Universidade de Aveiro Departamento de Química
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João Samuel de Almeida Pereira Patinha

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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.

À família, por todo o apoio…

o júri

presidente

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palavras-chave

Polihidroxialcanoatos, *Cupriavidus necator*, hidrolisado da pasta de papel.

resumo

O objetivo deste trabalho foi a utilização de hidrolisado de pasta de papel crua proveniente do processo kraft, uma matéria-prima renovável, como substrato para a produção de polihidroxialcanoatos (PHAs) por *Cupriavidus necator*. O hidrolisado obtido após uma hidrólise enzimática era uma solução com 84.06 $g \cdot L^{-1}$ de glucose e 20.22 $g \cdot L^{-1}$ de xilose.

As experiências preliminares foram realizadas usando dois meios de cultura, meios 1 e 2, com hidrolisado ou glucose como fonte de carbono. Duas estirpes de *C. necator*, NRRL B-4383 and DSM 545, foram crescidas em ambos os meios e os melhores resultados obtidos com hidrolisado foram com a NRRL B-4383 no meio 1, conseguindo uma biomassa máxima, uma taxa específica de crescimento e uma acumulação máxima de P(3HB) de 4.82 $g \cdot L^{-1}$, 0.305 h^{-1} e 26.51 %, respetivamente, enquanto que a DSM 545 obteve o seu melhor resultado no meio 2, conseguindo uma biomassa máxima, uma taxa específica de crescimento e uma acumulação máxima de P(3HB-co-3HV) de 4.07 $g \cdot L^{-1}$, 0.498 h^{-1} e 4.26 %. A estirpe DSM 545 foi a escolhida para continuar os testes devido ao seu rápido crescimento, à capacidade para consumir xilose e de produção de P(3HB-co-3HV) em vez de P(3HB), apesar da baixa acumulação. Foram realizados alguns ensaios no meio 2 com hidrolisado com diferentes concentrações de glucose (20 e 40 $g \cdot L^{-1}$). A estirpe DSM 545 cresceu melhor no meio com 20 $g \cdot L^{-1}$, consumindo todo o substrato, chegando a 3.26 $g \cdot L^{-1}$ de biomassa com uma taxa específica de crescimento de 0.137 h^{-1}, contrariamente à concentração máxima de biomassa de 1.99 $g \cdot L^{-1}$ e à taxa específica de crescimento de 0.139 h^{-1} obtidos no ensaio com 40 $g \cdot L^{-1}$. Ácidos láctico, acético e propiónico foram produzidos durante estes ensaios. Um ensaio em fed-batch foi realizado em Erlenmeyer com agitação para alimentar a cultura com hidrolisado. Contudo, a biomassa parou o seu crescimento logo após o pulso de hidrolisado devido à descida do pH para valores inibitórios, 4.97. De modo a controlar o pH, oxigénio, assim como a agitação e temperatura e para impor uma limitação de amónio, foi realizado um ensaio em reator. Uma concentração de biomassa máxima, taxa especifica de crescimento, acumulação máxima de P(3HB-co-3HV) e produtividade de 7.63 $g \cdot L^{-1}$, 0.209 h^{-1}, 6.5 % e 0.0126 $g_{PHA} \cdot L^{-1} h^{-1}$ foram conseguidos com uma taxa de consumo de glucose e xilose de 1.58 $g \cdot L^{-1} h^{-1}$ e 0.136 $g \cdot L^{-1} h^{-1}$, respetivamente. Contudo, devido à limitação de oxigénio, *C. necator* alterou o seu metabolismo para fermentativo, produzindo ácidos láctico, acético e propiónico numa concentração de 3.34 $g \cdot L^{-1}$, 1.63 $g \cdot L^{-1}$ e 6.77 $g \cdot L^{-1}$, respetivamente. O polímero extraído foi identificado como sendo P(3HB-co-3HV) com 21.51 % mol 3HV e foi caracterizado por DSC, tendo sido obtidos temperaturas de transição vítrea e de fusão, e uma cristalinidade de -3.60 °C, 123.41 °C e 27.17 %, respetivamente.

Este trabalho reconheceu a possibilidade de produção de PHAs usando hidrolisado de pasta de papel como substrato, pela primeira vez por *C. necator*.

keywords Polyhydroxyalkanoates, *Cupriavidus necator*, pulp hydrolysate.

abstract

The aim of this work was the application of hydrolysate of unbleached kraft pulp, a renewable feedstock, as substrate for the production of polyhydroxyalkanoates (PHAs) by *Cupriavidus necator*. The hydrolysate obtained after an enzymatic hydrolysis was a solution of 84.06 $g \cdot L^{-1}$ of glucose and 20.22 $g \cdot L^{-1}$ of xylose.

The preliminary experiments were performed using two media with different composition, media 1 and 2, with hydrolysate or glucose as carbon source. Two *C. necator* strains, NRRL B-4383 and DSM 545, were grown in both media and the best results obtained with hydrolysate were with NRRL B-4383 in medium 1, achieving a maximum biomass, specific growth rate and a maximum P(3HB) accumulation of 4.82 $g \cdot L^{-1}$, 0.305 h^{-1} and 26.51 %, respectively. DSM 545 reached its best result in medium 2, obtaining a maximum biomass, specific growth rate and P(3HB-co-3HV) accumulation of 4.07 $g \cdot L^{-1}$, 0.498 h^{-1} and 4.26 %. DSM 545 strain was chosen to proceed with further tests due to its fast growth, capacity to consume xylose and production of P(3HB-co-3HV) instead of P(3HB), despite the low accumulation. Some assays in medium 2 with hydrolysate with different glucose concentration, 20 and 40 $g \cdot L^{-1}$, were performed. DSM 545 grew better in medium with 20 $g \cdot L^{-1}$, consuming all the substrate, achieving 3.26 $g \cdot L^{-1}$ of biomass with a growth rate of 0.137 h^{-1}, contrary to the maximum biomass concentration and growth rate achieved in medium with 40 $g \cdot L^{-1}$, 1.99 $g \cdot L^{-1}$ and 0.139 h^{-1}. Lactic, acetic and propionic acids were produced during these assays. A fed-batch assay was performed in shake flask in order to feed the culture with hydrolysate. However, biomass stopped growing just after the hydrolysate pulse due to pH decrease to inhibitory values, 4.97. In order to control pH, oxygen, as well as stirring and temperature and to impose an ammonium limitation, an assay was performed in bioreactor. A maximum biomass, a specific growth rate and a maximum P(3HB-co-3HV) accumulation and productivity of 7.63 $g \cdot L^{-1}$, 0.209 h^{-1}, 6.5 % and 0.0126 $g_{PHA} \cdot L^{-1} h^{-1}$ were obtained with a maximum glucose and xylose uptake rates of 1.58 $g \cdot L^{-1} h^{-1}$ and 0.136 $g \cdot L^{-1} h^{-1}$, respectively. However, due to oxygen limitation, *C. necator* shifted its metabolism towards sugar fermentation, producing lactic, acetic and propionic acids achieving a maximum concentration of 3.34 $g \cdot L^{-1}$, 1.63 $g \cdot L^{-1}$ and 6.77 $g \cdot L^{-1}$, respectively. The polymer extracted was identified as P(3HB-co-3HV) with 21.51 % mol 3HV and was characterized by DSC, having obtained a glass transition and melting temperatures and a crystallinity of -3.60°C, 123.41 °C, and 27.17 %, respectively.

This work recognizes the production of PHAs using unbleached pulp hydrolysate as substrate, for the first time by *C. necator*.

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Abbreviations

3HAME	3-hydroxyalkanoate methyl ester
3HB	3-hydroxybutyric acid
3HBME	3-hydroxybutyrate methyl ester
Abs	Absorvance
BEKP	Bleached Eucalyptus Kraft Pulp
BG	β-glucosidase
CBHs	Cellobiohydrolases
CDW	Cell dry weight
CO_2	Carbon dioxide
DNS	3, 5-Dinitrosalicylic acid
DOC	Dissolved oxygen concentration
DSC	Differential Scanning Calorimetry
EGs	Endoglucanases
FPUs	Filter Paper Units
GC-FID	Gas Chromatography with Flame Ionization Detector
GMOs	Genetically Modified Organisms
HAc	Acetic acid
HAs	Hydroxyalkanoates
HLa	Lactic acid
HPLC	High Performance Liquid Chromatography
HPr	Propionic acid
ICI	Imperial Chemical Industries
IEA	International Energy Agency
ISA	Ionic Strength Adjuster
LCF	Lignocellulosic Feedstock
P(3HB)	Poly-3-hydroxybutyrate
P(3HB-co-3HV)	Poly-3-hydrobutyrate-co-3-hydroxyvalerate
P(3HHx)	Poly-3-hydroxyhexanoate

PBS	Phosphate Buffer Solution
PGW	Pressure Groundwood
PHA_{lcl}	Long-chain-length PHA
PHA_{mcl}	Medium-chain-length PHA
PHAs	Polyhydroxyalkanoates
PHA_{scl}	Short-chain-length PHA
PLA	Polylactic acid
RAIZ	Research Institute of Forest and Paper
r_{Glc}	Glucose uptake rate
r_{Xyl}	Xylose uptake rate
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SGW	Stone Groundwood
TEM	Transmission Electron Microscopy
TMP	Thermo-mechanical Pulping
UWF	Uncoated Woodfree
X	Biomass
$Y_{HAc/S}$	Acetic acid yield on substrate
$Y_{HLa/S}$	Lactic acid yield on substrate
$Y_{HPr/S}$	Propionic acid yield on substrate
$Y_{PHA/S}$	PHA yield on substrate
$Y_{X/S}$	Biomass yield on substrate

CHAPTER I – INTRODUCTION

I. General Introduction

Plastics are one of the most used and versatile materials for everyday use (Reddy et al. 2003). World's plastic production was estimated to be 260 million tons in 2007 (Rameshwari & Meenakshisundaram 2014) and surpass 300 million tons by 2015 (Reddy et al. 2013). For that purpose it is used an enormous quantity of petroleum in its production. It is also known that petroleum reserves are running low. Besides that, the greenhouse gases produced by the combustion of fuels and plastics originated from petroleum are accentuating global warming. Moreover, the excessive use of non-degradable plastics results in an increase of its accumulation in municipal's solid wastes. All these aspects started to concern researchers and industrials, creating the need to search for non-petroleum-based products and sustainable feedstocks (Octave & Thomas 2009). There are ongoing research projects, some of them already operating in an industrial scale regarding the production of biofuels and other products such as bioplastics (whose market is growing about 30% annually (Reddy et al. 2013)), namely bioethanol starting from feedstocks such as corn crops, and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV)) from wheat carbohydrates (Kamm & Kamm 2007).

The concept of using renewable and sustainable non-petroleum-based feedstocks to produce some products that are usually made from petroleum is the so-called biorefinery. The biggest source of biomass are the forests. Lignocellulosic biomass can undergo a pre-treatment as it is already done in the pulp and paper industry to release cellulose and hemicellulose from the lignin matrix as they could be used afterwards as feedstock for biorefineries to produce biopolymers such as polyhydroxyalkanoates.

Portucel, a pulp and papermaking factory located in Aveiro, produces huge volumes of pulp every year. Some of that pulp can be used as an inexpensive renewable feedstock after an enzymatic hydrolysis in order to produce fermentable sugars. Microorganisms, such as *Cupriavidus necator* can produce and accumulate polyhydroxyalkanoates, namely poly-3-hydroxybutyrate, P(3HB) using these sugars as carbon source.

II. Literature review

1. Biorefineries

Fuels as well as plastics are petroleum-based products used in a wide range of applications. The widespread use of plastics, estimated to surpass 300 million tons in 2015 (Reddy et al. 2013), and the excessive use of fuels started to concern the mankind due to the massive amounts of greenhouse gases produced through their combustion, which have a heavy role in global warming. Besides that, plastics result in enormous quantities of wastes in landfills (about 40% of produced plastics ends up in landfills) and in marine environments (Reddy et al. 2003). It is estimated a plastic consumption of about 60 kg per capita in European countries, per year (Reddy et al. 2003). Besides this, petroleum reserves are quickly decreasing, and if the consumption levels remain this high, reserves of oil could end in the next 5-6 decades (Liew et al. 2014; Mekala et al. 2014; BP Data).

To revert this situation, academic and industrial researchers started to search for alternatives in order to reduce the use of petroleum as the main source for fuels, energy and products like plastics, chemicals, among others. Additionally, environmental sustainable substitutes are also being searched. So, in 1970s, with the oil crisis, which inflated the oil prices (Figure 1), industries started to have an increasing interest in bioplastics as they could become economically competitive with conventional ones (Tan et al. 2014; EIA Bioenergy Report, 2012).

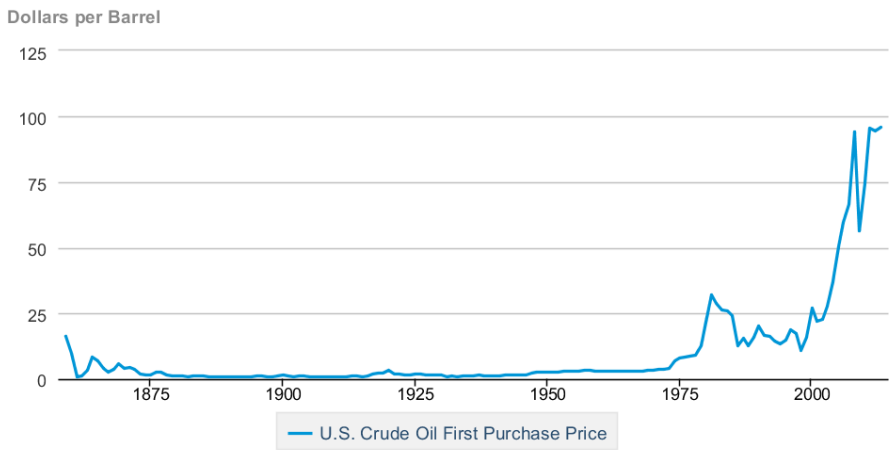


Figure 1 - Fluctuation of U.S. crude oil purchase prices. Data from IEA (2013).

Recently the biorefinery concept gained attention. Biorefinery, as described by Kamm et al. (2008), is a refinery that uses cheap renewable feedstocks as raw materials rich in sugars which are then converted into the desired products, formerly produced merely by petroleum-based refineries. In other words, International Energy Agency (IEA), defines "Biorefining" as the sustainable and synergetic processing of biomass into marketable food and feed ingredients, products (chemicals, materials) and energy (fuels, power, heat) (IEA 2014). Currently there are only few biorefineries working all around the world because some of the technologies used are not mature enough in addition to a non sustainable utilization of crops for food and non-food biomass and not full cooperation between the different industries to get an efficient use of whole biomass (IEA, 2014). Nevertheless, studies are being done in order to produce biopolymers as well as other compounds and to scale them up to industrial level. The global market for fermentation-derived fine chemicals in 2009 was $16 billion and it is estimated to have increased to $22 billion in 2013 (Jong & Gosselink 2014).

Biorefineries have been classified in different ways depending on some factors. Some of the most common classifications are described in the next topic.

1.1. Biorefineries classification

Biorefineries can be distinguished into two groups, namely energy-driven biorefineries and product-driven biorefineries. The first one aims the production of high volumes of relatively low-value energy (or fuels) out of biomass while the main goal of the second one consists of producing smaller amounts of relatively higher value-added biobased products out of biomass. In the product-driven biorefineries, besides the main product, residues are also utilized to produce energy for either internal or external use. Currently, these kind of biorefineries are not very common in an industrial scale due to the fact that some key operation processes are still being optimized. However, they show a huge potential for the future since it is believed that a future optimal sustainable use of biomass in a biorefinery will produce mainly energy, chemicals, and materials, depending on the raw material used (IEA, 2014; Liew et al. 2014; Cherubini et al. 2009; Cherubini 2010).

The most common classifications of biorefineries classifies them according to platforms, products, feedstocks or processes.

1.1.1. Type of platforms

Biorefineries can be classified according to the type of platforms included. Platforms represents the number of key intermediates between raw materials and final products. This platform concept is similar to the one used in the petrochemical industry, where the crude oil is fractionated into a large number of intermediates that are further processed in order to get energy and chemical products. Some of the main platforms which are recognized as energy-driven biorefineries are biogas, syngas, hydrogen, C_5 and C_6 sugars, lignin, oils, and electricity and heat (Cherubini et al. 2009).

1.1.2. Type of products

This classification can be grouped into two main sub-classes: energetic and non-energetic products. This way, energy-driven biorefineries use biomass to produce energy such as transportation biofuels (bioethanol, biodiesel, synthetic biofuels and biomethane), power and/or heat. Products can be sold as feed or in some situations can be converted into value-added biobased products, which improves economic and ecological performances of the full biomass supply chain. Product-driven biorefineries aim to generate biobased products, e.g. fine chemicals such as amino acids, organic acids and extracts, used in food, chemical or pharmaceutical industry; and animal feed and fiber products, among others, and residues that can be further processed in order to produce energy. Material products can be categorized as fertilizers, biohydrogen, glycerine, chemical and building blocks, polymers and resins, food, animal feed or biomaterials (Cherubini et al. 2009).

1.1.3. Type of feedstocks

This classification is based on the renewable raw material from which the marketable product is processed. Currently, biomass feedstock, a renewable carbon-based feedstock, comes from four main sectors: agriculture (dedicated crops and crop residues), forestry (wood, short-rotation plantations and log residues), industry and domestic activities, and aquaculture (algae and seaweed). It is possible to group these four sectors into two: dedicated feedstocks (agriculture, forestry and aquaculture) and residues.

Regarding this classification, some of the most common biorefineries are: sugar crops, starch crops, lignocellulosic crops, grasses, marine biomass (dedicated feedstocks) and oil-

based, lignocellulosic and organic (residues) (Cherubini et al. 2009; Cherubini 2010; Jong & Gosselink 2014).

1.1.4. Type of processes

Biorefineries can also be classified based on the main technological process used to convert biomass into the desired marketable product. In this way, the classification consists of four subgroups of processes: Mechanical/physical, Biochemical, Chemical and Thermochemical (Cherubini et al. 2009; Cherubini 2010).

1.1.5. Biofuel generations

Other common classification of biorefineries now-a-days is by the biofuel generation produced. Biofuels evolved from the first to fourth generation, primarily differing in feedstocks and production technologies (Liew et al. 2014). The 1st generation consists on the utilization of the whole plant (biomass) to produce biofuels, and competes directly with food and feed supply. An example of these biorefineries are those which use sugar crops (corn, wheat, sugar beet) to produce bioethanol or biodiesel (Kamm & Kamm 2007; Liew et al. 2014).

The 2nd generation uses lignocellulosic biomass, which do not compete with food or feed feedstocks so, it is not involved in fuel vs food debate. Lignocellulose is converted into fermentable sugars which are then fermented by yeast or bacteria into bioethanol and other chemicals (Liew et al. 2014; Visioli et al. 2014).

The 3rd generation is mainly derived from algae. Algae are one of the most photosynthetically efficient plants as they convert solar energy into chemical energy and store it in the form of oils. Algae have their advantages as reviewed recently by Demirbas (2009), however, better technology is needed to extract the oil. The technologies for the production of biofuels from oil are also not optimized, making this generation not economically viable yet (Liew et al. 2014).

The 4th generation consists of using genetically modified microbes in order to consume waste CO_2 in presence of sunlight – carbon negative process. This generation is causing some trouble in its implementation since it could origin environmental and ethical problems because of the possibility of concerns related to GMOs. Consequently the research on this generation is very limited (Liew et al. 2014).

1.2. Lignocellulosic biorefinery

Lignocellulosic biorefineries use lignocellulosic feedstock (LCF) such as woody biomass, agricultural residues (straw, stem and roots from food crops, the top ends of trees not used in paper manufacture or fast growing tall grasses) as the main source of carbon to convert it to the desired product (Mekala et al. 2014). About 90 % of the material resources used in the production process end up as waste with a high impact in the environment (Mekala et al. 2014).

There are two types of LCF biorefineries. The first one, called thermo-chemical, uses the whole biomass without separation of the components (cellulose, hemicellulose and lignin) by cracking and gasification to produce 2nd generation biofuel by Fischer-Tropsch method (Octave & Thomas 2009). The second one, and the relevant for this project, is the biological pathway, which consists on the use of soft methods such as enzymes and fermentation to transform biomass. The main difference between this last biorefinery type and a sugar one is the fact that in this type of biorefinery, a pre-treatment is needed in order to separate the cellulose from the lignin matrix (Octave & Thomas 2009). This step is critical because it has a direct impact in the final yield of the biorefinery, once lignin hinders the effect of enzymes and other treatments used *a posteriori* (Octave & Thomas 2009; Jong & Gosselink 2014).

After the pre-treatment (de-lignification), cellulose and hemicellulose suffers a hydrolysis to obtain C_5 and C_6 sugars. These sugars are then ready to be used in fermentations in order to be converted into biorefineries desired products (FitzPatrick et al. 2010).

There is a great variety of lignocellulosic biomass, however, from the Table 1 presented below, it is possible to understand that in a long-term view, the best biomass productivity, considering the same land area (ha) and time (year), is from algae and forests (Liu et al. 2012). However, algae have some disadvantages such as low biomass and the need of high capital investment in plant biorefineries (Demirbas 2011).

Table 1 – Recycle times and productivities of chemical/energy feedstock. n/a = not applied.

Feedstock	Recycle time	Standing biomass (tons/ha)	Biomass production (tons/(ha·year))
Algae	1 month	0.9	11.25
Agricultural crops	3-12 months	4.5	2.93
Temperate grasses	1 year	7.2	2.70
Tropical Forests	5-25 years	202.5	9.90
Boreal forests	25-80 years	90.0	3.60
Temperate evergreen	10-80 years	157.5	5.85
Oil, gas and coal	280 Ma	n/a	(0)

Woody biomass includes two groups of trees: gymnosperms (as known as coniferous woods or softwood) and the angiosperms (as known as hardwood). These types of trees are usually used in pulp and papermaking industry. Presently, there are about 10 softwood and 50 hardwood species existing naturally in Europe (Sjöström 1993). These wood types as well as their composition will be mentioned later (section 2).

Among the potential large-scale industrial biorefineries, the LCF biorefinery is a candidate of great success since the raw material used do not compete with food industries and exists in high amounts. Moreover, the products from the conversion of the fermentable sugars from lignocellulose can compete with conventional petroleum-based products (Kamm et al. 2008).

