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Integrated valorization of *Annona cherimola* Mill. seeds

DOCTORAL THESIS

Pedro Miguel Sena da Costa Branco
DOCTORATE IN CHEMISTRY - ORGANIC CHEMISTRY

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To all of you who didn't
allow me to give up

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«Eu sou aquele oculto e grande Cabo

A quem chamais vós outros Tormentório,

Que nunca a Ptolomeu, Pompónio, Estrabo,

Plínio e quantos passaram fui notório.»

Luís de Camões

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Resumo

Os resíduos agrícolas e das indústrias agro-alimentares são normalmente um problema não só ambiental, mas também económico. Assim, é necessária uma mudança de paradigma, que passa pela assumpção destes como matérias-primas da biorrefinaria. Neste trabalho utilizaram-se sementes de anona (*Annona cherimola* Mill.), ricas em lípidos (*ca.* 30%) e material lenhocelulósico, como exemplo de um resíduo actualmente sem qualquer valorização.

A fracção lipídica foi extraída com solvente, tendo o rendimento variado entre 13 e 28 g óleo/100 g biomassa, consoante o método e tempo de extracção e a pureza do solvente. Este óleo foi convertido em biodiesel (por transesterificação alcalina), tendo-se obtido 76 g FAME/100 g óleo. Este biodiesel demonstrou ser passível de incorporação em combustíveis comerciais, de acordo com os parâmetros da norma EN14214.

A restante fracção lenhocelulósica foi sujeita a dois processos de fraccionamento alternativos para recuperação selectiva da hemicelulose, por forma a obterem-se diferentes produtos. Para ambos os processos foram desenvolvidos modelos matemáticos empíricos, tendo em vista a sua utilização em estudos de *scale-up*.

Por autohidrólise obtiveram-se maioritariamente oligossacáridos (10 gL^{-1}) cujas propriedades sustentam potenciais aplicações alimentares e/ou farmacêuticas. O resíduo sólido resultante foi posteriormente sacarificado enzimaticamente, tendo-se obtido um rendimento de sacarificação máximo de 83%.

No hidrolisado obtido com ácido diluído destacam-se os monossacáridos, maioritariamente xilose (26 gL^{-1}), glucose (10 gL^{-1}) e arabinose (3 gL^{-1}), e um baixo teor de inibidores do crescimento microbiano. Este hidrolisado mostrou ser um meio de cultura apropriado para bactérias e consórcios microbianos, conduzindo à produção de exopolissacáridos. A conversão

máxima de monossacáridos em xantano foi de 0,87 g/g e a produtividade máxima de kefirano foi de 0.07 g.$(Lh)^{-1}$.

Este trabalho demonstra a exequibilidade técnica da utilização das sementes de anona como um resíduo passível de ser valorizado, abrindo novas perspectivas para a valorização deste tipo de materiais no âmbito da biorrefinaria.

Palavras chave

Biodiesel; Biorrefinaria; Exopolissacáridos; Fraccionamento; Materiais lenhocelulósicos; Oligossacáridos

Abstract

Agricultural and agro-industrial residues are often considered both an environmental and an economical problem. Therefore, a paradigm shift is needed, assuming residues as biorefinery feedstocks. In this work cherimoya (*Annona cherimola* Mill.) seeds, which are lipid-rich (*ca.* 30%) and have a significant lignocellulosic fraction, were used as an example of a residue without any current valorization.

Firstly, the lipid fraction was obtained by solvent extraction. Extraction yield varied from 13% to 28%, according to the extraction method and time, and solvent purity. This oil was converted into biodiesel (by base-catalyzed transesterification), yielding 76 g FAME/100 g oil. The obtained biodiesel is likely to be incorporated in the commercial chain, according to the EN14214 standard.

The remaining lignocellulosic fraction was subjected to two alternative fractionation processes for the selective recovery of hemicellulose, aiming different products. Empirical mathematical models were developed for both processes, aiming future scale-up.

Autohydrolysis rendered essentially oligosaccharides (10 gL^{-1}) with properties indicating potential food/feed/pharmacological applications. The remaining solid was enzymatically saccharified, reaching a saccharification yield of 83%.

The hydrolyzate obtained by dilute acid hydrolysis contained mostly monosaccharides, mainly xylose (26 gL^{-1}), glucose (10 gL^{-1}) and arabinose (3 gL^{-1}), and had low content of microbial growth inhibitors. This hydrolyzate has proven to be appropriate to be used as culture media for exopolisaccharide production, using bacteria or microbial consortia. The maximum conversion of monosaccharides into xanthan gum was 0.87 g/g and kefiran maximum productivity was 0.07 $g.(Lh)^{-1}$.

This work shows the technical feasibility of using cherimoya seeds, and materials as such, as potential feedstocks, opening new perspectives for upgrading them in the biorefinery framework.

Keywords

Biodiesel; Biorefinery; Exopolysaccharides; Fractionation; Lignocellulosic materials; Oligosaccharides

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This thesis is based on the following publications and communications:

- Papers in international scientific periodicals with referees

Pedro Costa Branco, Paula C. Castilho, Maria Fernanda Rosa, Juan Ferreira, *Characterization of* Annona cherimola *Mill. Seed Oil from Madeira Island: a Possible Biodiesel Feedstock*, **Journal of the American Oil Chemists' Society, Volume 87, pp. 429-436**, April 2010**, DOI 10.1007/s11746-009-1513-1**

P.C. Branco, A.M. Dionísio, I. Torrado, F. Carvalheiro, P.C. Castilho, L.C. Duarte, *Autohydrolysis of* Annona cherimola *Mill. seeds: Optimization, modeling and products characterization,* **Biochemical Engineering Journal, Volume 104, pp 2-9**, December 2015, **DOI 10.1016/j.bej.2015.06.006**

P.C. Branco, I. Torrado, F. Carvalheiro, P.C. Castilho, L.C. Duarte, *Optimization and mathematical modelling of cherimoya seeds dilute acid hydrolysis for the production of hemicellulosic hydrolyzates,* **(submitted)**

- Papers in peer reviewed conference proceedings

P.C. Branco, P.C. Castilho, J. Ferreira, *Extraction and Composition of Annona's Seed Oil and Its Potential Use for the Production of Biodiesel*, **Proceedings of Bioenergy: Challenges and Opportunities – International Conference and Exhibition on Bioenergy, Volume II, pp. 27-32**, Guimarães, Portugal, April 2008

Branco, P.C., Torrado, I., Klydjeneiro, Z., Pereira, C., Bogel-Lukasik, E., Carvalheiro, F., Castilho, P.C., Duarte, L.C., *Cherimoya seeds hemicellulosic hydrolysate as a culture medium for the production of kefiran*, **Proceedings**

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- Invited oral communications

Branco, P.C.; Torrado, I.; Dionísio, A.M.; Carvalheiro, F.; Duarte, L.C.; Castilho, P.C., *Wastes from agro-food industries as raw materials for the production of biofuels and bioproducts*, **Seminar on Renewable Energies and Environment**, Portalegre, Portugal, May 2014

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Branco, P.C., Duarte, L.C., Torrado, I., Roseiro, L.B., Pereira, C., Klydjeneiro, Z., Carvalheiro, F., Castilho, P.C., Annona cherimola *Mill seeds as a potential feedstock for the production of biofuels and bioproducts*, **International Workshop "Valorization of Mediterranean biowastes and effluents" IAMAW - The International Association of Mediterranean Agro-Industrial Wastes**, Santarém, Portugal, April 2012

- Posters in conferences

P.C. Branco, P.C. Castilho, M.F. Rosa, *Characterization of Triacylglycerols in* Annona cherimola *Mill. Seed Oil by NARP-HPLC-APCI-ITD-MS*, **1st PYCheM – Portuguese Young Chemist Meeting**, Lisboa, Portugal October 2008

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Biotechnical Processing of Lignocellulosic Raw Materials, Izmir, Turkey, September 2010

Pedro C. Branco, Ivone Torrado, Florbela Carvalheiro, Paula C. Castilho, Luís C. Duarte, *Optimisation and mathematical modelling of custard apple seeds dilute acid hydrolysis*, **Biomass derived pentoses: from biotechnology to fine chemistry**, Reims, France, November 2010

Branco, Pedro C., Torrado, Ivone, Carvalheiro, Florbela, Castilho, Paula C., Duarte, Luís C., *Production of Xanthan Gum Using Cherimoya Seeds' Hemicellulosic Hydrolysate As A Culture Medium*, **2nd Iberoamerican Congress on Biorefineries**, Jaén, Spain, April 2013

Dionísio, A.M.; Branco, P.C.; Torrado, I.; Roseiro, L.B.; Carvalheiro, F.; Castilho, P.C.; Duarte, L.C., *Production of oligosaccharides from Annona cherimola Mill. seeds: optimization and modelling*, **ChemPor2014: 12th International Chemical and Biological Engineering conference**, Oporto, Portugal, September 2014

Notation

A	Pre-exponential factor
Ac	Acetyl groups
ACN	Acyl carbon number
ADI	Acceptable daily intake
AIS	Acid insoluble residue
Ara	Arabinose
Arn	Arabinan
ASE	Accelerated solvent extraction
BVPI	Biotechnological valorization potential indicator
CDM	Chemically defined medium
CFPP	Cold filter plugging point
CPS	Capsular polysaccharide
CS	Combined severity factor
DAH	Dilute acid hydrolysis
DB	Number of double bonds
Deg. Pol.	Degree of polymerization
DP	Degradation products
DW	Dried sample weight
$\mathbf{E_a}$	Activation energy
ECN	Equivalent carbon number
EPS	Extracellular polysaccharide
EU	European Union
$\mathbf{F_1}$	Correction factor accounting for glucose degradation
$\mathbf{F_2}$	Correction factor accounting for xylose degradation
$\mathbf{F_3}$	Correction factor accounting for arabinose degradation
FA	Fatty acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFA	Free fatty acid
FOS	Fructo-oligosaccharides
FPU	Filter paper units

Fru	Fructose
Furf	Furfural
FT-IR	Fourrier transform infrared spectroscopy
Gal	Galactose
GalA	Galacturonic acid
GalOS	Galacto-oligosaccharides
GC	Gas chromatography
GDP	Gross domestic product
Glc	Glucose
GlcA	Glucoronic acid
GlcNAc	N-Acetylglucosamine
GLCOS	Gluco-oligosaccharides
Gn	Glucan
GulA	Guluronic acid
H	Moisture content
HAc	Acetic acid
Hemicel	Hemicellulose
HMF	5-Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
HSI	Hemicellulose selectivity index
$\mathbf{I_{cetane}}$	"Apparent cetane number"
i.d.	Internal diameter
IR	Infrared
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
JCBN	Joint Commission on Biochemical Nomenclature
JECFA	Joint WHO/FAO Expert Committee on Food Additives
k	Rate constant
KL	Klason lignin
LCF	Lignocellulose feedstock
$\boldsymbol{log\ R_O}$	Severity factor
LPS	Lipopolysaccharide
LSR	Liquid-to-solid ratio
Man	Mannose

ManA	Mannuronic acid
Monos	Monosaccharide
MS	Mass spectrometry
NDOS	Non-digestible oligosaccharides
NMR	Nuclear magnetic resonance
NREL	National Renewable Energy Laboratory
NRRL	Northern Regional Research Laboratory
OS	Oligosaccharide
PFE	Pressurized fluid extraction
PLE	Pressurized liquid extraction
pNPGU	*p*-nitrophenyl-β-D-glucopyranosidase units
$\mathbf{Q_K}$	Kefiran-biomass productivity
$\mathbf{Q_x}$	*Xanthomonas campestris* production rate
$\mathbf{Q_{Xant}}$	Xanthan gum productivity
Rha	Rhamnose
$\mathbf{R_O}$	Reaction ordinate
RT	Retention time
RTE	Room temperature extraction
SFC	Supercritical fluid extraction
TAG	Triacylglycerol
TFA	Trifluoroacetic acid
$\mathbf{T_{ref}}$	Temperature up to which the hydrolysis process is considered to be negligible
TI	Total inhibitors
TS	Total sugars
TS-TI	Total sugars minus total inhibitors
T(t)	Temperature as a function of time
$\mathbf{V_1}$	Volume of titrant spent on the blank assay
$\mathbf{V_2}$	Volume of titrant spent on sample analysis
W	Sample weight
WDCM	World Data Center for Microorganisms
WHO	World Health Organization
$\mathbf{W_{sol}}$	Solution weight

$\mathbf{X_1}$	Coded variable 1 of the Doehlert experimental design
$\mathbf{X_2}$	Coded variable 2 of the Doehlert experimental design
$\mathbf{[Xant]_{max}}$	Maximum xanthan gum concentration
$\mathbf{X_{ME}}$	Weight percentage of each individual methyl ester
Xn	Xylan
XOS	Xylo-oligosaccharides
Xyl	Xylose
Y	Obtained response of the Doehlert experimental design
$\mathbf{Y_K}$	Kefiran-cells yield
$\mathbf{Y_x}$	*Xanthomonas campestris* yield
$\mathbf{Y_{Xant}}$	Xanthan gum yield

Greek Symbols

α	Heating rate
β	Temperature at the beginning of the treatment
Δt	Time increment
μ_K	Kefiran-cells specific growth rate
μ_X	*Xanthomonas campestris* specific growth rate
ω	constant related to the influence of the temperature on the hydrolysis reaction

Scope and outline of the thesis

The research described in this thesis was carried out both in Centro de Química da Madeira, Universidade da Madeira (in the Natural Products research group) and Unidade de Bioenergia, Laboratório Nacional de Energia e Geologia (Biomass Deconstruction program), under the scope of residue valorization. The aim of these two groups is mainly to develop processes that lead to added value products from agricultural, forestry and agro-industrial products, by-products and residues. The main common denominator is the plant and its total utilization, in an integrated valorization strategy.

The underlying concept is the biorefinery that aims to fully exploit biomass for the production of added-value products with a zero waste approach. Specifically, this thesis aims to contribute to the conception of a sustainable biorefinery based on Madeira island agro-industrial residues.

The thesis is organized in eight chapters, in which the first is a general introduction to the subjects that are covered in the thesis and the last one is dedicated to a global conclusion and where future prospects are also debated. The remaining chapters correspond to publications that were the base for writing this thesis. Even though these chapters are interrelated, they must be seen as independent chapters that don't follow a timeline. This is the reason why some repetitions were difficult to be avoided. In fact, the chapters are presented in a logical order that reflects the several steps of a path towards the whole valorization of a feedstock, and this order does not reflect the chronology of the work.

- **Chapter 1** presents the biorefinery concept and the biorefinery's role on islands' sustainability. It then passes to biomass, focusing on oil seeds and the several fractions that can be obtained from them, the extraction methods and some processes for obtaining added-value products. It finishes with an insight on cherimoya (*Annona cherimola* Mill.), the plant used in this work.

Cherimoya seeds were chosen as feedstock because it's a relevant local agro-industrial residue in Madeira Island, besides being increasingly available in several other regions of the globe. Three different lots of seeds were used in this work and thus some slight differences are observed in their composition, which must be taken into account throughout this thesis. The first lot was used in chapter 2, the second one in chapters 3 and 5 and, finally, the third lot was used in chapters 4, 6 and 7.

- **Chapter 2** describes cherimoya seed oil characterization, its conversion into biodiesel (FAME) and the biodiesel's suitability according to EN14214, which regulates biodiesel's characteristics in the EU. This chapter has already been published *ipsis verbis* as a paper in JAOCS.

- **Chapter 3** is dedicated to the optimization of cherimoya seed oil extraction, as an application for the oil has been already described. The goal was to find the better solution for solvent extraction that mimics industrial operation, either regarding extraction yield and products composition. The composition of the extracted "cake" is especially evaluated and discussed, aiming for the next step in the whole valorization strategy.

- **Chapter 4** introduces a first option on the lignocellulosic fraction's valorization. Oligosaccharides can be a product *per se* and the method for obtaining them was optimized and modeled. Furthermore, the stability of the hydrolyzate and of the oligosaccharides was evaluated. The enzymatic digestibility of the remaining solid was also assessed regarding further valorization (focused on ethanol production). Most of this chapter has already been published in BEJ as research article.

Then, the thesis focuses the production of monosaccharides for further microbial conversion.

- **Chapter 5** describes the optimization and mathematical modeling of a process to obtain fermentable sugars from the lignocellulosic fraction, an alternative to oligosaccharides' production. The aim was a balance between

a high monosaccharide content and minimal microbial inhibitors production, regarding further upgrading using microorganisms. The content of this chapter has been submitted for publication.

- **Chapter 6** reports the production of an exopolysaccharide (used in cosmetics, pharmaceuticals, food…) by strains of a bacterial species, using the monosaccharide-rich hydrolyzate, obtained in optimized conditions.

- **Chapter 7** reports the use of the monosaccharide-rich hydrolyzate as a novel culture medium for the growth of kefir, a microbial consortium used in food industries.

- **Chapter 8** presents a general discussion of the main results of this thesis and discusses the foreseen research directions concerning cherimoya's seeds biotechnological upgrade.

CHAPTER 1

GENERAL INTRODUCTION

Summary

This chapter is a literature review focused on the key aspects addressed in this thesis.

An overview on topics like the biorefinery, its classifications and platforms, or some possible biorefinery feedstocks and products is made. Moreover, the role of the biorefinery in islands' context is also referred.

Finally, the chosen feedstock is presented and some applications reported for *Annona cherimola* Mill. are described. The valorization potential of *A. cherimola* seeds is also discussed.

