Universidade de Aveiro Departamento de Química

Ano 2019-2020

MARTA

BATISTA MORGADO

PRODUÇÃO DE BIOETANOL DE 2ª GERAÇÃO NUMA LÓGICA DE ECONOMIA CIRCULAR

2nd GENERATION BIOETHANOL PRODUCTION IN A CIRCULAR ECONOMY LOGIC

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, Ramo Industrial e Ambiental, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e da Doutora Luísa Alexandra Seuanes Serafim Martins Leal, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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o júri

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palavras-chave

bioetanol, pastas kraft, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, hidrólise enzimática, economia circular

resumo

A utilização excessiva de recursos fósseis leva não só à sua depleção, mas também a graves problemas ambientais. A produção de biocombustíveis de segunda geração, através de matérias primas não destinadas à alimentação humana, pode mitigar estes problemas, ao mesmo tempo que contribui para a redução da deposição de resíduos. Por conter grandes quantidades de celulose e hemiceluloses e estar disponível em larga escala, a biomassa lenhocelulósica é uma fonte promissora para a produção de bioetanol de segunda geração. Para que materiais lenhocelulósicos possam ser convertidos em etanol através de microrganismos fermentativos, têm de sofrer um pré-tratamento complexo e caro, para que a lenhina seja libertada e a celulose e a hemicelulose se tornem mais facilmente acessíveis à ação de enzimas celulolíticas. Sendo Portugal um país que possui uma vasta área florestal, a Indústria Papeleira assume um papel de destaque. Na produção de pasta de papel é empregue o processo kraft, que remove a lenhina da madeira, e afeta as hemiceluloses e a celulose. Desta forma, o processo kraft poderá atuar como pré-tratamento de biomassa lenhocelulósica, nomeadamente de resíduos da própria indústria papeleira, minimizando os custos do processo e contribuindo para a prática de uma lógica de Economia Circular que integra a Indústria Papeleira no conceito de biorefinaria. O objetivo deste trabalho foi estudar a viabilidade da produção de bioetanol a partir de pastas kraft de cascas de *Eucalyptus globulus* pelas leveduras *Saccharomyces cerevisiae* e *Scheffersomyces stiptis*. Após hidrólise enzimática das pastas fornecidas obteve-se um hidrolisado com concentrações de 60,5 g/L de glucose e 15,8 g/L de xilose, com um rendimento de 77 %. Fizeram-se ensaios em Erlenmeyer e obteve-se uma concentração de etanol de 18,13 g/L com a *S. cerevisiae* e de 17,49 g/L com a *S. stipitis*, com eficiências de conversão de 78,0 % e 65,0 %, respetivamente. Testou-se uma co-cultura das duas leveduras mas não se verificou um aumento significativo na produção de etanol. O aumento de escala da fermentação com *S. cerevisiae* num biorreactor com controlo de pH e um volume útil de 3 L resultou num aumento da concentração máxima de etanol para 20,37 g/L e consequentemente a eficiência aumentou para 85 %. Os resultados obtidos demonstram que a produção de bioetanol de segunda geração, através de hidrolisados de pastas de cascas de *E. globulus,* por mono-culturas de *S. cerevisiae* e *S. stipitis* utilizando o processo kraft como pré-tratamento é de facto um processo viável, que pode contribuir significativamente para o estabelecimento de biorefinarias integradas em indústrias papeleiras.

keywords

bioethanol, kraft pulps, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, enzymatic hydrolysis, circular economy

abstract

The excessive use of fossil resources leads not only to their depletion but also to serious environmental problems. The production of second generation biofuels from raw materials not intended for human consumption, can mitigate these problems while contributing to the reduction of waste disposal. Because of the large amounts of cellulose and hemicelluloses and its wide availability, lignocellulosic biomass (LCB) is a promising source for the production of second generation bioethanol. In order for lignocellulosic materials to be converted to ethanol through fermentative microorganisms, they must undergo complex and expensive pretreatment, to release lignin and to make cellulose and hemicelluloses more easily accessible to the subsequent action of cellulolytic enzymes responsible for releasing glucose monomers. Being Portugal a country with a vast forest area, Paper Industry assumes a prominent role. In the production of paper pulp is employed the kraft process, which removes lignin from wood, and affects hemicelluloses and cellulose. Thus, the kraft process can act as a pretreatment of lignocellulosic biomass, namely wastes from the paper industry itself, minimizing the process costs and contributing to the practice of a Circular Economy logic, which integrates the Pulp and Paper Industry into the biorefinery concept.

The aim of this work was accessing the viability of bioethanol production from kraft pulp of *Eucalyptus globulus* barks by the yeasts *Saccharomyces cerevisiae* and *Scheffersomyces stiptis*. After enzymatic hydrolysis of the provided pulps, a hydrolysate with concentrations of 60.5 g/L glucose and 15.8 g/L xylose was obtained, yielding 77 %. Erlenmeyer tests were carried out and ethanol concentrations of 18.13 g/L with *S. cerevisiae* and 17.49 g/L with *S. stipitis* were obtained, with conversion efficiencies of 78.0 % and 65.0 %, respectively. A co-culture of the two yeasts was tested but there was no significant increase in ethanol production. Scaling up fermentation with *S. cerevisiae* to a pH controlled bioreactor with a working volume of 3 L resulted in an increase in the maximum ethanol concentration to 20.37 g/L. Consequently, efficiency increased to 85 %.

The results obtained in this work demonstrate that the production of second generation bioethanol, through hydrolysates of *E. globulus* barks, by *S. cerevisiae* and *S. stipitis* mono-cultures using the kraft process as a pretreatment is indeed a viable process, which can contribute significantly to the establishment of integrated biorefineries in pulp and paper mills.

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List of abbreviations

μ - Specific growth rate

ADH – Alcohol dehyidrogenase

AGU – Anhydroglucose units

BGL – β-gluosidase

CBH – Cellobiohydrolase

CNG – Compressed natural gas

COD – Chemical oxygen demand

CQO – Chemical oxygen demand

EG – Endoglucanase

EtOH – Ethanol

EU – European Union

FFV – Flexible fuel vehicles

FPU – Filter paper unit

GFP – Green fluorescent protein

GHG – Greenhouse gas

GVA – Gross value added

HPLC – High performance liquid chromatography

LEX/SEC chromatography – Ligand-exchange/Size-exclusion chromatography

LPG - Liquefied petroleum gas

OD_{620} – Optical density at 620 nm

P&P – Pulp and paper

$Prod_{vol}$ - Volumetric ethanol productivity

Q-SSF – Quasi- simultaneous saccharification and fermentation

$r_{Glucose}$ – Volumetric glucose production rate

r_{Xylose} - Volumetric xylose production rate

R&D - Research and development

$r_{Ethanol}$ - Volumetric ethanol production rate

SHF – Simultaneous hydrolysis and fermentation

SM – Supplementary medium

SSF – Simultaneous saccharification and fermentation

XDH – Xylitol dehydrogenase

XK – Xylulokinase

XR – Xylose reductase

$Y_{Biomass/substrate}$ – Biomass yield

$Y_{Ethanol/substrate}$ – Ethanol yield

$Y_{Glucose}$ – Hydrolysis yield on glucose

$Y_{Hydrolysis}$ – Hydrolysis yield

YM medium – Yeast mould medium

Y_{Xylose} – Hydrolysis yield on xylose

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I. Introduction

1.1. Scope

Modern lifestyle requires an excessive consumption of fossil resources, which account for most of the world primary energy consumption (Mohr et al., 2015): fossil fuels such as coal, oil and natural gas provide about 95 % of the world's total energy and the demand has been increasing over the past years (Hu et al., 2018). Due to the fact that they are inexpensive to use and bring high profits for big companies, fossil fuels are overused. However, once they take millions of years to evolve, if their use is not limited, in some decades there will be not enough resources left to be exploited (Guo, 2016).

The dependence on fossil fuels has also led to many environmental issues, such as severe pollution and greenhouse gas (GHG) emissions. Thus, the development of renewable eco-friendly alternative energy sources is extremely necessary, and as technology and science advance, several new resources appear available.

Biofuels have proven to be an attractive alternative and therefore have undergone very rapid development and expansion compared to other options. In contrast to fossil fuels, biofuels are produced from biomass, which as long as the crop growth cycle is respected, is a renewable resource (Carneiro et al., 2017). A great focus is being put on the development of new technologies that enable the production of biofuels by non cost-intensive, profitable ways (Lucia, 2008), from sources not included in the food chain (1st generation biofuels). Bioethanol and biodiesel, two liquid fuels that can be used without major modifications in the current engines, are the main examples of several biofuels presently under study (Henrique et al., 2014).

Lignocellulosic biomass consists in a potential source for the production of second generation bioethanol, mainly due to the presence of high levels of cellulose and hemicellulose, its large availability and relatively low cost. Furthermore, it is a renewable resource that does not compete with food production or animal feed, thus avoiding the "food versus fuel" problem (Sindhu et al. 2016). Thence, the biorefinery concept, especially the lignocellulosic biorefinery, which practices a non-food feedstock based process, is now seen as key factor for the achievement of a sustainable and less fossil fuel dependent society, contributing to minimize the climate change problem as well (Mongkhonsiri et al., 2018).

Nowadays, many governments all over the world uphold the use of biofuels, and, obviously, each country takes benefit of its available biomass. For instance, in the United States, South America and Asia the most promising and abundant cellulosic residues are corn stover, sugarcane bagasse, and wheat and rice straw, respectively (Limayem & Ricke, 2012)

In Portugal, the primary sources of biomass are forest resources, which are still relatively abundant and if correctly explored can be renewable and sustainable, contributing to the development of rural areas as well as jobs creation (Nunes & Matias, 2017). Therefore, one of the solutions to reduce the consumption of fossil resources in Portugal consists in using forest biomass for the production of biofuels, being the most sustainable way the use of wastes resulting from wood and pulp and paper industrial processes.

1.2. The biorefinery concept and the circular economy

The intensive use and consumption of petroleum derivatives combined with the decrease of petroleum resources has been causing environmental and political concerns. In order to not only reduce the dependence on oil, but also to mitigate the climate changes caused by transportation and chemical sectors, alternative production chains are necessary. Hence, many governments all around the globe started to finance R&D activities aiming their development and implementation (Cherubini, 2010).

Biorefinery based techniques appear to be a promising alternative way to the use of fossil fuels. The biorefinery concept is a green approach of renewable raw biomass conversion processes to valuable bio-products that can progressively replace fossil oil refineries (Cherubini 2010; Figoli et al., 2016).

In this methodology, feedstock such as wood, grasses or corn are separated into their basic constituents (e.g., carbohydrates, proteins, triglycerides) which in turn can be converted into added value products, biofuels and chemicals, as seen in **Figure 1**. These products can be divided into two large groups: energy products – the ones that are used due to their energy content, providing heat, electricity or transportation service – and material products, which are valuable because of their chemistry and physical proprieties. The most important energy products, which can be produced in biorefineries, are biofuels such as bioethanol, biodiesel, syngas, hydrogen or biogas, whilst chemicals, organic acids, polymers, resins and biomaterials are the most relevant chemical and material goods (Cherubini, 2010). Compared with traditional petroleum derived products, bioproducts assure superior properties regarding renewability, biodegradability and nontoxicity, reducing the economic impact and promoting health benefits (Parawira, 2010).

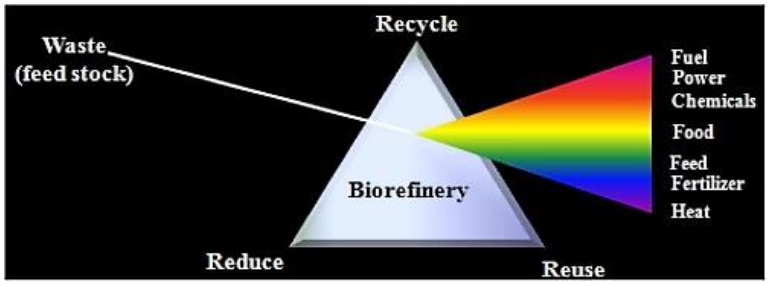


Figure 1: Schematic representation of the biorefinery concept (Mohan et al. 2016).

In addition to allowing the use of renewable natural resources to generate added value products, biorefineries enable the adoption of a circular economy system: contrary to the linear economy adopted in the past, the concept of circular economy fits the future management of global resources, since it encourages the prevention of virgin resources, the manufacturing of reusable products and lower generation of residues. Ultimately, circular economy exploits the potential of by-products or even used resources that would otherwise become wastes and sets them back into the economy (Liguori & Faraco, 2016).

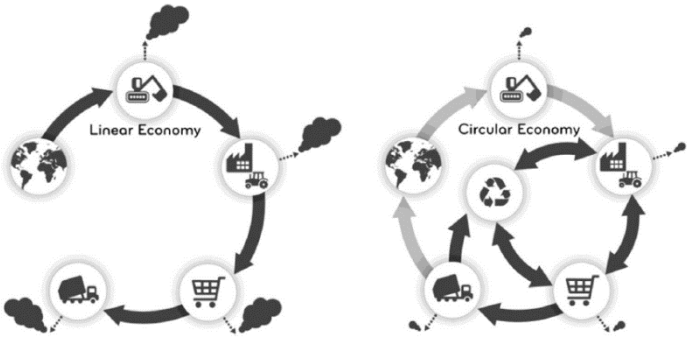


Figure 2: Schematic representation of both linear and circular economy models (Sauvé et al., 2016).

Figure 2 highlights the differences between linear and circular economy: whereas a linear economy model follows a straightforward process of extracting, producing, consuming and discarding, the circular economy model includes reusing, reducing and recycling steps, taking into account the environmental impact associated with resources consumption and processing (Sauvé et al., 2016). Apart from economic benefits, the development of a sustainable waste management results in a reduced amount of waste that are intended for landfills, or, in the worst cases, for uncontrolled disposal, improving the human and environmental health.

It is now widely recognized that biomass such as plant based raw materials have the potential to replace a large fraction of fossil resources as feedstocks for industrial processes in both energy and non-energy sectors (Cherubini, 2010). Therefore, the implementation of a biorefinery-based technology is a major step in the achievement of a crude oil independent or at least less dependent future with a sustainable economy based on natural eco-friendly feedstocks such as agricultural wastes, household wastes, forest residues and algae (Figoli et al., 2016).

1.2.1. Biorefineries classification

Different classifications of biorefineries have been proposed depending on various considerations, including:

- **Level of development**

A simple overview and classification of biorefineries subdivides them into three types: Phase I, Phase II and Phase III. Phase I biorefineries are integrated facilities with fixed processing capabilities limited to a single feedstock which is converted into a single major product. Phase II biorefineries are more advanced, since they possess the capability to produce various end products from a single feedstock. Phase III biorefineries are the most advanced, because they use a mix of biomass feedstocks to yield a wide variety of products by employing a combination of technologies (Clark et al., 2012).

Thus, an increase of flexibility concerning the number of feedstocks, conversion processes and final products is observed from Phase I to Phase III biorefineries, which arises as a need of the market demand.

- **Platform**

Platforms are key intermediates between feedstocks and final materials (Clark et al., 2012). They can be obtained by several conversion processes applied to various feedstocks, therefore platforms are recognized as the main pillars of the biorefinery classification. Platforms available to an energy-driven biorefinery include a) biogas (a mixture of mainly methane - CH_4 – and carbon dioxide - CO_2); b) syngas (a mix of carbon monoxide - CO – and hydrogen - H_2); c) hydrogen (H_2); d) C6 sugars (e.g., glucose, fructose, galactose: $C_6H_{12}O_6$); e) lignin; f) pyrolysis liquid; g) renewable oils (triglycerides); h) organic juice (liquid phase separated after pressing wet biomass, made of different chemicals) and also electricity and heat, which can be internally used to meet the energy needs of the biorefinery or sold to the grid (Cherubini et al., 2009).

- **Products**

Biorefineries can be grouped into energy-driven and material-driven biorefineries. The main goal of energy-driven biorefineries is the production of fuels, power, and/or heat from biomass. Products are sold as feed or can be upgraded to added-value bio-based products. On the other hand, product-driven biorefineries aim to generate one or more bio-based products from biomass, such as chemicals, biomaterials, lubricants and food and/or feed, processing residues that can be further processed or used to produce energy (Cherubini et al., 2009; Clark et al., 2012).

- **Feedstocks**

Feedstock is defined as the renewable raw material that is converted into value added products in a biorefinery (Cherubini et al., 2009). It can be classified as primary, secondary or tertiary: primary feedstocks relate to primary biomass, directly harvested from forest or agricultural land; secondary feedstocks are process residues, whereas tertiary feedstocks are post consumption wastes or residues (Speight, 2008).

Biomass can also be divided among two subgroups, according to **Table 1**. Unlike residues, dedicated feedstocks are specifically grown to provide raw materials to be used in bioprocesses.

Regarding the type of feedstock, biorefineries can be divided into three large groups (Cherubini et al., 2009):

1. The lignocellulosic feedstock biorefinery: uses nature-dry raw materials, that is, cellulose-containing biomass and wastes;
2. The whole crop biorefinery: uses cereals and cereal derived biomass as raw materials;
3. The green biorefinery: uses nature-wet biomass.

- **Processes**

The classification of biorefinery systems according to different conversion technological processes to obtain the pretended final marketable product identifies four main subgroups (Cherubini et al., 2009).

1. Mechanical/physical processes (e.g., pressing, milling, separation, distillation): do not interfere with the chemical structure of the components, preforming a size reduction or a separation of feedstock components;
2. Biochemical processes (e.g., anaerobic digestion, aerobic and anaerobic fermentation, enzymatic conversion): make use of microorganisms or enzymes, at mild conditions;
3. Chemical processes (e.g., hydrolysis, transesterification, hydrogenation, oxidation, pulping): chemical reactions occur to obtain a chemical modification of the substrate;
4. Thermochemical processes (e.g., pyrolysis, gasification, hydrothermal upgrading, combustion): biomass is altered by undergoing extreme conditions.

Table 1: Classification of biomass into dedicated feedstocks and residues in biorefinery systems (Clark et al., 2012).

Classification	Feedstock	Examples
Dedicated feedstocks	Sugar crops	Sugar beet, sugarcane
	Starch crops	Wheat, corn, sweert sorghum
	Lignocellulosic crops	Wood, short-rotation poplar, switchgras, *Miscanthus*
	Oil-based crops	Rapeseed, soy, palm oil, *Jatropha curcas*
	Grasses	Green plant materials, grass silage, immature cereals, plant shoots
	Marine biomass	Micro- and macroalgae, seaweed
Residues	Oil-based residues	Animal fat from food industries; used cooking oil from restaurants, households, and others
	Lignocellulosic residues	Crop residues, sawmill residues
	Organic residues and others	Organic urban waste, manure, wild fruits and crops

1.2.2. Lignocellulosic biomass

The main components making up lignocellulosic biomass are cellulose (35-50 %), hemicelluloses (20-30 %) and lignin (10-25 %) (Sjostrom, 1981), in addiction to fewer amounts of pectin, minerals and proteins (Wyman & Kumar, 2017). Covalent and hydrogen bonds that tightly link the carbohydrate component (cellulose and hemicellulose) to the lignin assure that the structure is highly robust (Limayem & Ricke, 2012).

