Universidade de Aveiro Departamento de Química
Ano 2018

RITA HENRIQUES REBOLA BRANCO

Produção de Bioetanol de 2ª Geração numa lógica de Economia Circular

2nd Generation Bioethanol Production in terms of Circular Economy

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, Ramo de Biotecnologia 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 Leal, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave

bioetanol, pasta Kraft, hidrólise e fermentação separadas, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, mono-cultura, co-cultura.

resumo

A Economia Circular (CE) é uma solução promissora para os fluxos de materiais e de energia que tornaram insustentável o sistema económico atual. As biorrefinarias são cruciais para a CE, uma vez que processam todas as frações da biomassa para a coprodução de vários produtos e energia, minimizando a geração de resíduos. O bioetanol, devido à menor emissão de gases de efeito de estufa, apresenta-se como uma potencial alternativa aos combustíveis fósseis, os quais têm um impacto negativo na saúde e no ambiente. O bioetanol é atualmente o biocombustível mais produzido e deriva quase na sua totalidade de culturas alimentares, levando a uma competição entre alimento e combustível. Em alternativa, o bioetanol de segunda geração é produzido a partir de biomassa lenhocelulósica (LCB), mas requer um pré-tratamento caro e tecnicamente difícil. Na indústria papeleira o cozimento Kraft remove a lenhina das madeiras e afeta as hemiceluloses, libertando a celulose podendo, por isso, ser considerado um pré-tratamento de LCB. Um pré-tratamento com base no cozimento Kraft, seguido por hidrólise dos polissacarídeos que podem sofrer subsequente fermentação, surge como uma abordagem promissora para valorizar resíduos da indústria da pasta e do papel, convertendo as fábricas existentes em biorrefinarias integradas.

O objetivo deste trabalho foi estudar a produção de bioetanol pelas leveduras *Saccharomyces cerevisiae* e *Scheffersomyces stipitis* a partir de pasta Kraft não branqueada de *Eucalyptus globulus*, adotando o cozimento Kraft como pré-tratamento da LCB. Da hidrólise enzimática da pasta Kraft resultaram hidrolisados com 65.4 ± 0.8 $g.L^{-1}$ de glucose e 16.0 ± 1.8 $g.L^{-1}$ de xilose, obtendo-se um rendimento de 95.6 ± 2.6 %. Nos ensaios em balão Erlenmeyer com mono-cultura, a levedura *S. cerevisiae* produziu uma maior quantidade de etanol que *S. stipitis*, obtendo-se 19.81 ± 0.15 $g.L^{-1}$, que corresponderam a um rendimento em etanol de 0.450 ± 0.009 $g.g^{-1}$, e uma produtividade de etanol de 2.01 ± 0.01 $g.L^{-1}.h^{-1}$. Nem a co-cultura sequencial nem a co-cultura simultânea das duas leveduras resultou numa melhoria significativa da produção de bioetanol quando comparadas com a mono-cultura de *S. cerevisiae*.

A fermentação do hidrolisado de pasta Kraft por *S. cerevisiae* em bioreator resultou numa concentração de etanol de 19.24 $g.L^{-1}$, num rendimento em etanol de 0.433 $g.g^{-1}$, e numa produtividade de etanol de 0.733 $g.L^{-1}.h^{-1}$. Também em bioreator, a utilização de co-cultura sequencial não resultou numa melhoria em relação à mono-cultura de *S. cerevisiae*.

O elevado rendimento e produtividade de etanol obtido na fermentação de hidrolisado de pasta Kraft de *E. globulus* pela mono-cultura de *S. cerevisiae* demonstra que este é um processo promissor para a produção de bioetanol de segunda geração. Tendo em conta estes resultados, a produção de bioetanol a partir de resíduos da indústria da pasta e do papel, como madeira de baixa qualidade, cascas e outros resíduos, bem como pasta de baixa qualidade e em excesso, é uma potencial oportunidade para a implementação de biorrefinarias integradas nas plantas fabris de cozimento Kraft.

keywords

bioethanol, Kraft pulp, separate hydrolysis and fermentation, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, mono-culture, co-culture.

abstract

Circular Economy (CE) is a promising solution for the unsustainable material and energy flow model of the current economic system. Biorefineries are crucial for CE as they process all fractions of biomass to co-produce a multiplicity of products and energy, minimizing waste generation. Bioethanol, with lower greenhouse gasses emissions, is a potential alternative to fossil fuels, which have negative impacts in health and environment. Bioethanol is currently the most produced biofuel and is almost entirely of first generation since it is produced from food crops, leading to food-fuel competition. In alternative, second generation bioethanol is produced from lignocellulosic biomass (LCB) but requires a costly and technically difficult pretreatment. In pulp and paper industry the Kraft pulping step removes lignin and targets hemicelluloses, releasing cellulose, and therefore can be considered as a pretreatment of LCB. A process based on Kraft pulping followed by the hydrolysis of polysaccharides and subsequent fermentation emerges as a promising approach to valorise wastes resulting from pulp and paper industry, converting the existing mills in integrated biorefineries.

The aim of this work was to study the production of bioethanol using yeast *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* from unbleached Kraft pulp of *Eucalyptus globulus,* exploiting the Kraft pulping process as LCB pretreatment.

Enzymatic hydrolysis of unbleached Kraft pulp of *E. globulus* released hydrolysates with 65.4 ± 0.8 $g.L^{-1}$ of glucose and 16.0 ± 1.8 $g.L^{-1}$ of xylose, corresponding to a yield of 95.6 ± 2.6 %. In Erlenmeyer flask assays, *S. cerevisiae* yeast showed higher ethanol concentration, 19.81 ± 0.15 $g.L^{-1}$, ethanol yield, 0.450 ± 0.009 $g.g^{-1}$, and ethanol productivity, 2.01 ± 0.01 $g.L^{-1}.h^{-1}$, than *Scheffersomyces stipitis*. Neither *S. cerevisiae* and *S. stipitis* sequential co-culture nor simultaneous co-culture showed a significant improvement in these parameters compared with *S. cerevisiae* mono-culture.

S. cerevisiae mono-culture fermentation of Kraft pulp hydrolysate in bioreactor resulted in an ethanol concentration of 19.24 $g.L^{-1}$, an ethanol yield of 0.433 $g.g^{-1}$, and an ethanol productivity of 0.733 $g.L^{-1}.h^{-1}$. Also, in bioreactor, sequential co-culture did not show any improvement from *S. cerevisiae* mono-culture.

The high ethanol yield and productivity obtained by *S. cerevisiae* mono-culture fermentation of *E. globulus* Kraft pulp hydrolysate show that this is a promising process for second generation bioethanol production. Also, given these results, it appears that producing bioethanol from pulp and paper industry wastes, such as low-quality wood, bark, and other rejects, as well as low-quality and excess pulp, is a potential opportunity for implementing integrated biorefineries in the existing Kraft pulp mills.

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List of abbreviations

AD	Anaerobic Digestion
ADH	Alcohol Dehydrogenase
ATP	Adenosine Triphosphate
BG	B-Glucosidases
CBH	Cellobiohydrolases
CBP	Consolidated Bioprocessing
CE	Circular Economy
COD	Chemical Oxygen Demand
DNS method	Dinitrosalicylic Acid method
EG	Endoglucanases
EMP	Embdem-Meyenhorf-Parnas Pathway
EU	European Union
FPU	Filter Paper Unit
GHG	Greenhouse Gases
HMF	5-Hydroxymethyl-2-Furaldehyde
HPLC	High Performance Liquid Chromatography
HSSL	Hardwood Spent Sulphite Liquor
LCB	Lignocellulosic Biomass
LPMO	Lytic Polysaccharide Monooxygenases
MSW	Municipal Solid Wastes
NA	Not Available
NAD^+/NADH	Nicotinamide Adenine Dinucleotide (oxidized/reduced forms)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NEG	Net Energy Gain
NS	Not Specified
OD_{620}	Optical Density at 620 nm
PDC	Pyruvate Decarboxylase
$Prod_{vol}$	Volumetric Ethanol Productivity
RES	Renewable Energy Sources
$r_{ethanol}$	Volumetric Ethanol Production Rate
$r_{glucose}$	Volumetric Glucose Consumption Rate
r_{xylose}	Volumetric Xylose Consumption Rate
SDS	Sodium Dodecyl Sulphate
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
US	United States
XDH	Xylitol Dehydrogenase

XR	Xylose Reductase
$Y_{biomass/substrate}$	Biomass Yield
$Y_{glucose}$	Hydrolysis Yield on Glucose
$Y_{hydrolysis}$	Hydrolysis Yield
$Y_{ethanol/substrate}$	Ethanol Yield
Y_{xylose}	Hydrolysis Yield on Xylose
YM medium	Yeast Mold medium
μ	Specific Growth Rate
$[Ethanol]_{max}$	Maximum Ethanol Concentration

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1. Introduction

1.1. General context

The current economic system is based on an unsustainable linear material and energy flow model. Circular Economy (CE) seems like a promising solution for this issue, since it proposes a cyclical material and energy flow (Geissdoerfer et al., 2017; Korhonen et al., 2018). Biorefineries are crucial for CE as they process all fractions of biomass to co-produce a multiplicity of products and energy in a closed loop, minimizing waste generation and sometimes also using waste as raw material (Mohan et al., 2016; Bauer et al., 2017; IEA Bioenergy Task 42, 2017).

Most developed nations depend on non-renewable energy sources such as fossil fuels, which have great impact on health and environment (Hussain et al., 2017; Singh, 2017). As a rapid growth of economies and world's population is expected in the next years, there is a pressing need to start using renewable energy sources, a key principle of CE (Ellen MacArthur Foundation, 2012; Amarasekara, 2014). Biofuels are a potential renewable energy source in replacing fossil fuels, particularly given their much lower greenhouse gases emissions (Babu et al., 2014; Liew et al., 2014).

Bioethanol is currently the most produced biofuel (REN21, 2017a). However, it is almost entirely produced from food crops like sugarcane and corn, i.e. first generation bioethanol, resulting in a food-fuel competition (Dutta et al., 2014; Manochio et al., 2017). Bioethanol can alternatively be produced from lignocellulosic biomass (LCB), known as second generation bioethanol, which does not rise concern about food availability (Zabed et al., 2016, 2017). However, second generation bioethanol is still not economically feasible on a large scale (Taha et al., 2016). Due to the recalcitrance of LCB, a costly and technically difficult pretreatment is required (Achinas and Euverink, 2016). Other key limitations include the high cost of the hydrolysis step and the inability of most well-known natural microorganisms to ferment both hexose and pentose sugars (Banerjee et al., 2010; Zabed et al., 2016).

Chemical pulping processes used in pulp and paper industry remove lignin and target hemicelluloses to some extent, releasing cellulose, and therefore can be considered as LCB pretreatment methods (Jönsson and Martín, 2016). Kraft pulping is a well-established and commercially proven technology, which can be exploited as pretreatment for subsequent

ethanol production, converting the pulp and paper industry into integrated biorefineries, increasing the opportunity of success of the ethanol production process (Monrroy et al., 2012; Phillips et al., 2013).

1.2. Objectives

The present work aimed to study the production of bioethanol from unbleached Kraft pulp of *Eucalyptus globulus*, exploiting the Kraft pulping process as a pretreatment of lignocellulosic biomass.

Firstly, unbleached Kraft pulp of *E. globulus* was enzymatically hydrolysed and then the obtained hydrolysate was used as substrate for ethanol fermentation. Erlenmeyer flask assays with mono-cultures of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* were performed to study the growth and ethanol production by the yeasts in synthetic medium and hydrolysate with and without supplementation of yeast extract. Then, assays of simultaneous and sequential co-cultures of *S. cerevisiae* and *S. stipitis* co-culture in hydrolysate supplemented with yeast extract were tested in Erlenmeyer flasks. Finally, mono-culture of *S. cerevisiae* in synthetic medium and in hydrolysate supplemented with yeast extract and sequential co-culture in hydrolysate supplemented with yeast extract were tested in bioreactor.

This work intended to evaluate the feasibility of producing bioethanol from pulp and paper industry wastes, such as low-quality wood, bark and other rejects, as well as and low-quality and excess pulp. In this way, integrated biorefineries could be implemented in the existing Kraft pulp mills.

2. State Of The Art

2.1. Circular Economy

The development of the current economic system is based on an extract-produce-use-dump material and energy flow model (Korhonen et al., 2018), which leads to environmental problems, such as air, water, and soil pollution, biodiversity loss, climate change, natural resources depletion, social inequality, and human health problems due to pollution (Geissdoerfer et al., 2017; Winans et al., 2017). This flow model, though meeting the current generation needs, compromises the ability of future generations to meet their ones, and therefore is unsustainable (Korhonen et al., 2018). Circular Economy (CE) gained importance in the past years due to the pressing need to address this sustainability issue, since it proposes a cyclical material and energy flow model as an alternative for the linear and open-ended contemporary economic system (Geissdoerfer et al., 2017; Korhonen et al., 2018). CE focuses on keeping materials available within a closed-loop system, through principles such as reuse, recycle, redesign, remanufacture, reduce and recover, instead of disposing them (Ritzén and Sandström, 2017; Winans et al., 2017). The increasing number of publications on this topic, with more than 100 articles published in 2016, compared to about 30 articles in 2014, shows the growing interest on CE (Geissdoerfer et al., 2017). The European Commission adopted the *Circular Economy Package* in 2015, which consists on a programme of actions that contribute to the implementation of CE in the European Union (EU) (European Commission, 2017). **Figure A1** in **Appendix A** presents a CE system diagram.

2.1.1. Bioeconomy and biorefineries

Bioeconomy can be defined as an economy that uses renewable biological resources, such as agricultural or forestry biomass and organic wastes, to produce materials, chemicals, fuels, and energy (Bauer et al., 2017; Maina et al., 2017). Bioeconomy is circular by nature, once it relies on the conversion of renewable carbon reserves into different end-products. Thus, bioeconomy can contribute to CE and offers an alternative to fossil-based products and energy. The latter fact is very important as the global population is rapidly growing and is expected to reach 9 billion by 2050, increasing the demand for food, feed, fuel, and other

products, and putting unparalleled pressure on the environment (Dupont-Inglis and Borg, 2017; Maina et al., 2017).

Biorefining and biorefineries promote an efficient use of resources and raw materials and therefore are essential to achieve the transition to a sustainable bioeconomy (Bauer et al., 2017). A biorefinery accepts various feedstocks and integrates several parallel processes and technologies (e.g. biotechnological, chemical, physical, and thermal) that convert the different fractions of biomass into a multiplicity of useful products and energy, allowing the biomass to be entirely utilized (Clark et al., 2012; Salles-Filho et al., 2016; Bauer et al., 2017). Biorefining is the best way to achieve a large-scale sustainable use of biomass in bioeconomy and is a crucial part of CE, as it closes loops of biomass, water and carbon through co-production of food/feed ingredients, biobased products and bioenergy from renewable resources in a clean and efficient way, maximizing biomass valorisation (IEA Bioenergy Task 42, 2014, 2017). One of the primary principles of CE is the elimination of waste (Ellen MacArthur Foundation, 2012), as the modern society faces escalating waste problems, both in developing and developed countries, causing alarming ecological pollution (Arevalo-Gallegos et al., 2017; Dugmore et al., 2017). Biorefineries can help in solving this problem by applying zero-waste conversion technologies (waste prevention), but also by using waste itself as raw material (waste management) (European Commission, 2015; Mohan et al., 2016).

2.2. Biofuels

Most developed nations depend on non-renewable energy derived from fossil fuels (crude oil, natural gas, coal, oil shale, and tar sand) and nuclear power. These energy sources do not renew themselves at a sufficient rate for a sustainable economic extraction (Singh, 2017). The global consumption of fossil fuels is causing several problems with great health and environmental impact, notably greenhouse gases (GHG) emission to the atmosphere, which leads to global warming (Hussain et al., 2017). As global energy consumption grew, the need for sustainable and pollutant-free energy sources, known as renewable energy sources (RES), emerged (Amarasekara, 2014; Hussain et al., 2017). Indeed, one of the key principles of CE is to be fuelled by RES (Ellen MacArthur Foundation, 2012). Bioenergy or biomass energy is a RES defined as the energy enclosed in products (solid, liquid or gaseous) derived from biomass (Ho et al., 2014). Biomass is obtained from recently living organisms

and based on the carbon cycle, containing chemical energy originally received from the sun (REN21, 2017a; Singh, 2017). Currently, bioenergy is the leading supplier to global renewable energy, and it can substitute fossil fuels in transport, heat or power generation, and combined heat and power generation (IEA Bioenergy Task 42, 2014; REN21, 2017a).

Biofuels are identified as a potential RES in replacing fossil fuels and have become one of the most promising sustainable forms of energy (Babu et al., 2014; Liew et al., 2014). They are defined as liquid, gas and solid fuels produced from biomass. A wide variety of fuels can be produced from biomass including solid biofuels like charcoal, liquid biofuels such as bioethanol, biodiesel, and biomethanol, and gaseous biofuels like biogas, syngas, and biohydrogen (Nigam and Singh, 2011; Amarasekara, 2014). The development of biofuels is primarily driven by the instability of world oil prices (Babu et al., 2014). The fact that, based on the present use of fossil fuel, the fossil fuel regeneration and discovery rates will at no time match the consumption rate, and that the larger part of fossil fuels reserves is located in a few countries, has led to an increasing research on biofuels (Pandey et al., 2011; Luque et al., 2016). Also, oil and gas reservoirs are getting deeper and harder to reach (Abdulrahman, 2013).

When produced and used in a sustainable way, biofuels result in no net increase in atmospheric CO_2, as the CO_2 deriving from biofuels combustion has recently been consumed by photosynthesis (Demirbas, 2017a; Jacob-Lopes and Zepka, 2017). However, besides the environmental benefit of lower GHG emission, biofuels provide independence and security of energy supply since they are produced from common biomass sources that are easily available and geographically more evenly distributed than fossil fuels, which allows for a domestic energy supply (Nigam and Singh, 2011; Demirbas, 2017b). The growing interest in biofuels by the scientific community is shown by the rising number of articles published on this topic. From 1990 to 2005 researchers were publishing about 50 additional biofuel-related papers compared to the previous year, but from 2005 onwards biofuel-related journal publications expanded at a rate of approximately 550 papers per year (Azadi et al., 2017).

Biomass can be converted to biofuels by different conversion technologies, which are divided in thermochemical and biochemical (Luque et al., 2016). Thermochemical technologies include *(i)* direct combustion of biomass to generate heat and electricity; *(ii)* gasification, where biomass is heated with partial oxygen to produce syngas (a mixture of mainly carbon monoxide and hydrogen) that is used to produce fuels and intermediate

chemicals; *(iii)* liquefaction, where biomass is subjected to high temperature and pressure, producing bio-oil, which can be further processed to generate other biofuels; and *(iv)* pyrolysis, which consists of thermal degradation of biomass in the absence of oxygen to produce charcoal, also known as biochar, bio-oil, and syngas (Liew et al., 2014; Singh, 2017). Biochemical conversion technologies are used to produce biofuels like *(i)* biogas (a mixture of methane and carbon dioxide), through anaerobic digestion (AD) of biomass; *(ii)* biodiesel, mixture of long-chain fatty acid methyl ester (FAME), by transesterification of triacylglycerol (e.g. plant oil, microalgae oil, and animal fats) with methanol; and *(iii)* bioethanol, which is produced by fermentation of sugars from biomass (Luque et al., 2016; Singh, 2017).

2.2.1. Generations of biofuels

Biofuels are classified in first, second, third, and fourth generation biofuels, which differ in the feedstock used (Acheampong et al., 2017). First generation biofuels are produced from feedstocks like sugar, starch, or vegetable oil derived from sugar crops (e.g. sugarcane, sugar beet, and sweet sorghum), starch crops (e.g. corn, wheat, and cassava) or oil crops (e.g. rapeseed, soybean, palm and coconut oil), respectively. These crops are sources of food for human consumption (Ho et al., 2014; Singh, 2017). First generation biofuels are also known as conventional biofuels, since their production involves conventional and well-established technologies, and are also already produced at commercial scale (Babu et al., 2014). The main disadvantage of this generation is the fact that it leads to competition over the utilisation of arable land for cultivation of food crops and biofuel feedstock, thus resulting in a food-fuel competition with increased food prices (Dutta et al., 2014).

Second generation biofuels, derived from lignocellulosic biomass (LCB), which is not used as food for human consumption, were identified as a way to surpass the aforesaid problems (Liew et al., 2014). Second generation biofuels have several advantages, the main advantage being the fact that they reduce the food versus fuel competition associated with first generation biofuels, as dedicated energy crops can be grown on marginal lands (i.e. lands which cannot be used efficiently to cultivate arable/edible crops), and municipal/industrial wastes and agricultural/forestry residues do not require using additional land (Dutta et al., 2014; Singh, 2017). However, second generation biofuels also present

disadvantages. LCB has a varied chemical composition and physical characteristics and is recalcitrant, which leads to some technical and economic challenges. In order to convert LCB to biofuels a costly pretreatment is required (Ho et al., 2014; Liew et al., 2014).

Third generation biofuels are biofuels derived from algae, which compared with first and second generation feedstocks, are highly efficient in converting solar to chemical energy, storing it in the form of oils, carbohydrates and proteins, and have a rapid growth rate when compared to any terrestrial plant (Dutta et al., 2014; Liew et al., 2014). Nevertheless, even when cultivated in wastewater, algae need vast amounts of water and nutrients, and third generation biofuels require the development of new technologies and improvement of the existing ones, from cultivation to harvesting and downstream processing (Ho et al., 2014; Singh, 2017). More recently, a fourth generation of biofuels has been suggested. This generation uses metabolic engineered algae for biofuel production. Genetic modification and metabolic engineering could be used to increase the lipid content and biomass yield. However, research on these biofuels is still at primary stage (Nigam and Singh, 2011; Dutta et al., 2014). These photosynthetic microorganisms could also be genetically optimized to capture larger amounts of CO_2. This improved CO_2 capture ability renders fourth-generation biofuels a carbon negative source of biofuel, rather than just carbon neutral (Acheampong et al., 2017). **Table 2.1** shows the feedstock and the main advantages and disadvantages of each generation.

Table 2.1 – Feedstock and mains advantages and disadvantages of each biofuel generation.

Generation	Feedstock	Advantage	Disadvantage
First	Sugar, starch or oil crops	Conventional and well-established technologies	Food versus fuel competition
Second	Lignocellulosic biomass	No food crop competition	Costly pretreatment of lignocellulosic feedstock
Third	Algae	High growth rate	Development of new technology
Fourth	Metabolic engineered algae	Increase the lipid content and CO_2 capture ability	Research still at primary stage

2.2.2. Biofuels for transportation

The global energy demand in transport accounts for around 28 % of the overall energy consumption and for 23 % of energy-related GHG emissions, being the end-use sector with the largest CO_2 emissions (IEA, 2016; REN21, 2017a). Oil accounts for 93 % of the final energy of the entire sector and road transport alone corresponds to 75 % of the total

energy used in the transport sector (REN21, 2017b, 2017a). Biofuels provide only around 4 % of global transport fuels (REN21, 2017a). In the EU, the *Renewable Energy Directive* (2009/28/EC) sets a target of at least 10 % of each Member State transport fuels to come from renewable sources such as biofuels by 2020, advanced biofuels accounting for twice this target (European Union, 2009).

In 2016, 135.3 billion litres of biofuel were produced. Bioethanol and biodiesel are the most commonly used liquid biofuels (Amarasekara, 2014; REN21, 2017a). **Figure 2.1** presents the evolution of biofuel mandates and bioethanol and biodiesel global production from 2004 to 2016. The US is the largest biofuel producers, followed by Brazil and then by Germany, Argentina, and China. The US and Brazil account for 70 % of the world biofuel production (REN21, 2017a). As a Member State of the EU, Portugal has the target of at least 10 % of transport fuels to come from renewable sources such as biofuels by 2020, as set by the *Renewable Energy Directive* (2009/28/EC). In Portugal, this goal is to be achieved by means of 89 % of biofuels and 11 % renewable electricity and by applying several policies promoting biofuel production (República Portuguesa, 2010). A promising path towards these targets might be the sustainable production of bioethanol.

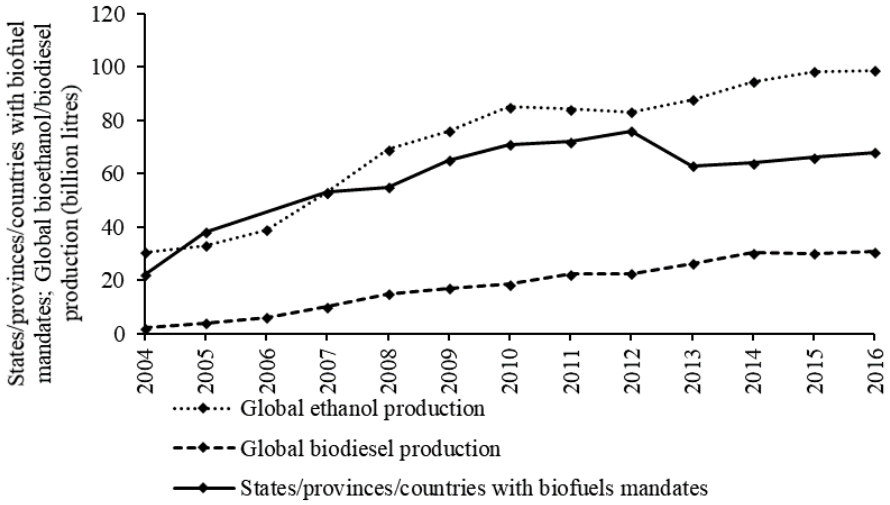

Figure 2.1 - Evolution of biofuel mandates and bioethanol and biodiesel global production from 2004 to 2016. Adapted from REN21, 2017a, 2017b

2.3. Bioethanol

Generally, ethanol can be classified into bio-based ethanol (bioethanol) and synthetic ethanol. Synthetic ethanol is produced through catalytic hydration of ethylene, which comes from cracking of crude oil or natural gas and generates toxic by-products. Bioethanol derives

mainly from biological fermentation of sugars present in biomass and represents the majority (90-95 %) of the globally produced ethanol (Yusoff et al., 2015; Sarris and Papanikolaou, 2016). Besides being an alternative fuel, bioethanol has other applications that include chemicals (e.g. diethyl ether, ethylene, propylene, acetaldehyde, and ethyl acetate), beverages, pharmaceuticals and cosmetics (Maity, 2015b; Sarris and Papanikolaou, 2016).

2.3.1. Bioethanol for transportation

Using bioethanol in spark ignition engines has many advantages, notably reducing CO_2 by recycling the CO_2 released when bioethanol is combusted (Balat, 2011). The high oxygen content of bioethanol promotes better combustion and lower exhaust emissions, such as particulate matters, CO and SO_x emissions (Sebayang et al., 2016). Bioethanol has a higher octane number when compared with gasoline, and consequently has a higher capacity to withstand compression before igniting. Therefore, bioethanol prevents premature ignition that causes knocking, which can damage the engine (Yusoff et al., 2015; Araújo et al., 2017). However, bioethanol has some disadvantages like its corrosiveness, lower vapour pressure (a problem when starting cold engine), and lower energy density (34 % lower than gasoline), which leads to a higher bioethanol consumption (Balat, 2011; Sebayang et al., 2016). In **Table 2.2** several properties of bioethanol and gasoline are depicted.

Table 2.2 - Physical and chemical properties of gasoline and bioethanol. Adapted from Yusoff et al., 2015; Sebayang et al., 2016

Property	**Gasoline**	**Bioethanol**
Chemical formula	C_4 to C_{12}	C_2H_5OH
Oxygen content (wt %)	0	34.73
Research octane number	90-100	108
Motor octane number	81-90	92
Density at 15 °C ($Kg.m^{-3}$)	750-765	785-809.9
Heat of vaporization ($MJ.kg^{-1}$)	0.32	0.92
Vapor Flammability Limits (vol %)	0.6-8	3.5-15
Reid vapour pressure at 37.8 °C (kPa)	53-60	17

Bioethanol can be used as pure hydrated ethanol, or in blends with gasoline, in which the percentage of anhydrous ethanol varies from 5 % (E5) to 85 % (E85) (Kang et al., 2014; Sarris and Papanikolaou, 2016). Anhydrous ethanol presents a water content of less than 1%, while hydrous ethanol water content ranges from 5 to 10 % (Sebayang et al., 2016). E10 (10 % bioethanol, 90 % gasoline), also known as gasohol, is the most commonly used blends in the EU (Balat, 2011; Kang et al., 2014).

2.3.2. Current bioethanol production

Bioethanol is currently the most produced biofuel (**Figure 2.1**), corresponding to about 73 % of the 135.3 billion litres of biofuel produced in 2016. **Figure 2.2** presents the worldwide bioethanol production in 2016 by country. The US and Brazil are the biggest ethanol producers, producing 58.8 and 27.4 % of the global ethanol production, respectively, while Europe produces 3.4 % of the global bioethanol (REN21, 2017a). According to the Portuguese *Third Progress Report (2013-2014)*, measuring the country's progress towards the targets of the *Renewable Energy Directive*, the totality of bioethanol used in Portugal in 2014 was imported (DGEG, 2015).

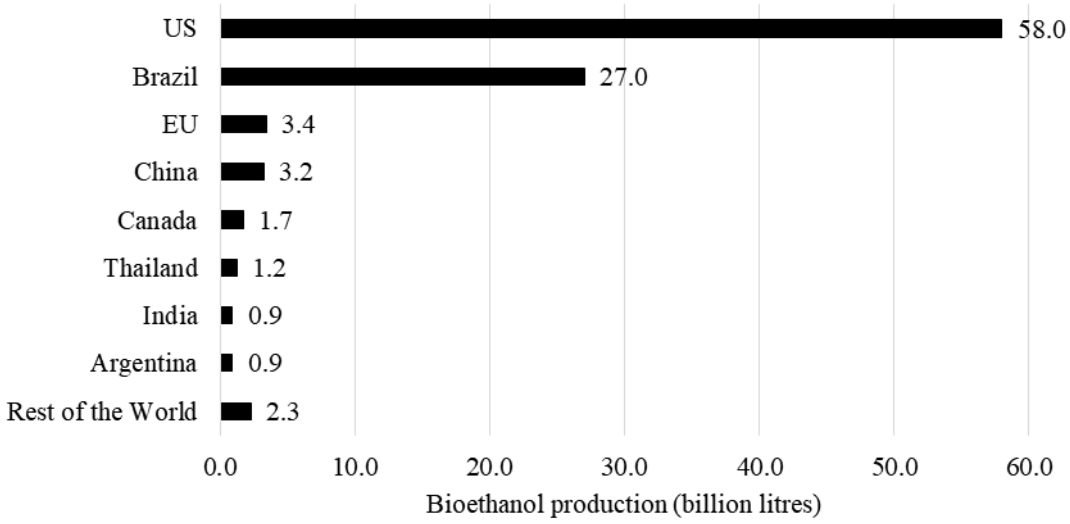

Figure 2.2 - Bioethanol production worldwide in 2016 by country. Adapted from REN21, 2017a

Bioethanol is presently produced almost entirely from food crops, i.e. first generation bioethanol (Thangavelu et al., 2016). Brazil uses sugarcane for bioethanol production, while the US and the EU produce bioethanol predominantly from corn and sugar beet, respectively (Manochio et al., 2017). **Table 2.3** presents a comparison of several parameters regarding bioethanol production from sugarcane, corn and sugar beet.

