater treatment plant was used as the inoculum. AS was considered a suitable source due to the presence of certain species of bacteria which have been known, to naturally accumulate PHA into bacterial cells. Moreover, the anaerobic digestion tests were carried out using cheese whey (CW) as a substrate from a cheese producer in central Lazio. The serum was stored at about 4 ° C until use. The parameters, expressed in g / l, of CW characterization are shown in the table below. As final goal of the project was to use rich-VFA anaerobic digestion effluent, during the preliminary tests, the reactor was fed with a synthetic mixture of volatile organic acids (VFAs) representing the carbon source for activated sludge used as inoculum. Considering that the acidogenic fermentation of agro-industrial by-products typically generates acetic, propionic and butyric acid as the most abundant products, the feeding of the preliminary tests contained a mixture that mimicking the real substrate, was prepared by mixing such acids in appropriate proportions. The mineral composition of the feeding is reported in Table 3.2 and Table 3.3

Table 3.2 Characterization of the parameters of cheese whey and activated sludge

Parameters	**CW**	**AS**
Units	**g/l**	**g/l**
pH	5,5 ± 0,3	-
Total solid (TS)	60 ± 5,8	17,2
Volatile solid (VS)	90 ± 4,9	14,05
Total organic carbon (TOC)	29 ± 2,2	6,97
Dissolved organic carbon (DOC)	25 ± 4,2	0,59
Soluble Ammonium (N-NH4)	0,2	0,033
Total ammonium (N-NH4)	0,25	2731
Soluble carbohydrate	18,36	234,3
Total carbohydrate	19,4	379,2
Total Nitrogen (TKN)	1,1 ± 0,2	255
Soluble Nitrogen (TKN)	1,2 ± 0,2	2325

Table 3.3 Mineral solution composition

Element	(mg/l)
$(NH_4)_2SO_4$	525,38
K_2HPO_4	86,58
KH_2PO_4	67,63
$MgSO_4.7H_2O$	301,2
$CaCl_2.2H_2O$	150,6
Na_2EDTA	9,04
$FeCl_3.6H_2O$	6,02
$ZnSO_4.7H_2O$	0,3
$MnCl_2.4H_2O$	0,09
H_3Bo_3	0,9
$CoCl_2.6H_2O$	0,6
$NiCl_2.H_2O$	0,06
Allylthiourea	30,12

3.3.2 Experimental set-up and preliminary test

This study aims to explore the capabilities of the mixed microbial communities (MMCs) to produce PHAs from complex substrates such as organic waste. The purpose of this research is to design a sequential process for the exploitation of biodegradable organic waste for the production of hydrogen, through Dark fermentation from a mix of cheese whey (substrate) and activated sludge from civil wastewater treatment plants (inoculum) and use the volatile fatty acids, a by-product of DF, for the production of Biopolymers. To achieve the main goal of the project an experimental set up and preliminary test were studied, in order to explore and optimize the PHA production and accumulation processes through: definition of operating conditions, extraction methods and optimal polymer characterization. The aim is an overall assessment of the combined production process of bio-H_2 and PHA (mass and energy balances) and in terms of sustainability and technological innovation.

3.3.3 Dark fermentation batch tests for bio-H_2 and VFAs production

The final goal of the project was to combine the production process, evaluating the effluent of the dark fermentation as feeding for the biopolymer production. The first tests for the biopolymer production were carried out with a synthetic mixture of volatile organic acids (VFAs) mimicking the real substrate. But a preliminary anaerobic batch test for bio-hydrogen production were conducted using the input matrices concentration described above (characterization of input matrices). The test was carried out in order to understand how the different operating conditions affect H_2 production and the fermentation products. Especially on the VFA typology and on the amount of the organic acids that are released during fermentation. The organic acids released was a fundamental parameter that has been evaluated in order to direct the feeding concentration for the biopolymer production in the next phase. The fermentation tests were set according to the operating conditions previously optimized by the research group and an experimental sketch with two factors (pH and composition of the mixture CW: AS) and two conditions was used. The two conditions are: pH = 5.5, 6.5; CW: AS = 100: 75, 50:50. The anaerobic digestion tests were conducted using as a substrate of the cheese whey (CW). The serum was stored at about 4 ° C until use. The batch reactor fermentation tests were conducted at 39 ± 1 ° C, using 1 l glass reactors (working volume 0.5 l), equipped with mechanical stirrers and connected to eudiometers for gas measurement using the principle of volume replacement.

3.3.4 Design, construction and put into operation of a lab-scale SBR for the enrichment of PHA producers

Once the VFA concentration in the effluent of the DF are characterized, a first set of experiments were carried out at La Sapienza University of Rome with the participation of ACEA SpA. An experimental set up was designed to evaluate the optimal operating conditions to enrich the PHA-accumulating biomass from the aerobic biological sludge collected at a full-scale WWTP located in Rome. The reactor was operated as a sequencing batch reactor (SBR) and the preliminary tests were carried out to analyses the effect of the FF conditions, which continuously selects for organisms capable of storing PHA during a short feast phase and consuming it during a long famine phase, on the selection of PHA accumulating microorganism and the PHA storage yields capacity. The reactor was fed with a mixture of acetic, propionic and butyric acid representing the carbon

source for activated sludge used as inoculum. Considering that the acidogenic fermentation of agro-industrial by-products typically generates acetic, propionic and butyric acid as the most abundant products, the feeding of the preliminary tests contained a mixture that mimicking the real substrate, was prepared by mixing such acids in appropriate proportions, as described above. The SBR cycle length is 8 h, managed according to the following structure: fill (10 min), settling (25 min), withdrawal (1 min), sludge retention time (SRT) was set at 4 days and the hydraulic retention time (HRT) at 1 day. The temperature is kept at 25±2 °C by the use of a magnetic stirrer with temperature control; pH is set in the range of 7-9, and dissolved oxygen kept in the range of 2-8 mg/L. Five OLR were tested: SBR 1/ SBR 2 (OLR 33 mmolC/ld), SBR 3 (OLR 100 mmolC/ld) SBR 4 (OLR 300 mmolC/ld) and SBR 5 (OLR 150 mmolC/ld). The SBR 1 test was conducted with a manual control system.

The 1 L glass reactor was operated at working volume of 0.5 l; it was kept under continuous mixing conditions by a magnetic stirrer, while oxygen was diffused through a porous stone connected to an external pump. The pH was monitored manually at 1-hour intervals and corrected with hydrochloric acid (HCl) previously diluted. Finally, through a probe connected to an oximeter, the concentration of dissolved oxygen (DO) was monitored constantly; with the same instrument was also possible to measure the temperature, which was set around 25 ±2 °C by the magnetic stirrer with temperature control. The 334 ml of activated sludge taken from the secondary sedimentation plant of a wastewater treatment plant operated by Acea, were used as inoculum for the first trial. For the subsequent tests the biomass selected from the SBR reactor was used. The feeding phase was carried out through an external pump controlled by a timer so that the pump added the fixed amount of feeding (166 ml) in 10 minutes. The nutrient solution was generally prepared in a 2 l conical flask, from which the 166 ml of feeding were taken daily, to which 2.5 ml of acetic acid diluted were added. The pH of the solution was corrected to values between 6 and 7. The sampling (40 ml) was carried out manually during the test at regular intervals to monitor nutrient consumption, or at the end of the reaction phase, always under mixing conditions. Once the magnetic stirrer has been switched off, the sludge has settled, then the supernatant is discharged (126 ml). The system was subsequently automated through the LabView software and it was therefore possible to carry out the tests continuously. The duration of the cycle was maintained for 8 hours. Each cycle was characterized from the 5 phases of the SBR, automatically controlled by the software: 10 minutes for feeding, 7 hours for the reaction phase, 5 minutes for the withdraw of

the mixed product, 20-30 minutes for sedimentation and 5 minutes for the discharge of the supernatant. Three timed pumps were connected and controlled by the LabView software, one for feeding, one for discharging the mixed sludge and one for discharging the supernatant.

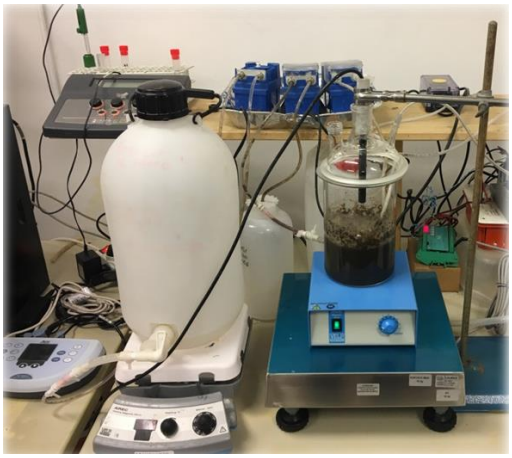


Figure 3.3 Experimental setup of SBR reactor

The SBR2 was operated at the same operating conditions as SBR1, with an operating volume of 0.5 l, hydraulic residence time (HRT) at 1 day, Sludge Retention Time (SRT) at 4 days, Organic Loading Rate (OLR) at 33 mmolC / ld and the C / N / P ratio at 100/8/1. The biomass selected from the SBR1 was used as inoculum. The nutrient solution was kept unchanged from the previous tests and was prepared in a 2 l conical flask, to which acetic acid diluted to 5 and 30% soda were added to correct the pH and bring it back to neutral conditions. The solution was then stored in a glass bottle, closed with a screw cap and connected to the feed pump.

After the second trial, progressively, the organic load of the feeding was increased, to emulate the concentration of the digestate of whey. For this purpose, a mix of pure acids was used, composed of acetic, propionic and butyric acid.

For SBR3 the operating volume was increased at 1 l; as an inoculum for the first trial, 668 ml of activated sludge were taken from the secondary sedimentation plant of the waste water treatment plant managed by Acea, while for the subsequent tests the biomass selected by the SBR reactor was used. HRT and SRT remained fixed at 1 and 4 days respectively, while the OLR was increased to 100 mmolC / ld and the C / N / P ratio to 100/12/1. For the feeding, 332 ml of nutrient solution were loaded to which acetic acid (28%), propionic acid (27%) and butyric acid (45%) were added.

For SBR4 the C / N / P ratio was brought back to 100/8/1, while the OLR was increased to 300 mmolC / ld, with a nutrient solution to which acetic acid (28%), acid was added propionic (27%) and butyric acid (45%). The other parameters remained unchanged. The biomass selected from SBR3 at 100 mmolC / ld was used as inoculum. A schematic resume of the overall process is reported below (Table 3.4)

Table 3.4 schematic resume of the overall process parameters

Parameters	**SBR1**	**SBR2**	**SBR3**	**SBR4**	**SBR5**
Activity period (d)	20	15	15	15	15
Cycle length (h)	8	8	8	8	8
OLR (mmolC/l d)	33	33	100	300	150
SRT (d)	4	4	4	4	4
HRT (d)	1	1	1	1	1
Fed	Acetic	Acetic	Acetic Propionic Butyric	Acetic Propionic Butyric	Acetic Propionic Butyric

3.3.5 Analytical methods

3.3.5.1 Total solid

The total suspended solids (SST) represent the quantity of solids present in suspension, which can be separated by filtration and determined gravimetrically after drying the filter up to constant weight (Analytical methods for water, notebooks 100 (1992-n.2), CNR-IRSA). For the analysis, ceramic cups and MCG filters (55 mm diameter, 1.2 μm porosity) were used, previously dried in an oven at 105 ° C and then weighed (P_f). After the sampling the sample was filtered by vacuum filtration, placed in an oven at 105 ° C for 24 hours and then weighed (P_{105}). The calculation of the SST (g / l) the formula was used is showed below:

$$\frac{P_{105} - P_f}{V}$$

The volatile suspended solids (SSV) are the part of the total suspended solids which is oxidized at 550 ° C and which corresponds approximately to the organic substance; they are then measured to evaluate the biomass concentration within the biological reactor. After the sample was dried in the oven at 105 ° C, was placed in a 550 ° C furnace and then weighed (P_{550}). For the calculation of the SSV (g / l) the following formula was used:

$$\frac{P_{550} - P_{105}}{V}$$

3.3.5.2 Volatile fatty acid

A fixed sampling mode has been established to determine the consumption of volatile fatty acids. During the course of the reaction phase 3 or 4 10 ml sludge samples were taken, at one-hour intervals, in order to properly describe the kinetics consumption of the VFA during the feast phase and the famine phase. The samples, appropriately diluted, were filtered with 0.2 μm filters and acidified with HCl hydrochloric acid diluted to 5, to bring the pH to values between 2 and 3. Subsequently 1 μl of sample was taken and analyzed through a gas chromatograph equipped with a flame ionization detector and a 30 m capillary column (TRB-WAX) with an internal diameter of 0.53 mm. The injector temperature is 250 ° C while the detector temperature is 270 ° C; the initial temperature of the column is 70 ° C and increases up to 220 ° C, with an increase of 10 ° C / min. Helium was used as a carrier gas, with a flow of 1.6 ml / min. In response to the instrument, for each sample analyzed, the chromatogram was obtained, which shows the peaks of the compounds identified by the analysis, and the data relating to the retention times and the surveyed area. From the retention time of each peak it is possible to deduce the identity of the eluted compound; from the area or height of the peaks it is possible to calculate the concentrations of the various compounds present in the analyzed sample. To derive the VFA concentration, is possible to use the value of the area obtained from the analysis within the equation of the calibration line, different for each acid. Analyzing the samples taken at different times, it was possible to determine the consumption of the fatty acids supplied as food for the biomass, for each of which the consumption kinetics were described, obtaining the order of the reaction and the respective constants of speed (k). For this purpose, reference was made to integral method, which requires that the concentrations

measured experimentally at different time points are replaced within the integrated expressions of reaction speed (Metcalf & Eddy, Engineering of waste water, 2006):

- Zero order kinetics $C_t = C_0 - k_0 t$

Where C_t is the value of the concentration of the acid obtained after the fermentation at the time t, C_0 is the value of the concentration of the acid at the beginning of the reaction, t is the time expressed in hours, while k_0 is the speed constant of the zero-order reaction (mol l-1s-1).

- First order Kinetics $\ln C_t = \ln C_0 - k_1 t$
- Where k_1 is the velocity constant of order one (s-1)
- Second order kinetics $\frac{1}{C_t} = \frac{1}{C_0} + k_2 t$
- Where k2 is the velocity constant of second order (mol-1s-1)
- Saturation kinetics $\frac{1}{t} \ln \frac{C_0}{C_t} = -\frac{1}{K} \frac{C_0 - C_t}{t} + \frac{k_s}{K}$

Where K is the semi-saturation constant, which represents the concentration value at which the reaction speed is equal to half of its maximum value, while k_s is the speed constant of the saturation reaction and represents the maximum reaction speed (mol l-1s-1).

The kinetics described have been linearized and represented on the respective planes; the data were interpolated with the ordinary least squares method, to determine in which of them the adaptation to the theoretical trend was characterized by the higher coefficient of determination R^2. From the parameters of the interpolating line the values of the velocity constants have been calculated.

3.3.5.3 Ammoniacal nitrogen

For the measurement of ammoniacal nitrogen the spectrophotometric Nessler method (Figure 3.4), or direct method, has been adopted, described in "Analytical methods for water", notebooks 100 (1992-n.2) of the CNR-IRSA. This method allows to determine the concentration of ammoniacal nitrogen in the range of 0.5-5 mg N-NH4 / l based on the reaction between the free ammonia present in the sample and the Nessler reagent (alkaline solution of potassium iodo-mercurate), which react forming colored complex, according to the report:

$$2\ (\ HgI_2 + KI\ ) + 2\ NH_3 \rightarrow 2\ (NH_3HgI_2) + 2\ KI$$

$$2\ (\ NH_3HgI_2\ ) \rightarrow NH_2Hg_2I_3 + NH_4I$$

The solution obtained assumes a more intense color at increasing concentration of the ammonical nitrogen:

Figure 3.4 Nesler method for the analysis of ammonium

The samples analyzed were taken during the trial at one-hour intervals and at the end of the reaction phase to assess the progress of ammoniacal nitrogen concentration and in order to determine the final amount of ammoniacal nitrogen present in the reactor at the end of the test. For the analysis, 1 ml of the soluble fraction of the sample (obtained by centrifuging the sample as it is at 4000 rpm for 10 minutes) was diluted into a flask, usually 20 ml for the 30 mmolC / ld tests and 100 ml for the higher concentration tests. Then 0.5 ml of Nessler's reagent and 1 drop of Seignette salt were added, to avoid possible interference with the measurements deriving from the possible precipitation of calcium and magnesium ions in the solution. After 20-25 minutes the spectrophotometric analysis was performed, using a spectrophotometer set with a wavelength of 410 nm. In parallel, the same procedure was followed for the blank test preparation, made with 20 ml of deionized water. The equation of the calibration line was used to obtain the concentration of ammoniacal nitrogen.

3.3.6 PHAs Extraction method and characterization

PHAs are stored inside bacterial cells as a carbon source as an intracellular product. Therefore, their extraction involves two steps: cell lysis, the cell membrane and wall breakdown, which allows the polymer recovery. The samples (10 ml) used for the extraction were taken both at the end of the feast phase, to reach the maximum accumulation of the polymers and at the end of the reaction phase. The samples were inserted into Pyrex glass flasks, left in the freezer for 24 hours and then subjected to freeze-drying for another 24 hours (shown in Figure 3.5).

Figure 3.5 Freeze-drying preparation

The freeze-dried sample was weighed and placed in glass vials closed by a screw cap. At each samples were added 1 ml of hydrochloric acid HCl at 37%, to carefully wet all the biomass in order to ensure the lysis of all the bacterial cells. The samples were then subjected to a first step of digestion in a digester at 100 ° C for 1 hour. Then 3 ml of a solution of methanol and 37% hydrochloric acid (previously prepared with 660 ml of methanol and 440 ml of HCl) were added, then the samples were mixed with a mechanical stirrer and then digested at 100 ° C for other 2 hours. After the cooling down were added 3 ml of dichloromethane, which allowed precipitation and extraction of esters. Finally, 1 ml of sample, taken from the bottom part of the stratified liquid, containing the esters, was placed in glass vials bottle with aluminum caps. After the digestion the samples were analyzed by gas chromatography - mass spectrometry, which combines a gas chromatograph with a mass spectrometer (Figure 3.6).

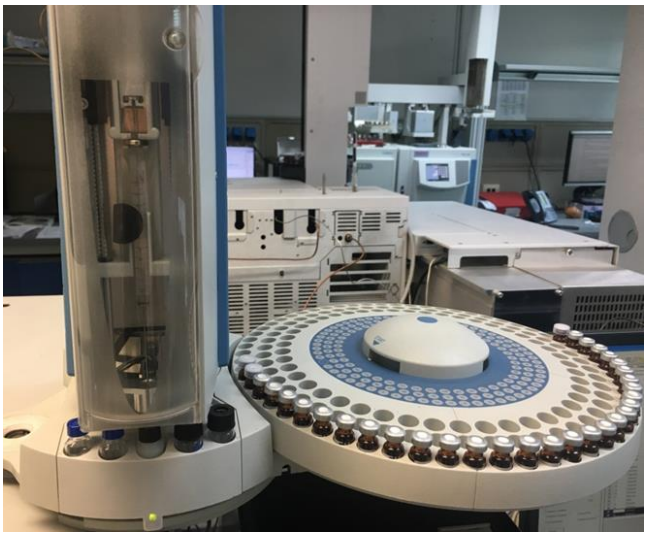


Figure 3.6 Gas chromatograph with a mass spectrometer

The samples were placed in an auto sampler system (Figure 2.6), programmed to take 1 µl of liquid each vials; this was injected into a gas chromatographic system consisting of a capillary column

(length 30 m, diameter 0.25 mm, internal film thickness 1 μm) placed in a thermostatic oven (280-300 ° C) and crossed by a flow of helium (flow 1.2 ml / min). The compounds of interest are separated from the chromatographic column. Once reached the spectrometer are positively ionized in the source for electronic impact (70 eV). The quadrupole subsequently, determines the various m/z ratios by supplying the relative chromatogram as a function of time. For the quantification, the method of the external standard was used, calibrating on the two compounds of interest. For the determination of PHB, reference was made to 3-hydroxybutyrate-methyl-ester. To perform the narrow instrumental and analytical repeatability tests, the analyzes were performed in replicate to assess the repeatability of the measurement obtained by the GC-MS. The replicate was used also for the sampling method, to evaluate the repeatability of the of the sampling, extraction and characterization methods. In the first case a percentage difference less than 10% was obtained, in the second case less than 20%, demonstrating the actual reliability of the instrumental measurements and the goodness of the extraction process.

3.3.7 Experimental data processing

To evaluate the efficiency of the process the kinetic and stoichiometric parameters were calculated. First of all, the active biomass content was calculated, X_A (molC / l), as the difference between the quantity of volatile suspended solids SSV (g / l) and produced PHA (g / l), according to the formula:

$$X_A = (VSS - PHA) \cdot 44{,}2$$

The chemical formula of standard biomass (C5H7O2N) was applied to calculate the biomass concentration on a basis of equivalent carbon moles, with a molecular weight of 44.2 g / Cmol (Oliveira et al., 2016).

The polymer content in biomass, % PHA (g PHA / g SSV), is given by the ratio between the quantity of PHA stored and the concentration of volatile solids (Valentino et al., 2014)

$$\%PHA = \frac{\mathrm{PHA}}{SSV} \cdot 100$$

The specific production rate of PHA, qPHA (molC PHA / molC X_A h), is calculated according to the formula:

$$q_{PHA} = \frac{PHA}{t \cdot X_A}$$

Where it is the length of the feast phase expressed in hours (Colombo et al., 2016).

The specific substrate consumption rate, $-q_s$ (molC ΔS / molC X_A h), is calculated according to the formula (Chen et al., 2017):

$$-q_s = \frac{\Delta S}{X_A \cdot t}$$

The storage yield, $Y_{PHA/S}$ or Y_{sto} (molC PHA / molC ΔS), is given by the ratio between the quantity of PHA stored ΔPHA and the amount of substrate removed ΔS (Chen et al., 2017):

$$Y_{PHA/S} = \frac{PHA}{\Delta S}$$

Finally, the observed yield $Y_{X/S}$ or Y_{obs} (molC X_A / molC ΔS), is given by the ratio between the amount of active biomass XA and the quantity of substrate removed ΔS (Chen et al., 2017):

$$Y_{X/S} = \frac{X_A}{\Delta S}$$

or from the ratio between the quantity of volatile suspended solids (SSV) and the amount of substrate removed ΔS (Valentino et al., 2014):

$$Y_{X/S} = \frac{VSS}{\Delta S}$$

3.4 Results and discussion

As described in the operating procedures, the biomass selection was carried out through a sequential batch reactor (SBR). For the finalization of the system, preliminary non-automated tests were carried out to assess the actual operation and optimization of the plant. The system was subsequently automated through the LabView software and it was therefore possible to carry out the tests continuously. Three cycles were performed which, for the same HRT, SRT and cycle length itself, differed for the organic load and the type of substrate supplied to the biomass.

In SBR1 and SBR2 the organic loading rate (OLR) was set at 33 mmolC / ld and was supplied through acetic acid. In the SBR3 the OLR was set at 100 mmolC / ld and was supplied through a

mix of pure synthetic acids consisting of acetic, propionic and butyric acid. Finally, it was decided to progressively increase the OLR of the feeding, at similar concentrations to those of the whey digestate; therefore, 300 mmolC / ld was supplied in the SBR4 through the synthetic acid mix already described.

The performance of the SBR was evaluated through the monitoring of dissolved oxygen (DO) during the tests, the measurement of total suspended solids (TSS) and volatile (VSS), the samples were taken at the end of the reaction phase, the trend of the ammoniacal nitrogen consumptions during the test, the concentration of the substrate in the form of volatile fatty acids (VFA) and the concentration of the polymers stored at a characteristic moment of the test, that is between the feast phase and the famine phase. The results obtained from the tests carried out are reported below.