Lignocellulosic material constitutes the world's largest bioethanol renewable resource (Limayem & Ricke, 2012), and is a potential source of starting materials for many industrial processes. The major advantages of this type of raw material are related to not interfere with food supplies, and to the lower environmental impact of derived chemicals. Furthermore, it is a dioxide carbon neutral material, since it does not affect the bulk of CO_2 in the atmosphere. The amount of CO_2 released during the combustion of biomass is balanced by the required amount for the growth process (Lucia, 2008).

There are several types of residual lignocellulosic biomass, including agricultural residues, herbaceous crops and woody tree species (Karimi et al., 2013), which differ in terms of composition. Cellulose, hemicelluloses and lignin content of some examples of lignocellulosic biomass is shown in **Table 2**.

Since lignocellulosic biomass is recalcitrant due to strong cellulose, hemicellulose and lignin covalent cross linkages and non-covalent forces, one or more biomass pretreatments need to be carried out in order to convert biomass into sugars for high-value chemicals. Those pretreatment processes alter the micro, macro and chemical structure of lignocellulose: lignin and hemicellulose are broken down, lignin is removed, hemicellulose is degraded, and the crystalline structure of cellulose is changed to improve the availability and release of cellulose. The pretreatments are classified as physical, chemical, physicochemical and biological and a combination of different processes can be used (Hongyan Chen et al., 2017). Examples of pretreatments of each category, as well as their advantages and disadvantages are summarized on **Table 3**.

Table 2: Chemical composition of agricultural residues and wastes (Lee et al., 2014).

Types of biomass	Lignocellulosic substrate	Cellulose (%)	Hemicelluloses (%)	Lignin (%)
Agricultural waste	Corncobs	45	35	15
	Wheat straw	30	50	15
	Barley straw	33-40	20-35	8-17
	Corn stover	39-42	22-28	18-22
	Nut shells	25-30	25-30	30-40
Energy crops	Empty fruit bunch	41	24	21.2
	Switch grass	45	31.4	12
Forestry waste	Hardwood stems	40-55	24-40	18-25
	Softwood stems	45-50	25-30	25-35
	Leaves	15-20	80-85	0
Industrial waste	Waste papers from chemical pulps	60-70	10-20	5-10
	Organic compound from wastewater solid	8-15	0	0

Table 3: Advantages and disadvantages of different pretreatments for LCB (Hongyan Chen et al., 2017).

Pretreatment methods		Advantages	Disadvantages
Physical pretreatment	Mechanical splintered	Reduce particle size and cellulose cristallinity	Cannot remove lignin and hemicelluloses, high energy
	Microwave	Simple operation, energy-efficient, short time	High cost
	Ultrasonic	Improve accessibility and reactivity of cellulose	Negative to enzymatic hydrolysis
	High-energy electron radiation	Reduce cellulose polymerization degree	High cost
	High-temperature pyrolysis	Decompose cellulose rapidly	Energy consumption, low productivity
Chemical pretreatment	Concentrated acid	High sugar conversion	Toxic and corrosive, high cost
	Dilute acid	Fast and do not need recycle acid	High temperature and pressure, formation of inhibitors
	Alkali pretreatment	Room temperature, destroy lignin	Less sugar degradation
	Oxidation pretreatment	Environmental, remove lignin effectively	High cost
	Organosolv pretreatment	Obtain pure lignin, cellulose and hemicelluloses	High cost, certain effects on environment and fermentation
	Ionic liquid pretreatment	Environmental, large temperature range	High cost
Physicochemical pretreatment	Steam explosion	Lignin transformation, hemicelluloses solubilisation	High temperature and pressure
	AFEX method CO_2 explosion	Cost effective, Increased surface area of cellulose, absence of inhibition substances formed	High cost, not efficient for high lignin content material
	Electrical catalysis	Does not produce inhibition compounds, cost-effective, increases surface area	High pressure, do not affect lignin and hemicelluloses, lower efficiency
Biological pretreatment	-	Degrades lignin and hemicellulose, low energy consumption	Low rate of hydrolysis

1.2.2.1. Cellulose

As a main component of the cell walls of higher plants, cellulose is the most abundant and highly important organic polymer and is widely used in industrial applications such as paper, textiles and pharmaceutical compounds (Heinze T, 2012; Klemm et al., 2005). Cellulose can be naturally found in trees, annual plants, animals, fungi, algae and bacteria; however, its main source is plant fibre, where cellulose acts as a structural element (Jedvert & Heinze, 2017).

Regardless of the source, the cellulose molecule has always the same molecular structure: cellulose is a simple linear polymer of β-(1→4)-linked D-glucose (D-glucopyranose) monomer units, the so-called anhydroglucose units in the chair conformation. The β-1,4-glycosidic bonds result in an alternate turning of cellulose chain axis by 180º, which means that cellobiose - a molecule made up of two covalently linked glucose molecules, also known as a β-(1→4)-linked disaccharide of D-glucopyranose – can be considered the basic cellulose forming unit (Jedvert & Heinze, 2017), as **Figure 3** represents. The degree of polymerization (number of glucose molecules in a cellulose chain) depends on the source and extraction method and ranges from 800 to 15000 glucose molecules (Chundawat et al. 2011; Karimi et al. 2012). Cellulose has plenty of hydroxyl groups. Interactions where oxygen atoms from these groups take part, in addition to oxygen-ring bonds, result in complex patterns of hydrogen bonds (Jedvert & Heinze, 2017).

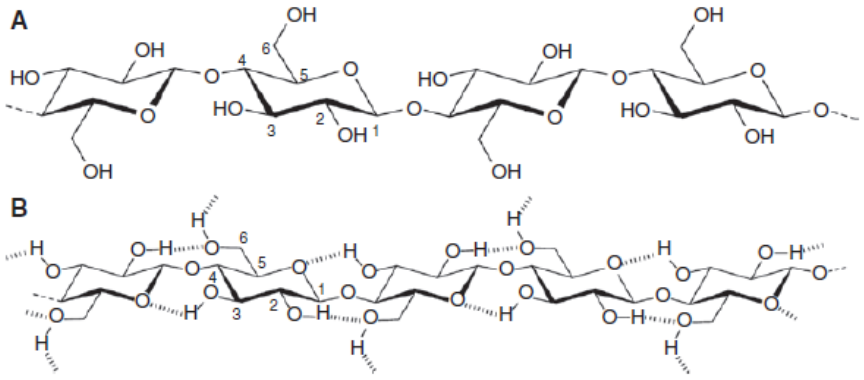


Figure 3: The chemical structure of cellulose. (A) The cellulose polymer, (B) Hydrogen bond pattern for cellulose (Tashiro and Kobayashi 1991).

Cellulose chains are very well organized with different structural levels: cellulose chains are aligned and connected to one another by hydrogen bonds to form microfibrills, which pack together as highly regular microcrystalline regions. These fibrils, in turn, organize themselves into structures of higher order, resulting into the assembly of layers of varying texture and density (Jedvert & Heinze, 2017).The periodic disruption of the long segments of ordered chain conformations by irregular shorter amorphous regions provides plants great strength and flexibility (Wyman & Kumar, 2017) . The fact that cellulose is highly insoluble is also an important feature for its structural function in plant cell walls (Ding, 2012).

1.2.2.2. Hemicelluloses

Hemicelluloses are available in almost all lignocellulosic materials along with cellulose (Karimi et al., 2013), usually making up about 15-30 % of the overall dry weight of lignocellulosic biomass (Wyman & Kumar, 2017). These amorphous, noncrystalline polymers are typically heteropolymers including hexoses (D-galactose, D-glucose and D-mannose) as well as pentoses (L-arabinose and D-xylose) (Limayem & Ricke, 2012; Wyman & Kumar, 2017). They also contain fewer amounts of other molecules such as methoxyl, acetyl, and free carboxyl groups (Wyman & Kumar, 2017) and may have uronic acids (sugar acids) as D-glucuronic, D-galacturonic and methylgalacturonic acids (Saha, 2003). Hemicelluloses, along with lignin, serve as a connection between the cellulose fibers and gives the cellulose-hemicellulose-lignin network more rigidity, forming a very strong composite material (Hendriks & Zeeman, 2009; Wyman & Kumar, 2017), as exemplified in **Figure 4**.

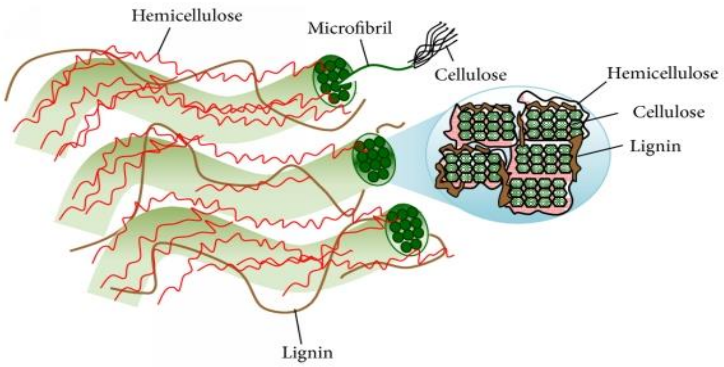


Figure 4: Plant cell wall structure and microfibrill cross-section (Lee et al., 2014).

Unlike cellulose, hemicelluloses composition varies depending on cell tissue and plant species, differing in type of glycosidic linkages, side chain composition and degree of polymerization (D. Fengel & G. Wegener, 1984; Jeffries, 1994). Branch frequencies also vary depending on the nature and the source of feedstocks (Limayem & Ricke, 2012). **Figure 5** shows two chemical structures of hemicelluloses: ii) Glucomannan where the dominant monomeric sugar in softwoods hemicelluloses is mannose, which is highly acetylated and contains galactose side groups and i) Xylan, the prevailing polysaccharide in hardwoods and agriculture residues, which is less acetylated and contains arabinose side groups (Karimi et al., 2013).

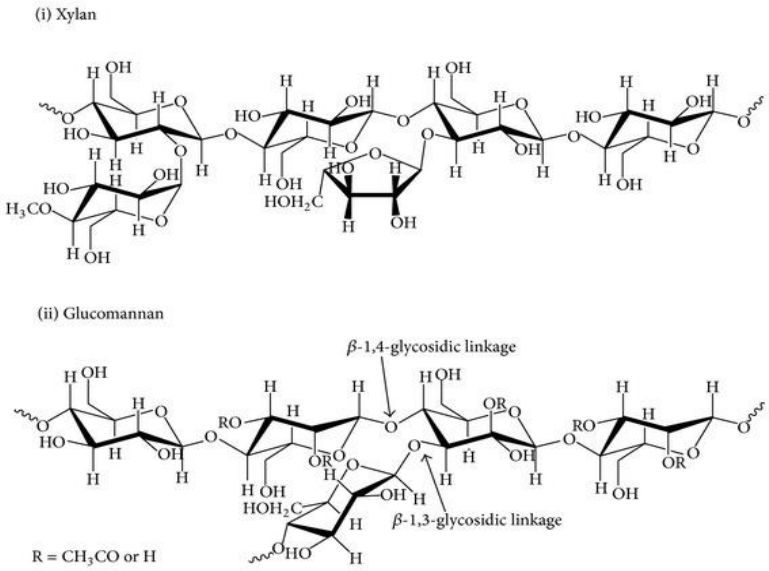


Figure 5: Examples of chemical structure of hemicelluloses: i) Xylan and ii) Glucomanan (Lee et al., 2014).

Because of the diversity of its sugars, hemicellulose requires a wide range of enzymes to be completely hydrolyzed into free monomers (Karimi et al., 2013).

1.2.2.3. Lignin

Lignin is the third major biopolymer making up the structural components in lignocellulosic biomass (Wyman & Kumar, 2017). This complex hydrophobic, cross-linked aromatic polymer (Hongyan Chen et al., 2017) is a complex phenylpropanoic acid polymer covalently linked to hemicellulosic xylans, having the function to bind cellulose

chains together and confering rigidity and compactness to the plant cell wall (Mielenz, 2001; Wyman & Kumar, 2017). Lignin is composed of three phenolic monomers of phenyl propionic alcohol namely coumaryl, coniferyl and sinapyl alcohols, whose chemical structures are represented in **Figure 6**. Three dimensional structure of lignin polymer is shown in **Figure 7**.

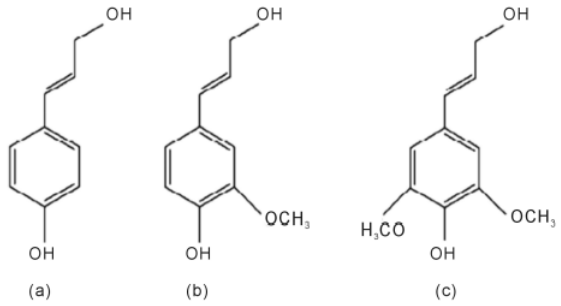


Figure 6: Structure units of lignin: (a) coumaryl alcohol, (b) coniferyl alcohol and (c) sinapyl alcohol (Gadhave et al., 2018).

Softwoods have the highest levels of lignin (25-40 %), followed by hardwoods (18-25 %) and agricultural residues (10-20 %) (Fengel & Wegener, 1984). However, the main distinction between softwoods, hardwoods and other lignocelluloses is originated from the difference in monomeric units and linkage types in lignin (over 10 inter-phenylpropane linkage type have been detected in lignin structure). These dissimilarities may result in significant differences concerning the susceptibility of the pretreatment techniques between hardwoods and softwoods (Karimi et al., 2013).

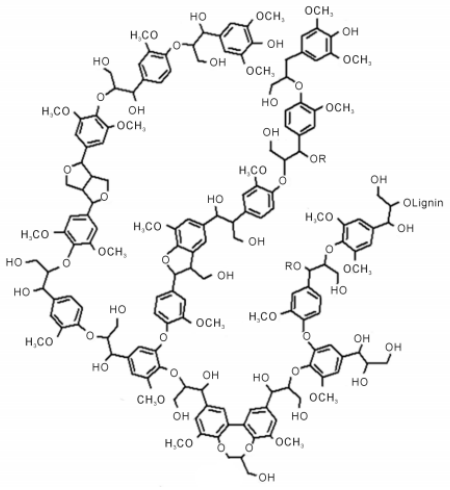


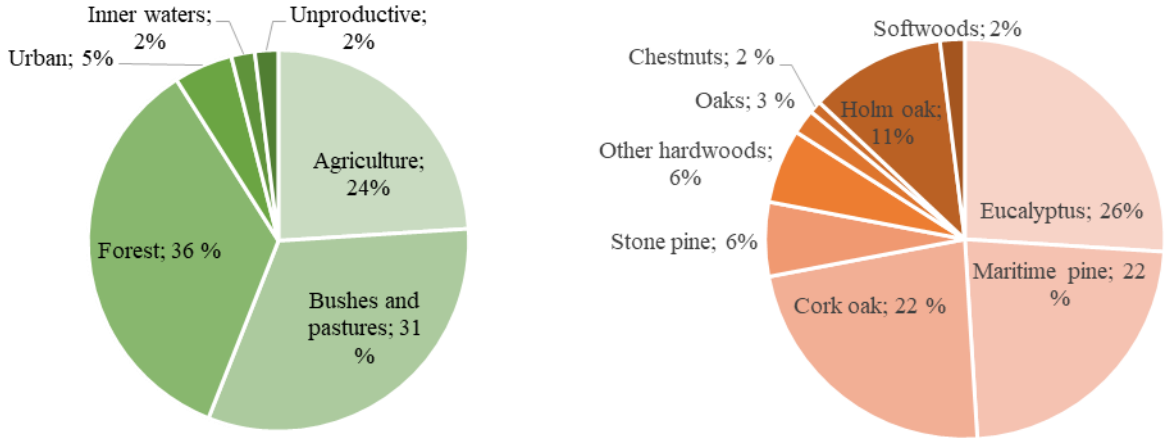
Figure 7: Three dimensional structure of lignin polymer (Retrieved from Gadhave et al., 2018).

1.2.2.4. Extractive materials

Even though lignocellulosic material is mainly composed of cellulose, hemicellulose and lignin, it also contains other so-called extractive materials. This group of compounds do not constitute cell walls or cell layers and can be extracted by polar or nonpolar organic solvents, water and water vapor (S. Wang & Luo, 2017) which can be divided into three large groups: aliphatic compounds (mainly fats, waxes and resins), terpenes and terpenoids (isoprene alcohols and ketones), and phenolic compounds (residues and byproducts of lignin biosynthesis) (Karimi et al., 2013; S. Wang & Luo, 2017). The type, structure and amount of extractives depend on the biomass category as well as the extraction time and methods (Fan et al., 1982; S. Wang & Luo, 2017).

1.3. Paper industry

According to the last *"Inventário Florestal Nacional"* (ICFN, 2019), in 2015, 36.2 % of the Portuguese territory was occupied by forest. Eucalyptus (namely the specie *E. globulus*) is the main forest resource, occupying a total area of 845 thousands ha, followed by cork oak (720 thousands ha) and finally by the maritime pine (713 thousands ha), having the occupied area by eucalyptus increased 59.1 ha between 2005 and 2015. **Figure 8 a** and **b** show, respectively, the distribution of land use and the percentage of occupation of several species in Portuguese mainland, by the year 2015.



(a) (b)

Figure 8: Data concerning the forest area in continental Portugal in 2015. **(a)**: distribution of the land use and **(b)**: distribution of forest area per specie/group of species (Adapted from ICFN, 2019)

In a country with such a high forest area, as Portugal, the paper industry is expected to represent a relevant sector of the industry. CELPA ("*Associação da Indústria Papeleira*") states that the gross value added (GVA) of the eucalypt based industrial paper sector is 1.4 % of the national GVA. Besides, exportations of P&P industry account for 4.9 % of the national exportations (CELPA, 2016).

On a global scale, the pulp and paper (P&P) industry has become one of the leading sectors due to its economic benefits (Kamali & Khodaparast, 2015). P&P industry

uses a panoply of woody and non-woody materials as feedstocks (Kamali et al., 2016) according to the most abundant resource in each country, as shown in **Figure 9**.

Figure 9: Main raw materials for P&P production in some main P&P producers (Kamali et al., 2016).

However, being one of the largest world industries entails some environmental impacts, not only due to the generated wastes and pollution, but also because of the water and energy outlay. Over the years, the utilization of paper industry products is growing, and the paper production is expected to increase hereafter (Toczyłowska-Mamińska, 2017). Furthermore, strict environmental rules are now imposed to pulp and paper industry.

As stated on "The state of the global paper industry" (Haggith et al., 2018), seven goals have been defined in order to transform paper production, trade and use, such as ensuring social responsibility, transparency and integrity, reducing greenhouse gas emissions and ensuring clean production.