Lignocellulosic biomass is used in huge quantities in pulp and paper industry worldwide (Bajpai 2012). These industries are a good place to install a biorefinery based on their products such as pulp which is the objective of this project.

2. Lignocellulosic biomass composition

Lignocellulose is the most abundant organic compound on Earth and represents the major portion of the world's annual production of renewable biomass (Jong & Gosselink 2014). Forests cover about 9.5 % of Earth's surface, which is about a third of the land area (Liu et al. 2012). It represents a global biomass production of about 150 billion tons annually (Jong & Gosselink 2014).

Lignocellulose is composed mainly by cellulose, hemicellulose and lignin. Depending on the species and origin, the percentage of constituents may vary. However, woody biomass usually have about 40-50 % of cellulose, 15-35 % of hemicellulose and 15-30 % of lignin (Jong & Gosselink 2014).

Table 2 shows the main constituents of both softwood and hardwood wood types.

Table 2 – Typical components of wood (%).

Component	Softwood	Hardwood
Cellulose	40-50	40-50
Hemicellulose	15-20	20-35
Lignin	23-33	16-25
Other organics	1-5	1-2
Inorganic as ash	0.2-0.5	0.2-0.5

2.1.1. Cellulose

Cellulose is a complex linear homopolysaccharide, consisting of 3000 or more β-(1→4) linked D-glucose units (Figure 2). It is the most abundant of all naturally occurring organic compounds, containing over 50 % of all the carbon in vegetation (Jong & Gosselink 2014). It can be converted to glucose and act as the major source of hexoses in woody feedstocks (Mekala et al. 2014). Due to hydrogen bonds, cellulose is highly crystalline and it has a fibrillar structure which makes the most difficult component of woody biomass to hydrolyse (Mekala et al. 2014; Sjöström 1993).

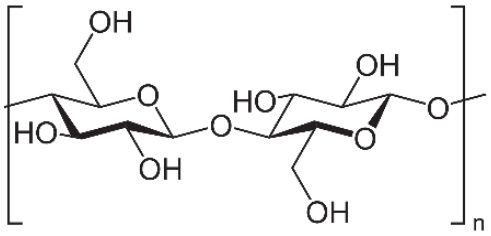


Figure 2 - Structure of cellulose.

2.1.2. Hemicellulose

Unlike cellulose, hemicellulose are heteropolymers composed of both five and six-carbon sugars, including xylose, glucose, mannose, arabinose, among others such as uronic acids. These compounds are mainly linked by β-(1→4) bonds, although other types can be found. Hemicellulose has an amorphous structure and it is easily breakable into its constituents, though they are usually bonded to cellulose, lignin and others by covalent and hydrogen bonds and by ionic and hydrophobic interactions. The most relevant hemicellulose is xylans, consisting about 20-30 % of the biomass of hardwoods and constituted mostly by xylose. Xylans do not have a defined structure since their monomers can be linked in many ways. Figure 3 showns an example of a xylan (O-acetyl-4-O-methylglucurono-β-D-xylan), normally present in hardwood species (Sjöström 1993; Mekala et al. 2014).

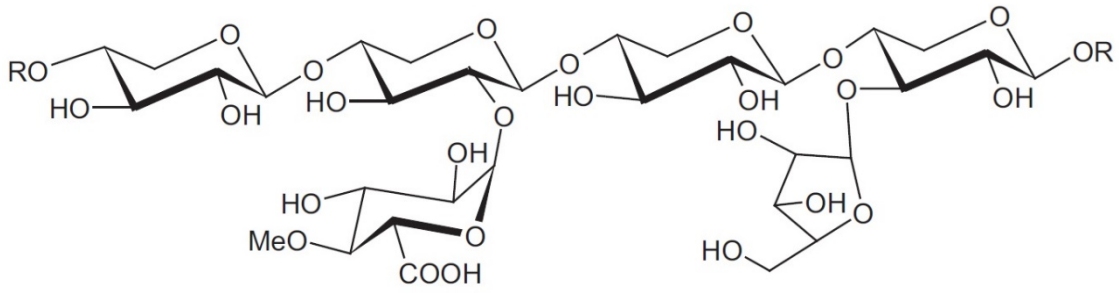


Figure 3 - Structure of O-acetyl-4-O-methylglucurono-β-D-xylan.

Xylose-based hemicellulose can be easily converted mainly into xylose residues (Sjöström 1993). Besides xylans, hardwood has also 2-5% of glucomannan, which can be hydrolysed mainly into residues of glucose and mannose (Sjöström 1993).

2.1.3. Lignin

Lignin is the third major constituent of woody biomass and acts as the "glue" that protects woody biomass from pathogens. Lignin is a major structural component of higher plants, conferring them their mechanical structure. It mainly consists of phenolic units (p-hydroxyphenyl-, guaiacyl- and syringyl- units, derived from the monolignols sinapyl-, coniferyl-, and coumaryl alcohol, respectively). In hardwoods, lignin is composed by guaiacyl and syringyl units (Figure 4) as the predominant building units (Sjöström 1993; Jong & Gosselink 2014).

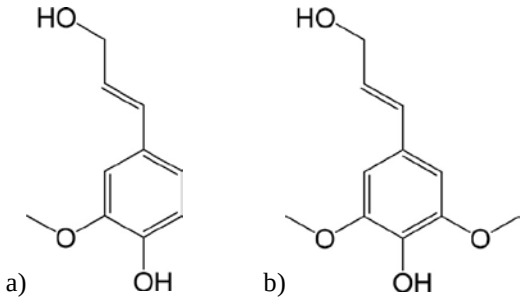


Figure 4 - Chemical strutures of phenylpropane units of lignin. a) Coniferyl alcohol; b) Sinapyl alcohol.

Lignin, after being separated from cellulose and hemicelluloses by the proper pre-treatment, can be combusted to produce "green electricity", power, fuel, steam or syngas and it can be used in chemical market as well (Berlin & Balakshin 2014).

2.1.4. Extractives

Extractives are a minor fraction (1-5 %) of wood components that are soluble in neutral organic solvents or water, and can be lipophilic or hydrophilic. The former includes, for example, resins, while the later, tannins, for instance. They are valuable raw materials for organic chemicals and play an important role in pulp and paper industry processes (Sjöström 1993).

3. Pulping and papermaking industry

Pulp and paper industry is dominated by North America, Northern Europe and East Asia and it is one of the largest industries in the world. About 380 million tons of paper and paper board are produced worldwide, annually. On average, one person uses about 60 kg of paper a year. The pulp and paper industry plays an important role in the country's economic growth (Bajpai 2012). In Portugal, for instance, according to Portucel Soporcel group, more than 95 % of their production is commercialized in 113 foreign markets (about 3 % of Portugal exportations), which represents an annual turnover of over 1500 million euros (Soporcel 2014).

In Portugal, there are two major groups that produce pulp and/or paper: Portucel Soporcel and Altri. Both groups use mainly hardwood species, more concretely *Eucalyptus globulus*. *E. globulus* is present in various countries, however, the portuguese strain is considered the best wood for pulp and paper production (Soporcel 2014; Altri 2014).

Since this project is done in association with RAIZ – Research Institute of Forest and Paper, which in turn is associated with Portucel Soporcel group as well as Universidade de Aveiro, Universidade de Coimbra and Instituto Superior de Agronomia de Lisboa (RAIZ 2014b), only the production process of the Portucel Soporcel group will be discussed in the following topic.

3.1. Portucel Soporcel group

Portucel Soporcel group has 3 facilities: Cacia, Setubal and Figueira da Foz. It is the best european and the 6th biggest world producer of Uncoated WoodFree (UWF) printing and writing paper. The group is also Europe's leading manufacturer, and one of the largest producers of Bleached Eucalyptus Kraft Pulp (BEKP) of the world (Soporcel 2014). It has

an annual production capacity for 1.6 million tons of paper and 1.4 million tons of pulp (of which 1.1 is turned into paper). Besides this, it generates 2.5 TWh of power. Portucel Soporcel is yet the owner of Navigator brand, the world's best-selling product in premium office paper segment (Soporcel 2014).

RAIZ works as a research center on forest and paper, located near Cacia's factory. More concretely, RAIZ works on achieving better productivities on eucalyptus forests, better fiber quality, reduce the cost of wood as well as implement a sustainable management of the forest. This research center intends also to obtain better pulp and paper quality, reduce the cost of production and minimize the environmental impact (RAIZ 2014a).

3.2. Pulping process

The pulping process starts in the forest with *E. globulus* wood being collected and transported to the factory. Here, it is turned into chips (chipping) after the logs had been debarked, since it is easier to separate the cellulose fibers from small chips rather than big ones. The pulping process can be of different types such as mechanical, chemical or chemimechanical, although the most common are the first two. Each one has its advantages and disadvantages. Mechanical process allows to obtain yields up to 85-95 % and are cheaper (about half) compared to chemical processes. However, mechanical pulps are weaker than chemical pulps and have a low resistance to aging which results in a tendency to discolour. The main mechanical pulping processes are Stone GroundWood Pulping (SGW), Pressure GroundWood Pulping (PGW) and Thermo-Mechanical Pulping (TMP) (Bajpai 2012). The yields of chemical processes are around 50 %, but the pulp produced can be used for high-strength papers and board as well as for packaging. Besides that, chemical pulps are more easily breached because the mechanical processes do not remove lignin (Bajpai 2012).

Chemical pulping is the process used to produce most of the paper sold in the world today. It consists on cooking the raw material in digesters using the Kraft (sulfate) or sulfite processes in order to remove non-cellulose wood components, leaving intact the cellulose fibers. The Kraft process is the most used chemical pulping process worldwide, as well as in Portucel Soporcel group, and it is schematically represented in Figure 5 (Moshkelani et al. 2013; Bajpai 2012).

The chemicals used in the Kraft process are sodium hydroxide (NaOH) and sodium sulfide (Na_2S) and it is operated in an almost closed cycle as it is shown in Figure 5 – the

recovery loop. The chemicals are recovered and then reused (white liquor) while the black liquor is evaporated and combusted to produce steam and electricity. Chemical processes produce more energy than they use, so the energy can be sold to the grid (Moshkelani et al. 2013; Bajpai 2012).

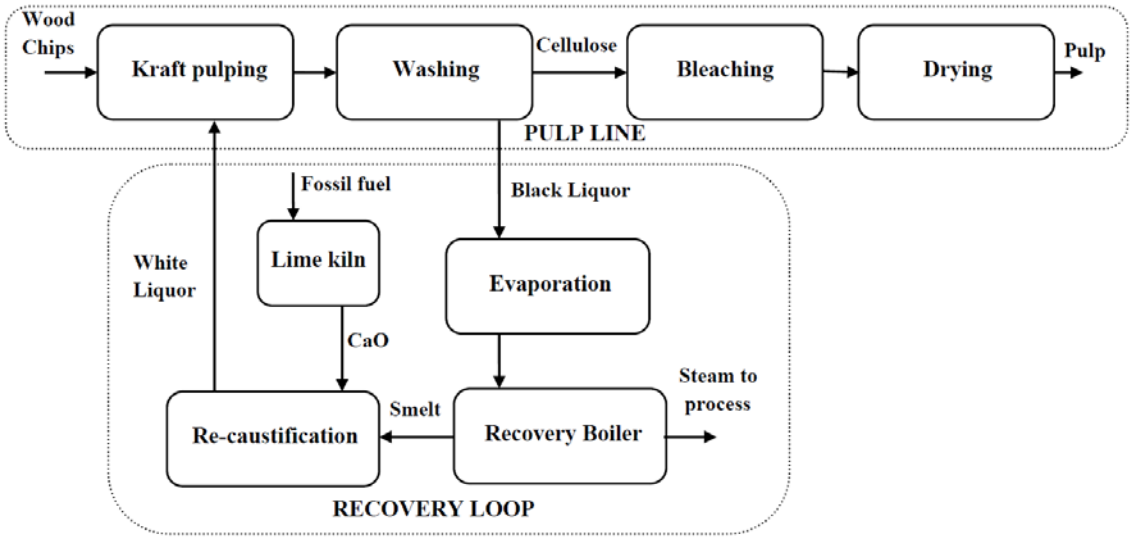


Figure 5 - Simplified schematic of Kraft process.

After pulp production, pulp is washed to remove impurities, obtaining the unbleached pulp. Residual spent cooking liquor from Kraft process is washed from the pulp using pulp washers, called brown stock washers. This step is critical so that chemicals can return to the recovery loop (Figure 5), avoiding losses (Moshkelani et al. 2013; Bajpai 2012).

For a papermaking factory, the pulp needs to be bleached to remove the small fraction of lignin and impurities which remained after cooking. This process turns the pulp whiter. For that, some chemicals are used in order to transform lignin into an alkali-soluble form. These chemicals, which act as oxidants, include oxygen (O_2), hydrogen peroxide (H_2O_2), ozone (O_3), sodium hypochlorite (NaClO), chlorine dioxide (ClO_2), chlorine (Cl) among others (Bajpai 2012). Enzymes had also gained much attention of pulp industries, especially for the bleaching step. With the use of enzymes, pulp and papermaking factories can increase the efficiency of other bleaching chemicals as well as reducing pollutants, namely organochloride compounds, discarded in effluents (Sharma et al. 2014). Besides this, the cost of this step and of pulp production could be reduced (Bhardwaj 1996). The most used enzymes are xylanases and laccases. Xylanases showed good results in cleaving bonds between lignin and residual xylan making this last one more accessible to other reagents for

its removal. Laccase is also being used as an oxidant to phenolic units and amine compounds of lignin, also facilitating its removal (Sharma et al. 2014). The main objective in a near future is to use exclusively enzymes in the bleaching process, allowing a total chlorine free bleaching step which will be called biobleaching. However, currently only about 10 % of all kraft pulp produced already use xylanases before bleaching (data from 2012). Suppliers such as Novozymes, Genecor, AB Enzymes, Diversa and Oji Paper are already producing and commercializing these enzymes (Bajpai 1999; Bajpai 2012).

Bleached pulp is then dried with steam and hot air. After this step, pulp is ready to go to a papermaking factory to be turn into paper (Moshkelani et al. 2013).

4. Enzymatic hydrolysis

Enzymes have been increasingly applied in a wide range of industrial sectors, namely, food, animal feed, beverage, baking, starch, fuel, laundry, textile, personal care and pulp and paper (Kirk et al. 2002), chemicals and pharmacology (Beilen & Li 2002), among others. Enzymes are already used in pulp and paper industries in order to give some special properties to the final product or to improve intermediate steps of the production. From the point of view of a biorefinery, unbleached pulp, which is constituted mainly by cellulose fibers but also by a smaller quantity of hemicellulose, can be hydrolysed in order to get fermentable sugars so that they can be converted into other products besides paper, such as chemicals and biopolymers like PHAs. For that, it is necessary to use some enzymes, cellulase, to turn the pulp into a liquid medium and to break cellulose fibers into glucose units, both required for the bacteria culture (Sharma et al. 2015; Gil et al. 2009; Cui et al. 2015; Bajpai 1999).

Cellulase consist of several key constituents such as cellobiohydrolases (CBHs), exocellulases which hydrolyse crystalline cellulose to generate predominantly cellobiose (β-1→4)-D-glucopyranosyl-D-glucopyranose), endoglucanases (EGs) which are endocellulases that cleave cellulose chain randomly along its length, and β-glucosidase (BG), as known as cellobiase, which hydrolyses cellobiose to D-glucose monomers (Quinlan et al. 2010). Actually, these types of enzymes are used as a mixture, which facilitates the hydrolysis of the pre-treated pulp into sugar monomers (Quinlan et al. 2010).

Data from RAIZ reports that the sugar content of hydrolysates after the enzymatic hydrolysis consists of 7.0 $g \cdot L^{-1}$ of disaccharides, 124 $g \cdot L^{-1}$ of glucose, 22 $g \cdot L^{-1}$ of xylose, and,

in minor quantities, 4.4 $g \cdot L^{-1}$ of dymers, 0.3 $g \cdot L^{-1}$ of fructose and 0.1 $g \cdot L^{-1}$ of mannose. Phosphate (0.1 $g \cdot L^{-1}$) and magnesium or calcium (2.7 $g \cdot L^{-1}$) were also detected. Organic acids (~1 $g \cdot L^{-1}$ of formic acid and citric acid in total) and furfural were in general not detected, which makes these pulp hydrolysates excellent potential candidates as carbon sources for biological conversions.

5. Polyhydroxyalkanoates

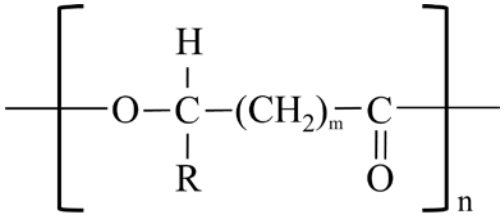
Bioplastics are polymers which have at least one monomer synthesized by bacterial transformation. The best studied bioplastics are polyhydroxyalkanoates (PHAs), polylactic acid (PLA), polybutylene succinate, polytrimethylene terephthalate and polyphenylene (Park et al. 2012; Reddy et al. 2013). Among the most popular bioplastics emerge PHAs, a group of polyesters composed by hydroxyalkanoates (HAs) monomers linked by ester bonds. There are a great variety of PHAs since at least 150 different HAs are known (Chen 2009). The best known PHA is poly-3-hydroxybutyrate (P(3HB)). The fact that there are so many HA, creates the possibility to tailor different types of biodegradable polymers with a wide range of applications (Reddy et al. 2003). There was an intense research in PHAs in the last 20 years. Data from Web of Science citation report (Thomson Reuters) showed that PHA-related documents have increased by almost 10-fold while citations have increased by more than 500-fold, accounting an average citation count of about 1100 citations per year (Tan et al. 2014). The HAs that constitutes the PHAs depends on the carbon source used and in the native biological pathway of the microorganism (Snell & Peoples 2009).

The first reference to PHAs, though not by this name, is dated from 1888 by Beijerinck who observed some refractile bodies inside bacterial cells (Shrivastav et al. 2013; Braunegg et al. 1998). However, it was in 1926 that Lemoigne was the first one to successfully identify P(3HB), after the isolation of lipid-like inclusions in *Bacillus megaterium* (Lemoigne 1926). In the middle of the 20th century, there were already enough studies on the genus *Bacillus* that suggest that P(3HB) had the function of storing carbon and energy as intracellular granules (Sudesh et al. 2000).

In 1973, P(3HB) was described as a bacterial storage material analogous to starch and glycogen on other species (Sudesh et al. 2000). In 1974, Wallen and Rohwedder reported the identification of others HAs other than 3HB (Sudesh et al. 2000). After that, in late 80s,

copolymers composed by different HAs, namely P(3HB-co-3HV) were discovered and the first steps towards industrial production of these biopolymers were done (Sudesh et al. 2000).

The most common PHAs are schematically represented in Figure 6 where their general chemical formula is showed. R represents the alkyl side group while the "n" represents the number of HAs in the PHA chain, corresponding to the degree of polymerization and it can range from 100 to some thousands monomers.



m	R group	Name
1	Methyl	Poly (3-hydroxybutyrate)
1	Ethyl	Poly (3-hydroxyvalerate)
1	Propyl	Poly (3-hydroxyhexanoate)

Figure 6 - PHA general formula and the most common HA. Note. m can be 1, 2 or 3. m=1 is the most common.

P(3HB), the simplest and the most widely studied PHA is constituted by 3-hydroxybutyrate (3HB) monomers. In 3HB, the alkyl group in the general formula mentioned above is a methyl group and it has an m=1. Other well-known PHAs are poly-3-hydroxyvalerate (P(3HV)) and poly-3-hydroxyhexanoate (P(3HHx)), which have a m=1 and a R group of R=CH_2CH_3 and R=$CH_2CH_2CH_3$ respectively (Figure 6) (Tan et al. 2014).

Depending on the number of carbon atoms of the HAs, PHAs are classified into three groups. The major two PHAs groups are short-chain-length (PHA_{scl}) and medium-chain-length (PHA_{mcl}). The other minor PHAs group is long-chain-length (PHA_{lcl}). PHA_{scl} are composed by monomers of 3 to 5 carbon atoms and have thermoplastic properties similar to polypropylene. On the other hand, PHA_{mcl} are composed by monomers of 6 to 14 carbon atoms and display elastic properties similar to rubber and elastomer (Park et al. 2012). PHA_{lcl} have more than 14 carbon atoms and are quite uncommon (Tan et al. 2014).

Research has been done in order to tailor different PHAs with wanted properties, playing with the percentage of different HAs or even assembling PHA_{scl} with PHA_{mcl}, obtaining a copolymer in both ways (Tan et al. 2014; Lu et al. 2009). The best known and most studied copolymer is P(3HB-co-3HV) (Lu et al. 2009).

5.1. Properties

PHAs are linear polymers which have from 100 to some thousands HAs in each chain and molecular weights ranging from 50.000 to 1.000.000 Da (Reddy et al. 2003). PHAs are biodegradable since they can be degraded to water and carbon dioxide in aerobic conditions and to methane anaerobically by microorganisms in various environments such as soil, water and sewage (Lee 1996; Shrivastav et al. 2013). This property is very important to reduce the packing waste in landfills as well as in the environment such as land or sea (Reddy et al. 2003). Thus, it opens new applications such as agriculture films, which degrades at the end of the season and do not have to be collected (Snell & Peoples 2009).

PHA are biocompatible and non-toxic since they do not cause severe immune reactions in soft tissues or blood of a host organism such a mammal, during degradation in the body (Shrivastav et al. 2013; Akaraonye et al. 2010). PHAs do not appear as storage materials in plants and animals but they are ubiquitous. They appear in some metabolic pathways however in small concentrations. In human blood, for instance, 3HB has concentrations between 0.3 and 1.3 mM (Shrivastav et al. 2013). So far, it had been shown that PHAs have been compatible with a wide range of tissues and there are already some products in the market in biomedical area (Shrivastav et al. 2013).

PHAs are chiral biopolymers once they are produced exclusively by R-hydroxyalkanoic acids, therefore they are optically active. Their depolymerized HAs are chemically interesting due to the chiral centre and two easily modified functional groups (–OH and –COOH). Consequently, HAs could work as starting materials for the synthesis of fine chemicals such as antibiotics, vitamins, macrolides and other novel chiral polyesters (Chen 2009).