3.4.1 Manual system SBR1 and SBR2 automatic LabVIEW system

As an inoculum for the first test, activated sludge was taken from the secondary sedimentation plant of the Roma Est, wastewater treatment plant managed by Acea. For the subsequent tests the biomass selected from the previous SBR reactor was used. The organic load was set at 33 mmolC / ld and was supplied by acetic acid. Several preliminary cycle studies were conducted to analyses the process, by measuring the concentration of dissolved oxygen (DO) inside the reactor and the consumption of the carbon source. To obtain the kinetics of consumption of acetic acid was selected the integral method, it was possible to derive the order of the reaction and the relative speed (k). Below (Table 3.5) the calculated values of slope (m) and intercept (q) of the straight lines, the coefficient of determination (R2) and the speed constant (k) are reported. In Figure 3.7 is shown the activity of the biomass during the 8 hours of the cycle. The biomass after the first cycle enhanced the acetic removal capacity, up to rich the complete removal in the two hours of the cycle study. The concentration of the DO varies according to the phases of the SBR cycle (Figure 3.8): the feeding phase is followed by a strong consumption of the oxygen due to the biomass activity. Those consumption causes in the first part of the cycle a decreasing of the DO concentration in the reactor up to 0 and 2 (mg/l), proof of the actual metabolic activity of the bacteria. Subsequently, when the carbon source is removed, a rapid increase of the DO is recorded (between 6 and 8 mg OD / l). The study of the DO during the cycle studies is necessary to predict the cycle of the feast and famine condition apply to the SBR and select the time point for the sampling.

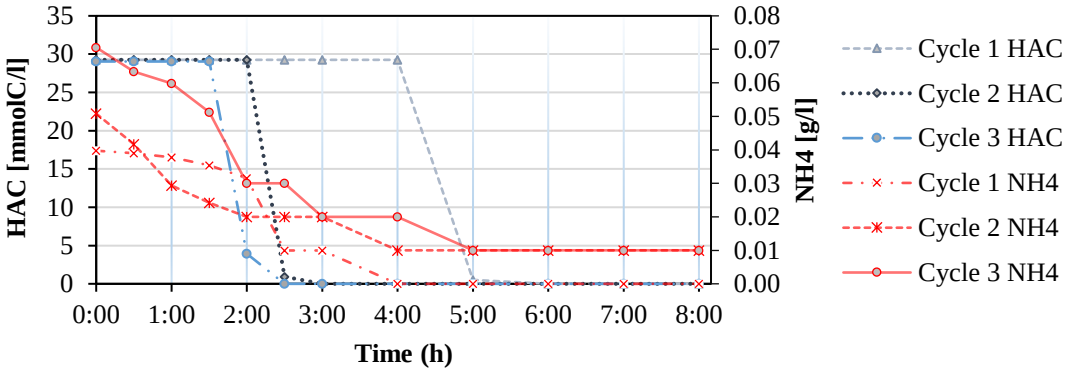


Figure 3.7 Non-automatized SBR Cycle study (OLR 33 mmolC/l)

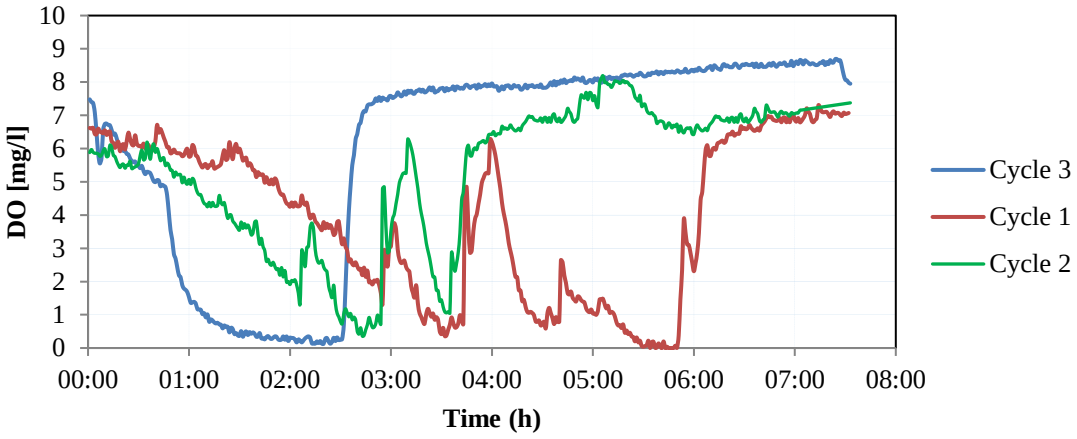


Figure 3.8 Dissolved oxygen profile during the Non-automatized SBR Cycle study

Table 3.5
Calculated parameters for determining the reaction order (integral method) of the kinetics of consumption of acetic acid

Order	m	q	R2	k
0	-10,280	25,940	0,761	10,280
1	-1,138	3,290	0,978	1,138
2	0,013	0,121	0,010	0,013
Saturation	0,067	-0,165	0,759	0,067

After the preliminary test the setup were automatized by a LabVIEW system and the second phase of the selection of PHA accumulation bacteria were carried out at the same OLR. For the SBR 2 the biomass collected from the SBR1 manual system. The preliminary study on the SBR were focused on the application of the feast and famine condition for the selection of the biomass. The cycle studies in Figure 3.9 showed that the biomass was maintained at 3 hours of feast phase, where all the nutriments were depleted and 5 hour of famine phase where the PHA accumulating bacteria are selected. The sequence of feast and famine phases is managed in order to apply pressure on the biomass, allowing the selection of only one capable of accumulating PHAs. In this cycle was not recorded a good response in terms of PHA accumulation capacity of the mixed culture (Figure 3.10), probably due the low carbon source fed at the T start of each cycle and at the preliminary condition applied. In Figure 3.11 is shown the effect of the feeding on the PHA composition, the SBR fed with only acetic acid as carbon source have reached a higher PHB content (> 85 %) than PHV (< 15 %). The preliminary study where necessary to fix the condition and for the biomass acclimation.

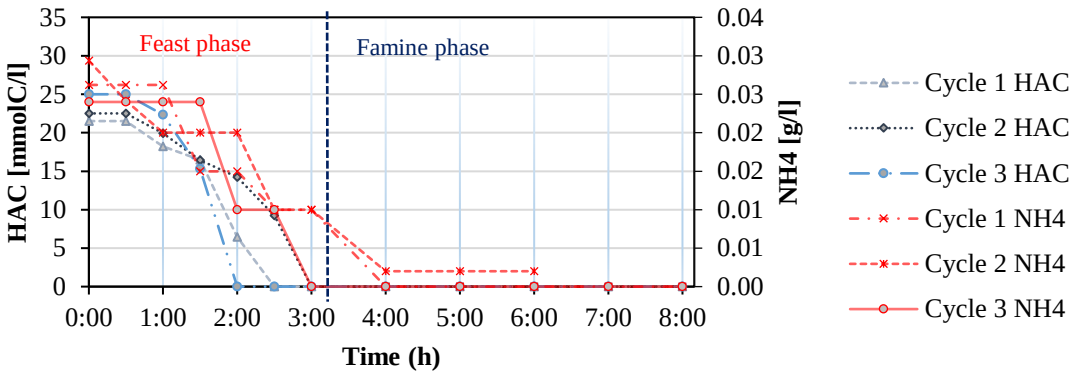


Figure 3.9 SBR Cycle study (OLR 33 mmolC/l): study of the fest and famine condition

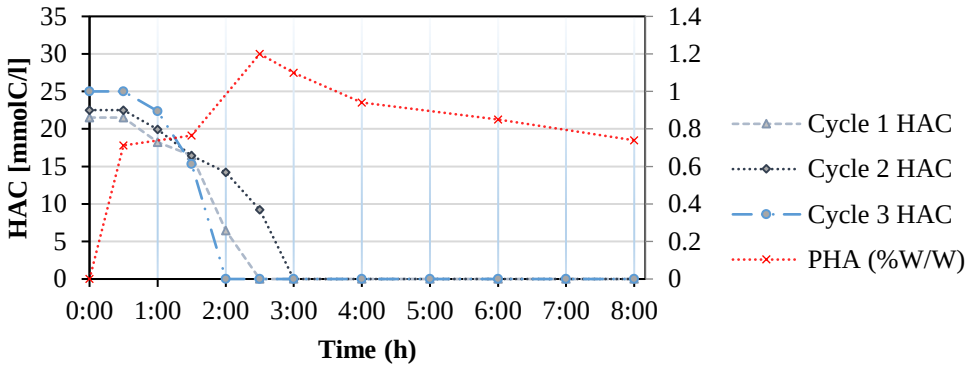


Figure 3.10 SBR Cycle study (OLR 33 mmolC/l): reletion of the VFA consumption and the PHA accumulation

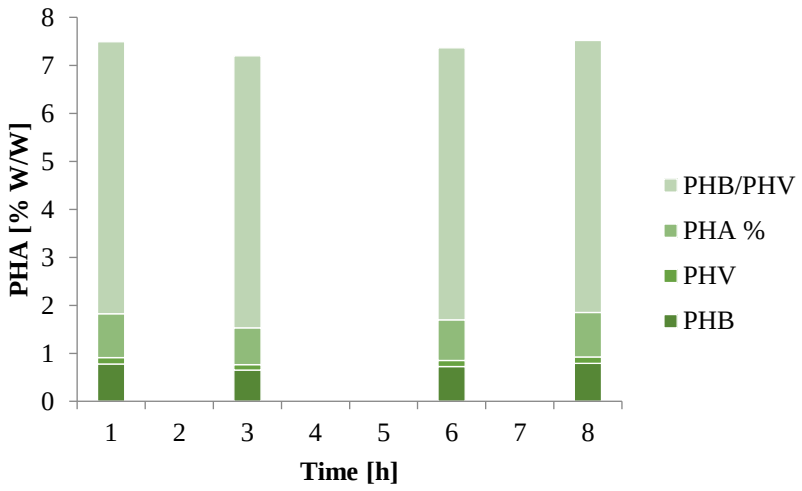


Figure 3.11 The influence of the feeding on the PHA composition during the cycle

3.4.2 SBR 3 – OLR 100 mmolC/l d

A laboratory-scale SBR, as described before, was carried out at 100 mmolC/l d (OLR). The 4 hours of feast phase corresponding to the depletion of the substrate after the feeding during the cycle test. The feast phase was indicated by a strong characteristic DO decrease and a fast DO increase after the complete depletion of the substrate (Figure 3.12). The feeding was characterized by a synthetic mix of VFA (100 mmolc/l D) composed of acetic, propionic and butyric acid at different concentration respectively (45, 30, 25 mmolC/l). The concentration of the single organic acid was calculated to emulate the concentration of the effluent of the Dark fermentation for the Bio hydrogen production. For all three acids the value greater than the coefficient of determination (R2) is obtained by applying a kinetic of order 0; the velocity constant is therefore equal to 9.1 mol 1-1s-1 for acetic acid, 7.35 mol l-1s-1 for propionic acid and 12.028 mol l-1s-1 for butyric acid (Table 3.6).

In Figure 3.12 is shown that the PHA production was observed during the uptake phase and was recorded a slow consumption of the PHA during the feast phase. After the depletion of the substrate the biomass degraded the accumulated polymer for the metabolic activities. The ability of the selected biomass to store PHA was reflected by a maximum accumulation percentage from 1 % up to 5 % (w/w) in 8 hours. A balanced relationship between cell growth and accumulation probably

led to the selection of accumulating biomass, triggered a progressive increase of accumulation capacity (Figure 3.13).

Table 3.6 Calculated parameters for determining the reaction order (integral method) of the kinetics of consumption of the VFA

Acetic acid				
Order	**m**	**Q**	**R^2**	**k**
0	-9,09963	28,72536919	0,9839	9,100
1	-1,04549	3,77934622	0,7981	1,045
2	-0,00771	0,053138458	0,0888	0,008
Saturation	0,02104	0,051649501	0,1714	0,021
Propionic acid				
Order	**m**	**Q**	**R^2**	**k**
0	-7,35009	27,38773558	0,9633	7,350
1	-0,85624	3,698091421	0,9413	0,856
2	0,01984	0,05874053	0,0615	0,020
Saturation	0,06211	-0,0661112	0,4572	0,062
Butyric acid				
Order	**m**	**Q**	**R^2**	**k**
0	-12,02814	47,01280338	0,9844	12,028
1	-0,90734	4,365561437	0,8477	0,907
2	0,00396	0,032826015	0,0224	0,004
Saturation	0,02771	-0,019676798	0,3734	0,028

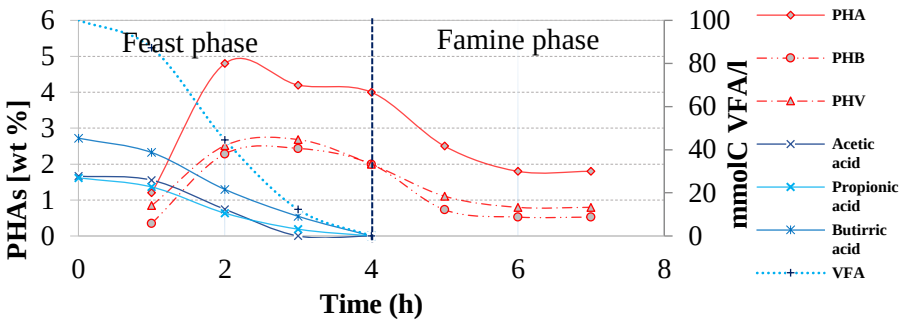


Figure 3.12 SBR 3 Cycle study (OLR 100 mmolC/l): study of the fest and famine condition and PHA accumulation

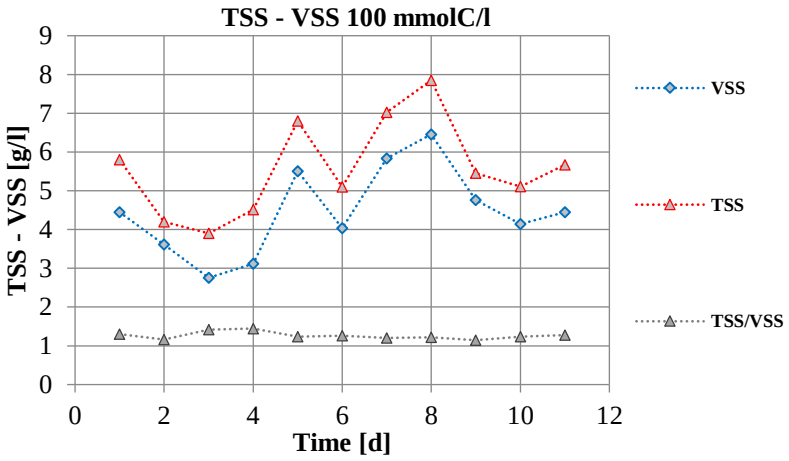


Figure 3.13 SST and SSV concentration trend for SBR3

The influence of the feeding on the PHA composition

The influence of the type of feeding on the PHA composition and distribution is shown in Figure 3.14; Figure 3.15. While all the SBR fed with only acetic acid as carbon source have reached a higher PHB content (> 85 %) than PHV (< 15 %). SBR 3 fed with the mix of VFA, the PHA composition has reached a higher PHV content almost up to the 50 % and the other 50 % was composed of PHB. This could due to the influence of the composition of the feeding, because adding butyric and propionic acid the biomass stored the polymer with a different composition and the PHB and the PHB content are almost the same. This is an interesting commercial point of view that needs further study to better manipulate the polymer composition.

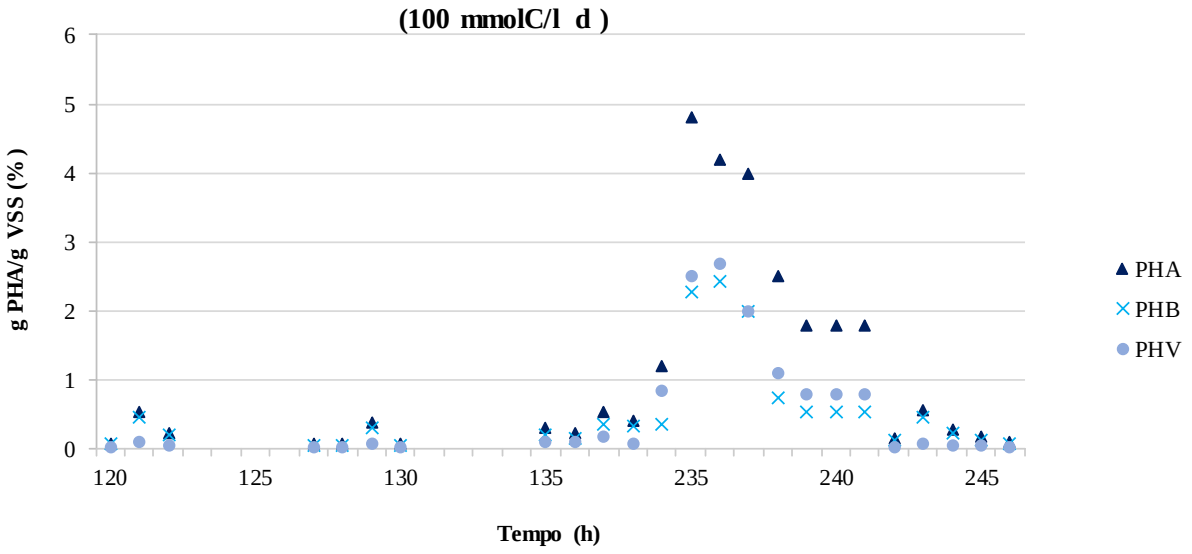


Figure 3.14 Distribution of the PHA accumulation during the 100 mmolC/l d SBR 4

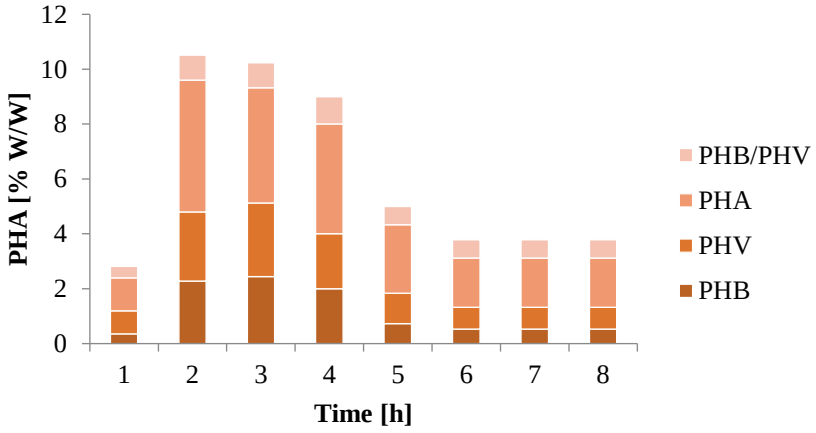


Figure 3.15 The influence of the feeding on the PHA composition during the cycle study

3.4.3 SBR 4 – OLR 300 mmolC/l d

A laboratory-scale SBR was carried out at 300 mmolC/l d (OLR). The progressively increasing of OLR was applied to evaluate the possible condition of application of the dark fermentation's effluent. No feast and famine condition were fixed in this SBR cycle due probably the high concentration of the feeding. A long feast phase corresponding to the depletion of the substrate after the feeding during the cycle test. The feast phase was indicated by a strong characteristic DO

decrease, but was not recorder the characteristic fast DO increase after the complete depletion of the substrate (Figure 3.16). At the end of the cycle the VFA were not depleted and this did not allow the application of selection pressure for the PHA accumulating bacteria.

The feeding was characterized by a synthetic mix of VFA (300 mmolc/l d) composed of acetic, propionic and butyric acid at different concentration respectively (85, 80, 135 mmolC/l). The concentration of the single organic acid was calculated to emulate the concentration of the effluent of the Dark fermentation for the Bio hydrogen production. In this case, for the acetic acid and the propionic acid, the greater value of the coefficient of determination (R2) is obtained by applying a saturation kinetics; the speed constant is therefore equal to 0.017 mol l-1s-1. For butyric acid, the major R2 occurs with a kinetic of order 1 and the speed constant is equal to 0.108 s-1 (Table 3.7).

In figure 2.16 is shown that the PHA production was observed during the uptake phase but with a low production efficiency and without any selection pressure applied trough the feast and famine condition. Confirming that probably the OLR was too concentrated for the optimal condition for the PHA accumulation and the biomass selection, is shown in Figure 3.17 that was not possible to control the biomass growth in the SBR at this concertation. The TSS reached a concentration higher than 10 g/l, instead the other conditions where the solid are stabilized after few cycles between 2-4 g/l.

Table 3.7 Calculated parameters for determining the reaction order (integral method) of the kinetics of consumption of the VFA

Acetic acid				
Ordine	**m**	**q**	**R2**	**k**
0	-5,88088	78,70077986	0,8845	5,881
1	-0,11738	4,43496588	0,9343	0,117
2	0,00246	0,010081391	0,9573	0,002
Saturation	0,01666	0,004866164	0,9807	0,017
Propionic acid				
Ordine	**m**	**q**	**R2**	**k**
0	-5,87870	78,1168449	0,5902	5,879
1	-0,10327	4,359443566	0,5618	0,103
2	0,00189	0,012780313	0,5101	0,002
Saturation	0,01741	-0,008466883	0,9689	0,017
Butyric acid				
Ordine	**m**	**q**	**R2**	**K**
0	-19,64436	279,3088662	0,9624	19,644
1	-0,10770	5,696254483	0,9771	0,108
2	0,00061	0,002923481	0,9745	0,001
Saturation	0,00492	-0,000890458	0,9751	0,005

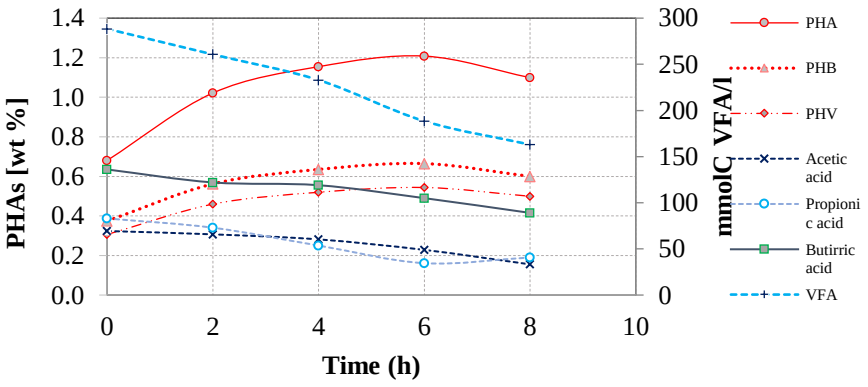


Figure 3.16 SBR 4 Cycle study (OLR 300 mmolC/l): study of the fest and famine condition and PHA accumulation

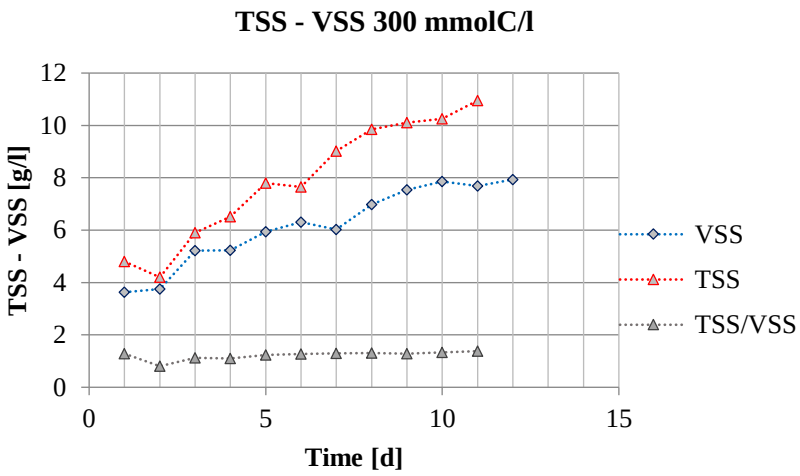
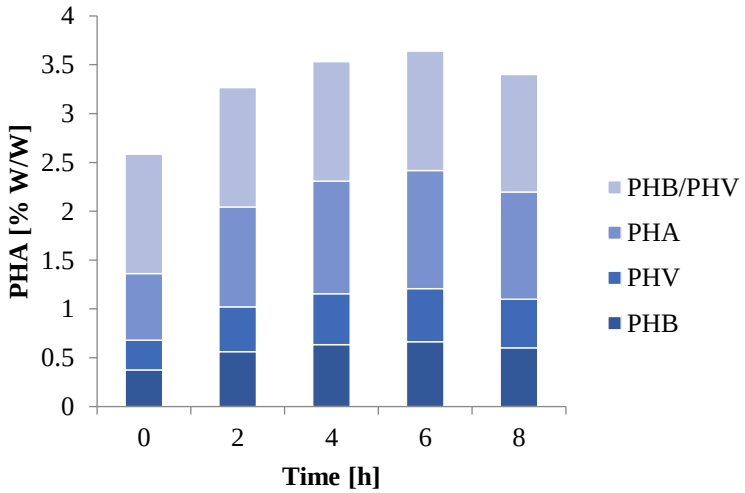


Figure 3.17 SST and SSV concentration trend for SBR4

The influence of the feeding on the PHA composition

The influence of the type of feeding on the PHA composition is shown in figure 2.8. While all the SBR fed with only acetic acid as carbon source have reached a higher PHB content (> 85 %) than PHV (< 15 %). SBR 3 fed with the mix of VFA, the PHA composition has reached a higher PHV content almost up to the 50 % and the other 50 % was composed of PHB, confirming what was found with the SBR fed with 100 mmolC/l.