In order to achieve these objectives and overcome the environmental issues, P&P mills go now beyond paper production: waste heat delivery to district heating systems and production of electricity and valuable chemicals such as ethanol are examples of additional measures adopted by the sector (Kamali & Khodaparast, 2015).

1.3.1. Paper manufacturing

The papermaking process can be defined by two essential steps: firstly, a fibrous raw material is converted into pulp. Subsequently, the pulp is converted into paper. Evidently, these steps divide into several stages, namely: raw material preparation, pulp manufacturing, pulp washing and screening, chemical recovery, bleaching, stock preparation and papermaking (Bajpai, 2010).

The pulp manufacturing stage will be scrutinized, once the aim of this work is the production of bioethanol from paper pulp.

1.3.1.1. Pulping process

Pulping is the process that reduces wood (or other lignocellulosic material) into a fibrous mass, the so-called pulp (Sixta et al., 2008). During this process, lignin is removed from the wood, and wood chips become individual cellulose fibers (Bajpai, 2015).

Table 4 summarizes the existing types of pulping processes, as well as the respective raw material and final use. The pulping process can be carried out mechanically, chemically or by a combination of mechanical and chemical processes. The resulting pulps have different proprieties according to the employed method, making them suited to particular products (Sixta et al., 2008).

It is worth mentioning that, in what paper manufacturing is concerned, the pulping process assumes the role of a pretreatment to the lignocellulosic material, once it alters its structure in order to improve cellulose availability. Therefore, no further pretreatment is required.

The chemical pulping process (i.e. kraft, soda and sulfite), in which raw materials are “cooked” in aqueous chemical solutions using elevated temperatures and pressure to extract pulp fibers (Bajpai, 2015), is the most applied worldwide (Sixta et al., 2008). Lignin and other materials of the interfiber matrix are degraded and dissolved; if 90 % of the lignin has been removed, the fibers can be subsequently separated without the need of mechanical action (Sixta et al., 2008). This enables the fibers to connect to each other by the formation of hydrogen bonds between their cellulosic surfaces during the papermaking process (Bajpai, 2010).

Table 4: Types of pulping processes (Bajpai, 2015).

Pulp grades		Raw material	End product use
Chemical pulps	Sulfite pulp	Softwoods and hardwoods	Fine and printing papers
	Kraft sulfate pulp	Softwoods and hardwoods	Bleached-printing and writing papers, paperboard, unbleached-heavy packaging papers, paperboard
	Dissolving pulp	Softwoods and hardwoods	Viscose rayon, cellophane, acetate fibers, and film
Semichemical pulps	Cold-caustic process	Softwoods and hardwoods	Newsprint and groundwood printing papers
	Neutral sulfite process	Hardwoods	Newsprint and groundwood printing papers
Mechanical pulps	Stone groundwood	Mainly softwoods	Corrugating medium
	Refiner mechanical (RMP)	Mainly softwoods	Newspaper and groundwood printing papers
	Termomechanical (TMP)	Mainly softwoods	Newspaper and groundwood printing papers
	Chemi-mechanical (CTMP)	Mainly softwoods	Newsprint, fine papers

Kraft process accounts for 91 % of the chemical pulping processes and 75 % of all produced pulp, due to its advantages concerning chemical recovery and pulp strength. In this process, wood chips are digested by an alkaline cooking liquor of sodium hydroxide and sodium sulfite (white liquor) in a digester. (Bajpai, 2015). The cooking process, the so-called delignification, is divided into three stages: the initial, bulk and residual or final phases. On the first phase, most of the lignin carbohydrate portion is lost, while α-aryl and β-aryl ether bonds in the phenolic units of lignin (15-25 % of native lignin) are cleaved. Then, in the bulk delignification phase, as the name itself suggests, the major part of the lignin is removed, as well as a small fraction of the carbohydrate content. At last, on the final phase, around 10-15 % of the native lignin is removed (Sixta et al., 2008). After cooking, both the resulting pulp and the black liquor (white liquor now

enriched with degraded wood components) are discharged under pressure into a blow tank: when the cooked chips impact on the blow tank, they disintegrate into fibers, the pulp (Bajpai, 2015; Sixta et al., 2008). The resulting pulp can undergo further delignification through bleaching processes (Sixta et al., 2008). The main factors that differentiate kraft process from sulfite process are the temperature and pH at which they are carried out: the sulfite process occurs at lower temperatures in an acid medium, while kraft process occurs at higher temperatures in alkaline medium. Even though cellulose is largely preserved in sulfite pulps, xylan is most stabilized in kraft pulps, this being the major advantage of kraft over sulfite pulps in case of hardwoods (Sixta et al., 2008).

Unbleached kraft pulp of *E. globulus* barks has been used as a substrate for bioethanol production (Branco et al., 2018). However, pulp consists of cellulose and hemicelluloses polysaccharides that constitute the fibers, so a hydrolysis step is required in order to convert these polysaccharides into fermentable monomeric sugars that can be metabolized by fermentative microorganisms. Cellulolytic enzymes assume a crucial role on this process, by allowing the breakage of the glycosidic bonds in cellulose and consequent release of glucose polysaccharides from the fibers, which are now available as a result of the pulping process. Cellulases (EC 3.2.1.4) are members of the hydrolases family of enzymes primarily produced by fungi, bacteria and yeasts (Chandel et al., 2012) and, rather than a single enzyme, are complex mixtures of cellobiohydrolases, endo β-glucanases and β-glucosidases. Firstly, endoglucanase (EG) acts on random sites of the cellulose chain and releases smaller fibres of oligosaccharides. Then cellobiohydrolase (CBH) acts on free ends to release cellobiose which is finally hydrolysed into glucose monomers by β-glucosidase (BGL).

1.4. Biofuels

Whereas before the 19th century wood and plant oil were the predominant fuels used worldwide respectively for heating and lighting, in 1905, fossil energy surpassed bioenergy and today fossil fuels are the major energy source and account for nearly 80 % of the word's energy needs (Guo et al., 2015)

It is fully acknowledged that the use of conventional fossil fuels entails several environmental concerns. Their combustion products are the main cause of problems such as the emission of greenhouse gases, which trap Earth's heat leading to the expansion of the greenhouse effect and consequent global warming. However, petroleum derived fuels are not harmful merely regarding the environment, since their overuse has been causing worldwide economical and geopolitical problems. For example, the high demand for fossil fuels and price increase of crude oil have been affecting the global economy over the past few decades (Gaurav et al., 2017). Moreover, by 2050, the planet population is expected to surpass the 9 billion people, and the energy demands to increase by 84 %, while the sources of fossil fuels and oil reserves are depleting faster and faster (Gaurav et al., 2017).

Numerous efforts are being made globally in order to mitigate the environmental impact of modern lifestyle. On December 11th, 1997, was adopted in Kyoto, Japan, the *Kyoto Protocol*, which came into force on February 16th 2005. This protocol is an international agreement linked to the United Nations Framework Convention on Climate Change, which involves 192 Parties and aims to stabilize GHG emissions by establishing maximum amounts of emissions that each country may emit during the commitment period. More recently, on November 4th 2016, *The Paris Agreement* entered into force. The long-term goal of the latter is to maintain the increase in global average temperature below 2°C above pre-industrial levels, while limiting the temperature increase to 1.5 °C (Kumar et al., 2018).

To overcome these problems and to meet what is required on the agreements above, alternatives as liquefied petroleum gas (LPG), compressed natural gas (CNG) and electricity for electric vehicles have emerged. Nevertheless, all of these candidates are still associated with significant disadvantages: they require engine modifications and a

new fuel infrastructure, which makes it difficult for them to achieve a competitive position on the market (Chang et al., 2017).

A solution that goes beyond these barriers is the utilization of biofuels (Chang et al., 2017). Biofuels can be defined as energy-enriched, biologically originated, non-fossil chemicals produced from biomass (Sampaio & Amado Gomes, 2017). Biomass derived fuels play a crucial role on the mitigation of the problems associated to petroleum derived fuels, such as the depletion of fossil resources and rising levels of atmospheric greenhouse gases. Furthermore, the development of the biofuels industry can create numerous job opportunities, improve the energy security in oil and gas major importing countries (Azadi et al., 2017), increase farm income and even promote research and development (Guo et al., 2015). In fact, the production and use of biofuels is already a reality that is gaining momentum all over the world. While in 1996 the amount of biofuels production in European Union (EU) was less than 500 ktoe, in 2007 an amount of 7000 ktoe was reached (Darda et al., 2018). Recently, the production of biofuels was estimated to be of 86000 kt/year, on a global scale. The dominant countries in biofuel production are the United States of America and Brazil (Rastogi & Shrivastava, 2017), while in Europe Germany and France are the major producers, followed by the Netherlands and Spain according to 2017 data (Darda et al., 2018). **Figure 10** shows the annual production of biofuels (production in thousand metric tons oil equivalent) in the EU.

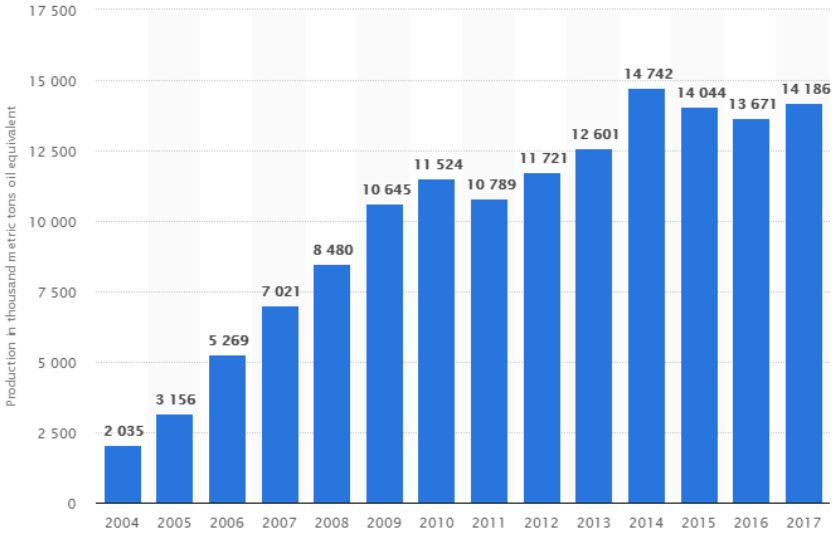


Figure 10: Annual production of biofuels in the European Union from 2004 to 2017 (NCES, 2018).

Currently, the goal of the European Union is that, by 2020, 10 % of the transportation fuels come from renewable resources. Furthermore, fuel suppliers are expected to reduce the GHG emissions by 6 % in comparison to 2010.

1.4.1. Biofuel generations

During the last two decades, four different generations of biofuels have been developed, according to their biomass feedstocks (Dutta et al., 2014). The four generations differ in terms of current/future availability, processing technology and sustainability level (Darda et al., 2018; Shuba & Kifle, 2018).

First generation biofuels are produced using food ingredients composed by carbohydrates, vegetable oils and animal fats (Rodionova et al., 2017) , such as, corn ethanol in the United States and sugarcane ethanol in Brazil (Sims et al., 2010). Despite being well studied and understood processes, sustainability and economic advantages of the production of first generation biofuels raised a lot of doubts. These issues are mainly related to the use of land and water for the production of fuel, which would otherwise be used for growing food goods. This farmland competition, along with the hypothetical increase of the food and animal feed prices, raises the “food for fuel” ethical concern. Production and processing expenses that often require government subsidies and fluctuating results of GHG reduction rate assessments are also factors that prevent widespread use of first generation biofuels (Sims et al., 2010).

The above mentioned “food versus fuel” dilemma associated to first generation biofuels encouraged the development of second generation biofuels, produced from non-food feedstocks, i.e., lignocellulosic biomass or industrial or urban resources. These latter include by-products such as cereal straw, sugar cane bagasse and forest residues as well as organic components of municipal solid wastes and even dedicated feedstocks. Lignocellulosic biomass can be converted to second generation biofuels biochemically or thermochemically. In both cases, a higher reduction of GHG emissions is achieved, in comparison to first generation biofuels (Darda et al., 2018; Dutta et al., 2014). Although lignocellulosic biomass is able to overcome the "food for fuel" problem, there are still some disadvantages concerning its utilization, such as the requirement of previous

pretreatment and more complex processing. Besides that, eventual simultaneous discard of lignin and hemicelluloses may cause environmental pollution and waste of resources (Chen & Qiu, 2010). To avoid that problem, processes that enable the valorisation of all components of lignocellulosic biomass are currently being developed. Lignin, for instance, has been used as a potential source of valuable chemicals such as lignin monomers and dimers, including phenols and alkylphenols (Wang et al., 2019).

Table 5: Pros and cons of each biofuel generation (Dutta et al., 2014).

Generation	Pros	Cons
First	GHG savings Simples and low cost conversion technology	Low yields Cause food crisis as a large portion of arable land is required for growing crops
Second	GHG savings Utilize food wastes as feed-stock No food crop competition Use of non-arable land for growing few energy crop	Costly pretreatment of lignocellulosic feedstock Highly advanced technology needs to be developed for cost effective conversion of biomass to fuel
Third	Easy to cultivate algae Higher growth rate No food crop competition Versatility: can use wastewater, seawater	More energy consumption for cultivation of algae (for mixing, filtration, centrifugation, etc.), low lipid content or biomass contamination problem in open pond system High cost of photo-bioreactor
Fourth	High yield lipid containing algae More CO_2 capture ability High production ratio	Initial investment is high Research is at its primary stage

Third generation biofuels utilize marine biomass, i.e., algae as feedstock (Rastogi & Shrivastava, 2017). Algae elevated lipid content can be easily converted to biofuel

namely biodiesel (Yang et al., 2015), and their higher photosynthesis and faster growth rates comparing to any terrestrial plant makes them a potential resource for biofuels production (Dutta et al., 2014). Another advantage concerning algae utilization relate to the facts that their cultivation can take place throughout the entire year (Alaswad et al., 2015) and that they avoid pesticides, herbicides and fertilizers once there is no need for arable lands for cultivation (Gaurav et al., 2017).

There is still a fourth generation of biofuels still under development. Metabolic engineered algae to allow higher yields and production rates (Dutta et al., 2014), photobiological solar fuels and electrofuels (Rastogi & Shrivastava, 2017) and carbon storage biomass (Yang et al., 2015) can be considered as such. All the advantages and disadvantages associated with each biofuel generation illustrated above are summarized in **Table 5**.

1.4.2. Bioethanol

Although virtually any organic molecule of the alcohol family can be used as a fuel, only methanol (CH_3OH) and ethanol (C_2H_5OH) fuels suit technical and economical requirements of internal combustion engines (Bala, 2005).

Ethanol (ethyl alcohol, CH_3-CH_2-OH or EtOH), often simply referred to as "alcohol", is a clear colorless liquid completely miscible with water which has a burning pungent taste and smell. It is widely used by humans and, besides alcoholic beverages, is present in medicines, lotions, mouthwashes, tonics, colognes, rubbing compounds and solvents. One of the most relevant and promising applications of ethanol is its use as a transportation fuel, which is allowed by its ability to be burned with oxygen in air according to equation 1 above (Demirbas, 2009; Wyman & Kumar, 2017):

$$C_2H_5OH + 3\ O_2 \rightarrow 2\ CO_2 + 3\ H_2O \quad (1)$$

Flammability (ethanol burns with a light blue flame), high octane number – around 103 – and high heat of vaporization – about 907 J/g compared to about 395 J/g for gasoline (Wyman & Hinman, 1990) makes it possible to employ higher compression

ratios in dedicated ethanol engines, leading to more efficiency in the use of ethanol relatively to gasoline in lower compression ratio engines (Kohse-Höinghaus et al., 2010; Wyman & Kumar, 2017). In what environmental impact is concerned, not only is bioethanol less toxic than any other fuel, but also remediation of possible leaks or splits is fairly easy, once ethanol is totally water soluble and microorganisms promptly metabolize it into carbon dioxide and water (Lynd et al., 1991). In what GHG emissions are concerned, type of feedstock, agricultural practices, site productivity and conversion technology influence the reduction rate, which implies emissions of cultivation, transport, conversion process and distribution (Micic & Jotanovic, 2015).

Despite the environmental benefits, bioethanol encompass some limitations, such as low cetane number - which makes it less appropriate for compression ignition engines – and low energy density. Also, ethanol preference for water is a problem in transportation through pipelines, which oftentimes accumulate water (Wyman & Kumar, 2017).

Bioethanol can be blended with gasoline at different ratios that influence the fuel proprieties: E10, E20, E25, E70, E85 and E95 are common ethanol blends which contain 10, 20, 25, 70, 85 and 95 % of ethanol, respectively. Flexible-fuel vehicles (FFV) now available in Europe, can work with an ethanol/gasoline blend containing up to 85 % of ethanol (Micic & Jotanovic, 2015).

1.4.2.1. Bioethanol from lignocellulosic biomass

Bioethanol can be produced from lignocellulosic biomass through two broad platforms: the sugar platform (biochemical conversion) and the syngas platform (thermochemical conversion), as outlined in **Figure 11**. Briefly, in the first one pretreated lignocellulosic biomass is broken down into simple sugars by cellulolytic enzymes, which are then fermented into ethanol. In the latter, the feedstock is gasified to produce syngas (synthesis gas, a fuel gas mixture of hydrogen, carbon monoxide and carbon dioxide). Syngas is posteriorly converted into ethanol chemically or biologically (Datta et al., 2011; Vohra et al., 2014).

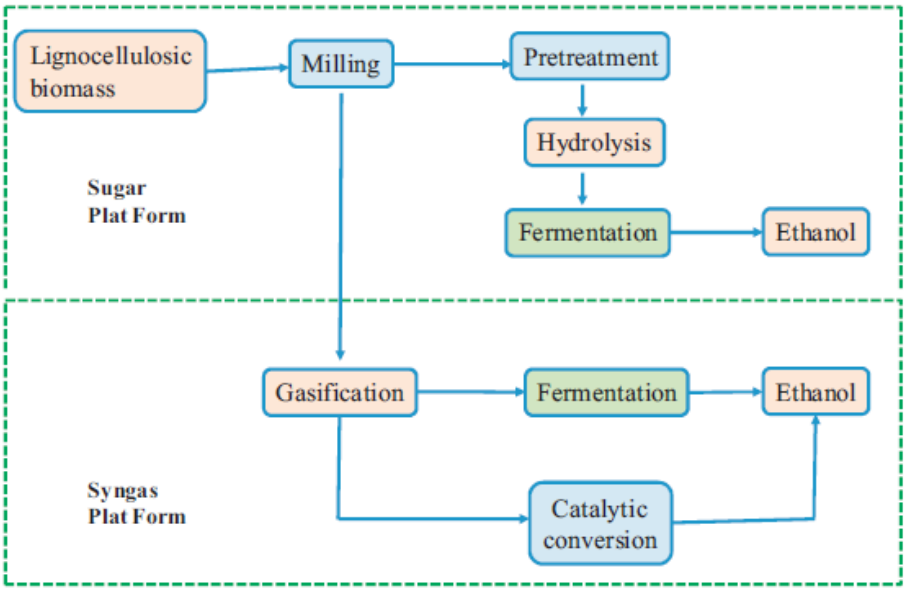


Figure 11: Main pathways for bioethanol production from lignocellulosic biomass (Vohra et al., 2014).

