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Ana Rita Pontes Pereira

OPTIMIZING THE SCALE UP BIOCHEMICAL PLATFORM BIOREFINERY FOR LACTIC ACID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Dezembro de 2020

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Tese no âmbito do doutoramento em Biociências, especialização em Biotecnologia orientada pelo Professor Doutor José António Couto Teixeira, pelo Professor Doutor António Manuel Veríssimo Pires, e pelo Doutor João Miguel dos Santos Almeida Nunes e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Dezembro de 2020

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Obrigada Diogo por seres o pilar da minha vida, pelo amor, por me fazeres acreditar que é sempre possível!

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Obrigada a todos os meus amigos, ao SPOT e aos de coimbra, pela amizade constante, pela motivação e por acreditarem sempre em mim!

Dedico este trabalho aos meus pais.

Rita

STATEMENT OF INTEGRITY |

I hereby declare having conducted this entire work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

ABSTRACT |

Optimizing the scale up biochemical platform biorefinery for lactic acid production from lignocellulosic biomass

Lignocellulosic biomass, such as forest and agriculture residues or dedicated energy crops, is a promising renewable feedstock for the production of advanced biofuels and chemical building blocks. Lactic acid (LA) has been identified as one with high potential, playing an essential role in industrial applications ranging from the food industry to life-sciences. Moreover, LA is widely used for producing green, biodegradable and biocompatible polylactic acid polymers (PLA).

In order to develop an efficient process for the production of LA from lignocellulosic biomass, complementary to the selection of the biomass, process optimization must be carried out. For this, three main operations have to be considered - (1) biomass pretreatment, (2) enzymatic saccharification to obtain fermentable sugar by cellulases and (3) the fermentation of sugars by suitable microorganisms to lactic acid.

The selection of the raw material as well as the development of the main process operations are the focus of this work.

The selection of the raw material was focused on evaluating two mixtures of lignocellulosic biomass (M1-4 and M2-3), forest ecosystems and biological resources from marginal land, in order to co-produce oligosaccharides, solid fuel and glucose under a biorefinery concept. The selection of mixtures was based on different criteria, namely, territorial distribution, fire risk during summer months and total sugar content. The two mixtures were submitted to autohydrolysis pretreatment under non-isothermal conditions (in the range of 190 °C - 240 °C corresponding to severity of 3.71 to 4.82). Both mixtures were compared in terms of fractionation (cellulose and lignin recoveries and hemicellulose solubilization) and for enzymatic susceptibility of cellulose. The highest xylan recoveries (62 and 69 %), as xylose and xylooligosaccharides, were achieved for both mixtures in the liquid phase at 206 °C. Moreover, enzymatic susceptibility of these pretreated mixtures was also improved from 45 to 90 % of glucose yield by increasing pretreatment severity and at 206 °C glucose yield from enzymatic hydrolysis resulted in 60.1 % and 73.7 % for M1-4 and M2-3, respectively, these results led to the selection of the mixture M2-3 for further processing.

The solid fraction of M2-3 resulted from autohydrolysis (AM2-3) at 206 ºC was subsequently delignified by uncatalyzed ethanol-organosolv process to recover hemicellulose, cellulose and lignin in separate streams. Three factors were evaluated in the experimental design of organosolv process: ethanol concentration (30–80%), temperature (160–200 ºC) and time (20–60 min). Organosolv process showed that the best compromise between lignin removal and cellulose preservation was obtained at highest temperature and ethanol concentration (p-value of 0.05). Maximal delignification (49.40%) was obtained at the highest severity condition (200ºC, 60 min, 80 % EtOH). Moreover, 35.32 g/L glucose, corresponding to a glucose yield of 49.65 %, was produced from enzymatic hydrolysis of delignified biomass. FTIR analysis of the isolated lignins (OL1–OL10) showed that the main lignin structure was not changed, while thermal analysis revealed T_g values from 73 to 85 ºC. All OL presented radical scavenging activity as high as the commercial antioxidant BHT.

Considering the glucose yield of solid fraction from AM2-3 and from organosolv, the last one did not increase enzymatic susceptibility and for this reason the following processes did not include this step. Whereas enzymatic susceptibility improved by increasing pretreatment severity, M2-3 was presented to autohydrolysis pretreatment at 226 ºC. The solid fraction (AM2-3) obtained was submitted to separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) for LA production. LA yield on glucose obtained for both assays was 1 g/g, although the volumetric productivity of SSF (2.5 g/Lh) was higher than SHF (0.8 g/Lh). Therefore, the SSF process was optimized through a factorial design to evaluate the effect of independent variables, solids load and enzyme-substrate ratio (ESR), on LA production. The maximum concentration of LA was obtained using the highest solids load (16 %) and with the highest ESR (54 FPU/g). Finally, scale up of LA was performed in a bioreactor under the optimized conditions in Erlenmeyer flasks, being obtained 61.74 g/L of LA at 44 h which corresponds to LA yield of 0.97 g/g.

In order to present a quantitative evaluation of the environmental loads associated with LA production from M2-3, it was compared with the lactic acid production from non-renewable resources and modeled using the Life Cycle Assessment method through SimaPro®. The life cycle approach took into account the raw material, transport,

pretreatment, saccharification and fermentation and LA recovery considering 1 tonne of LA as the functional unit. The major environmental savings obtained by replacing one tonne of fossil-based LA by biobased LA are : 4056.60 kg CO_2 eq. of global warming potential; 193.03 kBq U235 eq. of ionizing radiation potential; 3.78 kg C_2H_4 eq of photochemical oxidation potential; 0.73 kg PO4 3- eq freshwater eutrophication potential; 9569.40 kg 1,4-DB eq. of terrestrial ecotoxicity potential; 99.32 kg 1,4-DB eq. of fresh water aquatic ecotoxicity potential; 137.69 kg 1,4-DB eq. of marine aquatic ecotoxicity potential; 94.89 human toxicity potential and 126.63 m2 of land use. Auxiliary chemicals, electricity and enzyme used in the biobased LA production are most relevant to the total environmental impacts. Biobased LA production significantly reduces the impact on the environment, giving 60 % environmental savings compared to fossil-derived LA.

The results obtained in this work demonstrate the potential of lignocellulosic biomass as an unexploited raw material for an economical and environmental viable solution to produce lactic acid by fermentation.

SUMÁRIO |

Otimização do scale up da biorrefinaria da plataforma bioquímica para a produção de ácido láctico a partir de biomassa lenhocelulósica

A biomassa lenhocelulósica, como resíduos florestais e agrícolas ou culturas energéticas dedicadas, é uma matéria-prima renovável promissora para a produção de biocombustíveis avançados e químicos de plataforma. O ácido láctico (LA) foi identificado como um de elevado potencial, desempenhando um papel essencial em aplicações industriais, que vão desde a indústria alimentar até às ciências da vida. Além disso, o ácido láctico é amplamente utilizado na produção de polímeros de ácido poliláctico (PLA) verdes, biodegradáveis e biocompatíveis.

A fim de desenvolver um processo eficiente de produção de LA a partir de biomassa lenhocelulósica, complementar à seleção da biomassa, a otimização do processo deve ser realizada. Assim, três operações principais devem ser consideradas: (1) pré-tratamento da biomassa, (2) sacarificação enzimática para obter açúcares fermentáveis através de celulases e (3) fermentação de açúcares por microrganismos adequados ao ácido láctico. A seleção da matéria-prima, bem como o desenvolvimento das principais operações do processo são o foco deste trabalho.

A seleção da matéria-prima centrou-se na avaliação de duas misturas de biomassa lenhocelulósica (M1-4 e M2-3), ecossistemas florestais e vegetação natural, com o objetivo de coproduzir oligossacarídeos, combustível sólido e glucose sob um conceito de biorrefinaria. A seleção das misturas foi baseada em diferentes critérios, nomeadamente a distribuição territorial, risco de incêndio durante os meses de Verão e teor total de açúcar. As duas misturas foram submetidas a um pré-tratamento de autohidrólise em condições não isotérmicas (na gama de 190 ºC - 240 ºC correspondente a uma severidade de 3.71 a 4.82). Ambas as misturas foram comparadas em termos de fracionamento (recuperações de celulose e lenhina e solubilização de hemicelulose) e de suscetibilidade enzimática da celulose. As maiores recuperações de xilanos (62 e 69 %), como xilose e xilooligossacarídeos, foram obtidas para ambas as misturas na fase líquida a 206 ºC. Além disso, a suscetibilidade enzimática destas misturas pré-tratadas foi

também melhorada de 45 a 90 % em rendimento da glucose, com o aumento da severidade do pré-tratamento e, a 206 ℃, o rendimento de glucose da hidrólise enzimática resultou em 60.1 % e 73.7 % para M1-4 e M2-3, respetivamente, esses resultados levaram à seleção da mistura M2-3 para os processos posteriores.

A fração sólida resultante da auto-hidrólise (AM2-3) a 206 ℃ foi subsequentemente deslignificada pelo processo de etanol-organosolv não catalisado para recuperar hemicelulose, celulose e lenhina em fluxos separados. Foram avaliados três fatores no desenho experimental do processo organosolv: concentração de etanol (30-80 %), temperatura (160-200 ℃) e tempo (20-60 min). O processo organosolv mostrou que o melhor compromisso entre a remoção da lignina e a preservação da celulose foi obtido nas condições extremas de temperatura e concentração de etanol (*p*-value de 0.05). A deslignificação máxima (49.40%) foi obtida na condição de maior severidade (200 ℃, 60 min, 80 % EtOH). Além disso, 35.32 g/L de glucose, correspondendo a um rendimento de glucose de 49.65 %, foi produzida a partir da hidrólise enzimática da biomassa deslignificada. A análise FTIR das lenhinas isoladas (OL1-OL10) mostrou que a estrutura principal da lenhina não foi alterada, enquanto que a análise térmica revelou valores de T_g de 73 a 85 ℃. Todas as lenhinas (OL1-OL10) apresentavam atividade antioxidante tão elevada quanto o antioxidante comercial BHT.

Considerando o rendimento de glucose da fração sólida do AM2-3 e do organosolv, este último não aumentou a suscetibilidade enzimática e por esse motivo os processos a seguir não incluíram esta etapa. Considerando que a suscetibilidade enzimática melhorou com o aumento da severidade do pré-tratamento, M2-3 foi submetido ao pré-tratamento de auto-hidrólise a 226 ℃. A fração sólida (AM2-3) obtida foi submetida a hidrólise e fermentação em separado (SHF) e sacarificação e fermentação em simultâneo (SSF) para produção de LA. O rendimento de LA em glucose obtido para ambos os ensaios foi de 1 g/g, embora a produtividade volumétrica de SSF (2.5 g/Lh) tenha sido superior a SHF (0.8 g/Lh). Portanto, o processo SSF foi otimizado através de um desenho fatorial para avaliar o efeito das variáveis independentes, carga de sólidos e relação enzima-substrato (ESR), na produção de LA. A concentração máxima de LA foi obtida com a maior carga de sólidos (16 %) e com a maior ESR (54 FPU/g). Por fim, o aumento de escala do LA foi realizado em biorreator nas condições otimizadas nos frascos

Erlenmeyer, sendo obtido 61.74 g/L de LA às 44 h que corresponde a rendimento de LA de 0.97 g/g.

Para apresentar uma avaliação quantitativa das cargas ambientais associadas à produção de LA a partir de M2-3, esta foi comparada com a produção de ácido láctico a partir de recursos não renováveis e foi modelada utilizando o método de Avaliação do Ciclo de Vida através do SimaPro®. A abordagem do ciclo de vida teve em conta a matéria-prima, transporte, pré-tratamento, sacarificação e fermentação e recuperação de LA, considerando 1 tonelada de LA como a unidade funcional. As maiores poupanças ambientais obtidas através da substituição de uma tonelada de LA de base fóssil por LA de base biológica são : 4056.60 kg CO_2 eq. de potencial de aquecimento global; 193.03 kBq U235 eq. de potencial de radiação ionizante; 3.78 kg C_2H_4 eq de potencial de oxidação fotoquímica; 0.73 kg PO_4^{3-} eq de potencial de eutrofização de água doce; 9569.40 kg 1,4-DB eq. de potencial de ecotoxicidade terrestre; 99.32 kg 1,4-DB eq. de potencial de ecotoxicidade aquática de água doce; 137.69 kg 1,4-DB eq. de potencial de ecotoxicidade aquática marinha; 94.89 potencial de toxicidade humana e 126.63 m^2 de uso da terra. Os produtos químicos auxiliares, eletricidade e enzimas utilizados na produção de LA de base biológica são os mais relevantes para os impactos ambientais totais. A produção de LA de base biológica reduz significativamente o impacto sobre o ambiente, proporcionando 60 % de poupança ambiental em comparação com o LA de origem fóssil.

Os resultados obtidos neste trabalho demonstram o potencial da biomassa lignocelulósica como matéria-prima inexplorada para uma solução económica e ambientalmente viável para a produção de ácido lático por fermentação.

OUTPUTS |

Role:

Chief Research Officer of Association BLC3 - Technology and Innovation Campus

Board Member of Association BLC3 - Technology and Innovation Campus

Papers:

Pontes, R.; Michelin, M.; Romaní, A.; Teixeira, J.; Nunes, J. (2021). Assessment of the organosolv process in a mixture of autohydrolyzed unexploited lignocellulosic biomasses for an effective recovery and valorization of lignin. International journal of biological macromolecules (*under review to International Journal of Biological Macromolecules*).

Pontes, R., Romaní, A., Michelin, M., Domingues, L. Teixeira, J., Nunes, J. 2021. L-lactic acid production from multi-supply autohydrolyzed economically unexploited lignocellulosic biomass. Industrial Crops and Products 170.

Pontes, R., Romaní, Aloia, Michelin, M., Domingues, L., Teixeira, J., Nunes, J. (2018). Comparative autohydrolysis study of two mixtures of forest and marginal land resources for co-production of biofuels and value-added compounds. Renewable Energy 128, 20-29

Book chapter:

Pontes, R., Romaní, Aloia, Michelin, M., Domingues, L., Nunes, J., Teixeira, J. (2020). Biobased fuel and chemicals from lignocellulosic biomass- Prospects & Challenges. In: Mondal, S., Singh, S., Lahir, Y. (eds.), Emerging Trends in Environmental Biotechnology (*submitted*).

Conferences:

Pontes, R.; Michelin, M.; Romaní, A.; Domingues, L.; Teixeira, J.; Nunes, J. (2019). Biomass fractionation of forest and marginal land resources using autohydrolysis and organosolv processes for the lignin valorization (EUBCE 2019), Lisbon, 27-30 May, Portugal.

Pontes, R.; Ribeiro, S.; Alves, N.; Cancela, E.; Figo, S.; Nunes, J. (2019). Assessing the main lignocelluosic biomass resources in Portugal for biobased industries market (EUBCE 2019), Lisbon 27-30 May, Portugal.

Nunes, C.; **Pontes, R**.; Nunes, J. (2019). PinusResina- Evaluation of Higher Heating Value of Pine Resin Waste from Portugal for Energy application valorization (EUBCE 2019), Lisbon, 27-30 May, Portugal.

Pontes, R.; Romaní, A.; Michelin, M.; Domingues, L.; Teixeira, J.; Nunes, J. (2018). Multi-feedstock biorefinery for valorization of forest and marginal land resources: comparative autohydrolysis study (4-CIAB), Jaén, 24-26 October, Spain.

Projects Involved:

Project Biomass4Synthon: Straightening training, research and innovation capacities in the valorisation of bio-renewable resources (H2020-EU: 900 000, 00 Euros-2021 to 2023).

BioVino Project: Development of a cross-border strategy for the eco-sustainable recovery of waste biomass from the wine sector into integral biorefineries for biofuels and bioproducts production. (INTERREG Spain – Portugal; FEDER –European Regional Development Fund: 626 642, 00 Euros – 2018 to 2021).

ValorMais Project: Creation value in agri-food and forestry by-products (PDR2020: 18 500, 00 Euros - 2018 to 2020).

PinusResina Project: Identify / establish new value chains for the competitive and safe transformation and enhancement of *Pinus* resin in products with high added value (PDR2020: 163 839, 03 Euros - 2017 to 2021).

3iBioeconomia Project: Support System for Collective Actions - Transfer of Scientific and Technological Knowledge- New 2nd and 3rd generation integrated Biorefinery

system as a multi-input conversion system into three main outputs: bioenergy, biofuels and bioproducts with high levels of energy efficiency and zero waste (PT2020: 532 216,91 Euros - 2017 to 2020)

Presentations:

Discussing meeting, International Conference of Agricultural, agrifood and forestry by-products valorization- ValorMais Project, Polytechnic Institute of Castelo Branco (November, 2019).

New models of multifunctional forests and ecosystem services – PinusResina Project, RAIZ (October, 2019).

Efficient use of resources and sustainability through innovation - 3iBioeconomia Project, Tagus Valley (May, 2018).

PinusResina Project, Competence Center of Pinheiro-Bravo, Coimbra Agricultural School (May, 2018).

Application of new technologies, Smartagrifor Norte, School of Biotechnology (November, 2017).

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Symbols

T_{MAX}	Final temperature	°C
S_0	Severity	
SY	Solid yield	g/g
Y_G	Glucose yield	%
$Y_G(t)$	Glucose yield at time t	%
$t_{1/2}$	Time needed to reach 50% of glucose yield	h
G_t	Glucose concentration at time t	g/L
Y_L	Lignin yield	%
EE	Lignin extraction efficiency	%
DE	Delignification Extend	%
T	Temperature	°C
E	Ethanol Concentration	v/v
t	Time	h
G_{POT}	Glucose potential	g/L
T_g	Glass transition	°C
RSA	Radical Scavenging Activity	%
T_{onset}	Temperature of degradation	°C
$Y_{L/G}(t)$	Glucose to lactic acid yield at time t	g/g
$L(t)$	Lactic acid concentration at time t	g/L
$Y_{L/RM}(t)$	Raw material to lactic acid yield at time t	g/g
$Qp(t)$	volumetric productivity at time t	g/L h

Abbreviations

ADP	Abiotic Depletion Potential
AM	Autohydrolyzed Mixture
AP	Acidification Potential
ArOS	Arabinooligosaccharides
AcGOS	Acetyl groups
CBM	Carbohydrate Binding Module
CFCs	Chlorofluorocarbons
DSC	Differential Scanning Calorimetry
EH	Enzymatic Hydrolysis
EMP	Embden-Meyerhof-Parnas
EP	Eutrophication Potential
ETLA	Volatile Ethyl Ester
ESR	Enzyme-Substrate Ratio
F	Furfural
FPU	Filter Paper Units
FTIR	Fourier Transform Infrared spectroscopy
FWAETP	Freshwater Aquatic Ecotoxicity Potential
GOS	Glucooligosaccharides
GWP	Global Warming Potential
HCFCs	Halons and Hydrochlorofluorocarbons
HHVs	Higher Heating Values
HMF	hydroxymethylfurfural
HTP	Human Toxicity Potential
HVLV	High-Value Low-Volume
IRP	Ionizing Radiation Potential
KL	Klason Lignin
LA	Lactic Acid
LAB	LA Bacteria
LCB	Lignocellulosic biomass
LCM	Lignocellulosic Materials
LDA	Linear Discriminant Analysis
LU	Land Use
LVHV	Low-Value High-Volume
MAETP	Marine Aquatic Ecotoxicology Potential
ODP	Ozone Layer Depletion Potential
OL	Organosolv Lignin Fractions
OS	Oligosaccharides
PLA	Polylactic Acid
PM	Particulate Matter
POCP	Photochemical Oxidation Potential
PP	Pentose Phosphate
PK	Phosphoketolase
RSA	Radical Scavenging Activity
SHF	Separate Hydrolysis and Fermentation
SSCF	Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
SSR	Sum of squares of residuals
TETP	Terrestrial Ecotoxicity Potential
TGA	Thermogravimetric Analysis
WDP	Water Depletion Potential
XOS	Xylooligosaccharides

CHAPTER 0 |

General Introduction

| Context and motivation

The BLC3 Association (Biomass Lignocellulose and 3-3G, Microalgae) – Technology and Innovation Campus is a non-profit organization that began its activities in 2011, located in Oliveira do Hospital (Headquarters), main structure, Alfândega da fé (Entrepreneurship and R&TD Molecular Biology) and Macedo de Cavaleiros (Entrepreneurship in low density territories and Agrofood) (**Figure 1).**
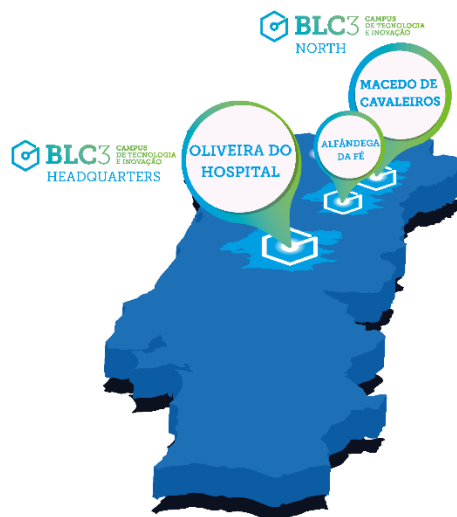
Figure 1- Illustrative map of the BLC3 Association locations.

BLC3 Evolution, Lda is a Spin-off of the BLC3 Association and is born in the pursuit of results and strategic positioning of valuation and industrialization of knowledge in the Waste-to-Value area, focusing on the development of engineering processes (design engineering) for the treatment and recovery of (1) lignocellulosic residues, based on concepts from biorefineries and bioprocesses; (2) and dangerous residues that are difficult to circulate.

The Centre Bio R&D Unit is a new and unique Unit within the BLC3 Association, recently rated "very good".

The members of the BLC3 Association are mainly of a technical-scientific background: Oliveira do Hospital School of Management and Technology, University of Coimbra, National Laboratory of Energy and Geology (LNEG), University of Minho, Porto Catholic University, Oliveira do Hospital Municipality, Institute of Catalysis and Petrochemistry of the Higher Council of Scientific Research of Spain. João Nunes, coordinator of the Centre Bio R&D Unit, is the founder of BLC3 Association and CEO of BLC3 Evolution where he merged the capacities of the Academic and Industrial partners to promote unique synergies for project development.

The BLC3 Association has been extremely engaged in collaborating with several entities and working groups in order to expand its network (55 entities from 9 European Countries). In 2013, BLC3 Association became the first Portuguese representative in the European Commission's Biobased Industries Consortium (BIC), and a member of the National Horizon 2020 Task Force and Analysis in the Societal Challenge 2 area: "Food Security, Sustainable Agriculture, Marine and Maritime Research and Bioeconomy". In 2014, the BLC3 Association integrated the Network of Business Incubators of the Central Region (RIERC) and in 2015 the National Network of Business Incubators (RNIE). It is also part of the RIS3 working group (Strategy of Intelligent Specialization: Center Region) and the network "Mentor" North and Center of Portugal, for entrepreneurship projects.

Its mission is to encourage social and economic growth of rural regions, through innovative and sustainable business initiatives. BLC3 Association is composed of four main MODULES (**Figure 2**) that are articulated to promote technology intensification and development of new knowledge for the territory and industries associated to lignocellulosic biomass and microalgae, namely:

1. "Centre Bio: Bioindustries, Bio-refineries and Bioproducts" technological Infrastructure where R&D activities are developed (main Module I);

2. Projects and Innovative Ideas Support Centre (CAPI) (Module II);

3. Incubator | BLC3 (Module III) and

4. Business Reception Centre (CAE) (Module IV).
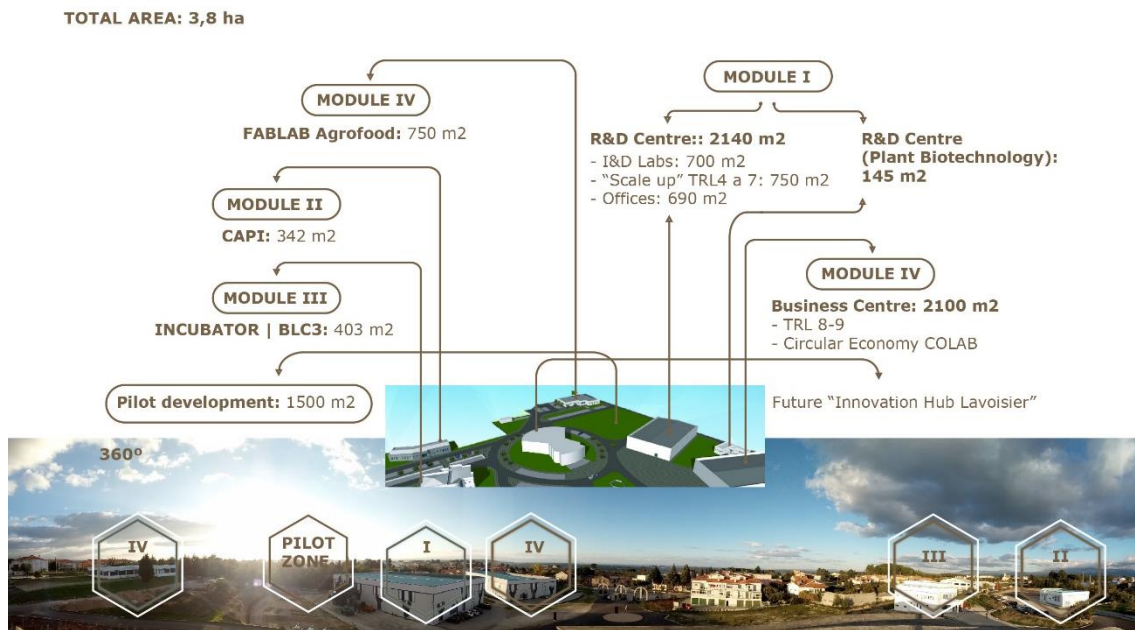
Figure 2- BLC3 Association Main Structure

The BLC3 Awards:

- 2018 (Finalist of the Green Award Project in the category "Efficient Resource Management";

- 2017 (Finalist at the European Commission Best Public Administration For Start Up in the category of Green Entrepreneurship);

- 2016 (most innovative European project winner of the Regiostars prize, European Commission, Sustainable Growth: Circular Economy category);

- 2015 (World Top 25 University Business Incubator and European Top 10 University Business Incubator, in a group of more than 370 incubators from 76 countries;

- 2014 (2nd place in the European Enterprise Promotion Awards (EEPA) in the area of "Supporting the development of ecological markets and resource efficiency".

With the creation of a Centre Bio R&D Unit (with the participation of the ESTGOH/IPC - School of Technology and Management of Oliveira do Hospital of the Polytechnic Institute of Coimbra), the BLC3 Association aims to bring Academia and industry closer together (Academia ↔ Interface (BLC3) ↔ Industry) and to support the transfer of knowledge and technology between higher education institutions and the industry in a targeted and results-oriented manner.

Centre Bio R&D Unit is composed of 29 integrated researchers (12 PhDs) and 11 other collaborators.

The Centre Bio R&D Unit has four main core strategic areas of action (**Figure 3**): 1. Citizenship: General education (including training, pedagogy and didactics for different age students – 3 to 16 years conducted under the Lab-I-DUCA project focused on the Bioeconomy and Circular Economy).

Support the creation of the ESTGOH/IPC Bioindustry Management course. The practical component of the course is lectured within the BLC3 premisses.

2. Energy and Territory: Biorefineries (expanding the sustainable use of the bioresources or biowastes, integrating to the well-known lignocellulosic biomass resources, other mass fluxes such as, agro-industrial, fabric industries, construction and urban effluents), industrial biotechnology, bio-processing technologies (industrial processes that rely on biological agents to promote the process), bioproducts (products manufactured using biological materials as raw material) biomaterials, bioplastics, biofuels, new bio-derived materials, bio-derived chemicals;

3. Agriculture and Food Technology: food engineering and technology; plant biotechnology; mycology; food biotechnology; biomass production technologies; development of high value products (food and pharmaceutical) and

4. Environment and Quality of life: Turnkey solutions for effluent treatment systems, recovery of degraded soils.

Figure 3- R&D Strategic areas.

Within the areas of action of BLC3 Association, the main motivation of this thesis was the development of advanced technologies for biomass conversion and valorisation

in cascade of value through biochemical biorefineries for the production of bioproducts, namely intermediate chemical building blocks, as succinic acid, lactic acid (LA), citric acid 2,3-butanediol, ethanol, n- butanol among others (Kumar and Longhurst, 2018), while simultaneously creating problem-solving solutions (creation of opportunities) in the areas of the energy and environmental sector related to forest residues and innovation strategies to sustainable use of territory ("Bioeconomy solutions"), considering the Circular Economy concepts.

In the business context, LA is an industrially important product with a large and expanding market valued at USD 2.64 billion in 2018 and is expected to grow at a compound annual growth rate (CAGR) of 18.7% from 2019 to 2025 ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.). The global production of LA is due to its multi-function properties, namely in polylactic acid (PLA), food and beverages, industrial, personal care, pharmaceuticals and others ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.). PLA segment emerged as a dominant application in 2018 accounting for 28.3% of the revenue share (**Figure 4**).
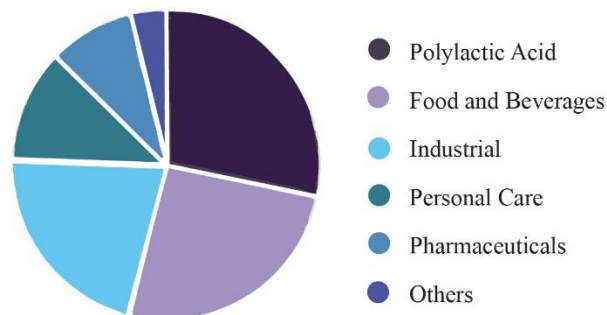
Figure 4- Global lactic acid revenue by application (%) of 2018("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.).

The search for sustainable packaging, as well as the change in consumer behavior paradigm, combined with the pollution generated by the production of plastics from non-renewable sources, is increasingly leading to the utilization of PLA in packing.

In addition, a strict ban on single-use plastics in many countries, such as the U.K., Taiwan, New Zealand, Zimbabwe, and several states of the U.S. (New York, California, and Hawaii), is significantly driving the demand for PLA in the packaging end-use segment. Furthermore, thermal and mechanical properties offered by PLA makes it a

suitable packaging material ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.).

Another large application of LA is in the food industry as it is recognized as safe (GRAS) food substance. LA is found in beverages, candy, meat, sauces, etc. for their mildly acidic taste (Dusselier et al., 2013). Traditional non-food uses of LA can be found in leather tanning, and industrial textile treatments is the third market with greater importance.

Pharmaceutical and personal care were the fastest-growing segment, taking into account PLA properties such as its effectiveness as a natural body constituent and chiral intermediate in pharmaceutical products, along with pH regulation and metal sequestration are likely to be the key factors that will contribute to its increasing use in the pharmaceutical industry (e.g., the use of Na-lactate in dialysis, Ca-lactate in calcium deficiency therapy, anti-acne treatments) (Dusselier et al., 2013).

Currently, LA is produced using different raw materials such as corn, sugarcane, and cassava (**Figure 5**). It has been estimated that about 90% of the LA worldwide production is done through fermentation (Tarraran and Mazzoli, 2018).
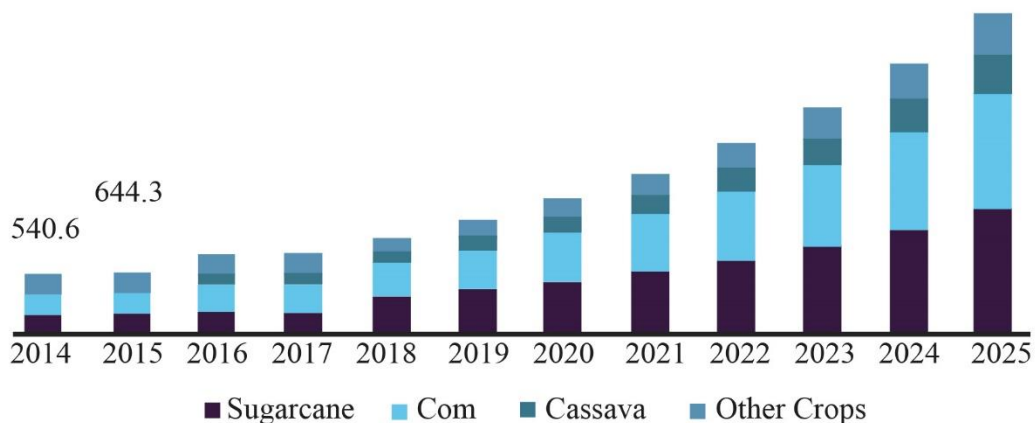
Figure 5- U.S. lactic acid market revenue, by raw material, from 2014 to 2025 (USD Million) ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.).

The viability of LA production by fermentation depends mainly on the cost of the raw materials between several factors. Lignocellulosic biomass (LCB) appears as an attractive option, due to their wide availability, low cost, renewability and no competition with food crops. The annual worldwide production of LCB has been estimated as more than 10^{10} MT (Cubas-Cano et al., 2018), the plant biomass production (90% is LCB) is

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approximately 200×10^9 tons per year, where $8\text{-}20 \times 10^9$ tons of the primary biomass remains potentially accessible (Abdel-Rahman et al., 2011). Several studies suggest the use of LCB as feedstock for lactic acid production. There is a recent trend of using forestry waste, agricultural waste, and industrial waste as feedstock, owing to their cost-effectiveness for large-scale fermentation ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.).

Some of the prominent players in the lactic acid are located on North America. Corbion attained the top market position in 2018, followed by NatureWorks LLC, Galactic, Henan Jindan Lactic Acid Technology Co., Ltd., and Synbra Technology BV ("Global Lactic Acid Market Size & Share Report, 2019-2025," n.d.).

| Research aims

The main aim of this thesis was the optimization of key processes for the scaling up of a biochemical platform to produce lactic acid using forest residues as raw material. More specifically, the objectives of this proposal were:

O1) To study the influence of raw material in the downstream processes. For this purpose, the following different lignocellulosic residues, from natural vegetation and forest ecosystems, were used: Broom (*Cytisus sp*.), Carqueja (*Genista tridentate*), Mimosa (*Acacia dealbata*), Rockrose (*Cistus ladanifer*), Eucalyptus (*Eucalyptus globulus*) and Pine (*Pinus pinaster*). These species are the source of most forest residues in Portugal, which are also responsible for the majority of forest fires.