Table 3.8 The influence of the feeding on the PHA composition during the cycle study



3.4.4 SBR 5 - 150 mmol C/l

A last SBR cycles were carried out at 150 mmolC/l. 3 cycle study were analyzed to evaluate the performance of the SBR (Figure 3.18). The max PHA content is reported below (Figure 3.19) not relevant data in terms of biomass selection are underlined from this last SBR.

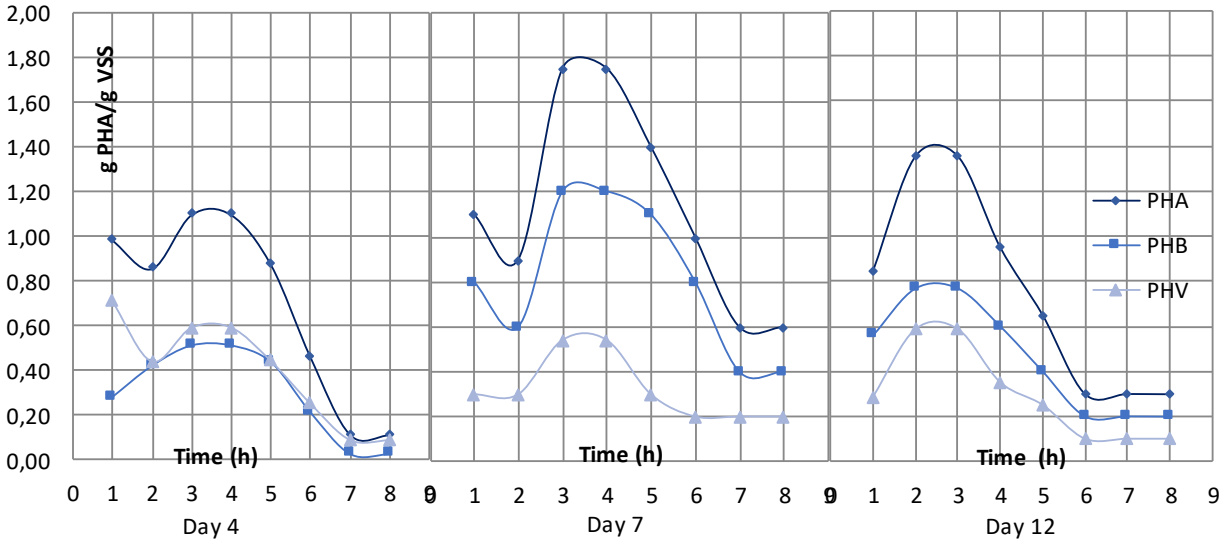


Figure 3.18 Compared cycle study SBR 5 (day: 4;7;12) 150 mmolc/l d

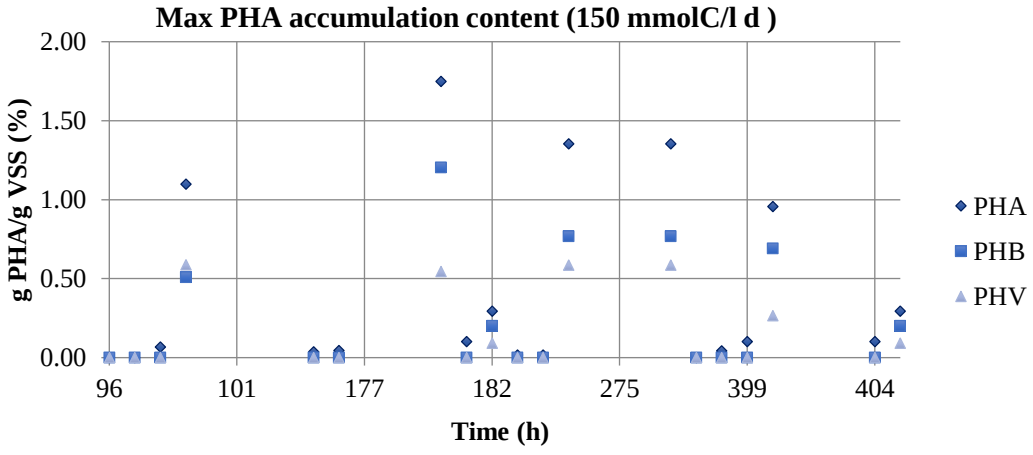


Figure 3.19 max PHA accumulation distribution SBR 5

3.4.5 Comparison of the different operating conditions

A comparison between SBR1 and SBR2 is useful for the purpose of assessing the current improvement in system performance, since these have been performed with the same operating conditions without (SBR1) and with (SBR2) automatic control.

In terms of biomass concentration, calculated as volatile suspended solids (SSV), the concentration of SSV for SBR1 had more variations than that the SBR2 (30 mmolC/l), which is quite stable already from the first days of testing. This is both because through the automated system, it was possible to control all the parameters continuously, which led to a better stabilization of the system. However, in both cases, the concentration of SSV is maintained stable. Is a sign that the cell growth is not prevalent. In the SBR 3 (100 mmolC/l) a balanced relationship between cell growth and accumulation probably led to the selection of accumulating biomass, after few cycles the concentration it was stabilized at 2 g/l (VSS). The SBR 4 (300 mmolC/l), due the high concentration of the feeding, has led an unbalanced relationship between cell growth and accumulation probably led to the selection of non-accumulating biomass, triggered a progressive loss of accumulation capacity. The compared concentration trend is reported in Figure 3.20.

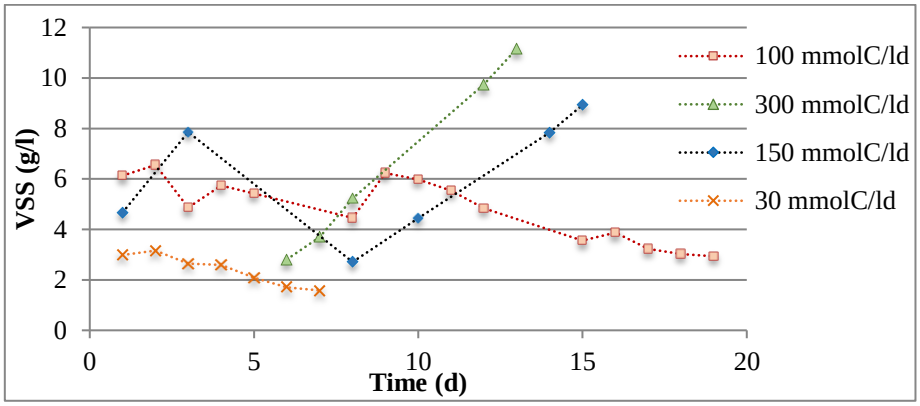


Figure 3.20 SBR SSV concentration trend compared

The appropriateness of the strategy for the biomass selection can be also assessed through the f / f ratio, given by the length of the feast phase over the entire cycle. We know from several studies that a f / f ratio less than 0.28 provides a competitive advantage to PHA accumulating microorganisms compared to non-accumulating ones, while a ratio greater than 0.55 favors bacterial growth at the expense of polymer storage (Freches, 2017). From Figure 3.21 it can be seen

that SBR2 and SBR 3 was possible to reach lower and more stable values of the f / f ratio below the limit threshold for the correct selection of biomass. The f/f ration compared with SBR 4 showed a stable result but above the limit underlining that it was a non-efficient trial.

The influence of the type of feeding on the PHA composition into the different SBR cycles is shown in Figure 3.22. While all the SBR fed with only acetic acid as carbon source (SBR 1 and SBR 2) have reached a higher PHB content (> 85 %) than PHV (< 15 %). SBR 3 and SBR 4 fed with the mix of VFA, the PHA composition has reached a higher PHV content almost up to the 50 % and the other 50 % was composed of PHB. This could due to the influence of the composition of the feeding, because adding butyric and propionic acid the biomass stored the polymer with a different composition and the PHB and the PHB content are almost the same. This is an interesting commercial point of view that needs further study to better manipulate the polymer composition.

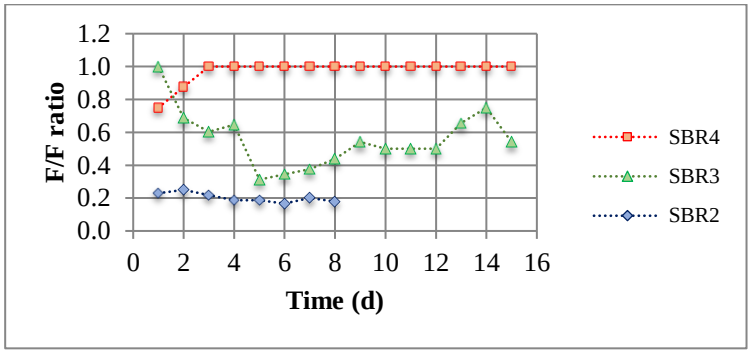


Figure 3.21 SBR F/F ratio compared

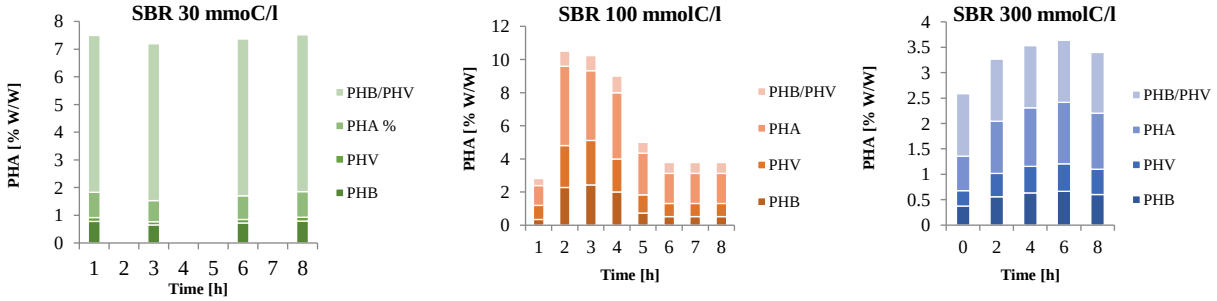


Figure 3.22 The influence of the feeding on the PHA composition compared

In Figure 3.23 is shown the overall SBR performance and the operative condition compared from 30 mmolC/l d to 300 mmolC/l d. As can be seen from the results, the operative conditions at OLR (100 mmolC VFA/Ld) and 8-hour cycle length have demonstrated a more efficient removal capacity, with a complete use of the VFA and a simultaneously PHA production increase from 1 to 5 (% g PHA/g Volatile Suspended Solid-VSS). The cycle study showed before and the overall SBR performance study, demonstrated the actual selection pressure conditions and biomass behavior by storing PHA during a short feast phase, reaching the max PHA content, and then consuming it during a long famine phase.

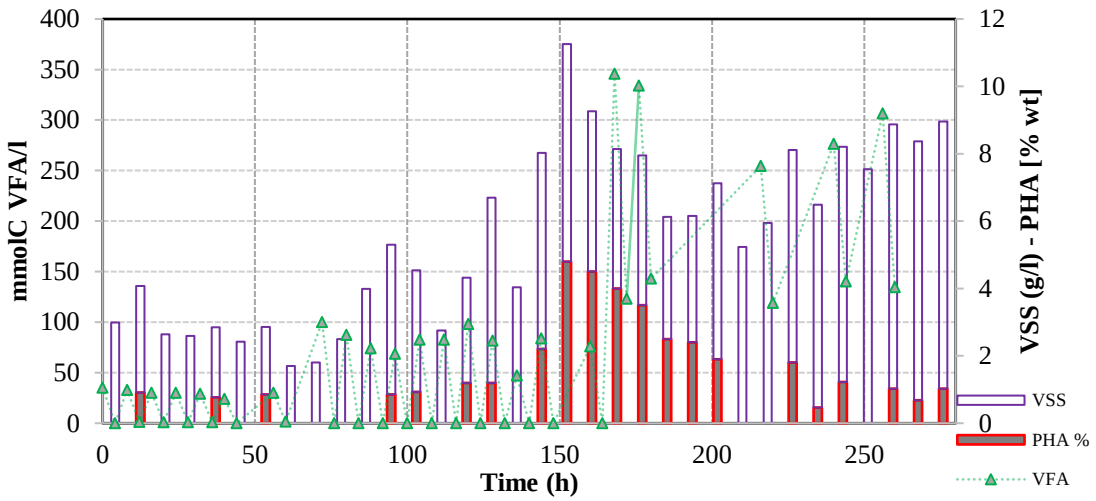


Figure 3.23 SBR overall process performance

3.5 Conclusion

The purpose of the present work was to study and implement a polyhydroxyalkanoate production system, in particular by optimizing the PHA accumulating biomass selection phase.

The manufacturing processes implemented so far at the industrial level use pure cultures of microorganisms and synthetic substrates to maximize the production of polymers, thus being very expensive and not very competitive on the market. The proposed process is based on the use of mixed microbial cultures coming from activated sludge, in order to obtain a reduction in process costs and an enhancement of materials otherwise destined for disposal. With regard to the substrate used in this research, it was decided to initially use synthetic acids, so as to allow the acclimatization and the selection of biomass with high process yields. Subsequently, the dark

fermentation process of whey for the production of biohydrogen is expected to be used as substrate of the digestate, in view of the integration of the two production processes.

As regards the efficiency of the process in terms of PHA productivity, the results that had reached the highest percentage of PHA content were with the SBR 3. However, the trends for SBR3 and SBR4 are extremely variable, while the productivity of SBR2 is around more or less constant values, a sign of greater system stability. The comparison of the three systems was also carried out in terms of the content of polymers stored within the biomass: the highest percentages were achieved in the first tests carried out with SBR3.

Considering the elements compared, it can be concluded that the operating conditions adopted with SBR3, with an organic load set at 100 mmolC / l d, were those that led to the best performance of the biomass selection and PHA production process, both in terms of productivity and polymer storage yields.

As a continuation of the work carried out, it is possible to gradually increase the organic load by successive steps, so as to allow the acclimatization and the correct selection of the biomass, and reach the concentrations of volatile fatty acids contained within the whey digestate. Once the optimal organic load concentrations have been reached for the SBR phase, it will be possible to proceed with the study of the stabilization of the process over several production cycles.

Once the PHA accumulating biomass selection phase (SBR phase) has been optimized, the Batch phase can be implemented, during which the polymer storage performance will be evaluated. The efficiency of the Batch system will mainly depend on the actual selection of the biomass carried out in the previous phase.

Finally, as a substrate for the production of PHA, the use of digestate from the dark fermentation process for the production of biohydrogen can be envisaged, integrating the two production processes.

4 Research activities for reaching RO2: Photofermentative biopolymer production by mixed culture of Purple phototrophic bacteria

4.1 Introduction

4.1.1 Biotechnological application of PPB

The Table 4.1 that will be illustrated below describes the multiple applications of these bacteria, testifying how high the adaptability of this species is.

Table 4.1 Biotechnological application of Purple Phototrophic Bacteria

Product	Application	Reference
Signle cell protein	Protein source	Sasaki et al., (1998)
S-adenosyl cystine	therapeutic marketing	Yamada et al., (1986)
Metal ion uptake	Metal recovery	Sanaa et al., (2006)
Vit B 12	Vitamin	Goldrick (2003) Sasikala et al., (1992)
Hydrogen	Fuel	Fedorov et al., (1998) Katsuda et al., (2000) Mahakhan et al., (2005) Zabut et al., (2005)
NH3	Fertilizer	Saaikala and Ramana (1990)
Carotenoid	Natural dye	Nopartnarporn et al., (1986)
5-Amino levulinic acid	Herbicide	Sasaki et al., (1997)
Biodegration of organic and inorgani compounds	Wastewater treatment	Blasco and Castillo (1993) Merugu et al., (2008) Vasavi et al., (2008)
Hopanoids	therapeutic	Nagumo et al., (1991) Brandl et al., (1991)
Poly-hydroxy Alkanoates	Biodegradable plastic	Serdyuk et al., (1993) Hashimoto et al., (1993) Kranz et al., (1997) Mahuya et al., (2005) Lorrungruang, (2006)
Enzymes	Food preparation Biotecnhnology	Scavetta et al., (2000) Srinivas et al., (2007)
Cytokinins	Plant growth Hormones	Serdyuk et al., (1993) Scavetta et al., (2000)

This family of proteobacteria (Rhodospirillaceae) can produce PHA through the photo-fermentation process. Most species are also capable of growing photoautotrophically with molecular hydrogen as donor. Not all PPB are PHA accumulators, but some notable PHA-accumulating genera reported to be dominant in agri-industrial sources include:

• α-Proteobacteria – *Rhodosporillum - rubrum sp.* was one of the first organisms used to study PHA metabolism, – Rhodopseudomonas, – Rhodobacter,

• β-Proteobacteria – Rhodocyclus

• *γ-Proteobacteria*: PPB capable of chemolithotrophic growth. – Family: *Chromatiaceae* - deposit sulfur inside the cell, – Family: *Ectothiorhodospiraceae* - deposit sulfur outside the cell. ∗ *Thiorhodospira* ∗ Thiolamprovum

• *δ-Proteobacteria*: No Phototrophic growth observed.

4.1.2 Integration of PHA and Biohydrogen production by PPB

Photo-fermentative production of different cultures of PNSB reported in literature are summed up below in Table 4.2. As can be seen from the literature data, the use of mixed cultures is very reduced compared to the numerous experiments carried out on selected specific cultivar. The PHA production yield of the mixed microbial cultures is still very low compared to the select strains, but the adaptive capacity of the latter can influence feeding strategies. A valid alternative is to study the most promising waste to be applied as a feeding of the production process, to reduce the costs and make the product expendable on the market. Quantitative studies on PHA metabolism by phototrophic purple non-sulfur bacteria are restricted only to a few species like Rhodospirillum rubrum, Rhodobacter sphaeroides, Rhodopseudomonas palustris and R. palustris, the widely known species of purple non-sulfur bacteria, accumulate P(3HB) but only to a limited scale (Kemavongse et al., 2007). It seems necessary to find alternative solutions that can reduce production costs and experiment on a large scale. Studying and supporting production by integrating the concept of biorefinery to the production chain. Such a waste management strategy is inspired by the concepts of green economy and is ultimately aimed at transforming waste treatment plants from "simple" plants into true biorefineries. In which the organic matter that is present in waste is converted into useful products with high added value

Table 4.2 PHA and Biohydrogen production under varying operating conditions

Microbial Inoculum sources	Main Carbon and nitrogen source	C/N ratio	Light intensity	PHB (% Dry cell weight)	Volumetric H_2 Yield (mLH_2/L)	References
Rhodobacter sphaeroides 17023 (wild type)	30 mM acetate and 7 mM glutamic acid	8.6	1500 lux of incandescent light	**70**	0	(Hustede et al., 1993)
Rhodobacter sphaeroides 17023 (wild type)	30 mM acetate and 7 mM glutamic acid	12.86	1500 lux of incandescent light	**24**	2310	(Hustede et al., 1993)
Enriched photoheterotrophic culture	DFE (11.61 g/L butyric, L 1.76 g/L propionic and 1.01 g/L acetic acid and 0.78 g/L total ammonia	10.63	3000 lux of LEDS and halogen lamps	**5**	1478 ± 17	**(Montiel-Corona et al., 2015)**
Rhodobacter capsulatus				**29**	1252 ± 20	
Rhodobacter sphaeroides O.U. 001 (DSM 5648)	Sugar refinery wastewater (30% v/v in medium)	-	200 W/m^2	**70.4**	648	(Yiğit et al., 1999)
Rhodobacter sphaeroides strain RV	40 mM Acetate only	-	5000 lux incandescent light	**38**	0	(Khatipov et al., 1998)

4.2 Experimental approach

Continuously stirred 10L jars were irradiated with IR to enrich a mixed culture high in PPB from wastewater. Once the 10L vials had turned purple, they were used as inoculum for a continuous 2L bench scale reactor that was used to test the ability to enrich PHA accumulating with IR in a defined synthetic media. Once a reasonable PHA accumulation was observed in the continuous reactor, its contents were used as inoculum for accumulation studies in batch tests to assess any effects of VFA input on PHA composition and accumulation. A pilot scale reactor was used to enrich wastewater for PHA-accumulating PPB at the same condition studied in the 2L reactor, grown in IR induced biofilms attached at the walls of the reactor. In Figure 4.1 is schematize the research approach. This study was carried in collaboration with the School of Chemical Engineering and Advanced Wastewater Management Centre (AWMC) of the University of Queensland (Australia).

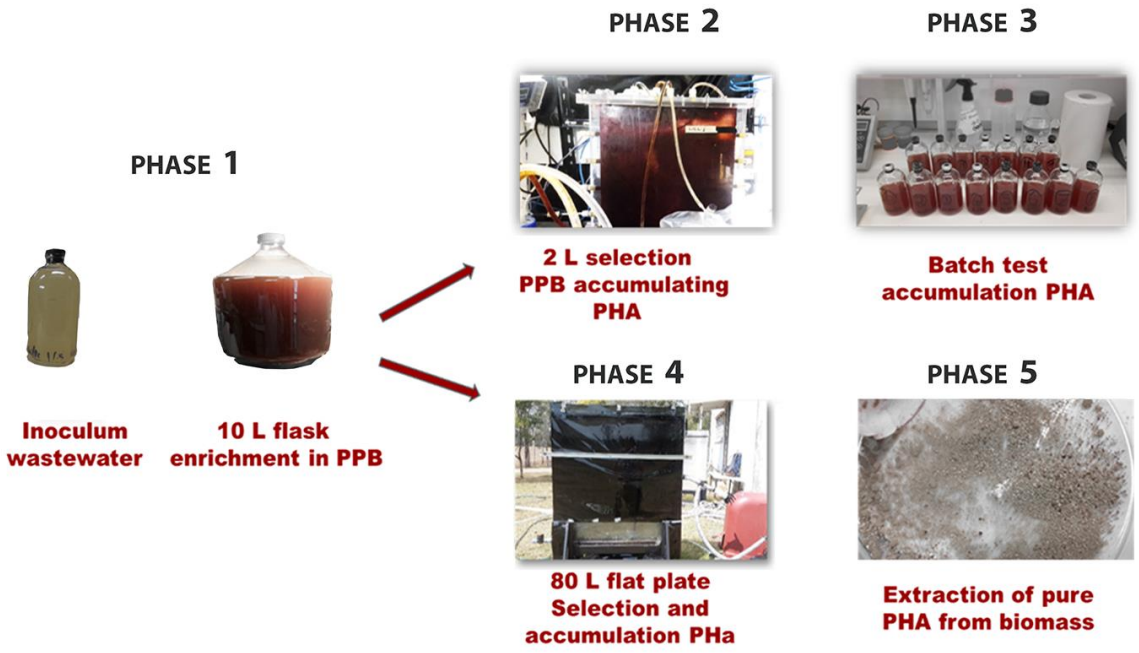


Figure 4.1 Schematic summary of the research approach

4.3 Materials and methods

4.3.1 Synthetic Media Preparation: Ormerod's Salts

A defined media (Table 4.3) was used in all synthetic media trials to simulate the domestic wastewater, as the media described by Ormerod *et al.*. The mixture was buffered to pH 7.12 with 667 mg.L-1 KH2PO4 and 900 mg.L-1 K2HPO4. Stock solutions of each individual salt were added to 10L of water that was continuously sparged with N2 to maintain anaerobic conditions.

Table 4.3 Synthetic media preparation

Mineral solution (1 l)		Trace element solution 1L	
Element	**mg**	**Element**	**mg/100 ml**
KH_2PO_4,	666	H_3BO_3	280
$MgSO_4$* $7H_2O$	200	$MnSO_4$*H_2O	173
$CaCl_2$*$2H_2O$	75	Na_2MoO_4* $2H_2O$,	75
$FeSO_4$*$7H_2O$	11.8	$ZnSO_4$*$7H_2O$,	24
EDTA,	20	$CuSO_4$*$5H_2O$.	4
NH_4Cl	764		
Yeast extract	30		

4.3.2 Wastewater characterization

PPB for inoculum were enriched and maintained using domestic wastewater as the sole source. The enrichment process is explained in section 3.3. 800L of wastewater was collected from pump station 118 in St. Lucia, Brisbane and stored at 4°C where it settled for 24 hours before use. The settling stage removed large solids that would foul the system, and the wastewater characteristics mimicked those of the output of a clarifier as outlined in Table 4.4.