In this work, biochemical conversion of lignocellulosic materials is going to be carried out, therefore sugar platform is going to be scrutinized. As previously mentioned, coupled with its global widespread availability, the great amount of celluloses and hemicelluloses present in lignocellulosic feedstocks makes them a potential substrate for the production of bioethanol. **Figure 12** represents the biochemical pathway for bioethanol obtainment from lignocellulosic materials, which encompasses four basic steps: pretreatment, hydrolysis (saccharification), fermentation and product separation/distillation.

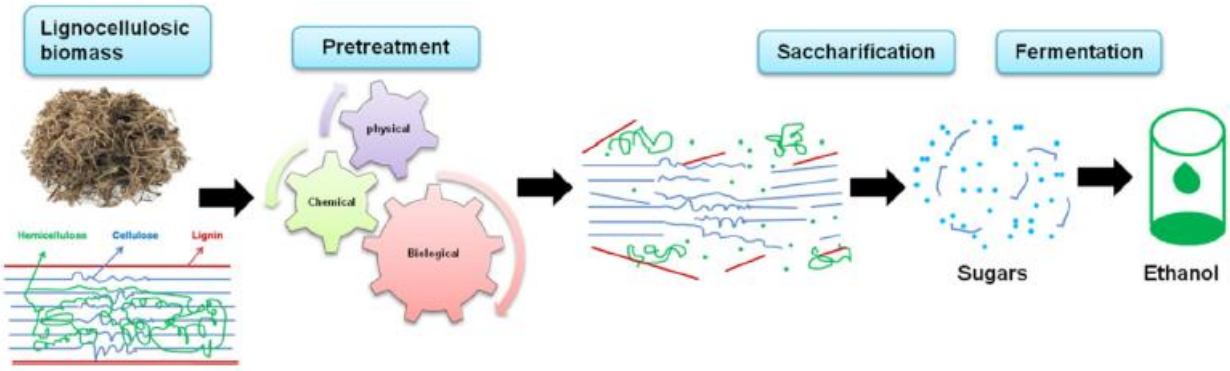


Figure 12: Fundamental stages of bioethanol biochemical production from lignocellulosic materials (Pandiyan et al., 2019).

In the specific case of this work, pulping by kraft process assumes the role of a pretreatment (Phillips et al., 2013), since it removes the existing lignin, which would

make feedstock resistant to enzymatic attack and unable to release sugars. Thus, the pretreatment step aims to enable higher saccharification yields with the least possible amount of enzymes required, inhibitors formation, loss of fermentable sugars and general process costs (Pandiyan et al., 2019; Phillips et al., 2013). Subsequently, polysaccharides fibers undergo the formerly described hydrolysis stage. Xylan and arabinan in hemicelluloses are broken down into xylose or arabinose C5 sugars, according to Equation 2, whereas the hydrolysis of cellulose and other hemicelluloses, polysaccharides of C6 sugars (galactose, mannose and also glucose) occurs according to Equation 3 (Wyman & Kumar, 2017):

$$(C_5H_8O_4)_n + n\ H_2O \rightarrow n\ C_5H_{10}O_5 \qquad (2)$$

$$(C_6H_{10}O_5)_n + n\ H_2O \rightarrow n\ C_6H_{12}O_6 \qquad (3)$$

After the fermentable hexoses and pentoses monomers have been released, the fermentation stage can be performed.

From a fermenting microorganism it is expected a high yield of bioethanol, broad substrate utilization range, resistance to inhibitors, minimal by-products formation and ability to withstand high sugars and alcohol concentrations, high temperatures and low pH (Banerjee et al., 2010) although these features hardly coexist in any wild organism (Sánchez & Cardona, 2008; Vohra et al., 2014). *S. cerevisiae*, along with *Zymomonas mobilis*, is the most used microorganism for bioethanol fermentation. In industrial processes, *S. cerevisiae* is the fittest microorganism to carry out fermentation of lignocellulosic hydrolysates due to its good performance, its robustness under harsh process conditions and the fact that is non-pathogenic. The hexoses glucose, mannose and fructose and the disaccharides sucrose and maltose are readily fermented by *S. cerevisiae* wild-type through the Emden-Meyerhof pathway of glycolysis, and D-galactose by the combined action of the Leloir pathway and glycolysis (van Maris et al., 2006) - **Figure 13**. In an initial stage, D-galactose and D-glucose are phosphorylated into glucose-6-P and D-mannose into mannose-6-P. All the hexoses are then converted in fructose-1,6-biP, to later become pyruvate and, finally, ethanol (van Maris et al., 2006).

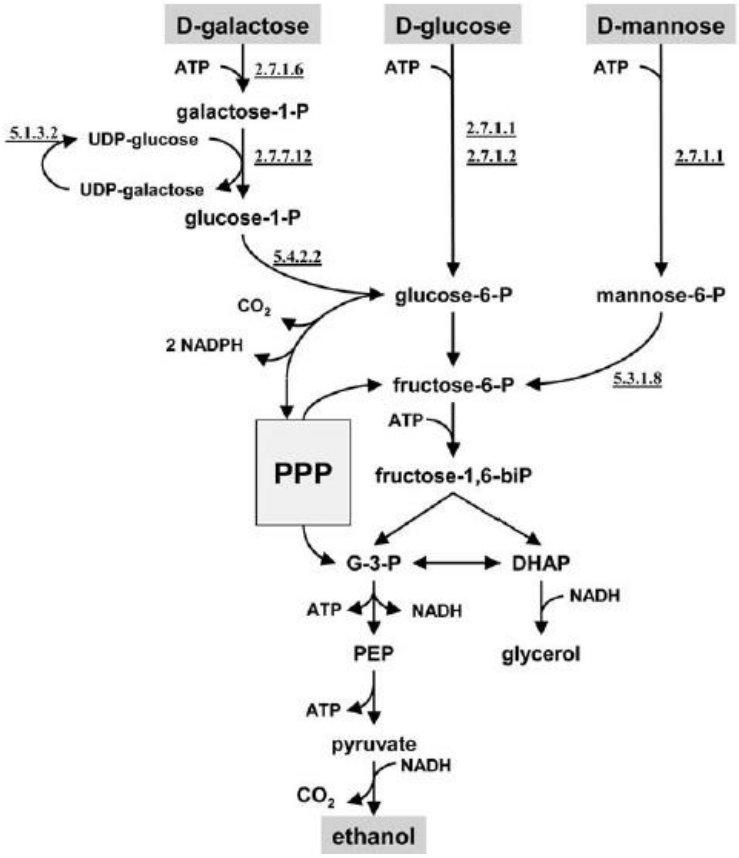


Figure 13: Hexoses catabolism of *S. cerevisiae*. Underlined EC numbers represent enzymes present in wild type *S. cerevisiae* metabolism. (van Maris et al., 2006).

However, both of the microorganisms lack the ability to ferment hemicellulose derived pentose sugars (van Maris et al., 2006; Vohra et al., 2014), which abound in a wide range of lignocellulosic biomass. The most employed approach to enable the fermentation of pentoses by *S. cerevisiae* is enhancing the expression of xylose reductase gene (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) genes by metabolic engineering.

Several studies are now dwelling on the fact that D-xylose might not to be sensed by *S. cerevisiae* in its media. Osiro et al. (2018) introduced a green fluorescent protein (GFP) into the yeast to study what kind of signal the yeast would give in the presence of xylose and measure the activation or repression of three sugar signaling routes by detecting the fluorescence. Using this biosensor system, it was shown that the poor consumption of xylose by *S. cerevisiae* is in fact a consequence of the inability of the yeast to sense xylose on its environment. This work further demonstrated that when the

yeast is genetically modified by adding a xylose pathway, high xylose concentration induces the same signal as for low glucose concentrations (< 5 g/L), similar to the response to carbon starvation due to a low level of nutrients, causing the low assimilation rates, as also shown by Bergdahl et al. (2012). On the other hand, when the yeast is engineered in order to change the recognition signal of xylose, the reaction becomes similar to that of high concentrations of glucose (50 g/L). There are now well-studied and efficient routes for D-xylose assimilation in metabolic engineered strains of *S. cerevisiae*, but the linkage between xylose fermentation and sugar signaling is still not entirely understood and requires further investigation.

Among the naturally occurring fermenting yeasts, *Schefersomyces stipitis* (formerly *Pichia stipitis*) stands out due to its ability to ferment xylose and other important hexoses (glucose, mannose and galactose) found in lignocellulosic material, with relatively high yield (Farias et al., 2014). Cellobiose and hemicellulose oligomers can also be fermented by this yeast, owing to its capability of producing cellulolytic and hemicellulolytic enzymes (Laplaza et al., 2007).

Whereas *S. cerevisiae* is a Crabtree positive yeast, the oxygen availability on the fermentation media is a determining factor in ethanol production by *S. stipitis* (Laplaza et al., 2007), rather than the composition of culture media and operational conditions. The alcohol dehydrogenase complex (ADH) is encoded by the genes ADH1 and ADH2 and is responsible for ethanol production in *S. stipitis.* The activity of ADH is induced by a decrease in the oxygen tension, but under strictly anaerobic conditions, almost no ethanol is produced and the strain cannot survive longer than one generation (Papini et al., 2012). Therefore, oxygen concentration must be controlled at microaerophilic conditions, so that carbon flux is not deviated to cell growth and ethanol production is not negatively affected (Farias et al., 2014).

When in aerobic conditions, ethanol is assimilated and becomes acetaldehyde which in turn is oxidized into acetate. These compounds are toxic to the cells and inhibit growth and fermentation, making them less tolerant to ethanol than *S. cerevisiae*. Therefore, ethanol absorption can be avoided by reducing the amount of available oxygen (Farias et al., 2014).

Currently, the main challenges associated with the upgrading of biochemical fermentation of lignocellulosic feedstocks are enzyme costs and performance, co-fermentation of pentoses and hexoses, toxins released during pretreatment stage, processing time and investment costs. In order to produce economically viable, sustainable bioethanol to compete with petroleum based fuels, conventional process stages need to be integrated into a consolidated process to minimize the production of inhibitory sugar derivatives and to achieve higher ethanol yields (Rastogi & Shrivastava, 2017).

Traditionally, on the bioconversion of lignocellulosic biomass to ethanol, a strategy of separate hydrolysis and fermentation (SHF) is employed. Simultaneous saccharification and fermentation (SSF) technique was proposed in order to avoid end-product inhibition associated with the hydrolysis step of SHF. In addition, SSF entails financial advantages once the number of required vessels decreases. However, the difference between the optimum temperature for the action of the enzyme and the optimum temperature for the growth of the organism is still an obstacle associated with SSF (Ebrahimi et al., 2017; Yang et al., 2016)

In quasi-simultaneous saccharification and fermentation (Q-SSF), the pretreated lignocellulosic raw material undergoes a pre-hydrolysis step at elevated temperatures, optimized for the used cellulases. During this stage, a certain amount of monomeric and oligomeric sugars is produced. After the pre-hydrolysis, the liquefied feedstock is cooled down to the optimal temperature for the yeast growth. This fermentation approach mitigates the hurdle related to disparate temperatures in SSF. Furthermore, facilitates mixing for saccharification and fermentation, by reducing the viscosity of the hydrolysate (Ebrahimi et al., 2017; Yang et al., 2016). A Q-SSF approach may be advantageous when working with recalcitrant raw materials, as is the case of lignocellulosic feedstocks, particularly wood materials. Zhu et al., (2015) produced bioethanol from forest harvest residue by Q-SSF with *S. cerevisiae* with a yield of 282 L/ton.

Throughout the world, research groups have been focused on studying and optimizing the bio-production of ethanol through the most varied types of lignocellulosic biomass, including paper pulp, food and agricultural wastes. Yields of ethanol production from different lignocellulosic feedstock are shown in **Table 6**, as well as the employed pre-treatment, fermentative microorganism and fermentation strategy carried out.

Despite being susceptible to high temperatures, high ethanol concentrations and unable to ferment pentose sugars, the yeast *S. cerevisiae* is definitely the most commonly employed microorganism in the bioproduction of ethanol, mostly due to its high ethanol generation yield and ability of fermenting a wide range of sugars besides the resistance to inhibitors (Mohd Azhar et al., 2017). Martínez-Patiño et. al (2018) applied a recombinant ethanologenic *Escherichia coli* MM160 on the co-fermentation of cellulosic and hemicellulosic sugars after detoxification of the enzymatic hydrolysates. This detoxification step was crucial in terms of producing non-toxic liquors, probably due to the removal of the most toxic phenolic compounds for *E. coli*. Furthermore, this work revealed that high sugar concentrations inhibit the metabolism of the microorganism causing incomplete sugar assimilation and, consequently, lower conversion yields. Agbogbo & Coward-Kelly (2008) studied fermentation results on lignocellulosic substrates by *S. stipitis*. The ethanol produced ranged from 6.0 to 41 g/l, at a yield of 0.31–0.48 g ethanol/g sugars consumed.

Concerning the bioconversion of lignocellulosic biomass, acid pretreatment is one of the most applied technologies on an industrial scale (Alvira et al., 2010). Nevertheless, organosolv pretreatment has been proving to be a promising method, allowing the separation of high purity cellulose, isolation of high quality lignin and higher efficiency of hemicelluloses fractionation (Salapa et al., 2017). It is worth highlighting the specific case of wood pulp fermentation, once kraft process is employed as a pretreatment itself.

Table 6: Yields of ethanol production from different lignocellulosic feedstock, employed pre-treatment, used microorganisms and fermentation strategies carried out.

Raw material	Substrate	Organism	Pre-treatment	Type of fermentation	Ethanol yield	Reference
Coconut coir fibers	48.6 % glucan on pre-treated solid	*S. cerevisiae*	Organosolv	SSF	8.97 g/L	(Ebrahimi et al., 2017)
Wheat straw	84.8 g/L glucose	*S. cerevisiae*	Organosolv	SHF	32.6 g/L	(Salapa et al., 2017)
Corn stover	57.1 % glucan on pre-treated solid	*S. cerevisiae*	Nitric acid	Q-SSF	22.4 g/L	(Ilgook et al., 2015)
Poplar pulp	31.5 g/L glucose	*S. cerevisiae*	Kraft pulping	SHF	9.54 g/L	(Przybysz Buzała et al., 2017)
Bleached pine pulp	37.3 g/L glucose	*S. cerevisiae*	Kraft pulping	SHF	18.4 g/L	(Przybysz Buzała et al., 2017)
Rice straw	80.6 % cellulose and 3.2 % hemicelluloses on pre-treated solid	*S. cerevisiae*	Microwave/Acid/ Alkali/H_2O_2 Pretreatment	SSF	57.3 g/L	(Shengdong et al., 2006)
Olive tree biomass	144 g/L glucose and 58.6 g/L xylose	Recombinant ethanologenic *E. coli*	Sulfuric acid	SHF	96.0 g/L	(Martínez-Patiño et al., 2018)
Corn stover	40 g/L glucose and 10 g/L xylose	*S. stipitis*	Sulfuric acid	SHF	25.0 g/L	(Agbogbo & Coward-Kelly, 2008)

2. Objectives

Wastes from the pulp and paper industry were used in order to produce bioethanol in a circular economy logic. Chips of eucalyptus barks separated during preparation of raw-material for usual pulping process undergone kraft pulping process. This process results in a pulp similar to that obtained under regular conditions, that is, when the wood is used to produce pulp.

The pulps, rich in celluloses and hemicelluloses, were subsequently hydrolyzed by means of cellulosic enzymes so as to obtain fermentable sugars. The resulting hydrolysate was characterized in terms of sugar concentration and presence of inhibitory compounds.

Subsequent pure culture fermentations with *S. cerevisiae*, *S. stipitis* and a co-culture of both were carried out in batch mode, firstly in Erlenmeyer flasks in order to optimize fermentation conditions. A scale-up to a 5 L bioreactor regarding the assays with *S. cerevisiae* and the co-culture was performed afterwards.

This work aims to serve as a proof of concept of the feasibility of using kraft pulps from eucalyptus barks, a waste feedstock, as a substrate in the production of bioethanol.

II. Material and methods

2.1. Feedstock

Wood chips of *E. globulus* barks were converted into unbleached pulp by applying kraft pulping process prior to this work at *RAIZ – Forest and Paper Research Institute*. The wood chips undergone a preliminary extraction step at *RAIZ* before the kraft pulping with ethanol:water carried out to decrease the amount of extractive materials originally present in the wood, aiming to improve the kraft process and the following hydrolysis of the obtained pulp. The unbleached pulp (**Figure 14**) used in this work was then washed and the complete characterization of the pulp provided by *RAIZ* is presented in **Appendix A**.

Figure 14: Unbleached kraft pulp from *E. globulus* barks.

2.1.1. Enzymatic hydrolysis of kraft pulp

933.68 g of unbleached kraft pulp were added to 2.216 L of sodium citrate buffer 0.05 N at 50 ºC. When the temperature stabilized, 100 mL of a cellulolytic enzymatic solution (Novozymes) with an enzymatic activity of 133.5 FPU/mL (corresponding to an enzymatic load of 25 FPU.g of carbohydrate^{-1}) was added. The mixture was incubated for a period of 24 h, with a stirring of 100 rpm at 50 ºC (**Figure 15**). Several samples were taken in order to monitor the concentration of reducing sugars, the temperature and pH during the time. If necessary, pH was adjusted to 4.9-5.1, through the addition of H_2SO_4 3 M or NaOH 1 M.

Figure 15: Unbleached Kraft pulp of *E. globulus* barks in citrate buffer solution during the hydrolysis process.

After 24h, the resulting hydrolysate was cooled and centrifuged for 25 min, at 5000 rpm at 4 ºC (Megafuge 16R, Thermo Scientific) to remove the solids fraction. The hydrolysate was then sterilized in an Uniclave 88 (AJC) autoclave at 121 ºC for 20 min. As the formation of a precipitate was observed, the hydrolysate was centrifuged once again, under the same conditions, and the supernatant was stored at -18 ºC in volumes of approximately 500 mL.

A spectrum between 300 and 800 nm of the hydrolysate was acquired with a spectrophotometer UVmini-1240 (Shimadzu) and an UVProbe 2.10 (Shimadzu) software in order to select the wavelength to measure biomass concentration in the fermentation assays.

2.2. Microorganisms

Saccharomyces cerevisiae PYCC 5246 (ATCC 24860) was gently supplied from Portuguese Yeast Culture Collection. *Scheffersomyces stipitis* NRRL Y-7124 was purchased by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. Both stock cultures were stored in 20 % (v/v) glycerol at -80 ºC, and the colonies used in this work were grown at 28 ºC and maintained at 4 ºC in YM Petri dishes.