O1.1) To define different criteria to the use of different biomass mixtures, according to the residual forest biomass mentioned before;

O1.2) To determine the composition of the feedstock in terms of cellulose (as glucan), xylan, arabinan, acetyl groups, klason lignin, extractives and ashes, and to evaluate the fractionation of biomass;

O2) To develop a pretreatment for fractionation of LCB in order to solubilize hemicellulose fraction and reduce the lignin content to improve saccharification taking into account the industrial scale up;

O2.1) To evaluate and optimize operational conditions of autohydrolysis pretreatment;

O2.2) To recover lignin from the autohydrolysis solid fraction by organosolv process, characterize it and evaluate its revalorization;

O3) To optimize Saccharification and Fermentation process of autohydrolysis solid fraction.

O3.1) Optimize different conditions of enzymatic saccharification in order to obtain fermentable sugars of autohydrolysis solid fraction;

O3.2) Optimize SHF and SSF processes;

O3.3) Produce lactic acid in a bioreactor by batch fermentation;

O4) To evaluate the viability of scaling up processes for further pilot and/or industrial applications (considering the technological performance, environmental impact and cost of a biochemical biorefinery over the whole life cycle).

| Outline of the thesis

This thesis is organized in 6 chapters, beyond the chapter 0 general introduction, which covers the research aims stated above, as follows and outlined on **Figure 6**:

- Chapter 1 provides an overview of different lignocellulosic biomass, pretreatments methods, saccharification and fermentation processes to produce lactic acid for further polymerization to polylactic acid.

- Chapter 2 describes the selection of two mixtures (M1-2 and M2-3) of different lignocellulosic biomass, namely, broom (*Cytisus sp.*), carqueja (*Genista tridentata*), mimosa (*Acacia dealbata*), rockrose (*Cistus ladanifer*), eucalyptus (*Eucalyptus globulus*) and pine (*Pinus pinaster*) residues. Biomass were collected in the Center Inner Region and milled, including branches and twigs with bark and leaves, for further use. Analytical assays for characterization were performed. The two mixtures suffered a pretreatment (hydrothermal treatment or autohydrolysis) to alter LCM recalcitrant structure and to improve enzymatic accessibility towards cellulose. Different conditions of maximum temperature

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(190 ºC, 196 ºC, 206 ºC, 216 ºC, 226 ºC and 240 ºC) were evaluated. Different approaches were employed for the interpretation of autohydrolysis data, including severity analysis.

- In Chapter 3, the solid fraction (enriched in cellulose and lignin) resulted from autohydrolysis, was submitted to an organosolv process to separate lignin from cellulose ("delignification"). Organosolv process was performed by different ethanol concentration (v/v), temperature and time. The precipitated lignin was recovered for further valorization. Different organosolv lignin fractions (OL1-OL10) were chemically and thermally characterized by Fourier Transform Infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Moreover, lignin fractions were analyzed for their antioxidant activity.

- Chapter 4 describes the optimization of SHF and SSF using the solid fraction resulting from autohydrolysis at $T_{MAX}226$ ºC. SHF and SSF were performed on Erlenmeyer flasks, with the enzyme (Cellic CTec2) and the microorganism *Lactobacillus rhamnosus*. Lactic acid, acetic acid, glucose and xylose were monitored during 48 hours. The parameters (percentage of solids and enzyme solid ratio) were optimized by a factorial design. After the conditions were optimized, experiments were carried out in an automated bioreactor in batch mode, with approximately 5 L of working volume.

- Chapter 5, defines the environmental impacts evaluated using Life Cycle Assessment method using SimaPro of biobased and fossil based LA production. The ecoinvent database of SimaPro was used for fossil based LA production while the inventory of biobased LA was performed using the results of the lab-scale experiments. The total environmental savings of the impact categories obtained upon replacing a ton of fossil-based LA with biobased LA were assessed and compared.

- In Chapter 6 the main conclusions are summarized and future perspectives from the current work are addressed.
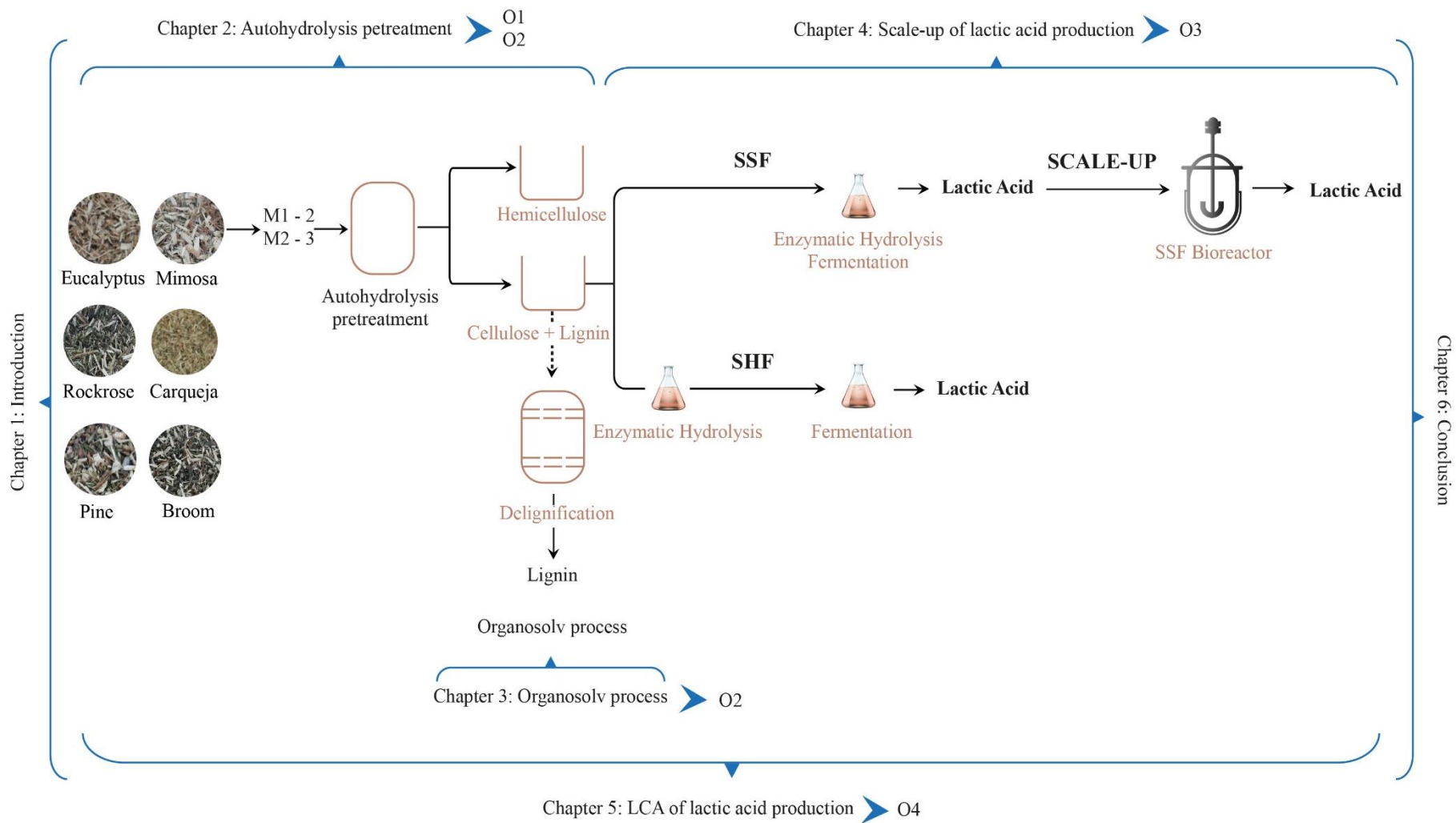
Figure 6- Representative scheme of the outline of the thesis divided by chapters and the proposed objectives (O).

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CHAPTER 1 |

Introduction

Abstract

Lignocellulosic biomass, such as forest and agriculture residues or dedicated energy crops, is a promising renewable feedstock for the production of advanced biofuels and chemical building blocks. Lactic acid has been identified as one with high potential, playing an essential role in industrial applications ranging from the food industry to life-sciences. Moreover, LA is widely used for producing green, biodegradable and biocompatible polylactic acid polymers (PLA).

Its biochemical production includes three main operations: (1) biomass pretreatment, (2) enzymatic saccharification to obtain fermentable sugar by cellulases and (3) the fermentation of sugars by suitable microorganisms to lactic acid. Special emphasis has been given to the recovery and recycling of cellulases because high enzyme costs are still a hurdle for large competitive lignocellulosic lactic acid production. The present chapter reviews the viability and industrial potential of the most recent advances in lactic acid production.

Keywords: Biochemical biorefinery, lignocellulosic biomass, pretreatment, lactic acid, lactic acid bacteria, polylactic acid

Contents

1.1. Brief introduction: Current status

Nowadays, the world energy requirements are largely met by fossil resources. These non-renewable sources are used for the manufacturing of fuels, fine chemicals, pharmaceuticals, detergents, synthetic fiber, plastics, pesticides, fertilizers, lubricants, solvents, waxes, coke, asphalt, among others, to meet the growing demand of the population (Yu et al., 2011). Moreover, the use of fossil resources contributes to the emission of greenhouse gases, increasing the level of carbon dioxide in the atmosphere, consequently contributing to global warming (Naik et al., 2010). During the last three decades, greenhouse gas emissions have already made the global temperature rise 0.6 °C, according to the intergovernmental panel on climate change (Bessou et al., 2011).

The economic development around the globe and the increasing shortage of petroleum reserves, coupled with these environmental problems, greenhouse gas emission and global warming, have boosted demand of sustainable resources for the production of renewable energy (John et al., 2009). Currently, only 10 % of the total energy is supplied by renewable energy including solar, wind and other renewable energy sources (Bessou et al., 2011).

The integrated biorefinery can provide a strong, self-dependent and sustainable alternative for the production of energy and high value-added products that could fulfill the demand for energy and chemicals, as shown in **Figure 7** (Alvarado-Morales et al., 2009).

Generally, a biorefinery includes several processes from biological, enzymatic to chemical technologies, where biomass feedstock is fractionated into main constituting components (cellulose, hemicellulose and lignin) for the conversion of a wide range of valuable products with applications in many industrial sectors, likewise to petroleum refineries (Carvalheiro et al., 2008; Yánez et al., 2014). Nevertheless, in contrast to petroleum refinery, a biorefinery uses renewable resources to produces fuels and chemicals that have lower impact on the environment (Fernando et al., 2006). Biorefineries work as a carbon "catch and release" method: as plants consume CO_2 through photosynthesis, the amount of CO_2 released from the Biorefinery is equal to the amount CO_2 caught by the plant. The development of new manufacturing concepts enables the management of environmental challenges, namely, the decrease in greenhouse gas emissions and the mitigation of global warming, as shown in **Figure 7**.

(González-Muñoz et al., 2012). However, in order to become a viable alternative to petroleum refinery, a biorefinery must be competitive, cost-effective (Ruiz et al., 2011) and must follow sustainable development principles such as: (i) utilization of green processing technologies, (ii) efficiency in the utilization of feedstocks (use of raw material as a whole avoiding waste generation), and (iii) limitation of energy consumption and environmental impact (Gullón et al., 2012).
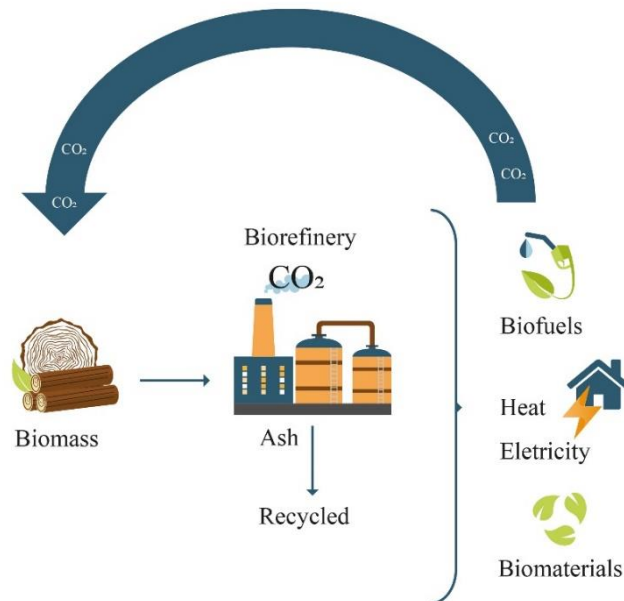
Figure 7- The fully integrated forest-biofuel-biomaterial-energy cycle for sustainable technologies-Biorefinery.

There is a considerable wide range of feedstock which can be used (Carvalheiro et al., 2008). Among the factors that play an important role in the selection of the most appropriate feedstock are geographical location, availability, transport costs and feedstock composition (Alvarado-Morales et al., 2009).

In a biorefinery, biomass can be converted into useful products using a combination of technologies and processes, that produce high-value low-volume (HVLV) and low-value high-volume (LVHV) products through a series of unit operations (Fernando et al., 2006); moreover, it can maximize the economic value of the biomass used while reducing the waste streams produced (Kaparaju et al., 2009). In this field, the profitability prospect from both (i) high value-added compounds in limited amounts and

(ii) lower added-value compounds at large scale fits in the scope of biorefineries (Gullón et al., 2012).

Consequently, a wide range of products can be made with different values and markets. To date, most emphasis has been on fuel production but is clear that there are many opportunities for chemical production, which will become increasingly significant in near future (Alvarado-Morales et al., 2009). Concerning the biobased products, building block chemicals as lactic acid can be used as monomers for the synthesis of biodegradable polymers, including polylactic acid (PLA)(Gross and Kalra, 2002; Lee et al., 2011). Other biobased products include: adhesives, cleaning compounds, detergents, dielectric fluid, dyes, hydraulic fluids, inks, lubricants, packaging materials, paints and coatings, paper and box board, plastic fillers, polymers, solvents and sorbents (Cherubini, 2010).

In this chapter we will introduce the lignocellulosic biomass structure followed by biomass pretreatment and saccharification and then we will discuss the fermentation of lactic acid from microorganisms.

1.2. Lignocellulosic biomass

Lignocellulosic biomass (LCB) is composed of cellulose (40-50 %), hemicellulose (25-30 %) and lignin (15-20 %) (Brethauer and Studer, 2015; González-Muñoz et al., 2012). Cellulose is a crystalline, liner polymer of D-glucose molecules bound together by β-1,4-glycosidic linkages with a high polymerization degree. Cellobiose are two units of glucose joined, since adjacent glucose molecule is rotated 180 º(Brethauer and Studer, 2015). Hemicellulose is a set of branched or linear heteropolymer, amorphous, made up of a variety of different pentoses (C5 sugars; xylose, arabinose), hexoses (C6 sugars; glucose, mannose, galactose) and uronic acid moieties, acetyl groups or esterified phenolic acids, with a polymerization degree several times lower than cellulose (Brethauer and Studer, 2015). Lignin is a three-dimensional, amorphous polymer of complex structure, made up of different phenyl-propane units ρ-coumaryl, coniferyl and sinapyl alcohol (Brethauer and Studer, 2015).
In the plant cell wall, the linear cellulose chains arrange themselves to microfibrils of 3-4 nm diameter held together by strong interchain hydrogen bonds and containing highly ordered crystalline structures as well as amorphous regions. Hemicellulose is attached to

the outside of the microfibrils by hydrogen bonding and is covalently linked to lignin by ester bonds (Brethauer and Studer, 2015).

Additionally, LCB contains non-structural components (including ashes, extractives, pectins and proteins) in lower proportions (Ares-Peón et al., 2013; Romaní et al., 2010).

The constituents of LCB varies between species and are distinct among hardwood and softwood, as shown on **Table 1**, which indicates an appropriated valorization.

Table 1- The contents of cellulose, hemicellulose, and lignin in different types of lignocellulosic biomass (LCB) (% dry weight).

LCB	Type	Cellulose (%)	Hemicellulose[1] (%)	Lignin (%)	Reference
Oak wood chips	hardwood	49.3	25.9	21.7	(Wee and Ryu, 2009)
Acacia dealbata	hardwood	43.03	18.83	22.41	(Yánez et al., 2014)
Switchgrass	-	37.38	23.03	20.65	(Brudecki et al., 2013)
Switchgrass	-	47.72	37.65	26.04	(Jensen et al., 2008)
Clover	hardwood	15.60	10.5	14.4	(Martín et al., 2008)
Ryegrass	hardwood	23.90	17.5	12.8	(Martín et al., 2008)
Aspen	hardwood	52.43	25.85	26.69	(Jensen et al., 2008)
Balsam	softwood	47.09	28.58	36.04	(Jensen et al., 2008)
Basswood	hardwood	43.99	25.12	28.44	(Jensen et al., 2008)
Red Maple	hardwood	43.18	32.90	36.49	(Jensen et al., 2008)
Eucalyptus globulus	hardwood	44.40	21.85	27.70	(Romaní et al., 2010)
Poplar branches	hardwood	40.50	22.48	14.7	(Nitsos et al., 2016)

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[1] Hemicellulose as a sum of xylan, arabinan, acetyl groups, mannan and galactan.

Table 1- The contents of cellulose, hemicellulose, and lignin in different types of lignocellulosic biomass (LCB) (% dry weight) (Cont.).

LCB	Type	Cellulose (%)	Hemicellulose[2] (%)	Lignin (%)	Reference
Vineyard pruning	hardwood	33.8	18.14	25.30	(Nitsos et al., 2016)
Pine wood sawdust	softwood	37.90	19.78	23.90	(Nitsos et al., 2016)
Paulownia tomentosa	hardwood	39.70	17.99	21.90	(Domínguez et al., 2017)
Rice straw	-	40.90	20.46	14.43	(Moniz et al., 2015)
Corncob	-	34.30	37.38	17.7	(Garrote et al., 2002)
Cytisus scoparius	hardwood	40.90	18.90	26.60	(González et al., 2010)
Pinus Pinaster	softwood	39.20	18.90	28.50	(González-Muñoz et al., 2012)
Ulex europaeus	hardwood	41.70	22.30	29.40	(Ares-Peón et al., 2013)
Acacia dealbata	hardwood	43.03	18.96	22.41	(Romaní et al., 2008)

LCB represents an abundant and renewable resource potentially suitable for the sustainable production of ethanol, chemicals, paper, pharmaceutical and biomaterials (Gullón et al., 2012; Ruiz et al., 2011).

1.3. Pretreatment methods

The chemical utilization of LCB can be carried out using two different approaches: (i) utilization as a whole (for example combustion, gasification or pyrolysis), or (ii) using methods based on the selective separation of its components (cellulose, hemicellulose, lignin) (Gullón et al., 2012).

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[2] Hemicellulose as a sum of xylan, arabinan, acetyl groups, mannan and galactan.

For the development of lignocellulosic biorefineries, suitable fractionation making use of environmentally friendly pretreatments is necessary. One of the main objectives of almost all pretreatments is the removal of lignin/hemicellulose to increase the cellulose accessibility for enzymatic hydrolysis or microorganisms (Karimi and Taherzadeh, 2016).

LCB have external and internal surfaces, where the total accessible surface area is the sum of both. External surface could be increased by size reduction, according to a typical physical pretreatment, since it depends on the size and shape. Internal surface depends on the pore size and distributions, and it decreases especially after drying. Thus, a suitable pretreatment should significantly increase the internal surfaces (Karimi and Taherzadeh, 2016) .

Therefore, the pretreatment is considered the first step in a biorefinery since it allows for the disruption of LCB complex structure and facilitates the enzymatic access to polysaccharides. Enzymatic saccharification of polysaccharides produces fermentable sugars able to be used for bioconversion to fuels and chemicals. Hence, two steps should be optimized, namely pretreatment and enzymatic hydrolysis (González-Muñoz et al., 2012).

A suitable pretreatment should fulfill the following requirements: (1) allow high carbohydrate recovery; (2) produce substrates with high digestibility towards the subsequent enzymatic hydrolysis; (3) avoid the formation of byproducts (phenolic acids, furfural or 5-hydroxymethylfurfural) that cause inhibition in the subsequent hydrolysis and/or fermentation, reducing or suppressing detoxification steps; (4) suitable for operation at high solid concentration, enabling the production of hydrolysates with high concentration of sugars; (5) to be cost effective, (6) limited energy consumption, (7) employ green solvents and (8) limited generation of wastes (Gregg and Saddler, 1996; Hama et al., 2015). The multiple methods proposed for LCB pretreatment can be grouped in different categories: physical, chemical, biological or a combination of all (**Table 2**). During the pretreatment, some sugars are converted into toxic inhibitors of microbial growth or enzymes, such as furan derivatives (mostly furfural and 5-hydroxymethylfurfural) and several phenolic compounds (namely vanillin) and from heavily acetylated polymers, as well as acetic acid are also formed, which is the frequent inhibitor present in plant-biomass hydrolysates (Duarte et al., 2013).

Table 2- Pretreatments physical, chemicals, biological or a combination of these

Pretreatment		Method	Advantages	Disadvantages
Physical (Chen et al., 2017; Kumar and Sharma, 2017; Kumari and Singh, 2018)	Mechanical	Dry crushing, wet crushing, vibrating ball mill grinding and comprehension.	Reduce the particle size to expand the contact surface;	Cannot remove lignin and hemicellulose; High cost.
	Microwave	Acceleration of ions and collision with other molecules with an alternating electric field.	Improve cellulose accessibility and reactivity; Enhance ability of lignocellulose feedstock to enzymes; Simple operation; Energy-efficient; Heat in short time;	High cost of equipment.
	Ultrasound	Cell disruption technique.	Improve accessibility and chemical reactivity of cellulose; Decompose hemicellulose	Limited effect on fine structure of cellulose; Negative effect on enzymatic hydrolysis.
	High-energy electron radiation	Emission of rays at the raw material.	Reduction of the degree of polymerization of cellulose; Loose cellulose structure; Improve hydrolysis and conversion rates; Increase moisture; Less pollution; Environmental friendly.	High degree of cellulose structure damage; High cost with high-energy electron radiation; Difficult large scale industrial production.
	High-temperature pyrolysis	Include pyrolysis and liquid hot water decomposition.	Decompose cellulose rapidly; Promote hydrolysis of raw material to oligosaccharides; High hydrolysis efficiency.	Limited microbial fermentation; Low solid content; Large energy consumption.

Table 2- Pretreatments physical, chemicals, biological or a combination of these (cont.).

Pretreatment		Method	Advantages	Disadvantages
Chemical (Chen et al., 2017; Kumar and Sharma, 2017; Kumari and Singh, 2018)	Concentrated acid pretreatment	Acid concentration >30%; 100 °C; 2-10 h; 1 atm.	High sugar conversion rates.	Highly toxic and corrosive acids; High operational and maintenance costs.
	Diluted acid pretreatment	Acid concentration <10 %; 100–240 °C; several minutes; > 10 atm.	Fast reaction; Suitable for continuous production (no need to recycle acid).	High temperature and pressure; Negative influence of degradation products on fermentation.
	Alkali pretreatment	NaOH, KOH Ca(OH)$_2$ and ammonium hydroxide, seconds to days, room temperature, atmospheric pressure.	Room temperature and natural atmospheric pressure; Increase internal area; Decrease polymerization degree and crystallinity; Break the chemical bonds between lignin and carbohydrates; Destroy lignin structure.	Less sugar degradation compared with acid pretreatment.
	Oxidation pretreatment	Degradation of lignocellulose by oxidant (ozonolysis, wet oxidation and photocatalysis).	Ozonolysis: Does not produce toxic substances and inhibitory compounds; Remove lignin at room temperature and normal pressure. Wet oxidation: Effective treatment compared with other methods. Photocatalysis: Cannot affect the final products.	Ozonolysis: High cost with large amount of ozone needed. Wet oxidation: High temperature and high pressure. Photocatalysis: Reduce reaction time.
	Ionic Liquid pretreatment	Cellulose "green solvent", no explosive or toxic gases are formed.	Large temperature range; Break non-covalent interactions of lignocellulose; Minimize products degradation; Environmental friendly.	High cost.
	Organosolv pretreatment	Organic solvents to degrade lignin and to break and decompose the internal chemical bonds of cellulose and hemicellulose.	Obtain pure lignin, cellulose and hemicellulose.	Expensive organic solvents; Effect on environmental and fermentation.

Table 2- Pretreatments physical, chemicals, biological or a combination of these (cont.).

Pretreatment		Method	Advantages	Disadvantages
Physicochemical (Chen et al., 2017; Kumar and Sharma, 2017; Kumari and Singh, 2018)	Steam explosion pretreatment	Hydrothermal pretreatment with high temperature steam; combines chemical effects and mechanical forces.	Lower environmental impact; Lower requirement for reaction conditions; Lower cost investment; Less hazards of chemical reagent; Complete sugar recovery.	High temperature and pressure; Degradation compounds could affect enzymatic hydrolysis.
	AFEX pretreatment	Combination of steam explosion with alkali treatment in liquid anhydrous ammonia with high temperature.	Increase surface area of cellulose; Improve enzyme accessibility; Remove hemicellulose and lignin partly; Absence of inhibitory substances.	High cost; Not efficient for raw material with high lignin content.
	CO_2 explosion treatment	Addition of CO_2 to steam explosion.	Improve enzymatic hydrolysis; Remove lignin; Does not produce inhibitory compounds; Nontoxic; Nonflammable; Easy recovery after extraction; Cost-effective.	High cost (higher than steam explosion and lower than AFEX).
	SO_2 explosion treatment	Similar to CO_2 explosion.	Low optimal temperature; Partial cellulose hydrolysis.	High requirements of the equipment; Large amount of degradation compounds.
	Electrical catalysis pretreatment	Use electrode material, namely titanium-based SnO_2 and CeO_2.	Increase surface area, Remove lignin, Does not produce inhibition compounds; Cleanliness; Cost-effective	Lower efficient.
Biological (Chen et al., 2017; Kumar and Sharma, 2017; Kumari and Singh, 2018)	Use fungi to degrade lignin (wood-rotting fungi)	High delignification efficiency; Mild conditions; Low energy consumption; Absence of pollution.	Need long cycle; Fungi use cellulose and hemicellulose to growth; Low activity of ligninolytic enzymes.	**Biological** (Chen et al., 2017; Kumar and Sharma, 2017; Kumari and Singh, 2018)

To overcome some drawbacks of the aforementioned pretreatment methods, a combination of two or more treatments for improving the process are also reported. An increased efficiency of sugar production, shortened process time and decreased formation of inhibitors is observed when a combination of pretreatment methods has been tried. These combinations of pretreatment would result in more commercial processes and higher yields (Kumari and Singh, 2018)

Considering LCB biorefineries, the key instrument is the efficient utilization of the major feedstock components and the development of viable technologies that allow efficient and clean fractionation to produce multiple commercially products to develop the so-called Bioeconomy (Menon and Rao, 2012; Nunes et al., 2012)

1.4. Lactic acid production by saccharification and fermentation process

There are four stages in production of lignocellulose-based lactic acid: pretreatment, saccharification, fermentation and product purification (Limayem and Ricke, 2012).

After pretreatment, enzymatic hydrolysis is used to convert cellulose and hemicellulose into monomeric sugars. When enzymatic hydrolysis and fermentation are performed sequentially, it is referred to as separate hydrolysis and fermentation (SHF). However, the two processes can be performed simultaneously, i.e. simultaneous saccharification and fermentation (SSF) (Öhgren et al., 2007). The latter is usually described as simultaneous saccharification and co-fermentation (SSCF) when the consumption of different sugars take place (Cubas-Cano et al., 2018).

SHF is the conventional method that allows hydrolysis process to work first to produce monosaccharide sugar, so this is available when the fermentation begins. Through this method, each process should operate at optimum conditions (Dahnum et al., 2015).SSF improves enzymatic hydrolysis because glucose is continuously removed by fermentation as soon is produced, thus preventing the sugars accumulation and enzyme end-product inhibition. Besides the reducing of end-product inhibition, reduces equipment cost, as saccharification and fermentation are performed in the same step and in the same reactor and have lower enzyme requirements (Gregg and Saddler, 1996; Wyman et al., 1992).

In recent years, cellulolytic enzymes have increasingly gained attention for lignocellulose hydrolysis. The main drawback is that the cost of enzymes significantly contributes to the total process cost (Cavka et al., 2014). According to the National Renewable Energy Laboratory's (NREL) 2011 report, the enzyme cost accounts for 15,7% of the total cost considering enzyme loading of 20 mg per gram glucan (Jin et al., 2012).

Enzymatic hydrolysis generally consists of three steps: adsorption of cellulase enzymes onto the surface of cellulose, the subsequent breakdown of cellulose to fermentable sugars and the desorption of the cellulase enzymes into de supernatant (Gregg and Saddler, 1996).The susceptibility of cellulosic substrates to enzymatic hydrolysis depends on (1) cellulose crystallinity, (2) degree of cellulose polymerization, (3) lignin content, and (4) surface area accessible to cellulases (Gregg and Saddler, 1996). There is adsorption of cellulases onto lignin due to hydrophobic interaction lignin-enzyme. Lignin is one of the major barriers in enzymatic hydrolysis, and thus, delignification improves cellulose saccharification, being greater than 90% of theoretical maximum and decreasing enzyme losses, as the solid fiber remaining with the residual lignin is highly susceptible to cellulose hydrolysis (Ruiz et al., 2011).

Although several factors affect hydrolysis rate, such as enzyme inhibition or recalcitrant substrate, complete hydrolysis could be obtained and the majority of cellulose added will be free on the solution (Gregg and Saddler, 1996).

Recovery and recycling of enzymes are the strategies that have been adopted to decrease the cost of overall enzymatic processes (John et al., 2009). An efficient cellulase recycling process should meet the following requirements: (1) high stable cellulase, (2) high hydrolysis efficiency, and (3) good control over the substrate adsorption/desorption processes (Gomes et al., 2015). A vast number of methods have been extensively investigated to recycle enzymes, such as: immobilization (Verardi et al., 2012); membrane separation; chromatography or affinity purification (Rashid et al., 2013); and re-adsorption with fresh substrate (Rodrigues et al., 2014).

Consequently, there are two complementary strategies to recover cellulases, one regarding the fraction of enzymes present in the liquid phase and the other one relating to the solid bound fraction (Gregg and Saddler, 1996; Rodrigues et al., 2014).

Free cellulases in bulk solution may be recovered by promoting its re-adsorption on fresh substrate. It is a simple and low-cost method of enzyme recovery after

lignocellulose bioconversion (Gregg and Saddler, 1996; Guo et al., 2015). Cellulases can be recovered through the exposure to fresh substrate, relying on their high capacity to adsorb the solid residue. Fresh substrate (usually the same amount of the first cycle) is added to the free cellulase suspension and the adsorption occurs (under optimal conditions). Afterwards, the overall suspension is filtered or centrifuged to separate the fresh substrate with bound cellulases from the product of hydrolysis. The solid is resuspended in buffer and supplemented with fresh β-glucosidase, allowing the next round of hydrolysis. The addition of fresh β-glucosidase is mandatory since it lacks the carbohydrate binding module (CBM) and cannot bind to cellulose substrate (Gomes et al., 2015). However, the process cost will increase due to supplementation of β-glucosidase (Guo et al., 2015).

Most cellulases are found free on liquid fraction, however the solid-bound cellulases may also be recovered, almost all desorption methods involve pH shift or addition of chemicals (alcohols or surfactants). The control of pH allows a substantial control over the cellulase adsorption/desorption onto the substrate. Alkaline environment increases the cellulases desorption. The amount of desorbed cellulase increased from 20% with an acidic neutral pH to 80% with alkaline pH (Gomes et al., 2015).

The addition of chemicals also showed influence on binding of cellulases to both lignin and cellulose, with the consequent improvement of hydrolysis and cellulase recovery. Among several detergents, Tween 80 led to enzyme desorption increase. However, polyhydric alcohols (ethylene glycol and glycerol) were efficient in cellulase desorption compared to surfactants (e.g. Tweens, Triton X-100) (Gomes et al., 2015; Guo et al., 2015).

Temperature is the major influence on cellulase recycling efficiency. Cellulase desorption increased when temperature raised from 24 to 45 ◦C but dropped rapidly in the range of 50 to 75 ◦C. Higher temperatures may favor a faster reaction rate but also lead to a faster denaturation (Gomes et al., 2015).

A significant reduction in the cost of cellulases is an urgent requirement to enable an economically sustainable utilization of lignocellulosic biomasses. Enzyme recycling depends on the amounts of lignin and cellulose, the affinity of cellulases for the substrate, the pH, the presence of additives and the temperature and, enzymes recycling is possible only as long as they are stable through several cycles (Gomes et al., 2015; Rodrigues et al., 2014).

LCB is a potential feedstock for the production of organic acids, such as lactic acid. Both cellulose and hemicellulose can be hydrolyzed into fermentable monosaccharides such as glucose and xylose and converted to lactic acid (Maas et al., 2006). Some successful examples of LA production from lignocellulosic biomasses are displayed on **Table 3**. Lactic acid production from renewable biomass is economic and environmental friendly, comparing with the lactic acid production from fossil resources, which is now widely accepted as unsustainable due to resources depletion and the accumulation of environmentally hazardous chemicals (John et al., 2009).

Table 3- Comparison of lignocellulosic feedstocks used for the production of lactic acid by lactic acid bacteria.

Strain	Feedstock	Pretreatment	Fermentation conditions	Concentration lactic acid (g/L)	Yield	Productivity (g/L h)	Reference
Lactobacillus rhamnosus CECT 288	Cellulosic biosludges (from Kraft pulp mill)	-	SSF in Fed.Batch mode 45 ℃ during 48h	39.4	0.36	0.82	(Romaní et al., 2008)
Lactobacillus. rhamnosus ATCC 7469	Paper sludge	-	SSF, incubation at 37 ℃ during 168 h	73.0	0.97	2.90	(Marques et al., 2008)
Lactobacillus sp. RKY2	Oak wood chips	Steam explosion	Enzymatic saccharification Continuous fermentation at 36 ℃	42.0	0.95	6.70	(Wee and Ryu, 2009)
Lactobacillus brevis	Corncob	Dilute-acid hydrolysis	Bath fermentation at 30 ℃ during 48 h	39.2	0.69	0.81	(Guo et al., 2010)
	Corn stover		Bath fermentation at 30 ℃ during 24 h	18.2	0.74	0.76	
Lactobacillus brevis ATCC 367	Corn stover	NaOH treatred	SSF, incubation at 37 ℃ during 36h	16.71	0.54	0.45	(Cui et al., 2011)
Lactobacillus rhamnosus				17.70	0.59	0.49	
Lactobacillus rhamnosus strain CASL	Cassava poder	-	SFF, incubation at 42 ℃ under static conditions during 24h	120.7	0.81	1.7	(Wang et al., 2010)
	Corn powder			138.9	0.97	1.9	
Bacillus sp. NL01	Corn stover	Steam explosion	Enzymatic saccharification Fed-batch Fermentation	75.0	0.75	1.04	(Ouyang et al., 2013)

[a] Volumetric productivity was calculated at 12h of fermentation.