Table 2.3 - Ethanol yield, potential yield, energy balance, net energy gain, avoided GHG emissions, and cost of bioethanol production from sugarcane, corn and sugar beet. Adapted from Manochio et al., 2017; Zabed et al., 2017

Feedstock	**Sugarcane**	**Corn**	**Sugar beet**
Ethanol yield (L.ton^{-1})	70–90	370–470	95–107
Potential yield (L.ha^{-1})	6470–6660	4180	1605–5500
Energy balance[a]	9.4	1.2	1.6
NEG[b] (GJ.ha^{-1})	101.7–203.4	1.3–3.8	9.8–19.6
Avoided GHG emissions (%)	69–89	−30–38	35–56
Production cost[c] (€.L^{-1})	0.21–0.36 (Brazil)	0.43–0.75 (US)	0.40–0.66 (EU)

NEG net energy gain

[a] fossil energy used during the production process divided by the renewable energy produced at the end of the process; [b] renewable energy produced at the end of the process minus the fossil energy used during the production process; [c] data collected from the period of 2006–2012

2.4. Second Generation Bioethanol Production

Second generation bioethanol, also known as lignocellulosic or cellulosic bioethanol, can be produced from different lignocellulosic biomass (LCB), including agricultural residues, energy crops, forest biomass and waste, and the organic fraction of municipal solid waste (MSW) (Zabed et al., 2017). Agricultural residues are the by-products of agriculture and its related industries. The majority of agricultural residues are corn stover, wheat straw, rice straw, and sugarcane bagasse (Kang et al., 2014; Zabed et al., 2016). Perennial grasses like miscanthus, switchgrass, reed canary grass, giant reed, and alfalfa are energy crops frequently studied for bioethanol production, as they present a minimal need of water, fertilizer and arable land (Zabed et al., 2016). Lignocellulosic bioethanol can also be produced from woody materials like softwoods, gymnosperm trees, or hardwoods, angiosperm trees. Also, different forestry wastes such as wood chips, branches, slashes, and sawdust can be used (Limayem and Ricke, 2012). Municipal and industrial wastes are another potential feedstock for bioethanol production. The use of these wastes is a form of waste management, reducing the environmental problems related with wastes like garbage household, food-processing by-products, black liquors and pulps (Limayem and Ricke, 2012; Zabed et al., 2017). The potential ethanol yield of different second generation bioethanol feedstocks is shown in **Table 2.4**.

Table 2.4 - Potential bioethanol yield of different second generation bioethanol feedstocks. Adapted from Zabed et al., 2017

Second generation bioethanol feedstock	**Agricultural residues**	**Perennial grasses**	**Forest biomass and waste**		**MSW**
			Softwoods	**Hardwoods**	
Potential ethanol yield (L.ton of biomass^{-1})	235–450	160–460	220–275	280–285	154

Bioethanol production from LCB can be achieved using one of two technologies, namely biochemical or thermochemical conversion (Rastogi and Shrivastava, 2017). Thermochemical conversion is achieved firstly by the production of syngas through LCB gasification at a much higher temperature than those used in biochemical conversion. Then, the CO, CO_2 and H_2 present in the syngas are converted to bioethanol either biologically through fermentation by anaerobic bacteria, usually by clostridia, or by chemical catalysis

using molybdenum disulphide (Kennes et al., 2016; Rastogi and Shrivastava, 2017). Biochemical conversion starts with a preliminary step of feedstock preparation that involves cleaning and size reduction by milling, grinding, or chopping, consuming a large amount of energy (Sebayang et al., 2016; Wong et al., 2017). Biochemical conversion of LCB to bioethanol is divided in four major steps (**Figure 2.3**): *(i)* pretreatment - to degrade lignocellulosic network into its fractions (i.e. cellulose, hemicelluloses, and lignin); *(ii)* hydrolysis – to obtain fermentable sugars from cellulose and hemicelluloses; *(iii)* microbial fermentation - to convert sugars into ethanol; and *(iv)* ethanol recovery and dehydration (Rocha-Meneses et al., 2017; Zabed et al., 2017). Although thermochemical conversion produces a cleaner fuel to be used directly in engines, the biochemical route is less cost intensive and more commonly used (Dutta et al., 2014; Rastogi and Shrivastava, 2017). This work will now focus on the second generation bioethanol biochemically produced via.

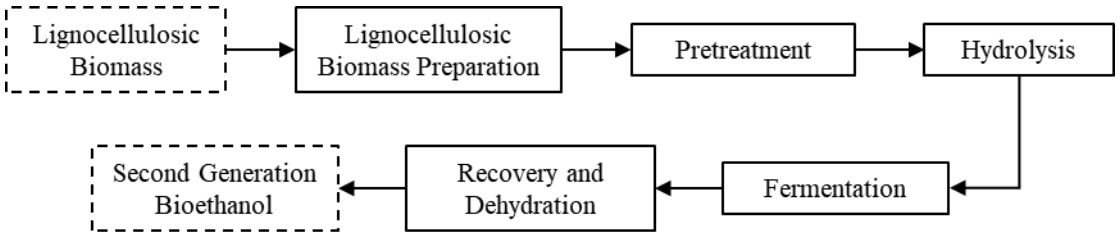

Figure 2.3 - Steps involved in biochemical conversion of LCB to bioethanol.

Second generation bioethanol has several advantages over the first generation that make it a more sustainable option for large scale biofuel production, and therefore investigation has been focused on it (Taha et al., 2016). LCB is the world's most abundant, evenly distributed and easily available organic material (Bhutto et al., 2017). Contrary to first generation feedstocks for bioethanol production, LCB does not rise concern about food availability, has a low and stable price and it practically does not demand extra land (Zabed et al., 2016). Also, second generation bioethanol was reported to lead to GHG emissions almost as low as the ones resulting from sugarcane bioethanol, that is nearly 90 % less than gasoline (Balat, 2011). Despite all the advantages aforementioned, bioethanol production from LCB is still not economically feasible on a large scale, as it faces a number of technical barriers that lead to commercially ineffective operational and production costs. Therefore, second generation bioethanol production costs (0.69-0.95 €.L^{-1}) exceed those of transportation fossil fuels and first generation bioethanol and biodiesel (Balat, 2011; Xiros et al., 2013; Taha et al., 2016). Due to the recalcitrance of LCB at least one costly pretreatment is required (Achinas and Euverink, 2016). Along with pretreatment, the

hydrolysis step also contributes for an increased cost in lignocellulosic bioethanol production (Banerjee et al., 2010). Two other key limitations are the formation of inhibitors during pretreatment and the inability of most well-known natural microorganisms to ferment both hexose and pentose sugars (Zabed et al., 2016).

There are already some facilities that produce cellulosic ethanol. These facilities are very important as they make way for further innovation through cost and operability evaluation based on real experience (Lynd et al., 2017). Some of the pioneer bioethanol plants are no longer running, showing the pressing need to learn more about the different steps involved in its production, and also to develop the industrial scaling process in order to obtain a technical-economic feasible process (Manochio et al., 2017). According to IEA Bioenergy's database on biofuels companies there are currently about 40 operational pilot or demonstration facilities producing second generation bioethanol for transportation (IEA Bioenergy Task 39, 2017a). **Table 2.5** lists 4 of those facilities.

Table 2.5 - Operational facilities producing second generation bioethanol for transportation.

Company	Country	Feedstock	Ethanol production ($ton.y^{-1}$)	Coproducts	Ref.
GranBio	Brazil	Sugarcane bagasse and straw	65,000	Power Heat	GranBio, 2014; Beta Renewables, 2017
LanzaTech	China	Steel flue gas	300	-	IEA Bioenergy Task 39, 2015; LanzaTech, 2017
POET-DSM Advanced Biofuels	US	Corn stover	75,000	Biogas Fischer–Tropsch fuels	POET-DSM Advanced Biofuels, 2016; IEA Bioenergy Task 39, 2017c
Raízen	Brazil	Sugarcane bagasse and straw	31,600	Power	Raízen; IEA Bioenergy Task 39, 2017b

2.4.1. Lignocellulosic biomass composition

In general, LCB is composed by cellulose (30–60 %), hemicelluloses (20–40 %), lignin (15–25 %), and small amounts of extractives and ash (Balat, 2011). Cellulose and hemicelluloses are polysaccharides that can be converted to fermentable sugars, which are then converted to bioethanol, while lignin is not used for bioethanol production, but is a source of high value-added aromatic products (Seidl and Goulart, 2016). Cellulose $(C_6H_{10}O_5)_x$ is a linear unbranched polysaccharide composed by monomers of D-glucose

linked by β-(1,4)-glycosidic bonds (Mood et al., 2013). The fibers of this homopolysaccharide are linked to each other by intra- and inter-molecular hydrogen bonds, resulting in a highly ordered crystalline structure. These crystalline regions are interrupted by amorphous regions (Silveira et al., 2015).

Hemicelluloses are shorter and highly branched heteropolysaccharides (Sarkar et al., 2012). These polymers are composed of different monomers that include pentoses (e.g. xylose, and arabinose), hexoses (e.g. mannose, glucose, galactose) and/or uronic acids (e.g. glucuronic, and galacturonic acids) (Mood et al., 2013). Different polysaccharides can compose hemicelluloses, like glucomannan, galactoglucomannan, and xylan. Hemicelluloses are mostly amorphous (Chen et al., 2017). Lignin is an amorphous, highly hydrophobic, non-polysaccharide polymer matrix. This polyphenolic compound has undefined molecular mass and is composed mainly by syringyl, guaiacyl and p-hydroxyphenol (Mood et al., 2013; Silveira et al., 2015). Ether linkages and also carbon–carbon bond connection link lignin's structural units, giving lignin a complex irregular structure (Chen et al., 2017). Cellulose, hemicelluloses, and lignin are bonded by non-covalent bonds and covalent cross-linkages, interacting closely with each other in a strong and intermeshed network (Sun et al., 2016). **Table 2.6** presents the cellulose, hemicelluloses and lignin contents of some LCB.

Table 2.6 - Cellulose, hemicelluloses and lignin composition of some lignocellulosic biomass.

Lignocellulosic biomass	**Composition (%, dry basis)**			**Ref.**
	Cellulose	**Hemicelluloses**	**Lignin**	
Energy crops				
Switchgrass	45	30	12	Hu and Ragauskas, 2012
Miscanthus	48	30	12	
Agricultural residues				
Corn stover	40.8	20.6	21.3	Loow et al., 2016
Sugarcane bagasse	41.0	30.1	21.1	
Forest biomass				
Softwood	40-45	25-29	30-60	Limayem and Ricke, 2012
Hardwood	45-47	25-40	20-25	
Municipal solid waste				
General MSW	33-49	9-16	10-14	Zabed et al., 2016
Newspapers	40–55	25–40	18–30	Balat, 2011
Chemical pulps	60–80	20–30	2–10	

2.4.2. Pretreatment

The carbohydrate polymers of LCB, i.e. cellulose and hemicelluloses, must be converted to simple sugars before fermentation, which is done by means of hydrolysis (Pandey et al., 2015). Hemicelluloses and lignin strongly interact with cellulose, decreasing its accessibility to the hydrolysis agent. Also, the crystalline structure of cellulose leads to a higher resistance to hydrolysis, and the presence of lignin limits enzymatic hydrolysis due to adsorption of enzymes (Sun et al., 2016). Hence, several pretreatments exist to reduce this recalcitrance through disruption of the LCB compact and complex structure, changing both its micro- and macro-structure. Pretreatment breaks down and removes lignin, i.e. delignification. Additionally, in this process, hemicelluloses degradation and decrease of cellulose crystallinity also occur (Bhutto et al., 2017; Chen et al., 2017). Pretreatments can be classified as physical (e.g. milling, grinding, and microwave), chemical (acid, alkali, ozonolysis, organosolv and ionic liquids), physicochemical (steam explosion, ammonia fiber explosion, CO_2 explosion, liquid hot water, and wet oxidation), or biological (Mood et al., 2013; Pandey et al., 2015).

Pretreatment is the biggest technological barrier for the development of a cost-effective bioprocess from LCB, accounting for about 40 % of the total cost of bioethanol production from LCB. When choosing a pretreatment and its configuration feedstock physical and chemical differences must be taken in account. Cost, energy performance, operating environment, presence of inhibitors, and process integration should also be considered (Bhutto et al., 2017). Diluted acid and steam explosion are two of the most commonly used pretreatment methods, since they present a low cost and yield good delignification. However, formation of inhibitors is associated with these pretreatments (Capolupo and Faraco, 2016; Loow et al., 2016; Rastogi and Shrivastava, 2017).

Depending on the pretreatment method and conditions several degradation products may be formed. These products often have an inhibitory effect in enzymes and microorganisms, affecting the ensuing biocatalytic processes of hydrolysis and fermentation. These inhibitors include *(i)* furfural and HMF (5-hydroxymethyl-2-furaldehyde), through degradation of hexoses and pentoses, respectively, which can then be further degraded to formic and levulinic acid; *(ii)* acetic acid originated from hemicellulose; and *(iii)* phenolic compounds from lignin degradation. Numerous detoxification technologies exist to remove inhibitors: *(i)* physical – evaporation, extraction, and adsorption; *(ii)* chemical –

neutralization, alkaline detoxification, and ionic exchange; and *(iii)* biological – enzymatic and microbial detoxification. Nevertheless, they represent an additional cost (Amarasekara, 2014; Jönsson and Martín, 2016).

2.4.3. Hydrolysis/Saccharification

At the end of the pretreatment, the solid residue, containing the majority of cellulose and remaining hemicelluloses and lignin, is separated from the liquid stream and sent to hydrolysis (Maity, 2015a). Hydrolysis, also known as saccharification, is a crucial step as it converts the cellulose and hemicelluloses in the pretreated biomass into their monomers, i.e. fermentable sugars, allowing their conversion into ethanol. This can be achieved either biologically (enzymatic hydrolysis) or chemically (acid hydrolysis) (Haldar et al., 2016; Singh and Chaudhary, 2017). The maximum theoretical yield of hexoses and pentoses is 1.111 kg and 1.136 kg per kg of hexose polymer and pentose polymer, respectively (Corbin et al., 2015). **Table 2.7** shows the hydrolysis agent and main advantages and disadvantages of the different hydrolysis processes.

Table 2.7 – Hydrolysis agent and main advantages and disadvantages of the different hydrolysis processes.

Hydrolysis	Concentrated acid	Diluted acid	Enzymatic
Hydrolysis agent	30-70 % H_2SO_4/HCl	2-5 % H_2SO_4/HCl	Cellulase and hemicellulase
Advantages	Low temperature High sugar yield	Low acid consumption	Mild conditions No inhibitors formation High sugar yield
Disadvantages	Large amounts of acids Equipment corrosion Environmental and cost issues	High temperature Formation of inhibitors Low sugar yield	High cost Slow reactions

2.4.3.1. Acid hydrolysis

Acid hydrolysis involves the use of acids, commonly sulphuric or hydrochloric acids, to break down cellulose and hemicellulose (Lima and Natalense, 2012). There are primarily two acid hydrolysis technologies - hydrolysis with concentrated acid or with diluted acid (Kennes et al., 2016). Concentrated acid hydrolysis can be performed at low temperatures and a high sugar yield is obtained (i.e. 90 % of the theoretical glucose yield) (Lima and Natalense, 2012). However, it requires great acid concentrations, usually of 30-70 %, which leads to equipment corrosion. Therefore, concentrated acid hydrolysis entails economic and environmental problems, which compromise its commercial application (Kennes et al.,

2016). Conversely, diluted acid hydrolysis requires a much lower amount of acid, 2-5 %, and is more commonly applied in industry (Lima and Natalense, 2012; Kamzon et al., 2016). However, it needs much higher temperature, about 200 °C, which leads to the formation of different inhibitory compounds, such as acetic acid, furfural, HMF, and phenols. These compounds not only negatively affect the following fermentation step, but also decrease the sugar yield (Haldar et al., 2016; Kennes et al., 2016).

2.4.3.2. Enzymatic hydrolysis

Hydrolysis can be catalysed by highly substrate specific enzymes that are able to convert the complex carbohydrates of LCB to simple monomers (Gupta and Verma, 2015). Enzymatic hydrolysis is usually the hydrolysis technique chosen for industrial applications, since it takes place at mild temperature and pH conditions (i.e. 50–60 °C and pH 4.5–5.5) (dos Santos et al., 2016). These conditions require less energy and do not lead to the formation of inhibitory compounds, neither to equipment corrosion (Gupta and Verma, 2015). Most importantly, enzymatic hydrolysis attains high yields, of 80-95 %, and as a reduced environmental impact (Kamzon et al., 2016). Cellulases and hemicellulases are the enzymes usually employed for hydrolysis of the pretreated LCB (Harris et al., 2014).

Cellulases are enzymes defined as hydrolases involved in cellulose hydrolysis, and usually comprise three complementary groups of enzymes able to hydrolyse the β-(1,4)-glycosidic bond between glucose units: endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BG) (Gupta et al., 2016). EG (endo-1,4-β-D-glucanases, EC 3.2.1.4) cleave the amorphous regions of cellulose. CBH (exo-1,4-β-D-glucanases, EC 3.2.1.91) hydrolyse the free ends of cellulose chain into the disaccharide cellobiose, and are divided in CBHI and CBHII that act on the reducing and non-reducing ends, respectively. BG hydrolyse cellobiose to produce glucose (dos Santos et al., 2016; Volynets et al., 2017). **Figure 2.4** presents a schematic representation of the enzymatic hydrolysis of cellulose. *Trichoderma reesei* is the most commonly used fungi for industrial cellulases production. Other fungi capable of producing cellulases is *Aspergillus* sp. and, therefore, has also been studied (dos Santos et al., 2016; Zhao et al., 2016).

Due to the heterogeneity of hemicellulose, an extensive number of hemicellulases, with different mechanisms of action, capable of hydrolysing the backbone and the side

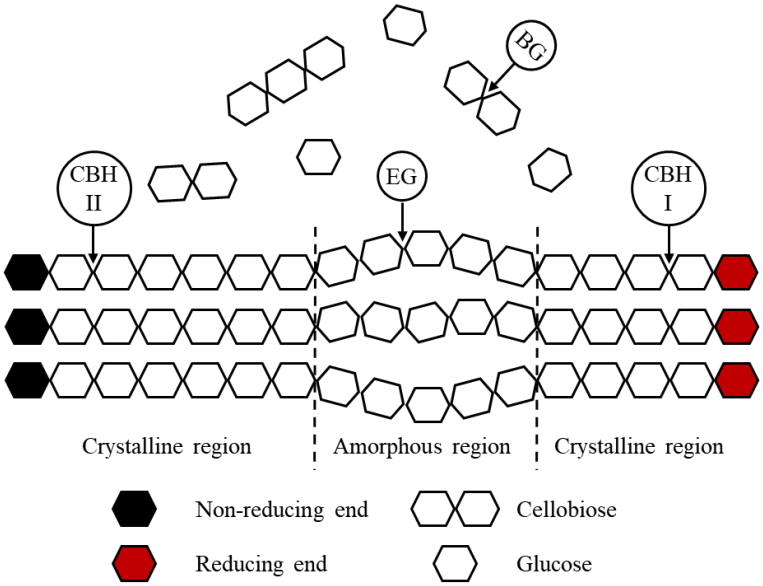

Figure 2.4 - Schematic representation of enzymatic hydrolysis of cellulose.

groups of the hemicelluloses, is required. The mechanism of hydrolysis of hemicelluloses is no yet fully understood because of hemicelluloses heterogeneity and hemicellulases system complexity (Gupta et al., 2016; Haldar et al., 2016). Xylan is the most abundant polysaccharide in hemicelluloses of LCB. Hence, xylanases, which hydrolyse the β-(1,4)-linkage between xylose units from the homopolymeric backbone structure of xylan, are the most important hemicellulases. These enzymes are defined as hydrolases and are commercially produced by filamentous fungi, like *Aspergillus niger* and *T. reesei*, which present enzyme levels much higher than those of yeasts and bacteria (Moreira and Filho, 2016; Escamilla-Alvarado et al., 2017). Xylanases include endo-1,4-β-xylanase (EC 3.2.1.8), which hydrolyses internal bonds in the xylan chain yielding xylooligomers, and exo-1,4-β-xylosidase (EC 3.2.1.37), which hydrolyses the xylooligomers to xylose (Haldar et al., 2016; Volynets et al., 2017).

Cellulases exhibit a synergistic action, i.e. the hydrolytic activity observed is higher than the sum of the hydrolytic activities of the individual enzyme components (Gupta et al., 2016). EG activity provides new accessible reducing and non-reducing chain ends for CBH, while CBH release cellobiose molecules to be hydrolysed by BG (Gupta and Verma, 2015). Hemicellulases of different families also act synergistically in order to achieve hemicelluloses complete breakdown (Gupta et al., 2016). Additionally, synergy between hemicellulases and cellulases has been observed, since hemicelluloses degradation increases

cellulose accessibility and removes xylo-oligomers, which inhibit cellulase enzyme activity (Jordan et al., 2012; Moreira and Filho, 2016).

Different enzymatic cocktails have been developed for cellulose and hemicelluloses degradation and are currently available to be used industrially for LCB hydrolysis. These cocktails were already used in other industries, including textile, for cotton softening and denim finishing, detergent, for colour care and cleaning, food industry, for mashing, and pulp and paper fiber improvement and modification (Xiros et al., 2013). Although substantial advances have already been made that allowed a decrease in enzyme cocktails price, the cost of enzymes, which is estimated to account for about 20 % of the ethanol production cost, is still a major limitation of enzymatic hydrolysis (Saini et al., 2015; dos Santos et al., 2016). Also, hydrolysis time is long (e.g. 1.5 days) due to the slowness of the reactions (Kamzon et al., 2016).

There are several factors that negatively affect enzymatic hydrolysis. Lignin and hemicelluloses present a structural barrier to enzymes, while a high cellulose crystallinity decreases the area for enzyme action. Enzymes can also unproductively bind to lignin. All these factors stress the importance of pretreatment (Volynets et al., 2017). The addition of surfactants, which bind to lignin instead of enzymes, increases the effectiveness of enzymatic hydrolysis, lowering the enzyme loading and, consequently, the processing cost (Haldar et al., 2016). Moreover, formic acid, furfural, and lignin degradation products generated during pretreatment are inhibitory to both cellulases and hemicellulases (Kubicek and Kubicek, 2016; Moreira and Filho, 2016). CBH and BG are strongly inhibited by their reaction products, cellobiose and glucose, respectively (Gupta et al., 2016).

2.4.4. Fermentation

The fermentable sugars resulting from saccharification are then converted into bioethanol by a diversity of microorganisms via fermentation (Rastogi and Shrivastava, 2017). The reaction of anaerobic conversion of hexoses and pentoses to ethanol can be expressed by **Equations 2.1** and **2.2**, respectively (Babu et al., 2014). The maximum theoretical yield of ethanol is 0.511 kg of ethanol, produced with 0.489 kg of CO_2, per kg of hexose and pentose (Kang et al., 2014).

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad \textbf{Equation 2.1}$$

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \qquad \textbf{Equation 2.2}$$

2.4.4.1. Fermentation configuration

There are different hydrolysis and fermentation bioprocess integrations. These configurations include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and still consolidated bioprocessing (CBP) (Saini et al., 2015). **Figure 2.5** presents the various configurations of the process for bioethanol production form LCB.

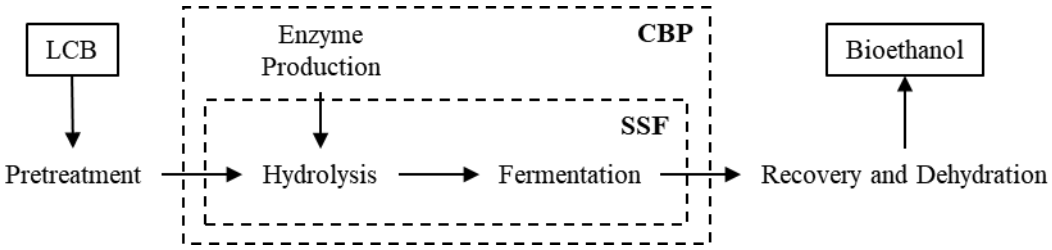

Figure 2.5 - Various configurations of the process for bioethanol production form LCB.

SHF consists of two consecutive steps: firstly the enzymatic hydrolysis, followed by microbial fermentation (Sarris and Papanikolaou, 2016). The main advantage of this configuration is the fact that both processes can take place under their optimal conditions, which is very important due to the difference in optimum working temperatures of hydrolytic enzymes and ethanol producing microbial strains, usually about 50 and 28-37 °C, respectively (Paulova et al., 2015). However, end product inhibition of BG, i.e. glucose, during the first step is a major drawback of SHF (Sarris and Papanikolaou, 2016).

In SSF hydrolysis and fermentation are performed simultaneously in the same vessel (Saggi and Dey, 2016). Since the hydrolysed sugars are immediately fermented into ethanol, end-product inhibition is eliminated, improving both enzymatic hydrolysis efficiency and the ethanol yield (Choudhary et al., 2016). Another advantage of this configuration is the lower number of steps and reactors, which leads to lower costs (Aditiya et al., 2016). Additionally, glucose's low concentration and its production in the presence of ethanol decreases the risk of contamination. Nevertheless, ethanol may inhibit cellulase activity (Choudhary et al., 2016). The major disadvantage of SSF is the difference in optimum temperature of the enzymes and microorganisms used. This issue could be solved by lowering the temperature, however this leads to reduced cellulase hydrolytic efficiency (Paulova et al., 2015). As reduction of the optimum temperature of cellulases via protein engineering is hard, SSF relies mainly on thermotolerant ethanologenic microbial strains (Choudhary et al., 2016). Several thermophilic/thermotolerant microorganisms belonging to

species like *Clostridium*, *Thermoanaerobacterium*, *Thermoanaerobacter*, and *Kluyveromyces* have been identified as potential lignocellulosic ethanol producers (Arora et al., 2015). However, these microorganisms usually have low tolerance to ethanol (Limayem and Ricke, 2012).

In CBP enzyme production, biomass hydrolysis, and fermentation occur in a single step. This configuration has a great capital investment reduction since utilities associated with enzyme production are eliminated. Although there is a reduced operational complexity as the three processes occur in a single vessel, CBP requires the development of highly engineered microbial strain capable of producing hydrolytic enzymes and with higher fermentation capacity (Choudhary et al., 2016).

2.4.4.2. Ethanologenic microorganisms

An ethanologenic microorganism should have certain important traits, such as *(i)* robust growth and simple growth requirement, so that a medium with inexpensive formulation can be used; *(ii)* tolerance to acidic pH or higher temperatures in order to retard contamination; *(iii)* high ethanol yield (> 90.0 % of theoretical); *(iv)* tolerance to high ethanol concentration (> 40.0 $g.L^{-1}$); *(v)* good ethanol productivity (> 1.0 $g.L^{-1}.h^{-1}$); and *(iv)* resistance to inhibitors, being able to grow in undiluted hydrolysates (Balat, 2011). There is a great number of microorganisms able to produce bioethanol, i.e. bacteria, yeast and filamentous fungi. Nevertheless, not all of them are efficient and feasible for ethanol fermentation, for example, filamentous fungi present low ethanol production rates and tolerance (Zabed et al., 2016).

Saccharomyces cerevisiae is the most commonly used microorganism for bioethanol production, being a robust and well-suited microorganism for fermentation of lignocellulosic hydrolysates. This yeast presents several traits that make it attractive for bioethanol production, like high ethanol yield, high ethanol and inhibitors tolerance, and the ability to ferment a wide range of hexoses (glucose, mannose, and galactose) and disaccharides (sucrose and maltose) (Zabed et al., 2016, 2017). The better-known fermentation bacteria is *Zymomonas mobilis*. This gram-negative bacterium is able to ferment glucose, sucrose and fructose to ethanol (Aditiya et al., 2016). When compared to *S. cerevisiae*, *Z. mobilis* has a higher ethanol yield and a much higher ethanol specific productivity, since it produces less biomass (Bisaria and Kondo, 2014). It also presents a higher ethanol tolerance. However, its

ability to ferment a narrow range of sugars and low tolerance to inhibitors like acetic acid still limits its application (Kang et al., 2014). Besides, *Z. mobilis* requires a neutral pH range, a common feature to most bacterial species (Limayem and Ricke, 2012; Avanthi et al., 2017).

One of the best approaches to reduce second generation bioethanol production costs is to use all sugars from LCB, including the ones obtained from the hemicelluloses (Faraco, 2013). This is very important, as hydrolysates present a high content in pentoses, mainly xylose, which can be about 25 % of the hydrolysate's sugar composition, followed by arabinose (dos Santos et al., 2016). Hence, the selection of a microorganism which can ferment both hexose and pentose sugars is required for an economical lignocellulosic ethanol production (Avanthi et al., 2017). Although *Z. mobilis* and *S. cerevisiae* are the most commonly employed microorganisms, they are incapable of fermenting pentose sugars (Aditiya et al., 2016). *Scheffersomyces stipitis*, *Candida shehatae* and *Pachysolan tannophilus* are the most promising yeasts that have the ability to use pentoses, in addition to hexoses (Saini et al., 2015). However, these yeasts require micro-aerophilic conditions, and are sensitive to low pH, inhibitors and high ethanol concentration. *S. stipitis* is the most promising pentose fermenting organism for industrial applications, presenting the best performance xylose fermentation with a higher ethanol yield (Limayem and Ricke, 2012; Faraco, 2013; Aditiya et al., 2016).

2.4.4.2.1. *Saccharomyces cerevisiae*

S. cerevisiae is a facultative anaerobic yeast that besides presenting ethanol yields of 90 to 95 % of the theoretical maximum, also tolerates a wide range of pH with acidic optimum, which decreases the probability of contamination (Madhavan et al., 2012; Tesfaw and Assefa, 2014). *S. cerevisiae*'s ideal temperature and optimum pH for fermentation range between 20-35 °C and 4.0-5.0, respectively (Azhar et al., 2017). **Table 2.8** compiles several studies were *S. cerevisiae* was applied for second generation bioethanol production.

Glucose uptake into the cell is mediated by different hexose transporters (high or low-affinity systems) (Madhavan et al., 2012). Glucose is metabolized by the Embdem-Meyenhorf-Parnas pathway (EMP), leading to the formation of 2 moles pyruvate per mole of glucose. Pyruvate can then enter the respiratory pathway, which leads to the production

Table 2.8 – Second generation bioethanol production in batch mode by *Saccharomyces cerevisiae* and/or *Scheffersomyces stipitis.*

Feedstock	Pretreatment	Hydrolysis	Microbial strain	Fermentation (configuration/T/ agitation/t)	Ethanol			Ref.
					[Ethanol] ($g.L^{-1}$)	$Y_{ethanol/substrate}$ (% of theroretical)	$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	
S. cerevisiae								
Cistus ladanifer	Steam explosion	Enzymatic	NCYC 1119	SHF/30 °C/130 rpm/24 h	15.0	61.0	0.6	Ferro et al., 2015
			PYCC 2613[b]	SSF/42 °C/130 rpm/72 h	16.1	69.8	0.2	
Paper primary sludge	No	Enzymatic	ATCC 26602[b]	SSF/38 °C/150 rpm/53 h	41.7	48.9	0.78	Mendes et al., 2016
Cynara cardunculus	Dilute acid (H_2SO_4)	Enzymatic	NCYC 1119	SHF/30 °C/130 rpm/24 h	11.5	52.5	0.48	Fernandes et al., 2018
			PYCC 2613[b]	SSF/42 °C/130 rpm/72 h	14.4	64.4	0.20	
Bamboo	Alkali (H_2O_2)	Enzymatic	SR8u[c]	SHF/30 °C/180 rpm/96 h	46	90.2	0.48	Yuan et al., 2018
Miscanthus floridulus	Alkali (NaOH)	Enzymatic	Ethanol Red™	SSF/40 °C/150 rpm/72 h	27.5	78.4	0.38	Yeh et al., 2016
S. stipitis								
HSSL	No	No	NRRL Y-7124	-/28 °C/180 rpm/28 h[d]	2.4	47.1	0.09	Pereira et al., 2012
Sugarcane bagasse	Acid (H_2SO_4)	No	NRRL Y-7124	-/30 °C/450 rpm/-[d]	15.03	72.5	0.30	Silva et al., 2016
Wheat straw	Alkali (NaOH)	Dilute Acid (H_2SO_4)	NCIM 3498	SHF/28 °C/-/48 h	9.61	64.53	0.200	Koti et al., 2016
			PSUV9[e]		11.93	75.95	0.240	
***S. cerevisiae* and *S. stipitis* simultaneous co-culture**								
Sugarcane bagasse	Dilute acid (HNO_3)	Enzymatic	VSI 1011 + NCIM 3498	SHF/32 ° C/120 rpm/36 h[d]	31.01	76.84	1.29[f]	Santosh et al., 2017
Prosopis juliflora	Chemical ($Na_2S_2O_4$)	Dilute Acid (H_2SO_4)	VS3 + NCIM 3498	SHF/30°C/-/72 h[d]	6.11	82.34	0.170[g]	Naseeruddin et al., 2017

HSSL hardwood spent sulphite liquor; [Ethanol] ethanol concentration; $Y_{ethanol/substrate}$ ethanol yield; $Prod_{vol}$ volumetric ethanol productivity
[a] or fermentation/conversion efficiency; [b] thermotolerant strain; [c] strain engineered for xylose fermentation; [d] detoxification prior to fermentation; [e] *S. stipitis* NCIM 3498 UV mutant; [f] calculated based on 24 h fermentation period; [g] calculated based on 36 h fermentation period

of biomass and is induced by the presence of oxygen, or the alcoholic fermentation, induced by anaerobic conditions and producing ethanol. Hence, in the absence of oxygen pyruvate is converted to ethanol by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). This regulation between respiration and fermentation is called Pasteur effect (Sarris and Papanikolaou, 2016). Beside CO_2, acetate and glycerol can be formed as by-products during alcoholic fermentation (Madhavan et al., 2012).

