Universidade de Aveiro Departamento de Química
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Production of bioplastics from hydrolysates of paper industry by *Haloferax mediterranei*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Luísa Seuanes Serafim, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e do Engenheiro Alexandre M. R. Gaspar, do Instituto de Investigação da Floresta e Papel – RAIZ.

o júri

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palavras-chave

Hidrolisado da pasta de papel, glucose, xilose, polihidroxialcanoatos, *Haloferax mediterranei*

resumo

O conceito de biorrefinaria tem atraído muita atenção na última década devido ao aumento das preocupações ao nível do uso de combustíveis fósseis. Neste contexto emergiu o uso de bioplásticos, nomeadamente polihidroxialcanoatos (PHA). Os PHA são plásticos biodegradáveis e biocompatíveis que podem ser obtidos a partir de matérias-primas renováveis e podem constituir uma solução alternativa para os plásticos convencionais. Neste trabalho, o hidrolisado da pasta de papel, proveninte do cozimento da madeira de *Eucalyptus globulus*, foi usado como substrato para a bactéria *Haloferax mediterranei* produtora de PHA. O hidrolisado é rico em açúcares simples, principalmente glucose (81,96 g/L) e xilose (20,90 g/L).

Realizaram-se vários ensaios em meio definido com glucose e xilose e em hidrolisado suplementado com sais e extracto de levedura. Testaram-se diferentes concentrações de glucose, nomeadamente, 10, 15, 20, 30 e 40 g/L. Os melhores resultados de acumulação (27,1 % of PHA) foram obtidos em meio hidrolisado com 10 g/L. Utilizando esta concentração, foram realizados ensaios em "fed-batch" e em "sequencing batch reactor", de modo a determinar a melhor estratégia de alimentação de *H. mediterranei*. A estratégia que levou à obtenção dos melhores resultados foi o ensaio em fed-batch com 24,7 % de PHA. Foi também testado o processo sem condições estéreis, em que se obteve um crescimento idêntico ao ensaio com esterilização. Por fim, foi realizado um ensaio em biorreactor, onde se observou um rápido crescimento (0,14 h^{-1}), com elevados consumos de glucose e xilose (0,368 g/(L.h) e 0,0947 g/(L.h), respectivamente). No entanto, foram obtidos 1,50 g/L de PHA, correspondendo a 16,1 % (92,52 % de 3HB e 3HV de 7,48 %) de % PHA. O polímero foi ainda caracterizado por DSC tendo-se obtido uma temperatura de transição vítrea de -6,07 ºC, uma temperatura de fusão de 156,3 ºC e uma entalpia de fusão de 63,07 J/g, valores que estão de acordo com a literatura.

Neste trabalho foi verificada pela primeira vez a adequação do hidrolisado da pasta de papel como meio para produção de PHA por *H. mediterranei*.

keywords Paper pulp hydrolysate, glucose, xylose, polyhydroxyalkanoates, *Haloferax mediterranei*

abstract

The biorefinery concept has attracted much attention over the last decade due to increasing concerns about the use of fossil resources. In this context emerged the use of bioplastics, namely polyhydroxyalkanoates (PHA). PHA are biocompatible and biodegradable plastics that can be obtained from renewable raw materials and can constitute an alternative solution to conventional plastics. In this work, hydrolysed cellulose pulp, coming from *Eucalyptus globulus* wood cooking, was used as substrate to the PHA-storing bacteria *Haloferax mediterranei*. The hydrolysed pulp is rich in simple sugars, mainly glucose (81.96 $g.L^{-1}$) and xylose (20.90 $g.L^{-1}$).

Tests were made in defined medium with glucose and xylose and in hydrolysate supplemented with salts and yeast extract. Different concentrations of glucose were tested, namely 10, 15, 20, 30 and 40 $g.L^{-1}$. The best accumulation results (27.1 % of PHA) were obtained in hydrolysate medium with 10 $g.L^{-1}$. Using this concentration, assays were performed in fed-batch and sequencing batch reactor conditions in order to determine the best feeding strategy. The strategy that led to the best results was fed-batch assay with 24.7 % of PHA. An assay without sterile conditions was performed, in which was obtained the same growth than in sterilization test. Finally it was performed an assay in a bioreactor and a fast growth (0.14 h^{-1}) with high glucose and xylose consumption rates (0.368 $g.L^{-1}.h^{-1}$ and 0.0947 $g.L^{-1}.h^{-1}$, respectively) were obtained. However 1.50 $g.L^{-1}$ of PHA, corresponding to 16.1 % (92.52 % of 3HB and 3HV of 7.48 %) of % PHA were observed. The polymer was further characterized by DSC with a glass transition temperature of -6.07 °C, a melting temperature of 156.3 °C and a melting enthalpy of 63.07 $J.g^{-1}$, values that are in accordance with the literature.

This work recognizes for the first time the suitability of the pulp paper hydrolysate as a substrate for PHA production by *H. mediterranei*.

Contents

Index of figures

Index of tables

Abbreviations

3HB	3-Hydroxybutyrate
3HV	3-Hydroxyvalerate
ACP	Acyl Carrier Protein
BEKP	Bleached Eucalyptus Kraft Pulp
CoA	Coenzyme A
CTMP	Chemi-Thermo-Mechanical Pulping
DSC	Differential Scanning Calorimeter
DSMZ	German Collection of Microorganisms and Cell Cultures (In German: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)
EIA	Energy Information Administration
EPA	United States Environmental Protection Agency
EPS	Exopolysaccharide
FPU/g_{HC}	Filter Paper Unit / Carbohydrates grams
GC-FID	Gas Chromatography – Flame Ionization Detector
[Glc]	Glucose concentration
$[Glc]_{cons}$	Glucose concentration consumed
$[Glc]_{in}$	Initial glucose concentration
HA	Hydroxyalkanoates
HPLC	High-Performance Liquid Chromatography
ICNF	Instituto da Conservação da Natureza e das Florestas (in English: Institute of Nature and Forestry Conservation)
lclPHA	long chain length PHA
mclPHA	medium chain length PHA
[P]	Polymer concentration
$[P]_{fi}$	Final polymer concentration
P(3HB)	Poly-3-hydroxybutyrate
P(3HB-*co*-3HV)	Poly-3hydroxybutyrate-*co*-3-hydroxyvalerate

P(3HB-*co*-3HV-*co*-4HB)	Poly-3hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate
P(4HB)	Poly-4-hydroxybutyrate
PGW	Pressure Groudwood Pulping
PHA	Polyhydroxyalkanoates
% PHA	PHA content
RAIZ	Instituto de Investigação da Floresta e do Papel (in English: Research Institute of Forest and Paper)
$\mathbf{r_{Glc}}$	Glucose consumption rate
$\mathbf{r_{Prod}}$	Polymer production rate
$\mathbf{r_{Xyl}}$	Xylose consumption rate
[S]	Substrate concentration
$\mathbf{[S]_{cons}}$	Substrate concentration consumed
$\mathbf{[S]_{in}}$	Initial substrate concentration
SBR	Sequencing Batch Reactor
sclPHA	short chain length PHA
SGW	Stone Groudwood Pulping
TMP	Thermo-Mechanical Pulping
USA	United States of America
UWF	Uncoated WoodFree
VSS	Volatile Suspended Solids
[X]	Biomass concentration
$\mathbf{[X]_{fi}}$	Final biomass concentration
$\mathbf{[X]_{in}}$	Initial biomass concentration
[Xyl]	Xylose concentration
$\mathbf{[Xyl]_{cons}}$	Xylose concentration consumed
$\mathbf{[Xyl]_{in}}$	Initial xylose concentration
μ	Specific growth rate
$\mathbf{\mu_{max}}$	Maximum specific growth rate

CHAPTER I

Introduction

I. General introduction

During the last century there was not only a significant increase in operating mass of fossil fuels and pollution levels, as well as the fluctuation in the price per barrel and a consequent dwindling oil reserves, leading to serious problems both environmentally and economic (Owen et al. 2010). All these factors point to a need for a progressive increase in the use of renewable sources as an alternative to fossil fuels – biorefinery (Octave & Thomas 2009; Carreira et al. 2011).

One of these applications is the bioplastics, plastics obtained from raw materials (usually derived from biomass), which differ from conventional plastics because of their biocompatibility and biodegradability (Bugnicourt et al. 2014). Among the various kinds of bioplastics, are polyhydroxyalkanoates (PHA) that have attracted more interest in the substitution of plastics derived from fossil fuels (Steinbuchel 2005). PHA polyesters are several hydroxyalkanoates (HA) synthesized by various species of microorganisms, including bacteria, as a source of carbon and energy reserve (Philip et al. 2007). Poly-3-hydroxybutyrate, P(3HB), was the first PHA to be discovered (Lemoigne 1926) and is also the most studied and well characterized (Steinbuchel & Fuchtenbusch 1998). However, there are another PHA with better properties, for instance the copolymer Poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate, P(3HB-*co*-3HV), which is a bioplastic less crystalline, more resistant and more flexible than P(3HB) (Lu et al. 2008).

Nevertheless their production is expensive, still unable to compete commercially with conventional synthetic petrochemical-based polymers. One of the best factors that contributes to the high costs is the carbon source (Choi & Lee 1999). In response to this many efforts have been made to find cheaper sources, like for example extruded rice bran (Huang et al. 2006), whey (Koller et al. 2007b), vinasse (Bhattacharyya et al. 2012), among others.

In this work it will be used pulp from pulp industry to produce PHA, due to its composition rich in sugars such as glucose and xylose, which makes it a raw material with biotechnological interest. To make this possible, it will be used the microorganism *Haloferax mediterranei*, an archabacteria that grows with high salts concentrations, which reduces significantly the costs of sterilization.

II. State of the art

1. Biorefinery

Petroleum is the raw material most frequently used. Nevertheless, it is neither sustainable, because of its limited reserves, nor environmental friendly (Kamm & Kamm 2007). Since the beginning of the industrialization, fossil fuels have been largely used in many applications (Owen et al. 2010). Herewith, the concept of petroleum refinery arises, which comprises a lot of different and complicated processes with various possible configurations (Al-Shammari & Ba-Shammakh 2011). Many efforts have been made to improve and optimize refinery process, in order to maximize company profit margins and to keep on in the competitive market (Al-Shammari & Ba-Shammakh 2011). Nevertheless, this strong dependence on fossil fuels, due to the high consumption of petroleum derivatives products, can lead to a decrease of petroleum resources, an increase of petroleum price and a bigger environmental and politics concerns (Steinbuchel 2005; Cherubini 2010). Data from Energy Information Administration (EIA) shows the increase of imported crude oil prices since 1990 until September 2014 (Figure 1). Besides the crude oil price is decreasing during the last four years, there is a significant increase it in last thirty years, around 500 % (EIA).

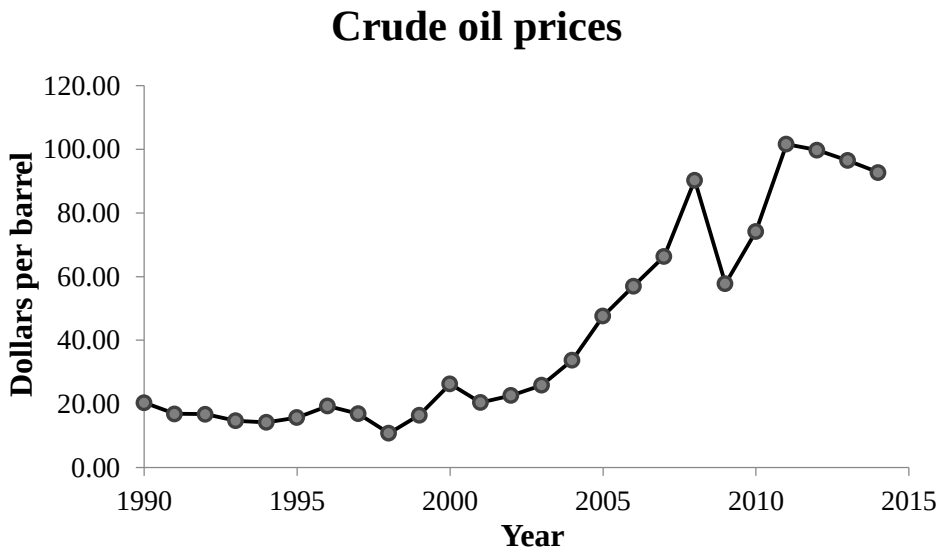


Figure 1: Fluctuation of crude oil prices since 1990 (EIA).

Furthermore, the emissions of greenhouse gases are unbalancing the Earth's climate, due to emission of, for instance, carbon dioxide , methane and nitrous oxide from fossil fuels combustion and changes of land-use as a result of human activities (Piemonte 2012). Therefore, it is important to seek for alternatives to the oil economy that use renewable resources. Consequently the biorefinery concept emerged (Octave & Thomas 2009). A biorefinery consists on an installation which integrates biomass conversion processes and equipment to produce energy (e.g. biofuels, heat), molecules (e.g. fine chemistry, cosmetics), materials (e.g. bioplastics, composites) and further on from biomass (Octave & Thomas 2009). A biorefinery consists in the utilization of different technologies to convert biomass resources, like wood, grasses or corn, into building blocks, such as carbohydrates, proteins or triglycerides, and these can be converted into value-added products, biofuels or chemicals (Cherubini 2010). The biorefinery concept is analogous to petroleum refineries, which produce multiple fuels and petroleum derivatives products (Cherubini 2010).

1.1 Generations of biorefinery

In order to reduce the disadvantages and to improve the benefits of the proceeding generation, several generations of biofuels have been developed. However, the appearance of these new generations have also came with new challenges (Holden & Gilpin 2013).

Biorefinery can be classified depending on their biomass feedstock and according to different technologies which support.

1.1.1 First generation.

First generation biorefineries use conventional technologies and their principal products are biofuels obtained from sugar, starch, vegetable oils and animal fats (Demirbas 2009). The main feedstocks comprise seeds and grains, like wheat or corn (Cherubini 2010) and the main products are bioethanol, biodiesel, starch-derived biogas, and others (Demirbas 2009). The great advantage of this is the high sugar and oil content of feedstocks and how easy it is to convert these into the different products, such as bioethanol. However, this type of biorefineries use food crops, raising concerns about world hunger and shortages of some foods (Cherubini 2010).

1.1.2 Second generation

Second generation uses advanced technologies and include biofuels obtained from lignocellulosic materials, such as agriculture residues, forestry residues or industry residues, non-food crops, wood, and others (Cherubini 2010; Demirbas 2009). Contrarily to the first generation that only uses a small portion of plant, the second generation biorefinery uses the whole plant as feedstock, except the edible part (Cherubini 2010). However, the land needed to food crops is occupied by cultivation of terrestrial plants (Goh & Lee 2010).

1.1.3 Third generation

Third generation is based on marine algae and seaweeds (Goh & Lee 2010), in other words, consists on a conversion of algae in biofuels (Demirbas 2009). Macroalgae contain high contents of sugar which can be used for the production of biofuels, such as bioethanol (Goh & Lee 2010). Furthermore, algae converts solar energy into chemical energy very efficiently, thus, it is easier to convert biomass in biofuels in third generation than in first and second generation biorefinery (Liew et al. 2014). Nevertheless, this type of biorefinery requires the development of technology to extract the oil. Due to the low efficiency of the harvesting technology, the biofuel production is not, yet, economical viable (Liew et al. 2014).

1.1.4 Fourth generation

Fourth generation biorefineries uses genetically modified feedstocks and algae metabolically engineered to biofuel production (Holden & Gilpin 2013). In this biorefinery recombinant DNA and other biological and bioengineering techniques, like introduction, deletion and modification of algal metabolic networks, are used for direct modification of cellular metabolism and properties and to create or enhance biofuel production (Lü et al. 2011). Fourth generation biorefineries are still poorly studied, namely in terms of practical performance (technical and economic aspects) (Liew et al. 2014).

There are several pros and cons (Table 1) from the first to fourth generation in terms of technical and economic performance, social impact (as food competition), feedstock potential (as availability and oil content), among others.

Table 1: Advantages and disadvantages of different generations of biofuel (adapted from Liew et al. 2014).

Fuel	Advantages	Disadvantages
Petroleum fuel	• High availability • Established technologies	• Depletion of fossil fuel • Causing climate change • Fluctuation of fossil fuel price • Higher carbon footprint than biofuel
First generation biofuel	• Biodegradable • Energy security	• Competition of land use • Blending with conventional fuel • Highest carbon footprint compared with other generations of biofuel, but lower than petroleum
Second generation biofuel	• No food competition • Production of high-value added products • Energy security	• Complex processes are required • Conversion technologies are under development • Low conversion as compared with petroleum fuel
Third generation biofuel	• High oil yield • No food nor land competition • No toxic content • Energy security	• High processing cost • Production technology is under development • Difficulty in harvesting and processing
Fourth generation biofuel	• Carbon negative biofuel • Energy security	• Lack of study on this practical performance in terms of technical and economic aspects • Still in research and development stage

2. Bioplastics

One of the most important applications of fossil resources is the production of plastics, which have a great role on the major part of industries, from food and hygiene to medicine. Due to their versatility and low production costs, these plastics are promising substitutes compared to other materials such as paper, glass and wood (Urtuvia et al. 2014). Synthetic plastics can be manipulated in terms of their chemical structure, and thus acquire different forms, due to be produced by an synthetic manner (Reddy et al. 2003).

These plastics are resistant to degradation and have some versatile characteristics of lightness, durability and resistance (Khanna & Srivastava 2005a). Plastics appear with a better coating and packaging properties than other materials, such as glass or paper (Lange & Wyser 2003). Nevertheless, the polluting oil exploration associated with the production of plastic, and problems about the accumulation of recalcitrant plastics in the environment are a worldwide problem. The half-life of plastics is up to > 500 years (Urtuvia et al. 2014). So, it is imperative to find an alternative to them (Lee et al. 1991). Therefore, there are many efforts to discover and develop degradable plastics in order to enhance the biodegradability of the plastics in landfills and composts (Urtuvia et al. 2014). These bioplastics differ from conventional plastics due to their raw material, because they can be produced from a wide range of renewable resources, namely wastes or non-food competing sources. The main advantage in terms economics for industries and municipalities is the saving of raw materials and the reduction in production costs (Bugnicourt et al. 2014). Furthermore bioplastics are biodegradable, because some of these plastics can be degraded by the microorganisms which synthesize them as internal carbon and energy sources (Steinbuchel 2005). It is estimated that in 2016 the production of bioplastics will increase up to 500% of its current capacity (European Bioplastics).

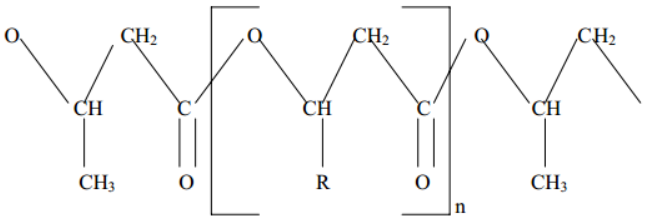
2.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are an example of bioplastics with an interesting potential for the substitution of fossil fuels-derived plastics (Steinbuchel 2005). Biodegradability, biocompatibility, chemical-diversity and manufacture from renewable carbon resources are the principal characteristics that distinguish PHA from petroleum-derived plastics (Boopathy 2000; Tan et al. 2014). PHA have also promising properties such as water insolubility, resistance to hydrolytic degradation, resistance to ultra-violet, solubility in chloroform and other chlorinated hydrocarbons and are not toxic (Bugnicourt et al. 2014).

PHA are polyesters of various hydroxyalkanoates (HA) synthetized by several species of microorganisms, namely bacteria, as a carbon and energy source, by, for example, fermentation (Philip et al. 2007). PHA are composed by fatty acids (R)-hydroxyalkanoates, R group can be just one hydrogen atom or vary from one (short chain)

over fourteen carbons (long chain), and depending on this they have different denominations, as shown in Figure 2 (Madison & Huisman 1999).

N varies between 600 and 35000



Group R	Denomination
Hydrogen	Poly(3-hydroxypropionate)
Methyl	Poly(3-hydroxybutyrate)
Ethyl	Poly(3-hydroxyvalerate)
Propyl	Poly(3-hydroxyhexanoate)
Pentyl	Poly(3-hydroxyoctanoate)
Nonyl	Poly(3-hydroxydodecanoate)

Figure 2: Structure of polyhydroxyalkanoates (adapted from Khanna & Srivastava 2005a).