Table 3- Comparison of lignocellulosic feedstocks used for the production of lactic acid by lactic acid bacteria.

Strain	Feedstock	Pretreatment	Fermentation conditions	Concentration lactic acid (g/L)	Yield	Productivity (g/L h)	Reference
Lactobacillus rhamnosus ATCC 7469	Brewer's spent grain	Enzymatic saccharification	Fermentation with yeast extract addition, incubation at 37 ºC during 36h	-	0.82	0.67[a]	(Pejin et al., 2017)
Bacillus coagulans strain IPE22	Wheat straw	Sulfuric acid	Simultaneous saccharification and co-fermentation during 60h	38.73	0.46	0.65	(Zhang et al., 2014)
Lactobacillus plantarum NCIMB 8826	Delignified hardwood kraft pulp (pulverized)	-	SSF in Fed.Batch mode, incubation at 37 ºC	102.3	0.88	2.29	(Hama et al., 2015)
Lactobacillus rhamnosus ATCC-10863	Mixture of softwood pre-hydrolysate and paper mill	Prehydrolysate (Hot-water)	SSF, incubation at 37 ºC during 96h	59.63	0.83	0.62	(Shi et al., n.d.)
Pediococcus acidilactici DQ2	Corn stover	Dry sulfuric acid	SSF, with 8h of prehydrolysis, incubation at 50 º C during 96h	101.9	0.77	1.06	(Zhao et al., 2013)
Lactobacillus pentosus	Sugarcane bagasse	Sulfuric acid	SSF in Fed.Batch mode, incubation at 37 ºC during 96 h	72.75	0.61	1.01	(Unrean, 2018)
Lactobacillus delbrueckii	Cassava starch	Enzymatic saccharification	*Immobilized cells,* Fermentation in continuous mode during 144 h	39.6	0.93	0.33	(John et al., 2007)
Rhizopus sp. MK-96-1196	Corncob	Enzymatic saccharification	*Incubation at 30 ºC during 120 h*	26.00	0.78	0.27	(Miura et al., 2004)
Lactobacillus delbrueckii NRRL B-445	*Eucalyptus globulus*	Delignified in acetic acid-water-HCl	*SSF with addition of fresh substrate and cellulases, incubation at 45 ºC during 124 h*	62.00	-	0.50	(Moldes et al., 2000)

[a] Volumetric productivity was calculated at 12h of fermentation.

Lactic acid (LA) is the simplest hydroxyl acid and presents a hydroxyl group adjacent to the carboxyl group (2-hydroxypropanoic acid, CH3-CH(OH)-COH)) (Cubas-Cano et al., 2018). Due to the chirality of LA, there are two optical forms L(+) and D(-): the first is produced in humans, other mammals and bacterial systems, the other is only produced in bacterial systems (Garlotta, 2002). Pure isomers are more valuable than the racemic mixture, since each one has a specific industrial role. Considering food and pharmaceutical industries, L-LA have a preference as it can be metabolized by the human body due to the presence of L-lactate dehydrogenase. In view of green chemistry, both isomers can be used in the production of acetaldehyde, propylene glycol, propionic acid, acrylic acid, 2,3-pentadione and mainly polylactic acid (PLA) (Cubas-Cano et al., 2018).

LA is industrially produced either by chemical synthesis or by microbial fermentation, 90% of LA produced commercially is made by bacterial fermentation, while only 10% is produced synthetically by the hydrolysis of lactonitrile (Schmidt and Padukone, 1997; Hofvendahl and Hahn-Hägerdal, 2000; John, Madhavan Nampoothiri and Pandey, 2007).

LA bacteria (LAB) are included in the order Lactobacillales by 16S rRNA sequencing (Cubas-Cano et al., 2018). They are a group of Gram-positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major metabolic end product from fermentation of sugars. LAB contains six families with 38 genera and the main species cover *Lactococcus* (*Lc*), *Lactobacillus* (*Lb*), *Leuconostoc* (*Leu*), *Pediococcus* (*Ped*), *Enterococcus* and *Streptococcus* (*Str*). There are also some non-LAB that produce LA with significant yields, namely, *Escherichia coli*, *Corynebacterium glutamicum* and, among them, several *Bacillus* strains (Cubas-Cano et al., 2018). Even as the filamentous fungus *Rhizopus oryzae* is another natural producer, that has the advantage of growing on mineral medium and carbon sources such as starch or xylose (Sauer et al., 2008).

LAB have high acid tolerance and survive pH 5 and lower. Their acid tolerance gives them competitive advantage over other bacteria. The optimal temperature for growth varies between the genera from 20 ºC to 45 º. Most of them are considered GRAS (generally regarded as safe) (Hofvendahl and Hahn–Hägerdal, 2000).

LAB have complex nutrients requirements, due to their limited ability to synthetize B-vitamins and amino acids. Therefore, they are naturally found in nutrient-rich environments such as in plants, milk and inside the human and animal bodies. A

complex natural environment renders microorganism able to metabolize many different carbohydrates (Hofvendahl and Hahn–Hägerdal, 2000).

The metabolic pathway involved in LA production are the Embden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate (PP) pathway and the phosphoketolase (PK). Thus, some LAB have one or more of these metabolic pathways and the conversion yield and energy generated depends on the strain and the growth conditions (Cubas-Cano et al., 2018). Based on this, LAB can be classified as homolactic LAB, obligately and facultative heterolactic LAB (Cubas-Cano et al., 2018).

Homolactic LAB converts hexoses to pyruvate which is reduced to LA, via the EMP pathway. The maximum theoretical yield is two molecules of LA per molecule of glucose, generating two ATP (**Figure 8**) (Cubas-Cano et al., 2018; Maas et al., 2006; Tarraran and Mazzoli, 2018). Nevertheless, some strains have the ability of converting glucose via PP pathway. In this case, the maximum theoretical yield is 1.67 mol LA per mol of glucose (Cubas-Cano et al., 2018). For obligately heterolactic LAB equimolar amounts of LA, carbon dioxide and ethanol or acetate are formed from glucose via the PK pathway, as shown on **Figure 8**. The maximum theoretical yield is 1 mol of LA per mol of hexose or pentose. The facultative heterolactic LAB metabolize hexoses via the EM pathway and pentoses via PK pathway and for this reason, acetic acid is not produced until all the glucose is completely consumed, changing from homo- to heterofermentative. The maximum yield is 1 and 2 mol of LA per mol of pentoses and hexoses, respectively (Cubas-Cano et al., 2018).
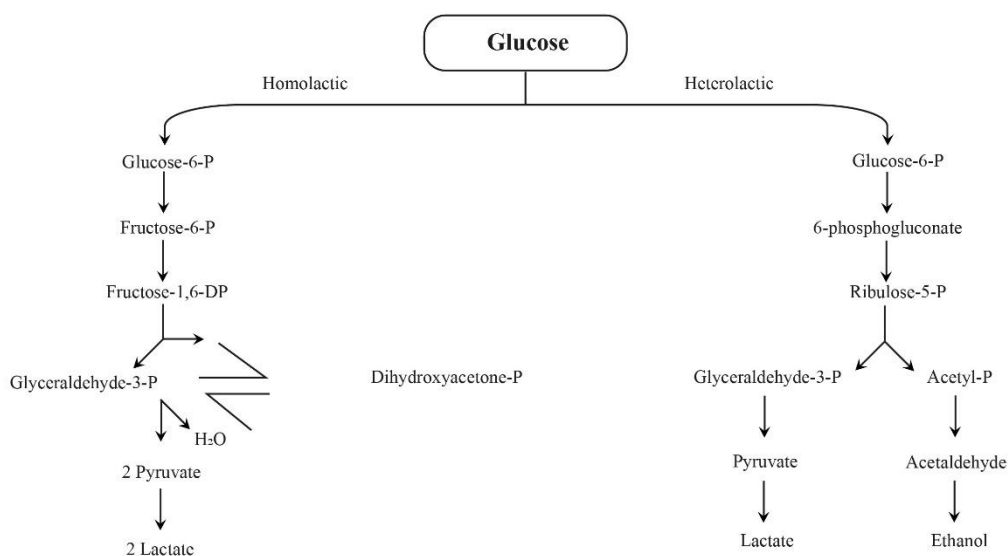
Figure 8- Generalized scheme for the fermentation of glucose to lactic acid bacteria.

The conventional method to produce lactic acid is an anaerobic fermentation in batch reactors, and, in order to keep the fermentation, the acid produced is neutralized with alkali or removed from the fermentation system (John, 2011).

LA is an interesting end-product due to its (i) high market price, (ii) growing demand, (iii) high fermentation yield (theoretical product yield in homolactic fermentations) and (iv) wide range of applications (Moldes, Alonso and Parajó, 2000).

The most important application of LA may yet be as a raw material for producing the polymer polylactic acid (PLA). However, the use of LA has a wide range of applications, such as acidulant, flavoring and preservative in the food, pharmaceutical, leather and textile industries, and for the production of other base chemicals (Schmidt and Padukone, 1997; Hofvendahl and Hahn–Hägerdal, 2000; John, Madhavan Nampoothiri and Pandey, 2007)

1.5. Polylactic Acid

PLA is one of the most important commercial biodegradable thermoplastic polymer. Not only does it has a favorable melting point (around 160–180 °C, below the temperatures at which natural fibers start to degrade), but it is also versatile (many applications in several areas), suitable for melt processing (injection molding), biodegradable, thermoplastic and very strong (González et al., 2010; John et al., 2009). This polymer is an appropriate material for the manufacture of fibers, nonwoven fabrics and films and its use for packing is also starting to gain interest (Cubas-Cano et al., 2018).

Recently, Naturworks LLC, the major producer of PLA, has doubled the production capacity of its PLA plant in Blair (Nebraska, USA) reaching 140.000 metric tons/year. Environmental concerns and legislatives incentives, particularly in the European Union (EU) and the United States (US), tend to promote the use of biodegradable plastics (González et al., 2010).

The mixtures of both isomers (L-LA and D-LA) are used in polymerization of PLA. However, the resulting polymer is amorphous and unstable. Nevertheless, using optically pure L-LA and D-LA, highly crystalline poly-D-(-)-lactic acid (PDLA) or poly-L-(+)-lactic acid (PLLA) is formed (Cubas-Cano et al., 2018).

The synthesis of lactic acid into PLA can follow two different routes of polymerization (**Figure 9**). Lactic acid can be polymerized to yield a low-molecular

weight, and through the use of coupling agents the molecular weight will be increased. The second route is to collect, purify and ring-open polymerize lactide to yield high molecular weight PLA (Garlotta, 2002).
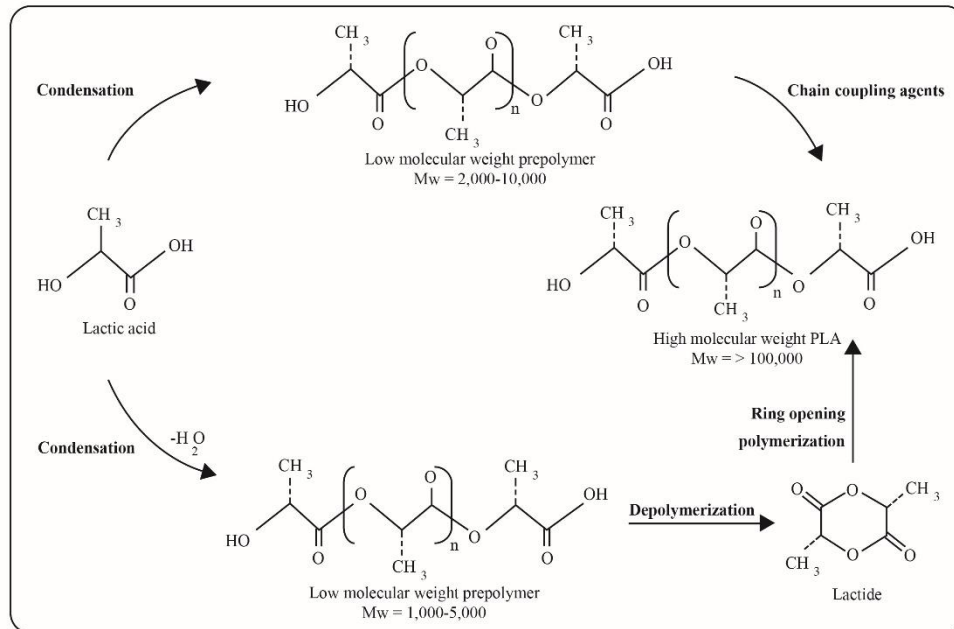
Figure 9- Synthesis routes of PLA.

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1.6. Conclusions

Lactic acid production from lignocellulosic biomass has received a lot of attention in recent years. The cost of the raw material is one of the most significant factors affecting the viability of LA production, making the selecting of the appropriate raw material a key factor for the profitability of the process. From process point of view, choosing the right configuration will increase the yield and productivity. Considering the microorganism, the ability to produce optically pure LA remains one of the major obstacles to reach cost-effective LA production.

Thus, a chemical platform for the valorization of lignocellulosic biomass, aiming at the production of biobased chemicals, with high value, can contribute to reduce the world's dependence on fossil fuels while also increasing the viability of the biorefining industry.

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CHAPTER 2 |

Fractionation of multi-lignocellulosic resources: Autohydrolysis pretreatment

Abstract

This chapter was focused on evaluating two mixtures (M1-4 and M2-3) of lignocellulosic biomass, forest ecosystems and biological resources from marginal land, in order to co-produce oligosaccharides, solid fuel and glucose under a biorefinery concept. The selection of mixtures was based on different criteria, namely, territorial distribution, fire risk during summer months and total sugar content. The two mixtures were submitted to autohydrolysis pretreatment under non-isothermal conditions (in the range of 190 ℃ - 240 ℃ corresponding to severity of 3.71 to 4.82). Both mixtures (AM1-4 and AM2-3) were compared in terms of fractionation (cellulose and lignin recoveries and hemicellulose solubilization), analyzed for thermal properties (high heating values) and for enzymatic susceptibility of cellulose. The highest xylan recoveries (62 and 69 %), as xylose and xylooligosaccharides, were achieved for both mixtures in the liquid phase at 206 ℃. Autohydrolysis pretreatment increased the high heating values of the two mixtures presenting an alternative use of solid fraction as solid fuel. Moreover, enzymatic susceptibility of these pretreated mixtures was also improved from 45 to 90 % of glucose yield by increasing pretreatment severity.

Keywords: Multi-feedstock, lignocellulosic resource, autohydrolysis, enzymatic hydrolysis, biorefinery, solid fuel

This chapter is based on the following paper:

Contents

2.1. Introduction

In Portugal, the territory is divided in wood and uncultivated land (22 %); forest of pure and mixed stands of *Pinus pinaster* and *Eucalyptus globulus* (21 %); and farm land (33 %), mainly composed by olive groves, vineyards and orchards, that generate significant amount of lignocellulosic residues (Duarte et al., 2013). Approximately half of the national territory (40 % to 50 %) consists of poor soils with no potential for profitable agricultural use. On average, 60 % to 70 % of the total fires take place in forested and uncultivated areas, resulting in a loss of roughly 800 million Euros annually (Nunes et al., 2012) . So far, there is no sustainable alternative for the use of this territory and no viable solution for forest residues valorization (Nunes et al., 2012) . Social and economic benefits could be achieved from the utilization of these raw materials in order to develop the so-called bioeconomy, which would boost the creation of new rural jobs (Gullón et al., 2012).

Forest and agricultural residues are the most important sources of lignocellulosic materials (LCM) (Cherubini, 2010) . LCM are the most abundant renewable resource in the world, generated at high rate (González-Muñoz et al., 2012) and suitable for production of energy, biofuels, chemicals, paper, pharmaceuticals and biomaterials (Gullón et al., 2012; Ruiz et al., 2011). LCM are composed by 50 % to 60 % carbohydrates, namely, cellulose and hemicellulose and 10 % to 30% of lignin, together with non-structural components (including ashes, extractives, pectin and proteins) in lower proportions (Ares-Peón et al., 2013; Romaní et al., 2010a, 2013). Nevertheless, the conversion of LCM into chemicals is one of the main challenges for biomass processing due to their complex three-dimensional structure, requiring multidisciplinary approaches to achieve their integrated benefit (González-Muñoz et al., 2012).

The chemical utilization of LCM can be carried out using two different approaches: (i) utilization as a whole (for example combustion, gasification or pyrolysis), or (ii) using methods based on the selective separation of its components (cellulose, hemicellulose, lignin) (Gullón et al., 2012). The latter process can be based on multistep processing, starting with separation of easily recovered fractions (extractives and hemicellulose) from the more resistant ones (cellulose and lignin), which could be further fractioned by means of more aggressive treatments (González-Muñoz et al., 2012).

Hydrothermal pretreatment has been successfully applied to LCM. This eco-

friendly process, also known as autohydrolysis, requires no other reagents than water and high temperature, which enables a wide variety of reactions without the need of a catalyst (Domínguez et al., 2017; Xu et al., 2017). The autohydrolysis reaction solubilizes selectively hemicellulose into oligosaccharides and promotes lower liberation of compounds derived from lignin and cellulose, as well as hemicellulose degradation products (Silva-Fernandes et al., 2015). The main compounds found in the remaining solid fraction are cellulose and sulfur-free lignin. Cellulose can be subjected to enzymatic hydrolysis to produce glucose, an important input for biofuels and biochemicals (Patel et al., 2017; Silva-Fernandes et al., 2015).

The use of feedstock mixtures rather than a single raw material can minimize the problems related to biomass availability, seasonality, price volatility and storage. In this work, broom (*Cytisus sp.*), carqueja (*Genista tridentate*), mimosa (*Acacia dealbata*), rockrose (*Cistus ladanifer*), eucalyptus (*Eucalyptus globulus*) and pine (*Pinus pinaster*) were identified as the most important sources of forest fire cases in Portugal. Since the security supply for biorefineries and the sustainability of exploration are key factors to ensure the industrialization of these systems, the aim of this chapter was to evaluate feedstock mixtures fractionation to supply a biorefinery throughout the year. Two feedstock mixtures were selected and subjected to autohydrolysis treatment in the range of 190 ºC – 240 ºC, in order to evaluate and compare the pretreatment effect on fractionation of feedstock mixtures by hemicellulose solubilization. Besides oligosaccharides, two other alternatives were evaluated for valorization of pretreated feedstock mixtures: solid fuel production and enzymatic saccharification of cellulose into glucose.

2.2. Materials and methods

2.2.1. Raw materials and criteria of feedstocks mixture

Lignocellulosic biomass collecting through the year was divided into four quarters, considering the biomass from 1st and 4th quarters (winter months) as mixture 1-4 (M1-4) and from 2nd and 3rd quarters (summer months) as mixture 2-3 (M2-3). The M1-4 and M2-3 were set with different lignocellulosic biomasses from forest ecosystems (A) (namely, eucalyptus and pine) and from marginal land (B) (namely, broom, carqueja,

mimosa and rockrose). The criteria for the formulation of (A) and (B) were taking into account their proportion of territorial occupation, based on the National Portuguese Forest Inventory (ICNF, 2013). These percentages were considered to establish the proportion of biomass for M2-3. The collection of biological resources from marginal land (B) during winter months (1st and 4th quarters) provide reduction of fire risks in the summer months (2nd and 3rd quarters) therefore, a preference factor of 2:1 of biomass from marginal land (B) to forest ecosystem (A) was considered in order to establish the M1-4 (**Table 4**). The percentage of eucalyptus and pine present in M1-4 and M2-3 was also based on area in the Portuguese territory for these species (Lavoie et al., 2010). For the percentages of biomass from marginal land, the weight ratio among broom, mimosa, carqueja and rockrose was calculated as a function of total sugars content (Duarte et al., 2013), since there is no information available regarding territorial distribution of biological resources from marginal land.

The mixtures M1-4 and M2-3 were prepared, homogenized and then characterized as described below. The raw material resulting from forest management practices, namely, broom, carqueja, mimosa, rockrose, eucalyptus and pine, were collected in the Center Region of Portugal, from a location with the same type of soil. The lignocellulosic materials included branches and twigs with barks and leaves. The raw materials were air-dried, milled and sieved between 0.25 to 0.40 mm using a vibratory sieve shaker (40 and 60 mesh). After that, the samples were homogenized in a single lot to avoid compositional differences among aliquots and were stored in polypropylene bags at room temperature.

Table 4- Proportion of the two mixtures from lignocellulosic resources stablished by quarter

Type Lignocellulosic resources		Mixtures proportion (%)	
		M1-4	**M2-3**
(A) forest ecosystems	Pine (*Pinus pinaster*)	16.6	24.6
	Eucalyptus (*Eucalyptus globulus*)	18.8	27.9
	Total	**35.4**	**52.5**
(B) biological resources from marginal land	broom (*Cytisus sp.*)	18.8	13.8
	mimosa (*Acacia dealbata*)	17.0	12.5
	carqueja (*Genista tridentata*)	14.6	10.7
	rockrose (*Cistus ladanifer*)	14.2	10.4
	Total	**64.6**	**47.5**

2.2.2. Analysis of raw material

Analytical assays were performed according to the following methods: moisture (TAPPI T-206-om-88 m), ash content (T-244-om-93), extractives (TAPPI-264-om-88 m) and quantitative acid hydrolysis with 72% w/w sulphuric acid (T-249-em-85).

The hydrolysates from acid hydrolysis were analyzed by high performance liquid chromatography (HPLC) for sugars (glucose, xylose and arabinose) and acetic acid using the column Aminex HPX-87H (conditions: refractive index detector; flow rate of 0.6 mL/min at 60 ºC; 0.005 M H_2SO_4 as mobile phase) and HPX-87P column for mannose and galactose analysis (conditions: refractive index detector; flow rate of 0.6 mL/min at 85 ºC; H_2O as mobile phase). The concentrations of sugars and acetic acid were employed to calculate the contents of cellulose and hemicellulose. The Klason lignin content was gravimetrically measured from the insoluble solid residue obtained after the quantitative acid hydrolysis. Analyses were carried out in triplicate. The analytical methods used in this work and the scheme of the whole process are shown in **Figure 10**.
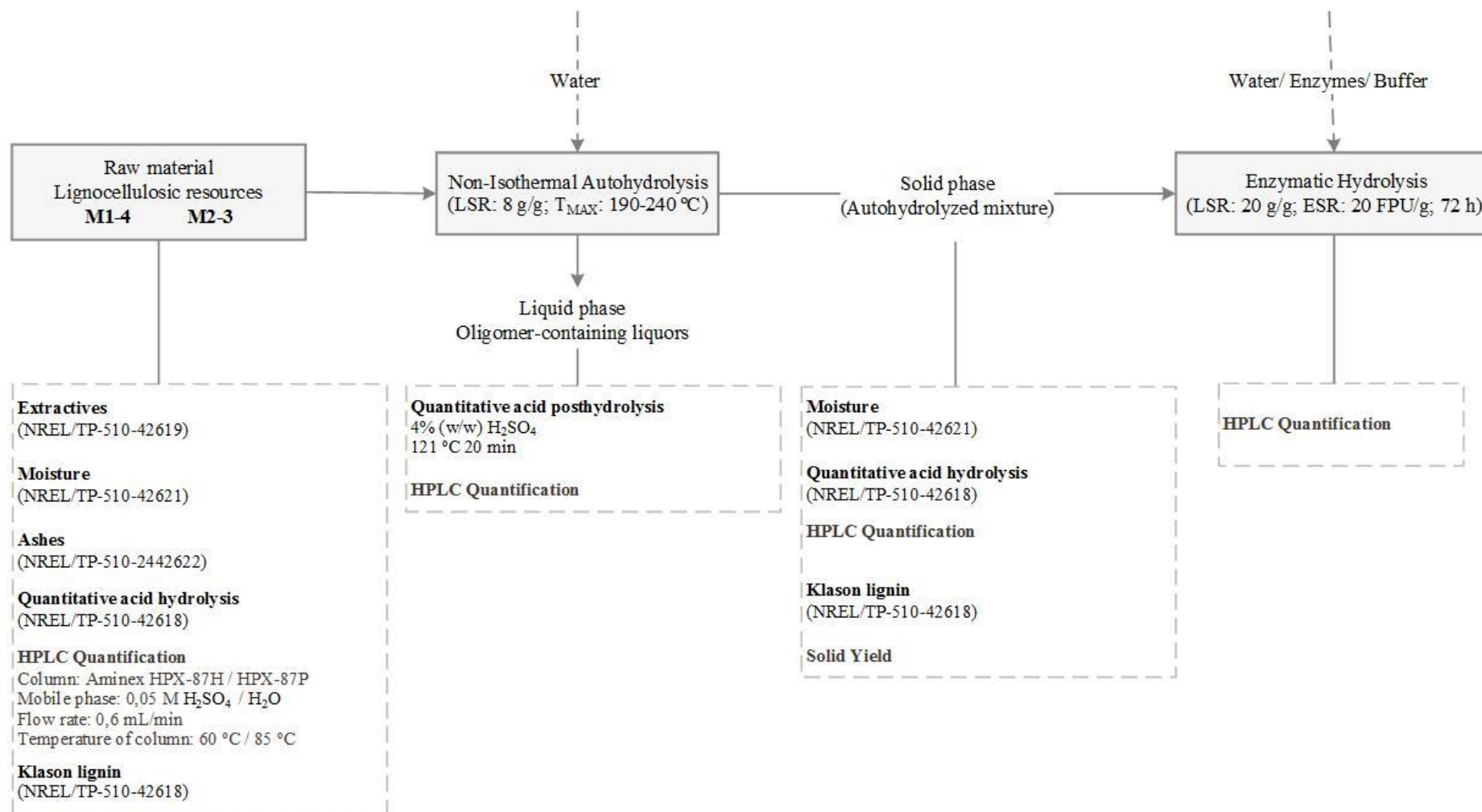
Figure 10- Flow chart of whole process and analytical methods used in this work.

2.2.3. Non-isothermal autohydrolysis pretreatment of the lignocellulosic mixtures (M1-4 and M2-3): solid and liquid phases composition

The autohydrolysis pretreatment was performed in a 2 L stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature controller (model 4848). Each sample (M1-4 or M2-3) was mixed at liquid to solid ratio (LSR) of 8 kg of water/kg of oven-dry raw material. In autohydrolysis experiments, the reaction media was stirred at 150 rpm and heated by an external jacket, following the standard heating temperature-time profile to reach the desired maximum temperature, and the reactor was rapidly cooled-down through water recirculation by an internal loop (**Figure 11**).

For each mixture, several non-isothermal conditions were tested, reaching final temperatures (T_{MAX}) of 190, 196, 206, 216, 226 and 240 ºC. Once the target temperature was reached, the media were immediately cooled and filtered.

The intensity of autohydrolysis pretreatments can be expressed in terms of "severity" (S_0), defined as the logarithm of R_0 (Lavoie et al., 2010), which was calculated using the expression, Equation (1):

$$(1)$$

$$S_0 = \log R_0 = \log \left[R_{0\ HEATING} + R_{0\ COOLING} \right]$$

$$= \log \left[\int_0^{t_{MAX}} \exp \left(\frac{T\ (t) - T_{REF}}{\omega} \right) \, dt \right]$$

$$+ \log \left[\int_0^{t_F} \exp \left(\frac{T'\ (t) - T_{REF}}{\omega} \right) \, dt \right]$$

According to this equation, S_0 includes the combined effects of temperature and reaction time along the periods of heating and cooling. In Eq. (1), t_{MAX} (min) is the time needed to achieve T_{MAX} (K), t_F (min) is the time needed for the whole heating-cooling period, whereas $T\ (t)$ and $T'\ (t)$ represent the temperature profiles in heating and cooling (**Figure 11**), respectively. Calculations were made assuming the values reported in literature for ω and T_{REF} (14.75 K and 373.15 K, respectively). The range of studied temperatures was 190 ºC to 240 ºC corresponding to severities (S_0) of 3.71 and 4.82, respectively.
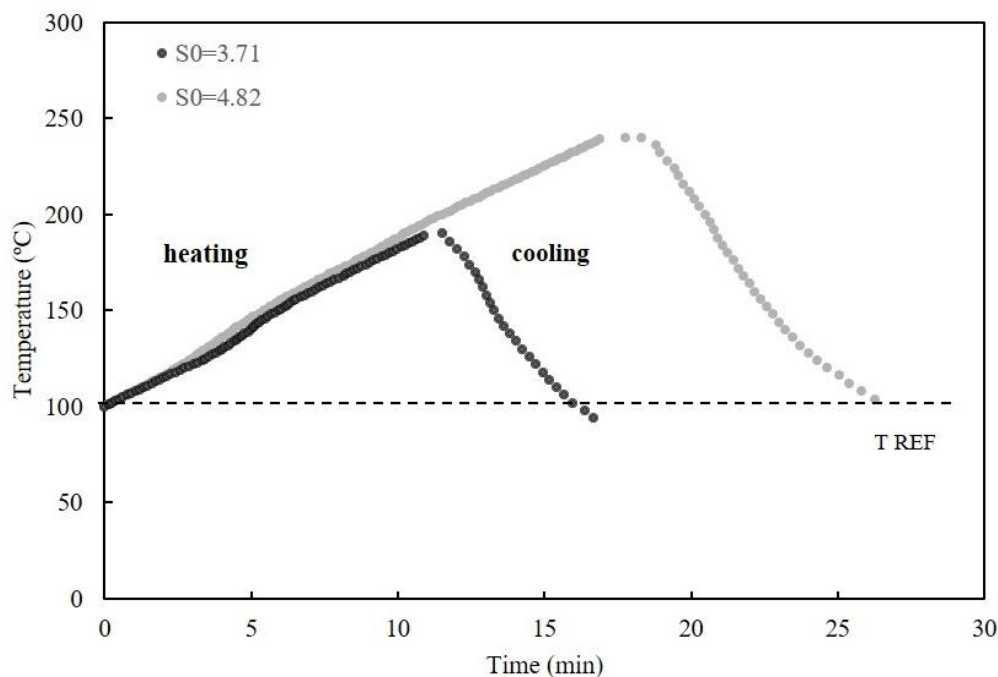
Figure 11-Figure 2- Heating and cooling temperature profiles of autohydrolysis assay carried out at S0 = 3.71 and 4.82. TREF: temperature of reference, 100 ºC.

Operational conditions were evaluated to maximize the concentration of hemicellulose derived compounds in liquid fraction and to improve enzymatic susceptibility of the glucan present in solid fraction.

The solid fraction was washed with distilled water and used to measure the solid yield of the autohydrolysis stage ($SY,$ kg $_{autohydrolyzed\ mixture}$/100 kg $_{raw\ material}$, oven-dry basis) and analyzed for chemical composition as described in Section 2.2. An aliquot of autohydrolysis liquid phase was filtered through 0.2 μm membranes and used for direct HPLC determination of glucose, xylose, arabinose, acetic acid, hydroxymethyl (HMF) and furfural (F), using the same method specified above. A second aliquot was subjected to quantitative acid posthydrolysis (4 % w/w sulphuric acid at 121 ºC for 20 min), filtered through 0.2 μm membranes and analyzed in HPLC for oligosaccharides quantification.

2.2.4. Enzymatic hydrolysis of solid fraction from autohydrolysis pretreatment

Enzymatic hydrolysis (EH) of autohydrolyzed mixtures were carried out at 50 ºC and pH 4.85 (0.05 N sodium citrate buffer) in 100 mL Erlenmeyer flasks with 50 mL of volume in orbital agitation (150 rpm) using Cellic CTec2 (Novozymes, Bagsvaerd,

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Denmark). The enzyme activity was 120 FPU/mL. (measured as described by (Ghose, 1987)). The conditions employed were 5 % of oven-dry autohydrolyzed mixtures, enzyme to substrate ratio, denoted as ESR = 20 FPU/g $_{\text{autohydrolyzed mixture}}$ on dry basis. The reaction time of enzymatic hydrolysis ranged from 0 h to 72 h. At selected times, samples were withdrawn from the media, centrifuged, filtered and analyzed by HPLC for glucose and cellobiose. The results achieved in the EH were expressed in terms of glucose yield (Y_G) (%), calculated using the following Equation (2):

(2)

$$\% \, Y_{G=} \ \frac{[Glucose] + 1.053 \, [Cellobiose]}{1.111 \, f [Biomass]} \ 100$$

Where [Glucose] is the glucose concentration (g/L), [Cellobiose] is the cellobiose concentration (g/L), [Biomass] is the dry biomass (or LCM) concentration (g/L), f is cellulose fraction in dry biomass (g/g), the multiplication factor, 1.053, converts cellobiose to equivalent glucose. In all experiments, cellobiose was not detected.

2.2.5. Determination of higher heating values of autohydrolyzed mixture

Samples of dried biomass were analyzed for Higher Heating Values (HHVs) using an automatic adiabatic bomb calorimeter (Parr calorimeter Type 6200), in accordance with Jessup et al. (R.S. Jessup, 1960) . The interior surface of the bomb was washed with distilled water and collected in a beaker. The bomb washings were titrated with a standard sodium carbonate solution (0.0709 N).

2.2.6. Statistical analysis

Linear Discriminant Analysis (LDA) was computed using STATGRAPHICS Centurion XVI.I, with a significance level of 5 %. The sources of variation for the raw material composition were cellulose (as glucan), xylan, arabinan, acetyl groups, mannan and galactan (as hemicellulose), Klason lignin, ashes and extractives for M1-4 and M2-3.

2.3. Results and Discussion

2.3.1. Raw material mixture criteria

Several lignocellulosic residues have been individually studied to be used as raw material for biorefineries, nonetheless, the availability, seasonality, variability, price volatility and storage of biomass supply may be the major constraints on the use of these raw materials. In this work, the use of different biomass sources may overcome these problems. Thus, the analysis of availability, security supply and seasonality (based on fire prevention) of lignocellulosic resources in Portugal were considered for the mixture definition.

The total territorial area generating residues (ICNF, 2013) within the work focus corresponds to 52.5 % of forest ecosystems (A) and 47.5 % of biological resources from marginal land (B). Since eucalyptus and pine are the main ecosystems in Portugal that generate residues with high potential for the biorefineries, these two species were selected to represent (A) . Whereas their distribution is according to territorial area (ICNF, 2013) , where 725 thousands (46.9%) of hectares correspond to pine and 820 thousands (53.2%) of hectares to eucalyptus. Regarding (B), the selection criteria was based on total sugars content of broom (66.7%), carqueja (51.7%), mimosa (60.4%) and rockrose (50.2%) (Duarte et al., 2013). Based on the different criteria, two different mixtures, M1-4 and M2-3, were established taking into account the higher fire risk in Portugal during the summer months (2nd and 3rd quarters) and the importance of collecting biological resources from marginal land during winter months (1st and 4th quarters). In general, 60 to 70% of forest fires occurs in woods and uncultivated areas (Duarte et al., 2013). Therefore, **Table 5** shows the final proportions of biomasses assembled for M1-4 and M2-3.

