Universidade de Aveiro Departamento de Química

Ano 2021

Manuel João Afecto Gonçalves

Economia Circular: Produção de Bioetanol a partir de resíduos da indústria papeleira

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Dissertação 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 Professora Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e coorientação da Professora Doutora Luísa Alexandra Seuanes Serafim Leal, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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o júri

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palavras-chave

Bioetanol, Economia Circular, fermentação, industria da pasta e do papel, pasta Kraft, reutilização de leveduras, extrato de levedura.

resumo

Grandes quantidades de resíduos são geradas diariamente pela indústria da pasta e do papel, como por exemplo as cascas das árvores que são consideradas um material lenhocelulósico. A biomassa lenhocelulósica, que é composta maioritariamente por celulose, hemiceluloses e lenhina, é uma matéria-prima primordial para a produção de biocombustíveis devido às suas características renováveis e aos seus níveis de carbono. O bioetanol é o principal biocombustível produzido em todo o mundo e a sua produção passa geralmente por diferentes passos: o pré-tratamento, a hidrólise, a fermentação e por fim a destilação e purificação. Estes processos têm altos custos associados o que dificulta a sua produção à escala industrial.

O conceito de Economia Circular aplicado à indústria do papel através da integração de uma biorrefinaria produtora de bioetanol pode ser uma abordagem promissora para diminuir os custos associados à sua produção, devido à disponibilidade da matéria-prima e às tecnologias já implementadas para processamento da biomassa lenhocelulósica.

O principal objetivo deste trabalho é o estudo da produção de bioetanol a partir de hidrolisados enzimáticos de pasta kraft proveniente de cascas de *Eucalyptus globulus* nomeadamente no melhoramento do processo pela redução dos seus custos associados. Assim, ureia e Fermaid O™ foram estudados como uma alternativa económica para substituir ou reduzir a utilização do extrato de levedura. Também, a reutilização das leveduras resultantes do meio de fermentação, foi estudada como suplementação, através da produção de extrato de levedura por autólise, e ainda como inóculo em fermentações sucessivas.

A fermentação utilizando o extrato de levedura produzido demonstrou os melhores resultados na produção de bioetanol nos ensaios de teste de suplementações em Erlenmeyer. Comparando os ensaios com o extrato de levedura produzido e o comercial verifica-se um aumento da concentração máxima de etanol de 45.31 ± 1.24 g L^{-1} para 48.26 ± 0.94 g L^{-1}, na produtividade de 1.59 ± 0.04 g L^{-1} h^{-1} para 1.82 ± 0.04 g L^{-1} h^{-1} e no rendimento de 72.72 ± 1.32 % para 76.73 ± 4.53 %. O aumento de escala para biorreator, suplementado com extrato de levedura comercial, forneceu a máxima concentração de etanol obtida em todo o estudo, de 61.05 g L^{-1}.

Este estudo indica que a produção de bioetanol a partir de hidrolisados de pasta de kraft de cascas de *E. globulus* é viável pela implementação de um modelo de economia circular nas indústrias da pasta e do papel. No entanto, são necessárias otimizações no processo para aumentar os rendimentos obtidos e os lucros desta produção.

Keywords Bioethanol, Circular Economy, fermentation, pulp and paper industry, Kraft pulp, yeast recycling, yeast extract.

Abstract

High quantities of wastes are generated by the pulp and paper industry every day, such as the wood barks which are lignocellulosic materials. The lignocellulosic biomass, mainly composed of cellulose, hemicelluloses, and lignin, is a primordial feedstock for biofuels production due to its renewable properties and high carbon source. Bioethanol is the major biofuel produced worldwide and its production goes through several steps: the pretreatment, the hydrolysis, the fermentation, and lastly a distillation and purification step. These processes have high associated costs which oppress this production at an industrial scale.

The circular economy applied to the pulp and paper industry, through the integration of a bioethanol producing biorefinery, may be a promising approach to decrease the overall bioethanol production costs, due to the feedstock availability and the implemented technologies to process the lignocellulosic biomass.

The main objective of this work was to study the production of bioethanol from the enzymatic hydrolysate of *Eucalyptus globulus* barks kraft pulp, namely this process improvement by reducing the overall costs associated. Therefore, urea and Fermaid O™ were studied as economical alternatives to replace or reduce yeast extract utilization. Also, the spent yeast from the fermentation broth was studied for reutilization as supplementation, through the production of yeast extract by autolysis, and as inoculum (in successive fermentations).

The fermentation using the produced yeast extract presented the highest ethanol performance in the Erlenmeyer assays, testing different supplementations. Comparing the results with produced yeast extract, and commercial yeast extract, there is an improvement of the maximum ethanol concentration from 45.31 ± 1.24 g L^{-1} to 48.26 ± 0.94 g L^{-1}, of the productivity from 1.59 ± 0.04 g L^{-1} h^{-1} to 1.82 ± 0.04 g L^{-1} h^{-1} and of the ethanol yield from 72.72 ± 1.32 % to 76.73 ± 4.53 %. The scale-up to a bioreactor provided the highest ethanol concentration of 61.05 g L^{-1}.

This study indicates that bioethanol production from *E. globulus* barks kraft pulp hydrolysates could be viable by implementing a circular economy model into the pulp and paper industry. However, several optimizations are still required to improve the yields obtained and increase the profits of this production.

Index

Figures Index

Tables Index

Abrevitation Index

BG β-glucosidases

CBH Cellobiohydrolases

CBP Consolidated bioprocessing

CE Circular economy

CSL Corn steep liquor

CW Cheese whey

EBR *E. globulus* bark residues

EG Endoglucanase

FAME Fatty acid methyl esters

FAN Free amino nitrogen

HPLC High-performance liquid chromatography

LCB Lignocellulosic biomass

MTBE Methyl tertiary butyl ether

$Prod_{vol}$ Volumetric ethanol productivity

PSSF Pre-saccharification plus simultaneous saccharification and fermentation

$r_{ethanol}$ Volumetric production rate

$r_{glucose}$ Volumetric consumption rate

RYE Raw yeast extract

SHF Separate hydrolysis and fermentation

SSF Simultaneous saccharification and fermentation

$Y_{ethanol}$ (%) Ethanol yield,

$Y_{ethanol/substrate}$ Ethanol/substrate yield

1. Introduction

The high development of the industry, the new consumption habits, and the growth of the population are arising concerns about the scarcity of natural resources, the emission of pollutants, and the generation of wastes as well as global climate change. Circular Economy (CE) is a fresh new business model created to redress the current linear economic model (based on the extraction, production, consumption and deposition), where a policy of reuse, recovery, recycling, and repairing during the use cycle of a product is followed (Ferreira et al., 2019).

The pulp and paper industry has been growing over the years, increasing the production and the generation of wastes, such as rejects from pulping, bleaching and, washing processes, where most of these wastes are potential air, soil, and water pollutants (Mandeep et al., 2020). However, these residues, considered lignocellulosic biomass, are favorable renewable resources to be applied into the second-generation biorefineries, where bio-based products and biofuels can be generated through sustainable processes (Liguori & Faraco, 2016).

Biofuels are a promising way to fight fossil fuels dependency and bioethanol is the most common renewable fuel used (Gray et al., 2006). Bioethanol production is mainly from the first generation biorefineries, using food crops as feedstock and creating a competition between food and fuels. Nevertheless, lignocellulosic biomass (LCB) is an alternative non-food feedstock available in large quantities to produce this biofuel (Hemansi et al., 2019). The increasing of this production profitability at an industrial scale is a major concern of diverse works with the development of technological advances. LCB recalcitrance requires a highly expensive pretreatment, and its further hydrolysis also carts high costs Thus, they aim to reduce the high costs associated with the several steps required to convert the LCB into fermentable sugars necessary for microbial fermentation (Rastogi & Shrivastava, 2017; Zabed et al., 2017). Regardless, these integrated processes' overall costs can be softened by replacing some high-cost nutritional fermentation media supplements such as yeast extract, with low-cost alternatives (Maddipati et al., 2011; F. B. Pereira et al., 2010). Also, the fermentation yeasts can be reused to decrease the operational time and costs associated with preparation of microbial cultures for inoculations (Basso et al., 2008; Hama et al., 2018).

Chemical pulping processes in the pulp and paper industry can be considered as good LCB pretreatment methods since these processes perform delignification of the raw materials: they release celluloses and some hemicelluloses in the solid fraction and the lignin is removed (Jönsson & Martín, 2016). With the integration of biorefineries into the pulp and paper industries, since the kraft pulping process is already implemented, the overall production costs and eventually the high investment necessary could be overtaken increasing the competitivity of this biofuel production and its feasibility at an industrial scale (Monrroy et al., 2012; Phillips et al., 2013).

Herein, the bioethanol production from *Eucalypus globulus* barks kraft pulp is studied, aiming to reduce the overall production costs by replacing usual fermentation supplementation for a more economical alternatives and by the reutilization of the spent yeast cells.

2. State of the Art

2.1. Bioethanol

The classification of ethanol is usually differentiated into two categories: synthetic ethanol produced through catalytic hydration of ethylene, usually provided from cracking crude oil or natural gas, generating toxic by-products, and bioethanol (bio-based ethanol) which derives from the biological fermentation of sugars provided from organic biomass. Bioethanol represents the majority of the ethanol produced globally, and besides its major appliance as an alternative fuel, bioethanol is also very important in different industries namely chemical, beverages, pharmaceutical, and cosmetics (Kheybari et al., 2019; Sarris & Papanikolaou, 2016).

Ethanol is a promising energy source over gasoline due to its advantageous properties (Lynd, 1996; Zabed et al., 2017). Bioethanol is a less environmentally harmful fuel containing 34.7% oxygen, resulting in higher combustion efficiency while reducing the emission of particulate and nitrogen oxides. Furthermore, the reduced amount of sulfur in bioethanol, which lowers the total sulfur amount in gasoline-ethanol blended fuels, contributes to reducing the emissions of sulfur oxide, which is carcinogenic and improves acid rain formation. This blended fuel will also decrease the use of methyl tertiary butyl ether (MTBE) which is added to gasoline as an octane enhancer resulting in cleaner combustion with reduced carbon monoxide (CO) and carbon dioxide (CO_2) emissions (Pickett et al., 2008; Zabed et al., 2017).

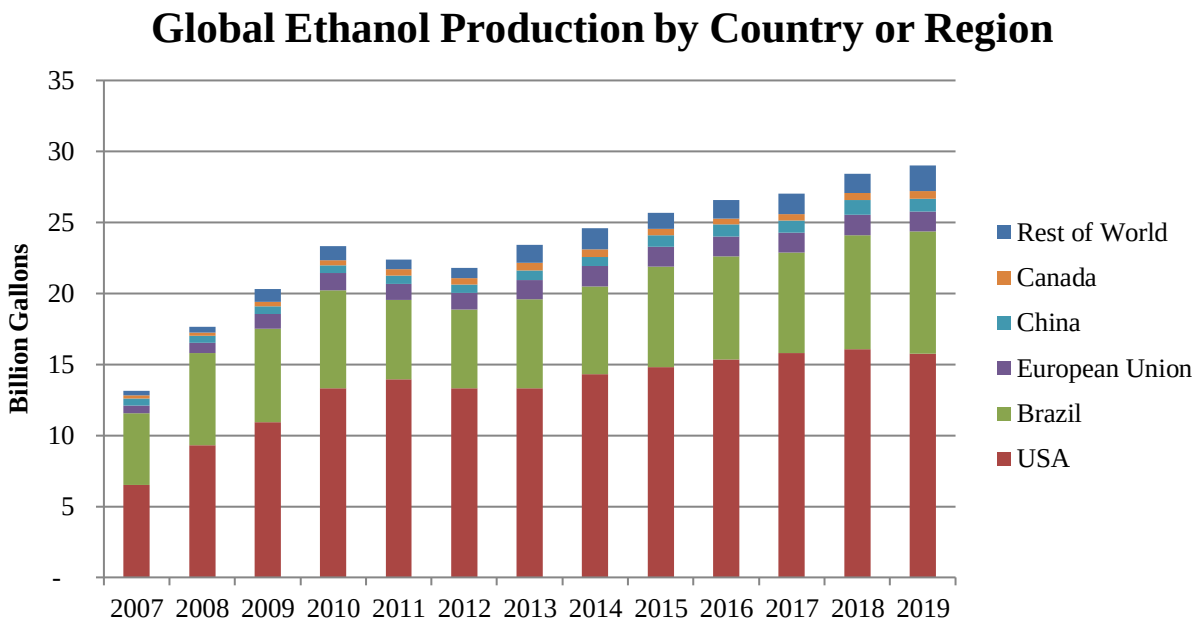

Figure 2.1 – Evolution of ethanol production worldwide. Source: Renewable Fuels Association (RFA, 2020)

Figure 2.1 represents ethanol production by county over the years. Nowadays, bioethanol is mainly produced from food crops being the US and Brazil the producing leaders of this biofuel, mainly using corn and sugarcane respectively as feedstocks (Rastogi & Shrivastava, 2017; RFA, 2020).

2.2 Second Generation Bioethanol

Biofuels, such as bioethanol can be classified as first, second, third, and fourth-generation biofuels, depending on the feedstock used in their production. First-generation biofuels use food source material, either seeds or oils with high amounts of starch and sugars that can be processed to produce fatty acid methyl esters (FAME), like biodiesel and glycerol or biofuels such as bioethanol, or biobutanol. Second-generation biofuels rely on the nonedible part of plants to produce FAME, biofuels, and other bio-based chemicals. Third-generation biofuels use algae and other seaweed to produce FAME, biofuels, and other biochemicals (Dahman et al., 2019; Dutta et al., 2014). Fourth-generation biofuels are a newly proposed type of biofuels that uses genetic and metabolic engineering on algae to increase the lipid content and biomass yield for biofuel production (Dutta et al., 2014).

Second-generation bioethanol based on non-food raw materials is commonly produced from lignocellulosic biomass (LCB) but other industrial byproducts such as

whey and crude glycerol can also be used as feedstock for this biofuel production (Robak & Balcerek, 2018). The used lignocellulosic feedstocks include agricultural residues that are the by-products resulting from agriculture and their related industries and also woody materials such as hardwoods and softwoods, including all their processing residues such as wood chips, barks, slashes, or sawdust. Municipal and industrial wastes are also used as second-generation bioethanol feedstock, promoting waste management (Limayem & Ricke, 2012; Zabed et al., 2016).

This bioethanol production does not compete with food chain supplies, and the biomass used is usually inexpensive and readily and locally available (Robak & Balcerek, 2018). These advantages originated a focus on research and investigation to produce these biofuels to optimize the process and reduce the overall production costs. Hence, novel technologies are being developed to overtake the present operational barriers such as LCB recalcitrance. The current pretreatments required are expensive and form fermentation inhibitors, optimizing them to increase its bioethanol yields while decreasing the costs, aiming to achieve an economically feasible process (Taha et al., 2016; Zabed et al., 2017).

Lignocellulosic bioethanol has two methodologies developed for its production, thermochemical or biochemical conversion.

In the thermochemical conversion, the LCB is converted into syngas through gasification at high temperatures. The CO, CO_2, and H_2 in syngas are later converted into bioethanol through fermentation by anaerobic bacteria or using molybdenum by chemical catalysis (Kennes et al., 2016; Rastogi & Shrivastava, 2017).

The biochemical conversion performed by microorganisms will be the focus of this study.

The biochemical conversion, the most commonly used in industry, begins with feedstock preparation, principally remaining on debarking and size reducing the raw materials. Therefore, this method relies on four major processes for the conversion of the LCB into bioethanol. First, the pretreatment which will degrade the complex lignocellulosic structure into its major components (cellulose, hemicellulose, and lignin); second the hydrolysis to convert the polysaccharides from LCB into fermentable sugars; third the fermentation, performed by microorganisms to convert the monosaccharides into ethanol; and fourth the ethanol recovery and dehydration to isolate and purify the produced ethanol (Hemansi et al., 2019; Zabed et al., 2016).

2.2.1 Lignocellulosic Biomass

Plants dry matter is considered LCB and it is a potential source of feedstock for bioconversion into biofuel and other value-added products due to its abundant availability and renewable nature (Anwar et al., 2014).

LCB's major components are polysaccharides (cellulose and hemicelluloses), and the aromatic biomolecule lignin. The structural composition of LCB in the cell wall is presented in Figure 2.2.

Cellulose, which comprises 40-50% of LCB is the major component. This homopolymer consists of D-glucopyranose monomers linked by β-(1,4)-glycosidic bonds in a linear chain. The present repeating regular unit is a cellobiose residue composed of two molecules of glucose (S. R. Pereira et al., 2013). Its polymer chains associated generate cellulose fibers that are linked together by several intra- and inter-molecular hydrogen bonds. Thus, a bundled arrangement and highly ordered crystalline structure is created, generating high insolubility and high resistance to most organic solvents (H. Chen et al., 2017; Mood et al., 2013).

Hemicelluloses, being the second most abundant component in LCB, comprising 20-30%, combine several heteropolymers such as arabinoxylans, glucomannans, galactomannans, xylans, and xyloglucans. These heteropolymers are composed of diverse units of pentoses such as arabinose, xylose, and hexoses, such as mannose, galactose, fucose and glucose. Due to the low degree of polymerization and the absence of crystalline regions, the degradation of these polymers into their former monosaccharides is easily obtained compared to cellulose. Furthermore, hemicellulose structural composition varies between different kinds of plants due to their genetic variability, presenting, for example, a majority of glucomannans in softwood species and xylans in hardwood species (H. Chen et al., 2017; Hafiz et al., 2013; Rahmati et al., 2020).

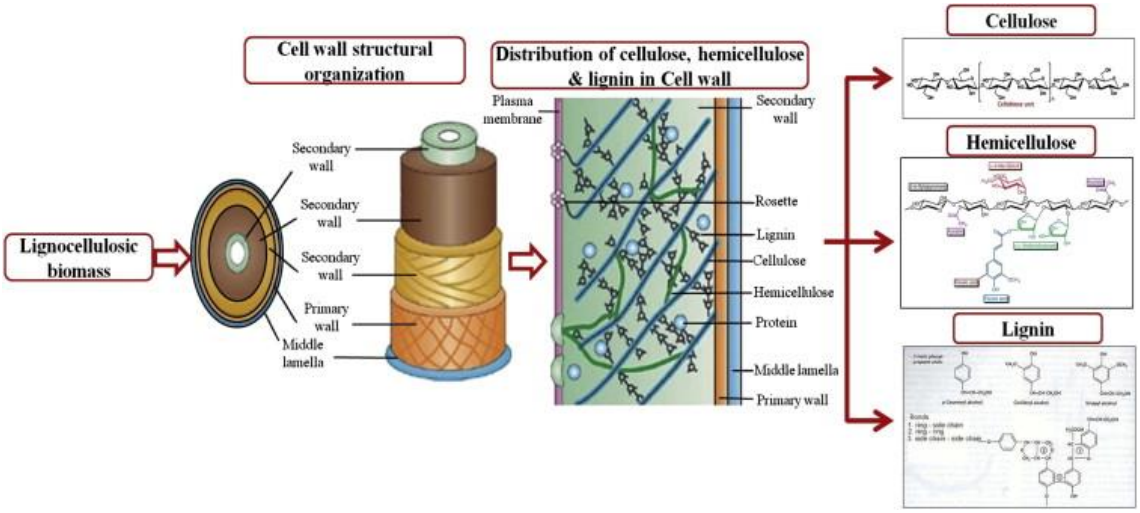

Figure 2.2 - Diagrammatic illustration of the framework of lignocellulose. (Menon & Rao, 2012)

Lignin is a complex three-dimensional aromatic macrobiomolecule consisting of phenylpropane structures linked to each other by irregular coupling of C-C and C-O, that differ according to the raw material source, and the growing conditions of the plant, which comprises 15 to 30% of the LCB dry weight. Syringyl monomer (S) derived from sinapyl alcohol, guaiacyl monomer (G) derived from coniferyl alcohol, and *p*-phenyl monomer (H) derived from coumaryl alcohol are the main three basic structural monomers present in this biomolecule (Alzagameem et al., 2017; H. Chen, 2014; Mood et al., 2013). Lignin acts similar to glue between the cellular network generating compressive strength on plant tissue and the individual fibers, therefore increasing the cell-wall toughness and simultaneously playing a big role concerning biological resistance against insects and pathogens (Rahmati et al., 2020).