Thus, depending on the number of carbon atoms in the side chain, some authors divide PHA into three groups: short chain length PHA (sclPHA), which contains three to five carbon atoms; medium chain length PHA (mclPHA), which contains six to fourteen carbon atoms (Zinn et al. 2001) and long chain length PHA (lclPHA), which contains more than fourteen carbon atoms (Tan et al. 2014). Due to their high degree of crystallinity, sclPHA are presented as hard and brittle plastics, while mclPHA, due to their low degree of crystallinity, low tensile strength and low melting point, present themselves as more flexible plastics (Philip et al. 2007).

PHA can be produced from a wide range of feedstocks, such as renewable sources (e.g. sucrose, starch, cellulose, triglycerides, hemicelluloses), sub-products (e.g. molasses, whey, glycerol, rice bran), organic acids (e.g. propionic acid, 4-hydroxybutyric acid), fossil resources (e.g. methane, mineral oil, lignite) and wastes (e.g. wastewater, palm oil mill, activated sludge effluents) (Urtuvia et al. 2014).

PHA are synthesized and stored by the microorganisms in the cell cytoplasm, into granules insoluble in water, and may account for 90 % of cell dry weight (Reddy et al. 2003). The production and commercialization of PHA are done by microbial fermentation, using a two-stage production process. Firstly, there is a microbial fermentation consuming a renewable feedstock in which microbes accumulate PHA intracellulary. After the end of the fermentation, microbial cells are harvested and then PHA are extracted from cells, in which there is a separation of non-PHA content from PHA content by chemical or enzymatic digestion of non-PHA component of the microbial cell (Snell & Peoples 2009).

Once extracted from the cell, PHA exhibit thermoplastic and elastomeric properties (Steinbuchel 2005).

2.1.1 Examples of PHA

Among the various existing polyhydroxyalkanoates the poly-3-hydroxybutyrate, P(3HB), can be highlighted as the most studied and well characterized bioplastic. P(3HB) is a homopolymer of 3-hydroxybutyrate and was the first PHA discovered, in 1926 by Lemoigne, in inclusion bodies of *Bacillus megaterium* (Lemoigne 1926). The molecular weight of P(3HB) can differs and depends on the producing microorganism, conditions of growth and also extraction method (Bugnicourt et al. 2014). P(3HB) has mechanical properties similar to conventional plastics, such as propylene and polyethylene (Khanna & Srivastava 2005a) but it can be distinguished by its completely degradation to water and carbon dioxide under aerobic conditions (Harding et al. 2007). However, P(3HB) have some disadvantages like the price of production and the poor mechanical properties (Philip et al. 2007). P(3HB) has had limited use mainly because of its intrinsic brittleness (Bugnicourt et al. 2014).

In addition to this, another PHA stands out, the copolymer poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate, P(3HB-*co*-3HV), a less crystalline plastic than P(3HB) but more tough and flexible, with a lower boiling point (Steinbuchel & Fuchtenbusch 1998). In general, copolymers, polymers constituted by two different types of monomers, due to their low crystal structure, are easier to degrade when compared with the homopolymers, like P(3HB) (Reddy et al. 2003). However, when the content of 3HV is low, P(3HB-*co*-3HV) are stiff and brittle like P(3HB), with poor impact strength. This brittleness has been reported as an obstacle to the industrial applications of these materials. Many efforts have been made to manipulate these mechanical properties. One of the best solutions found was the production of copolymeric materials, as P(3HB-*co*-3HV) with a higher content of 3HV, or mcl-PHA copolymer. This decreases the stiffness and the brittleness properties of bioplastics and increase the flexibility and the tensile strength and toughness (Laycock et al. 2014).

2.1.2 Metabolic pathways of PHA production

PHA-producing organisms can use a wide range of carbon sources to convert them in different kinds of PHA. These carbon sources could be saccharides (e.g. fructose, maltose, lactose, xylose, arabinose), n-alkanes (e.g. hexane, octane, dodecane), n-alkanoic acids (e.g. acetic acid, propionic acid, butyric acid, valeric acid, oleic acid), n-alcohols (e.g. methanol, ethanol, glycerol) and gases (e.g. methane and carbon dioxide) (Tan et al. 2014). PHA biosynthetic pathways depend on the substrate (carbon source) and the microbial strain used. These are linked with bacterium's central metabolic pathways, such as glycolysis, Krebs cycle, β-oxidation, *de novo* fatty acids synthesis, amino acid catabolism, Calvin cycle and serine pathway (Tan et al. 2014).

The synthesis of PHA from feedstock rich in sugars begins with glycolysis of the sugar to pyruvate. The obtained pyruvate is converted to acetyl coenzyme A (acetyl-CoA) by action of enzyme pyruvate dehydrogenase (PDH). Then there is the condensation of two molecules of acetyl Co-A into acetoacetyl coenzyme A (acetoacetyl-CoA) through the action of β-ketothiolase. After, acetoacetyl-CoA reductase converts acetoacetyl-CoA into monomers of 3-hydroxyacyl-CoA and finally the polymerization of these into monomers of PHA by PHA synthase occurs (Gumel et al. 2013). To make this possible, three enzymes coded by three different gens, organized in a single operon as *phbCAB* are necessary. Thus, *phbA* encodes for β-ketothiolase, *phbB* encodes for acetoacetyl-CoA reductase and *phbC* encodes for PHA synthase (Fukui et al. 1998). The synthesis of P(3HB) is schematized in Figure 3.

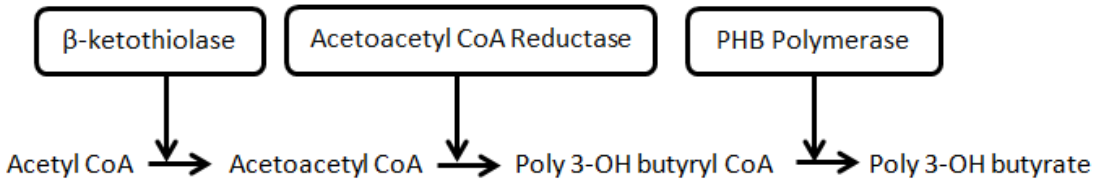


Figure 3: Biosynthetic pathway of poly(3-hydroxybutyrate) (Reddy et al. 2003).

The synthesis of PHA from fatty acids follows the β-oxidation and can be used to produce mcl-PHA. At first the β-oxidation occurs, where fatty acids are converted into acyl CoA, who is then converted to 3-hydroxyacyl-CoA. Finally, this is converted into PHA

under synthase catalysis. The enzymes involved in this pathway are 3-ketoacyl-CoA reductase, epimerase and (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I (Laycock et al. 2014). On the other hand, other pathway can occur from simple sugars like glucose, sucrose or fructose. The intermediates 3-hydroxyacyl from the biosynthesis pathway of fatty acids are converted to 3-hydroxyacyl-ACP by action of 3-hydroxyacyl-ACP-CoA transferase (encoded by *Pha*G), which are then converted into 3-hydroxyacyl-CoA and thus PHA (Laycock et al. 2014; Philip et al. 2007).

The synthesis of PHA can be done using NADPH-dependent acetoacetyl-CoA reductase to oxidize (S)-(+)-3-hydroxybutyryl-CoA. Other pathways can be used for the synthesis of alternative copolymers. For instance biosynthetic pathways to form P(4HB) (Laycock et al. 2014).

2.1.3 Applications

PHA have a wide range of applications afforded by their different characteristics and properties. In an initial phase, PHA were used mainly in the manufacture of bottles and films for biodegradable packaging materials (Steinbuchel & Fuchtenbusch 1998). Packaging is one of the biggest polymer processing industries. Despite environmental problems, the European polymer packaging market is increasing in the order of millions of tons per year. In the future, due to environmental concerns, namely the depletion of petroleum and the increase of greenhouse gases, will receive growing attention cheap and biodegradable polymeric products (Bugnicourt et al. 2014).

Nowadays, PHA are used in several applications similar to conventional plastics, that include household, consumer, catering products and disposable materials, like razors, utensils, diapers, feminine hygiene products, cosmetic containers, medical disposable devices, and so on (Khanna & Srivastava 2005a; Bugnicourt et al. 2014). In medicine, PHA are particularly used for biodegradable controlled release drugs, antibiotics, hormones, pesticides and herbicides carrier, and it is also used as osteosynthetic materials in the stimulation of bone growth, bone plates, stitches, replacement of blood vessels, and many other applications in terms of tissue engineering (Philip et al. 2007). PHA is also considered as pharmaceutically-active compound and currently investigated as potential anti-HIV drugs, anti-cancer drugs, or antibiotics (Tan et al. 2014).

Despite the huge range of features and benefits presented by the PHA production, the high costs involved in their production and the great potential of plastics derived from fossil fuels, have proved an obstacle to its implementation in the market (Choi & Lee 1999).

2.1.4 PHA producer organisms

PHA-producing organisms can be divided into two groups, based on the culture conditions required. The first group comprises the microorganisms whose growth requires the limitation of an essential nutrient, such as nitrogen (N), phosphorus (P), magnesium (Mg), potassium (K), oxygen (O) or sulfate (S) and carbon source in excess. This group includes microorganisms such as *Cupriavidus necator*, *Protomonas extorquens*, *Pseudomonas oleovorans*, among others (Lee 1996b). The second group comprises the microorganisms that do not require a nutrient limitation and can accumulate polymer during their growth. This group includes microorganisms such as *Alcaligenes latus*, recombinant *Escherichia coli* (Lee 1996b) and *Haloferax mediterranei* (Lillo & Rodriguez-Valera 1990), among others.

Nowadays the industrial production of PHA is largely based on pure cultures due to its high PHA production capacity. Despite pure cultures are predominantly used, PHA production based on mixed cultures has been proposed as a means of lowering production costs, because in this case no reactor sterilization is necessary and the culture is able to use various complex and cheap waste feedstocks (Laycock et al. 2014). In the other hand pure cultures need more expensive substrates, greater production control and sterilization equipment, consequently require a higher investment (Serafim et al. 2008a).

2.2.4.1 *Cupriavidus necator*

Cupriavidus necator, formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*, is a Gram-negative bacteria considered model concerning the production of PHA, due to be the most widely studied microorganism in this area (Sichwart et al. 2011) and due to its ability to accumulate large amounts of P(3HB) from simple carbon sources (Khanna & Srivastava 2005b).

The production of PHA by *C. necator* occurs in the presence of high concentrations of carbon source, like glucose, fructose or acetic acid (Khanna & Srivastava 2005a) and with limitation of certain nutrients (Cavalheiro et al. 2012). Fereidouni et al. (2011) studied the growth of *C. necator* and the production of P(3HB) from different carbon sources and verified that this microorganism synthetizes PHA only from simple carbon sources, being the fructose the sugar that allow for higher concentration of biomass and polymer. Besides that, it was shown a significant impact of nitrogen limitation, which varies since 25 % of P(3HB) of cell dry weight in normal nitrogen concentration to 64 % in limiting conditions (Fereidouni et al. 2011). Beyond fructose, Khanna and Srivastava (2005) showed that glucose was also an efficient carbon source to growth of *C. necator* and to produce P(3HB). Although glucose do not permit a higher yield than fructose, it has a significant contribution in biomass growth, presenting itself like a good compromise between P(3HB) production yield and production cost associated with carbon source (Khanna & Srivastava 2005b).

2.2.4.2 Genetically modified organisms – Recombinant *Escherichia coli*

Some bacteria, like *Escherichia coli*, are incapable of producing PHA naturally (Reddy et al. 2003). However, this microorganism presents many advantages that make it one of the best host and commercial producer of PHA (Li et al. 2007). The fast growth and high cell density, the accumulation of large amounts of PHA and the ability to utilize several inexpensive carbon sources are the main advantages. Furthermore, this microorganism has a relatively easy capacity of PHA purification, which reduces the costs of the PHA granules purification since it lacks the intracellular depolymerases that degrade the accumulated PHA (Lee & Choi 2001). Besides this, *E. coli* is well studied in terms of its physiology, biochemistry and genetics (Sudesh et al. 2000). Production of PHA in recombinant *E. coli* is currently made by introduction of genes (*phaCAB* operon) (Sudesh et al. 2000) from *C. necator* (Khanna & Srivastava 2005a). Studies showed that recombinant *E. coli* can accumulate up to 90 % of P(3HB) of dry cell weight (Ahn et al. 2000). Furthermore, economic analysis done by Wegen et al. (1998) showed that using recombinant *E. coli* it is possible to reduce the production costs of PHA.

2.2.4.3 *Haloferax mediterranei*

The discovery that halophilic microorganisms could also produce PHA promoted the process of competitive production at industrial scale (Bhattacharyya et al. 2012). Halophiles, microorganisms that require high concentrations of salt for growth and survival (Fernandez-Castillo et al. 1986), belong to the *Archaea* domain (Lillo & Rodriguez-Valera 1990). Despite halobacteria being classically associated to a very slow growing using a very limited range of organic compounds, such as amino acids as an energy source, studies emphasize the existence of substrates, as sugars, capable of providing rapid growth (Anton et al. 1988). Due to survival capacity of these microorganisms at high salt concentrations, risks of microbial contamination are significantly reduced. This facilitates the production process, because it is not necessary to consider sterility maintenance, reducing the associated costs of PHA synthesis (Huang et al. 2006).

Although halobacteria family includes 30 genus, only some genus are capable of producing PHA, and among them there are *Haloferax*, *Haloarcula*, *Haloquadratum*, *Haloterrigena*, *Halorhabdus*, *Halobiforma* and *Halopiger* (Bhattacharyya et al. 2012). Studies showed that *Haloferax mediterranei* is the best known PHA halobacteria producer until now. This is due to high growth rates, due to metabolic versatility and due to genetic stability (Bhattacharyya et al. 2012). Beyond intracellular polymers production, *H. mediterranei* also produces extracellular polymers. Thus, halobacteria origins a polymeric substance that gives the colonies a typical mucous character and it is responsible for the appearance of a superficial layer in colonies. This polymeric substance can be obtained from liquid crop supernatant, after agitation, by cold precipitation of ethanol (Anton et al. 1988).

Since 1986 many studies focused on the best way to increase the PHA production by *H. mediterranei* and make this production sustainable. Fernandez-Castillo et al. (1986) studied the production of PHA by several halobacteria from diverse carbon sources (glucose, glycerol, sodium citrate, cellobiose and lactose) and different amounts of salts (15, 20, 25 and 30 % of salt) under same conditions of temperature (37 ºC) and pH (7.2). They concluded that *H. mediterranei* was the best halobacteria producer, that the glucose was the best carbon source for this microorganism, achieving P(3HB) concentrations of 17 % of dry weight, and the P(3HB) production was favored by lower salt concentrations,

because it was with the 15 % of salt which have reached the best production of P(3HB) (38 %). Lillo and Rodriguez-Valera (1990) proved that glucose and starch were the best carbon sources, with 30.9 % and 32.4 % of P(3HB) respectively, and limiting the phosphorus concentration to 0.00375 % the total P(3HB) production ($g.L^{-1}$) and the P(3HB) yield (g.g-1) would be higher. Furthermore they showed the influence of several parameters in growth and P(3HB) accumulation, concluding that the optimal temperature, pH and marine salts concentrations were 45 ºC, 7.2 and 25 %, respectively. D'Souza et al. (1997) also tested the effect of salt concentration in *H. mediterranei* growth, from salt concentrations of 0 % to 20 %. They observed that, with glucose as carbon source, the growth of halobacteria was only possible with salt concentrations of at least 10 %, being the growth in 10 % NaCl medium similar to 20 % NaCl. In contrast, by the same parameters, no growth was observed in 5 % NaCl and in basal salts medium. Huang et al. (2006) tested the use of rice bran and cornstarch as substrate to produce PHA with *H. mediterranei*. They concluded that the archaebacteria could only use extruded rice bran and extruded cornstarch to growth and accumulate PHA. The results showed that with extruded cornstarch, and in a 5-liter jar fermentor, *H. mediterranei* accumulated 38.7 % of PHA with a PHA concentration of 24.2 $g.L^{-1}$, while with a mixture of extruded rice bran and extruded cornstarch (1:8) she accumulated 55.6 % of PHA and a PHA concentration of 77.8 $g.L^{-1}$. They also compared these values with glucose such as control (27 % of PHA content and 23 $g.L^{-1}$ of PHA concentration). Although previous studies reported that the best growth temperature for *H. mediterranei* was 45 ºC (Lillo & Rodriguez-Valera 1990), Huang et al. suggested that the 37 ºC was the more advantageous temperature because it saves energy cost during long-term continuous culture and it saves oxygen cost in the high cell density culture (lower temperature promotes a higher saturated oxygen concentration in the broth). Don et al. (2006) studied PHA production but performed polymer characterization. Bacterial growths were made in presence of glucose as carbon source (1 $g.L^{-1}$) and in optimal conditions to *H. mediterranei* (temperature of 37 ºC and pH of 7.0). A high cell density of 85.8 $g.L^{-1}$ and a PHA content of 48.6 % were obtained. The obtained PHA was characterized by FTIR and NMR spectrophotometry showing that the polymer was P(3HB-*co*-3HV). Koller et al. (2007a; 2007b) published two studies about P(3HB-*co*-3HV) production from hydrolyzed whey. In one of them, bacterial growth occurred with at 36 ºC and at pH in a range of 6.8 to 7.0. Among several archaebacterias studied, *H.*

mediterranei presented the best PHA production (50 %), with a maximal PHA concentration of 5.5 $g.L^{-1}$. In the other published study, the assays were made at 37 ºC and pH 7.0. While in the first case P(3HB-*co*-8%-3HV) was obtained, in the second both P(3HB-*co*-6%-3HV) and P(3HB-*co*-21.8%-3HV-*co*-5.1%-4HB) were formed. Furthermore, the PHA content was 72.8 % of P(3HB-*co*-3HV) and 87.5 % of P(3HB-*co*-3HV-*co*-4HB), with higher PHA concentrations of 12.2 and 14.7 $g.L^{-1}$, respectively.

More recently, Bhattacharyya et al. (2012) tested the production of P(3HB-*co*-3HV) from vinasse by *H. mediterranei*. The growth of *H. mediterranei* in raw vinasse and pre-treated vinasse was tested at different concentrations of substrate, at a temperature 37 ºC and pH 7.2. The results showed that higher concentrations of raw vinasse inhibited the growth of halobacteria and in contrast the pre-treated vinasse allowed for the growth at concentrations of 25 % and 50 % while inhibition occurs with 75 % and 100 % pre-treated vinasse. The obtained results pointed to a PHA content of 70 % from 25 % pre-treated vinasse and 66 % from 50 % pre-treated vinasse and a PHA concentration of 19.7 $g.L^{-1}$ and 17.4 $g.L^{-1}$, respectively. The polymer was identified as P(3HB-*co*-3HV) and quantified through several techniques, such as UV–vis spectroscopy, gas chromatography, differential scanning calorimetry and nuclear magnetic resonance spectroscopy. Zhao et al. (2013) identified a gene cluster involved in exopolysaccharide (EPS) biosynthesis in *H. mediterranei*. The assays were performed at 37 ºC with 20 $g.L^{-1}$ starch as a carbon source. The results showed that the deficiency in EPS biosynthesis decreased the viscosity of culture broth, and hence increased the dissolved oxygen content and decreased the foaming propensity. Furthermore the P(3HB-*co*-3HV) concentration in the mutant strain (21.28 $g.L^{-1}$) was nearly 20 % higher than the wild-type strain (17.80 $g.L^{-1}$). Bhattacharyya et al. (2014) tested the use of rice-based ethanol stillage to P(3HB-*co*-3HV) production by *H. mediterranei*. The tests were done in Erlenmeyers flasks at 37 ºC, 180 rpm and with a pH of 7.2. At the end of fermentation, PHA concentration of 16.42 $g.L^{-1}$ was obtained, corresponding to 71 % of PHA/CWD.

The summary of evolution of state of art of *H. mediterranei* is represented on Table 2.

Table 2: PHA production by *Haloferax mediterranei*.