The consideration of feedstock mixtures have been previously studied only in few works (Jensen et al., 2008; Silva-Fernandes et al., 2015; Thomsen and Haugaard-Nielsen, 2008). In some of these cases, the criteria of mixture were based on the importance of these raw materials for the region, such as mixture of eucalyptus, wheat straw and olive tree pruning in Southern Europe, prepared in different combinations to be tested (Silva-Fernandes et al., 2015). In other cases, the mixture was prepared in equivalent amounts (clover and ryegrass) (Martín et al., 2008) due to its importance in many agroecosystems

(Martín et al., 2008) , as well three relative proportions of wheat straw and clover-grass were studied (Thomsen and Haugaard-Nielsen, 2008). Moreover, the consideration of more than one species of crops stands and/or forest feedstock supplemented with energy crops (such as switchgrass) to increase biomass yield have been previously studied by Jensen et al., where five species were mixed (50/50 wt.%) in 10 possible combinations (Jensen et al., 2008) .

2.3.2. Chemical characterization of M1-4 and M2-3 mixtures

Chemical characterization (**Table 5**) of the two mixtures proposed was carried out revealing a very similar composition, although the content of each fraction slightly varied according to the contribution of the predominant feedstock (described in **Table 4**). Glucan was the polysaccharide found in higher amount and similar concentrations were found in both mixtures. Among the hemicellulose components, the xylan was found in the highest amount in the two mixtures, reaching 16.58 g/100g $_{raw\ material}$ of M1-4 and 17.48 g/100g $_{raw\ material}$ of M2-3.

Table 5- Chemical composition of feedstock mixtures (M1-4 and M2-3) (expressed in g/100g of raw material in oven-dry basis ± standard deviation on three replicate determinations).

Components	Feedstock mixtures	
	M1-4	M2-3
Cellulose (as Glucan)	34.17 ± 1.14	34.63 ± 0.18
Hemicellulose		
Xylan	16.58 ± 0.62	17.48 ± 0.45
Arabinan	1.36 ± 0.21	1.27 ± 0.03
Acetyl groups	2.13 ± 0.01	1.95 ± 0.29
Mannan	3.19 ± 0.03	3.31 ± 0.01
Galactan	1.43 ± 0.02	1.26 ± 0.03
Klason lignin	30.05 ± 0.00	31.76 ± 0.00
Ash	1.29 ± 0.08	1.19 ± 0.03
Extractives	10.51 ± 0.10	9.23 ± 0.31

Arabinan and acetyl groups were identified, although in lower proportions in both mixtures, approximately 2 g/100g $_{raw\ material}$. Mannan showed concentration around 3 g/100g $_{raw\ material}$ for both mixtures and galactan was detected in low concentrations approximately 1 g/100g $_{raw\ material}$. Klason lignin was the second highest fraction in the mixtures, namely 30 % for M1-4 and 32 % for M2-3. Ashes were quantified and correspond to about 1 % for both mixtures. Extractives correspond approximately to 10 % in both mixtures. As seen in **Table 5**, there were no significant differences regarding chemical composition between M1-4 and M2-3 (p-value > 0.05).

Nevertheless, the study of mixture of different species is still scarce. Previous reports already studied the species that compose M1-4 and M2-3 individually, namely, *Acacia dealbata* (Yánez et al., 2014), *Cytisus sp* (Dusselier et al., 2013) , *Pinus pinaster* (González-Muñoz et al., 2012) and *Eucalyptus globulus* (Romaní et al., 2014). In these studies, the cellulose content (as glucan) was higher than 40 %, while M1-4 and M2-3 presented lower content of cellulose, around 35 %. This fact can be explained due to extractives content, as the mixtures comprised branches and twigs with bark and leaves (which contain high ash content) (Dibdiakova et al., 2015), since the aim of this work was the integral valorization of these lignocellulosic resources.

Despite the similar composition of M1-4 and M2-3, the outcome of pretreatment could be different due to their diverse origin (hardwood, softwood and bush), making it necessary to analyze the pretreatment effect on both mixtures.

2.3.3. Effect of autohydrolysis pretreatment on fractionation of M1-4 and M2-3 mixtures

The conditions of pretreatment (190 ℃ to 240 ℃) were chosen based on reported data by Romaní et al. (Romaní et al., 2010a) and Silva-Fernandes et al. (Silva-Fernandes et al., 2015). For integral valorization of biomass, all fractions should be considered (Gullón et al., 2012). Thus, in this work, fractionation of two mixtures was evaluated in order to recover the hemicellulose as oligosaccharides and to improve the enzymatic saccharification of cellulose and/or use the solid fraction as solid fuel.

2.3.3.1. Solid phase composition after autohydrolysis pretreatment

Chemical composition of solid phase after autohydrolysis pretreatment is shown in **Table 6**. The solid yield (SY) decreased with severity increase and varied from 62.37 - 75.25 g /100 g raw material for M1-4 and 62.33 - 76.70 g/100 g raw material for M2-3, which is in agreement with previous works under similar conditions for other hardwoods (Domínguez et al., 2017; Romaní et al., 2014).
The glucan content varied in the range of 40.96 – 47.00 g glucan/100 g autohydrolyzed mixture (on dry basis) for pretreated M1-4 and 41.07 - 48.47 g glucan/100 g autohydrolyzed mixture (on dry basis) for pretreated M2-3. Thus, the percentage of glucan that remained in the solid fraction was very similar after pretreatment for both mixtures, presenting an average of glucan recovery about 84.86 % and 88.68 % (expressed as g of glucan per 100 g of autohydrolyzed mixture) for M1-4 and M2-3, respectively, which reveals the selectivity of this pretreatment.

In addition, the content of lignin after pretreatment varied in the range of 35.76 - 47.82 and 37.04 - 44.56 g lignin/ 100 g autohydrolyzed mixture of M1-4 and M2-3, respectively. The average recovery was high for the two mixtures, 96.36% and 90.36% (expressed as g of lignin per 100 g of autohydrolyzed mixture) for M1-4 and M2-3, respectively.

However, lignin content followed a typical pattern for both mixtures, up to a temperature of 206 ℃ the remaining lignin in the solid fraction increased, from this temperature forward, lignin content decreased and it increased again under the most severe condition (240 ℃). This behavior was also studied by Moniz et al. with autohydrolyzed rice straw where until 210 ℃ the remaining lignin was close to 100 % of the initial amount, from 210 ℃ onwards showed a decreased around 30 % and it increased again for the most severe conditions (Moniz et al., 2015). The lignin increase is common of autohydrolysis pretreatment due to condensation reactions between lignin, sugars and degradation products (HMF and F) leading to the formation of insoluble compounds that are quantified as Klason lignin (Pereira Ramos, 2003; Romaní et al., 2010a)

Under most severe conditions (S_0=4.15) glucan and lignin represent more than 84% of the solid fraction, and the combined amounts of these fractions matched the one contained in the raw material. These results are comparable with the results obtained by Silva-Fernandes et al. in which at the same conditions glucan and lignin contain 85% of the solid fraction (Silva-Fernandes et al., 2015).

The hemicellulose in the pretreated mixtures, namely, xylan showed a steadily decrease with the severity of pretreatment and it was the most solubilized fraction, since it was totally solubilized for both mixtures at temperatures higher than 226 ºC. The same was reported by Silva-Fernandes et al. in which under most severe conditions 93-95% of xylan was solubilized in liquid phase (Silva-Fernandes et al., 2015). Patel et al. studied a different pretreatment (dilute acid pretreatment) to solubilize the hemicellulose fraction in which revealed that almost, all the hemicellulose content was hydrolyzed, obtained only 0.4 % in the solid fraction. However, this pretreatment is not ecofriendly, since it requires an additional detoxification step, increasing the process cost (Patel et al., 2017). The data described above indicate that autohydrolysis pretreatment in an appropriate process for the selective fractionation of both mixtures in which showed high hemicellulose solubilization, directly proportional to autohydrolysis severity, while cellulose and lignin were usually retained in the solid fraction.

2.3.3.1. Composition of liquid phase resulting from autohydrolysis pretreatment

Autohydrolysis process allows substantial fractionation of components, namely oligosaccharides, monosaccharides, acetyl groups from hemicellulose, and degradation products of released sugars as furfural and hydroxymethylfurfural.

The liquid phase composition of the two mixtures (M1-4 and M2-3) is presented in **Table 6**, in which the products recovered were represented in three groups: oligosaccharides (OS), including glucooligosaccharides (GOS), xylooligosaccharides (XOS), arabinooligosaccharides (ArOS) and acetyl groups (AcGOS); monosaccharides, as well as glucose, xylose and arabinose; and other by-products such as organic acids (acetic acid) and furans (HMF and F).

Based on the previous reports (Romaní et al., 2010a), the concentrations (in g/L) of the liquid phase derived principally from hemicellulose fractions. Hence, the main compounds were XOS and xylose. The maximal XOS concentrations (10.8 g/L and 14.3 g/L) were obtained at 190 ºC and 206 ºC for M1-4 and M2-3, respectively, representing 47.7 % and 58.1 % of the total compounds presented in the liquid phase. Therefore, at these conditions of autohydrolysis pretreatment, 47.4 % and 59.8 % of xylan solubilization was recovered as XOS for M1-4 and M2-3, respectively. These results can be compared with reported data in literature in which 60 % of the compounds identified

in the liquid phase were XOS, achieved at maximal concentration, at S_0 of 3.99 (Domínguez et al., 2017).

At more severe conditions $S_0 > 4.15$ the concentration of XOS decreased until reached 0.33 g/L and 0.49 g/L for M1-4 and M2-3, respectively. XOS started to degrade into xylose, in which M1-4 achieved 7.9 g/L of maximal xylose concentration at 226 ºC ($S_0=4.60$) and M2-3 obtained 3.5 g/L at $S_0= 4.38$.

The highest xylan solubilization as a sum of xylose and XOS (62.2 % and 68.6 % for M1-4 and M2-3, respectively) was obtained at $T_{MAX} = 206$ ºC for both mixtures. This result is consistent with Romaní et al. (Romaní et al., 2010a) in which, at mild conditions ($T_{MAX}=210$ ºC) 76 % of xylan can be recovered as xylose and XOS.

Consequently, the highest furfural concentration was 2.4 g/L for M1-4 and 2.9 for M2-3 g/L at $S_0= 4.82$. The highest HMF concentration was also found at the same severity, in which 1.1 g/L was obtained for both mixtures. Acetic acid raised the maximum at $S_0 > 4.38$ of 4.9 g/L and 4.7 g/L for M1-4 and M2-3, respectively. The harsher conditions of pretreatment led to an increase of inhibitor compounds, as F, HMF, and acetic acid.

Table 6- Solid yield and chemical composition of pretreated solids and liquid fraction of mixtures M1-4 and M2-3 after autohydrolysis pretreatment

Yield and Components	Temperature (ºC) or S_0											
	190 or 3.71		196 or 3.93		206 or 4.15		216 or 4.38		226 or 4.60		240 or 4.82	
	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3
Solid yield (g/100g raw material on dry basis)	75.25	76.70	73.93	75.8	67.61	71.24	62.15	65.04	62.43	66.78	62.37	62.33
Solid phase composition (g/100 g autohydrolyzed mixture on oven-dry basis)												
Gn (glucan)	40.96	41.07	41.96	40.59	42.12	41.15	47.0	46.69	44.09	48.47	43.13	48.03
Xn (xylan)	6.97	6.64	4.1	2.59	1.26	2.3	1.52	2.72	0	0.72	0	0
KL (Klason lignin)	35.76	37.04	40.72	39.15	47.44	43.17	44.7	40.75	44.09	43.53	47.82	44.56
Liquid phase composition (g/L)												
GOS (Gluco-oligomers)	2.36	1.35	0.47	1.51	0	1.72	0	1.62	0.08	0.87	0.89	0.8
XOS (Xylo-oligomers)	10.82	10.51	10.15	13.59	9.35	14.33	5.68	10.37	0.23	0.95	0.33	0.49
ArOS (Arabinosyl moieties in oligomers)	2.92	0.92	0	0.09	0	0	0	0	0	0.05	0	0.05
AcGOS (Acetyl groups in oligomers)	4.12	2.09	2.81	2.31	3.14	2.17	3.72	1.54	3.7	0.52	0	0
G (Glucose)	1.18	0.98	2.41	1.13	3.06	1.07	2.87	0.97	2.11	1.23	0.63	0.6
X (Xylose)	0.51	0.5	2.98	1.63	4.72	2.11	7.17	3.45	7.94	2.06	0.28	0.41
Ar (Arabinose)	0.66	0.93	2.83	1.59	4.09	1.76	2.22	1.27	1.08	0.1	0.12	0.07
AcA (Acetic acid)	0	0	0.17	0.85	0.36	1.15	0.57	2.15	0.93	4.68	4.85	4.42
HMF (Hydroxymethylfurfural)	0.06	0.01	0.08	0.11	0.19	0.14	0.32	0.17	0.58	0.21	1.13	1.12
F (Furfural)	0.05	0	0.06	0.19	0.30	0.21	0.81	0.31	1.77	1.09	2.35	2.91

The concentration of F is higher than HMF, because the first is attributed to the degradation of xylose while HMF is obtained through C6 degradation, namely glucose. As mentioned before, glucose was also present in low amounts varying between 0.6 to 3.1 g/L for M1-4 and 0.6 to 1.2 g/L for M2-3, which represented on average 3.6 % of glucan solubilization for M1-4 and 1.7 % for M2-3. The results obtained in this work re in agreement with reported data using *Paulownia tomentosa* wood in which <4 % of glucan was recovered in liquid phase (Domínguez et al., 2017). As seen in **Table 6**, M1-4 and M2-3 showed differences in XOS and xylose concentration. This fact was probably influenced by the intrinsic features of lignocellulosic material, since M1-4 is composed by a higher percentage of residues from bush (as broom, mimosa, carqueja and rockrose) than M2-3. The main fractions recovered in the liquid phase, xylose and XOS, can be used for value-added compounds production as xylitol, lactic acid and ethanol obtained by fermentation and/or directly as prebiotic (Costa et al., 2017; Gullón et al., 2014; Ma et al., 2016).

As previously reported, at $T_{MAX} = 206 °C$, high percentages of hemicellulose were removed but also primary degradation products (F and HMF) were kept at relatively low levels, which could be achieved by applying pretreatment conditions of moderate severity. Although pretreatment improves enzymatic access to cellulose for further fermentation, it generates byproducts decomposition which may affect negatively fermentation (Siqueira and Reginatto, 2015).

2.3.4. Energy production of pretreated M1-4 and M2-3 mixtures

HHVs of pretreated mixtures were analyzed in order to evaluate the influence of pretreatment and compare their behavior as solid fuel. These results are displayed in **Figure 12**. The untreated mixtures were analyzed and showed HHVs of 17.23 MJ/kg and 17.26 MJ/kg for M1-4 and M2-3, respectively. HHV of pretreated samples increased with severity, achieving maximal values of 20.4 MJ/kg and 20.5 MJ/kg, for M1-4 and M2-3, respectively. The results obtained in this work are in agreement with reported data using softwood chips in which the HHV of the original wood was 17.9 MJ/kg and with temperature increase (autohydrolysis pretreatment) reached 20.5 MJ/kg (Pu et al., 2013). Leaching processes with water and acetic acid were also used to increase the HHVs of

six different biomasses (fast growing timber species and oil pal biomass), achieving values in the range of 16.52-18.47 MJ/kg (Chin et al., 2015).
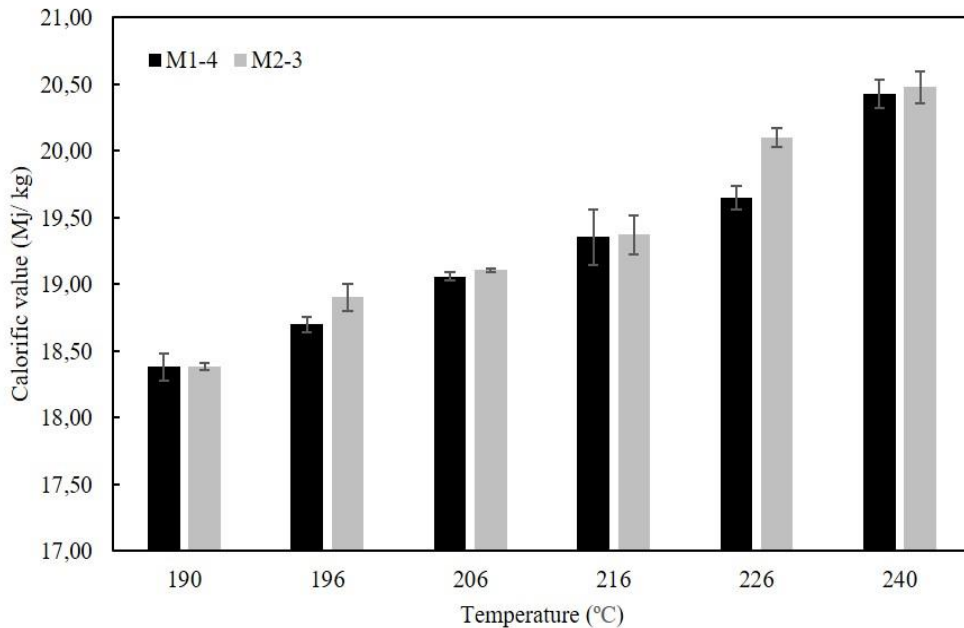
Figure 12- Evaluation of calorific value (MJ/kg) in different T_{MAX} for M1-4 and M2-3.

This behavior is related to the increase of lignin content in the samples (**Table 6**) as a consequence of temperature rise, since lignin presents higher calorific value (20.4 MJ/kg) than cellulose (16.5 MJ/kg) and hemicellulose (13.9 MJ/kg) (Kim et al., 2016; Pu et al., 2013). The HHVs are higher for raw materials as hardwoods and softwoods than for non-wood biomass being linearly related with lignin content (Demirbaş, 2000).

The HHV obtained in this work showed suitability of these mixtures as solid fuels when compared with other biomasses (López et al., 2012). Nevertheless the use of these mixtures as solid fuel for energy content in combustion process or the alternative use as glucose source, for liquid biofuels production should be carefully analyzed and evaluated, in order to the overall net benefit (Pu et al., 2013).

2.3.5. Enzymatic saccharification of pretreated M1-4 and M2-3 mixtures

Two mixtures of pretreated lignocellulosic biomass by autohydrolysis were also suitable for glucose production, the main carbon source to produce several industrial products. Thus, cellulosic fraction can be saccharified for sugar production using enzymes. Autohydrolysis pretreatment improves the enzymatic saccharification due to the

structural alteration, as result of hemicellulosic fraction solubilization. In this sense, the solid fraction obtained from autohydrolysis was used as substrate in the assays of enzymatic hydrolysis in order to evaluate the susceptibility of pretreated biomass for glucose production. Time course of glucose yield for the two mixtures in the selected autohydrolysis conditions studied in this work (S_0: 3.71 - 4.82) is displayed in **Figure 13** (a) and (b). As seen in **Figure 13**, kinetics of enzymatic hydrolysis followed a typical pattern. Therefore, values of glucose yield obtained from enzymatic hydrolysis in this set of experiments were fitted to the Holtzapple empirical equation (3) (Holtzapple et al., 1984):

(3)

$$Y_{Gt} = Y_{GMAX} \times \frac{t}{t + t_{1/2}}$$

Where Y_{Gt} is the glucose yield at time t, Y_{GMAX} is the maximum glucose yield achievable at infinite reaction time, and $t_{1/2}$ (h) measures the reaction time needed to reach 50% of glucose yield.

The representation of calculated and experimental data (**Figure 13**) and the values of R^2 (**Table 7**) showed the goodness of adjustment to the empirical model. These results showed that the severity of pretreatment increased the glucose yield and reduced the time of hydrolysis. The reaction time needed to reach 50 % of glucose yield ($t_{1/2}$) was lower for M1-4 than for M2-3, in all the cases, except for the lowest (190 ℃) and highest (240 ℃) T_{MAX}. As evident in **Figure 13**, the harshness of pretreatment had a positive effect on the susceptibility of pretreated biomass to enzymatic hydrolysis. Glucose yield increased from 45.30 % to 89.94 % and from 42.46 % to 81.78 %, for M1-4 and M2-3, respectively, at 72h of enzymatic hydrolysis (**Table 7**). Considering only autohydrolyzed *Eucalyptus globulus* wood, Romaní et al. (Romaní et al., 2010b) reported a glucose yield of 100% at $T_{MAX} > 210$ ℃.

Table 7- Glucose concentration, G_{72}, and glucose yield, Y_{G72}, at reaction time of 72 h, maximal glucose yield, Y_{GMAX}, time needed to achieve ½ of Y_{GMAX}, $t_{1/2}$ and coefficient of determination R^2.

T_{MAX} (ºC) / S_0	Substrate	G_{72} (g/L)	Y_{G72} (%)	Y_{GMAX} (%)	$t_{1/2}$ (h)	R^2
190 or 3.71	M1-4	10.04	45.30	73.21	45.68	0.98
	M2-3	9.44	42.46	48.15	10.47	0.95
196 or 3.93	M1-4	10.26	45.09	51.86	11.67	1.00
	M2-3	16.21	73.70	100.0	39.11	0.96
206 or 4.15	M1-4	13.81	60.18	71.08	13.54	1.00
	M2-3	16.47	73.72	100.0	35.61	0.97
216 or 4.38	M1-4	19.91	77.97	85.33	5.77	1.00
	M2-3	21.15	85.54	100.0	23.45	0.97
226 or 4.60	M1-4	21.51	89.82	92.26	5.98	0.99
	M2-3	19.59	74.43	87.26	21.25	0.96
240 or 4.82	M1-4	21.11	89.94	99.87	6.07	1.00
	M2-3	21.34	81.78	81.28	1.93	0.99

There was a greater difference of glucose yield at T_{MAX} 196 ºC between M1-4 (45.09%) and M2-3 (73.70%) at 72h. On the other hand, M1-4 reached glucose yield higher than M2-3 at $T_{MAX} > 226$ ºC.
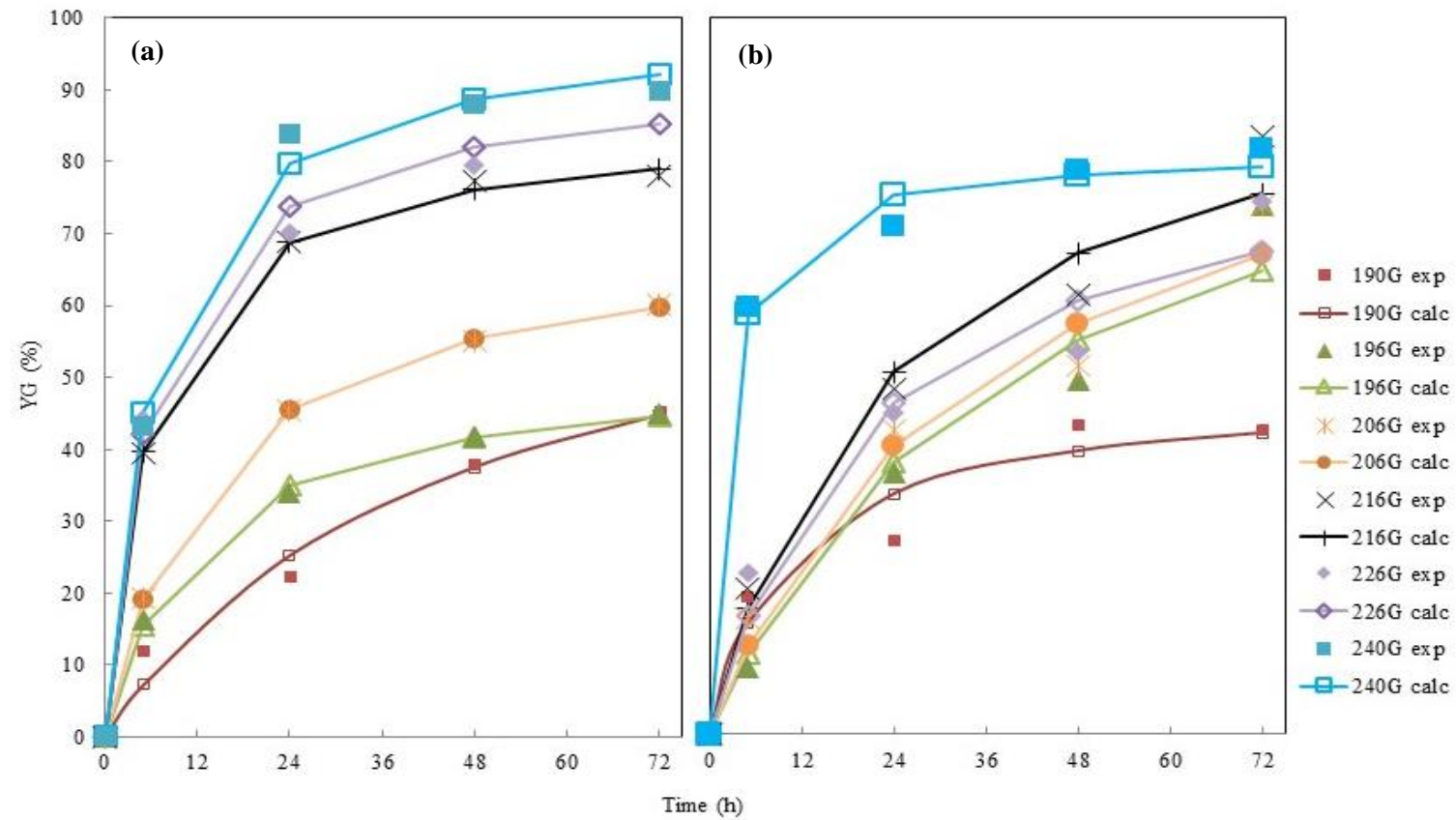
Figure 13- Yield of glucose Y_G (%) at autohydrolysis conditions (T_{MAX}) in the range 190 ℃ to 240 ℃ for M 1-4 (a) and M 2-3 (b).

An increase in the autohydrolysis severity (S_0) from 3.71 to 4.82 allowed glucose concentration to increase from 10 g/L to 21 g/L, approximately, at 72 h of hydrolysis for the two mixtures (**Table 7**). The similar behavior was reported by Domínguez et al. (Domínguez et al., 2017), using *Paulownia tomentosa* as biomass, where an increase in the autohydrolysis severity from S_0 3.31 to 4.82 allowed a five-fold increment in glucose concentration to 27.5 g/L at 120 h.

In general, enzymatic hydrolysis is an efficient process without generation of any toxic waste and does not contain fermentation inhibitors, which reveals a promising strategy to obtain higher glucose yield (Singh et al., 2013).

2.3.6. Overall balance of M1-4 and M2-3

Considering the results obtained in this chapter, **Figure 14** compares the fractionation effect of autohydrolysis pretreatment on the two feedstock mixtures. The highest hemicellulose solubilization (as XOS and xylose) was observed at $T_{MAX} = 206\,°C$, as glucose yield of enzymatic hydrolysis higher than 60% for both mixtures (**Table 7**). As seen in **Figure 14**, the value-added compound obtained in separated streams was of 19.1 kg of glucose for M1-4 and 24.1 kg of glucose for M2-3. Overall yield of glucose for M1-4 and M2-3 was 50% and 63%, respectively. These results can be compared with reported data in literature using the same pretreatment, in which 76% and 63% of glucose yield at $S_0 = 4.13$ were achieved from brewers's spent grain and corn husk, respectively (Michelin and Teixeira, 2016). At the same condition, overall yield of xylose was 62% and 69% for M1-4 and M2-3, respectively. The results obtained for xylose yield can be favorably compared with data reported by Nitsos et al., that obtained around 60% yield (S_0 3.8 - 4.01) for poplar and grapevine, respectively (Nitsos et al., 2016). The data described above indicate that autohydrolysis at 206 °C is an appropriate process for the selective fractionation of mixtures obtaining a solid fraction composed mainly by glucan and lignin, and high solubilization of hemicellulose into the liquid phase with minimum formation of degradation products. Cellulose was subjected to enzymatic hydrolysis and could be further processed for biological conversion into biofuels, biochemical or biomaterials as single or in combination with sugars obtained from liquid phase. The remaining lignin can simply be used for co-generation of energy in a biorefinery context or exploited for other high value applications.

(a)

Water
800 kg

Mixture 1-4 (M1-4) —100 kg→ Autohydrolysis 206 °C

Gn: 34.17 kg
Xn: 16.58 kg
Ar: 1.36 kg
AcA groups: 2.13 kg
KL: 30.05 kg

Solid phase

Gn: 27.80 kg
Xn: 0.85 kg
KL: 32.07 kg

Water/ Buffer   Cellic Cetec2

Enzymatic saccharification
5 % solids, 20 FPU/g

Glucose: 19.15 kg

Liquid phase

GO: 0 kg
XO: 7.78 kg
AcGO: 2.61 kg
G: 2.55 kg
X: 3.93 kg
Ar: 3.40 kg
AcA: 0.30 kg

(b)

Water
800 kg

Mixture 2-3 (M2-3) —100 kg→ Autohydrolysis 206 °C

Gn: 34.63 kg
Xn: 17.48 kg
Ar: 1.27 kg
AcA groups: 1.95 kg
KL: 31.7 kg

Solid phase

Gn: 29.32 kg
Xn: 1.64 kg
KL: 30.75 kg

Water/ Buffer   Cellic Cetec2

Enzymatic saccharification
5 % solids, 20 FPU/g

Glucose: 24.13 kg

Liquid phase

GO: 1.43 kg
XO: 11.88 kg
AcGO: 1.80 kg
G: 0.89 kg
X: 1.75 kg
Ar: 1.46 kg
AcA: 0.95 kg

Figure 14- Overall balance of M1-4 (a) and M2-3 (b) for autohydrolysis and saccharification processing at T_{MAX} = 206 °C (results expressed in kg/100kg raw material) oven dry basis.

2.4. Conclusions

This chapter proved an efficient fractionation of the selected mixtures (M1-4 and M2-3) by autohydrolysis, demonstrating that the criteria selected, besides representing an actual scenario of lignocellulosic biomass selection, results in valuable outcomes. Mixed feedstocks evaluated in this work showed a similar behavior to autohydrolysis treatment, however different yields of value-added compounds were co-produced. One condition ($T_{MAX} = 206\ ^\circ C$ or $S_0 = 4.15$) could be selected for the treatment of both biomass mixtures. Since under this condition, recoveries of 62.2% and 68.8% of hemicellulose-derived compounds (as XOS and xylose) were obtained for M1-4 and M2-3, respectively. Likewise, glucose yield from enzymatic hydrolysis resulted in 60.1% for M1-4 and 73.7% for M2-3. Alternatively, autohydrolyzed pretreated biomass may be used for solid fuel as high heating values of 20.5 MJ/kg were attained.

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CHAPTER 3 |

Organosolv process of a mixture of autohydrolyzed biomass

Abstract

Mixture of autohydrolyzed unexploited lignocellulosic biomass (AM2-3) was delignified by uncatalyzed ethanol-organosolv process to recover hemicellulose, cellulose and lignin in separate streams. Three factors were evaluated in the experimental design of organosolv process: ethanol concentration (30–80%), temperature (160–200ºC) and time (20–60 min). Organosolv process showed that the best compromise between lignin removal and cellulose preservation was obtained at high temperature and ethanol concentration (p-value of 0.05). Maximal delignification (49.40%) was obtained at the highest severity condition (200ºC, 60 min, 80% EtOH). Moreover, 35.32 g/L glucose, correspondig to a glucose yield of 49.65%, was produced from enzymatic hydrolysis of delignified biomass. FTIR analysis of the isolated lignins (OL1–OL10) showed that the main lignin structure was not changed, while thermal analysis revealed T_g values from 73 to 85 ºC. All OL presented radical scavenging activity as high as the commercial antioxidant BHT.

Keywords: Lignocellulosic feedstock, organosolv delignification process, lignin.

Contents

3.1. Introduction

Lignocellulosic biomass (LCB) has proven to be a promising resource for sustainable development towards a biobased society, and is a key option for biorefinery concepts. Nevertheless, some factors such as feedstock type and availability, time of harvest, transportation costs, and storage have been identified as main drawbacks for the feasibility of biorefineries. Thus, the combination of mixtures of unexploited biomasses from marginal land could be a solution for the sustainable biorefinery supply when compared to single feedstock (Astner et al., 2015). Furthermore, the economic viability of the future lignocellulosic biorefinery depends on the conversion of all fractions, namely, cellulose, hemicellulose and lignin to value-added compounds, based on a cascade use concept (Azadi et al., 2013). Thus, the selective separation of these components enables their efficient utilization (Romaní et al., 2011).

One potential strategy proposed as a first step is the hemicellulose solubilization by hydrothermal processing (or autohydrolysis), with hot and compressed water (Cybulska et al., 2017). This technology, under optimized conditions, enables a high production of soluble hemicellulosic oligosaccharides without causing significant dissolution of cellulose and lignin, while remaining in the solid fraction (Moniz et al., 2015; Romaní et al., 2011).

For further fractionation, the removal of lignin before hydrolysis of cellulose in glucose and subsequent fermentation of fermentable sugars avoids problems related to the non-specific binding of cellulolytic enzymes to lignin and reprecipitation of soluble lignin, which could make cellulose fibers more recalcitrant to enzyme digestion reducing fermentation yield (Brudecki et al., 2013; Romaní et al., 2011).

Among the different processes able to break lignin bonds from cellulose, chemical treatments are particularly effective for lignin extraction/recovery, in particular the organosolv delignification process, which is highly optimized for this purpose (Cybulska et al., 2017; Maniet et al., 2017). The Organosolv process is based on organic solvents that break bonds such as β-aryl ether and aryl glycerol-β-aryl ether in the lignin macromolecule, causing lignin alteration (Vargas et al., 2016). To date, different combinations of solvents (e.g. methanol, ethanol, acetone, and/or organic acids) and many treatment conditions have been studied for autohydrolyzed biomass (Amiri and Karimi, 2016; Michelin et al., 2018; Moniz et al., 2015; Romaní et al., 2011; Vargas et al., 2016;

Zhu et al., 2015). Nevertheless, ethanol is the most used due to its low cost and easy recovery, with promising results (Brudecki et al., 2013; Moniz et al., 2015). In the studied cases, and considering our knowledge and the available literature, it is the first time the organosolv process was used to pretreat complex mixtures of autohydrolyzed lignocellulosic biomass, especially in the scope of the raw material from marginal lands without economic activity and relevant uses.