In minor proportions, LCB has also proteins such as structural proteins, zymoproteins, and hydrophobic proteins, pigments, pectin, and ashes in its composition (H. Chen, 2014).

As presented above, LCB structure is constituted by groups of cellulose strands formed in semicrystalline microfibrils, which are cross-linked with hemicellulose in a lignin matrix. Fibers and microfibrils are produced via different intra and intermolecular hydrogen bonds between this complex matrix. Those characteristics are responsible for the recalcitrance of the lignocellulosic materials to chemical and biological attacks either by microorganisms or by enzymes (Rahmati et al., 2020).

The three major components proportion in LCB varies depending on the genetic variability among the different species (Table 2.1) (Hafiz et al., 2013; Sánchez, 2009).

Feedstocks such as agricultural residues (straw of cereals grain crops) (García et al., 2016; Hafiz et al., 2013), forest residues (X. Li et al., 2015; Rahmati et al., 2020; Vassilev et al., 2012; Wang et al., 2018; J. Yu et al., 2017), and food processing residues (husks, shells, cobs, or bagasse) (Hafiz et al., 2013; Rahmati et al., 2020; Vassilev et al., 2012) have shown promising cellulose and hemicellulose amounts for bioethanol production (Anwar et al., 2014; García et al., 2016; Menon & Rao, 2012). Pulp and paper industry production lines also produce enormous amounts of residues. Mechanical pulping mils only use about 90% of the tree during the mechanical pulping process leaving tons of bark and wood residues as wastes (Menind et al., 2012). Low-quality Kraft pulp, spent sulfite liquor, and pulp and paper sludge are also some residues produced by this type of industry. Meanwhile, these residues can be recognized as potential LCB resources for bioethanol production due to their high polysaccharides content (Branco et al., 2019).

Table 2.1 – Chemical composition of different LCB feedstock

Lignocellulosic Materials		Composition (%)			References
		Cellulose	Hemicellulose	Lignin	
Agricultural Residues	Corn stover	38	26	19	(Hafiz et al., 2013)
	Rice straw	52.3	32.8	14.9	(García et al., 2016)
	Wheat sraw	44.5	33.2	22.3	(García et al., 2016)
Forest Residues	Eucalyptus wood	41	28	26	(Wang et al., 2018)
	Pine wood	46	24	27	(J. Yu et al., 2017)
	Pine Sawdust	45.9	26.4	27.7	(Vassilev et al., 2012)
	Oak wood	43	22	35	(J. Yu et al., 2017)
	Spruce wood	47	22	29	(J. Yu et al., 2017)
	Olive tree biomass	21	17	21	(Rahmati et al., 2020)
	Wood barks	25.2	30.3	44.5	(Vassilev et al., 2012)
	Bamboo	45	24	20	(X. Li et al., 2015)
	Paper (pulp wastes)	74.3	17.1	8.6	(Vassilev et al., 2012)
Food processing Residues	Sugarcane bagasse	42	25	20	(Hafiz et al., 2013)
	Almond Shells	32	35	29	(Rahmati et al., 2020)
	Corn cobs	48.1	37.2	14.7	(Vassilev et al., 2012)
	Sunflower shells	56.5	28	15.5	(Vassilev et al., 2012)

2.2.2 Pretreatments

Since the raw LCB reveals extremely high recalcitrance to enzymes, an effective pretreatment process is crucial to facilitate the access of the enzymes to the cellulose and hemicelluloses polysaccharides for their hydrolysis into fermentable sugars (Silveira et al., 2015; Sun et al., 2016). Besides the importance of these processes in breaking of the LCB matrix recalcitrance, they require optimization since they correspond to the most expensive step of the global process. Each method results in different yields and products because of the approach in deconstructing the physicochemical properties from the lignocellulosic structures (Seidl & Goulart, 2016). Nevertheless, for selecting the good and most cost-effective pretreatment strategy, it is essential to consider the aspects such as 1) the low formation of inhibitor compounds for the subsequent hydrolysis and fermentation, 2) the decrease of the enzyme loading for efficient hydrolysis, 3) the prevention of sugar losses, and 4) the possibility to recover several compounds which can further be converted into value-added products, while always considering the minimum energy consumption (Silveira et al., 2015).

Pretreatments are usually divided into four main categories: physical, chemical, physicochemical, and biological. Physical methods usually involve mechanical and radiation intervention on the biomass which will help to decrease the particles size while increasing the surface area and affecting the cellulose crystallinity. However, these methods are usually applied at a preliminary preparation of the substrate before pretreatment or in combination with other pretreatment methods (Kumar et al., 2020; Kumari & Singh, 2018). Otherwise, chemical pretreatments will alter the native chemical structure of LCB and are mediated by acids, alkali, ionic liquids, organic solvents, ammonia, and ozone (Kumar et al., 2020). Physicochemical pretreatments usually combine specific parameters from both physical and chemical methods. With this strategy, the benefits of both methods can be taken into advantage (Brodeur et al., 2011; Rahmati et al., 2020). Biological pretreatment methods to degrade lignin and eventually hemicelluloses use different micro-organisms (white, brown, and soft-rot fungi) or lignin-degrading enzymes such as peroxidases and laccase (Kumar et al., 2020; Rahmati et al., 2020). Some of the most used chemical, physicochemical and biological pretreatments are summarized in Table 2.2.

Besides the high cost of the pretreatment process, another big obstacle is the formation of unwanted byproducts. The appearance of these substances will depend on

the pretreatment method, the severity and the feedstock. Nevertheless, these compounds may be phenolic compounds, and other aromatics, aliphatic acids (formic, acetic, and levulinic acids), furan aldehydes (furfural and HMF), inorganic ions, and bioalcohols or other pretreatment by-products with a negative impact on the ethanol yield, acting as inhibitors of microorganisms and/or enzymes activities (Jönsson et al., 2013; Jönsson & Martín, 2016).

Table 2.2 – Overview of pretreatment methods for lignocellulosic feedstocks, adapted from (Jönsson & Martín, 2016)

Pretreatments	Main effect	Used chemicals	By-product formation
Acid-based methods	Hydrolysis of hemicelluloses to monosaccharides	Involves catalysts such as H_2SO_4, SO_2, HCl, H_3PO_4	Aliphatic carboxylic acids, compounds furans, etc
Mild alkaline methods	Removal of lignin and a minor part of hemicelluloses	Involve alkali such as NaOH, $Ca(OH)_2$,NH_3	Acetic acid, hydroxy acids, dicarboxylic acids, phenolic compounds
Oxidative methods	Removal of lignin and part of hemicelluloses	Involve oxidants such as H_2O_2 and O_2 (alkaline conditions), and O_3	Aldonic and aldaric acids, furoic acid, phenolic acids, acetic acid
Chemical pulping processes	Methods that target lignin and to some extent hemicelluloses	Kraft pulping, sulfite pulping, soda pulping, organosolv pulping	Aliphatic acids
Alternative solvents	Dissolution of Specific lignocellulosic components or the whole biomass	Ionic liquids	Dependent on solvent and conditions
Hydrothermal processing	Solubilization of hemicelluloses without complete hydrolysis	No additives	Acetic acid, minor amounts of furan aldehydes

Although this obstacle will increase the costs of the global process, several strategies were developed to reduce or eliminate these inhibitors. Detoxification treatments with chemical additives (as precipitation agent), sulphite addition (as a reducing agent), activated carbon adsorption, liquid-liquid extraction, and lignin-blocking agents (for avoiding non-productive enzyme binding to lignin in subsequent hydrolysis

step) are the most commonly used methods (Kim, 2018). The use of specific microorganisms with the capacity of eliminating these byproducts (biodetoxification) is an environmentally friendly alternative strategy. Another method can be the selection of an alternative feedstock or the genetic modification of the plants to decrease the lignin amount. The selection of microorganisms with high resistance to inhibitors and with high fermentative capacity is a field still under study where these characteristics can be achieved by adaptive evolution or by genetic and metabolomic engineering (Jönsson & Martín, 2016; Kim, 2018).

2.2.3 Hydrolysis

After the pretreatment process, the solid fraction, which contains the sugar polysaccharides, and any remaining lignin is submitted to a hydrolysis process. The hydrolysis or saccharification is very significant for bioethanol production since converting cellulose and hemicellulose polysaccharides into their fermentable monosaccharides is mandatory for obtaining bioethanol (Haldar et al., 2016). There are mainly two ways to perform this process, a chemical (acidic) and a biological (enzymatic) way (Table 2.3) (Singh & Chaundhary, 2016).

2.2.3.1 Acid Hydrolysis

Acidic hydrolysis reaction can be made using either diluted or concentrated acid. Concentrated acid hydrolysis commonly uses acid concentrations between 10-30% (Binod et al., 2011; Haldar et al., 2016). However, these high concentrations of acid are highly corrosive to the equipment and require the use of expensive corrosive-resistant reactors. Acid recovery is also needed to lower the total hydrolysis costs and make the process economically feasible (Wijaya et al., 2014). Another major drawback is the environmental concerns associated with the use of high acid concentrations, which are highly toxic, making the process environmentally harmful (Liao et al., 2006). On the other way, diluted acid hydrolysis uses much lower acid concentrations (2-5%) but it requires higher temperatures (between 170-230 ºC) (Iranmahboob et al., 2002) to hydrolyze cellulose effectively. These temperatures will generate several inhibitory compounds affecting the following fermentation step decreasing the ethanol yields (Sebayang et al., 2016).

2.2.3.2 Enzymatic Hydrolysis

Enzymatic hydrolysis is carried out by highly specific enzymes resulting in converting these complex carbohydrates into monosaccharides. This method is usually chosen by industry because of the mild conditions needed since these enzymes' optimal temperatures are between 45-50 ºC and pH is around 4.8. Within these conditions, the corrosion problem is not occurring (Binod et al., 2011). Also, these conditions generate higher sugar yields (80-95%) with lower energy consumption and a reduced environmental impact (Kamzon et al., 2016).

Cellulose hydrolysis (Figure 2.3) is carried out by a highly specific enzyme family, the cellulases. Three distinct classes of cellulases work synergistically to degrade cellulose into glucose: 1) endoglucanases (EGs) that hydrolyze internal β-1,4-glucosidic linkages randomly in the cellulose chain, 2) cellobiohydrolases (CBHs or exoglucanases) that present two different forms, CHI and CHII, that progress along the cellulose chain cleaving the cellobiose disaccharides from the reducting and non-reducting ends, respectively, and 3) β-glucosidases (BGs) which hydrolyze cellobiose into glucose by cleaving off glucose units from cello-oligosaccharides (Binod et al., 2011; Volynets et al., 2017).

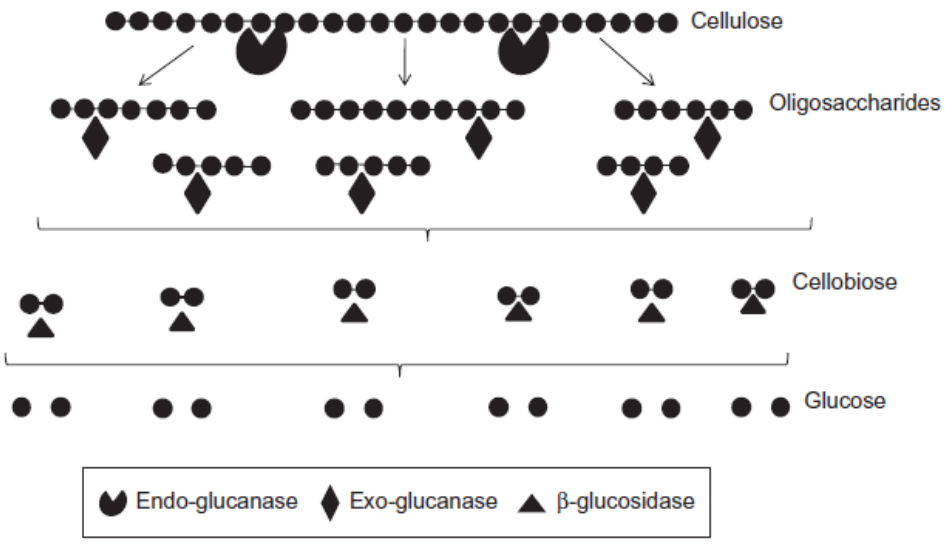

Figure 2.3 - Schematic representation of cellulose enzymatic hydrolysis (Binod et al., 2011)

Hemicelluloses structures are more heterogeneous and complex chains than cellulose, presenting different monosaccharides and linkages. Xylan hydrolysis is carried out by the xylanases enzymatic group: 1) Endo-1, 4-β-xylanases cleave the glycosidic bonds in the xylan backbone, releasing xylooligosaccharides, 2) β-xylosidase acts upon cellobiose and other short oligosaccharides, 3) α-arabinofuranosidase and α-glucuronidase remove from the xylan backbone, the arabinose and the 4-O-methyl glucuronic acid substituent, respectively, 4) esterases act upon ester linkages between xylose and acetic acid or between arabinose side chain residues and phenolic acids (Binod et al., 2011; Volynets et al., 2017). Figure 2.4 presents a diagram of hemicelluloses enzymatic hydrolysis.

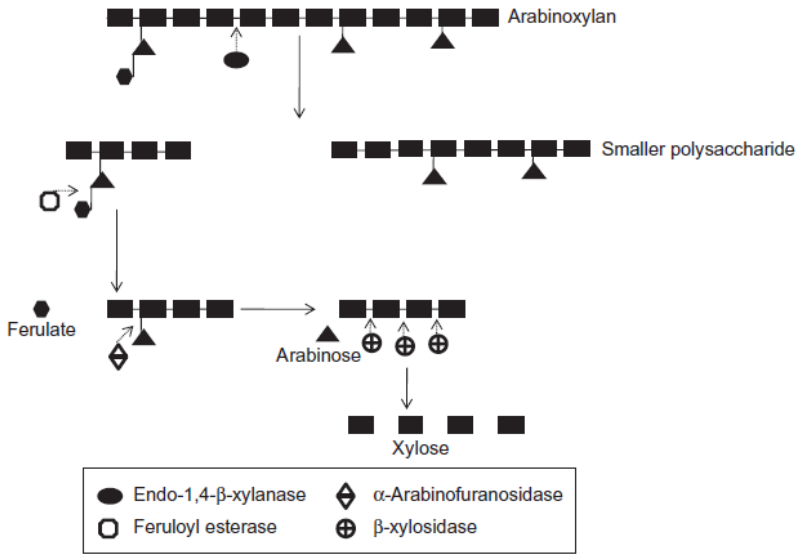

Figure 2.4 – Schematic representation of hemicellulose enzymatic hydrolysis (Binod et al., 2011)

Nevertheless, to successfully hydrolyze LCB, several enzymatic commercial solutions were developed and optimized, being already sold and used in different types of industries such as textile, detergent, and pulp and paper industries (Xiros et al., 2013).

Several factors affect the LCB enzymatic hydrolysis negatively. Lignin works as a structural barrier to enzymes protecting cellulose and allowing high cellulose crystallinity areas, decreasing the enzymes' access to the celluloses surface area. Hereupon, the LCB pretreatment demonstrates crucial importance (Volynets et al., 2017).

In addition, the monosaccharides and short oligosaccharides resulting from enzymatic hydrolysis generate a product inhibitory effect on the enzymes (Kim, 2018).

Table 2.3 – Advantages and disadvantages of hydrolysis, adapted from (Branco et al., 2019)

Hydrolysis	Concentrated Acid	Diluted Acid	Enzymatic
Hydrolysis agent	30–70% H_2SO_4/HCl	2–5% H_2SO_4/HCl	Cellulases and hemicellulases
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 inhibitor Low sugar yield	High cost Slow reactions

2.2.4 Alcoholic Fermentation

Yeast, bacteria, or even fungus can produce ethanol through alcoholic fermentation when feed on simple sugars. This reaction occurs in anaerobic conditions converting the hexoses and pentoses from hydrolysis into ethanol and CO_2 (Kang et al., 2014; Taherzadeh et al., 2014). Equation 2.1 represents the conversion reaction of hexoses:

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

In the fermentation reaction, the theoretical ethanol maximum yield is 0.511 kg per kg of sugar, also generating 0.489 kg of CO_2 during the process (Kang et al., 2014).

2.2.5 Bioethanol production configurations: SHF, SSF, and Consolidated Bioprocessing

Separate hydrolysis and fermentation (SHF) is a conventional two-step process: in the first step, there is the enzymatic hydrolysis of the LCB providing reducing sugars, the fermentable monosaccharides, for feeding the second step, where microorganisms ferment sugars to produce ethanol (Taherzadeh et al., 2014). This methodology's main advantage is the possibility to apply the optimal temperature and pH conditions in each one of both processes, which is important since enzymes and ethanologenic microorganisms optimal working temperatures are about 50 ºC and 28-37 ºC, respectively (Volynets et al., 2017; Xiros et al., 2013). On the other hand, during SHF, cellulases will

suffer product inhibition from glucose and cellobiose accumulation during the saccharification step, decreasing its yield (Xiros et al., 2013). Another possible problem during SHF is contamination since this method involves more manipulation steps and there is always a risk of contamination in a dilute solution of sugar (Taherzadeh et al., 2014).

Simultaneous saccharification and fermentation (SSF) is a one-step combination of enzymatic hydrolysis and fermentation. The ethanologenic microorganisms are present in the same vessel where the saccharification is occurring. The monosaccharides originated by the enzymatic hydrolysis are directly consumed by the ethanologenic microorganisms through fermentation, producing bioethanol. The end product inhibition is relieved by the fermenting microorganisms resulting in a lower enzyme loading requirement. Also, fewer vessels are required in this method and there will be less contaminations in enzymatic hydrolysis due to the presence of ethanol. Since sugars are consumed directly by fermentation microorganisms, they are not available to foreign organisms (Saini et al., 2015; Taherzadeh et al., 2014). The major drawback in SSF is the difference in optimal temperature conditions between the hydrolyzing enzymes and the fermenting microorganisms. Consequently, the use of thermotolerant yeasts like *Kluyveromyces marixianus*, some strains of *S. cerevisiae*, and *Pichia kudriavzevii* in SSF was studied due to their ability to ferment at higher temperatures (Choudhary et al., 2016; N. Hu et al., 2012).