S. cerevisiae is a Crabtree-positive yeast and therefore if glucose is available in excess it metabolizes sugar through fermentation even if oxygen is present (Papini et al., 2012). Also, in a mixture of glucose and other sugars, *S. cerevisiae* cannot metabolize the other sugars in the mixture until glucose is depleted, a phenomenon called glucose repression (Kim et al., 2012). *S. cerevisiae* presents a much higher ethanol tolerance when compared with other microorganisms. Several reasons have been pointed out to explain this fact, including faster production of ATP compared to respiration, ethanol's growth inhibitory effect on other microorganisms, and the ability of *S. cerevisiae* cells to respire ethanol when the concentration of fermentable sugars has dropped. Nevertheless, at the end of fermentation ethanol can reach inhibitory concentrations (Snoek et al., 2016). **Figure B1** in **Appendix B** presents glucose metabolism of *S. cerevisiae*.

It was found that aeration, especially in the beginning of the fermentation, could increase *S. cerevisiae*'s fermentation efficiency and ethanol tolerance. Oxygen is required for the synthesis of unsaturated fatty acids, ergosterol, and trehalose, which are related to membrane integrity, protecting it under stress conditions, like high ethanol concentration (Deesuth et al., 2016; Thani et al., 2017). *S. cerevisiae* presents an innate tolerance to hydrolysate inhibitors, however it can only tolerate certain levels of this compounds, which can inhibit yeast growth and fermentation. Besides, hydrolysate detoxification, metabolic engineering by overexpression of genes encoding enzymes that confer resistance toward specific inhibitors and also yeast adaptation to inhibitors have been studied to solve this issue (Xiros et al., 2013; Tesfaw and Assefa, 2014). The fact that it does not ferment pentoses, is one of the main challenges of applying *S. cerevisiae* for lignocellulosic ethanol production (Moysés et al., 2016). *S. cerevisiae* does not present specific xylose transporters, although xylose can enter the cell through hexose transporters. However, the latter present a very low affinity for xylose and hence the expression of heterologous xylose transporters is required (Nogué and Karhumaa, 2015). Although *S. cerevisiae* possesses the enzymes needed for the

complete metabolic pathway of xylose, their expression levels are very low (Pereira et al., 2013). Expression of heterologous genes coding enzymes involved in the xylose fermentation pathway has been researched, however inadequate metabolic responses, including xylitol accumulation, lower ethanol yields, and lower tolerance to inhibitors, have been observed. Evolutionary engineering through *S. cerevisiae* adaptation to a xylose medium was also investigated, however it is a slow process (Moysés et al., 2016; Kwak and Jin, 2017). Simultaneous (**Table 2.8**) or sequential co-culture of *S. cerevisiae* with a xylose fermenting microorganism is another alternative to solve this problem (Paulova et al., 2015).

2.4.4.2.2. *Scheffersomyces stipitis*

S. stipitis, formerly known as *Pichia stipitis* (Kurtzman and Suzuki, 2010), is a yeast isolated from decaying wood and the larvae of wood inhabiting insects able to ferment glucose, xylose, mannose, galactose and cellobiose, but not arabinose (Agbogbo and Coward-Kelly, 2008). Additionally, this yeast produces cellulases and hemicellulases (Jeffries et al., 2007). *S. stipitis* optimum fermentation temperature and pH range between 25-33 °C and 4.5-5.5, respectively (Agbogbo and Coward-Kelly, 2008). Although, *S. stipitis* presents the highest ethanol yields, near the theoretical maximum, from xylose under oxygen limited conditions (Veras et al., 2017), its specific ethanol productivity is at least five-fold lower than that obtained from *S. cerevisiae* when cultivated on glucose (Agbogbo et al., 2006). In **Table 2.8** different studies applying *S. stipitis* for second generation bioethanol production are summarized.

Xylose enters the cell either by low-affinity and high-affinity proton symport systems that operate simultaneously and is first reduced to xylitol by xylose reductase (XR), which is then oxidised to xylulose by xylitol dehydrogenase (XDH) (Agbogbo and Coward-Kelly, 2008). Subsequently, xylulose is phosphorylated by the enzyme xylulokinase and the resulting product enters the pentose phosphate pathway, whose products enter EMP, being converted to pyruvate. Pyruvate is then converted to ethanol by PDC and ADH (Gírio et al., 2010). Generally, XR is NADPH cofactor specific whereas XDH is NAD^+ cofactor specific, which leads to cofactor imbalance and, consequently, xylitol accumulation (Agbogbo and Coward-Kelly, 2008). However, *S. stipitis*' XR is specific for both NADPH and NADH, having a minimal xylitol accumulation (Yablochkova et al., 2003; Veras et al., 2017). This yeast also presents other pathways that provide a redox sink for coping with cofactor in-

balance (Agbogbo and Coward-Kelly, 2008). **Figure B2** in **Appendix B** presents *S. stipitis*' xylose metabolism.

Contrary to *S. cerevisiae*, which regulates fermentation depending on sugar concentration, *S. stipitis* induces fermentative activity in response to oxygen levels. *S. stipitis* is a Crabtree-negative yeast, showing a fully respiratory metabolism under aerobic conditions, even if glucose is present in excess, favouring cell growth. *S. stipitis* produces ethanol when oxygen becomes limiting, since reduced oxygen tension induces PDC and ADH activity (Papini et al., 2012). Nevertheless, under strictly anaerobic conditions, this yeast grows just one generation before growth and ethanol production stop (Acevedo et al., 2017). Hence, a high ethanol yield is associated to a narrow range of oxygen concentrations, i.e. microaerophilic conditions. The need of oxygenation for efficient ethanol production must be related to sugar transport, growth, or an unimpaired mitochondrial function (Silva et al., 2012, 2016). The optimum oxygen concentration in the bioreactor is the main bottleneck of *S. stipitis* bioethanol production (Pereira et al., 2013).

When culture medium contains both glucose and xylose, glucose repression through inhibition of xylose transport occurs (Agbogbo and Coward-Kelly, 2008). This leads to a preference for glucose as substrate, being consumed before xylose fermentation starts (Agbogbo et al., 2006). Another drawback of *S. stipitis* is the consumption of ethanol in the presence of oxygen while considerable amount of xylose is still in the medium (Harner et al., 2015). Besides inhibition by ethanol, inhibition of *S. stipitis* by high substrate concentrations has also been reported (Farias et al., 2014). *S. stipitis* is highly sensitive to several inhibitors, namely formic, acetic and levulinic acids, phenolics, and furfural (Pereira et al., 2013). Yeast adaptation is frequently used to improved *S. stipitis* tolerance to inhibitors. However, ethanol productivity from xylose has not yet matched *S. cerevisiae* performance (Harner et al., 2015).

2.4.5. Recovery and dehydration

Generally, at the end of the fermentation step, the fermentation broth contains only about 5 wt % of bioethanol, a low value when compared with first generation bioethanol, which can reach 12 wt % (Singh and Rangaiah, 2017). Lignocellulosic bioethanol recovery is achieved by distillation, a technique already well developed for first generation bioethanol (Amarasekara, 2014). The fermentation broth is first distilled in a stripper column that

concentrates ethanol to above 20 wt %, and then the ethanol stream is further concentrated in a rectifier column to no more than 95.6 wt % ethanol in water, due to the formation of a minimum boiling azeotrope at 78.15 °C and 1 atm (Ramaswamy et al., 2013; Singh and Rangaiah, 2017). Distillation is energy-intensive, accounting for 60−80% of total separation cost of bioethanol from water, particularly due to low ethanol concentration in the broth (Singh and Rangaiah, 2017). It is important to obtain ethanol concentrations higher than 4 wt % in order to have a lower energy demand in the recovery step. In this context, membrane technology appears as a more energy efficient process (Ramaswamy et al., 2013).

In order to achieve anhydrous ethanol (> 99.5 wt % ethanol) that is able to be blended with gasoline, a dehydration step after distillation is required (Singh and Rangaiah, 2017). As it happens with distillation, dehydration techniques are well developed for first generation bioethanol and are generally applicable to cellulosic ethanol. In the past, dehydration was usually achieved by azeotropic distillation (Amarasekara, 2014). Due to the high energy demand, azeotropic distillation has been replaced by adsorption with zeolite molecular sieves (Ramaswamy et al., 2013).

2.5. Pulp and paper industry

The pulp and paper industry is one of the largest industries in the world (Bajpai, 2012). In 2015, about 400 million tons of paper and paperboard were produced worldwide. Of the 188 million tonnes of virgin pulp produced, 93 % were produced from wood, while the remaining were produced from other raw materials like straw, bamboo, and bagasse (FAO, 2017). The manufacturing of pulp starts with wood preparation through debarking and chipping of wood logs in order to obtain wood chips that then proceed to the pulping process. The resulting bark and other rejects (e.g. branches, shives, and fine chips) are burned for energy production (Holik, 2013). After pulp production, the pulp, i.e. cellulosic fibers, presents a brown colour, as lignin is still present, and therefore is usually bleached before being sent to the papermaking process (Bajpai, 2012). Besides papermaking, pulp has other applications including textile, plastics (e.g. acetate and nitrate) and chemicals (e.g. cellulosic ethers). Cellulosic fibers present several advantages including abundance, renewable character, and biodegradability (Belgacem and Pizzi, 2016).

The pulping process can be chemical, semi-chemical, or mechanical. Mechanical pulping processes present the highest pulp yield, 85-95 %, and are cheaper (Belgacem and

Pizzi, 2016). These processes aim to mechanically separate the wood fibers. However, most of the lignin is kept in the fibers, yielding a pulp with low strength properties. Semichemical pulping processes are the combination between mild chemical and mechanical treatments and present pulp yields between 65 and 85 % (Bajpai, 2012). Chemical pulping alone represents about 77 % of the virgin pulps produced globally (FAO, 2017). Contrary to mechanical pulping, chemical pulping goal is delignification through lignin chemical degradation and solubilisation. Although chemical pulping processes present the lowest yields, about 45–60 %, pulps with better strength properties are produced. The most commonly used chemical pulping processes are sulphite and Kraft pulping (Holik, 2013; Belgacem and Pizzi, 2016). Sulphite pulping processes can be classified as acidic sulphite, bisulphite, neutral sulphite or alkaline sulphite and yield pulps easier to bleach but with lower strength than Kraft pulping (Belgacem and Pizzi, 2016).

Kraft pulping or sulfate process corresponds to more than 95 % of the chemical pulps produced (FAO, 2017) and consists on the reaction of an alkaline aqueous solution containing caustic soda (NaOH) and sodium sulphide (Na_2S) (i.e. white liquor) at high temperature (150-170 °C) with lignin. When this solution contacts with the wood chips, hydroxide and hydrosulfide anions react with lignin, which is partly depolymerized into phenolic fragments, whose dissolution removes almost 90 % of the lignin from the wood. Kraft fibers have a very high mechanical quality and can be obtained from any wood, unlike sulphite fibers. However, Kraft cooking conditions lead to hemicelluloses and some cellulose loss as well as lower cellulose degree of polymerization (Belgacem and Pizzi, 2016). At the end of the process, besides Kraft pulp, black liquor is also obtained. The black liquor is firstly concentrated by evaporation and then burned in a recovery boiler, producing energy to fuel the pulp mill and allowing the recovery of the chemicals used in the process, therefore providing an almost closed system with minimal pollution (Holik, 2013).

In Portugal, the pulp and paper is a growing industry of great importance for the country's economy. In 2015, the sales of the Portuguese pulp and paper industry corresponded to almost 4 billion euros, being mainly an exporting industry, responsible for 5 % of the country's exportations. In 2015, 2.662 million tons of virgin pulp were produced in Portugal, of which about 2.5 million tons were Kraft pulps (CELPA, 2016; INE, 2017). Portugal is the third biggest producer of pulp in the Confederation of European Paper

Industries, an association that represents 92 % of the European pulp and paper industry production (CEPI, 2017).

Eucalypt, a hardwood, is used to produce approximately 94 % of the pulps in Portugal, with *Eucalyptus globulus* being the most commonly used species (CELPA, 2016; Celbi, 2017). Hardwoods and softwoods have different compositions. While xylan is the predominant hemicellulose in hardwoods, softwoods contain mainly galactoglucomannan. Xylan are more resistant to the Kraft pulping conditions, explaining the higher pulp yield of hardwood Kraft pulp. Hardwoods also present a lower lignin content than softwoods and hardwood lignin also contains less carbon-carbon bonds, which are not affected by the cooking conditions. Hence, lignin removal is easier with hardwood (Belgacem and Pizzi, 2016). **Table 2.9** presents *E. globulus* plantation wood chemical composition.

Table 2.9 - *Eucalyptus globulus* plantation wood chemical composition (wt %). Adapted from Pereira et al., 2013

Cellulose	**Hemicellulose**		**Lignin**	**Extractives**	**Ash**
	Glucuronoxylan	**Glucomannan**			
50.0–53.0	18.0–24.0	2.0–4.0	19.0–22.0	1.0–2.7	0.3–0.5

2.5.1. Bioethanol production from Kraft pulp

Although chemical pulping processes are primarily used for manufacturing of paper and cellulose derivatives, they can be considered as LCB pretreatment methods since they remove lignin and even target hemicelluloses to some extent (Jönsson and Martín, 2016). Hence, Kraft pulping can be exploited as a pretreatment for ethanol production. Different studies have already proven the feasibility of Kraft pulping as a LCB pretreatment by obtaining hydrolysates with sugar profiles adequate for fermentation through enzymatic hydrolysis of Kraft pulp (Buzała et al., 2015, 2017a). Besides, Kraft pulping as many advantages including low sugar degradation, pulps are free of inhibitors like furfural and HMF, and the chemicals used are recovered and energy is produced in the recovery boiler (Monrroy et al., 2012; Buzała et al., 2015).

Edgardo et al. (2008) isolated a thermotolerant *S. cerevisiae* strain and tested it for SSF of *Pinus radiata* bleached Kraft pulp. Enzymatic hydrolysis was achieved using Celluclast 1.5 L (cellulases) supplemented with Novozym 188 (β-glucosidase) and 28 g ethanol.L^{-1} were obtained with a yield corresponding to 62 % of the theoretical. Alternatively, the authors tested organosolv as pretreatment, yielding 22 g ethanol.L^{-1} (73% of the theoretical). Using the same enzymatic cocktails and fermenting strain, Monrroy et al.

(2012) investigated the SSF of several *E. globulus* Kraft pulps. The ethanol concentrations and yields varied between 30-38 g ethanol.L^{-1} and 86-100 % (pulp basis), respectively.

Ko et al. (2012) used a mixture of endoglucanase, cellobiohydrolases, and xylanase and a *S. cerevisiae* strain for SSF of unbleached Kraft pulps of eucalypt and acacia, obtaining ethanol concentrations of 5.67 and 5.88 g.L^{-1} and cellulose conversions of 33.35 and 34.53 %, respectively. The authors did the same experiment using acid steam-explosion as pretreatment instead, obtaining higher ethanol concentration and cellulose conversion. Bauer and Gibbons (2012) tested the SSF of Kraft pulp using different dosages of Celluclast 1.5 L supplemented with Novozym 188 for hydrolysis and *S. cerevisiae* or *Candida molischiana* for ethanol fermentation. Increasing enzyme dosage resulted in increased ethanol concentration and yield. The ethanol concentrations obtained with *S. cerevisiae* and *C. molischiana* were 14.24-17.90 and 12.51-17.54 g.L^{-1}, respectively, and the ethanol yields were 68.33-85.90 and 60.00-84.17 % of the theoretical.

Wistara et al. (2016) investigated SSF of Kraft pulp of Jabon wood with different lignin content and freeness, concluding that higher yield of ethanol and cellulose conversion were obtained for lower lignin contents and higher pulp freeness. Ethanol yield varied between 2.89 and 16.39 % v/w and cellulose conversion ranged from approximately 5 to 24 %. Huang et al. (2017) produced bioethanol from Kraft pulp of bamboo residues through SHF. Novozymes Cellic CTec2 cellulase and Cellic HTec2 hemicellulase were used for hydrolysis, leading to an enzymatic hydrolysis efficiency of glucan and xylan of 91.5 and 93.2 %, respectively. Sequential fermentation with *S. cerevisiae* and *S. stipitis* was applied for ethanol fermentation. *S. cerevisia*e produced 43.5 g.L^{-1} of ethanol from glucose, while *S. stipitis* produced to 11.1 g.L^{-1} from xylose. Production efficiencies of ethanol from glucose and xylose were 84.4 and 76.6 %, respectively.

Amoah et al. (2017) investigated the production of bioethanol from hardwood unbleached Kraft pulp through CBP. A *S. cerevisiae* strain expressing five cellulase genes was applied, producing 0.71 g ethanol.L^{-1} with a yield of 41.2 % of theoretical. Buzała et al. (2017b) tested the production of bioethanol from different Kraft pulps through SHF. Enzymatic hydrolysis using enzyme preparation NS-22086 (cellulase and xynalase) yielded between 63.51-105.95 % (pulp basis). Ethanol yield from the five hardwood unbleached pulps used ranged from 0.24-0.26 g.g d.w. $pulp^{-1}$. For the pine (i.e. softwood) unbleached and bleached pulps, ethanol yields of 0.46 g.g d.w. $pulp^{-1}$ and 0.04 g.g d.w. $pulp^{-1}$ were

obtained, respectively. The production of biogas through AD of the obtained stillage was suggested.

High technical risk and high capital investment are two of the major drawbacks of industrial scale bioethanol production from LCB. Kraft mills already possess the technologies and equipment required for pretreatment of LCB, which are well-established and have been proven commercially for decades, including Kraft pulping and wood preparation. Hence, applying pulp and paper industry processes for bioethanol production in an integrated biorefinery seems like a promising solution for the aforementioned bottlenecks, increasing the opportunity of success of the ethanol production process (Monrroy et al., 2012; Phillips et al., 2013). Kraft pulping and subsequent hydrolysis and bioethanol production seem like a promising approach to valorise wastes resulting from this industry, such as low-quality wood, bark and other rejects, and low-quality and excess pulp, as well as to diversify the products and increase the profitability of pulp and paper industry. **Figure C1** in **Appendix C** presents a possible integrated biorefinery for the production of pulp, energy, second generation bioethanol and biogas from eucalypt wood, exploiting Kraft pulping as LCB pretreatment.

3. Material and Methods

3.1. Feedstock

3.1.1. Characterization of unbleached Kraft pulp

The unbleached Kraft pulp of *Eucalyptus globulus* (**Figure 3.1**) used in this work was previously washed and analysed at *RAIZ – Instituto de Investigação da Floresta e do Papel*. A more complete characterization of the pulp is shown in **Appendix D**.

Figure 3.1 - Unbleached Kraft pulp of *Eucalyptus globulus.*

3.1.2. Enzymatic hydrolysis of unbleached Kraft pulp

For the enzymatic hydrolysis, an enzymatic activity of 194.6 $FPU.mL^{-1}$ was used, corresponding to an enzymatic load of 25 $FPU.g\ of\ carbohydrate^{-1}$ (calculations described in **Appendix D**). 200.0 g of unbleached Kraft pulp and 2.354 L of distilled water (**Figure 3.2**) were placed in a bath at 50 °C. When the temperature stabilized, 200.0 mL of enzymatic cocktail Novozymes NS were added. The mixture was incubated for 24 h with a stirring of 100 rpm and at a temperature of 50 °C. Temperature and pH were monitored after 30 and 60 minutes of hydrolysis and then every hour, except during the night time. When necessary temperature and pH were adjusted to 45-50 °C and 4.5-5.5, respectively. The pH was adjusted through the addition H_2SO_4 3 M and NaOH 2 M solutions. Throughout the hydrolysis, samples were also taken to monitor reducing sugars.

After 24 h the hydrolysis was stopped by cooling the hydrolysate, then it was vacuum filtered to remove the existing solids (filter paper 545 with particle retention of 12-15 μm, VWR). The filtered hydrolysate (**Figure 3.3**) was sterilized in an autoclave Uniclave 88 (AJC) for 20 min at 121 °C. The formation of a precipitate was observed, which was then separated from the hydrolysate by centrifugation (Megafuge 16R, Thermo Scientific) for 1h

Figure 3.2 - Unbleached Kraft pulp and water in hydrolysis cup before hydrolysis.

Figure 3.3 - Filtered hydrolysate.

at 5000 rpm and 4 °C. The hydrolysate was sterilized again and stored at -20 °C in volumes of 300 mL. Before storage, samples were taken from the hydrolysate to analyse pH, dry weight, chemical oxygen demand (COD), glucose, xylose, ethanol, acetic acid, propionic acid, butyric acid, lactic acid, and valeric acid. A spectrum of the hydrolysate was acquired between 400 and 700 nm using a spectrophotometer UVmini-1240 (Shimadzu) and UVProbe 2.10 software (Shimadzu).

3.2. Microorganisms

Scheffersomyces stipitis NRRL Y-7124 was gently supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. *Saccharomyces cerevisiae* PYCC 5246 (ATCC 24860) was gently supplied by Portuguese Yeast Culture Collection. The stock cultures of *S. cerevisiae* and *S. stipitis* were stored in 20 % (v/v) glycerol at -80 °C. *S. cerevisiae* and *S. stipitis* cultures were grown at 28 °C and maintained at 4 °C in YM plates.

3.3. Media and stock solutions

The pH of all media and solutions used in this work was adjusted to 5.5 before autoclaving.

3.3.1. Yeast Mold medium

Yeast Mold (YM) liquid medium (3.0 $g.L^{-1}$ yeast extract, 3.0 $g.L^{-1}$ malt extract, 5.0 $g.L^{-1}$ peptone, and 10.0 $g.L^{-1}$ glucose) was used for pre-inocula and inocula preparation. YM solid medium, with 20.0 $g.L^{-1}$ of agar, was used for strain maintenance.

3.3.2. Supplementary medium

Two supplementary media were used in this work. Supplementary medium 1 (SM1) was composed by $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, and yeast extract. Supplementary medium 2 (SM2) had the same composition except for the yeast extract. To avoid salt precipitation due to complexation, each supplementary medium was prepared in two separate solutions, one containing $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$, and the other the remaining components. The concentration of these solutions was such that the components' concentration in the fermentations working volume were as shown in **Table 3.1**. SM1 was either used to supplement the hydrolysate or a sugar solution mimicking the sugars concentrations in the hydrolysate (64 $g.L^{-1}$ glucose and 12 $g.L^{-1}$ xylose), while SM2 was only used for hydrolysate supplementation.

Table 3.1 - Concentration in the fermentations working volume of the supplementary media components.

	Concentration ($g.L^{-1}$)	
Component	**SM1**	**SM2**
$(NH_4)_2HPO_4$	2.0	2.0
$(NH_4)_2SO_4$	1.0	1.0
$MgSO_4.7H_2O$	0.5	0.5
Yeast extract	2.5	-

3.4. Erlenmeyer flask assays

The Erlenmeyer flask assays were carried out in batch mode in 250 mL Erlenmeyer flasks with a working volume of 100 mL and incubated at 28 °C and 180 rpm. Throughout the fermentations, samples were taken to determine biomass, glucose, xylose, ethanol and pH. The COD of some selected samples was also quantified. All assays were performed in duplicate.

3.4.1. Pre-inocula and inocula

The pre-inocula were performed in 50 mL Falcon tubes with 10 mL of YM liquid medium by transferring a single colony from a maintenance YM plate to YM liquid medium, which were then incubated at 28 °C and 180 rpm during 24 h. At the end of this period, the inocula were prepared in 100 mL Erlenmeyer flasks with 40 mL of YM liquid medium by transferring, to new YM liquid medium, a volume of pre-inoculum that allowed an initial biomass concentration of 0.200 $g.L^{-1}$. The inocula were incubated at 28 °C and 180 rpm for 14 h.

3.4.2. Assays with mono-cultures of *S. cerevisiae* and *S. stipitis*

For each yeast strain, three Erlenmeyer flask assays, with a 100 mL working volume each, were performed:

- Synthetic medium assay prepared with 65 % (v/v) of sugar solution and 25 % (v/v) of SM 1;
- Hydrolysate supplemented with SM1 assay prepared with 65 % (v/v) of hydrolysate and 25 % (v/v) of SM 1;
- Hydrolysate supplemented with SM2 assay prepared with 65 % (v/v) of hydrolysate and 25 % (v/v) of SM 2.

Each assay was inoculated with a volume of inoculum that allowed to obtain an initial biomass concentration of 0.200 $g.L^{-1}$ for both strains. The final volume of each assay was adjusted with NaCl solution (0.9 %).

3.4.3. Assays with co-cultures of *S. cerevisiae* and *S. stipitis*

Assays with co-cultures of *S. cerevisiae* and *S. stipitis* were carried out in Erlenmeyer flasks of 100 mL working volume with 65 % (v/v) hydrolysate supplemented with 25 % (v/v) SM1. Two types of co-cultures were studied: simultaneous and sequential. In the assay with simultaneous co-culture both strains were added to the fermentation medium simultaneously in 1:1 ratio. A volume of inoculum that allowed for an initial biomass concentration of 0.100 $g.L^{-1}$ was used for each strain. After inoculation, NaCl 0.9 % (m/v) was added in order to obtain a final volume of 100 mL. In the assay with sequential co-culture, *S. cerevisiae* was firstly inoculated in a volume that allowed for an initial biomass concentration of 0.200 $g.L^{-1}$. Then, the working volume was adjusted to 100 mL with NaCl

0.9 % (m/v). *S. stipitis* was inoculated after 24 h of fermentation, with a volume of inoculum that allowed an initial concentration of this yeast of 0.200 $g.L^{-1}$.

3.5. Bioreactor fermentation

The bioreactor fermentations were carried out in batch mode, in a 5 L bioreactor BIOSTAT® Aplus (Sartorius Stedim Biotech) with a working volume of 3 L (**Figure 3.4**), automatic control of temperature and pH using micro DCU system (Sartorius Stedim Systems) and data acquisition by MFCS/DA 3.0 system (Sartorius Stedim Systems). The pH was measured using an electrode EasyFerm Plus K8 325 (Hamilton) and controlled to 5.50 ± 0.05 through the addition of KOH 5 M e de H_2SO_4 1 M. The temperature was controlled at 28 °C and the stirring 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. Throughout the fermentations, samples were taken to monitor biomass, glucose, xylose, and ethanol.

Figure 3.4 – Bioreactor fermentation.

3.5.1. Pre-inoculum and inoculum

The pre-inocula were performed in 100 mL Erlenmeyer flasks with 40 mL of YM liquid medium by transferring two colonies from a maintenance YM plate to YM liquid medium, which were then incubated at 28 °C and 180 rpm during 24 h. At the end of this period, the inocula were prepared in 500 mL Erlenmeyer flasks with 300 mL of YM liquid medium by transferring, to new YM liquid medium, a volume of pre-inoculum that allowed an initial biomass concentration of 0.300 $g.L^{-1}$. The inocula were incubated at 28 °C and 180 rpm for 14 h.

3.5.2. Assays with mono-cultures of *S. cerevisiae*

The fermentation media were inoculated with 10 % (v/v) of inoculum in order to obtain an initial biomass concentration of 0.200 $g.L^{-1}$. Two assays were performed with the following media:

- Synthetic medium assay prepared with 65 % (v/v) of sugar solution and 25 % (v/v) of SM 1;
- Hydrolysate assay prepared with 65 % (v/v) of hydrolysate and 25 % (v/v) of SM1.

3.5.3. Assay with sequential co-culture of *S. cerevisiae* and *S. stipitis*

The co-culture assay was carried out in 65 % (v/v) hydrolysate supplemented with 25 % (v/v) SM1. The fermentation medium was firstly inoculated with 10% (v/v) of inoculum of *S. cerevisiae* in order to obtain an initial biomass concentration in the working volume of 0.200 $g.L^{-1}$. *S. stipitis* was inoculated after 24 h of fermentation with the total volume of inoculum to attain the maximum concentration of *S. stipitis* in the fermentation medium. From 15 minutes before the inoculation of *S. stipitis* until the end of the fermentation the bioreactor was aerated using a ring sparger and an air flow of 50 $mL.min^{-1}$ (4800 series flow controller, Brooks).

3.6. Asepsis and sterility

To ensure aseptic and sterile conditions, all media and material were sterilized by autoclaving (Uniclave 88 AJC) for 20 min at 121 °C. All procedures involving the microorganisms, except for the sample collection in the bioreactor assays and the inoculation of *S. stipitis* in the co-culture bioreactor assay, were carried in a laminar air flow cabinet (BBH4 Braun Horizontal). At the end of each assay, the absence of contamination was assessed by plating the samples in YM solid medium. Sampling in the bioreactor assays was made using a manual sampler, containing a sampling tube and a suctioning syringe with a 0.22 µm nylon filter (**Figure 3.5**). The inoculation with *S. stipitis* was done using a system composed by a hollow stainless-steel tube connected to a glass tubing connector and a pump (Watson-Marlow SciQ 400).

Figure 3.5 – Sampling tube for sample collection in bioreactor fermentations.

3.7. Analytical methods

3.7.1. Reducing sugars

Reducing sugars were analyzed by the dinitrosalicylic acid (DNS) method (Miller, 1959). The enzymes present in the hydrolysate were firstly inactivated by heating the samples at 100 °C for 5 min. The samples were then centrifuged for 20 min at 5000 rpm. The supernatant was collected and centrifuged for 10 min at 5000 rpm. 5 mL of DNS reagent were added to 1 mL of properly diluted sample. The mixture was heated at 100 °C for 5 min and the reaction was stopped by cooling the mixture. After adding 10 mL of distillate water to the mixture, the absorbance was measured at 540 nm. The calibration was done with glucose in concentrations between 0–5 $g.L^{-1}$.

3.7.2. pH

The pH of the samples from the Erlenmeyer flask fermentations was measured using an electrode InPro 3030/200 (Mettler Toledo) and a benchtop meter sensION+ MM340 (Hach).

3.7.3. Biomass quantification

The biomass was monitored measuring the optical density at 620 nm (OD_{620}) using a spectrophotometer UVmini-1240 (Shimadzu) and further conversion into biomass concentration using a calibration curve of OD_{620} versus biomass dry weight. A different calibration curve was made for each yeast strain and for each medium, due to the differences in the absorbance of each medium (see **Appendix E** for one example). The calibration curve

for the co-culture assays was the linear regression obtained after plotting together the data from the calibration curves of *S. cerevisiae* and *S. stipitis* in hydrolysate.

3.7.4. Glucose, xylose, ethanol and short chain organic acids quantification

Glucose, xylose, and ethanol were analysed by HPLC (High Performance Liquid Chromatography). Samples were centrifuged for 5 min at 13000 rpm (MiniSpin, Eppendorf) to remove the biomass, then the supernatant was collected and stored at -20 °C. Before analysis, it was properly diluted, acidified with 4 % (v/v) H_2SO_4 0.25 M and filtered through modified nylon 0.20 µm centrifugal filter (VWR) by centrifugation for 10 min at 8000 rpm (MiniSpin, Eppendorf). Then, the samples were injected to a Rezex ROA-Organic Acid H+ (8 %) 50 x 7,8 mm ion-exchange column (Phenomenex), with an oven Gecko 2000 set at 65 °C, 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, with a flow rate of 0.5 $mL.min^{-1}$. The HPLC system also consisted of an autosampler L-2200 (VWR-Hitachi), a pump L-2130 (VWR-Hitachi), and 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 area versus the compound concentration in the corresponding standard. The calibration curve was done with solutions containing the different compounds in concentrations between 0 and 5 $g.L^{-1}$.

3.7.5. Chemical oxygen demand

The COD was measured with Spectroquant Kit (Merck) and the solutions used were prepared according to Standard Methods (Clesceri et al., 1999). Before analysis, samples were centrifuged for 5 min at 13000 rpm (MiniSpin, Eppendorf) to remove the biomass, then the supernatant was collected and stored at -20 °C. 1.2 mL of digestive solution ($K_2Cr_2O_7$, $HgSO_4$, H_2SO_4 and H_2O) and 2.8 mL of acid solution (H_2SO_4 and $AgSO_4$) were added to 2 mL of sample properly diluted. The mix was incubated at 150 ºC for 2 h in a termoreactor Spectroquant TR 620. After cooling, the absorbance was measured in a spectrometer Spectroquant Picco. The calibration was done with glucose with COD concentrations between 0–1 $g.L^{-1}$.