Year	Strain	Carbon Source	Temp.	pH	Kind of PHA	% PHA	[PHA] ($g.L^{-1}$)	Reference
1986	*H. mediterranei* ATCC 33500	Glucose (10 $g.L^{-1}$), yeast extract (1 $g.L^{-1}$), 25 % salts	37 ºC	7.2	P(3HB)	17		Fernandez-Castillo et al. 1986
1990	*H. mediterranei* ATCC 33500	Glucose (1 %), 25 % marine salts	38 ºC	7.2	P(3HB)	30.9	3.09	Lillo & Rodriguez-Valera 1990
		Glucose (2 %), 25 % marine salts	38 ºC	7.2	P(3HB)	20.8	4.16	
		Glucose (5 %), 25 % marine salts	38 ºC	7.2	P(3HB)	6.6	3.28	
		Glucose (10 %), 25 % marine salts	38 ºC	7.2	P(3HB)	3.5	3.52	
2006	*H. mediterranei* ATCC 33500	Glucose	37 ºC	6.9 - 7.1	P(3HB-*co*-3HV)	27	23	Huang et al. 2006
		Extruded rice bran	37 ºC	6.9 - 7.1	P(3HB-*co*-3HV)	55.6	77.8	
		Extruded wheat bran	37 ºC	6.9 - 7.1	P(3HB-*co*-3HV)	40.2	52.7	
2006	*H. mediterranei* ATCC 33500	Glucose (1 $g.L^{-1}$)	37 ºC	7.0	P(3HB-*co*-3HV)	48.6	85.8	Don et al. 2006
2007	*H. mediterranei* DSM 1411	Hydrolyzed whey	36 ºC	6.8 - 7.0	P(3HB-*co*-3HV)	50	5.5	Koller et al. 2007b
2007	*H. mediterranei* DSM 1411	Hydrolyzed whey	37 ºC	7.0	P(3HB-*co*-3HV)/ P(3HB-*co*-3HV-*co*-4HB)	72.8/ 87.5	12.2/ 14.7	Koller et al. 2007a
2012	*H. mediterranei* DSM 1411	25 % pre-treated vinasse	37 ºC	7.2	P(3HB-*co*-3HV)	70	19.7	Bhattacharyya et al. 2012
		50 % pre-treated vinasse	37 ºC	7.2	P(3HB-*co*-3HV)	66	17.4	
2013	*H. mediterranei* ATCC 33500	Starch (20 $g.L^{-1}$)	37 ºC	7.0	P(3HB-*co*-3HV)	44.5	17.8	Zhao et al. 2013
	H. mediterranei ATCC 33500 (mutant)	Starch (20 $g.L^{-1}$)	37 ºC	7.0	P(3HB-*co*-3HV)	48	21.28	
2014	*H. mediterranei* DSM 1411	Rice-based ethanol stillage	37 ºC	7.2	P(3HB-*co*-3HV)	71	16.42	Bhattacharyya et al. 2014

Some of these studies have also been estimated the PHA production cost. It is known that the cost of carbon substrate affect the PHA cost and further that the production can be considerably lowered when agricultural wastes (whey, molasses, among others) are used (Choi & Lee 1997). For example, studies made by Koller et al. (2007b) estimated a P(3HB-*co*-3HV) production cost from whey lactose of 2.82 € per kilogram, significantly lower compared with that of recombinant *E. coli* from the same carbon source (4.00 €/kg) (Quillaguaman et al. 2010). Beyond the carbon source, also the recovery of the polymer has a significantly impact in PHA cost production. This was verified by Choi and Lee (1997) that compared the price of P(3HB) production using two different recuperation methods: by surfactant-hypochlorite digestion and by dispersion treatment of chloroform and hypochlorite, with a reduction of costs in the order of 2.45 € per kg P(3HB).

2.1.5 PHA recovery

After fermentation, it is necessary to separate cells from the growth medium at the end of a high cell density cultivation process (Madkour et al. 2013), through conventional procedures such as centrifugation, filtration or flocculation-centrifugation. Only after that the cells are disrupted and the polymer recovered (Lee 1996a).

Cells can be disrupted through the application of heat, which affects the firmness of cells by the denaturation of cell proteins and by the destabilization of the outer membrane. Also the use of alkaline solutions, such as sodium hydroxide, or through the use of a sodium chloride salt solution can lyse cells. Cells can suffer also freezing/thawing cycles, which allow to the mechanical disruption of the cells by ice crystal formation during freezing (Madkour et al. 2013).

The separation of PHA from cells debris could be done by several different methods. The most common is the extraction of the polymer with organic solvents, a simple and rapid method that extract the polymer by improving the cellular membrane permeability and subsequent solubilization of the PHA (Gumel et al. 2013). This method is based in the water insolubility of PHA, but solubility in a limited number of organic solvents. Principal recovery agents are chloroform, dichloroethane, methylene chloride, acetone or some solvent mixtures, such as chloroform/methanol and dichloromethane/ethanol. However, solvent extractions are still very expensive and are

very dangerous to humans and to the environment due their toxicity, they are uneconomic and are only applicable in laboratory (Madkour et al. 2013).

Another way of recovering PHA is through chemical disruption (chemical digestion), which uses chemicals such sodium hypochlorite and surfactants, like sodium dodecyl sulfate (SDS), to disrupt the microbial cells and consequent release of cell content (Madkour et al. 2013). In this method there is no degradation of the polymer and it could be applied to large volumes and high cell densities (Gumel et al. 2013).

Other way to recovery PHA is through enzymatic cell disruption (enzymatic digestion) using some types of enzymes (proteases, nucleases, lysozyme and lipases) to hydrolyze proteins and other polymers of the bacterial cell mass and initiate cell lysis, having no effect (or minor effect) on PHA. This method allows to the reduction of chemicals use and a high recovery rate and purity of the polymer. However, the cost of enzymes is high (Gumel et al. 2013; Madkour et al. 2013).

On the other hand, there are some methods that do not resort to chemicals. Mechanical disruption is an example of this, being usually used in laboratory to release PHA from bacterial cells on a small scale. There are many types of mechanical disruption, for example by bead mill, by high pressure homogenization or by sonication. However these methods are not viable in industrial large scale (Gumel et al. 2013; Madkour et al. 2013).

Supercritical fluids, such as CO_2 could also be used to recovery PHA. This method has several advantages because supercritical CO_2 is readily available and nonflammable, has low reactivity, toxicity and a moderate critical temperature and pressure and allow to a low cost chemical treatment. Nevertheless, this method requires strict process parameters and further chemicals for a high degree of extraction (Gumel et al. 2013; Madkour et al. 2013).

Other methods, such as cell fragility, air classification, dissolved-air flotation and spontaneous release of PHA granules have been developed to improve the PHA recovery either in laboratory level or in industrial level (Madkour et al. 2013). The complete PHA recovery process is illustrated in Figure 4.

In specific case of *H. mediterranei*, the first step is a centrifugation to separate the PHA from cells. The pretreatment of cells is made through the use of a sodium chloride salt solution. Finally the release of PHA from cells is usually done by an extraction of the

polymer with organic solvents, mainly chloroform (Bhattacharyya et al. 2012; Koller et al. 2007a; Koller et al. 2007b; Don et al. 2006; Huang et al. 2006; Lillo & Rodriguez-Valera 1990).

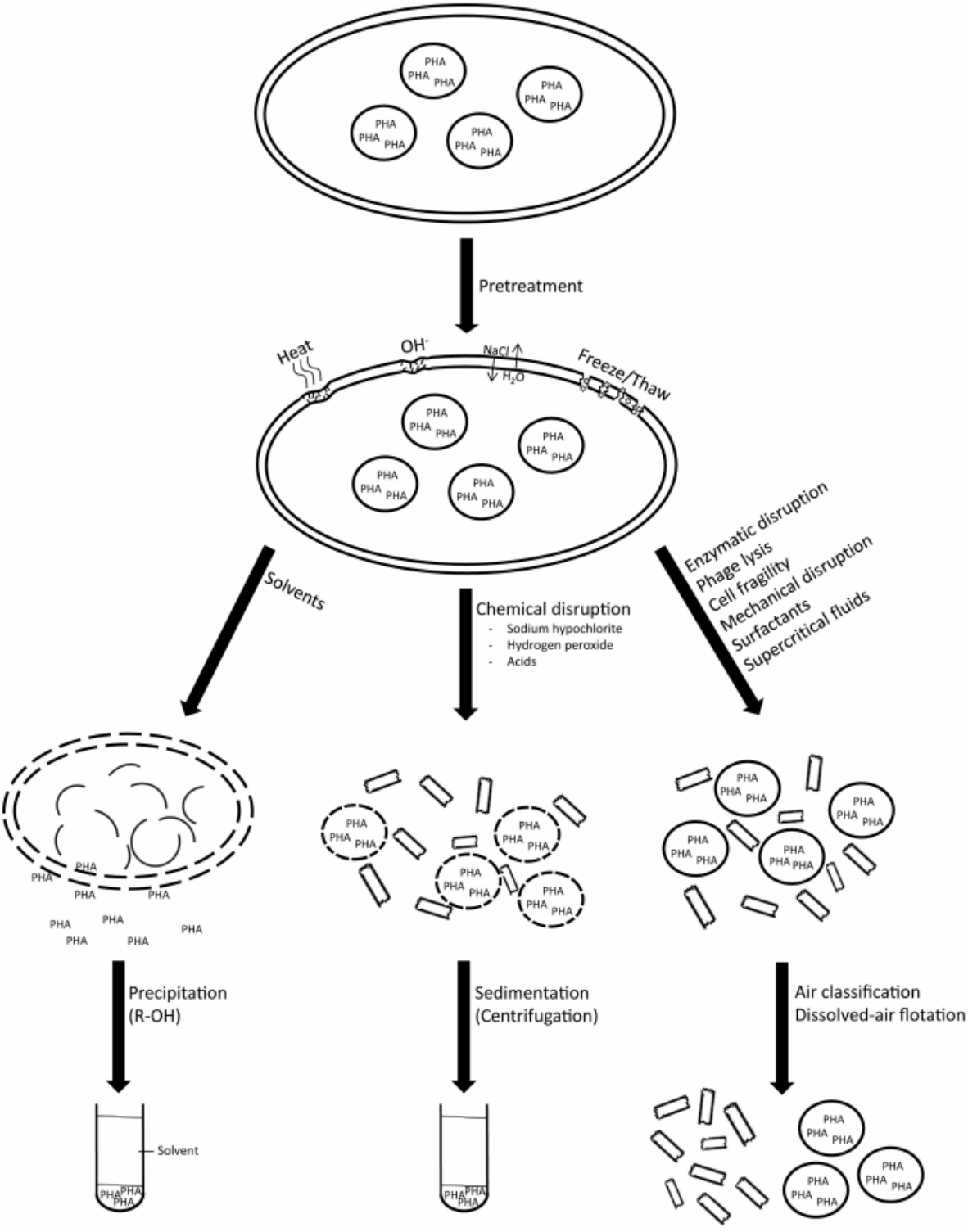


Figure 4: Principal methods for PHA recovery from bacterial cells (Madkour et al. 2013).

3. Pulp and paper industry

The pulp and paper industry is one of the biggest industries in the world, with main focus in the North America (United States and Canada), Northern Europe (Finland and Sweden), East Asia (Japan) and further Australia and Latin America, reaching levels of global production of paper and paper board in the order of 380 million tons (Bajpai 2012). This industry uses advanced chemical and mechanical technologies to produce high quality products and support the lifestyles of the global economy, presenting a key role in the country's economic growth. Many efforts have been made to reduce the manufacturing costs through the development of new technologies (Viikari & Lantto 2002).

3.1 Portugal

The activity of pulp and paper industry strongly contributes to Portuguese economic growth, once exportations are higher than importations. This sector exports mainly to countries like Spain, Germany, Italy, France and United Kingdom (Celpa).

Portuguese paper industry markedly progressed in the last few years, in dealing and addressing the problems of pollution and the environment in general. Thus, there has been made an industrial and environmental management in terms of CO_2 emissions, energy consumption, gas consumption, biomass utilization and water consume. The main measures adopted by this industry were replacing fossil fuels with biomass, substitution of fuel oil by natural gas, new biological treatment of wastewater, improving the energy efficiency of processes, attenuation of noise and nuisance odors, proper management of forest areas, use of improved varieties of eucalyptus and preventing and fighting forest fires (Celpa).

Eucalyptus globulus is one of the most important tree species in the world and in Portugal has a great impact in pulp production (Potts et al. 2004; Águas et al. 2014). The fast growth associated with good wood properties lead to a bleached pulp production for printing quality (Catry et al. 2013). *E. globulus* is native from Australia, but it can be found in many regions around the world, namely in Portugal, one of the countries with the largest areas of plantation of *E. globulus*. This specie was introduced in Portugal in the middle of the 19th century, recording an huge increase over the years and it is, nowadays, the most widespread tree species in the country (Águas et al. 2014). Data from Instituto da

Conservação da Natureza e das Florestas (ICNF) indicates that *E. globulus* is the most dominant specie in Portugal, occupying 26 % of Portugal forest cover (corresponding to 812 thousand ha), followed by *Pinus pinaster* with 23 % (corresponding to 737 thousand ha) and then by *Quercus ilex* with ~23 % (corresponding to 714 thousand ha) (Figure 5). Furthermore, the Portuguese Forest Inventory shows that the area occupied by softwood species corresponds to 31 % of the Portuguese forest and the remainder (69 %) is occupied by hardwoods (ICNF 2013).

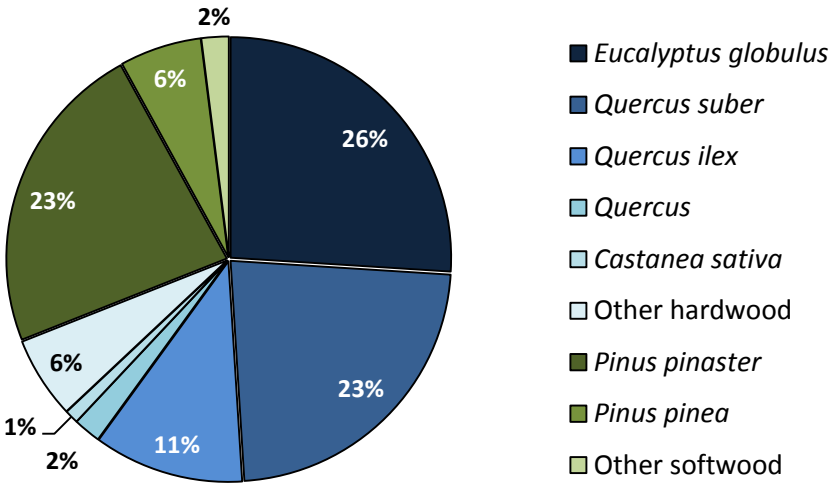


Figure 5: Distribution of total areas by species / species group (ICNF 2013).

In Portugal the most part of *E. globulus* wood is sold to pulp mills, which supply papermaking industries all around the world (Catry et al. 2013).

3.1.1 Portucel Soporcel group

Portucel Soporcel group is one of the biggest producers in Europe and one of the biggest production industries of Bleached Eucalyptus Kraft Pulp (BEKP) in the world. It was the first European producer of Uncoated WoodFree (UWF). The group has its facilities in Figueira da Foz, Cacia and Setubal and they are trying to spread to Mozambique. Portucel Soporcel group has, presently, a capacity of production of 1.6 millions of tons of paper by year, 1.4 millions of tons of pulp by year, of which 1.1

millions of tons are integrated into paper, and also 2.5 TWh by year of electric energy, reaching a business volume of more than 1.5 billion of euros per year.

Last years, the main goals of Portucel Soporcel group were the study of favorable conditions of eucalyptus growth in Portugal and improvement of paper production to printing and writing. The company is responsible for the production of Navigator®, the world's leading brand in the *premium* segment of office paper, and others like Pioneer®, Explorer®, Soporset® and Inaset®. Group sales are destined for 113 countries on five continents, especially Europe and the United States of America (USA). Furthermore, the group manages woodlands of about 120 000 hectares promoting the appreciation and protection of national forest (Soporcel 2011).

3.1.1.1 RAIZ

RAIZ is the Research Institute of Forest and Paper associated to Portucel Soporcel group and Universities of Aveiro, Coimbra and Lisbon. Its principal goal is to increase the competitiveness of paper and forest sectors through research, technological support and specialized formation. Furthermore it aims to transform knowledge in technology in order to increase forest productivity, enhance the quality of fiber produced (reduce production costs and improve the quality of the paper) and implement sustainable forest management (RAIZ).

In terms of forest investigation the main objectives are to increase the productivity of eucalyptus forest and improve the quality of fiber produced. Furthermore, it is pretended to implement sustainable forest management from economic, environmental and social point of view and decrease the cost of wood. Investigation was developed in several areas, including biotechnology, propagation of plants, forest protection (pests, diseases and natural vegetation), biometrics, bioenergy, among others.

In terms of industrial investigation the main objectives are to improve the quality of pulp and paper; decrease the cost of production and minimize environmental impact, being developed investigation in areas like kraft cooking, bioenergy, bleaching pulp, clean technologies in the pulp industry, quality of pulp and papermaking applications, recycling of solid waste from the pulp industry, among others (RAIZ).

3.2 Wood composition

Wood is an organic material composed, mainly, by carbon, hydrogen and oxygen and by other inorganic elements in small amounts, such as nitrogen, sodium, potassium, calcium, magnesium and silicon, as represented in Table 3 (Sixta 2006).

Table 3: Elementary composition of wood (adapted from Sixta 2006).

Element	Content (%)
Carbon (C)	49
Hydrogen (H_2)	6
Oxygen (O_2)	44
Nitrogen (N_2)	<1
Inorganic elements (Na, K, Ca, Mg, Si)	<<1

These elements form three different polymers: cellulose, hemicellulose and lignin. The proportion of these three major components in wood is about 50, 25 and 25%, respectively, whereas these contents can vary among wood species (Whitacre 2011). For instance, in softwood these contents are about 44% of cellulose, 22% of hemicellulose and 28% of lignin (Pan et al. 2005). In addition, there is a little percentage (approximately 1.5–5.0%) of compounds that are extractable with organic solvents, such as resin acids, fats, tannins, flavanoids, among others (Whitacre 2011).

Trees species can be subdivided into two groups, depending on the mode of plant reproduction: gymnosperms and angiosperms. Although all trees reproduce by producing seeds, the seed structure varies. Softwoods trees are gymnosperms because let seeds fall to the ground, with no covering. On the other hand, hardwood trees are angiosperms because they produce seeds with some sort of covering, which can be, for example, a fruit or an hard shell (Sjostrom 1993).

3.2.1 Cellulose

The cellulose consists in a homopolymer with successive units of D-glucose (Lakshmidevi & Muthukumar 2010), linked via β-1,4 glycosidic linkages, this is, the hydroxyl group attached to carbon 1 of one of the monomers, is linked to the carbon 4 of the adjacent monomer (Claassen et al. 1999), such represented in Figure 6.

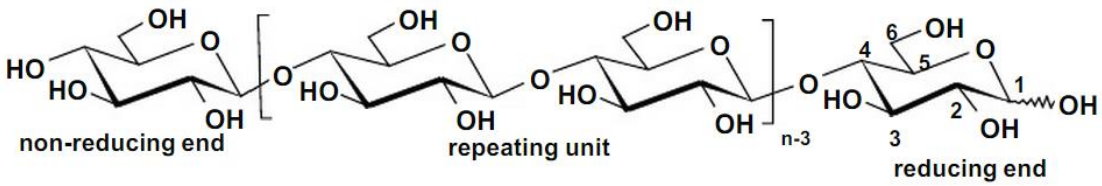


Figure 6: Chemical structure of cellulose (Sixta 2006).

Cellulose units are assembled in a first layer of microfibrils, then fibrils and finally through hydrogen bounding between linear molecules, thus becoming in a strong microcrystalline structure. Furthermore, cellulose fibrils are associated with lignin, hemicelluloses and other materials, giving it a complex and heterogeneous structure (Whitacre 2011). This molecule has intramolecular hydrogen bonds that confer chain stiffness and intermolecular hydrogen bonds that confer cellulose crystallinity (Sixta 2006). Due to its characteristics, cellulose is considered as an inexhaustible and unique source of new materials for a wide number of applications (Carreira et al. 2011), such as biofuels production, among others (Huber et al. 2006). Most of the available cellulose is produced by plants; however some microorganisms, like some algae, fungi or bacteria, can produce extracellular cellulose form (Carreira et al. 2011). Despite this, most bacteria cannot utilize directly cellulose molecules, being necessary a previously hydrolysis pre-treatment, in order to reduce the polymer to monomers. This step is essential so that glucose can be metabolized into the desired product, like for example, bacterial PHA production (Nduko et al. 2012).