Consolidated bioprocessing (CBP) is another technique where enzyme production, biomass hydrolysis, and fermentation are performed in a single step. This is a promising configuration for reducing capital investment since the enzyme production costs are eliminated. However, CBP requires cooperation between enzyme production and ethanol fermentation species or the development of a genetically modified ethanologenic microorganism with enzyme production capacity (Choudhary et al., 2016). Amoah et al. 2017 developed a genetically engineered yeast with cellulase production capacity with an ethanol production yield of 91.2% with ionic liquid-pretreated bagasse. Further studies are necessary to optimize this species to have consistent results among different biomass (Amoah et al., 2017).

2.2.5.1 Ethanologenic Microorganisms

The fermentation process to obtain bioethanol is performed by ethanologenic microorganisms that should incorporate several characteristics to provide efficient bioethanol production. A large number of microorganisms, like bacteria, yeast, and filamentous fungi can produce bioethanol through ethanolic fermentation, however, not all of them have enough efficiency in ethanol production to be applied in an industrial operation (Zabed et al., 2016). Efficient producers should attain high growth rates with simple requirements allowing the use of inexpensive media, be tolerant to acidic pH or high temperatures to decrease the probability of contaminations. M. Balat 2011 stated that such microorganisms should provide high ethanol yields with values above 90% of the theoretical one and ethanol productivity above 1.0 g L^{-1} h^{-1}. Also, they must be tolerant to ethanol concentrations higher than 40.0 g L^{-1} and have the ability to grow in undiluted hydrolysates showing high resistance to inhibitors (Balat, 2011).

Saccharomyces cerevisiae is the most common and widely used microorganism in ethanol production. This species is robust and well suited to ferment lignocellulosic hydrolysates with high productivity and high ethanol yield (90% to 95% of the theoretical maximum). It has a high tolerance to a wide range of pH, decreasing the possibility of contaminations in the medium, and presents tolerance to ethanol and sugar concentrations decreasing their inhibition effects in fermentation. *S. cerevisiae* can use a wide range of hexoses such as glucose, mannose, and galactose, and also some disaccharides such as sucrose and maltose. However, this yeast lacks a metabolic mechanism to ferment pentoses into ethanol (due to the lack of enzymes that convert xylose to xylulose), like xylose and arabinose which can reach about 25% of the total amount of sugars in the hydrolysate. This is an important aspect to improve lignocellulosic bioethanol production costs and efficiency at an industrial operation (Azhar et al., 2017; Zabed et al., 2016).

Scheffersomyces stipitis (formerly known as *Pichia stipitis*) is included in a group of yeasts with the natural capacity of fermenting xylose while accumulating low amounts of other by-products such as xylitol (Ruchala et al., 2020). This yeast is one of the most efficient organisms for xylose and, in general, lignocellulose fermentation, achieving an ethanol yield of 80% from lignocellulosic sugars (M. Liang et al., 2014; Ruchala et al., 2020). However, *S. stipitis* has some major drawbacks such as low fermentation rates, low ethanol and inhibitors tolerance, and the inability to grow anaerobically. Several studies selected *S. stipitis* strains with increased ethanol and inhibitor tolerance obtaining

higher ethanol productivity. Further studies are required to overtake these drawbacks since this organism has an extremely high potential for industrial fermentation (Ruchala et al., 2020).

A novel strain isolated with high potential for second-generation biofuel production is *Spathaspora passalidarum*. This yeast is a natural xylose-consuming yeast with its growth and ethanol production yield being strongly influenced by the oxygen availability in the fermentation medium. When in aerobic conditions, the biomass formation is favoured, whereas when the oxygen availability is decreased to reduced levels, the ethanol production increases. Under anaerobic conditions, this yeast is also capable of efficiently convert xylose into ethanol (Veras et al., 2017) and co-ferment xylose, glucose, and some oligomeric sugars like cellobiose. (Du et al., 2019; Long et al., 2012; H. Yu et al., 2017). The ethanol yields of this species from xylose are promising, producing up to 0.48 g ethanol g^{-1} xylose, under anaerobic conditions, contrary to other xylose fermentation species that usually need a controlled oxygen rate to successfully metabolize this sugar (Selim et al., 2020). When fermenting other sugars, *S. passalidarum* showed 0.42 g g^{-1} yield in a mixed sugar fermentation of xylose, cellobiose, and glucose. From a lignocellulosic hydrolysate, an adapted strain accumulated up to 39 g L^{-1} bioethanol with a 0.37 g g^{-1} yield (Long et al., 2012). However, this microorganism presents a high sensibility to the chemical inhibitors released throughout the preparation of hemicellulosic hydrolysates. Several strains of *S. passalidarum* are being studied to overcome this issue (Selim et al., 2020). Hou and Yao presented a strong strain, produced through hybridization of an *S. cerevisiae* and a UV-mutagenized *S. passalidarum*, which was able to grow on furfurals and many other inhibitors of wheat straw hydrolysate while producing up to 0.40 g g^{-1} ethanol (Hou & Yao, 2012). Morales et al. developed an adapted strain with high tolerance to acetic acid. This strain was obtained by UV irradiation followed by successive growing assays of the strain under high concentrations of acetic acid. The ethanol yield obtained by this species was 0.48 g g^{-1} and in a non-detoxified hydrolysate of *Eucalyptus globulus*, this strain co-fermented mixed sugars of xylose, glucose, and cellobiose under microaerobic conditions with a yield of 0.39 g g^{-1} ethanol (Morales et al., 2017). In Table 2.4, ethanologenic microorganisms are presented in different studies with the respective fermentation conditions.

Table 2.4 – Ethanologenic microorganisms in different fermentation conditions

Microorganism	Hydrolysate	Substrates	Inhibitors	Fermentation Conditions	Bioethanol ($g\ L^{-1}$)	Bioethanol yield	Ref.
S. cerevisiae	10% Dilute acid pretreated Corn stove mixed with 20% liquified Corn	Cellobiose 3.37 $g\ L^{-1}$ Glucose 9 $g\ L^{-1}$ Xylose 6.82 $g\ L^{-1}$ Glycerol 6.58 $g\ L^{-1}$	Acetic acid 0.95 $g\ L^{-1}$	SHF in Shaking Flask	104.9	80.47%	(S. Chen et al., 2018)
	E. globulus Kraft Pulp	78% Cellulose 19% Hemicelluloses 2.4% Lignin		SHF in Shaking Flask	19.81	0.45 $g\ g^{-1}$	(Branco et al., 2020)
S. stipitis	Syntetic Medium	40 $g\ L^{-1}$ Xylose	-	Bioreactor with oxygen-limited conditions	16.48	0.45 $g\ g^{-1}$	(Veras et al., 2017)
	E. globulus Kraft Pulp	78% Cellulose 19% Hemicelluloses 2.4% Lignin		SHF in Shaking Flask	17.50	0.333 $g\ g^{-1}$	(Branco et al., 2020)
	Hardwood spent sulfite liquor			Bioreactor with two-stage aeration	12.2	0.39 $g\ g^{-1}$	(Henriques et al., 2018)
S. Passalidarum	Syntetic Medium	40 $g\ L^{-1}$ Xylose	-	Bioreactor with oxygen-limited conditions	16.36	0.44 $g\ g^{-1}$	(Veras et al., 2017)
	Corncob	76.74 $g\ L^{-1}$ Glucose 46.23 $g\ L^{-1}$ Xylose	-	SHCF	42.46	72.12%	(H. Yu et al., 2017)
	E. globulus Autohydrolysate	80 $g\ L^{-1}$ Glucose 6.1 $g\ L^{-1}$ Xylose 1.4g L^{-1} Cellobiose	Acetic acid 2.7 $g\ L^{-1}$ Furfural 0.4 $g\ L^{-1}$ HMF 0.17 $g\ L^{-1}$ Formic acid 0.31 $g\ L^{-1}$	SSCF	-	0.39 $g\ g^{-1}$	(Morales et al., 2017)

2.2.5.2 Economic alternatives to fermentation supplementation

In the bioethanol production process, the product concentration, yields, and the overall process economics are directly influenced by the composition of the fermentation medium (Comelli et al., 2016). Yeasts require specific nutrients such as nitrogen, trace elements, or vitamins in adequate amounts to decrease the fermentation time and increase ethanol levels (F. B. Pereira et al., 2010). However, the fermentation media is often supplemented with yeast extract or peptone as nitrogen sources increasing the production cost to an unrealistic level for an industrial process. Therefore, it is crucial to discover cost-effective nutrients to support the nutritional requirements of yeast to obtain optimal growth and fermentation rates (Kadam & Newman, 1997; F. B. Pereira et al., 2010).

A rich and effective nutritional supplement to replace yeast extract and peptone is corn steep liquor (CSL) which is a major by-product of corn starch processing. CSL is a low-cost source of proteins, amino acids, minerals, vitamins, and trace elements that can be used as a fermentation supplement (F. B. Pereira et al., 2010). Maddipati et al. 2011 used 20 g L^{-1} CSL as a low-cost nutrient source in syngas fermentation obtaining over 40% more ethanol production compared to the yeast extract medium (Maddipati et al., 2011). Pereira et al. 2010 optimized the fermentation media using CSL as well with a concentration of 44.3 g L^{-1}. The supplementation medium was complemented with 2.3 g L^{-1} urea, 3.8 g L^{-1} $MgSO_4 \cdot 7H_20$, and 0.03 g L^{-1} $CuSO_4 \cdot 5H_2O$ obtaining an ethanol yield of 93%. Other agro-industrial wastes can be used as nutrient supplementation for fermentation like cheese whey (CW) and raw yeast extract (RYE). Kelbert et al. 2015 obtained 50.04 g L^{-1} of ethanol after 24 h of fermentation in a SSF of *Eucalyptus globulus* wood supplemented with glucose, CSL, CW, RYE, and urea (Kelbert et al., 2015). Likewise CSL, urea is also a low-cost free amino nitrogen source reported with positive effects in fermentation outputs (F. B. Pereira et al., 2010). Appiah-Nkansah et al. 2018 showed increases when supplementing with 16 mM urea obtaining an ethanol production of 20.25% (v v^{-1}) and 96% fermentation efficiency from sweet sorghum juice and sorghum starch (Appiah-Nkansah et al., 2018). Ammonium sulfate is another nitrogen source available for supplementing fermentations. Li et al. 2017, in corn starch fermentation, combined urea and ammonium sulfate (4.08 g L^{-1} and 3.44 g L^{-1}, respectively) to reduce the amount of yeast extract in supplementation media (2 g L^{-1} to 0.6 g L^{-1}) improving the ethanol yield and the fermentation efficiency compared to the essays with only 2 g L^{-1} of yeast extract (Z. Li et al., 2017).

Magnesium supplementation generates a positive effect on yeast ethanol tolerance by protecting cells during fermentation by a mechanism that decreases the permeability of the plasmatic membrane under ethanol stress conditions (C. Hu et al., 2003). Zinc is another essential element to the normal growth, metabolism, and physiology of yeasts playing a big role in controlling the cellular metabolic processes increasing ethanol fermentation under cellular stress conditions (Zhao & Bai, 2012). A mineral-based supplement can also be performed to obtain a successful alcoholic fermentation with low-cost supplementation. Comelli et al. 2016 obtained an ethanol yield of 0.42 g g^{-1} on soft drinks wastewaters using a mineral-based medium (10.6 g L^{-1} $(NH_4)_2HPO_4$, 6.4 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 7.5 mg L^{-1} $ZnSO_4 \cdot 7H_2O$). The ethanol yield obtained from yeast extract was about 8% higher than the mineral-based supplementation, probably because of the carbon sources present in it. However, supplementing the fermentation with a mineral-based medium is six times cheaper than with the yeast extract, demonstrating an economically convenient supplementation alternative.

Several cost-effective supplementation methods for alcoholic fermentation have been shown, however, the optimization of this supplementation media is required for each different feedstock since the hydrolysate composition varies among the species (Comelli et al., 2016).

2.2.5.3 Batch vs Fed-Batch Fermentation

Fermentation processes can be classified into batch, fed-batch, and continuous operation, depending on how the substrate is added to the fermentation vessel. These methods are applicable in industrial fermentation processes of sugar and starch material, being fed-batch and continuous modes of operating dominant in the bioethanol production market (Taherzadeh et al., 2014).

In the batch fermentation method, the process occurs in a closed culture system where the biomass, substrate, and nutrients are added into the fermenter vessel before the initiation of the fermentation. During the fermentation, no media is removed from the vessel, and the products are only harvested at the end of the process (Phukoetphim et al., 2017). Batch technology can be preferred due to ease of operation enabling the use of unskilled labour and the low cost of controlling and monitoring the system, lowering the risk of financial loss. However, this mode has disadvantages when the microorganisms present a slow-growing rate or are strongly affected by substrate inhibition, leading to an

increased lag time or even a total inhibition of the metabolism. Several strategies were used to overtake these problems such as the use of high initial cell density to decrease the quantity of substrate per microorganism, and by increasing the tolerance of the microorganisms to the inhibitors either by adaptation of the cells to the medium (S. R. Pereira et al., 2015), or by genetic engineering (Phukoetphim et al., 2017; Taherzadeh et al., 2014).

Other approaches are used to improve batch fermentation such as the use of several bioreactors in staggered intervals to keep a continuous feed of the products to the distillation system, and cell recycling, separating the yeast from previous fermentation to be used in the following one (Taherzadeh et al., 2014).

In the fed-batch process, all the substrate and the nutrients required are added through the fermentation, continuously or intermittently from the beginning of the process, or halfway point throughout a bach process. Usually, the initial substrate concentration is very low in this method, and the feeding medium is fed when most of the initial substrate is consumed. By this approach, the total substrate in the fermenter can be increased while its concentration is maintained low to reduce the negative effects of osmotic pressure on the microorganisms (Phukoetphim et al., 2017). Thus, the fed-batch method reduces of the substrate and end-product inhibition, consequently having higher ethanol productivity, lower fermentation time, and higher saccharification rate (Chang et al., 2012).

Fed-batch can be monitored through feedback control, otherwise, any deviation occurring is not detected and maximum fermentation rates are not obtained. However, there are several processes without feedback control where the feed is added on a predetermined fixed schedule. The feed rate of the substrate is also very important for maintaining bioconvertible inhibitors such as furfural and HMF at low concentrations in the bioreactor through a low rate feeding (Taherzadeh et al., 2014).

Several authors have evaluated a comparison between batch and fed-batch methods, concluding that the fed-batch process enhances bioethanol productivity. Chang et al. 2012 performed corncob hydrolysate fermentation in batch and fed-batch modes and obtained an increase in ethanol concentration from 23.0 g L^{-1} in batch fermentation to 32.3 g L^{-1} in fed-batch fermentation (Chang et al., 2012). In another study, Chang et al. 2018 increased the initial glucose concentration in the batch and fed-batch processes obtaining 102 g L^{-1} ethanol in batch fermentation before the substrate inhibition started. In fed-batch, the concentration increased to 130 g L^{-1} with an ethanol yield of 0.51 g

ethanol g^{-1} glucose presenting an increasing 12% and 13% of the ethanol concentrations and conversion rate, respectively, when compared to batch process (Chang et al., 2018). Phukoetphim et al. 2017 in sweet sorghum juice fermentations obtained an increase of ethanol productivity from 1.56 g L^{-1} h^{-1} to 2.35 g L^{-1} h^{-1} in batch and fed-batch, respectively (Phukoetphim et al., 2017).

2.2.5.3.1 Yeasts Reutilization from the Fermentation Medium

Bioethanol production from LCB feasibility at an industrial scale depends on an efficient and cost-effective production with strategies that focus on increasing the yields and productivity of the process. Yeast cells from the fermentation broth can be reused as inoculum or as nutrients to improve the production process and to ensure the competitiveness of the lignocellulosic ethanol production (Gray et al., 2006; Hama et al., 2018).

a) Yeasts as inoculum

Yeasts reutilization as inoculum is made by collecting these cells after a batch fermentation, separating the fermentation broth, and reusing the cells in the following fermentation process. This practice is common among the industrial processes where the yeasts are recycled for several consecutive batches, to reduce the time and costs of the inoculum preparation (F. B. Pereira et al., 2012). For instance, in brewing processes yeasts can be reused up to 20 times, depending on the particular brewery. In Brazilian bioethanol biorefineries yeasts are recycled by centrifugation, and in some cases, the recycling extends for the entire sugarcane harvesting season (Basso et al., 2008; Hama et al., 2018).

Cell recycle batch fermentation (CRBF) is a yeast recycling method based on the separation of the microorganisms by centrifugation followed by the direct transference of the resuspended cell pellet into the new fermentation vessel (F. B. Pereira et al., 2012). Meanwhile, to improve cell viability and ethanol production, Hama et al. 2018 added 0.125% CSL in each cycle, performing a six-cycle fermentation in SSF configuration. Nutrient supplementation in each cycle improved the ethanol productivity resulting in ethanol titers of 63.5-67.7 g L^{-1} (Hama et al., 2018).

However, for yeasts reutilization in lignocellulosic bioethanol production by CBP, an extra low-speed centrifugation was required, for removing the remaining

lignocellulosic residues, a solid fraction, since lignin left in these residues could inhibit cellulase activity decreasing the saccharification efficiency. Matano et al. 2013 maintained cell viability in this process, during five cycles, obtaining an ethanol titter of 42.2 g L^{-1} corresponding to 86.3% of the theoretical yield (Matano et al., 2013). Pereira et al. 2012 developed a strategy to improve cell viability based on transferring only a fraction of the biomass to inoculate the following fermentation when the final viability of the cells at the end of a fermentation cycle was inferior to 50%. This technique allowed to perform a fifteen-cycle fermentation with ethanol productivity of 3.5 g L^{-1} h^{-1} (F. B. Pereira et al., 2012).

b) Yeasts as nutrients – Yeast extract production

Yeast biomass autolysates or hydrolysates are a potential source of nutrients for supplementing microorganisms growing medium due to their high level of protein, vitamin B complex, and minerals (Ferreira et al., 2010). Furthermore, the yeast extract, which is commonly used to supplement the fermentation medium, is usually manufactured from spent yeast of the brewing industry due to its inexpensive costs (Jacob, Striegel, et al., 2019).

In commercial yeast extract production, after lysis of the cells, the yeasts go through several processes, including solid-liquid separation, clarification, Maillard reaction, debittering, formulation concentration, and drying. Most of these processes are used to remove the bitter and objectionable taste of the yeast hydrolysate since yeast extract is used as a flavoring agent and nutritional supplement in diverse foods (In et al., 2005).