PHAs have also material properties similar from thermoplastics to elastomers, depending on the homopolymer or copolymer in case, as shown in Table 3. P(3HB), for example, is a thermoplastic with a high degree of crystallinity and has properties similar to polypropylene. On the other hand P(3HB-co-3HV) is more flexible and tougher than P(3HB) and is used for a wide variety of applications (Lee 1996).

Table 3 – Comparison of physical properties of PHA and polypropylene. (P(3HB-co-3HV) contains 20% of 3HV).

Properties	P(3HB)	P(3HB-co-3HV)	Polypropylene (PP)	PHA_{scl}	PHA_{mcl}
Melting temperature (°C)	177	145	176	53-80	30-80
Glass transition temperature (°C)	2	-1	-10	-148 to 4	-40 to 150
Crystallinity (%)	60	56	50-70	40-80	20-40
Tensile strength (MPa)	43	20	34-38	43	20
Extension to break (%)	5	50	400	6-1000	300-450

Besides all the characteristics mention before, PHAs are also hydrophobic, hence insoluble in water, and crystalline in nature (Shrivastav et al. 2013). PHAs are also easily thermoprocessable and their properties are easy to control, modulating some variations in the process or changing molecular weight of the polymer composition (Shrivastav et al. 2013). This, along with the biocompatibility and biodegradability properties, makes PHAs a good material for drug delivery systems (Shrivastav et al. 2013).

PHAs can be blended with other biobased/biodegradable/biocompatible and non-toxic materials or even with some petroleum resins, in order to enhance some desired properties such as better biomedical applications or some new biobased materials (Shrivastav et al. 2013; Snell & Peoples 2009). Moreover, PHAs have piezoelectric properties and some PHAs are oxygen impermeable (Akaraonye et al. 2010). It has also been reported that some HAs appear to have antimicrobial and antiviral properties, important for pharmaceutical industry, for instance. One of these HAs is 3-hydroxy-n-phenylalkanoic acid that had been used to effectively attack *Listeria monocytogenes* which is a ubiquitous microorganism able to multiply at refrigeration temperatures (Chen 2009).

5.2. Applications

PHAs are being used in a wide range of applications because of their extensive number of properties, as well as the possibility to either chemically modify PHAs or blending them with other (bio)polymers. Currently, the major areas in the market for PHAs are packaging, coating and as raw material for other materials. However, PHAs have been used for almost every usage of conventional plastic from packaging to medical applications for the reason that PHAs present properties from stiff and brittle to rubber-like materials (Verlinden et al. 2007; Poirier et al. 1995).

Studies over the past decades showed that PHAs can be used for applications such as packaging films and bottles, as a coating for fibrous materials such as paper, as long term slow-release encapsulation of drugs (Shrivastav et al. 2013) or insecticides and herbicides (Akaraonye et al. 2010), as biomedical devices, as a source for the synthesis of enantiomerically pure substrates for pharmaceutical (such as antibiotics, macrolides, dendrimers (Chen 2009)) or agrochemical industries, among many others (Poirier et al. 1995). PHAs can also be a good substitute of conventional petrochemical products in applications such as moulded goods, performance additives, foils, films and diaphragms (Akaraonye et al. 2010). Beyond that, it had been successfully tested in feeding trials with sheep, pigs and chicks (Snell & Peoples 2009). Also, PHAs can be used in printing (in toners) and photographic industry since they are easily stained (Chen 2009) and as other bulk chemicals like heat sensitive adhesives, latex and smart gels (Chen 2009). In medical, biomedical and orthopaedic area, PHAs can have potential applications in sutures, regeneration devices, bone plates and bone fracture fixation, bone marrow scaffolds, ligament and tendon grafts, bone graft substitutes, heart valve scaffolds, artificial cartilage tissue, tissue adhesives, among others (Akaraonye et al. 2010; Verlinden et al. 2007; Chen 2009). It has already been described some applications such as diapers because of their biodegradability (Verlinden et al. 2007). PHAs have also been used in textile industry when PHA is processed as fibers, like nylons (Chen 2009). They had been tested in the form of oligomers to be used as food supplements for obtaining ketone bodies (Chen 2009). Some steps had been made in protein purification field too (Chen 2009). Some composites of PHAs are already used in electronic products like mobile phones. They have also potential agricultural applications such as encapsulation of seeds and fertilizers for slow release as well as for biodegradable plastic films for crop protection (Verlinden et al. 2007).

Recent studies presented PHAs as a possible source of biofuels, namely 3-hydroxybutyrate methyl ester (3HBME) and medium chain length hydroxyalkanoate methyl ester (3HAME), after acid catalysed hydrolysis of P(3HB) and PHA_{mcl}, respectively. These methyl esters have combustion heat values (20 and 30 $kJ \cdot g^{-1}$ respectively) very similar to ethanol (27 $kJ \cdot g^{-1}$). However the production costs are not competitive enough with the current petroleum fuels (Chen 2009).

5.3. PHA producing microorganisms

About 300 known species, from at least 70 genera were characterized as capable of producing and accumulating PHAs (Reddy et al. 2003; Suriyamongkol et al. 2007; Lu et al. 2009; Rameshwari & Meenakshisundaram 2014; Tan et al. 2014), including gram-negative and gram-positive bacteria, some archaea and even plants (Tan et al. 2014; Akaraonye et al. 2010). Bacteria produce PHAs to promote their long-term survival under nutrient-scarce conditions (Tan et al. 2014). Some producers can accumulate PHAs up to 90 % of their cell dry weight and it functions like a carbon and energy reserve (Reddy et al. 2003). PHAs are displayed as mobile, amorphous, lipid granules in cytoplasm (Tan et al. 2014). Some authors divide PHA producers into two major groups – ones that produce PHA exclusively in conditions of excess of the carbon source when in the limitation of other nutrients (Nitrogen, Phosphorus, Sulphur, Oxygen, and Magnesium) or when an excess of polymer precursors exists. The second group can produce PHA alongside growth in cultivation medium (Akaraonye et al. 2010). In accordance with this classification, bacteria in the first group are, for instance, *Rhodopseudomonas palustris* ,*Methylobacterium organophilum* and *Cupriavidus necator* (Akaraonye et al. 2010; Tan et al. 2014), even though the production of PHA alongside the bacteria growth had also been reported in literature, for instance in *C. necator* H16 (Shimizu et al. 2013). Bacteria of the second group includes, for example, *Azohydromonas lata* (formely *Alcaligenes latus*), *Pseudomonas Putida*, *Azotobacter vinelandii* and recombinant *Escherichia coli* containing the PHA biosynthetic genes (Akaraonye et al. 2010).

In the last years, there have been reported many studies about metabolic engineering of some native producers and other non-native producers bacteria like *E. coli* in order to achieve high PHA production. Some characteristics such as high growth rate, the possibility to achieve high cell density from several inexpensive carbon sources and also the easy purification of the polymer gives *E. coli* its attractiveness (Leong et al. 2014; Akaraonye et al. 2010).

Besides pure cultures, there are some research groups working with mixed cultures, regarding the production of PHA using mixed cultures but it will not be mention on this project. It will just be mention a production of PHA up to 89 % of CDW within 7.6 h by open mixed cultures, namely γ-proteobacterium (Saharan et al. 2014).

Besides bacteria, there is an enormous interest in research and optimization for industrial use of some species of plants after the first demonstrated in 1992 with the accumulation of P(3HB) in the cytoplasm of cells of a metabolic engineered *Arabidopsis thaliana* (Poirier et al. 1992; Lee 1996). Since then, many agricultural crops such as corn, sugar beet, sugarcane, switchgrass, among others were studied. Depending on the species, they were able to accumulate up to about 40 % of dry weight in *A. thaliana* (Bohmert et al. 2000). However, severe changes in metabolism as a result from the production of P(3HB) were observed (Bohmert et al. 2000). PHA levels of 3.7 %, 1.88 %, 5.66 % and 14 % of dry weight of the tissue were reported in leaf samples of switchgrass, sugarcane, corn and *Arabidopsis* respectively, using plastids (Snell & Peoples 2009). Some companies had special interest in these kind of plants as is the case of Metabolix, which is working principally with switchgrass in order to obtain high levels of P(3HB) to make the process industrially viable (Metabolix 2014).

It is shown in Table 4, some of the best results achieved by producers of PHA so far. Results from *C. necator, E. coli* and *Haloferax mediterranei* can be highlighted. This last one had been very studied lately due to the fact that it can grow with salt concentrations up to 25 %, which reduces the cost of sterilization of the process (Saharan et al. 2014).

Table 4 – Main achievements in PHA production by bacteria, recombinant E.coli, Archaea and plants.

Producer	Carbon source	PHA	Culture time (h)	Cell concentration ($g \cdot L^{-1}$)	[PHA] ($g \cdot L^{-1}$)	PHA content (%)	Productivity ($g \cdot L^{-1} h^{-1}$)	Reference
C. necator	Glucose	P(3HB)	50	164	121	76	2.42	(Kim et al. 1994)
	Glucose+ propionic acid	P(3HB-co-3HV)	46	158	117	74	2.55	(Byrom 1992)
	Carbon dioxide (CO_2)	P(3HB)	40	91.3	61.9	67.8	1.55	(Tanaka et al. 1995)
	Glucose	P(3HB)		281	232	80	3.14	(Ryu et al. 1997)
Azohydromonas lata	Sucrose	P(3HB)	18	142	68.4	48	4.0	(Yamane et al. 1996)
	Sucrose	P(3HB)		112	98.7	88	4.94	(Wang & Lee 1997)
Azotobacter vinelandii	Glucose	P(3HB)	47	40.1	32	79.8	0.68	(Page & Cornish 1993)
Methylobacterium organophilum	Methanol	P(3HB)	70	250	130	52	1.86	(Kim et al. 1996)
***Pseudomonas* strain K**	Methanol	P(3HB)	170	233	149	64	0.88	(Suzuki et al. 1986)
Bacillus megaterium		P(3HB)				50		(Shahid et al. 2013)
Halomonas boliviensis	Glucose	P(3HB)		44	35.6	81	1.1	(Quillaguamán et al. 2008)
Recombinant *E. coli*	Glucose	P(3HB)	39	101.4	81.2	80.1	2.08	(Lee et al. 1994)
XL1-blue (*A. Lata*)	Glucose	P(3HB)	31	194.1	141.6	73	4.63	(Choi et al. 1998)
GCSC4401 (*A. Lata*)	Whey (lactose)	P(3HB)	37	194	168.8	87	4.60	(Ahn et al. 2001)
XL1-blue (*A. Lata*)	Glucose+ propionoc acid	P(3HB-co-3HV)	55	203.1	158.8	78.2	2.88	(Choi & Lee 1999)
Haloarcula sp. IRU1	Glucose	P(3HB)				63		(Taran & Amirkhani 2010)
Haloferax mediterranei	Extruded cornstarch / extruded rice bran (8:1)	P(3HB-co-3HV)		140	77.8	55.6		(Huang et al. 2006)
	Hydrolized whey	P(3HB-co-3HV)				72.8	0.09	(Koller et al. 2008)
***Arabidopsis* (plastids)**		P(3HB)				40		(Bohmert et al. 2000)
		P(3HB)				14		(Nawrath et al. 1994)
Switchgrass		P(3HB)				3.7		(Somleva et al. 2008)
Phaeodactylum tricornutum		P(3HB)				10.6		(Hempel et al. 2011)

5.3.1. *Cupriavidus necator*

C. necator (formerly known as *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha*) is a soil gram-negative bacterium. It is rod-shaped and measures about 0.7-0.9 by 0.9-1.3 µm. It moves due to 2 to 10 flagella. It divides by binary fission. The best conditions to grow *C. necator* were improved over the years, but the best results of growth and PHA production consists on a pH between 6.8 and 7.0 and a temperature of either 28 °C or 34 °C (Makkar & Casida 1987; Cavalheiro et al. 2012).

C. necator is the principal organism model for PHA production since it can produce high amounts of PHA. There were studies where PHA contents reached up to 96 % of CDW (Sheppard et al. 1994) although the normal PHA content for this bacterium is about 80-90 %. The highest biomass concentrations of *C. necator* and P(3HB) concentration were obtained by Ryu et al. (1997), 281 $g{\cdot}L^{-1}$ and 232 $g{\cdot}L^{-1}$, respectively, after 74 h of culture in a 60 L reactor. High cell density fed-batch fermentation was performed with glucose being controlled between 0 and 20 $g{\cdot}L^{-1}$. P(3HB) accumulation was induced by phosphate limitation. P(3HB) productivity was 3.14 $g{\cdot}L^{-1}{\cdot}h^{-1}$ with the P(3HB) yield from glucose of 0.38 $g{\cdot}g^{-1}$. High values of cell and P(3HB) concentrations were also reported by Kim et al. (1994), 164 $g{\cdot}L^{-1}$ and 121 $g{\cdot}L^{-1}$, respectively, after 50 h of culture. A fed-batch culture technique was performed with a glucose concentration controlled between 10 and 20 $g{\cdot}L^{-1}$ and ammonium limitation was implemented after biomass concentration reached 70 $g{\cdot}L^{-1}$ to induce P(3HB) accumulation. P(3HB) productivity in this experiment was 2.42 $g{\cdot}L^{-1}{\cdot}h^{-1}$ with the yield of 0.3 g P(3HB)/g glucose.

C. necator is being used by some major companies around the world (Tianjin Northern Food, ICI, Metabolix) to industrially produce P(3HB) and P(3HB-co-3HV) (Chen 2009). *C. necator* can produce P(3HB) from different carbon sources like sugars (i.e. glucose, fructose and sucrose), fatty acids (i.e. acetic acid, butyric acid, propionic acid, lactic acid) or by the fixation of CO_2 through the Calvin Cycle since it is a hydrogen-oxidizing bacterium (Tan et al. 2014; Akaraonye et al. 2010; Chakraborty et al. 2012). Besides these, *C. necator* has been tested to produce PHA from various renewable inexpensive carbon sources such as waste potato starch, palm kernel oil, olive oil, corn oil (Akaraonye et al. 2010), waste glycerol from biodiesel production (Cavalheiro et al. 2009), spent coffee grounds oil (Cruz et al. 2014), protein hydrolysates (Bormann et al. 1998), corn syrup (Daneshi et al. 2010), among many others.

5.4. Metabolic pathways

Several metabolic pathways known to produce PHA, depending on the microorganism and/or on the carbon source available are already known. PHA biosynthetic pathways are intrinsically associated with the central metabolic pathways including glycolysis, Krebs Cycle, β-oxidation, *de novo* fatty acids synthesis, amino acids catabolism, Calvin Cycle, and serine pathway. Acetyl-CoA is the most notably intermediate among many common others shared between PHA and these metabolic pathways (Tan et al. 2014).

The PHA biosynthetic pathways are shown in Figure 7.

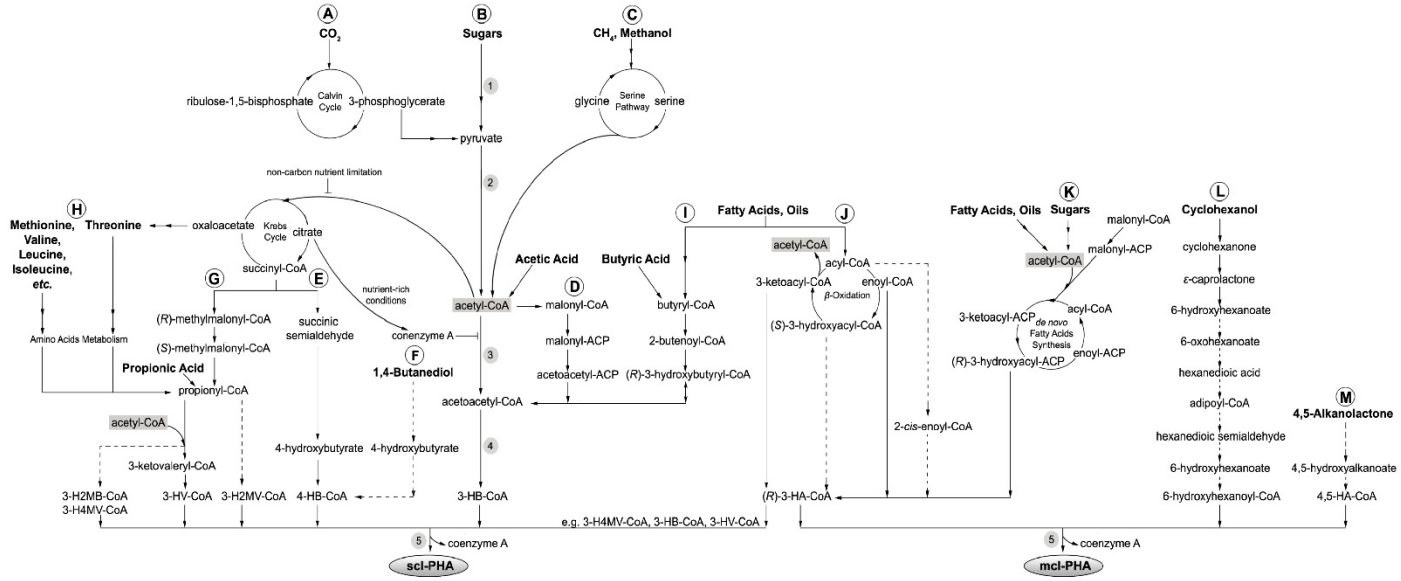


Figure 7 - PHA biosynthetic pathways. Dotted lines represent putative pathways. Numbers represent enzymes. A to J represents various routes of PHA_{scl} synthesis and J to M, routes of PHA_{mcl} synthesis.

Acting as biocatalysts, PHA producers can use a wide range of carbon sources in order to obtain PHAs. These carbon sources include saccharides (glucose, fructose, maltose, lactose, xylose, arabinose), n-alkanes (hexane, octane), n-alkanoic acids (acetic acid, propionic acid, butyric acid, valeric acid, oleic acid), n-alcohols (methanol, ethanol, glycerol), and gases (methane and carbon dioxide). Some bacteria can also produce PHA from waste streams since they provide a free source of carbons. Some of waste streams tested with good results were waste frying oil, vinegar waste, agricultural waste, crude glycerol from biodiesel production, among others (Tan et al. 2014).

Gram-negative bacteria such as *C. necator*, *A. lata* and *Burkholderia* sp. can produce PHA_{scl} from sugars or fatty acids, for example (Tan et al. 2014). Starting from glucose, the pathway for PHA production consists generally on the glycolysis of glucose into pyruvate

(1 of Figure 7) which is then converted to acetyl-CoA by aerobic growth (2 of Figure 7) (Tan et al. 2014). If another carbon source is used, it is processed via the Entner–Doudoroff pathway and Krebs Cycle until it is converted into acetyl-CoA and then it continues the PHA pathway from here (Braunegg et al. 1998). P(3HB) pathway (bigger in the bottom of Figure 8) is constituted by three key enzymes, each one encoded by a different gene (Figure 8): β-ketothiolase (encoded by PhaA), NADPH-dependent acetoacetyl-CoA reductase (encoded by PhaB) and PHA synthase (encoded by PhaC). The first reaction is catalyzed by β-ketothiolase (number 3 in Figure 7) to convert two molecules of acetyl-CoA into acetoacetyl-CoA. Then, acetoacetyl-CoA is reduced to (R)-3-hydroxybutyril-CoA by NADPH-dependent acetoacetyl-CoA reductase (number 4 in Figure 7). Finally, PHA synthase (number 5 in Figure 7) polymerizes 3-hydroxybutyril-CoA into P(3HB) (pathway B in Figure 7) (Lu et al. 2009; Tan et al. 2014; Reddy et al. 2003).

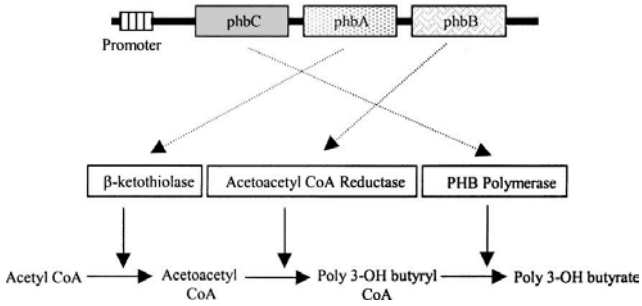


Figure 8 - Biosynthetic pathway of P(3HB) and PHA synthase enzymes and their encoding genes.

As mentioned earlier, some bacteria such as *C. necator* need nutrient limitation (section 5.3) to produce PHAs. Under nutrient-rich conditions, acetyl-CoA enters the Krebs Cycle for energy production and cell growth as a result of the inhibition of β-ketothiolase and the consequent blockade of PHA synthesis caused by high amounts of coenzyme A from Krebs Cycle. On the other hand, under unbalanced nutrients conditions, coenzyme A levels are non-inhibitory and acetyl-CoA is channelled towards PHA synthesis pathway for PHA accumulation (Tan et al. 2014; Braunegg et al. 1998).

5.5. PHA extraction and purification

PHA is accumulated in the cytoplasm of the cell, contrary to what happens with most of biotechnological products produced by microorganisms, like antibiotics, pharmaceuticals, vitamins, proteins, antibodies, among others. The main step of the separation process is the extraction of PHA granules. There are plenty of different methods of extraction, but the most studied methods consist on solvent extraction, chemical and enzymatic digestion, mechanical disruption, supercritical fluids, cell fragility, and recently, air classification and dissolved-air flotation (Jacquel et al. 2008).

The oldest and most used extraction method is the solvent extraction (Jacquel et al. 2008; Kunasundari 2011). Solvent extraction includes two steps, first, the cell membrane permeability is modified, allowing the release and solubilisation of PHAs. This step is sometimes followed by a non-solvent precipitation (Jacquel et al. 2008; Kunasundari 2011).

Besides solvent extraction, there are other methods such as digestion methods and mechanical disruption. Digestion methods consist on the solubilisation of the cellular materials surrounding PHA granules, the non-PHA cellular mass. This digestion can be chemical (sodium hypochlorite or surfactants such as SDS, Triton X-100, palmitoyl carnitine, among others) or enzymatic (especially proteases). Some mechanical disruption methods, namely bead milling and high-pressure homogenization, can also be used as an extraction method and are already used in large scale cell disruption in pharmaceutical and biotechnological industries (Kunasundari 2011).