The products commonly obtained by this process include sulphur-free lignin fragments, which are useful for the production of lignin-based high value products due to their purity and low molecular weight (Moniz et al., 2015). Therefore, aside from its current use as solid fuel, potential applications for lignin , such as copolymers and polymers blends (Doherty et al., 2011), cosmetic (Vinardell et al., 2011), resins (Stewart, 2008), adhesives (Reddy and Yang, 2005), carbon fibers, fillers, encapsulants, pharmaceutical intermediates and oxygenated aromatic compounds could be developed (Astner et al., 2015).

Furthermore, lignin separation could reduce problems in wastewater treatment from the environmental point of view and improve the economic value of the process (Amiri and Karimi, 2016). In order to find new lignin valorization routes, it is essential to perform an extensively characterization of this fraction, since lignin demonstrates different properties according to lignocellulosic biomass, pretreatment and isolation process (Martín-Sampedro et al., 2019).

In this context, the present original study aimed to extract lignin from mixtures of autohydrolyzed forest and marginal lands lignocellulosic biomasses resources by organosolv delignification process, using ethanol/water mixture. The purpose of using a mixture of biomasses is to have sufficient resources available to supply a biorefinery throughout the year, since the security supply and the sustainability of exploration are key factors to ensure the industrialization of these systems (Pontes et al., 2018). The criteria for the mixture of biomasses was based on territory distribution, fire risk during summer months and total sugar content (Pontes et al., 2018).

For organosolv evaluation, the effects of operational conditions (temperature, time and ethanol concentration) were evaluated by experimental design. This chapter has extended to the study of the physicochemical and antioxidant properties of isolated lignins for further valorization and follow up of the enzymatic hydrolysis of the recovered cellulosic fraction.

3.2. Materials and methods

3.2.1. Lignocellulosic biomass

Mixture of autohydrolyzed unexploited lignocellulosic biomasses from forest and marginal lands sources (AM2-3) (branches and twigs with bark and leaves of Eucalyptus (*Eucalyptus globulus*), Pine (*Pinus pinaster*), Broom (*Cytisus sp.*), Carqueja (*Genista tridentata*), Mimosa (*Acacia dealbata*), and Rockrose (*Cistus ladanifer*)) was used as raw material. The criterion of feedstocks mixture was previously studied (Pontes et al., 2018). Taking into account the reported data, for the present study, the mixture M2-3 (mixture 2nd and 3rd quarters), which represent 52.5 % of forest ecosystems (24.6 % of pine, 27.9 % of eucalyptus) and 47.5 % of biological resources from marginal land (13.8 % broom, 12.5 % mimosa, 10.7 % carqueja, 10.4 % rockrose) was selected. The raw materials were air-dried, milled and sieved between 0.250 to 0.400 mm using a vibratory sieve shaker (40 and 60 mesh). Afterwards, the samples were homogenized in a single lot to avoid heterogeneity compositional among aliquots and stored in polypropylene bags at room temperature.

3.2.2. Organosolv delignification process

M2-3 was submitted to an autohydrolysis pretreatment, following the standard heating temperature-time profile up to reach the final temperature (T_{MAX}) of 206 ºC, as previously reported and studied by the authors (Pontes et al., 2018). The solid fraction resulting from autohydrolysis pretreatment was named AM2-3.

The AM2-3 biomass obtained after autohydrolysis pretreatment under optimized conditions was subjected to organosolv delignification process to isolate the lignin fraction. A solid to liquid ratio (SLR) of 1:8 (w/w) was used and all reactions were performed in a 2 L stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature controller (model 4848). The treatment was performed using uncatalyzed ethanol/water solutions according to the design of experiments (DOE) described in the next section.

After reaction, the solid phase was separated from liquid phase (black liquor) by vacuum filtration using Whatman® Nº1 and washed twice with ethanol/water solution (at

the same concentration of the treatment). The washing ethanol/water solution and black liquor were combined to recover the lignin. The organosolv lignin fractions were recovered by precipitation after addition of three volumes of water acidified with acetic acid to pH 2.0. The precipitated lignins were centrifuged, washed, and freeze-dried.

On the other hand, the delignified solids were washed with 10 g of NaOH (1%, w/w)/g of solids at room temperature plus washes until pH 7 (approximately 1 L of distilled water/g of solids), in order to remove adsorbed lignin from the pretreated solids as described at (Romaní et al., 2016). Washed delignified solids were air-dried at 45 ℃. The yield of lignin or solid residue (Y_L) was determined according to Eq (1).

(1)

$$Y_L(\%) = \frac{m_{recovered}}{m_{AM2-3}} \times 100$$

Where $m_{recovered}$ is the mass of lignin or solid residue recovered after the organosolv process (g), m_{AM2-3} is the mass of the AM2-3 biomass used for the treatment (g).

The lignin extraction efficiency (EE) was determined according to Eq. (2).

(2)

$$EE\ (\%) = \frac{Y}{KL_{AM2-3}} \times 100$$

Where Y is the yield of lignin extracted in the organosolv process (%), KL_{AM2-3} is the quantity of the Klason lignin of the AM2-3 biomass (%).

The delignification extent (DE) was determined by Eq. (3).

(3)

$$DE\ (\%) = \left\{ \frac{KL_{AM2-3} - \left[KL_{delignified\ AM2-3} \left(Y_{delignified\ AM2-3}/100 \right) \right]}{KL_{AM2-3}} \right\} \times 100$$

Where KL_{AM2-3} is the quantity of Klason lignin in the AM2-3 biomass (%), $KL_{delignified\ AM2-3}$ is the quantity of the Klason lignin in the solids after organosolv process (%) and $Y_{delignified\ AM2-3}$ is the yield of solid residue (%).

3.2.2.1. Experimental design

The experiments were performed using the design of experiments (DOE) to evaluate the effect of temperature, time and ethanol concentration on lignin removal for its valorization and cellulose preservation for further enzymatic hydrolysis.

A DOE used was a cubic experimental design procedure with three factors of two levels each (2^3) and using the two central points for error evaluation. Three independent variables were evaluated: temperature (T, ºC) [160 (-1), 180 (0), 200 (+1)], time (t, min) [20 (-1), 40 (0), 60 (+1)] and ethanol concentration (E, v/v) [30 (-1), 55 (0), 80 (1)]. The scheme of the experimental design is summarized in **Figure 15**.
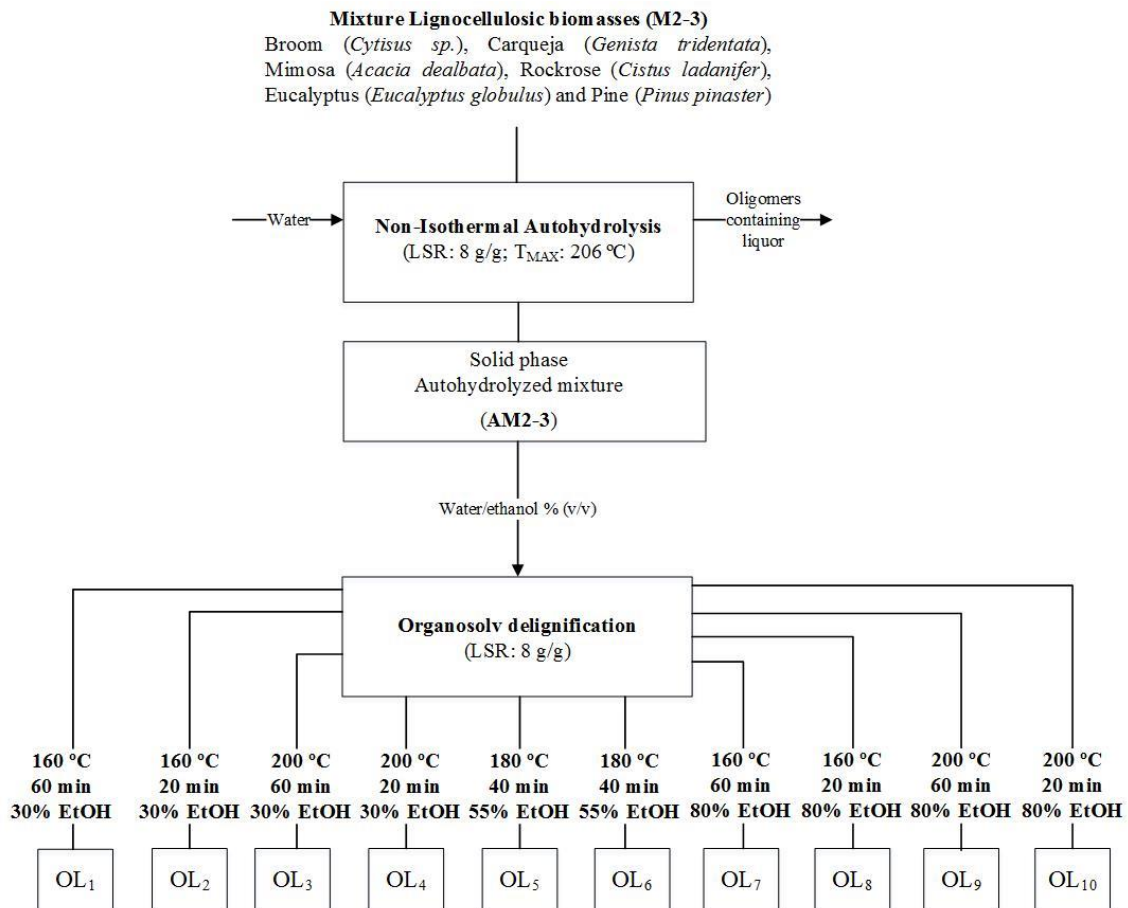
Figure 15-Scheme of the organosolv delignification process.

The analyzed responses (dependent variables) were solid residue yield, lignin yield, delignification extend, lignin extraction efficiency and cellulose content. Experimental results were evaluated with STATISTICA (data analysis software system),

version 12 from StatSoft, Inc. (2014) (www.statsoft.com) for analysis of variance (ANOVA), modeling the responses, regression coefficients and graphical analysis. Sum of squares of residuals (SSR) was chosen for statistical significance and computation of standard error. Statistical analyses were performed with a 95% significance level.

3.2.3. Enzymatic hydrolysis of delignified biomass

The delignified solid recovered after the organosolv process at the highest severity condition (200 ℃, 60 min and 80 % of ethanol concentration) was subjected to enzymatic hydrolysis (EH). Hydrolysis was conducted in a 100 ml Erlenmeyer flasks with the Cellic CTec2 enzyme (Novozymes, Bagsvaerd, Denmark) at 50 ℃ and pH 4.8 (0.05 N sodium citrate buffer), in orbital agitation at 150 rpm. The enzyme activity was 120 FPU/mL (measured as described by Ghose) (Ghose, 1987). The percentage of solids in the enzymatic hydrolysis assays was 10 %, with an enzyme to substrate ratio (ESR) of 20 FPU/g of substrate (or delignified solid). The reaction time of enzymatic hydrolysis varied in the range 0–72h. The samples were withdrawn from the media at desired times (0 h, 5 h, 25 h, 48 h and 72 h). The samples were centrifugated and analyzed by HPLC as described in 2.4 section – Chemical composition of material. In order to determine the enzymatic susceptibility of delignified biomass, glucose yield (Y_G) was calculated as follows (Romaní et al., 2011):

(4)

$$Y_G = 100 \times \frac{G_{t-}G_{t-0}}{G_{POT}}$$

Where G_t is the glucose concentration (g/L) achieved at time t, G_{t-0} is the glucose concentration at the beginning of the experiments, and G_{POT} means the potential glucose concentration (calculated assuming total cellulose conversion into glucose). G_{POT} was calculated by equation 5 (Romaní et al., 2011):

(5)

$$G_{POT} = \frac{Gn}{100} \times \frac{180}{162} \times \frac{\rho}{LSR + 1 - \frac{KL}{100}}$$

Whereas Gn is the glucan content of solid residue recovered after organosolv process (kg glucan/ 100 kg solid residue, on dry basis), 180/162 is a stoichiometric factor, ρ is the density of the reaction medium (average value, 1005 g/L), LSR is the liquid-to-solid ratio, in this case for 10 % of solids is 10, and KL is the Klason lignin content of solid residue.

3.2.4. Chemical analysis of biomass composition

The aliquots of lignin and delignified solids were chemically analyzed. Analytical assays were performed according to the following National Renewable Energy Laboratory (NREL) methods: moisture (NREL/TP-510-42621)) and quantitative acid hydrolysis with 72% w/w sulphuric acid (NREL/TP-510-42618).

The hydrolysates from acid hydrolysis were analyzed by high performance liquid chromatography (HPLC) for sugars (glucose, xylose, arabinose), acetic acid, hydroxymethylfurfural (HMF) and furfural (F) using the columns Aminex HPX-87H (conditions: refractive index detector; flow rate of 0.6 mL/min at 60 ºC; 0.005 M H_2SO_4 as mobile phase). The concentrations of sugars and acetic acid were employed to calculate the contents of cellulose and hemicellulose. The solid phase from the quantitative acid hydrolysis was considered as Klason lignin. Analyses were carried out in triplicate.

3.2.5. Lignin characterization

3.2.5.1. Fourier-transform infrared spectroscopy (FT-IR)

The organosolv lignins were analyzed by a FTIR spectrometer (IRAffinity -1S Shimadzu) operating in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} recorded over 20 scans according to Hansen et al. 2016 (Hansen et al., 2016).

3.2.5.2. Thermal analysis: thermo gravimetric analysis (TGA) and differential scanning calorimetric (DSC)

Thermo gravimetric analysis (TGA) and differential scanning calorimetric (DSC) of organosolv lignins were carried out in Perkin Elmer TGA 4000 and DSC 6000

equipment, respectively, in order to evaluate their thermal behaviors. TGA was performed with about 10 mg of lignin, with a heating rate of 10 ºC/min under nitrogen atmosphere, and temperature ranging from 20 ºC to 800 ºC. DSC analysis was carried out according to a standard heat-cool-heat experiment at a constant heating rate of 20 ºC/min. Lignin samples were heated from 0 ºC to 120 ºC and then, cooled until 0 ºC for a new heating up to 200 ºC, under a nitrogen atmosphere. The measurements were made in triplicate using 4 mg of lignin samples placed in a hermetically sealed DSC pan, the top of each sealed pan was punctured to allow volatiles to escape. Glass transition (T_g) was calculated by using the half-height technique in the transition region.

3.2.5.3. Antioxidant activities

3.2.5.3. DPPH free radical scavenging

The DPPH scavenging activity of lignins was determined according to the slightly modified method of Blois (Blois, 1958) that is based on its ability to act as free radical scavenger. Commercial antioxidants like 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and butylated hydroxytoluene (BHT) were used as reference. Briefly, 150 M solution of DPPH was prepared and diluted in ethanol to get an absorbance of 0.700 at 517 nm. A volume of 200 μL of this solution was added to 25 μL of lignin samples dispersed in 80 % ethanol at different concentrations (0.05–1 mg/mL). These solutions were incubated in the dark for 30 min at room temperature. The decrease of the solution absorbance, due to proton donating activity, was measured using a UV–Vis spectrophotometer against a control sample (200 μL of DPPH mixed with 25 μL of ethanol 80 %). The percentage of radical scavenging activity (*RSA*) was calculated using the Eq. (6) (Michelin et al., 2018):

(6)

$$RSA\ (\%) = \left[\frac{\left(A_{control} - A_{sample}\right)}{A_{control}}\right] \times 100$$

where $A_{control}$ is the absorbance of the control sample and A_{sample} is the absorbance of the lignin sample. The IC_{50} (half maximal inhibitory concentration) value was

calculated as the concentration of the compounds that causes 50% reduction in the DPPH color (also referred as inhibition). All experiments were carried out in triplicate.

3.2.5.3.2. ABTS radical scavenging activity

The ABTS radical scavenging activity of lignins was determined according to the slightly modified method of Magalhães et al. (Magalhães et al., 2014), that is based on the ability of antioxidants to interact with the ABTS radical, descreasing its absorbance. Commercial antioxidants like trolox and BHT were used as reference at the same concentrations as the samples. ABTS was dissolved in water to a 7 mM concentration, and ABTS radical cation (ABTS^{*+}) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate in the dark, at room temperature for 12–16 h before use. ABTS^{*+} solution was diluted with water to get an absorbance of 0.700 at 734 nm. For each sample, six concentrations were analyzed, in duplicate, to establish a relationship between the radical scavenging activity (RSA) and the antioxidant concentration (mg/mL). Blanks were run in each assay. The absorbance was read after 6 min of reaction in a 96-well microplate format using Synergy HT spectrophotometer. The percentage of radical scavenging activity (RSA) was calculated using the previous Eq. (4).

3.3. Results and discussion

3.3.1. Organosolv lignin extraction

AM2-3 solid fraction used in this study was chemically analyzed and its composition was as follow: (41.87 ± 2.36) % cellulose, (6.30 ± 0.35) % xylan, (50.62 ± 1.07) % Klason lignin and the remaining minor compounds were ashes and soluble lignin. In the liquid fraction, 68.80 % of hemicellulose was recovered (mainly composed by XOS and xylose). This condition was selected taking into account the high hemicellulose solubilization and higher preservation of cellulose and lignin in the solid fraction, as described in the previous work (Pontes et al., 2018). Thus, an organosolv process after an autohydrolysis pretreatment may provide an efficient fractionation of autohydrolyzed

biomass by means of the separation of cellulose and lignin, which allows the valorization of all fractions.

To maximize the delignification of AM2-3 during organosolv process, the effects of time, temperature and ethanol concentration on the delignification extent (**Table 8**) and lignin yield (**Table 9**) were investigated. The best results according to delignification (49.40 %), lignin yield (24.47 %) and lignin extraction efficiency (48.34 %) were obtained on experiment 9 (L9), at high ethanol concentration (80 %), temperature (200 ºC) and time (60 min).

Table 8- Chemical composition of solid fractions after organosolv delignification process of AM2-3.

Experiment	Ethanol Concentration (%, v/v)	Temperature (ºC)	Time (min)	Solid Yield (%)	Cellulose[a] (%)	Lignin[b] (%)	Delignification (%)
1	30 (-1)	160 (-1)	60 (+1)	83.78	55.46	38.03	37.06
2	30 (-1)	160 (-1)	20 (0)	86.55	54.10	36.37	37.82
3	30 (-1)	200 (+1)	60 (+1)	75.78	60.28	38.23	42.76
4	30 (-1)	200 (+1)	20 (0)	80.31	61.33	39.73	36.97
5	55 (0)	180 (0)	40 (-1)	79.11	62.57	36.48	42.98
6	55 (0)	180 (0)	40 (-1)	77.64	62.93	35.63	45.35
7	80 (+1)	160 (-1)	60 (+1)	78.86	58.55	37.09	42.21
8	80 (+1)	160 (-1)	20 (0)	81.25	56.40	35.92	42.35
9	80 (+1)	200 (+1)	60 (+1)	75.53	67.92	33.91	49.40
10	80 (+1)	200 (+1)	20 (0)	77.32	68.81	33.98	48.09

[a] Estimated from glucan content
[b] Klason lignin

Although these results showed a solid fraction with less lignin content, the delignification was no higher than 50%. According to Obama et al. (Obama et al., 2012) this fact can be explained by the autohydrolysis pretreatment, where lignin was deconstructed to enable the lignin-carbohydrate bonds during organosolv; however, the formation of small fragments of lignin are associated with repolymerization reactions (C-C linkages), leading to negative effects on the delignification, resulting in a lignin fraction difficult to extract even under harsh conditions (Obama et al., 2012). Amendola et al.

(Amendola et al., 2012) also reported a low delignification rate, that is, from an autohydrolyzed grape stalks residue containing 56% lignin and 27.9% cellulose, only 6.7% solids were solubilized in the organosolv process, lignin precipitation corresponded to 28% of the total solids of the liquor and 2.3% of grape stalks lignin. The authors also attributed this behavior to a possible lignin repolimerization or even to the use of conditions not severe enough to hydrolyze stalks lignin. Vargas et al. (Vargas et al., 2016) also showed a high fraction of lignin (24–29 %) remaining in the cellulosic fraction after the organosolv process of an autohydrolyzed barley straw, a less recalcitrant biomass. Other authors have reported the presence of residual lignin in the cellulosic fraction after sequential autohydrolysis-organosolv pretreatment, independently of the biomass nature or pretreatment severity (Michelin et al., 2018; Romaní et al., n.d.; Ruiz et al., 2012; Zhu et al., 2015), possibly due to the lignin repolymerization.

Table 9- Chemical composition of precipited lignin after organosolv process of AM2-3.

Experiment	Ethanol Concentration (v/v)	Temperature (ºC)	Time (min)	Lignin Yield[a] (%)	Lignin [b] (%)	Carbohydrate[c] (%)	Extraction efficiency (%)
1	30 (-1)	160 (-1)	60 (+1)	16.22	78.26	n.d.	32.05
2	30 (-1)	160 (-1)	20 (0)	13.45	86.45	n.d.	26.57
3	30 (-1)	200 (+1)	60 (+1)	24.22	88.00	n.d.	47.85
4	30 (-1)	200 (+1)	20 (0)	19.69	86.72	n.d.	38.90
5	55 (0)	180 (0)	40 (-1)	20.89	91.71	n.d.	41.27
6	55 (0)	180 (0)	40 (-1)	22.36	92.71	n.d.	44.16
7	80 (+1)	160 (-1)	60 (+1)	21.14	87.40	n.d.	41.76
8	80 (+1)	160 (-1)	20 (0)	18.75	89.46	n.d.	37.05
9	80 (+1)	200 (+1)	60 (+1)	24.47	92.31	n.d.	48.34
10	80 (+1)	200 (+1)	20 (0)	22.68	85.60	n.d.	44.80

[a] Calculated by difference
[b] Klason lignin
[c] n.d.: not detected (glucose, xylose, arabinose and acetic acid).

It is important to highlight that in the current work a complex mixture of biomass was used, including hardwood, softwood and bush from forest and marginal land resources, which could have hindered delignification. This is the first report using

organosolv process in complex mixtures of autohydrolyzed biomasses. The purpose of using multi-supply raw biomass, as reported in a previous work by Pontes et al. (Pontes et al., 2018), is to provide enough feedstocks for biorefineries throughout the year, increasing the sustainability of the value chain in terms of biomass (not pressure in the same feedstock) and also avoiding the risk of forest fire, commonly related to these biomasses. However, probably more than one step delignification will be required to improve the lignin removal, such as the extended delignification reported by Wen et al. (Wen et al., 2013), as well as a more severe process.

According to the Pareto diagrams (**Figure 16**), where EtOH corresponds to ethanol concentration, T for temperature and t means time, temperature and ethanol concentration were the factors with the greatest effect for the response on lignin yield, indicating that the lignin recovery process is favored at higher temperature and ethanol concentration. Delignification is positively influenced by ethanol concentration. For a confidence level of 95% (*p*-value of 0.05) lignin extraction efficiency and cellulose content are favored first by higher temperature and secondly by higher ethanol concentration. Therefore, the best compromise between lignin removal and cellulose preservation was obtained at high temperature and ethanol concentration.

The *p*-values, obtained from ANOVA, for the different factors and interactions of each response are presented in **Table 10**. These values indicate the significance of the factor or interaction to the model, for confidence levels of 95%. The significant factors for the model are in bold in **Table 10**. For all the measured responses, the most significant effect was from changes in temperature, possessing a confidence level higher than 99.99%, with the exception of delignification. The second-most significant level on the responses was caused by ethanol concentration that presents *p*-values of 0.02.

For evidence of this consistence, the plot of predicted versus observed data demonstrated a good correlation between the predicted responses and those observed for solid residue and lignin yields ($R^2= 0.95$), delignification ($R^2= 0.92$), lignin extraction efficiency ($R^2= 0.95$) and cellulose content ($R^2= 0.96$).
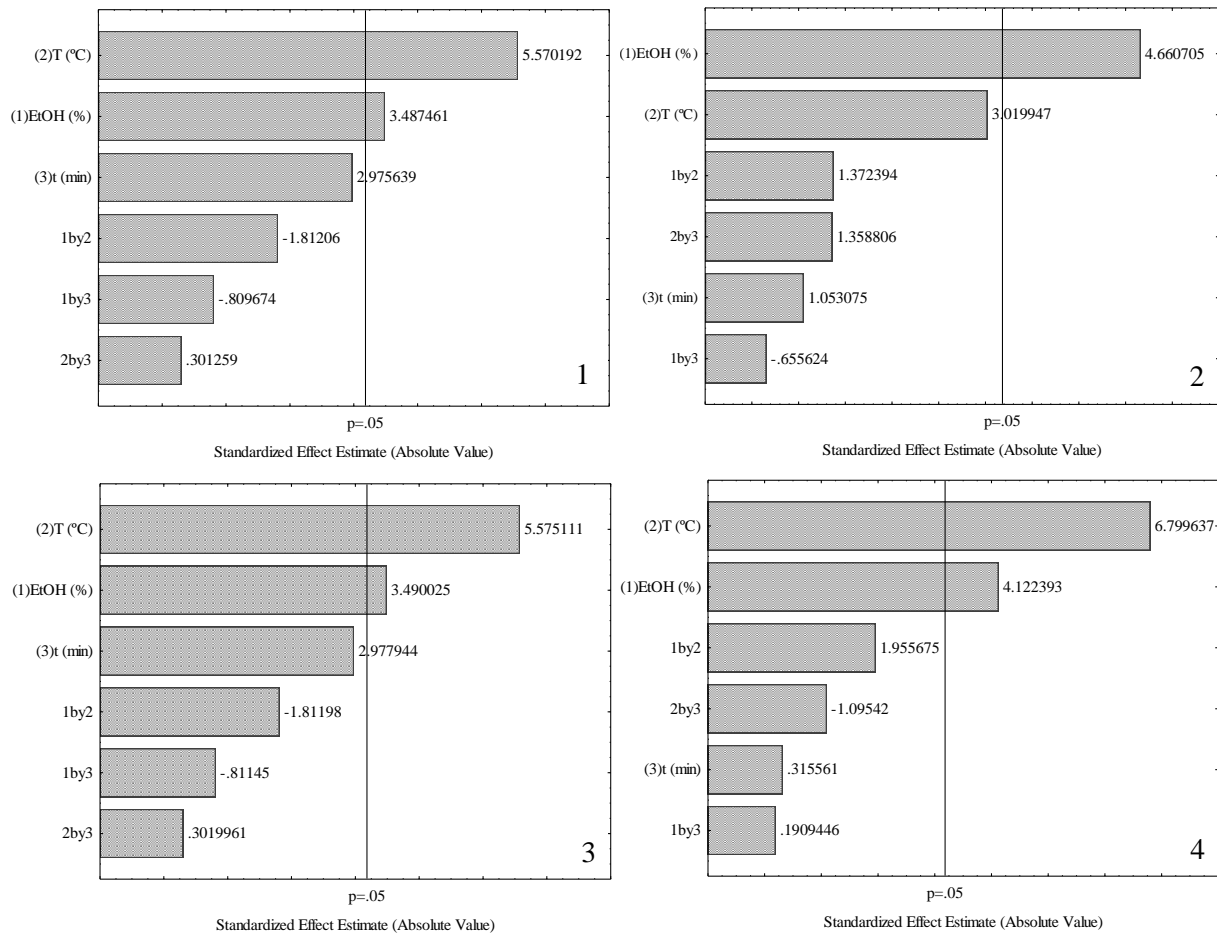
Figure 16- Pareto diagrams of the responses (1- Lignin yield, 2- Delignification, 3- Lignin extraction efficiency, 4- Cellulose content) from the cubic experimental design. (1) EtOH (%) denotes the ethanol concentration, (2) T (°C) the temperature, and (3) t (min) the time, 1by2 the interation between EtOH and T, 1by3 interation between EtOH and t, 2by3 interation between T and t.

Lignin samples, recovered by precipitation, for all samples, showed high purity with no detection of carbohydrates (**Table 9**). Regarding to glucan preservation, the values of cellulose content on solid residues were an average of 60% for all conditions, being that of 68% for the optimal condition. Vallejos et al. (2015) (Vallejos et al., 2015) have reported a higher delignification than the results obtained in this work, and then glucan preservation. However, a less recalcitrant biomass was used (sugarcane bagasse), as well as an autohydrolyzed biomass with much lower lignin content (26%).

Table 10- ANOVA table for the studied responses.

Source of variation	*p*-values				
	Solid yield	Lignin yield	Delignification extend	Extraction efficiency	Cellulose content
EtOH	**0.0398**	**0.0398**	**0.0186**	**0.0398**	**0.0259**
T	**0.0114**	**0.0114**	0.0568	**0.0114**	**0.0065**
t	0.0588	0.0588	0.3696	0.0587	0.7730
EtOHT	0.1676	0.1676	0.2635	0.1677	0.1455
EtOHt	0.4774	0.4774	0.5589	0.4765	0.8608
Tt	0.7829	0.7829	0.2673	0.7824	0.3534

3.3.2. Enzymatic Hydrolysis (EH) of delignified biomass mixture

The glucose yield (Y_G) after 72 h of enzymatic hydrolysis is shown in **Figure 17**, using the solid fraction resulted from the optimal condition (L9) at 10 % solids load. This condition was selected taking into account the lignin content, since lower lignin content could be related with higher enzymatic susceptibility of cellulose, as cellulases can irreversibly bind to lignin (Obama et al., 2012).

In the current work, it was obtained the maximum Y_G of 53 %, being the maximum glucose concentration of 37.51 g/L, as shown in the **Figure 17**.
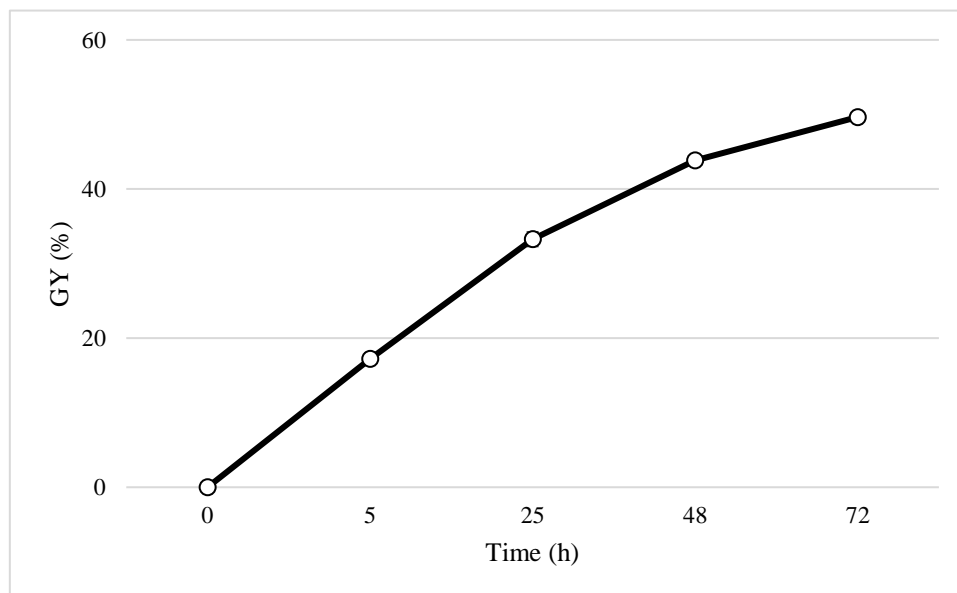
Figure 17- Glucose yield (Y_G %) during enzymatic hydrolysis of the solid fraction from organosolv treatment for the 10 % solids load (the standard desviation is presented on the graph).

However, the fraction of cellulose was not completely hydrolyzed to glucose, probably due to the presence of repolymerized lignin in the solids. These results are in agreement with Santos et al. (Santos et al., 2019) who reported the EH yield as a function

of the insoluble lignin content of the pretreated solids, showing that the higher the lignin content, the lower the EH yield. On the other hand, Obama et al. (Obama et al., 2012) used a Miscanthus biomass in two combined treatments of autohydrolysis + organosolv (AO), and enzymatic hydrolysis + organosolv (EO). The authors obtained a maximum of CGC (cellulose-to-glucose conversion) of approximately 40 % , in conditions similar to those of this work (AO2), and this conversion increased to approximately 60 % with enzymatic prehydrolysis step instead of autohydrolysis (EO2) (Obama et al., 2012). As previously mentioned, autohydrolysis promotes the repolymerization of small lignin fragments that are formed during pretreatment, resulting in a fraction of lignin that is more difficult to remove from cellulose (Obama et al., 2012).

Nonetheless, other authors also used different pretreatments and obtained low yields, as reported by Mesa el at. (Mesa et al., 2011), who submitted sugarcane bagasse to acid pretreated before organosolv process, under different operational conditions, obtaining the maximum glucose yield of 29 %, at the most severe condition of 195 ºC, 60 minutes and 60 % of ethanol concentration. However, Hallac et al. (Hallac et al., 2010) using ethanol organosolv treatment of *Buddleja davidii,* obtained around 60 % of CGC with a material with 21 % of lignin content. They also tested other condition with less lignin, and a higher yield was obtained, but the relationship between lignin content and enzymatic hydrolysis was not proportional. For this reason, they have shown that there are other factors beyond lignin content influencing enzymatic hydrolysis. Wang et al. (Wang et al., 2017) studied the use of organosolv on Eucalyptus wood and reported from 54.65 % to 88. 59 % Y_G for organosolv pretreated solids, and verified that the saccharification ratio increased with the increment of the time (60–120 min) and temperature (200–220 ºC), which was related to the hemicellulose degradation and cleavage of LCC (lignin carbohydrate complex) bonds, with partial depolymerization and removal of lignin, improving the cellulase accessibility to cellulose and consequently the enzymatic hydrolysis. Furthermore, Huijgen et al. (Huijgen et al., 2012) found that increasing the temperature of organosolv treatment from 200 ºC to 210 ºC for prehydrolyzed wheat straw biomass, under the same conditions as in the current work, increased the enzymatic glucose yield from 67 % to 90 %.