2.3. Media and stock solutions

The pH of all media and solutions used in this work was adjusted to 5.5 before sterilization in an Uniclave 88 (AJC) autoclave at 121 ºC for 20 min.

2.3.1. Yeast Mould medium

Yeast Mould (YM) solid medium, with 20.0 g/L of agar was used for strain maintenance. YM liquid medium (3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone and 10.0 g/L glucose) was used for pre-inocula and inocula preparation.

2.3.2. Supplementary medium

Supplementary medium (SM) was prepared in two separate solutions in order to avoid salt precipitation due to complexation, one containing $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ and the other one containing $MgSO_4 \cdot 7H_2O$ and yeast extract. The concentration of these components on the fermentations working volume is shown in **Table 7**:

Table 7: Concentration of SM components.

Component	Concentration (g/L)
$(NH_4)_2HPO_4$	2.0
$(NH_4)_2SO_4$	1.0
$MgSO_4 \cdot 7H_2O$	0.5
Yeast extract	2.5

SM was used to supplement two solutions: the hydrolysate, and a concentrated glucose solution mimicking the hydrolysate, used to perform synthetic media assays with *S. cerevisiae*. In both types of assays, the initial sugar concentration was adjusted to 40 g/L.

2.4. Erlenmeyer flask assays

Fermentation assays were performed using SM containing glucose (25 % v/v) and hydrolysate (65 % v/v). All assays were performed in 250 mL Erlenmeyer flasks with a

working volume of 100 mL containing and incubated at 180 rpm and 28 ºC. Two replicas were made for each assay. Throughout the fermentations, samples were taken to monitor pH and biomass concentration. Then, samples were centrifuged for 5 min at 13000 rpm (MiniSpin, Eppendorf) and the supernatant was stored at -18 ºC for prior determination of glucose, xylose, and ethanol concentrations.

2.4.1. Pre-inocula and inocula

Pre-inocula were prepared by transferring a single colony from a maintenance YM plate to 10 mL of YM liquid medium in 50 mL Falcon tubes, which were incubated at 28 ºC and 180 rpm for 24h. At the end of this period, the inocula were prepared by transferring a volume of pre-inoculum that allowed an initial biomass concentration of 0.200 g/L to 40 mL of new YM medium, on 100 mL Erlenmeyer flasks. The inocula were incubated at 28 ºC and 180 rpm for 14h.

2.4.2. Fermentation assays with cultures of *S. cerevisiae* or *S. stipitis*

Each assay was inoculated with a volume of inoculum that allowed an initial biomass concentration of approximately 0.200 g/L. The final fermentation volume (100 mL) was adjusted with a NaCl solution (0.9 %).

2.4.3. Fermentation assays with sequential co-cultures of *S. cerevisiae* and *S. stipitis*

Co-culture assays started with the inoculation of *S. cerevisiae* in a volume that allowed an initial biomass concentration of 0.200 g/L. Then, the working volume was adjusted to 100 mL with NaCl 0.9 % (m/v). Regarding the inoculation of *S. stipitis*, two different approaches were followed. One in the presence of *S, cerevisiae*, with *S. stipitis* being inoculated after 24h of fermentation, in a volume that allowed an initial biomass concentration of 0.100 g/L of this yeast. The other in the absence of *S. cerevisiae* by removing it after 24 h of fermentation by centrifugation for 20 min, at 5000 rpm at 4 ºC (Megafuge 16R, Thermo Scientific) under sterile conditions. Then, *S. stipitis* was inoculated in a volume that allowed an initial biomass concentration of 0.200 g/L of this yeast.

2.5. Bioreactor assays

The bioreactor fermentation assays were performed in batch mode, in a 5L bioreactor BIOSTAT® Aplus (Sartorius Stedim Biotech®) with a working volume of 3L. Temperature and pH were automatically controlled by DCU system (Sartorius Stedim Systems®) and data was acquired by MFCS/DA 3.0 (Sartorious Stedim Systems®). Temperature was maintained at 28 ºC. The pH was measured using an electrode EasyFerm Plus K8 325 (Hamilton) and controlled to 5.5 ± 0.1 through the addition of KOH 5 M and H_2SO_4 1 M. The stirring was imposed at 180 rpm through two 6-blade disk impellers and 4 baffles. A 0.2 µm Midisart® 2000 PTFE filter (Sartorius Stedim Biotech) was installed in a nozzle in the cover plate to exhaust the gases from the headspace of the bioreactor. When necessary, an antifoaming agent 10 % (v/v) (BDH® Prolabo® Antifoam Silicone 426 R) was added.

Throughout the fermentations, samples were taken to monitor pH and biomass concentration. Then, samples were centrifuged for 5 min at 13000 rpm (MiniSpin, Eppendorf) and the supernatant was store at -18 ºC prior determination of glucose, xylose, and ethanol concentrations.

2.5.1. Pre-inocula and inocula

Pre-inocula were prepared by transferring two colonies from a maintenance YM plate to 40 mL of YM liquid medium in 100 mL Erlenmeyers, which were then incubated at 28 ºC and 180 rpm for 24 h. At the end of this period, the inocula were prepared by transferring a volume of pre-inoculum that allowed an initial biomass concentration of 0.300 g/L to 300 mL of new YM medium, on 500 mL Erlenmeyer flasks. The inocula were incubated at 28 ºC and 180 rpm for 14 h.

2.5.2. Fermentation assays with cultures of *S. cerevisiae*

Two assays were performed with 25 % (v/v) SM and one was supplemented with 6 5 % (v/v) of sugar solution and the other with 65 % (v/v) of hydrolysate. Each assay was inoculated with a volume of inoculum that allowed an initial biomass concentration of approximately 0.200 g/L.

2.5.3. Fermentation assay with sequential co-cultures of *S. cerevisiae* and *S. stipitis*

Co-cultures assays were performed with 65 % (v/v) of hydrolysate and 25 % (v/v) of SM. *S. cerevisiae* was first inoculated in a volume that allowed an initial biomass concentration of 0.200 g/L. *S. stipitis* was inoculated after 24 h of fermentation, in a volume that allowed an initial biomass concentration of 0.100 g/L of this yeast. After the inoculation of *S. stiptis*, the assay was divided in two aeration stages: during the first 8h the medium was aerated and the dissolved oxygen tension (DOT) controlled to approximatey 50 %. In the remaining hours, the air flow was interrupted.

2.6. Analytical methods

2.6.1. Reducing sugars

Reducing sugars in the obtained hydrolysate were analyzed by the dinitrosalicylic acid (DNS) method (Miller, 1958). First, the hydrolytic enzymes were inactivated by heating samples at 100 ºC for 5 min. Then, samples were centrifuged for 10 min at 5000 rpm. The supernatant was collected and centrifuged for 10 min at 5000 rpm. 3 mL of DNS reagent were added to 1 mL of properly diluted supernatant. The mixture was heated at 100 ºC for 5 min and the reaction was stopped by cooling the mixture. After adding 20 mL of distillate water to the mixture, the absorbance was measured at 540 nm. The calibration curve was done with glucose standards with concentrations between 0-5 g/L.

2.6.1. pH

A InPro 3030/200 (Mettler Toledo) sensor connected to a benchtop meter sensION+ MM340 (Hach) was used to measure pH.

2.6.2. Biomass

The biomass concentration was monitored by measuring the optical density at 620 nm (OD_{620}), using an UVmini-1240 (Shimadzu) spectrophotometer. The optical density value was further converted into biomass concentration by the proper calibration curve

of OD_{620} versus biomass dry weight, obtained for both yeasts and respective co-cultures, in each fermentation media.

2.6.3. Glucose, xylose, and ethanol

Glucose, xylose, and ethanol were analyzed by High Performance Liquid Chromatography (HPLC). Before analysis, samples were properly diluted, and acidified with 4 % (v/v) H_2SO_4 0.25 M for decreasing pH to 1-3, if necessary. Diluted samples were then filtered through modified nylon 0.20 µm centrifugal filter (VWR) by centrifugation for 10 min at 8000 rpm (MiniSpin, Eppendorf). Then, samples were injected into a Rezex ROA-Organic Acid H^+ (8 %) 50 x 7.8 mm ion-exchange column (Phenomenex), with a Gecko 2000 oven set at 65 ºC, and a refraction index detector L-2490 (VWR-Hitachi). The injection volume was 10 µL and the eluent was H_2SO_4 0.01 N, at a flow rate of 0.5 mL/min. The HPLC system consisted of an autosampler L-2200 (VWR-Hitachi), a pump L-2130 (VWR-Hitachi), and a data acquisition and processing system EZChrom Elite (Agilent Technologies). The concentration of the analysed compounds was determined using a calibration curve of the compound peak areas versus the compound concentration in a set of standard solutions, containing the target compounds in concentrations between 0-5 g/L.

2.6.4. Chemical oxygen demand

The Chemical oxygen demand (COD) was measured with Spectroquant Kit (Merck) and the solutions used were prepared according to the Standard Methods for the Examination of Water and Wastewater: 1.2 mL of a digestive aqueous solution and 2.8 mL of acid solution were added to 2.0 mL of properly diluted sample. The mixture was incubated at 150 ºC for 2 h in a termoreactor Spectroquant TR 620. After cooling, absorbance was measured in a spectrometer Spectroquant Picco. The calibration was made with glucose with concentrations in the range of 0-1 g COD /L.

2.6.5. Ligand-exchange/size-exclusion chromatography (LEX/SEC)

The hydrolysate was analysed by semi-preparative ligand-exchange/size-exclusion chromatography (LEX/SEC) on a high-performance liquid chromatograph

equipped with a Shodex sugar KS 2002 column (300 mm of length and 20 mm of internal diameter) from Showa Denko K. K. (Tokyo, Japan). The column was maintained at 30 º C, the injected sample volume was 500 µL and ultrapure water was used as eluent at a flow rate of 2.80 mL/min. A refractive index detector (Knauer K-2401, Berlin, Germany) was used. To obtain the retention time corresponding to the different degrees of polymerization, a standard solution containing fructose, glucose, sucrose and melezitose (20 mg/mL) was injected, using the same chromatographic conditions used for the samples separation.

2.7. Calculation methods

2.7.1. Enzymatic hydrolysis

The hydrolysis total yield, $Y_{Hydolysis}$ (%), was calculated according to the obtained glucose and xylose concentrations and the potential glucose and xylose concentrations in the unbleached kraft pulp – **Equation 2.1**. The hydrolysis yields on glucose and xylose were calculated using **Equation 2.2** and **Equation 2.3**, respectively. The final values are the average value of the four performed hydrolysis.

$$Y_{\text{Hydrolysis}}\,(\%) = \frac{[\text{Glucose}]_{\text{obtained}} + [\text{Xylose}]_{\text{obtained}}}{[\text{Glucose}]_{\text{potencial}} + [\text{Xylose}]_{\text{potential}}} \times 100 \qquad \textbf{Equation 2.1}$$

$$Y_{\text{Glucose}}\,(\%) = \frac{[\text{Glucose}]_{\text{obtained}}}{[\text{Glucose}]_{\text{potencial}}} \times 100 \qquad \textbf{Equation 2.2}$$

$$Y_{\text{Xylose}}\,(\%) = \frac{[\text{Xylose}]_{\text{obtained}}}{[\text{Xylose}]_{\text{potential}}} \times 100 \qquad \textbf{Equation 2.3}$$

2.7.2. Fermentation assays

Specific growth rate, μ (h^{-1}), was calculated by determining the slope of the linear regression obtained after plotting the natural logarithm of biomass concentration versus time during the exponential phase of the yeasts growth.

Volumetric glucose consumption rate $r_{glucose,}$ ($g.L^{-1}.h^{-1}$), and volumetric xylose consumption rate, r_{xylose} ($g.L^{-1}.h^{-1}$), were calculated by determining the module of the slope of the linear regression obtained after plotting, respectively, glucose and xylose concentration versus time, during the time their consumption had an approximately linear behavior. Similarly, the volumetric ethanol production rate, $r_{ethanol}$ ($g.L^{-1}.h^{-1}$), was calculated by determining the slope of the linear regression obtained after plotting ethanol concentration versus time, for the period in which ethanol production was approximately linear.

The volumetric ethanol productivity, $Prod_{vol}$ ($g.L^{-1}.h^{-1}$), from the beginning of the fermentation until maximum ethanol concentration was achieved was calculated using **Equation 2.4**.

$$Prod_{vol} = \frac{\Delta[Ethanol]}{\Delta t} \qquad \textbf{Equation 2.4}$$

The ethanol yield, $Y_{ethanol/substrate}$ ($g.g^{-1}$), and the biomass yield, $Y_{biomass/substrate}$ ($g.g^{-1}$) were calculated using **Equations 2.5** and **2.6**, respectively, considering both glucose and xylose as substrates. $Y_{ethanol/substrate}$ and $Y_{biomass/substrate}$ were calculated considering the time between the beginning of the fermentation until the maximum ethanol concentration was achieved.

$$Y_{ethanol/substrate} = -\frac{\Delta[\text{Ethanol}]}{\Delta[\text{Substrate}]} \qquad \textbf{Equation 2.5}$$

$$Y_{biomass/substrate} = -\frac{\Delta[\text{Biomass}]}{\Delta[\text{Substrate}]} \qquad \textbf{Equation 2.6}$$

To determine the conversion efficiency (%), a maximum/ theoretical ethanol yield of 0.511 $g.g^{-1}$ (Kang et al., 2014) was considered – **Equation 2.7**.

$$Conversion\ efficiency\ (\%) = \frac{Y_{ethanol/substrate}}{Y_{theoretical\ maximum}} \times 100 \qquad \textbf{Equation 2.8}$$

Consumed sugars (%) was calculated through the ratio between the final and initial sugar concentrations - **Equation 2.9**.

$$Consumed\ sugars\ (\%) = 1 - \frac{[glucose]f - [xylose]f}{[glucose]i - [xylose]i} \times 100 \qquad \textbf{Equation 2.9}$$

III. Results and discussion

3.1. Enzymatic hydrolysis of unbleached kraft pulp of *E. globulus* barks

Figure 16 shows the evolution of temperature, reducing sugars, pH and yield during the hydrolysis assays. Four assays were performed in order to obtain a high volume of hydrolysate.

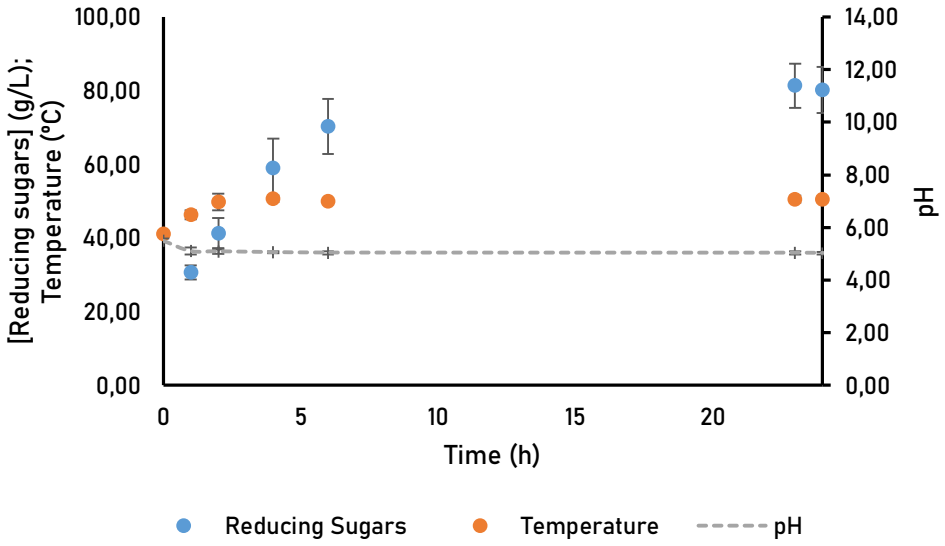


Figure 16: Temperature, reducing sugars and pH during hydrolysis assay.

Temperature took 2 h to stabilize at 50 ºC. The pH value was between 5.57 and 4.90 and, after 4 h of hydrolysis, stabilized around 5.05. The concentration of reducing sugars, as well as the hydrolysis yield, increased over time: among the four assays, the maximum values obtained were 85.34 $g.L^{-1}$ and 83.4 %, respectively. The total hydrolysis yield was 80.0 %. Yields of 78.8 % and 87.1 % were achieved regarding glucose and xylose. Branco et al., (2018) obtained glucose and xylose yields of 96.1 % and 94.0 % using unbleached kraft pulps of *E. globulus* wood hydrolysed by cellulolytic enzymes under similar hydrolysis conditions to the present work.

The yield of glucose from kraft cellulosic pulps depends not only on the pre-treatment pulps have undergone, but also on the origin of the pulp and its residual content of the lignin, which is represented by the Kappa number. The Kappa number of the unbleached kraft pulps used in this work was 15.2 (data provided by *RAIZ* – **Appendix A**).

Table 18 compares the total reducing sugars concentration obtained in this work with values reported for different cellulosic kraft pulps of eucalyptus, poplar, beech (hardwoods) and pine (softwoods) found in literature.

Theoretical glucose yield was slightly lower of those obtained by Buzała et al., (2017) for other hardwoods, probably because of hydrolysis inhibitors, more abundant in the utilised barks. The theoretical yield is also lower than that regarding the hydrolysis of *E. globulus* wood kraft pulps. In this work, a hydrolysate with a higher concentration of reducing sugars was obtained, which is beneficial regarding the volume of hydrolysate required for each fermentation assay and facilitates the storage of the hydrolysate itself.

Table 8: Kappa number, cellulose, hemicellulose and lignin content (% d.w.) of different cellulosic kraft pulps and total reducing sugars (g/L) in their hydrolysates.

Feedstock	Kappa number	Cellulose (% d.w.)	Hemicelluloses (% d.w.)	Lignin (% d.w.)	Y_{Glu} (% theoretical)	Total reducing sugars (g/L)	Ref.
Eucalyptus barks* (*E. globulus)*	15.2	83.0	14.7	2.3	80.0	80.2	This work
Eucalyptus wood (*E. globulus*)	16.0	82.7	14.6	2.7	96.1	81.5	(Branco et al., 2018)
Pine (*Pinus sylvestris)*	31.4	91.1	4.1	4.7	80.4	14.5	(Buzała et al., 2017)
Poplar (*Populus tremula)*	15.4	95.6	2.1	2.3	89.7	16.5	(Buzała et al., 2017)
Beech (*Fagus sylvatica*)	25.8	91.2	4.85	3.9	89.4	16.8	(Buzała et al., 2017)

*The pulp undergone an ethanol:water extraction that decreased the amount of extractive materials.

3.2. Ligand-exchange/size-exclusion chromatography (LEX/SEC) analysis of the hydrolysate

Semi-preparative ligand-exchange/size-exlusion analysis could give further insight about the composition of the hydrolysate. This prediction was made based on the molecular weight and charge of the compounds separated by the LEX/SEC, which were eluted with different retention times depending on their ionic charge and their size.