Solvent extraction, in particular using chloroform, is commonly used in laboratory scale due to its simplicity and rapidity (Kunasundari 2011). Although the PHAs obtained by this method can have high purity and high molecular weight with very limited degradation of the polymer, this method can not be used in large scale. Solvent extraction has various drawbacks such as the hazards connected with halogenated solvents due to their toxicity and volatility and the cost associated with the large volumes of solvent needed (Jacquel et al. 2008; Kunasundari 2011).

Some of the best extraction results reported in literature for *C. necator* are presented in Table 5. Although some good results had been reported using other extraction methods for *C. necator*, the extraction using chloroform done by Fiorese et al. (2009) can be highlighted with a recovery yield of 96 % and a purity of 95 %.

Table 5 – Best PHA extraction results for *C. necator*. nd –not defined.

Method	Purity (%)	Recovery (%)	Reference
Solvent extraction – Chloroform	95	96	(Fiorese et al. 2009)
Solvent extraction – 1,2-Propylene carbonate	84	95	(Fiorese et al. 2009)
Solvent extraction – Methylene chloride	98	nd	(Zinn et al. 2003)
Digestion with surfactant – SDS	>95	>90	(Kim et al. 2003)
Digestion with sodium hypochlorite	98	nd	(Berger et al. 1989)
Digestion with sodium hypochlorite and chloroform	90-97	91-99	(Hahn et al. 1994; Ryu et al. 2000)
Digestion: Triton X-100 - sodium hypochlorite	98	nd	(Ramsay et al. 1990)
Enzymatic digestion – Bromelain	89	nd	(Kapritchkoff et al. 2006)
Enzymatic digestion – Pancreatin	90	nd	(Kapritchkoff et al. 2006)
Supercritical fluid – CO_2	nd	89	(Hejazi et al. 2003)
Air classification	95	85	(Noda 1995)

To increase the purity level of the polymer, sometimes a purification step is added to the procedure. This purification usually involves a hydrogen peroxide treatment combined with enzymes or chelating agents. Another purification method is using ozone. However, in most cases, this step is not necessary, unless a higher purity for medical use, for example, is requested (Jacquel et al. 2008).

5.6. State of industrial production of PHA

Currently, some PHA industries are already labouring at pilot and industrial scale (Chen 2009). However, it is very difficult to compete with petroleum-based plastics, once the PHAs prices are still very high, because of the carbon sources used in the fermentation and accumulation steps and petroleum price (Figure 1) did not increase significantly which would increase the price of petroleum-based plastics. Many studies were made in order to lower the prices of PHAs, using cheap carbon sources such as some industrial by-products or other carbon feedstocks instead of pure carbon sources. Summing up, there is a huge effort to implement the biorefinery concept in bioplastics production (Chen 2009).

PHA factories started to get noticed in 1980s. The first ones were Imperial Chemical Industries (ICI), in UK (P(3HB-co-3HV)) and Chemie Linz GmbH, in Austria (P(3HB)). Nevertheless, PHAs had so much potential that companies as Monsanto, BASF, P&G, Biomers, ADM, Mitsubishi, among others, tried their luck in this business but some of them had to closure their projects related to PHAs since they did not achieved their goals. In 2008, when the oil barrel reached $140, two biobased polymers come into play. They were PLA and PHAs. So PHAs started to move to an industrial scale. Currently, about 75 000 tons PHAs are produced per year for a few companies: Meredian, Bio-on, Tianjin Green Bio, Metabolix, among others. The main companies are shown in Table 6 along with their production volumes and species used (Chen 2009).

Table 6 – Main PHA companies.

Period	Company	Country	Polymer	Trade name	Capacity (tons/year)	Price (€/kg)	Application	Microorganism
1980s-1990s	Chemie Linz	Austria	P(3HB)	n/a	20-100	----	Packaging and drug delivery	*A. latus*
1980s	ICI	UK	P(3HB-co-3HV)	n/a	300	----	Packaging	*C. necator* 11599
1980s-present	Metabolix	USA	P(3HB)	Mirel	10 000	n/a	Raw materials and Packaging	rec. *E. coli* K12 *C. necator*
1990s-present	Biomer	Germany	P(3HB)	Biomer	1 000	3.00-5.00	Packaging and drug delivery	*A. latus*
1990s-present	P&G Chemicals/ Kanela	USA/Japan	P(3HB-co-3HH)	Nodax	20 000-50 000	2.50	Packaging	rec. *C. necator* $phaC_{Ac}$ / *E. coli*
1990s-present	Tepha INC	USA	P(4HB)	Tephaflex, TephElast	n/a	n/a	Medical bio-implants	
1990s-present	Mitsubishi Gas Chemical	Japan	P(3HB)	Biogreen	10 000	2.50-3.00	n/a	
1990s-present	PHB Industrial	Brazil	P(3HB)	Biocycle	50-100	n/a	Raw materials	*Bacillus spp.*
1990s-present	TianAn Biologic Material Poly-one	China	P(3HB-co-3HV)	ENMAT	2 000-10 000	3.26	Raw materials	
1990s-present	Jiangsu Nation Group	China	P(3HB)	n/a	Pilot scale	n/a	Raw materials	rec. *E. coli* phbCAB+vgb
2004-present	Tianjin Green Bio-science (+DSM)	China	P(3HB-4HB)	Green Bio	10 000	n/a	Raw materials and Packaging	rec. *E. coli* phbCAB
2005-2012	ADM (with Metabolix) - Telles	USA	P(3HB)	Mvera/ Mirel	50 000	1.50	Raw materials	Transgenic plants
2007-present	Meredian	USA	several PHA	Meredian	15 000 (272 000 announced)	n/a	Raw materials	
2008-present	Bio-On	Italy	unclear	Minerv-PHA	10 000	n/a	Raw materials	
1998-present	BioMatera	Canada	P(3HB), P(3HB-co-3HV)	n/a	n/a	n/a	Raw materials	
2003-present	Newlight Technologies	USA	unclear	AirCarbon	100 000	n/a	Raw materials	

6. Objectives

From a biorefinery point of view, the main objective of this project was to use unbleached pulp from the pulp and paper industry as a renewable carbon source rich in sugars to produce PHAs. Starting with pulp, an enzymatic hydrolysis was performed to obtain monosaccharides, mainly glucose and xylose. The growth of *C. necator* was studied in mineral media and in hydrolysate supplemented with necessary nutrients in order to compare the biomass growth and PHA production. A fed-batch assay in reactor with controlled conditions (pH, temperature, dissolved oxygen, stirring) was tested in order to increase the amount of polymer produced. Nutrient limitation was applied in reactor to optimize PHA production and accumulation by *C. necator* in hydrolysate. The extracted polymers were analysed in order to determine their physic and mechanical properties.

CHAPTER II – MATERIALS AND METHODS

1. Raw material

1.1. Pulp

Eucalyptus globulus unbleached pulp was provided by RAIZ, a research institute from Portucel Soporcel group. The characterization of the pulp is shown in Table 7.

Table 7 – Characterization of E. globulus unbleached pulp. Data provided by RAIZ.

Humidity (%)	Lignin (%)	Inert (%)	Organics (%)	Cellulose (%)	Hemicellulose (%)
72.00	2	0	98	78.4	19.6

1.2. Enzymatic hydrolysis

Taking into account the available enzymes in RAIZ and the best results of a previous work about this subject (Cabral 2014), a mixture of cellulases (namely Novozymes NS-22192) (20 FPUs) was chosen in order to hydrolyse the *E. globulus* unbleached pulp.

Two parallel hydrolysis of 3 L of volume each were performed. In order to do that, a rigorously measured amount of unbleached pulp (1071.4 g) equivalent of 300.0 g of dry sample were added to 2028.6 mL of ultrapure water and 200 mL of the enzymatic solution was prepared. The 200 mL of the enzymatic solution consists on an enzyme dosage of 20 $FPU/g_{carbohydrate}$ (58.51 mL of enzyme) made up with ultrapure water.

Then, the mixture and the enzymatic solution were heated to 50 ºC separately, before the beginning of the hydrolysis.

The hydrolysis took 24 h at 50 °C with constant stirring (100 rpm, 45 ° pitched three-bladed turbine impeller) and pH was maintained around 4.5-5.5 (the optimal pH range for the enzyme) with HCl 37 % and NaOH (100 $g \cdot L^{-1}$). Temperature and pH were measured before and at time zero (the beginning of the hydrolysis); every half an hour during the first two hours; every hour until the seventh hour and then at hours 23 and 24. Every time it was needed, temperature and pH were adjusted to the optimal value. Samples from the final 24 h were collected and heated for 5 min at 100 °C to stop the hydrolysis and then centrifuged at 2000 rpm for 5 min and stored at -20 °C until the supernatant analysis.

After 24 h of hydrolysis, the solution was filtered under vacuum with paper filters in order to remove debris of the hydrolysis. Then, hydrolysate was stored in plastic bottles and placed at -20 °C. The right amount of volume was only unfrozen when it was necessary to prepare the media.

2. Hydrolysate preparation

The hydrolysate was autoclaved at 121 °C for 21 min. However, since it showed to form a precipitate after the first autoclave stage, an additional step to remove precipitate was done. The autoclaved hydrolysate with the precipitate was centrifuged at 5000 rpm at 4 °C for 30 min (Heraeus Megafuge 16R centrifuge) and then decanted and autoclaved again. A sample of supernatant of every step of this preparation was collected and analysed with High Performance Liquid Chromatography (HPLC) (section 11.3) to determine the sugars concentration in every step.

3. Acid hydrolysis of hydrolysate precipitate

Due to the formation of a precipitate during the hydrolysate preparation, an acid hydrolysis of the precipitate was necessary in order to understand its content.

The precipitate was harvested after a centrifugation (5000 rpm at 4 °C for 30 min) of the autoclaved hydrolysate. For the acid hydrolysis, 6 mg of lyophilized precipitate were weighted and 200 µL of H_2SO_4 (72 % (w/w)) were added in appropriate Pyrex tubes. The mixture was left for 3 h at room temperature with occasional stirring. After, 2.2 mL of distilled water were added to dilute the solution to a H_2SO_4 concentration of 1 M. The solution was incubated at 100 °C for 2.5 h. Lastly, this solution was diluted 1:5 and analysed by HPLC (section 11.3).

4. Microorganism

Two strains of *Cupriavidus necator* were used in this project. NRRL B-4383 was kindly provided by the Agricultural Research Service Culture Collection of United States Department of Agriculture. DSM 545, on the other hand, was kindly given by Universidade Lisboa, Instituto Superior Técnico.

5. Media

All media used were sterilized in autoclave at 121 °C for 21 minutes before inoculation.

In the assays with hydrolysate as carbon source, glucose was replaced by hydrolysate with the appropriate dilution to achieve the desired concentration of sugars.

5.1. Inocula and seed media

5.1.1. Lauria-Bertani (LB) medium

LB medium was used to revive the culture from stock cultures, for solid medium, and for pre-inocula. The composition of LB medium is shown in Table 8.

Table 8 – LB medium composition.

Components (Brand)	**Concentration ($g \cdot L^{-1}$)**
Tripton (OXOID)	10.00
Yeast extract (OXOID)	5.00
Sodium chloride (VWR)	10.00
Agar* (JMVP)	15.00

(*) – Agar was used only for solid media.

5.1.2. Seed medium

Seed medium was used for *C. necator* DSM 545 as an adaptation medium. The composition of seed medium is presented in Table 9 and Table 10.

Table 9 – Seed medium composition.

Components (Brand)	**Concentration (per L)**
KH_2PO_4 (Sigma)	1.50 g
$(NH_4)_2SO_4$ (Pronalab)	1.00 g
$MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)	0.20 g
Na_2HPO_4 (Panreac)	4.76 g
D(+) Glucose (Scharlau)	10.00 g
Micronutrients solution	1.0 mL

Table 10 - Micronutrients solution composition.

Components	Concentration (per L)
$FeSO_4 \cdot 7H_2O$	10.00 g
$ZnSO_4 \cdot 7H_2O$	2.25 g
$CuSO_4 \cdot 5H_2O$	1.00 g
$MnSO_4 \cdot 4H_2O$	0.50 g
$CaCl_2 \cdot 2H_2O$	2.00 g
$Na_2B_4O_7 \cdot 10H_2O$	0.23 g
$(NH_4)_6Mo_7O_{24}$	0.10 g
35% HCl	10.00 mL

Both $MgSO_4 \cdot 7H_2O$ and glucose were autoclaved separately from the rest of the medium to avoid precipitation and added to the medium, after cooling, under sterilized conditions.

5.2. Growth media

Two different growth media were used during this project, medium 1 and medium 2.

5.2.1. Medium 1

The composition of the medium 1 is described in Table 11 and Table 12.

Table 11 – Medium 1 composition.

Components (Brand)	Concentration (per L)
KH_2PO_4 (Sigma)	2.30 g
Na_2HPO_4 (Panreac)	2.30 g
NH_4Cl (VWR)	1.00 g
$MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)	0.50 g
$NaHCO_3$ (JMGS)	0.50 g
$CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich)	10.0 mg
$C_6H_{14}N_2O_7$	22.0 mg
Carbon source – D(+) Glucose/ Hydrolysate*	20.00 g/ 250.0 mL
$FeCl_3$ (Lancaster)	30.0 mg
Micronutrients solution	5.0 mL

Table 12 – Micronutrients solution composition.

Components	Concentration ($g \cdot L^{-1}$)
$ZnSO_4 \cdot 7H_2O$	0.10
$MnCl_2 \cdot 4H_2O$	0.03
H_3BO_3	0.30
$CoCl_2 \cdot 6H_2O$	0.20
$CuCl_2 \cdot 2H_2O$	0.01
$NiCl \cdot 6H_2O$	0.02
Na_2MoO_4	0.03

KH_2PO_4 and Na_2HPO_4, $FeCl_3$ and glucose or hydrolysate were autoclaved separately from the rest of the medium as well as the micronutrients solution to avoid precipitation. The solutions were added together after cooling, under asepsis conditions.

5.2.2. Medium 2

The composition of the medium 2 is described in Table 13 and Table 10.

Table 13 – Medium 2 composition.

Components (Laboratory)	Concentration (per L)
$MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)	1.20 g
$(NH_4)_2SO_4$ (Pronalab)	4.00 g
KH_2PO_4 (Sigma)	13.3 g
Citric acid (Panreac)	1.70 g
Carbon source – D(+) Glucose/ Hydrolysate	20.00 g/ 250.0 mL
Micronutrients solution*	5.0 mL

(*) – Micronutrients solution is described in Table 10.

$MgSO_4 \cdot 7H_2O$ and glucose or hydrolysate were autoclaved separately from the rest of the medium to avoid precipitation and added to the medium, after cooling, under sterilized conditions.

6. Stock cultures

To make the stocks cultures, the strains were grown in LB medium (section 7.2) for 72 h from solid medium. The grown culture was inoculated in LB medium and incubated for 24 h (section 7.3.1). Aliquots of 2 mL of biomass suspension were centrifuged at 13000 rpm for 30 s to separate the biomass from the medium. The supernatant was discarded and the pellet was ressuspended in 600 µL of fresh LB medium with 15 % glycerol and stored in sterile crio-vials at -80 °C.

7. Inocula preparation

7.1. Solid medium

Petri dishes filled with solid LB medium, were renewed monthly and they were inoculated by streaking using one colony of the plate from the previous month. The plates were incubated for 72 h at 28 °C before getting stored at 4 °C.

7.2. Pre-inocula

Pre-inocula were done in 100 mL Erlenmeyers with 25 mL of LB medium. A couple of colonies from solid media were used to inoculate the medium and were incubated in an orbital shaker (Certomat's) for 48-72 h at 180 rpm at 28 °C.

7.3. Inocula

Two types of inoculum were used: LB inoculum and seed medium inoculum. Each one was tested and used in different occasions. In a general way, for medium 1, the inoculum used was directly the LB medium. For medium 2, an inoculum in seed medium was also done after the one in LB medium in order to adapt the culture.

7.3.1. LB inocula

A small volume (1.0-2.0 mL) of pre-inoculum was transferred to 100 mL Erlenmeyers with 50 mL of LB medium. After incubating for 24-48 h at 28 °C at 180 rpm in an orbital shaker (Certomat's) the inoculum was ready.

7.3.2. Seed inocula

8 mL of LB inoculum were used to inoculate 120 mL of seed medium, incubated for 24/48 h at 30 °C in an orbital incubator (Stuart orbital incubator SI500).

7.3.3. Reactor inocula

The inocula for reactor assays were prepared by inoculating 15 mL of LB inoculum into 500 mL Erlenmeyers with 235 mL of seed medium and incubated for 48 h at 180 rpm at 34 °C in an orbital incubator (Stuart orbital incubator SI500).

8. Flask assays

All Erlenmeyer assays were performed in Erlenmeyers of 250 and 500 mL with half of the volume of medium. Each strain was tested in medium 1 and medium 2 either with glucose or hydrolysate. Also, different sugar concentrations were tested. These assays were performed for 2-6 days at 30 °C in an orbital incubator (Stuart orbital incubator SI500) at 180 rpm. All shake flasks experiments were conducted in duplicate to confirm the precision of the results.

9. Reactor assays

pH-stat fed-batch assays were performed in a 5 L Sartorius Biostat A Plus reactor (Figure 9).

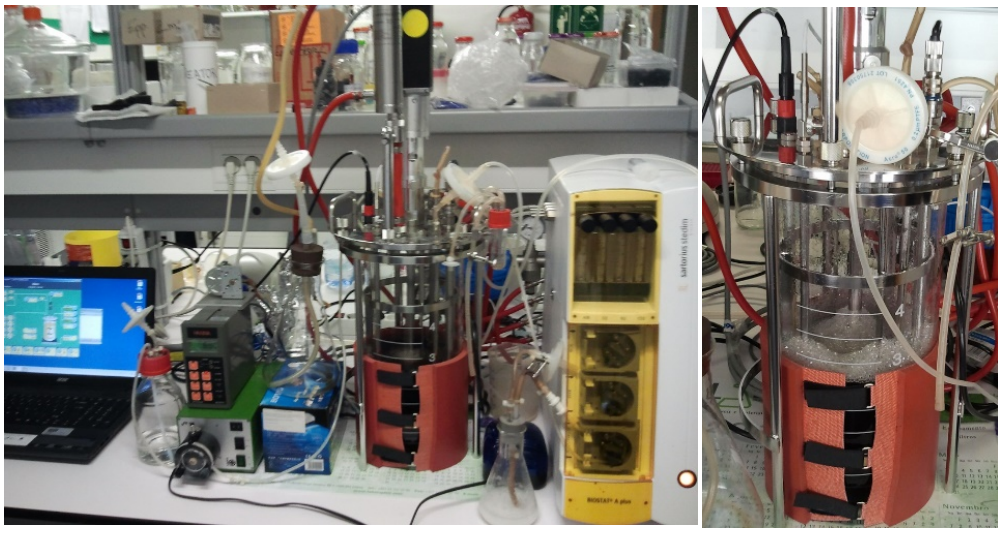


Figure 9 – Reactor used.

The culture vessel was equipped with two six-bladed disk-turbine impellers. The reactor was inoculated with 10 % (v/v) of *C. necator* DSM 545 in seed medium to reach an initial volume of 2.5 L in the reactor. The temperature and pH were controlled at 34 °C and 6.80, respectively. pH was monitored by a pH sensor (Hamilton) and controlled using H_2SO_4 (1 M) and 25 % (v/v) NH_4OH until a certain point of the culture growth when a limitation of ammonium was imposed in order to promote accumulation of PHA. At this point, NH_4OH was replaced by KOH (5 M). Dissolved oxygen concentration (DOC) was monitored by an O_2 sensor (Hamilton Oxyferm O_2 sensor) and controlled in cascade mode with the stirring speed, maintaining the aeration rate constant. In the beginning, the DOC was controlled above 20 % saturation using an aeration rate of half the top flow of the pump (Sera air 550R plus) until the stirring speed reached its maximum value and then the pump was set to pump its maximum volume. Later on, when the stirring speed reached the top speed and the DOC decreased to 0.0 %, an external air flow was supplied in order to increase the oxygen availability. Foaming was controlled using 10 % anti-foam (VWR) when necessary.

10. Sampling

Sample of 2 mL were taken under aseptic conditions to measure optical density (OD) to monitor the culture growth. A sample of 1 mL was centrifuged at 13000 rpm for 5 min (Eppendorf mini spin) to separate the pellet of biomass from the liquid phase. The supernatant was stored at -20 °C before HPLC analysis. The pellet of biomass was washed twice with NaCl 0.9 % (v/v) and stored at -20 °C to be analysed with gas-chromatography with flame ionization detector (GC-FID).

For the reactor assay, samples of about 5 mL were collected. OD was measured and the remaining volume was centrifuged to separate biomass from the supernatant. Supernatant was stored at -20 °C for HPLC and ammonium analysis and biomass was stored at -20 °C for GC-FID analysis.

11. Analytical methods

11.1. pH

pH of the flask assays was measured with a pH sensor coupled with a Hanna pH 211 Microprocessor pH meter.

11.2. Dry weight determination

The cell growth was monitored by measuring OD in a UV-Vis spectrophotometer (Shimadzu UVmini - 1240) at 650 nm. The values were maintained between 0.090 and 0.300 with appropriate dilutions to maintain the linearity of the measurements.

A calibration curve was done in order to relate the values of OD with the correspondent biomass concentration. A sample of about 9/15 mL was harvested from the culture in the stationary phase of the growth to filter in triplicate. A sample of 3/5 mL was filtered in cellulose nitrate membrane filters (Whatman) with a 0.2 µm of pore and a diameter of 47 mm and it was washed with 9/15 mL (the triple of the volume filtered) of distilled water. Then, filters were left to dry at 100 °C in an oven (Memmert), until constant weight (usually 3 days). These measurements were performed in triplicate. At the same time, different dilutions were done and the respective OD was read. A calibration curve relating the value of biomass concentration of the specific dilution to its respective OD was done (appendix A). Using these curves, it was possible to follow the biomass growth during the assays as well as to determinate the culture growth phases.