1.1 The Biorefinery

1.1.1 The Biorefinery concept

The biorefinery concept can be considered as an evolution of concepts like "Green Chemistry" or Chemurgy. Biorefinery is an overall concept of an integrated and diversified industrial processing facility where biomass feedstocks are converted into a wide range of valuable products, much likewise to petroleum refineries. In these facilities carbohydrates, oils, lignin, and other materials are recovered from biomass and converted into fuels, high value chemicals and other materials, with a zero waste approach (Fig. 1-1). Pulp and paper mills, corn wet and dry mills that produce multiple products from biomass can be categorized as biorefineries. In the Mediterranean area, including Portugal, olive-pomace oil extraction facilities and wine distilleries are examples of proto-biorefineries since they produce a wide combination of food, feed and power.[1,2]

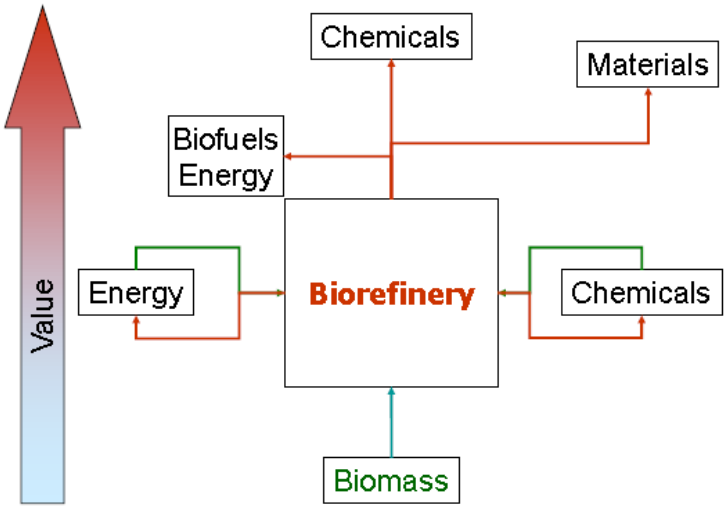

Figure 1-1 The biorefinery concept (reprinted from [3])

In comparison to petroleum refineries, biorefineries have to operate with a wider variety of feedstocks and a larger range of processing technologies, which may hinder overall economics. Furthermore, biomass as a feedstock is much bulkier than fossil fuels. By integrating the production of higher value

bioproducts with the biorefinery's fuel and power output, overall profitability and productivity of all energy related products are potentially improved. Increased productivity and efficiency can also be achieved through actions that decrease the overall energy amount of the biorefinery's unit operations. For example, maximizing the use of all feedstock components, byproducts and waste streams and common processing operations, materials and equipment, can cut down production costs.[2]

1.1.2 Biorefinery platforms

Biorefinery platforms can be distinguished depending on i) the raw materials; ii) which products are obtained; and iii) the technological processes used.

Biorefinery platforms, based only on main raw materials,[4] are: i) Lignocellulose Feedstock (LCF) based biorefinery, that uses nature-dry raw material (wood, straw etc.); ii) whole crop biorefinery, that uses cereals; and iii) Green biorefineries, that use nature-wet biomasses such as green grass, alfalfa, immature cereal, etc.

Alternatively, when the obtained products are considered, two categories are accounted: i) energy-driven biorefineries; and ii) material-driven biorefinery.[5]

Finally, according to the National Renewable Energy Laboratory, USA (NREL),[6] biorefineries are divided according to the processes used. In a first approach four platforms were considered: i) sugar (biochemical); ii) syngas (thermochemical); iii) biogas; or iv) carbon-rich chains (namely oils). As the concept evolved, currently only the first two platforms are usually recognized, being the latter two somehow incorporated/considered particular cases of the biochemical platform. In fact, any platform can incorporate other platforms (*e. g.* anaerobic digestion for wastewater treatment or the thermochemical transformation of lignin within the sugar platform biorefinery).

Table 1-1 Traditional division of the biorefinery into four platforms and the main processes used in each of them

Platform	Main processes
Biochemical (sugar)	Chemical and enzymatic hydrolysis
	Biotransformation
	Chemical transformation
Biogas	Anaerobic digestion
Thermochemical (syngas)	Gasification
	Pyrolysis
Carbon-rich chains	Transesterification

Although these classification systems are useful to understand the main differences between different facilities, they only reflect a partial view on the biorefinery. In fact, none of them really reflects the current view on the biorefinery concept, as these must be flexible installations dealing with multiple feedstocks, multiple technologies and multiple products, similarly to today's oil refinery.

1.1.3 Biorefineries and islands' sustainability

Islands' and outmost regions' economies are usually constrained by geographical and social factors. Their peripheral position, emphasized local cultural patterns and close dependence of the quantity and quality of available natural resources on the social system, make them particular cases within their national reality.[7] The economy of the Madeira archipelago is dominated by the tertiary sector, namely by tourism. In the secondary sector, representing 10% of Madeira's Gross Domestic Product (GDP), craftwork products (mainly destined for foreign markets) coexist with regional oriented consumption products (like dairy products, wine or bakery products) and with construction. Agriculture represents a low cut of the regional economy, being dominated by Madeira wine and banana production, and biomass residues in the archipelago were estimated to represent more than 140,000 ton per year.[8,9] These residues, together with other proto-biorefinery residues (*e.g.* from wheat processing or the production of beer, wine or other alcoholic beverages) could possibly be integrated in a biorefinery framework, contributing, for example, to lower the energetic dependence of the region and to the development of the

industrial tissue, like what has been done in other outmost regions, *e.g.* Reunion Island.[10]

1.2 Biorefinery feedstocks

1.2.1 Biomass oil sources for biodiesel production

There are four main categories of oily sources used for biodiesel production: i) edible and non-edible vegetable oils; ii) animal fats; iii) used oily materials (mainly waste cooking oils); and, emerging in recent years, iv) algal oils. Edible oils (like sunflower, soybean, canola/rapeseed, palm or coconut) are still the main feedstock for biodiesel production, potentially representing a problem as the world's population is growing, as well as the human consumption of such oils. Therefore, the use of non-edible oils is of great importance, both socially and economically, due to their lower cost.[11,12]

Several non-edible oils have been surveyed for their potential application as biodiesel feedstock and some of them are already being used for this purpose (*e.g.* jatropha or castor oils). Within this category of vegetable oils, there are various residues/by-products of common commercial crops, *e.g.* tobacco or cotton seed oils.[11]

The search for new sources of non-edible oils, particularly among the agricultural or agro-industrial residues, is then not only necessary but almost mandatory in a socio-economic point of view. The inclusion of such stream within the biorefinery framework would be of great benefit both upstream (as a residue would become a by-product) and downstream (as a low value feedstock would be converted into a high added-value product).

1.2.1.1 Vegetable oil extraction procedures

The most efficient industrial process commonly used for vegetable oil extraction is solvent extraction. Although it presents some security problems and causes the emission of volatile organic compounds to the

atmosphere, solvent extraction is still the most used method. Alternatively, for obtaining a chemicals free product, mechanical pressing is the method of choice, usually used for vegetable oil extraction. Pressing also better preserves the native properties, namely the organoleptic properties, when relevant. For instance, mechanical pressing is used prior to solvent extraction in some industries (*e.g.* olive oil).[13]

At laboratory level, Soxhlet extraction is the most widely used method for fat removal from solid matrices. Moreover, official methods are based on Soxhlet extraction. However, Soxhlet extraction is a slow method. To overcome this shortcoming, several approaches have been tested. These include pressurized liquid extraction (PLE) or pressurized fluid extraction (PFE), which is also known by the trade name "Accelerated Solvent Extraction" (ASE – Dionex, Sunnyvale, CA, USA), supercritical fluid extraction (SFC), mainly using supercritical CO_2, microwave- or ultrasonic-assisted Soxhlet extraction, or even enzyme-assisted aqueous based extraction.[14-20]

Despite all these developments, it has been concluded that the scale-up of these methods is hardly feasible and solvent extraction is still the most common method used industrially, with hexane being the most adequate solvent. For example, the extraction of oil from olive bagasse (as it is currently carried out in one of the major such industries in Portugal, UCASUL) and the extraction of microalgae lipids with hexane has been shown to be the best alternative among the solvent systems tested, reinforced by the simplicity of the process.[21]

1.2.1.2 Biodiesel production processes

Biofuels, and particularly biodiesel, are considered to be divided in three generations. First-generation biodiesel is produced from edible vegetable oils, second-generation biodiesel is obtained mainly from non-edible sources and algae are the feedstock for third-generation biodiesel.[22]

The first approach for substituting fossil diesel by a cleaner, and compatible, fuel was transforming vegetable oils (namely their triacylglycerols) into fatty acid methyl esters (FAME) and glycerol, exclusively in a chemical process called transesterification (Fig. 1-2). In this process an alkaline homogeneous catalyst is usually employed and the main limitation is the free fatty acid (FFA) content of the oil. High amounts of FFA in the oil leads to the formation of soap, caused by the reaction of FFA with the basic catalyst, instead of FAME formation. However, this is still the usual process for producing biodiesel.[23]

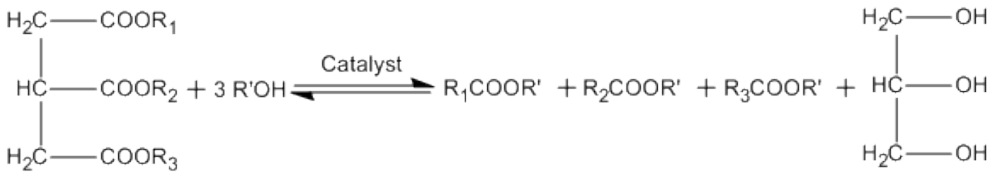

Figure 1-2 Simplified transesterification reaction

Several other transesterification processes were then developed either to overcome the limitations of base-catalyzed transesterification (*e.g.* homogeneous acid-catalyzed transesterification)[23] or to simplify the separation and regeneration of the catalyst (heterogeneous acid- or base-catalyzed transesterification).[23-25] These heterogeneous catalysts also have the advantage of tolerating more extreme reaction conditions (like higher temperatures) and higher yields are achieved.[23] Immobilized enzymes can also be used as catalysts for transesterification with several advantages, like milder reaction conditions (room temperature), high substrate specificity, the ability to convert both FFA and triacylglycerols in one single step, lower alcohol to oil ratios or easier product separation and recovery.[26] Non-catalytic transesterification processes have also been reported (*e.g.* using supercritical alcohol), as well as microwave- or ultrasonic-assisted transesterification processes.[27-29]

Besides transesterification there are other processes to obtain biodiesel from biomass, yielding a biofuel that is chemically similar to fossil diesel, instead of FAME biodiesel. This kind of biodiesel can be obtained by: i)

hydrogenation of oils and fats; ii) thermal cracking (pyrolysis) of oils, fats and lignocellulosic materials; or iii) by gasification of lignocellulosic materials followed by Fischer-Tropsch reaction.[23,25,30,31]

1.2.2 The lignocellulosic materials

Lignocellulosic biomass is the most abundant organic material in nature. Annually 10 to 50 billion tons of lignocellulosics (dry mass) are produced worldwide, according to estimates, accounting for about half of the global biomass yield.[32,33] Sources of lignocellulosic biomass include wood, agricultural residues, aquatic plants, grasses, and other vegetable matter. In general, lignocellulosics have been included in the term biomass, but this term has broader implications as it also includes living substances such as animal tissue and bones. Lignocellulosic materials have also been called photomass because they are a result of photosynthetic processes.[34]

1.2.2.1 Composition of lignocellulosic materials

Lignocellulosic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, extractives, and ashes (Fig. 1-3).[35] The term "holocellulose" is often used to describe the total carbohydrate contained in a plant or residue. Holocellulose is therefore comprised of cellulose and hemicellulose in lignocellulosic materials.

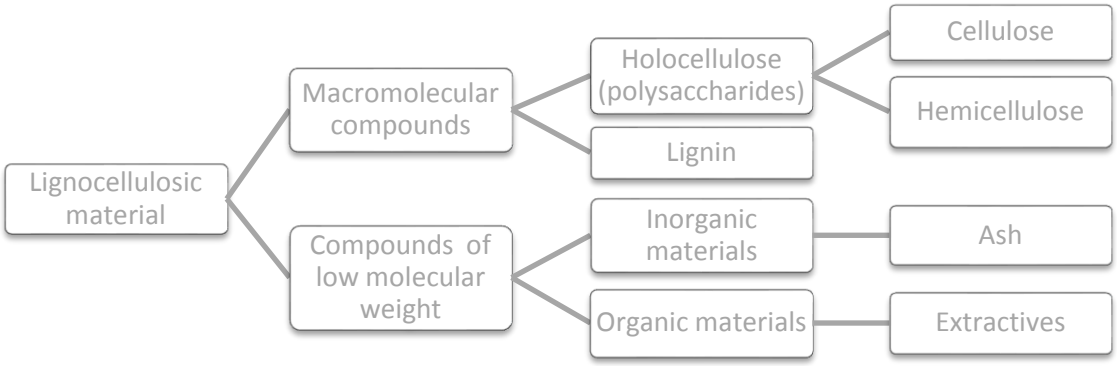

Figure 1-3 Composition of lignocellulosic materials (adapted from [35])

Lignocellulosic materials constitute most of the available biomass in nature. Their main components are cellulose (a glucose-only polymer), comprising

about 25 to 55%), hemicellulose (a sugar heteropolymer consisting predominantly of pentoses or hexoses, depending on the biological origin), 10 to 35% and lignin (a polymer of several phenolic compounds, whose chemical composition is also dependent of the biological origin), which accounts for 10 to 35%. Some materials also have a significant content of extractives. Hemicellulose is then a sugar polymer whose types and distribution depend on the particular lignocellulosic material.[2,36,37]

1.2.2.1.1 Cellulose

Cellulose, a highly stable glucose polymer, is the most abundant organic compound on earth as it is the main component of plant cellular walls.[37,38] Cellulose is a linear biopolymer of anhydroglucopyranose, connected by β(1→4) glycosidic bonds with the chemical formula $(C_6H_{10}O_5)_n$, where "n" is the degree of polymerization of the polymer (Fig. 1-4).[32,39] As the degree of polymerization increases, various properties of the cellulose also change. However, the basic building block of cellulose is actually cellobiose, a dimer of two-glucose unit.[40] The chains are held together by intra- and intermolecular hydrogen bonds between oxygen of alternating glycosidic bond in one glucan chain and the primary hydroxyl groups at position 6 of glycosyl residues in another chain to form thin, flattened, rod-like structures that are referred to as microfibrils.[33,41]

The cellulose microfibrils are bound to each other and to hemicellulose polymers by hydrogen bonding and there is no evidence of covalent linkage between cellulose and other cell wall constituents. This leads to bundling of cellulose molecules into microfibrils, which in turn form fibrils and finally cellulose fibers. Cellulose microfibrils contain regions with highly oriented molecules or less oriented microfibrils called crystalline and amorphous regions respectively. The crystallinity index of cellulose, i.e. degree of microfibrils orientation, is highly variable and depends on the source and age of the tissue.[2]

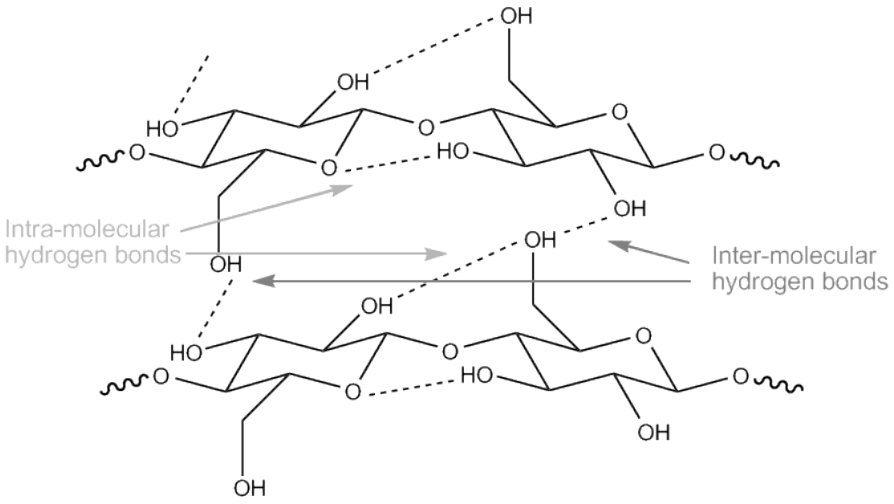

Figure 1-4 The β-linked glucopyranoside residues and the inter- and intra-molecular bonding of cellulose

Cellulose can be chemically hydrolyzed by diluted or concentrated acid, or enzymatically.[38]

1.2.2.1.2 Hemicelluloses

The term hemicellulose is applied to cell wall polysaccharides which occur in close association with cellulose, especially in lignified tissues, and belong to a group of heterogeneous polysaccharides. It is often restricted to substances extracted with alkaline reagents.[32,38,42] Hemicellulose is a highly branched heteropolymer containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose) and uronic acids (D-glucuronic acid).[43-45] Hemicellulose is more easily hydrolyzed than cellulose.[38]

The most relevant hemicelluloses are xylans and glucomannans, with xylans being the most abundant. Xylans are the main hemicellulose components of secondary cell walls of hardwoods and herbaceous plants, usually constituting about 20 to 30% of the biomass, and reaching up to 50% in some tissues of grasses and cereals. Xylans are usually available in huge amounts as by-products from forestry and wood production, pulp and paper mills or agriculture and agro-industries. Mannan type hemicelluloses, like glucomannans and galactoglucomannans, are the major hemicellulosic

components of the secondary wall of softwoods whereas in hardwoods they occur in minor amounts.[39]

1.2.2.1.3 Lignin

Lignin is a complex polymer that maintains the stiffness of trees and plants and it's the most abundant natural non-carbohydrate organic compound in fibrous materials.[32] Generally, softwoods contain more lignin than hardwoods. Lignin is a very complex molecule constructed of phenylpropane units linked in a three-dimensional structure.[38,41] Although the primary structural elements in lignin have been largely clarified, many aspects of its chemistry remain unclear.