3.8. Calculation methods

3.8.1. Enzymatic hydrolysis

The hydrolysis yield, $Y_{Hydrolysis}$ ($g.g^{-1}$), was calculated using **Equation 3.1** according to the glucose and xylose concentrations obtained and the potential glucose and xylose in the unbleached Kraft pulp (calculated in **Appendix F**). The hydrolysis yield on glucose and hydrolysis yield on xylose were calculated using **Equation 3.4** and **3.3**, respectively. The final values resulted from the calculation of the average and standard deviation of the four hydrolysis performed.

$$Y_{Enzymatic\ hydrolysis}\ (\%\ theoretical) = \frac{glucose\ obtained + xylose\ obtained}{potential\ glucose + potential\ xylose} \times 100 \qquad \textbf{Equation 3.1}$$

$$Y_{Glucose}\ (\%\ theoretical) = \frac{glucose\ obtained}{potential\ glucose} \times 100 \qquad \textbf{Equation 3.2}$$

$$Y_{Xylose}\ (\%\ theoretical) = \frac{xylose\ obtained}{potential\ xylose} \times 100 \qquad \textbf{Equation 3.3}$$

3.8.2. Fermentation assays

The 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.

The volumetric glucose consumption rate, $r_{glucose}$ ($g.L^{-1}.h^{-1}$), was calculated by determining the module of the slope of the linear regression obtained after plotting glucose concentration versus time for the time in which glucose consumption had an approximately linear behaviour. The volumetric xylose consumption rate, r_{xylose} ($g.L^{-1}.h^{-1}$), was calculated in the same way, by plotting xylose concentration versus time.

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 time in which ethanol production had a linear behaviour.

The volumetric ethanol productivity, $Prod_{vol}$ ($g.L^{-1}.h^{-1}$), was calculated using **Equation 3.4**, from the beginning of the fermentation until maximum ethanol concentration was achieved.

$$Prod_{vol} = \frac{\Delta[Ethanol]}{\Delta t} \qquad \textbf{Equation 3.4}$$

The ethanol yield, $Y_{ethanol/substrate}$ ($g.g^{-1}$), and the biomass yield, $Y_{biomass/substrate}$ ($g.g^{-1}$), were calculated using **Equation 3.5** and **3.6**, respectively, considering both glucose and xylose as substrate. $Y_{ethanol/substrate}$ and $Y_{biomass/substrate}$ were calculated from the beginning of the fermentation until the maximum ethanol concentration was achieved.

$$Y_{ethanol/substrate} = -\frac{\Delta[ethanol]}{\Delta[substrate]} \qquad \textbf{Equation 3.5}$$

$$Y_{biomass/substrate} = -\frac{\Delta[biomass]}{\Delta[substrate]} \qquad \textbf{Equation 3.6}$$

The conversion efficiency (%) was determined using **Equation 3.7**, considering a maximum theoretical ethanol yield ($Y_{theoretical\ max}$) of 0.511 $g.g^{-1}$ (Kang et al., 2014).

$$Conversion\ efficiency\ (\%) = \frac{Y_{ethanol/substrate}}{Y_{theoretical\ max}} \times 100 \qquad \textbf{Equation 3.7}$$

The consumed sugars (%) were calculated from the beginning of the fermentation until the maximum ethanol concentration was achieved (**Equation 3.8**).

$$Consumed\ sugars\ (\%) = \left(1 - \frac{[glucose]_f + [xylose]_f}{[glucose]_i + [xylose]_i}\right) \times 100 \qquad \textbf{Equation 3.8}$$

The final values for the Erlenmeyer flask fermentations resulted from the calculation of the average and standard deviation of the duplicates performed for each assay.

4. Results and Discussion

4.1. Enzymatic hydrolysis of unbleached Kraft pulp

In this work, four assays of enzymatic hydrolysis of unbleached Kraft pulp of *Eucalyptus globulus*, all under the same conditions, were performed. The evolution of temperature, reducing sugars, and pH was very similar in all assays. **Figure 4.1** shows the evolution of these parameters for hydrolysis 1, and the results of the other three assays are shown in **Appendix G**. During the assays, the temperature increased in the first 2 h of hydrolysis and stabilized around 50 °C, while the pH oscillated in the first hour of hydrolysis and then stabilized around 4.86. There was an increase in the concentration of reducing sugars over the hydrolysis time until 78.1 $g.L^{-1}$.

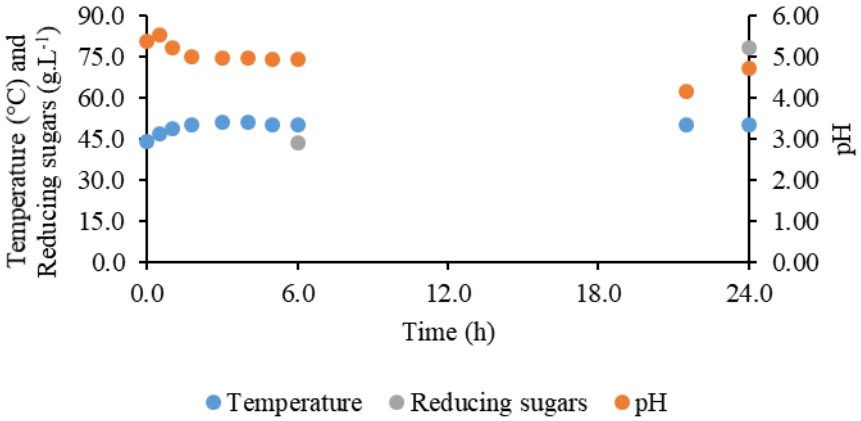

Figure 4.1 – Evolution of temperature, reducing sugars, and pH in enzymatic hydrolysis 1.

Hydrolysates with 65.45 ± 0.80 $g.L^{-1}$ of glucose and 16.02 ± 1.75 $g.L^{-1}$ of xylose were obtained, corresponding to a hydrolysis yield of 95.6 ± 2.6 %. **Table 4.1** shows the results obtained in enzymatic hydrolysis assays of Kraft pulps of different origins and of *E. globulus* wood with different pretreatments found in the literature. The yields on glucose and xylose achieved in this work, 96.1 ± 3.6 and 94.0 ± 7.1 %, are very similar to those reported by Huang et al. (2017), 91.5 and 93.2 %, using unbleached Kraft pulp of *Phyllostachys heterocycle* as feedstock. The average yield on glucose was higher than the results obtained by Buzała et al. (2017b), 54.3-92.7 %, for the enzymatic hydrolysis of several Kraft pulps. The yields obtained in the present work were also higher than those obtained by Arévalo et al. (2017) and Martín-Sampedro et al. (2015), who studied the enzymatic hydrolysis of *E. globulus* wood with autohydrolysis or diluted acid hydrolysis and biological and autohydrolysis pretreatments, respectively.

Table 4.1 – Comparison between the results of the enzymatic hydrolysis of this work and the results of enzymatic hydrolysis in literature.

Enzymatic cocktail	**Enzymes**	**Hydrolysis conditions (T/pH/duration)**	**Feedstock**	**$Y_{glucose}$ (% theoretical)**	**Y_{xylose} (% theoretical)**	**Ref**
Novozymes NS	Cellulases and hemicellulases	45-50 °C/4.5-5.5/24 h	Unbleached Kraft pulp of *E. globulus*	96.1 ± 3.6	94.0 ± 7.1	This work
Novozymes NS-22086	Cellulases and xylanases	50 °C/5.0/72 h	Unbleached Kraft pulp of *Populus tremula*	82.7	-	Buzała et al., 2017b
			Unbleached Kraft pulp of *Betula pendula*	81.2	-	
			Unbleached Kraft pulp of *Fagus sylvatica*	81.6	-	
			Unbleached Kraft pulp of *Pinus sylvestrii*	54.3	-	
			Bleached Kraft pulp of *Pinus sylvestrii*	92.7	-	
Novozymes Cellic CTec2 + Novozymes Cellic HTec2	Cellulase + Hemicellulase	50 °C/4.8/48 h	Unbleached Kraft pulp of *Phyllostachys heterocycle* residues	91.5	93.2	Huang et al., 2017
Novozymes NS-22086	Cellulases and xylanases	50 °C/5.0/48 h	Unbleached Kraft pulp of *Triticum aestivum* L.	88.8	-	Buzała et al., 2017a
Vlan Biotech Group Cocktail	Cellulase	50 °C/5.0/10 h	Bleached Kraft pulp of Hardwood	61.0	-	Liu et al., 2016
Novozymes NS-22128 + Novozymes NS-22128	Cellulase + β-glucosidase	60 °C/-/72 h	Autohydrolysis-pretreated *E. globulus*	75.9	-	Arévalo et al., 2017
			Diluted Acid Hydrolysis-pretreated *E. globulus*	80.0	-	
Novozymes Novozym 188 + Novozymes Celluclast 1.5L	β-glucosidase + Cellulolytic complex	50 °C/4.8/72 h	Biologically and autohydrolysis-pretreated *E. globulus*	31.0–34.2	23.6–29.0	Martín-Sampedro et al., 2015

Before storage the hydrolysate was sterilized by autoclave. During the sterilization, the formation of a precipitate was observed (**Figure 4.2**). Alves (2015) also reported the formation of a precipitate when a similar hydrolysate was autoclaved. After an acidic hydrolysis of the precipitated, Alves (2015) verified that it was mainly composed by low molecular weight xylans. These xylans were polysaccharides not fully hydrolysed, which is consistent with the hydrolysis yield lower than 100 %.

Figure 4.2 – Precipitate formed after hydrolysate sterilization.

4.2. Hydrolysate characterization

From the four enzymatic assays performed, the obtained hydrolysates were mixed and a final volume of about 8.4 L was obtained. The composition of the mixture of the four hydrolysates in glucose and xylose was 64.37 $g.L^{-1}$ and 11.75 $g.L^{-1}$, respectively, and no ethanol, acetic acid, propionic acid, butyric acid, lactic acid, and valeric acid were detected by HPLC. The mixture had a pH of 4.52, a dry weight of 0.750 $g.L^{-1}$, and a COD of 120 g $O_2.L^{-1}$. To allow biomass quantification by DO at 620 nm and to determine if the hydrolysate presented any absorbance near 620 nm a spectrum of the hydrolysate was acquired (**Appendix H**). The hydrolysate did not show a significant absorbance at 620 nm and therefore it would not interfere at this wavelength. From now on, the mixture of hydrolysates will be designated by hydrolysate.

4.3. Erlenmeyer flask assays

Erlenmeyer flask assays with mono-cultures of *S. cerevisiae* and *S. stipitis* were initially investigated. In these first assays, growth and fermentation of the yeasts in synthetic medium and hydrolysate were studied, and it was accessed if the supplementation of the hydrolysate with yeast extract was required. Fermentation of the hydrolysate by *S. cerevisiae* was studied because of its high efficiency in bioethanol production, including high ethanol yields and high tolerance to ethanol and inhibitors usually present in hydrolysates of LCB. However, *S. cerevisiae* does not ferment pentoses, such as xylose, which is present in the hydrolysate obtained from Kraft pulp (11.75 $g.L^{-1}$). Hence, *S. stipits*, the most promising hexose and pentose-fermenting yeast for ethanol production, was also investigated in this work. Despite the sensitivity of *S. stipitis* to low pH, inhibitors presence and high ethanol concentrations, using this yeast would allow for the fermentation of both glucose and xylose present in the hydrolysate.

4.3.1. Assays with *S. cerevisiae*

Three assays were performed with *S. cerevisiae*: one in synthetic medium with SM1 and a sugar solution that mimic the hydrolysate; other in hydrolysate supplemented with SM1; and another in hydrolysate supplemented with SM2. SM1 and SM2 had the same composition except for the yeast extract, which was only present in SM1, allowing to assess the necessity of using yeast extract. **Figure 4.3** shows the evolution of pH and concentrations of glucose, xylose, ethanol, and biomass in all three assays and **Table 4.2** the kinetic and stoichiometric parameters calculated for these assays.

In the synthetic medium assay, glucose exhaustion and maximum ethanol concentration, 16.88 ± 1.05 $g.L^{-1}$, with an ethanol yield of 0.388 ± 0.028 $g.g^{-1}$, were detected after 23.0 h of fermentation (**Figure 4.3A**). However, considering the $r_{glucose}$ and $r_{ethanol}$, 4.57 ± 0.00 and 1.81 ± 0.16 $g.L^{-1}.h^{-1}$, respectively, it was assumed that glucose exhaustion and maximum ethanol occurred earlier around 13.0 h. In this assay, a μ of 0.447 ± 0.002 h^{-1}, a $Prod_{vol}$ of 1.30 ± 0.08 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ of 0.092 ± 0.003 $g.g^{-1}$, and a conversion efficiency of 76.1 ± 5.6 % were achieved. Biomass concentration increased initially while glucose was being consumed and then stabilized around 4.25 $g.L^{-1}$ until 28.5 h, when ethanol started being consumed for biomass production and a slight increase in biomass concentration was observed. In the last 4.0 h of fermentation, 7.39 $g.L^{-1}$ of ethanol were

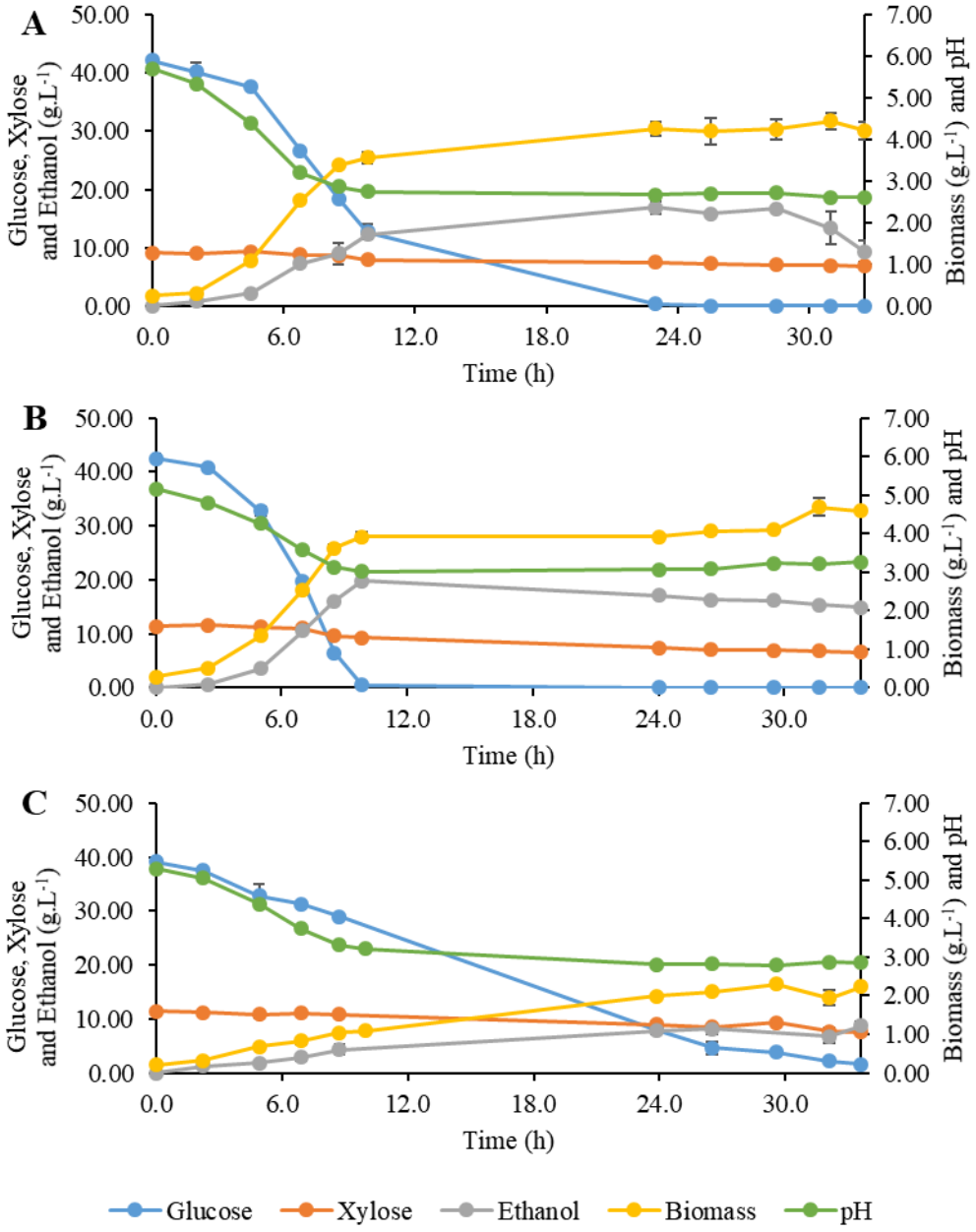

Figure 4.3 - Evolution of pH and concentration of glucose, xylose, ethanol, and biomass in Erlenmeyer flask assays with *S. cerevisiae*. A- Synthetic medium; B- Hydrolysate supplemented with SM1; C- Hydrolysate supplemented with SM2.

consumed. Indeed, one of the reasons pointed out to explain the high tolerance to ethanol by *S. cerevisiae* is its ability to respire ethanol when the concentration of fermentable sugars has dropped (Snoek et al., 2016). pH decreased simultaneously with the increase in biomass and ethanol concentrations and glucose consumption (**Figure 4.3A**). This decrease probably resulted from the production of CO_2 during cell growth and alcoholic fermentation. When CO_2 dissolves in water it forms carbonic acid (H_2CO_3), causing the pH to drop to values as low as 2.75. Although, *S. cerevisiae* PYCC 5246 (ATCC 24860) presents a high pH

tolerance, this yeast strain is known to be affected when the pH drops below 4 (Geng et al., 2010). Also, a slight consumption of xylose was detected, with very low r_{xylose} (**Table 4.2**), since *S. cerevisiae* presents very low expression levels of the enzymes required for the complete metabolic pathway of xylose (Pereira et al., 2013).

Table 4.2 – Kinetic and stoichiometric parameters calculated for Erlenmeyer flask assays with *S. cerevisiae*.

Parameter	**Assay**		
	Synthetic medium	**Hydrolysate SM1**	**Hydrolysate SM2**
μ (h^{-1})	0.447 ± 0.002	0.360 ± 0.008	0.203 ± 0.023
$[Ethanol]_{max}$ ($g.L^{-1}$)	16.88 ± 1.05	19.81 ± 0.15	8.72 ± 0.79
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	4.57 ± 0.00	6.91 ± 0.20	1.24 ± 0.03
r_{xylose} ($g.L^{-1}.h^{-1}$)	0.237 ± 0.050	0.193 ± 0.003	0.129 ± 0.002
$r_{ethanol}$ ($g.L^{-1}.h^{-1}$)	1.81 ± 0.16	3.40 ± 0.05	0.299 ± 0.006
$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	1.30 ± 0.08[a]	2.01 ± 0.01	0.260 ± 0.024
$Y_{ethanol/substrate}$ ($g.g^{-1}$)	0.388 ± 0.028	0.450 ± 0.009	0.211 ± 0.020
$Y_{biomass/substrate}$ ($g.g^{-1}$)	0.092 ± 0.003	0.081 ± 0.004	0.049 ± 0.000
Conversion efficiency (%)	76.1 ± 5.6	88.3 ± 1.7	41.4 ± 3.8
Consumed sugars (%)	84.7 ± 0.4	81.8 ± 0.4	81.8 ± 0.4

[a] calculated for 13.0 h of fermentation period

In the assays with hydrolysate supplemented with SM1, the fermentation time required to reach maximum ethanol concentration decreased to 9.8 h, coinciding with glucose exhaustion (**Figure 4.3B**). Biomass concentration increased initially while glucose was being consumed and then stabilized at 3.92 $g.L^{-1}$ until around 24.0 h, when ethanol started to be reassimilated, resulting in biomass concentration increase. In this assay, not only a higher maximum ethanol concentration, 19.81 ± 0.15 $g.L^{-1}$, was attained in a shorter fermentation time, but also higher values of $r_{ethanol}$, 3.40 ± 0.05 $g.L^{-1}.h^{-1}$, $Prod_{vol}$, 2.01 ± 0.01 $g.L^{-1}.h^{-1}$, and $Y_{ethanol/substrate}$, 0.450 ± 0.009 $g.g^{-1}$, were observed. On the other hand, in this assay, lower μ and $Y_{biomass/substrate}$ were observed, 0.360 ± 0.008 h^{-1} and 0.081 ± 0.004 $g.g^{-1}$, respectively (**Table 4.2**). It appears that in this medium, *S. cerevisiae* was able to deviate its carbon flux more towards alcoholic fermentation and less towards growth. These results were in accordance to Geng et al. (2010) that studied the tolerance of different *S. cerevisiae* strains to some stress factors, including inhibitors usually present in LCB hydrolysates (i.e. formic acid, acetic acid, furfural, and HMF). *S. cerevisiae* PYCC 5246 (ATCC 24860), the strain used in the present work, showed a high inhibitor resistance (Geng et al. 2010). In this assay, a decrease in pH, simultaneously with the increase in biomass and ethanol

concentrations and glucose consumption, and a slight consumption of xylose with very low r_{xylose} were observed (**Figure 4.3B**).

Figure 4.4 shows the evolution of COD determined according to Standard Methods and COD calculated from the concentrations of glucose, xylose and ethanol obtained in the HPLC (calculations explained in **Appendix H**) in the hydrolysate supplemented with SM1 assay. The evolution of Experimental COD and Calculated COD got closer as the fermentation progressed. The higher decrease observed on Experimental COD could be an indication that *S. cerevisiae* consumed other compounds present in the hydrolysate than glucose and xylose. *S. cerevisiae* strains can assimilate glucose, maltose, raffinose, and ethanol. Most strains of *S. cerevisiae* ferment sugars like glucose, fructose, mannose, maltose, maltotriose, raffinose, trehalose and xylulose. Some organic acids, and polyhydroxy alcohols are also fermentable (Batt and Tortorello, 2014). *S. cerevisiae* PYCC 5246 in specific is known to ferment glucose, maltose, raffinose, and xylulose, and to grow on glucose, ethanol, maltose, trehalose, and raffinose (Jeppsson et al., 1996; Portuguese Yeast Culture Collection, 2018). The increase in biomass concentration after glucose exhaustion could also be explained by the consumption of other compounds present in the hydrolysate medium, as well as the lower decrease on ethanol concentration compared with the assay in synthetic medium.

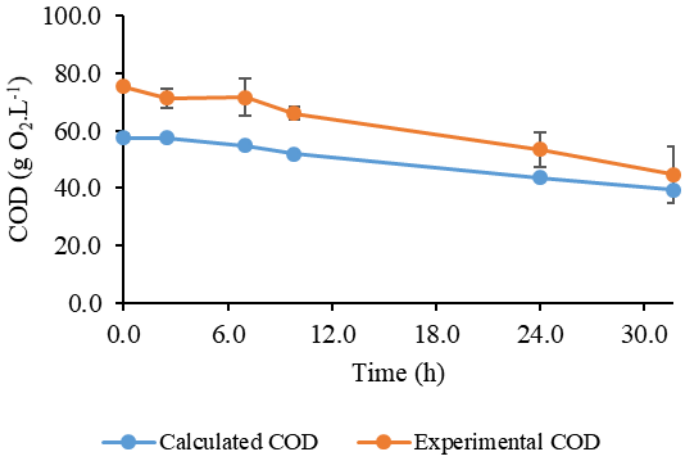

Figure 4.4 – Evolution of COD in the assay with *S. cerevisiae* in hydrolysate supplemented with SM1.

In the assay in hydrolysate supplemented with SM2, a maximum ethanol concentration of only 8.72 ± 0.79 $g.L^{-1}$ was attained after 33.6 h of fermentation, with a lower ethanol yield, 0.211 ± 0.020 $g.g^{-1}$. By this time there were still 1.62 ± 0.09 $g.L^{-1}$ of glucose in the medium (**Figure 4.3C**). In the absence of yeast extract, all the kinetic and stoichiometric parameters, with exception of the percentage of consumed sugars, were the

lowest of the three assays (**Table 4.2**). Yeast extract has as complex composition, providing nitrogenous compounds, carbon, sulphur, trace nutrients, vitamin B complex and other important growth factors (Sigma-Aldrich, 2018). Therefore, the fact that both growth and ethanol fermentation decreased when yeast extract was not supplemented indicates that the hydrolysate lacks one or more of these nutrients required for these two metabolic pathways in *S. cerevisiae*. The consumed sugars at the time of maximum ethanol were the same in both assays in hydrolysate, 81.8 ± 0.4 %. However, at this time in the hydrolysate supplemented with SM2 the biomass and ethanol concentrations were lower (**Figure 4.3C**). Probably, sugars are being redirected to metabolic pathways other than biomass and ethanol formation, possibly pathways required for maintenance to withstand the limiting conditions. Hence, hydrolysate supplementation with yeast extract is required to achieve a better growth and fermentation by *S. cerevisiae*.

Although a high conversion efficiency, 88.3 ± 1.7 %, was achieved in the hydrolysate supplemented with SM1 assay, most xylose was still present in the fermentation medium by the end of the fermentation.

4.3.2. Assays with *S. stipitis*

One of the best approaches to reduce second generation bioethanol production costs is to use all sugars from LCB, including the ones obtained from the hemicelluloses. However, in the assays with *S. cerevisiae* the main sugar resulting from the hemicellulose fraction of LCB, i.e. xylose, was not being used and, consequently, the sugars resulting from LCB were not entirely utilized for ethanol production. Thus, fermentation with *Scheffersomyces stipitis* NRRL Y-7124, a hexose- and pentose-fermenting yeast, was evaluated. The assays with *S. stipitis* were similar to those with *S. cerevisiae*: one in SM1 and sugar solution, other in hydrolysate supplemented with SM1, and another in hydrolysate supplemented with SM2. **Figure 4.5** shows the evolution of pH and concentrations of glucose, xylose, ethanol, and biomass in all three assays and **Table 4.3** the kinetic and stoichiometric parameters calculated for assays in synthetic medium and hydrolysate supplemented with SM1.

In the synthetic medium assay, a maximum ethanol concentration, 14.40 ± 0.28 $g.L^{-1}$, was observed after 31.8 h, when glucose exhaustion was detected (**Figure 4.5A**). In this assay, a μ of 0.334 ± 0.016 h^{-1}, a $r_{glucose}$ of 1.98 ± 0.07 $g.L^{-1}.h^{-1}$, a $r_{ethanol}$ of 0.582 ± 0.026 $g.L^{-1}.h^{-1}$, a $Prod_{vol}$ of 0.533 ± 0.015 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ 0.123 ± 0.003 $g.g^{-1}$, and a $Y_{ethanol/substrate}$ of 0.323 ± 0.005 $g.g^{-1}$, corresponding to a conversion efficiency of 63.3 ± 0.9 %, were achieved (**Table 4.3**). Contrary to what was expected only a slight decrease in xylose concentration was observed, with an r_{xylose} of 0.225 ± 0.070 $g.L^{-1}.h^{-1}$, similar to the observed in *S. cerevisiae* assay in synthetic medium. Instead, reassimilation of 1.00 $g.L^{-1}$ of ethanol was observed after 33.8 h, with simultaneous biomass growth (**Figure 4.5A**). Indeed, a drawback of *S. stipitis* is the consumption of ethanol in the presence of oxygen while a considerable amount of xylose is still present in the medium as previously observed by Harner et al. (2015). Also, the fact that the pH dropped to values of 2.63, due to the high amount of biomass produced while glucose was being consumed, might inhibited *S. stipitis* from using xylose. Slininger et al. (1990) determined that the optimum pH range of 4.0 – 7.0 for growth and fermentation of *S. stipitis* NRRL Y-7124 on xylose. The higher cell growth in this fermentation, with a maximum biomass concentration of 6.27 ± 0.10 $g.L^{-1}$, arises from the fact that *S. stipitis*, contrary to *S. cerevisiae,* is a Crabtree-negative yeast, favouring cell growth over ethanol production under aerobic conditions, even if glucose is present in excess

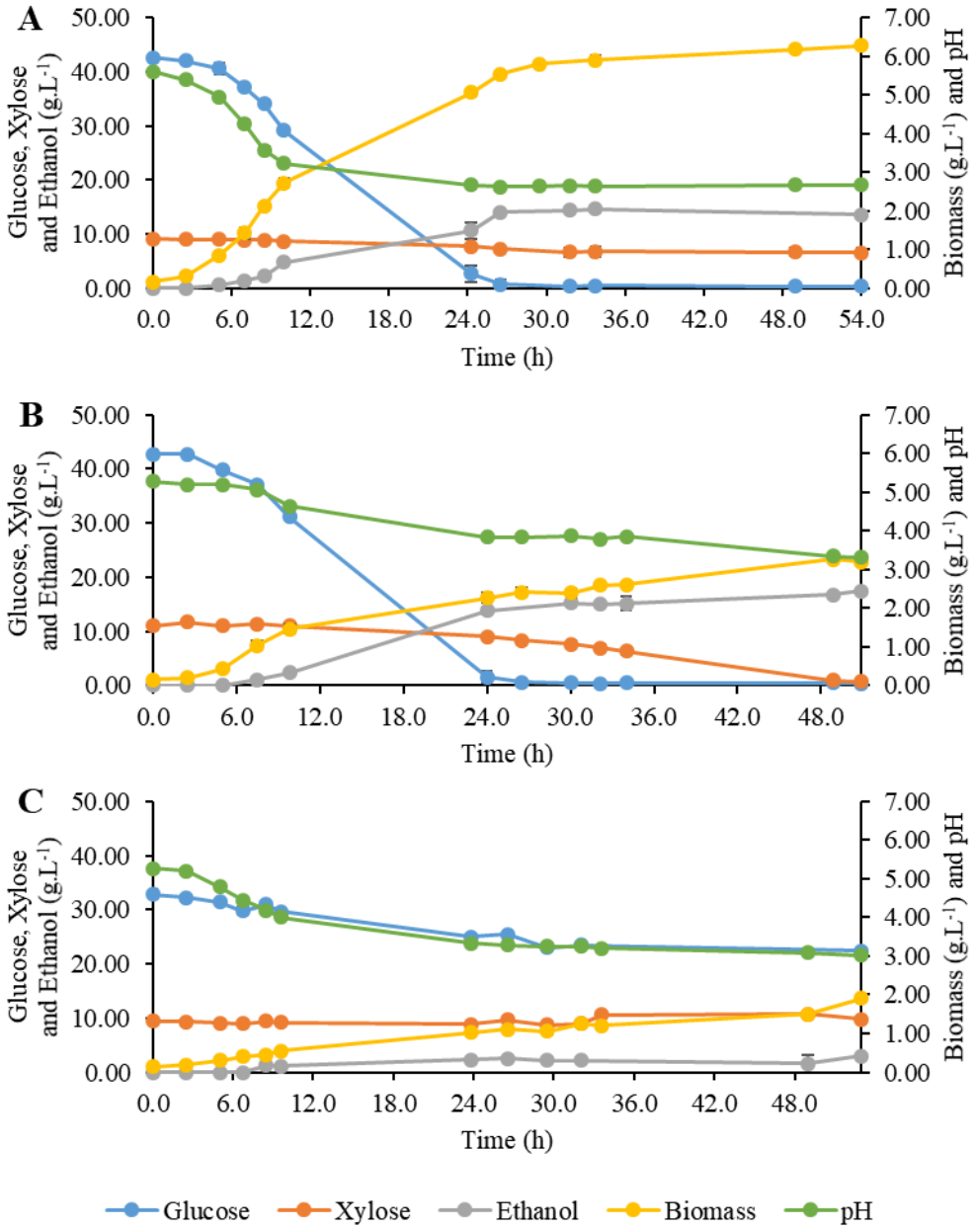

Figure 4.5 - Evolution of pH and concentrations of glucose, xylose, ethanol, and biomass in Erlenmeyer flask assays with *S. stipitis*. A- Synthetic medium; B- Hydrolysate supplemented with SM1; C- Hydrolysate supplemented with SM2.

(Papini et al., 2012). In fact, in the assay with *S. stipitis* in synthetic medium a much higher biomass yield, but lower ethanol productivity, conversion efficiency and maximum ethanol concentration, than in the assay with *S. cerevisiae* in synthetic medium were observed.