3.2.2 Hemicelluloses

Hemicelluloses are branched heteropolysaccharides, composed by sugar units of five carbons (pentoses), as xylose and arabinose units; six carbons (hexoses), as glucose, galactose and mannose units; hexuronic acids, as glucuronic acid and deoxy-hexoses, as rhamnose units. The main chain of the hemicelluloses can be composed by only one unit like xylans, or it can be composed by two or more units like glucomannans (Sixta 2006).

By the fact that hemicelluloses are much branched, some wood polysaccharides are readily soluble in water. The composition of hemicelluloses is comprised between 20 and 30 % of dry weight of wood and both the composition and the structure differ from

softwood to hardwood (Sjostrom 1993). Furthermore, both type and amount of hemicelluloses varies widely, depending on plant materials, type of tissues, growth stage, growth conditions, storage and method of extraction (Ek et al. 2009).

Hemicelluloses increase the strength of paper, especially tensile, burst and fold, and improve the pulp yield. Compared with cellulose, hemicelluloses have a lower degree of polymerization (number of units that make the polymer), with an average number about 100-200 sugar units per hemicellulose molecule compared to 1000-10000 glucose units per cellulose molecule. Moreover, hemicelluloses are more soluble and labile than cellulose, being more susceptible to chemical degradation (Biermann 1996).

3.2.3 Lignin

Lignin is a complex phenolic polymer, formed by polymerization of three precursors: *p*-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol, linked by ether linkages (Figure 7) (Sixta 2006; Sjostrom 1993).

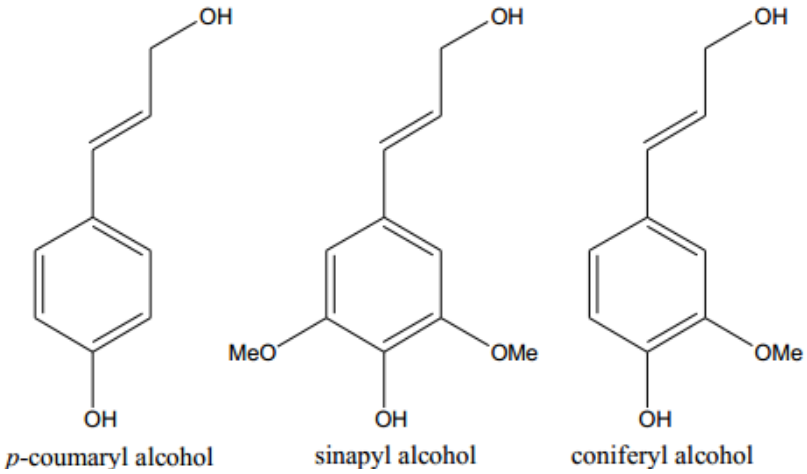


Figure 7: Chemical structure of lignin precursors (adapted from Santos et al. 2013).

This is the most abundant aromatic polymer and, next to cellulose, is the second most abundant organic polymeric substance in plants (Whitacre 2011). Generally, the content of lignin in different plants can vary between 20 and 40 % (Sixta 2006). In gymnosperms (softwoods) the main lignin precursor is coniferyl alcohol while in angiosperms (hardwoods) the main lignin precursor is sinapyl alcohol. On the other hand,

p-coumaryl alcohol is a precursor that less contributes to the structure of lignin (Sjostrom 1993). Lignin is considered adhesive or binder in wood because it holds the fibers together. During process of chemical pulping it is necessary to remove this polymer in order to separate the fibers (Biermann 1996).

Lignin is not a linear polymer like cellulose and is not a branched polymer like hemicelluloses, but it is a three-dimensional net, in which the monomers are randomly distributed and connected by different linkages, namely ether bonds (C-O-C) or carbon-carbon bonds (C-C) (Ek et al. 2009).

3.3 Description of pulping processes

3.3.1 Wood preparation

There are several raw materials that can be used in a pulp and paper mill, however wood is the main raw material used to manufacture pulp. Manufacturing of pulp begins with raw material preparation. For wood, the process starts with entrance into a pulp and paper mill as logs or chips. In first case, several operations are responsible to convert the logs into wood chips. For that, logs are transported to the slasher to cut them into desired lengths, followed by debarking, chipping, chip screening and conveyance to storage (Bajpai 2012; EPA 2010).

3.3.2 Pulping process

During the pulping process occurs the separation of wood chips into individual cellulose fibers, by removing the lignin from the wood (EPA 2010). The two principal processes to make this possible are chemical or mechanical pulping. Mechanical pulp has the main advantage of having a high yield of fiber (about 90 % compared with 50 % from chemical pulp). Moreover, chemical pulp has higher strength properties and has fibers which are more easily broken (Bajpai 2012). Types of pulping processes are presented in Figure 8.

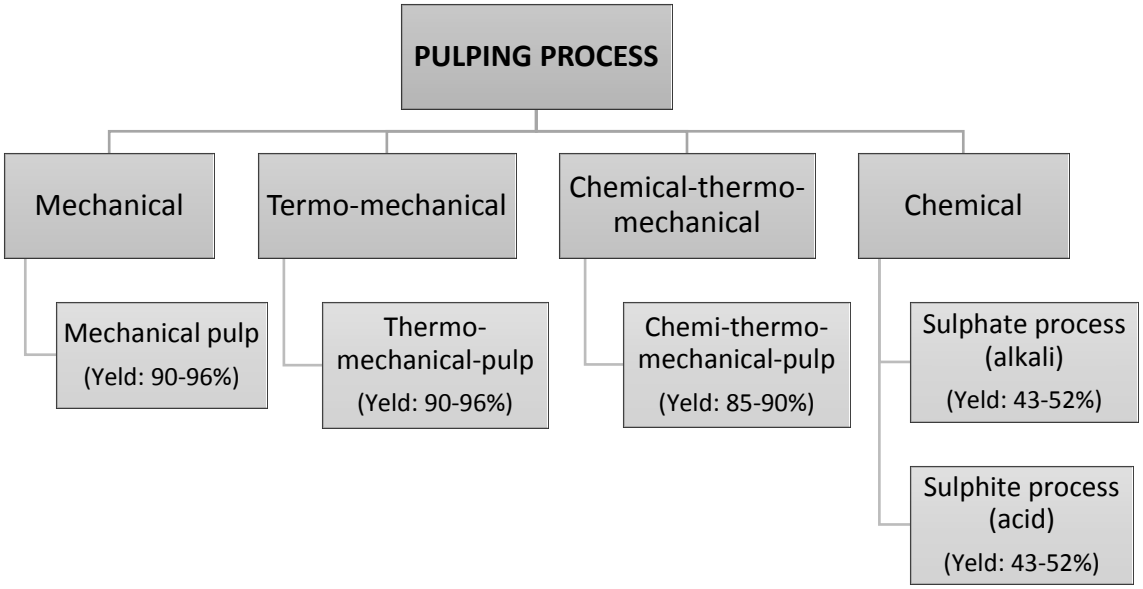


Figure 8: Types of pulping processes (sappi).

3.3.2.1 Mechanical pulping

During the mechanical pulping occurs the separation of pulp fibers from wood through physical energy, namely by grinding or shredding, which separates the fibers and it is responsible for breaking the bounds between fibers and for the release of fiber bundles, single fibers and fiber fragments. However, there are other mechanical processes that use chemical or thermal energy to pretreat raw material (Bajpai 2012; EPA 2010). The main disadvantages of mechanical pulping are the strong damage of fibers, the several impurities of pulp mass, the yellowish color and the low light resistance (sappi). Among the several types of mechanical pulping it stands out thermo-mechanical pulping (TMP), chemi-thermo-mechanical pulping (CTMP) (sappi) and others like stone groudwood pulping (SGW) and pressure groudwood pulping (PGW) (EPA 2010).

3.3.2.2 Chemical pulping

The main objective of chemical pulping consists of lignin removal in order to set the fibers free. For this, it proceeds to a baking with a chemical solution and also high pressures and temperatures (Bajpai 2012; sappi).

Chemical pulping can be done in different ways depending on the chemical used.

The sulphate process, also called kraft process, is the most common pulping process used in worldwide and digests the wood trough addition of an alkaline solution of sodium hydroxide (NaOH) and sodium sulfide (Na_2S). The chemical pulping starts with the addition of this alkaline solution (cooking liquor) with wood chips into a digester. When wood chips were "cooked", it occurs the discharge under pressure into a blow tank. Finally, the pulp and spent cooking liquor are separated in a series of brown stock washers (EPA 2010). This process can be applied to all types of wood species, but, although it allows to the processing of strongly resinous wood types, this requires expensive installations and intensive use of chemicals (sappi). Kraft process can produce a high variety of pulps, which can be used for packaging and high-strength papers and board (Bajpai 2012).

The sulphite process uses different chemicals to remove lignin. The cooking liquor are an acid mixture composed by sulfurous acid (H_2SO_3) and bisulfite ion (HSO_3^-) (EPA 2010). In this process, cooking liquid penetrates into the wood and degrades the lignin bonds between wood fibers, being the lignin converted into a water-soluble substance that can be washed out (sappi). The use of sulphite pulping permits the production of many different types and qualities of pulps for a broad range of applications (Bajpai 2012).

Making a brief comparison between the two chemical processes, we can infer that sulfite pulps have less color than sulfate pulps and can be bleached more easily, but are not as strong (EPA 2010).

3.3.3 Pulp bleaching

The bleaching process is responsible to remove the brown or brownish color of pulp (caused by residual lignin), to obtain the brightness required for white papers. This is made by the addition of several chemicals, such as chlorine (Cl_2), chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), oxygen (O_2), caustic and sodium hypochlorite (NaOH). The content of these chemicals vary depending on the end use of the product (EPA 2010; sappi). During the pulp bleaching, the chemicals are added to the pulp in stages in the bleaching towers, in which it occurs the removal of spent bleaching chemicals. Washer effluent is then collected and either re-used in other stages as wash water or sent to wastewater treatment (EPA 2010). Due to the negative impact caused by some chlorine

containing decomposition products, it has been developed chlorine-free processes of pulp bleaching, called as Totally Chlorine Free (TCF) (sappi).

3.4 Enzymatic hydrolysis of paper pulp

After pulping, pulp is usually conducted for the production of paper, passing, therefore, for a process of pulp bleaching in order to be possible to obtain the white color of paper. However, several efforts have been made to find other applications for this pulp, such as the production of chemical products. To make this possible, it is necessary to proceed to an enzymatic hydrolysis so that glucose could be available and could be obtained a liquid medium that allow the microorganisms to growth. Thus, cellulose can be hydrolyzed by specific enzymes - cellulases - which are produced by several microorganisms such as bacteria or fungi (Harrison et al. 2013). These enzymes are usually a mixture of several enzymes, constituted essentially by endoglucanases, exoglucanases and β-glucosidases, being the exoglucanases the responsible to catalyze the major of bond-cleavages during the hydrolysis (Santos & Gouveia 2009). Endoglucanases hydrolyze intermolecular 1,4-β-glucosidic bonds of cellulose, creating free chain ends, and could be measured through decrease of viscosity caused by cleavage of linkages; exoglucanases cleave cellulose chains to release glucose or cellobiose (disaccharide constituted by two glucose molecules, product of incomplete hydrolysis of cellulose); β-glucosidases act to release glucose units from cellobiose (Dumitriu 2005; Steffien et al. 2014). The hydrolysis occurs in mild conditions of temperature (T=45-50 ºC) and pH (pH=4.5-5.5) and requires a pre-treatment to provide cellulose from enzymatic attack (Santos & Gouveia 2009). However, in this process, it can appear some inhibitors compounds, such as furfural and 5-hydroxymethylfurfural, weak organic acids or phenol derivatives, which can constrain the enzymatic hydrolysis and the fermentation (Santos & Gouveia 2009; Steffien et al. 2014).

At the end of hydrolysis the monosaccharides are available for microorganisms. Data provided by RAIZ show that the mainly monosaccharides present in pulp are glucose (124 $g.L^{-1}$) and xylose (22 $g.L^{-1}$) and in minor quantities, fructose (0.3 $g.L^{-1}$) and mannose (0.1 $g.L^{-1}$).

4. Objective

In large papermaking industries, enzymatic hydrolysis has been used as a way of obtaining raw material for the production of industrial-interest molecules, namely base molecules alternatives to molecules from fossil resources. Thus, the goal of this project is to obtain monosaccharides, namely of glucose and xylose, by pulp enzymatic hydrolysis for later use in bioprocesses to polyhydroxyalkanoates production using *H. mediterranei* bacteria. For that, it will be done assays in defined medium and growths in hydrolysate medium supplemented with defined medium compounds. At the end of fermentation, they will be made several assays to analyze the consumption of sugars and the PHA production and to extract and identify the polymer obtained. On the other hand, it will be done the optimization of process, namely in terms of quantity of sugars in the medium to use, in order to increase the productivity of biomass and polymer.

CHAPTER II

Material and methodology

1. Raw material - Pulp

The unbleached pulp was provided by RAIZ, an affiliation of the Portucel Soporcel group.

1.1 Enzymatic hydrolysis of pulp

An enzymatic hydrolysis was applied to unbleached pulp to obtain the monosaccharides required to the growth of microorganisms. To prepare 3 L of hydrolysate, 1071.4 g of unbleached pulp were weighed and added to 2028.6 mL of ultrapure water (Appendix 1.) in a bath at 50 °C and stirred at 100 rpm with a mechanical stirrer with helical blade. 58.8 ml of enzyme (Novozyme NS (20 FPU/g_{HC})) were added to a 200 mL volumetric flask and the volume was made up with ultrapure water. The enzyme solution was heated at 50 ºC and, then, added to the suspension of pulp. Enzymatic hydrolysis was carried out for 24 h, with pH controlled with hydrochloric acid 0.10 M between 4.5 and 5.5, and temperature around 50 °C. During the first six hours, pH and temperature were monitored each hour, and, then, at 23h and 24h after the beginning of hydrolysis. At the end of hydrolysis, the hydrolysate was filtered by vacuum twice to remove some solid wastes.

1.2 Sulfuric acid hydrolysis of the hydrolysate precipitate

The hydrolysate when autoclaved formed a precipitate that needed to be removed. Then, the hydrolysate was autoclaved and then centrifuged at 5000 rpm, 30 min, at 4 ºC and the solid phase was discarded and the supernatant collected and once again autoclaved. In order to investigate the precipitate composition, an acidic hydrolysis was performed. 200 µL of 72 % (p/p) sulfuric acid were added to 6 mg of precipitate and were kept for three hours at room temperature with occasional stirring. Then, 2.2 mL distilled water were added to dilute the sample until a final H_2SO_4 concentration of 1M and the mixture was incubated for two and a half hours at 100 ºC. Finally, the samples were diluted 1:5 and analyzed by High-Performance Liquid Chromatography (HPLC).

2. Microorganism

In this work the microorganism *Haloferax mediterranei* DSM 1411 was used and was purchased from the German Collection of Microorganisms and Cell Cultures, DSMZ - *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*.

The strain was kept in Petri dishes with solid DSMZ 372 medium through monthly streaking through growth for 96 hours at 37 °C, and subsequent storage under refrigeration at 4 °C.

3. Culture media

Two different media for *H. mediterranei* were used in the present study. The first medium, DSMZ 372 (Table 4), was used to revival the lyophilized culture, to grow the pre-inocula and as solid medium (agar). The defined medium (Table 5) was used in other tests.

Table 4: Composition of DSMZ 372 medium.

Compound	Quantity to 1L of medium
Yeast extract	5.00 g
Casamino acids	5.00 g
Sodium glutamate	1.00 g
Potassium chloride	2.00 g
Sodium citrate	3.00 g
Magnesium sulfate heptahydrate	20.00 g
Sodium chloride	200.00 g
Iron (II) chloride tetrahydrate	36.00 mg
Manganese (II) chloride tetrahydrate	0.36 mg
Agar	**20.00 g**

Table 5: Composition of Fang 2010 medium (Fang et al. 2010).

Compound	Quantity to 1L of medium
Glucose	10.00 g
Sodium chloride	156.00 g
Magnesium chloride hexahydrate	13.00 g
Magnesium sulfate heptahydrate	20.00 g
Calcium chloride hexahydrate	1.00 g
Potassium chloride	4.00 g
Sodium hydrogen carbonate	0.20 g
Sodium bromide	0.50 g
Yeast extract	5.00 g

When pulp hydrolysate was used as carbon source, it substituted glucose in defined medium. To prepare each media, the respective components were added to distilled water at constant stirring. Then pH was adjusted to 7.00 and the media was autoclaved for 20 min at 121 ºC. The exception was sugars in defined medium that needed to be sterilized separately and added under aseptically conditions.

4. Assays

4.1 Pre-inocula preparation

Pre-inocula were conducted in shake flasks of 100 ml and prepared according to Table 6, by sequentially transferring the indicated amount of pre-inoculum after the indicated time of incubation at 180 rpm and 37 °C.

Table 6: Conditions of pre-inocula.

Pre-inoculum	Conditions	Incubation time
1	2 colonies + 30 mL DSMZ 372 medium	72 h
2	10 mL pre-inoculum 1 + 50 mL DSMZ 372 medium	72 h
3	10 mL pre-inoculum 2 + 50 mL Defined medium	72 h
4	10 mL pre-inoculum 3 + 50 mL assay medium	72-96 h

4.2 Shake flasks studies

For this assays in shake flasks 10 % of pre-inoculum 4 were transferred to 250 ml flasks, with assay medium with a working volume up to 125 mL. To each sample it was measured the optical density at 520 nm (UVmini-1240, Shimadzu) using as control the medium assay. A solution of 10 % NaCl was used for dilutions necessary. The pH was also monitored. It was further removed 1 mL of each sample and was centrifuged at 13000 rpm, for 5 minutes. The supernatant was moved into a new eppendorf and both supernatant and precipitate were frozen at -4 °C under refrigeration. The supernatant was used to determine the sugars concentration. The precipitate was used to polymer quantification. At the end of sampling, extraction of polymer from the remaining culture was made in order to form films.

In fed-batch assay, 11 mL of hydrolysate with 156 $g.L^{-1}$ of NaCl was introduced to restore initial sugars concentration (10 $g.L^{-1}$ of glucose), while in SBR assay, fermentation medium has been distributed amongst sterile falcon, centrifuged at 1500 rpm, at 20 °C for 40 minutes. The supernatant was discarded and the pellet was resuspended in 125 mL of fresh hydrolysate medium with 10 $g.L^{-1}$ of glucose. In assay without sterile conditions, growth medium was not autoclaved and the inoculation and the sampling were not made in laminar flow chamber as usual.

4.3 Bioreactor studies

A bioreactor with 5 L of capacity was operated in fed-batch. The pre-inocula preparation was similar to described previously (section 4.1), but the volume was changed in the last two pre-inoculums. The third pre-inoculum was prepared with 20 mL of pre-inoculum 2 more 100 mL of defined medium and the fourth pre-inoculum was prepared with 50 mL of pre-inoculum 3 more 250 mL of assay medium. The reactor was operated from an initial volume of 2500 ml of hydrolysate medium with 10 $g.L^{-1}$ of glucose, at 37 ° C. The culture pH was maintained at 7.0-7.2 by the addition of 2.0 M NaOH or 2.0 M H_2SO_4. The dissolved oxygen concentration was automatically controlled at 20 % by the variation of the stirring speed (200 - 800 rpm). The air flow rate was kept constant during the assay, by an extra air bomb.

Samples of 5 mL were collected to measure the optical density at 520 nm and, then, were centrifuged at 13000 rpm during 5 minutes. Both supernatant and precipitate were frozen at -4 ºC under refrigeration, for further analysis of sugars and polymer quantification, respectively. At the end of fermentation, the remaining biomass was used to extract the polymer.

5. Analytical methods

5.1 Biomass concentration

Biomass concentration was determined as volatile suspended solids (VSS). A sample of 20 mL was collected and centrifuged at 11000 rpm during 20 minutes. The supernatant was discarded and 0.5 mL of NaCl 10 % solution were added to resuspend the

precipitate. The obtained precipitate was dried at 105 ºC until constant weight (approximately three days). Afterwards the sample was reduced to ashes at 550 ºC during two hours. The final concentration could be obtained by difference between mass after 105 ºC treatment and mass after 550 ºC treatment.