11.3. Sugars and organic acids quantification

Sugars and organic acids were quantified by HPLC. Standards with known sugars (glucose and xylose) and organic acids concentrations were used in order to obtain a calibration curve. Supernatant samples were diluted with distilled water to a maximum sugar concentration of 5 $g \cdot L^{-1}$ and a volume of 650 µL was transferred to appropriate eppendorfs (Spin-X polypropylene centrifuge tube filter with a pore of 0.22 µm). 50 µL of H_2SO_4 (usually 0.25 M) were added to adjust the pH between 1 and 3 to fulfil the specifications of the column used. 700 µL of standards and above prepared samples were centrifuged at 8000 rpm for 10 min in a microcentrifuge (Eppendorf mini spin). Then, 600 µL were transferred into appropriate vials to be analysed in HPLC (Hitachi HPLC, Biorad Aminex HPX-87H column, over Gecko 2000, Detector RI Hitachi U-2490) at 40 °C in runs of 20 min with an eluent of H_2SO_4 in MilliQ water (0.01 N) as mobile phase at a flow rate of 0.6 $mL \cdot min^{-1}$.

11.4. Ammonium quantification

Ammonium was quantified using an NH_4^+ selective electrode (96 63 C Crison electrode coupled with a Hanna pH 211 Microprocessor pH meter). A calibration curve was drawn relating the symmetrical base-10 logarithm of NH_4^+ concentrations with the respective potential value (Appendix B). Several dilutions of an NH_4Cl solution (NH_4^+ 0.1 M) were done in order to obtain the standards. 100 µL of an ionic strength adjuster (ISA) ($MgSO_4$ 1 M) was added to a sample of 1 mL (standards and samples) to adjust the ionic strength. The value of the potential (mV) was noted 4 min after start the reading.

11.5. PHA quantification

PHA content and its concentration was determined by GC-FID using the adapted method of Lemos et al (2006). The biomass was lyophilized for 24 h (VirTis benchtop K). Then, lyophilized biomass was incubated in 1 mL solutions of heptadecane (internal standard) dissolved with chloroform (1:1) and acidic methanol (20 %) at 100 °C for 3.5 h. Then, the organic phase was extracted and injected in GC-FID (Bruker 400-GC; Bruker BR-SWAX column (30 m × 0.25 mm × 0.25 µm) with hydrogen as carrier gas (14.5 Psi)). An injection split of 0.50 µL was used at 280 °C with a proportion split of 1:5. The heating programme used was 40 °C with an increase of 20 $°C \cdot min^{-1}$ until 100 °C, followed by an increase of 3 $°C \cdot min^{-1}$ until 155 °C and 20 $°C \cdot min^{-1}$ until 220 °C. The temperature of the detector was 250 °C. The calibration of 3HB and 3HV was done using standards of P(3HB/3HV) commercial polymer (88%/12%, Aldrich).

12. Microscopy

12.1. Sample preparation

Samples were fixed accordingly with the adapted method of Amann et al (1990). A sample of 1 mL was harvested from the reactor and centrifuged at 8000 rpm for 5 min to separate the biomass from the rest of the medium maintaining the integrity of the cells. The supernatant was discarded and the biomass was ressuspended in 1xPBS and centrifuged at 8000 rpm for 5 min. This wash was performed three times. Centrifuged biomass was ressuspended in 500 µL of 1xPBS and 500 µL of formaldehyde (4 %) was then added to the eppendorf. This was incubated overnight at 4 °C. The fixed sample was centrifuged at 8000

rpm for 5 min at 4 °C and the supernatant was discarded. The sample was ressuspended in 500 µL of ice cold 1xPBS and 500 µL of ice cold ethanol (96 % (v/v)) was added. The sample was stored at -20 °C.

12.2. Scanning Electron Microscopy

Scanning electron microscopy (SEM) images were obtained using a field-emission gun (FEG) SEM Hitachi SU-70 microscope operated at 15 kV and equipped with an energy-dispersive X-ray (EDX) spectroscopy accessory (EDX detector: Bruker AXS; Software: Quantax). For SEM analysis, centrifuged samples were placed on a metallic aluminium stub, left to dry for 24 h at room temperature and then coated with evaporated carbon just before being analysed.

12.3. Fluorescence Microscopy

Fluorescence images to observe the polymer accumulation in the cells were made with an AXIO imager equipped with Zeiss lens coupled to a shutter HXP 120C. The samples were coloured with Nile Blue stain since it presents a red/orange colour when in the presence of PHA granules under fluorescence light (Ostle & Holt 1982; Kitamura & Doi 1994).

The preparation of the samples was performed accordingly with the adapted method of Gameiro (2010). A sample of 1 mL from the reactor was harvested and centrifuged at 10000 rpm for 5 min to separate the biomass from the medium. The medium was discarded and the pellet was ressuspended in 1 mL of NaCl (0.9 %). 50 µL of Nile blue solution was added to 500 µL of sample and it was incubated at 55 °C for 15 min, protected from light. A few drops of this sample was then put in a slide and it was observed in the microscope using epifluorescence.

12.4. Contrast Phase Microscopy

Contrast phase microscopy images were taken in an optic microscope Olympus BX51 using uEye software.

13. Polymer extraction

At the end of the bioreactor operational period, the remaining medium was centrifuged at 5000 rpm for 30 min at 4 °C (Heraeus Megafuge 16R centrifuge) and the obtained biomass was lyophilized for 72 h and the rest of the medium was discarded. Then, the biomass was ressuspended in chloroform (30-50 $mL \cdot g^{-1}$) and incubated in closed erlenmeyers in an orbital sacker for 72 h at 180 rpm at 30 °C. Lastly, a vacuum filtration was performed using glass membrane filters (Specanalitica) with a diameter of 47 mm and a pore of 1.2 µm in order to separate the biomass debris from the solubilized polymer. The solution was left to evaporate to obtain the desired polymer.

14. Differential Scanning Calorimetry (DSC)

DSC of the polymer obtained at the end of the bioreactor assay was performed in a PerkinElmer Diamond DSC. A sample of approximately 5 mg was weighted into 60 µL stainless steel pan and it was operated with a pressure limit of 24 bar. The heating programme consisted in heating three times from -30 °C to 200 °C with an increase of 10 $°C \cdot min^{-1}$ with two cooling's between them: the first cooling from 200 °C to -30 °C at 100 $°C \cdot min^{-1}$ and the second one from 200 °C to -30 °C at 10 $°C \cdot min^{-1}$.

15. Calculation

15.1. Specific growth rate

The specific growth rate was calculated using the Equation 1 which describes the microbial growth.

Equation 1

$$\mu = \frac{1}{X} \times \frac{dX}{dt} \ (h^{-1})$$

By integration and linearization of Equation 1 it is possible to obtain the specific growth rate, µ.

Equation 2

$$\ln(X_i) = \mu \cdot t + \ln(X_0)$$

In equation 2, X corresponds to biomass concentration and t to time. To obtain the maximum specific growth rate, μ_{max}, only the exponential phase is considered. 0 and i

corresponds to the beginning and ending of the exponential phase, respectively. μ_{max} corresponds to the slope of the linearization obtained.

15.2. Substrate uptake rate

The volumetric substrate uptake rate, r_{Glc} and r_{Xyl} were calculated from the variation of glucose (ΔGlc) and xylose (ΔXyl) concentrations respectively, over time (Δt) (Equation 3 and Equation 4).

Equation 3

$$r_{Glc} = -\frac{\Delta Glc}{\Delta t}\ (gL^{-1}h^{-1})$$

Equation 4

$$r_{Xyl} = -\frac{\Delta Xyl}{\Delta t}\ (gL^{-1}h^{-1})$$

15.3. Product formation rate

The volumetric product formation rate, r_P, was calculated from the variation of PHA concentration (ΔP) over time (Δt) (Equation 5).

Equation 5

$$r_P = \frac{\Delta P}{\Delta t}\ (gL^{-1}h^{-1})$$

15.4. Biomass yield on substrate

The biomass yield on substrate, $Y_{(X/S)}$, was calculated dividing the amount of biomass (ΔX) produced by the total substrate (glucose and xylose) consumed (ΔS) (Equation 6). The biomass chemical formula used was $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$ proposed by Ishizaki & Tanaka (1990).

Equation 6

$$Y_{(X/S)} = \frac{\Delta X}{\Delta Glc + \Delta Xyl}\ (Cmol_X Cmol_S^{-1})$$

15.5. PHA yield on substrate

The PHA yield on substrate, $Y_{(PHA/S)}$, was calculated dividing the amount of PHA (ΔPHA) produced by the total substrate (glucose and xylose) consumed (ΔS) (Equation 7).

Equation 7

$$Y_{(PHA/S)} = \frac{\Delta PHA}{\Delta Glc + \Delta Xyl} \ (Cmol_{PHA} Cmol_S^{-1})$$

15.6. Organic acids yield on substrate

The lactic (HLa), acetic (HAc) and propionic (HPr) acids yield on substrate, $Y_{(HLa/S)}$, $Y_{(HAc/S)}$, $Y_{(HPr/S)}$ respectively, were calculated dividing the amount of each acid (ΔAC) produced individually by the total substrate (glucose and xylose) consumed (ΔS) (Equation 8).

Equation 8

$$Y_{(AC/S)} = \frac{\Delta AC}{\Delta Glc + \Delta Xyl} \ (Cmol_{AC} Cmol_S^{-1})$$

15.7. DSC crystallinity

Crystallinity of the polymer was calculated accordingly to Equation 9.

Equation 9

$$\%\ Crystallinity = \frac{\Delta Hm - \Delta Hc}{\Delta Hm^{\circ}} \times 100$$

ΔHm and ΔHc corresponds to the melting and cold crystallization (in terms of $J{\cdot}g^{-1}$), respectively, and ΔHm° is the reference value for P(3HB) of heat of melting if the polymer was 100 % crystalline and this value is 146.0 $J{\cdot}g^{-1}$ accordingly to Barham et al (1984).

CHAPTER III – RESULTS AND DISCUSSION

1. Hydrolysate preparation and analysis

E. globulus unbleached pulp was hydrolysed in order to obtain a solution of sugars that could be fermented by microorganisms. 3 L of enzymatic hydrolysis was performed in duplicate, in a total of 6 L of hydrolysate (calculations about weights and volumes of the enzymatic hydrolysis for a volume of 3 L is shown in appendix C).

The hydrolysate was autoclaved to be used in the medium. However, with the appearance of a precipitate (Figure 10), a centrifugation followed by a second sterilization by autoclave was tested in order to verify if a significant quantity of sugars was lost during that procedure. An alternative method to prepare the hydrolysate was centrifugation under sterile conditions, which means a higher process time and the sterilization after could not be ensured.

Figure 10 – Precipitate obtained after autoclaving the hydrolysate (left) and after centrifuging the previous solution (right).

The obtained hydrolysate was analysed in every step during its preparation until it was ready to be added to the medium: at end of hydrolysis, after filtration, after the first sterilization by autoclave, after centrifugation and after the second sterilization. The results of sugar quantification, analysed by HPLC are shown in Table 14.

Table 14 – Sugar quantification of hydrolysate throughout its preparation.

Procedure step	Glucose concentration ($g \cdot L^{-1}$)	Xylose concentration ($g \cdot L^{-1}$)
After the hydrolysis	83.08 ± 2.52	17.13 ± 0.06
After the filtration	83.68 ± 1.96	17.84 ± 0.29
After 1st autoclave	85.24 ± 4.69	19.32 ± 1.72
After centrifugation	83.09 ± 0.85	18.30 ± 0.24
After 2nd autoclave	84.06 ± 1.00	20.22 ± 0.26

These values are consistent with Cabral (2014), who obtained 92.40 $g \cdot L^{-1}$ of glucose and 23.57 $g \cdot L^{-1}$ of xylose, but with an enzyme dosage of 35 FPUs/ $g_{carbohydrate}$ instead of the 20 FPUs/ $g_{carbohydrate}$ used in this study.

By analysing the results from Table 14, the concentration of sugars remained practically unaltered during the procedure, with a variation of approximately 1 $g \cdot L^{-1}$ of glucose and 3 $g \cdot L^{-1}$ of xylose. This was not a significant concentration loss for the process taking into account the high values of concentration, however, at an industrial scale this procedure would lead to an increase on the hydrolysate cost due to energy and extra time necessary.

The precipitate formed (Figure 10) was analysed by HPLC after an acidic hydrolysis to determine its composition. The solution obtained after acidic hydrolysis of the precipitate contained mostly xylose and a small amount of glucose (1.21 ± 0.07 $g \cdot L^{-1}$ of xylose and 0.53 ± 0.22 $g \cdot L^{-1}$ of glucose). This represents a proportion of approximately 2.3 g of xylose to 1 g of glucose which indicates the presence of hemicelluloses in precipitate, most provably xylans, considering the high content of xylose related to glucose. Similar results were obtained by Silva et al (1998) with hemicellulose hydrolysis. This can be explained due to presence of cellulases and the lack of xylanases in the enzymatic solution used in the hydrolysis that were designed to cleavage especially cellulose and not hemicelluloses.

2. Preliminary tests

A pre-inoculum growth (Figure 11) was followed to evaluate when it was ready to be used as inoculum. *C. necator* had a lag phase of about 48h before entering in exponential phase. This information was important to schedule the different inocula, since the beginning of exponential phase is considered the appropriated moment to inoculate a fresh medium and start a new growth.

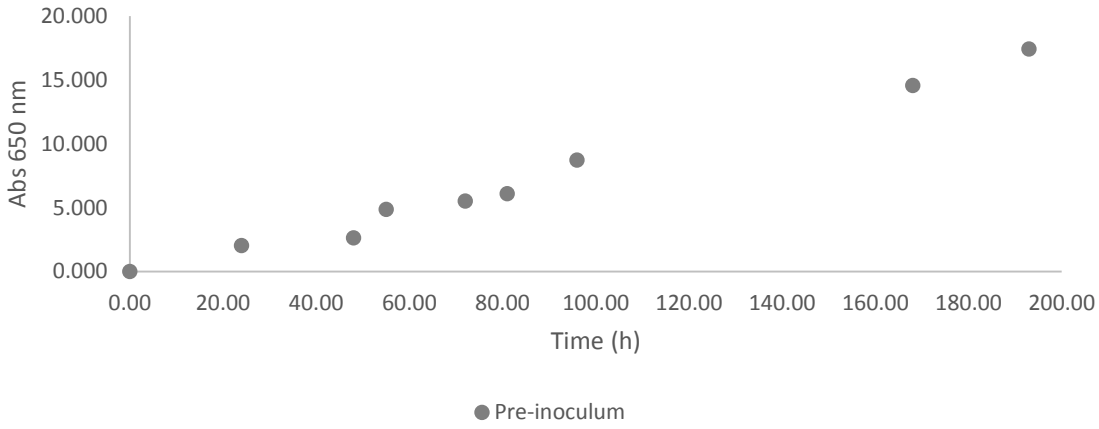


Figure 11 – Pre-inoculum assay in LB medium.

3. Shake flask assays

The results of the assays performed in this project will be discussed into two main parts. The first one englobes the preliminary tests, where the medium composition (media 1 and 2) was tested for each strain (NRRL B-4383 and DSM 545) (section 3.1) and the possibility to grow the chosen strain in medium with hydrolysate with different concentrations of glucose (20 $g \cdot L^{-1}$ and 40 $g \cdot L^{-1}$) (section 3.2). The second part refers essentially to tests in fed-batch conditions in flask (section 3.3) and in bioreactor (sections 4), using hydrolysate.

It is important to emphasize that *C. necator* accumulates PHA mainly in limited nutrient conditions, more concretely ammonium. For this reason, biomass concentration will be the objective of preliminary tests since the more biomass produced, the more polymer could be accumulated in a posterior phase. Even though a polymer extraction was performed mostly of the times at the end of the fermentations, non-relevant results were achieved, as expected, since no limitation was imposed in flask assays. Nevertheless, GC-FID analysis of some samples of preliminary tests were also done.

3.1. Strain selection

Some preliminary tests with both *C. necator* strains, NRRL B-4383 and DSM 545, were performed in media 1 and 2, with a glucose concentration of 20 $g \cdot L^{-1}$, in order to understand in which medium each strain produced more biomass. In these preliminary tests, inocula were done in LB medium. *C. necator* NRRL B-4383 was tested in medium 1 (Figure 12) and in medium 2 (Figure 13) while *C. necator* DSM 545 was also tested in medium 1 (Figure

14) and in medium 2 (Figure 15). All these tests were performed in medium with hydrolysate (solid dots connected by a continuous line, in figures) with corresponding controls with glucose (empty dots connected by a discontinuous line). The assay with NRRL B-4383 in medium 2 (Figure 13) does not show PHA concentration due to the low biomass concentration obtained, insufficient for GC-FID analysis.

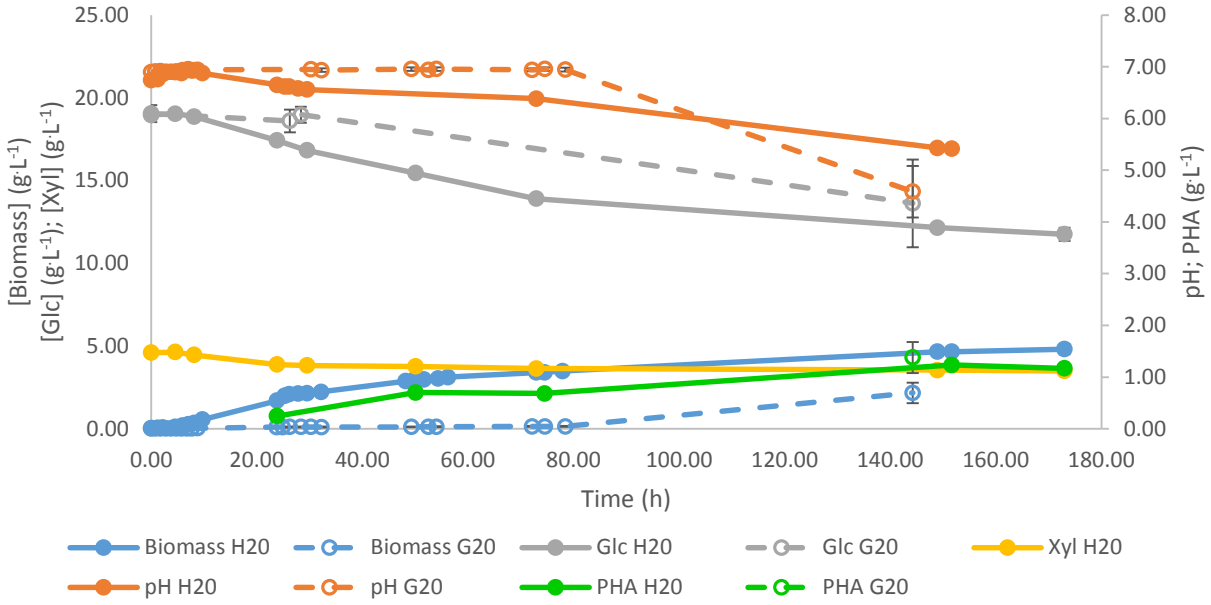


Figure 12 – Evolution of biomass, pH, PHA and sugars concentration during the assay with NRRL B-4383 in medium 1 with hydrolysate (H20) using a glucose concentration of 20 g·L-1 and the corresponding control with glucose (G20).

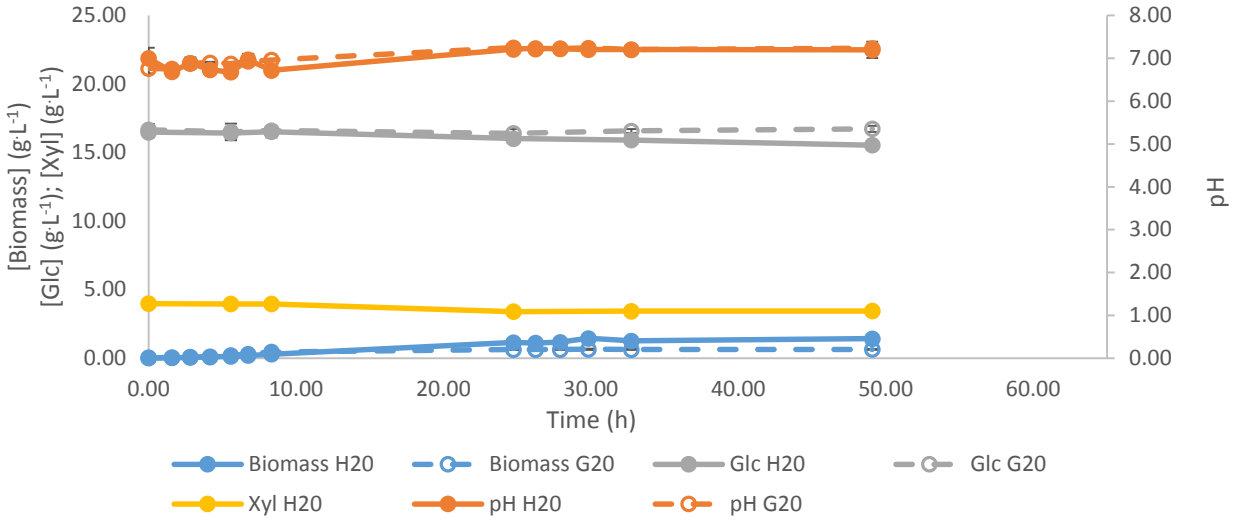


Figure 13 – Evolution of biomass, pH and sugars concentration during the assay with NRRL B-4383 in medium 2 with hydrolysate (H20) using a glucose concentration of 20 g·L-1 and the corresponding control with glucose (G20).