3.3.3. Organosolv lignin characterization

3.3.3.1. FTIR spectral analysis

The influence of the organosolv process conditions in the chemical structure of the OL was investigated by FTIR. FTIR spectra of different lignin fractions are showed on **Figure 18**, the bands were normalized and the intensity of the highest band equal 1. The band assignments are presented on **Table 11**. Characteristic lignin bands for O-H stretching (3412-3460 cm^{-1}), C-H stretching (3000-2842 cm^{-1}) and aromatic skeletal vibrations (1593-1605 cm^{-1} and 1505-1515 cm^{-1}) were observed for all fractions (Faix, 1991). The high-purity of these lignin fractions is also observed with absence of cellulose band (1150 cm^{-1}) (Vaz, 2016).
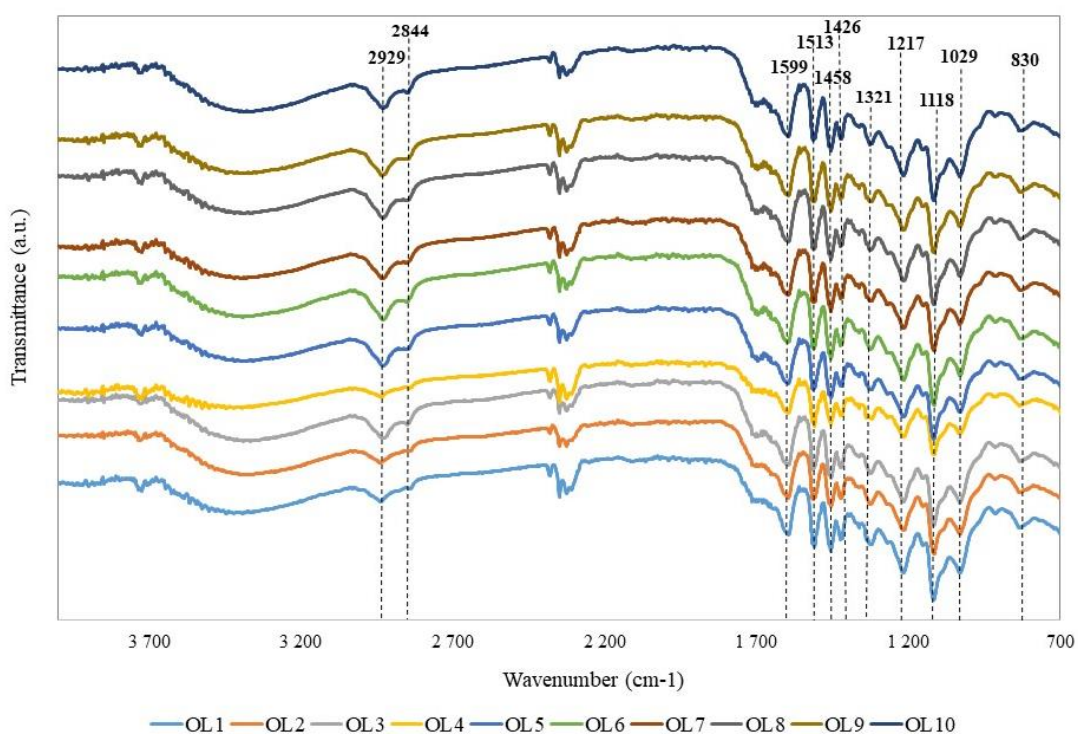
Figure 18- FTIR spectra of the different ethanol organosolv lignins (OL1-OL10) in a wavenumber from 3000-700 cm-1.

Furthermore, the spectra and the intensity of the bands were very similar for the different fractions of lignin from organosolv, which confirmed that the structure of lignin did not change with the different conditions of organosolv pretreatment.

Table 11- Assignments of FTIR bands of lignin (Faix, 1991).

OL_1	OL_2	OL_3	OL_4	OL_5	OL_6	OL_7	OL_8	OL_9	OL_{10}	Assignments
				Bands (cm^{-1})						
3420	3414	3420	3447	3416	3420	3420	3420	3420	3420	O-H stretch
2938	2938	2930	2938	2928	2930	2934	2930	2932	2932	C-H stretch in methyl and methylene groups
1593	1595	1595	1595	1595	1595	1595	1593	1595	1595	aromatic skeletal vibrations plus C = O stretch; S > G; G condensed > G etherified
1508	1514	1514	1508	1514	1514	1508	1508	1508	1514	aromatic skeletal vibrations; G > S
1460	1460	1462	1460	1462	1460	1462	1460	1462	1460	C-H deformations; asym.in -CH_3 and -CH_2-
1423	1423	1423	1423	1423	1423	1423	1423	1423	1423	aromatic skeletal vibrations combined with C-H in-plane deform.
1325	1325	1325	1325	1325	1325	1325	1325	1325	1325	S ring plus G ring condensed; (i.e. G ring substituted in pos. 5)
1221	1221	1221	1221	1221	1221	1221	1221	1221	1221	C-C plus C-O plus C = O stretch; G condensed > G etherified
1086	1086	1086	1086	1086	1086	1086	1086	1086	1086	C-O deformation in secondary alcohols and aliphatic ethers
1030	1030	1030	1030	1030	1030	1030	1030	1030	1030	aromatic C-H in-plane deformation, G > S; plus C-O deform, in primary alcohols; plus C = O stretch (unconj.)
833	829	833	833	829	829	831	831	827	829	C-H out-of-plane in positions 2,5, and 6 of G units

3.3.3.3. Thermal analysis

Thermal analysis includes some techniques that establish a connection between temperature and physical properties of materials. TGA was used to determine the thermal stability of the lignin samples (OL_1-OL_{10}). The onset temperature of degradation (T_{onset}), the temperature at the maximum rate of decomposition (T_{max}) and the mass remaining at 800 ºC for the several lignin fractions are listed in **Table 12**. The values of T_{onset} varies from 234.30 – 262.45 ºC for the fractions obtained from organosolv. The lowest

temperature corresponded to the less severe condition (experiment 2) and higher temperature to the second most severe condition (experiment 10).

Table 12- Thermal analysis of lignins.

Samples	T_{onset} (ºC)	$T_{max.}$ (ºC)	Residue at 800 ºC (%)	T_g (ºC)
OL_1	246.23 ± 0.50	359.23 ± 2.04	38.38 ± 0.12	84.78 ± 2.26
OL_2	234.30 ± 0.37	365.41 ± 1.68	36.84 ± 0.27	80.67 ± 2.04
OL_3	258.60 ± 0.00	367.75 ± 3.45	34.61 ± 1.16	80.01
OL_4	255.68 ± 2.87	371.86 ± 8.66	33.89 ± 1.30	83.93
OL_5	247.29 ± 2.00	380.55 ± 1.97	29.61 ± 0.18	76.77 ± 1.73
OL_6	245.17 ± 2.25	367.18 ± 7.16	34.88 ± 0.66	72.87 ± 0.05
OL_7	252.59 ± 0.00	372.74 ± 0.00	28.71 ± 0.29	n.d.
OL_8	247.29 ± 0.75	364.00 ± 0.98	30.97 ± 0.05	n.d.
OL_9	248.26 ± 2.12	366.91 ± 3.89	30.78 ± 0.14	n.d.
OL_{10}	262.45 ± 2.80	359.58 ± 0.44	29.64 ± 0.47	n.d.

The values expressed are the average of three replicates
n.d.: not detected

Sebio-Punal et al. (Sebio-Puñal et al., 2012) observed lignin degradation temperature of approximately 200 ºC for hardwood (*Castannea sativa*, *Eucaliptus globulus* and *Quercus robur*), and for softwood (*Pinus pinaster* and *Pinus sylvestris*) in the range of 290–300 ºC, this fact was explained by the different behavior of softwood and hardwood.

In this sense, the onset temperature around 225 ºC was also reported by Martín-Sampedro et al. (Martín-Sampedro et al., 2019) for a hardwood lignin, namely organosolv lignin from *Robinia pseudoacacia L.* In the current study, which involves a mixture of hardwood and softwood, the T_{onset} obtained are between these values. However, the results obtained do not represent the behavior of lignin, since the separation process produces chemically significant changes in its structure and removes the interactions between fractions (Caballero et al., 1996). Nevertheless, T_{onset} is an important parameter for the thermal properties of the material, which can limit its application in plastics (Li and McDonald, 2014).

Regarding the T_{max}, these ranged from 359.23 to 380.55 ºC. Wen et al. (Wen et al., 2012) verified maximal degradation of an organosolv lignin from birch, a hardwood, at 355.7 ºC, suggesting that more stable lignin structures, such as condensed lignin

structures, were formed after the organosolv process, as also reported by the authors. Nonetheless, Martín-Sampedro et al. (Martín-Sampedro et al., 2019) reported the maximal lignin degradation at 475 ℃ for hardwood. As mentioned before the T_{max} from OL of the current work, which involves a mixture of hardwood and softwood, are among these values.

For all the samples the mass loss increased until the temperature of 800 ℃ where the mass loss curves became relatively flat. The mass remaining at 800 ℃ varies from 28.71 % to 38.38 %. This is in agreement with the work of Ross et al. (Ross et al., 2012) which reported at 800 ℃ the mass remaining between 34.7 % and 36.9 % for lignin extracted from wheat straw, triticale straw and flax shives with ionic liquid. This amount of residue is also comparable to that reported by Elshafie et al. (Elshafie et al., 2020) for lignin extracted from the black liquor.

In general, as reported by Prime et al.(Prime et al., 2009), if the polymeric portion of a material is relatively stable, up to 300 ℃, only a low molecular mass will be lost; between 300 ℃ and 600 ℃, most polymers will degrade and volatilize; and above 600 ℃, some polymers based on aromatic structures will not be converted into gaseous products and will form a char residue, as was verified in OL samples of the current work.

The glass transition temperature (T_g) is an important property to use lignin as a polymer for industrial application and limits its usability in the glassy state (for example, polystyrene and epoxy) or in the rubbery state (for example, polysoprene or polybutadiene). At T_g (on heating), the glassy state changes to the rubbery or melt state. Nonetheless, the glass transition does not take place at a precise temperature, but in a temperature range, thus being a kinetic transition (Menczel et al., 2009). For this reason, T_g is defined as the midpoint of the linear variation of the polymer heat capacity and, taking into account this inflexion point, is the maximum of the first derivative curve.

In this study, to determine the T_g for the different lignin fractions from organosolv process, it was applied a first heating to remove the thermal history in all lignin fractions. Values of T_g obtained from DSC are given in **Table 12**. The studied lignins had a glass transition temperature between 73–85 ℃, and it was not possible to detect T_g in treatment with the highest ethanol concentration (80%). The results showed that, in general, the increase in treatment severity led to a decrease in T_g values.

The low values of T_g are related to the decline in the rigid aromatic backbone of macromolecules due to the replacement of hydroxyl groups (namely phenolic hydoxyl groups) by ester, which lead to a reduction in the number of hydrogen bonds in the lignin

molecule, implying an increase of the free volume and thus the mobility of the chains (Gordobil et al., 2017; Li and McDonald, 2014).

In general T_g of lignin has been reported to be between 90 and 180 ℃ (Lora, 2008; Lora and Glasser, 2002), but lower T_g has been reported for organosolv lignin (Hansen et al., 2016; Huijgen et al., 2014; Hussin et al., 2013; Michelin et al., 2018). For example, Hussin et al. (2013) reported T_g value for soda lignin of 81.79 ℃, while for kraft and organosolv lignins were 64.62 ℃ and 51.65 ℃, respectively, suggesting that the relation between the molecular weight of lignin samples and the free volume can affect the T_g values (Hussin et al., 2013) . Li and McDonald (2014) studied the fractionation of commercial lignin with methanol and reported a lower T_g for the methanol soluble lignin fractions, 117 ℃ for Indulin AT lignin (softwood kraft) and 132 ℃ for Protobind 1000 lignin (agricultural fiber soda pulp), than the original lignin, which was related to the fewer condensed structures of the methanol soluble lignin fractions (Li and McDonald, 2014). On the other hand, Martín-Sampedro (Martín-Sampedro et al., 2019) showed a T_g at 138 ℃ for lignin obtained from organosolv treatment. Low T_g temperatures, as those of the current work, may be related to more thermoplastic lignins. Thus, these OL are more susceptible to be mixed with synthetic and bio-based polymers (Martín-Sampedro et al., 2019).

3.3.3.5. Radical scavenging activities

In the present study the antioxidant properties of the different lignin fractions were characterized using two different tests. The results of the DPPH and ABTS radical-scavenging activity are presented in **Table 13** in terms of IC_{50}. IC_{50} measured the concentration of the tested antioxidant sample required for a 50% inhibition of radical species. The lower is this value the higher is the radical scavenging activity of the compounds tested.

Radical scavenging activity of DPPH of all OL fractions did not strongly differ between samples (IC_{50} from 0.14 to 0.21 mg/mL). Although their antioxidant potential was lower than that of Trolox (0.06 mg/mL), they were comparable to BHT (0.16 mg/mL), commercial antioxidants often used as standards. **Figure 19** compares the RSA of L9 (the optimal condition for OL extraction), L4 (OL with the lowest IC_{50}) and the

standards trolox and BHT, confirming the antioxidant potential of OL is comparable to BHT. The same behavior was obtained for radical scavenging activity of ABTS.

Table 13- Summary of IC50 values of lignin samples.

Samples	IC_{50} in DPPH radical-scavenging activity (mg/mL)	IC_{50} in ABTS radical-scavenging activity (mg/mL)
OL_1	0.16 ± 0.01	0.31 ± 0.02
OL_2	0.18 ± 0.00	0.46 ± 0.06
OL_3	0.17 ± 0.00	0.23 ± 0.01
OL_4	0.14 ± 0.00	0.24 ± 0.01
OL_5	0.21 ± 0.00	0.26 ± 0.00
OL_6	0.19 ± 0.02	0.23 ± 0.00
OL_7	0.16 ± 0.00	0.48 ± 0.00
OL_8	0.18 ± 0.00	0.40 ± 0.01
OL_9	0.17 ± 0.001	0.40 ± 0.01
OL_{10}	0.20 ± 0.01	0.37 ± 0.02
Trolox	0.06 ± 0.00	0.13 ± 0.00
BHT	0.16 ± 0.00	0.13 ± 0.00

It has been reported (Ross et al., 2012) that the antioxidant activity is related to the lignin extraction technique. Phenolic functionalities present in the chemical structure as well as the molecular weight (Mw), which could be affected by the extraction process. For example, Béghin et al. (2015), reported IC_{50} of around 0.31 mg/mL for organosolv lignin and 0.37 mg/mL for alkali lignin (DPPH method), that showed to be related with the Mw, where the organosolv lignin samples presented Mw of 1590 – 2810 g/mol and alkali lignin of 3160 – 5090 g/mol (Véronique et al., 2015). Michelin et al. (2018) also reported low IC_{50} for organosolv lignin (0.17 – 0.26 mg/mL, DPPH method) (Michelin et al., 2018). On the other hand, An et al. (An et al., 2017) have approached the fractionation of enzymatic hydrolysis lignin by organic solvent extraction as strategy for enhancing its antioxidant performance.
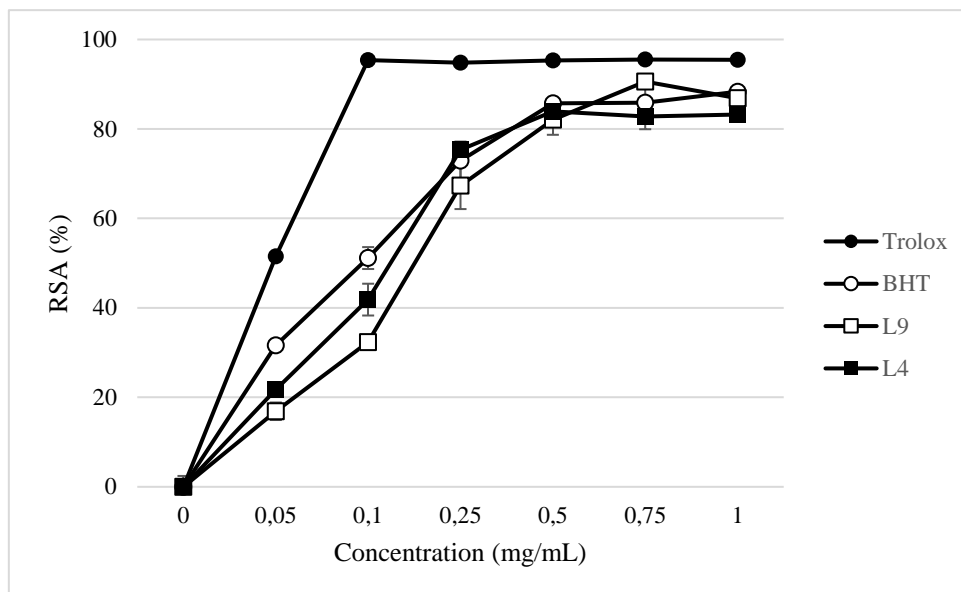
Figure 19- Radical scavenging activities of OL samples (OL4 and OL9) compared with commercial antioxidants (BHT and Trolox) for DPPH assay.

3.4. Conclusion

AM2-3 was further processed by an organosolv treatment aiming at improving lignin extraction. Considering the results obtained in this work, experiment 9 (ethanol concentration 80 %, temperature 200 ºC, time 60 min) allowed a high delignification (49.40 %) with a lignin yield of 24.47 % and lignin extraction efficiency of 48.34 %.

Moreover, the isolated lignin samples (OL1-OL10) showed high purity and also kept the main structure, according to FTIR results. Nonetheless, the organosolv process influenced the thermal properties of the lignin, becoming more thermoplastic and, for this reason, more susceptible to be used as bio-based polymers. Regarding to antioxidant activity, the lignin fractions showed antioxidant activity as high as the commercial antioxidant BHT. The Y_G of enzymatic hydrolysis was 53 % at 10% solids load, however this yield could be higher if the cellulose fraction did not have the presence of repolymerized lignin.

This chapter showed that the application of the organosolv process to previously autohydrolyzed biomass makes possible to produce a high-quality lignin, with high purity and structure relatively unaltered.

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CHAPTER 4 |

Scale-up of lactic acid production

Abstract

This chapter was focused on evaluating a mixture of lignocellulosic biomass in order to produce lactic acid (LA) by *Lactobacillus rhamnosus* within a multi-supply biorefinery scheme. The mixture of lignocellulosic biomass was submitted to autohydrolysis pretreatment under non-isothermal regime at 226 ℃ (corresponding to a severity of 4.15) yielding a glucan recovery of 93 % in the solid phase.

Two different strategies were assayed for LA production, namely separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The glucose to LA yield obtained for both assays was 1 g/g, although the volumetric productivity of SSF (2.5 g/Lh) was higher than SHF (0.8 g/Lh). Therefore, the SSF process was optimized through a factorial design to evaluate the effect of the independent variables, solids load and enzyme-substrate ratio (ESR), on LA production. The maximum concentration of LA was obtained using the highest solids load (16 %) and with the highest ESR (54 FPU/g). Finally, scale up of LA was performed in a bioreactor under the optimized conditions of Erlenmeyer, obtaining 61.74 g/L of LA at 44 h which corresponds to LA yield of 0.97 g/g.

This chapter is based on the following paper:

Pontes, R., Romaní, A., Michelin, M., Domingues, L. Teixeira, J., Nunes, J. 2021. Scale-up of lactic acid production from multi-supply autohydrolyzed economically unexploited lignocellulosic biomass *(submitted to Industrial Crops and Products).*

Contents

4.1. Introduction

The demand for lactic acid (LA) is expected to increase due to new processing technologies and further fields of application, namely biodegradable polymers, green solvent and oxygenated, fine and commodity chemicals (Ziadi et al., 2020). Traditionally LA is used in food industry as a preservative, acidulant, flavoring agent or pH buffer (Jawad et al., 2013; Pejin et al., 2017). LA (2-hydroxypropanoic acid) is a chiral molecule with two optical enantiomers, L-(+)-LA and D-(-)-LA, produced via either chemical synthesis or microbial fermentation (Choi et al., 2019; Mora-Villalobos et al., 2020) Through microbial fermentation an optically pure LA can be obtained by choosing an appropriate strain of lactic acid bacteria (LAB).

LAB are non-sporuling, non-motile, acid-tolerant, non-respiring but aerotolerant, catalase-negative, Gram-positive cocci or rods and recognized as safe (GRAS). They are characterized to produce LA as the major end product of sugars derived from carbohydrate (Mora-Villalobos et al., 2020).Some LAB species are capable of synthetizing only one of the two enantiomers, most of them produce the L-(+) enantiomer, partly because the D-(-) cannot be metabolized by animal cells. As an example, *L. rhamnosus* produces L-(+)-LA (Cubas-Cano et al., 2018; Mora-Villalobos et al., 2020). Furthermore, pure isomers are more valuable than the racemic mixture due to each isomer's specific industrial application (Pejin et al., 2017).

Lignocellulosic biomass (LCB) has gained a lot of interest for LA production. Currently, corn and cassava were commercially used as raw material, however, the cost of feedstock is more than 50% of the overall production cost (Unrean, 2018). Other LCB also have been studied and reviewed, namely from sorghum, corncob, bagasse and wheat straw (Alexandri et al., 2019; Tarraran and Mazzoli, 2018). Therefore, is crucial to select a low-cost feedstock, obtained in large quantities, unexploited or exploited inefficiently and renewable to make LA production cost-effective (Unrean, 2018).

In the present chapter we explore unexploited LCB sources, namely forest ecosystems and biological resources from marginal land, in a mixture regime, not studied so far. LCB is primarily composed of the two carbohydrate polymers, cellulose and hemicellulose, and the non-carbohydrate phenolic polymer (lignin). Thus, the microorganism should be selected for the corresponding fermentation, for example, glucose is widespread metabolized, but most microorganisms lack the ability to utilize

xylose. In this regard, mixed-culture fermentation systems have been studied using LAB to produce LA. In a typical mixed-culture system, microorganisms that are suitable for each sugar are involved (Zhang et al., 2018).

Still, there are several technical barriers to use lignocellulosic biomass, namely pretreatment to cleave the complex structure (Zhang et al., 2018; Zhou et al., 2016). An effective and ecofriendly pretreatment of LCB is required to overcome its intrinsic recalcitrant nature prior to the production of LA. In this case, a hydrothermal pretreatment was selected for being an ecofriendly process (only requires high temperature and water). This pretreatment promotes hemicellulose solubilization, cellulose decrystallization, reduces the lignin-recalcitrant behavior, while increases the surface accessibility for hydrolysis, in this work for enzymatic hydrolysis (Romaní et al., 2010; Sarip et al., 2016).

Enzymatic hydrolysis is the most promising process to obtain a high yield of fermentable sugars from pretreated lignocellulosic biomass allowing LAB to use them as a carbon source (Abdel-Rahman et al., 2011; Siqueira et al., 2020).

Fermentation technology is another important factor that affects the production and scalability of LA production. Both Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are process configurations used for the biochemical process. In SHF, saccharification and fermentation are carried out in two steps, while in SSF they are combined in one (Müller et al., 2017). The first one allows for optimum temperature for cellulases and producer cells, although the duration of the process is longer (Maslova et al., 2019). High solid load should also be favorable, as it can yield high titer of LA (Unrean, 2018). SSF reduces reactor volume, processing time, and feedback inhibition and consequently increases productivity (Marulanda et al., 2019). Microorganisms in the SSF system can readily consume the sugars, maintaining the sugar concentrations at a low level reducing the osmotic pressure of cells, while significantly reducing the feedback inhibition of the enzymes (Chen et al., 2019a). The disadvantage of SSF lies in the difference of optimum temperature and pH required for saccharification and fermentation, as well as LA inhibition of the enzymes (Zhang et al., 2018).

Despite attracting a lot of attention, the large-scale production of LA from lignocellulosic biomass has not yet been commercialized. The scalability limitations of LA depend on the type of lignocellulosic biomass, pretreatment, saccharification, strains and fermentation process. For any pretreatment process to be commercially feasible, energy costs and solvent requirements for the separation, depolymerization, and conversion of lignocellulose must be taken into account (Grewal and Khare, 2018). In the

present chapter, the performance of SSF and SHF of autohydrolysis pretreated lignocellulosic biomass for LA production was evaluated. SSF process, according to an enzyme-substrate ratio (ESR) and solids load, was assessed by experimental design and a predictive model was developed. Finally, the SSF process to maximize LA production efficiency was performed in 5L stirred tank bioreactor. This work aims to fulfil the knowledge gap regarding to the production of LA from multi-supply lignocellulosic biomass for flagship and industrial projects. One of the most important challenge for industrialization of LA production from lignocellulosic biomass is the security supply of the raw material. Take into account this challenge and necessity of the market and to increase the private investment in this field, the work was focused:

1. Sustainable raw material, with different lignocellulosic biomasses from forest ecosystems namely, eucalyptus (*Eucalyptus globulus*) and pine (*Pinus pinaster*) and from biological resources from marginal land, namely broom (*Cytisus sp.*), carqueja (*Genista tridentate*), mimosa (*Acacia dealbata*) and rockrose (*Cistus ladanifer*), without competitive with feed and food sectors and without commercial exploitation. Marginal lands in Portugal, representing 22% of the total territory without economic activity use, are associated with the paradigm of the rural fires, as they increase the fuel available in the land resulting in a loss of roughly 800 million Euros annually (Pontes et al., 2018);

2. Security supply: a mixture of raw materials was used in this work, which represents possible scenarios for a multi-supply biorefinery concept from residues and biological resources without commercial exploitation. The mixture defined in this work is a result of previous work (Pontes et al., 2018). This type of multi-supply concept is aligned with the principles of circular and sustainable bio economy European action plan, defined by European Commission (European Commission, 2018).

4.2. Materials and methods

4.2.1. Lignocellulosic biomass and pretreatment

Mixture of autohydrolyzed forest and marginal lands lignocellulosic biomass residues, namely M2-3 (mixture 2nd and 3rd quarters) was used as raw material. The mixture M2-3 represents 52.5 % of forest ecosystems (24.6 % of pine (*Pinus pinaster*) and 27.9 % of eucalyptus (*Eucalyptus globulus*)), and 47.5 % of biological resources from

marginal land (13.8 % broom (*Cytisus sp.*), 12.5 % mimosa (*Acacia dealbata*), 10.7 % carqueja (*Genista tridentata*) and 10.4 % rockrose (*Cistus ladanifer*)). The criterion of feedstocks mixture was previously reported in the study by Pontes and co-authors (Pontes et al., 2018). The raw materials were air-dried, milled and sieved between 0.250 to 0.400 mm using a vibratory sieve shaker (40 and 60 mesh). Afterwards, the samples were mixed in order to obtain a homogeneous lot and avoid compositional heterogeneity between the aliquots, and stored in polypropylene bags at room temperature.

M2-3 was submitted to an autohydrolysis pretreatment, following the standard heating temperature-time profile up to reach the final temperature (T_{MAX}) of 226 ℃, as reported by Pontes and co-authors (Pontes et al., 2018). The solid fraction obtained from the autohydrolysis pretreatment was named AM2-3.

4.2.2. Microorganism, culture media and inoculum preparation

Lactobacillus rhamnosus (ATCC American Collection 7469) was the organism used in this study. Stock cultures were maintained at -80 ℃ in the pre-culture MRS broth media with 20 % (v/v) glycerol. The inoculum for SSF and SHF assays was prepared by transfering the cells to 150 mL of MRS broth media placed in 250 mL erlenmeyer flasks. After 7 h of shaking at 37 ℃ and 150 rpm, a volume of 15 mL of this was transferred to the same amount of MRS medium, which was incubated again for 12 h under the same conditions. MRS broth media contained 20 g/L glucose, 5 g/L yeast extract, 10 g/L meat extract, 10 g/L enzymatic digest of casein, 5 g/L sodium acetate, 2 g/L diamonium citrate, 2 g/L K2HPO4, 0.2 g/L MgSO4.

4.2.3. Simultaneous and separated hydrolysis and fermentation in shake flask

The solid fraction AM2-3 was subjected to batch SSF and SHF. Both assays were carried out in 100 mL Erlenmeyer flasks with a final volume of 50 mL, 10 % of solids load and with approximately 50 g/L of potential glucose of AM2-3, taking into account the glucan content in the pretreated biomass, and nutrients (according to MRS broth without glucose), previously sterilized at 121 ℃ for 15 min. Commercial enzyme Cellic CTec2 (Novozymes, Bagsvaerd, Denmark) presented an enzymatic activity of 120 FPU/mL (measured as described by Ghose, 1987) (Ghose, 1987) and was employed with

an ESR of 25 FPU/g of substrate. The inoculum size of *L. rhamnosus* was 10 % (v/v). The experiment was conducted at 37 ºC, 150 rpm, for 28 h and to prevent acidification of fermentation medium caused by LA production, 0.6 g CaCO$_3$ per gram of initial glucose concentration was added to the medium. SHF was carried out like SSF, except for the enzymatic hydrolysis step, which was carried out for 41.5 h at 48 ºC and pH 4.8 previous to the fermentation.

The samples were withdrawn from the media at the desired times, centrifugated and analyzed by HPLC as described in 2.6 section – Chemical composition of material. The following expressions were calculated according to (Romaní et al., 2008) :

(1)

$$G_{POT} = \frac{Gn}{100} \times \frac{180}{162} \times \frac{\rho}{LSR + 1 - \frac{KL}{100}}$$

Whereas *G$_{POT}$* means glucose potential, Gn is the glucan content of solid residue recovered after autohydrolysis pretreatment (g glucan/100 g solid residue, on dry basis), 180/162 is a stoichiometric factor, ρ is the density of the reaction medium (average value, 1005 g/L), LSR is the liquid-solid ratio, KL is the Klason lignin content of solid residue

(2)

$$Y_G = \left(1 - \frac{\text{Residual glucose (g)}}{G_{POT}})\right)$$

Considering *Y$_G$* as glucose yield and residual glucose, the glucose remaining after fermentation.

(3)

$$Y_{\frac{L}{G}}(t) = \frac{L(t)}{G_{POT}}$$

Whereas *Y$_{L/G}$(t)* means glucose to lactic acid yield and and *L(t)* is the LA concentration at time *t* expressed as g lactic acid/L.

(4)

$$Y_{\frac{L}{RM}}(t) = \frac{L(t) \times (LSR + 1)}{1000}$$

Considering $Y_{L/RM}(t)$ as raw material to lactic acid yield.

(5)

$$Qp(t) = \frac{L(t)}{t}$$

Whereas *Qp(t)* is lactic acid volumetric productivity.

4.2.4. Experimental design and statistical analysis

Experiments were carried out according to the design generated by the software to study the effect of two factors (independent variables): solids load and ESR on LA concentration and glucose to LA yield (dependent variables or responses). This experimental design resulted in 11 with two factors at three levels, with three center points. The real and coded experimental variables are presented in **Table 14**.

Table 14. Value (real and coded) of experimental variables used in the experimental design.

Independent variables	Levels		
	-1	0	1
Solids load (%) (x_1)	8	12	16
ESR (FPU/g) (x_2)	18	36	54

The process behavior can be explained by a quadratic equation form, between independent variables and responses:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k}\sum_{j=i}^{k} b_{ij} x_i x_j + \varepsilon$$

Where y represents the predicted responses, namely yLA(24) represents LA concentration and yYL/G(24) glucose to LA yield. Whereas b0, bi ,bii, and bij means offset term, linear effect, quadratic effect and interaction effect respectively. The independents variables xi and xj represent solids load (SL) and enzyme to solid ratio (ESR). The factor ε is random error or allows for discrepancies or uncertainties between predicted and measured values.

4.2.5. Data fitting

Experimental results were evaluated with STATISTICA (data analysis software system), version 12 from StatSoft, Inc. (2014) (www.statsoft.com) for analysis of variance (ANOVA), regression coefficients, modeling the responses and graphical analysis. Sum of squares of residuals (SSR) was chosen for statistical significance and computation of standard error. Statistical analyses were performed with a 95% significance level.

4.2.6. Simultaneous saccharification and fermentation in bioreactor

The condition selected for scaling up was based on the experimental design assay (condition 11). Batch SSF was conducted in 2 L of a 5 L Bioreator BioFlo/CelliGen115 for 48 h. SSF was carried out with a LSR of 6 g/g, ESR of 54 FPU/g and 10 % (v/v) inoculum size, and conducted at 37 ºC, 150 rpm, and pH 4.8 controlled with 5 M NaOH. At given fermentation times, samples were withdrawn from the media and centrifuged. The concentrations of glucose, xylose, lactic acid and acetic acid were measured by HPLC in the supernatants (see Section 2.6 for details). The parameters were calculated according to the expressions described in Section 2.3).

4.2.7. Chemical composition of materials

Analytical assays were performed according to the following methods: moisture (NREL/TP-510-42621) T-206-om-88 m) and quantitative acid hydrolysis with 72 % w/w sulphuric acid (NREL/TP-510-42618). The solid phase from the quantitative acid hydrolysis was considered as Klason lignin. The hydrolysates from acid hydrolysis were analyzed by high performance liquid chromatography (HPLC) for sugars (glucose, xylose), lactic acid and acetic acid using the columns Aminex HPX-87H (conditions: refractive index detector; flow rate of 0.6 mL/min at 60 ºC; 0.005 M H_2SO_4 as mobile phase). Analyses were carried out in duplicate.

4.3. Results and discussion

4.3.1. Effect of autohydrolysis pretreatment on M2-3 lignocellulosic biomass mixture

To evaluate the potential of pretreatment biomass for LA production, M2-3 was submitted to autohydrolysis treatment to obtain an enriched solid phase (or AM2-3) composed by 48.47 ± 0.34 % of cellulose (measured as glucan), 0.72 ± 0.10 % of xylan and 43.53 ± 0.45 % of Klason lignin. The liquid phase enriched in hemicellulose-derived compounds was composed mainly by oligosaccharides (OS), including 0.87 g/L glucooligosaccharides (GOS), 0.95 g/L xylooligosaccharides (XOS), 0.05 g/L arabinooligosaccharides (ArOS) and 0.52 g/L acetyl groups (AcGOS). The content of monosaccharides in autohydrolysis liquor was 2.23 g/L of glucose, 2.06 g/L of xylose and 0.1 g/L of arabinose; and other by-products such as organic acids, 4.68 g/L of acetic acid, and furans (0.21 g/L of hydroxymethlyfurfural and 1.09 g/L of furfural).

Therefore, at these conditions the recovery of glucan and lignin in the solid phase was 93.47 % and 91.53 %, respectively. The xylan solubilization as a sum of xylose and XOS was 12.63 %.