Table 4.4 Domestic wastewater characteristics

Property	Value
TCOD [mg/l]	430
SCOD [mg/l]	245
NH4 [mg/l]	47
pH	7.4

4.3.3 PPB Enrichment phase from wastewater

The initial phase of the project is the selection of biomass directly from waste (Figure 4.2). Through the use of the only waste water the bacterial component belonging to the microbiological group of the PPB is selected and enriched. Waste water was collected at a local treatment plant and immediately stored in a refrigerated environment at 4 ° C. A 10 L reactor is inoculated, continuously stirred by a magnetic stirrer and maintains the anaerobic conditions by means of nitrogen flushing to a duration of 10 min. The incubation takes place under constant illumination of the reactor with 150 w fluorescent lamps and the reactor cover with an absorbing UV-VIS. The material absorbs 90% of the light waves below 790 nm. This is the best way to achieve positive results in the reactor by forming a good bacterial colony at 10 days of incubation. This operation is repeated weekly for the maintenance of an always optimized inoculum to be taken for the following phases

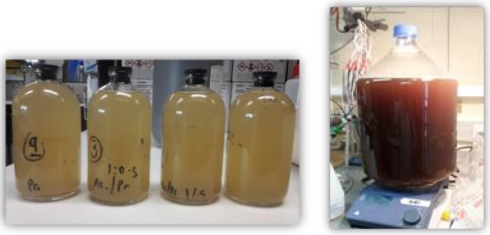


Figure 4.2 PPB Enrichment phase

4.3.4 Enrichment phase in a 2 L Anaerobic membrane photobioreactor (PAnMBR) setup

A 2 L anaerobic membrane photobioreactor (PAnMBR) (working volume 1.5L) (Figure 4.3) was used to study the potential using of the feast-famine cycles to select PHA-accumulators. The feeding regime was characterized by a high concentration of carbon and nitrogen limitation. The reactor works continuously through the Labview interface which allows to perform the phases that occur during the test. The phases are: the cycle time (12 hours), the discharge phase (30 min) and the load phase (30 min). The operating conditions set are: organic load rate (OLR)-(1000 mgCOD / l d), sludge retention time (SRT)-(10 days), hydraulic retention time (HRT)-(1 day). The nutritive solution is a synthetic reproduction of the wastewater with the addition of a specific source of carbon as natural fatty acids (Acetic acid and Propionic acid ratio 1: 1 and 3:1). The samples are carried out daily and characterized, the following analyzes are performed: TCOD, SCOD, VFA, SST / SSV, PHA%. The selection of the PHA, the biomass is taken from the reactor and is supplied

inoculum ready for the batch accumulation step. The figure below shows the schematic PAnMBR configuration. A 2 L rectangular acrylic PAnMBR as seen in was equipped with a submerged flat sheet membrane with 0.45 mm pore size and 0.12 m2 surface area (Kubota, Osaka, Japan) was anaerobically illuminated at 50 W m_2 with IR light (IR 96 LED Illuminator for Night Vision Camera, St. Louis, MO, USA). Illumination intensity was measured on the outside of the reactor wall with an IR light sensor (PAS Port™, Roseville, CA, USA). The illuminated surface/volume ratio was 18 m2 m_3. The width, length and height of the photo-bioreactor were 2, 34 and 40 cm. Liquid height was maintained at 32cm and periodically drained to 28 before filling. The reactor was continuously mixed with an internal gas recycle of 6 L min_1 by a vacuum pump (KNF Neuberger Laboport N86KT.18 (3), Trenton, NJ, USA) via a condensate trap the gas recycle loop also functioned to minimize biofilm formation on the inner wall and membrane. The reactor was operated in a semi-batch configuration. A pump (Watson Marlow 120U/DM2 pump, Wilmington, MA, USA) periodically fed refrigerated feed into the reactor. The time between feeding cycles, as well as the composition of the feed was altered over the course of the experiment as outlined in section. A pressure sensor (GE 5000 Series Pressure Transmitter, Fairfield, CO, USA) in the side of the reactor was used as input for a level switch at the site of the reactor which controlled the when the influent and effluent pump (WELCO peristaltic pump WPM1-S2AA-BP, Tokyo, Japan) turned off, the level switch was calibrated to drain to 1L and fill to 1.5L of liquid contents. Effluent removal was therefore semi-continuous. Reactor pressure was not set but remained above atmospheric pressure as indicated by a tipping bucket gas counter. pH (TPS minichem pH, Brendale, QLD, Australia) and temperature (TPS minichem temperature, Brendale, QLD, Australia) were measured and recorded.

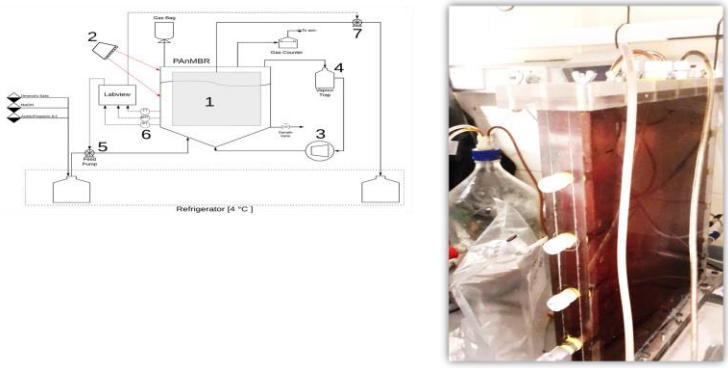


Figure 4.3 2 L PAnMBR PPB selection

4.3.4.1 Operative conditions and timeline

The preliminary tests were carried out by the research group of the Advanced Water Management Center (AWMC) department of the University of Queensland. The first tests of the 2 L PAnMBR started with the study of how the production of polymers and the selection of biomass is influenced by the length of the operating cycle and the ratio between the carbon sources. The operative conditions studied in the early stages were the 6 h cycle length and the fixed carbon source ratio at acetic acid/propionic acid (80/20 %). After a start-up period of 31 days a healthy culture had been established, and PPB started to visibly dominate. Initially the cycle time was set to 6 hours and 34 days, after start-up the activity, the trial was conducted. Subsequently different cycle length was compared in terms of selection of PHA accumulating bacteria. The timer was set to 12 hours to allow for a longer accumulation phase and more convenient sampling schedule. Increasing the cycle time also halved the number of cycles that went unmeasured overnight. A comparison of operating conditions between the cycles is outlined in Table 4.5.

Table 4.5 Comparison of operating conditions

Cycle time [hours]	Ratio C feed [Hac/Hpr %]	HRT [days]	SRT [days]	NH_4N loading rate [mg_N/L/d]	OLR [mg_{SCOD}/L/d]
6	80/20	1.5	15	33.3	446.7
12	80/20	1.5	30	33.3	666.7
12	50/50	1.5	10	33.3	666.7
24	80/20	1.5	10	33.3	666.7

4.3.5 Accumulation batch test

Using the biomass taken from the selection reactor, batch reactors are inoculated to study the polymer storage capacity of the selected biomass (Figure 4.4). It has been analyzed how the accumulation is affected by feeding the biomass with 4 different substrates. The production of PHA in the biomass could change in terms of productivity and percentage yield. Biomass from either the continuous reactor in section was mixed with Ormerod's salts and a range of feeds in 150mL shaker flasks and placed in a custom incubator containing an LSETM shaker set to 130RPM. Four Infra-red LED arrays irradiated the flasks at 820 and 870 nm. Once media was prepared biomass and SCOD were added at t0 and the flasks were flushed with N2 for 5 minutes after sealing with 20mm aluminum crimp caps (SUPELCO 508500) to ensure an anaerobic environment. Samples are taken every six hours and the duration of the test is 35 hours. The temperature is constantly maintained by a heater. The analysis performed after sampling are: TCOD, SCOD, VFA, SST / SSV, PHA% and pH.

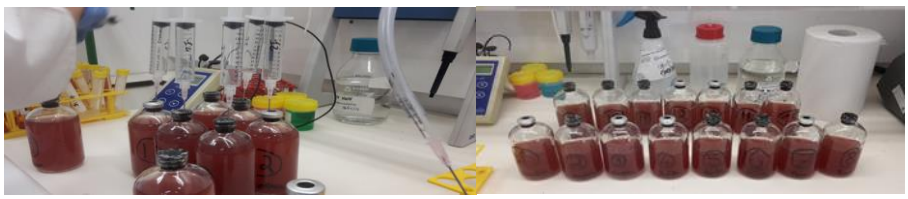


Figure 4.4 Accumulation batch test preparation

4.3.5.1 Accumulation Trial - VFA Comparison

Stock solutions of acetic (HAc), propionic (HPr) and formic (HFor) acid were prepared and neutralized to pH 7 with NaOH prior to the trial and stored at 4 °C. 500 mg SCOD VFA/L of inoculum and VFA stock in that order before sealing and sparging the vials as outlined in the section above. The experiment was performed in parallel triplicate with a total of 15 vials prepared. The operating conditions set in the batch tests are summarized below in Table 4.6.

Table 4.6 Operating condition of the batch test

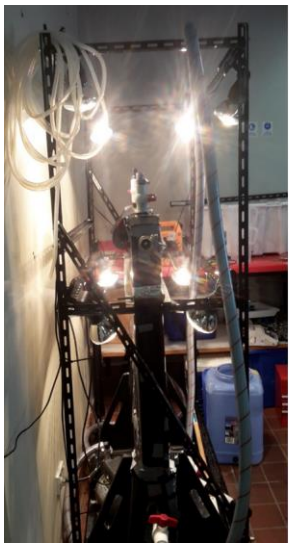
Operative condition		
Vials	n	15
C conc feed	mg SCOD VFA/L	500
C Feed strategy	n	VFA
	1--3	Hac
	4--6	Hpr
	7--9	Hac/Hpr ratio 1:1
	10--12	Hform
	13--15	Blanck
N conc feed	/	/

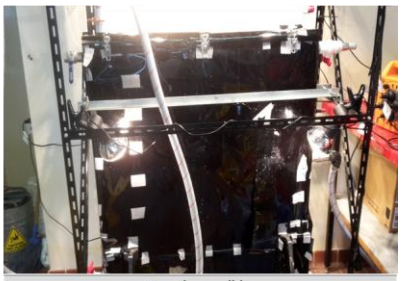
S/I Ratio	%	83/13
cycle lenght	h	32
Time point interval	h	6

4.3.6 Lab-scale Photobioreactor

A large-scale flat plate photobioreactor was studied to apply the same operative condition for the production of PHA and for the selection of the accumulating bacteria. Two different way were applied: the first one was a 60 L flat plate lab-scale photobioreactor with a 24 h illumination and the second was an 80 L flat plate pilot scale photobioreactor and the investigation was carried out under the natural light/dark cycle. Flat-plate photobioreactors have received much attention for cultivation of photosynthetic microorganisms due to their large illumination surface area. Generally, flat-plate photobioreactors are made of transparent materials for maximum utilization of solar light energy. Study the PHA accumulating capacity of PPBs, grown in IR induced biofilms attached at the walls of the reactor and compare the results with the 2 L continuous reactor. Irradiating 60 L photobioreactor (Figure 4.5) with IR light at an intensity of 18W.m-2. Sheets were used to filter light, only transmitting bands > 800nm wavelengths on the wall, to lead and the PPB to growth. A pump was used to continuously mix the reactor liquid contents at flowrate and the culture dominated by PPB would attach to walls. To drain the reactor, a manual valve was used to divert the recycle line to the drain and it is also used for sampling. The operative condition, summarize below, are divided into different phases: 1) use the 30 % of inoculum taken from the enrichment phase to start the test; 2) the fed phase is the 70 % of the total volume of the reactor at fixed concentration of carbon source (500 mg COD VFA/L); 3) the reaction phase is characterized by the activity of bacterial metabolism, that after an acclimation period, starts to consume the available SCOD. Subsequently, due the external light, the biomass starts to attached to the wall and develops a biofilm growth on it. The feeding regime imposed, based on feast and famine condition with limitation of nitrogen source, should lead the PPB towards the accumulation of PHA instead of growth; 4) The drain phase starts every end of the cycle with a slow flowrate and the reactor is drained of the 70 % of the total volume; 6) The harvest of the biomass is carried out whipping

directly the 70 % of the biofilm from the wall, the rest of the PPB is scratched of in the liquid phase becoming the inoculum for the next cycle.





Operative condition		
Vol	l	80
I/S Ratio	%	30
C:N	g COD/g N	100:10:01
Conc Fed	mg COD VFA/l	500
	% Hac/Hpr	80/20
Withdraw	%	65
	Vol (l)	52
Fed phase	%	65
	Vol (l)	52

Figure 4.5 60 L Lab-scale Photobioreactor

Improve the PPB and PHA harvest directly from the walls of 60 L reactor as a really concentrated biofilm matter, it is an attempt to solve the problems of need for a large amount of polymer and the additional problem of the difficult achievement of high production percentages.

4.3.7 Out-door Photobioreactor

One of the most critical points is the cost of energy for the 24 h illumination that does not allow the project to be sustainable being an important cost item. One of the applicable solutions is to take advantage of the natural light through a development of an out-door pilot scale system (Figure 4.6). The tests were conducted under the same operative condition and with the same experimental set up illuminated by a natural light/dark cycle and uncontrolled temperature.

Figure 4.6 Out-door pilot scale Photobioreactor

4.3.8 Analytical methods

TCOD and SCOD were determined by COD cell tests (Merck, 1.14541.0001, Darmstadt, Germany). Dissolved NH4eN, NO2eN and PO4eP were determined by a QuikChem8000 Flow Injection Analyzer (FIA) (Hach Company, Loveland, USA). Temperature and pH were measured using an Oakton pH 11 Series (Vernon Hill, IL, USA). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined by filtering sample through a 0.45μm filter, then drying the filter at 105 °C for 24 h before 17 weighting it to determine the TSS. The VSS was determined after 2 h in a furnace at 550 °C. NH4 was determined using sulfuric acid, potassium sulfate and copper sulfate catalyst in a block digester (Lachat BD-46, Hach Company, Loveland, CO, USA). All soluble constituents were determined after filtering with a 0.45 μm membrane filter (Millipore, Millex®-HP, Merck Group, Darmstadt, Germany).

4.3.9 Extraction and qualitative/quantitative characterization of produced the polymers

PHA samples were prepared by centrifuging reactor samples at 3725 RPM for 20 minutes before removing the supernatant and freezing the pellet for a minimum of 3 hours at -20 °C. Frozen pellets were then freeze-dried at -50 °C and 0.2 bar for 24 hours before drying in an oven for 1 hour at 60 °C. A specific amount of dried sludge is weighed out onto a weighing paper on an analytical balance and then added to a pre-cleaned and properly labeled Hach tube. Approximately 20mg of dried sludge is required per PHA sample, and approximately 10mg per glycogen sample. The sludge weight is recorded accurately for each PHA or glycogen sample through use of a 4 decimal point analytical balance. Each sample is properly labeled with both the initials and number.

4.3.9.1 Reagents

Reagents required for this analysis are as follows:

- Chloroform-(HPLC-grade)

- 3% sulphuric acid in methanol (HPLC grade) with 100-600mg/L sodium benzoate
    - In 1L Schott bottle add 970ml (768g, d=0.7914g/mL) of methanol, then put a magnetic bar into the bottle; put the Schott bottle in a bucket with ice water and put it on a stirrer; while stirring the solution add 30 mL (55.2g, d=1.84g/mL)

sulphuric acid very slowly so that there is no splashing at all. Let the solution stir until it has cooled down to room temperature. Add the required amount of sodium benzoate (100-600mg/L) and stir until dissolved. Put the cleaned dispenser onto the bottle and prime the dispenser.

4.3.9.2 Standards

A set of standards is also required for PHA analysis. These standards are first weighed onto weighing paper and then transferred into labeled Hach Tubes. Weighing these samples can be very difficult, since only a small amount of each standards is required. The standards used are stored in the fridge, is a copolymer of R-3-hydroxybutyric acid and R-3-hydroxyvaleric acid (7:3). There should be a set of 6 standards corresponding approximately to the following set of weights (0-2.5 mg)

4.3.9.3 Sample digestion

After all samples and standards have been weighed, the reagents are added. For each PHA sample add 2mL of 3% sulphuric acid and 2mL of chloroform. Put the digestion block into the fume hood, install the timer and heat the digestion block up to 100^{o}C. When that temperature has been reached carefully insert the sample tubes into the digestion block and wait until the temperature has been equilibrated to 100^{o}C again. Set the on/off times on the timer and start the timer. PHA samples require 20 hours at 100^{o}C. When the digestion is complete, allow the samples to cool for at least 3 hours or better let the samples cool overnight. Add 1mL milli-Q water into each sample and vortex. Allow sample to settle for at least 1 hour. With a Pasteur pipette extract about 1mL of the bottom phase of the sample and transfer it into a labeled GC vial. Add about 5 pieces of molecular sieve, cap the GC vial and shake.

4.3.9.4 Sample characterization

1mL of the chloroform phase was extracted with disposable glass Pasteur pipettes and transferred to glass chromatography vials containing molecular sieve before analysis with gas chromatography. PHA monomers were determined by gas chromatography equipped with a flame ionization detector (detection temperature 300°C, injection temperature 250°C) and non-polar capillary column (DB-5, 30m x 0.25mm with a 0.25μm film) using 1mL/min high purity helium

as the carrier gas at a 1:10 injection split (injection volume 0.5μL) oven cycle was as follows: 90oC for 1 min; 10oC/min to 120oC, 45oC /min to 270oC, hold for 3min.

4.3.10 Polymer characterization - Mechanical and thermal property

4.3.10.1 2-butanol pure PHA extraction

The biomass was dried at 70 °C in a vacuum oven overnight and digested for 1 h at 140 °C in a vacuum high-pressure reactor in butanol. The mechanical stirring and the chemical solvent allow the pure PHA to be extracted from the biomass and after the digestion and the cooling down, it was allowed to evaporate the butanol under fume hood overnight.

4.3.10.2 Chloroform purification

After the extraction the biomass need to be purified to have the most accurate analyzes possible. It proceeded with 1 h at 70 °C chloroform digestion with a condenser under fume hood and after cooling down the sample has been filtered with nylon paper filters and vacuum pump subsequently was necessary to let the chloroform evaporate under fume hood. The whole process is schematizing below (Figure 4.7).

Figure 4.7 Process of the chemical PHA extraction

4.3.10.3 Acetone – methanol purification

PPB are generally referred to as “purple” bacteria due to the presence of carotenoids, and can range from orange to purple in color. The organic pigments are soluble in chloroform and the resulting matrix after extraction and purification was a mixture of carotenoids and PHA. The first purification was not enough to make a pure material, it was not possible to separate the pigments from the polymers, for this reason a second purification was carried out with chemical solvent Acetone and methanol (7:3), after chemical treatment was filtered and dried under the fume hood, obtaining a purified material as showed in the figure below (Figure 4.8)

Figure 4.8 Pure PHA purification

4.3.11 Differential scanning calorimetry (DSC)

A differential scanning calorimetry Q2000(TA Instruments) under a constant nitrogen flow of 50 ml/min was used to determine the thermal properties of the polymer. Samples of 2-3 mg were placed in a sealed aluminum pan and were analyzed with standard DSC heating and cooling scans. The melting temperature and the crystallization temperature were determined from the heating and cooling cycle.

4.3.12 Thermal Gravimetric Analysis (TGA)

TGA methods were applied was for routine TGA (Q2000 TA Instrument), and this was performed using dried biomass sub-samples (grain size < 0.71 mm), recovered polymer, or process residuals (3–8 mg). The heating rate, initially under nitrogen atmosphere from room temperature, was 10°C/min to 550°C, with a 10-min isothermal hold at 103°C. The hold was used to eliminate and quantify residual water content. After 2 min at 550°C, the inert atmosphere was switched to air and the temperature was maintained at 550°C for 30 min before cooling. Weight loss was followed

under the elevated temperature with isothermal conditions. The crystallization temperature (Tc), melting temperature (Tm), were determined from the DSC curves.

4.3.13 Nuclear magnetic resonance

Spectroscopy (NMR, e.g. 1 H NMR and 13 C NMR) is a diagnostic tool that is used for PHA structural analysis and metabolic pathway studies.

4.4 Results and discussion

4.4.1 Performance of the enrichment phase in a 2 L PAnMBR

The figure 3.7 shows the TCOD, SCOD, NH4-N, PHB%wt, PHV%wt, PHA%wt and PHB:PHV ratio results for the continuous 2L PAnMBR reactor. It is clear that the SCOD removal and TCOD level were reasonably steady in the reactor. Different operating conditions were assessed and compare, and in all of the different phases is attested that the efficiency of removal of the SCOD was effective. Moreover, it has been studied how the length of the cycle affects the selection of microorganisms. The reason why the cycle has been extended to 12 h and after to 24 h, was to impose a stronger feast and famine regime to the biomass. As can be seen from the graph, during the 6 hours cycle length, it has been obtained good results in terms of removal of COD, NH4 and accumulation of polymers, but the length of the cycle was not enough to impose a strong feast and famine condition. SCOD and NH4 have been consumed at the end of the cycle, this means that no famine phase was impose in the reactor. The final goal of the selection phase in a 2L PAnMBR is to increase the bacterial population with higher PHA accumulating capacity not only a higher yield in PHA concentration.