Table 4.3 - Kinetic and stoichiometric parameters calculated for Erlenmeyer flask assays with *S. stipitis* in synthetic medium and hydrolysate supplemented with SM1.

Parameter	**Assay**	
	Synthetic medium	**Hydrolysate SM1**
μ (h^{-1})	0.334 ± 0.016	0.336 ± 0.000
$[Ethanol]_{max}$ ($g.L^{-1}$)	14.40 ± 0.28	17.50 ± 0.09
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	1.98 ± 0.07	1.98 ± 0.03
r_{xylose} ($g.L^{-1}.h^{-1}$)	0.225 ± 0.070	0.349 ± 0.017
$r_{ethanol}$ ($g.L^{-1}.h^{-1}$)	0.582 ± 0.026	0.752 ± 0.031
$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	0.533 ± 0.015	0.344 ± 0.002
$Y_{ethanol/substrate}$ ($g.g^{-1}$)	0.323 ± 0.005	0.333 ± 0.011
$Y_{biomass/substrate}$ ($g.g^{-1}$)	0.123 ± 0.003	0.058 ± 0.001
Conversion efficiency (%)	63.3 ± 0.9	65.3 ± 2.2
Consumed sugars (%)	84.5 ± 2.1	97.9 ± 0.2

Contrary to the synthetic medium, in the hydrolysate supplemented with SM1 assay both glucose and xylose were fully consumed (**Figure 4.5B**), yielding a higher percentage of consumed sugars (**Table 4.3**). Glucose was exhausted around 30.0 h, identically to the assay in synthetic media, after that, the consumption rate of xylose increased. When the culture medium contains both glucose and xylose, glucose repression occurs and there is a preference for glucose as substrate (Agbogbo et al., 2006; Agbogbo and Coward-Kelly, 2008). The $r_{glucose}$ observed was very close to the observed in the synthetic medium assay, while there was an increase in r_{xylose} (**Table 4.3**). Ethanol concentration increased throughout the fermentation, with a maximum ethanol concentration of 17.50 ± 0.09 $g.L^{-1}$ after 50.8 h. However, the production rate of ethanol decreased when glucose was depleted and xylose consumption started (**Figure 4.5B**). Biomass concentration also kept increasing, although more slowly, when xylose uptake started. The maximum biomass concentration obtained was 3.26 ± 0.02 $g.L^{-1}$, which led to a lower decrease in pH in the latter to 3.35. The lower decrease might be related with the fact that, when fermenting hydrolysate, *S. stipitis* was able to metabolize xylose. Comparing the results obtained with the synthetic medium and hydrolysate SM1 (**Table 4.3**), it appears that, like in *S. cerevisiae* assays, the hydrolysate resulted in a deviation of the carbon flux more towards ethanol fermentation and less towards growth. In hydrolysate SM1 assay, higher maximum ethanol concentration, $r_{ethanol}$, 0.752 ± 0.031 $g.L^{-1}.h^{-1}$, and $Y_{ethanol/substrate}$, 0.333 ± 0.011 $g.g^{-1}$ were attained. In this assay μ was very close to the synthetic medium, but $Y_{biomass/substrate}$ was half the observed in the latter. Although

a lower maximum ethanol concentration was obtained in the synthetic medium assay, a higher productivity was observed due to the fact that maximum ethanol was reached 19.0 h earlier than in hydrolysate SM1 assay.

Figure 4.6 shows the evolution of Experimental COD and Calculated COD in the hydrolysate supplemented with SM1 assay. The difference between both CODs is approximately the same during the fermentation, indicating that, in fact, only glucose and xylose were being consumed by *S. stipitis*.

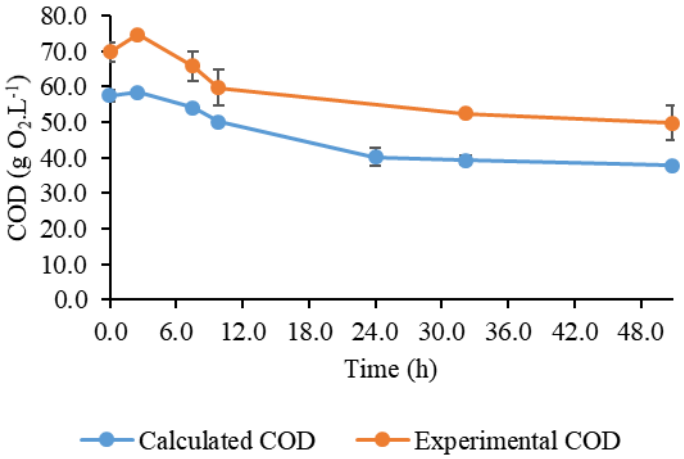

Figure 4.6 - Evolution of COD in the assay with *S. stipitis* in hydrolysate supplemented with SM1.

When fermentation of hydrolysate supplemented with SM1 with *S. stipitis* was tested, the percentage of consumed sugars was higher than in the assay with *S. cerevisiae* using the same medium, given that both glucose and xylose were metabolized. A higher r_{xylose} was also observed. However, in the latter, higher biomass and ethanol yield were observed, suggesting that, in the assay with *S. stipitis*, carbon flow was deviated for pathways other than growth and ethanol production. It is known that *S. stipitis* is highly sensitive to several inhibitors present in hydrolysates, namely formic, acetic and levulinic acids, phenolics, and furfural. Although yeasts can detoxify microbial inhibitors, such as furfural and HMF, the detoxification mechanisms compete for key enzymes and cofactors needed to channel the carbon flow to respiration or ethanol production (Slininger et al., 2009; Pereira et al., 2013).

The fermentation in hydrolysate supplemented with SM2 was not successful, as almost no ethanol production was observed, and biomass growth was low (**Figure 4.5C**). Hence, it was not possible to determine kinetic and stoichiometric parameters. By the end of the fermentation, 76.2 ± 3.5 % of the sugars were still present in the medium. Slininger et

al. (2009) studied the impact of culture nutrition in inhibitor tolerance of *S. stipitis* NRRL Y-7124, concluding that enrichment of the culture medium with amino acids enhanced the ability of cells to resist furfural and HMF exposure. The authors reported that addition of vitamins, namely biotin, to the fermentation medium improves biomass growth of *S. stipitis* NRRL Y-7124. Also by supplying a nitrogen source, such as amino acids, and minerals, like Fe, Mn, Mg, Ca, Zn, and others, bioethanol and biomass production by this yeast could also be enhanced (Slininger et al. 2009). Therefore, the unsuccessful results obtained in the assay in hydrolysate supplemented with SM2 probably were a consequence of the lack of yeast extract, which provides nitrogenous compounds, trace nutrients, and vitamin B complex. Supplementation with yeast extract is essential for *S. stipitis* growth and fermentation in the hydrolysate.

Table 4.4 compares the results obtained with *S. stipitis* NRRL Y-7124 with other studies using different feedstocks for ethanol production. The ethanol yield achieved in this work, 0.333 ± 0.011 $g.g^{-1}$, was lower than the reported by most studies, possibly because in the majority of these studies there was a preliminary detoxification step to remove inhibitory compounds. Still, the obtained yield was higher than the values reported by Germec and Turhan (2018) and Pereira et al. (2015) by *S. stipitis* fermentation of tea processing waste, 0.173 $g.g^{-1}$, and *E. globulus* HSSL, 0.13 and 0.16 $g.g^{-1}$, respectively. Germec and Turhan (2018), Kleingesinds et al. (2018), and Fonseca et al. (2018) achieved higher ethanol productivities, 0.40-1.02 $g.L^{-1}.h^{-1}$, than in the present work, 0.344 ± 0.002 $g.L^{-1}.h^{-1}$. Nevertheless, the productivity achieved by the fermentation of *E. globulus* Kraft pulp hydrolysate with *S. stipitis* is higher than in the studies by Silva et al. (2016), Kundu and Lee (2016), Koti et al. (2016), and Pereira et al. (2015), 0.029-0.330 $g.L^{-1}.h^{-1}$.

Table 4.4 - Results of ethanol production by *S. stipitis* from different feedstocks.

Feedstock	**Pretreatment**	**Detoxification**	**Hydrolysis**	**Microbial strain**	**[Ethanol] ($g.L^{-1}$)**	**$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)**	**$Y_{ethanol/substrate}$ ($g.g^{-1}$)**	**Ref.**
E. globulus[a]	Kraft pulping	No	Enzymatic	NRRL Y-7124	17.50 ± 0.09	0.344 ± 0.002	0.333 ± 0.011	This work
Corncob	Acid (H_2SO_4)	No	Enzymatic	CBS 6054	26.60	1.02	0.37	Kleingesinds et al., 2018
Tea processing waste	Acid (H_2SO_4)	Activated charcoal	No	ATCC 58784	3.82	0.49	0.173	Germec and Turhan, 2018
				ATCC 58785	2.47	0.40	0.173	
Rice straw	Acid (H_2SO_4)	Biological (baker's yeast)	No	NRRL Y-7124	23.0	0.52	0.24	Fonseca et al., 2018
Sugarcane bagasse	Acid (H_2SO_4)	CaO + Acquapol WW® biopolymer	No	NRRL Y-7124	15.03	0.30	0.37	Silva et al., 2016
E. globulus	Oxalic acid	Electrodialysis-XAD resin	No	CBS 6054	7.83	0.163	0.40	Kundu and Lee, 2016
Wheat straw	Alkali (NaOH)	Overliming ($Ca(OH)_2$)	Enzymatic	NCIM 3498	8.28 ± 0.54	0.230 ± 0.005	0.380 ± 0.006	Koti et al., 2016
				PSUV9[b]	12.15 ± 0.57	0.330 ± 0.011	0.450 ± 0.009	
E. globulus HSSL	No	No	No	NRRL Y-7124	1.76	0.029	0.13	Pereira et al., 2015
				C_4 isolate[c]	4.60	0.051	0.16	
Sugarcane bagasse	Acid (H_2SO_4)	No	No	NRRL Y-7124[d]	28.08 ± 0.01	0.346 ± 0.009	0.371 ± 0.001	Gutiérrez-Rivera et al., 2015
Wheat straw	Ozonolysis	Cold water wash	Enzymatic	DSM 3651	11.6	NA	0.41	Bellido et al., 2013

HSSL hardwood spent sulphite liquor *NA* not available; SDS sodium dodecyl sulphate
[a] hydrolysate supplemented with SM1; [b] *S. stipitis* NCIM 3498 UV mutant; [c] *S. stipitis* NRRL Y-7124 adapted to HSSL; [d] adapted to sugarcane hydrolysate

4.3.3. Assays with co-cultures of *S. cerevisiae* and *S. stipitis*

The results obtained in the previous assays confirmed the possibility of using hydrolysate from unbleached Kraft pulp as substrate for bioethanol production by *S. cerevisiae* and *S. stipitis*. The results also showed that xylose, as expected, was consumed in a very small amount by *S. cerevisiae* but *S. stipitis*, a known pentose-consuming yeast, showed the same preferences towards sugars, being the former a faster glucose consumer than the later. *S. stipitis* also showed low ethanol yield and slow ethanol production when compared with *S. cerevisiae*. Considering these findings, the next step on studying bioethanol production from the hydrolysate was the use of co-cultures of the two yeasts in order to enhance the consumption of sugars until fully exhaustion of glucose and xylose and, consequently, increase the amount of ethanol produced. By maximizing substrate utilization, it is expected to reduce process costs. Also, co-culture is expected to increase the sugars consumption and ethanol production ratios and, consequently, reduce the fermentation time. *S. cerevisiae* and *S. stipitis* is the most frequently used combination in co-culture fermentations, because the pH and temperature required for both yeasts are compatible (Chen, 2011). The fermentation medium chosen for the co-culture assays was hydrolysate supplemented with SM1, since it was the medium containing hydrolysate that yielded the best results for both *S. cerevisiae* and *S. stipitis*. **Figure 4.7** shows the evolution of pH and concentrations of glucose, xylose, ethanol, and biomass in these assays and **Table 4.5** shows the stoichiometric and kinetic parameters.

Firstly, sequential co-culture was evaluated. In this assay, in order to aliviate repression of xylose consumption by the availability of glucose in *S. stipitis*, the hexose-fermenting yeast, *S. cerevisiae*, was inoculated first due to its higher glucose consumption rate and ethanol yield. After glucose depletion, *S. stipitis* was inoculated to consume the remaining xylose. In this fermentation a maximum ethanol concentration of 20.03 ± 0.61 $g.L^{-1}$ was obtained after 23.7 h of fermentation, at the same time that glucose exhaustion was detected (**Figure 4.7A**). However, given the $r_{glucose}$ and $r_{ethanol}$, 5.13 ± 0.19 and 1.90 ± 0.07 $g.L^{-1}.h^{-1}$, respectively, it was assumed that glucose exhaustion and maximum ethanol occurred around 13.5 h of fermentation. Hence, maximum ethanol was achieved before *S. stipitis* inoculation, when only *S. cerevisiae* was present in the medium, and remained constant until the end of the fermentation. Thus, the ethanol yield observed in sequential co-culture, 0.464 ± 0.009 $g.g^{-1}$, was similar to the obtained in *S. cerevisiae* mono-culture in the

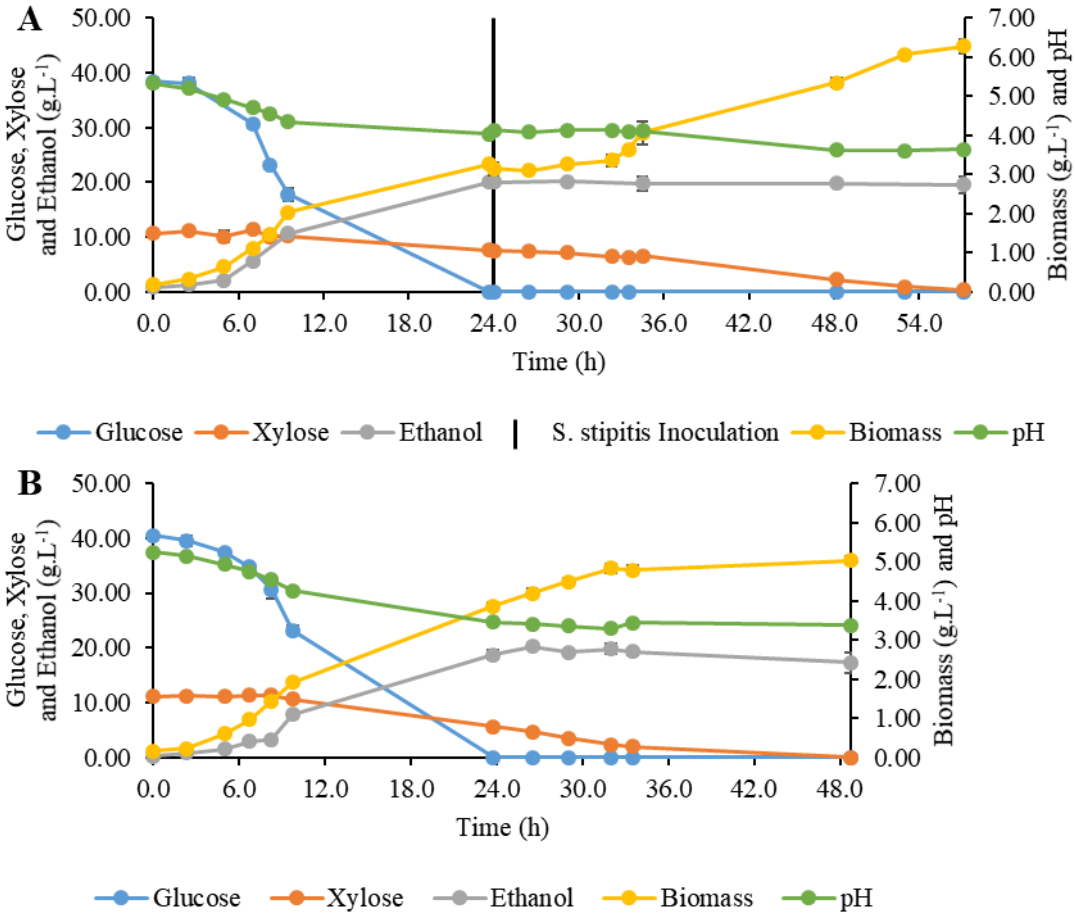

Figure 4.7 - Evolution of pH and concentration of glucose, xylose, ethanol, and biomass in Erlenmeyer flask assays with co-culture. A- Sequential co-culture; B- Simultaneous co-culture.

same medium, 0.450 ± 0.009 $g.g^{-1}$, and the consumed sugars were of only 84.3 ± 0.3 % (**Table 4.5**). *S. stipitis* was inoculated at 24 h of fermentation, when glucose was already exhausted. Since the pH at the time of the inoculation was sligtly higher than 4, xylose was expected to be metabolized. Xylose was consumed slowly until 34.5 h of fermentation and, then, the consumption rate increased to 0.309 ± 0.003 $g.L^{-1}.h^{-1}$, a value higher than in *S. cerevisiae* mono-culture in hydrolysate supplemented with SM1, but still lower than the observed in *S. stipitis* mono-culture assay using the same medium. Coincidently, a second lag fase was observed in the first 8 h after *S. stipitis* inoculation and then biomass grew exponentially, with simultaneous pH decrease (**Figure 4.7A**). This lag fase was longer than the observed in the assay with *S. stipitis* using hydrolysate supplemented with SM1, since the yeast had to adapt to the medium with a lower pH. Contrary to the mono-culture, in the sequential co-culture assay no glucose was present in the medium at the time of *S. stipitis* inoculation. Hence, the yeast, whose inoculum was performed in a medium that contained

glucose but no xylose, had to synthesize the enzymatic machinery required for xylose metabolization. Considering the small amount of xylose consumed and the great increase in biomass observed, the latter could additionally be explained by growth of *S. cerevisiae* due to the consumption of other compounds present in the hydrolysate medium.

After *S. stipitis* inoculation, no ethanol production was observed, possibly because of inhibiton by ethanol. Although the ethanol concentration at the time of *S. stipitis* inoculation was only of 20.03 ± 0.61 $g.L^{-1}$, this value can already be inhibitory to the yeast metabolism. Gutiérrez-Rivera et al. (2015) investigated the sequential co-culture of the two yeasts for ethanol production from sugarcane bagasse, and observed that *S. stipitis* was inhibited when ethanol concentrations were still lower than 30 $g.L^{-1}$ due to the strain low ethanol tolerance. For this reason, some authors promoted the recovery of ethanol through distillation prior to *S. stipitis* inoculation (Chu et al., 2014; Huang et al., 2017; Patel et al., 2017). Additionally, the inhibitory and toxic compounds present in LCB hydrolysates could also represent a negative impact on co-culture fermentation (Chen, 2011).

Table 4.5 - Kinetic and stoichiometric parameters calculated for Erlenmeyer flask assays with co-culture.

Parameter	**Assay**	
	Sequential co-culture	**Simultaneous co-culture**
$[Ethanol]_{max}$ ($g.L^{-1}$)	20.03 ± 0.61	20.25 ± 0.29
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	5.13 ± 0.19	3.83 ± 0.36
r_{xylose} ($g.L^{-1}.h^{-1}$)	0.309 ± 0.003	0.410 ± 0.003
$r_{ethanol}$ ($g.L^{-1}.h^{-1}$)	1.90 ± 0.07	0.925 ± 0.027
$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	1.42 ± 0.05[a]	0.754 ± 0.021
$Y_{ethanol/substrate}$ ($g.g^{-1}$)	0.464 ± 0.009	0.426 ± 0.016
$Y_{biomass/substrate}$ ($g.g^{-1}$)	0.074 ± 0.001	0.086 ± 0.002
Conversion efficiency (%)	91.0 ± 1.8	83.6 ± 3.1
Consumed sugars (%)	84.3 ± 0.3	90.8 ± 0.3

[a] calculated for 13.5 h of fermentation period

Although a high conversion efficiency was obtained in sequential co-culture, it corresponded to the one of *S. cerevisiae* mono-culture. Hence, simultaneous co-culture was investigated next. In this culture both yeasts were inoculated at the same time. Glucose exhaustion was detected around 23.8 h of fermentation (**Figure 4.7B**), but given the $r_{glucose}$, 3.83 ± 0.36 $g.L^{-1}.h^{-1}$, it is probable that glucose was fully consumed around 16.0 h of fermentation. Therefore, when *S. cerevisiae* and *S. stipitis* were cultivated simultaneously,

glucose was consumed slower than when *S. cerevisiae* was alone, but faster than the mono-culture of *S. stipitis*. As it was observed in the assays with mono-culture of *S. stipitis*, using the same medium, the consumption rate of xylose increased after glucose exhaustion due to the end of glucose repression, and xylose was fully consumed by the end of the fermentation. The r_{xylose} observed, 0.410 ± 0.003 $g.L^{-1}.h^{-1}$, was the highest among all the Erlenmeyer flask assays. The maximum ethanol concentration, 20.25 ± 0.29 $g.L^{-1}$, achieved after 26.5 h, was similar to the observed in sequential co-culture and *S. cerevisiae* mono-culture in hydrolysate supplemented with SM1, although a higher percentage of consumed sugars, 90.8 ± 0.3 %, was registered. Nevertheless, due to slower glucose consumption, a lower $r_{ethanol}$ of 0.925 ± 0.027 and productivity of 0.754 ± 0.021 $g.L^{-1}.h^{-1}$ were observed and the maximum ethanol concentration was achieved 13 h later. A lower ethanol yield, 0.426 ± 0.016 $g.g^{-1}$, but higher biomass yield, 0.086 ± 0.002 $g.g^{-1}$, were observed (**Table 4.5**). Therefore, it can be concluded that carbon flow was deviated more towards growth when both yeasts were present in the fermentation medium, most likely by *S. stipitis*, since, contrary to *S. cerevisiae*, it is a Crabtree-negative yeast (Papini et al., 2012). Biomass grew rapidly while glucose was being used as substrate but this rate decreased slightly when xylose uptake started, because *S. stipitis* was most likely the only yeast growing from xylose consumption. Around 32.0 h, after maximum ethanol yield was attained and xylose concentration dropped, biomass stabilized and at the end of the fermentation grew very slightly and a slow ethanol consumption was observed (**Figure 4.7B**). Ethanol consumption after sugar exhaustion in simultaneous co-culture was already reported in the literature (Santosh et al., 2017; Sreemahadevan et al., 2018).

Table 4.6 shows the ethanol concentration, productivities, and yields obtained by simultaneous co-cultures of *S. cerevisiae* and *S. stipitis* in different studies compared with mono-cultures. In all studies, including the present work, simultaneous co-culture resulted in an enhanced ethanol production than *S. stipitis* mono-culture. Similar to this work, the results obtained by Iram et al. (2018) and Xin et al. (2010) did not indicate a significant improvement in ethanol concentration, productivity, or yield when simultaneous co-culture was used instead of *S. cerevisiae* mono-culture. On the other hand, in the studies by Unrean and Khajeeram (2016), Gutiérrez-Rivera et al. (2015), Singh et al. (2014), and Suriyachai et al. (2013) an increase of these parameters was reported when using simultaneous co-cultures. While productivity values differ a lot from one study to the other, 0.54-1.39 $g.L^{-1}.h^{-1}$, the

ethanol yield achieved by simultaneous co-culture in the present work, 0.426 ± 0.016 $g.g^{-1}$, is very similar to the reported by Gutiérrez-Rivera et al. (2015), Singh et al. (2014), and Xin et al. (2010). However, it was lower than in the studies by Iram et al. (2018), Unrean and Khajeeram (2016), and (Suriyachai et al., 2013).

When comparing simultaneous and sequential co-cultures of *S. cerevisiae* and *S. stipitis* for the fermentation of hydrolysate obtained from *Prosopis juliflora*, Naseeruddin et al. (2017) reported slightly lower ethanol yields on the first case, suggesting that when both yeasts were inoculated at the same time, the utilization of hexose and pentose was less efficient due to the competition for sugars between the organisms. Unrean and Khajeeram (2016) suggested a way of improving fermentation efficiency of simultaneous co-culture by adjusting the proportion of cell ratio to the sugars available in biomass feedstock. Karagöz and Özkan (2014) reported that, when using medium containing 20 $g.L^{-1}$ of glucose and 10 $g.L^{-1}$ of xylose, by increasing the proportion of *S. stipitis*, ethanol production and xylose consumption were improved.

Table 4.6 – Results of bioethanol production by simultaneous co-culture of *S. cerevisiae* and *S. stipitis* and mono-cultures from different substrates.

Feedstock	Culture type	[Ethanol] ($g.L^{-1}$)	$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	$Y_{ethanol/substrate}$ ($g.g^{-1}$)	Ref.
E. globulus Kraft pulp hydrolysate[a]	*S. stiptis* mono-culture	17.50 ± 0.09	0.344 ± 0.002	0.333 ± 0.011	This work
	S. cerevisiae mono-culture	19.81 ± 0.15	2.01 ± 0.01	0.450 ± 0.009	
	S. cerevisiae and S. stipitis co-culture	20.25 ± 0.29	0.754 ± 0.021	0.426 ± 0.016	
Alkali-pretreated sugarcane bagasse hydrolysate	*S. stiptis* mono-culture	NA	NA	0.289	Iram et al., 2018
	S. cerevisiae mono-culture	NA	NA	0.493	
	S. cerevisiae and S. stipitis co-culture	NA	NA	0.489	
Acid-pretreated sugarcane bagasse hydrolysate	*S. cerevisiae* mono-culture	42.17 ± 0.65	0.87 ± 0.03	0.44 ± 0.02	Unrean and Khajeeram, 2016
	S. cerevisiae and S. stipitis co-culture	46.68 ± 0.09	0.97 ± 0.00	0.46 ± 0.03	
Acid-pretreated sugarcane bagasse[b]	*S. stiptis* mono-culture	28.08 ± 0.01	0.346 ± 0.009	0.371 ± 0.001	Gutiérrez-Rivera et al., 2015
	S. cerevisiae mono-culture	22.15 ± 0.22	1.477 ± 0.146	0.364 ± 0.005	
	S. cerevisiae and S. stipitis co-culture	30.52 ± 0.19	1.39 ± 0.01	0.400 ± 0.009	
Microwave alkali-pretreated rice husk hydrolysate	*S. stiptis* mono-culture	12.2 ± 1.2	0.36	0.35	Singh et al., 2014
	S. cerevisiae mono-culture	14.0 ± 1.3	0.36	0.39	
	S. cerevisiae and S. stipitis co-culture	20.8 ± 1.5	0.54	0.40	
Alkali-pretreated rice straw hydrolysate	*S. stiptis* mono-culture	12.75 ± 1.28	NA	0.42	Suriyachai et al., 2013
	S. cerevisiae mono-culture	12.17 ± 0.61	NA	0.41	
	S. cerevisiae and S. stipitis co-culture	13.38 ± 0.34	NA	0.47	
SDS-pretreated newspaper hydrolysate	*S. stiptis* mono-culture	13.45	0.46	0.41	Xin et al., 2010
	S. cerevisiae mono-culture	14.29	1.79	0.43	
	S. cerevisiae and S. stipitis co-culture	14.03	1.28	0.42	

NA not available; *SDS* sodium dodecyl sulphate; [a] hydrolysate supplemented with SM1; [b] yeast strains adapted to sugarcane hydrolysate

4.4. Bioreactor assays

The effect of scaling up the process from Erlenmeyer flask to bioreactor on growth and fermentation was studied. In the bioreactor fermentations, three assays were performed: one with *S. cerevisiae* in synthetic medium; an assay with *S. cerevisiae* in hydrolysate supplemented with SM1 (the assay with the highest ethanol yield until now); and an assay with sequential co-culture of *S. cerevisiae* and *S. stipitis* in hydrolysate supplemented with SM1. In this last assay it was expected a better control of aeration allowing to establish microaerophilic conditions after *S. stipitis* inoculation. In the bioreactor assays, the pH was controlled to 5.5, which is within the optimum pH range for fermentation by both yeasts. **Figure 4.8** shows the evolution of pH and concentrations of glucose, xylose, ethanol, and biomass in all three assays

In the synthetic medium assay, a μ of 0.300 h^{-1}, a $r_{glucose}$ of 5.42 $g.L^{-1}.h^{-1}$, a r_{xylose} of 0.304 $g.L^{-1}.h^{-1}$, a Pro_{vol} of 1.35 $g\text{-}L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ 0.110 $g.g^{-1}$, and $Y_{ethanol/substrate}$ of 0.474 g.g-1, corresponding to a conversion efficiency of 93.0 %, were achieved (**Table 4.7**). In this assay, glucose was depleted after 12.7 h (**Figure 4.8A**). Although maximum ethanol concentration, 20.42 $g.L^{-1}$, was detected after 23.4 h, it appears it was achieved shortly after glucose exhaustion, around 15 h. After maximum ethanol was attained, ethanol concentration started to decrease slightly reaching 18.12 $g.L^{-1}$ by the end of fermentation. Biomass concentration increased while glucose was being consumed and then stabilized around 4.85 $g.L^{-1}$ until the end of fermentation. Base addition to the fermentation medium stopped when biomass growth and ethanol production stopped. These two microbial processes release CO_2 causing pH decrease and, consequently, leading to the automatic addition of base to maintain a constant pH. Compared with the corresponding assay in Erlenmeyer, this process in bioreactor resulted in higher $r_{glucose}$, maximum ethanol concentration, and ethanol and biomass yields. The $r_{ethanol}$ and ethanol productivity were very similar in both fermentations and maximum ethanol was achieved around the same time: 13.0 h in the Erlenmeyer assay and 15.0 h in the bioreactor assay. The better results can be justified by the fact that in the Erlenmeyer assay, pH decreased from 5.71 to 2.75 in the first 9.8 h of fermentation, reaching a pH inhibitory to *S. cerevisiae*, while in the bioreactor assay the pH was kept at 5.50 ± 0.05 for the entire fermentation.

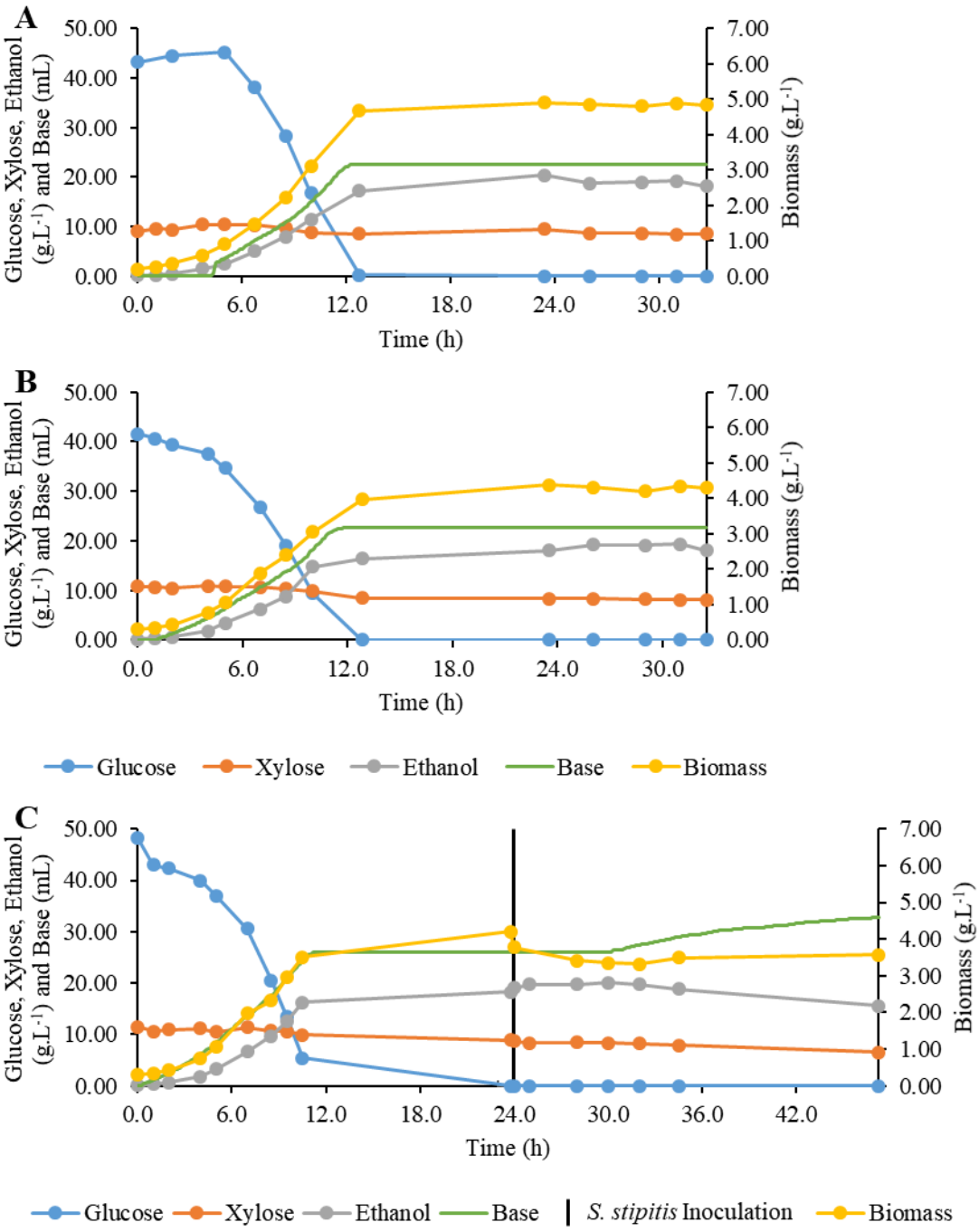

Figure 4.8 - Evolution of pH and concentration of glucose, xylose, ethanol, and biomass in bioreactor assays. A- *S. cerevisiae* in synthetic media; B- *S. cerevisiae* in hydrolysate supplemented with SM1; C- Sequential co-culture.