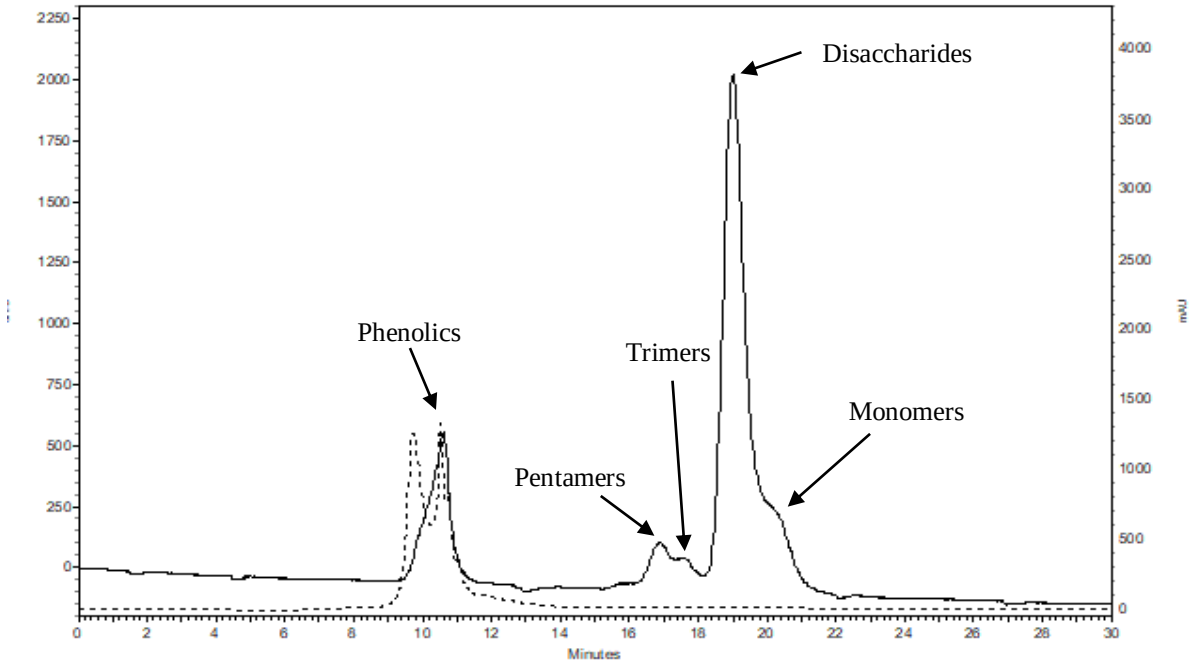


Figure 17: LEX/SEC chromatogram of the hydrolysate (dilution 1:4): --------- 280 nm and —— refractive index.

The association between the eluted compounds and their retention times was made based on the work of (Prozil et al., 2012). Lignin derived phenolic compounds could be the first to be eluted (10.6 min) due to its negative charge which is repelled by the column. Then, some uncharged sugar oligomers compounds could start to be eluted, starting with the pentamers at 16.9 min, followed by the trimmers at 17.6 min. Finally, dimers and monomeric sugars probably corresponded to the retention time of 19.0 min. The two last peaks overlapped in the chromatogram, not allowing for a clear distinction among them – **Figure 17** and **Table 9**. The disaccharides and other oligomers that possibly remained unhydrolysed, negatively affect the ethanol yield, especially in fermentation assays with *S. cerevisiae*, which unlike *S. stipitis* lacks the ability of degrading cellobiose and other

cellulose oligomers (Hu et al., 2016; Laplaza et al., 2007). Another problem for the posterior ethanol production is the considerable amount of phenolic compounds in the hydrolysate, that could correspond to the retention time of 10.6 min. Phenolics result from lignin degradation and can negatively affect the metabolism of fermenting microorganisms, acting as fermentation inhibitory agents and decreasing ethanol yield and fermentation productivity (Roque et al., 2019).

Table 9: Prediction of the composition of the hydrolysate, based on LEX/SEC analysis.

	Phenolics	**Pentamers**	**Trimers**	**Disacharides and monomers**
Retention time (min)	10.6	16.9	17.6	19.0
Content (%)	17.5	5.82	3.24	68.6

3.3. Assays with cultures of *S. cerevisiae*

S. cerevisiae is known to be the most effective microorganism when it comes to fermenting sugars to ethanol, mainly due to its high ethanol resistance and high tolerance to inhibitors. Hence, ethanol production with this yeast was firstly accessed in this work.

3.3.1 Erlenmeyer flask assays

The feasibility of ethanol production from hydrolysates of *E. globulus* barks by *S. cerevisiae* was firstly accessed by performing Erlenmeyer flask assays.

3.3.1.1. Erlenmeyer flask assay of *S. cerevisiae* in synthetic media

An Erlenmeyer flask fermentation assay with synthetic media (**Figure 18**) was performed in order to evaluate the capability of *S. cerevisiae* to successfully ferment the glucose in the experimental conditions that would be utilised with the hydrolysate.

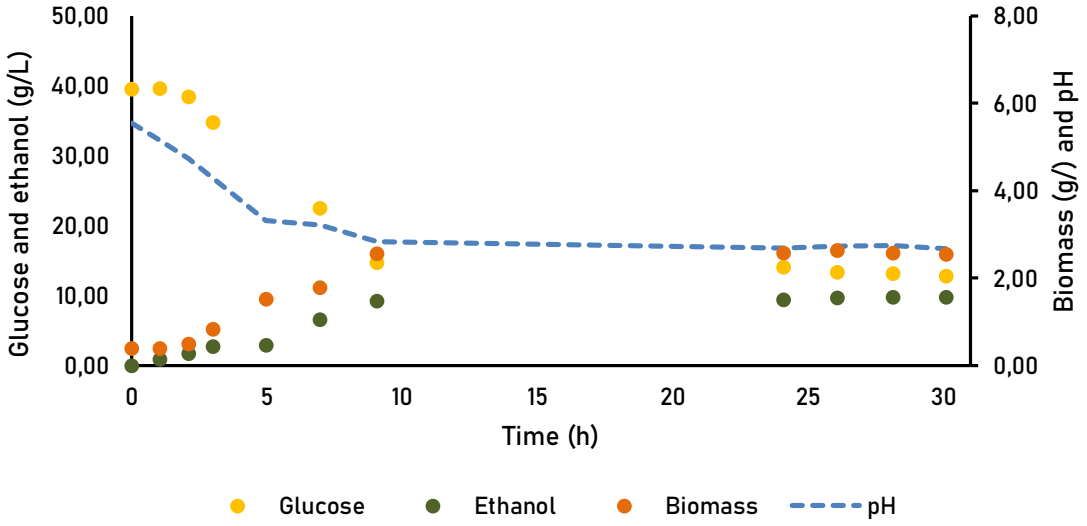


Figure 18: Evolution of pH and concentrations of glucose, ethanol and biomass in Erlenmeyer flask assay with *S. cerevisiae* in synthetic medium.

The lag phase lasted for 2.1 h, followed by an exponential growth phase of 6 h, in which a specific growth rate, μ, of 0.178 h^{-1} was achieved. Simultaneously, glucose consumption and ethanol production showed a linear behaviour. Seemingly, by 9 h fermentation was already concluded, since no significant biomass growth, glucose consumption or ethanol production were further observed. The pH decreased from 5.54

to 2.83 during the lag and exponential phases of yeast growth. This probably resulted formation of carbonic acid (H_2CO_3) which resulted from the dissolution of the CO_2 (released during cell growth and alcoholic fermentation) in water. The incomplete glucose consumption probably resulted from the low pH of 2.83 that has been reached, which is out of the yeast's optimal pH range that goes from 4 to 6. Until 9.1 h, 28.8 g/L of glucose or 62.8 % of the initial glucose added to the medium were consumed at a rate of 3.13 $g.L^{-1}.h^{-1}$. Up to 30 h of the assay, sugar exhaustion was not observed and 12.8 g/L of glucose were still left in the medium.

In this assay, a $Prod_{vol}$ of 1.03 $g.L^{-1}.h.^{-1}$, a $Y_{biomass/substrate}$ of 0.0943 $g.g^{-1}$ and a conversion efficiency of 67 % were achieved.

3.3.1.2. Erlenmeyer flask assay of mono-cultures of *S. cerevisiae* in hydrolysate

After studying the performance of *S. cerevisiae* in a synthetic medium, an essay in the same conditions using the hydrolysate instead of a sugar solution was conducted – **Figure 19**.

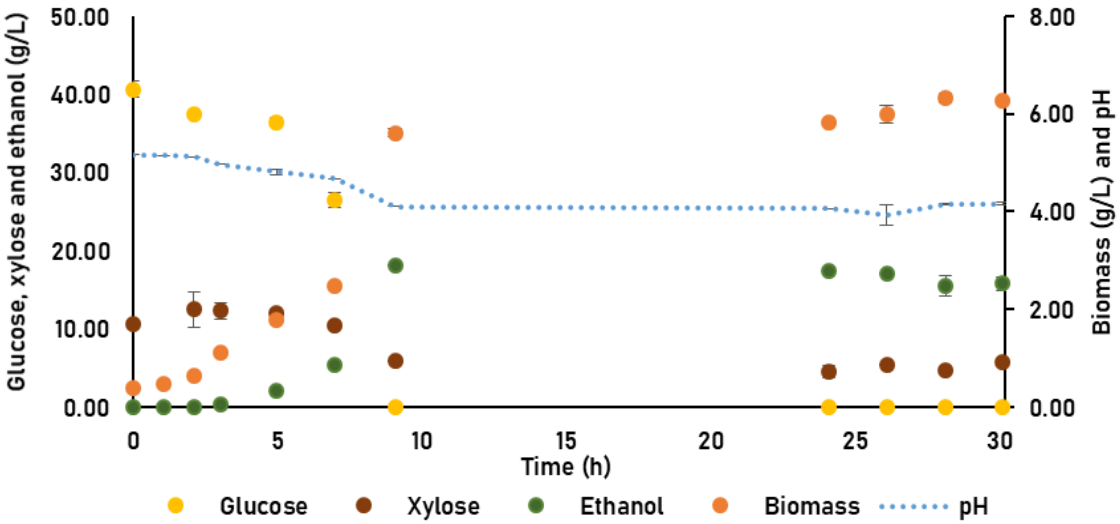


Figure 19: Evolution of pH and concentrations of glucose, xylose, ethanol and biomass in Erlenmeyer flask assay with *S. cerevisiae* in hydrolysate.

Similarly to the synthetic media assay, the lag phase of *S. cerevisiae* growth took 2.1 h and the exponential phase 7.0 h. Biomass concentration increased exponentially

while glucose was consumed at a rate of 3.89 $g.L^{-1}.h^{-1}$. Growth reached the stationary phase with a concentration of 5.81 g/L when glucose was completely exhausted at 9.1 h of fermentation, corresponding to the maximum concentration of ethanol detected by HPLC, 18.13 g/L. At 26 h, a slight increase in biomass and decrease of ethanol were observed, which indicates that *S. cerevisiae* might have shifted from rapid growth on glucose to slow growth on ethanol (Stahl et al., 2004). A slight decrease on the xylose concentration was also noticed, although more than half of the initial xylose present in the hydrolysate remained in the medium. The maximum biomass concentration obtained was 6.3 g/L in the last hours of the assay, which is more than double of the value obtained in the synthetic media assay. This is reflected by the maximum specific growth rate of 0.280 h^{-1} of the hydrolysate assay, which was higher than in the synthetic medium, 0.178 h^{-1}. Despite pH was not controlled during this assay, the citrate buffer used in the hydrolysis did not allow high variations in the pH (from 5.2 to 4.2), which benefited the viability of the fermentation. In this assay, a $Prod_{vol}$ of 1.99 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ of 0.115 $g.g^{-1}$ and a conversion efficiency of 78 % were achieved.

Ethanol production was significantly higher when using hydrolysate of kraft pulp of *E. globulus* barks, indicating this could be a promising substrate for bioethanol production. More efficient sugar consumption in the hydrolysate also relies on the fact that *S. cerevisiae* PYCC 5246 shows a slight capability of xylose utilization. Overall, a higher fermentation yield was achieved in the hydrolysate assay, proving the feasibility of the hydrolysate for bioethanol production.

3.3.2. Bioreactor assays

After attesting the feasibility of producing bioethanol from hydrolysates of *E. globulus* barks using *S. cerevisiae*, the process was scaled-up to a 3 L bioreactor.

3.3.2.1. *S. cerevisiae* in synthetic medium

A bioreactor fermentation assay with synthetic media (**Figure 20**) was performed in order to evaluate the capability of *S. cerevisiae* to successfully produce ethanol on a 3 L working volume and with pH control at 5.5.

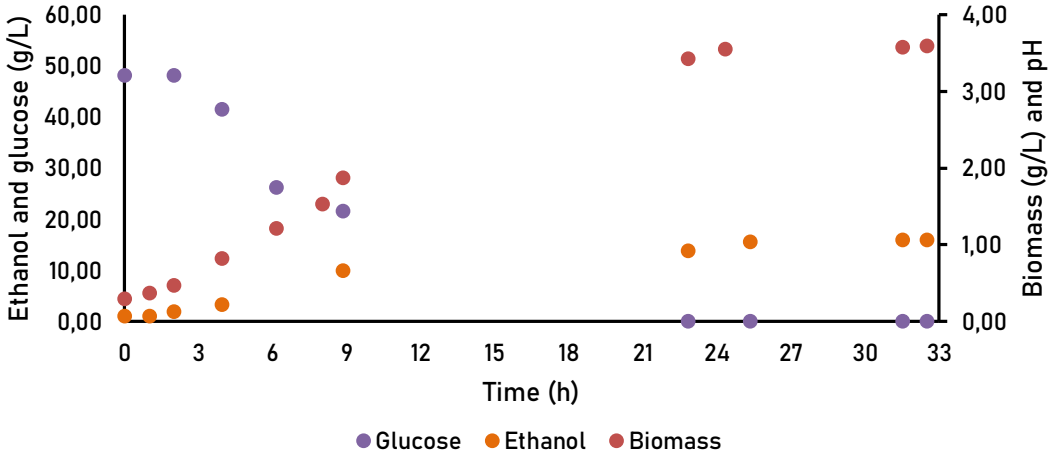


Figure 20: Evolution of concentrations of glucose, ethanol and biomass in bioreactor assay with *S. cerevisiae* in synthetic medium.

Although glucose exhaustion was detected by 22.8 h, the fermentation was completed only after 25 h, since 1.7 g of ethanol was still produced. Certainly this was due to carbon sources from the yeast extract. At 25.4 h, no glucose was detected in the media, and ethanol had already reached its maximum concentration, 15.9 $g.L^{-1}$.

Lag phase was not detected in this assay. Biomass concentration increased exponentially from 1.0 h from 6.2 h with a specific growth rate of 0.236 h^{-1}. By 25.4 h ethanol reached its maximum detected concentration, 15.9 $g.L^{-1}$, after glucose being exhausted from the medium. Due to pH control, no inhibition by very low pH values was observed.

Up to the end of the assay, which lasted 32 h, no ethanol reassimilation was observed to favour biomass growth. In this assay, a $Prod_{vol}$ of 1.13 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ of 0.0651 $g.g^{-1}$ and a conversion efficiency of 56 % were achieved.

3.3.2.2. *S. cerevisiae* in hydrolysate

After the bioreactor fermentation assay with the synthetic media (**Figure 21**) the capability of *S. cerevisiae* to successfully ferment the glucose in hydrolysate of *E. globulus* barks was evaluated on a 3 L working volume and with pH control at 5.5.

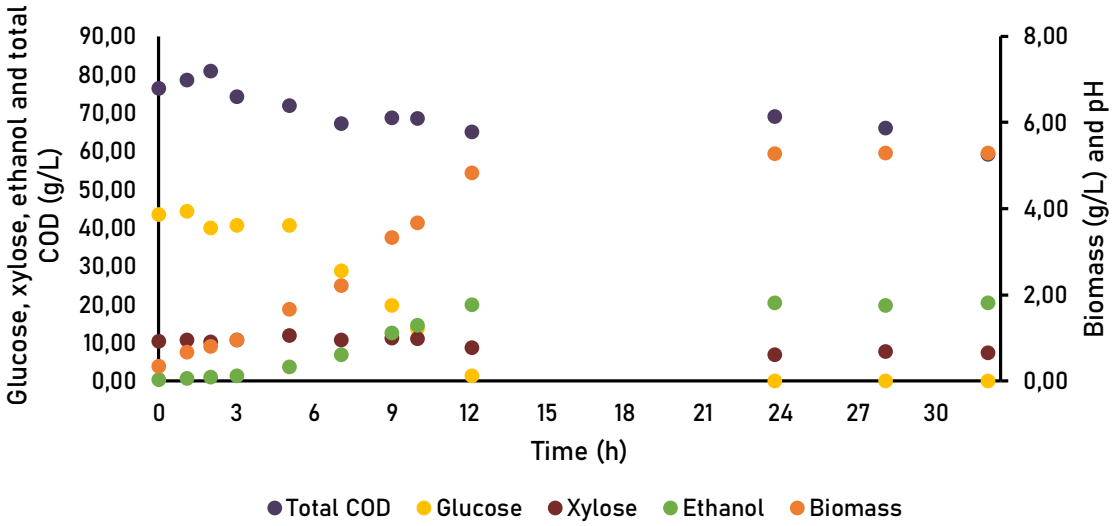


Figure 21: Evolution of concentration of total COD, glucose, ethanol and biomass in bioreactor assay with *S. cerevisiae* in hydrolysate.

Up to the first 12 h of fermentation, glucose in the media was almost completely assimilated by *S. cerevisiae*. By this time, ethanol concentration had already reached its maximum value, 20.4 $g.L^{-1}$. A slight decrease of xylose was observed, corresponding to 15 % of the total initial xylose.

Biomass concentration increased exponentially with a specific growth rate of 0.176 h^{-1}, which indicates a slower growth compared to the synthetic media assay and to the corresponding Erlenmeyer flask assay. The maximum biomass concentration obtained was also inferior to that obtained in the aforementioned assays. Since less ethanol was produced in those assays, this might indicate that carbon influx was more deviated towards ethanol production than to growth in the bioreactor with the hydrolysate.

Until the end of the assay, which lasted for 32 h, no ethanol reassimilation was observed, contrary to what happened in the Erlenmeyer flask assay. Ethanol reassimilation by *S. cerevisiae* is favoured by the increase of dissolved oxygen tension. In the bioreactor, the dissolved oxygen was lower, mainly due to a lower area of liquid-

air interface in relation to the total fermentation volume. Sampling procedures also contribute to a higher dissolved oxygen tension in the Erlenmeyer.