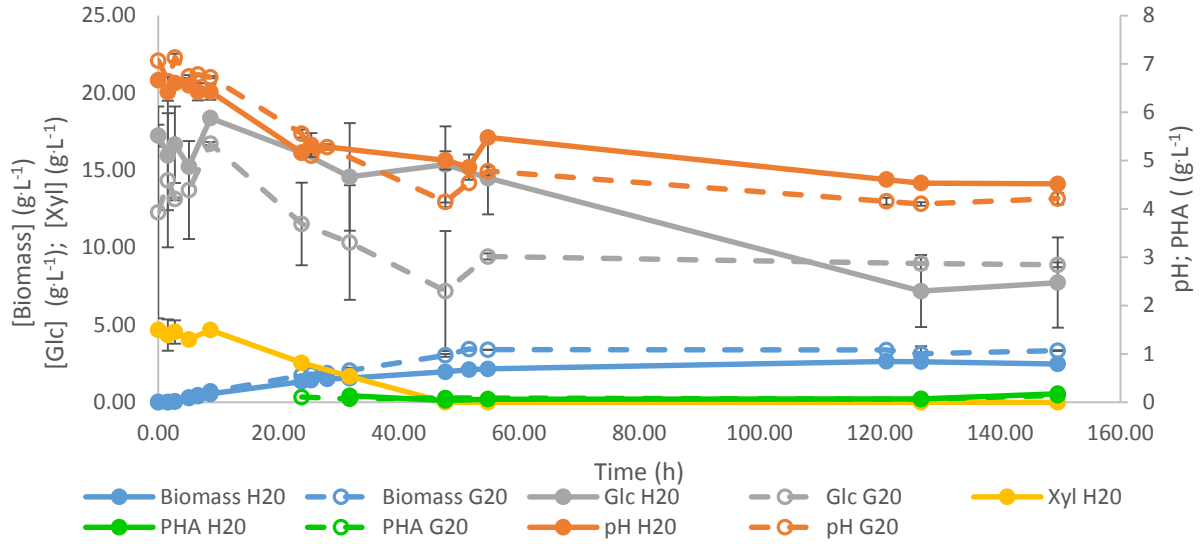


Figure 14 – Evolution of biomass, pH, PHA and sugars concentration during the assay with DSM 545 in medium 1 with hydrolysate (H20) using a glucose concentration of 20 g·L^{-1} and the corresponding control with glucose (G20).

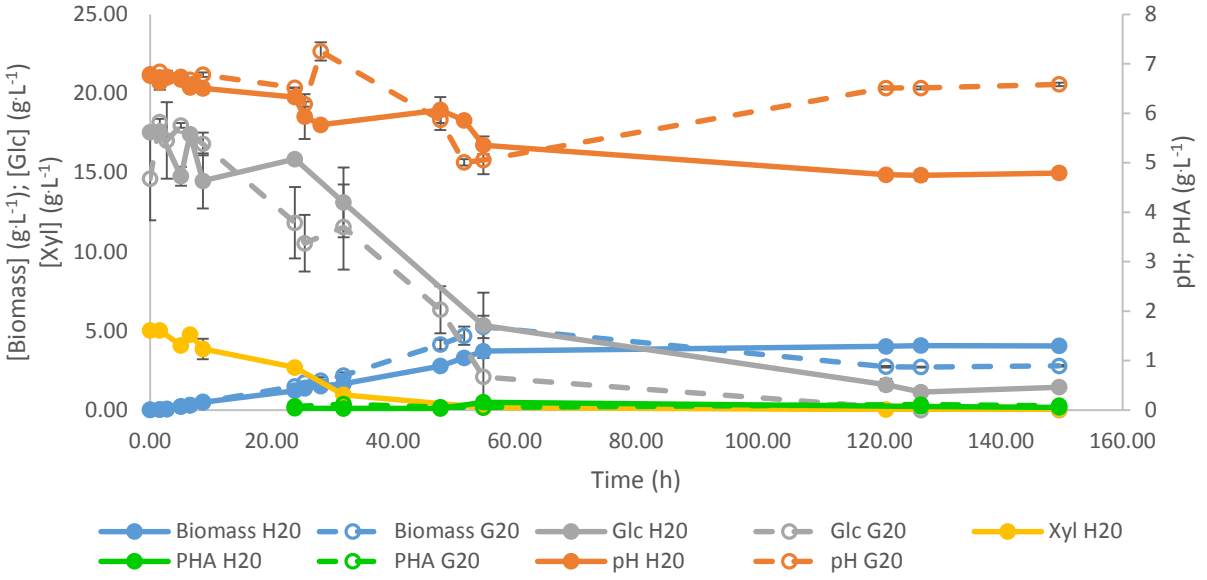


Figure 15 – Evolution of biomass, pH, PHA and sugars concentration during the assay with DSM 545 in medium 2 with hydrolysate (H20) using a glucose concentration of 20 g·L^{-1} and the corresponding control with glucose (G20).

From the assays presented above, some parameters were calculated (Table 15), such as maximum specific growth rate, μ_{max}; maximum biomass concentration achieved, $[X]_{max}$; .volumetric uptake rate of glucose and xylose, r_{Glc} and r_{Xyl}, respectively; biomass yield on substrate, $Y_{X/S}$; PHA yield on substrate, $Y_{PHA/S}$; and maximum PHA accumulation achieved.

Table 15 – Parameters from strain selection assays. n/a – not applied.

Strain	Medium	Substrate	μ_{max} (h^{-1})	$[X]_{max}$ ($g \cdot L^{-1}$)	r_{Glc} ($g \cdot L^{-1} h^{-1}$)	r_{Xyl} ($g \cdot L^{-1} h^{-1}$)	$Y_{X/S}$ ($Cmol \cdot Cmol^{-1}$)	$Y_{PHA/S}$ ($Cmol \cdot Cmol^{-1}$)	PHA_{max} (%)
NRRL B-4383	1	Hydrolysate	0.305 ± 0.001	4.82 ± 0.31	0.090 ± 0.010	0.038 ± 0.003	0.725 ± 0.051	0.196	26.51
NRRL B-4383	1	Glucose	0.082 ± 0.059	2.18 ± 0.62	0.044 ± 0.196	n/a	0.503 ± 0.159	0.355	63.50
NRRL B-4383	2	Hydrolysate	0.258 ± 0.010	1.45 ± 0.06	0.026 ± 0.010	0.014 ± 0.004	1.40 ± 0.724	-	-
NRRL B-4383	2	Glucose	0.315 ± 0.008	0.66 ± 0.04	0.008 ± 0.002	n/a	3.34 ± 1.44	-	-
DSM 545	1	Hydrolysate	0.688 ± 0.015	2.65 ± 0.13	0.162 ± 0.133	0.131 ± 0.019	0.220 ± 0.034	0.001	8.72
DSM 545	1	Glucose	0.626 ± 0.010	3.44 ± 0.08	0.284 ± 0.167	n/a	0.309 ± 0.030	0.007	6.23
DSM 545	2	Hydrolysate	0.498 ± 0.005	4.07 ± 0.24	0.338 ± 0.045	0.132 ± 0.003	0.242 ± 0.001	0.008	4.26
DSM 545	2	Glucose	0.472 ± 0.011	5.25 ± 0.70	0.295 ± 0.048	n/a	0.528 ± 0.052	0.006	5.45

Taking into account the experimental results, both strains of *C. necator* were able to grow and accumulate PHA in medium with hydrolysate of unbleached kraft pulp. NRRL B-4383 strain grew better in medium 1, resulting in a maximum biomass of 4.82 ± 0.31 $g \cdot L^{-1}$, more than the double of control, 2.18 ± 0.62 $g \cdot L^{-1}$. In medium 2, NRRL B-4383 practically did not grow, achieving 1.45 ± 0.06 $g \cdot L^{-1}$ of biomass with hydrolysate while control got a maximum of 0.66 ± 0.31 $g \cdot L^{-1}$. DSM 545, on the other hand, grew in both media but it achieved higher biomass concentration in medium 2 with hydrolysate, 4.07 ± 0.24 $g \cdot L^{-1}$, a concentration a little smaller than in control, where it reached 5.25 ± 0.70 $g \cdot L^{-1}$. In medium 1, DSM 545 obtained a maximum biomass of 2.65 ± 0.13 $g \cdot L^{-1}$, a value slightly lower than in control, 3.44 ± 0.08 $g \cdot L^{-1}$. In addition, some assays were most probably inhibited by pH, interfering with the results, since they reached values under 5.00 in some tests with DSM 545 (Figure 14 and Figure 15), which is considered inhibitory for this bacterium (Makkar & Casida 1987; Zahari et al. 2012) though they had yet substrate available. Moreover, considering the first 60 h of the assays of each strain in its best medium, DSM 545 showed a faster growth, resulting in a biomass concentration of 3.72 $g \cdot L^{-1}$ in medium 2, in contrast with 3.12 $g \cdot L^{-1}$ of NRRL B-4383 in medium 1. Both strains reached a higher μ in medium 1 than medium 2, however, DSM 545 reached higher values than NRRL B-4383. DSM 545 reached 0.688 ± 0.015 h^{-1} in medium 1 with hydrolysate and 0.498 ± 0.005 h^{-1} in medium 2. On the other hand, NRRL B-4383 reached 0.305 ±0.001 h^{-1} in medium 1 with hydrolysate and 0.258 ±0.010 h^{-1} in medium 2.

Both strains were able to accumulate PHA, although in small quantities, as it was expected. The exception, which can be highlighted, was the accumulation of 26.51 % (1.24 $g \cdot L^{-1}$) of P(3HB) by NRRL B-4383 in medium 1 as well as the control of the same assay, where the culture accumulated 63.50 % of P(3HB). PHA accumulation in NRRL B-4383 in medium 2 was not detected by GC-FID, due to the low biomass achieved during that assay. DSM 545 was not able to accumulate more than 9 % of PHA, however, it accumulated co-polymer P(3HB-co-3HV) with a fraction of approximately 24 % of HV. P(3HB-co-3HV) is referred for being produced by this bacterium strain in literature (Marangoni et al. 2002; Agustín Martinez et al. 2015). This co-polymer production is being studied in most cases under nitrogen limitation by the addition of propionic acid into the medium in low concentrations to promote the production of the HV fraction (Du et al. 2001). In these assays, DSM 545 produced P(3HB-co-3HV) without any external addition of fatty acids. A co-

polymer with a high fraction of HV is more valuable than P(3HB), since it can be applied in a broader range of applications (Tan et al. 2014; Chen 2009).

About substrate uptake rates and yields in assays with hydrolysate, although NRRL B-4383 had not consumed neither glucose nor xylose at the same high rates as DSM 545, it achieved much higher biomass and PHA yields in medium 1. While NRRL B-4383 reached a biomass yield of 0.725 $Cmol_X \cdot Cmol_S^{-1}$ in medium 1, DSM 545 only achieved a biomass yield of 0.220 $Cmol_X \cdot Cmol_S^{-1}$ in medium 1 and 0.242 $Cmol_X \cdot Cmol_S^{-1}$ in medium 2. In the assay with NRRL B-4383 in medium 2, although biomass yields were calculated, due to the small amount of biomass produced and the low concentration of sugars consumed, the yields were not accurate, being much higher than the yield of other tests. Moreover, DSM 545 consumed glucose at a rate of 0.162 $g \cdot L^{-1}h^{-1}$ in medium 1 and 0.338 $g \cdot L^{-1}h^{-1}$ in medium 2 while NRRL B-4383 consumed glucose at a rate of 0.090 $g \cdot L^{-1}h^{-1}$ in medium 1, less than half of the former. About xylose, NRRL B-4383 practically did not consume any xylose, just a difference of about 1.0 $g \cdot L^{-1}$ during each assay was observed. This represented a xylose uptake of 0.038 $g \cdot L^{-1}h^{-1}$ in medium 1 and 0.014 $g \cdot L^{-1}h^{-1}$ in medium 2. On the other hand, DSM 545 consumed all the xylose after 55 h, with an uptake rate of 0.131 $g \cdot L^{-1}h^{-1}$ in medium 1 and 0.132 $g \cdot L^{-1}h^{-1}$ in medium 2. About PHA yields, since DSM 545 produced a small amount of PHA with a maximum accumulation of 8.72 % in medium 1 its yields were also low. The maximum PHA yield of this strain was 0.0075 $Cmol_{PHA} \cdot Cmol_S^{-1}$, achieved in medium 2. On the other hand, NRRL B-4383 achieved a PHA yield of 0.196 $Cmol_{PHA} \cdot Cmol_S^{-1}$ in medium 1, significantly higher than DSM 545.

Bearing all these aspects into consideration, DSM 545 was the strain selected to be used in further tests due to its fast growth; the capability to consume xylose, which corresponds to 20 % of the hydrolysate sugars; the ability to produce and accumulate P(3HB-co-3HV) instead of P(3HB); as well as some satisfactory results from literature referring *C. necator* DSM 545 using inexpensive or renewable substrates to produce PHA, such as waste glycerol, corn syrup, cheese whey and hydrolysed molasses (Cavalheiro et al. 2009; Daneshi et al. 2010; Povolo et al. 2010; Baei 2009; Du et al. 2012).

3.2. Effect of sugars concentration

Taking into account the results from strain selection, DSM 545 was tested in medium 2 with glucose concentrations of 20 $g \cdot L^{-1}$ and 40 $g \cdot L^{-1}$ using hydrolysate, H20 (Figure 16) and H40 (Figure 17), respectively. As control, a growth in medium 2 with 20 $g \cdot L^{-1}$ of glucose, G20, was performed. For these assays, an inoculum in seed medium was prepared contrary to the assays tested with LB medium in section 3.1, so that the culture could adapt better to the growth medium and to reduce the duration of lag phase. Besides this, some acids were produced during this assay, namely lactic, acetic and propionic acids, and the evolution of their concentrations are shown in Figure 18. PHA was not quantified because this test was very similar to the assay with medium 2 with hydrolysate from strain selection, where DSM 545 accumulated just a small amount of PHA (4.26 %) and the objective of this experiment was to compare the culture growth in medium with different sugars concentrations, and to observe if there was any growth inhibition associated with high sugars concentration.

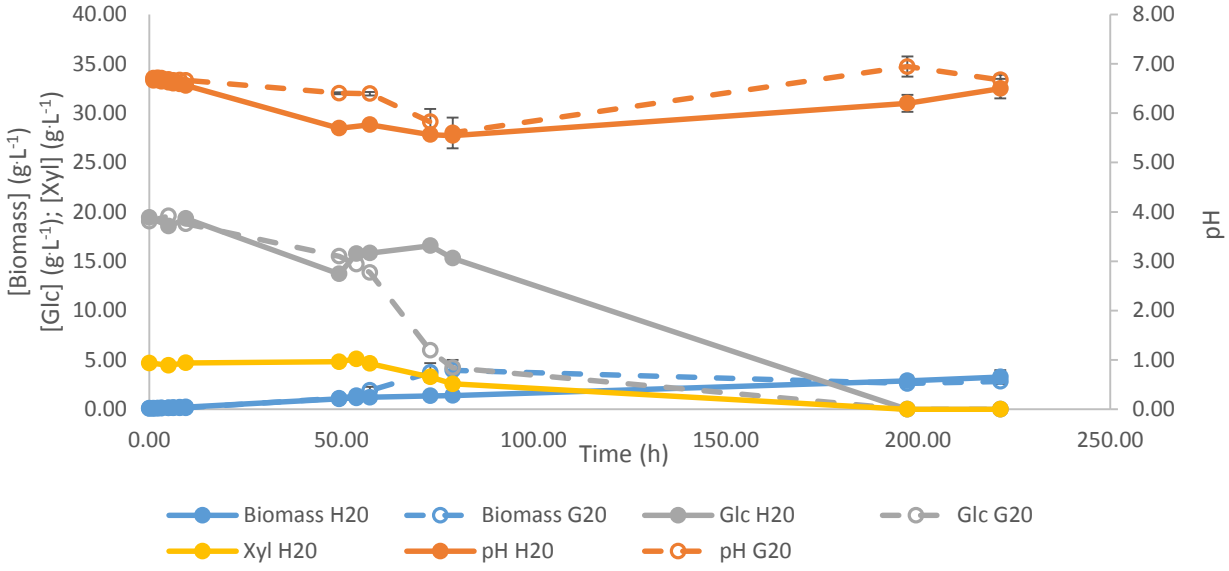


Figure 16 – Evolution of biomass, pH, and sugars concentration during the assay with DSM 545 in medium 2 with hydrolysate with a glucose concentration of 20 $g \cdot L^{-1}$ (H20), and control (G20).

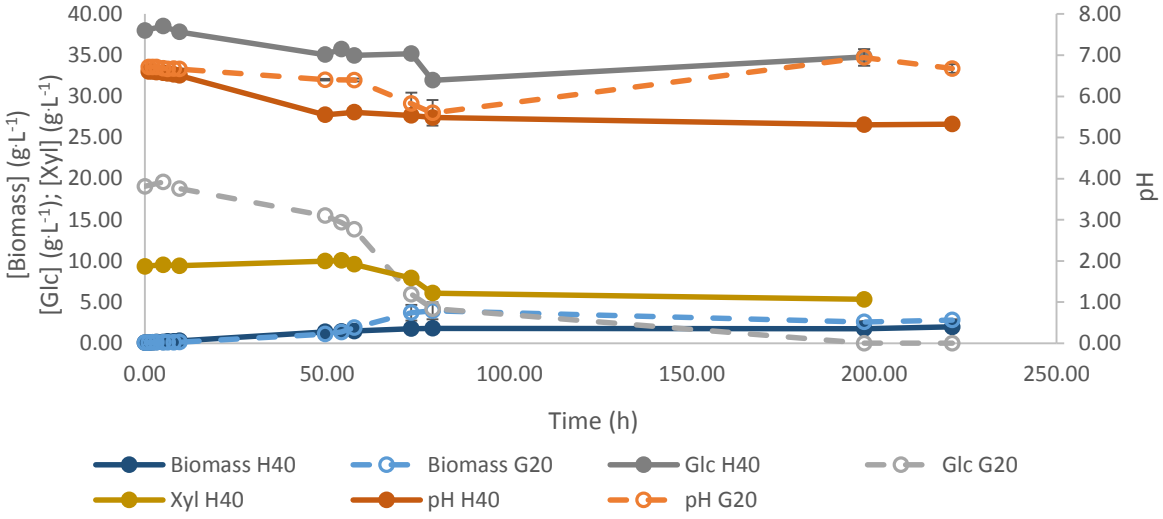


Figure 17 – Evolution of biomass, pH, and sugars concentration during the assay with DSM 545 in medium 2 with hydrolysate with a glucose concentration of 40 g·L^{-1} (H40), and control (G20).

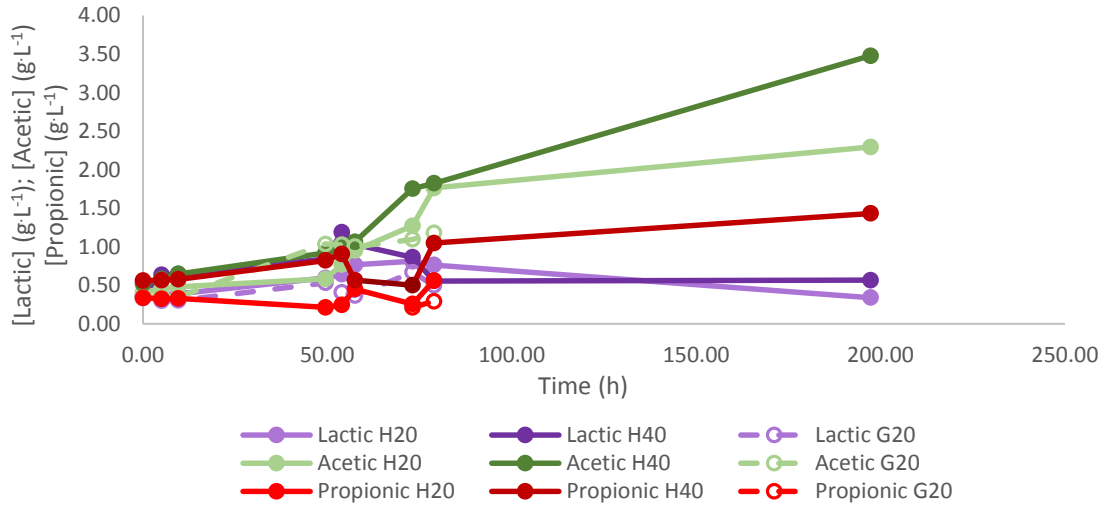


Figure 18 - Evolution of lactic, acetic and propionic acid concentrations during the assay with DSM 545 in medium 2 with hydrolysate with a glucose concentration of 20 g·L^{-1} (H20) and 40 g·L^{-1} (H40), and control (G20).

Some parameters from these assays were calculated and are shown in Table 16.

Table 16 – Maximum biomass concentration, μ_{max}, volumetric substrate uptake rates, and biomass and lactic, acetic and propionic acids yields on substrate from the assays with different sugars concentrations using DSM 545.

Assay	H20	H40	G20
$[X]_{max}$ ($g \cdot L^{-1}$)	3.26 ± 0.70	1.99 ± 0.19	3.95 ± 1.03
μ_{max} (h^{-1})	0.137 ± 0.017	0.139 ± 0.002	0.065 ± 0.008
r_{Glc} ($g \cdot L^{-1} h^{-1}$)	0.053	0.062	0.205
r_{Xyl} ($g \cdot L^{-1} h^{-1}$)	0.097	0.151	n/a
$Y_{X/S}$ ($Cmol \cdot Cmol^{-1}$)	0.167	0.236	0.328
$Y_{HLa/S}$ ($Cmol \cdot Cmol^{-1}$)	0.112	0.366	0.0272
$Y_{HAc/S}$ ($Cmol \cdot Cmol^{-1}$)	0.0779	0.411	0.0566
$Y_{HPr/S}$ ($Cmol \cdot Cmol^{-1}$)	0.0429	0.147	0.0237

Glucose and xylose were consumed at similar rates in both H20 and H40 assays, 0.053 $g \cdot L^{-1} h^{-1}$ of glucose and 0.097 $g \cdot L^{-1} h^{-1}$ of xylose in H20 and 0.062 $g \cdot L^{-1} h^{-1}$ of glucose and 0.151 $g \cdot L^{-1} h^{-1}$ of xylose in H40, although these values were approximately half of the respective rates observed in section 3.1. However, only H20 consumed all the glucose and the xylose available, contrary to H40, where 35 $g \cdot L^{-1}$ of glucose and 5 $g \cdot L^{-1}$ of xylose were still present at the end of the assay.

These assays showed that DSM 545 could growth in both H20 and H40. In both assays a μ of 0.14 h^{-1} was achieved, almost the double achieved in G20, but about three times lower than in the assay with DSM 545 in medium 2 using hydrolysate, 0.498 h^{-1} (section 3.1). It was also about half the value obtained by Cavalheiro et al (2009) using waste glycerol (0.30 h^{-1}). H20 showed a continuous growth reaching 3.26 $g \cdot L^{-1}$ of biomass after 220 h while H40 entered in stationary phase at around 80 h, achieving a maximum of 1.99 $g \cdot L^{-1}$ at 220 h. A higher amount of biomass obtained with a lower concentration of glucose was also observed by Kim et al (1994). H40 entered in stationary phase and the concentration of some acids started to increase, particularly acetic and propionic acids, which could have inhibited its growth.