The importance of lignin in plants should be considered from different aspects, *e.g.* its role in plant development, contribution to mechanical strength and protection from degradation. From the biorefinery point of view, lignin has always been considered as an important barrier to polysaccharide utilization.[46] Lignins are composed by blocks of nine carbon units derived from substituted cinnamyl alcohol; that is, *p*-coumaryl, coniferyl, and sinapyl alcohols. Most lignins also contain some esterified aromatic carboxylic acids (*p*-hydroxycinnamic – or *p*-coumaric – acids) – Fig. 1-5.[32,34,38]

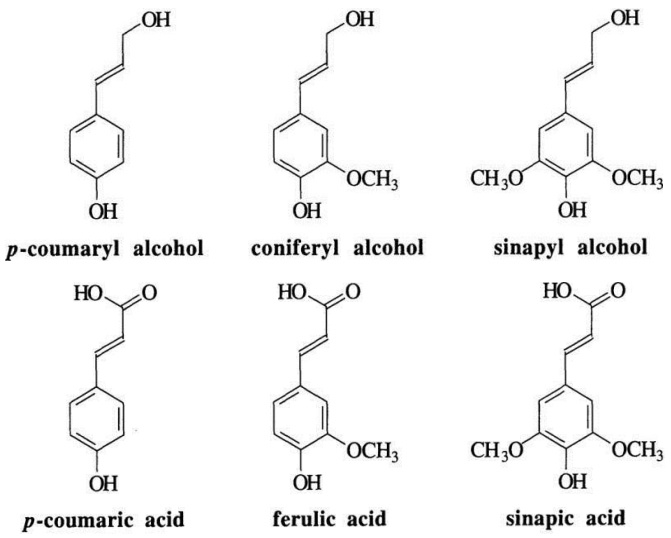

Figure 1-5 Chemical structure of lignin precursors

The lack of enzymatic control during lignin polymerization (formation), results in an almost random series of bonding and a very complex structure. The existence of strong carbon-carbon (C-C) and ether (C-O-C) linkages in lignin (Fig 1-6) diminishes its susceptibility to chemical disruption. Lignins are always associated with hemicellulose, not only in intimate physical mixture, but also anchored to the latter by actual covalent bonds. Soluble lignin-carbohydrate complexes have been isolated from lignocellulosic material.[38]

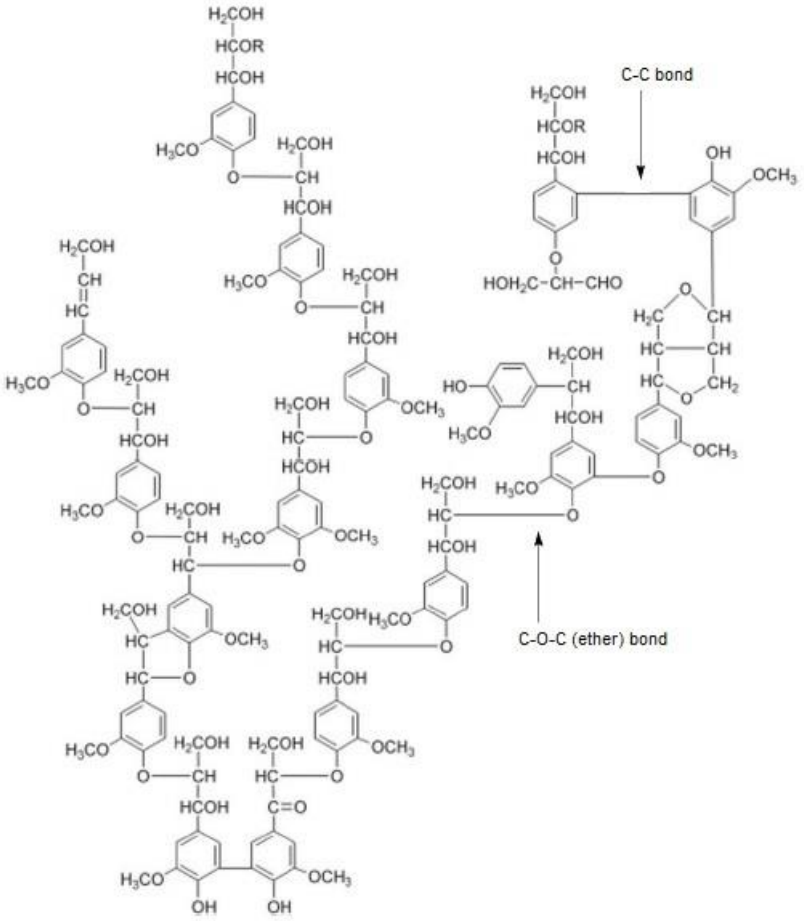

Figure 1-6 Proposed structural model for spruce lignin (adapted from [47])

1.2.2.1.4 Compounds of low molecular weight/extractives

Biomass compounds that are soluble in neutral organic solvents or water and are primarily composed of cyclic hydrocarbons are usually referred as extractives.[34] They usually represent a minor fraction (between 1 and 5%) of lignocellulosic materials. They contain a large number of both lipophilic and

hydrophilic constituents. The extractives can be classified in four groups: (a) terpenoids and steroids, (b) fats and waxes, (c) phenolic constituents, and (d) inorganic components.

1.3 Biochemical platform processes

The biochemical platform can be described by basically using 4 types of processes: i) biomass fractionation processes; ii)enzyme-based processes (most noteworthy, cellulose hydrolysis); iii) fermentation processes; iv) down-stream purification processes.

The later three are unspecific for the biorefinery and are common to most of the existing biotechnological industries, such as yeast or antibiotics production. Conversely, the biomass fractionation processes are rather specific for the biorefinery.

Previously called pretreatment processes, as their aim was to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials, they have now evolved to be called fractionation processes, as their aim has been enlarged to selectively fractionate the components in order to make a more efficient use of the feedstock.

Nevertheless, either called “fractionation” or “pretreatment”, these processes must meet the following requirements: i) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; ii) avoid the degradation or loss of carbohydrates and lignin; iii) avoid the formation of inhibitory byproducts to the subsequent hydrolysis and fermentation processes; and iv) be cost-effective. Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials.[48]

There are many such processes ranging from acid to alkali-based processes, from solvent to ionic-liquid based processes, operating under mild to harsh

temperature and pressure conditions.[1] A schematic explanation is presented in figure 1-7.

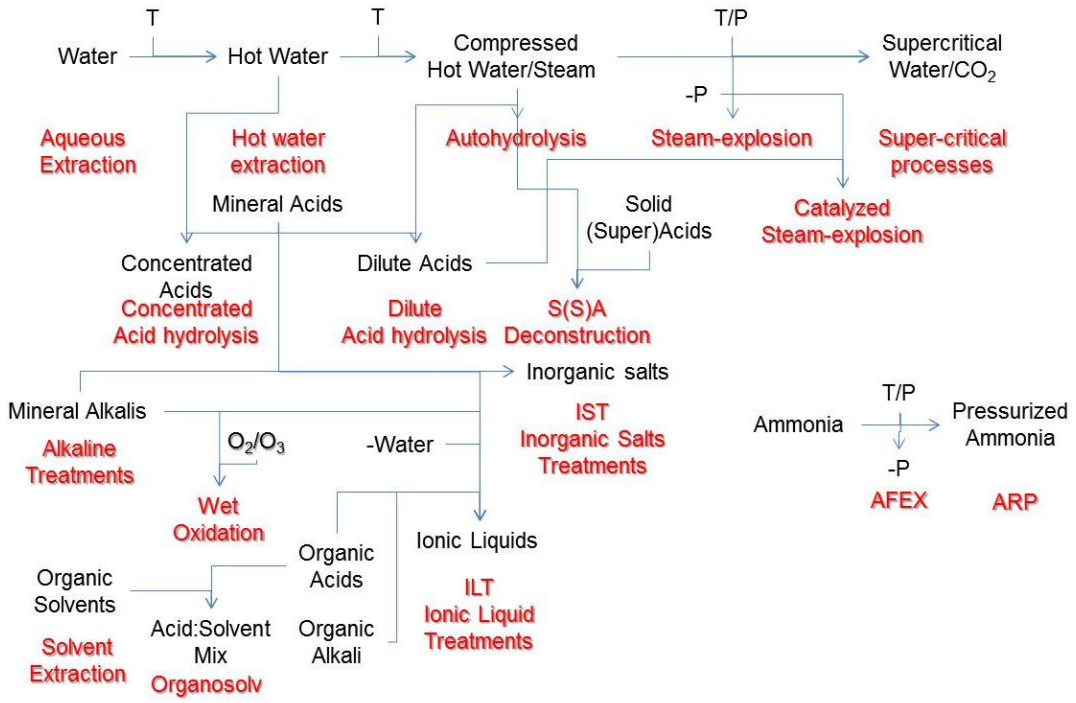

Figure 1-7 Different biomass fractionation processes and their associations

All these processes have many specific (dis)advantages regarding its use within the biorefinery that must be studied in depth for each feedstock and, most important, taking special attention to the target products. As such, there is not a single solution to fit all needs.

In this work, the focus is on sugar recovery from the hemicellulosic fraction and, as such, acid(neutral)-based processes are more useful and will be presented and studied in detail.

1.3.1 Acid hydrolysis

Hydrolysis involves cleaving the polymers of cellulose and hemicellulose into their monomers. As said before, softwood hemicellulose is mainly composed of mannose and the dominant sugar in hemicellulose derived from hardwood and crop residues is usually xylose. Complete hydrolysis, chemical or enzymatic, of cellulose results in glucose, whereas the hemicellulose gives

rise to several pentoses and hexoses. Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicellulose polymers.[45]

This process of hydrolysis can be divided in two general approaches, based on concentrate-acid/"low"-temperature and dilute-acid/high-temperature hydrolysis. Sulfuric acid is the most common acid employed although other mineral acids such as hydrochloric, nitric and trifluoroacetic (TFA) acids have also been assayed. In dilute-acid processes the use of phosphoric acid and weak organic acids has also been reported.[44]

1.3.1.1 Concentrated-acid hydrolysis

Hydrolysis of lignocellulosics by concentrated sulfuric or hydrochloric acids is a relatively old process, as Braconnot in 1819 first discovered that cellulose can be converted to fermentable sugars by concentrated acids.[49] Concentrated acid processes enable the hydrolysis of both hemicelluloses and cellulose. The solubilization of polysaccharides is reached using different acid concentrations, like 72% H_2SO_4, 41% HCl or 100% TFA. HCl and TFA have the advantage to be easier recovered.[50] Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion.[48]

1.3.1.2 Dilute-acid hydrolysis

Among the chemical hydrolysis methods, dilute-acid hydrolysis (DAH) is probably the most commonly applied. DAH is a simple and rapid method commonly used for biomass hydrolysis.[51] This process has, in relation to the above, fewer problems of corrosion of the equipment.[52] DAH, also called the pre-hydrolysis, is a method commonly used to solubilize the hemicelluloses. It is a method that can either be used as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolyzing lignocellulosics

into sugars. The first established DAH process was probably the Scholler process. This was a batch process, in which the wood material was kept in 0.5% sulfuric acid at 170 ºC and 20 bar for approximately 45 minutes.[53] Nowadays, most of DAH processes are performed in a batch mode with a retention time of a few minutes.

Sulfuric acid is the most widely used, apparently because it is cheap and effective, although HCl, HNO_3, HF and H_3PO_4 are also used. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve further cellulose hydrolysis. High temperature in dilute acid treatment is favorable for cellulose hydrolysis.[48]

Nevertheless, DAH was not a preferable pre-treatment for cellulose hydrolysis since the high temperatures required for cellulose hydrolysis also lead to formation of very high amount of degradation products. Typical sulfuric acid concentrations for hemicellulose hydrolysis are in the range 0.5–1.5% and temperatures range from 121 to 160 ºC, or even higher. From hemicelluloses, dilute-acid processes yield sugar recoveries from 70% up to >95%. However, for both dilute- and concentrate-acid hydrolysis approaches, the acid has to be removed/neutralized before fermentation, yielding large amounts of waste.[54]

Compared to the concentrate-acid hydrolysis, one of the advantages of dilute-acid hydrolysis is the relatively low acid consumption, leading to less energy demands for acid recovery. Under controlled conditions, the levels of degradation compounds produced can also be low.

1.3.2 Autohydrolysis

Autohydrolysys is a hydrothermal process aiming to recover most of the hemicellulosic fraction but avoiding the use of acid catalysts. As it only uses water and heat, besides biomass, this process can be considered to be within the so called “green chemistry” concept.[44]

In the autohydrolysis process high yields of hemicelluloses recovery can be achieved (usually above 80%). However, sugars are obtained as oligomers, which is an advantage when oligosaccharides are the desired product. Cellulose and lignin are not significantly affected in the autohydrolysis process and the obtained liquid fraction presents a high HIS (Hemicellulose Selectivity Index).[54] The hemicellulosic liquor is therefore basically free of chemical contaminants, easing products purification. On the other hand, the solid residue is rich in lignin and cellulose, more prone to valorization, *e.g.* into cellulosic ethanol.[1,44] Furthermore, with autohydrolysis corrosion problems are reduced, due to the moderate pH, and further neutralization, acid catalyst recovery and precipitates removal steps are not needed.[44]

The chemical reactions present on the autohydrolysis process, namely those involved in hemicelluloses hydrolysis, are catalyzed by the hydronium ion (H_3O^+) formed in the first phase of the process, due to water self-ionization. The hydrolysis of acetyl groups and glycosidic bonds also contribute to H_3O^+ formation. It has been suggested recently that uronic acids may also have a contribution, but their role in the hydrolysis process is still to be unveiled.[44]

Hemicelluloses hydrolysis is triggered by the protonation of the glycosidic bond. This promotes the rupture of the polysaccharide chain, resulting in the formation of an unstable carbocation that reacts with a water molecule to form the stable saccharide and the consequent regeneration of the hydronium ion. The formed saccharide can either be an oligomer or a monomer, if this rupture occurs on a terminal position of the chain.[55] The mechanism for this reaction is represented in figure 1-8 for a terminal hexose.

1.4 Biorefinery products

Biorefinery products can be obtained from all biomass fractions.

Cellulose upgrade is not difficult *per se*, yielding easily fermentable glucose, *e. g.* into second generation bioethanol, by hydrolysis.

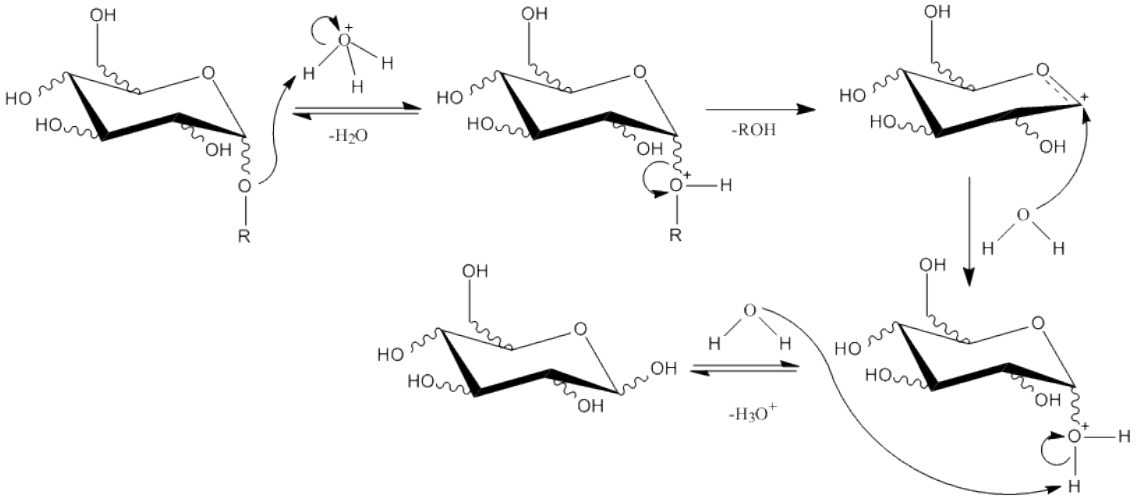

Figure 1-8 Polysaccharide hydrolysis mechanism

Due to its phenolic nature, lignin does not yield fermentable sugars, but many diverse added-value phenolic based products can be produced. Furthermore, residual/non-converted lignin can be used to fuel the biorefinery.

The hemicellulosic fraction, due to its heterogeneous nature, is not easily upgradable and is usually considered the major hindrance for cellulose enzymatic hydrolysis. Therefore, many efforts are being put forward for its effective removal.[56] Readily available oligosaccharides or monomeric sugars (foremost pentoses) can be produced from this fraction. Yet, these monosaccharides are not as easily fermented as glucose, and xylose fermentation into ethanol is still one of the major scientific hurdles to be overcome. Nevertheless, the sugars obtained from hemicelluloses create an opportunity for the production of many chemicals.[57] Several of them possess significant added-value and have not been yet fully exploited, which can contribute to the biorefinery's sustainability. Nevertheless, it is critical in commercialization of any co-product that the market is not flooded with single products produced on a grand scale, simply because they are tied to large biorefineries. Therefore, the careful selection of co-products to be produced in a biorefinery plant is an important task.[39,58]

1.4.1 Oligosaccharides as valuable compounds

Oligosaccharides (OS) are, according to the IUPAC-IUB's Joint Commission on Biochemical Nomenclature (JCBN), hydrolysable sugars, of low molecular weight, consisting in 2 to 10 units of monosaccharides [59-61] connected by glycosidic bonds between the anomeric carbon of one monosaccharide and an hydroxyl group of another. However, several authors broaden the definition to carbohydrates with a degree of polymerization up to 60.[62-64]

The physico-chemical characteristics of OS, like their ability to retain moisture (of great convenience for controlling microbial contamination in food), make them useful, for example, in the food industry. They are soluble in water and slightly sweeter than sucrose, even though they are low in calories (1.5-2.0 $kcal.g^{-1}$ against 4.0 $kcal.g^{-1}$ for sucrose), making them a useful substitute to sucrose for people with diabetes. Furthermore, typically OS are highly stable up to 100 ºC and over a wide range of pH values (from 2.5 to 8) and have freezing-point depression properties, making them suitable for usage in carbonated drinks and for food processing.[65] Their relatively high molecular weight, mainly when compared to mono- and disaccharides, make them good thickening agents, besides enhancing food taste and causing a good mouth feel.[61,66]

Among the several OS with high dietary and economic value, the non-digestible oligosaccharides (NDOS) are of great importance. Their main feature is the presence of monosaccharide units which are non-hydrolyzable by human digestive enzymes. These non-digestible units may then reach the intestine and be used to feed the bacteria in the gut flora, acting like prebiotic agents.[66,67] The most representative classes of NDOSs, their chemical structure and degree of polymerization are presented in Table 1-2.

In the pharmaceutical industry OS are also very common, with applications as immunomodulators or as substrate regulators in the major metabolic

pathways, as well as in viral or bacterial infections, as cellular adhesives, as signal transducers or in intracellular communication.[65-68]

Table 1-2 Main groups of OS, their structure and degree of polymerization (adapted from [59])

Source	Classification	Characteristic chemical structure	Deg. Pol.
Lactose	Galacto-oligosaccharides	α-D-Glc-(1→4)-(β-D-Gal-(1→6)-)$_n$	2 to 5
	Lactulose	β-D-Gal-(1→4)-β-D-Fru	2
Sucrose	Lacto-sucrose	β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-Fru	3
	Isomaltulose oligosaccharides	(α-D-Glc-(1→6)-D-Fru)$_n$	4 to 8
	Fructo-oligosaccharides	α-D-Glc-(1→2)-(β-D-Fru-(1→2)-)$_n$	2 to 4
Inulin		β-D-Fru-(1→2)-(β-D-Fru-(1→2)-)$_n$	2 to 9
		α-D-Glc-(1→2)-(β-D-Fru-(1→2)-)$_n$	3 to 10
Starch	Malto-oligosaccharides	(α-D-Glc-(1→4)-)$_n$	2 to 7
	Gentio-oligosaccharides	(β-D-Glc-(1→6)-)$_n$	2 to 5
	Isomalto-oligosaccharides	(α-D-Glc-(1→6)-)$_n$	2 to 5
	Ciclodextrins	(α-D-Glc-(1→4)-)$_n$	6 to 12
Xilans	Xilo-oligosaccharides	(β-Xyl-(1→4)-)$_n$	2 to 9
Pectin	Soy oligosaccharides	(α-D-Gal-(1→6)-)$_n$-α-D-Glc-(1→2)-β-D-Fru	3 or 4

The OS market is nowadays an important market (and still expanding) dominated by the lactose derived NDOS like lactulose or galacto-oligosaccharides (GalOS), followed by fructo-oligosaccharides (FOS). In 1996 xylo-oligosaccharides (XOS) represented less than 1% of the market (worldwide), however the demand for XOS has been largely increasing since then.[69,70]

1.4.1.1 Oligosaccharides production processes

Oligosaccharides can be produced from various polysaccharide sources (Table 1-1) both by physical, chemical or enzymatic depolymerization and by chemical or enzymatic synthesis.[67]

The use of enzymes in order to modify polysaccharides and potentiate their biotechnological applications is of great interest, as these proteins are highly specific both to their substrates and their products. Among the enzymatic processes used to produce OS hydrolases, isomerases and transferases are the most common enzymes. For example, lactulose is produced from lactose using isomerases under alkaline conditions and using inulinase FOS can be

obtained from inulin.[59,66] Xilanases are used to obtain XOS from agro-industrial by-products/residues like corn cobs, sugarcane or cassava.[59,67]

Enzyme catalyzed synthesis is an interesting alternative to the classic chemical methods due to the high regioselectivity and stereochemical control of the products.[71] In this process two types of enzymes, glycosidades and glycosyltransferases, are used in order to produce OS hardly obtained by common enzymatic depolymerization or hydrolyzation methods,[72] *e.g.* the production of FOS from sucrose and GalOS from lactose.[66] However, the yield of these enzymatic synthesis are still low and the development of new functional food ingredients has potentiated the search for new biotechnological processes to obtain OS, as the market demands more and more of them due to their peculiar properties.