4.4.2 6-hours cycle to 12-hours cycle, ratio of acetic and propionic 4:1 (Period 1-2-3)

It appears that the 6-hour cycle times had effectively selected a culture of PHA accumulators since PHA as %wt steadily increased after 38 days of operation (from 1% to 14% between days 30 and 48). This is confirmed by previous studies which shows the change in relative abundances between low accumulators (1%wt at day 30) and good accumulators (14%wt at day 48) with the promotion of known accumulators *Rhodopseudomonas sp* and *Phyllobacteriaceae* as well as a decrease in *Fermicutes*. After the 12-hour cycle time was introduced the PHA accumulation as %wt appeared

to decrease. The unreasonably long SRT of 30 days meant that various slow growing organisms could have competed with PPB, potentially explaining the decrease in PHA fraction. After correcting the SRT, the period of 12-hour cycles (period 2 - 3) exhibited steady SCOD removal of approximately 500mg per cycle, and complete removal of 17mgN/L of ammonia can be seen in period 3, suggesting that roughly 330mg/L of SCOD was converted into storage product during a typical cycle (66% of COD uptake). From period 1 onwards PHA accumulated to a reasonably high level (24%wt) and had a very stable and favorable PHB:PHV ratio (3.28:1 ±0.274) (Figure 4.9)

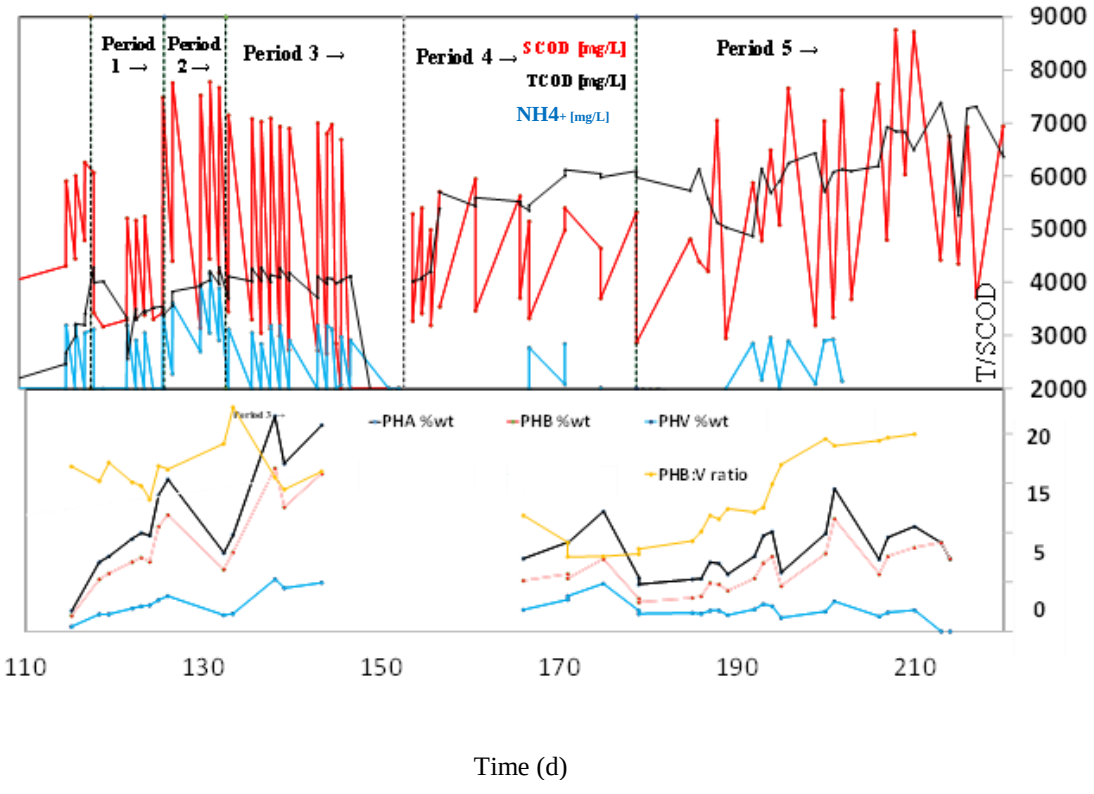


Figure 4.9 Performance of the enrichment phase in a 2 L PAnMBR

4.4.3 12-hours to 12-hours at ratio of acetic and propionic 1:1 (Period 4)

The 12-h cycle being evaluated as optimal was also maintained for test of period 4. In this period another condition was examined, the ratio between acetic and propionic acid has been changed from 4:1 to 1:1 to analyze the effect on the selection of biomass but, above all, on the characteristics of the polymers produced. A different VFA feeding could affects polymer composition and as shown in the graph, the ratio between PHB and PHV is lower compared to those of the previous period. This is due to the feeding composition which, having a higher propionic concentration, pushes the bacteria to accumulate the carbon with a higher PHV percentage than the other tests. The PHB:PHV ratio was stable (1.52:1) and peaks of PHV production yields are reached at this stage up to 5 $\%_{wt.}$

4.4.4 12-hours cycle to 24-hours cycle, ratio of acetic and propionic 4:1 (Period 5)

After 25 days of selection, condition fixed at 12 h cycle, it has been changed to the 24 h cycle for the period 5. The ratio between acetic and propionic acid was restored at 4:1 for a better comparison of the best performance of the reactor. SCOD removal and TCOD level were reasonably steady in the reactor and there was a good response from the biomass in terms of accumulation. It appears that the 24-hour cycle times had effectively selected a culture of PHA accumulators since PHA as %wt steadily increased (from 3% to 15% between days 86 and 93). The period 5 of 24-hour cycles exhibited a SCOD removal of approximately 680mg per cycle, and complete removal of ammonia, suggesting that roughly 331mg/L of SCOD was converted into storage product during a typical cycle (66% of COD uptake). Compared to the previous period it can be seen how the increase of the concentration of the feeding has led to an alteration of the stability in the reactor. In fact, the consumption of the SCOD is not always constant and the VSS have a growing trend (from 3 to 4 gVSS/L between days 186 and 225) due the higher disposition of carbon source and the SRT fixed at 10 days. Regarding the selection of microorganisms, the increase up to 15% of PHA gives positive results and moreover, given the imposition of the feast and famine condition, it is possible to evaluate a doable achievement of the maximum accumulation in the middle of the cycle, considering that each sampling was taken at the end of the cycle. Whereas, there are not many

studies of the use of PPB mixed crops for the production of PHA. Initial studies with PMCs also applied the FF strategies to obtain PHA storing phototropic bacteria, and by using acetate as a carbon source. The PHA accumulation values, reported by Fradinho et al., 2013, were 20 and 30 % g PHA/ g VSS obtained under continuous illumination. A study of Fradinho et al., 2016, reported a contents of 60% PHA/VSS in accumulation tests conducted with high light availability, while in the selector reactor, operated at lowlight availability, the PHA content averaged 3–5%. Subsequent studies will have to focus on evaluating the data of the entire cycle in order to have a more accurate result on the maximum accumulation point, which can probably reach values higher than 15% previously reported. Compared the results with the results obtained from bibliographic research, this study has achieved promising goals for future applications. The results are shown in the Figure 4.10.

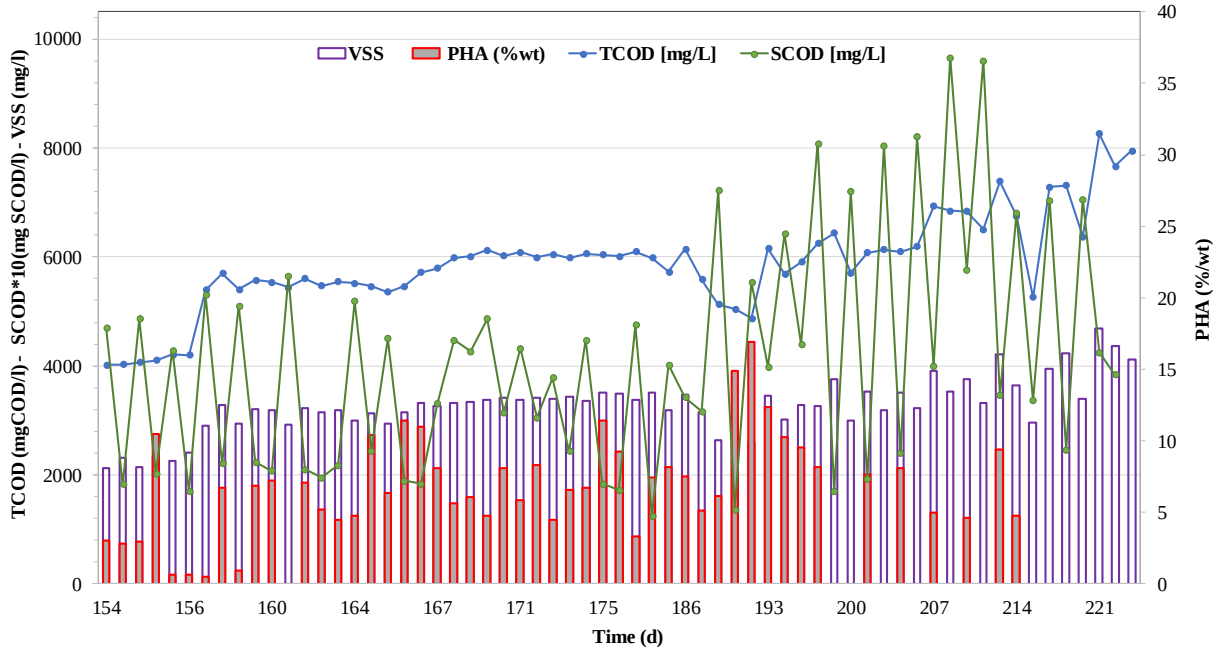


Figure 4.10 Performance of the enrichment phase in a 2 L PAnMBR (Period 4 – 5)

4.4.5 Batch test

4.4.5.1 Effect of VFA substrate on PHA accumulation

Figure 4.11 shows the TCOD, SCOD, PHA%wt pH, and temperature data for VFA accumulation batch tests described in previous sections. The overall process and results of the batch test are shown in Figure 4.12. The TCOD remained stable over the period of the trial (32 hours) suggesting that no significant amount of COD was lost as gaseous by-products. The NH4N remained below 0.3 mgN/L during the entire trial (data not shown) suggesting that growth did not occur. Temperature was well maintained between 32 and 34 C. During the 32 hours' pH was maintained at 7. The relative trends in uptake rates were linear and Propionic acid was removed more slowly than acetic acid, which is in line with the results conducted in the continuous reactor discussed earlier. The maximum accumulation point is reached by the tests fed with acetic acid, reaching from 4% to 14%. In all the other tests with the other acids was reach only the maximum of 8% by the test fed with mix of acetic and propionic acid. An initial lag phase in a complete nitrogen limitation is detected in the first 18 hours, underlined by an increase in the accumulation only after 24 hours. This unexpected phase of biomass acclimatization is probably due to a previous temporary stress condition of the biomass in the continuous reactor from where the inoculum was taken

Accumulation Batch Test 27/09/2018

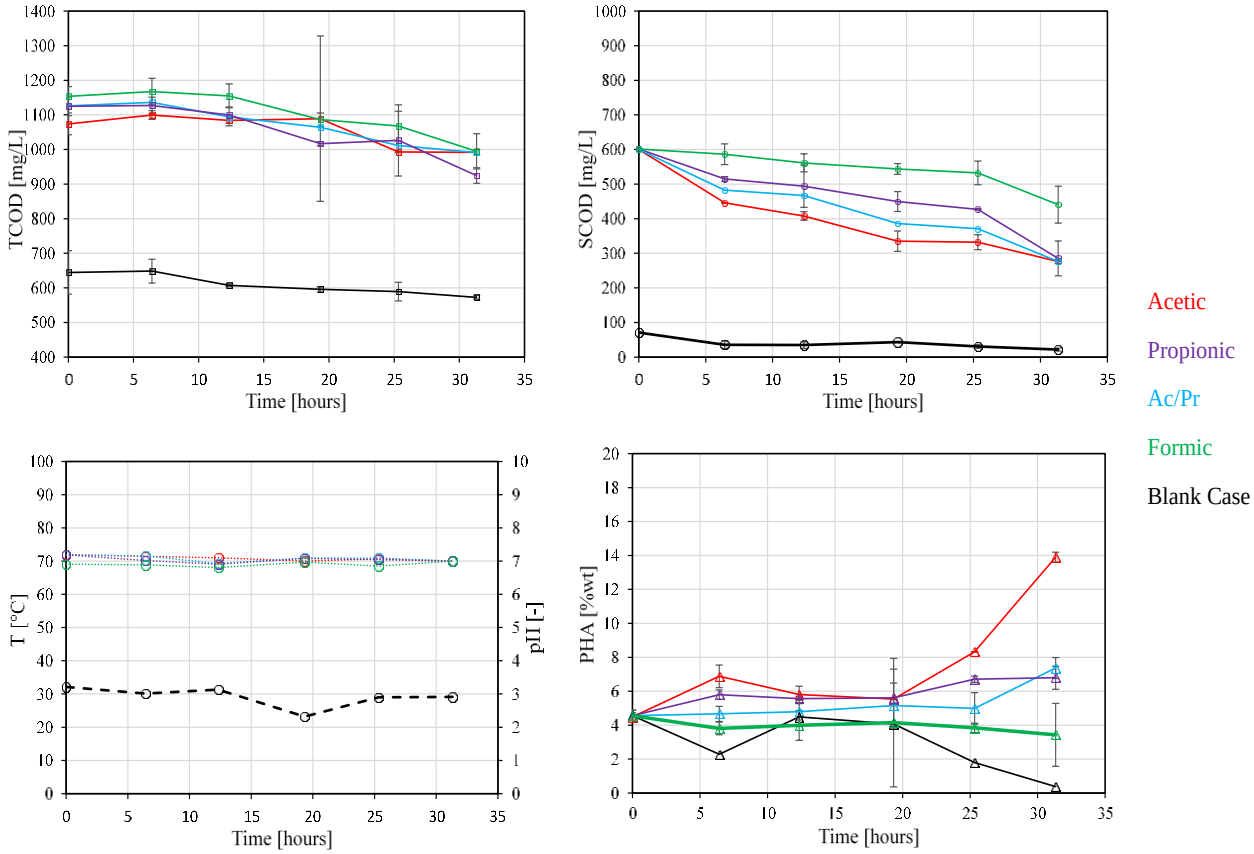


Figure 4.11 Results of the accumulation batch test

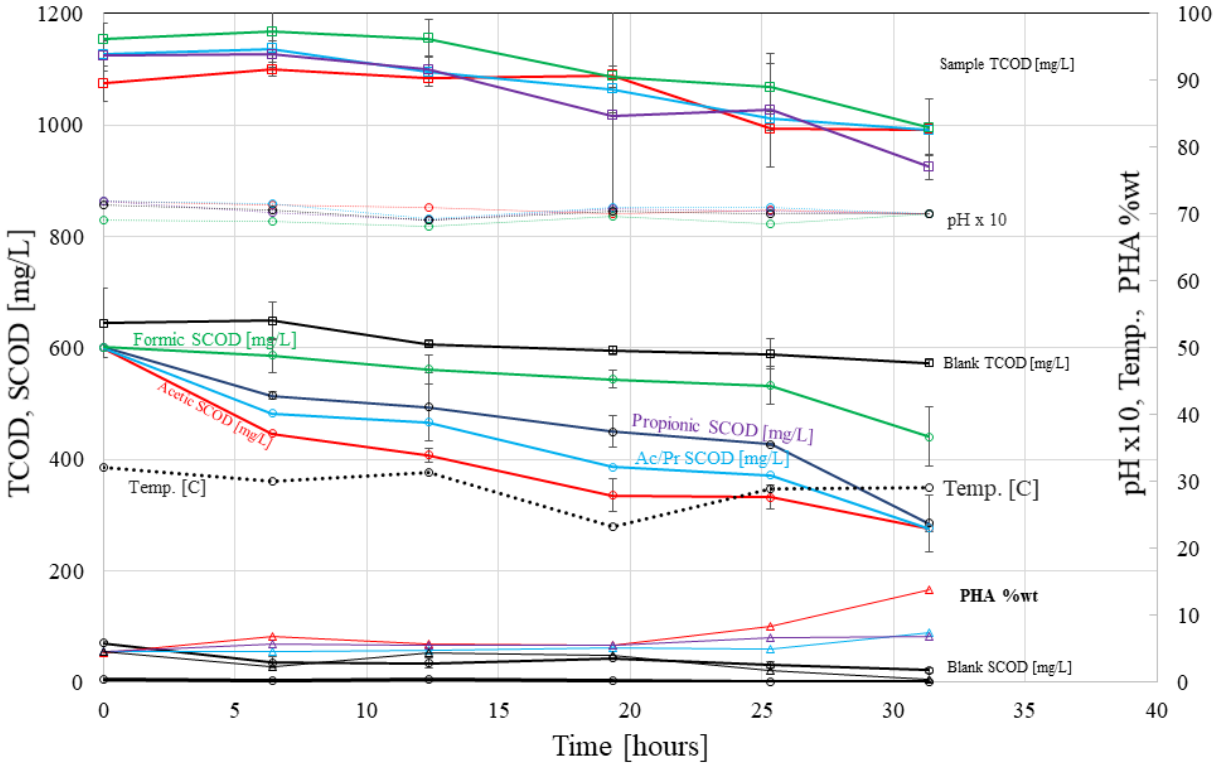


Figure 4.12 Overall batch test results

4.4.6 60 L Flat plate photobioreactor

4.4.6.1 Lab-scale photobioreactor

In a flat plate 60 L Lab scale reactor was study the PHA accumulating capacity of PPBs, grown in IR induced biofilms attached at the walls of the reactor. Applying the optimized operative condition of the PAnMBR to increase the PHA production, was improved the PPB and PHA harvest directly from the walls of the reactor as a really concentrated biofilm matter. This is an important assessment for economic and commercial purposes to obtain a product already separated from the liquid part directly in the production reactor. The results obtained from the laboratory tests have been very positive results in terms of biomass adaptation to the new conditions, recording during the daily activity tests a good capacity of growth and selection of the PPB on the walls of the reactor. As is shown from the figure 3.11 an example of a daily test activity of the reactor is reported, during five days of cycle the TCOD decrease from 1025 to 650 mg TCOD/l this explains that much of the biomass has abandoned the liquid phase and is adhered to the reactor walls. Moreover, the test activity showed a removal efficiency of 107 mg COD/l d with 80 % of removal

of the duration of the cycle and a complete removal of ammonia. Is possible to grow PPB biofilm on the wall of reactor with high removal COD and ammonia capacity. No positive results have yet been found in PHA production (Max accumulation 3 %wt) but it was first necessary to understand the adaptability of biomass to the photobioreactor. New tests will be carried out to optimize production.

4.4.6.2 Out-door Photobioreactor

To limit the costs due to artificial lighting 24 hours, the use of a 60-liter out-door photobioreactor was analyzed under the same conditions. The only varied operating conditions is the natural light, the reactor were illuminated by a natural light/dark cycle and uncontrolled temperature. The results obtained from the laboratory tests have been very positive results in terms of biomass adaptation to the new conditions, recording during the cycle tests a good capacity of growth and selection of the PPB on the walls of the reactor (Figure 4.13). The test activity showed a removal efficiency of 75 mg COD/l d with 90 % of removal of the duration of the cycle and a complete removal of ammonia. In the last week the test was compromised by an algae contamination that imposed a strong competition to the PPB. No relevant result was observed about PHA accumulation (max accumulation 2 %wt).

Cycle test

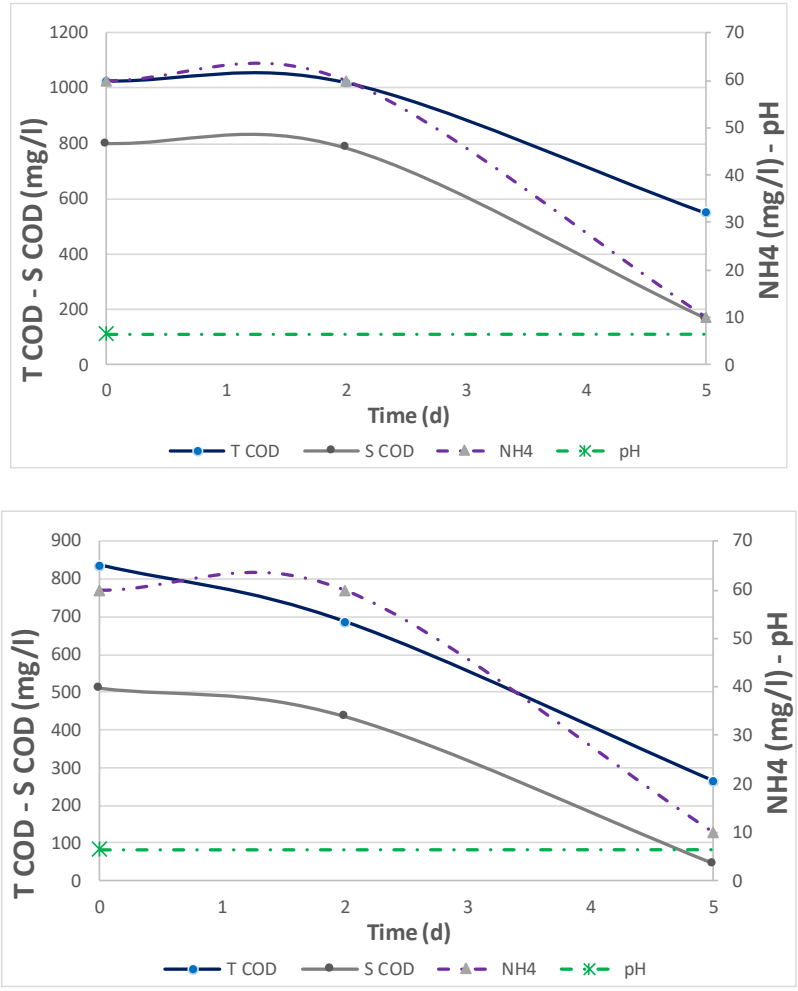


Figure 4.13 Cycle test of out-door photobioreactor

4.4.7 Performance compared: 2 L PanMBR and 60 L Lab/outdoor Photobioreactor

The la scale and out-door photobioreactor confirmed the capacity for an attached biofilm induced by IR and the removal rate of COD and Nh4 but not for the PPB selection and for the PHA accumulation (Table 4.7).

Table 4.7 Performance compared

PAnMBR 12 h			**PAnMBR 24 h**		
mg COD/l/d removed	% COD removed	max PHA %	mg COD/l/d removed	% COD removed	max PHA %
516,5	61	9	399,5	50,9	16
Out-door 60 L			**Lab-scale 60 L**		
mg COD/l/d removed	% COD removed	max PHA %	mg COD/l/d removed	% COD removed	max PHA %
75	90	2	107	80	3

4.4.8 Polymer extraction: pure PHA

The overall mass balance of the pure PHA extraction is shown in Table 4.8. Is reported the weight and the percentage of recovery from the chloroform extraction and the acetone-methanol extraction necessary process for the purification of the pure PHA from the carotenoids.

Table 4.8 Overall mass balance

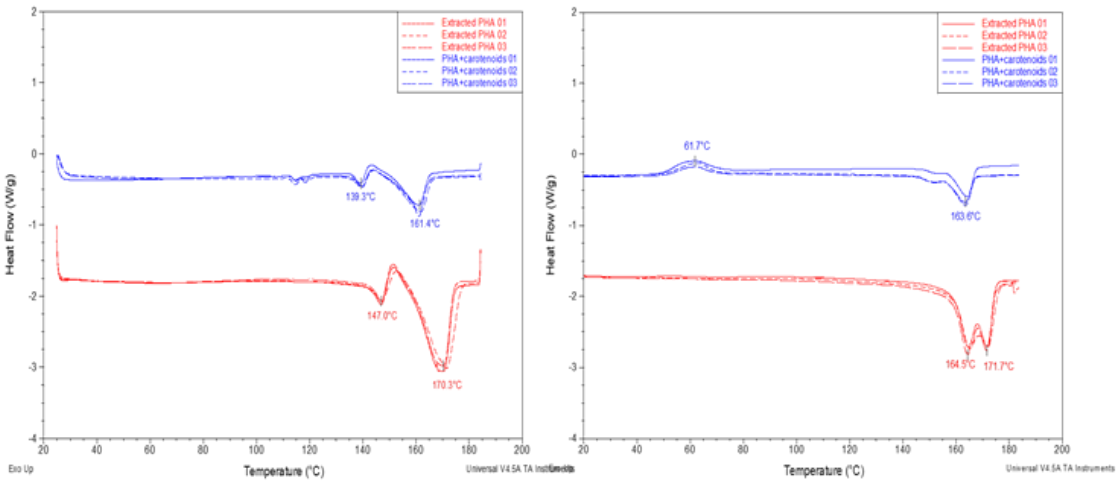
Phase		**Weight**	**Matter**
-	%	g	-
Vacuum oven (dry)	100	89.7	Biomass, PHA, carotenoids
2-butanol extraction (dry)	35.7	32	PHA, carotenoids and residual biomass
Chloroform extraction (filtered and dry)	18.4	16.5	PHA /carotenoids
Aceton/methanol extraction	6.1	5.5	PHA

(filtred and dry)

4.4.9 Polymer characterization

4.4.9.1 Differential scanning calorimetry (DSC)

1st heating ramp (as extracted) **2nd heating ramp**



Cooling ramp (crystalisation) **20C heating ramp (glass transition)**

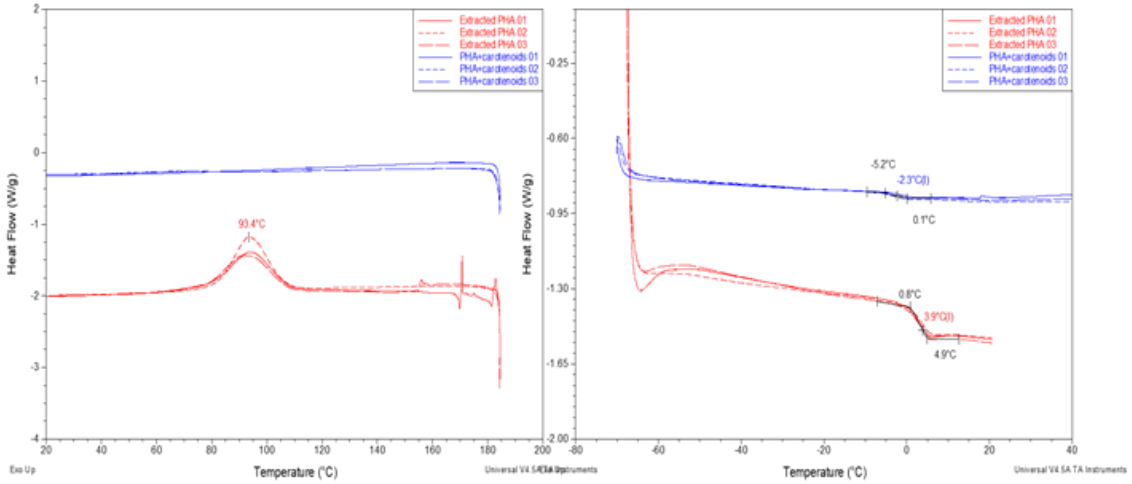


Figure 4.14 Differential scanning calorimetry (DSC) results

Two heating and two cooling cycles were performed so that useful comparisons could be made. The heating and cooling cycles are described before. The DSC curves were used to determine the crystallization temperature (Tc) and the melting temperature (Tm) of the polymer. The samples were analyzed before and after purification from carotenoids to compare the different results. All the relevant polymer characterization is shown in Figure 4.14.