Table 4.7 - Kinetic and stoichiometric parameters calculated for bioreactor assays.

Parameter	**Assay**		
	***S. cerevisiae* in synthetic medium**	***S. cerevisiae* in hydrolysate**	**Sequential co-culture in hydrolysate**
μ (h^{-1})	0.300	0.251	-
$[Ethanol]_{max}$ ($g.L^{-1}$)	20.42	19.24	20.13
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	5.42	4.66	5.24
r_{xylose} ($g.L^{-1}.h^{-1}$)	0.304	0.385	0.388
$r_{ethanol}$ ($g.L^{-1}.h^{-1}$)	1.77	1.77	2.13
$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)	1.35[a]	0.733	0.668
$Y_{ethanol/substrate}$ ($g.g^{-1}$)	0.474	0.433	0.391
$Y_{biomass/substrate}$ ($g.g^{-1}$)	0.110	0.091	0.060
Conversion efficiency (%)	93.0	84.8	76.6
Consumed sugars (%)	81.8	84.1	86.0

[a] calculated for 15.0 h of fermentation period

In the assay with hydrolysate supplemented with SM1, biomass grew until 12.8 h of fermentation, when glucose was fully consumed, and then stabilized around 4.30 $g.L^{-1}$ (**Figure 4.8B**). Ethanol concentration increased rapidly until 9.3 h, with an $r_{ethanol}$ of 1.77 $g.h^{-1}.L^{-1}$, and when glucose was exhausted, 16.39 $g.L^{-1}$ of ethanol were present in the medium. Maximum ethanol concentration of 19.24 $g.L^{-1}$ was observed 13.2 h after glucose depletion. Xylose concentration decreased very slightly during this period of fermentation. Indeed, a slight production of ethanol from xylose by this *S. cerevisiae* PYCC 5246 (ATCC 24860) was observed by Beck et al. (1990). Also, other compounds of the hydrolysate could have been fermented, since the results from COD (**Figure 4.4**) indicated that *S. cerevisiae* PYCC 5246 could metabolize compounds present in the hydrolysate other than glucose and xylose. Yu et al. (1995) and Jeppsson et al. (1996) reported that *S. cerevisiae* ATCC 24860 is able to ferment xylulose. This particular strain, *S. cerevisiae* PYCC 5246, is known to ferment glucose, maltose, and raffinose (Portuguese Yeast Culture Collection, 2018). *S. cerevisiae* strains in general are known to ferment sugars like fructose, mannose, maltose, maltotriose, and xylulose besides some organic acids, and polyhydroxy alcohols (Batt and Tortorello, 2014). After the maximum ethanol was achieved, ethanol concentration stabilized until the last 1.5 h of the fermentation period, when it decreased 1.25 $g.L^{-1}$ (**Figure 4.8B**). Similar to the assay in synthetic medium, base was added to the fermentation medium while biomass and ethanol concentrations increased. In both fermentations, a total of 22.6

mL of base were added. In this assay, a μ of 0.251 h^{-1}, a $r_{glucose}$ of 4.66 $g.L^{-1}.h^{-1}$, a r_{xylose} of 0.385 $g.L^{-1}.h^{-1}$, a $Prod_{vol}$ of 0.733 $g.L^{-1}.h^{-1}$, a $Y_{biomass/substrate}$ 0.091 $g.g^{-1}$, and $Y_{ethanol/substrate}$ of 0.433 g.g-1, corresponding to a conversion efficiency of 84.8 %, were achieved (**Table 4.7**).

Although the maximum values of ethanol concentration achieved in both fermentations by *S. cerevisiae* mono-culture in hydrolysate supplemented with SM1 were very close. However, in the Erlenmeyer fermentation, higher consumption and production rates, as well as higher ethanol productivity, ethanol yield and, consequently, conversion efficiency were achieved (**Table 4.2**). The performance of a bioreactor depends on transportation parameters related with hydrodynamics and properties of mass and heat transfer, which are strongly scale-dependent. The scale-up of biological processes faces problems in obtaining a perfect mixture, because gradients of velocity and concentration of nutrients and products are formed, leading to decreased yields and productivities (da Fonseca and Teixeira, 2007). No problems were observed while increasing the scale of *S. cerevisiae* mono-culture fermentation in synthetic media, however, the hydrolysate has a more complex composition than synthetic medium and contains some particulate suspended matter. Hence, a thirty-fold increase in scale results in a higher number of suspended particles, which may hinder mass transfer, possibly resulting in the decrease of consumption and production rates and ethanol yield.

Table 4.8 presents the results of different *S. cerevisiae* mono-culture fermentations using the same microbial strain, pretreatment and/or feedstock as in the present work. Zambare et al. (2012 and 2011) used *S. cerevisiae* ATCC 24860 and a SHF configuration for ethanol production from *Spartina pectinata* and corn stover, respectively. Both studies reported lower ethanol concentrations and productivities than the present work. However, a higher ethanol yield was obtained from extrusion-pretreated corn stover hydrolysates fermentation, 0.47 $g.g^{-1}$. On the other hand, extrusion-pretreated *Spartina pectinata* hydrolysate fermentation resulted in an ethanol yield of 0.426 $g.g^{-1}$, close to the obtained in this work, 0.433 $g.g^{-1}$.

Buzała et al. (2017c) studied the possibility of using several Kraft hardwood and softwood pulps as substrate for ethanol production using a SHF configuration. Buzała and co-workers reported low ethanol yields compared with the present work, especially when *P. sylvestrii* was used as feedstock. Most studies using Kraft pulping as pretreatment of LCB for ethanol production applied a SSF configuration. In all the studies in which volumetric

ethanol productivities where reported or possible to be determined, values lower than the obtained in this work were reported, 0.010-0.392 $g.L^{-1}.h^{-1}$. Like in the present work, Ko et al. (2012) and Monrroy et al. (2012) used *E. globulus* as feedstock, reporting lower ethanol yields, 0.042 g.g d.w. $wood^{-1}$ and 0.469 g.g d.w. $pulp^{-1}$, respectively. Bauer and Gibbons (2012) observed ethanol yields very close to the present work, 0.438 ± 0.027 $g.g^{-1}$, while in the remaining assays lower ethanol yields were achieved.

Kelbert et al. (2016) applied autohydrolysis as pretreatment and used SHF configuration for production of ethanol from *E. globulus*. Although a higher ethanol concentration was achieved, 39.7 $g.L^{-1}$, lower ethanol productivity and yield were obtained, 0.551 $g.L^{-1}.h^{-1}$ and 0.38 $g.g^{-1}$, when compared with this work, 0.733 $g.L^{-1}.h^{-1}$ and 0.433 $g.g^{-1}$. Pereira et al. (2014) investigated different yeast strains for ethanol production from hydrothermal-pretreated *E. globulus*, without hydrolysis step: using the strain PE-2, a productivity of 0.78 ± 0.01 $g.L^{-1}.h^{-1}$, similar to this work but a higher yield of 0.48 ± 0.00 $g.g^{-1}$, were achieved, while using CAT1 resulted in lower ethanol productivity of 0.49 ± 0.00 $g.L^{-1}.h^{-1}$ and higher ethanol yield of 0.47 ± 0.00 $g.g^{-1}$. *S. cerevisiae* CA1162 yielded a lower productivity of 0.25 ± 0.00 $g.L^{-1}.h^{-1}$ and a close ethanol yield of 0.42 ± 0.01 $g.g^{-1}$.

Table 4.8 - Comparison between the results obtained for *S. cerevisiae* mono-culture fermentations in this work and in the literature.

Feedstock	**Pretreatment**	**Hydrolysis**	**Fermentation configuration**	**Microbial strain**	**[Ethanol] ($g.L^{-1}$)**	**$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)**	**$Y_{ethanol/substrate}$**	**Ref.**
E. globulus[b]	Kraft pulping	Enzymatic	SHF	PYCC 5246 or ATCC 24860	19.24	0.733	0.433 $g.g^{-1}$ 0.529 g.g d.w. $pulp^{-1}$[b] 0.275 g.g d.w. $wood^{-1}$[c]	This work
Spartina pectinata	Extrusion	Enzymatic	SHF	ATCC 24860	7.2	0.10	0.426 $g.g^{-1}$	Zambare et al., 2012
Corn stover	Extrusion	Enzymatic	SHF	ATCC 24860	6.2	0.086	0.47 $g.g^{-1}$	Zambare et al., 2011
P. tremula	Kraft pulping	Enzymatic	SHF	NS	NA	NA	0.26 g.g d.w. $pulp^{-1}$	Buzała et al., 2017c
B. pendula					NA	NA	0.25 g.g d.w. $pulp^{-1}$	
F. sylvatica					NA	NA	0.26 g.g d.w. $pulp^{-1}$	
P. sylvestrii					NA	NA	0.04 g.g d.w. $pulp^{-1}$	
NS	Kraft pulping	-	CBP	MT8-1[d]	0.7	0.010	0.210 $g.g^{-1}$	Amoah et al., 2017
NS	Kraft pulping	Enzymatic	SSF	NRRL Y-2034	17.90 ± 0.99	0.25 ± 0.015	0.438 ± 0.027 $g.g^{-1}$	Bauer and Gibbons, 2012
Acacia confusa	Kraft pulping	Enzymatic	SSF	D5A	5.88	0.035	0.045 g.g d.w. $wood^{-1}$	Ko et al., 2012
E. globulus					5.67	0.032	0.042 g.g d.w. $wood^{-1}$	
E. globulus	Kraft pulping	Enzymatic	SSF	IR2T9[e]	NA	NA	0.469 g.g d.w. $pulp^{-1}$	Monrroy et al., 2012

Table 4.8 - Comparison between the results obtained for *S. cerevisiae* mono-culture fermentations in this work and in the literature (cont.).

Feedstock	**Pretreatment**	**Hydrolysis**	**Fermentation configuration**	**Microbial strain**	**[Ethanol] ($g.L^{-1}$)**	**$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)**	**$Y_{ethanol/substrate}$**	**Ref.**
NS[d]	Kraft pulping	Enzymatic	SSF	IR2-9a[e]	28	0.392	0.316 $g.g^{-1}$	Edgardo et al., 2008
E. globulus	Autohydrolysis	Enzymatic	SHF	PE-2	39.7	0.551	0.38 $g.g^{-1}$	Kelbert et al., 2016
E. globulus	Hydrothermal	No	-	PE-2	54.6 ± 0.3	0.78 ± 0.01	0.48 ± 0.00 $g.g^{-1}$	Pereira et al., 2014
				CAT1	53.8 ± 0.2	0.49 ± 0.00	0.47 ± 0.00 $g.g^{-1}$	
				CA1162	41.1 ± 0.1	0.25 ± 0.00	0.42 ± 0.01 $g.g^{-1}$	

NA not available; NS not specified
[a] hydrolysate supplemented with SM1; [b] 1.22 g ethanol.g d. w. $pulp^{-1}$; [c] 0.52 g d.w pulp.g d.w. wood (data provided by *RAIZ – Instituto de Investigação da Floresta e do Papel*); [d] transformed to express five cellulase genes; [e] thermal acclimatized

The first 24.0 h of the sequential co-culture assay in hydrolysate supplemented with SM1 in bioreactor were common to the *S. cerevisiae* mono-culture assay in bioreactor in the same medium. Hence, it was observed that the evolution of the fermentation in this period was very similar in both assays (**Figure 4.8B** and **C**). In fact, the fermentation parameters of the two assays that were determined in the first 24.0 h ($r_{glucose}$, r_{xylose}, and $r_{ethanol}$), were close (**Table 4.7**). At 24.0 h of fermentation, when all glucose had been consumed, *S. stipitis* was inoculated in the fermentation medium. After inoculation of *S. stipitis* a slight consumption of xylose was observed and ethanol concentration increased very slowly, from 19.10 $g.L^{-1}$ to 20.13 $g.L^{-1}$ reaching this maximum ethanol concentration at 30.0 h (**Figure 4.8C**). Therefore, contrary to the sequential co-culture assay in Erlenmeyer, ethanol production was verified after *S. stipitis* inoculation in the sequential co-culture assay in bioreactor. However, the maximum ethanol concentration was only slightly higher than the observed with *S. cerevisiae* mono-culture in hydrolysate SM1 assays in both scales, and lower ethanol productivity, 0.668 $g.L^{-1}.g^{-1}$, and ethanol yield, 0.391 $g.g^{-1}$, were observed. In the bioreactor assays, the pH was automatically controlled to 5.50 ± 0.05, hence and improved xylose fermentation to ethanol was expected. The low xylose consumption observed may be due to the *S. stipitis* inoculum, which was performed in a medium that contained glucose but no xylose, forcing the culture to synthesize or at least to activate the enzymatic machinery required for xylose metabolization. Additionally, the air flow of 50 $mL.min^{-1}$, corresponding to an aeration rate of 0.02 vvm, may not have been sufficiently low to promote xylose fermentation. After maximum ethanol concentration was attained, ethanol consumption was observed until the end of the fermentation with a higher rate, 0.266 g $ethanol.L^{-1}.h^{-1}$, than xylose consumption, 0.108 g $xylose.L^{-1}.h^{-1}$. These consumptions resulted in an increase in biomass concentration, with simultaneous pH decrease, reinforcing that the aeration of the medium was not adequate for ethanol production by *S. stipitis*.

Table 4.9 presents the results of different sequential co-culture of *S. cerevisiae* and *S. stipitis* fermentations. Ethanol concentration varied significantly between studies, 12.6-59.6 $g.L^{-1}$, given the different ammounts of glucose and xylose in the beginning of each fermentation. Volumetric productivity also presented a wide range of values. Nevertheless, a high ethanol productivity was achieved in this work, 0.671 $g.L^{-1}.h^{-1}$, only surpassed by the sequential co-culture fermentation of unbleached Kraft pulp of *P. heterocycle* (Huang et al., 2017) and green liquor-pretreated corn stover hydrolysates (Chu et al., 2014), 0.910 and 1.57

$g.L^{-1}.h^{-1}$, respectively. Certainly a higher volumetric productivity could have been achieved if *S. stipitis* was inoculated imediately after glucose exhaustion, at 12 h instead at 24 h of fermentation. The ethanol yield achieved was slightly lower than in most assays, most likely due to inadequate aeration conditions and inoculum preparation for xylose fermentation by *S. stipitis*. Also, in most of these assays a step of distillation was performed before *S. stipitis* inoculation, which avoided *S. stipitis* inhibition by ethanol.

Table 4.9 – Comparison between the results obtained for *S. cerevisiae* and *S. stipitis* sequential co-culture in this work and in the literature.

Feedstock	**[Ethanol] ($g.L^{-1}$)**	**$Prod_{vol}$ ($g.L^{-1}.h^{-1}$)**	**$Y_{ethanol/substrate}$ ($g.g^{-1}$)**	**Ref.**
Unbleached Kraft pulp of *E. globulus* hydrolysate	20.13	0.671	0.391	This work
Unbleached Kraft pulp of *P. heterocycle* residues hydrolysate[a]	54.6	0.910	0.411	Huang et al., 2017
Briquetted sugar beet pulp hydrolysate	12.6	0.175	NA	Berlowska et al., 2017
Ammonia-pretreated sugarcane bagasse hydrolysate[a]	13.0	0.271	0.419	Patel et al., 2017
Acid-pretreated corncob hydrolysate[b,c]	27.0	0.281	NA	Brar et al., 2016
Green liquor-pretreated corn stover hydrolysate[a]	59.6	1.57	0.463	Chu et al., 2014
Alkali-pretreated rice straw[b,d]	19.1	0.273	0.370	Li et al., 2011

NA not available
[a] ethanol distillation prior to *S. stipitis* inoculation; [b] inactivation of *S. cerevisiae* prior to *S. stipitis* inoculation; [c] detoxified by activated charcoal; [d] SSF

5. Conclusions

The present work aimed to study the production of second generation bioethanol from unbleached Kraft pulp of *Eucalyptus globulus*, exploiting the Kraft pulping process as lignocellulosic biomass pretreatment.

Firstly, unbleached Kraft pulp of *E. globulus* was enzymatically hydrolysed. Hydrolysates with 65.4 ± 0.8 g L^{-1} of glucose and 16.0 ± 1.8 g L^{-1} of xylose were obtained, with a hydrolysis yield of 95.6 ± 2.6 %.

The results from Erlenmeyer flask assays of Kraft pulp hydrolysate with mono-cultures of *S. cerevisiae* and *S. stipitis* showed that supplementation of the hydrolysate with yeast extract is required to achieve a better growth and fermentation by both yeasts. *S. stipitis* was more strongly affected by the absence of yeast extract in the supplementation media. *S. cerevisiae* mono-culture fermentation of hydrolysate resulted in higher maximum ethanol concentration, 19.81 ± 0.15 $g.L^{-1}$, ethanol yield, 0.450 ± 0.009 $g.g^{-1}$, and ethanol productivity, 2.01 ± 0.01 $g.L^{-1}.h^{-1}$, than *S. stipitis* mono-culture.

In Erlenmeyer flask assays with sequential co-culture of *S. cerevisiae* and *S. stipitis* no improvement in alcoholic fermentation was observed compared with *S. cerevisiae* mono-culture, as no ethanol production was observed after inoculation with *S. stipitis*. Simultaneous co-culture resulted in the consumption of both glucose and xylose, yielding a higher percentage of consumed sugars. However, similar ethanol concentration to *S. cerevisiae* mono-culture, 20.25 ± 0.29 $g.L^{-1}$, and lower ethanol yield and productivity were achieved, 0.426 ± 0.016 $g.g^{-1}$ and 0.754 ± 0.021 $g.L^{-1}.h^{-1}$.

S. cerevisiae mono-culture fermentation of Kraft pulp hydrolysate in bioreactor resulted in a maximum ethanol concentration of 19.24 $g.L^{-1}$, an ethanol yield of 0.433 $g.g^{-1}$, and an ethanol productivity of 0.733 $g.L^{-1}.h^{-1}$. The lower yield and productivity compared with the corresponding assay in Erlenmeyer probably resulted from mass transfer problems.

Ethanol production was observed after *S. stipitis* inoculation in sequential co-culture in hydrolysate at bioreactor scale. However, no improvement in ethanol yield and productivities were observed, as xylose consumption was very slow and ethanol consumption for growth was observed.

The good fermentation results, especially high ethanol yield and productivity obtained by *S. cerevisiae* mono-culture fermentation of *E. globulus* Kraft pulp hydrolysate,

show that this is a promising process for second generation bioethanol production. These results also show that that producing bioethanol from pulp and paper industry wastes, such as low-quality wood, bark and other rejects, as well as low-quality and excess pulp, is a potential opportunity for implementing integrated biorefineries in the existing Kraft pulp mills.

6. Future Work

Considering the good results obtained in this work, in the future, instead of good-quality *Eucalyptus globulus* Kraft pulp, low-quality Kraft pulp or Kraft pulp obtained from wastes, like low-quality wood, bark and other rejects, should be tested for enzymatic hydrolysis and subsequent ethanol production. A detailed characterization of hydrolysate composition should also be obtained in order to access which other compounds present in the hydrolysate can be used for ethanol production and if there are, in fact, inhibitory compounds, particularly for *S. stipitis*. According to the results obtained, different detoxification processes and their effect on fermentation could be investigated.

In the case of *S. cerevisiae* mono-culture fermentation, ethanol production stops clearly because of glucose exhaustion and not because of ethanol inhibitory concentrations. Hence, fed-batch mode should be studied and optimized next. Anaerobic conditions for ethanol production can be tested in order to minimize *S. cerevisiae* growth. *S. cerevisiae* and *S. stipitis* sequential co-culture should also be further investigated. In this fermentation, ethanol distillation prior to *S. stipitis* inoculation and optimization of aeration conditions for xylose fermentation by *S. stipitis* could be studied. The medium for *S. stipitis* pre-inoculum and inoculum preparation should contain xylose instead of glucose.

References

Abdulrahman, R. The Utilization Of Underbalanced Drilling Technology May Minimize Tight Gas Reservoir Formation Damage: A Review Study. Int. J. Eng. Trends Technol. 2013, 5 (1), 10–12.

Acevedo, A.; Conejeros, R.; Aroca, G. Ethanol Production Improvement Driven by Genome-Scale Metabolic Modeling and Sensitivity Analysis in *Scheffersomyces stipitis*. PLoS One 2017, 12 (6), 1–26.

Acheampong, M.; Ertem, F. C.; Kappler, B.; Neubauer, P. In Pursuit of Sustainable Development Goal (SDG) Number 7: Will Biofuels Be Reliable? Renew. Sustain. Energy Rev. 2017, 75, 927–937.

Achinas, S.; Euverink, G. J. W. Consolidated Briefing of Biochemical Ethanol Production from Lignocellulosic Biomass. Electron. J. Biotechnol. 2016, 23, 44–53.

Aditiya, H. B.; Mahlia, T. M. I.; Chong, W. T.; Nur, H.; Sebayang, A. H. Second Generation Bioethanol Production: A Critical Review. Renew. Sustain. Energy Rev. 2016, 66, 631–653.

Agbogbo, F. K.; Coward-Kelly, G. Cellulosic Ethanol Production Using the Naturally Occurring Xylose-Fermenting Yeast, *Pichia stipitis*. Biotechnol. Lett. 2008, 30 (9), 1515–1524.

Agbogbo, F. K.; Coward-Kelly, G.; Torry-Smith, M.; Wenger, K. S. Fermentation of Glucose/Xylose Mixtures Using *Pichia stipitis*. Process Biochem. 2006, 41 (11), 2333–2336.

Alves, C. A. A. Estudo Do Comportamento Fermentativo de Actinobacillus Succinogenes Para a Produção de Ácido Succínico e Ácido Lático, Universidade de Aveiro, 2015.

Amarasekara, A. S. Handbook of Cellulosic Ethanol, 1st ed.; John Wiley & Sons, 2014.

Amoah, J.; Ishizue, N.; Ishizaki, M.; Yasuda, M.; Takahashi, K.; Ninomiya, K.; Yamada, R.; Kondo, A.; Ogino, C. Development and Evaluation of Consolidated Bioprocessing Yeast for Ethanol Production from Ionic Liquid-Pretreated Bagasse. Bioresour. Technol. 2017, 245, 1413–1420.

Araújo, K.; Mahajan, D.; Kerr, R.; da Silva, M. Global Biofuels at the Crossroads: An Overview of Technical, Policy, and Investment Complexities in the Sustainability of Biofuel Development. Agriculture 2017, 7 (4), 1–22.

Arevalo-Gallegos, A.; Ahmad, Z.; Asgher, M.; Parra-Saldivar, R.; Iqbal, H. M. N. Lignocellulose: A Sustainable Material to Produce Value-Added Products with a Zero Waste Approach — A Review. Int. J. Biol. Macromol. 2017, 99, 308–318.

Arévalo, C.; Freer, J.; Naulin, P. A.; Barrera, N. P.; Troncoso, E.; Araya, J.; Peña-Farfal, C.; del P. Castillo, R. Study of the Ultrastructure of *Eucalyptus globulus* Wood Substrates Subjected to Auto-Hydrolysis and Diluted Acid Hydrolysis Pre-Treatments and Its Influence on Enzymatic Hydrolysis. Bioenergy Res. 2017, 10 (3), 714–727.

Arora, R.; Behera, S.; Kumar, S. Bioprospecting Thermophilic/Thermotolerant Microbes for Production of Lignocellulosic Ethanol: A Future Perspective. Renew. Sustain. Energy Rev. 2015, 51, 699–717.

Avanthi, A.; Kumar, S.; Sherpa, K. C.; Banerjee, R. Bioconversion of Hemicelluloses of Lignocellulosic Biomass to Ethanol: An Attempt to Utilize Pentose Sugars. Biofuels 2017, 8 (4), 431–444.

Azadi, P.; Malina, R.; Barrett, S. R. H.; Kraft, M. The Evolution of the Biofuel Science. Renew. Sustain. Energy Rev. 2017, 76, 1479–1484.

Azhar, S. H. M.; Abdulla, R.; Jambo, S. A.; Marbawi, H.; Gansau, J. A.; Mohd Faik, A. A.; Rodrigues, K. F. Yeasts in Sustainable Bioethanol Production: A Review. Biochem. Biophys. Reports 2017, 10, 52–61.

Babu, V.; Thapliyal, A.; Patel, G. K. Biofuels Production, 1st ed.; John Wiley & Sons, 2014.

Bajpai, P. Biotechnology for Pulp and Paper Processing, 1st ed.; Springer Science & Business Media, 2012.

Balat, M. Production of Bioethanol from Lignocellulosic Materials via the Biochemical Pathway: A Review. Energy Convers. Manag. 2011, 52 (2), 858–875.

Banerjee, S.; Mudliar, S.; Sen, R.; Giri, B.; Satpute, D.; Chakrabarti, T.; Pandey, R. A. Commercializing Lignocellulosic Bioethanol: Technology Bottlenecks and Possible Remedies. Biofuels, Bioprod. Biorefining 2010, 4 (1), 77–93.

Batt, C. A.; Tortorello, M.-L. Encyclopedia of Food Microbiology, 2nd ed.; Academic Press, 2014.

Bauer, F.; Coenen, L.; Hansen, T.; McCormick, K.; Palgan, Y. V. Technological Innovation Systems for Biorefineries: A Review of the Literature. Biofuels, Bioprod. Biorefining 2017, 11 (3), 534–548.

Bauer, N. A.; Gibbons, W. R. Saccharification versus Simultaneous Saccharification and Fermentation of Kraft Pulp. Int. J. Agric. Biol. Eng. 2012, 5 (1), 48–55.

Beck, M. J.; Johnson, R. D.; Baker, C. S. Ethanol Production from Glucose/Xylose Mixes by Incorporating Microbes in Selected Fermentation Schemes. Appl. Biochem. Biotechnol. 1990, 24–25 (1), 415–424.

Belgacem, N.; Pizzi, A. Lignocellulosic Fibers and Wood Handbook: Renewable Materials for Today's Environment, 1st ed.; John Wiley & Sons, 2016.

Bellido, C.; González-Benito, G.; Coca, M.; Lucas, S.; García-Cubero, M. T. Influence of Aeration on Bioethanol Production from Ozonized Wheat Straw Hydrolysates Using *Pichia stipitis*. Bioresour. Technol. 2013, 133, 51–58.

Berlowska, J.; Pielech-Przybylska, K.; Balcerek, M.; Cieciura, W.; Borowski, S.; Kregiel, D. Integrated Bioethanol Fermentation/Anaerobic Digestion for Valorization of Sugar Beet Pulp. Energies 2017, 10 (9).

Beta Renewables. Projects/GranBio http://www.betarenewables.com/en/projects/granbio (accessed Dec 4, 2017).

Bhutto, A. W.; Qureshi, K.; Harijan, K.; Abro, R.; Abbas, T.; Bazmi, A. A.; Karim, S.; Yu, G. Insight into Progress in Pre-Treatment of Lignocellulosic Biomass. Energy 2017, 122, 724–745.

Bisaria, V. S.; Kondo, A. Bioprocessing of Renewable Resources to Commodity Bioproducts, 1st ed.; John Wiley & Sons, 2014.

Brar, K. K.; Kaur, S.; Chadha, B. S. A Novel Staggered Hybrid SSF Approach for Efficient Conversion of Cellulose/Hemicellulosic Fractions of Corncob into Ethanol. Renew. Energy 2016, 98, 16–22.

Buzała, K.; Przybysz, P.; Rosicka-Kaczmarek, J.; Kalinowska, H. Production of Glucose-Rich Enzymatic Hydrolysates from Cellulosic Pulps. Cellulose 2015, 22 (1), 663–674.

Buzała, K. P.; Kalinowska, H.; Przybysz, P.; Małachowska, E. Conversion of Various Types of Lignocellulosic Biomass to Fermentable Sugars Using Kraft Pulping and Enzymatic Hydrolysis. Wood Sci. Technol. 2017a, 51 (4), 873–885.

Buzała, K. P.; Kalinowska, H.; Małachowska, E.; Przybysz, P. The Utility of Selected Kraft Hardwood and Softwood Pulps for Fuel Ethanol Production. Ind. Crops Prod. 2017b, 108, 824–830.

Buzała, K. P.; Kalinowska, H.; Małachowska, E.; Przybysz, P. The Utility of Selected Kraft Hardwood and

Softwood Pulps for Fuel Ethanol Production. Ind. Crops Prod. 2017c, 108 (August), 824–830.

Capolupo, L.; Faraco, V. Green Methods of Lignocellulose Pretreatment for Biorefinery Development. Appl. Microbiol. Biotechnol. 2016, 100 (22), 9451–9467.

Celbi. O Eucalipto http://www.celbi.pt/pt/oeucalipto/20/ (accessed Dec 29, 2017).

CELPA. Boletim Estatístico Indústria Papeleira Portuguesa 2015; 2016.

CEPI. Key Statistics 2016 - European Pulp & Paper Industry; 2017.

Chen, H.; Liu, J.; Chang, X.; Chen, D.; Xue, Y.; Liu, P.; Lin, H.; Han, S. A Review on the Pretreatment of Lignocellulose for High-Value Chemicals. Fuel Process. Technol. 2017, 160, 196–206.

Chen, Y. Development and Application of Co-Culture for Ethanol Production by Co-Fermentation of Glucose and Xylose: A Systematic Review. J. Ind. Microbiol. Biotechnol. 2011, 38 (5), 581–597.

Choudhary, J.; Singh, S.; Nain, L. Thermotolerant Fermenting Yeasts for Simultaneous Saccharification Fermentation of Lignocellulosic Biomass. Electron. J. Biotechnol. 2016, 21, 82–92.

Chu, Q.; Li, X.; Yang, D.; Xu, Y.; Ouyang, J.; Yu, S.; Yong, Q. Corn Stover Bioconversion by Green Liquor Pretreatment and a Selected Liquid Fermentation Strategy. BioResources 2014, 9 (4), 7681–7695.

Clark, J. H.; Luque, R.; Matharu, A. S. Green Chemistry, Biofuels, and Biorefinery. Annu. Rev. Chem. Biomol. Eng. 2012, 3 (1), 183–207.

Clesceri, L. S.; Greenbaerg, A. E.; Eaton, A. D. Standard Methods for Examination of Water and Wastewater, 20th ed.; American Public Health Association, American Water Works Association, Water Environment Federation, 1999.

Corbin, K. R.; Hsieh, Y. S. Y.; Betts, N. S.; Byrt, C. S.; Henderson, M.; Stork, J.; DeBolt, S.; Fincher, G. B.; Burton, R. A. Grape Marc as a Source of Carbohydrates for Bioethanol: Chemical Composition, Pre-Treatment and Saccharification. Bioresour. Technol. 2015, 193, 76–83.

Deesuth, O.; Laopaiboon, P.; Laopaiboon, L. High Ethanol Production under Optimal Aeration Conditions and Yeast Composition in a Very High Gravity Fermentation from Sweet Sorghum Juice by *Saccharomyces cerevisiae*. Ind. Crops Prod. 2016, 92, 263–270.

Demirbas, A. The Social, Economic, and Environmental Importance of Biofuels in the Future. Energy Sources, Part B Econ. Plan. Policy 2017a, 12 (1), 47–55.