In this assay, a $Prod_{vol}$ of 1.64 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ of 0.112 $g.g^{-1}$ and a conversion efficiency of 85 % were achieved. Relatively to the corresponding Erlenmeyer flask assay, $Prod_{vol}$ was inferior which indicates that more time was necessary to reach the maximum ethanol concentration. $Y_{biomass/substrate}$ was also lower, indicating that a lower amount of the carbon flux was directed into growth, and instead to the fermentative metabolic pathway, which is confirmed by the higher amount of ethanol produced. In the assay with *S. cerevisiae* with the hydrolysate in the bioreactor the conversion efficiency was higher. As in both assays all glucose was totally exhausted, the increase of the conversion efficiency, probably resulted from the additional xylose conversion, which was slightly favoured in the bioreactor.

Table 10 compares kinetic parameters relating to assays with cultures of *S. cerevisiae* in both synthetic media and hydrolysate. Similar to the Erlenmeyer flask corresponding assays, a higher concentration of ethanol was achieved in the hydrolysate media. Also, glucose was consumed faster as well as ethanol was produced faster than in the synthetic media, as indicated by $r_{glucose}$ and $r_{ethanol}$. Besides, specific growth rate was higher in the bioreactor assay, indicating more carbon was directed into growth than into ethanol production.

These results indicate that most likely a component of the hydrolysate is favouring the growth and fermentation rates of *S. cerevisiae.* In order to access this hypothesis, chemical oxygen demand (COD) assays were performed in strategic samples taken throughout the fermentation assay (**Figure 21**) and in the hydrolysate itself. Samples removal of biomass was performed before analysing COD.

Total chemical oxygen demand decreased on the first 12 h of the assay, which can be explained by the yeasts cell growth and the release of CO_2 associated with the production of ethanol. On the last hours of the assay, between 23.8 h and 32.8 h, another decrease in the total COD value was observed. No biomass or ethanol were further produced, nor a depletion of xylose was noticed, which might indicate that *S. cerevisiae* is consuming an unknown carbon source present on the hydrolysate and utilising it for cell maintenance. Analysis of the evolution of the total chemical oxygen demand during the assay and the calculation of glucose, xylose and ethanol contributions to the total CQO revealed that, as expected, there are additional carbon sources beyond glucose, xylose or

even ethanol that *S. cerevisiae* might be utilising as well. *S. cerevisiae* PYCC 5264 is known to ferment maltose, raffinose and xylulose besides glucose, and to grow in maltose and trehalose besides glucose and ethanol.

3.4 Assays with cultures of *S. stipitis*

After verifying that *S. cerevisiae* could only ferment a small part of xylose present in the hydrolysate, an assay with the pentose fermenting yeast *S. stipitis* were performed. Despite being less tolerant to hydrolysate inhibitors and to ethanol, naturally occurring *S. stipitis* has the ability to utilise xylose much more efficiently than *S. cerevisiae*, therefore increasing ethanol production yield.

3.4.1. Erlenmeyer flask assay of cultures of *S. stipitis* in hydrolysate

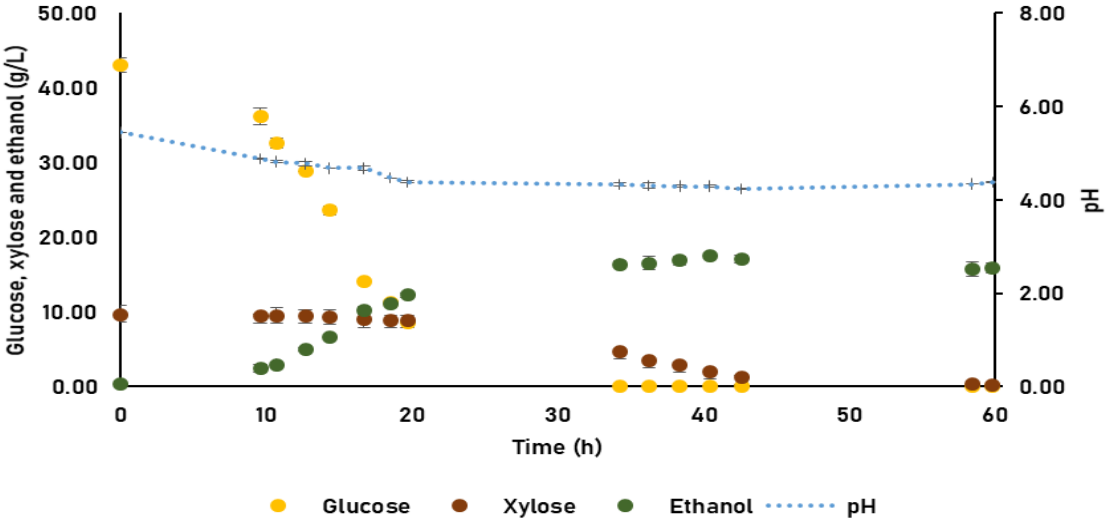


Figure 22: Evolution of pH and concentrations of glucose, xylose, ethanol and biomass in Erlenmeyer flask assay with *S. stipitis* in hydrolysate.

In the assay with *S. stipitis* in hydrolysate the biomass concentration could not be measured over time due to flocculation, **Figure 23,** and therefore determination of the parameters associated to growth could not be calculated. This was also reported by Danielle et al., (2016) and was associated with adverse environmental factors that cause cells to aggregate when in media containing harmful compounds, as could be the case of hydrolysates (Silva et al., 2014).

When feeding a mixture of glucose/xylose of 43/10 $g.L^{-1}$, glucose is the preferred substrate, once a sequential consumption of glucose and xylose was observed – **Figure**

22. This observation is corroborated by the work of Gutiérrez-Rivera et al. (2012) in which *S. stipitis* NRRL Y-7124 was also used to produce ethanol using mixtures of glucose and xylose. They verified that in mixtures with high sugar concentrations there was a sequential sugar consumption. However, when using mixtures with a low glucose/xylose concentration (25/10 g-L^{-1}), simultaneous sugar consumption was observed, which happens due to competition for the transport system.

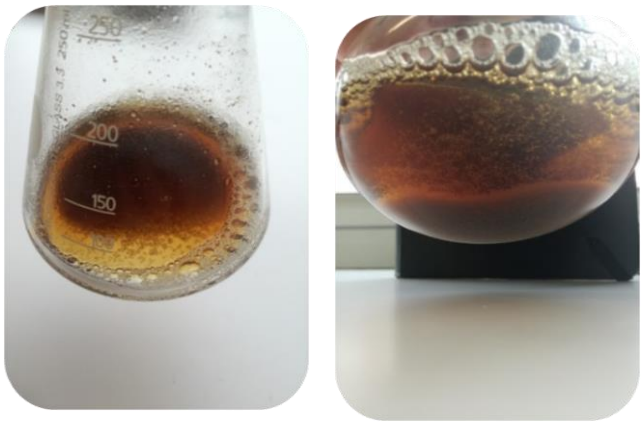


Figure 23: Flocculated *S. stipitis* in hydrolysate Erlenmeyer flask assays, at 18.5h of fermentation.

In this assay, all the sugars in the hydrolysate were consumed up to 45 h of fermentation. Glucose was firstly consumed by *S. stipitis* at a rate of 2.88 $g.L^{-1}.h^{-1}$, and its exhaustion was detected at 34.2 h of fermentation. After the depletion of glucose, xylose started to be utilised and was totally consumed at a rate of 0.402 $g.L^{-1}.h^{-1}$. *S. stipitis* further demonstrated faster glucose consumption than xylose. After 33 h of assay, a slight ethanol production was observed (0.68 $g.L^{-1}$) at the expense of a significant xylose consumption (3.64 $g.L^{-1}$). In this assay, a $Prod_{vol}$ of 0.433 $g.L^{-1}.h^{-1}$ and a conversion efficiency of 65 % were achieved.

Table 10 shows the fermentation kinetic parameters obtained in Erlenmeyer flask assays for both *S. cerevisiae* and *S. stipitis*. A higher concentration of ethanol, as well as a higher ethanol production rate were achieved in the assay using *S. cerevisiae*. The percentage of consumed sugars was higher in *S. stipitis* assay, due to the xylose consumption by *S. stipitis* which hardly happens with *S. cerevisiae*. In contrast, sugar uptake by *S. stipitis* is considerably slower, as reflected in the values of glucose and xylose consumption rate which are lower in *S. stipitis* assay.

The results obtained in the assays with *S. cerevisiae* and *S. stipitis* showed that *S. cerevisiae* is the fittest yeast to produce ethanol using hydrolysates from *E. globulus* at an industrial level, among the two studied in this work. Regardless *S. stipitis* being capable of fermenting xylose and therefore showing a higher sugar uptake than *S. cerevisiae*, the latter enables a higher produced ethanol concentration (18.1 g/L) in a shorter period, as expressed by the higher $r_{ethanol}$ (3.92 $g.L^{-1}.h^{-1}$).

3.5. Sequential co-culture of both *S. cerevisiae* and *S. stipitis*

Previous assays using *S. cerevisiae* and *S. stiptis* established that both of the yeasts could perform fermentation of hydrolysates from *E. globulus* barks kraft pulps. *S. cerevisiae* consumed all the glucose and 15 % of the xylose in the hydrolysate, and *S. stipitis* was able to deplete all the sugars available. Both strains were able to produce ethanol, corresponding the former to 18.1 $g.L^{.1}$ and the latter to 17.5 $g.L^{-1}$.

Hereupon, co-culture of both *S. cerevisiae* and *S. stipitis* was conducted aiming to the optimization of ethanol production. Panchal et al. (1988) studied the repression of xylose utilization by glucose presence in xylose fermenting yeasts and demonstrated that, in mixed sugar fermentation, *S. stipitis* requires the glucose concentration to be lower than 2 % (w/v) before significant xylose consumption can start. Taking this into account, and also that *S. stipitis* requires an aerated medium to grow, a sequential co-culture was performed, to avoid oxygen consumption by *S. cerevisiae*.

Fermentations where total glucose consumption was promoted by *S. cerevisiae*, were inoculated with *S. stipitis* in the fermentation broth in order to carry out further xylose consumption and maximize ethanol production.

3.5.1. Erlenmeyer flask assay of sequential co-culture of *S. cerevisiae* and *S. stipitis* in hydrolysate

Two co-culture assays were carried out in media containing hydrolysate. On the first one, *S. cerevisiae* was not removed from the fermentation broth before the inoculation of *S. stipitis* (assay A) - **Figure 24A**. On the other assay, *S. cerevisiae* was removed before the inoculation of *S. stipitis* (assay B) – **Figure 24B**.

Regarding assay A, after a lag phase of 3.2 h, *S. cerevisiae* grew exponentially with a specific growth rate of 0.249 h^{-1} until 9.0 h. As expected based on previous Erlenmeyer flask assays, glucose consumption rate, 1.85 $g.L^{-1}.h^{-1}$, was significantly higher than that concerning xylose, 0.21 $g.L^{-1}.h^{-1}$. At the end of the first fermentation stage, which lasted for 24h, no glucose was detected by HPLC, and only 9.9 % of the xylose had been consumed. In this stage, a $Y_{biomass/substrate}$ of 1.10 $g.g^{-1}$ and a conversion efficiency of 76 % were achieved. On the second fermentation stage, which started when *S. stipitis* was inoculated, a significant growth was detected, and biomass concentration

increased 2.2 g/L in 8.9 h. Nevertheless, the slight decrease in xylose concentration (1.07 g/L) did not appear to be sufficient to justify such an increase of biomass.

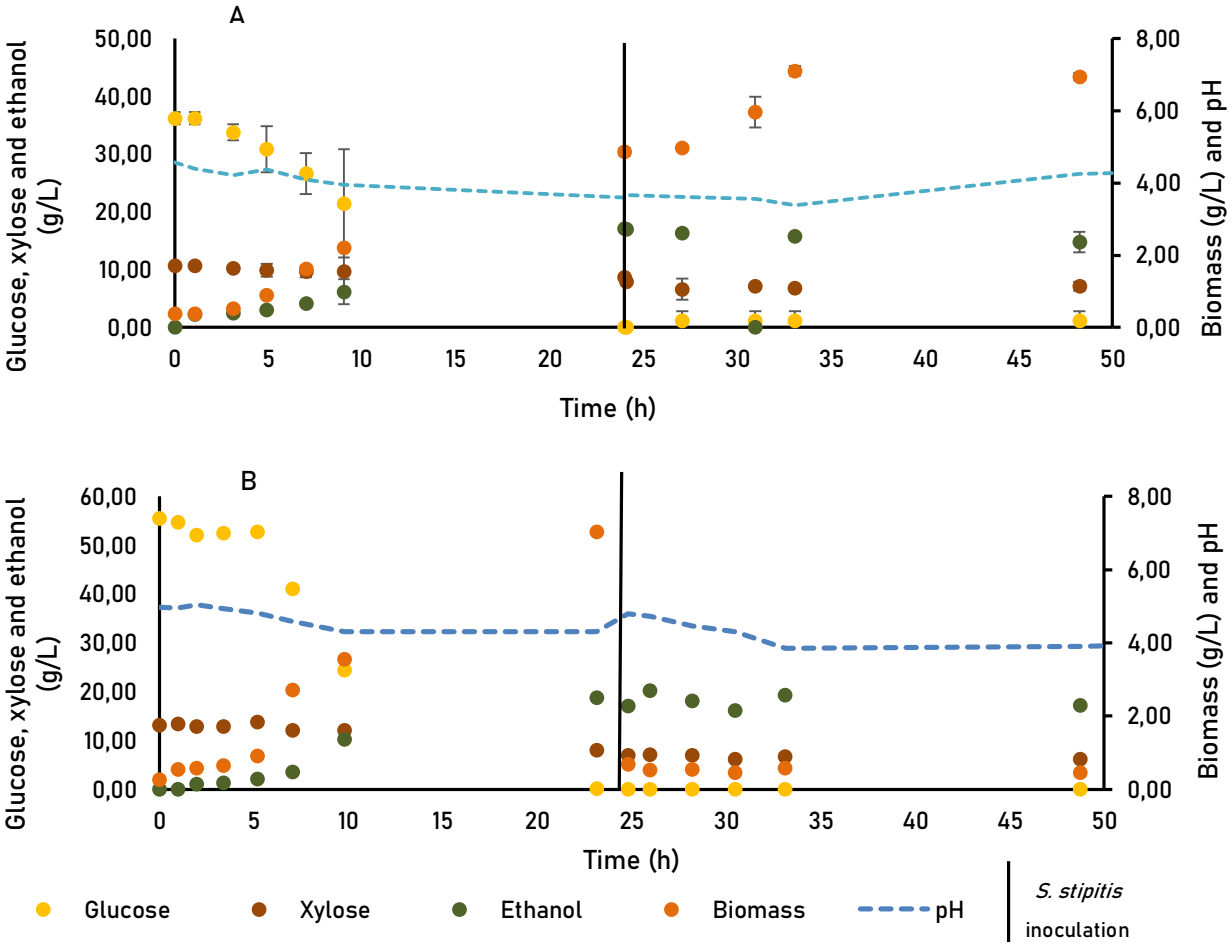


Figure 24: Evolution of concentration of glucose, ethanol and biomass in Erlenmeyer flask assay with two co-culture of *S. cerevisiae* and *S. stipitis*: without **(A)** and with **(B)** removal of *S. cerevisiae* of the fermentation broth before the inoculation of *S. stipitis*.

Assay **B** was performed in order to study *S. stipitis* behaviour in a media without glucose and containing a considerably amount of ethanol. In this case, *S. stipitis* was not able to grow using the hydrolysate fermentation media. Given that no biomass growth or sugar consumption was observed after the inoculation of *S. stipitis* (24.8 h), it is inferred that the already existing amount of ethanol in the media precluded the yeast activity. Ethanol alters cell membrane organization and permeability, therefore causing damage to the cell membrane and ultimately stopping the yeasts growth. Similar results regarding ethanol inhibition on *S. stipitis* were obtained by (Gutiérrez-Rivera et al., 2012) while

studying the conversion efficiency of glucose/xylose mixtures for ethanol production by *S. stipitis* NRRL Y-7124. In this work, the yeast metabolism was inhibited by ethanol concentrations below 30 $g.L^{-1}$. Taking this into account, the observed biomass growth is most likely due to *S. cerevisae*, which, besides xylose and ethanol, is possibly feeding on yeast extract coming from the inoculum of *S. stipitis*.

3.5.2. Bioreactor sequential co-culture of *S. cerevisiae* and *S. stipitis* in hydrolysate

A sequential co-culture assay (**Figure 25**) was performed in the bioreactor, since it allows to control the dissolved oxygen tension (DOT) in the fermentation medium. The control of the DOT assumes a crucial role in fermentation assays with *S. stipitis* once this yeast produces ethanol under microaerophilic conditions (Degn et al., 1992).

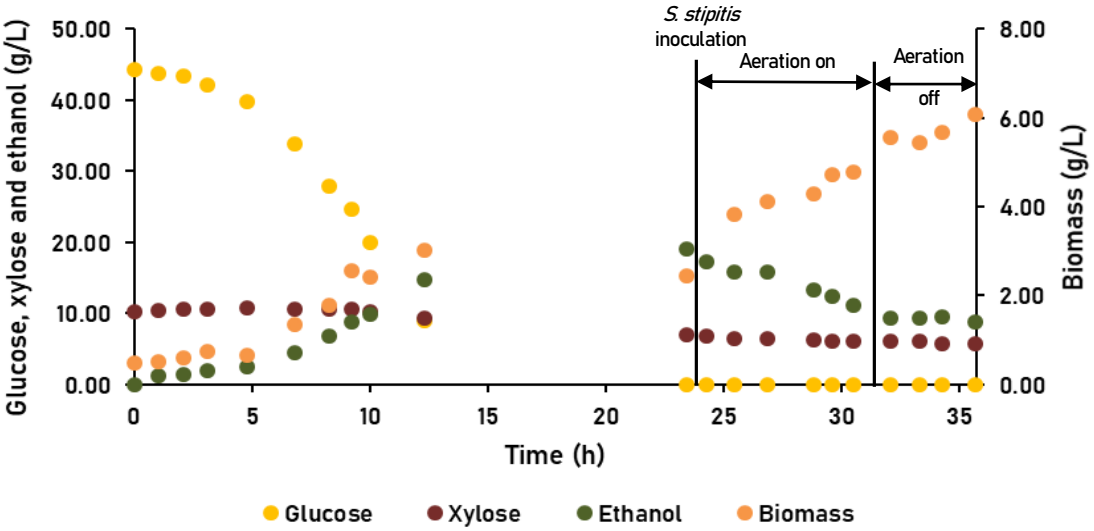


Figure 25: Evolution of concentration of glucose, ethanol and biomass in bioreactor assay with co-cultures of *S. cerevisiae* and *S. stipitis*.