The biomass yield from substrate in H40 (0.236 $Cmol \cdot Cmol^{-1}$) was higher than in H20 (0.167 $Cmol \cdot Cmol^{-1}$) but lower than the control (0.328 $Cmol \cdot Cmol^{-1}$). Comparing these results with those from the previous section, inoculated with LB medium, it can be concluded

that these assays inoculated with seed medium showed a slower growth, although they achieved practically the same biomass concentration.

A noticeable production of fatty acids, namely, lactic, acetic and propionic acids were measured by HPLC (Figure 18). It was clear that the more hydrolysate used in the medium, the higher the concentration of those acids. Thus, acid production yields were also higher in H40 compared with H20. This probably resulted from an oxygen limitation. These acids are usually produced as intermediates of metabolism under oxygen limitation and could be an explanation for the presence of HV units in assays with DSM 545 described in section 3.1 (Yang et al. 2010; Grousseau et al. 2014; Magdouli et al. 2015; Du et al. 2004; Chakraborty et al. 2012).

Other tests could be performed and an experiment could be designed in order to produce PHA in a two stage strategy. Biomass and organic acids could be produced in a first stage, without reaching inhibitory concentrations of fatty acids and, then, switch to a second stage where an ammonium limitation could be imposed to promote PHA accumulation. This way, the boundary costs that are associated with the use of these acids to promote and increase the PHA accumulation would be much less, which could make the PHA industrial production much more competitive (Grousseau et al. 2014).

3.3. Fed-batch assay

After the preliminary tests, a shake flask fed-batch experiment was performed (Figure 19) in the medium (section 3.1) and glucose concentration (section 3.2) where DSM 545 grew better in order to provide substrate so that the assay do not end due to substrate depletion as in the assays with DSM 545 in H20, from last section (section 3.2). An inoculum of DSM 545 was prepared in seed medium and inoculated in medium 2 with hydrolysate and a glucose concentration of 20 $g \cdot L^{-1}$. The culture grew until the sugars were completely consumed, at 48 h, when a maximum biomass concentration of 7.39 ± 0.38 $g \cdot L^{-1}$ was obtained, achieving a μ of 0.198 ± 0.009 h^{-1} in the exponential phase, a value slightly higher than the previous assays (0.14 h^{-1}). This maximum biomass concentration obtained is higher than the maximum biomass achieved with *C. necator* DSM 545 in some works with other renewable feedstocks, such as hydrolyzed whey (4.5 $g \cdot L^{-1}$) (Marangoni et al. 2002), corn and palm oil (3.0-4.3 $g \cdot L^{-1}$) (Fukui & Doi 1998), for example. On the other hand, a slightly higher biomass concentration was achieved with palm kernel oil (7.5 $g \cdot L^{-1}$) (Lee et al. 2008).

Glucose and xylose were consumed at a rate of 0.942 $g \cdot L^{-1}h^{-1}$ and 0.156 $g \cdot L^{-1}h^{-1}$, respectively. pH decreased to 5 before the end of first pulse, but it did not inhibited so markedly the biomass growth as in previous assays.

At 48 h, a pulse of hydrolysate was added aseptically and glucose and xylose increased their concentrations to 17.98 and 5.03 $g \cdot L^{-1}$ respectively. 6 h after pulse addition, the biomass stopped its growth because pH decreased to 4.97 and also perhaps due to the propionic acid concentration, 10.50 $g \cdot L^{-1}$ which is higher than values considered inhibitory, 1.5 to 3.0 $g \cdot L^{-1}$, accordingly with literature (Grousseau et al. 2014; Ramsay et al. 1990).

Acids were once again produced as it was observed in previous tests (section 3.2) reaching values of about 4.1 $g \cdot L^{-1}$ of acetic acid, 0.8 $g \cdot L^{-1}$ of lactic acid and over 11.0 $g \cdot L^{-1}$ of propionic acid.

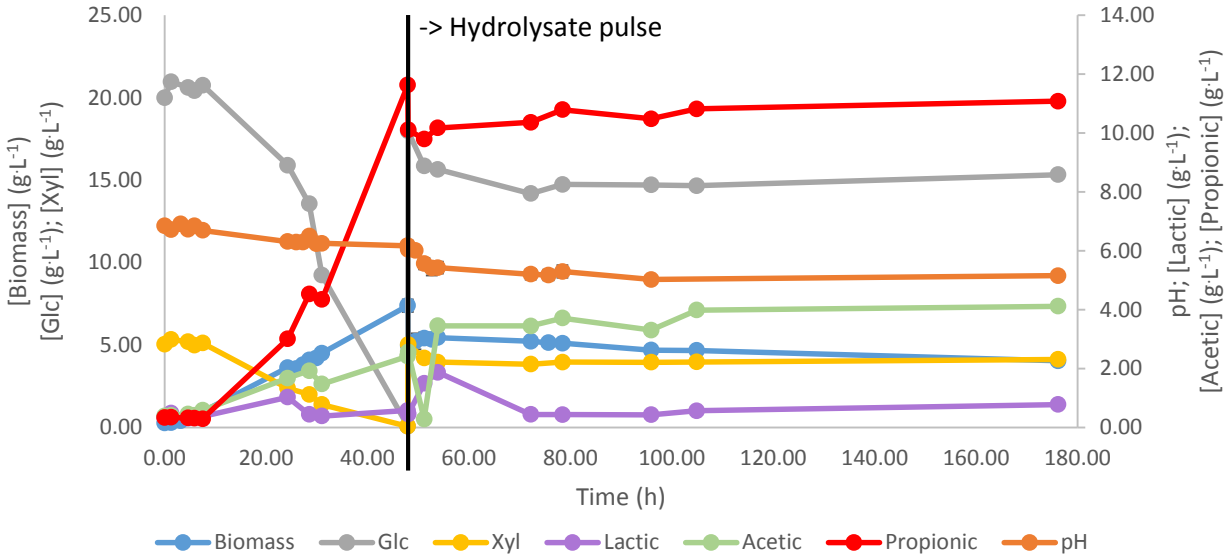


Figure 19 – Evolution of biomass, pH, sugars and acids concentrations during the fed-batch assay in medium 2 with DSM 545.

However, this assay did not accomplish the objective proposed, since cells stopped its growth after the hydrolysate pulse due to pH decrease. To obtain higher biomass concentration and PHA production, it is important to control pH in an optimal interval for culture growth and PHA accumulation, in order to avoid growth inhibition by pH. Moreover, acids formation as a result of a possible oxygen limitation also require an efficient oxygen control. In order to control pH, oxygen, as well as stirring and temperature, the next assays were performed in a bioreactor.

4. Fed-batch bioreactor assays

The first assay was performed to test the bioreactor (Figure 9) itself as well as to understand the behaviour of the culture in a higher operating volume. The evolution of sugars consuption, biomass growth, PHA and organic acids production is shown in Figure 20.

After a lag phase of nearly 24 h, the culture started to grow exponentially with a μ of 0.424 h^{-1}. This value is nearly 2-fold the values calculated in preliminary tests inoculated with seed medium and slightly higher than the values of the literature (Cavalheiro et al. 2009; Mozumder et al. 2014). The biomass achieved a maximum biomass concentration of 3.80 $g{\cdot}L^{-1}$ at 33.50 h. The DOC rapidly decreased to the minimum set value of 20 % and the stirring speed started to increase from the minimum value of 150 rpm to the maximum 460 rpm (the maximum of the reactor). This value is very low compared with the maximum values found in the literature (Cavalheiro et al. 2009; Mozumder et al. 2014; Kim et al. 1994) which used reactors that reach stirring speed up to 1600 rpm (around 3.5 times higher). This influenced the results because 12 h after the culture entered in exponential phase it was under oxygen limitation since it was consuming all oxygen supplied to the reactor and this affects the PHA production as shown by Lefebvre et al (1997) where a decrease of about 25 % in PHA productivity was obtained under low DOC, 1-4 %, compared with control with a DOC of 50-70 %.

Also, there was a delay in the addition of the second pulse, due to problems of sugar detection by DNS. The pulse was added with a delay of about 18 h. This resulted in a loss of viability of the biomass.

The analysis by GC-FID results showed that PHA achieved a maximum content and concentration of 13.1 % and 0.50 $g{\cdot}L^{-1}$ respectively, at 33.50 h.

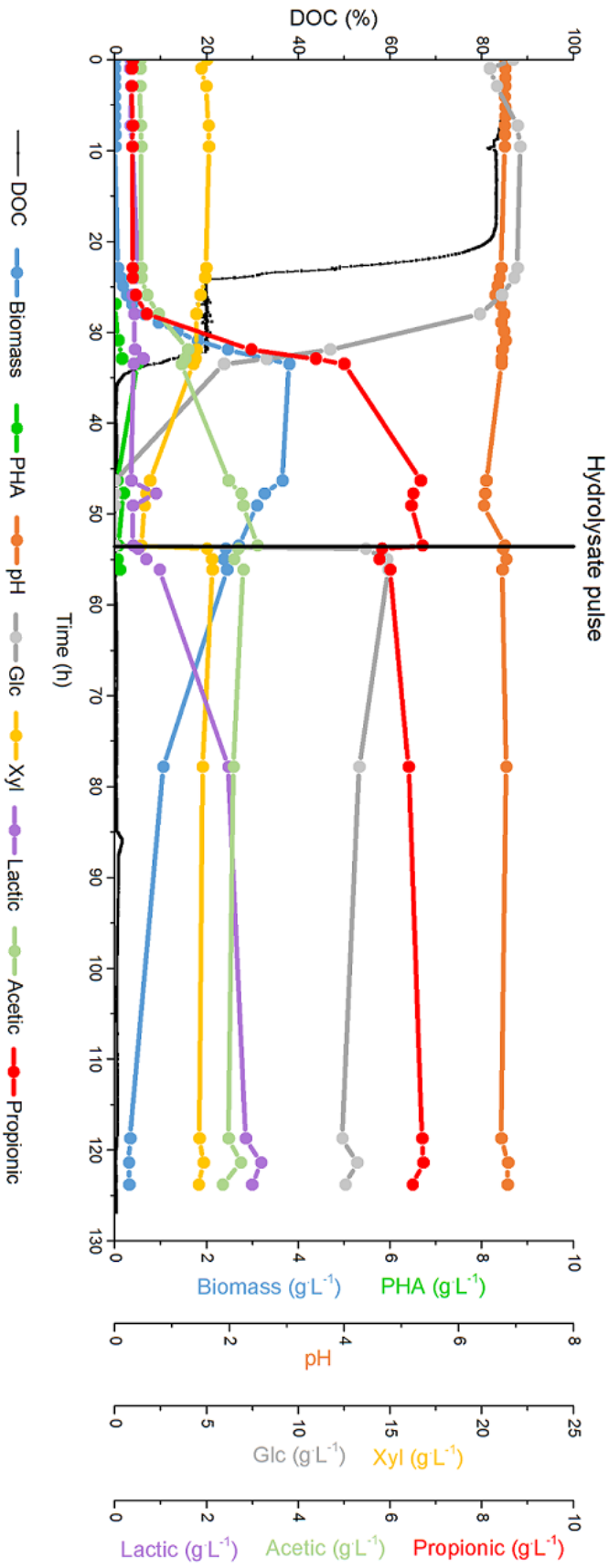


Figure 20 – Evolution of biomass growth, PHA and organic acids production, sugars consumption, and pH and DOC, in the first assay in bioreactor.

Then, a second fed-batch assay was performed in order to achieve higher biomass and PHA accumulation, taking into account the pulse delay from the previously assay and imposing an ammonium limitation to promote the PHA accumulation. The results are shown in Figure 21 and parameters such as maximum biomass, growth rate, substrates uptake and yields of each hydrolysate pulse are shown in Table 17.

The culture started its growth after a lag phase of about 26 h, nearly the same time as the last assay (24 h), followed once again by a DOC decrease. After that time, the exponential phase started with a μ of $0.209 \pm 0.005\ h^{-1}$ before the second hydrolysate pulse at 34.6 h and continued with a μ of $0.203 \pm 0.003\ h^{-1}$ for at least 4 h after the addition of the second pulse. These specific growth rates are in accordance with the results obtained by Mozumder et al (2014) achieving a μ of $0.21\ h^{-1}$ in assays with an initial glucose concentration of $20\ g{\cdot}L^{-1}$. After 37.5 h, DOC, which was at 20 %, continued its declining when the stirring speed reached its highest value, to values under 0.8 % at 39.4 h, what is inhibitory for PHA production as described by Lefebvre et al (1997). At this point, lactic, acetic and propionic acids started to be produced.

Although no information on the production of these acids by *C. necator* was found in literature, this may possibly be related with oxygen limitation, meaning that the culture shifted its metabolism towards sugar fermentation.

The production of organic acids together with low DOC values are known to inhibit PHA production and biomass growth (Lefebvre et al. 1997; Chung et al. 1997; Kim et al. 1992). Everything was done to try to revert the situation about DOC. A new air pump with more capacity than the one used in the former assay (Figure 20) was used and when the DOC reached 0.00 % another air stream was added to increase this value, however with no significant results.

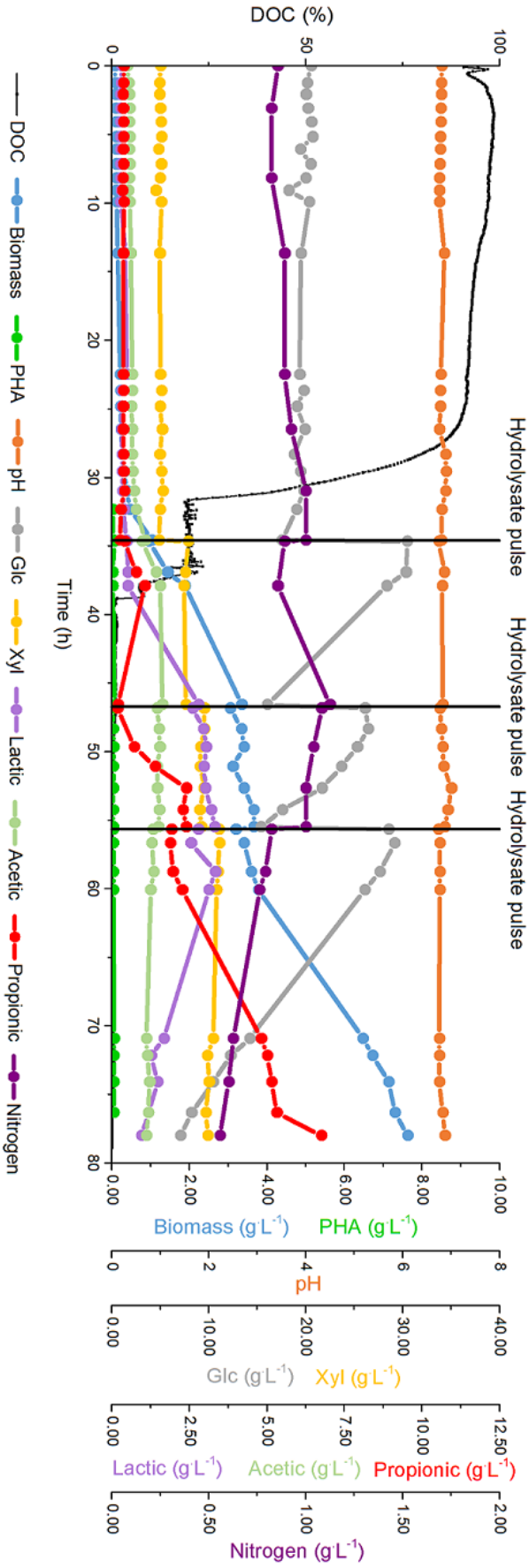


Figure 21 – Evolution of biomass growth, PHA and organic acids production, sugars consumption, and pH and DOC, in the second assay in bioreactor.

Glucose and xylose uptake rates when the culture reached exponential phase, were 0.649 $g \cdot L^{-1}h^{-1}$ and 0.102 $g \cdot L^{-1}h^{-1}$, respectively, increasing these values to 1.46 $g \cdot L^{-1}h^{-1}$ and 0.136 $g \cdot L^{-1}h^{-1}$, respectively after the 2nd pulse. These values are higher than those obtained in the former assays, probably due to pH control.

At 46.7 h, the 3rd pulse was added, and ammonium limitation was imposed, changing the NH_4OH (25 %) to KOH (5 M) to control pH. At this point (46.7 h), for 8.8 h, until the next pulse, biomass grew very slowly at 0.015 ± 0.005 h^{-1}, probably because of the low DOC and the presence of organic acids which could inhibited the culture. However, glucose continued to be consumed at a high rate of 1.58 $g \cdot L^{-1}h^{-1}$ while xylose dropped uptake rate to 0.020 $g \cdot L^{-1}h^{-1}$. Once the biomass practically did not alter the concentration value as well as PHA concentration, these sugars may had been channelled to acids production, namely lactic acid and propionic acid, which increased their concentrations from 2.61 to 3.32 $g \cdot L^{-1}$ and from 0.19 to 2.40 $g \cdot L^{-1}$ respectively, confirming the fermentative metabolism.

Since biomass did not grow significantly during that period, a very small amount of nitrogen was utilized, decreasing from 1.08 just to 1.00 $g \cdot L^{-1}$. At 55.5 h, and considering the glucose uptake rate, a 4th pulse of hydrolysate was added to the reactor. Biomass continued to grow after this pulse with a μ of 0.041 ± 0.001 h^{-1}. Glucose and xylose uptake rates were 1.09 $g \cdot L^{-1}h^{-1}$ and 0.059 $g \cdot L^{-1}h^{-1}$ respectively. In this interval, biomass reached its maximum concentration obtained in this assay, 7.63 $g \cdot L^{-1}$.

Regarding organic acids, lactic acid concentration increased to 3.34 $g \cdot L^{-1}$ at 58.8 h and then the concentration started to decrease, remaining at 0.96 $g \cdot L^{-1}$ at the end of the fermentation. Acetic acid had a similar behaviour, increasing to 1.63 $g \cdot L^{-1}$ at 46.6 h and ended with 1.11 $g \cdot L^{-1}$. Propionic acid, on the other hand, was produced until the end of the fermentation, achieving its maximum concentration of 6.77 $g \cdot L^{-1}$ at 78 h. This value is considered inhibitory for biomass growth in the literature (Kim et al. 1992; Chakraborty et al. 2009; Chung et al. 1997) and could be an explanation for the results obtained in the last 4 hours with a deceleration of biomass growth.

PHA was analysed and only a maximum accumulation of 6.5 % of P(3HB-co-3HV) was achieved at 34.7 h, just before DOC decreased to values under 20 %. After that, PHA storage remained at values under 2 %, which is explained with the incomplete ammonium limitation, ending with 0.56 $g \cdot L^{-1}$ of nitrogen at 78 h. However, a maximum productivity of 0.0126 $g_{PHA} \cdot L^{-1}h^{-1}$ was achieved in the first 34.7 h. This productivity value was very low compared

with other results of *C. necator* from literature, such as 0.0675 $g_{PHA} \cdot L^{-1}h^{-1}$ obtained with corn syrup (Daneshi et al. 2010) or 0.13 $g_{PHA} \cdot L^{-1}h^{-1}$ with a constant feeding strategy for fructose and nitrogen (Patwardhan & Srivastava 2004) or 0.84 $g_{PHA} \cdot L^{-1}h^{-1}$ obtained with waste glycerol (Cavalheiro et al. 2009). Regarding other works with forest biomass hydrolysates using *Burkholderia cepacia*, a productivity of 0.02-0.03 $g_{PHA} \cdot L^{-1}h^{-1}$ was achieved (Keenan et al. 2006; Keenan et al. 2004), which is about just 2-2.5 times the productivity achieved in this assay. Despite the low results, *C. necator* appears to be a good microorganism to continue the studies with unbleached pulp hydrolysate.

Table 17 – Growth rates, sugars uptakes and biomass and acids yields from substrate from the second reactor assay.

	1st pulse	2nd pulse	3rd pulse	4th pulse
μ (h^{-1})	0.209 ± 0.006	0.203 ± 0.003	0.015 ± 0.005	0.041 ± 0.001
r_{Glc} ($g \cdot L^{-1}h^{-1}$)	0.649	1.46	1.58	1.09
r_{Xyl} ($g \cdot L^{-1}h^{-1}$)	0.102	0.136	0.020	0.059
$Y_{X/S}$ ($Cmol \cdot Cmol^{-1}$)	0.385	0.206	0.0689	0.247
$Y_{HAc/S}$ ($Cmol \cdot Cmol^{-1}$)	0.186	0.0443	0.0034	0.0248*
$Y_{HLa/S}$ ($Cmol \cdot Cmol^{-1}$)	0.0408	0.158	0.0638	0.533*
$Y_{HPr/S}$ ($Cmol \cdot Cmol^{-1}$)	0.0579	0.306*2	0.244	0.259

Note: (*) - corresponds to the yield of acetic and lactic acids respectively, until the culture started consuming these organic acids, at 58.75 h. (*2) - corresponds to the yield of propionic acid until the culture started consuming it, at 37.92 h; after the pulse, the culture produced propionic acid again. HAc – Acetic acid; HLa – Lactic acid; HPr – Propionic acid.

A sample taken at 78 h was fixated to be observed in contrast phase microscopy and stained with Nile blue to be observed under fluorescence.

Some images obtained with phase contrast microscopy are shown in Figure 22.

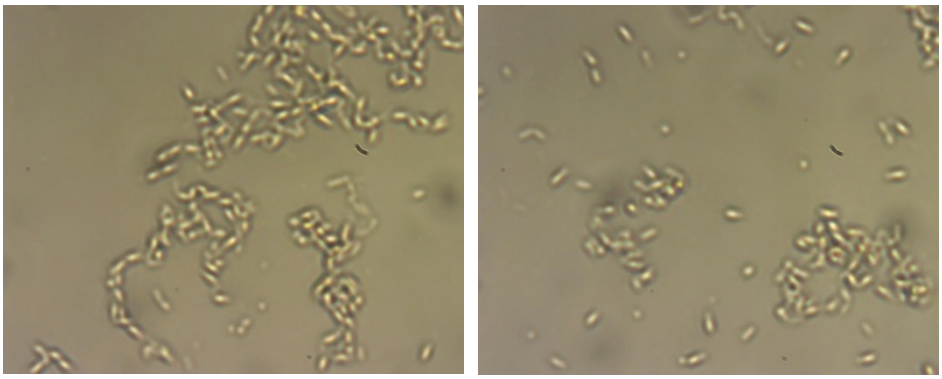


Figure 22 – Contrast phase images of the sample taken at 78 h of the reactor assay. Magnification of 1000x.