Demirbas, A. Tomorrow's Biofuels: Goals and Hopes. Energy Sources, Part A Recover. Util. Environ. Eff. 2017b, 39 (7), 673–679.

DGEG. Third Progress Report (2013-2014) - Portugal; 2015.

Dugmore, T. I. J.; Clark, J. H.; Bustamante, J.; Houghton, J. A.; Matharu, A. S. Valorisation of Biowastes for the Production of Green Materials Using Chemical Methods. Top. Curr. Chem. 2017, 375 (2), 46.

Dupont-Inglis, J.; Borg, A. Destination Bioeconomy - The Path towards a Smarter, More Sustainable Future. N. Biotechnol. 2017.

Dutta, K.; Daverey, A.; Lin, J.-G. Evolution Retrospective for Alternative Fuels: First to Fourth Generation. Renew. Energy 2014, 69, 114–122.

Edgardo, A.; Carolina, P.; Manuel, R.; Juanita, F.; Baeza, J. Selection of Thermotolerant Yeast Strains *Saccharomyces cerevisiae* for Bioethanol Production. Enzyme Microb. Technol. 2008, 43 (2), 120–123.

Ellen MacArthur Foundation. Towards the Circular Economy Vol. 1: An Economic and Business Rationale

for an Accelerated Transition; 2012.

Ellen MacArthur Foundation. Circular Economy System Diagram https://www.ellenmacarthurfoundation.org/circular-economy/interactive-diagram (accessed Nov 19, 2017).

Escamilla-Alvarado, C.; Pérez-Pimienta, J. A.; Ponce-Noyola, T.; Poggi-Varaldo, H. M. An Overview of the Enzyme Potential in Bioenergy-Producing Biorefineries. J. Chem. Technol. Biotechnol. 2017, 92 (5), 906–924.

European Commission. Closing the Loop - An EU Action Plan for the Circular Economy. Off. J. Eur. Union 2015.

European Commission. Circular Economy http://ec.europa.eu/environment/circular-economy/index_en.htm (accessed Nov 3, 2017).

European Union. Directive 2009/28/EC on the Promotion of the Use of Energy from Renewable Sources and Amending and Subsequently Repealing Directives 2001/77/EC and 2003/30/EC; 2009.

FAO. Yearbook of Forest Products 2015; 2017.

Faraco, V. Lignocellulose Conversion: Enzymatic and Microbial Tools for Bioethanol Production, 1st ed.; Springer Science & Business Media, 2013.

Farias, D.; De Andrade, R. R.; Maugeri-Filho, F. Kinetic Modeling of Ethanol Production by *Scheffersomyces stipitis* from Xylose. Appl. Biochem. Biotechnol. 2014, 172 (1), 361–379.

Fernandes, M. C.; Ferro, M. D.; Paulino, A. F. C.; Chaves, H. T.; Evtuguin, D. V.; Xavier, A. M. R. B. Comparative Study on Hydrolysis and Bioethanol Production from Cardoon and Rockrose Pretreated by Dilute Acid Hydrolysis. Ind. Crops Prod. 2018, 111, 633–641.

Ferro, M. D.; Fernandes, M. C.; Paulino, A. F. C.; Prozil, S. O.; Gravitis, J.; Evtuguin, D. V.; Xavier, A. M. R. B. Bioethanol Production from Steam Explosion Pretreated and Alkali Extracted *Cistus ladanifer* (Rockrose). Biochem. Eng. J. 2015, 104, 98–105.

Fonseca, B. G.; Mateo, S.; Moya, A. J.; Roberto, I. C. Biotreatment Optimization of Rice Straw Hydrolyzates for Ethanolic Fermentation with *Scheffersomyces stipitis*. Biomass and Bioenergy 2018, 112 (March 2017), 19–28.

da Fonseca, M. M.; Teixeira, J. A. Reatores Biológicos; Lidel, 2007.

Geissdoerfer, M.; Savaget, P.; Bocken, N. M. P.; Hultink, E. J. The Circular Economy – A New Sustainability Paradigm? J. Clean. Prod. 2017, 143, 757–768.

Geng, A.; Wang, Z.; Lai, K. S.; Tan, M. W. Y. Bioenergy II: Comparison of Laboratory and Industrial *Saccharomyces cerevisiae* Strains for Their Stress Tolerance. Int. J. Chem. React. Eng. 2010, 8 (A87).

Germec, M.; Turhan, I. Ethanol Production from Acid-Pretreated and Detoxified Tea Processing Waste and Its Modeling. Fuel 2018, 231 (January), 101–109.

Gírio, F. M.; Fonseca, C.; Carvalheiro, F.; Duarte, L. C.; Marques, S.; Bogel-Łukasik, R. Hemicelluloses for Fuel Ethanol: A Review. Bioresour. Technol. 2010, 101 (13), 4775–4800.

GranBio. GranBio inicia produção de etanol de segunda geração http://www.granbio.com.br/site/granbio-inicia-producao-de-etanol-de-segunda-geracao/ (accessed Dec 4, 2017).

Gupta, A.; Verma, J. P. Sustainable Bio-Ethanol Production from Agro-Residues: A Review. Renew. Sustain.

Energy Rev. 2015, 41, 550–567.

Gupta, V. K.; Kubicek, C. P.; Berrin, J. G.; Wilson, D. W.; Couturier, M.; Berlin, A.; Filho, E. X. F.; Ezeji, T. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. Trends Biochem. Sci. 2016, 41 (7), 633–645.

Gutiérrez-Rivera, B.; Waliszewski-Kubiak, K.; Carvajal-Zarrabal, O.; Aguilar-Uscanga, M. G. Conversion Efficiency of Glucose/Xylose Mixtures for Ethanol Production Using *Saccharomyces cerevisiae* ITV01 and *Pichia stipitis* NRRL Y-7124. J. Chem. Technol. Biotechnol. 2012, 87 (2), 263–270.

Gutiérrez-Rivera, B.; Ortiz-Muñiz, B.; Gómez-Rodríguez, J.; Cárdenas-Cágal, A.; Domínguez González, J. M.; Aguilar-Uscanga, M. G. Bioethanol Production from Hydrolyzed Sugarcane Bagasse Supplemented with Molasses "B" in a Mixed Yeast Culture. Renew. Energy 2015, 74, 399–405.

Haldar, D.; Sen, D.; Gayen, K. A Review on the Production of Fermentable Sugars from Lignocellulosic Biomass through Conventional and Enzymatic Route—a Comparison. Int. J. Green Energy 2016, 13 (12), 1232–1253.

Harner, N. K.; Wen, X.; Bajwa, P. K.; Austin, G. D.; Ho, C. Y.; Habash, M. B.; Trevors, J. T.; Lee, H. Genetic Improvement of Native Xylose-Fermenting Yeasts for Ethanol Production. J. Ind. Microbiol. Biotechnol. 2015, 42 (1), 1–20.

Harris, P. V.; Xu, F.; Kreel, N. E.; Kang, C.; Fukuyama, S. New Enzyme Insights Drive Advances in Commercial Ethanol Production. Curr. Opin. Chem. Biol. 2014, 19 (1), 162–170.

Ho, D. P.; Ngo, H. H.; Guo, W. A Mini Review on Renewable Sources for Biofuel. Bioresour. Technol. 2014, 169, 742–749.

Holik, H. Handbook of Paper and Board, 2nd ed.; Wiley-VCH, 2013.

Hu, F.; Ragauskas, A. Pretreatment and Lignocellulosic Chemistry. Bioenergy Res. 2012, 5 (4), 1043–1066.

Huang, C.; He, J.; Chang, H. min; Jameel, H.; Yong, Q. Coproduction of Ethanol and Lignosulfonate From Moso Bamboo Residues by Fermentation and Sulfomethylation. Waste and Biomass Valorization 2017, 8 (3), 965–974.

Hussain, A.; Arif, S. M.; Aslam, M. Emerging Renewable and Sustainable Energy Technologies: State of the Art. Renew. Sustain. Energy Rev. 2017, 71, 12–28.

IEA. World Energy Outlook 2016 Part B: Special Focus on Renewable Energy; 2016.

IEA Bioenergy Task 39. Project Waste Gas to Fuel http://demoplants21.bioenergy2020.eu/projects/info/3262 (accessed Dec 12, 2017).

IEA Bioenergy Task 39. Database on facilities for the production of advanced liquid and gaseous biofuels for transport http://demoplants.bioenergy2020.eu/ (accessed Dec 13, 2017a).

IEA Bioenergy Task 39. Project Brazil http://demoplants21.bioenergy2020.eu/projects/info/3286 (accessed Dec 13, 2017b).

IEA Bioenergy Task 39. Project Project Liberty http://demoplants21.bioenergy2020.eu/projects/info/3196 (accessed Dec 13, 2017c).

IEA Bioenergy Task 42. IEA Bioenergy Task42 Biorefining Brochure; 2014.

IEA Bioenergy Task 42. Newsletter IEA Bioenergy Task42 - Number 2, August; 2017.

INE. Estatísticas Agrícolas 2016; 2017.

Iram, M.; Asghar, U.; Irfan, M.; Huma, Z.; Jamil, S.; Nadeem, M.; Syed, Q. Production of Bioethanol from Sugarcane Bagasse Using Yeast Strains: A Kinetic Study. Energy Sources, Part A Recover. Util. Environ. Eff. 2018, 40 (3), 364–372.

Jacob-Lopes, E.; Zepka, L. Q. Frontiers in Bioenergy and Biofuels, 1st ed.; InTech, 2017.

Jeffries, T. W.; Grigoriev, I. V.; Grimwood, J.; Laplaza, J. M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; Shapiro, H.; et al. Genome Sequence of the Lignocellulose-Bioconverting and Xylose-Fermenting Yeast *Pichia stipitis*. Nat. Biotechnol. 2007, 25 (3), 319–326.

Jeppsson, H.; Yu, S.; Hahn-Hagerdal, B. Xylulose and Glucose Fermentation by *Saccharomyces cerevisiae* in Chemostat Culture. Appl. Environ. mMcrobiology 1996, 62 (5), 1705–1709.

Jönsson, L. J.; Martín, C. Pretreatment of Lignocellulose: Formation of Inhibitory by-Products and Strategies for Minimizing Their Effects. Bioresour. Technol. 2016, 199, 103–112.

Jordan, D. B.; Bowman, M. J.; Braker, J. D.; Dien, B. S.; Hector, R. E.; Lee, C. C.; Mertens, J. A.; Wagschal, K. Plant Cell Walls to Ethanol. Biochem. J. 2012, 442 (2), 241–252.

Kamzon, M. A.; Abderafi, S.; Bounahmidi, T. Promising Bioethanol Processes for Developing a Biorefinery in the Moroccan Sugar Industry. Int. J. Hydrogen Energy 2016, 41 (45), 20880–20896.

Kang, Q.; Appels, L.; Tan, T.; Dewil, R. Bioethanol from Lignocellulosic Biomass: Current Findings Determine Research Priorities. Sci. World J. 2014, 2014, 1–13.

Karagöz, P.; Özkan, M. Ethanol Production from Wheat Straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* Co-Culture in Batch and Continuous System. Bioresour. Technol. 2014, 158, 286–293.

Kelbert, M.; RomanÃ-, A.; Coelho, E.; Pereira, F. B.; Teixeira, J. A.; Domingues, L. Simultaneous Saccharification and Fermentation of Hydrothermal Pretreated Lignocellulosic Biomass: Evaluation of Process Performance Under Multiple Stress Conditions. Bioenergy Res. 2016, 9 (3), 750–762.

Kennes, D.; Abubackar, H. N.; Diaz, M.; Veiga, M. C.; Kennes, C. Bioethanol Production from Biomass: Carbohydrate vs Syngas Fermentation. J. Chem. Technol. Biotechnol. 2016, 91 (2), 304–317.

Kim, S. R.; Ha, S. J.; Wei, N.; Oh, E. J.; Jin, Y.-S. Simultaneous Co-Fermentation of Mixed Sugars: A Promising Strategy for Producing Cellulosic Ethanol. Trends Biotechnol. 2012, 30 (5), 274–282.

Kleingesinds, E. K.; José, Á. H. M.; Brumano, L. P.; Silva-Fernandes, T.; Rodrigues, D.; Rodrigues, R. C. L. B. Intensification of Bioethanol Production by Using Tween 80 to Enhance Dilute Acid Pretreatment and Enzymatic Saccharification of Corncob. Ind. Crops Prod. 2018, 124 (July), 166–176.

Ko, C. H.; Wang, Y. N.; Chang, F. C.; Chen, J. J.; Chen, W. H.; Hwang, W. S. Potentials of Lignocellulosic Bioethanols Produced from Hardwood in Taiwan. Energy 2012, 44 (1), 329–334.

Korhonen, J.; Honkasalo, A.; Seppälä, J. Circular Economy: The Concept and Its Limitations. Ecol. Econ. 2018, 143, 37–46.

Koti, S.; Govumoni, S. P.; Gentela, J.; Rao, L. V. Enhanced Bioethanol Production from Wheat Straw Hemicellulose by Mutant Strains of Pentose Fermenting Organisms *Pichia stipitis* and Candida Shehatae. Springerplus 2016, 5 (1), 1–9.

Kubicek, C. P.; Kubicek, E. M. Enzymatic Deconstruction of Plant Biomass by Fungal Enzymes. Curr. Opin. Chem. Biol. 2016, 35, 51–57.

Kundu, C.; Lee, J. W. Bioethanol Production from Detoxified Hydrolysate and the Characterization of Oxalic Acid Pretreated Eucalyptus (*Eucalyptus globulus*) Biomass. Ind. Crops Prod. 2016, 83, 322–328.

Kurtzman, C. P.; Suzuki, M. Phylogenetic Analysis of Ascomycete Yeasts That Form Coenzyme Q-9 and the Proposal of the New Genera Babjeviella, Meyerozyma, Millerozyma, Priceomyces, and Scheffersomyces. Mycoscience 2010, 51 (1), 2–14.

Kwak, S.; Jin, Y.-S. Production of Fuels and Chemicals from Xylose by Engineered *Saccharomyces cerevisiae*: A Review and Perspective. Microb. Cell Fact. 2017, 16 (1), 82.

LanzaTech. LanzaTech Facilities http://www.lanzatech.com/facilities/ (accessed Dec 4, 2017).

Li, Y.; Park, J. yil; Shiroma, R.; Tokuyasu, K. Bioethanol Production from Rice Straw by a Sequential Use of *Saccharomyces cerevisiae* and *Pichia stipitis* with Heat Inactivation of *Saccharomyces cerevisiae* Cells Prior to Xylose Fermentation. J. Biosci. Bioeng. 2011, 111 (6), 682–686.

Liew, W. H.; Hassim, M. H.; Ng, D. K. S. Review of Evolution, Technology and Sustainability Assessments of Biofuel Production. J. Clean. Prod. 2014, 71, 11–29.

Lima, M. A. P.; Natalense, A. P. P. Bioethanol, 1st ed.; InTech, 2012.

Limayem, A.; Ricke, S. C. Lignocellulosic Biomass for Bioethanol Production: Current Perspectives, Potential Issues and Future Prospects. Prog. Energy Combust. Sci. 2012, 38 (4), 449–467.

Liu, W.; Wang, B.; Hou, Q.; Chen, W.; Wu, M. Effects of Fibrillation on the Wood Fibers' Enzymatic Hydrolysis Enhanced by Mechanical Refining. Bioresour. Technol. 2016, 206, 99–103.

Loow, Y. L.; Wu, T. Y.; Jahim, J. M.; Mohammad, A. W.; Teoh, W. H. Typical Conversion of Lignocellulosic Biomass into Reducing Sugars Using Dilute Acid Hydrolysis and Alkaline Pretreatment. Cellulose 2016, 23 (3), 1491–1520.

Luque, R.; Lin, C. S. K.; Wilson, K.; Clark, J. Handbook of Biofuels Production: Processes and Technologies, 2nd ed.; Elsevier Ltd, 2016.

Lynd, L. R.; Liang, X.; Biddy, M. J.; Allee, A.; Cai, H.; Foust, T.; Himmel, M. E.; Laser, M. S.; Wang, M.; Wyman, C. E. Cellulosic Ethanol: Status and Innovation. Curr. Opin. Biotechnol. 2017, 45, 202–211.

Madhavan, A.; Srivastava, A.; Kondo, A.; Bisaria, V. S. Bioconversion of Lignocellulose-Derived Sugars to Ethanol by Engineered *Saccharomyces cerevisiae*. Crit. Rev. Biotechnol. 2012, 32 (1), 22–48.

Maina, S.; Kachrimanidou, V.; Koutinas, A. A Roadmap towards a Circular and Sustainable Bioeconomy through Waste Valorization. Curr. Opin. Green Sustain. Chem. 2017, 8, 18–23.

Maity, S. K. Opportunities, Recent Trends and Challenges of Integrated Biorefinery: Part I. Renew. Sustain. Energy Rev. 2015a, 43, 1427–1445.

Maity, S. K. Opportunities, Recent Trends and Challenges of Integrated Biorefinery: Part II. Renew. Sustain. Energy Rev. 2015b, 43, 1446–1466.

Manochio, C.; Andrade, B. R.; Rodriguez, R. P.; Moraes, B. S. Ethanol from Biomass: A Comparative Overview. Renew. Sustain. Energy Rev. 2017, 80, 743–755.

Martín-Sampedro, R.; Fillat, Ú.; Ibarra, D.; Eugenio, M. E. Use of New Endophytic Fungi as Pretreatment to Enhance Enzymatic Saccharification of *Eucalyptus globulus*. Bioresour. Technol. 2015, 196, 383–390.

Mendes, C. V. T.; Cruz, C. H. G.; Reis, D. F. N.; Carvalho, M. G. V. S.; Rocha, J. M. S. Integrated Bioconversion of Pulp and Paper Primary Sludge to Second Generation Bioethanol Using *Saccharomyces*

cerevisiae ATCC 26602. Bioresour. Technol. 2016, 220, 161–167.

Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 1959, 31 (3), 426–428.

Mohan, S. V.; Nikhil, G. N.; Chiranjeevi, P.; Reddy, C. N.; Rohit, M. V.; Kumar, A. N.; Sarkar, O. Waste Biorefinery Models towards Sustainable Circular Bioeconomy: Critical Review and Future Perspectives. Bioresour. Technol. 2016, 215, 2–12.

Monrroy, M.; García, J.-R.; Mendonça, R. T.; Baeza, J.; Freer, J. Kraft Pulping Of *Eucalyptus globulus* As A Pretreatment For Bioethanol Production By Simultaneous Saccharification And Fermentation. J. Chil. Chem. Soc. 2012, 57 (2), 1113–1117.

Mood, S. H.; Golfeshan, A. H.; Tabatabaei, M.; Jouzani, G. S.; Najafi, G. H.; Gholami, M.; Ardjmand, M. Lignocellulosic Biomass to Bioethanol, a Comprehensive Review with a Focus on Pretreatment. Renew. Sustain. Energy Rev. 2013, 27, 77–93.

Moreira, L. R. S.; Filho, E. X. F. Insights into the Mechanism of Enzymatic Hydrolysis of Xylan. Appl. Microbiol. Biotechnol. 2016, 100 (12), 5205–5214.

Moysés, D. N.; Reis, V. C. B.; de Almeida, J. R. M.; de Moraes, L. M. P.; Torres, F. A. G. Xylose Fermentation by *Saccharomyces cerevisiae*: Challenges and Prospects. Int. J. Mol. Sci. 2016, 17 (3), 1–18.

Naseeruddin, S.; Desai, S.; Rao, L. V. Ethanol Production from Lignocellulosic Substrate *Prosopis Juliflora*. Renew. Energy 2017, 103, 701–707.

Nigam, P. S.; Singh, A. Production of Liquid Biofuels from Renewable Resources. Prog. Energy Combust. Sci. 2011, 37 (1), 52–68.

Nogué, V. S.; Karhumaa, K. Xylose Fermentation as a Challenge for Commercialization of Lignocellulosic Fuels and Chemicals. Biotechnol. Lett. 2015, 37 (4), 761–772.

Pandey, A.; Larroche, C.; Ricke, S. C.; Dussap, C.-G.; Gnansounou, E. Biofuels: Alternative Feedstocks and Conversion Processes, 1st ed.; Academic Press, 2011.

Pandey, A.; Negi, S.; Binod, P.; Larroche, C. Pretreatment of Biomass: Processes and Technologies, 1st ed.; Elsevier, 2015.

Papini, M.; Nookaew, I.; Uhlén, M.; Nielsen, J. *Scheffersomyces stipitis*: A Comparative Systems Biology Study with the Crabtree Positive Yeast *Saccharomyces cerevisiae*. Microb. Cell Fact. 2012, 11, 1–16.

Patel, H.; Chapla, D.; Shah, A. Bioconversion of Pretreated Sugarcane Bagasse Using Enzymatic and Acid Followed by Enzymatic Hydrolysis Approaches for Bioethanol Production. Renew. Energy 2017, 109, 323–331.

Paulova, L.; Patakova, P.; Branska, B.; Rychtera, M.; Melzoch, K. Lignocellulosic Ethanol: Technology Design and Its Impact on Process Efficiency. Biotechnol. Adv. 2015, 33 (6), 1091–1107.

Pereira, F. B.; Romaní, A.; Ruiz, H. A.; Teixeira, J. A.; Domingues, L. Industrial Robust Yeast Isolates with Great Potential for Fermentation of Lignocellulosic Biomass. Bioresour. Technol. 2014, 161, 192–199.

Pereira, S. R.; Ivanuuša, Š.; Evtuguin, D. V.; Serafim, L. S.; Xavier, A. M. R. B. Biological Treatment of Eucalypt Spent Sulphite Liquors: A Way to Boost the Production of Second Generation Bioethanol. Bioresour. Technol. 2012, 103 (1), 131–135.

Pereira, S. R.; Portugal-Nunes, D. J.; Evtuguin, D. V.; Serafim, L. S.; Xavier, A. M. R. B. Advances in Ethanol

Production from Hardwood Spent Sulphite Liquors. Process Biochem. 2013, 48 (2), 272–282.

Pereira, S. R.; Sànchez I Nogué, V.; Frazão, C. J. R.; Serafim, L. S.; Gorwa-Grauslund, M. F.; Xavier, A. M. R. B. Adaptation of *Scheffersomyces stipitis* to Hardwood Spent Sulfite Liquor by Evolutionary Engineering Grant Stanley. Biotechnol. Biofuels 2015, 8 (1), 1–8.

Phillips, R. B.; Jameel, H.; Chang, H. M. Integration of Pulp and Paper Technology with Bioethanol Production. Biotechnol. Biofuels 2013, 6 (1), 1.

POET-DSM Advanced Biofuels. Project LIBERTY in "ramp-up" phase http://poet-dsm.com/pr/project-liberty-in-ramp-up-phase (accessed Dec 13, 2017).

Portuguese Yeast Culture Collection. PYCC 5246 http://pycc.bio-aware.com/BioloMICS.aspx?Table=PYCC strains (accessed Aug 11, 2018).

Raízen. Tecnologia em energia renovável: Etanol de segunda geração https://www.raizen.com.br/energia-do-futuro-tecnologia-em-energia-renovavel/etanol-de-segunda-geracao (accessed Dec 4, 2017).

Ramaswamy, S.; Huang, H.-J.; Ramarao, B. V. Separation and Purification Technologies in Biorefineries, 1st ed.; John Wiley & Sons, 2013.

Rastogi, M.; Shrivastava, S. Recent Advances in Second Generation Bioethanol Production: An Insight to Pretreatment, Saccharification and Fermentation Processes. Renew. Sustain. Energy Rev. 2017, 80, 330–340.

REN21. Renewables 2017 Global Status Report; 2017a.

REN21. Renewables Global Futures Report: Great Debates towards 100% Renewable Energy; 2017b.

República Portuguesa. National Renewable Energy Action Plan - Portugal; 2010.

Ritzén, S.; Sandström, G. Ö. Barriers to the Circular Economy - Integration of Perspectives and Domains. Procedia CIRP 2017, 64, 7–12.

Rocha-Meneses, L.; Raud, M.; Orupõld, K.; Kikas, T. Second-Generation Bioethanol Production: A Review of Strategies for Waste Valorisation. Agron. Res. 2017, 15 (3), 830–847.

Saggi, S. K.; Dey, P. An Overview of Simultaneous Saccharification and Fermentation of Starchy and Lignocellulosic Biomass for Bio-Ethanol Production. Biofuels 2016, 1–13.

Saini, J. K.; Saini, R.; Tewari, L. Lignocellulosic Agriculture Wastes as Biomass Feedstocks for Second-Generation Bioethanol Production: Concepts and Recent Developments. 3 Biotech 2015, 5 (4), 337–353.

Salles-Filho, S. L. M.; Cortez, L. A. B.; da Silveira, J. M. F. J.; Trindade, S. C.; Fonseca, M. da G. D. Global Bioethanol: Evolution, Risks, and Uncertainties, 1st ed.; Academic Press, 2016.

dos Santos, L. V.; Grassi, M. C. de B.; Gallardo, J. C. M.; Pirolla, R. A. S.; Calderón, L. L.; de Carvalho-Netto, O. V.; Parreiras, L. S.; Camargo, E. L. O.; Drezza, A. L.; Missawa, S. K.; et al. Second-Generation Ethanol: The Need Is Becoming a Reality. Ind. Biotechnol. 2016, 12 (1), 40–57.

Santosh, I.; Ashtavinayak, P.; Amol, D.; Sanjay, P. Enhanced Bioethanol Production from Different Sugarcane Bagasse Cultivars Using Co-Culture of *Saccharomyces cerevisiae* and *Scheffersomyces* (*Pichia*) *stipitis*. J. Environ. Chem. Eng. 2017, 5 (3), 2861–2868.

Sarkar, N.; Ghosh, S. K.; Bannerjee, S.; Aikat, K. Bioethanol Production from Agricultural Wastes: An Overview. Renew. Energy 2012, 37 (1), 19–27.

Sarris, D.; Papanikolaou, S. Biotechnological Production of Ethanol: Biochemistry, Processes and

Technologies. Eng. Life Sci. 2016, 16 (4), 307–329.

Sebayang, A. H.; Masjuki, H. H.; Ong, H. C.; Dharma, S.; Silitonga, A. S.; Mahlia, T. M. I.; Aditiya, H. B. A Perspective on Bioethanol Production from Biomass as Alternative Fuel for Spark Ignition Engine. RSC Adv. 2016, 6 (18), 14964–14992.

Seidl, P. R.; Goulart, A. K. Pretreatment Processes for Lignocellulosic Biomass Conversion to Biofuels and Bioproducts. Curr. Opin. Green Sustain. Chem. 2016, 2, 48–53.

Sigma-Aldrich. Yeast Extracts https://www.sigmaaldrich.com/analytical-chromatography/microbiology/microbiology-products.html?TablePage=8657623 (accessed Aug 11, 2018).

Silva, D. D. V.; Dussán, K. J.; Hernández, V.; da Silva, S. S.; Cardona, C. A.; Felipe, M. das G. de A. Effect of Volumetric Oxygen Transfer Coefficient (KLa) on Ethanol Production Performance by *Scheffersomyces stipitis* on Hemicellulosic Sugarcane Bagasse Hydrolysate. Biochem. Eng. J. 2016, 112, 249–257.

Silva, J. P. A.; Mussatto, S. I.; Roberto, I. C.; Teixeira, J. A. Fermentation Medium and Oxygen Transfer Conditions That Maximize the Xylose Conversion to Ethanol by *Pichia stipitis*. Renew. Energy 2012, 37 (1), 259–265.

Silveira, M. H. L.; Morais, A. R. C.; Lopes, A. M. da C.; Olekszyszen, D. N.; Bogel-Łukasik, R.; Andreaus, J.; Ramos, L. P. Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. ChemSusChem 2015, 8 (20), 3366–3390.

Singh, A.; Rangaiah, G. P. Review of Technological Advances in Bioethanol Recovery and Dehydration. Ind. Eng. Chem. Res. 2017, 56 (18), 5147–5163.

Singh, A.; Bajar, S.; Bishnoi, N. R. Enzymatic Hydrolysis of Microwave Alkali Pretreated Rice Husk for Ethanol Production by *Saccharomyces cerevisiae*, *Scheffersomyces stipitis* and Their Co-Culture. Fuel 2014, 116, 699–702.

Singh, L. K.; Chaudhary, G. Advances in Biofeedstocks and Biofuels, Volume 1: Biofeedstocks and Their Processing, 1st ed.; Scrivener Publishing, 2017.

Singh, R. L. Applied Environmental Science and Engineering for a Sustainable Future: Principles and Applications of Environmental Biotechnology for a Sustainable Future, 1st ed.; Springer Science & Business Media, 2017.

Slininger, J. P.; Bothast, R. J.; Ladisch, M. R.; Okos, M. R. Optimum Ph and Temperature Conditions for Xylose Fermentation by *Pichia stipitis*. Biotechnol. Bioeng. 1990, 35 (7), 727–731.

Slininger, P. J.; Dien, B. S.; Gorsich, S. W.; Liu, Z. L. Nitrogen Source and Mineral Optimization Enhance D-Xylose Conversion to Ethanol by the Yeast *Pichia stipitis* NRRL Y-7124. Appl. Microbiol. Biotechnol. 2006, 72 (6), 1285–1296.

Slininger, P. J.; Gorsich, S. W.; Liu, Z. L. Culture Nutrition and Physiology Impact the Inhibitor Tolerance of the Yeast *Pichia stipitis* NRRL Y-7124. Biotechnol. Bioeng. 2009, 102 (3), 778–790.

Snoek, T.; Verstrepen, K. J.; Voordeckers, K. How Do Yeast Cells Become Tolerant to High Ethanol Concentrations? Curr. Genet. 2016, 62 (3), 475–480.

Sreemahadevan, S.; Singh, V.; Roychoudhury, P. K.; Ahammad, S. Z. Mathematical Modeling, Simulation and Validation for Co-Fermentation of Glucose and Xylose by *Saccharomyces cerevisiae* and

Scheffersomyces stipitis. Biomass and Bioenergy 2018, 110, 17–24.

Sun, S.; Sun, S.; Cao, X.; Sun, R. The Role of Pretreatment in Improving the Enzymatic Hydrolysis of Lignocellulosic Materials. Bioresour. Technol. 2016, 199, 49–58.

Suriyachai, N.; Weerasaia, K.; Laosiripojana, N.; Champreda, V.; Unrean, P. Optimized Simultaneous Saccharification and Co-Fermentation of Rice Straw for Ethanol Production by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* Co-Culture Using Design of Experiments. Bioresour. Technol. 2013, 142, 171–178.

Taha, M.; Foda, M.; Shahsavari, E.; Aburto-Medina, A.; Adetutu, E.; Ball, A. Commercial Feasibility of Lignocellulose Biodegradation: Possibilities and Challenges. Curr. Opin. Biotechnol. 2016, 38, 190–197.

Tesfaw, A.; Assefa, F. Current Trends in Bioethanol Production by *Saccharomyces cerevisiae*: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Imobilization. Int. Sch. Res. Not. 2014, 2014, 1–11.

Thangavelu, S. K.; Ahmed, A. S.; Ani, F. N. Review on Bioethanol as Alternative Fuel for Spark Ignition Engines. Renew. Sustain. Energy Rev. 2016, 56, 820–835.

Thani, A.; Laopaiboon, P.; Laopaiboon, L. Improvement of a Continuous Ethanol Fermentation from Sweet Sorghum Stem Juice Using a Cell Recycling System. J. Biotechnol. 2017, 251, 21–29.

Unrean, P.; Khajeeram, S. Optimization and Techno-Economic Assessment of High-Solid Fed-Batch Saccharification and Ethanol Fermentation by *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* Consortium. Renew. Energy 2016, 99, 1062–1072.

Veras, H. C. T.; Parachin, N. S.; Almeida, J. R. M. Comparative Assessment of Fermentative Capacity of Different Xylose-Consuming Yeasts. Microb. Cell Fact. 2017, 16 (1), 1–8.

Volynets, B.; Ein-Mozaffari, F.; Dahman, Y. Biomass Processing into Ethanol: Pretreatment, Enzymatic Hydrolysis, Fermentation, Rheology, and Mixing. Green Process. Synth. 2017, 6 (1), 1–22.

Winans, K.; Kendall, A.; Deng, H. The History and Current Applications of the Circular Economy Concept. Renew. Sustain. Energy Rev. 2017, 68, 825–833.