The first step for obtaining yeast extract is the lysis of the yeast cells. On an industrial scale cell mill, ultrasonic sonotrode, and autolysis methods can be used to disrupt the cell wall presenting differences in the chemical composition of each resulting extract. The free amino nitrogen (FAN), which is the source of nitrogen supplementation for fermentation, can be obtained in higher concentrations by autolysis (45.1 mg g^{-1}) compared to the cell mill (16.5 mg g^{-1}) and the ultrasonic sonotrode (25.8 mg g^{-1}). Thus, autolysis can be considered a good choice in this process to obtain the best yeast extract for fermentation applications (Jacob, Hutzler, et al., 2019). Berlowska et al. 2017 also showed that autolysis can be successfully chemically induced using saponins from *Quillaja saponaria* being a simple and inexpensive process to be used in industrial

productions (Berlowska et al., 2017). However, the most cost-effective method should be chosen since a yeast extract (RYE, for example) can be completed by other nitrogen sources to complement the nitrogen supplementation (Kelbert et al., 2015).

Therefore, in bioethanol production, post-fermentation yeast biomass can also be used to produce yeast extract. This process will reuse the spent yeasts used in the whole bioethanol production while lowering the costs associated with nutritional supplementation (Berlowska et al., 2017).

2.3 Bioethanol production from kraft pulp in a Circular Economy approach

The twenty-first-century society follows consumerism ideals with a take-make-consume-disposal flow of material generating high environmental problems such as climate change, depletion of natural resources, earth's biodiversity decrease, and massive environmental pollution. These challenges are motivating society to act and incorporate a circular economy (CE) concept which is regenerative by design (Sheldon, 2020). CE defends the preservation of natural resources and the attempt of reducing waste formation by their recycling and reuse. This concept is implicit in the bio-based economy that strives to replace the use of fossil resources as the raw materials for the production of fuels, commodity chemicals, and materials, such as plastics, for using renewable biomass (Ferreira et al., 2019; Sheldon, 2020).

The pulp and paper industry is very dependent on natural resources, consuming large quantities of water, energy, and wood fibers, concerning the integrity of forest ecosystems (Toppinen et al., 2017). The wood preparation for pulp manufacturing begins with debarking and chipping of logs obtaining the raw material for the pulping production, the wood chips. Meanwhile, the resulting barks, branches, fine chips, and other wood rejects are usually burned for energy production (Bajpai, 2015). However, these rejects are a strong source of lignocellulosic biomass making them a potential feedstock for second-generation biorefineries since wood barks have about 25% and 30% of cellulose and hemicelluloses, respectively. Furthermore other rejects like sawdust have about 45% and 26% of cellulose and hemicelluloses, respectively (Branco et al., 2019; Vassilev et al., 2012).

Meantime, there are diverse pulping methods for wood delignification that can be applied in the pulping processes and are classified in chemical, semi-chemical, and mechanical. Kraft pulping, one of the chemical processes, dominating the industry, was

reported as representing 91% of chemical pulping and 75% of all pulp produced (Bajpai, 2015). This method consists of a reaction with an alkaline solution of caustic soda (NaOH) and sodium sulfide (Na_2S) at high temperatures and pH where the wood materials are submerged. The hydroxide and hydrosulfide ions react with the lignocellulosic fibers causing a separation of the structural linkages between lignin and carbohydrates and a disruption in the lignin structure. Thus, the majority of lignin and some parts of the hemicelluloses are degraded and solubilized in the alkaline solution (Monrroy et al., 2012).

Chemical pulping methods, like kraft pulping, are primarily used for paper production and they perform the removal of lignin from LCB, the delignification. Since pulping processes expose cellulose fibers of LCB they can be considered as LCB pretreatment methods (Jönsson & Martín, 2016). The Kraft pulping feasibility as LCB pretreatment has already been shown since it generated hydrolysates with optimum sugar profiles for fermentation through enzymatic hydrolysis. Still, this method presents several advantages like the inexistence of inhibitors like furfural and HMF and in a boiler recovery, chemicals and energy can be produced from the resultant wastes (Buzała et al., 2015; Huang et al., 2015; Przybysz Buzała, Kalinowska, Przybysz, et al., 2017).

The kraft pulp hydrolysates fermentation also has been studied by several authors to develop an optimized process for bioethanol production. Aierkentai et al. 2017 produced bioethanol from alkali-pretreated softwood bleached kraft pulp by applying successive enzymatic saccharification and fermentation, with Cellic CTec 2 and the recombinant pYBGA 1 yeast. This recombinant *S. cerevisiae* IFO4308 yeast expresses β-glucosidase in the extracellular fluid and besides the cell wall (X. Liang et al., 2013)) obtaining a bioethanol yield of 93.3% (Aierkentai et al., 2017). Buzala et al. 2017 obtained an ethanol yield of 0.46 g g^{-1} (d.w.) from pine (softwood) bleached kraft pulp. Using *S. cerevisiae* in a modified reactor with the attachment of a distillation column, they obtained an integrated wood-to-ethanol conversion (Przybysz Buzała, Kalinowska, Małachowska, et al., 2017).

Huang et al. 2017 used kraft pulp of bamboo residues to produce bioethanol. This biomass was enzymatically hydrolyzed with the commercial Novozymes cellulases and hemicellulases CTec2 and HTec2 with an efficiency of 91.5 and 93.2% for glucan and xylan, respectively. Sequential fermentation for cellulose and hemicellulose consumption was applied using *S. cerevisiae* and *S. stipitis* obtaining an ethanol production efficiency of 84.4 and 76.6% from glucose and xylose, respectively (Huang et al., 2017). Bauer and

Gibbons performed Kraft pulp SSF testing different enzyme loadings of Celluclast 1.5 L supplemented with Novozym 188 and the performance of *S. cerevisiae* and *Candida molischiana* for ethanol fermentation. The obtained ethanol concentrations were 14.24 – 17.90 and 12.51 – 17.54 g L^{-1} for *S. cerevisiae* and *C. molischiana*, respectively, and ethanol yields of 68.33-85.90 % and 60.00-84.17 % of the theoretical, where the increasings in the bioethanol production were resultant from the increasing of the enzyme loading (Bauer & Gibbons, 2012).

Eucalyptus, a hardwood, is the main species cultivated in fast-growing wood plantations comprehending 26% of the total plantations for industrial purposes (Dillen et al., 2016). Therefore, in Portugal, *Eucalyptus* genus dominates the pulp and paper industry and is used to produce about 92% of the pulps where *E. globulus* is the most used species since its composition provides good kraft cooking, fiber morphology, and handsheet properties (CELPA, 2019; Neiva et al., 2015).

Eucalyptus kraft pulp conversion to bioethanol has also been a focus of research. Monrroy et al. 2012 evaluated the kraft pulping process as pretreatment, reaching a delignification of over 78%. Though SSF at 10% substrate consistency they obtained a maximum ethanol yield of 78% wood basis with a pretreated pulp at 155 ºC, 15% alkali active, and 60 min reaction (Monrroy et al., 2012). Branco et al. 2020 also performed the enzymatic hydrolysis and fermentation of bleached *E. globulus*. 96.1% and 94.0% of glucose and xylose yields, respectively, were obtained where *S. cerevisiae* and *S. stipitis* were used in the fermentation process. The best bioethanol yield presented was 0.433 g g^{-1} with ethanol productivity of 0.733 g L^{-1} h^{-1} using *S.cerevisiae* alone in the fermentation vessel (Branco et al., 2020).

Ko et al. 2012 used an SSF process conducted by *S. cerevisiae* D5A to produce bioethanol from Eucalyptus pulps obtaining, after 170h of fermentation, 65.21 g of ethanol (per kg oven-dried wood) (Ko et al., 2012). Guigou et al. 2019 used eucalyptus sawdust to produce ethanol. The sawdust suffered a combination of pretreatments to improve the hydrolysis process. The best hydrolysis parameters were obtained by autohydrolysis followed by kraft pulping, converting 71% of cellulose, with cellulose hydrolysis of 95%. In this process, lignin and xylose released during pretreatment could be recovered with a rate of 99% and 85%, respectively. Ethanol yields were 215 L of ethanol per tonne of sawdust, with an ethanol conversion rate of 81% (Guigou et al., 2019). In another work, Guigou et al. 2017 also produced bioethanol from the hemicellulose released and extracted with the green liquor before the Eucalyptus kraft

pulping. This waste fermentation presented a yield of 0.19 g g^{-1} and sugar consumption of 89% (Guigou et al., 2017). Gomes et al. 2021 produced bioethanol using *E. globulus* bark residues (EBR) as feedstock. EBR was pretreated using an autohydrolysis process and fermented with a pre-saccharification plus simultaneous saccharification and fermentation (PSSF) configuration. The maximum ethanol concentration and the higher ethanol yield obtained were respectively 38.7 g L^{-1} and 78% of the theoretical yield, performing a 48h pre-saccharification cycle and using nutrient supplementation of the fermentation medium (Gomes et al., 2021). Bioethanol production from different pulp and paper industry wastes is presented in Table 2.5.

The high capital investment and high technical risk are major drawbacks and reduce the interest of financial investing in LCB industrial production. The creation of a kraft pulp mill for bioethanol production is not economically viable and competitive. However, kraft mills from pulp and paper industries already have the required technologies and equipment for wood preparation and kraft pulping with the capacity and infrastructure of handling biomass on the scale of 1000 ton per day. The integration of a biorefinery into pulp and paper industries is a solution to reduce the initial project costs for the implementation of an LCB biorefinery (Monrroy et al., 2012; Zhu & Pan, 2010). Other aspects strategies mentioned above such as the use of low-cost sources to supplement the fermentation medium, the yeast reutilization for inoculum and as a nutrient, and the reutilization of hemicellulose recovered in green liquor before pulping combined with the use of pulp and paper industry wastes, as feedstock, will decrease the overall costs associated with bioethanol production while increasing its rentability generating a potentially profitable biorefinery.

Table 2.5 – Bioethanol production using pulp and paper industry wastes

Microorganism	Feedstock	Pretreatment	Hydrolysis	Fermentation Configuration	Bioethanol	Bioethanol Yield	Reference
***S. cerevisiae* pYBGA1**	Softwood bleached kraft pulp	Alkali-treatment	Cellic CTec2 cellulases	SUSF	6.1 g L^{-1}	93.3%	(Aierkentai et al., 2017)
S. cerevisiae	Pine softwood	Bleached kraft pulping	Enzymatic mix	Modified bioreactor with an attached distillation system	246.45 g L^{-1}	0.46 g g^{-1}	(Przybysz Buzała, Kalinowska, Małachowska, et al., 2017)
S. cerevisiae ***S. stipitis***	Moso bamboo residues	Kraft pulping	CTec2 cellulases HTec2 hemicellulases	Sequential glucose and xylose fermentation in shaking flasks	43.5 g L^{-1} from glucose 11.1 g L^{-1} from xylose	84.4% from glucose 76.6% from xylose	(Huang et al., 2017)
***S. cerevisiae* IR2T9**	*E. globulus*	Kraft pulping	Celluclast 1.5 L cellulases Novozym 188 β-glucosidase	SSF	202 g kg^{-1} wood	78%	(Monrroy et al., 2012)
***S. cerevisiae* PYCC 5246**	*E. globulus*	Kraft pulping	Cellulase and hemicellulases cocktail	Bioreactor	19.24 g L^{-1}	0.433g g^{-1}	(Branco et al., 2020)
***S. cerevisiae* PE2**	*E. grandis* sawdust	Autohydrolysis and Kraft pulping	Celic CTec2 cellulase	PSSF	57 g L^{-1}	215 L ton^{-1} of sawdust	(Guigou et al., 2019)
***S. cerevisiae* Ethanol-Red®**	*E. globulus* bark residues	Autohydrolysis	Cellic CTec2 cellulase	PSSF	38.7 g L^{-1}	73.14%	(Gomes et al., 2021)

3 Materials and Methods

3.1 Raw Material

In all the performed assays, the carbon source was a hydrolysate obtained from an enzymatic hydrolysis of *E. globulus* barks kraft pulp. The *E. globulus* barks kraft pulp was kindly provided by RAIZ – Instituto de Investigação da Floresta e Papel under the Inpactus project. This kraft pulp was submitted to an enzymatic hydrolysis by Mariana Amândio in the Laboratory of Biotechnology Engineering, CICECO – Aveiro Institute of Materials with an enzymatic cocktail also provided by this institute.

3.2 Media and stock solutions

The pH of all media and solutions used were adjusted to 5.5 before sterilization. In order to avoid contaminations, these media and solutions were sterilized by autoclaving (AJC Uniclave 88) at 121 ºC for 20 minutes.

3.2.1 YM media (yeast media)

The liquid YM media was constituted by glucose 10.0 g L^{-1}, peptone 5.0 g L^{-1}, yeast extract 3.0 g L^{-1}, and malt extract 3.0 g L^{-1}. The composition of the solid YM media used for strain maintenance was the same as the liquid, with the addition of agar 20 g L^{-1}.

3.3 Microorganism and inoculums

3.3.1 Microorganism

The microorganism studied in this work was Ethanol Red®, a *Saccharomyces cerevisiae* modified strain. This yeast was generously provided by Leaf by Lesaffre Advanced Fermentations (Marcq-en-Baroeul, France).

This yeast culture was maintained in Petri dishes with solid YM media, at 4 ºC, and were monthly replicated to keep the viability of the microorganisms.

3.3.2 Pre-Inoculum

In the Erlenmeyer assays, the pre-inoculum was performed by transferring a single colony of the Ethanol Red® from the maintenance cultures in YM plates into 10 mL liquid YM media, in 50 mL Falcon tubes. The yeasts were incubated in an orbital shaker at 180 rpm, in a controlled temperature room at 28 ºC for a period of 24h. All the pre-inoculums were performed in duplicate.

3.3.3 Inoculum

In the Erlenmeyer assays, the inoculum was performed in 100 mL Erlenmeyers with a volume of 40 mL of YM medium. The volume of pre-inoculum transferred to the Erlenmeyers was calculated to assure an initial optical density at 620 nm of approximately 0.400. Posteriorly, the inoculums were incubated as described before, for a period of 14h. All the inoculums were carried out in duplicate.

3.4 Erlenmeyer Fermentations

3.4.1 Assays with different supplementation

Several batch fermentations were performed in 250 mL Erlenmeyers with a working volume of 100 mL, containing 85% (v v^{-1}) of hydrolysate, 10% (v v^{-1}) of inoculum, and 5% (v v^{-1}) of supplementation. This supplementation was made as described (Amândio et al., 2021; Branco et al., 2020) containing 2.5 g L^{-1} yeast extract as nitrogen source, and a salts solution (Salts) containing 2.0 g·L^{-1} $(NH_4)_2HPO_4$, 1.0 g·L^{-1} $(NH_4)_2SO_4$, and 0.5 g·L^{-1} $MgSO_4 \cdot 7H_2O$ and used as a control assay. Other different supplementations were tested changing the nitrogen source and/or salts solution according to Table 3.1. One last assay was made according to control conditions but changing the commercial yeast extract by produced yeast extract. All the fermentation assays were carried out in duplicates and sampling was made during the time of fermentations.

Table 3.1 - Alternative economic supplementation configurations used in fermentation assays (+ represents the use of the total concentration indicated, - represents the absence of this component in the supplementation solution).

Assays	*Yeast Extract (2.5 g L^{-1})*	*Urea (3.0 g L^{-1})*	*Salts*	*Fermaid O™ (2.5 g L^{-1})*
Control	+	-	+	-
Urea	-	+	-	-
Urea + salts	-	+	+	-
Reduction I	½ Concentration	½ Concentration	+	-
Reduction II	¼ Concentration	¾ Concentration	+	-
Fermaid O™	-	-	+	+

3.4.2 Assays with inoculum reutilization

The spent yeast from the Erlenmeyer fermentation assays was reutilized as an inoculum using as medium control fermentation conditions. During this approach, the yeast was reutilized throughout five consecutive fermentation assays.

After 24h of the first assay, it was finished, and the fermentation broth was centrifugated for 10 min, at 4 ºC and 4000 g in 50 mL Falcon flasks. After that, the supernatant was discarded, and the pellet containing the biomass was resuspended in YM medium. A fraction of this biomass suspension was used to re-inoculate the following assay under the same inoculum concentrations. The following assays were performed reusing the inoculum in the same conditions. All the process was performed under sterilized conditions.

3.5 Biorreactor Fermentation

A bioreactor assay was performed in a bioreactor Biostat® Aplus with 5 L capacity (Sartorius Stedim Biotech®) (Figure 3.1) with automatic control of temperature (28 ºC), agitation (180 rpm) and pH of 5.5, using a working volume of 2 L. Fermentation media composition was the described for previous control assay. The pre inoculum was performed, transferring about 2 colonies of yeast to 100 mL Erlenmeyer's with 50 mL YM medium and incubating for 24 h. The inoculum was prepared by adding a certain pre inoculum volume, into 500 mL Erlenmeyer with 200 mL YM medium, allowing to obtain an optical density at 620 nm of around 0.400. It was incubated for 14 h and after that, a

certain volume was inoculated in the bioreactor, guaranteeing an initial optical density around 0.500.

After inoculation, sampling was made along with the assay that lasted for 50 h.

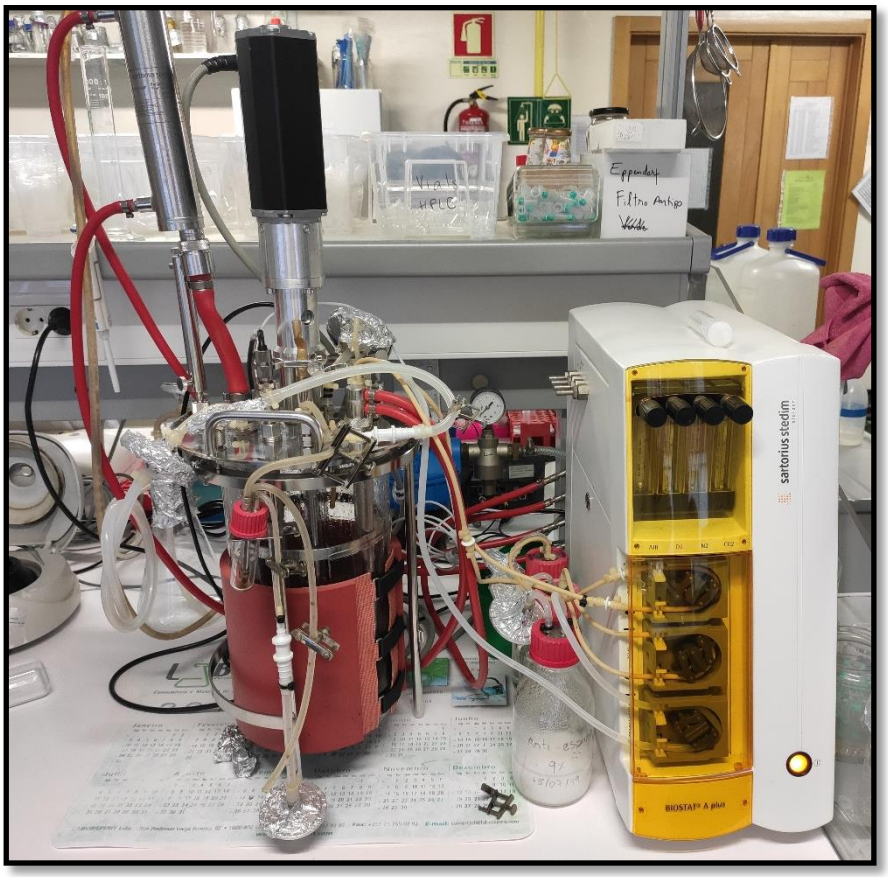

Figure 3.1 – Bioreactor fermentation

3.6 Yeast Extract Production

The spent yeast resulting from the bioreactor fermentation was carefully separated from the fermentation broth in the end of fermentation (50 h). This separation was performed by centrifugation in 400 mL centrifuge bottles for 10 min at 4ºC and 4000 g. After discarding the supernatant, the biomass was resuspended in distilled water at a concentration of 300 g L^{-1}. This biomass suspension was separated to be treated in three different processes to obtain a yeast lysate as a yeast extract substitute.