As an alternative to the enzymatic methods, autohydrolysis is described as a promising method for obtaining XOS from agro-industrial by-products/residues like brewery spent grain (BSG) and corn stover or from *Arundo donax*, an energy crop endemic from the Mediterranean area (and an invasive species in Madeira Island).[73-76] Within the biorefinery framework, hydrothermal processes and acid hydrolysis are the most efficient methods for obtaining OS, preferably the former, as previously explained.[1]

1.4.2 Microbial polysaccharides as biorefinery products

Polysaccharides are natural, non-toxic and biodegradable polymers that may cover the surface of most cells and play important roles in various biological mechanisms such as immune response, adhesion, infection and signal transduction. Investigations on the alternative treatments applied by different cultures throughout the history revealed the fact that the utilized plants and fungi were rich in bioactive polysaccharides with proven immunomodulatory activity and health promoting effects in the treatment of inflammatory diseases and cancer.[77]

1.4.2.1 Microbial polysaccharides

Biopolymers often play important roles in nature. Conservation of genetic information, defense against hazardous environmental factors, storage of carbon-based macromolecules and energy production or reduction are examples of properties that contribute to maintain cell viability. Microbial polysaccharides are high molecular weight carbohydrate polymers present either at the outer membrane as lipopolysaccharides (LPS), that mainly determine the immunogenic properties, secreted as capsular polysaccharides (CPS), forming a discrete surface layer (capsule) associated with the cell surface, or excreted as extracellular polysaccharides (EPS) that are only loosely connected with the cell surface. CPSs are usually associated with functions directly related with pathogenicity, like resistance to specific and nonspecific host immunity and adherence. EPSs fulfill a variety of diverse functions including adhesion, cell-to-cell interactions, biofilm formation and cell protection against environmental extremes.[77]

Considerable diversity can be observed in polysaccharide composition and structure. They are generally classified as homopolysaccharides or heteropolysaccharides, based on their monomeric composition. Homopolysaccharides are composed exclusively of one type of monosaccharide repeating unit, where sugar monomers are either bound to form linear chains (pullulan, levan, curdlan or bacterial cellulose) or ramified chains (dextran). Hetero-polysaccharides are composed of two or more types of monosaccharides and are usually present as multiple copies of oligosaccharides, containing three to eight residues (gellan – Figure 1-9 – or xanthan).[77]

The microorganisms used as industrial producers of extracellular polysaccharides are, essentially, bacteria. Species of *Xanthomonas*, *Leuconostoc*, *Sphingomonas* and *Alcaligenes* which produce, respectively, xanthan, dextran, gellan and curdlan are the best known and most industrially used (Table 1-3).

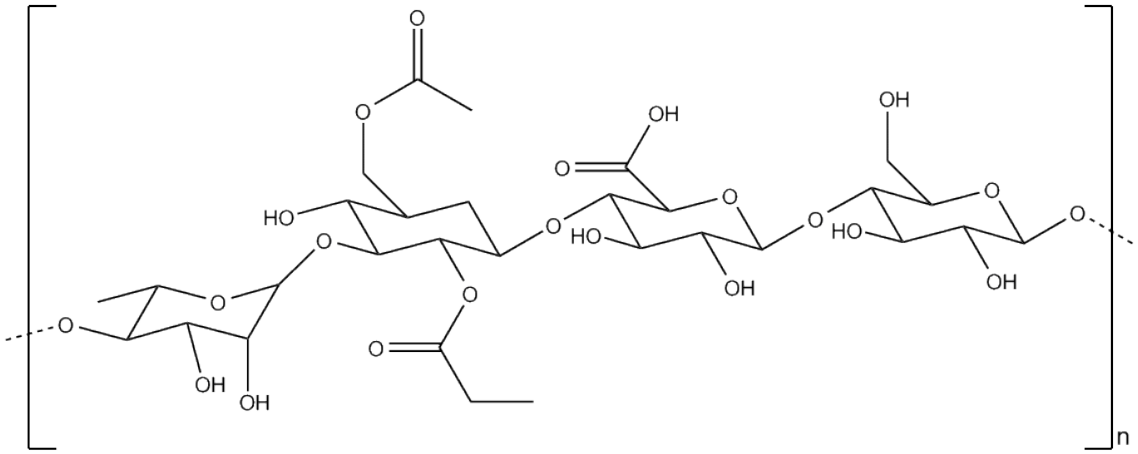

Figure 1-9 Native structure of gellan (adapted from [78])

Table 1-3 Main bacterial polysaccharides (adapted from[77])

EPS	Monomers	Characteristics of Chemical Structure	Organism
Alginate	Guluronic acid Mannuronic acid	Blocks of β-1→4-linked D-ManA residues, blocks of α-1→4-linked L-GulA residues, and blocks with these uronic acids in random or alternating order	*Pseudomonas aeruginosa* *Azotobacter vinelandii*
Cellulose	Glucose	β-1→4-D-glucan	*Gluconacetobacter xylinus*
Curdlan	Glucose	β-1→3-D-glucan	*Alcaligenes faecalis* *Cellulomonas flauigena*
Dextran	Glucose	D-glucan linked by α-1→,6-glycosidic bonds; some 1→2-, 1→3-, or 1→4-bonds are also present in some dextrans	*Leuconostoc mesenteroides*
Gellan	Glucose Rhamnose Glucuronic acid	Partially O-acetylated polymer of D-Glc-β-1→4-D-GlcA-β-1→4-D-Glc-β-1→4-L-rhamnose tetrasaccharide units connected by α-1→,3-glycosidic bonds	*Sphingomonas paucimobilis*
Hyaluronan	Glucuronic acid N-Acetylglucosamine	Repeating units of β-1,4-linked disaccharides of D-GlcNAc-β-1→3-D-GlcA	*Ps. aeruginosa* *Pasteurella multocida*
Levan	Fructose	β-2→6-*D*-fructan	*Bacillus subtilis* *Zymomonas mobilis* *Halomonas sp.*
Xanthan	Glucose Mannose Glucuronic acid	β-1→4-D-glucan with D-Man-β-1→4-D-GlcA-α-1→2- D-Man sidechain. Approximately 50% of terminal Man residues are pyruvated and the internal Man residue is acetylated at C-6.	*Xanthomonas campestris*

1.4.2.2 Microbial exopolysaccharides production processes

Fermentation is a very versatile process technology for producing added value products such as microbial biopolymers. Exopolysaccharides all share one common feature both interesting in terms of physicochemical properties

and challenging and complex to deal with. Operating parameters and system properties, like temperature, agitation, fermenter design, pH, substrate or aeration highly influence fermentation performance and biological properties.[79] Moreover, besides the fermentation conditions, the chemical structure, monomer composition and physico-chemical and rheological properties of the final product also change with the type of strain. This in turn allows the industrial production of polysaccharides with desired specifications *via* controlling the fermentation conditions, choosing feasible feedstocks and using high-level producer strains.

Fermentations for EPS production are batch, fed-batch or continuous processes depending on the microbial system used. In most cases, optimum values of temperature and pH for biomass formation and EPS production differ considerably so that typical fermentations start with the growth phase followed by the production phase. In addition, considerable changes in the rheological properties occur during the course of fermentation due to EPS production. This results in a highly viscous and non-Newtonian broth which may not only cause serious problems of mixing, heat transfer, and oxygen supply but also give rise to instabilities in the quality of the end product. This is a common technical difficulty in commercial xanthan and pullulan production processes.[77]

1.4.2.3 *Xanthomonas campestris*

Xanthomonas is a genus of the family *Pseudomonaceae*. With the exception of the bacterium *X. maltophilia* all other organisms of this genus are pathogenic. The Gram-negative aerobic bacteria *X. campestris* pv. *campestris* (*Xcc*) is a phytopathogen that causes black rot (Fig. 1-10) in crucifers, and is considered one of the most important necrotrophic bacterial diseases occurring worldwide on all *Brassica*, other members of the *Brassicaceae*, and a few wild *Capparales* species.[80,81] The virulence of *X. campestris* depends upon a number of factors, including the ability to

produce exopolysaccharides and extra-cellular enzymes (such as protease, endoglucanase, pectinase and mannanase).[81,82]

Figure 1-10 *X. campestris* pv. *campestris* caused black rot in cabbage leaf (Photo by David B. Langston, University of Georgia, USA)[83]

X. campestris was originally divided into five races designated 0, 1, 2, 3 and 4. Later studies further refined the designation of *Xcc* races into 1–6; the designation "0" became "6" to avoid the implication that race "0" was avirulent. It was also found that *Xcc* races 1 (62%) and 4 (32%) were predominant and that the other races were rare and often host-specific.[80]

Besides carbohydrate solutions, *X. campestris* has been reported to grow satisfactorily and to produce xanthan in crude complex media such as molasses, hydrolyzed cereal grains, hydrolyzed acid whey, flour and bran, corn syrup and starch.[84]

1.4.2.4 Xanthan production

Xanthan gum is a non-linear anionic microbial hetero-polysaccharide produced by aerobic fermentation of *Xanthomonas campestris* and other *Xanthomonas* species, whose molecular weight exceeds 106 KDa. Xanthan gum, an exo-polysaccharide, is one of the major commercial biopolymers produced.[82,85-91]

The primary structure of xanthan was established in 1975, and consists of 1,4 linked β-D-glucose residues, having a trisaccharide side chain of D-mannose-D-glucuronic acid-D-mannose attached to alternate D-glucose units of the main chain at a 2:2:1 ratio (Fig 1-11).[82] The chemical structure of the polymer skeleton is therefore identical to that of cellulose. The anionic character of this polymer is due to the presence of glucuronic, acetic and pyruvic acid groups in the side chain.[82,92,93]

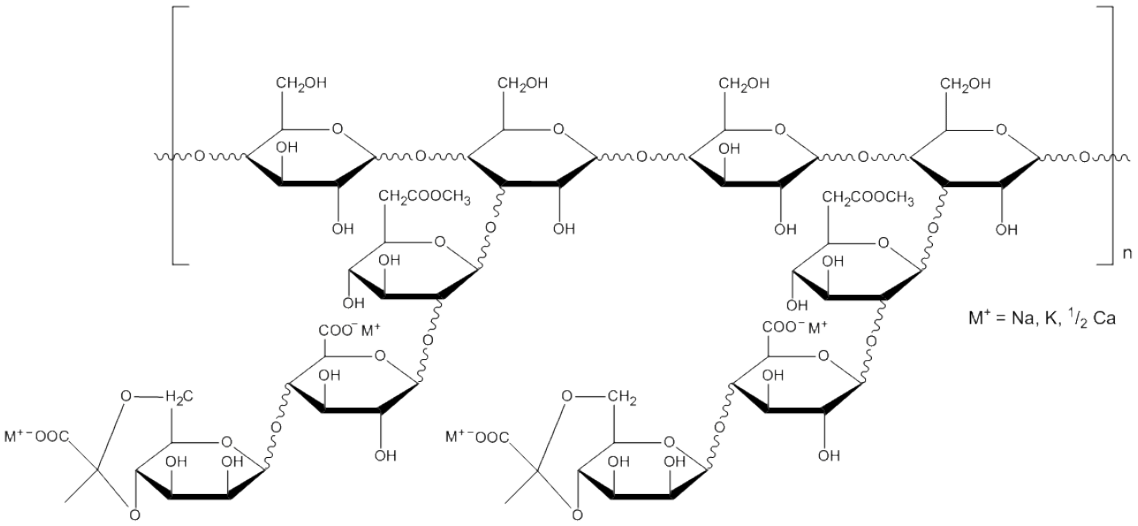

Figure 1-11 Structure of extracellular polysaccharide of *X. campestris* (adapted from[82])

Among the microbial gums, xanthan is the most important industrially as it occupies a prominent place in the market. Due to its rheological properties (that are quite different and unusual), such as a high degree of pseudoplasticity, high viscosity even at low concentrations, stability and compatibility with most metallic salts, excellent solubility and stability in acidic and alkaline solutions and resistance to degradation at elevated temperatures and various pH levels, xanthan gum can be found in a huge variety of food and cosmetic products.[79,92] Xanthan gum was evaluated by FDA (Food and Drug Administration) in 1969, allowing its use in the production of foods. Food and Agriculture Organization (FAO) and the World Health Organization (WHO) also regulated its use in 1990.[79] Xanthan gum is also classified as E415 in the European List of Permitted Food Additives. According to the JECFA (Joint WHO/FAO Expert Committee on Food Additives), it has the status of ADI-non-specified (Acceptable Daily

Intake), *i.e.*, no quantitative limitation is stated, and, as such, xanthan gum is recognized as a non-toxic additive for human consumption. The gum exhibits many advantages as a thickener, stabilizer, gelling agent and suspending agent, being a common ingredient in creams, artificial juices, sauces for salads, meat, chicken or fish, as well as in syrups and coverings for ice creams and desserts.[87,88,92,94]

The major producers of xanthan in the US are Merck, Kelco, and Pfizer. In Europe, Rhone Poulenc, Mero-Rousselot-Santia and Sanofi-Elf are the major producers in France and Jungbunzlauer is the main producer in Austria. In China, the major producer of xanthan gum is Saidy Chemical. The market capitalization of xanthan gum was approximately US$270 million in 2010, and projections for 2015 exceed US$400 million. To supply the various sectors of consumption more than 86,000 tons/year of xanthan gum were being produced back in 2010.[92]

1.4.2.5 Kefir and Kefiran

Kefir is an acidic and mildly alcoholic fermented dairy product that is believed to have functional properties.[95] It is generally considered to have high nutritional, biological and dietetic values. In addition, the microbes constituting the Kefir grains produce lactic acid and other biologically active compounds that increase the storage capability of milk and inhibit the growth of undesirable and pathogenic microbes.[96]

Kefir inoculum is a mix culture of various yeast species of the genera *Kluyveromyces*, *Candida*, *Saccharomyces*, *Pichia* and some lactic acid bacteria of the genera *Lactobacillus, Lactococcus* and *Leuconostoc*.[97,98] Kefir, besides its inherent high nutritional value and antimicrobial activity, ferments lactose, and therefore whey, a waste of negligible cost and rich in lactose, that could be used as raw material for kefir production.[96,99-103]

Kefir grains (Fig. 1-12) are a natural mixed culture starter, where the diverse microbial population represents a pattern of symbiotic community.

Traditionally kefir grains have been used for centuries in many countries, especially in Eastern Europe, as the natural starter in the production of the unique self-carbonated dairy beverage known as kefir.

Figure 1-12 Milk Kefir "grains"(reprinted from [104])

However, many studies indicate also their potential novel applications in bread production, other fermented drinks and as cheese starter,[105] but polysaccharide (Kefiran) production may be the most attractive novel application.

Kefiran is a polysaccharide composed of glucose and galactose (1:1) presenting a hexasaccharide repeating unit (Fig. 1-13).[106]

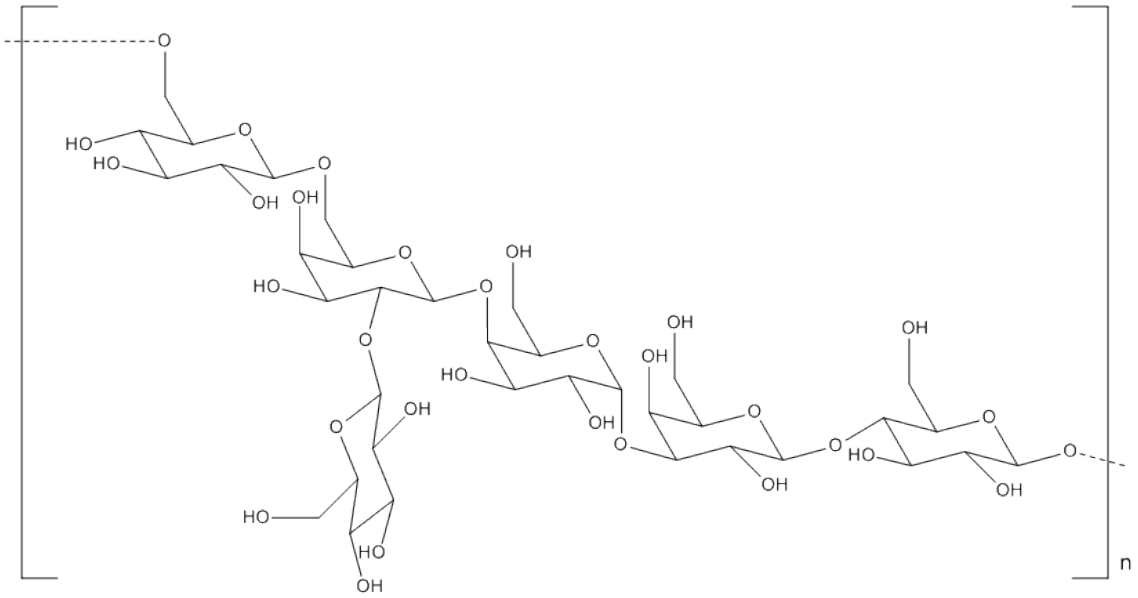

Figure 1-13 Putative structure of Kefiran (adapted from[106])

The average molecular weight and the z-average radius of gyration of a sample may vary, but have been reported to be 760 KDa and 39.9 nm, respectively. Many biological activities are being discovered, like positive changes in blood pressure and serum components (with cholesterol, TAG and FFA being the most important), antimicrobial and anticarcinogenic activities or anti-cytotoxic effects.[107-109]

1.5 *Annona cherimola* Mill.