In order to determine the amount of biomass in the assay, a calibration curve was constructed by measuring the optical density at 520 nm and the VSS of several dilutions of the liquid culture.

5.2 Concentration of sugars

The concentration of sugars was determined by using HPLC. Samples were centrifuged at 8000 rpm during 10-20 minutes, in eppendorfs with a filter with a pore diameter of 0.22 μm and then analyzed on HPLC (Hitachi HPLC, Biorad Aminex HPX-87H column, oven Gecko 2000 40 °C Detector RI Hitachi U-2490) at a temperature of 40 °C for 20 minutes with an eluent (H_2SO_4 0.01 N) at a flow rate of 0.6 $ml.min^{-1}$. The pH of samples was adjusted to a range of 1-3, with addition of a H_2SO_4 0.25 M solution. A calibration curve was constructed using standards of glucose and xylose with the concentrations described in Table 7.

Table 7: Glucose and xylose concentrations of patterns used in HPLC.

Pattern	**[Glc]** ($g.L^{-1}$)	**[Xyl]** ($g.L^{-1}$)
1	10.00	5.01
2	5.00	2.50
3	4.00	2.00
4	2.50	1.25
5	2.00	1.00
6	1.00	0.50
7	0.50	0.25
8	0.25	0.13

5.3 PHA concentration

PHA concentration was measured by GC-FID based on the method of Lemos et al. 2006. Lyophilized biomass was incubated at 100 ºC during 3.5 hours with heptadecane solutions dissolved with chloroform (1:1) and acidic methanol (20 %). After digestion, the organic phase of each sample was extracted and injected in a gas chromatograph with

flame ionization detector (GC-FID, Bruker 400-GC) and a Bruker BR-SWAX column (30 m x 0.25 mm x 0.25 μm) using hydrogen as carrier gas (14.5 Psi). An injection split of 0.50 μL at 280 ºC with a split proportion of 1:5 were used and the oven temperature program was 40 °C; 20 °C.min^{-1} until 100 °C; 3 °C.min^{-1} until 155 °C and 20 °C.min^{-1} until 220 °C. The detector temperature was 250 ºC. 3HB and 3HV calibration was made through the use of standard commercial P(3HB-*co*-3HV) polymer (88 % / 12 %, Aldrich).

5.4 Extraction of polymer

In order to extract the polymer from the culture, the cell suspension was centrifuged at 11000 rpm for 20 minutes. The supernatant was discarded and the cells were washed with a 20 mL of 0.9 % NaCl solution. The biomass was resuspended and centrifuged again under the same conditions. The supernatant was discarded and the solid phase was ressuspended in 30 mL of chloroform. After being transferred to covered Erlenmeyers, samples were incubated at 28 °C and 180 rpm. After three days of incubation, a vacuum filtration was performed, with glass fiber filter with a pore diameter of 47 μm. The solid phase was discarded and the chloroform was left to evaporate to thereby obtain the desired film.

5.5 Fluorescence microscopy

The method of Nile Blue was applied to some samples taken in order to visualize the accumulation of P(3HB-*co*-3HV) by fluorescence microscopy. 50 μL of Nile Blue were added to 500 μL of sample and the mixture was incubated at 55 °C for 15 minutes. Then it was centrifuged at 3000 rpm for 5 minutes and the supernatant was subsequently discarded. The pellet was resuspended in 0.5 mL of NaCl 10 %, and centrifuged at 3000 rpm, 5 minutes. The supernatant was discarded and the pellet was resuspended in a solution of 8 % of acetic acid in NaCl 10 %. The sample was centrifuged for 1 minute at 3000 rpm. The supernatant was discarded and resuspended in NaCl 10 %. Finally, an aliquot of the prepared sample on a slide was placed for viewing in the microscope (AXIO Imager, Zeiss lenses and shutter HXP 120C), and some images were captured for analysis of the accumulation of P(3HB-*co*-3HV) (adapted from Gameiro 2010).

5.6 Thermal characterization of polymer

Thermal characterization was performed on a Mettler TA 4000 System instrument that consisted of a DSC-30 Differential Scanning Calorimeter, TGA-50 furnace with M3 microbalance, and TA72 GraphWare software. Differential scanning calorimetry (DSC) samples of approximately 5 mg were weighed into 40 mL aluminum pans with an empty pan as reference. Measurements were carried out under 40 mL.min^{-1} nitrogen flow rate as follows: first, second, and third heating from -30 to 200 ºC at 10 ºC.min^{-1}; first cooling (quenching after the first heating) from 200 to -30 ºC at 100 ºC.min^{-1} and the second cooling from 200 to -30 ºC at 10 ºC.min^{-1}. Glass transition temperatures (Tg), melting temperatures (Tm) and melting enthalpy (ΔH_m) were reported from the second heating scan (Koller et al. 2007a; Koller et al. 2007b).

6. Data processing

6.1 Maximum specific growth rate

The maximum specific growth rate (μ_{max}) of *H. mediterranei* was obtained using the equation that describes the microbial growth:

$$\mu_{max} = \frac{1}{X} \cdot \frac{dX}{dt} \quad (h^{-1})$$

(Equation 1)

By linearization and integration of Equation 1, it was possible to calculate the specific growth rate, "μ",:

$$\underbrace{\ln(X_i)}_{\mathbf{y}} = \underbrace{\mu}_{\mathbf{m}} \cdot \underbrace{t}_{\mathbf{x}} + \underbrace{\ln(X_0)}_{\mathbf{b}}$$

(Equation 2)

where "X" is the biomass concentration and "t" is the time. The integration time is taken from "0", which corresponds to the beginning of the exponential phase, and "i" which corresponds to the final instant of the exponential phase, respectively. After the linearization, the maximum specific growth rate corresponded to the slope.

6.2 Substrate consumption rate

The volumetric substrate consumption rate, r_s, was calculated from the variation of substrate concentration (ΔS) over time (Δt):

$$r_s = -\frac{\Delta S}{\Delta t} \quad (g.L^{-1}.h^{-1})$$

(Equation 3)

6.3 Product formation rate

The volumetric product formation rate, r_p, was calculated from the variation of concentration of PHA (ΔP) over time (Δt):

$$r_p = \frac{\Delta P}{\Delta t} \quad (g.L^{-1}.h^{-1})$$

(Equation 4)

The specific product formation rate, q_P, was calculated from the volumetric product formation rate (r_p) and biomass concentration (X):

$$q_p = \frac{1}{X}.\frac{\Delta P}{\Delta t} = -\frac{r_p}{X} \quad (g.g^{-1}.h^{-1})$$

(Equation 5)

6.4 PHA concentration

The PHA concentration ($g.L^{-1}$) can be calculated from the PHA content (% PHA) and biomass concentration ($g.L^{-1}$) in the medium.

$$[PHA] = \frac{\%\ PHA}{[X]} \quad (g.L^{-1})$$

(Equation 6)

CHAPTER III

Results and discussion

1. Hydrolysis of pulp

The pulp hydrolysate has a great potential in terms of biorefinery application, due to its high content of sugars, namely glucose and xylose. These sugars can be consumed and converted by microorganisms to produce several products, like biopolymers. This study investigated the use of pulp from *E. globulus* for the production of biopolymers, but this method could be applied to wood coking from other species of trees with high sugar content. Thus, different types of pulps without the desired quality for the production of paper could be valorized. Furthermore, this concept could be applied to factories that only produce pulp paper or with the paper production stopped. In these cases, the only option for the factories is to sell the pulp paper. In this way the factories could convert the obtained pulp in chemical products with high added value and thus monetize your business.

In order to make monomeric sugars available for microorganisms, it is necessary to introduce a hydrolytic process. Taking into account previous studies conducted between Aveiro University and RAIZ (Cabral 2014), the enzymatic hydrolysis of pulp was chosen and five different enzyme preparations were tested: Cellic Ctec 2, Optimase, Serzym 42, Celluclast 1,5 L and Novozyme NS. The best results were found for the enzyme Cellic Ctec 2 (load 35 FPU/g_{HC}) and followed by Novozyme NS (20 FPU/g_{HC}). Due to unavailability of Cellic Ctec 2 enzyme, Novozyme NS was chosen to be used in this work. The hydrolysate obtained was analyzed and sugar concentrations were determined by DNS method and HPLC. The results are shown in Table 8.

Table 8: Sugars concentration obtained by DNS method and HPLC on the hydrolysate obtained.

Analysis method	**Load** (FPU)	**[Glc]** ($g.L^{-1}$)	**[Xyl]** ($g.L^{-1}$)	**[Reducing Sugars]** ($g.L^{-1}$)
DNS	35	--	--	107.0
	25	--	--	93.0
	15	--	--	81.0
HPLC	35	92.4	23.6	116.0

As it can be seen in Table 8, the concentration of reducing sugars by DNS method (107.0 $g.L^{-1}$) was below the sugars concentration determined by HPLC (116.0 $g.L^{-1}$).

These results showed that the method of HPLC was more efficient in sugar determination, since it detects and quantifies all of sugar monomers present in hydrolysate, while the DNS method only detects and quantifies reducing sugars.

Before its use for microbial growth, the hydrolysate was submitted to sterilization and autoclaved. Since there was a probability of occurring sugar degradation in this process, several steps were tested and samples were collected along the process. The process consisted in a first sterilization by autoclave, then, since a precipitate was formed, the suspension was centrifuged in order to remove the solid phase. Finally the supernatant was autoclaved. The concentration of sugars in each sample is shown in Table 9.

Table 9: Total sugars concentration in different steps of hydrolysate pretreatment determined by HPLC.

Sample	**[Glc]** ($g.L^{-1}$)	**[Xyl]** ($g.L^{-1}$)	**[Total sugars]** ($g.L^{-1}$)
Before 1st Sterilization	76.73	17.02	93.75
After 1st Sterilization	79.91	19.33	99.24
After Centrifuge	80.00	19.35	99.35
After 2nd Sterilization	81.96	20.90	102.86

The composition of the obtained precipitate during the sterilization step (Figure 9) was analyzed, after submitted to a sulfuric acid hydrolysis. The obtained results were 0.74 $g.L^{-1}$ of glucose and 3.77 $g.L^{-1}$ of xylose. The obtained results showed that the precipitate was related with the presence of oligosaccharides that probably resulted from xylans.

The results of Table 9 showed that with the hydrolysate pretreatment, glucose and xylose concentrations increased, probably due to evaporation of water. In a small scale, this pre-treatment can be advantageous, because it reduces the contamination risks. But, once in this work a high saline medium was required, in which only microorganisms such as *H. mediterranei* survive, there is low risk of contamination. The sterilization of the hydrolysate was not necessary, which on a larger scale means savings in terms of cost, energy and time.

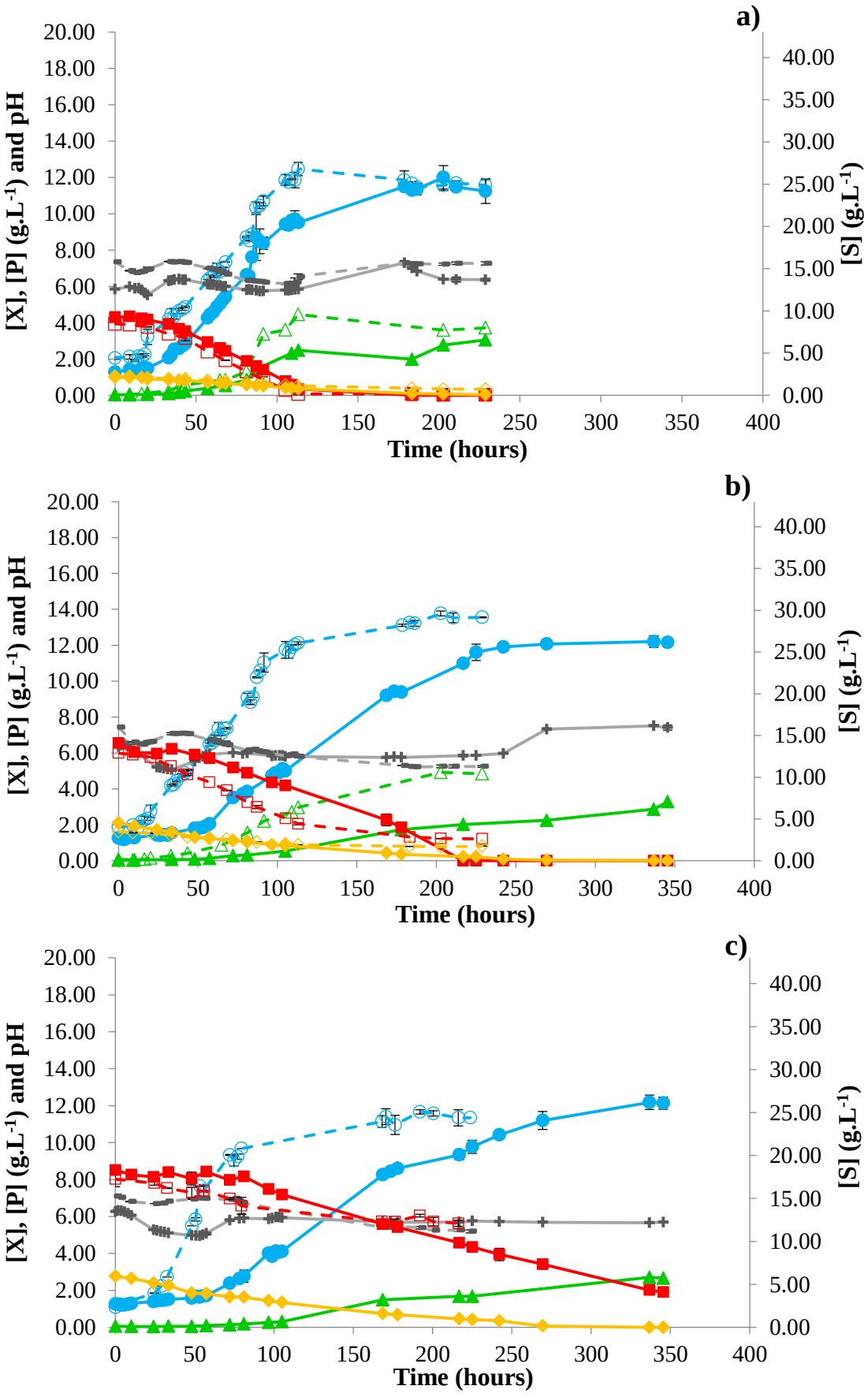
Nevertheless, in the preliminary tests, the sterilized hydrolysate was used with a glucose concentration of 81.96 $g.L^{-1}$ and 20.10 $g.L^{-1}$ for xylose.

Figure 9: Hydrolysate precipitate.

2. PHA production tests

2.1 Influence of the sugar concentration

Different concentrations of sugars were tested in order to determine the ideal conditions for the growth of *H. mediterranei*. Glucose concentrations of 10, 15, 20, 30 and 40 $g.L^{-1}$, both in hydrolysate and in defined media were tested and biomass concentration, [X], sugar consumption, [S], and PHA production, [P], were followed in the different assays. Only in assays in defined medium with 10 and 15 $g.L^{-1}$ xylose was added as carbon source. In defined medium with 20 $g.L^{-1}$ the polymer was not possible to quantify by GC-FID. The results of all tests are shown in Figure 10. Parameters as fermentation time, initial and final biomass concentrations ($[X]_{in}$ and $[X]_{fi}$), maximum specific growth rate (μ_{max}), glucose and xylose concentrations consumed ($[Glc]_{cons}$ and $[Xyl]_{cons}$), glucose and xylose consumption growth rates (r_{Glc} and r_{Xyl}), final polymer concentration ($[P_{fi}]$), polymer formation rate (r_{Prod}), PHA content (% PHA) and percentage of 3HV (% 3HV) of defined and hydrolysate media for all the tests are shown in Table 10.


a)
[X], [P] (g.L-1) and pH
[S] (g.L-1)
Time (hours)
b)
[X], [P] (g.L-1) and pH
[S] (g.L-1)
Time (hours)
c)
[X], [P] (g.L-1) and pH
[S] (g.L-1)
Time (hours)

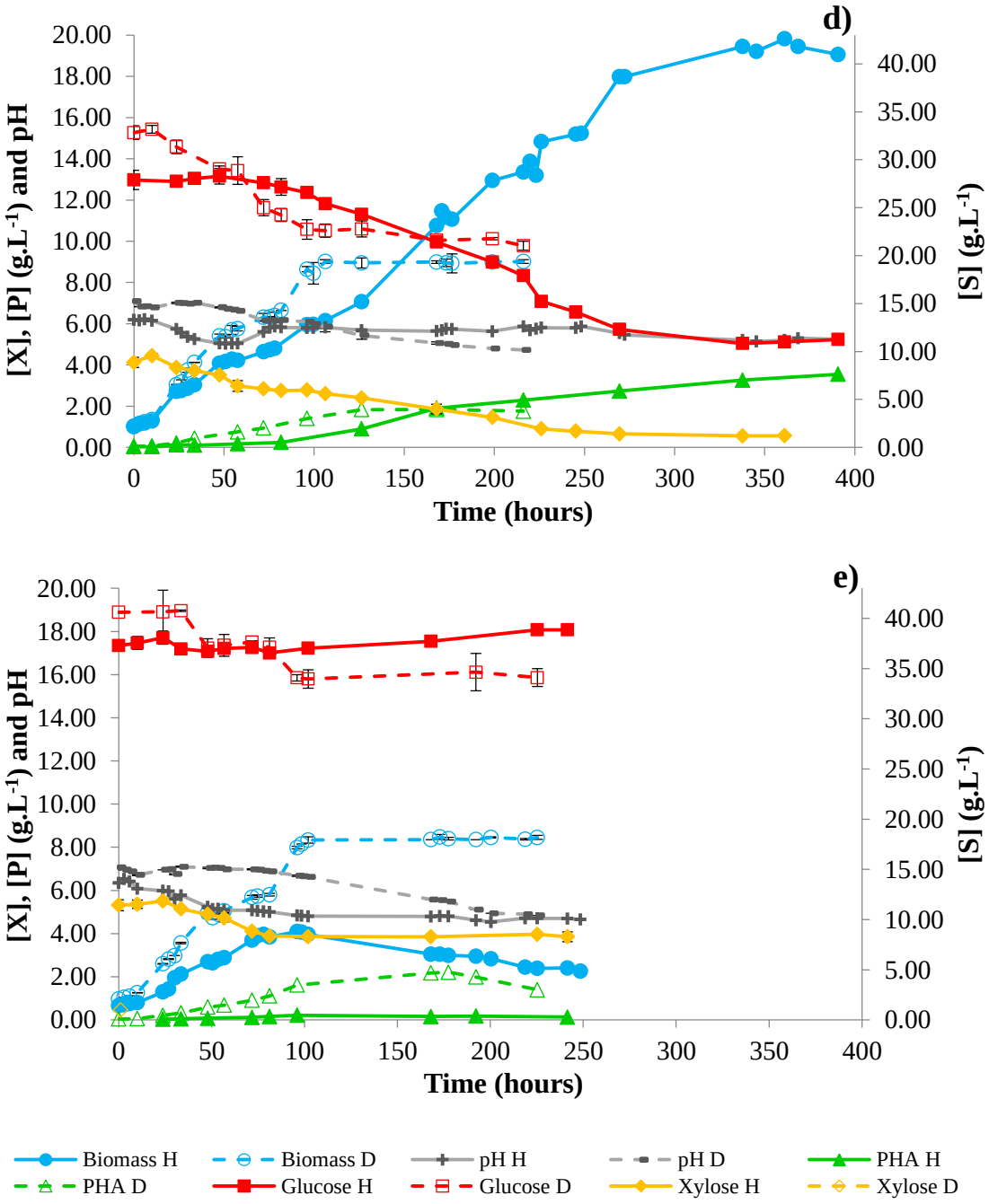


Figure 10: Evolution of pH and concentration of biomass ([X]), P(3HB-*co*-3HV) ([P]), glucose and xylose ([S]) during the PHA production test, by *H. mediterranei* in defined (D) and hydrolysate (H) media with **a)** 10 g.L^{-1} of glucose and 2.5 g.L^{-1} of xylose, **b)** 15 g.L^{-1} of glucose and 3.8 g.L^{-1} of xylose, **c)** 20 g.L^{-1} of glucose and 5.0 g.L^{-1} of xylose, **d)** 30 g.L^{-1} of glucose and 7.6 g.L^{-1} of xylose and **e)** 40 g.L^{-1} of glucose and 10.0 g.L^{-1} of xylose. Standard deviation indicated on the calculated average biomass concentration of biological samples (test in duplicate).