On the first stage of the assay only *S. cerevisiae* was present. Glucose was fully exhausted. Although the moment when glucose ran out was not detected, it was consumed at a 3.61 $g.L^{-1}.h^{-1}$ rate up to 12h. 30 % of the xylose was consumed by *S. cerevisiae* at a rate of 0.403 $g.L^{-1}.h^{-1}$ until 2 h. When *S. stipitis* was added, aeration in the bioreactor was turned on and DOT was maintained at approximately 30 %. Until de end of the assay, 1.51 g/L of xylose were consumed by *S. stipitis*. Plus, a significant decrease of 51 % in the ethanol concentration was observed, along with a spike on the biomass concentration, indicating that under aerobic conditions ethanol is reassimilated by this yeast. Reassimilated ethanol is oxidized into acetaldehyde, a known inhibitor of a wide range of metabolic activities (Degn et al., 1992). Therefore, *S. stipitis* activity stopped, and no xylose or ethanol consumption was further observed, neither significant biomass growth. Despite the addition of a pentose fermenting yeast, no further ethanol production occurred. However, *S. cerevisae* was still able to achieve a maximum of 19.1 g/L of ethanol.

3.6. Overall results discussion

The highest concentration of ethanol obtained was in the assay with a culture of *S. cerevisiae* in the bioreactor in hydrolysate supplemented with SM. 46.6 g/L of glucose were consumed in 12 h, corresponding to a $Y_{P/S}$ of 0.434 $g.L^{-1}.h^{-1}$ – **Table 10**. This means that from 130 g of dry pulp (or 606 g of wet pulp), 61.2 g of ethanol were obtained. Regarding bioethanol production from eucalyptus hydrolysates, similar results were obtained by Mcintosh et al. (2016) using *Eucalyptus grandis* biomass pre-treated by dilute acid and steam explosion: in this work, 19.14 g/L of ethanol were obtained using an industrial strain of *S. cerevisiae*, corresponding to a $Y_{Ethanol/substrate}$ of 0.430 $g.L^{-1}.h^{-1}$. Cunha et al. (2017) studied bioethanol production by *S. cerevisiae* CEN.PK113-PD from hydrolysates of *E. globulus* pretreated with autohydrolysis supplemented with cheese whey aiming to valorise two raw materials and produce high concentrations of ethanol. In their work, 93 $g.L^{-1}$ of ethanol were obtained.

In the present work, the best results concerning xylose consumption by *S. cerevisiae* PYCC 5264 were observed in the Erlenmeyer assay in hydrolysate, where 4.9 $g.L^{-1}$ of xylose were consumed in 30 h. These results showed that, in fact, this strain is advantageous when it comes to xylose consumption. Gong et al., (1983) analysed xylose consumption capability of 21 strains and species of *S. cerevisiae*. Among them, the best results were obtained with *S. cerevisiae* ATCC 26497, which in 3 days consumed 1.6 $g.L^{-1}$ of xylose, from an initial concentration of 50 $g.L^{-1}$. In another work, *S. cerevisiae* BY4741 was able to ferment 2 $g.L^{-1}$ of xylose in 10 days Träff et al. (2002). More recently, Sharma et al. (2018) used *S. cerevisiae* ITCC 8246 to ferment paddy straws and a consumption of 3.7 $g.L^{-1}$ of xylose was observed. But better results were obtained with *S. cerevisiae* YB2625 (Cheng et al., 2018) which consumed 15.2 $g.L^{-1}$ of xylose from in 96 h.

S. stiptis showed to be effective in the production of ethanol from the utilised hydrolysate, having produced 17.5 $g.L^{-1}$ of ethanol. Scordia et al., (2012) used *S. stipitis* CBS 6054 to produce ethanol from giant reed hydrolysates (*Arundo donax* L.) and obtained 8.20 $g.L^{-1}$ in 48 h. When using this strain to ferment hydrolysates of *Miscanthus giganteus*, 20.2 $g.L^{-1}$ were obtained (Scordia et al., 2013). Kumar et al. (2009) obtained slightly better results by using *S. stipitis* NCIM-3497 to ferment hydrolysates of water

hyacinth (*Eichhornia crassipes*) and obtained 21.2 $g.L^{-1}$ of ethanol, corresponding to 72.83 % of total xylose utilisation.

The use of a co-culture of *S. cerevisiae* and *S. sipitis* did not favour ethanol production in this work. Similar conclusions were drawn by Gutiérrez-Rivera et al., (2012) who also did not take significant advantage of *S. stipitis* addition after total glucose consumption by *S. cerevisiae*. More recently, Rojas-Chamorro et al. (2019) also reported a total glucose consumption by *S. cerevisiae* and negligible further sugar consumption when using this co-culture to produce ethanol from the slurry of pretreated brewers spent grain.

However, more promising results were achieved by Singh et al. (2013) using hydrolysates of alkali pretreated rice husks. With a 1:1 proportion of *S. cerevisiae* and *S. stipitis*, Singh obtained 20 $g.L^{-1}$ of ethanol with 78.4 % of sugars conversion. Another example of a successful co-culture of these yeasts is the work of Yadav et al. (2011), which produced 12 $g.L^{-1}$ of bioethanol from concentrated and detoxified rice straw hydrolysates.

Table 10: Comparative results of the assays in the present work.

	S. cerevisiae				***S. stipitis***	**Co-culture of *S. cerevisiae* and *S. stipitis***	
	Erlenmeyer		**Bioreactor**		**Erlenmeyer**	**Erlenmeyer**	**Bioreactor**
	Synthetic media (a)	Hydrolysate	Synthetic media (a)	Hydrolysate	Hydrolysate (b)	Hydrolysate (c)	Hydrolysate (c)
μ (h^{-1})	0.178	0.280	0.192	0.176	-	0.249	0.148
$[Ethanol]_{max}$ ($g.L^{-1}$)	9.2	18.1	15.93	20.4	17.5	18.1	19.1
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	3.13	3.90	4.14	5.47	2.88	1.85	3.60
r_{xylose} ($g.L^{-1}.h^{-1}$)	-	0.515	-	0.364	0.402	0.21	0.402
$r_{ethanol}$ ($g.L^{-1}.h^{-1}$)	1.53	3.92	1.21	2.34	9.02	0.76	1.65
Prod. vol. ($g.L^{-1}.h^{-1}$)	1.02	2.00	1.13	1.64	0.433	-	-
$Y_{Ethanol/substrate}$ ($g.g^{-1}$)	0.345	0.399	0.288	0.434	0.332	0.386	0.382
$Y_{Xylose/substrate}$ ($g.g^{-1}$)	0.0958	0.155	0.0651	0.112	-	0.104	0.122
Conversion efficiency (%)	67.4	78.0	56.4	84.9	65.0	75.6	74.8
Consumed sugars (%)	67.7	88.4	100	87.1	96.2	56.0	91.7
Consumed sugars (g/L)	26.8	45.5	48.1	46.9	52.6	40.3	50.0

(a) Xylose was not added in the fermentation medium; (b) Yeast flocculation has appeared; (c) Maximum ethanol was not detected.

IV. Conclusions

The present work aimed to produce second generation bioethanol using hydrolysates of *E. globulus* barks, and to add value to the residues generated by the Pulp and Paper sector, one of the most relevant in the Portuguese industry. To accomplish such purpose, leftovers of the pulping process were used as a substrate, and the employed pre-treatment was the kraft process, already integrated in the existing paper factories.

The viability of this process was accessed using two fermentative yeasts, *S. cerevisiae* and *S. stipitis*. The former is known for achieving high fermentation yields and for being tolerant to inhibitors, harsh environmental conditions and to ethanol itself. The latter, despite being less tolerant to inhibitors and to ethanol, has the ability of fermenting pentoses, unlike *S. cerevisiae*.

The results of this work showed that hydrolysates of *E. globulus* barks are a promising substrate for the production of second generation bioethanol by *S. cerevisiae*. Despite not utilizing pentoses such as xylose to produce ethanol, assays with *S. cerevisiae* resulted in higher ethanol/sugar yields, ethanol productivities and conversion efficiencies than those with *S. stipitis*. The most promising results were achieved in the bioreactor assay in which 20.37 $g.L^{-1}$ of ethanol were obtained, with an ethanol/sugars yield of 0.434 $g.g^{-1}$ and a conversion efficiency of 85 %. These results surpass those of the corresponding Erlenmeyer assays due to a better regulation of the fermentation operational conditions (pH, temperature and stirring rate) of the bioreactor.

As future work, a fed-batch fermentation approach should be investigated, aiming to maximize ethanol production in each assay. Pentose fermentation with metabolic engineered *S. cerevisiae* should be tested in order to maximize sugar intake and, consequently, ethanol production.

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Appendixes

Appendix A – Hydrolysis calculations

Table 11 shows the characterization, provided by *RAIZ*, in terms of percentage of lignin, sugars, cellulose, hemicellulose and moisture of the *E. globulus* unbleached pulp.

Table 11: Characterization of the *E. globulus* unbleached pulp (%)

Kappa index	Lignin	Sugars	Cellulose	Hemicellulose	Moisture
15.2	2.3	97.7	83	15	73.2

Previous hydrolysis assays established that 250 g of dry kraft pulp ($m_{dry\ pulp}$) should be hydrolysed with 200 mL of enzymatic solution (ES), in a total volume of 3.0 L ($V_{Hydrolysis}$).

250 g of dry pulp correspond to 932.8 g of moist pulp, which were added to each hydrolysis assay. Calculations were made basing on the moisture content (MC) of the pulp:

$$m_{moist\ pulp} = \frac{\text{Dry weight (g)}}{1-\text{MC}} = \frac{250}{1-0.732} = 932.8\ \text{g} \qquad \textbf{Equation A.1}$$

The volume of citrate buffer necessary to attain the intended hydrolysis volume of 3.0 L is determined by the volumes of enzymatic solution and water in the pulp. – **Equation A.2**.

$$V_{buffer}\ (\text{L}) = V_{Hydrolysis} - (V_{ES} + V_{water\ in\ pulp}) \qquad \textbf{Equation A.2}$$

In turn, the volume of water in the pulp is calculated subtracting the mass of dry pulp from the mass of moist pulp. To obtain the volume of water, its density is taken into consideration:

$$V_{\text{water in pulp}}\ (\text{mL}) = \frac{m\ moist\ pulp\ (g) - m\ dry\ pulp\ (g)}{\rho\ \text{water}\ (\text{g/mL})}$$

$$= \frac{932.8 - 250}{1} = 682.8\ \text{mL} \qquad \textbf{Equation A.3}$$

Therefore, **Equation A.2** comes:

$$V_{\text{buffer}}\ (\text{L}) = V_{\text{Hydrolysis}} - (V_{\text{ES}} + V_{\text{water in pulp}})$$

$$= 3.0 - (0.200 + 682.8) \times 10^{-3} = 2.218\ \text{L}$$

The volume of enzyme (V_{Enzyme}) necessary to prepare a 200 mL enzymatic solution takes into account the enzymatic activity of the enzymatic cocktail and the mass of the carbohydrates in the kraft pulp (m_{ch}) is given by **Equation A.3** and **Equation A.4**.

$$\text{Enzymatic load (FPU)} = \text{Enzymatic load (FPU.g ch}^{-1}) \times m_{ch} \qquad \textbf{Equation A.3}$$

$$= \text{Enzymatic load (FPU.g ch}^{-1}) \times m_{\text{dry pulp}} \times \text{Sugars}$$

$$= 25 \times 250 \times 0.977 = 6106\ \text{FPU}$$

$$V_{\text{Enzyme}} = \frac{Enzymatic\ load\ (FPU)}{Enzymatic\ load\ (FPU.mL-1)} = \frac{6106}{133.5} = 45.738\ \text{mL} \qquad \textbf{Equation A.4}$$

Appendix B – Calculation of potential glucose and xylose in the kraft pulp

Equation B1 represents the hydrolysis of cellulose. The hydrolysis of hemicelluloses are expressed by the hydrolysis of xylan and are represented by **Equation B2**. The maximum theoretical yield of glucose, $Y_{Glu/Cellulose}$ is given by the ratio between the molecular weight of glucose and the molecular weight of the anhydroglucose of cellulose. Therefore, 1.111 g of glucose result from 1 g of cellulose. The maximum theoretical yield of xylose, $Y_{Xyl/Hemicellulose,}$ is given by the molecular weight of xylose and the molecular weight of the anhydroxyloses. Hence, 1.136 g of xylose are released from 1 g of hemicelluloses.

$$(C_6H_{10}O_5)_n + {}_nH_2O \longrightarrow {}_nC_6H_{12}O_6 \qquad \textbf{Equation B1}$$

$$(C_5H_8O_4)_n + {}_nH_2O \longrightarrow {}_nC_5H_{10}O_5 \qquad \textbf{Equation B2}$$

The kraft pulp used in the present work had 83 % of cellulose and 15 % of hemicelluloses is 97.7 % of sugars – **Table A1**. Therefore, the percentage of cellulose and xylose among the sugars in the pulp is 85 and 15 %, respectively. Once the mass of carbohydrates is 244 g, the mass of cellulose and hemicelluloses is 207.8 and 36.5 g, respectively.

The mass of cellulose is used to calculate the potential glucose, corresponding to the mass of glucose obtained if all the cellulose present in the pulp were completely hydrolysed into glucose, taking into account the maximum theoretical yield of glucose – **Equation B3**. Similarly, the potential xylose is calculated with the mass of hemicelluloses and the theoretical yield of xylose – **Equation B4**.

$$\text{Potential glucose (g)} = m_{cellulose}\,(g) \times Y_{Glu/Cellulose}\,(g.g^{-1}) = 230.8\ g \qquad \textbf{Equation B3}$$

$$\text{Potential xylose (g)} = m_{xylose}\,(g) \times Y_{Xyl/Hemicellulose}\,(g.g^{-1}) = 41.5\ g \qquad \textbf{Equation B4}$$

Appendix C – Calculations of chemical oxygen demand

COD corresponding to ethanol, xylose and glucose were calculated by the ratio of the molecular weight of the number of moles of oxygen required to oxidize the given compound and the molecular weight of the number of moles of oxidized compound.

Therefore, **Equations C1-3**, respectively, express COD of ethanol, xylose and glucose, inferred through the oxidation reactions of those compounds, **Equations C4-6**, respectively.

$$COD_{ethanol} = \frac{3 \times 32}{46} = 2.09 \text{ g } O_2 \text{ / g ethanol} \qquad \textbf{Equation C1}$$

$$COD_{xylose} = \frac{5 \times 32}{150} = 1.07 \text{ g } O_2 \text{ / g xylose} \qquad \textbf{Equation C2}$$

$$COD_{glucose} = \frac{6 \times 32}{180} = 1.07 \text{ g } O_2 \text{ / g glucose} \qquad \textbf{Equation C3}$$

$$C_2H_6O + 3O_2 \longrightarrow 2CO_2 + 3H_2O \qquad \textbf{Equation C4}$$

$$C_6H_{10}O_6 + 5O_2 \longrightarrow 5CO_2 + 5H_2O \qquad \textbf{Equation C5}$$

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \qquad \textbf{Equation C6}$$

Appendix D – Spectrum of the hydrolysate

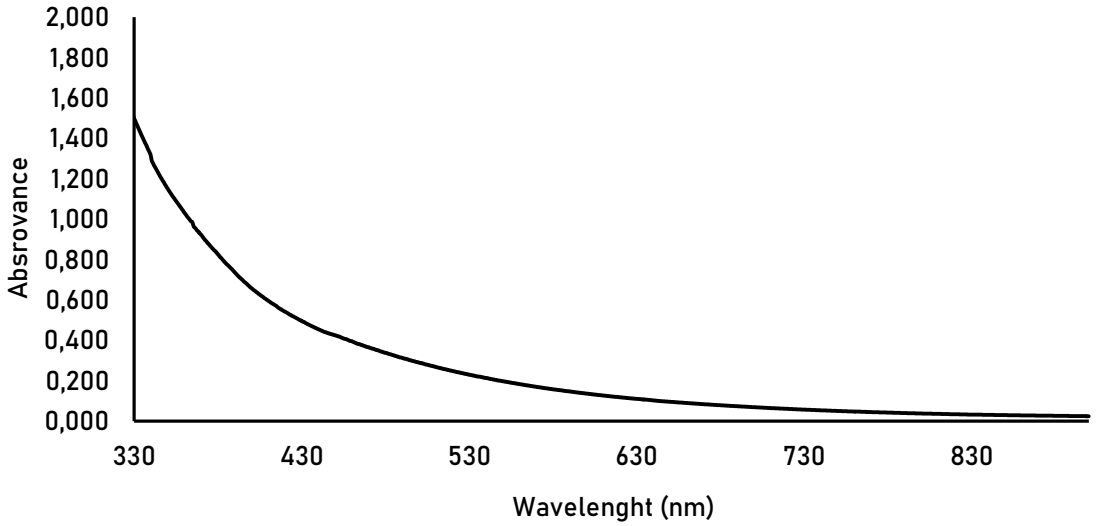

Figure 26: UV-Vis spectrum of the hydrolysate, between 330 and 900 nm.

Appendix E – Biomass growth calibration curve

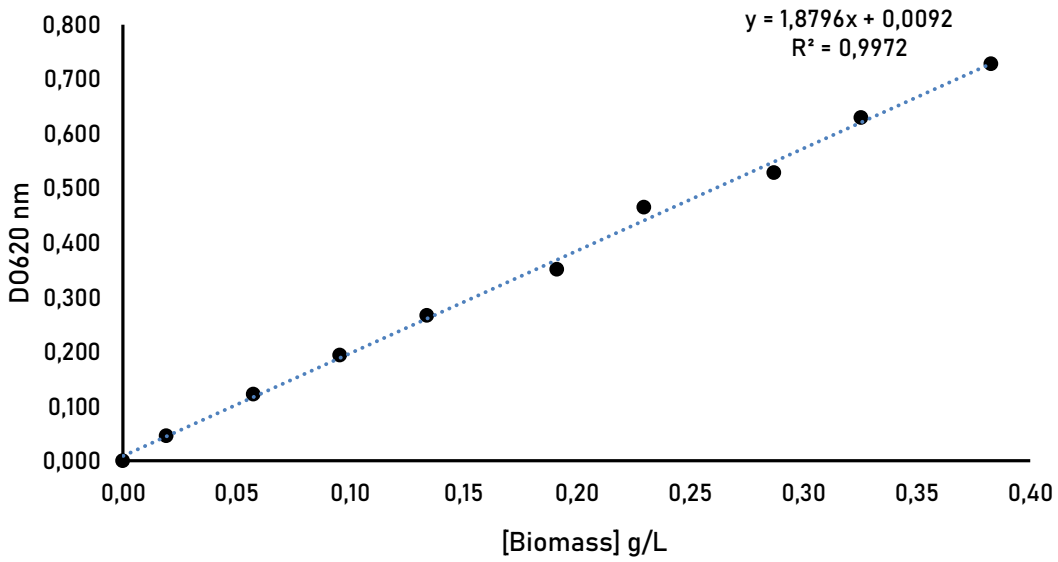

Figure 27: Example of a calibration curve of biomass growth made for *S. cerevisiae* in hydrolysate supplemented with SM.