Cherimoya (*Annona cherimola* Mill.) belongs to the *Annonaceae*, one of the largest tropical and subtropical families of trees, shrubs and lianas that include about 130 genera and 2300 species with a worldwide distribution, 900 of which are found in the tropics.[110]

A. cherimola is a species found in different subtropical areas around the world.[111] Cherimoya has hermaphroditic flowers with a gynoecium and an androecium of pyramidal form surrounded by two whorls of three petals, the inner small and scale-like and the outer green-like and fleshy. The gynoecium is located in the center of the conic receptacle and is composed of up to 300 carpels, each carpel containing a single ovule that will form a syncarp after fertilization.[110] The cherimoya fruit is known for its exceptional taste. The sub acid flesh is creamy white, soft, juicy, sweet and very fragrant, with a custard-like consistency.

Cherimoya seems to be originated in the inter-Andean valleys of southern Ecuador and northern Peru, although Spain is currently the major world producer of cherimoyas with about 3000 ha (33,000 tons/year) followed by Chile with about 1000 ha.[110,112]; Equador, Peru, Bolivia, Mexico and Portugal are other relevant producers (approx. 50,000 ton/yr, worldwide).[113] Specifically, in Madeira Island (Portugal) its production doubled in the last 5 years, reaching 1,100 ton in 2013, and has a considerable impact on regional agricultural economy, being now the third crop in terms of cultivated area.[114,115]

Three genera (*Annona*, *Rollinia* and *Asimina*) of the *Annonaceae* family contain species with edible fruits, such as cherimoya (*A. cherimola*), sugar apple (*A. squamosa*), atemoya (a hybrid between *A. cherimola* and *A. squamosa*), soursop (*A. muricata*), custard apple (*A. reticulata*), ilama (*A. macroprophyllata*), soncoya (*A. purpurea*), rollinia (*Rollinia mucosa*) or pawpaw (*Asimina triloba*). Atemoya and, especially, cherimoya are the most important worldwide both commercially and for local consumption.[110,116] This group of fruits presents increasing important socio-economic values within the producing countries, but considering international market it recently presents more and more importance, because of their status as exotic fruit showing excellent qualities, including the functional value, such as vitamins, antioxidants and other functional properties.[117]

Cherimoyas are also produced at a limited commercial scale in other Latin American countries where most of the cherimoya fruits sold or consumed are collected from the wild or from backyard trees. The increase in consumption of *Annonaceae* may be intensified if the producing countries implement an aggressive marketing program demonstrating its nutritional and functional qualities for consumers' health.[117] The increasing production and industrialization of this fruit will yield a novel waste, the seeds, which are oil-rich (approx. 30% w/w). The remaining lignocellulosic fraction can have potential advantages if upgraded within the biorefinery framework.

Some reported applications for cherimoya, other than its consumption as fresh fruit, are summarized in table 1-4. It's noticeable that *A. cherimola* has aroused great interest in the last 5 years.

In Madeira Island four cultivars of *A. cherimola* have been identified, namely Madeira, Funchal, Perry Vidal and Mateus,[135,136] with some reported differences among them. The main differences regarding fruits of these four cultivars are summarized in Table 1-5.

Table 1-4 Potential applications for *A. cherimola*

Target fraction	Product/application	Year	Technology	Economic sector	Ref.
Bark	Antimicrobial	1990	Extraction	Pharmaceutical	[118]
Stems	Anti-tumoral	1999	Extraction	Pharmaceutical	[119]
Leaves	Anti-fungal	2000	Extraction	Agriculture	[120]
Seeds	Anti-tumoral	2005	Extraction	Pharmaceutical	[121-123]
Fruit	Anti-protozoan	2006	Extraction	Pharmaceutical	[124]
Fruit	Anti-bacterial	2009	Extraction	Pharmaceutical	[125]
Pulp	Anti-oxidant	2011	Extraction	Pharmaceutical	[126]
Leaves	Anti-depressant	2012	Extraction	Pharmaceutical	[127]
Fruit	Anti-tumoral	2012	Extraction	Pharmaceutical	[128]
Bark	Energy	2012	Combustion	Energy	[129]
Peel/pulp	Anti-oxidant	2012	Extraction	Pharmaceutical	[111]
Fruit	Anti-fungal	2012	Extraction	Pharmaceutical	[130]
Leaves	Anti-hypercholesterolemial	2013	Decoction	Pharmaceutical	[131]
Pulp	Anti-fungal	2013	Extraction	Pharmaceutical	[132]
Seeds	Insecticides	2014	Milling	Agriculture	[133]
Leaves	Anti-depressant	2014	Extraction	Pharmaceutical	[134]

Table 1-5 Main differences reported for the fruits of the four Madeira Island cultivars of *A. cherimola*

	Madeira	Funchal	Perry Vidal	Mateus
Color	Light green	Yellow-green	Dark olive green	Dark olive green
Seed index[a]	6	7	8	9
^{o}Brix[b]	19.7	18.9	21.0	17.5

[a]number of seeds per 100 g of pulp; [b]measure of sugar content (1 ^{o}Bx = 1 g of sucrose per 100 g of solution)

A large variability is also observed in fruit weight, ranging from 100 g to 2 kg, with an average weight of 450 g.[135]

1.5.1 Cherimoya seeds' BVPI

A simple and objective way to measure the suitability of a given lignocellulosic material is to use the biotechnological valorization potential indicator (BVPI). This tool considers biological and physico-chemical factors, as well as economical, technological and geographical factors, to quantitatively estimate the biotechnological valorization potential of lignocellulosic materials. The assigned values range from 0 (highly undesirable) to 3 (strong positive impact), according to a predefined grid.[137] High scores are given if, for example, a material has low water content, is available in large quantities or if it has no current application.

In a first approach the BVPI score for cherimoya seeds in the Madeira Island context is somewhat low (Table 1-6). This score can be increased if some political measures (such as economic support to increase regional energetic self-sufficiency and sustainability) would be adopted and because the seasonality issue is somewhat overcome, as seeds are not perishable and can easily be stored. Other than in Madeira Island, if a more global context is considered, larger quantities are available: 3,400 ha of cultivated area and a production of around 35,000 tons of fruit a year, only in the coastal areas of the provinces of Granada and Málaga, in Spain.[138,139]

Table 1-6 BVPI scores for cherimoya seeds in Madeira Island (base score) and globally (potential)

	Base score	Potential score
Biological nature	1	1
Macromolecular composition	1	1
Water content	3	3
Physical characteristics	2	2
Seasonality	2	2
Economic value	2	2
Market dependency	2	2
Current technology/destination	3	3
Development stage of upgrade technology	0	2
Available quantities	0	1
Geographical concentration	0	1
Political or legal constraints	1	2
BVPI	**17**	**22**

Thus, considering this broader scenario, the BVPI for *Annona cherimola* Mill. puts this residue at the level of the top-listed Portuguese agro-industrial by-products, like rice husks, brewery's spent grain or carob pulp , with scores of 26, 25 and 23, respectively,[137] hence having great potential for valorization within the biorefinery framework. As a comparison, BVPI scores of 28 are given to sugar cane bagasse in Brazil or corn stover in the USA, two emerging residues prone to be used as biorefinery feedstocks.

Cherimoya seeds, due to the limited available amounts, are not prone to be biorefinery feedstock *per se*. In fact, as mentioned above, a biorefinery should be a multi-feedstock, multi-process and multi-product industrial installation. In Madeira Island many other possible biorefinery feedstocks are available, and large amounts of forest residues, wheat bran, BSG,

sugarcane residues or grape seeds are produced, besides the yet unquantified banana tree residues. In this sense, cherimoya seeds might be integrated in a flexible biorefinery and the feasibility of introducing this material as a feedstock must be studied.

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CHAPTER 2

CHARACTERIZATION OF *ANNONA CHERIMOLA* MILL. SEED OIL FROM MADEIRA ISLAND – A POSSIBLE BIODIESEL FEEDSTOCK

Summary

In this chapter, the possibility of using annona seed oil as an added value product, namely as a source of biodiesel, is explored. Milled annona seeds were extracted with hexane at room temperature (72 hours) and at solvent boiling point (6 hours). Oil content was found to be 25% and 22.4% respectively. The oil was characterized in terms of lipid composition (HPLC-APCI-MS and 13C-NMR), resistance to oxidation and acidity index. FAME composition was determined by GC-MS and 5 major peaks were identified. Production of biodiesel from annona's seed oil was achieved by base-catalyzed transesterification. Density, viscosity, acid value, cold filter plugging point, cloud point and oxidation stability were measured. Iodine value and "apparent cetane number" were calculated. Density, viscosity, acid value, iodine value, cold filter plugging point and cloud point were within EN14214 specifications and calculated "apparent cetane number" was also indicative of a suitable product.

2.1 Introduction

Residual agricultural products and food-processing by-products or wastes are often considered a problem. After processing or human consumption a great part of plant materials remain without any application.[1] The conversion of such materials into valuable resources can be a good contribution to residue reduction. In this sense, residue valorization has become of great interest from an economical point of view.

Fruit seeds are often considered waste by some industries and several studies have been carried out to evaluate their suitability as a source of biodiesel.[2-4] This application can introduce great economical interest as industrial waste is converted into a useful by-product. In Madeira island *Annona cherimola* Mill. is consumed as fresh fruit and part of the production is exported. However, since this is a very sensitive fruit, approximately half of the total production is lost and 500 tons of waste are produced every year, which are usually disposed off or used as fertilizer. Seeds represent about 2.5% of the residue weight and may be used as a source of biodiesel. Seeds of other annona species (*Annona squamosa* and *Annona muricata*) have been surveyed for its possible use as source of biodiesel.[5] *Annona cherimola* fruit production is largely developed in some Latin American countries, therefore large quantities of residue might be available.

Biodiesel (fatty acid alkyl esters) produced from vegetable oils has been considered a viable alternative for fossil diesel. As dedicated agro-production can have disastrous economic consequences there has been great discussion about using soils for energy crops instead of using them for feeding purposes. Therefore waste cooking oils and non-edible vegetable oils are considered a potential source of alternative fuels,[3,4] as they do not compete with food crops for the occupation of the soil. Waste seed oil can be considered a non-edible vegetable oil, although in some African or Middle

East countries pumpkin and melon seeds (waste products after the removal of the pulp and peel) are used as cooking oils.[1]

Oil characterization is essential to evaluate its potential applications, such as cosmetics, nutrition or fuel industries.[6,7] Chromatographic methods with mass spectrometric detection are well established to determine lipid composition. GC-MS is widely used to determine fatty acid (FA) composition, mainly as their methyl esters (FAME), and HPLC-MS has been used for triacyglycerol (TAG) characterization. ^{13}C-NMR can also be used to quantify free fatty acids (FFA) and partial acylglycerols, which are normally present as traces.[8]

Regarding fuel applications, the measurement of the oil's stability to oxidation and its acidity index can be screening methods to predict the suitability of the oil as a raw material for biodiesel production. Oils with low oxidation stability cannot be stored for a long time before conversion and it will probably result in a final product that most certainly is not itself resistant to oxidation processes. On the other hand, high contents in FFA (thus resulting in high acidity index) are inconvenient for base-catalyzed transesterification processes, since the catalyst is consumed in a saponification reaction and higher amounts of base are needed, lowering biodiesel yield beside emulsifying the final product hindering glycerol separation.[9]

2.2 Methods

2.2.1 Materials

Cherimoya seeds were provided by AGRIPÉROLA – Cooperativa Agrícola, C.R.L. (Funchal, Portugal). Hexane (95%) and Acetonitrile (LC-MaScan) were purchased from LAB-SCAN Analytical Sciences (Dublin, Ireland), methanol (99.8%), sodium chloride (analytical reagent), sodium hydroxide pellets (analytical reagent) and ethanol absolute (UV-IR-HPLC) from PANREAC (Barcelona, Spain), hydrochloric acid (37%) and diethyl ether

(99.5%) from Riedel-de Haën (Seelze, Germany), propan-2-ol (HPLC Gradient grade) from Fisher Scientific (Loughborough, UK), hexane (for HPLC) from Acros Organics (New Jersey, USA), potassium hydroxide (analytical reagent) and anhydrous magnesium sulphate (analytical reagent) from Merck (Darmstadt, Germany), boron trifluoride methanol complex from BDH (Poole, England), glyceryl trilaureate (≈99%), glyceryl tripalmitate (≈99%) and glyceryl trioeleate (>99%) from Sigma (Steinheim, Germany) and glyceryl tristearate (>99%) from Fluka (Steinheim, Germany).

2.2.2 Oil extraction and characterization

2.2.2.1 Extraction procedures

Dry seeds were powdered in an IKA® Werke (Staufen, Germany) MF10 basic mill. Powdered cherimoya seeds (400 g) were added to 4 L of hexane and stirred for 72 h at room temperature (≈25 ºC). In parallel, an extraction at solvent boiling temperature (6 h) was carried out for comparison. Both hexane extracts were then filtered and evaporated in a rotary evaporator to eliminate solvent.

2.2.2.2 Determination of the FFA content by titrimetry

The acidity index of fresh oil was calculated according to ISO 660 standard method.[10]

2.2.2.3 Oil resistance to oxidation

FT-IR spectrum of the recently extracted oil was registered in a Mattson (Madison, WI, USA) Infinity Series FT-IR using KBr cells, accumulating 32 scans from 4000 cm^{-1} to 600 cm^{-1}, with a scan resolution of 4 cm^{-1}. Fresh oil (10 g) was placed in a covered Petri dish and kept at 70 ºC. FT-IR spectra were registered every 24 h as described before.

Induction period and stability curves were determined according to EN1411 procedures in a Metrohm (Herisau, Switzerland) Rancimat® model 743 equipment.[11]

2.2.2.4 Determination of FA composition

FA were converted to FAME and determined according to the analytical methods described in regulations EEC/2568/91 and EEC/1429/92 of the European Union Commission for olive oil.[12,13]

2.2.2.5 FAME analysis

FAME composition was determined by CG-MS,[12,13] using a Varian (Walnut Creek, CA, USA) Star 3400 Cx Series II gas chromatograph equipped with Varian (Walnut Creek, CA, USA) Saturn III mass selective detector and Saturn GC-MS workstation software. A J&W (Rancho Cordova, CA, USA) DB-wax (30 m) column with 0.25 mm (i.d.) and 0.25 μm thickness coating film was used. Operating conditions: injector temperature – 240 ºC; initial temperature (column oven) – 70 ºC; heating ramp – 10 ºC.min^{-1} to 180 ºC, 10 minutes at 180 ºC, 10 ºC.min^{-1} to 220 ºC and 10 minutes at 220 ºC. The ion trap detector was set as follows: transfer line temperature 220 ºC; manifold and trap temperatures 180 ºC; mass range m/z 35-350; emission current 15 mA. The electron multiplier was set in the relative mode to the auto tune procedures. All mass spectra were acquired in the electron impact mode (E_i = 70 eV; source temperature, 180 ºC). The sample injection volume was 1 μL.

2.2.2.6 Evaluation of the lipidic composition by ^{13}C-NMR

The oil, without any treatment, was dissolved in $CDCl_3$ and analyzed by ^{13}C-NMR. Spectra were recorded on a Bruker (Rheinstetten, Germany) AVANCE 400 II+ operating at 100.61 MHz, equipped with a 5 mm BBO probe. Chemical shifts (δ) are all referred to internal tetramethylsilane. The spectra were recorded at room temperature with 2 s relaxation decay, a 45º

excitation pulse, 2.36 s acquisition time, a sweep total with of 24,038 Hz and 32K acquisition points to yield a digital resolution of 0.212 Hz/point.[14]

2.2.2.7 Determination of TAG structure by HPLC-APCI-MS

TAG composition was determined using a Dionex (Germering, Germany) UltiMate 3000 series chromatograph equipped with a Phenomenex Gemini C18 column (250 × 3.0 mm, 5 μm particle size) and a Bruker (Bremen, Germany) Esquire 6000 mass detector, using positive-mode atmospheric pressure chemical ionization (APCI) with an ion-trap mass analyser. The samples and column were kept at 30 ºC, and a gradient elution was effected by changing the mobile phase composition from acetonitrile/ethanol (90:10) to 18% acetonitrile in 90 min. The mobile phase flow was 0.7 mL.min^{-1}. 3% and 0.5% (w/v) solutions of the oil and standards (respectively) dissolved in acetonitrile/propan-2-ol/hexane (2:2:1) were prepared and 20 μL was injected.[15]

2.2.3 Biodiesel production and characterization

2.2.3.1 Biodiesel production procedure

Freshly obtained oil (50 mL) was heated to 60 ºC in a round bottom flask. Sodium hydroxide (0.175 g) was dissolved in 175 mL of methanol and added to the oil. Reaction was kept under reflux (55 – 60 ºC) for 1 hour. The reaction mixture was then transferred to a separatory funnel and left to cool down to room temperature. At this point two phases were observed. Biodiesel formed was washed with distilled water (15% of biodiesel's volume) and the aqueous phase was discarded. The biodiesel was then washed with 0.5% aqueous HCl (5% of biodiesel's initial volume) and again with water (10% of the initial volume of biodiesel) to remove soaps, discarding the aqueous phases in both steps. Finally, obtained biodiesel was dried with anhydrous magnesium sulfate, filtered and excess water was evaporated in a rotary evaporator.

2.2.3.2 Biodiesel characteristics

The following parameters were determined: density (picnometer method), kinematic viscosity (Ubbelohde capillary viscometer), iodine number (gas chromatography), acid value (titrimetry), cold filter plugging point (CFPP), cloud point, oxidation stability (Rancimat® method) and calculated "apparent cetane number" (Klopfenstein's equation).

Density was measured at 15 ºC following the procedures indicated on UNE-EN 14214 and kinematic viscosity was measured at 40 ºC, according to EN ISO 3104 standard method.[16,17] Iodine number was obtained by gas chromatography, as indicated by UNE-EN 14214;[17] GC-MS conditions were the same as described for FAME analysis. The procedure for calculating the acid value is the same as indicated for the oil's acidity index.[10] CFPP was determined according to EN 116 standard method and cloud point was determined according to ASTM D 2500 standard method.[18,19] Oxidation stability procedure was the same as previously described for the oil.