The yeast extract production was performed through 3 different methods: 1) an incubation with temperature solely, 2) an incubation assisted with the addition of ethyl acetate and NaCl, and 3) a simple autoclave protocol (Zarei et al., 2016).

1) The biomass suspension was diluted with distilled water for a concentration of 150 g L^{-1} in a Erlenmeyer, with a working volume of approximately 100 mL. Then the Erlenmeyer was incubated in an incubation chamber for 24h at 50 ºC, and 100 rpm. After the 24h incubation, the process was terminated with an 80 ºC water bath for 30 min (Tanguler & Erten, 2008).

2) This method followed the same procedure but with the addition of ethyl acetate and NaCl with the concentrations of 0.0051 mol L^{-1} and 0.0086 mol L^{-1}, respectively, while resuspending the spent yeast after initial centrifugation (Jacob, Hutzler, et al., 2019).

3) This method began with the separation of the spent yeast by centrifugation for 10 min at 4000 g and 4 ºC. Then the biomass was resuspended in distilled water in a concentration of 250 g L^{-1}. The yeast suspension was submitted to an autoclave cycle of 10 min at 115 ºC, followed by a fast cooling in an ice bath. After the cooling, the cell debris was separated by centrifugation at 4000 g and 4 ºC for 10 min. The supernatant was held and recentrifuged in the same conditions. Both pellets with the cell debris were discarded while the supernatant was kept. This solution was again submitted to an autoclave cycle in the described conditions, also followed by a fast cooling in an ice bath (Zarei et al., 2016).

In all the processes described above, the remaining solutions were lyophilized and a free powder was recovered and weighted.

3.7 Analytical Methods

3.7.1 Biomass

The biomass quantification was monitored spectrophotometrically (Shimadzu UVmini-1240) by measuring the optical density at 620 nm. The optical density was converted in biomass concentration through a calibration curve (Appendix A - Figure A1) of optical density *vs* biomass dry weight.

The samples analyzed were diluted with NaCl 0.9% (m V^{-1}) to obtain an optical density value inside the validity range of the Beer-lambert law.

3.7.2 pH

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

3.7.3 Glucose, Xylose, and Ethanol quantification

Glucose, xylose, and ethanol were quantified through high-performance liquid chromatography (HPLC). The samples from the fermentation assays were firstly diluted in order to obtain their sugar concentrations between the 1 and 5 g L^{-1}. Then the samples were centrifuged (VWR®) in modified nylon 0.20 µm centrifuge filters (VWR®) for 10 min at 8000 rpm. After filtration, 10 µL of the samples were injected in a HPLC Hitachi® LaChrom Elite composed by a column Rezex™ ROA-Organic Acid H+ (8%) H-0138-K0 (Phenomenex®), with a Gecko 2000 oven operating at 65 ºC, and a refraction index detector (L-2490 Hitachi® LaChrom Elite), using an 0.005 N H_2SO_4 solution as eluent with a flow rate of 0.5 mL min^{-1}.

The concentration of all the compounds was calculated using a calibration curve (Appendix B - Figure B1) of the compound peak area *versus* the compound concentration of their respective standard. The standard solutions contained all the compounds analyzed in concentrations between 0 and 5 g L^{-1}.

3.7.4 Protein quantification

The protein amount at the produced yeast extract was quantified by the Bradford assay (Kruger, 2009). A Bradford stock solution was made (since it had a longer shelf life) by dissolving 350 mg of Coomassie Brilliant Blue in 100 mL of ethanol 95% and then diluting with 200 mL of phosphoric acid 88%. A working solution of 500 mL to perform the assay was made by diluting 30 mL of the stock solution with 15 mL of ethanol 95%, 30 mL of phosphoric acid 88% and 425 mL of distilled water. The working solution was then filtered with Whatman No1 paper filters (Kielkopf et al., 2020) and kept in the dark.

The calibration curve (Appendix C - Figure C1) was made with bovine serum albumin (BSA). A gradual dilution with distilled water was made between 0.1 mg mL^{-1}

and 1.0 mg mL^{-1}, where our white was distilled water. To each vessel, 100 µL of each dilution was added with 5 mL of the Bradford working solution and incubated for 5 min. The optical density (595 nm) was read in a spectrophotometer (Shimadzu UVmini-1240). The yeast extract samples were diluted with distilled water in 20 mg mL^{-1} solutions and 100 µL of them were incubated for 5 min in 5.0 mL Bradford working solution. The optical density was also read between 5 and 60 min of incubation. The commercial yeast extract was also analyzed in the same conditions and used as control (Kruger, 2009).

3.7.5 Nitrogen and Carbon quantification

The nitrogen and carbon quantification of the produced yeast extract was quantified through Elementary Analysis performed in the (Truspec 630-200-200) by Dra Maria Manuela Marques in the Chemistry Department of the University of Aveiro. Under 10 mg of each sample were measured and processed under a combustion furnace temperature of 1075 ºC with an afterburner temperature 850 ºC. The nitrogen and the carbon were detected by thermal conductivity and infrared absorption, respectively.

3.8 Calculation methods

3.8.1 Specific growth rate

During the exponential phase, the linear regression from plotting the natural logarithm of biomass concentration *versus* the interval of time where that growth occurred was obtained. The specific growth rate, μ (h^{-1}) was then calculated through the determination of the slope of this linear regression.

3.8.2 Volumetric consumption and production rates

The volumetric rates calculation indicates the variation of the concentration along the time following equation as represented in the Equation 3.1:

$$dC = r \cdot dt \qquad \textbf{Equation 3.1}$$

The r in the equation is representing the volumetric rate (g L^{-1} h^{-1}) and the *C* and *t* are representing the concentration (g L^{-1}) and time (h), respectively.

From the plotting of the concentration variation, the time with a linear behavior was selected and a linear regression was performed. The volumetric consumption rate, $r_{glucose}$ was calculated from the module of the slope of this linear regression of the glucose *versus* time plot linear zone. The volumetric production rate, $r_{ethanol}$ was calculated by determining the slope of the linear regression of the ethanol *versus* time plot linear zone.

3.8.3 Volumetric ethanol productivity

The volumetric ethanol productivity, $Prod_{vol}$ was calculated following the Equation 3.2, considering the differences from the end of the fermentation's lag phase until the achievement of maximum ethanol concentration.

$$Prod_{vol}\ (g\ L^{-1}h^{-1}) = \frac{\Delta[Ethanol]}{\Delta t} \qquad \textbf{Equation 3.2}$$

3.8.4 Ethanol yield

The ethanol yield, $Y_{ethanol}$ (%) was calculated considering the maximum theoretical ethanol yield of 0.511 g g^{-1} (Kang et al., 2014) presented above (section 2.2.4, Equation 2.1).

Firstly, the ethanol/substrate yield, $Y_{ethanol/substrate}$ was calculated following the Equation 3.3, from the beginning of the assay until the maximum ethanol was achieved. Both glucose and xylose were considered as substrates.

$$Y_{ethanol/substrate} = \frac{\Delta[ethanol]}{\Delta[substrate]} \qquad \textbf{Equation 3.3}$$

The $Y_{ethanol}$ was then calculated in relation to the maximum theoretical value, following the Equation 3.4.

$$Y_{ethanol} = \frac{Y_{ethanol/substrate}}{Maximum\ theoretical\ value\ (0.511)} \times 100 \qquad \textbf{Equation 3.4}$$

The final values used in the calculations were performed using the average values of the duplicates for all the Erlenmeyer fermentation assays.

4 Results and discussion

A circular economy strategy was applied to the pulp and paper industry to valorize a side stream from these industries namely the *Eucalyptus* barks. The hydrolysate derived from enzymatic hydrolysis of bark kraft pulp of *E. globulus* was used as raw material for the whole study.

Ethanol Red is a commercial *S. cerevisiae* strain industrially optimized for biofuel production with high performance in bioethanol production and high resistance to inhibitors (Amândio et al., 2021; Demeke et al., 2013; Kossatz et al., 2017). This yeast was utilized in all the experimental assays since Amândio and co-workers previously selected it (Amândio et al., 2021).

4.1 Effect of different supplementations on bioethanol production

Lignocellulosic bioethanol production using bark kraft pulp hydrolysates as a carbon source was already published (Amândio et al., 2021). However, process optimization is always one of the main targets in the industry. Previous work from Amândio et al. 2021 utilized a supplementation composed of yeast extract and mineral salts to obtain a successful fermentation medium (Amândio et al., 2021). Although ensuring the high performance of yeasts in fermentation, the supplements added to the fermentation media increase the overall price of the process. Yeast extract, the most used supplement in yeast fermentation, is the primary source of nitrogen and other need micronutrients. Although, it has an impact on the final costs of fermentation processes.

The objective of this study was to find a more economical supplementation solution for hydrolysate fermentation. Several Erlenmeyer fermentation assays were performed to study the effect of different supplementations on the fermentation media, namely on bioethanol production.

4.1.1 Control Assay with commercial yeast extract

A fermentation assay using the described supplementation of 2.5 g L^{-1} of yeast extract (presented in Table 3.1 at section 3.4.1) was designated as a control assay, and it was performed to compare with the following experiments. Besides yeast extract, also

salts supplementation was tested, according to Amândio et al. 2021. (Amândio et al., 2021; Branco et al., 2020).

In Figure 4.1, biomass growth, glucose consumption, and ethanol production are presented. Xylose was not consumed as expected since this yeast is not able to consume xylose, a C5 sugar. A lag phase of 4.5 h is evidenced. A significant glucose consumption started after the lag phase corresponding to an exponential growth phase, and the specific growth rate of 0.155 ± 0.012 h^{-1} was achieved. The glucose depletion was occuring before 24h with a $r_{glucose}$ of 11.9 ± 0.59 g L^{-1} h^{-1}, providing a maximum ethanol concentration of 45.31 ± 1.24 g L^{-1} at 28.5 h, a productivity of 2.01 ± 0.06 g L^{-1} h^{-1} and an ethanol yield of 72.72 ± 1.32 % (Table 4.1). The pH slightly decreased during this period due to the CO_2 produced during the alcoholic fermentation (Coote & Kirsop, 1976). These results are lower than the Amândio et al 2021 promising results in similar conditions (initial glucose concentration of 120.0 g L^{-1} instead of 111.0 g L^{-1}) with a maximum ethanol concentration of 50.8 g L^{-1} at 20.5 h, productivity of 2.48 g L^{-1} h^{-1} and an ethanol yield of 81.0 % (Amândio et al., 2021).

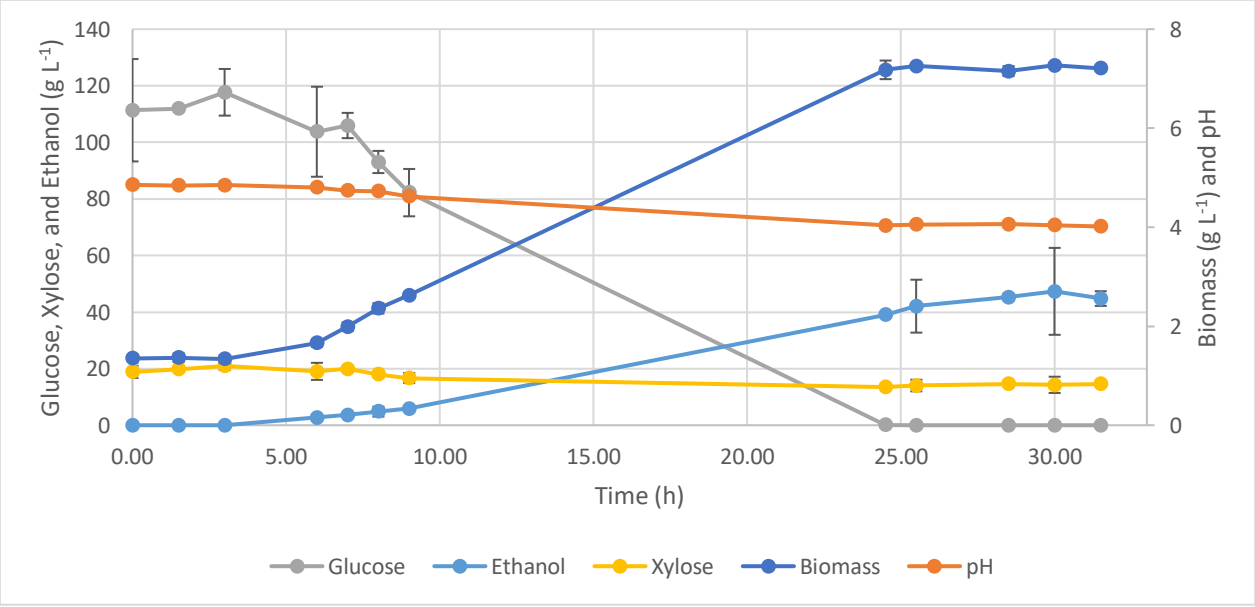

Figure 4.1 – Glucose, xylose, ethanol, biomass, and pH profiles during control fermentation assay.

4.1.2 Study of urea effect as supplementation

Urea was already studied as an organic economical nitrogen source to replace the use of yeast extract (Raposo et al., 2017). Due to this, it was tested as an economical option in this work for evaluating the possibility of lowering the costs of the bioprocesses.

According to Raposo et al. 2017, urea alone (3.0 g L^{-1}) was effective as supplementation, so urea 3.0 g L^{-1} was solely applied into the fermentation media to evaluate its effectiveness as a nitrogen source.

In this work, supplementation only with urea provided overall results significantly lower. In Figure 4.2, the biomass, glucose, and ethanol profiles are presented. The lag phase was similar to the control assay, but the growth was slower, achieving a specific growth rate of only 0.060 ± 0.006 h^{-1}. The glucose consumption was delayed with a $r_{glucose}$ of 3.43 ± 0.10 g L^{-1} h^{-1} reaching its depletion only after 48 h. The bioethanol production was slower with a $r_{ethanol}$ of 1.18 ± 0.13 g L^{-1} h^{-1}. The maximum ethanol concentration was 42.12 ± 1.50 g L^{-1} at 53 h with a productivity of 0.86 ± 0.02 g L^{-1} h^{-1} and a yield of 65.56 ± 0.82 % (Table 4.1). These rates resulted in a tardy glucose depletion, reached only after 48h of the beginning of the assay. Consequently, the maximum ethanol concentration was registered only after 53h of fermentation. However, the maximum ethanol concentration obtained of 42.12 g L^{-1}, is over the theoretical 40 g L^{-1} necessary to provide an efficient distillation process as stated by Zhang and co-workers in 2010 (Zhang & Lynd, 2010).

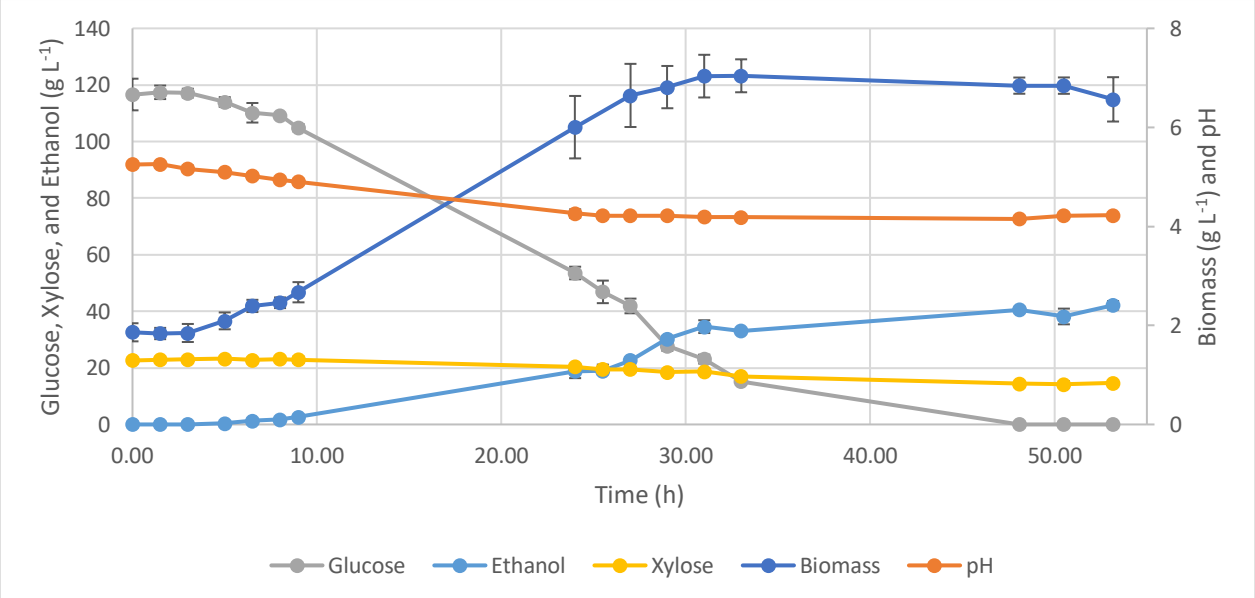

Figure 4.2– Glucose, xylose, ethanol, biomass, and pH profiles during Urea assay.

Raposo et al. 2017 produced bioethanol from carob extract using urea with a concentration of 3.0 g L^{-1} as supplementation. The bioethanol yield obtained was much higher, approximately 94 %, a value very closed to the maximum theoretical level. However, the initial sugars level was much higher, starting with the concentration of sugars of approximately 230 g L^{-1}. Of course, the maximum ethanol obtained in that study, 107.3 g L^{-1}, was only obtained after 76h of fermentation but provided an interesting productivity of 1.41 g L^{-1} h^{-1} (Raposo et al., 2017).

Ambitioning the improvement of the supplementation efficiency, urea was combined with the salts solution (ammonia salts and $MgSO_4 \cdot 7H_2O$) used in the control assay. Initially, as seen in Figure 4.3, the biomass growth seems to increase, starting the exponential phase after 4.5 h. However, the glucose consumption only started at a high rate near the end of the assay. The $r_{glucose}$ obtained was 1.33 ± 0.06 g L^{-1} h^{-1}. This low consumption rate resulted in a remaining final glucose concentration of almost half of the initial. The ethanol production only started after 25 h of fermentation at a slow rate of 0.48 ± 0.03 g L^{-1} h^{-1}. Consequently, the maximum ethanol concentration was significantly lower, producing only 14.6 g L^{-1} of ethanol after 52 h with a productivity of 0.33 ± 0.04 g L^{-1} h^{-1}. The ethanol yield of about 54.5% was also low (Table 4.1). Nonetheless, at the end of the assay, a significant increase in the bioethanol concentration can be seen, indicating a beginning of exponential production. However, this increase was only after 52 h of fermentation, requiring an extension of the assay. This prolongation would harshly decrease the bioethanol productivity, and consequently, the process performance was against our study purpose.