4.4.9.2 Thermal Gravimetric Analysis (TGA)

As is shown in Figure 4.15, the curve of the PPB-PHA before carotenoids removal showed the presence of impurities which thermally degrade across the temperature range. A 36.5% weight loss was observed from the 260 – 300 region that is the PHA region referenced by the TGA curve of TianAn PHBV. This was carried out to verify that the polymer produced was comparable with polymers of the same family already analyzed.

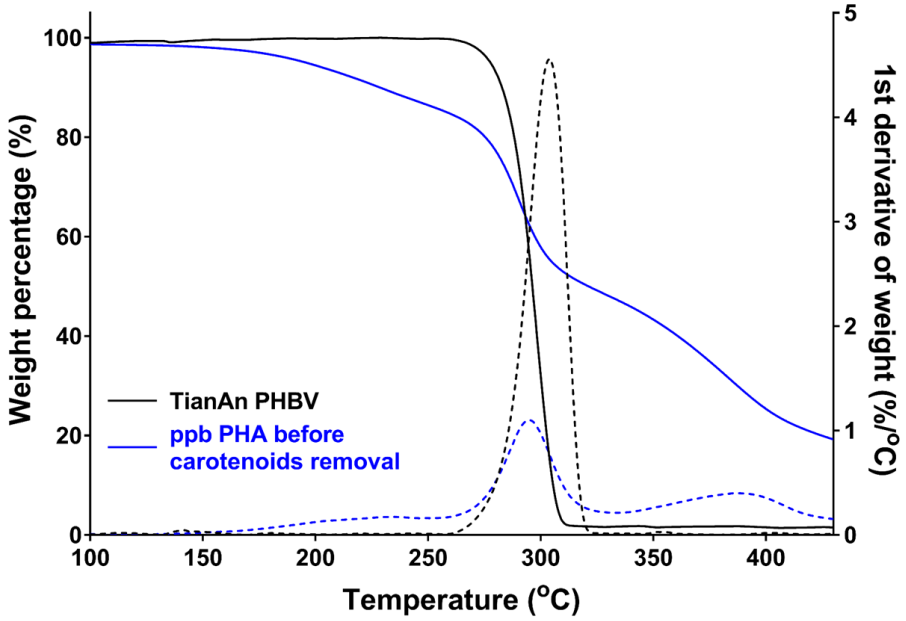


Figure 4.15 Thermal Gravimetric Analysis (TGA) results

4.4.9.3 Nuclear magnetic resonance (NMR)

The NMR analysis were carried both, before the carotenoid extraction and after the extraction. Through the spectroscope results was possible to show a success in carotenoids removal, yielding a clean PHBV spectra (Figure 4.16). After the carotenoid extraction the results showed a better shape of the peaks (tale to tale) and a clear selection of the target peaks. The matrix effect was reduced and made the carotenoid removal efficiency more reliable. The resulting HV content was estimated to be 5 mol%:

$$mol\%\ HV = \frac{peak\ integral\ (HV\ unit\ "7")}{peak\ integral\ (HV\ unit\ "7")\ +\ peak\ integral\ (HB\ unit\ "3")}$$

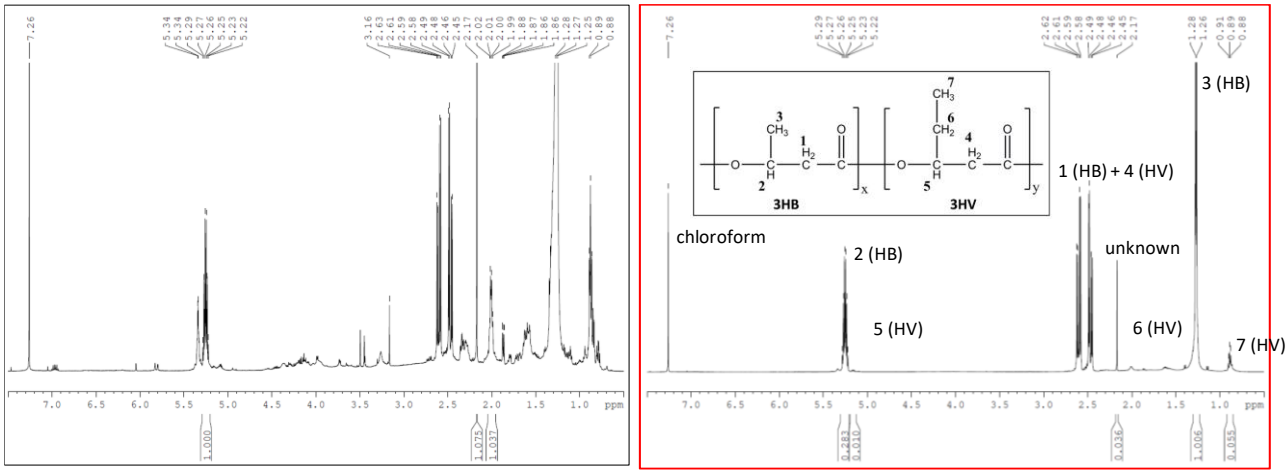


Figure 4.16 Nuclear magnetic resonance (NMR) results

4.4.9.4 Carotenoids extraction and characterization

The carotenoids extraction was carried out with chemical solvent Acetone and methanol (7:3), after chemical treatment the samples were filtered and dried under the fume hood, obtaining a purified material, the liquid part was stored and characterized. UV-vis spectrophotometer was used to characterize the carotenoid and, as shown in the Figure 4.17, the peaks are present in the carotenoid region. It was not possible to quantify and specify the type of carotenoid in the samples, but the detention of the peaks in the carotenoid region strengthens the possibility of these.

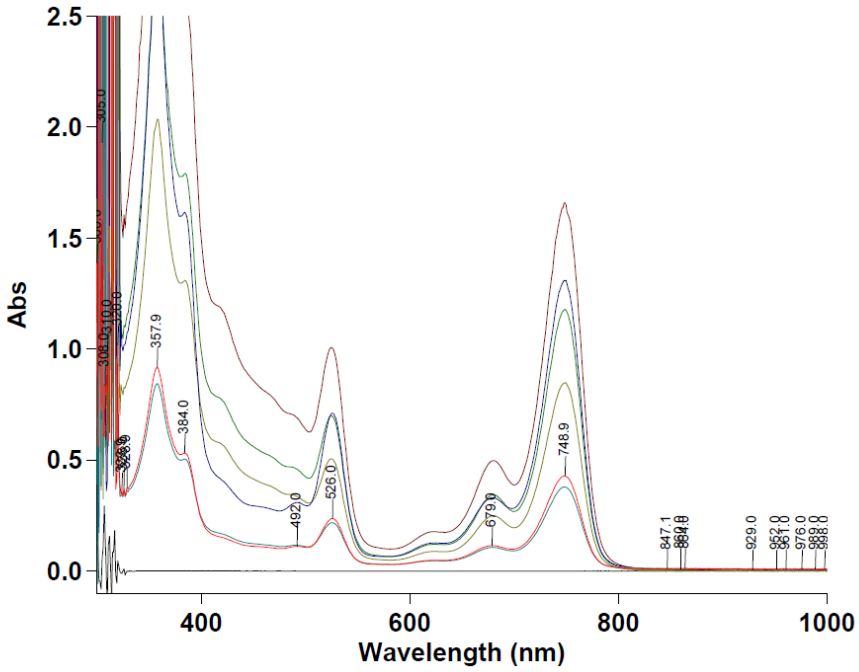


Figure 4.17 the peaks in the carotenoid region (UV-vis spectrophotometer results)

4.5 Conclusion

The purpose of the present work was to study the enrichment of PHA-accumulating PPB, study how the VFA feed affects polymer composition and optimize the operative condition and apply to a large-scale reactor. The development of a specific method for the Polymer characterization: through a qualitative/quantitative characterization, mechanical and thermal property, a study of the molecular weight and chemical structure. An innovative experimental setup for the production of PHA is proposed. The use of a mixed culture of PPB has given good results in terms of removal of COD and NH3 and has shown good biopolymer accumulation performance. Moreover, the capacity of anaerobic growth in photobioreactor it allows to decrease drastically the production cost. By removing the oxygen supplying and by the use of the sun light during the process, the PPB are strongly considered for future research and application, for the decreasing of the production cost. For the possibility of compensating the low yield of mixed culture, with a lower process cost, the PPB are an interesting research challenge for industrial application in the biopolymer market.

The operative condition studied in this research had shown a potential capacity of PPB in the PHA accumulation process, reaching more than 15 % (W/W) content of PHA in the microbial cells and 61 % of COD removal. Even if the biopolymer content was not the highest in literature, was a promising and innovating results for the novel photobioreactor setup and for the study of the mixed PPB culture. There are not many studies on the PPB mixed culture reaching more than 15 % of PHA content in the cells. The results of the batch tests have confirmed the same accumulation capacity of the culture selected strengthens the potential application of this experimental setup.

Specifically, there is a correlation between the cycle length and the PHA accumulation. The 24 h cycle length and the 10 days SRT had a negative effect on the PHA accumulation (relative to the previous settings 12h/10d SRT), that had shown better performance in terms of selection and accumulation. It is necessary to analyse the feast and famine condition in each cycle to determine the maximum PHA accumulation. Was not possible to do specific cycle study to better underline behaviour and the metabolic activities during the cycle, the next steps of the process it will be to focus on the cycle studies to reach the maximum accumulation point and the time of the harvest. Making the production of PHAs at an industrial level economically sustainable is the greatest scientific challenge worldwide and in this research. The application of the mixed PPB culture and

this novel setup, are a strongly promising process that needs future studies and research in order to contribute on the industrial application in the global biopolymer market.

5 Research activities for reaching RO3: - Photofermentative biopolymer production by mixed culture of Purple phototrophic bacteria from fermented molasses

5.1 Introduction

A new approach relying on the photosynthetic activity of mixed consortia has been explored recently (Fradinho et al., 2013). As it is said previously, the higher costs for the production of PHA that are mainly due the high costs of used carbon sources (e.g., glucose and sucrose), the low productivity and the low efficiency of extraction process of PHAs from microbial biomasses (Mozumder et al., 2015) and to the oxygen supply. Integrate the PHA production with bio refineries can represent a strategy to reduce the actual production costs using the biorefinery (crude oil) by-products glycerol, spent vegetable oils and low-grade biodiesel) as low-cost substrates for PHA production microorganisms (Jiang et al., 2012).

An alternative without the use of oxygen and with the use of real waste has been proposed in this research, eliminating the costly need for aeration during ADF enrichments and the use of expensive substrate. In such a system, photosynthetic bacteria uptake an external carbon source, in the form of acetate, during the feast phase using light as an energy source PHB was produced at the same time as a sink of NADH, given that no electron acceptor was present. Various low-cost waste is investigated as carbon source for the biopolymer production like cheese whey, wastewater from olive mills, molasses and mill effluent (Marangoni et al., 2002; Pozo et al., 2002).

5.1.1 PPB-PHA production through complex waste feedstock

Molasses represent a suitable cheap substrate which could be used for the biopolymer production process. Sugarcane is one of Australia's largest crops and accounts for a significant percentage of the crops produced in Queensland's coastal agricultural production areas. The main product of sugarcane is raw sugar, made from the juice of the cane, and molasses which is a by-product of the sugarcane refining process (AgriFuture-Sugarcane 2017) There are three ethanol refinery facilities in Australia located in Queensland and New South Wales, however only one of these, in

Queensland, processed molasses derived from sugarcane to produce ethanol (AgriFuture-Sugarcane 2017).

The use of sugar cane molasses (as a by-product the sugar refinery industry with a very high sugar content - over 50% in dry weight) has been studied for the production of PHA from mixed microbial cultures in a three-step process in a study by Albuquerque et al., 2007. Several studies have analyzed the use of molasses for the production of biopolymers through the use of pure PPB cultures. From the results of bibliographic research, it is difficult to find research concerning the use of PPB mixed cultures and molasses for PHA production. Photosynthetic purple non-sulfur bacteria (PNSB) can produce hydrogen through photofermentation using a variety of organic compounds. A study carried out by Sagir et al., 2017 was focused on the capacities of PNSB to produce biohydrogen from sugar-based wastes. This study explained that the low yield of hydrogen and biomass indicates that the bacterium directs the reducing equivalents from the use of sucrose to other metabolic pathways. One of these pathways could be, instead to produce hydrogen, the poly-b-hydroxybutyrate (PHB) production which is usually observed in the presence of excess carbon source, as a future study PHB measurements will be carried out for a better understanding of the sucrose metabolism (Cetin et al., 2012; Sagir et al., 2017). Another study has reported a single-stage photofermentation process carried out using four different PNSB on pure sucrose and sugar beet molasses for hydrogen production and further study on PHB production by Sagir 2017.

Also, in view of the fact that, so far, PMCs have only been tested with synthetic feed, a study by Fradinho et al., 2019 has evaluated for the first time the possibility of using a real waste, fermented cheese whey (FCW), as feedstock for PHA production in the accumulator reactor. For this reason, in this study a single-stage photo fermentation process and biopolymer production from sugar-rich waste like molasses was analyzed. An alternative of the conventional biopolymer production setup was carried out trying to minimize the cost of the oxygen supply and the use of expensive substrate.

5.2 Experimental approach

The Study of PHA accumulating capacity of PPBs growing under the natural light/dark cycle evaluating the effect of using molasses as sole carbon source, were tested in an out-door 60 l flat plate photobioreactor (**phase 4**). The out-door reactor (phase 4) was inoculated with the biomass taken from the lab-photobioreactor (**Phase 1**), this lab set up was previously described and developed in collaboration with the School of Chemical Engineering and Advanced Wastewater Management Centre (AWMC) of the University of Queensland (Australia). The lab-photobioreactor (phase 1) was fed with artificial feedstock (acetic acid), (estimated PHA content 25-30 %).

To achieve the set objectives, the following research activities have been planned:

- **60 l lab-scale photobioreactor (phase 1)** fed with artificial substrate selecting rich-PHA PPB accumulating mixed bacteria, was used to select the inoculum to be reused for the fallowing phases
- **PHA extraction test-tube study (phase 2);**
- **accumulation batch test** (**phase 3**) to study the effect of different limiting medium condition (N, Mg, S) on the PHA accumulation capacity, the batch tests were fed with acetic acid as a sole artificial carbon source;
- **Out-door 60 l flat plate photobioreactor** (**phase 4**) fed with real complex feedstock (sugar cane molasses) in a single stage process.

The inoculum for all phases was taken from a 60 L lab-scale photobioreactor (phase 1) used for the selection of PPB accumulating PHA. The reactor of phase 1 was fed with artificial carbon source with acetic acid as a sole feedstock and 24 h artificial illumination. In parallel with the out-door photobioreactor different test were carried out: The second phase carried out was a PHA extraction test-tube study (phase 2) with the freeze-dried PPB biomass taken from the lab-scale flat plate photobioreactor (phase 1) (estimated PHA content 25-30 %). Determining the optimum extraction procedure, specific to PPB-PHA based on the Mw of PHA and investigating the effect of carotenoids on PHA extractability and properties. An accumulation batch test (phase 3) with three different limiting medium condition, to study the selected PPB PHA accumulation capacity in absence of N, Mg and S.

The preliminary batch tests and the PHA extraction test-tube were useful to achieve the optimized operative conditions and knowledge that was used in the next stages of the project and the use of real complex substrate as the only source of carbon. In the Figure 5.1 below is summarized the experimental approach of the research and the main objectives and feedstock in Table 5.1.

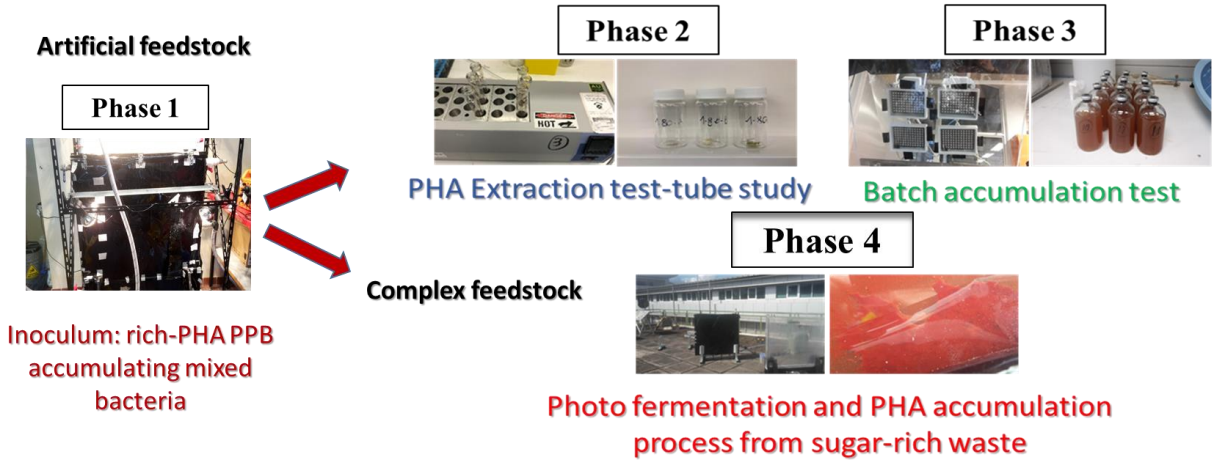


Figure 5.1 the experimental approach of the research

Table 5.1 Summarize of the principal objectives and feedstock of the experimental setup

Set up	Phases	Objectives	Feedstock
Lab-scale flat plate photobioreactor	Phase 1	➢ Selection of rich-PHA PPB accumulation mixed culture ➢ Inoculum preparation	Acetic acid
PHA extraction test-tube study	Phase 2	➢ Determining the optimum extraction procedure specific to PPB-PHA	-
Accumulation batch tests	Phase 3	➢ Effect of different limiting conditions	Acetic acid
Out-door flat plate photobioreactor	Phase 4	➢ PHA accumulating capacity of PPBs growing under the natural light/dark cycle evaluating the effect of using molasses as sole carbon source	Sugar cane molasses

5.3 Material and method

5.3.1 Synthetic Media Preparation: Ormerod's Salts

A defined media (Table 3.3) was used in all synthetic media trials to simulate the domestic wastewater, as the media described by Ormerod et al. The mixture was buffered to pH 7.12 with 667 mg.L-1 KH2PO4 and 900 mg.L-1 K2HPO4. Stock solutions of each individual salt were added to 10L of water that was continuously sparged with N2 to maintain anaerobic conditions

5.3.2 Wastewater characterization

PPB for inoculum were enriched and maintained using domestic wastewater as the sole source. The enrichment process is explained in section 3.3. 800L of wastewater was collected from pump station 118 in St. Lucia, Brisbane and stored at 4 °C where it settled for 24 hours before use. The settling stage removed large solids that would foul the system, and the wastewater characteristics mimicked those of the output of a clarifier as outlined in Table 3.4.

5.3.3 Complex feedstock characterization: sugar cane molasses

A complex sugar feedstock was used as the only carbon source for the photofermentation in a single stage process. Through the fermentation of the sugar cane molasses, the different concentration of VFAs for each cycle is produced. The VFAs were used in the second phase for the PHA accumulation and the mean property of sugar cane molasses are reported in Figure 5.2.

Property	Mean Value
TCOD [g /L]	814.8
NH3-N [mg /L]	0.37
PO4-P [mg /L]	0.14
TS [%]	72
VS [%]	52
K [mg/g]	29
Ca [mg/g]	7.3
Mg [mg/g]	6.6
S [mg/g]	6.4
pH	7

Figure 5.2 sugar cane molasses characterization

5.3.4 Accumulation batch test

5.3.4.1 Starving phase 24 h

Using the selected accumulating mixed PPB culture, taken as inoculum from the 60 L flat plate reactor (50 ml attached biomass), was setup an anaerobic endogenous respiration batch test (Starving phase), where the biomass consumed all the remaining nutriments and, when the

nutriments are depleted, consumed the accumulated polymers. After 24 h of starving phase the PHA content should decrease (to 5-10%) and the inoculum is ready to carry out the batch test. The 50 ml at average conc. (TS 8 g/l) was diluted in 450 ml of RO water at final conc. (TS 8 g/l). Due the 30 % PHA content (roughly), 1.44 g COD-PHA/l conc. In the biomass. The addition of 0.04 g NH4 is required to provide enough feeding to consume the PHA in the cells. After 24 h, once the analysis will testify the depletion of ammonium and phosphate and the PHA content decrease to 5-10 %, the biomass was use as inoculum for the accumulation batch test. Analysis required: SCOD, TCOD, pH, PHA accumulation (To-TF) and ammonium and phosphate kit.

5.3.4.2 Batch test

Biomass taken from (24 h starving phase) was tested with three distinct limiting medium condition, to study the selected PPB PHA accumulation capacity in absence of N, Mg and S. The biomass was mixed with a specific limiting Ormerod's salts (previously prepared) in a 160mL shaker flasks (working volume 140 ml) placed in a custom incubator containing an LSETM shaker set to 130RPM. Four Infra-red LED arrays irradiated the flasks at 820 and 870 nm. The setup is shown in Figure 5.3. Before mixing with the biomass, the three different Stock solutions was prepared, at same carbon source concentration (500 mgCOD/L of Acetic acid) and with distinct limiting condition:

1. Absence of N
2. Absence of Mg
3. Absence of S

Once the different mineral stock solution and the acetic acid were ready, were added to each flask:

- 134.75 ml of mineral solution + HAC and (flush with N2)
- 5.25 ml of Inoculum

Before adding all the medium in the flask, was necessary to check the pH and neutralized at pH 6 (with NaOH) and flush the flasks with N2 for 5 minutes. Then was added the inoculum and it was flushed with N2 again. After that, the flasks were sealed with 20mm aluminium crimp caps

(SUPELCO 508500) to ensure an anaerobic environment. The experiment was performed in parallel and triplicate with a total of 12 flasks. The operating conditions set in the batch tests are summarized below.

Figure 5.3 Batch test setup

5.3.4.3 Analysis required

At each time point (every 5 h) remove the flasks from the shaker take samples with a 10mL syringe (Terumo 1.38*0.3mm needle). Prior to sampling the syringes has to be cleaned with milli-Q water and dried before filling with nitrogen gas. The N2 gas in the syringe is added into the vials to maintain overpressure before each reading. At each time point 10 ml sampling is required to run the requested analysis: (SCOD, TCOD, FIA, VFA, RA and PHA extraction). About TSS-VSS To-TF is enough (ICP – VFA, IC Sulfate) (Table 5.2).

Table 5.2 Analytical method required for the batch test

Analytical methods	
TCOD/SCOD	COD cell test
NH4/PO4	FIA
T/pH	T/pH meter
TS/VS	drying 105/550 °C
PHA	GC-MS

5.3.5 Accumulation Trial - VFA Comparison

Stock solutions without N, Mg and S were prepared and neutralized to pH 7 with NaOH prior to the trial and stored at 4 °C. The inoculum and VFA stock of acetic were added. The experiment was performed in parallel triplicate with a total of 12 vials prepared. The operating conditions set in the batch tests are summarized below in Table 5.3

Table 5.3 operating condition of the batch test

Operative condition		
Vials	n	12
C conc.	mg SCOD HAC/L	500
C Feed strategy	n	**VFA**
	1-3	N limit
	4-6	Mg limit
	7-9	S limit
	8-12	Control
N conc feed	/	/
S/I Ratio	%	83/13
cycle lenght	h	30
Sampling time point	h	5

5.3.6 Study of a single-stage photo fermentation process and biopolymer production from sugar-rich waste

5.3.6.1 Objectives of the study

The principal objectives of this studies:

- Study the PHA accumulating capacity of PPBs growing under the natural light/dark cycle
- Evaluate the effect of using molasses as sole carbon source:

- Photoferentation process
- Possible dark fermentation process (natural light/dark cycle)
- VFA production from fermented sugar
- PHA accumulation from VFA

- Apply the optimized operative condition of the lab photobioreactor fed with artificial feeding and light
- Compare the performance in terms of COD removal, PHA and biomass production

5.3.6.2 Operative conditions

A large scale 60 L out-door flat plate photobioreactor was studied to apply the same operative condition for the production of PHA and for the selection of the accumulating bacteria that were studied in lab-scale. The investigation was carried out under the natural light/dark cycle with a complex sugar feedstock: sugar cane molasses.