Table 10: Parameters of the tests in defined and hydrolysate media with 10, 15, 20, 30 and 40 $g.L^{-1}$ of glucose.

	Ferm. time	**$[X]_{in}$** ($g.L^{-1}$)	**$[X]_{fi}$** ($g.L^{-1}$)	**μ_{max}** (h^{-1})	**$[Glc]_{cons}$** ($g.L^{-1}$)	**r_{Glc}** ($g.L^{-1}.h^{-1}$)	**$[Xyl]_{cons}$** ($g.L^{-1}$)	**r_{Xyl}** ($g.L^{-1}.h^{-1}$)	**$[P]_{fi}$** ($g.L^{-1}$)	**r_{Prod}** ($g.L^{-1}.h^{-1}$)	**% PHA**	**% 3HV**
Hydrolysate 10 $g.L^{-1}$	229	1.27	11.25	0.0237	9.34	0.0926	2.18	0.0127	3.05	0.0345	27.1	16.2
Def. Medium 10 $g.L^{-1}$	229	2.06	11.58	0.0156	8.40	0.0903	1.41	0.0108	3.72	0.0788	32.1	11.4
Hydrolysate 15 $g.L^{-1}$	346	1.26	12.16	0.0094	14.07	0.0665	4.54	0.0229	3.29	0.0101	27.0	13.8
Def. Medium 15 $g.L^{-1}$	229	1.86	13.56	0.0159	10.28	0.0869	1.80	0.0147	4.85	0.0290	35.8	11.4
Hydrolysate 20 $g.L^{-1}$	346	1.27	12.14	0.0102	14.20	0.0479	5.94	0.0274	2.66	0.0097	21.9	16.2
Def. Medium 20 $g.L^{-1}$	201	1.09	11.59	0.0470	5.15	0.0553	---	---	---	---	---	---
Hydrolysate 30 $g.L^{-1}$	391	1.00	19.06	0.0081	16.02	0.0751	9.57	0.0289	3.55	0.0105	19.5	17.8
Def. Medium 30 $g.L^{-1}$	216	1.04	9.02	0.0129	12.16	0.1236	---	---	1.75	0.0156	19.5	10.7
Hydrolysate 40 $g.L^{-1}$	249	0.66	2.27	0.0148	3.85	0.0164	4.21	0.0598	0.13	0.0024	5.6	14.9
Def. Medium 40 $g.L^{-1}$	225	0.96	8.46	0.0142	8.30	0.0784	---	---	1.40	0.0194	16.5	10.3

By analyzing Figure 10 and Table 10, compare the *H. mediterranei* growth and PHA storage with different substrate concentrations. Considering the fermentation time, growth in hydrolysate medium with 15, 20 and 30 $g.L^{-1}$ required higher fermentation times (346 – 391 hours) than in hydrolysate medium with 10 and 40 $g.L^{-1}$ (229 – 249 hours). This may be due to the difficulty of culture in adapting to the medium, probably due to high decrease of pH to values around 5.0. In these assays, the lag phase was much higher than in other tests: in tests with 15 and 20 $g.L^{-1}$ the lag phase had duration of 57 hours and in the test with 30 $g.L^{-1}$, 78 hours. In assays in hydrolysate medium with 10 and 40 $g.L^{-1}$ the lag phase had durations of 20 and 10 hours, respectively. These last values were in accordance with results obtained in defined medium, which showed lag phases between 10 and 18 hours and fermentation times between 201 and 229 hours.

In experiments with 10, 15, 20 and 30 $g.L^{-1}$ in hydrolysate medium, pH decreased during the lag phase and increased when exponential phase started. After that, pH remained constant until the end of test, with the exception of tests in 10 and 15 $g.L^{-1}$ which showed pH variations during this phase, only stabilizing in stationary phase. In the experiment in hydrolysate medium with 40 $g.L^{-1}$, pH decreased during the entire assay. In assays in hydrolysate medium with 20, 30 and 40 $g.L^{-1}$ a significantly decrease to very low pH values, 4.66 - 5.70, was observed. This decrease throughout the tests, reaching values far below to what is considered ideal for this strain (4.66 < 7.20) might led to growth inhibition, and consequently the beginning of stationary phase. These values were in accordance with results obtained in defined medium, because in defined medium pH also decreased during lag phase and increased in the beginning of exponential phase. Only in the experiment using defined medium with 10 $g.L^{-1}$ growth seemed to not be inhibited by decrease of pH.

Glucose was depleted only in assays in hydrolysate medium with 10 and 15 $g.L^{-1}$, which led to growth arrest and a consequent entry into the stationary phase. Although in hydrolysate medium with 20 and 30 $g.L^{-1}$, the values of $[Glc]_{cons}$ were higher (14.20 $g.L^{-1}$ and 16.02 $g.L^{-1}$, respectively), the consumption was lower, because in the first case it corresponded to 76.93 % of the $[Glc]_{in}$, and in the second case to 53.90 %. In hydrolysate medium with 30 $g.L^{-1}$, the glucose consumption stopped at 338 hours. The lowest glucose consumption was observed in hydrolysate medium with 40 $g.L^{-1}$, which resulted in consumption percentage of just 10.11 % of $[Glc]_{in}$ and r_{Glc} of 0.0164 $g.L^{-1}.h^{-1}$, constituting

the hypothesis of substrate inhibition. The best r_{Glc} was obtained in hydrolysate medium with 10 $g.L^{-1}$, with r_{Glc} of 0.926 $g.L^{-1}.h^{-1}$. This tendency was found in tests with defined medium, where the experiment with 10 $g.L^{-1}$ resulted in higher consumption with glucose fully consumed, with r_{Glc} of 0.0903 $g.L^{-1}.h^{-1}$. The lowest $[Glc]_{cons}$ in defined medium was found in the experiment with 40 $g.L^{-1}$, with a percentage of glucose consumption of 22.25 % of $[Glc]_{in}$. Taking into account these results and the culture behavior over time, it can be concluded that the growth in hydrolysate and defined media with 40 $g.L^{-1}$ might have been inhibited by substrate. This can be explained by the ratio substrate/biomass, which was much higher than in other experiments. In general, experiments on hydrolysate medium presented a glucose consumption rate lower than in the experiments with defined medium. This may be due to the existence of compounds in hydrolysate that could inhibit *H. mediterranei* growth.

The best $[Xyl]_{cons}$ were obtained in hydrolysate medium with 10, 15 and 20 $g.L^{-1}$, in which exhaustion of xylose was observed, which might resulted in growth arrest and a consequent entry into the stationary phase. In hydrolysate medium with 30 $g.L^{-1}$ just remained 1.22 $g.L^{-1}$ of xylose at the end of fermentation. As observed to glucose, xylose consumption also stopped with the beginning of stationary phase. Only in hydrolysate medium with 40 $g.L^{-1}$ the consumption of this sugar was not significant, because only 35.53 % of initial xylose concentration, $[Xyl]_{in}$, were consumed. In this case, the xylose was consumed during the exponential phase. Although the hydrolysate medium with 40 $g.L^{-1}$ registered the worst consumption, the xylose uptake was fast, resulting in a high xylose consumption rate, 0.0598 $g.L^{-1}.h^{-1}$. In defined medium with 10 and 15 $g.L^{-1}$ the xylose was not totally consumed and the r_{Xyl} were lower than in hydrolysate medium (0.0108 and 0.0147 $g.L^{-1}.h^{-1}$ in defined medium, respectively, and 0.0127 and 0.0229 $g.L^{-1}.h^{-1}$ in hydrolysate medium, respectively).

In terms of $[X]_{in}$, this value was kept similar in all assays in hydrolysate medium (1.00 - 1.27 $g.L^{-1}$), with exception of the assay with 40 $g.L^{-1}$ (0.66 $g.L^{-1}$). In this case, a lower value was obtained, which might influence growth. In this case, inoculum concentration might not be enough for a good adaptation of the culture and thus its growth was hampered, reaching the lowest $[X]_{fi}$ and the worst sugar consumption rates. On the other hand, assays with the highest $[X]_{fi}$ in hydrolysate medium with 15, 20 and 30 $g.L^{-1}$ were obtained, being the latter the test with the highest $[X]_{fi}$ (19,06 $g.L^{-1}$). The μ_{max} was

similar in all the tests, with the exception of hydrolysate medium of 10 $g.L^{-1}$, with the best μ_{max} of 0.0237 h^{-1}. In contrast, the assays in hydrolysate medium with 15 and 30 $g.L^{-1}$ were those with the lowest values of μ_{max} (0.0094 and 0.0081 h^{-1}, respectively). Comparing with assays in defined medium, the biggest difference was found between hydrolysate and defined media with 30 $g.L^{-1}$, because the growth was very different between both. In defined medium, *H. mediterranei* had a similar behavior when compared with other tests in defined medium, achieving a $[X]_{fi}$ of 9.02 $g.L^{-1}$. On the other hand, in hydrolysate medium, *H. mediterranei* achieve a much higher $[X]_{fi}$ (19.06 $g.L^{-1}$) and much different from achieved in defined medium.

The PHA produced was identified and quantified by GC-FID as P(3HB-*co*-3HV). As can be seen in Figure 10, polymer production was associated to growth, because PHA production and biomass growth occurred simultaneously. The highest final polymer concentration, $[P]_{fi}$, were obtained in hydrolysate media with 10, 15 and 30 $g.L^{-1}$, reaching values higher than 3.00 $g.L^{-1}$. The best value was obtained in hydrolysate medium with 30 $g.L^{-1}$ (3.55 $g.L^{-1}$). On the other hand, the worst result was obtained in hydrolysate media with 40 $g.L^{-1}$ (0.13 $g.L^{-1}$), as expected. In tests in hydrolysate medium with 10, 15, 20 and 30 $g.L^{-1}$, [P] increased until the end of the assays. However, in the test in hydrolysate medium with 40 $g.L^{-1}$, [P] decreases during stationary phase, probably due to the consumption of the polymer, because there external sugars consumption stopped during this phase. In terms of r_{Prod}, the best results were obtained in hydrolysate media with 10 $g.L^{-1}$ (0.0345 $g.L^{-1}.h^{-1}$). In hydrolysate medium with 40 $g.L^{-1}$, this value was very low (0.0024 $g.L^{-1}.h^{-1}$). Comparing with assays in defined medium, $[P]_{fi}$ and r_{Prod} were higher in defined than in hydrolysate medium, with the exception of the experience with 30 $g.L^{-1}$ that registered a $[P]_{fi}$ lower than in hydrolysate medium. The values of % PHA were higher in hydrolysate media with 10 and 15 $g.L^{-1}$ (27.1 and 27.0 %). In the case of hydrolysate medium with 40 $g.L^{-1}$ this was very low (5.6 %). Despite $[P]_{fi}$ obtained in hydrolysate medium with 30 $g.L^{-1}$ was high, % PHA was low. This is due to the fact that in this test a $[X]_{fi}$ significantly higher was obtained than in the others tests, resulting in a consequent increase in polymer concentration. The highest % 3HV was obtained in hydrolysate medium with 30 $g.L^{-1}$, this means that polymer obtained in this assay presented the best processing properties (Serafim et al. 2008b). Regarding the assays in defined medium, %

PHA was higher in defined than in hydrolysate medium, but polymer films obtained in hydrolysate medium had higher % 3HV.

The tests performed confirmed the ability of *H. mediterranei* to grow and produce P(3HB-*co*-3HV) from glucose. Moreover, these tests showed that the xylose can also be used by this bacterium demonstrating the capability of the pulp hydrolysate can be used as substrate for the production of PHA.

Taking into account the results obtained in all of tests with different concentrations, the concentration of 10 $g.L^{-1}$ was selected as the best, due to high values of μ_{max}, $[Glc]_{cons}$ and $[Xyl]_{cons}$ (total depletion of glucose and xylose), r_{Glc}, r_{Prod}, % PHA and % 3HV. This concentration was considered as optimal and used in the following tests.

In Table 11 summarized some results of growths in shaker flasks obtained in this work and also data reported in literature. The strain used was the same in all tests (DSM 1411) but with different growth media. Bhattacharyya et al. (2012) used NaCl 200 $g.L^{-1}$ (while in this study 156 $g.L^{-1}$ of NaCl was added), $MgSO_4.7H_2O$ 20 $g.L^{-1}$ (the same used in this study), KCl 2 $g.L^{-1}$ (half of the amount used in this study), $C_5H_8NNaO_4$ 1 $g.L^{-1}$, KH_2PO_4 0.0375 $g.L^{-1}$, $FeSO_4.7H_2O$ 0.05 $g.L^{-1}$, yeast extract 1 $g.L^{-1}$ (five times less than the amount used in this study) and vinasse as carbon source. Studies by Parnaudeau et al. (2008) showed that sugarcane vinasse is composed by significant amounts of sucrose as well as oxalate, lactate, malate and pyruvate. These differences in the media composition could result in some differences between the results of this work and the work of Bhattacharyya et al. (2012).

Table 11: Parameters of the tests in 20 $g.L^{-1}$ and 30 $g.L^{-1}$ hydrolysate media and data from literature.

	$[S]_{in}$ ($g.L^{-1}$)	μ_{max} (h^{-1})	$[P]_{fi}$ ($g.L^{-1}$)	r_P ($g.L^{-1}.h^{-1}$)	q_P ($mg_P.g_X^{-1}.h^{-1}$)	% PHA
Hydrolysate 20 $g.L^{-1}$ (present study)	25.0	0.0102	2.66	0.0097	3.35	21.9
Hydrolysate 30 $g.L^{-1}$ (present study)	37.7	0.0064	3.55	0.0105	0.86	19.5
25% pre-treated vinasse (Bhattacharyya et al. 2012)	22.6	0.130	19.7	0.210	7.30	70.0
50% pre-treated vinasse (Bhattacharyya et al. 2012)	32.9	0.120	17.4	0.180	6.90	66.0

As can be seen from analysis of the Table 11, and comparing the tests with 25 % and 50 % of pretreated vinasse, it was possible to conclude that with the increase of carbon source concentration there was a decrease of $[P]_{fi}$ and % PHA. On the other hand, in the tests with hydrolysate medium with 20 and 30 $g.L^{-1}$ of glucose, $[P]_{fi}$ increased with the increase of the carbon source concentration. This can be explained by the fact that in the assay in hydrolysate medium with 30 $g.L^{-1}$ of glucose has been a great increase in biomass concentration, [X], significantly higher than that obtained in the medium with 20 $g.L^{-1}$. Thus was achieved also a higher $[P]_{fi}$. However, in terms of % PHA this value decreases, such as occurred in the testes reported in the literature. In this way it was possible to conclude that in these cases the increase of carbon source concentration produced an inhibitory effect in polymer production.

Making a brief comparison between this study and studies provided by Bhattacharyya et al. (2012) it can be seen that the results obtained in this work were worst, because from the same amount of substrate was not possible to accumulate the quantity of polymer such as reported in the literature, the values were much lower. The explanations may be the carbon source that was different in both assays (glucose and xylose in the present study and sucrose in the case of literature); in the test operating conditions, which although they are the same can be influenced by the types of equipment used and also by used inoculum, in which case reported in the literature it was concentrated by centrifugation at 10000 rpm for 12 minutes, which causes an increase in the initial concentration of the growth and possibly better results either growth or accumulation.

2.2 Influence of feeding strategy

Since tests in hydrolysate medium with 10 $g.L^{-1}$ growth ceased possibly due to lack of carbon source, an assay was conducted to test fed-batch conditions, by given a hydrolysate pulse of 10 $g.L^{-1}$ to restore the initial concentration of sugars. Another solution in order to avoid growth arrest due to depletion of sugars was resuspend the biomass into fresh medium, simulating a Sequencing Batch Reactor, SBR. The results of both tests are shown in Figure 11 and Table 12.

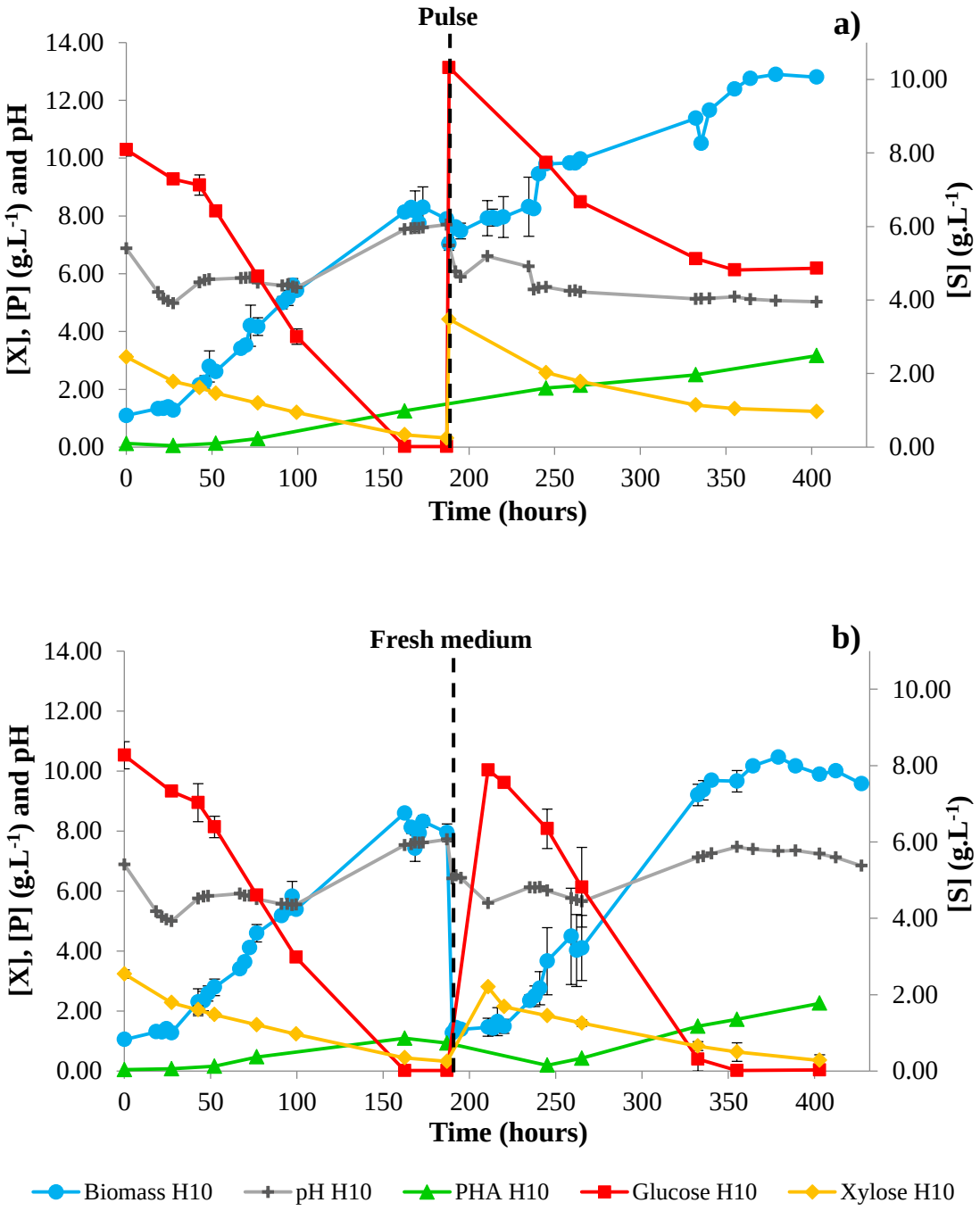


Figure 11: Evolution of pH and concentration of biomass ([X]), P(3HB-co-3HV) ([P]), glucose and xylose ([S]) during the PHA production test, by *H. mediterranei* in hydrolysate medium with 10 $g.L^{-1}$ of glucose and 2.5 $g.L^{-1}$ of xylose**, a)** in fed-batch and **b)** in SBR conditions. Standard deviation indicated on the calculated average biomass concentration of biological samples (test in duplicate).

Table 12: Parameters of the tests in hydrolysate medium with 10 $g.L^{-1}$ and in fed-batch and SBR conditions.