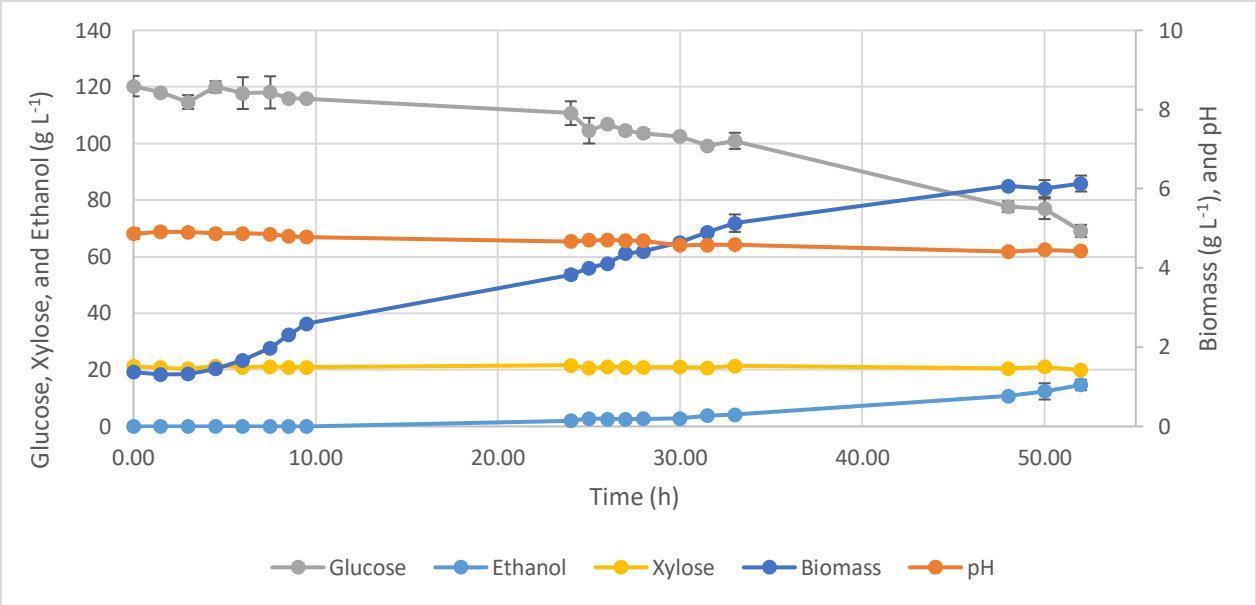

Figure 4.3– Glucose, xylose, ethanol, biomass, and pH profiles during Urea + Salts assay

Recently Tareen et al. 2021 used urea and some salts (2.0 g L^{-1} KH_2PO_4 and 5.0 g L^{-1} $MgSO_4$) as a low-cost nitrogen alternative to yeast extract and peptone. Firstly, the urea concentration was tested in a synthetic medium with 220 g L^{-1} of glucose. The ethanol yield achieved was 84%, and it was similar between the supplementation with yeast extract and peptone and constituted of 1.0 g L^{-1} urea and salts. After 72h of fermentation, the maximum ethanol concentration of 70.53 g L^{-1} was achieved. When performing SSF of oil palm trunk with the same supplementation conditions, authors obtained an ethanol yield of 81%. However, this was 16% lower than the assay supplemented with yeast extract and peptone as nutrient sources (Tareen et al., 2021).

Urea can be supplemented together with yeast extract to improve the fermentation performance (Appiah-Nkansah et al., 2018), and eventually reducing the yeast extract while maintaining the ethanol production (Z. Li et al., 2017). In this context, the reduction of yeast extract and urea concentrations was studied with their complementation.

A new assay with half of the concentration of yeast extract (1.25 g L^{-1}) and urea (1.5 g L^{-1}) was performed with the salts solution, already described. The biomass growth was similar to the control assay (Figure 4.4, a)). It started to grow exponentially after a 4.5 h lag phase with a specific growth rate of 0.164 ± 0.004 h^{-1}. However, the glucose consumption was delayed with a $r_{glucose}$ of 6.2 ± 0.13 g L^{-1} h^{-1}. This resulted in its depletion only after 25.5 h. The maximum ethanol concentration was 44.27 ± 4.32 g L^{-1} obtained after 30 h and, with a productivity of 1.74 ± 0.17 g L^{-1} h^{-1} and a yield of 69.75 ± 4.39 % (Table 4.1). The pH levels decreased from 5.22 ± 0.02 to 3.89 ± 0.00, stabilizing in this

value simultaneously as the maximum ethanol was obtained. It can be explained by the ending of ethanol production and, consequently, the ending of CO_2 release from alcoholic fermentation (Coote & Kirsop, 1976). In this experiment, biomass production was favored, increasing from 7.44 g L^{-1} to 9.17 g L^{-1} in comparison with the control assay. This could explain the lower ethanol yield. Nevertheless, the final ethanol concentration obtained was above the value estimated to a profitable ethanol distillation process (Zhang & Lynd, 2010).

Another assay was performed to evaluate a lower concentration of yeast extract complemented with urea. In this study, a supplementation with a quarter of the yeast extract (0.625 g L^{-1}), three quarters of the previous urea (2.25 g L^{-1}), and the salts solution was applied. Figure 4.4 b) shows the biomass growth was similar. It started to grow exponentially after 4.5 h of the beginning of the fermentation with a specific growth rate of 0.124 ± 0.004 h^{-1}. After 25.5 h of fermentation, glucose depletion was achieved (Figure 4.4, b)). The $r_{glucose}$ and the $r_{ethanol}$ were 5.89 ± 0.37 g L^{-1} h^{-1} and 2.22 ± 0.06 g L^{-1} h^{-1}, respectively. The maximum ethanol concentration obtained was 48.21 ± 5.75 g L^{-1} after 28 h with a productivity of 2.19 ± 0.26 g L^{-1} h^{-1} an ethanol yield of 71.38 ± 3.79 %. The results obtained were slightly higher than the assay using half of the yeast extract concentration (Table 4.1). Also, the specific growth rate was somewhat lower, indicating a higher promotion of fermentation.

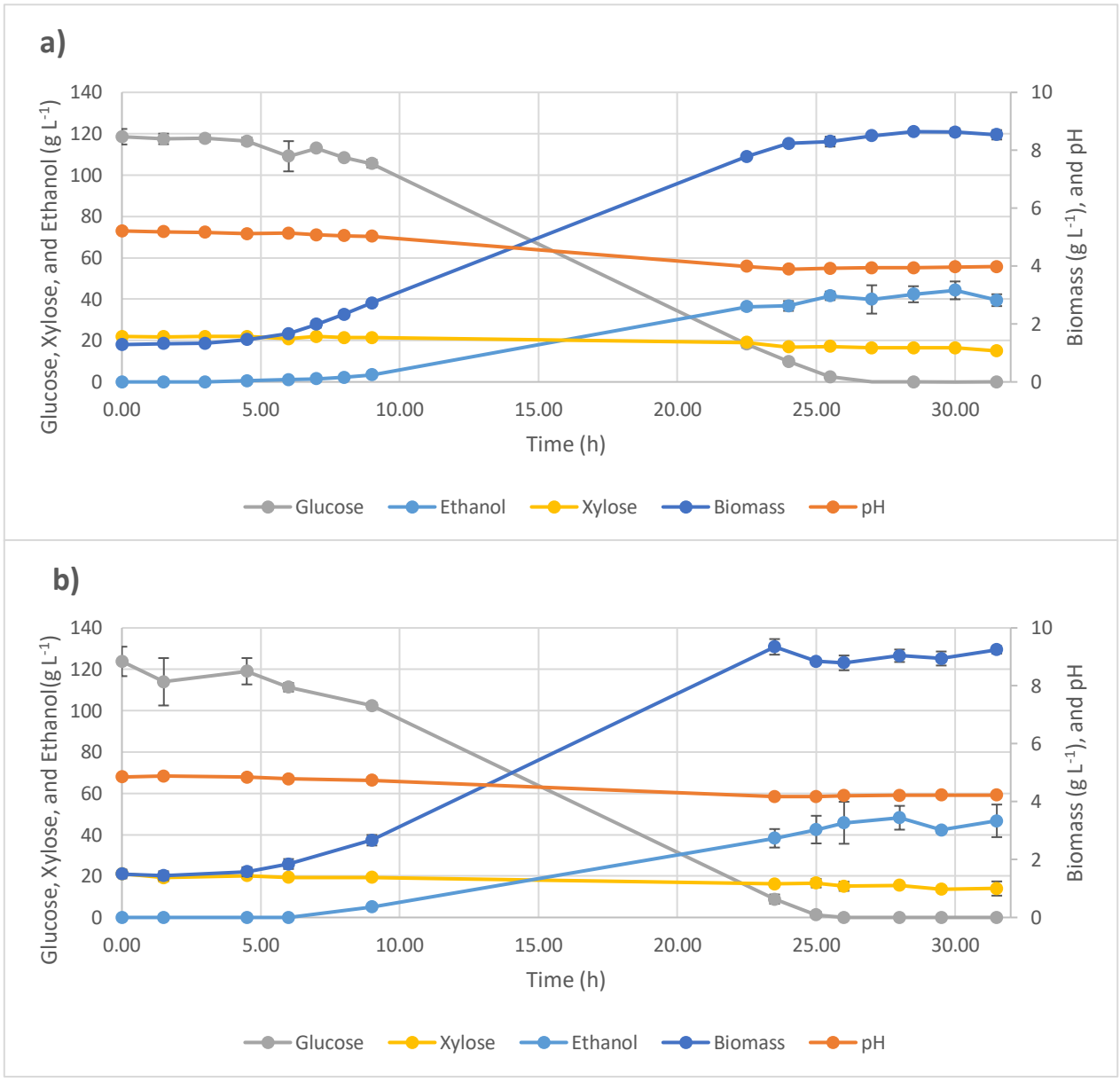

Figure 4.4 – Glucose, xylose, ethanol, biomass, and pH profiles in Reduction assays I (a) and II (b), respectively.

Appiah-Nkansah et al. 2018 studied the effect of urea (0.96 g L^{-1}) supplementation in SSF of sorghum mashes containing 4.1 g L^{-1} yeast extract. In that study, ethanol production generally increased when urea supplemented the mashes. The ethanol levels from supplemented mashes were generally different from the non-supplemented in 5% level of significance based on pairwise comparisons. They obtained a maximum ethanol concentration of 159.77 g L^{-1} with a yield of 96 % after 72 h of fermentation. This shows a good combination of these two nitrogen sources in bioethanol production (Appiah-Nkansah et al., 2018).

Li et al. 2017 optimized the nitrogen supplementation in bioethanol production by *Saccharomyces cerevisiae* in very high gravity fermentations of corn starch. In such a study, they reduced the amount of yeast extract used in the fermentation supplementation by adding urea and ammonium sulphate. They used 2.0 % yeast extract in supplementation as a control, where an ethanol yield of 84.5 % was obtained. Then, when complementing the supplementation with 4.08 g L^{-1} urea and 3.44 g L^{-1} ammonium sulphate they could reduce the use of yeast extract to 0.6 % while having a yield in ethanol production of 84.2% after 72 h (Z. Li et al., 2017). These results also complement our study, showing that yeast extract can be reduced in the fermentation media by replacing it with other nitrogen sources like urea and ammonium salts while maintaining a high bioethanol production.

4.1.3 Fermaid O™ as Yeast Extract substitute in supplementation

Yeast extract is the intracellular content of yeasts composed of a different mixture of amino acids, vitamins and other growth enhancers. This nutrient was shown to directly influence fermentation yield (Hakobyan et al., 2012; Zhang et al., 2003). Yeast lysates are an economical alternative, obtained from the yeasts' lysis without specific hydrolysis of the internal proteins into amino acids. However, hydrolysis is often promoted during the lysis process by endogenous yeast enzymes. The resulting yeast lysate will have similar characteristics as a commercial yeast extract specialized for biomass growth and fermentation enhancement (York & Ingram, 1996; Zarei et al., 2016).

Fermaid O™ is an example of an economic yeast lysate that is often used in the winemaking industry as a fermentation supplement. A new attempt was assayed experimenting this commercial yeast extract. Fermaid O™ substituted the control assay commercial yeast extract supplementation to study its effect on bioethanol production from *E. globulus* barks kraft pulp hydrolysates.

In this assay, the lag phase lasted 5.5 h and after this time, the glucose consumption began (Figure 4.5), with a consumption rate $r_{glucose}$ of 9.61 ± 0.25 g L^{-1} h^{-1}. At this period, the biomass also reached its exponential growth phase, achieving a specific growth rate of 0.131 ± 0.008 h^{-1}. Similar to the control assay, the glucose depletion occurred before 24 h, corresponding to the stabilization of the biomass growth and ethanol production. After 25 h, 39.29 ± 3.07 g L^{-1} of ethanol was produced with a productivity of 2.12 ± 0.17 g L^{-1} h^{-1} and yield of 73.42 ± 4.92 % (Table 4.1). The biomass achieved a

maximum concentration of 11.42 ± 0.26 g L^{-1}. The ethanol yield could be higher since occurred a prevalence of the biomass growth over the alcoholic fermentation.

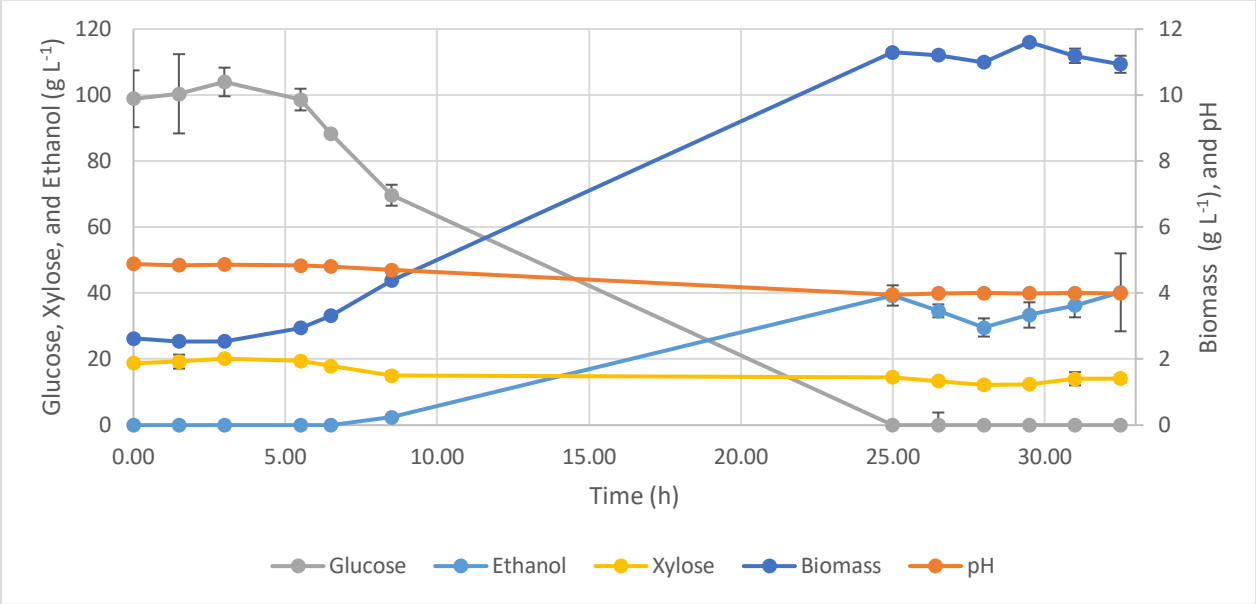

Figure 4.5 – Glucose, xylose, ethanol, biomass, and pH profiles in the assay supplemented with Fermaid O™ + Salts.

Beigbeder et al. 2021 supplemented fermentation from high concentration sugar beet molasses with 4 g L^{-1} of Fermaid O™, presenting higher ethanol yields than our study. Using an initial sugar concentration of 125 g L^{-1}, the authors achieved a maximum ethanol concentration of 54 g L^{-1} and a yield of 84.5%. However, at their assays, the ethanol production only began after 24h of lag phase. This lag phase delayed the achievement of the maximum ethanol concentration to only after 72 h. When increasing the initial glucose concentration from 125 g L^{-1} to 225 g L^{-1}, their ethanol concentration increased, but the yields decreased from 85% to 80%. This yield decrease could be caused by the increase of osmotic stress derived from the increase of sugars present in the fermentation system. (Beigbeder et al., 2021).

Still, Fermaid O™ has shown good potential for bioethanol production, and with further optimization, the ethanol production can be enhanced. Also, this study presents a high efficiency of yeast lysates performance in fermentation as an alternative to a more expensive commercial yeast extract.

According to the previous results, the fermentation performance between the control assay and the Reduction II were similar. Further optimization of the urea and yeast extracts concentrations for a maximum bioethanol production is required (Table 4.1).

Table 4.1 – Fermentation kinetics from the economical supplementation assays.

Assay	μ (h^{-1})	$r_{glucose}$ ($g\ L^{-1}\ h^{-1}$)	$r_{ethanol}$ ($g\ L^{-1}\ h^{-1}$)	$[Ethanol]_{max}$ ($g\ L^{-1}$)	$Prod_{vol}$ ($g\ L^{-1}\ h^{-1}$)	$Y_{ethanol}$ (%)
Control	0.155 ± 0.012	11.86 ± 0.59	5.18 ± 0.94	45.31 ± 1.24 (28.5 h)	2.01 ± 0.06	72.72 ± 1.32
Urea	0.060 ± 0.006	3.43 ± 0.10	1.18 ± 0.13	42.12 ± 1.50 (53 h)	0.86 ± 0.02	65.57 ± 0.82
Urea + Salts	0.118 ± 0.006	1.33 ± 0.06	0.48 ± 0.03	14.65 ± 1.84 (52 h)	0.33 ± 0.04	55.4 ± 14.00
Reduction I	0.164 ± 0.004	6.20 ± 0.13	2.31 ± 0.12	44.27 ± 4.32 (30 h)	1.74 ± 0.17	69.75 ± 4.39
Reduction II	0.124 ± 0.004	5.89 ± 0.37	2.22 ± 0.06	48.21 ± 5.75 (28 h)	2.19 ± 0.26	71.38 ± 3.79
Fermaid O™	0.131 ± 0.008	9.61 ± 0.25	2.17 ± 0.10	39.29 ± 3.07 (25 h)	2.12 ± 0.16	73.42 ± 4.92

4.1.4 Bioreactor assay

Following the previous results, the supplementation from the control assay provided the best fermentation performance. Thus, the process requires to scale-up to integrate the industry and verify this fermentation feasibility at larger scales.