For each individual methyl ester "apparent cetane number" ($I_{cetane,ME}$) can be estimated by using Klopfenstein's equation (Eq. 2-1).[20] "Apparent cetane number" can then be predicted using Equation 2-2 as the average of the product of each individual methylester contribution by its percentage in the mixture.[21]

$$\boldsymbol{I_{cetane,ME} = 58.1 + 2.8 \times \frac{(n-8)}{2} - 15.9 \times DB} \qquad \textbf{(Equation 2-1)}$$

$$\boldsymbol{I_{cetane} = \sum X_{ME} \times I_{cetane,ME}} \qquad \textbf{(Equation 2-2)}$$

where n is the number of carbons in the acyl chain, DB the number of double bonds and X_{ME} the weight percentage of individual methyl ester.

2.3 Results and discussion

The oil content of *Annona cherimola* seeds was found to be 25.1% (in terms of neutral lipids), obtained by extraction with n-hexane at room temperature, while extraction at boiling temperature afforded only 22.4% of

neutral lipids. These quantities are far below canola but above soybean oils, some usual sources of vegetable oil for biodiesel production.[22]

2.3.1 Oil characterization

The acidity number for the freshly extracted oil was 2.2 mg KOH.g^{-1}. This value is consistent with other raw vegetable oils suited for biodiesel production. Yet this value should be reduced to values below 1.0 mg KOH.g^{-1} prior to alkali catalyzed transesterification. The most widely used method to reduce FFA content is the acid catalyzed pre-esterification.[23]

The FT-IR spectrum of freshly obtained oil (Fig. 2-1a) shows a weak band at 3474 cm^{-1}, due to an overtone band of carbonyl group stretching.[24] The bands at 3008 cm^{-1}, 1653 cm^{-1} and 917 cm^{-1}, indicate the presence of cis unsaturated fatty acids and are due respectively to C–H, C=C stretching and C=C deformation (out-of-plane) in olefin groups.[25,26]

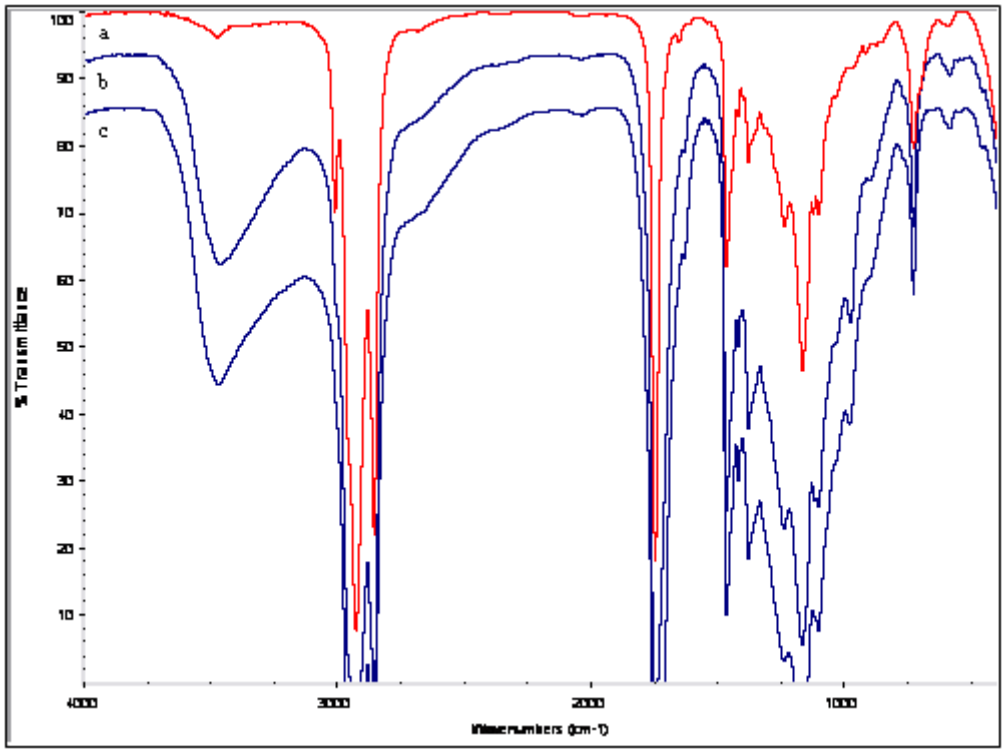

Figure 2-1 FT-IR spectra of **a** freshly obtained oil and after **b** 8 days and **c** 12 days under oxidative conditions

The high degree of unsaturation of *Annona cherimola* seed oil makes it susceptible to oxidation. Although the traditional methods for establishing

the oxidative state of oil and fats are chemical methods based on the measurement of the concentration of the main products generated in the process, such as peroxide value, anisidine value and iodine value, we present some observations based on infrared spectroscopy, since good correlations can be established between this and the traditional processes. FT-IR methods are increasingly being accepted for the evaluation and monitoring of oxidation processes in transformer and motor oils as well as vegetable oils, in replacement of the costly, labor and time consuming chemical methods. These spectrometric methods combine speed of analysis, high sensitivity, precision and reproducibility, and require minimum amount of sample.[27-29]

The comparison of the IR spectra of the oil initially and after 8 and 12 days under oxidative conditions (Fig. 2-1a, b and c) show some changes that indicate the oxidative process. At approximately 3500 cm^{-1}, an intense broad band appears after 8 days, indicating the presence of hydroperoxides. This band gets even more intense after 12 days. The disappearance, after 8 days, of the peak at approximately 3000 cm^{-1} means that the double bonds are fully oxidized. The band corresponding to carbonyl stretching at 1746 cm^{-1} has a drift to 1743 cm^{-1} after 12 days. This indicates the formation of FFA, as oxidation product of triglycerides. Finally, the disappearance of the band at 1119 cm^{-1} means that the degree of saturation rises, since the intensity of this band is inversely proportional to the degree of saturation.[25,26] A new band is observed at 986 cm^{-1}; this is absent from the spectrum of the freshly obtained oil and appears during the oxidation process. This band has been associated with bending vibrations of CH trans-trans-conjugated unsaturated fatty acids.

The standard test method to determine oxidation stability for biodiesel products is the Rancimat® method. The test was conducted for comparison with the previous results and the induction period for cherimoya seed oil was 3.96 hours.

These results indicate that the oil should not be kept stored for a long period, thus avoiding oxidation and consequently the increase of the FFA content, which will decrease the yield when converting the oil into biodiesel.

2.3.2 FA composition

Five major peaks were found in the GC-MS chromatogram of FAME. The peaks were identified respectively as C16:0 (RT = 22.36 min; M+ = 270 m/z), C18:0 (RT = 28.48 min; M+ = 298 m/z), C18:1 (RT = 29.21 min; M+ = 296 m/z), C18:2 (RT = 30.44 min; M+ = 294 m/z) and C18:3 (RT = 32.19 min; M+ = 292 m/z), and their relative abundance is presented in Table 2-1.

When compared with some leading sources of biodiesel (Table 2-1) cherimoya seed oil has a higher content in palmitic acid, almost equal parts of oleic and linoleic acids (much different of soybean, rapeseed and sunflower oils) and a low content in linolenic acid.[30,31] This composition indicates that cherimoya seed oil is a potential biodiesel source and as stable to oxidation as soybean and sunflower oils.

Table 2-1 FA composition of cherimoya seed oil and other vegetable oils used as biodiesel sources (%)

	Cherimoya seed oil	Soybean oil[a]	Rapeseed oil[a]	Sunflower oil[a]
Palmitic acid (C16:0)	19.99	11.75	3.49	6.08
Stearic acid (C18:0)	4.16	3.15	0.85	3.26
Oleic acid (C18:1)	38.58	23.26	64.40	16.93
Linoleic acid (C18:2)	35.97	55.53	22.30	73.73
Linolenic acid (C18:3)	1.31	6.31	8.23	0.00

[a]Ma and Hanna[31]

2.3.3 ^{13}C-NMR analysis

The spectrum of cherimoya seed oil (Fig. 2-2) shows mainly the characteristic signals of TAG. 1,3-DAG signals are of very low intensity (so low that the signals of the glyceride carbons at ca. 65 ppm cannot be integrated) and 1,2-DAG (signals in the glyceride carbon region at ca. 72 ppm) and FFA (carbonyl resonance at ca. 176 ppm) are detectable as traces.[8]

Although ^{13}C-NMR can provide essential structural information about TAG, concerning the structure of the acyl groups and their distribution in the glycerol backbone, it was used in this study mainly to confirm that cherimoya seed oil contained essentially TAG.

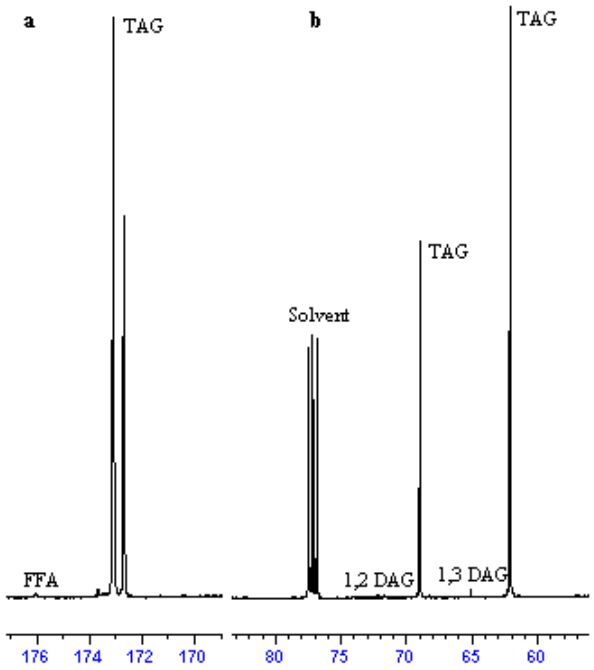

Figure 2-2 ^{13}C-NMR spectrum (100.61 MHz) of **a** the carbonyl carbons and **b** the glycerol carbons of cherimoya seed oil

2.3.4 TAG structure

Eleven peaks, affording seventeen TAG, were identified in the HPLC-APCI-MS chromatogram of the oil (Fig. 2-3). TAG elution in RP-HPLC is affected by both the combined number of carbon atoms in the acyl chain (ACN – acyl carbon number) and the number of double bonds, n, in the molecule; as some critical groups (TAG with the same equivalent carbon number – ECN) are usually difficult to separate, some peaks were a result of a co-eluted mixture of up to three TAG.[32]

The identification of the molecular species was mainly based on the mass spectral data, the $[M+H]^+$ ion provided molecular weight (MW) information, whereas the information on the FA residues was given by the DAG fragment ions ($[M-RCOOH]^+$ ion). Positional identification of the FA in the

glycerol moiety were possible taking into account some considerations:[14] seed oils normally have PUFA in the sn-2 position and the differences between sn-1 and sn-3 are very small, although less abundant FA tend to appear in the sn-3 position; the position of the FA in the glycerol backbone affects the elution order, with unsaturated FA in the sn-2 position causing the TAG to elute before the TAG with the same FA in the sn-1 or sn-3 positions; positional isomers can be identified from the relative intensities of the DAG fragment ions, as the least abundant DAG fragment ion is due to the loss of the FA from the sn-2 position. This last consideration is quite useful to differentiate between mixed symmetric and mixed asymmetric TAG (ABA and AAB type of TAG) because the AA/AB ratio of the DAG fragment ions is extremely different, being lower than 1 when B is in the sn-2 position.

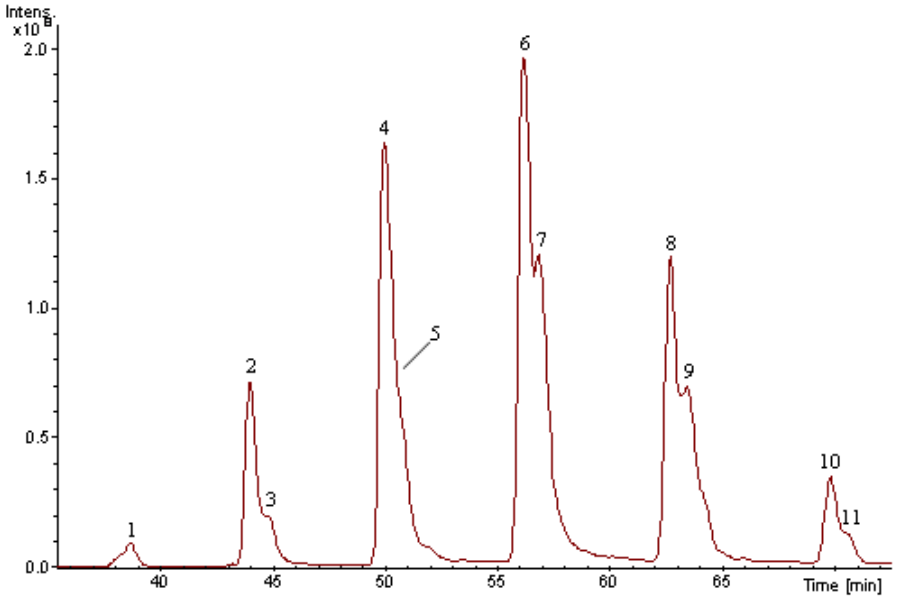

Figure 2-3 Reconstructed ion chromatogram of TAG from cherimoya seed oil achieved by HPLC–APCI–MS

The suggested FA combinations (Table 2-2) show that the four main types of TAG were distinguished: AAA, monoacid type (LLL and OOO); AAB, mixed asymmetric type (PLL, OLL, OOL, LLS and OOS); ABA, mixed symmetric type (LLnL, LOL, OLO, OPO and OSO); ABC mixed asymmetric type (OLnL, PLO, OLS, OLE and POS).

Table 2-2 Molecular species identification of the TAG of Annona seed oil

Peak #	RT (min)	ECN	TAG	$[M+H]^+$	DAG	$[M-R_1COO]^+$	DAG	$[M-R_2COO]^+$	DAG	$[M-R_3COO]^+$
1	38.5	40	LLnL	877.8	LnL	597.5	LL	599.6		
2	43.9	42	LLL	879.8	LL	599.6				
3	44.7	42	OLnL	879.9	LnL	597.6	OL	601.6	OLn	599.6
4	49.8	44	LOL	881.9	OL	601.6	LL	599.5		
5	50.6	44	PLL	855.9	LL	599.6			PL	575.6
			OLL	881.9	LL	599.6			OL	601.6
6	56.0	46	OLO	883.9	OL	601.6	OO	603.6		
7	56.8	46	PLO	857.9	LO	601.6	PO	577.6	PL	575.6
			OOL	883.9	OL	601.6			OO	603.6
			LLS		SL	603.6			LL	599.6
8	62.6	48	OOO	885.9	OO	603.6				
9	63.1	48	OPO	859.8	PO	577.6	OO	603.6		
			OLS	885.9	LS	603.6	OS	605.6	OL	601.6
10	69.7	50	OSO	887.9	OS	605.6	OO	603.6		
			OLA	913.9	LA	631.7	OA	633.7	OL	601.6
11	70.5	50	POS	861.9	OS	605.6	PS	579.6	PO	577.5
			OOS	887.9	OS	605.6			OO	603.6

*L, linoleic; Ln, linolenic; O, oleic; P, palmitic; S, stearic; A, arachidic; ECN, equivalent carbon number; RT, retention time

2.3.5 Conversion of the oil into biodiesel and its properties

The amount of FAME obtained by this method was 75.88 g / 100 g of seed oil.

EN 14214 establishes the requirements for biodiesel quality.[17] The parameters determined and calculated for biodiesel quality are shown in Table 2-3.

Table 2-3 Properties of the biodiesel produced

	Biodiesel	EN 14214 limits
Density, 15 °C (g.cm^{-3})	0.871	0.860-0.900
Viscosity, 40 °C (cSt)	4.4	3.5-5.0
Acid value (mg KOH.g^{-1})	0.3	< 0.5
Iodine number	99[a]	< 120
Oxidation stability, 110 °C (h)	1.17	> 6
CFPP (°C)	-5	-
Cloud point (°C)	1	-
"Apparent cetane number"	53[a]	> 51

[a]calculated

Cetane number is related to the ignition of the fuel and a low cetane number indicates that the combustion is not complete. Consequently, part of the fuel remains in the combustion chamber and it will produce more energy than needed when burned, wearing off the engine. As the equations used to predict cetane number are not appropriate to biodiesel, some correlations were developed for methyl esters.[20] A fuel with high "apparent cetane number" (no. 2 diesel fuel has a cetane number of 46) is better for the cold start of the engine, allows a quick warming of the engine and reduces noise and gas emissions to the atmosphere.[33] Biodiesel produced from cherimoya seed oil has a calculated "apparent cetane number" of 53 (EN 14214 establishes a minimum value of 51).[17]

The iodine value evaluates the number of double bonds, quantifying the unsaturation degree of the fuel, indicating its tendency to oxidation. The iodine number of the biodiesel produced was below the EN 14214 upper limit.[17]

Acidity value determined was also below the EN 14214 upper limit, indicating low quantity of FFA in the produced biodiesel.[17]

FAME composition of the biodiesel produced is very similar to the FAME composition of the oil (Table 2-1), thus indicating that the method for biodiesel production does not promote isomerization or hydrogenation. The predominance of unsaturated FAME makes the biodiesel susceptible to oxidation, but it also balances the viscosity of the fuel, allowing an optimal flow throughout the system. Due to the higher content of saturated fatty acids (when compared to other oils commonly used for the production of biodiesel) it would be expected that FAME derived from *A. cherimola* oil would have worse low temperature properties. The results show that these properties are in fact similar to those exhibited by biodiesels produced from soybean or rapeseed oils.[34]

2.4 Conclusions

Oil extraction procedures demonstrated that percolation at room temperature yields more oil than at solvent boiling temperature (25.1% vs. 22.4%). Although the energy balance favors room temperature, it must be taken into count that time is also a very important parameter.

The lipid content of *Annona cherimola* Mill. seeds is suitable to make this a promising source of biodiesel, as confirmed by the evaluated parameters of the methyl esters. The high degree of unsaturation (all TAG include oleic and/or linoleic acids and FAME analysis confirmed the predominance of these FA) makes the biodiesel produced from this oil susceptible to oxidation, but it also improves (reduces) its viscosity. The low oxidation stability can be overcome by introducing additives into the final product.

Biodiesel production can be a contribution to avoid waste disposal. However, this is a seasonable crop and it must be conjugated with other sources of biodiesel, such as waste cooking oil, another residue with high economic value.

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CHAPTER 3

OPTIMIZATION OF OIL EXTRACTION AND PRODUCTS CHARACTERIZATION

Summary

In this chapter, the extraction of oil from cherimoya seeds was studied using three different extraction methods, namely accelerated solvent extraction, Soxhlet extraction and room temperature extraction. Furthermore, extraction yields using analytical grade hexane and commercial hexane are compared. The maximum extraction yield was 28.4 g of oil/ 100 g of dried feedstock.