	Ferm. time	**$[X]_{in}$** ($g.L^{-1}$)	**$[X]_{fi}$** ($g.L^{-1}$)	**μ_{max}** (h^{-1})	**$[Glc]_{cons}$** ($g.L^{-1}$)	**r_{Glc}** ($g.L^{-1}.h^{-1}$)	**$[Xyl]_{cons}$** ($g.L^{-1}$)	**r_{Xyl}** ($g.L^{-1}.h^{-1}$)	**$[P]_{fi}$** ($g.L^{-1}$)	**r_{Prod}** ($g.L^{-1}.h^{-1}$)	**% PHA**	**% 3HV**
Hydrolysate 10 $g.L^{-1}$	229	1.27	11.25	0.0237	9.34	0.0926	2.18	0.0127	3.05	0.0345	27.1	16.2
Fed-Batch	403	1.09	12.81	0.0165	13.53	0.0467	4.72	0.0137	3.16	0.0085	24.7	18.1
SBR	427	1.05	9.57	0.0121/ 0.0134	16.11	0.0615	4.21	0.0116	2.26	0.0112	22.8	14.2

By analyzing Figure 11 and Table 12 both tests can be compared. The lag phase was similar in both tests and in hydrolysate medium with 10 $g.L^{-1}$, as expected. During the first part of the growth it was consumed 8.07 $g.L^{-1}$ of glucose in the fed-batch assay and 8.26 $g.L^{-1}$ in SBR assay. These values were lower when compared with the hydrolysate experiment with 10 $g.L^{-1}$, that registered $[Glc]_{cons}$ of 9.34 $g.L^{-1}$. This could explain the lower values of $[X]_{fi}$ in both fed-batch and SBR tests (~ 8.30 $g.L^{-1}$ comparative with 11.50 $g.L^{-1}$ in hydrolysate medium with 10 $g.L^{-1}$). $[Xyl]_{cons}$ was similar in all of assays, reaching values between 2.18 and 2.29 $g.L^{-1}$.

In fed-batch assay, the addition of pulse of 10 $g.L^{-1}$ promoted an increase in $[X]_{fi}$. Contrary to what would be expected, after this pulse, the culture showed again a lag phase. This can be explained by the fact that the culture was at least about 25 hours without glucose before the new pulse addition. Thus, a new adaptation to new conditions was necessary. Then, the bacteria started the exponential phase with μ lower than in first pulse and resulted in a low biomass concentration, probably due to the low pH values. In first part of the growth, glucose was consumed at a rate of 0.0594 $g.L^{-1}.h^{-1}$, while in the second part this value decreased to 0.0360 $g.L^{-1}.h^{-1}$. The opposite happened with xylose consumption that registered an increase of r_{Xyl} after second pulse (0.0106 $g.L^{-1}.h^{-1}$ after and 0.0159 $g.L^{-1}.h^{-1}$ before second pulse). In terms of polymer production, the big difference was in r_{Prod} that was much lower in this case (0.0085 $g.L^{-1}.h^{-1}$ compared to 0.0345 $g.L^{-1}.h^{-1}$ in hydrolysate medium with 10 $g.L^{-1}$). Nevertheless, in this assay higher polymer was obtained with higher % 3HV.

In assay with SBR conditions at 190 hours the exhausted fermentation medium was taken by centrifuging at 5000 rpm, for 40 minutes at 20 °C. The supernatant was discarded and the precipitate resuspended into fresh medium. However, the [X] suffered a large decrease due to poor separation of precipitate and supernatant, as a result of low centrifuge speed. Thus, the culture had a new lag phase for two reasons: to adapt to a fresh medium and as happened in assay in fed-batch conditions, probably it was at least 25 hours without glucose before resuspension of biomass. Both r_{Glc} and r_{Xyl} were higher in second part of the growth than in first part. Before first pulse glucose and xylose were consumed at rates of 0.0589 and 0.0104 $g.L^{-1}.h^{-1}$ and before second pulse at rates of 0.0657 and 0.0112 $g.L^{-1}.h^{-1}$. In terms of polymer production, $[P]_{fi}$ and % PHA were lower than in fed-batch

experiments, because [P] decreased significantly with resuspension of biomass. However, r_{Prod} was higher (0.0112 $g.L^{-1}.h^{-1}$).

Thereby as to avoid growth arrest due to depletion of the sugars, the most effective method is to carry out a test in fed-batch conditions, since it achieved better results of $[X]_{fi}$, μ_{max}, $[P]_{fi}$, % PHA and % 3 HV than the test in SBR conditions.

2.3 Assay without sterile conditions

H. mediterranei is known to require high concentration of sodium chloride to grow. In such high saline conditions, the probability of growing contaminants is low. Thus, an assay without sterilization was performed. The results are shown in Figure 12 and Table 13. The inocula preparation was made in the same way as in the previous tests, but the rest of the procedure was made without sterilization: the growth medium was not autoclaved and the inoculation and the sampling were not made in laminar flow chamber as usual.

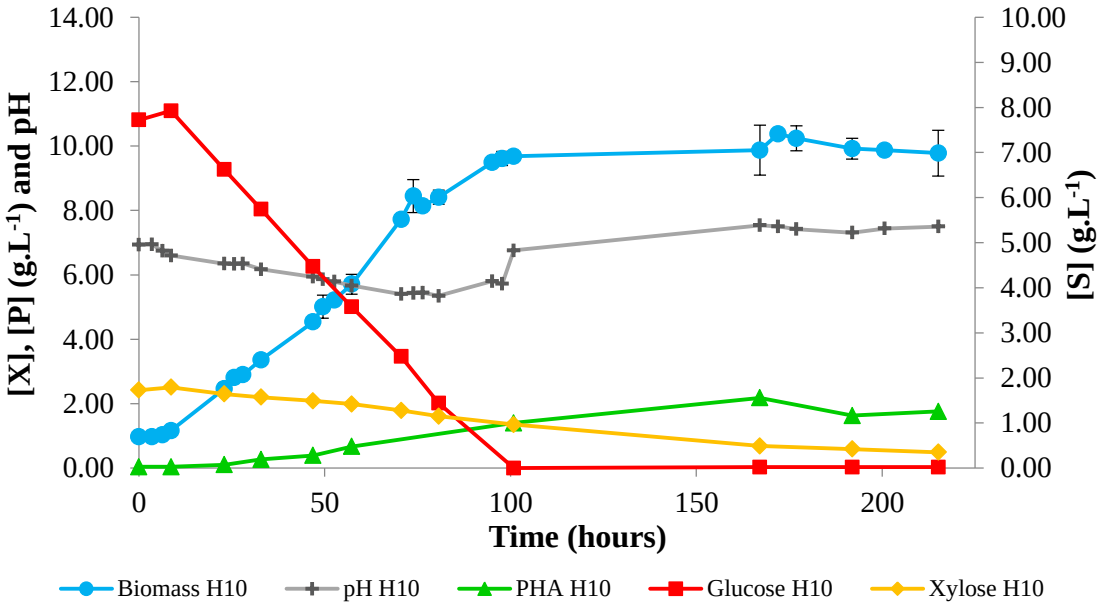


Figure 12: Evolution of pH and concentration of biomass ([X]), P(3HB-*co*-3HV) ([P]), glucose and xylose ([S]) during the PHA production test, by *H. mediterranei* in hydrolysate medium with 10 $g.L^{-1}$ of glucose and 2.5 $g.L^{-1}$ of xylose without sterilization. Standard deviation indicated on the calculated average biomass concentration of biological samples (test in duplicate).

Table 13: Parameters of the tests in hydrolysate medium with 10 $g.L^{-1}$ and without sterilization.

	Ferm. time	**$[X]_{in}$** ($g.L^{-1}$)	**$[X]_{fi}$** ($g.L^{-1}$)	**μ_{max}** (h^{-1})	**$[Glc]_{cons}$** ($g.L^{-1}$)	**r_{Glc}** ($g.L^{-1}.h^{-1}$)	**$[Xyl]_{cons}$** ($g.L^{-1}$)	**r_{Xyl}** ($g.L^{-1}.h^{-1}$)	**$[P]_{fi}$** ($g.L^{-1}$)	**r_{Prod}** ($g.L^{-1}.h^{-1}$)	**% PHA**	**% 3HV**
Hydrolysate 10 $g.L^{-1}$	229	1.27	11.25	0.0237	9.34	0.0926	2.18	0.0127	3.05	0.0345	27.1	16.2
Without sterilization	215	0.97	9.78	0.0226	7.90	0.0869	1.44	0.0127	1.76	0.0145	18.0	12.5

In the assay without sterilization, *H. mediterranei* presented a similar behavior to hydrolysate medium with 10 $g.L^{-1}$, with μ_{max}, r_{Glc} and r_{Xyl} approximately equal. However, in this case $[X]_{fi}$ were lower. This can be explained by the lower concentration of glucose supplied, since in this assay only 7.93 $g.L^{-1}$ of glucose were added, while in the test in hydrolysate medium with 10 gL^{-1}, 9.34 $g.L^{-1}$ of glucose were supplied. The same happened with xylose. In this experiment only 1.79 $g.L^{-1}$ of xylose were introduced, while in the other assay it were added 2.18 $g.L^{-1}$. Furthermore, in test without sterile conditions, xylose was not depleted, having been consumed only 80.45 % of $[Xyl]_{in}$. The polymer production was also very affected. In this case only 1.76 $g.L^{-1}$ of PHA was obtained with % PHA of 18.0 %, while in hydrolysate medium with 10 $g.L^{-1}$ it were registered 3.05 $g.L^{-1}$ with 27.1 % of PHA. Despite in this assay the polymer production was lower than in hydrolysate medium with 10 $g.L^{-1}$, in terms of growth it is possible to conclude that this was not significantly affected.

2.4 Assay in bioreactor

The previous assays allowed choosing the best conditions for PHA production and growth of *H. mediterranei* using pulp hydrolysate: fed-batch with pulses of 10 $g.L^{-1}$ of glucose. The results obtained along the assay in bioreactor are shown in Figure 13.

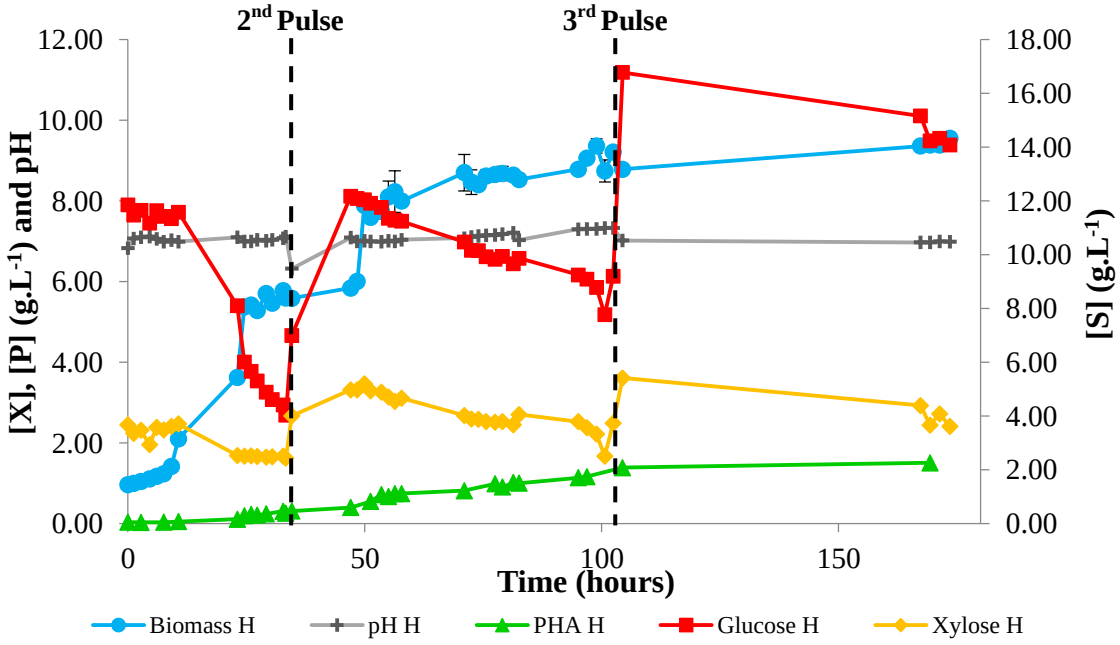
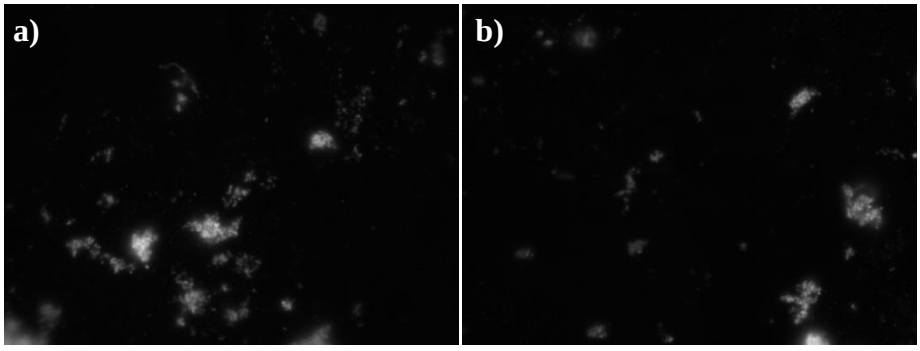


Figure 13: Evolution of pH and concentration of biomass ([X]), P(3HB-*co*-3HV) ([P]), glucose and xylose ([S]) during the bioreactor assay by *H. mediterranei* in hydrolysate medium with 10 $g.L^{-1}$ of glucose and 2.5 $g.L^{-1}$ of xylose. Standard deviation indicated on the calculated average biomass concentration of biological samples (test in duplicate).

In this assay, $[X]_{in}$ was 0.97 $g.L^{-1}$. After a lag phase of 9 hours, [X] increased exponentially up to 6.00 $g.L^{-1}$ at 25 hours. At 35 hours a second pulse of hydrolysate (10 $g.L^{-1}$) was added and at 104 hours a third pulse under the same conditions. $[X]_{fi}$ was 9.55 $g.L^{-1}$ at the end of test at 174 hours. Considering the variation of the total biomass in the exponential phase, there was a first μ of 0.0698 h^{-1}. After a second pulse, a second μ was determined, presenting a value much lower than first (0.0140 h^{-1}). After a third pulse, the growth stopped and so it was not possible to calculate a third μ. In terms of sugars, the $[Glc]_{in}$ and $[Xyl]_{in}$ after first pulse were 11.85 $g.L^{-1}$ and 3.68 $g.L^{-1}$, respectively, which remained constant during the lag phase of biomass growth. After that, *H. mediterranei* started sugars consumption and there was a decrease up to 4.03 $g.L^{-1}$ and 2.44 $g.L^{-1}$, respectively, to 33 hours, which corresponds to r_{Glc} and r_{Xyl} of 0.368 $g.L^{-1}.h^{-1}$ and 0.0947 $g.L^{-1}.h^{-1}$. With second pulse addition, sugar concentration increased up to 12.17 $g.L^{-1}$ and 4.97 $g.L^{-1}$, respectively. It was registered again sugars consumption rates, but were lower than in first part. In the case of glucose, this value was 0.0679 $g.L^{-1}.h^{-1}$ and in the case of xylose was 0.0357 $g.L^{-1}.h^{-1}$. Finally after third pulse, r_{Glc} and r_{Xyl} reached values of 0.0329 $g.L^{-1}.h^{-1}$ and 0.0220 $g.L^{-1}.h^{-1}$, respectively. Although they have been provided three carbon source pulses, the values of [P] and % PHA were very low when compared with previous assays. In this case the $[P]_{fi}$ obtained was 1.50 $g.L^{-1}$ and the final % PHA was 16.0 % (92.5 % of 3HB and 3HV of 7.5 %). Also the r_{Prod} (0.0144 $g.L^{-1}.h^{-1}$) was lower than previous assays. In this last assay the lowest content in 3HV was recorded. PHA accumulation was also observed at certain times of the assay through fluorescence microscopy using Nile Blue staining. The images allow observing polymer granules as bright white dots (Figure 14).



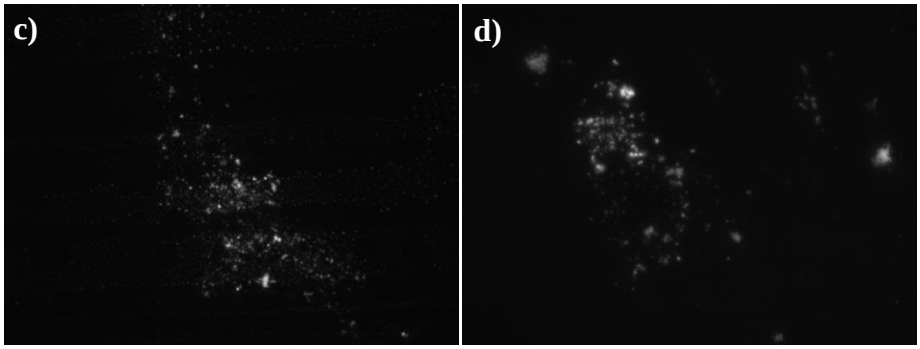


Figure 14: Fluorescence microscopy images with Nile Blue of the *H. mediterranei* bioreactor growth in hydrolysate medium with 10 $g.L^{-1}$ of glucose (Figure 13). The images **a)** and **b)** correspond to 33 hours with 5.60 $g.L^{-1}$ of biomass and 0.26 $g.L^{-1}$ of polymer; the images **c)** and **d)** correspond to 174 hours with 9.56 $g.L^{-1}$ of biomass and 1.50 $g.L^{-1}$ of polymer (Magnification 1000x).

In Table 14 some results obtained in this work are compared with results reported in literature. The strain used was the same in all tests (DSM 1411 / ATCC 33500). NaCl and carbon source quantities used were different in all of the studies: 156 $g.L^{-1}$ of NaCl, 10 $g.L^{-1}$ of glucose and 2.5 $g.L^{-1}$ of xylose in the present study; 194 $g.L^{-1}$ of NaCl and 10 and 20 $g.L^{-1}$ of glucose in the study of Lillo & Rodriguez-Valera (1990); 234 $g.L^{-1}$ of NaCl and 1 $g.L^{-1}$ of glucose in the study of Don et al. (2006); 110 $g.L^{-1}$ of NaCl and 20 $g.L^{-1}$ of starch in the study of Zhao et al. (2013) and 156 $g.L^{-1}$ of NaCl and whey lactose in studies of Koller et al. (2007b and 2008). The bioreactor conditions were similar: 37 ºC of temperature (with the exception of the Lillo & Rodriguez-Valera (1990) study that used 38 ºC); 7.0 – 7.2 of pH; 100 % of dissolved oxygen, controlled by increasing the air flow rate or agitation speed, with the exception of present study that controlled the dissolved oxygen concentration at 20 %, by the variation of the stirring speed and kept constant the air flow rate; Don et al. (2006) study that used constant agitation of 800 rpm and air flow rate of 10 $L.min^{-1}$ and the exception of Koller et al. (2008) that used oxygen tension corresponding to 50 % of air saturation during balanced growth and 30-40 % of air saturation during predominant PHA formation with control by adjustment of the agitation speed at constant aeration of 10 $mL.min^{-1}$. Only the Lillo & Rodriguez-Valera (1990) study was operated in batch. The Zhao et al. (2013) study initially operated the reactor in batch but from 26.0 hours to end, stock medium (129 $g.L^{-1}$ of NaCl, 45.45 $g.L^{-1}$ of NH_4Cl and 500 $g.L^{-1}$ of starch) was supplied at a constant speed of 10.5 $mL.h^{-1}$.

Table 14: Parameters of the test in bioreactor (hydrolysate medium with 10 $g.L^{-1}$ of glucose) and data from literature.