However, the fermentation performance may not follow the same behavior when applied in larger quantities (Hewitt & Nienow, 2007). The scale-up from Erlenmeyer to 5 L bioreactor was performed with the described supplementation and using a working volume of 2 L. In the bioreactor, the fermentation conditions were the same except the pH that was controlled at 5.5. In Figure 4.6, a very short lag phase was observed since the glucose consumption and biomass growth started fast, followed by bioethanol production. Startng from a higher sugar concentration could provide a maximum biomass concentration of 9.14 g L^{-1}, compared to 7.25 g L^{-1} in the control assay. However, the specific growth rate of 0.131 h^{-1} was slower than 0.155 h^{-1} in the control assay and the glucose depletion occurred after 19 h of the beginning of the assay with a $r_{glucose}$ of 8.42 g L^{-1} h^{-1}, slower than 11.86 g L^{-1} in the control. Concerning ethanol production, it stopped after 22 h, achieving its maximum of 61.05 g L^{-1} with a productivity of 3.49 g L^{-1} h^{-1} and a yield of 83%. With this assay, even with the initial glucose concentration a little higher (18 g L^{-1}), it was evident that the scale-up of the process was efficient. The ethanol yield, the maximum ethanol concentration, and the productivity increased 10 %, 16,2 g L^{-1} and

1.19 g L^{-1} h^{-1}, respectively, from the Erlenmeyer control assay. These results suggest the feasibility of this fermentation in larger scales.

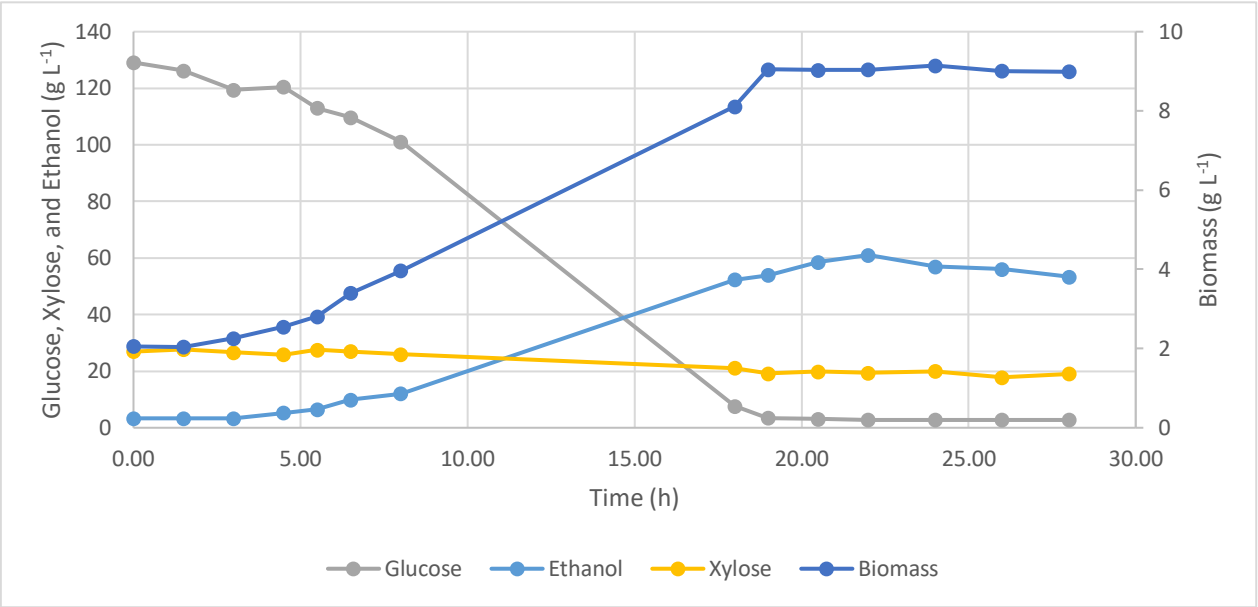

Figure 4.6 – Glucose, xylose, ethanol, and biomass profiles in the bioreactor assay (pH = 5.5, 180 rpm, T = 28º C)

4.2 Yeast reutilization

At bioethanol production industrial processes, the resultant biomass from the fermentation assays is often a side stream. Following a circular economic strategy, the spent yeast from the fermentation processes should be reused for additional income.

This yeast can be reused as supplementation by producing yeast extract and also as inoculum. Two different approaches were studied in order to reduce the costs of the supplementation and the costs and time associated with the inoculum preparation step. This strategy will also reduce the costs associated with biomass disposal.

4.2.1 Yeast reutilization as supplementation

Yeast extract is usually described as the remaining soluble intracellular contents (primarily amino acids, peptides and nucleotides) from yeast cells after their lysis (In et al., 2005; Jacob, Striegel, et al., 2019). The disruption of the intracellular content can be performed by chemical or enzymatic hydrolysis, either by the endogenous enzymes (autolysis) or by the application of exogenous enzymes (In et al., 2005; Tanguler & Erten, 2008). The remaining extract is composed of amino acids, vitamin B complex and minerals, a very nutritional source for microorganisms' growth and their related products formation (Ferreira et al., 2010).

In the previous assays of testing alternative nitrogen sources to replace the yeast extract, none of the supplementations applied performed well without using yeast extract. This way, a potential economical way to replace commercial yeast extract is producing its own. The spent yeast from fermentation can be processed to produce yeast extract while reducing the yeast biomass side stream following the circular economy model with the reutilization of produced wastes to increase profitability.

4.2.1.1 Yeast extract production

Three different methods to produce yeast extract from the spent yeast cells were studied. The biomass used was collected just after ending the bioreactor assay. This intends to preview the process in the industry by using the biomass wastes and using yeast cells that suffered usual stresses during fermentation.

The yeast lysis on the first two methods (Figure 4.7 a) and b)) was based on the yeast's autolysis process where the own yeast enzymes disrupt the yeast cell, followed by heating the solution to inactivate the enzymatic activity (Tanguler & Erten, 2008). In the second method, the addition of ethyl acetate presumably would inhibit possible contaminants (Jacob, Striegel, et al., 2019). In the third method (Figure 4.7 c)), the increased heat and pressure in the autoclave should damage the yeast cell walls, and the following cooling should induce a heat shock causing their rupture (Zarei et al., 2016).

The final protein concentration quantification was performed by the Bradford method due to its simplicity, quickness, sensitivity, and it's relatively free from interference by most commonly used biochemicals (Hammond & Kruger, 1988). The commercial yeast extract and the Fermaid O™ were also analyzed for comparison. Using

BSA to perform a calibration curve (Appendix C - Figure C1), the validation interval was between 0.10 g L^{-1} and 1.00 g L^{-1} of protein in the solution. Each yeast extract solution was prepared for a total concentration of 20.0 g L^{-1}, with protein between 0.5 % and 5.0 %, for being detected in the calibration curve. Only the autoclave method presented results in the validation interval, with a protein content of 3.12%. The same assay was performed with the commercial yeast extract and with the yeast lysate Fermaid O™, and both presented results under the validation interval. This demonstrated that both yeast extracts produced through autolysis and the commercial yeast extracts had a protein content below 0.5 % (Table 4.2).

The really low percentage of protein certainly can be explained through the hydrolysis of the proteins into free amino acids by the endogenous enzymes during the autolysis process, (Tanguler & Erten, 2008). As the Coomassie blue dye used in the Bradford assay does not react with free amino acids, these amino acids were not quantified evens as valuable nutrients (Krohn, 2011). The autoclave process showed a higher amount of protein which complies with the non-enzymatic autolysis results since, in this process the yeast cell walls were broken only through physical processes. Also, the nitrogen amount present on the autolysis samples tends to be free amino nitrogen, whereas in the autoclave process can be still in polypeptides configuration.

The nitrogen and carbon content of the extracts produced were measured through elementary analysis (Table 4.2). The yeast extract produced with the autolysis induced only through temperature was the one with the higher percentage of nitrogen and carbon, with 12.18 % and 40.92 %, respectively. The commercial yeast extract presented similar results, with 38.98 % and 10.98 % carbon and nitrogen, respectively.

The autolysis induced only through temperature (Figure 4.7 a) was selected for further studies since it presented the best results. Also, it followed a simpler and economical protocol, which is the objective to implement on larger scales.

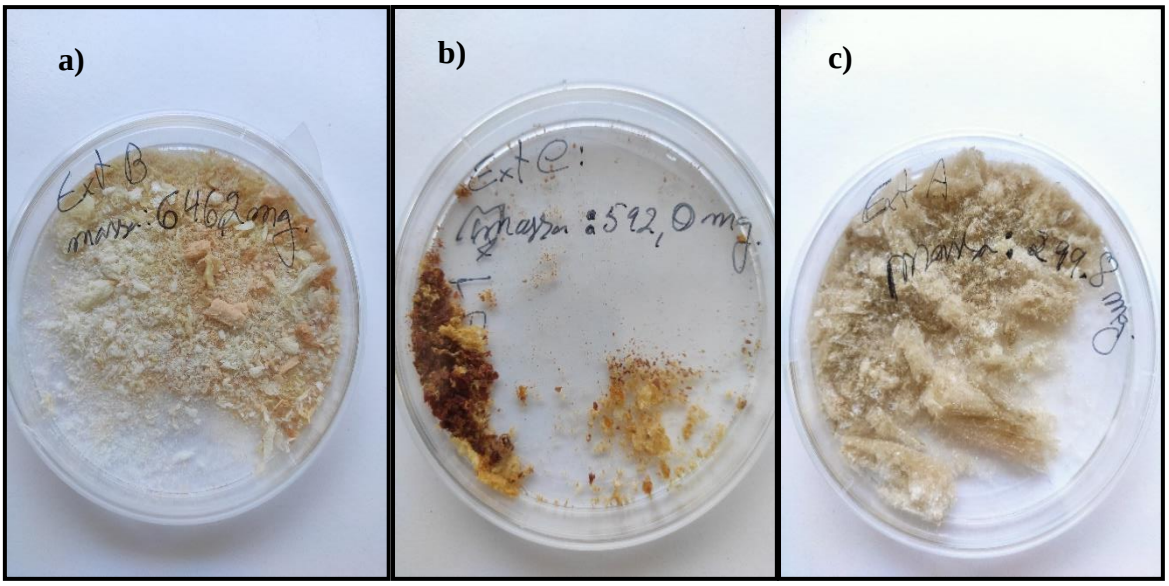

Figure 4.7 – Produced yeast extracts. a) represents the autoclave method, b) the autolysis method by temperature, c) the autolysis with addition of ethyl acetate and NaCl

Table 4.2 – Protein, nitrogen, and carbon contents in the commercial and produced yeast extracts.

	Autoclave	**Autolysis (Tº)**	**Autolysis (Ethyl acetate)**	**Commercial**	**Fermaid O™**
Protein (%)	3.12	<0.5%	<0.5%	<0.5%	<0.5%
Nitrogen (%)	9.25 ± 0.00	12.18 ± 0.12	9.93 ± 0.07	10.98 ± 0.06	9.99 ± 0.18
Carbon (%)	38.61 ± 0.16	40.93 ± 0.04	32.75 ± 0.01	38.99 ± 0.27	40.54 ± 0.31
Hydrogen (%)	5.56 ± 0.04	6.36 ± 0.16	5.99 ± 0.28	5.94 ± 0.16	5.93 ± 0.14

4.2.1.2 Fermentation assay with the produced yeast extract

The extract selected above was tested in an Erlenmeyer assay, using the same conditions as the control assay to compare its performance. In Figure 4.8 is observed that the lag phase lasted 4.5 h, and the biomass grew with a specific growth rate of 0.141 ± 0.005 h^{-1}. The glucose consumption was slightly delayed with a $r_{glucose}$ of 5.99 ± 0.11 g L^{-1} h^{-1}. However, glucose depletion occurred also before the 24 h. The ethanol production occurred with a $r_{ethanol}$ of 2.63 ± 0.08 g L^{-1} h^{-1}. The ethanol production stopped after 26.5 h achieving its maximum concentration of 48.26 ± 0.94 g L^{-1} with a productivity of 2.35 ± 0.05 g L^{-1} h^{-1} and a yield of 76.73 ± 4.53 %. Our produced yeast extract improved the

results in comparison with the control assay and with Fermaid O™, a simpler yeast lysate. The success of the produced yeast extract in the bioethanol production confirms its potential for replacing the commercial yeast extract. Thus, the results are competitive since the price of yeast extract is high compared to the manufacturing costs of this extract, evidencing the feasibility of this process in larger-scale bioethanol facilities to implant a sustainable circular economy strategy, without lowering the profits.

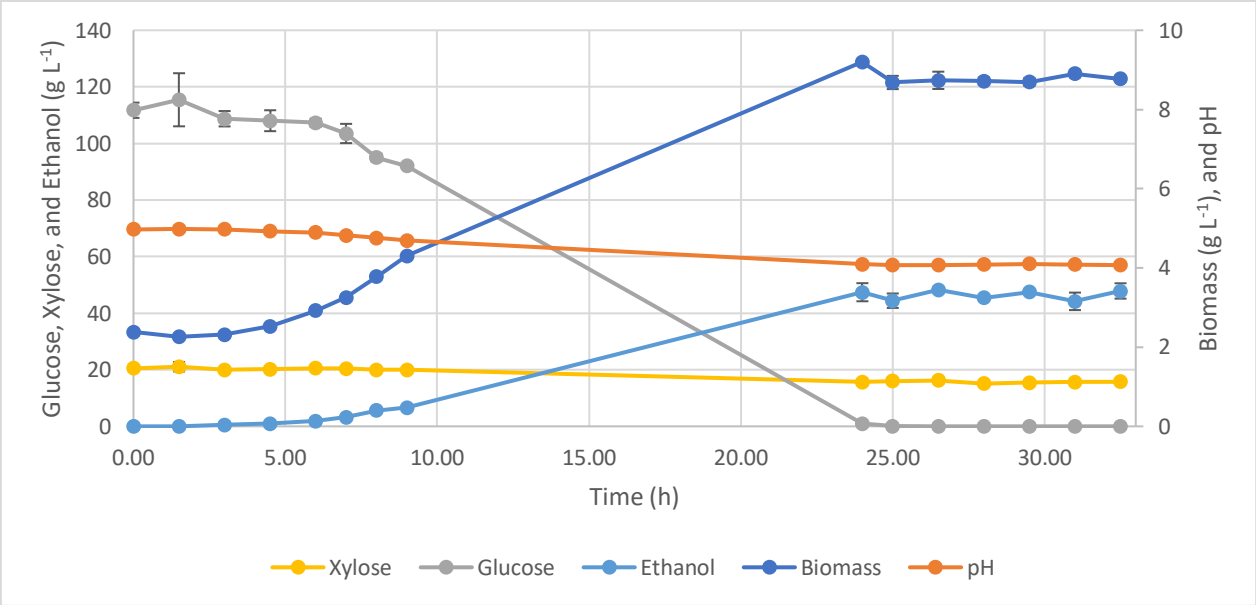

Figure 4.8 – Glucose, xylose, ethanol, biomass, and pH profiles in the assay using the produced yeast extract as supplementation.

Sridee et al. 2011, studied the use of dried spent yeast as an alternative to commercial yeast extract when fermenting sweet sorghum juice. In their study, the dried spent yeast presented 40% less nitrogen amount than the commercial yeast extract. This can highlight the importance of the hydrolysis of the peptides to obtain a higher amount of free amino nitrogen. With an initial total sugar of 280 g L^{-1} and supplementing the fermentation media with 8 g L^{-1} of dried spent yeast, they obtained an ethanol yield of 86% with a maximum ethanol concentration of 105.4 g L^{-1} (Sridee et al., 2011). Suwanapong et al. 2013 also performed a very high gravity fermentation of sweet sorghum juice hydrolysates using dried spent yeast as a nitrogen source. Nonetheless, the aim was to improve the available nitrogen by decomposing the protein content through acid hydrolysis. When supplementing the fermentation media with 21 g L^{-1} of their yeast hydrolysate, they obtained an ethanol yield of 88% with a maximum ethanol concentration of 98 g L^{-1} (Suwanapong et al., 2013). These authors obtained a higher

fermentation performance, but they required a higher dried yeast concentration and used a potentially higher nutritious hydrolysate (the sorghym juice hydrolysate is richer than the bark pulp hydrolysate). This studies also suggests a further optimization the produced yeast extract concentration used in our study to improve the ethanol yields.

Kawa-Rygielska et Pietrzak 2014 recycled spent brewer's yeast as a nitrogen source in high gravity maize mashes fermentation. Their best results were achieved supplementing the mashes with 30g of wet spent yeast per kg of mashes and then hydrolyzed with proteases to increase the free amino acids available nitrogen for the yeast fermentation. Between the 24 h and the 48 h, their exponential ethanol production phase ended with an ethanol yield of 90.34 % which was significantly higher than the 76.73 % obtained with our produced yeast extract. However, the use of proteolytic enzymes increases the costs of the yeast extract production compared with our process, contradicting the purpose of our study (Kawa-Rygielska & Pietrzak, 2014).

Nevertheless, the raw materials and the fermentation configurations were completely different. The produced extract in this current study is promissory to manufacture and utilize as a primary organic nitrogen source. Remarkably, the produced yeast extract potentially presents lower production costs compared to the literature processes that utilize exogenous enzymes or chemicals to hydrolyze the yeast peptides. Furthermore, this process is reutilizing a side stream of the fermentation process while replacing a costly supplement and remaining with high ethanol concentrations and yields.

4.2.2 Yeast reutilization as inoculum

Inoculum preparation before fermentation assays requires an additional time-consuming step in the whole process. Moreover, it represents additional costs, such as the growing media and the other supplements needed to grow the biomass. In order to evaluate potential cell reuse in batch fermentations, the biomass was reused into successive assays, skipping the additional inoculum steps while maintaining high ethanol production.

The five consecutive batch fermentations were performed using *E. globulus* barks hydrolyzed kraft pulp in Erlenmeyer flasks and control assay supplementation, and only the first had proper inoculum since the others had centrifuged cells. In the first assay (Figure 4.9 a)) it was observed a lag phase of 4.5 h with a biomass specific growth rate of 0.189 ± 0.007 h^{-1}. The glucose consumption followed a $r_{glucose}$ of 7.59 ± 0.25 g L^{-1} h^{-1}

and achieved its depletion before 20 h. After 21 h, the maximum ethanol concentration of 55.69 ± 3.05 g L^{-1}, productivity of 3.38 ± 0.19 g L^{-1} h^{-1} and an ethanol yield of 88.72 ± 7.56% were obtained (Table 4.2).

The second assay (Figure 4.9 b)) had a similar biomass growth with a lag phase of 4 h and a specific growth rate of 0.201 ± 0.015 h-1. The bioethanol production performance was also maintained achieving the maximum ethanol concentration of 55.25 ± 2.29 g L^{-1} at the same period with a productivity of 3.15 ± 0.13 g L^{-1} h^{-1} and a yield of 89.34 ± 3.34 %.