4.3.2. Simultaneous and separated hydrolysis and fermentation of AM2-3: preliminary assays

The solid fraction obtained from the autohydrolysis pretreatment (AM2.3), enriched with cellulose content was submitted to enzymatic hydrolysis and fermentation. SSF and SHF were conducted with 10 % of solids load (w/v) with the same enzyme loading in batch mode at 37 ºC for 52 h (except for the saccharification step in SHF which was performed at 48 ºC) as presented in the **Figure 20**. The non-detection of residual sugar showed that glucose was consumed rapidly and suggests that all of it was converted to LA. Additionally, the slower rate of glucose released during SSF implies a slower LA production, in the first 7 h, compared to SHF. Nonetheless, there was a lag phase (approximately 2 h) on the onset of glucose assimilation and LA production for both assays.
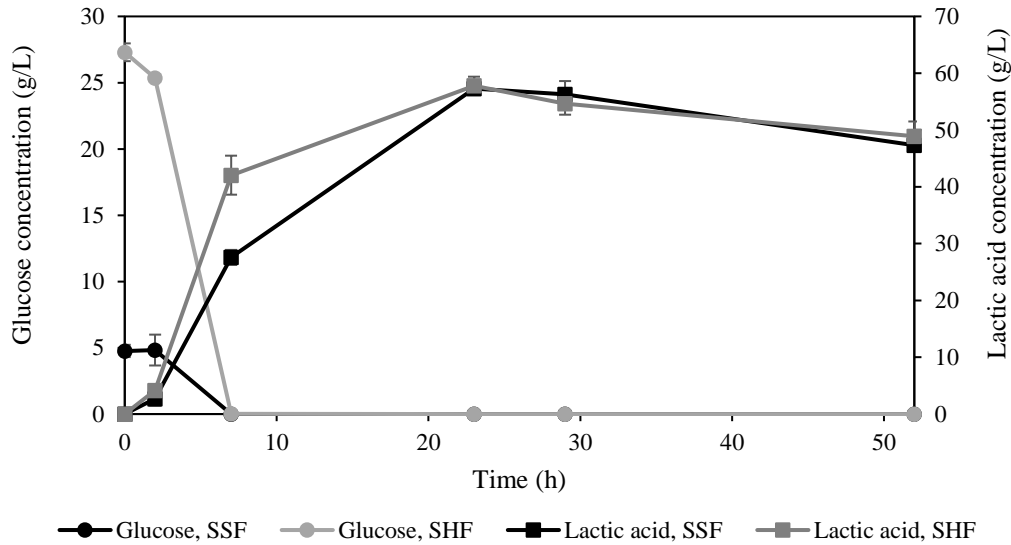
Figure 20. Comparison of the SHF and SSF processes during LA fermentation using *L. rhamnosus.*

At the end of fermentation both processes reached the same titers of LA, as reported by Muller et al. (Müller et al., 2017). The maximum LA concentration was 57.29 ± 0.97 g/L for SSF and 57.78 ± 1.61 g/L for SHF, both achieved at 24 h of fermentation. The glucose was comply consumed and metabolised to LA demonstrating that the processing of this raw material does not yield strong inhibitors to *L.rhamnosus*.

The $Y_{L/G}(t)$ at 24 h was 1 g/g for both assays. However, as in enzymatic hydrolysis, during SHF, was carried out for 48 h before fermentation, the volumetric productivity, at 24 h, was 0.81 g/Lh, 3 folds lower than that obtained on SFF. For this reason, the SSF demonstrated a remarkable advantage over the SHF, a phenomenon also reported by Zhou et al. (Zhou et al., 2016).

Comparing our results with those of Marques et al. (Marques et al., 2017), they reported a maximum of LA yield ($Y_{L/G}$) of 0.78 g/g, using MRS medium containing 56 g/L of glucose with *L. rhamnosus* in SSF, which was 1.3 times less than the yield reported in our work with a complex raw material. Our results were also more promising than those reported by Cui and co-authors, which obtained a LA yield ($Y_{L/G}$) of 0.70 g/g and a productivity of 0.58 g/Lh from NaOH-treated corn stover with a mixed culture of *L. rhamnosus* and *L. brevis* (Cui et al., 2011). Due to the very attractive LA yield and productivity under SSF, an experimental design based on the load of AM2-3 solids and

cellulase was carried out in order to improve LA production and is presented in the next section.

4.3.3. Optimization of simultaneous saccharification and fermentation: Experimental design

Highly efficient SSF process is expected when sugars are released into the medium, by enzymatic hydrolysis, and quickly converted to LA by *L. rhamnosus*. Thus, a high solid loading can favor the process as long as it yields a higher LA titer.

The present experimental design has been carried out to optimize the process parameters (solids load and ESR) for maximizing the production and yield during the conversion of lignocellulosic biomass into lactic acid by *L.rhamnosus*.

The dependent responses evaluated in this work were *L(24)* and $Y_{L/G}$ *(24)*, nonetheless other variables were also calculated and listed in Table 15 (such as $Y_{L/RM}$ *(24)* and *Qp(24)*.

Regarding to *L(24)* the quadratic model explained the role of each variable and their interaction on lactic acid production, as follows:

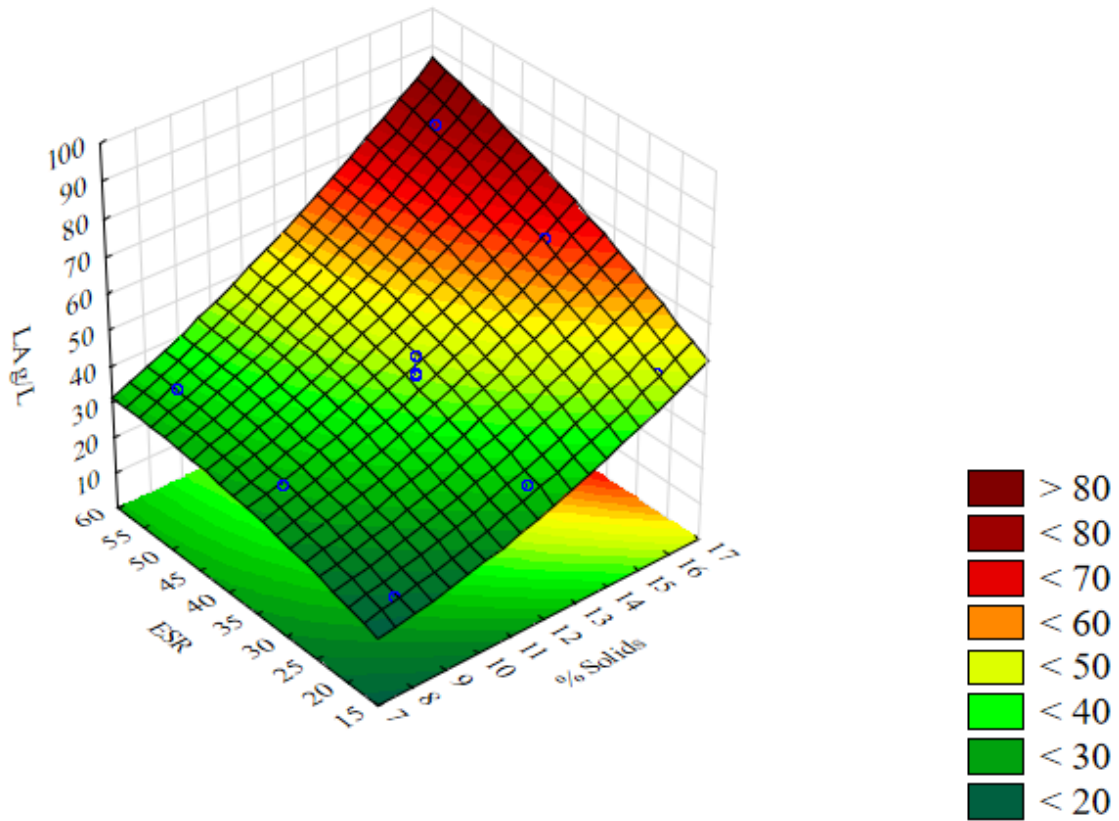$$L(24)\ (g/L) = 45.22 + 17.09 * SL + 10.33 * ESR + 3.92 * SL * ESR$$

The statistical significance of Equation, namely the significance of each coefficient, was checked by analysis of variance (ANOVA). The terms having *p -value* < 0.05 are identified as significant, smaller the values of *|p|* more significant is the correlation with the corresponding coefficient (**Table 15**). Significant interactions between SL and ESR has also been demonstrated by ANOVA) which indicates that SL and ESR did not work independently.

Table 15. ANOVA for *L(24)* produced response from the optimizing design.

Effect	Sum of squares	Degree of freedom	Mean squares	*F*-ratio	*P* value
Solids load (SL)	1752.87	1	1752.87	319.71	0.00001
Enzyme to solid ratio (ESR)	640.23	1	640.23	116.77	0.00012
SL. ESR	61.46	1	61.46	11.21	0.02037
Pure error	27.41	5	5.48		
Total	2506.56	10			
		$R^2=0.9891$			
		Adjusted $R^2=0.9781$			

The regression model in Equation fitted the data adequately since the value of the coefficient of determination R2 was high (0.99), meaning that 99 % of the total variation was explained by the model. This suggested a satisfactory representation of the process model and a good correlation between the experimental and predicted values.

The response surface graphic (**Figure 21**) also proved the positive significant interaction between SL and ESR, which means that the higher the solids load and the ESR the greater the LA concentration. Thus, to obtain higher LA concentration (> 77.83 g/L) the SL and ESR should be increased. On the other hand, as described, the lower values of LA concentration were obtained with lower SL and ESR.

Figure 21. Response surface for *L(24)* with variable solids loads and ESR.

Concerning to $Y_{L/G}$ *(24)* the linear model explained the role of each variable on lactic acid yield, as follows:

$$Y_{\frac{L}{G}}(24)(g/L) = 83.12 + 6.39 * SL + 18.91 * ESR$$

The statistical significance of Equation, namely the significance of each coefficient, was also checked by analysis of variance (ANOVA). The terms having *p - value* < 0.05 are identified as significant (**Table 16**).

Table 16. ANOVA for $Y_{L/G}(24)$ produced response from the optimizing design.

Effect	Sum of squares	Degree of freedom	Mean squares	F-ratio	P value
Solids load (SL)	245.16	1	245.16	13.59	0.01421
Enzyme to solid ratio (ESR)	2145.48	1	2145.48	118.89	0.00011
Pure error	90.23	5	18.05		
Total	2552.16	10			
		$R^2=0.9647$			
		Adjusted $R^2=0.9293$			

The regression model in Equation fitted the data adequately since the value of the coefficient of determination R^2 was high (0.96) which is an indicative of a good agreement between experimental results and predicted values and suggested that only 4% of the variation was not explained by the model.

The response surface graphic (**Figure 22**) showed the dependence of variables SL and ESR on $Y_{L/G}(24)$ but also exhibited insignificant interaction between SL and ESR. As shown on **Table 16**, both variables are significant, nonetheless, $Y_{L/G}(24)$ was mainly influenced by ESR.
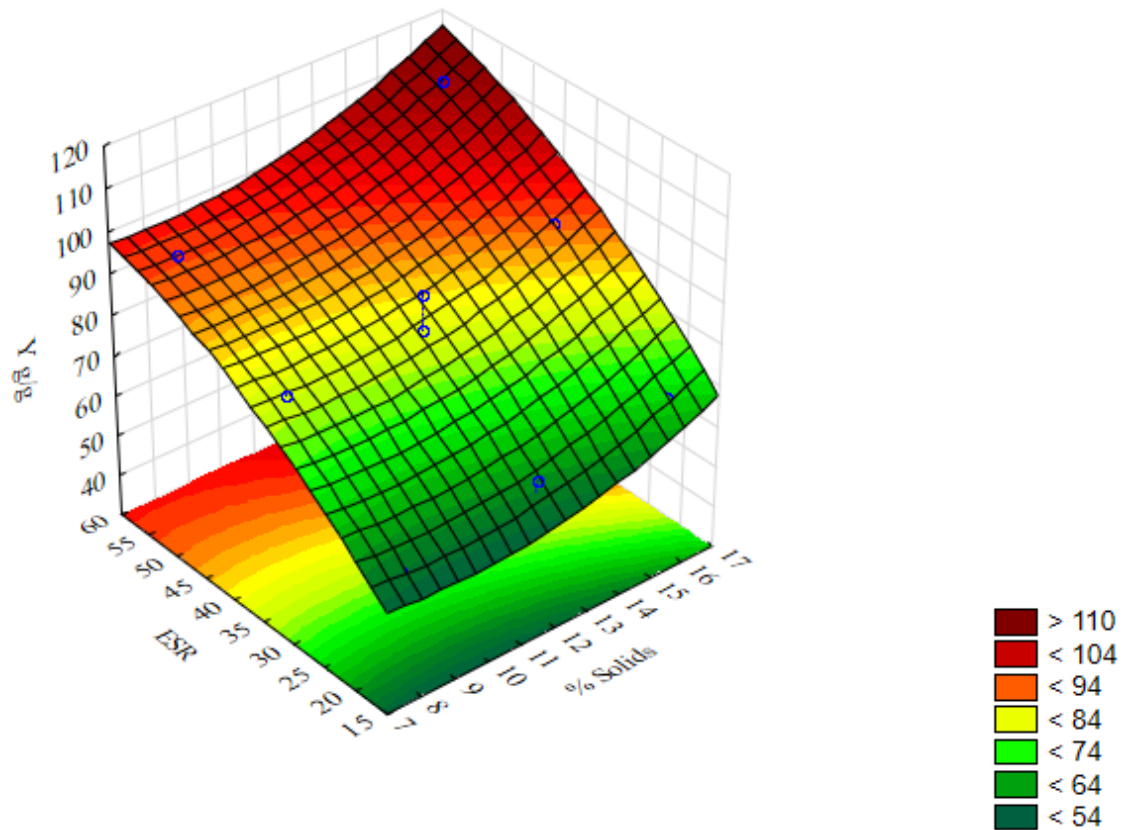
Figure 22. Response surface $Y_{L/G}$ *(24)* with variable solids loads and ESR.

The higher *L(24)* and $Y_{L/G}$ *(24)* was obtained at extreme conditions, namely in the experiment 11, with the highest concentration of LA 77.83 g/L and maximum LA yield of 1 g/g obtained with 16% of SL and 54 FPU/g of ESR.

Clearly, the statistical projection for the highest LA production was reached in the direction of higher solids load (16 %) and higher ESR (54 FPU/g). This relationship was expected since it followed directly from the fact that a higher enzyme load may be required to hydrolyze a higher concentration of substrate and thus increase the level of glucose available as substrate for the *L. rhamnosus* fermentation.

These results were very favorable when compared to the literature with the same SSF batch process. For example, Marques et al. obtained a maximum LA yield of 0.97 g/g and a productivity of 2.90 g/Lh using recycled paper sludge, a less recalcitrant raw material,with *L. rhamnosus* (Marques et al., 2008), while Shi et al. reached a LA yield of 0.83 g/g and a productivity of 0.62 g/Lh through a mixture of softwood pre-hydrolysate and paper mill with *L. rhamnosus* (Shi et al., 2015) . Zhao et al. reported a LA yield of

0.77 g/g, in this case with corn stover, using *Pediococcus acidilactici* (Zhao et al., 2013) and Chen et al. obtained a highest productivity of 2.74 g/Lh and LA yield of 0.88 g/g from cassava bagasse (starch + cellulose + hemicellulose) using simultaneous saccharification and co-fermentation (Chen et al., 2020).

4.3.4. Lactic acid production in bioreactor

In order to validate the optimized values of the process variables for the maximum production of LA, SSF process was carried out in the 5 L bioreactor. Firstly M2-3 was submitted to autohydrolysis pretreatment at 226 ℃.

SSF was carried out with 16 % solids load and ESR of 54 FPU/g. As the mixture is very dense due to the high load of solids, at first the agitation was kept as high as possible, but after the inoculation the agitation was reduced and kept at 150 rpm. The first sample was taken one hour after the onset of the process. Cell growth could not be quantified because of the biomass particles in the fermentation medium.

In this experiment, two stages were observed (**Figure 23**), (1) glucose concentration decrease sharply and LA was generated at high rate (5.64 g/Lh at 6 h) during the first 6 h (2) enzymatic hydrolyis was the limiting step and the rate of LA production decreased to less than half (2.25 g/Lh at 24 h). After 19 h, glucose was almost completely depleted and xylan was simultaneously hydrolysed, releasing xylose in the medium, although *L.rhamnosus* was not able to consume xylose and, for this reason, it remained in the medium (Khanongnuch et al., 2021). Acetic acid was found in the medium, as this compound was present in the raw material AM2-3 and also in the nutrients of the MRS broth, and remained constant throughout the fermentation process.
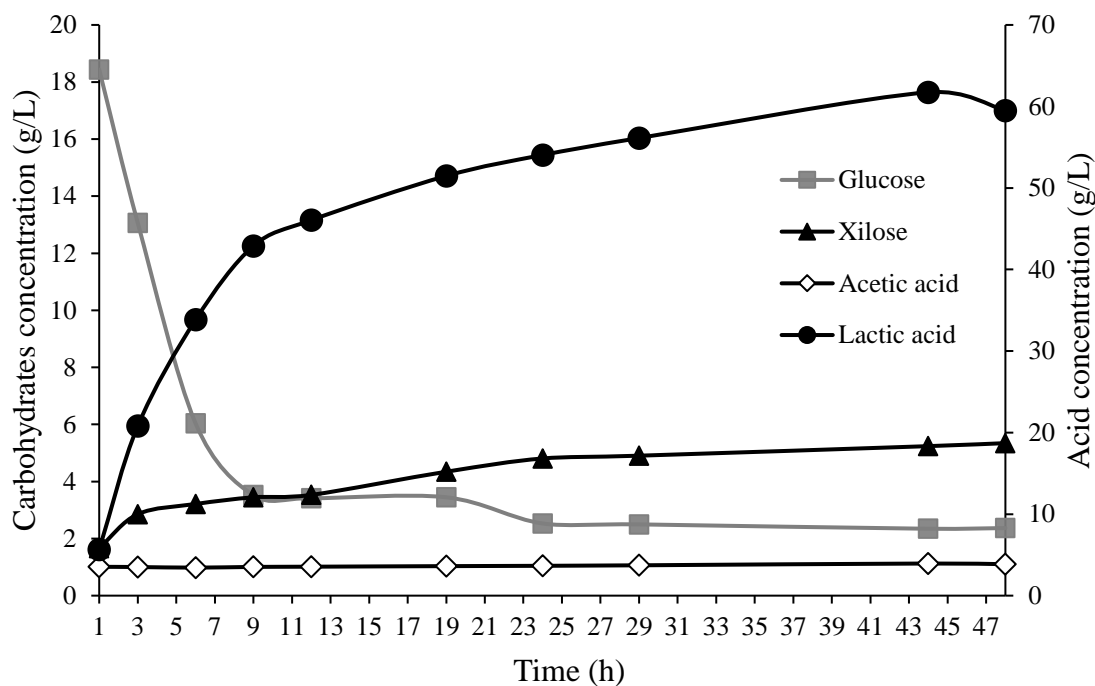
Figure 23. Time course of glucose, xylose, lactic acid and acetic acid for the bioreactor experiment.

The maximum L-LA concentration obtained was 61.74 g/L at 44, with optical purity of 98% and LA yield ($Y_{L/G}$) of 0.97 g/g. This means that starting from 100 g of dry AM2-3, 45.07 g of LA can be obtained with a volumetric productivity of 1.40 g/Lh. Few works have carried out LA production in bioreactor, still our results outperformed those of other studies carried out using similar raw materials and batch process in bioreactor. As an example, Unrean reported a LA yield of 0.61 g/g and a productivity of 1.01 g/Lh in optimal fed-batch SSF bioreactor with pretreated sugarcane bagasse, enzyme and cell combinatorial feeding (Unrean, 2018).

As verified, the yield was not 100 %, as reported on shake flasks assays (**Table 17**) because, in this case, the glucose was not completely consumed, with a concentration of 2 g/L of residual glucose being found at the end fermentation. These results, as well as those reported by Marques et al. (Marques et al., 2017), demonstrated an effective inhibition during the LA fermentation by the accumulated product, for LA concentrations equal to or above 62 g/L in SSF from recycled paper sludge with *L. rhamnosus* (Marques et al., 2017). These were also in agreement with Iyer and Lee (Iyer and Lee, 1999), who observed a decline in volumetric productivity of more than 6 times, in SSF from cellulose with *L. rhamnosus*, with LA concentrations higher than 65 g/L (Iyer and Lee, 1999). However, in the previous shake-flask experiments, (0.5 L) (**Table 15**) no inhibition was

observed and LA production reached 77.83 g/L. This difference could be explained by pH as the inhibitory threshold for total LA concentration should be dependent on pH value. In fact, at bioreactor scale the pH was maintained at 4.8 with NaOH and at experiment carried out in Erlenmeyer flask , $CaCO_3$ was used to buffer the media.

Table 17. Main experimental data from 0.05 L in Erlenmeyer flask and 2 L in bioreactor of batch process of *L.rhamnosus*. The fermentations conditions were 16 % solids load and 54 FPU/g of ESR. The values correspond to the end of fermentation, 24 h and 44h respectively.

Parameteres	Erlenmeyer flask 0.05 L	Bioreactor 2 L
L **(g/L)**	77.83	61.74
$Y_{L/G}$ **(g/g)**	1.11	0.97
$Y_{L/RM}$ **(g/g)**	0.56	0.45
Qp **(g/Lh)**	3.24	1.40

Conversely, LA production was negatively affected by scale-up, although the ratio of solids load and ESR were maintained. Scale up causes two important changes, (1) the mixing behavior and consequently mass transfer which lead to a less availability of nutrients for the cells and (2) the shear stress affect intrinsic resistance of LAB. Shear stress is caused by agitation, and, for this reason, the choose of the appropriate stirrer speed and design is essential (Ziadi et al., 2020). Thus, the decrease in more than 2-fold in lactic acid productivity could be related to the impeller three-paddle helix used to promote the homogenization of the media which caused shear stress. The same was reported by Ziadi et al. that showed a decrease in LA productivity during scale-up attributed to the high shear stress caused by the turbine type Rushton. Furthermore, the geometric and hydrodynamic of the bioreactor causes changes in fermentation (Ziadi et al., 2020). Despite some differences reported in the scabiliy, it was possible to develop a LA process with a yield close to 1g/g.

4.3.5. Mass balance and process comparison

Mass balance of the LA produced in a bioreactor from forest and marginal land resources using autohydrolysis as a pretreatment is shown in **Figure 24**. For 1000 g of raw material, 667.8 g of solid phase could be obtained after pretreament. After SSF, 300 g of L-LA was obtained. Ongoing advances of LA production from lignocellulosic biomass are listed on **Table 18**.
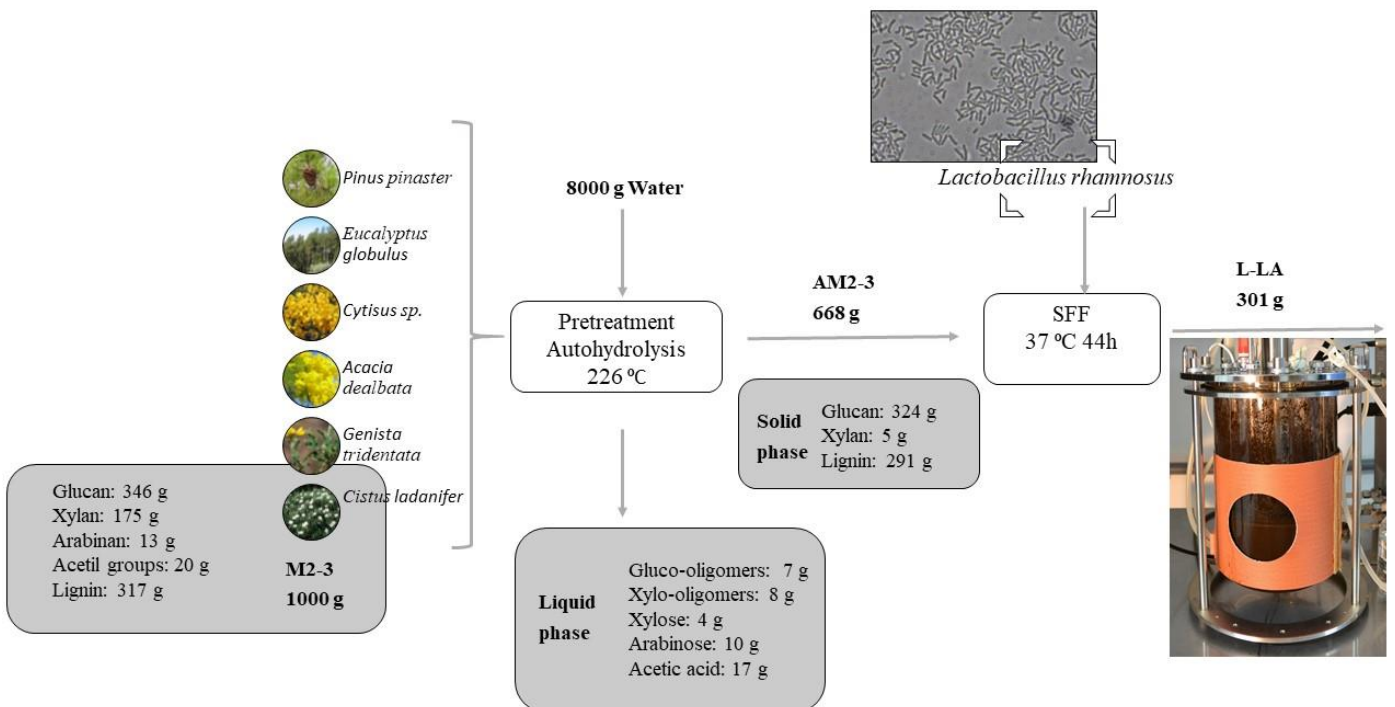
Figure 24. Mass balance of the optimized process for L-LA production.

From the current researchers for LA production from lignocellulosic biomass can be seen two different approaches. First the selected raw material and second the pretreatment used. Most of them are dependent from on single raw material and considering the supply is important to guarantee the sustainability, availability, seasonality, price volatility and storage (Pontes et al., 2018). The other important approach is the pretreatment, most of them suffered from costly and environmental

unfriendly processes. Autohydrolysis requires no other reagents than water and high temperature (Xu et al., 2017). Considering the reported, our results based on a new approaches (multi-supply lignocellulosic biomass) showed a greater possibility to produce LA with higher yields. This fact demonstrates which is possible to realize a flagship and industrial project with security supply and sustainability (based on multi-supply lignocellulosic biomass from a mixture of forest ecosystems and biological resources from marginal land).

Table 18. Comparative analysis of lignocellulosic biomass conversion into lactic acid.

Microorganism	Feedstock	Pretreatment	Fermention	Lactic acid			Reference
				C (g/L)	Y (g/g)[a]	P (g/L h)	
L. delbrueckii subsp. Bulgaricus (ATCC 11842)	*Pinus sp.*	Mild oxidative organosolv	SSF	36.4	0.40	0.51	(Karnaouri et al., 2020)
B. coagulans	Rice straw	Dilute ethylediamine	Fed-batch SSF	92.5	0.58	2.01	(Chen et al., 2019b)
L.pentosus	Sugarcane bagasse	Stream pretreatment with 0.5 % H_2SO_4	SSF	72.75	0.24	1.01	(Unrean, 2018)
L. rhamnosus ATCC 7469	Recycled paper sludge	-	SSF	53	0.20	-	(Marques et al., 2017)
Bacillus sp. NL01	Corn stover	Steam explosion	Fed-batch SSF	75.0	0.50	1.04	(Ouyang et al., 2013)
L.rhamnosus ATCC 7469	Brewer's spent grain	-	SHF	21.29	0.04	-	(Pejin et al., 2017)
B.coagulans strain IPE22	Wheat straw	Sulfuric acid	SSCF	38.7	0.46	0.65	(Zhang et al., 2014)
L. plantarum ATCC-10863	Mixture of softwood pre-hydrolysate and paper mill sludge	Hot-water	SSF	62.12	0.37	0.64	(Shi et al., 2015)
L. rhamnosus	Lignocellulosic mixture at 226 C	Autohydrolysis	SSF	61.74	0.45	1.40	Our study

C: Concentration
Y: Yield
P: Productivity
SSF: Simultaneous saccharification and fermentation
SHF: Separated Hydrolysis and Fermentation
SSCF: Simultaneous saccharification and co-fermentation
[a] g/g of pretreated raw material

4.4. Conclusion

The present chapter demonstrated an optimized batch SSF for the production of LA from an autohydrolyzed mixture of lignocellulosic biomass, from forest ecosystems and biological resources from marginal land not studied so far, by *L. rhamnosus*. SHF and SSF were successfully conducted on Erlenmeyer flasks and the glucose to LA yield obtained was 1 g/g for both assays, although the volumetric productivity was 3-fold higher for SSF. We demonstrated, through a factorial design with 11 experiments, with SFF process, there was a significant influence of the solids load and the ESR on the final LA concentration. This means that the higher the solids load and the ESR, the greater the LA concentration. The optimized condition was validated in a bioreactor. The achieved LA concentration (61.74 g/L), glucose to LA yield (0.97 g/g) and volumetric productivity (1.40 g/Lh) offers a process strategy to explore unexploited lignocellulosic biomass based LA production process for the industrialization of multi-supply biorefinery concept, contributing to the circular and sustainable bioeconomy development.

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CHAPTER 5 |

Life cycle assessment of lactic acid production

Abstract

Lactic acid (LA) demand is expected to increase due to new processing technologies and further several fields of application. The lactic acid production from unexploited lignocellulosic biomass was compared with the lactic acid production from non-renewable resources and it was modeled using the Life Cycle Assessment method through SimaPro®. The life cycle approach took into account the raw material, transport, pretreatment, saccharification and fermentation and LA recovery considering 1 tonne of LA as the functional unit. The life cycle inventory of the biobased LA was obtained from lab-scale experiments while the fossil-based LA was obtained from the ecoinvent database of SimaPro®. The major environmental savings obtained by replacing one tonne of fossil-based LA by biobased LA are : 4056.60 kg CO_2 eq. of global warming potential; 193.03 kBq U235 eq. of ionizing radiation potential; 3.78 kg C_2H_4 eq. of photochemical oxidation potential; 0.73 kg PO_4^{3-} eq. freshwater eutrophication potential; 9569.40 kg 1,4-DB eq. of terrestrial ecotoxicity potential; 99.32 kg 1,4-DB eq. of fresh water aquatic ecotoxicity potential; 137.69 kg 1,4-DB eq. of marine aquatic ecotoxicity potential; 94.89 human toxicity potential and 126.63 m^2 of land use. Auxiliary chemicals, electricity and enzyme used in the biobased LA production are most relevant to the total environmental impacts. Thus, biobased LA production significantly reduces the impact on the environment of LA production, giving 60 % environmental savings compared to fossil-derived LA.

Contents

5.1. Introduction

The concerns about global warming and the depletion of fossil reserves, associated with environmental impacts, are driving researchers to investigate the potential substitution of the current petrochemically derived building blocks with equivalent molecules obtained from renewable resources (Corma et al., 2007). Nonetheless, the environmental impacts of the growing demand for petroleum-derived chemicals and fuels can be mitigated by the use of lignocellulosic based biochemicals and biofuels, while also meeting the societal needs for greener products (Mandegari et al., 2017).

Lignocellulose biomass conversion should follow a biorefinery concept for carbohydrate conversion into fuels, chemicals, and energy, where a production of value-added compounds addressed to market demand, can impart robustness to the production facility business model, and exploit the full value presented by all fractions of lignocellulose, in a sustainable and environmentally favorable way (Mandegari et al., 2017).

The conversion of biomass to chemicals and other value-added products may create higher economic and environmental benefits compared to their conversion by synthetic route. More specifically, some recent investigations have shown that upgrading sugars and biomass into chemicals and polymers could lead to two-four fold more added value, when compared to the production of biofuels using a constant quantity of a given feedstock (Khoshnevisan et al., 2020).

Ethanol, lactic acid (LA), propionic acid, and succinic acid, among others, are some examples of the most important building blocks which can be produced from lignocellulosic biomass via biotechnological routes (Khoshnevisan et al., 2020).

In this context, LA is a key example of a platform molecule in biochemical production which has recently gained significant interest (Päivi et al., 2014). LA, the simplest hydroxy carboxylic acid is a bulk chemical with two optically active enantiomers D-(−) and L-(+). LA is a platform chemical with wide industrial applications demonstrating strong market growth, namely (i) in the food and beverage sector as a preservative acidulant, flavoring agent, pH buffer or for packing; (ii) generated multiple commodity and intermediate chemicals, for example pyruvic acid, acetaldehyde; (iii) biodegradable polymers such as poly lactic acid (PLA); (iv) exploited in pharmaceutical industry, for example internal drug dosing; (v) manufactured of hygiene products for cosmetic industry

(Daful et al., 2016; Jawad et al., 2013; Pejin et al., 2017). Global LA production is estimated to be in excess of 500 000 tonne per year (t/year) (Mandegari et al., 2017).

Current fermentation-based processes for LA production from lignocellulosic biomass are characterized by several technology barriers, namely (i) efficient separation of lignocellulosic components (cellulose, hemicellulose and lignin)(Ruiz et al., 2011); (ii) convention LA purification methods generate large quantities of gypsum as a by–product, with obvious environmental burdens since up to 1 kg gypsum per 1 kg LA produced was reported (Dusselier et al., 2013; Morales et al., 2015) and (iii) expensive purification requirements. In order to overcome these constraints, the selected pretreatment should start with separation of easily recovered fractions through a hydrothermal treatment as autohydrolysis(Yánez et al., 2014). Regarding the purification process, gypsum-free reactive distillation is a promising alternative and an environmentally friendly technology, whereby LA can be recovered at high purity (Mandegari et al., 2017).

Technologies to produce LA from lignocellulosic derived sugars are the least advanced, while the use of sugars from starch, sugar crops and whey are more commercially available (Daful et al., 2016).

In this study, as the first attempt, we considered a lactic acid production from lignocellulosic biomass whose concept was previously proved and tested at lab scale. Overall, the production routes of biobased and fossil based LA were thoroughly analyzed and compared -following a systematic approach- to support decisions towards the establishment of bioeconomy. Mass flow modeling and life cycle assessment (LCA) were performed in order to assess the feasibility of developing a biorefinery valorizing unexploited lignocellulosic biomass as a useful resource.

LCA has been widely used as a tool for the assessment of environmental performance of different products and services. According to ISO 14040-44 (2006), the main phases of an LCA are (i) goal & scope definition: where the product or service to be assessed is defined and a functional basis for comparison is chosen, (ii) inventory analysis: where the details on the data used for the assessment are discussed, (iii) impact assessment: where the effects of the resource use and the generated emissions are quantified into a limited number of impact categories, and (iv) interpretation of the results: where results are reported in the most informative way, along with the opportunities to reduce the impact of the product(s) or service(s) (Parajuli et al., 2017).

To the best knowledge of the authors, LCA of lactic acid production has only been investigated in a limited number of instances where studies focused predominantly on the

environmental performance of PLA, and specific consequences on GHG emissions and energy utilization of PLA production from corn and sugarcane.

The aim of this study is to present a quantitative evaluation of the environmental loads associated with (i) LA production from unexploited lignocellulosic biomass and (ii) LA produced from non-renewable resources. Two scenarios were identified and rigorous process models were developed using SimaPro Version 8 software (Aspen Technology Inc., Burlington, MA, USA).