Flat-plate photobioreactors have received much attention for cultivation of photosynthetic microorganisms due to their large illumination surface area. Generally, flat-plate photobioreactors are made of transparent materials for maximum utilization of solar light energy. Studying the PHA accumulating capacity of PPBs, grown in IR induced biofilms attached at the walls of the reactor and compare the results with the 2 L continuous reactor, it was possible to have a complete view of the process. Irradiating 60 L photobioreactor (Figure 5.4) with solar light, the same sheets of the other setup were used to filter light, only transmitting bands > 800nm wavelengths on the wall, to lead and allow the PPB to growth. A pump was used to mix the reactor liquid contents at flowrate and the culture dominated by PPB would attach to walls. An alternating time activation system was used to switch on/off the pump every 30 minutes. It was explained the positive effect on the growth in previous studies of the Australian research group. To drain the reactor, a manual valve was used to divert the recycle line to the drain and it is also used for sampling. The operative condition, summarize below (Table 5.4), are divided into different phases: 1) **Inoculum:** use the 30 % of inoculum taken from the selection lab-scale flat plate photobioreactor phase (just start-up phase), 10 % of the liquid of the drain phase each cycle; 2) **Fed phase:** is the 70 % of the total volume (start-up phase) 90 % each cycle of the reactor at fixed concentration of carbon source (500/1000/2000 mg COD/L); 3) **Reaction phase:** is characterized by the activity of bacterial metabolism, that after an acclimation period, starts to

ferment the available SCOD and produce the VFAs. Subsequently, due the external light, the biomass starts to attached to the wall and develops a biofilm growth on it. The feeding regime imposed, based on feast and famine condition with limitation of nitrogen source, should lead the PPB towards the accumulation of PHA from the produced VFAs instead of growth; 4) **Drain phase:** starts every end of the cycle with a slow flowrate and the reactor is drained of the 100 % of the total volume; 6) **Harvest:** of the biomass is carried out whipping directly the 100 % of the biofilm from the wall, the rest of the PPB is scratched of in the liquid phase. 10% of the liquid phase becoming the inoculum for the next cycle.

Table 5.4 60 L out-door flat plate photobioreactor operative condition

Operative condition		
Vol	l	60
Withdraw	%	100
	d	
Cycle length		7
Fed conc.	mg COD Molasses/l	1000 + 500
		1000 + 2000
		2500

Figure 5.4 single-stage 60 L flat plate out-door photo fermentation

5.3.6.3 Overall process parameters

Being the first study with the selected biomass in the laboratory fed through a real substrate, an initial settlement period was carried out to figure it out the adaptation of the mixed PPB culture at the complex sugar feeding. Below are reported in Table 5.5 a resume of the overall process

parameters, from the startup phase until the end of the cycle. The SRT was fixed at 7 days the conditions that was studied is how the concentration of the feeding affect the process with particular attention on the fermentation and accumulation of the polymer. Three different concentration were evaluated: the startup phase was carried out with 500 mgCOD/l of molasses. After few cycles of a good response of the biomass in terms of COD removal, a coupled/uncoupled strategy feeding was carried out in two different COD spike. The first spike added at the T zero at 1000 mgCOD/l concentration and N (35 mg/l), that after 4 days when the ammonia and the carbon source were depleted, the second spike of 500 mgCOD/l was added without N. Than a cycle study was carried out with the same coupled/uncoupled fed strategy but at increased concentration (1000 + 2000 mgCOD/l). After few cycles the last feeding concentration that was evaluated was a single carbon spike of 2500 mgCOD/l with also an increase of the ammonia (from 33 mg/l to 50 mg/l of NH_4Cl).

Table 5.5 Resume of overall process parameters

Feed	SRT	Conc. Fed	NH4Cl	Temp
-	[days]	[mg COD/ l]	[mg/l]	°C
Molasses	7	500	33.3	30-40
Molasses	7	1000+500	33.3	30-40
Molasses	7	1000 + 2000	50	30-40
Molasses	7	2500	50	30-40

5.3.7 PHA extraction test-tube study

5.3.7.1 Objectives of the study

The principal objectives of these studies:

- Determine the optimum extraction procedure specific to PPB-PHA based on the Molecular weight (Mw) of PHA

- Investigate the effect of carotenoids on PHA extractability and properties

5.3.7.2 Experimental protocol

A freeze-dried PPB (Figure 5.6) biomass taken from the lab-scale flat plate photobioreactor (estimated PHA content 25-30 %) has been submitted to the analyses. Seven different extraction temperatures were analyzed for 45min (from 30°C to 90°C) with chloroform. 20 mg biomass/mL were weighted and added the extraction glass tube (Figure 5.5). Filtration was used to separate residue biomass. After the extraction the purification phase was carried out. For carotenoids removal were tested three different chemicals: using acetone/methanol mix or Tetrahydrofuran (THF) or diethyl ether. The study was carried out to determine the best solvent for the carotenoids extraction. The extraction was also repeated in reverse, before the carotenoid extraction and then the PHA extraction to evaluate if the process is affected from one of the steps. The test matrix of the study is reported in Table 5.6.

Table 5.6 Test matrix of PHA extraction test-tube

Study	Removal of carotenoids	Solvents for carotenoid extraction	Extraction temperature (^{o}C)
1	After Extraction	1. Acetone/methanol 2. THF 3. Diethyl ether	30, 40, 50, 60, 70, 80, 90
2	Before Extraction	1. Acetone/methanol 2. THF 3. Diethyl ether	30, 40, 50, 60, 70, 80, 90

Figure 5.6 A freeze-dried PPB biomass

Figure 5.5 Digester and glass extraction tubes

5.4 Results and discussion

5.4.1 Batch test

5.4.1.1 The Effect of different limiting condition on PHA accumulation

Tested limiting condition:

1. Absence of N
2. Absence of Mg
3. Absence of S

In Figure 5.7 is shown the TCOD, SCOD, PHA %wt pH, and temperature data for VFA accumulation batch tests described in previous sections. The overall process and results of the batch test are shown in figure 3.10. The TCOD remained stable over the period of the trial (30 hours) suggesting that no significant amount of COD was lost as gaseous by-products. Temperature was well maintained between 25 and 35 °C and the pH dropped from 7 up to 10 when the acetic was depleted. The relative trends in uptake rates were linear and the acetic acid, the only carbon source was complete depleted at the end of the test. Is recorded a similar situation for the control and the test with absence of Mg, instead a different situation for the S limitation where the acetic was consumed before than the other test. An initial lag phase in a complete nitrogen limitation is detected in the first 10 hours, underlined by an increase in the accumulation only after 10 hours.

This unexpected phase of biomass acclimatization is probably due to a previous temporary stress condition of the biomass in the continuous reactor from where the inoculum was taken.

The maximum accumulation point is reached by the tests fed with acetic acid and with absence of N, reaching from 15 % to 28 %. In all the other tests with the other limitation condition, the PHA accumulation was less. The response in term of PHA accumulation was for Mg limitation from 15 % up to 24 % than for the control from 15 % up to 22 %. The S limitation was the fast condition that reached the maximum PHA accumulation point, at 11 h was from 15 % up to 25 %, but at the end of the cycle went back to 15 %. Regarding the NH4Cl in the tests where was present, after 6 hours was completed depleted for all of the tests. The overall process results are shown in Figure 5.8 and it points out that the N limitation condition, even there was an initial lag phase, was the best performance in terms of max PHA accumulation point. The results of the test, in addition showed and strengthened the selection efficiency of PPB accumulating PHA in the lab-flat plate photobioreactor, underlined how the PPB can be a fundamental resource in the recovery of biomaterials from waste research.

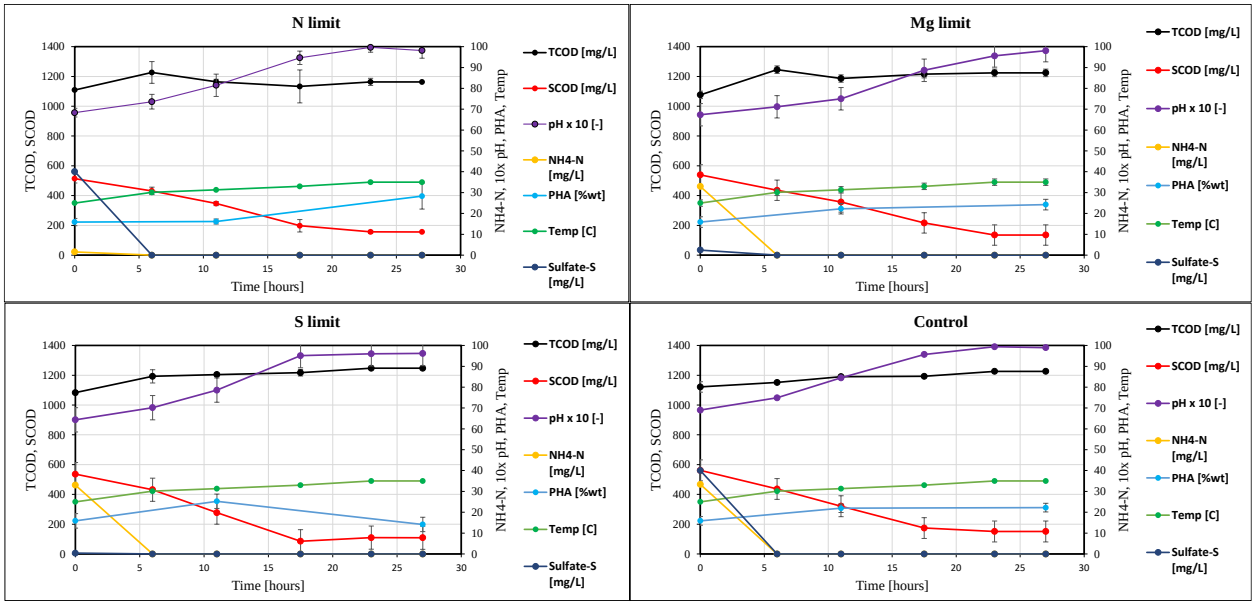


Figure 5.7 Results of the accumulation batch test

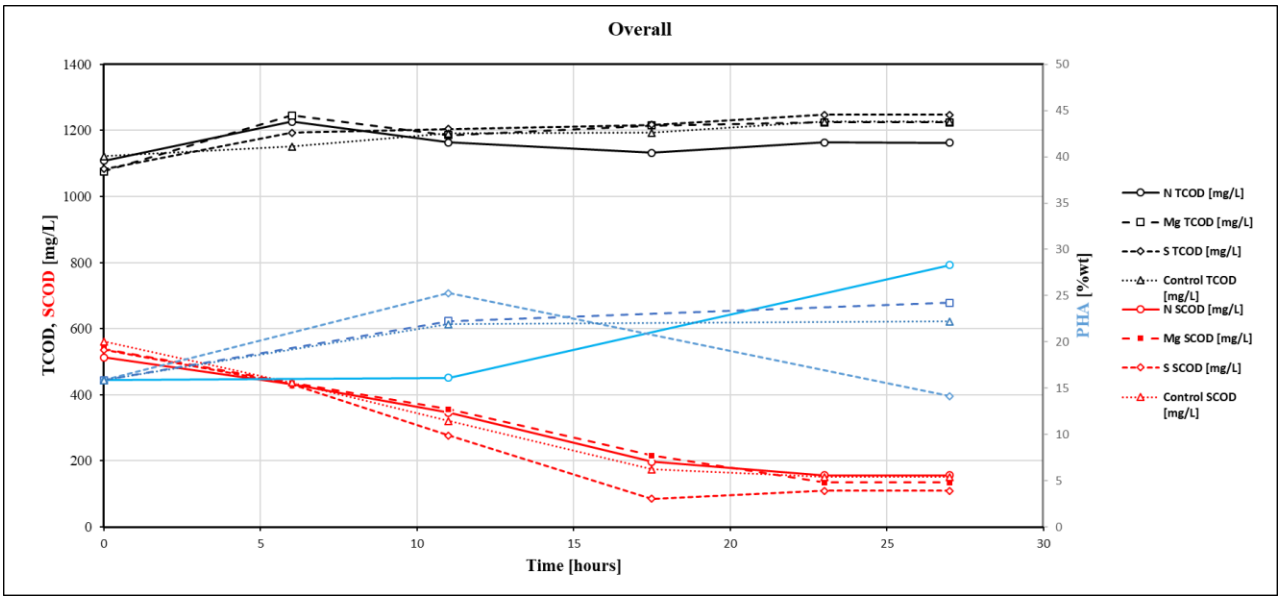


Figure 5.8 Overall results of the accumulation batch test

5.4.2 Study of a single-stage photo fermentation process from sugar-rich waste

5.4.2.1 The startup phase feeding concentration 500 mgCOD/l

The first cycle study of the reactor was carried out to start to understand how the biomass adapts to a new substrate. The PPB inoculum taken from the selection lab-scale photobioreactor, was never tested before with a real waste. Was necessary to test a startup phase to understand how to change and test different operative conditions. Starting with a low concentration molasses feeding (500 mgCOD/l) the reactor was inoculated with the 30 % of selected PPB rich in PHA accumulating biomass. Every cycle a mineral solution was added to the molasses to complete the feeding requirements of the biomass. The first condition that has been studied was the fermentation process. The sampling time point was at 1 h and the analyses required were the High-Performance Liquid Chromatography (HPlC) for the sugar and the Gas chromatography for the VFAs. As is shown in Figure 5.9 at the beginning of the cycle 500 mgCOD/l of molasses were added, for this reason at T0 the results of the HPLC showed 50 mg/l of glucose. The results showed the presence of the peak of sucrose and fructose but was not possible to separate the peak for this cycle. Roughly the concentration of the sugars was: 8 % of glucose (50 mg/l), 35 % of sucrose (175 mg/l) and 10 % of fructose (60 mg/l). By the fermentation of the main sugar the production of VFAs is reported below in the figure. 58 mg/l of formic acid, 56 mg/l of acetic acid and 23 mg/l of lactic acid were

produced. It was found a low concentration also of propionic and succinic acid. All the VFAs were completely consumed in three hours and the acetic was the first acid that has been consumed (2 h), the propionic acid was the last one (3 h). In the startup phase was not conducted the PHA extraction due the preliminary study of the fermentation process.

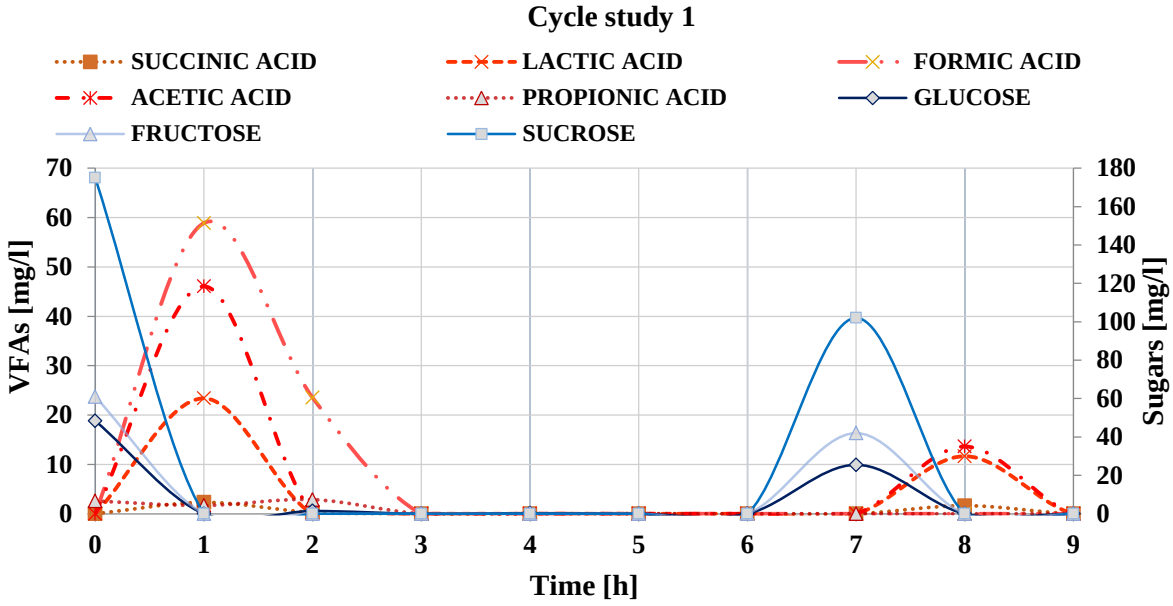


Figure 5.9 Cycle study startup phase: sugar fermentation and VFAs production

5.4.2.2 Phase 2: two carbon spikes Feeding (1000+500 mgCOD/l)

After few days of the start-up phase it was considered necessary to increase the concentration of the feeding. This phase was carried out with two different spike of carbon source, the first one (1000 mgCOD/l) was coupled with N and C added at the same time and second spike (500 mgCOD/l) was uncoupled, the carbon source was added without the nitrogen when the first spike was depleted. The uncoupled feeding strategy was applied to try to increase the PHA accumulation. In the first coupled spike phase was tested if the N feed was enough to grow the required biomass attached on the wall of the reactor and if the C/N ration influenced the PHA content. With the second uncoupled spike, was tested the effect directly on the PHA accumulation, it is supposed that in a complete absence of N all the second spike of C it is converted into biopolymer storage. To verify this assumption, the cycle test was carried out to control the fermentation process, VFAs production and the conversion of organic acids into biopolymer. As described above the HPLC was used for the sugar detention, GC-FID for the VFAs and CG-MS for the PHA characterization. The decreasing of the SCOD showed a good response of the biomass that start to consume the

molasses, in addition the TCOD decreasing in parallel underlined that the biomass is start to grown as a biofilm on the wall of the reactor. As it is shown in Figure 5.10 the first spike was added at T0 and the second after 5 days, concurrently with the additions of the spikes of carbon is recorded a production of VFAs due the fermentation process. As was reported, in the previous cycle, the acetic acid is one of the main products of the fermentation to follow lactic and formic acid. The coupled/uncoupled strategy has demonstrated an effect on the biopolymer accumulation, raising the PHA content from 3 % up to 10 %. Looking furthered into the biopolymer results, is shown in Figure 5.11 that were not significant difference between the PHA content in the attached biomass than the suspended biomass. But it is possible to evaluate that were difference between the PHA composition, in the suspended biomass the PHV content is significantly higher than the attached biomass and the PHB/PHV ratio is consequently lower.

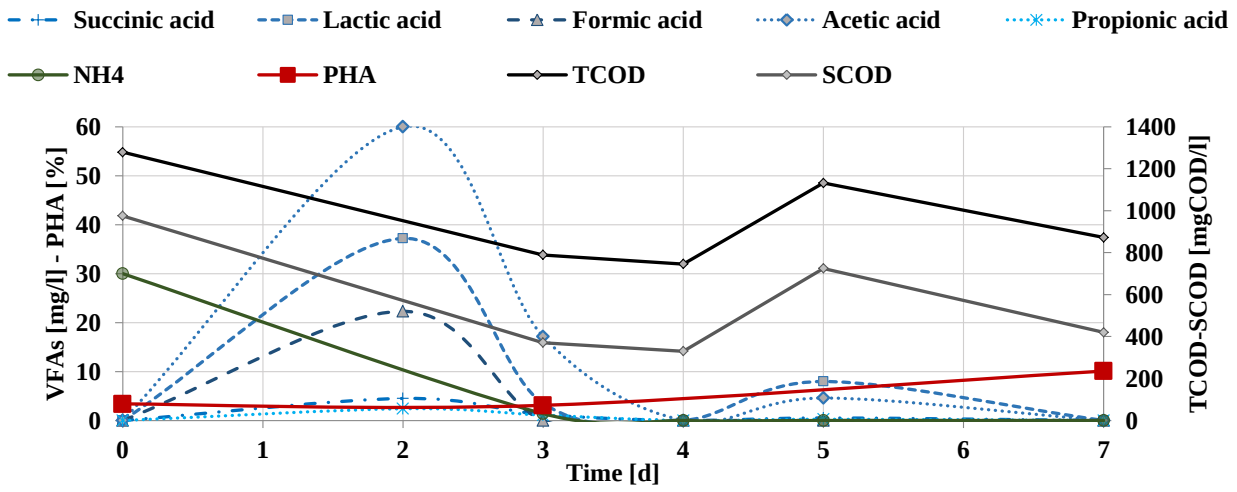


Figure 5.10 Cycle study Phase 2: sugar fermentation, VFAs production and PHA content

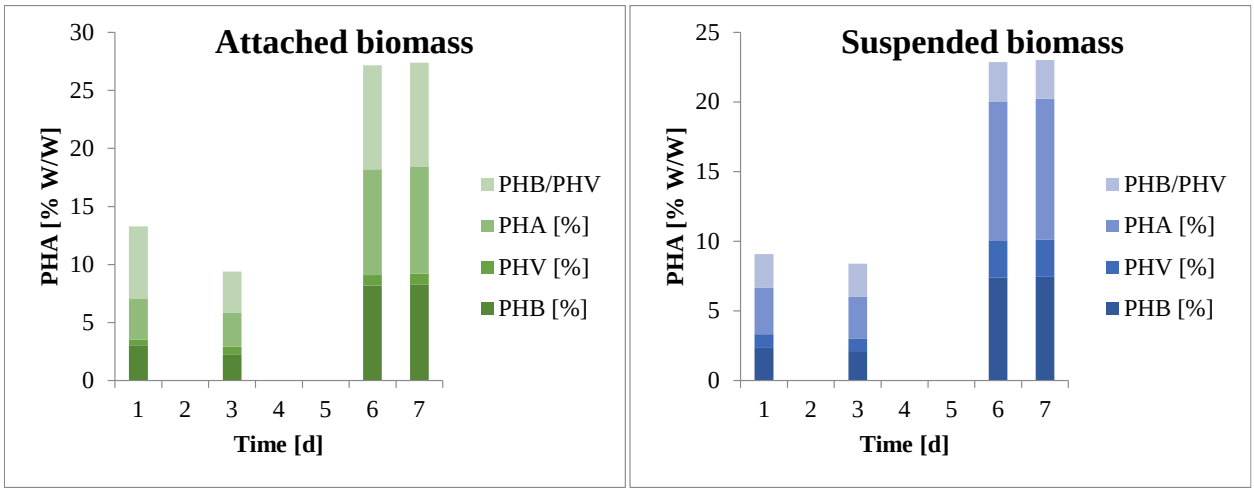


Figure 5.11 The influence of the feeding on the PHA composition during the cycle study: attached and suspended biomass compared

5.4.2.3 Phase 3: two carbon spikes Feeding (1000+2000 mgCOD/l)

After few cycles of the two-carbon spike Feeding phase (1000+500 mgCOD/l) a third phase was carried out to reach the optimization of the process and the maximum accumulation point of PHA. Analyzing the results, it was concluded that probably the biomass needed more VFAs concentration to accumulate raise the PHA content. In the third phase was carried out a coupled/uncoupled feeding strategy with the first spike of carbon source up to 1000 mgCOD/l and the second when all the C and N were depleted just with carbon at 2000 mgCOD/l. The cycle test was carried out to control the fermentation process, VFAs production and the conversion of organic acids into biopolymer. As described above the HPLC was used for the sugar detention, GC-FID for the VFAs and CG-MS for the PHA characterization. The decreasing of the SCOD showed a good response of the biomass that start to consume the molasses, in addition the TCOD decreasing in parallel underlined that the biomass is start to grown as a biofilm on the wall of the reactor. As it is shown in Figure 5.12 the first spike was added at T0 and the second after 4 days, concurrently with the additions of the spikes of carbon is recorded a production of VFAs due the fermentation process. As it shown from the figure the fermentation at the first spike is not associated with an increase of VFAs concentration as expected. But the second spike is associated a substantial increase of VFAs concentration that is suitable for the PHA accumulation. 90 mg/l of acetic, 70 mg/l of lactic acid and 20 mg/l of propionic acid were the VFAs manly produced. After the consumption of the VFAs the PHA content raise up to 13 % due the absence of N. The consumption of COD correlated with

the VFAs depletion is shown in Figure 5.13. The composition of polymer produced is influenced by the type of feeding that is generated from the fermentation, as it is possible to see in Figure 5.14 at the end of the cycle the content of PHV increase due the correlation with the propionic acid that was formed with the second spike of carbon. Comparing with bibliographic results, the PHA concentrations obtained from this study using complex waste as a carbon source, are very promising. The only reported study to date, that produces PHA from real waste by PPB mixed culture with different experimental setups, is reported by fradinho et al., 2019. The PHA content reported were from 10 to 25% in the test with synthetic fermented cheese whey and from10 to 20% with real fermented cheese whey (Fradinho et al., 2019).