In the third assay, the fermentation performance started to decrease. The biomass grew with a specific growth rate of 0.165 ± 0.015 h^{-1}, achieving its exponential growth phase after a 4.5 h lag phase, coinciding with the beginning of the glucose consumption (Figure 4.9 c)). The glucose depletion was achieved before 24 h, achieving the maximum ethanol concentration of 51.89 ± 4.08 g L^{-1} at the same time. A lower productivity and ethanol yield of 2.71 ± 0.21 g L^{-1} h^{-1} and 80.21 ± 1.19 % were obtained, respectively.

At the fourth assay, the yeast cell significantly decreased the fermentation activity, possibly due to cell senescence. The lag phase increased to 6h and the bioethanol production only began after 7.5h of the beginning of the assay (Figure 4.9 d)). The ethanol yield decreased to 57.55 ± 4.16 %, with a productivity of 2.27 ± 0.04 g L^{-1} h^{-1} and a maximum ethanol concentration of 38.66 ± 0.60 g L^{-1}. The biomass growth was maintained, allowing the possibility of its restoring it with new cells.

The fifth and last inoculum reutilization assay showed an agreement with the yeast recovery since the bioethanol production increased. The lag phase decreased to 4.5 h with the beginning of the glucose consumption after 3 h of fermentation (Figure 4.9 e)). The specific growth rate achieved was 0.203 ± 0.022 h^{-1} with a biomass concentration of 9.93 ± 0.38 g L^{-1}. The ethanol production began with glucose consumption and reached its maximum of 53.57 ± 2.22 g L^{-1} after 24 h with a productivity of 2.75 ± 0.11 g L^{-1} h^{-1} an ethanol yield of 88.44 ± 0.91 % (Table 4.3).

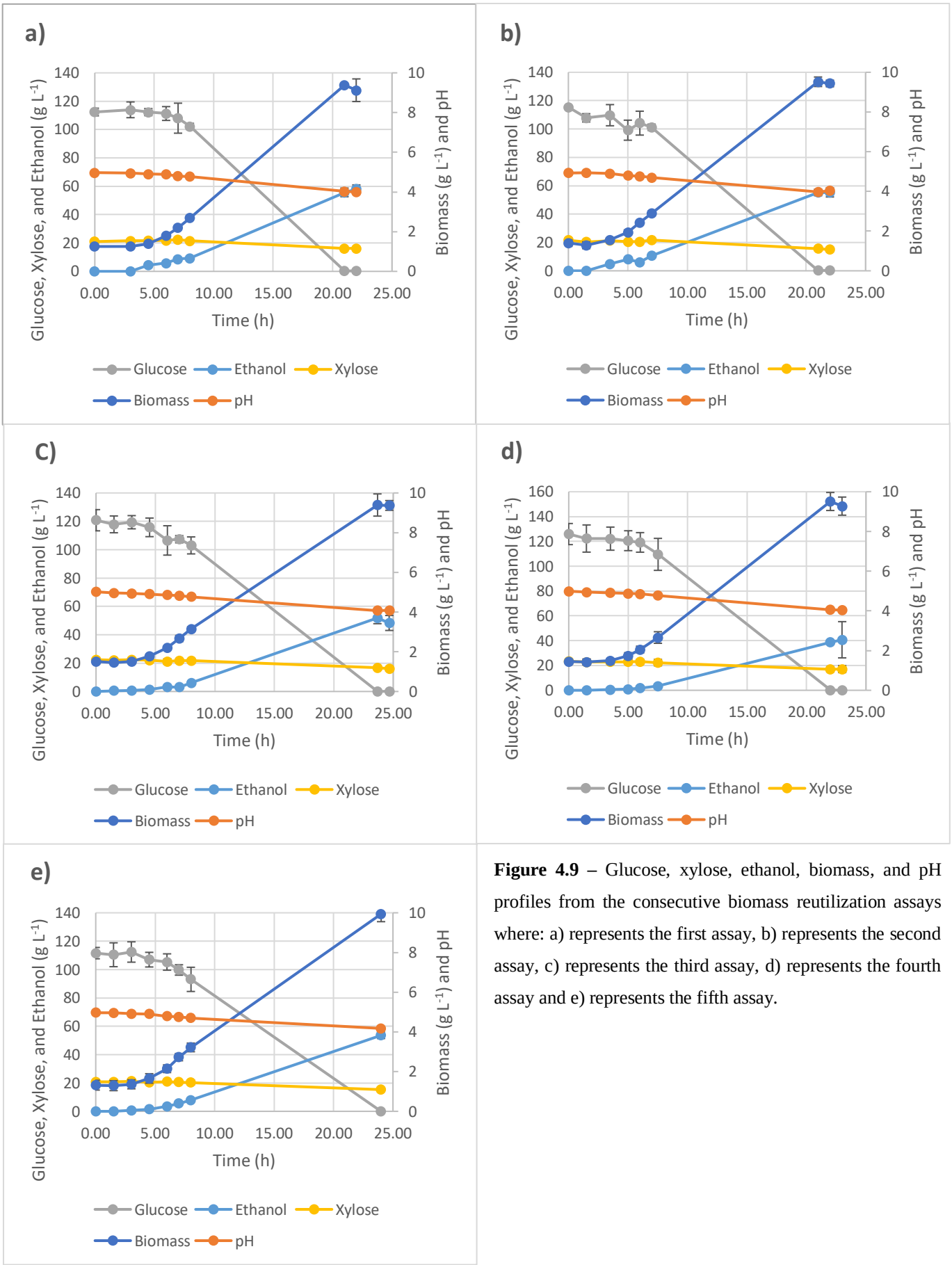

Figure 4.9 – Glucose, xylose, ethanol, biomass, and pH profiles from the consecutive biomass reutilization assays where: a) represents the first assay, b) represents the second assay, c) represents the third assay, d) represents the fourth assay and e) represents the fifth assay.

Pereira et al. 2012 performed a cell recycle batch fermentation for 15 consecutive cycles using a fermentation medium with a high glucose concentration (300 to 400 g L^{-1}). This study realized that cell viability harshly decreased after the fifth fermentation cycle, reducing ethanol yield. However, they managed to restore the viability of the yeasts, by after the fifth assay, transferring only a small fraction of the spent yeast to the following assay (F. B. Pereira et al., 2012). This could explain the fermentation activity recovered in our study, since the yeast inoculated through assays was only 10% of the fermentation working volume, suggesting that the ethanol yield from fourth to fifth assay increased since the biomass was composed with new yeast cells with higher fermentation activity. Besides that, centrifugation may not be enough to successfully separate some inhibitors, namely the residual lignin. Matano et al. 2012 used two-step centrifugation to increase the residual lignin removal over five consecutive fermentation batches of hydrothermally pretreated rice straws. However, a modified cellulase expressing yeast strain improved the ethanol yield to 86.3% with a maximum bioethanol concentration of 42.2 g L^{-1} (Matano et al., 2013). Even more, yeast cells adaptation to some inhibitory compounds present at the lignocellulosic biomass during the assays may increase their resistance to its toxicity. This process can increase ethanol productivity under those toxic compounds, maintaining the ethanol yields over the fermentation cycles (Landaeta et al., 2013; Silva et al., 2016).

Table 4.3 – Fermentation kinetics in the fermentation with successive biomass reutilization as inoculum

Assay	μ (h^{-1})	$r_{glucose}$ (g L^{-1} h^{-1})	$[Biomass]_{max}$ (g L^{-1})	$[Ethanol]_{max}$ (g L^{-1})	$Prod_{vol}$ (g L^{-1} h^{-1})	$Y_{ethanol}$ (%)
First	0.189 ± 0.007	7.59 ± 0.25	9.12 ± 0.04	55.69 ± 3.05 (21 h)	3.38 ± 0.19	88.72 ± 7.56
Second	0.201 ± 0.015	7.04 ± 0.24	9.44 ± 0.24	55.25 ± 2.29 (21 h)	3.16 ± 0.13	89.34 ± 3.34
Third	0.165 ± 0.007	6.18 ± 0.30	9.37 ± 0.25	51.89 ± 4.08 (24 h)	2.71 ± 0.21	80.21 ± 1.19
Fourth	0.169 ± 0.002	7.48 ± 0.09	9.28 ± 0.45	38.66 ± 0.60 (22 h)	2.27 ± 0.04	57.55 ± 4.16
Fifth	0.203 ± 0.022	5.70 ± 0.21	9.94 ± 0.38	53.57 ± 2.22 (24 h)	2.75 ± 0.11	88.44 ± 0.91

5 Conclusions

This work focused on studying and improving the bioethanol production process using an unbleached kraft pulp hydrolysate from *E. globulus* barks, following a circular economical strategy.

Supplementing the fermentation process with specific nutrients such as nitrogen, trace elements, or vitamins is crucial to increase the fermentation performance. The costs in supplementation are high since expensive nitrogen sources are used, usually yeast extract, decreasing the overall profit from this bioethanol production process.

The replacement or reduction of yeast extract was studied using urea, yeast lysate Fermaid O™, and a produced yeast extract. Urea has been shown as supplement for acholic fermentation, but for an industrial process, better performance in bioethanol production is required. The presence of yeast extract was shown to be essential to complement the urea supplementation since it highly boosted ethanol yields, even at low levels.

The replacement of the commercial yeast extract with other economical yeast lysates like Fermaid O™ is feasible. However, further experiments are required to adjust the optimal concentrations to increase the fermentation performance since it enhanced biomass growth.

The spent yeast from the fermentation processes can be reutilized to produce yeast extract and reduce the costs associated with the disposal of this side stream. A simple autolysis process induced only through temperature was selected to efficient rupturing the yeast cell walls and recovering of the intracellular nutrients. Yeast extract prepared with this process has shown really competitive results in bioethanol production.

The spent yeast can also be successfully reused as inoculum, taking into account the cell viability and the fermentative activity of the yeast cells. Besides the promising results in bioethanol production at the first assays, the ethanol yields began to decrease, probably due to the yeast cells' senescence or the accumulation of inhibitors transferred between assays.

Several strategies could be used in lignocellulosic bioethanol production to reduce the costs associated with the whole process. Considering the circular economy model, the reuse of yeast cells is promising to decrease the overall costs and reduce the wastes generation. The production of yeast extract from the spent yeast reduces this side stream and all the costs associated with its disposal while reintegrating it back into the process.

6 Future Work

Considering the results obtained during this work, the bioethanol production from *Eucalyptus globulus* barks kraft pulp hydrolysates is feasible but further optimization is required to be produced successfully at an industrial process. The supplementation of the fermentation media should be optimized by combining the produced yeast extract and the urea at optimal concentrations. Then, the optimal fermentation could be tested in other fermentation configurations such as fed-batch or SSF to improve the bioethanol production. The selected configuration should be scaled up to produce larger quantities of ethanol. Also, a detailed characterization of the produced yeast extract should be done to evaluate the sources of the nitrogen content and other nutrients present in the extract.

The spent yeast reutilization should be optimized to improve the process of separation of the yeast cells from the fermentation broth. Also, the fermentation media between assays could be analyzed for the detection of inhibitors to evaluate its accumulation over the successive fermentations.

A scale up should be performed, paying attention to the correct separation of the yeast cells from some lignocellulosic residues that remain in the reactor.

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Appendixes

Appendix A – Biomass calibration curve

Figure A1 represents the biomass calibration curve of the optical density 620 nm *versus* the biomass dry weight for the Ethanol Red® in the *E. globulus* barks kraft pulp hydrolysate.

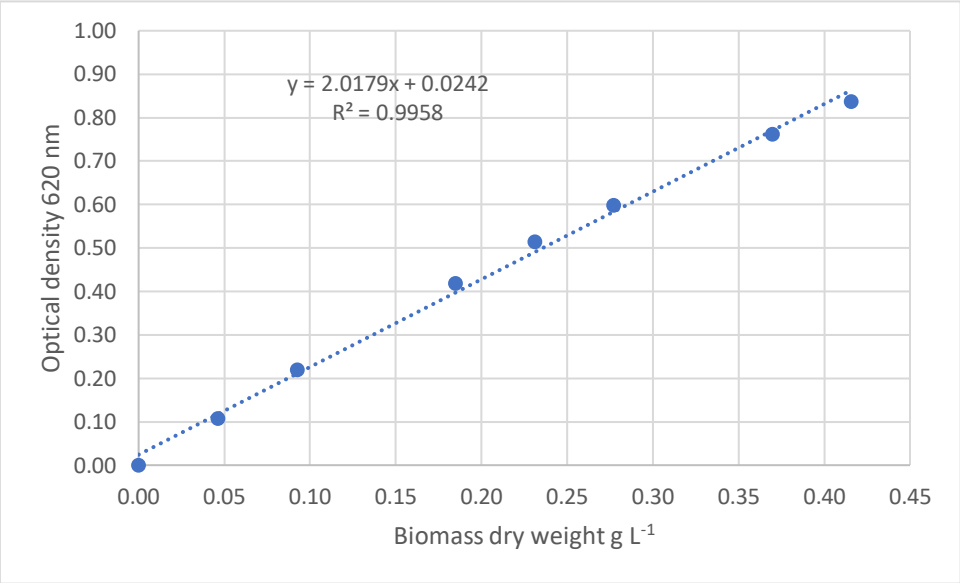

Figure A1 – Calibration curve of the optical density at 620 nm *versus* biomass dry weight for Ethanol Red® in the hydrolysate.

Appendix B – HPLC calibration curves (example)

In Figure B1 is represented an example of the calibration curves of the compound peak *versus* the compound concentration of the compound sample.

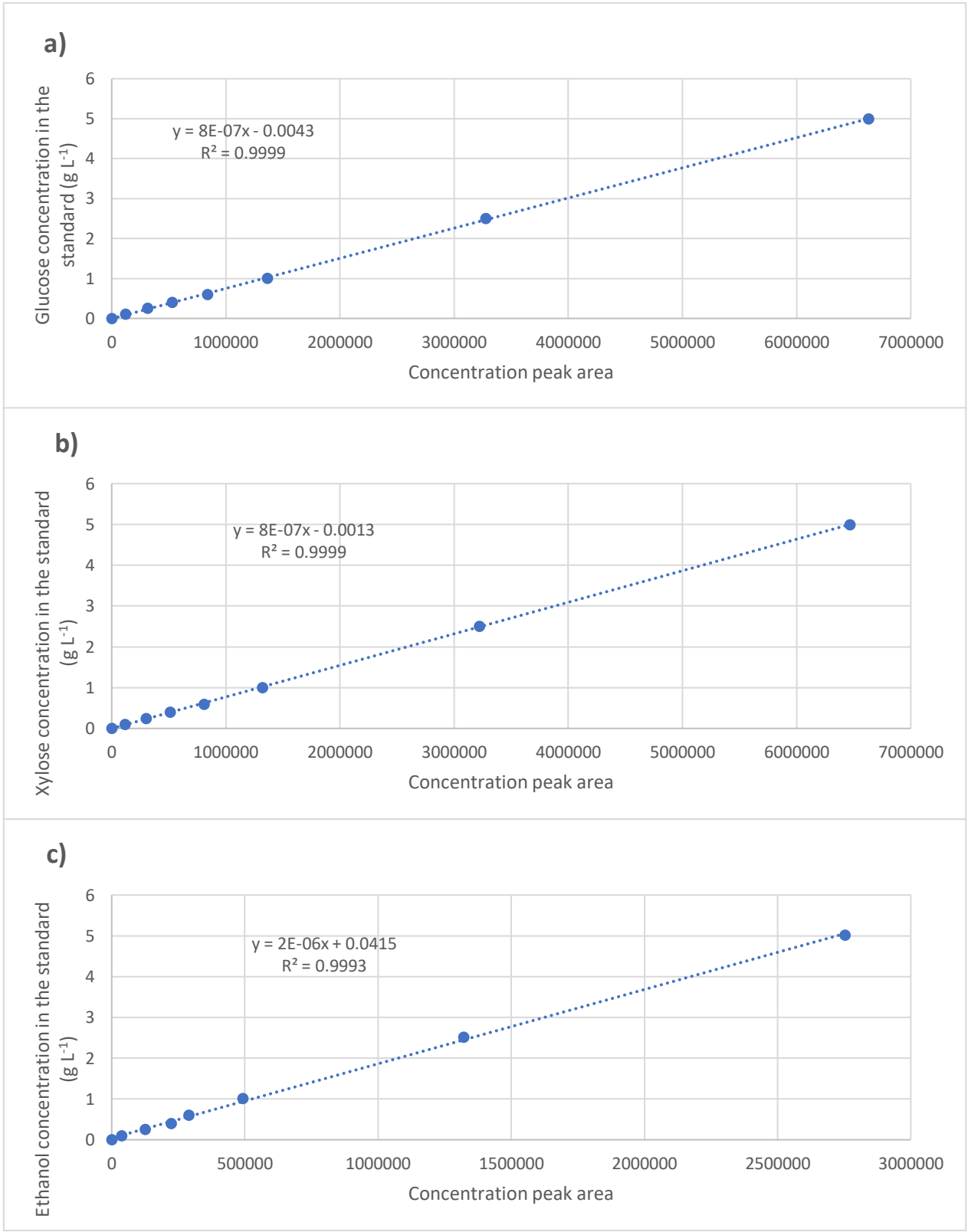

Figure B1 – HPLC calibration curve for glucose (a), xylose (b), and ethanol (c)

Appendix C – Calibration curve for Bradford assay

In Figure C1 is represented the calibration curve used for the Bradford assay with BSA concentration *versus* the optical density at 595 nm.

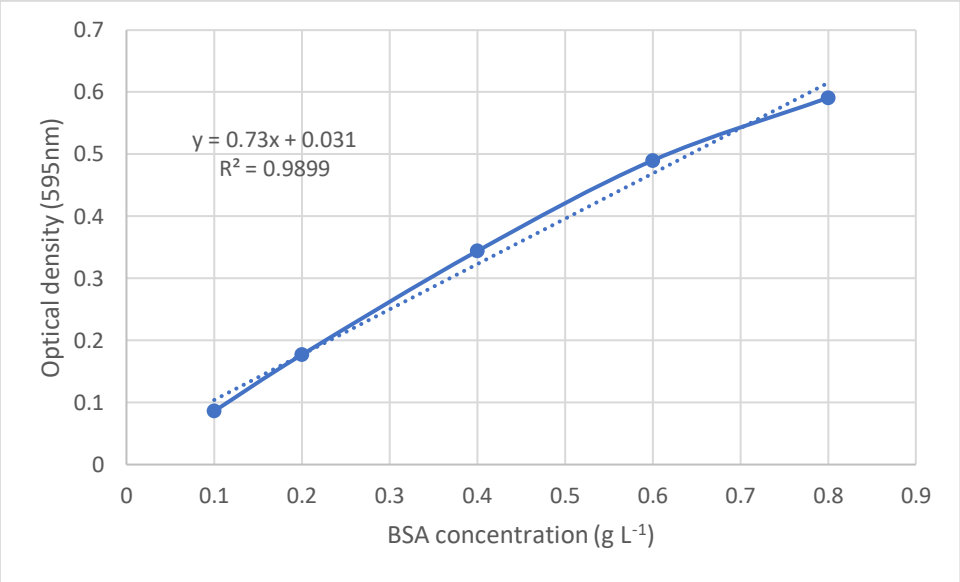

Figure C1 – BSA calibration curve for Bradford assay