Wistara, N. J.; Pelawi, R.; Fatriasari, W. The Effect of Lignin Content and Freeness of Pulp on the Bioethanol Productivity of Jabon Wood. Waste and Biomass Valorization 2016, 7 (5), 1141–1146.

Wong, J. W.-C.; Tyagi, R. D.; Pandey, A. Current Developments in Biotechnology and Bioengineering: Solid Waste Management, 1st ed.; Elsevier, 2017.

Xin, F.; Geng, A.; Chen, M. L.; Gum, M. J. M. Enzymatic Hydrolysis of Sodium Dodecyl Sulphate (SDS)-Pretreated Newspaper for Cellulosic Ethanol Production by *Saccharomyces cerevisiae* and *Pichia stipitis*. Appl. Biochem. Biotechnol. 2010, 162 (4), 1052–1064.

Xiros, C.; Topakas, E.; Christakopoulos, P. Hydrolysis and Fermentation for Cellulosic Ethanol Production. Adv. Bioenergy Sustain. Chall. 2013, 2 (December), 11–31.

Yablochkova, E. N.; Bolotnikova, O. I.; Mikhailova, N. P.; Nemova, N. N.; Ginak, A. I. The Activity of Xylose Reductase and Xylitol Dehydrogenase in Yeasts. Microbiology 2003, 72 (4), 414–417.

Yeh, R. H.; Lin, Y. S.; Wang, T. H.; Kuan, W. C.; Lee, W. C. Bioethanol Production from Pretreated *Miscanthus floridulus* Biomass by Simultaneous Saccharification and Fermentation. Biomass and Bioenergy 2016, 94, 110–116.

Yu, S.; Jeppsson, H.; Hahn-Hägerdal, B. Xylulose Fermentation by *Saccharomyces cerevisiae* and Xylose-

Fermenting Yeast Strains. Appl. Microbiol. Biotechnol. 1995, 44 (3–4), 314–320.

Yuan, Z.; Wen, Y.; Kapu, N. S. Ethanol Production from Bamboo Using Mild Alkaline Pre-Extraction Followed by Alkaline Hydrogen Peroxide Pretreatment. Bioresour. Technol. 2018, 247, 242–249.

Yusoff, M. N. A. M.; Zulkifli, N. W. M.; Masum, B. M.; Masjuki, H. H. Feasibility of Bioethanol and Biobutanol as Transportation Fuel in Spark-Ignition Engine: A Review. RSC Adv. 2015, 5 (121), 100184–100211.

Zabed, H.; Sahu, J. N.; Boyce, A. N.; Faruq, G. Fuel Ethanol Production from Lignocellulosic Biomass: An Overview on Feedstocks and Technological Approaches. Renew. Sustain. Energy Rev. 2016, 66, 751–774.

Zabed, H.; Sahu, J. N.; Suely, A.; Boyce, A. N.; Faruq, G. Bioethanol Production from Renewable Sources: Current Perspectives and Technological Progress. Renew. Sustain. Energy Rev. 2017, 71, 475–501.

Zambare, V. P.; Bhalla, A.; Muthukumarappan, K.; Sani, R. K.; Christopher, L. P. Bioprocessing of Agricultural Residues to Ethanol Utilizing a Cellulolytic Extremophile. Extremophiles 2011, 15 (5), 611–618.

Zambare, V. P.; Zambare, A. V.; Barh, D.; Christopher, L. P. Optimization of Enzymatic Hydrolysis of Prairie Cordgrass for Improved Ethanol Production. J. Renew. Sustain. Energy 2012, 4 (3), 033118-1-033118-8.

Zhao, X.; Xiong, L.; Zhang, M.; Bai, F. Towards Efficient Bioethanol Production from Agricultural and Forestry Residues: Exploration of Unique Natural Microorganisms in Combination with Advanced Strain Engineering. Bioresour. Technol. 2016, 215, 84–91.

Appendixes

Appendix A – Circular economy

Figure A1 presents a CE diagram.

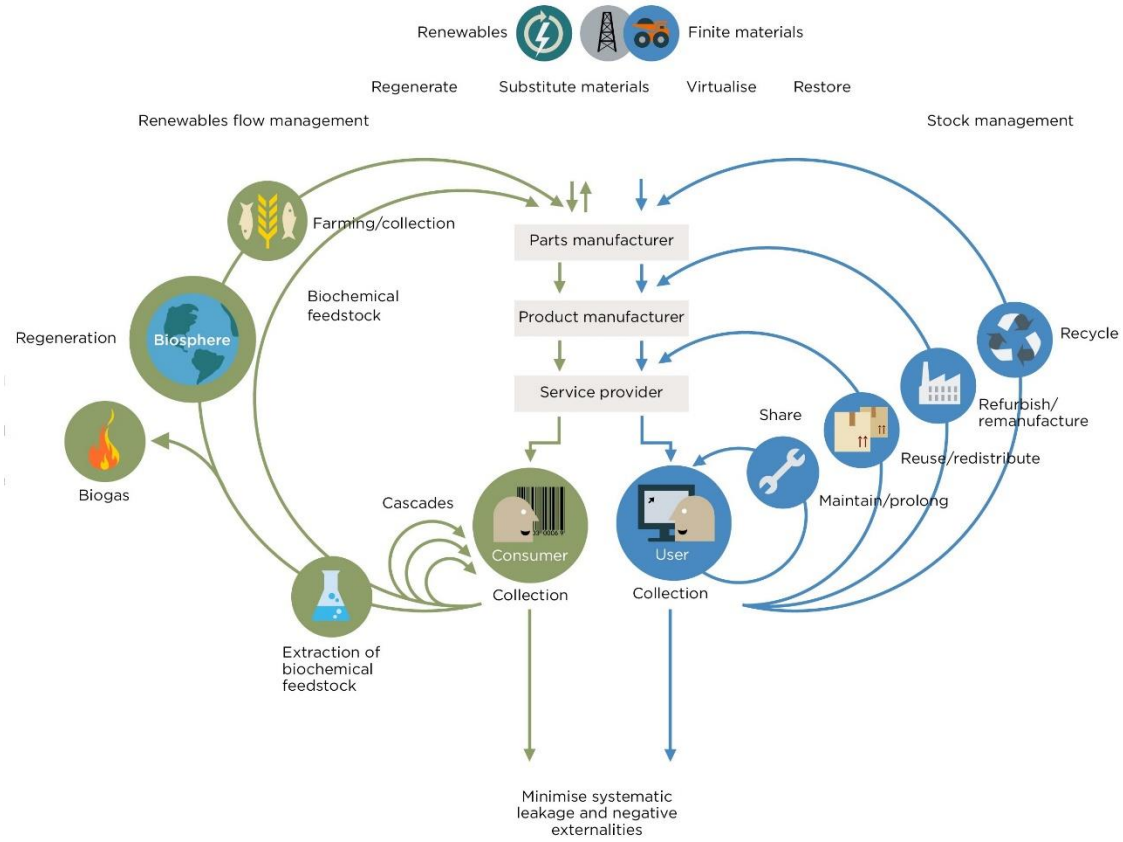

Figure A1 – Circular economy system diagram. Adapted from Ellen MacArthur Foundation, 2017

Appendix B – Sugar metabolism

Figure B1 and **B2** show an overview of *S. cerevisiae*'s glucose metabolism and *S. stipitis*' xylose metabolism, respectively.

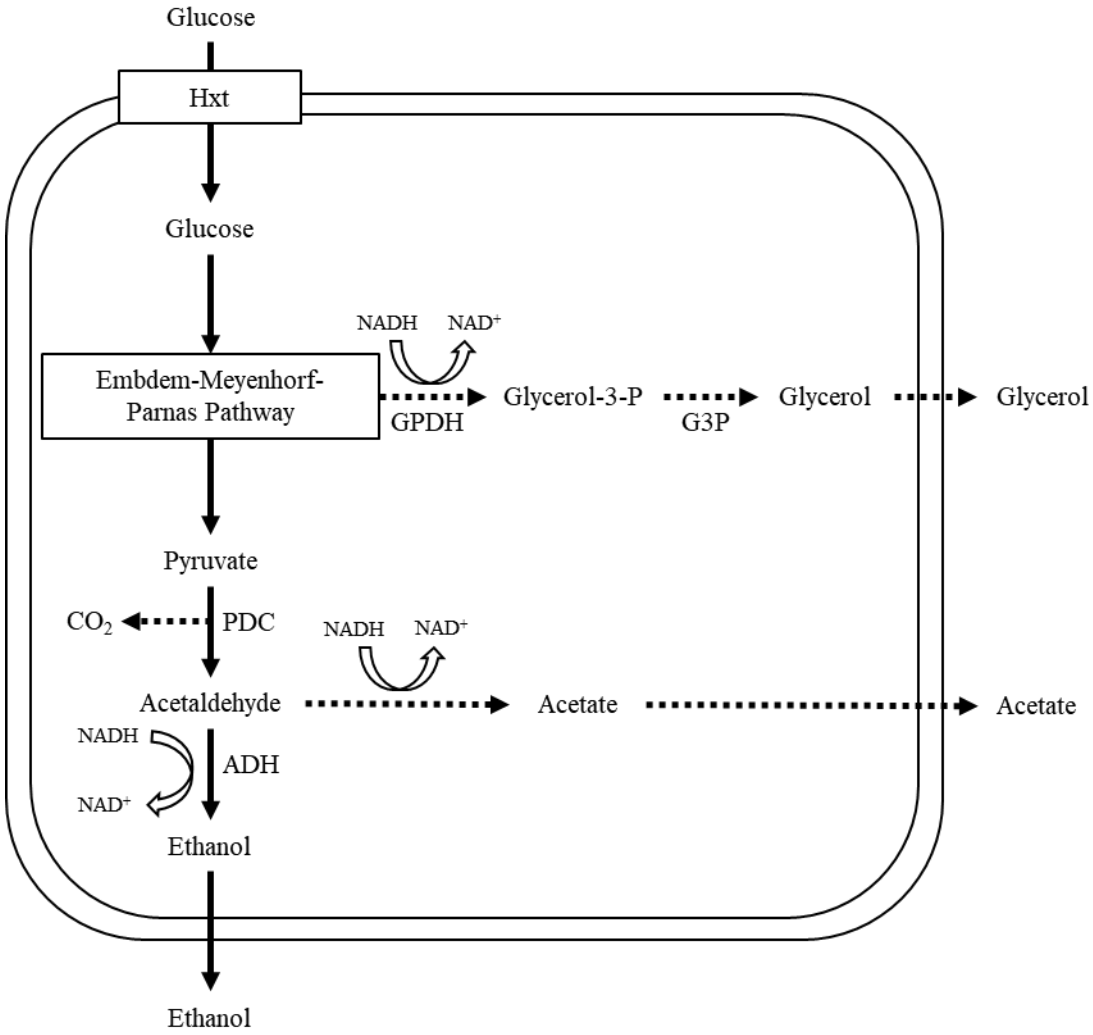

Figure B1 – Overview of glucose metabolism by *Saccharomyces cerevisiae*. Hxt – Hexose transporter; PDC - Pyruvate decarboxylase; ADH - Alcohol dehydrogenase; GPDH - Glycerol-3-phosphate dehydrogenase; G3P - glycerol-3-phosphate phosphatase. Adapted from Madhavan et al., 2012; Sarris and Papanikolaou, 2016

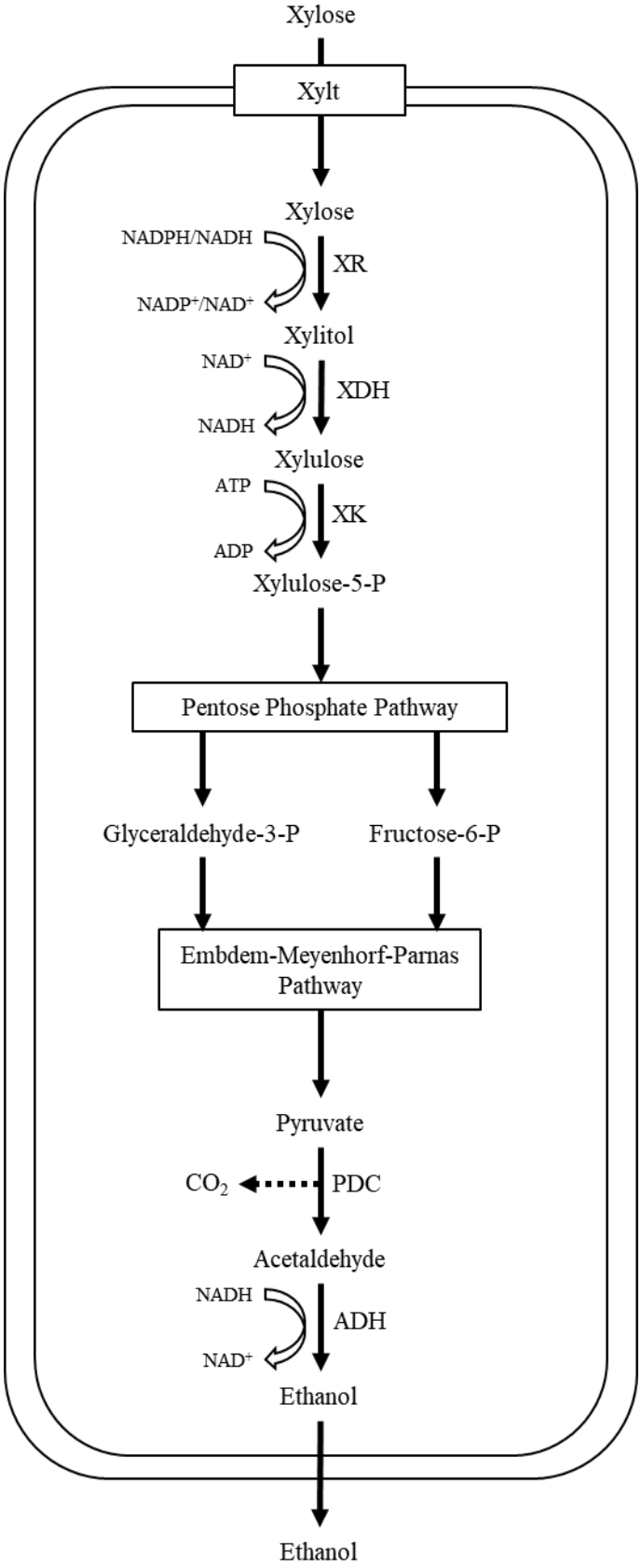

Figure B2 - Overview of xylose metabolism by *Scheffersomyces stipitis*. Xylt – Xylose transporter; XR - Xylose reductase ; XDH - Xylitol dehydrogenase; XK - Xylulokinase; PDC - Pyruvate decarboxylase; ADH - Alcohol dehydrogenase. Adapted from Agbogbo and Coward-Kelly, 2008; Gírio et al., 2010

Appendix C – Integrated biorefinery

Figure C1 presents a possible integrated biorefinery for the production of pulp, energy, second generation bioethanol, and biogas from eucalypt wood, using Kraft pulping as LCB pretreatment.

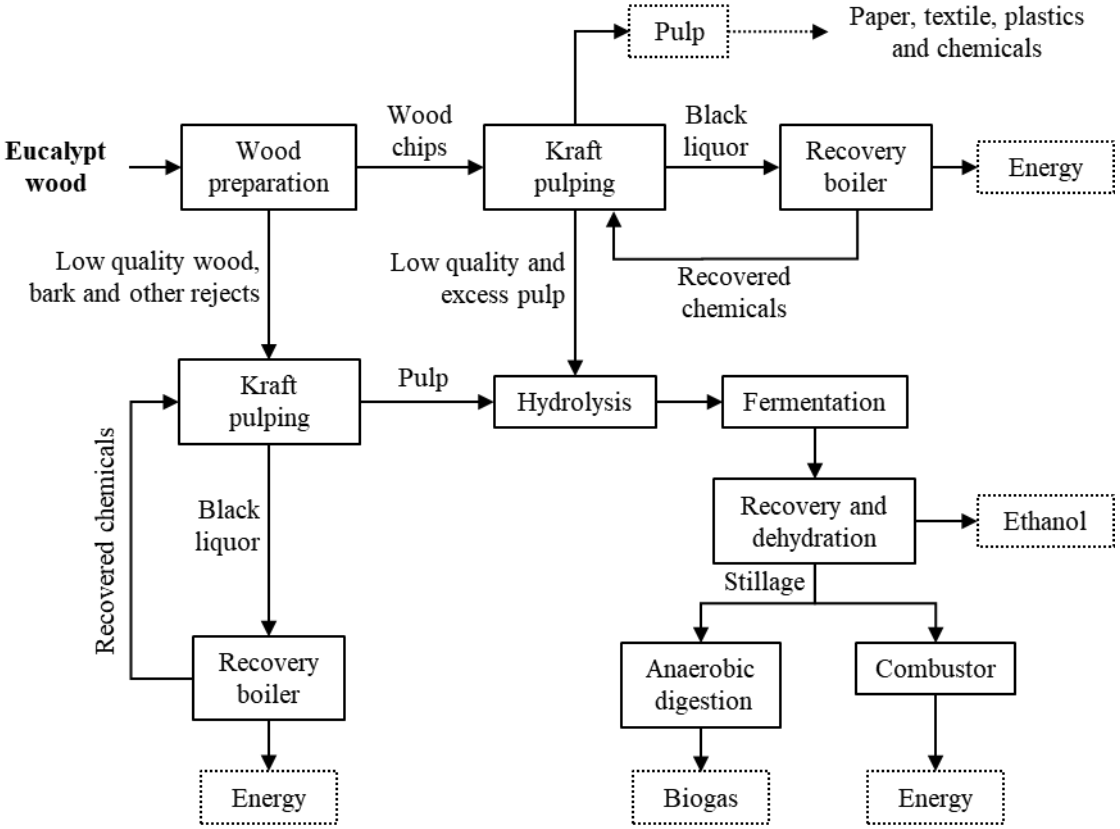

Figure C1 – Schematic representation of an integrated biorefinery for the production of pulp, energy, second generation bioethanol, and biogas.

Appendix D - Calculations for enzymatic hydrolysis of unbleached Kraft pulp

Table D1 shows the characterization of the unbleached Kraft pulp of *Eucalyptus globulus* used in this work.

Table D1 - Characterization of unbleached Kraft pulp of *Eucalyptus globulus* (%).

Moisture	Lignin	Organics	Cellulose	Hemicelluloses
69.02	2.4	97.6	78	19

Data provided by *RAIZ – Instituto de Investigação da Floresta e do Papel*

Based on previous studies and in order to obtain an efficient mixing it was established, for each hydrolysis, a total hydrolysis volume ($V_{hydrolysis}$), a volume of enzymatic solution (V_{ES}), and a mass of dry unbleached Kraft pulp ($m_{dry\ pulp}$) of 3.0 L, 200.0 mL and 200.0 g, respectively.

The unbleached Kraft pulp used in this work presented 69.02 % of moisture (M) (**Table D1**), therefore the mass of unbleached Kraft pulp (m_{pulp}) weighted should be 645.7 g (**Equation D1**).

$$m_{pulp}(g) = \frac{m_{dry\ pulp}\ (g)}{1 - M} = \frac{200.0}{1 - 0.6902} = 645.7\ g \qquad \textbf{Equation D1}$$

The unbleached Kraft pulp was placed in a bath at 50 °C and 2.354 L of water were added. In order to know the volume of water that needed to be added to the pulp, the volume of weighted pulp that corresponds to water in the form of moisture ($V_{water\ in\ pulp}$) was determined (**Equation D2**).

$$V_{water\ in\ pulp}(mL) = \frac{m_{pulp}(g) - m_{dry\ pulp}(g)}{\rho_{water}\ (g.mL^{-1})} = \frac{645.7 - 200.0}{1} = 445.7\ mL \qquad \textbf{Equation D2}$$

Then, the volume of water (V_{water}) to be added to the pulp in order to make up total hydrolysis volume of 3.0 L was determined given the water existent in the weighted pulp and the volume of enzymatic solution added (**Equation D3**).

$$V_{water}(L) = V_{hydrolysis}(L) - \left(V_{water\ in\ pulp}(mL) + V_{ES}(mL)\right) \times 10^{-3} = 3.0 - (445.7 + 200.0) \times 10^{-3} = 2.354\ L \qquad \textbf{Equation D3}$$

Subsequently, an enzymatic solution of 200.0 mL containing 25.08 mL of enzymatic cocktail Novozymes NS was prepared. To calculate the volume of enzymatic preparation (V_E) needed to hydrolyse the pulp the mass of carbohydrate (m_{ch}) present in the unbleached Kraft pulp and the enzymatic activity of the enzymatic cocktail used were taken in account (**Equation D4-D6**).

$$m_{ch}(g) = m_{dry\ pulp}(g) \times Organics = 200.0 \times 0.976 = 195\ g \qquad \textbf{Equation D4}$$

$$Enzymatic\ load\ (FPU) = Enzymatic\ load\ (FPU.g\ carbohydrate^{-1}) \times m_{ch}(g) = 25 \times 195 = 4880\ FPU \qquad \textbf{Equation D5}$$

$$V_E(mL) = \frac{Enzymatic\ load\ (FPU)}{Enzymatic\ activity\ (FPU.mL^{-1})} = \frac{4880}{194.6} = 25.08\ mL \qquad \textbf{Equation D6}$$

Appendix E – Biomass calibration curve (example)

Figure E1 shows the calibration curve of OD_{620} versus biomass dry weight for *S. stipitis* in synthetic medium.

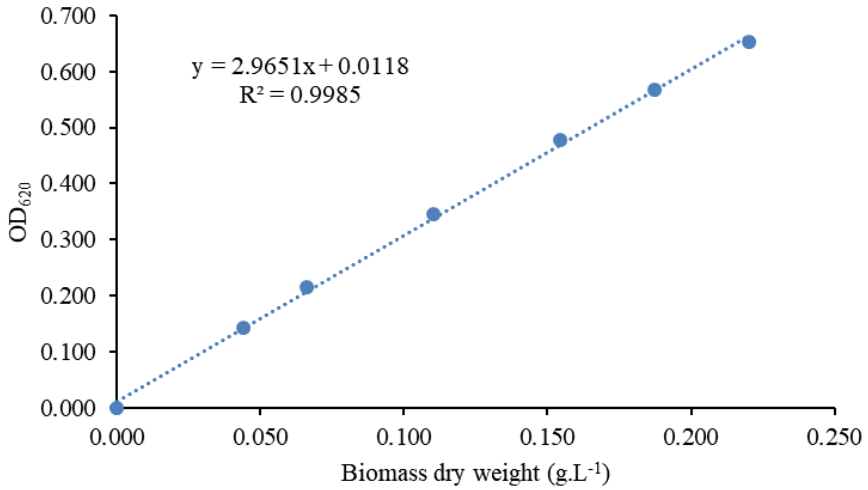

Figure E1 - Calibration curve of OD_{620} versus biomass dry weight for *S. stipitis* in synthetic medium.

Appendix F – Calculation of the potential glucose and xylose in the unbleached Kraft pulp

The hydrolysis of cellulose is expressed by **Equation F1**, while the hydrolysis of hemicelluloses can be expressed by the hydrolysis of xylan (**Equation F2**). Hence, the maximum theoretical yield of glucose ($Y_{Glu/Cellulose}$) is 1.111 g per g of cellulose, resulting

from the ratio of the molecular weight of glucose per molecular weight of anhydroglucoses that make up cellulose (180/162). Similarly, the maximum theoretical yield of xylose ($Y_{Xyl/Hemicelluloses}$) is 1.136 g per g of xylan, the molecular weight ratio of 150/132 for xylose and anhydroxyloses, respectively.

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \qquad \textbf{Equation F1}$$

$$(C_5H_8O_4)_n + nH_2O \rightarrow nC_5H_{10}O_5 \qquad \textbf{Equation F2}$$

The unbleached Kraft pulp used in this work presented 97.6 % of organics, 78 % cellulose, and 19 % hemicelluloses (**Table D1**). Hence, the percentage of organics that corresponded to cellulose and hemicelluloses was 80 and 19 %, respectively (**Equation F3** and **F4**).

$$Cellulose_{\,\%\ of\ organics} = \frac{Cellulose}{Organics} \times 100 = \frac{0.78}{0.976} \times 100 = 80\ \% \qquad \textbf{Equation F3}$$

$$Hemicelluloses_{\,\%\ of\ organics} = \frac{Hemicelluloses}{Organics} \times 100 = \frac{0.19}{0.976} \times 100 = 19\ \% \qquad \textbf{Equation F4}$$

The mass of cellulose ($m_{cellulose}$) and hemicelluloses ($m_{hemicelluloses}$) was then determined considering the mass of carbohydrate (**Equation F5** and **B.6**).

$$m_{cellulose}(g) = m_{ch}(g) \times Cellulose_{\,\%\ of\ organics} = 195 \times 0.80 = 156\ g \qquad \textbf{Equation F5}$$

$$m_{hemicelluloses}(g) = m_{ch}(g) \times Hemicelluloses_{\,\%\ of\ organics} = 195 \times 0.19 = 38\ g \qquad \textbf{Equation F6}$$

To calculate the potential glucose, which corresponds to the mass of glucose obtained if the cellulose present in the pulp were fully hydrolysed in glucose, the mass of cellulose and the maximum theoretical yield of glucose were taken in account (**Equation F7**). Correspondingly, to calculate the potential xylose the mass of hemicelluloses and the maximum theoretical yield of xylose were taken in account (**Equation F8**).

$$Potential\ glucose\ (g) = m_{Cellulose}(g) \times Y_{Glu/Cellulose}\ (g.g^{-1})$$
$$= 156 \times 1.111 = 173.3\ g$$

Equation F7

$$Potential\ xylose\ (g)$$
$$= m_{Hemicelluloses}(g) \times Y_{Xyl/Hemicelluloses}\ (g.g^{-1})$$
$$= 38 \times 1.136 = 43.2\ g$$

Equation F8

Appendix G – Evolution and results of enzymatic hydrolysis

Figure G1 shows the evolution of temperature, reducing sugars, and pH in enzymatic hydrolysis 2 to 4. **Table G1** presents the results of the four enzymatic hydrolysis assays.

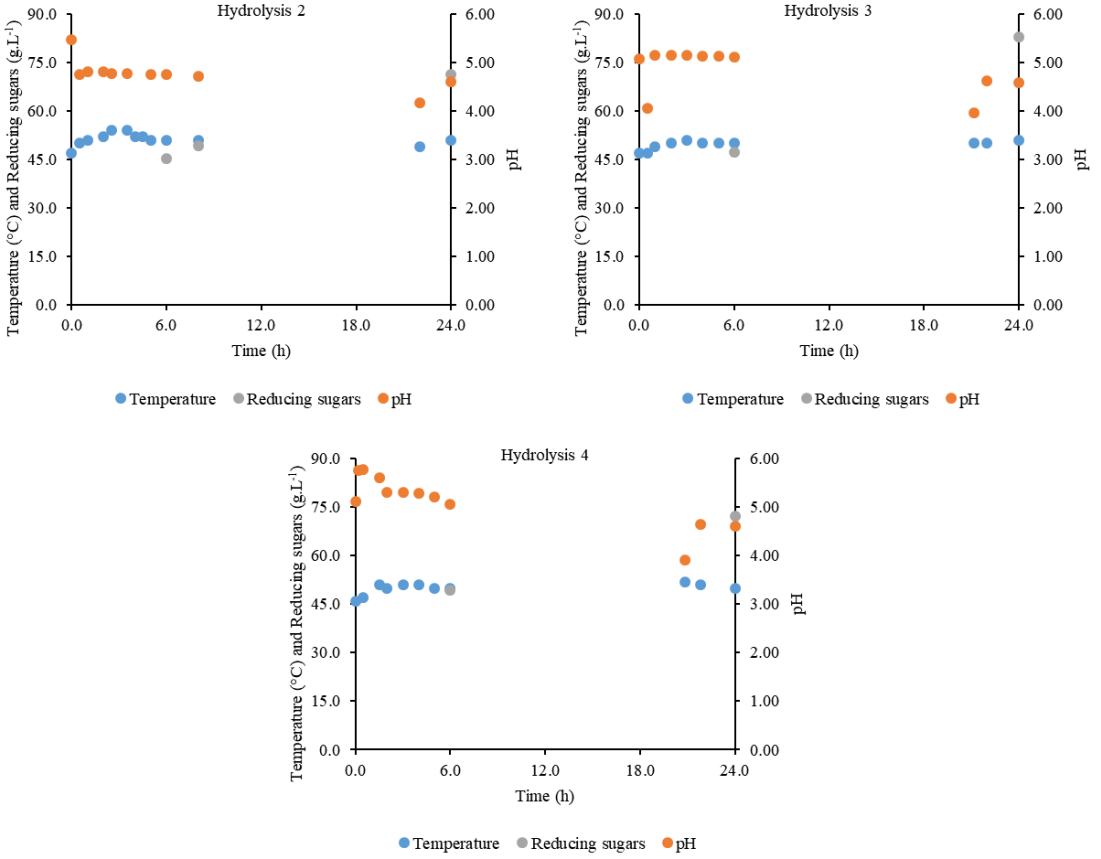

Figure G1 - Evolution of temperature, reducing sugars, and pH in enzymatic hydrolysis 2 to 4.

Table G1 – Results of the four assays of enzymatic hydrolysis.

Hydrolysis	[Glucose] ($g.L^{-1}$)	[Xylose] ($g.L^{-1}$)	$Y_{Hydrolysis}$ (%)	$Y_{Glucose}$ (%)	Y_{Xylose} (%)
1	66.3	18.6	95.7	93.3	100
2	65.4	15.4	98.2	99.3	94.0
3	65.9	16.2	91.4	91.6	90.5
4	64.2	13.8	97.2	99.9	86.2

Appendix H – Spectrum of the Hydrolysate

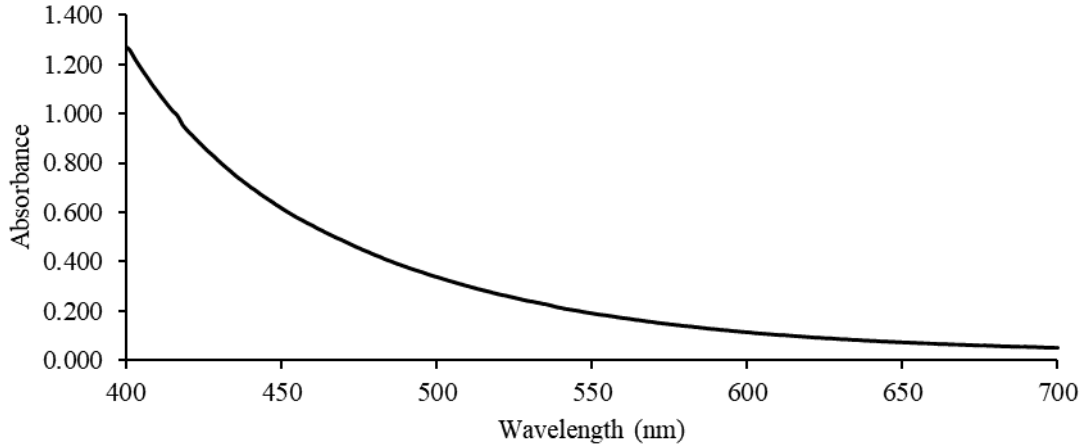

Figure H1 – Hydrolysate spectrum between 400 and 700 nm.

Appendix I – Chemical oxygen demand of glucose, xylose, and ethanol

The oxidation reaction of glucose, xylose, and ethanol are expressed by **Equation I1**, **I2**, and **I3**, respectively.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \textbf{Equation I1}$$

$$C_5H_{10}O_5 + 5O_2 \rightarrow 5CO_2 + 5H_2O \qquad \textbf{Equation I2}$$

$$C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O \qquad \textbf{Equation I3}$$

The COD of a compound results from the ratio of the molecular weight of the number moles of oxygen required to oxidize the compound per molecular weight of the number of moles of oxidized compound. COD of glucose, xylose, and ethanol are expressed by **Equation I4**, **I5**, and **I6**, respectively.

$$COD_{Glucose} = \frac{6 \times 32}{180} = 1.07\ g\ O_2/g\ glucose \qquad \textbf{Equation I4}$$

$$COD_{Xylose} = \frac{5 \times 32}{150} = 1.07\ g\ O_2/g\ xylose \qquad \textbf{Equation I5}$$

$$COD_{Ethanol} = \frac{3 \times 32}{46} = 2.09\ g\ O_2/g\ ethanol \qquad \textbf{Equation I6}$$