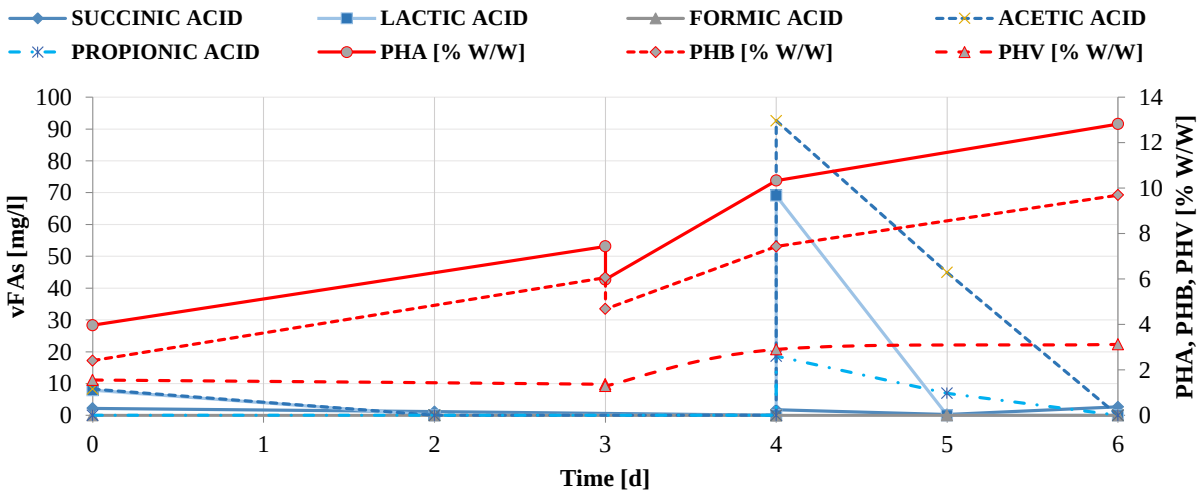


Figure 5.12 Cycle study Phase 3: sugar fermentation, VFAs production and PHA content

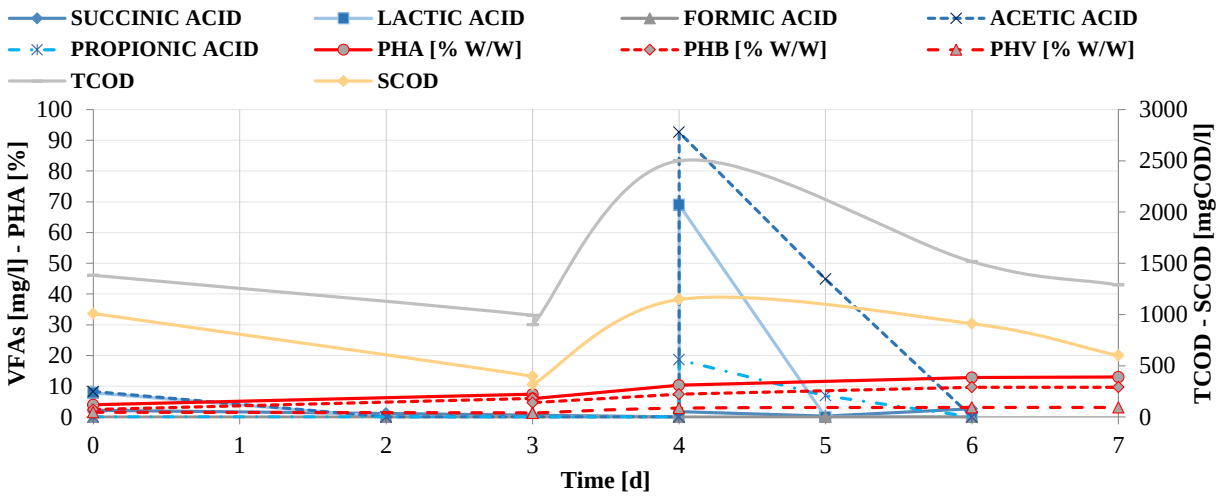


Figure 5.13 Cycle study Phase 3: sugar fermentation, VFAs production and PHA content and COD consumption

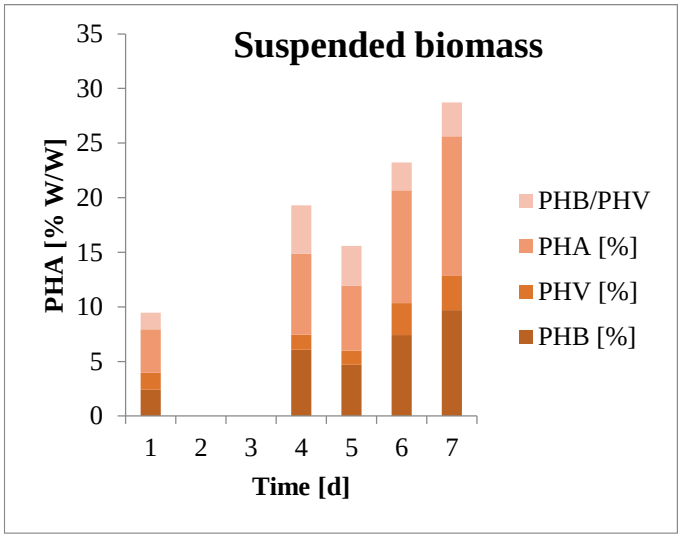


Figure 5.14 The influence of the feeding on the PHA composition during the cycle study

5.4.2.4 Comparison of the different operating condition

A comparison between the startup phase, phase 2 and phase 3 were carried out with the purpose of assessing the current improvement in system performance and evaluate the applied different condition. Overall process is shown in Figure 5.15 the average of total COD removed is 60 % per cycle, the N removal is 100 % per cycle but the PHA content at 13 % was reached just with the phase 3. The second uncoupled feeding at 2000 mgCOD/l had a good effect on the PHA accumulation content. The influence of feeding on the PHA composition is compared in Figure 5.16

were the results shown a slow increase during the cycle of the PHV when the propionic acid is produced from the fermentation. When the acetic is the main acid produced in the fermentation step the PHB is distinctly higher than the PHV. It can be concluded that there was a significantly correlation between propionic acid and composition of PHV and in general is possible to manipulate the polymer composition from the feeding concentration.

The overall process and the 3 different phases are summarized below in Table 5.7 Overall photofermentation process summarized. The increasing of the feeding had an evident impact on the growth of the biomass on the wall where the last harvest was roughly 285 g (wet weight). The coupled/uncoupled strategy affected the PHA content that raised up to 13 % (w/w). The point of weakness of the process that has to be optimize is the COD match parameters that further study needs to solve. The COD match % (Tot COD in/Tot COD out) in all the studied phases, is relevant high. This parameter showed a high loss of COD during the process probably due biogas production. High losses of COD could have a negative effect on the PHA accumulation and it need more investigation.

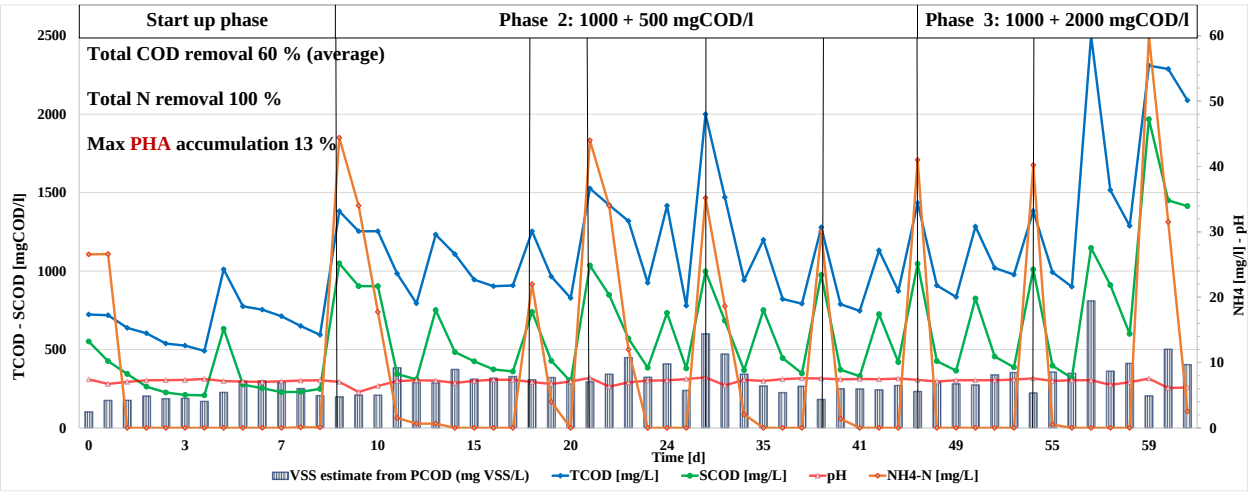


Figure 5.15 Overall process of single-stage photo fermentation from sugar-rich waste

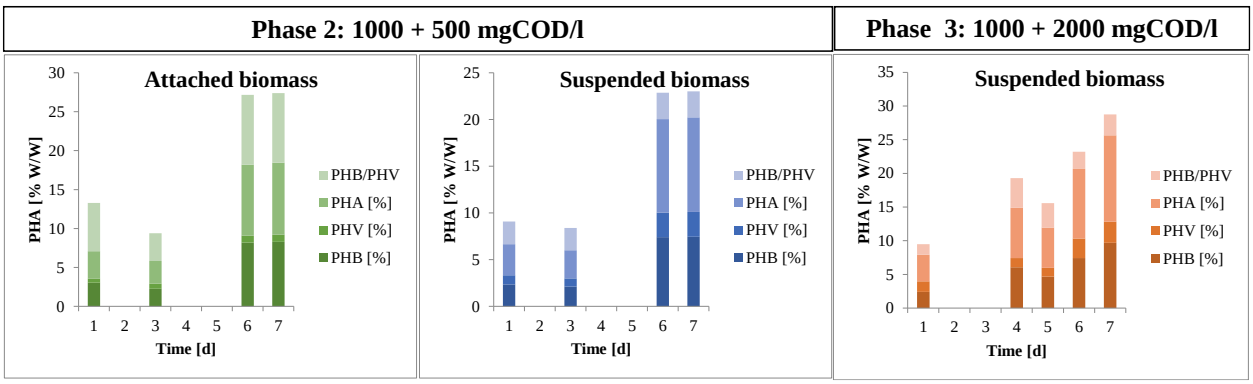


Figure 5.16 The influence of the feeding on the PHA composition compared

Table 5.7 Overall photofermentation process summarized

Cycle (#)	Run Time (d)	Total COD in (g)	Total COD out (g)	Total harvest (g)	COD Match %	TCOD removal %	SCOD removal %	PHA %
1	9,2	104,0	35,6			41,3	60,6	2
5	38,1	191,9	70,3	186	0,37	52,9	63,1	3
6	44,4	144,7	89,7	200	0,62	41,7	66,2	10
7	52,2	163,1	83,9	195	0,51	41,8	65,1	5
8	58,9	233,0	109,8	285,3	0,47	34,9	68,5	13

5.4.3 Polymer extraction and characterization

5.4.3.1 Thermal Gravimetric Analysis (TGA)

As described before the TGA methodology for the PHA detection is less accurate than the GC-MS analysis that can specify the type of polymer and the specific concentration. But is a fast screening to roughly know the presence of the polymer and an average percentage of PHA content. The GC-MS required a long process (freeze-dry, long sample preparation and more than 20 hours' extraction) for a more accurate response, the TGA was useful for a preliminary screening during the cycle. When the results of TGA showed the presence of good quantity of PHA, subsequent analysis were carried out to specify the PHB/PHV content produced.

The Figure 5.17, shows the TGA results at the beginning of the second phase of the single stage fermentation photobioreactor. Was a preliminary study to evaluate the PHA content. The curve of the PHA showed the presence of impurities which thermally degrade across the temperature range. A weight loss was observed from the 250 – 320 region that is the PHA region referenced by the TGA curve of commercial PHA. This was carried out to verify that the polymer produced was

comparable with polymers of the same family already analyzed. After this analysis different cycle study of the reactor were carried out to deepen investigations.

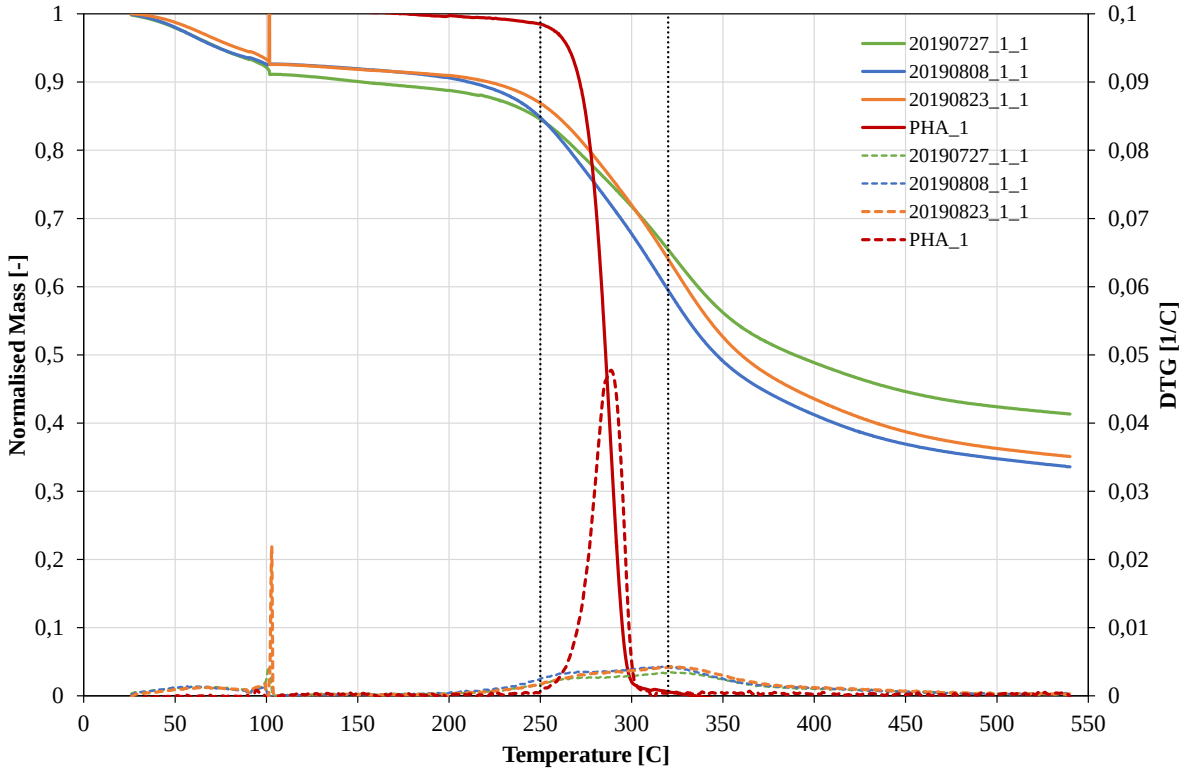


Figure 5.17 TGA results of the single stage fermentation photobioreactor

5.4.4 PHA extraction test-tube study

All the results of the PHA extraction test-tube study are summarized below in Table 5.8. At each PHA chloroform extraction temperature 3 different solvent were tested for the carotenoids extraction. Further analysis is required for the optimization of the extraction but higher temperature and the acetone/methanol extraction seems to be the best combination in terms of recovery, purification and molecular weight.

Molecular weight is one of the most important properties of polymeric materials, as it can significantly affect physical and mechanical characteristics. PHA molecules with higher molecular weights are desirable for practical applications, especially considering that decreases in molecular weight have been reported during PHA extraction and purification processes (Ramsay et al., 1990).

Table 5.8 PHA extraction test-tube study

Carotenoid Solvent Extraction →	**Acetone/Methanol ***			**THF**			**Diethyl ether**		
PHA Extraction in Chloroform Temperature	**Mn (kDa)**	**Mw (kDa)**	**PDI**	**Mn (kDa)**	**Mw (kDa)**	**PDI**	**Mn (kDa)**	**Mw (kDa)**	**PDI**
30°C	762 (±271)	**1,924 (±120)**	2.9 (±1.2)	583 (±14)	1,771 (±92)	3.0 (±0.1)	534 (±31)	1,744 (±89)	3.3 (±0.1)
40°C	905 (±110)	**2,061 (±54)**	2.3 (±0.2)	547 (±35)	1,989 (±80)	3.6 (±0.1)	579 (±2)	1,950 (±40)	3.4 (±0.1)
50°C	676 (±190)	**2,038 (±76)**	3.2 (±0.7)	540 (±25)	2,036 (±47)	3.8 (±0.2)	483 (±37)	1,955 (±37)	4.1 (±0.4)
60°C	844 (±171)	**2,111 (±82)**	2.6 (±0.5)	626 (±56)	2,058 (±55)	3.3 (±0.2)	593 (±28)	2,037 (±44)	3.4 (±0.1)
70°C	863 (±205)	**2,233 (±102)**	2.7 (±0.7)	575 (±55)	2,125 (±29)	3.7 (±0.3)	566 (±26)	2,077 (±75)	3.7 (±0.1)
80°C	895 (±225)	**2,316 (±146)**	2.8 (±0.7)	579 (±57)	2,139 (±99)	3.7 (±0.2)	605 (±55)	2,167 (±69)	3.6 (±0.2)
90°C	884 (±245)	**2,321 (±172)**	2.8 (±0.6)	549 (±26)	2,164 (±61)	4.0 (±0.2)	595 (±21)	2,217 (±38)	3.7 (±0.2)

A study conducted by Ramsay et al., 1990, has studied the Photosynthetic purple bacteria strains were cultured in growth medium and intracellular PHAs under growth conditions were analysed. The number-average molecular weight of the purified PHAs was between 3,000 and 994,000 g/mol, and the PDI was between 1.5 and 6.7. The PDI values were lower in photosynthetic purple bacteria that synthesized lower molecular weights of PHAs such as *Rps. marina, Ros. marina and Ros. goensis*. The results of this study, showed in the table above, correspond with was what found in the bibliography research. Moreover, another interesting result, is that the molecular weight values are obtained from mixed bacterial cultures and not from pure cultures, as in the above mentioned article. This showed how it is possible to obtain a high molecular weight polymer through mixed bacterial cultures with good chemical-physical characteristics.

5.5 Conclusion

Exploring for the first time in the research group the use of selected PHA-accumulating PPB with a real and complex feedstock, the objectives set have been achieved. The purpose of the present work was the study of a new experimental setup carried out to test a single-stage photo fermentation process from sugar-rich waste and biopolymer production. The results shown a good adaptation of the biomass to a new type of feeding and new applied condition. The biomass selected in the lab-scale flat plate reactor was used in a new set up: single stage out-door flat plate photobioreactor for the fermentation of molasses and biopolymer production. the conditions that have mainly changed compared to the laboratory-scale reactor are: natural lighting with day/night cycles, complex sugar feeding and the fermentation process. The operative condition studied in this research had shown a potential capacity of PPB in the PHA accumulation process from fermented molasses, reaching more than 13 % (W/W) content of PHA in the microbial cells and 61 % of COD removal. Even if the biopolymer content was not the highest in literature, was a promising and innovating results for the novel out-door 60 L photobioreactor setup and for the study of the mixed PPB culture. There are not many studies on the PPB mixed culture reaching more than 13 % of PHA content in the cells from fermented molasses with an out-door large scale reactor.

The results of the batch tests have confirmed the same accumulation capacity of the lab-selected culture strengthens the potential application of this experimental setup reaching more than 28 % of PHA content. Furthermore, the N limitation batch test has shown the greater effect on the PHA

accumulation, for this reason was applied the coupled/uncoupled feeding strategy characterize of two separate spike of C feeding, the first one combined with N the second alone.

Specifically, the average of total COD removed is 60 % per cycle, the N removal is 100 % per cycle but the PHA content at 13 % was reached just with the phase 3 (1000 + 2000 mgCOD/l). The second uncoupled feeding at 2000 mgCOD/l had a good effect on the PHA accumulation content. The influence of feeding on the PHA composition showed a slow increase of the PHV during the cycle when the propionic acid is produced from the fermentation. When the acetic is the main acid produced in the fermentation step, the PHB is distinctly higher than the PHV. It can be concluded that there was a significantly correlation between propionic acid and composition of PHV and in general is possible to manipulate the polymer composition from the feeding concentration.

The PPB are strongly considered for future research and application, for the possibility of compensating the low yield of mixed culture, with a lower process cost, the PPB are an interesting research challenge for industrial application in the biopolymer market. By removing the oxygen supplying, by the use of the sun light during the process and by the using of complex waste like molasses as a feedstock, the production cost of the process is strongly decreased. The final scope of this research was to explore new ways of producing low-cost polymers to make the industrial production of these bioplastics economically feasible.

6 General conclusion e future prospective

6.1 General conclusion

The research on the application of mixed microbial cultures (aerobic and anaerobic) for PHA production, have not yet achieved satisfactory results and needs further investigations applying waste complex feedstock in the process. The experimental campaign of this project was developed in order to acquire the information necessary to derive the kinetics that describe the production processes of biopolymers, not only to evaluate the yields but also in qualitative terms, by different engineering processing. Two different mixed culture were compared: MMC (AS) taken from a WWTP and the PPB enriched from wastewater. Two different set up were tested: an aerobic SBR for the selection of the biomass and an anaerobic flat plate photobioreactor. It can be concluded that the operating conditions adopted with SBR3, with an organic load set at 100 mmolC / ld, were those that led to the best performance of the biomass selection and PHA production process, both

in terms of productivity and polymer storage yields. PHA was reflected by a maximum accumulation percentage from 1 % up to 5 % (w/w) in 8 hours and a complete removal of C and N from the reactor.

The operative condition studied in this research had shown a potential capacity of PPB in the PHA accumulation process. A continuous 2-L bench scale photo-anaerobic membrane reactor (PAnMBR), reaching more than 15 % (W/W) content of PHA in the microbial cells and 61 % of COD removal and a complete N removal in each cycle.

The operative condition studied for the application of a complex sugar feedstock, in this research had shown a potential capacity of PPB in the PHA accumulation process from fermented molasses, reaching more than 13 % (W/W) content of PHA in the microbial cells and 61 % of COD removal. But The results of the batch test, with synthetic media, has confirmed the same accumulation capacity of the lab-selected culture strengthens the potential application of this experimental setup reaching more than 28 % of PHA content. The outcome of this study underlined the PPB as one of the main interesting mixed culture for industrial application in the biopolymer market and represent a significative challenge for the related research.

6.2 Future prospective

Further research is required to consolidate the achieved outcome and is necessary to understand the complex biopolymer production process and to obtain a better understanding of the unknown metabolic pathways in microbial mixed culture. Better knowledge and experience in the field of PHAs is the basis to be reached for the integration of the biorefinery concept in the commercial production chain. However, as previously mentioned, a paradigm shift in wastewater treatment could lead to rethinking the operation of these plants on the basis of the principles of the green economy and the concepts of biorefinery, directing the treatment process to the production of new materials, to recovery of elements such as nitrogen, and the production of biofuels. The outcome of this study underlined the PPB as one of the main interesting research challenges for industrial application in the biopolymer market. The numerous applications of these bacterial species and the ability to adapt to different conditions make these bacteria an innovative resource. The possibility

of exploring many types of organic waste in the production of biopolymers opens up large and relevant research scenarios, that have not yet been investigated.

A future perspective that seems to be the most interesting in this project is the integration of the process scheme with co-digestion of activated sludge (inoculum) and whey (substrate) for the production of bio-H_2 with the PPB capacity on the biopolymer production. As was explained before, the rich-VFA effluent produced by the dark fermentation process, it can be reinvested as a feed for the PPB-biopolymer production phase. Optimizing the two research paths that have given better results and studying the feasibility of an industrial application, a valid alternative to the conventional production of biopolymers can be found. By removing the oxygen supplying, by the use of the sun light during the process and by the using of complex waste like the effluent of the dark fermentation, the production cost of the process is strongly decreased.

Investigating the feasibility of applying the bio-refinery approach to bio-sludge from WWT and organic waste, through the analysis of a combined process for converting organic waste (agro-industrial wastes and sewage sludge) into biofuels (bio-H_2) and biomaterials (biopolymers).

7 References

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