No significant differences were observed for the compositions of the extracted seed cakes, and the obtained oils were chemically very similar, with oleic and linoleic acids as the main fatty acids (in almost equal parts), followed by palmitic, stearic and linolenic acids, respectively.

3.1 Introduction

Cherimoya seeds are the residues of industrial processing (*e.g.* alcoholic beverages production) and of rejected fruits at the production and handling sites. They can account for approximately 3% of the fruit weight and contains a very significant amount of oil. Since the seeds are known to be an important source for acetogenins, a class of alkaloids exhibiting a broad range biological activities (*e.g.* cytotoxicity),[1-4] the oil extracted from them cannot be used as an edible oil and, therefore, its potential for biodiesel production has been evaluated.[5]

The reference method for oil extraction is using a Soxhlet apparatus with analytical grade *n*-hexane.[6] The most common industrial methods are mechanical pressing (*e.g.* extra virgin olive oil) and solvent extraction (*e.g.* olive "pomace" oil). In the former, the oil bearing material (with or without a preheating step in indirectly heated conditioners) is fed in one end of a cylinder where a power-driven worm conveyor forces the material to the other end of the cylinder and out against resistance. The pressure exerted in the process squeezes out the oil. After pressing, some oil is left in the oily material and, therefore, this is an expensive and inefficient method. Solvent extraction is considered cheaper and more efficient than mechanical pressing, being the most common method used. The oily material is treated in a multistage counter current process with solvent until the remaining oil content is reduced to the lowest possible level. The mixture of oil and solvent is separated by distillation and the solvent is recycled into the extraction process.[7]

Hexane is typically used for solvent extraction of seed oil, but benzene and petroleum ether are also employed. In any case the used solvents are of lower purity, when compared to the reference method.

Thus, it is important to acknowledge that the extraction at industrial level will yield different liquid and solid streams, that have to be studied in detail, as these will be the real industrial materials.

The recovered solvent /oil mixture is called "miscella" and the extracted biomass is usually known as "cake" ("pomace" in olive oil extraction) and is mostly used as fuel and as cattle feed.[8,9] Since cherimoya seeds are considered as toxic, the cake resulting from oil extraction cannot be used for animal feed, and other applications, preferably of more added value, must be found. One option is its use within the biorefinery framework. Its potential for these applications is mainly determined by its chemical composition, which must be thoroughly evaluated.

3.2 Methods

3.2.1 Materials

Cherimoya seeds were taken from fresh fruits, washed and dried in an oven at 35 ºC to constant weight. Seeds were roughly ground with a blender before extraction.

Commercial grade hexane was gently provided by UCASUL – União de Cooperativas Agrícolas do Sul, U.C.R:L (Alvito, Portugal). Analytical grade *n*-hexane (>95%) was purchased from Carlo Erba (Rodano, Italy), sulfuric acid (95-97%), D-(+)-xylose (≥99%), levulinic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma-Aldrich (Steinheim, Germany), D-(+)-glucose (>99.5%) was purchased from Duchefa Biochemie (Haarlem, The Netherlands), formic acid (98%), potassium sulfate (≥99%) and titanium(IV) oxide (99.0-100.5%) were purchased from PANREAC (Barcelona, Spain), boric acid (≥99.8%) and methyl red were purchased from Riedel-de Haën (Seelze, Germany), L-(+)-arabinose (≥99%), acetic acid (>99.8%), copper(II) sulfate (≥99%), hydrochloric acid (37%), stearic acid (≥97%), methylene blue and anhydrous magnesium sulfate were purchased from Merck (Darmstadt, Germany) and sodium hydroxide was purchased from Eka (Bohus, Sweden).

3.2.2 Oil extraction procedures

Three extraction methods and two solvent purities were tested: ASE (exclusively with analytical grade *n*-hexane), Soxhlet extraction and room temperature (shake flask) extraction (RTE).

3.2.2.1 Accelerated solvent extraction (ASE)

In 100 mL ASE cells, 30 g of ground seeds and anhydrous magnesium sulfate (on the cell edges to complete the cell's volume) were inserted, with cellulose frits separating the layers. Three cycles were performed with a static time of 10 minutes, a flush volume of 100% and a purge time of 1 minute, using a 100 mL cell. Three different extraction temperatures were tested: 40 ºC, 80 ºC and 105 ºC. The solvent was evaporated in a rotary evaporator. All extractions were performed in duplicate.

3.2.2.2 Soxhlet extraction

A 125 mL extractor was coupled to a 250 mL round bottom flask and 10 g of ground seeds were placed inside an extraction thimble, inside the extractor. Either analytical grade or commercial grade hexane were added in a liquid-to-solid ratio (LSR) of 15 $mL.g^{-1}$. Four extraction times were tested – 10 min (1 cycle), 1 h, 2 h and 4 h – with analytical grade *n*-hexane. With commercial grade hexane 1, 3, 4 and 6 h were tested. The solvent was evaporated in a rotary evaporator. All extractions were performed in duplicate.

3.2.2.3 Room temperature extraction (RTE)

In 500 mL Schott flasks, 25 g of crushed seeds and 250 mL of hexane, both commercial and analytical grade, were mixed (LSR = 10 $mL.g^{-1}$). An Infors HT (Bottmingen, Switzerland) orbital shaker was used at 150 rpm. Extraction times were 6 h, 24 h, 48 h, 72 h and 120 h for analytical grade *n*-hexane and 24 h, 48 h and 72 h for commercial hexane. The solid was filtered and the solvent was evaporated in a rotary evaporator. All extractions were performed in duplicate.

3.2.3 Analytical assays

3.2.3.1 Cake analysis

The seed cakes corresponding to the best extraction yields were selected for analysis. All solid samples were milled with an IKA® Werke (Staufen, Germany) MF10 basic mill and screened to retain the fraction under 0.5 mm. Ash content was evaluated according to the corresponding National Renewable Energy Laboratory (NREL) protocol.[10] Solid residues were chemically characterized (in terms of structural polysaccharides and lignin) by quantitative acid hydrolysis followed by HPLC analysis using a Bio-Rad (Hercules, CA, USA) Aminex HPX-87H column, according to the NREL protocols.[11-13] Protein content was determined according to the Kjehdal method, adapted from the ISO 8968 standard, using the general conversion factor.[14]

3.2.3.1.1 Ash determination

Porcelain crucibles were dried in a muffle furnace at 550 ºC for at least 5 h and weighted after cooling in a desiccator. The solid samples (2 g) were placed in the tared crucibles and dried to constant weight in an oven at 105 ºC for moisture quantification. The dried samples were then burned inside the crucibles with a heating plate, transferred to the muffle furnace and kept at 550 ºC to constant weight. Determinations were performed in duplicate.

3.2.3.1.2 Quantitative acid hydrolysis

Sulfuric acid (72% w/w, 5 mL) was added to test tubes containing 0.5 g of solid sample. The reaction mixture was kept for 1 h at 30 ºC in a Memmert (Schwabach, Germany) W350 water bath, with occasional manual stirring. The content of the tubes was then transferred to 250 mL Schott flasks, diluted with water to a H_2SO_4 concentration of 4% (w/w) and put to react at 121 ºC for 1 h inside an autoclave. After cooling down, the mixture was

filtered through previously dried (in a muffle furnace) and weighted sintered glass crucibles (#3 porosity). The remaining solid (corresponding to Klason lignin) was dried in an oven at 105 ºC to constant weight and then burned in a muffle furnace for ash correction.

The monosaccharides (glucose, xylose and arabinose), aliphatic acids (formic, acetic and levulinic), and furan derivatives (HMF and furfural) present in the liquid fraction were quantified by high-performance liquid chromatography (HPLC) using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Hercules, CA) in an Agilent 1100 series HPLC system (Santa Clara, CA, USA) equipped with a refractive index detector (G1362A) controlled at 35 °C and a diode array detector (G1315B). The mobile phase was H2SO4 5 mM, the column temperature 50 °C, and the flow rate 0.6 mL.min^{-1}. The system was equipped with a Micro-Guard Cation-H Refill Cartridge from Bio-Rad before the HPX-87H column. Injection volume was 5 μL. All samples were filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters prior to analysis.

3.2.3.1.3 Quantification of structural polysaccharides and lignin in solid samples

The moisture content (H, %) of the samples was calculated using the following expression:

$$\boldsymbol{H = \frac{wet\ sample\ weight\ (g) - dry\ sample\ weight\ (g)}{wet\ sample\ weight\ (g)} \times 100} \qquad \textbf{(Equation 3-1)}$$

The ash content (Ash, %) of the samples was calculated using the following expression:

$$Ash = \frac{Ash\ weight\ (g)}{dry\ sample\ weight\ (g)} \times 100 \qquad \textbf{(Equation 3-2)}$$

The concentrations of Glc (glucose), Xyl (xylose), Ara (arabinose) and HAc (acetic acid) in the liquors resulting from the quantitative acid hydrolysis of raw materials and solid wastes were used for the calculation of glucan (Gn), xylan (Xn), arabinan (Arn) and acetyl groups (Ac) content (%), respectively.

The acid-insoluble residue, after correction for the ash content, was quantified as Klason lignin (KL). During the quantitative acid hydrolysis, a significant percentage of the monosaccharides is degraded, so correction factors are introduced to account for the losses.

$$Gn = F_1 \times \frac{100}{1005} \times \frac{162}{180} \times \frac{Glc \times W_{sol}}{DW}$$ **(Equation 3-3)**

$$Xn = F_2 \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Xyl \times W_{sol}}{DW}$$ **(Equation 3-4)**

$$Arn = F_3 \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Ara \times W_{sol}}{DW}$$ **(Equation 3-5)**

$$Ac = \frac{100}{1005} \times \frac{60}{61} \times \frac{HAc \times W_{sol}}{DW}$$ **(Equation 3-6)**

$$KL = \frac{AIS - Ash}{DW} \times 100$$ **(Equation 3-7)**

Where,

Gn, Xn, Arn, Ac and KL are the concentrations of glucan, xylan, arabinan, acetyl groups and Klason lignin (g per 100 g of dry solid) respectively;

Glc, Xyl, Ara and HAc are the concentrations of glucose, xylose, arabinose, and acetic acid in liquors ($g.L^{-1}$), respectively;

The terms $\frac{162}{180}, \frac{132}{150}, \frac{60}{61}$ are stoichiometric conversion factors of monomers into polysaccharides;

F_1, F_2 and F_3 are correction factors accounting for sugar degradation (usually 1.027, 1.096 and 1.049, respectively);[15]

W_{sol} and DW are the weights of the solution and dried sample used in the test, respectively (g);

AIS and Ash are the weight of the acid-insoluble residue of the sample and its ash content, respectively (g).

3.2.3.1.4 Protein content

Samples (0.5 g) were placed inside digestion tubes and 20 mL of H_2SO_4 and 10 g of the catalyzing mixture (93% potassium sulfate, 3% copper sulfate, 3% titanium oxide and 1% stearic acid – w/w) were added. The tubes were placed in a Tecator (Höganäs, Sweden) Digestion System 6 Model 1007 unit, capped with a fumes extraction system. After 30 min the temperature was raised to 420 ºC and kept for 1.5 h. After cooling, 100 mL of water was added and the tubes were placed in a Tecator (Höganäs, Sweden) Kjeltec System Model 1026 distillation unit, which automatically added 50% (w/V) NaOH (75 mL). The released ammonia was distilled by steam distillation and collected using 4% (w/V) boric acid (50 mL). Ammonia quantitation was determined by acid-base titration using 0.1 N hydrochloric acid as titrant and Tashiro's indicator (end point at pH 4.8). The indicator was prepared by mixing 2 g of methyl red and 1 g of methylene blue in ethanol to a final volume of 100 mL. A blank analysis was also performed.

The protein amount is calculated using the following expression:

$$Crude\ Protein\ (\%) = \frac{(V_2 - V_1)}{W_{samp}} \times 0.14 \times 6.25 \qquad \textbf{(Equation 3-8)}$$

Where,

V_1 and V_2 are the titrant volumes spent on the blank assay and on sample analysis, respectively, and W_{samp} is the sample weight.

3.2.3.2 Oil composition

Oil composition was evaluated by gas chromatography (GC) after transesterification,[16] using a Varian (Walnut Creek, CA, USA) 3000 gas chromatograph equipped with a flame ionization detector (FID). A Supelco (Bellefonte, PA, USA) Supelcowax (30 m) column with 0.32 mm (internal diameter – i.d.) and 0.32 μm thickness coating film was used. Operating conditions: injector temperature – 280 ºC; initial temperature (column oven)

– 175 ºC (kept for the initial 5 min); heating ramp – 2.5 ºC.min^{-1} to 235 ºC and 20 minutes at 235 ºC; detector temperature – 300 ºC; column pressure – 12 psi. A split ratio of 160:1 was used. The carrier gas was helium (N45, Air Liquide) with a 1.5 cm^3.min^{-1} flow, as well as the make-up gas (at a 20 cm^3.min^{-1} flow). Hydrogen (N50, Air Liquide) and air (K, Air Liquide) at 300 cm^3.min^{-1} were used to fuel the flame. Methyl esters were identified by comparison to a standard mixture. Peak areas were integrated automatically by a Varian (Walnut Creek, CA, USA) 4290 integrator and expressed as percentages of the corrected total response. Selected samples (corresponding to the best yields obtained for RTE and Soxhlet extractions) were methylated in duplicate and each duplicate was injected twice.

3.3 Results and discussion

3.3.1 Soxhlet extraction *vs* RTE *vs* ASE

The extraction results obtained for the different methods are presented in figure 3-1. Soxhlet extraction is less time consuming than the RTE method. After 6 hours the extraction yield is close to the highest yields obtained for RTE. RTE reached a plateau after 48 hours of extraction, thus indicating that extending that time is unnecessary. Even though there are studies regarding the application of ASE to oil extraction,[17] this technique has not proven to be efficient, in the tested conditions. Maximum oil extraction (>28%) was obtained for RTE after 120 hours. However, the yield gain (<0.5%) does not justify extending the extraction beyond 48 h.

Although RTE affords the maximum quantity of oil, reaching "saturation" after 48 h (indicating that it is not necessary to further extend the extraction time), this method is very slow. Even though Soxhlet extraction is considered a slow method, in 1/8 of the time the yield obtained is 95% of the higher quantity of oil obtained by RTE. Considering the usual batch processes used in industry, this less time consuming process seems to be

preferable. However, energy balances should be performed in order to determinate the most economically viable method.

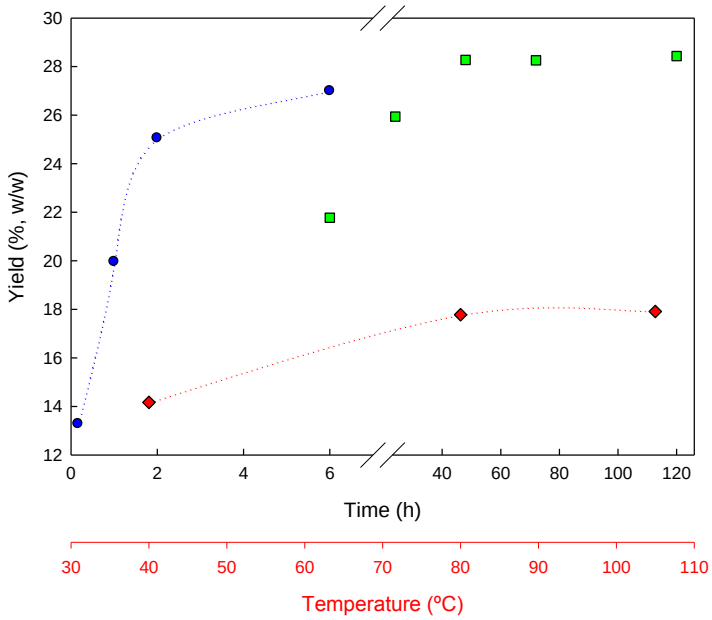

Figure 3-1 Extraction yields obtained for the three methods tested (● Soxhlet Extraction, ■ RTE, ◆ ASE)

3.3.2 Commercial *vs* analytical grade hexane

Hexane grade (commercial hexane is typically used in industrial processes), as expected, affects the extraction yield. Nevertheless, RTE is less sensitive to solvent purity than Soxhlet extraction. The best oil recovery using commercial grade hexane (>27%) was obtained for RTE after 72 hours (Fig. 3-2a). The yield loss in RTE was only 4%, whilst in Soxhlet the oil recovery was 68% of the maximum yield achieved (*ca.* 27%) with analytical grade *n*-hexane (Fig. 3-2b).

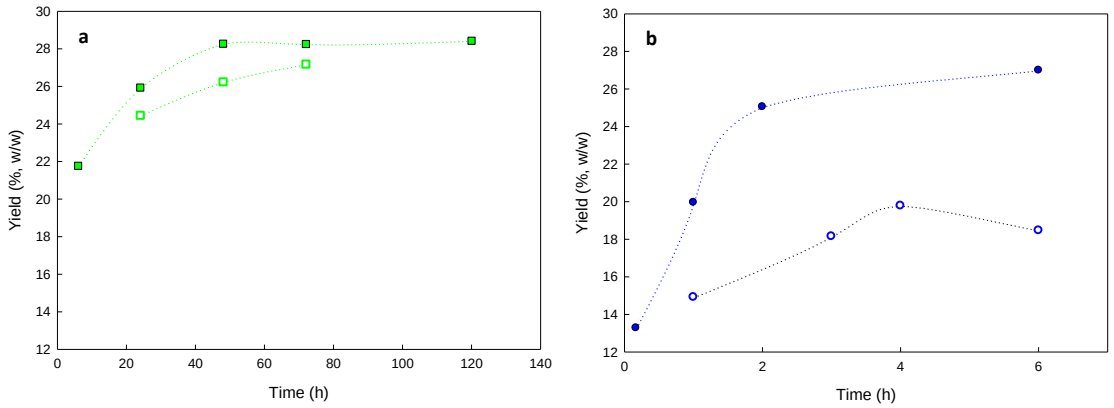

Figure 3-2 Comparison between commercial (open symbols) and pro analysis (solid symbols) hexane for **a** RTE and **b** Soxhlet extractions

3.3.3 Oil composition

The compositional analysis of the oils where the maximum yield was obtained (48 h for RTE – 28.3% yield– and 6 h for Soxhlet extraction – 27.0% yield–, both with analytical grade *n*-hexane), and the corresponding oils obtained in the same conditions but with commercial hexane, did not present any significant differences (Table 3-1). Oleic and linoleic acids were the major components, in almost equal parts, followed by palmitic acid. Besides the five major FA, other minor fatty acids were identified. All oils presented trace amounts (below 0.5%) of arachidic (C20:0) and eicosenoic (C20:1) acids. Palmitoleic acid (C16:1) was also found in one of the tested samples (*ca.* 0,1%).