	Ferm. type	**$[X]_{fi}$** ($g.L^{-1}$)	**μ_{max}** (h^{-1})	**$[S]_{in}$** ($g.L^{-1}$)	**$[S]_{cons.}$** ($g.L^{-1}$)	**r_S** ($g.L^{-1}.h^{-1}$)	**$[P]_{fi}$** ($g.L^{-1}$)	**r_{Prod}** ($g.L^{-1}.h^{-1}$)	**% PHA**
Hydrolysate 10 $g.L^{-1}$ (bioreactor, present study)	Fed-Batch	9.56	0.0271	15.5	20.4	0.144	1.50	0.014	16.0
Glucose 1 % (Lillo & Rodriguez-Valera 1990)	Batch	n.d.	n.d.	n.d.	n.d.	n.d.	3.09	n.d.	30.9
Glucose 2 % (Lillo & Rodriguez-Valera 1990)	Batch	n.d.	n.d.	16.2	15.7	0.418	4.16	0.149	20.8
Glucose 1 $g.L^{-1}$ (Don et al. 2006)	Fed-Batch	n.d.	n.d.	n.d.	n.d.	n.d.	85.80	n.d.	48.6
Whey lactose (Koller et al. 2007b)	Fed-Batch	n.d.	0.11	n.d.	n.d.	n.d.	5.50	0.050	50.0
Whey lactose (Koller et al. 2008)	Fed-Batch	n.d.	0.10	n.d.	n.d.	n.d.	12.20	0.090	73.0
Starch 20 $g.L^{-1}$ – Wild strain (Zhao et al. 2013)	Fed-Batch	38.9	0.0365	18.5	16.0	0.323	17.80	0.334	44.5
Starch 20 $g.L^{-1}$ – Mutant strain (Zhao et al. 2013)	Fed-Batch	44.0	0.0484	18.0	15.1	0.314	21.28	0.420	48.0

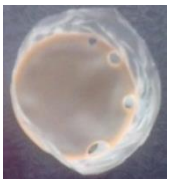
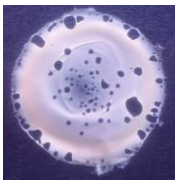
n.d. - not defined

Analyzing the results of Table 14 the best results in terms of polymer production were obtained by Don et al. (2006), with a $[P]_{fi}$ of 85.80 $g.L^{-1}$ and a % PHA of 48.6 %. Zhao et al. (2013) used a *H. mediterranei* mutant deficient in EPS biosynthesis and observed a remarkable reduction of medium viscosity and an increase of oxygen availability with a decrease in foam formation. As a result polymer production increased from 17.80 $g.L^{-1}$ to 21.28 $g.L^{-1}$ and the % PHA from 44.5 % to 48.0 %. In terms of % PHA these results were very similar with Don et al. (2006) study. The results obtained by Lillo & Rodriguez-Valera (1990) showed that from the various concentrations used (1%, 2%, 5% and 10%), 2% corresponded to the best results of PHA production despite the % PHA lower than 1%. In the present study all the parameters tested ($[X]_{fi}$, μ_{max}, $[S]_{in}$, r_S, $[P]_{fi}$, r_{Prod} and % PHA) were lower than the other studies, due to poor growth of *H. mediterranei*. This can be explained by the possible existence of inhibitors present in the hydrolysate which could influence growth and accumulation capacity. Koller et al. (2007b and 2008) tested PHA production from whey lactose. In these studies the best results of μ (0.11 and 0.10 h^{-1}) and % PHA (50.0 and 73.0 %) were obtained. The results proved that the conversion of whey into a polymer with excellent characteristics by *H. mediterranei* was viable, cheap and with simple downstream processing.

3. PHA extraction and characterization

The extraction of PHA from biomass at the end of all assays was carried out using chloroform, despite the ability of *H. mediterranei* to easily lise in a hypotonic medium (Koller et al. 2010). The extraction procedure followed had some differences concerning the biomass separation from the reactional medium, the biomass was always lyophilized with the exception of that obtained at the end of initial assay in defined medium with 10 $g.L^{-1}$ of glucose. In order to compare the effect of lyophilization, part of biomass from test with defined medium with 15 $g.L^{-1}$ of glucose was lyophilized and other part did not. Also, due some difficulties in recovering the majority of biomass, different centrifugation speeds were tested. The use or not of lyophilization, centrifugation speed, mass of polymer obtained and pictures of polymer films are shown in Table 15.

Table 15: Polymer films obtained by extraction of the polymer produced by *H. mediterranei* in defined and hydrolysate media.

Assay	**Lyophiliz.**	**Centrifug. Speed** (rpm)	$\mathbf{m_{polymer\ film}}$ (g)	**Polymer film**
Def. medium 10 $g.L^{-1}$	No	5000	0.0066	
Hydrolysate 10 $g.L^{-1}$	Yes	5000	0.0824	
Def. medium 15 $g.L^{-1}$	No/Yes	5000	0.0631/0.1297	
Hydrolysate 15 $g.L^{-1}$	Yes	5000	0.1402	
Def. medium 20 $g.L^{-1}$	Yes	11000	0.1622	
Hydrolysate 20 $g.L^{-1}$	Yes	5000	0.1764	
Def. medium 30 $g.L^{-1}$	Yes	11000	0.1282	
Hydrolysate 30 $g.L^{-1}$	Yes	11000	0.1233	

Def. medium 40 $g.L^{-1}$	Yes	11000	0.1837	
Hydrolysate 40 $g.L^{-1}$	Yes	11000	0.0577	
Hydrolysate 10 $g.L^{-1}$ - SBR	Yes	5000	0.1114	
Hydrolysate 10 $g.L^{-1}$ - Without sterilization	Yes	5000	0.0653	
Hydrolysate 10 $g.L^{-1}$ - Bioreactor	Yes	11000	0.1623	

Considering the results of test conducted in defined medium with 15 $g.L^{-1}$, the lyophilization step had a significant effect on the efficiency of extraction, because using the same extraction volume it was possible to obtain a polymer with twice the mass. This can be explained by the absence of water to facilitate contact between chloroform and biomass and consequently with the granules of PHA. Likewise, the increase in the centrifugal speed applied in the assays in defined medium with 20 $g.L^{-1}$, in defined and hydrolysate media with 30 and 40 $g.L^{-1}$ and in bioreactor assay increased the efficiency of separation between biomass and supernatant and in addition there is no loss of biomass, and consequently polymer, as significant in the extraction process.

As it can be observed in the pictures, some obtained polymer films have a reddish color. This color is due to the production of pigments by *H. mediterranei*, existing in cell membrane and responsible for reddish appearance. These pigments belong to the group of C_{50} carotenoids well as C_{45} carotenoids. Among these, bacterioruberin, that belongs to C_{50}

carotenoid, is the most common and increases the rigidity and mechanical strength. In order to remove the reddish color, a wash with acetone must be taken to solubilize the pigments (Fang et al. 2010).

In the case of *H. mediterranei*, lysis can also easily occur after resuspending the cells in distilled water. Thus, PHA granules can be easily collected after slow centrifugation. This results in a simple procedure for the PHA extraction, reducing the steps of downstream processing of PHA recovery and associated costs (Bhattacharyya et al. 2014; Koller et al. 2010). Extractions performed in this work with chloroform were only preliminary tests to try to extract as much polymer as possible but in the future extractions should be carried out with water.

The polymer film obtained at the end of the assay in the bioreactor was characterized by DSC. The obtained thermogram (Figure 15) showed a glass transition temperature (T_g) of - 6.07 ºC, a melting temperature (T_m) of 156.31 ºC and a melting enthalpy (ΔH_m) of 63.07 $J.g^{-1}$ (Table 16).

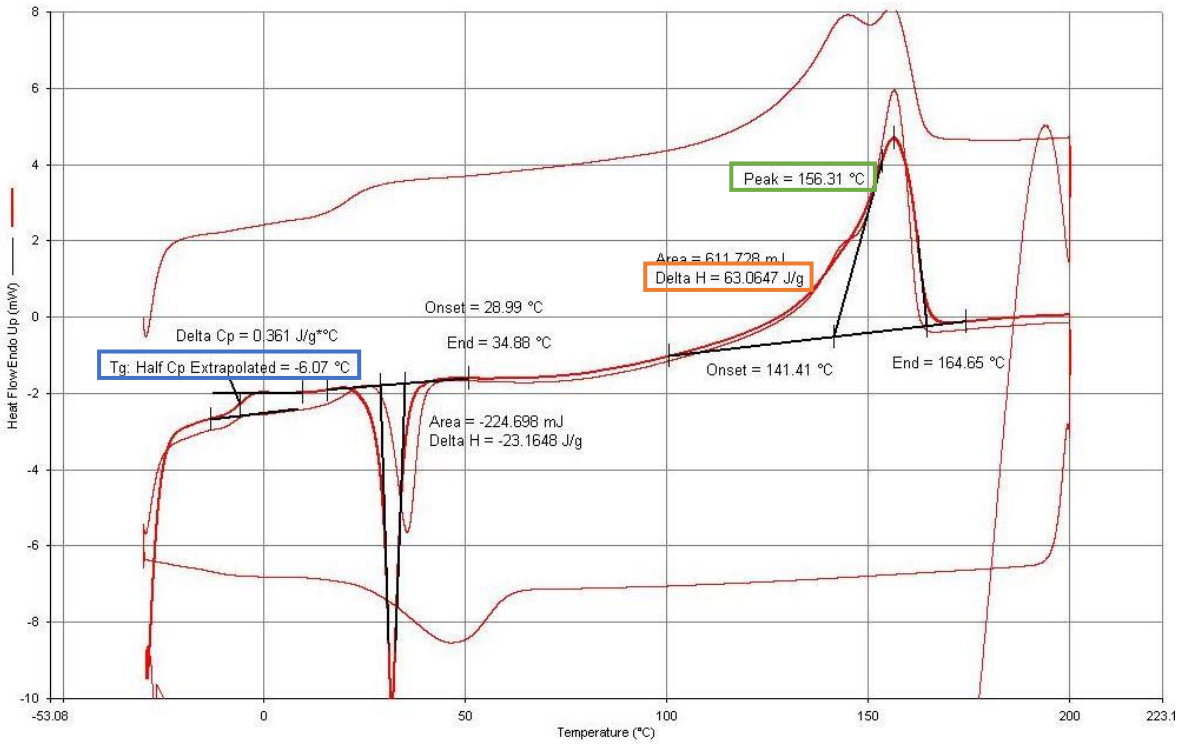


Figure 15: Thermogram obtained by DSC of polymer obtained at the end of the assay in bioreactor. Glass transition temperature (t_g: Half C_p Extrapolated – Blue rectangle), melting enthalpy (Delta H – Orange rectangle) and melting temperature (Peak – Green rectangle) were obtained from second heating.

By assuming that for all the PHA analyzed, P(3HB-*co*-3HV) with 100% of crystallinity presents a melting enthalpy of 132 $J.g^{-1}$ (Serafim et al. 2008b), the percentage of crystallinity was 47.78 %. Table 16 compares the values obtained by DSC with data from literature.

Table 16: Characterization of PHA obtained in this study and other values reported in the literature.

Microorganism	% 3HV	T_g (ºC)	T_{m1} (ºC)	T_{m2} (ºC)	ΔH_m ($J.g^{-1}$)	% crystallinity	Reference
Haloferax mediterranei	**7.5**	**-6.1**	**156.3**	**n.d.**	**63.1**	**47.8**	**Present study**
	6	6.0	150.8	158.9	n.d.	n.d.	(Koller et al. 2007a)
	8-10	7.0	149.7	160.7	n.d.	n.d.	(Koller et al. 2007b)
	10.8	1.4	133.6	144.7	n.d.	n.d.	(Don et al. 2006)
Azotobacter chroococum	0	3.5	179.1	n.d.	82.9	62.8	(Savenkova et al. 2000)
	8	-0.6	164.3	n.d.	75.6	57.3	
	10	-1.7	163.2	n.d.	77.6	58.8	
	13	-0.9	161.0	n.d.	60.6	45.9	
	17	-4.4	124.4	n.d.	51.2	38.8	
	18	-1.0	123.0	n.d.	58.1	44.0	
	20	-6.3	116.4	n.d.	40.8	30.9	
Burkholderia cepacia	0	4	177.0	n.d.	88.1	66.7	(Mitomo et al. 1999)
	43	-8	73.0	n.d.	27.9	21.1	
	67	-11	81.0	n.d.	43.8	33.3	
	86	-15	100.0	n.d.	71.9	54.5	
	90	-16	103.0	n.d.	73.6	55.8	
Ralstonia eutropha	0	6.0	175.0	n.d.	80.0	60.6	(Wang et al. 2001)
	45	-1.3	86.3	n.d.	40.3	30.5	
	49	-1.7	74.9	n.d.	50.4	38.2	
	70	-7.6	89.5	n.d.	71.7	54.3	

n.d. - not defined

The results obtained in the present work are in agreement with those presented in the literature also obtained with *H. mediterranei* (Koller et al. 2007a; Koller et al. 2007b; Don et al. 2006). Compared to Azotobacter chroococum with the same percentage of 3HV (8 %), H. mediterranei presents a melting temperature and percentage of crystallinity lower, which indicates that the copolymer produced by this microorganism has better resistance properties (Savenkova et al. 2000). By analyzing the results obtained in literature (Savenkova et al. 2000; Mitomo et al. 1999; Wang et al. 2001), the melting temperature and percentage of crystallinity values of the homopolymer P(3HB) are higher

than those of the copolymer. In addition, increasing 3HV fraction (until 50 %) decreases these values, which contribute to the increase of the degree of polymer processability. According to the study carried out by Serafim et al. (2008b) it is possible to estimate the percentage value of 3HV as function of values of glass transition temperature, melting temperature and percentage of crystallinity. Thus, considering that in this study was obtained a T_g of -6.07 ºC, T_m of 156.31 ºC and % of crystallinity of 47.78, it would be expected that the percentage of 3HV was comprised between 14 and 25 %. However, this was not what it was observed, since the polymer obtained contained only 7.5 % of 3HV. This difference may have to do with the polymer purification would not be completed.

CHAPTER IV

Conclusions

In this work the production of PHA by *H. mediterranei* from hydrolysate of the paper industry was investigated. The pulp was subjected to enzymatic hydrolysis and thus it was obtained the hydrolysate with free monomers. The sugar content of hydrolysate was characterized by HPLC which revealed glucose (81.96 $g.L^{-1}$) and xylose (20.90 $g.L^{-1}$) as the main constituent monosaccharides.

Initially different glucose concentrations (10, 15, 20, 30 and 40 $g.L^{-1}$) to find its influence the growth and PHA produce of *H. mediterranei* were tested. The assays in hydrolysate media with 10 and 15 $g.L^{-1}$ presented the best results, with $[P]_{fi}$ of 3.05 and 3.29 $g.L^{-1}$ and % PHA of 27.1 and 27.0 %, respectively. In terms of μ, r_{Glc} and r_{Prod} it was also the assay in hydrolysate medium with 10 $g.L^{-1}$ that provided the best results (0.0237 h^{-1}, 0.0926 $g_G.L^{-1}.h^{-1}$ and 0.0345 $g_P.L^{-1}.h^{-1}$, respectively). Nevertheless, the assay in hydrolysate medium with 30 $g.L^{-1}$ of glucose presented the best results in terms of biomass, reaching a $[X]_{fi}$ of 19.06 $g.L^{-1}$ (while in the other assays this value was between 11 and 13 $g.L^{-1}$). As a consequence of this significant increase in biomass, also the [P] was high (3.55 $g.L^{-1}$). However, the % PHA was just 19.5 %. From the analysis of the results it was also possible to conclude that high sugar concentration could inhibit the cell growth, which becomes clearly visible in the assay with 40 $g.L^{-1}$. Thus, the concentration 10 $g.L^{-1}$ of glucose was selected to be used in the following tests.

Due to total depletion of the sugars in previous assays, two different ways to provide more carbon source were tested. First an assay in fed-batch conditions was tested, with a hydrolysate pulse with 10 $g.L^{-1}$. The other way was resuspend the biomass into fresh medium as in a SBR. However this assay has not been successfully performed because centrifugation was not efficient. Thereby the best results resulted from the test in fed-batch, with a $[P]_{fi}$ of 3.16 $g.L^{-1}$ and a % PHA of 24.7 %.

Once *H. mediterranei* requires media with high concentrations of salts, a test without sterilization conditions was also carried out to prove that there is no risk of contamination. This assay was similar to what happened in previous assays in hydrolysate medium with 10 $g.L^{-1}$. However there was a decrease of various parameters, like $[X]_{fi}$ (9.79 $g.L^{-1}$), $[P]_{fi}$ (1.76 $g.L^{-1}$) and % PHA (18.0 %) due to lower [Glc] and [Xyl] supplied to this assay.

Finally an assay in bioreactor of 5 L, operated in fed-batch conditions, with the addition of two pulses of hydrolysate medium with 10 $g.L^{-1}$ of glucose was performed. In

this assay the best results in terms of μ (0.140 h^{-1}), r_{Glc} and r_{Xyl} (0.368 $g_G.L^{-1}.h^{-1}$ and 0.0947 $g_X.L^{-1}.h^{-1}$, respectively) were obtained and in less time than previous assays (174 hours). However the results of polymer production were lower, probably due to the initial low [X] (0.99 $g.L^{-1}$), which affected both growth and polymer production. In this case, the polymer was produced at r_{Prod} of 0.0144 $g_P.L^{-1}.h^{-1}$, having obtained $[P]_{fi}$ of 1.50 $g.L^{-1}$ with 16.0 % of % PHA (92.52 % of 3HB and 3HV of 7.48 %). The polymer was also analyzed by DSC to make a thermal analysis characterization of polymer. The obtained thermogram showed that the glass transition temperature (T_g) was - 6.07 ºC, the melting temperature (T_m) was 156.31 ºC and the melting enthalpy (ΔH_m) was 63.07 $J.g^{-1}$. These values are in accordance with the literature.

In summary, in this study the use of the hydrolysate from the paper industry as a medium for PHA production by *H. mediterranei* was evaluated. The results are a valuable contribution to the valorization of this industrial product and contribute to cost reduction in the PHA production due to the use of a halophile microorganism.

Future work proposals

Following these results of this study some suggestions are made to complete the research done so far:

- to study the PHA production using hydrolysate from wood wastes and/or wood with lower quality for paper industry;
- to study the composition of hydrolysate, to find out if in fact there are inhibitory compounds;
- to control of the pH in assays to prevent inhibition by low pH;
- to carry out further production assays in bioreactor to optimize the conditions for enhanced accumulation of PHA;
- to test extraction of PHA from *H. mediterranei* using hyposaline media as water.

CHAPTER V

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CHAPTER VI

Appendix

1. Preparation of hydrolysate

To the preparation of hydrolysate it was necessary calculate the mass of pulp to use. Firstly was calculated the dry mass (m_{dry}, g) through the required solution volume ($V_{solution}$, L) and hydrocarbons concentration (C_{HC}, $g.L^{-1}$):

$$m_{dry} = V_{solution} \times C_{HC} = 3.0\ L\ \times 100\ \ g/L = 300\ g$$

The hydrocarbons mass (m_{HC}, g) could be also calculated through the dry mass (m_{dry}, g) and the organic percentage (Organics, %) (provided by RAIZ):

$$m_{HC} = m_{dry} \times Organics = 300g\ \times 0.98 = 294\ g$$

To obtain the final mass (m_{final}, g) was necessary considering the humidity percentage (H, %) (provided by RAIZ):

$$m_{final} = \frac{m_{dry}}{1 - H} = \frac{300\ g}{1 - 0.72} = 1071.4\ g$$

Having obtained the mass of the value of pulp to be used, it was calculated the volume of enzyme preparation (V_{enzyme}, mL) put in a 200 mL flask, through the enzyme loading (C_E, FPU/g_{HC}), mass hydrocarbons (m_{HC}, g) and enzyme activity (A_{enz}, FPU/mL_{enzyme}):

$$C_z = C_E \times m_{HC} = 20\ \ FPU/g_{HC} \times 294\ g = 5880\ FPU$$

$$V_{enzyme} = \frac{C_Z}{A_{enz}} = \frac{5880\ FPU}{100.5\ \ FPU/mL_{enzyme}} = 58.51\ mL$$

Finally, to obtain the water volume (V_{water}, L) to add to the pulp and to make up the 3.0 L of solution ($V_{solution}$, L), it was required be in attention the parameters as dry and final mass (m_{dry} and m_{final}, g) (calculated early) and solution and enzyme solution volumes ($V_{solution}$ and $V_{enzyme\ solution}$, mL) (defined early):

$$V_{sample} = m_{final} - m_{dry} = 1071.4\ g - 300.0\ g = 771.4\ mL$$

$$V_{water} = V_{solution} - \left(V_{sample} + V_{enzyme\ solution}\right) \times 1 \cdot 10^{-3}$$
$$= 3.0\ L - (771.4\ mL + 200.0\ mL) = 2.0286\ L$$