In both images a tenuous bright greenish colour in the cells could be observed, typical of refractive material like PHA.

The images taken using fluorescence microscopy are present in Figure 23.

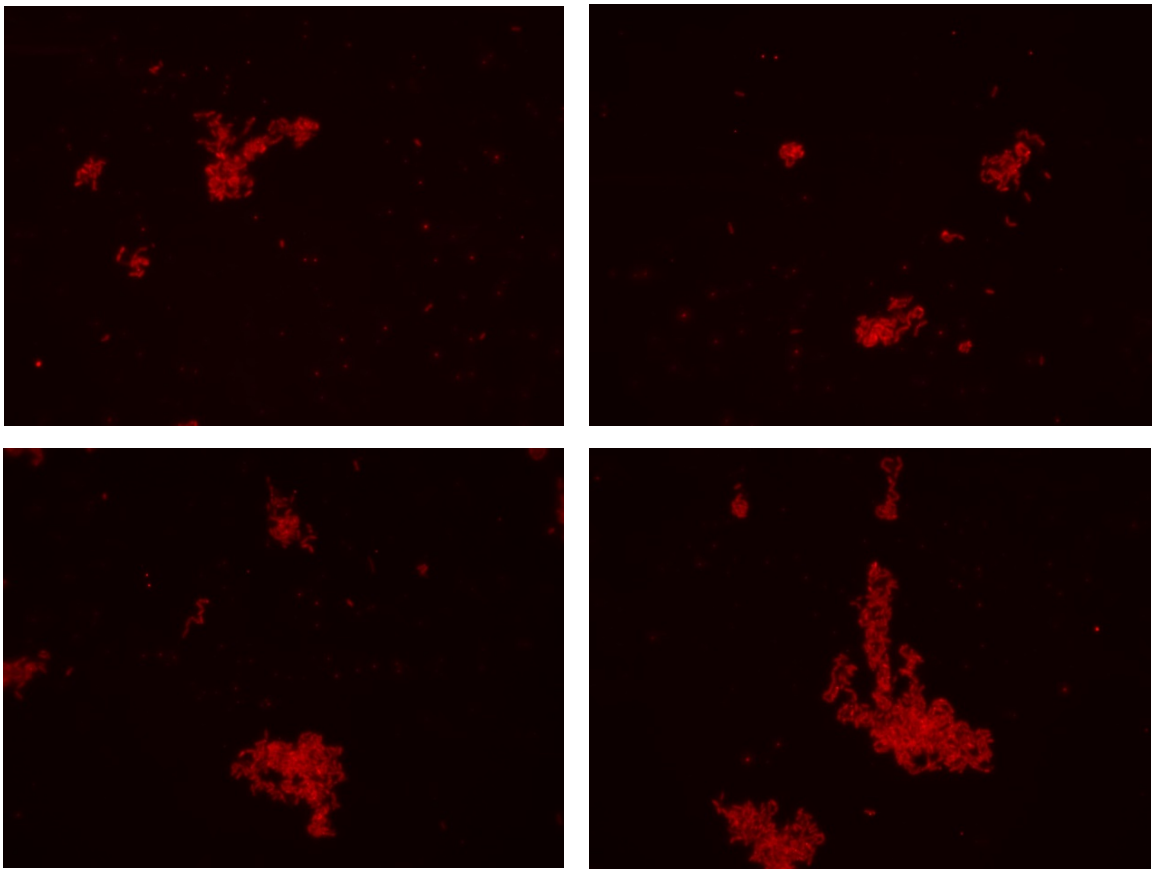


Figure 23 –Nile blue fluorescence images from the sample taken at 78 h of the reactor assay, taken under epifluorescence. Magnification of 1000x.

Cells showed weak fluorescence, confirming the low values of PHA content obtained.

Another sample was harvested for SEM observation. SEM images are presented in Figure 24.

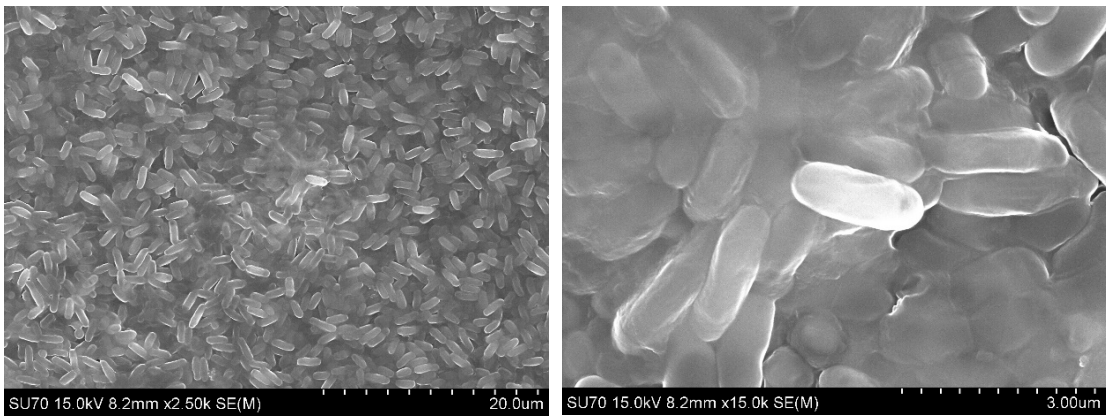


Figure 24 – SEM images of sample taken at 78 h. 2500x and 15000x of magnification, respectively. The bar scale is related to each image (20.0 and 3.00 µ, respectively)

A vast amount of very identical cells can be observed, practically all with the same size (about 2.0 µm × 0.7 µm), very similar to other images of *C. necator* from literature (Cavalheiro et al. 2012; Tian et al. 2005). In SEM images it is not possible to observe any granules of PHA, as this technique can only observe the surface of the sample and not the interior. Some TEM images of a slice of cells would be interesting to be observed to see the granules of PHA.

5. Thermal analysis

A film (Figure 25) of 45.9 mg of P(3HB-co-3HV) was obtained from the biomass of the second reactor assay, after an extraction with chloroform. The polymer was used to be analysed by Differential Scanning Calorimetry (DSC).

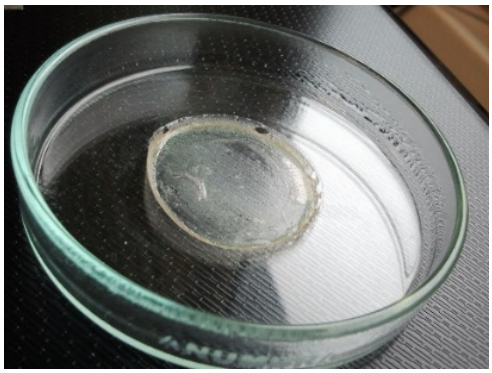


Figure 25 – PHA obtained from the end of fermentation.

The polymer extracted from the reactor was analysed by DSC (Figure 28 from section D from appendix) and the results are present in Table 18.

Table 18 – DSC PHA results.

Glass transition temperature	-3.60 °C
Melting temperature	123.41 °C
Crystallization temperature	60.71 °C
Crystallinity	27.17 %

Accordingly with GC-FID analysis, the PHA obtained was P(3HB-co-3HV) with 21.51 % mol 3HV. The results, obtained from a sample of PHA with 4.430 mg, are in accordance

with literature (Pereira et al. 2008; Luzier 1992). While Luzier (1992) describes a melting point of 130 °C and a crystallinity of 35 % for a P(3HB-co-3HV) with 20 % mol 3HV, Pereira et al (2008) refers to a melting temperature of 123.88 °C for a P(3HB-co-3HV) with 22 % mol 3HV. The melting temperature obtained fits in the interval mentioned, as well as the HV fraction. Moreover, Luzier (1992), Pereira et al.(2008) and Grousseau et al (2014) refer that the higher is the 3HV fraction in the polymer, the lower is the melting temperature and crystallinity, improving its flexibility and resistance without affecting its degradation temperature and, consequently resulting in good processability, giving this product a higher commercial price. About crystallinity, the value obtained is quite low, however, it is supposed to be lowered by the higher 3HV fraction, and so, the value 27.17 % is acceptable compared with 35 % obtained by Luzier (1992) with a lower HV fraction.

CHAPTER IV – CONCLUSION AND FURTHER WORK

1. Conclusions

In this work, the production of PHA by *C. necator* using unbleached pulp hydrolysate from paper industry was tested for the first time. Two strains of *C. necator*, namely NRRL B-4383 and DSM 545, were used and both demonstrated to be capable of growing and accumulating PHA using hydrolysate as substrate.

Flask assays with different media compositions, media 1 and 2, with a glucose concentration of 20 $g \cdot L^{-1}$ using hydrolysate, were performed with each strain. NRRL B-4383 achieved a maximum biomass of 4.82 ± 0.31 $g \cdot L^{-1}$ with a μ_{max} of 0.305 ± 0.001 h^{-1} in medium 1. A maximum P(3HB) accumulation of 26.51 % of cell weight was obtained during this assay. On the other hand, DSM 545, in medium 2, achieved a maximum biomass concentration of 4.07 ± 0.24 $g \cdot L^{-1}$ reaching a μ_{max} of 0.498 ± 0.005 h^{-1}. A maximum of 4.26 % of P(3HB-co-3HV) was accumulated in this assay. Glucose and xylose were consumed by NRRL B-4383 at an uptake rate of 0.090 ± 0.010 $g \cdot L^{-1}h^{-1}$ and 0.038 ± 0.003 $g \cdot L^{-1}h^{-1}$, respectively, achieving a biomass and PHA yields on substrate of 0.725 $Cmol \cdot Cmol^{-1}$ and 0.196 $Cmol \cdot Cmol^{-1}$, respectively. DSM consumed glucose and xylose at a rate of 0.338 ± 0.045 $g \cdot L^{-1}h^{-1}$ and 0.132 ± 0.003 $g \cdot L^{-1}h^{-1}$. Also, Biomass and PHA yields on substrate of 0.242 $Cmol \cdot Cmol^{-1}$ and 0.0075 $Cmol \cdot Cmol^{-1}$ were achieved by DSM 545, respectively.

DSM 545 was the strain chosen to proceed with further tests, despite the low accumulation of polymer in these flask assays, which had not any nutrient limitation, due to the fact that DSM 545 demonstrated a faster growth, the capacity to consume xylose, adding to the production of P(3HB-co-3HV) instead of P(3HB), produced by NRRL B-4383.

Some assays with different glucose concentration, 20 $g \cdot L^{-1}$ and 40 $g \cdot L^{-1}$, were performed in medium 2 using hydrolysate to study the effect of sugar concentration in DSM 545 growth. The culture achieved more biomass in the assay with 20 $g \cdot L^{-1}$, 3.26 ± 0.70 $g \cdot L^{-1}$, compared with the assay with 40 $g \cdot L^{-1}$, 1.99 ± 0.19 $g \cdot L^{-1}$. These tests demonstrated that although it achieved lower biomass in the end of the fermentation of the assay with 40 $g \cdot L^{-1}$, this concentration of sugars was not inhibitory for this strain. Also, DSM 545 consumed all the sugars in the assay with 20 $g \cdot L^{-1}$ and organic acids were produced in both assays.

Fed-batch assays were performed in flask and in reactor so that the culture did not stop its growth due to sugars depletion. After some tests, a pH-stat fed-batch assay in reactor was performed and a maximum biomass concentration of 7.63 $g \cdot L^{-1}$ was obtained, reaching a μ_{max} of 0.209 h^{-1}. Glucose and xylose were consumed at a maximum uptake rate of 1.58 $g \cdot L^{-}$

$^{1}h^{-1}$ and 0.136 $g{\cdot}L^{-1}h^{-1}$, respectively. Regarding PHA, due to the limitation of the stirring speed of the reactor, which maintained DOC values over 20 % for only about 12 hours, and to the incomplete limitation of ammonium, no high values of PHA were achieved. A maximum P(3HB-co-3HV) of 6.5 % was obtained just before the DOC decreased to values under 20 %, which represented a maximum productivity of 0.0126 $g_{PHA}{\cdot}L^{-1}h^{-1}$ at 34.7 h. Other metabolites were produced meanwhile, such as lactic acid, acetic acid and propionic acid, with their maximum yields from substrate of 0.533, 0.186 and 0.306 $Cmol{\cdot}Cmol^{-1}$ respectively. P(3HB-co-3HV) with 21.51 % mol of HV was obtained in the end of the fermentation and its analysis by DSC showed good thermal properties, which are in accordance with literature and have some interesting applications due to its properties.

Summing up, hydrolysate from paper industries showed to be a renewable resource which could be applied in biorefineries regarding the high sugar concentrations. *C. necator* demonstrated that it could use this hydrolysate to grow as well as to produce PHA, despite the low accumulation values obtained due to factors referred earlier. Moreover, it accumulated P(3HB-co-3HV) with a high content of HV, which has some enhanced properties compared with P(3HB), being applied in more valuable areas.

2. Further work

Regarding the results obtained during this project, some further work can be proposed to improve the obtained results:

- An assay in a reactor which could reach a higher stirring speed, of at least 1000 rpm, with a better aeration would be interesting to study, where an ammonium limitation could be imposed and a higher PHA accumulation could be obtained to realise if this process is cost-effective.
- The promising results obtained with NRRL B-4383 in flask assays would be also interesting to study in higher working volumes.
- As DSM 545 produced lactic, acetic and propionic acids, a two-stage strategy could be studied in order to promote the biomass growth and acids production (being careful with the inhibitory effect of those) in a first stage and then proceed with an ammonium limitation to promote the PHA accumulation, which would have high content of HV, due to the acids produced earlier.

CHAPTER V – REFERENCES

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CHAPTER VI – APPENDIX

A. Biomass quantification

A calibration curve of biomass vs Abs is shown in Figure 26.

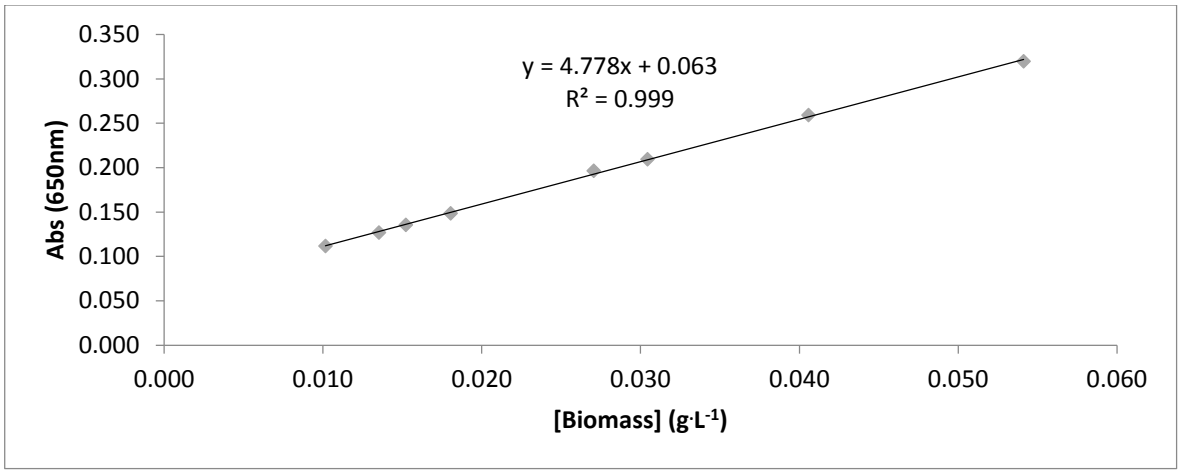

Figure 26 – Biomass vs Abs calibration curve.

From the linearization of the curve it is possible to know the slope (m) and interception (b) values on the equation y=mx+b. Replacing this values, it is possible to calculate the biomass accordingly with the Equation 10:

Equation 10

$$[X] = \frac{Abs - 0.063}{4.778}\ g{\cdot}L^{-1}$$

B. Ammonium quantification

A calibration curve of symmetrical base-10 logarithm of NH_4^+ concentrations vs potential value is shown in Figure 27.

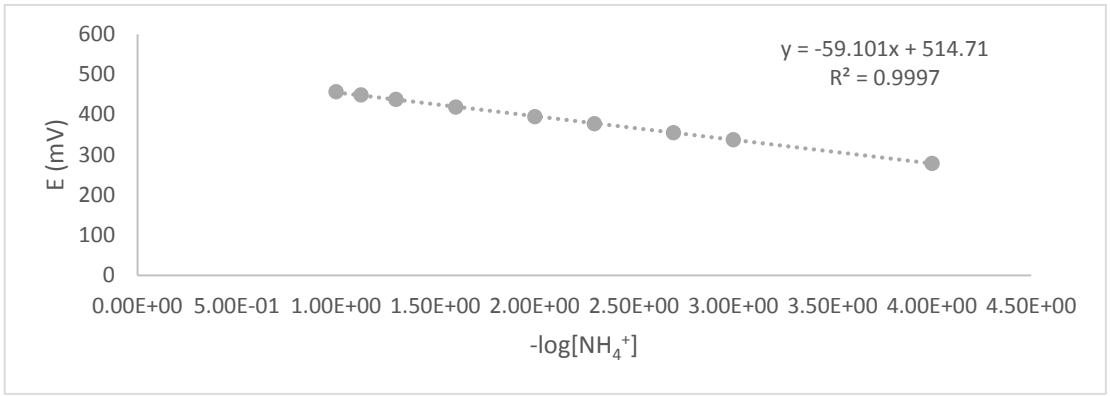

Figure 27 – Ammonium calibration curve.

C. Enzymatic hydrolysis

Data from RAIZ indicated that the unbleached pulp used in this project had a humidity content of 72 %. For the enzymatic hydrolysis it was needed 300.0 g of dry pulp accordingly with the process usually done there, so, the mass of unbleached pulp should be

$$m_{unbleached\ pulp} = \frac{300.0}{1 - 0.72} = 1071.4\ g$$

which represents a volume of

$$V_{unbleached\ pulp} = 1071.4 \times 0.72 = 771.4\ mL$$

To reach 3 L of volume, it was missing

$$3000 - 771.4 = 2228.6\ mL$$

From those, 200 mL were reserved for the enzymatic solution, so,

$$2228.6 - 200 = 2028.6\ mL$$

of ultrapure water were added to the previously weighted unbleached pulp.

About the enzymatic solution, data from RAIZ reports that the enzyme used had an enzymatic activity of 100.50 FPU/mL_{enzyme}, and since it was wanted an enzyme dosage of 20 FPUs/ $g_{carbohydrate}$ and taking into account that 98% of the dry pulp consists of organic matter (data from RAIZ),

$$300.0 \times 0.98 = 294.0\ g_{\text{carbohydrate}}$$

$$294.0 \times 20 = 5880\ FPUs$$

$$\frac{5880}{100.50} = 58.51\ mL_{enzyme}$$

The volume of 200 mL reserved for the enzymatic solution was made up with ultrapure water.

D. DSC graph

DSC polymer analyses was performed and the correspondent graph is shown in Figure 28.

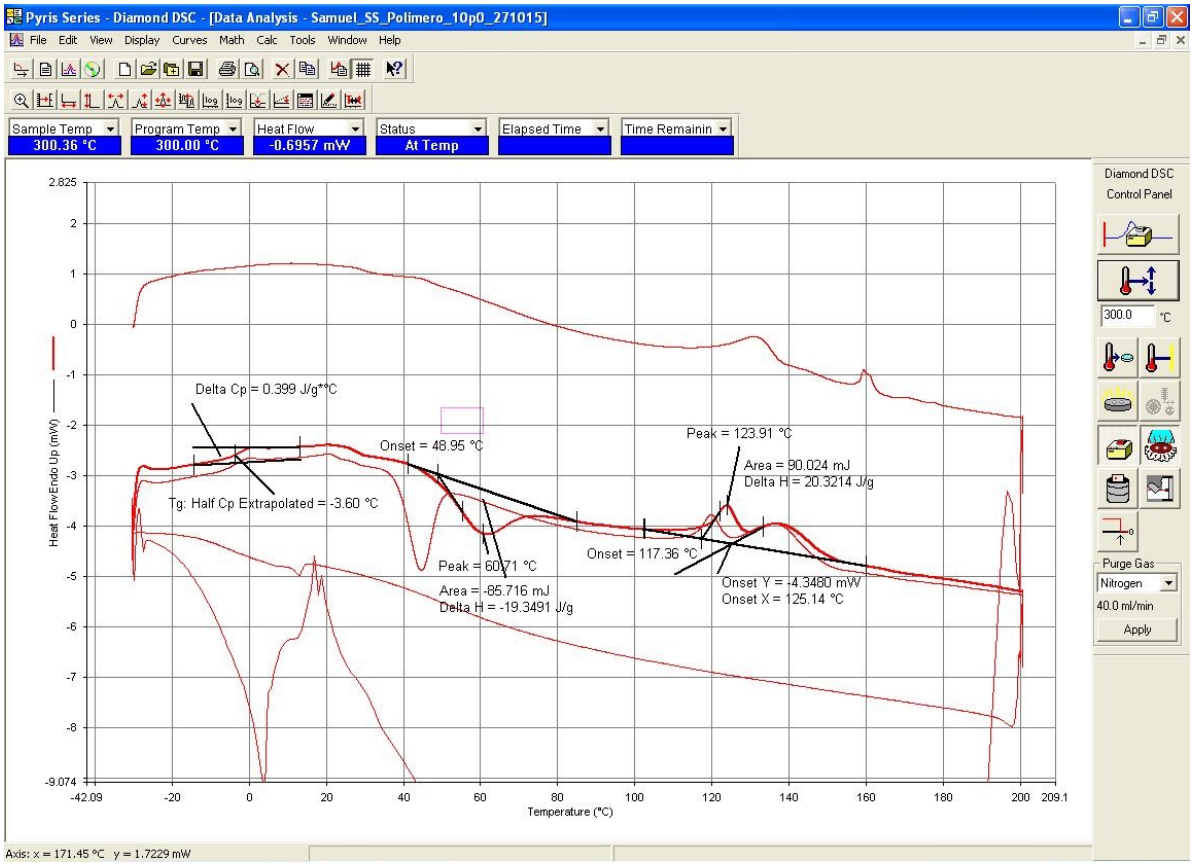

Figure 28 – Analysed DSC thermogram.