5.2. Materials and methods

This LCA study was based on ISO 14040 (ISO 2006) ("ISO - ISO 14040:2006 - Environmental management — Life cycle assessment — Principles and framework," n.d.) and ISO 14044 (ISO 2006) ("ISO - ISO 14044:2006 - Environmental management — Life cycle assessment — Requirements and guidelines," n.d.), which is expressed by four steps: (1) goal and scope definition of LCA, (2) life cycle inventory analysis (LCI), (3) life cycle impact assessment (LCIA) and (4) life cycle interpretation.

5.3. Goal and scope definition

The goal of this life cycle assessment was to compare and quantify the environmental impacts caused by LA production. The scope, the full system boundary and process flow diagram of scenario 1, is schematized in **Figure 25**. It includes two scenarios of LA production from lignocellulosic biomass and from non-renewable resources. Then the following two scenarios are presented:

Sc1- (A-SSF-LA): Lactic acid production through autohydrolysis pretreatment (A) followed by simultaneous saccharification and fermentation process (SSF). SSF was carried out with a percentage of solids (S) of 16 % and enzyme to substrate ratio (ESR) of 54 FPU/g (E). LA was separated and purified from the media and the solid fraction (waste) was subsequently valued.

Sc2- (Ace-LA): Lactic acid was produced from acetaldehyde (Ace),

A time scope from 2018 to 2035 was considered as temporal boundary and Portugal was selected as the geographical scope.

Functional unit (FU) is a key element in LCA studies functioning as a reference to which inputs to the system and outputs are related. Several FUs have been used, although the correct selection should be done based on the objectives of the study (Khoshnevisan et al., 2020). To provide a reference flow for the two scenarios," 1 tonne of LA produced" was defined as the FU. The biobased and fossil based LA are assumed to be functionally equivalent because they can be used in the same way.
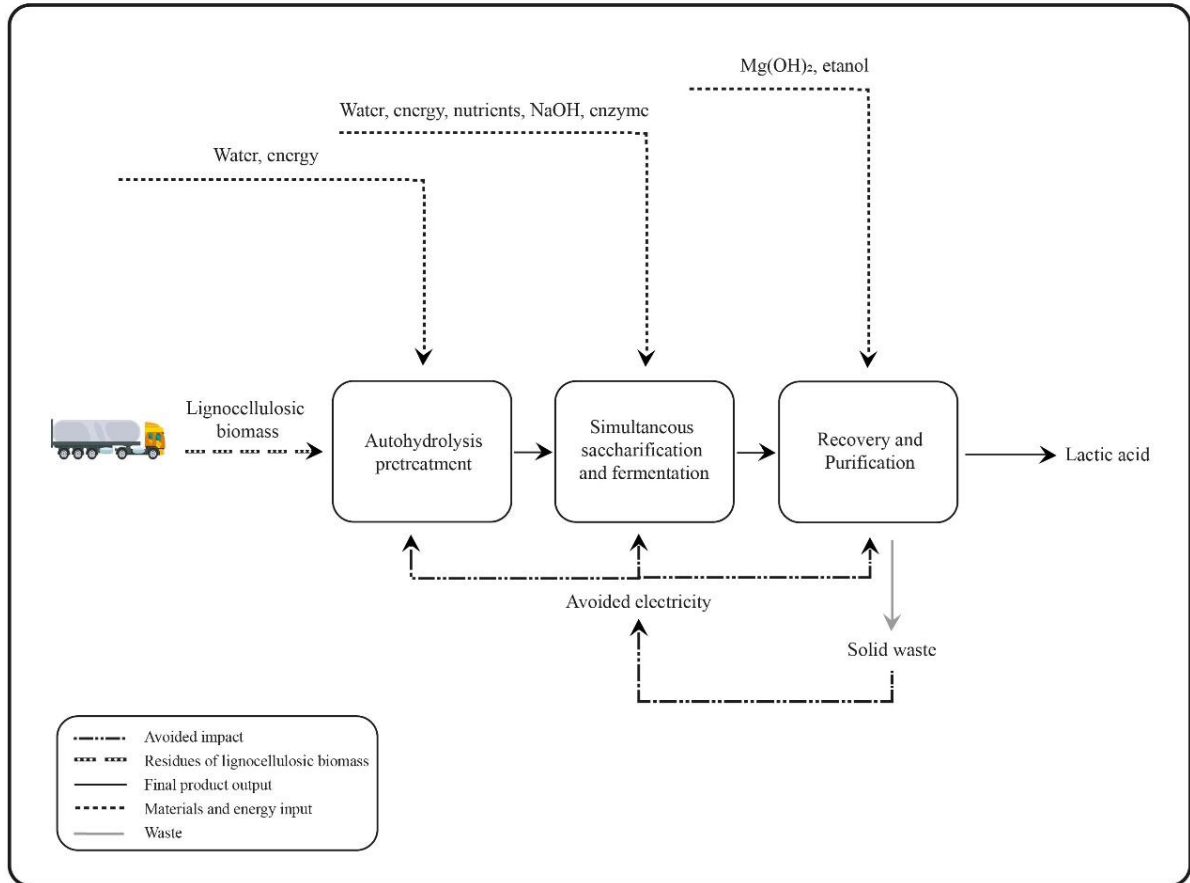
Figure 25- Schematic block flow diagram of scenario 1.

5.4. Life Cycle Inventory Analysis (LCI)

5.4.1. Scenario 1

The inventory data used in the current scenario was composed of background and foreground data. The foreground data related to LA production through autohydrolysis and SSF were obtained from the lab-scale experiments. The inventory of LA separation was developed using data from literature. The background LCA data concerning the production and processing of materials, chemicals, and energy carriers were retrieved from the Ecoinvent 3.4 databased under consequential modeling. The resources and utilities used and generated waste are given on **Table 19**. Brief descriptions of the lab experiments and the processes on which the LCA model was based are presented in the subsequent sections.

Table 19- Input and output of LA production from lignocellulosic biomass, based on 1 tonne of biomass per batch process.

Lactic acid (LA) kg	298.5
Resources (kg)	
Lignocellulosic biomass	1 000
Water	15 744
Nutrients	28.82
NaOH	60
R_3N	7.23
$Mg(OH)_2$	16.03
Ethanol	4.07
Enzyme, Cellulase, Novozyme	112.2
Utilities (kWh)	
Electricity	26.55
Waste (kg)	
Solid waste	146

5.4.1.1. Raw material process

A mixture of autohydrolyzed unexploited lignocellulosic biomasses from forest and marginal lands resources (branches and twigs with bark and leaves of Eucalyptus (*Eucalyptus globulus*), Pine (*Pinus pinaster*), Broom (*Cytisus sp.*), Carqueja (*Genista tridentata*), Mimosa (*Acacia dealbata*), and Rockrose (*Cistus ladanifer*)) was used as raw material. The criterion of feedstocks mixture was previously studied (Pontes et al., 2018). Taking into account the reported data, for the present study, the mixture M2-3 (mixture 2nd and 3rd quarters), which represent 52.5 % of forest ecosystems (24.6 % of pine, 27.9 % of eucalyptus) and 47.5 % of biological resources from marginal land (13.8 % broom, 12.5 % mimosa, 10.7 % carqueja, 10.4 % rockrose) was selected. The raw materials were air-dried, milled and sieved between 0.250 to 0.400 mm using a vibratory sieve shaker (40 and 60 mesh). Afterwards, the samples were homogenized in a single lot to avoid composition heterogeneity among aliquots and stored in polypropylene bags at room temperature.

5.4.1.2. Transport process

The transport process was selected according to two different paths, namely, from the forest to the collection point (10 km) by a 10 tonnes truck capacity and the collection point to the industrial unit (30 km) by a 24 tonnes truck capacity, taking into account the tortuosity coefficient of 1.2.

On these routes the truck was considered full on the outward journey and empty on the return.

5.4.1.3. Autohydrolysis pretreatment

The autohydrolysis pretreatment was performed in a reactor equipped with a temperature controller (model 4848). The mixture was mixed at liquid to solid ratio (LSR) of 8 kg of water/kg of oven-dry raw material. In autohydrolysis experiments, the reaction media was stirred at 150 rpm and heated by an external jacket, following the standard heating temperature-time profile to reach the desired maximum temperature (226 ºC), through non-isothermal conditions. Once the target temperature was reached, the media

were immediately cooled and filtered. The solid fraction was washed and used in the followed process.

5.4.1.4. Simultaneous saccharification and fermentation (SSF)

Batch simultaneous saccharification and fermentation was conducted in a bioreactor for 44 h. SSF was carried out with a LSR of 6 g/g, ESR (enzyme to substrate ratio) of 54 FPU/g and 10 % (v/v) inoculum size and it was conducted at 37 ºC, agitation rate at 150 rpm, and pH 4.8 controlled with 5M of NaOH.

5.4.1.5. Downstream process for LA recovery

One of the most important and challenging steps at the biorefining of the biopulp is the downstream strategy implemented to recover the produced LA from the fermentation broth. This step plays an important role because the fermentation broth includes both organic and inorganic components, considering that a successful purification and acidification step would increase the profitability of the whole process. Several processes for LA recovery have been studied and reported, such as, direct distillation, nanofiltration, adsorption, membrane separation, ion-exchange resins, solvent electrodialysis and reactive distillation (Daful et al., 2016; Khoshnevisan et al., 2020).

Reactive distillation was the process modeled in the present work, since it has shown promising results at the industrial scale. As reported by Daful et al. and Mandegari et al. LA recovery involved the reaction with $Mg(OH)_2$, as neutralizer, which formed Mg-lactate. The latter reacted with trimethylamine (R_3N) to form $Mg(OH)_2$ and R_3N-lactate. Thus $Mg(OH)_2$ could be recycled back to the reactor. So reactive distillation was applied for LA purification, which involved two catalyzed reversible reactions, esterification and hydrolysis. In the esterification column LA liberated from the R_3N-lactate was converted to the more volatile ethyl ester (ETLA) using ethanol and this ethyl ester was then removed from the column by distillation. Pure LA was subsequently recovered by hydrolysis of the ester with water, in a hydrolysis reactive distillation column. In further consideration, the ethanol from the top of hydrolysis reactive distillation could be purified by normal distillation and recycled back to the esterification section (Daful et al., 2016; Mandegari et al., 2017).

Accordingly, an acid recovery of 99.5% was assumed (Mandegari et al., 2017).

5.4.1.6. Electricity

The valorization of the solid fraction, obtained from LA recovery, was converted to energy resulting in 117 kWh, according to Khoshvenevisan et al. (Khoshnevisan et al., 2020). This electricity generated was described as avoided impact (negative values), which means could be subtracted from the induced impacts.

5.4.2. Scenario 2

The inventory data used in the current scenario were taken from the Ecoinvent database of SimaPro software. The process was modelled for the production of lactic acid from acetaldehyde in Europe. Raw material preparation involved extraction of crude oil and refining to produce ethane. Ethane was then used to make ethylene and then converted to acetaldehyde (CH_3CHO). Acetaldehyde was then used as a raw material to produce lactonitrile (acetaldehyde cyanohydrin) by a catalytic reaction of acetaldehyde with hydrogen cyanide (HCN) in the presence of a base catalyst under high pressure. Crude lactonitrile was then purified by distillation and subsequently hydrolyzed to LA by H_2SO_4 (Narayanan et al., 2004; Pal et al., 2009). LA was then esterified using methanol to produce methyl lactate, which was removed and purified by distillation and hydrolyzed by water under acid catalyst to produce LA and the methanol, which is recycled. The chemical synthesis route produced a racemic mixture of dl-lactic acid (Daful et al., 2016). Raw materials were modelled with a stoichiometric calculation. Emissions were estimated. Energy consumptions, infrastructure and transports were calculated with standard values.

5.5. Life Cycle Impact Assessment (LCIA)

The software package SimaPro was used as tool for the environmental impact assessment and the impact categories evaluated were presented in **Table 20** and were selected from ReCiPe Midpoint (H) methodology for the environmental impact assessment.

Table 20- Environmental impact categories considered for comparison of biobased LA and fossil based LA production.

| Symbol	Measured in	Description
ADP	Mj	Abiotic depletion
AP	kg SO_2 eq. (SO$_2$: dióxido de enxofre)	Acidification potential
FWAETP	kg 1,4-DB eq.	Freshwater aquatic ecotoxicity potential
FWEP	kg PO_4^{3-} eq. (PO$_4$:phosphate)	Freshwater eutrophication potential
GWP	kg CO_2 eq.	Global warming potential
HTP	kg 1,4-DB eq. (DB:dichlorobenzene)	Human toxicity potential
IRP	kBq U_{235} eq.	Ionizing radiation potential
LU	m^2	Land use
MAETP	kg 1,4-DB eq.	Marine aquatic ecotoxicology potential
MEP	Kg NO_3^-	Marine eutrophication potential
ODP	kg CFC-11 eq. (CFC:chlorofluorocarbon)	Ozone layer depletion potential
POCP	Kg C_2H_4 eq. (C$_2$H$_4$: ethylene)	Photochemical oxidation potential
PM	kg PM_{10} eq.	Particle matter formation potential
TETP	kg 1,4-DB eq.	Terrestrial ecotoxicity potential
WDP	m^3	Water depletion potential

5.6. Results and discussion

The fifteen environmental impact categories, depicted in **Table 20**, have been assessed and evaluated for both biobased LA and fossil based LA and discussed in the following sections.

5.6.1. Global warming potential (GWP)

The characterization model as established by the Intergovernmental Panel on Climate Change (IPCC) was selected for the development of characterization factors. Factors were expressed as Global Warming Potential for time horizon 100 years (GWP100), in kg carbon dioxide equivalent/kg emission.

GWP is the ability of greenhouse gases to trap heat in the atmosphere relative to carbon dioxide, which serves as the reference gas. The global warming potential of biobased and fossil-based LA are 1131.92 and 5188.52 kg CO_2 eq./tonne of LA respectively, giving total environmental saving of 4056.60 kg CO_2 eq./tonne of LA, i.e., reducing the environmental burden by 78 %. On the other hand, the main GWP contribution for the biobased LA comes from the SSF process, as depicted in **Figure 26**, due to the enzymes and nutrients required for the saccharification. Furthermore, it was considered that the selected raw material promoted a biogenic dioxide carbon storage of 6.03 kg CO_2eq. /tonne of LA resulting the photosynthesis process (the lignocellulosic biomass mixture used in the work represent a biogenic dioxide carbon storage of -1.870 kgCO_2eq. /tonne). These results were generally consistent with Mandegari et al which obtained 3800 kg CO_2 eq./tonne of LA of GWP_{100} and the difference was allocated to the sugarcane cultivation and processing (Mandegari et al., 2017). In the same manner, Parajuli et al. for the production of 1 tonne of biobased lactic acid from alfalfa obtained 3080 kg CO_2 eq. using attributional life cycle assessment approach (Parajuli et al., 2017).
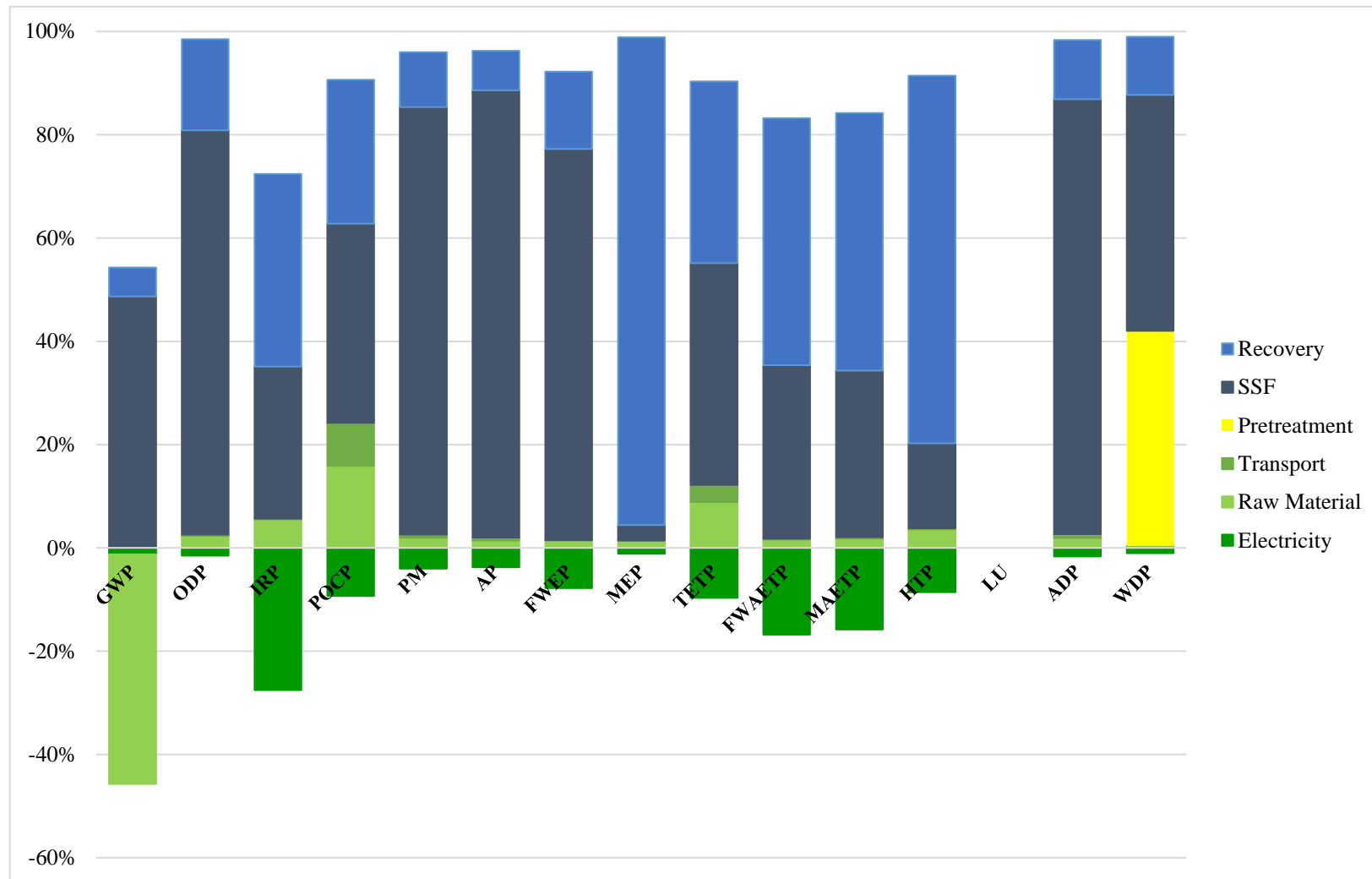
Figure 26- Environmental impacts for the production of 1kg of biobased LA production according to each process.

5.6.2. Ozone layer depletion potential (ODP)

The characterization model was developed by the World Meteorological Organization (WMO) and defines ozone depletion potential of different gases (kg CFC-11 equivalent/ kg emission).

Ozone-depleting gases damage the stratospheric ozone or the 'ozone layer'. There are no certainties as to the combined effects of different gases on the stratosphere, and all chlorinated and brominated compounds that are stable enough to reach the stratosphere can have an effect. Chlorofluorocarbons (CFCs), halons and hydrochlorofluorocarbons (HCFCs) are the major causes of ozone depletion. The ozone layer depletion potential for biobased and fossil based LA production are 3.78×10^{-3} and 1.34×10^{-3} kg CFC-11 eq. per tonne of LA produced respectively, which means there was no environmental saving on the production of biobased LA. As shown in **Figure 26**, SSF process was the major contributor for ODP, due to the enzyme and nutrients used. However, the recovery process also contributes through Mg $(OH)_2$ and trimethylamine.

5.6.3. Ionizing radiation potential (IRP)

Ionizing radiation is an impact category in LCA relating to the damage to human health and ecosystems that is linked to the emissions of radionuclides throughout a process. The ionizing radiation potentials of biobased and fossil based LA productions are 21.94 and 214.98 kBq U235 eq. per tonne of LA, respectively, the biobased LA process reducing the environmental burden by 90 %. As shown in **Figure 26**, SSF and recovery process were the major contributor for IRP.

5.6.4. Photochemical oxidation potential (POCP)

The model is developed by Jenkin & Hayman and Derwent and defines photochemical oxidation expressed in kg ethylene equivalents per kg emission.

Ozone is protective in the stratosphere, but on the ground-level it is toxic to humans in high concentrations. Also known as photochemical ozone, it is formed by the reaction of volatile organic compounds and nitrogen oxides in the presence of heat and sunlight. The photochemical oxidant creation potential measures the potential for the creation of photo-chemical (summer) smog due to the reaction of relevant chemical compounds

when exposed to sunlight. The POCP for biobased and fossil based LA productions were 3.61 and 7.38 kg C_2H_4 eq. per tonne of LA produced respectively, giving a net environmental saving potential of 3.78 kg C_2H_4 eq. per tonne of biobased LA produced. All the processes contributed to POCP with exception of pretreatment as shown on **Figure 26**.

5.6.5. Particulate Matter Formation (PM)

Particulate matter (PM) is a complex mixture of extremely small particles. Particle pollution can be made up of a number of components, including acids (such as nitrates and sulfates), organic chemicals, metals, and soil or dust particles. A multitude of health problems, especially of the respiratory tract, are linked to particulate pollution. PM is measured in PM10 equivalents, i.e. particles with a size of 10 μm. The potential particulate matter formation for the biobased LA production was 7.89 and that of fossil based LA 4.83 kg PM10 eq. per tonne of LA produced. In this case there was no total environmental saving on replacing the fossil-based LA with biobased LA.

5.6.6. Acidification potential (AP)

Acidification potential (AP) is expressed in kg SO_2 equivalents per kg emission and the applied model was developed by Huijbregts (Huijbregts et al., 2003). Acidic gases such as sulfur dioxide (SO_2) react with water in the atmosphere to form 'acid rain', causing ecosystem damage of varying degrees, depending on the nature of the landscape ecosystems.

The AP for the biobased LA production was 25.62 and for the fossil based LA 10.27 kg SO_2 eq. per tonne of LA produced. As shown in **Figure 26**, SSF process was the major contributor for AP because the enzyme production with contributed about 20 % to acidification, considering the waterborne emissions such as phosphate, caused by the consumption of phosphate fertilizers and di-ammonium phosphate used for enzyme production (Mandegari et al., 2017).

5.6.7. Eutrophication potential (EP)

Eutrophication potential was developed by Heijungs et al and is expressed in kg PO$_4$ equivalents per kg emission (de Bruijn et al., 2002). Eutrophication was the accumulation of chemical nutrients in an ecosystem which led to abnormal productivity. In this study, EP was considered in two categories, namely, Freshwater eutrophication (FWEP) and Marine eutrophication (MEP). The FWEP of biobased and fossil based LA productions are 0.64 and 1.37 kg P eq. per tonne of LA respectively, the biobased LA reducing the environmental burden by 53 %. The MEP for biobased and fossil based LA productions were 0.33 and 0.19 kg N eq. per tonne of LA produced respectively. As described in **Figure 26**, recovery process was the major contributor for EP, mainly due to the presence of Mg(OH)$_2$ and trimethylamine.

5.6.8. Terrestrial ecotoxicity potential (TETP), Freshwater aquatic ecotoxicity potential (FWAETP), Marine aquatic ecotoxicology potential (MAETP) and Human toxicity potential (HTP)

Characterization factors, expressed as Human Toxicity Potentials (HTP), are calculated with USES-LCA, describing fate, exposure and effects of toxic substances for an infinite time horizon. For each toxic substance HTP's are expressed as 1,4-dichlorobenzene equivalents/ kg emission. Regarding to TETP, FWAETP, MAETP and HTP, the production of biobased LA reduces the environmental burden by 77%, 66 %, 67 % and 62 % respectively.

5.6.9. Abiotic depletion potential (ADP)

This impact category is concerned with protection of human welfare, human health and ecosystem health. This impact category indicator is related to extraction of minerals and fossil fuels due to inputs in the system. The Abiotic Depletion Factor (ADF) is determined for each extraction of minerals (kg antimony equivalents/kg extraction) based on concentration reserves and rate of deaccumulation. Abiotic depletion of fossil fuels is related to the Lower Heating Value (LHV) expressed in MJ per kg of m^3 fossil fuel. The reason for taking the LHV is that fossil fuels are considered to be fully substitutable. The

ADP of biobased and fossil based LA productions are 2043.05 and 1820.02 kg fossil fuel eq. per tonne of LA. As shown in **Figure 26**, SSF process was the major contributor for AP.

5.6.10. Water depletion potential (WDP)

The water depletion potential is higher for the biobased LA production, 183.97 m3, than its equivalent from fossil based LA 69.67 m3. This is due to the impact associated with the lignocellulosic pretreatment, namely autohydrolysis. This process only required water and temperature to solubilize hemicellulose fraction.

5.6.11. Land Use (LU)

The LU impact was not considered in the results because there was no land use potential for agricultural land occupation (ALO) and urban land occupation (ULO). The raw material selected originated from wastes generated from forest and marginal land, in other words, the life cycle of the process, begins only at the waste collection stage. Taking into account the use of waste, environmental impacts up to the generation of the waste are not considered and they are part of the production process, e.g. roundwood.

Land use for biobased chemicals was generally expected to be higher than that for equivalent fossil based products (Hermann et al., 2007; Lammens et al., 2011) , although this was not verified in this study.

5.6.12. Life Cycle Interpretation

Considering the environmental impacts in 15 categories, the biobased-LA showed to reduce the environmental burden in 9, described in **Figure 27**.

Regarding to the raw material, as the feedstock was an unexploited lignocellulosic biomass resulted from forest residues and marginal land, the impact LU was not verified. However, in vast majority of the studies, the main contributor of the selected impact categories was the biomass production (Parajuli et al., 2017). This result highlights the importance of the strategy presented in this work for the selection of the biomass.

The processes that demonstrated to be more relevant for the total environmental impacts were SFF and LA recovery. This can be explained by the auxiliary chemicals

used in the biobased LA production, namely enzymes, nutrients and reagents for reactive distillation. Recovery and recycling of enzymes are strategies that should be adopted to improve the life cycle assessment. An efficient cellulase recycling process should meet the following requirements: (1) high stable cellulase activity, (2) high hydrolysis efficiency, and (3) good control over the substrate adsorption/desorption processes (Gomes et al., 2015). A vast number of methods have been extensively investigated to recycle enzymes, such as: immobilization (Verardi et al., 2012); membrane separation; chromatography or affinity purification (Rashid et al., 2013); and re-adsorption with fresh substrate (Rodrigues et al., 2014). As reported by Ramos et al., under the most favorable conditions, the recovery process can reduce the dosage by 30-50 % without compromising glucose yields (Ramos et al., 1993).
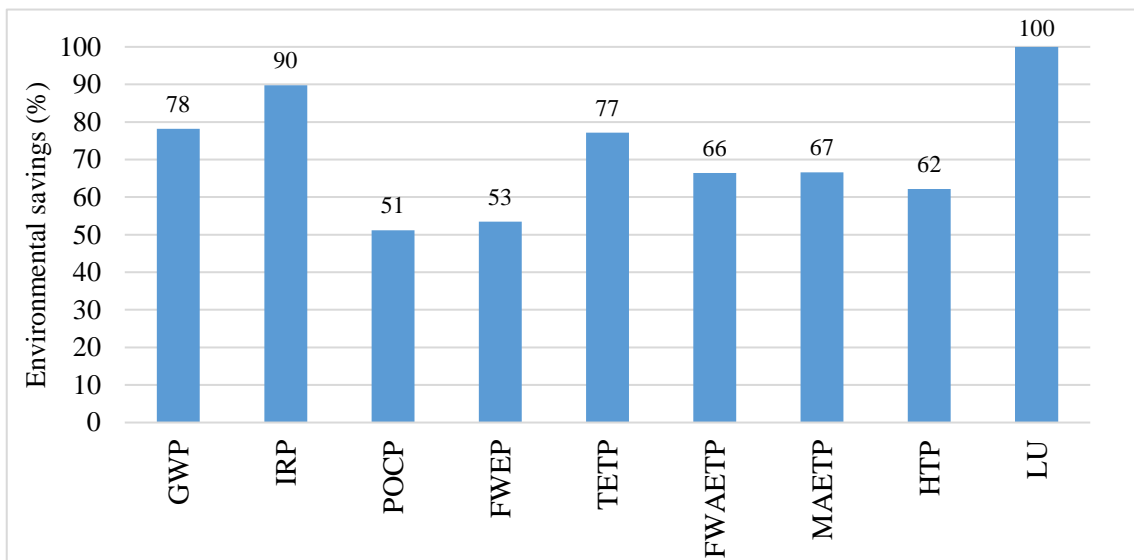
Figure 27- Environmental savings in different impact categories upon replacing one kg of LA produced from fossil resources with equivalent biobased LA.

As reported by Parajuli et al., the production of enzyme was one of the major environmental hotspots (Parajuli et al., 2017). Furthermore, to decrease the environmental impact a significant reduction in the cost of cellulases in an urgent requirement to enable an economically sustainable utilization of lignocellulosic biomass (Gomes et al., 2015; Rodrigues et al., 2014).

Regarding nutrients, lactic acid bacteria have complex nutrients requirements, due to their limited ability to synthetize B-vitamins and amino acids (Hofvendahl and Hahn–Hägerdal, 2000). Thus the development of nutrient media more sustainable is one of the

main objectives. As an example, fishery wastes have been studied as a nutrient source for lactic acid production (Gao et al., 2006).

5.7. Conclusion

This study evaluated the environmental performance of LA produced from lignocellulosic biomass and from non-renewable resources. From this analysis it has been found that biobased LA has a positive effect in nine environmental impact categories among the fifteen considered. The dominant environmental impacts of the analyzed categories from which large environmental savings obtained per tonne of LA produced are global warming potential (4056.60 kg CO_2 eq.), ionizing radiation potential (193.03 kBq U235 eq.), Terrestrial ecotoxicity (9569.40 kg 1,4-DB eq.) and land use (126.63 m^2). About 60 % of the environmental burdens of most of the environmental impact categories of the fossil based LA can be reduced upon replacing with its biobased equivalent. SSF and recovery process were the major contributors to the environmental impacts. Thus, enzyme recovery as well the use of sustainable nutrient supplementation could be an opportunity to lower the environmental footprint of biobased LA production. Briefly, the biobased LA is environmentally friendlier than the fossil based LA.

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CHAPTER 6 |

General conclusions and future perspectives

6.1. General conclusions

This chapter contains the main conclusions from the present thesis. Nonetheless, the detailed conclusions can be found at the end of each individual chapter.

The main outcome of the developed work was the demonstration of the feasibility of the production of lactic acid from multi supply lignocellulosic biomass, namely natural vegetation and forest residues, with high yield of conversion. A 40 folds scale up of the process was also done.

The highlights on the results obtained on this thesis are summarized below:

- Mixtures of lignocellulosic biomass were selected that allow for a positive outcome of its use on the production of lactic acid. The selected mixtures M1-4 and M2-3 from natural vegetation and forest ecosystems, namely, broom (*Cytisus sp.*), carqueja (*Genista tridentate*), mimosa (*Acacia dealbata*), rockrose (*Cistus ladanifer*), eucalyptus (*Eucalyptus globulus*) and pine (*Pinus pinaster*), represent an effective scenario to be used on the development of lignocellulosic biomass biorefineries in Portugal;

- When pretreated by autohydrolysis, both mixtures showed similar behavior and at T_{MAX} = 206 ℃ recoveries of 62.2 % and 68.8 % of hemicellulose-derived compounds (as XOS and xylose) were obtained for M1-4 and M2-3;

- Under this condition glucose yield from enzymatic hydrolysis resulted in 60.1 % and 73.7 % for M1-4 and M2-3, respectively; these results led to the selection of the mixture M2-3 for further processing;

- Autohydrolyzed M2-3 (AM2-3), solid fraction, was subsequently pretreated by organosolv process, through a factorial design, ethanol concentration 80 %, temperature 200 ℃ and time 60 min allowed a high delignification (49.40 %) with a lignin yield of 24.47 % and lignin extraction efficiency of 48.34 %;

- The delignified biomass was submitted to enzymatic hydrolysis and a glucose yield of 49.65 % was obtained;

- The isolated lignin samples obtained (OL1-OL10) showed the potential to be used as bio-based polymers because presented low T_g temperatures which was related with more thermoplastic properties;

- Comparing the glucose yield of solid fraction resulted from autohydrolysis and from organosolv, delignified biomass, the last one did not increased enzymatic susceptibility and for this reason the following processes did not include organosolv treatment;

- SSF and SHF was conducted on the solid fraction resulted from the autohydrolysis of M2-3 for the production of LA by *L. rhamnosus*. For both conditions, the yield of glucose into LA was s 1 g/g, but the volumetric productivity was 3 fold higher for SSF;

- Optimal conditions for LA production by SSF were obtained with 16 % solids load and ESR 54 FPU/g, allowing for a concentration of LA of 77.83 g/L, with an yield of 1 g/g and productivity of 3.24 g/Lh;

- The optimized condition was scaled up to a 5 L bioreactor and the achieved LA concentration was 61.74 g/L, glucose to LA yield 0.97 g/g and volumetric productivity 1.40 g/Lh;

- The environmental performance of LA produced from M2-3 when compared with LA obtained from non-renewable resources demonstrated large environmental savings per tonne of LA produced in several parameters – 78 % savings in global warming potential, 90 % savings in ionizing radiation potential, 77 % savings in Terrestrial ecotoxicity and 100 % savings in land use.

Overall, it was demonstrated that the use of mixture M2-3, processed by autohydrolysis followed by simultaneous saccharification and fermentation, offers a strategy to produce LA from lignocellulosic biomass with high conversion. Furthermore,

biobased LA production significantly reduces 60 % the environmental impact of the production process as compared to fossil-derived LA.

6.2. Future perspectives

This chapter presents recommendations/ suggestion for future work, taking into account the many questions that have emerged during the development of the thesis. Future perspectives on lactic acid production from lignocellulosic biomass resources are listed below:

- LAB are extremely fastidious and require one or more amino acids to grow. The rich nutrients supplements required for LA fermentation not only increase the raw materials costs, but also make the final product recovery and purification difficult and costly. Thus, the nutrient supplementation with residues should be developed and optimized;

- More research and studies should be done to decrease the enzyme loading, while maintaining the high solids, which could improve the economic and environmental viability of the process. Thus, this work could be integrated with other PhD research, in BLC3, in cooperation with University of Coimbra (the same research group present in this thesis), which intends to develop a bacterial consortium to produce enzymes, isolated from the digestive tract of a ruminant, that has the ability to degrade the different fractions of lignocellulosic biomass to increase the performance in pretreatment and fermentation processes;

- For a future work, fed-batch or a continuous fermentation of LA should also be investigated in order to improve the productivity, reduce the substrate inhibition and also offer a process more economically viable;

- Work to develop a tolerance strain of *L. rhamnosus* through metabolic engineering to tolerate higher LA concentrations and enhance the LA production should also be done.