Table 3-1 Percentages of the major fatty acids (FA) present in the selected oils

	RTE (48h)		Soxhlet (6h)	
	pro analysis	**commercial**	***pro analysis***	**commercial**
Palmitic acid (C16:0)	15.17±1.51	16.65±0.41	15.96±0.39	16.65±0.29
Stearic acid (C18:0)	3.86±0.14	3.70±0.24	3.77±0.04	3.62±0.04
Oleic acid (C18:1)	39.52±0.77	38.40±0.38	39.15±0.25	38.27±0.07
Linoleic acid (C18:2)	39.16±0.40	38.83±0.11	38.83±0.16	39.14±0.01
Linolenic acid (C18:3)	1.80±0.01	1.87±0.07	1.81±0.08	1.85±0.01

3.3.4 Solid residues analysis

The composition of the solid residues did not differ significantly. All solids contained (w/w, dry basis) 21.1-21.7% cellulose (measured as glucan), 20.5-21.4% hemicellulose, 25.3-26.1% Klason lignin, 22.1-23.5% protein, 2.3-2.9% ash and 4.0 to 8.5% of extractives and others (calculated by difference).

This composition is in line with other oily seed "cakes" and several other lignocellulosic materials. In comparison to rapeseed "cake", it has lower holocellulose content (mostly due to a lower hemicellulose content), the basis for lignocellulosic sugars production.[18] It has higher protein content than

the reported cereal straws (e.g. wheat and rice), reaching values close to the content of BSG, which can be an advantageous trait, namely for the production of fermentation media.[19]

3.4 Conclusions

Cherimoya seeds present a very significant quantity of lipids that can easily be recovered using hexane. The amount of oil recovered after extraction is solvent purity- and method-dependent. On the other hand, oil composition does not seem to be affected by the choice of the method or by solvent's purity, yielding oil (mainly composed of oleic and linoleic acids) and a solid residue of lignocellulosic nature.

The "cake" obtained after oil extraction has a suitable composition for further processing. Like other lignocellulosic materials, its polysaccharide content is apparently suitable for recovery.

Both these streams can be upgraded within the biorefinery framework.

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CHAPTER 4

AUTOHYDROLYSIS OF *ANNONA CHERIMOLA* MILL. SEEDS: OPTIMIZATION, MODELING AND PRODUCTS CHARACTERIZATION

Summary

In this chapter, the selective hemicelluloses removal by autohydrolysis was optimized aiming to maximize the yield of oligosaccharides with potential applications in food, pharmaceutical and cosmetic industries. A maximum of 10.4 $g.L^{-1}$ of oligosaccharides was obtained.

The process kinetics is presented, modelled (based on the Arrhenius equation) and its scale-up is discussed. The hydrolyzate shelf-life was evaluated and the produced oligosaccharides are stable at room temperature for, at least, 3 weeks. Furthermore, all oligosaccharides are also stable at 100 ºC for 1 h, in pH values between 1 and 11, enabling their industrial processing, and at 37 ºC for 3 h, in pH values between 1 and 3, thus indicating its potential classification as non-digestible oligosaccharides. The remaining cellulose enriched solids presented an increased enzymatic digestibility (as a function of the autohydrolysis severity) that assures its efficient use in subsequent processes (e.g. bioethanol production).

4.1 Introduction

Annona cherimola Mill. seeds are the main residue of the industrial processing of cherimoya. Currently it has no significant use being considered a waste disposable problem.

The seeds have a significant oil content (up to 30%) that can be used for biodiesel production.[1] Moreover, *Annonaceae* are well spread all over the globe and cherimoya, being one of the most important species of this family, can maybe be considered a model for other *Annonaceae*.

Oil extraction yields a lignocellulosic fraction, the seed cake, which must be further upgraded. The presence of xyloglucans in the seeds of another *Annona* species, namely *A. muricata*, has been reported,[2] and hence the presence of these compounds is also expected in the lignocellulosic fraction of cherimoya seeds. This is a strong indication that this residue can be used to produce novel xylo-oligosaccharides (XOS), thus assuring a potential valorization of cherimoya seeds within the biorefinery. In fact, oligosaccharides' (OS) demand has increased significantly and, thus, the search for new OS, and specifically of novel XOS sources is currently a relevant endeavor, as it can lead to novel products with applications in food, pharmaceutical and cosmetic industries.[3,4]

The autohydrolysis process is considered to be the most appropriate choice for the selective separation of hemicelluloses and the maximization of the OS production.[3,4] This process has many significant advantages e.g. reduced production of monosaccharides,[5] but the optimal operation conditions depend significantly from the composition of the feedstock.

There are several tools to help to better understand and control the behavior of the autohydrolysis process,[6] namely the use of the reaction ordinate (R_O – Equation 4-1) and formal kinetic models. The severity factor, log R_O, was developed to compare steam explosion and autohydrolysis treatments of

lignocellulosic materials between different conditions and equipment,[7,8] and is given by the following equation, for non-isothermal conditions:

$$R_0 = \int_0^t exp\left(\frac{T(t)-T_{ref}}{\omega}\right) \cdot dt \qquad \textbf{(Equation 4-1)}$$

where T(t) is the temperature as a function of time, T_{ref} is the temperature up to which the hydrolysis process is considered to be negligible (usually 100 ºC) and ω is a term that can be related to a conventional energy of activation, typically 14.75.[8,9]

Nevertheless, the application of the severity factor can be subjected to criticism, as it is observed that dissimilar responses may be obtained for similar severity factors, even if the operational conditions (e.g. heating/cooling profiles, liquid to solid ratio, target - maximum attained-temperature), are not that diverse. Therefore, formal kinetic models are usually preferable, as the severity factor does not give any mechanistic insight.[8]

The determination of the kinetic parameters is a powerful tool for scale-up procedures, either to the pilot and demonstration levels or to a later commercial phase,[10] but the rigorous kinetic modeling of acid catalyzed hemicelluloses' degradation is very complex. Several constraints do exist, like the uneven production and diffusion of the hydronium ions through the heterogeneous and complex polymeric matrix, the occurrence of side reactions with other components of the matrix (e.g. lignin), the limited access of the H_3O^+ ions to the glycosidic bonds due to steric hindrance, or the surface area availability and accessibility of the substrate during the reaction,[6,11] that make the system difficult to model in detail. Therefore, simplifications have to be made to overcome these constraints. The main assumption is that polysaccharide degradation (and also the subsequent reactions) is irreversible and follow a first order kinetic. It is also considered that the system is pseudo-homogenous. Furthermore, the kinetics is assumed to follow the Arrhenius equation (Equation 4-2):

$$k = Ae^{-\frac{E_a}{RT}}$$ **(Equation 4-2)**

where A is the pre-exponential factor, E_a the activation energy, R the ideal gas constant and T the temperature. Therefore, the determination of the Arrhenius parameters requires extensive experimental data to fit Equation 4-2, namely, the study of the process kinetics at diverse temperatures, what is typically laborious, expensive and time consuming, and clearly an alternative, more efficient, approach is required.

In order to maximize OS yield a series of non-isothermal autohydrolysis was carried out, varying the maximum temperature of the treatment. The obtained results were successfully fitted to a kinetic model, aiming to predict the behavior of the process. Also, bearing in mind a zero waste goal, it is important to characterize both the liquid and solid fractions resulting from the autohydrolysis process. Therefore, expedite methods for evaluating OS stability were applied and the enzymatic digestibility of the cellulose-enriched solid fractions were evaluated.

4.2 Methods

4.2.1 Materials

Seeds were provided frozen by J. Faria & Filhos Lda. (Funchal, Portugal). Upon collection seeds were washed and dried to constant weight. After drying, the seeds were stored at room temperature until further processing. The seeds were ground using a simple blender and its oil extracted with commercial hexane. Seeds were then dried in an oven, further milled with an IKA® Werke (Staufen, Germany) MF10 basic mill and screened to retain the fraction under 1.5 mm.

Sulfuric acid (95-97%), D-(+)-xylose (≥99%), levulinic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma-Aldrich (Steinheim, Germany), hydrochloric acid (37%), citric acid monohydrate (99.5-100.5%), L-(+)-arabinose (≥99%), acetic acid (>99.8%)

and potassium chloride (>99.5%) were purchased from Merck (Darmstadt, Germany), D-(+)-glucose (>99.5%) was purchased from Duchefa Biochemie (Haarlem, The Netherlands), formic acid (98%) was purchased from PANREAC (Barcelona, Spain), sodium hydrogen phosphate heptahydrate (99.5-100.5%) was purchased from Riedel-de Haën (Seelze, Germany), Celluclast® 1.5L and Novozyme 188 were purchased from Novozymes (Bagsvaerd, Denmark) and sodium hydroxide was purchased from eka (Bohus, Sweden).

4.2.2 Autohydrolysis treatments

Experiments were carried out using a Parr Instruments Company (Moline, IL, USA) T316SS two liter steel reactor heated externaly. Two turbines of four blades each agitated the mixture and cold water, through an internal serpentine, cooled the system when necessary. Temperature and agitation were monitored and controlled with a Parr Instruments Company (Moline, IL, USA) model 4842 PID controller. Agitation was set to 150 rpm. Pressure was measured by the same controller (Figure 4-1).

Figure 4-1 Apparatus used for the autohydrolysis treatments

In each treatment milled seeds and water were mixed in a liquid-to-solid ratio (LSR) of 5 (w/w) to a total mass of 1.2 kg. The experiments were conducted under non-isothermal conditions to reach target temperatures

ranging from 150 to 230 ºC. Heating profiles were obtained for all treatments. After a rapid cooling, (typically less than two minutes to reach temperatures below 100 ºC), liquid and solid phases were separated by pressing (up to 200 bar) with a Sotel (Portugal) manual oil press. The solid residues were washed with 500 mL of distilled water and pressed once again.

For comparison purposes, the severity of the treatments was estimated by calculating the log R_O (equation 4-1) based on the measured temperature profiles data.

4.2.3 Scanning electron microscopy

Samples were sputter-coated with gold/palladium and observed using different magnifications with a Philips XL30 FEG Scanning Electron Microscope (Eindhoven, The Netherlands) at 10kV.

Both the cherimoya seed cake and the solid residue obtained after autohydrolysis at 190 ºC were observed.

4.2.4 Mathematical modeling

As the temperature is time dependent, no analytical solution is possible to be obtained and the system has to be solved numerically. Numerical integration was carried out by implementing Euler's method in Microsoft Excel® 2010. The Microsoft Excel® 2010 Solver tool was used to estimate the kinetic model parameters that fit the experimental data, by minimizing the sum of square deviations. An increment (Δt) of 0.012 minutes was used.[12,13]

4.2.5 Analytical procedures

Both the raw material and the solid residues obtained after autohydrolysis were characterized according to standard NREL protocols and ISO standards as described in section 3.2.3.[14-18]

The liquid fractions were also characterized using standard NREL protocols.[18,19] Sulfuric acid was added to 100 mL of the liquid fraction to a final concentration of 4% (w/w). The samples were hydrolyzed in an autoclave for 1 h at 121 ºC. After cooling the hydrolyzates were filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters before HPLC analysis, as previously described. This procedure was performed at least in duplicate. Oligosaccharides' concentration was calculated from the increase in sugar monomers, before and after acid post-hydrolysis.

4.2.6 Stability of the liquid fraction and OS

To establish the shelf life of the hydrolysate obtained in optimal conditions, 500 mL were filtered through Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters to Schott flasks (under non-sterile conditions) and kept in a dry place at room temperature away from light. At defined intervals a sample was taken, hydrolyzed and analyzed according to NREL standard protocol, as previously described in section 4.2.5.[18]

To evaluate the OS' stability to pH and temperature the hydrolyzate was filtered through Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters and evaporated to dryness in a rotary evaporator (Büchi, Switzerland) at 35 ºC (down to 0.015 bar) to recover OS. The evaporated sample was dissolved to obtain concentrations of 30 $g.L^{-1}$, using diverse buffer solutions (Table 4-1).

Table 4-1 Composition of the buffers used for the evaluation

pH	Solution A	Solution B	$V_{Sol\ A}$ (mL)	$V_{Sol\ B}$ (mL)	V_{Total} (mL)
1*,[a]	0.2 M KCl	0.2 M HCl	50.0	97.0	200.0
2*,[a]	0.2 M KCl	0.2 M HCl	50.0	10.6	200.0
3[a]	0.1 M citric acid	0.2 M Na_2HPO_4	39.8	10,2	100.0
7[a]	0.1 M citric acid	0.2 M Na_2HPO_4	6.5	43.6	100.,0
11*,[b]	0.05 M Na_2HPO_4	0.1 M NaOH	100.0	8.2	108.2

[a]Gomori;[17] [b]Robinson and Stokes;[18] *these are not buffer solutions but will be considered as so, similarly to what is described in the literature

The solutions were thermostatically kept for 3 h at 37 ºC (at pH 1 - 3, away from sunlight, simulating human digestion) and 1 h at 100 ºC (simulating cooking procedures) in an Memmert (Schwabach, Germany) WNB 22 oil bath and then analyzed by HPLC (Aminex HPX-87H column) as previously described.[18]

4.2.7 Enzymatic digestibility of the remaining solid

Enzymatic digestibility was evaluated according to NREL standard protocol.[22]

The reaction mixture contained 0.15 g of frozen biomass (dry weight basis), 5 mL of sodium citrate buffer (0.1 M, pH 4.8), 100 μL of sodium azide solution (2% w/v), as an anti-microbial agent, and Celluclast® 1.5L and Novozyme 188 enzymes in prescribed amounts to obtain 60 FPU/g and 64 pNPGU/g of dry biomass, respectively. The total volume was adjusted to 10 mL with water.

Each biomass sample was hydrolyzed in duplicate. A biomass blank (without enzymes) and an enzyme blank (without biomass) were also carried out. The blanks were used to correct the results to any free saccharides present in the biomass and eventual products inherent to the reaction conditions or to determine the amount of glucose present in the enzymes.

The hydrolysis was carried out in a Comecta (Barcelona, Spain) 200D orbital shaker at 50 ºC and 250 rpm for 72 h. After this period, the samples were immersed in a water bath at 90 ºC for 5 min in order to inactivate the enzymes. The samples were then filtered through Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters and analysed by HPLC (Aminex Aminex HPX-87H column) as previously described.[18]

4.3 Results and discussion

4.3.1 Autohydrolysis liquors and solid residues composition

In order to understand the behavior of the autohydrolysis of cherimoya seed cake, the process was carried out under non-isothermal conditions and the composition of liquid and solid phases was evaluated for all experiments.

On the liquid phase, OS concentration reached 10.4 g.L^{-1} at the optimal experimental condition, 190 ºC, log R_O = 3.60 (Fig. 4-2). Monosaccharides formation follows a similar pattern and their maximum yield is achieved at the same severity. For more severe conditions sugars degradation becomes predominant over sugar production from oligosaccharides. In fact, degradation products (DP) – namely furans and formic acid – formation is clearly the main reaction for high severity conditions. Acetic acid formation is a consequence of acetyl groups' removal from the hemicellulose moiety and, therefore, it is correlated with the data presented in table 4-2 regarding residual solids composition.

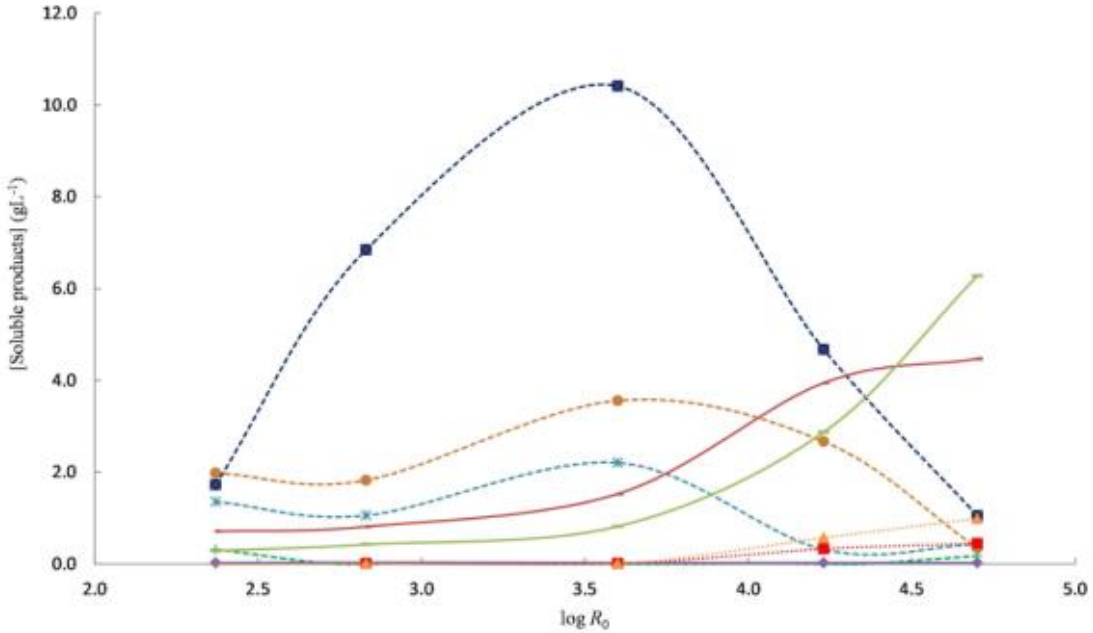

Figure 4-2 Soluble products' concentrations (g.L^{-1}) in the hydrolyzates as a function of log R_O. ■ OS,* Glucose, ● Xylose, + Arabinose, - Acetic acid, ¯ Formic acid, ♦ Levulinic acid, ■ HMF and ▲ Furfural (lines are used for eye guidance only)

The severity condition leading to the highest production of oligosaccharides

(log R_O = 3.60) is lower than for other lignocellulosic materials,[10,12,23] that typically present values close or above 4, thus indicating that this material is less recalcitrant to hydrolysis and it will require milder/economical operational conditions at industrial level.

The concentrations of soluble compounds, namely monosaccharides and degradation compounds, such as 5-hydroxymethylfurfural (HMF) and furfural, do not impose excessive purification problems. So it will be possible to easily purify OS *e.g.* using a membrane technology based process as formerly described,[24] or the simpler evaporative concentration process (see below).

Table 4-2 Seed cake composition (g/100g dry matter), and solid yield (%) and solid residues composition (g/100g dry matter) obtained in the autohydrolysis assays of cherimoya seeds

Target temperature (ºC)		**150**	**170**	**190**	**210**	**230**
	log R_O	**2.37**	**2.83**	**3.60**	**4.23**	**4.70**
	Solid Yield	96.01	83.12	78.04	58.61	59.17
Seed cake						
Glucan	22.05	24.39	24.83	33.78	39.68	38.31
Xylan	14.17	12.56	12.34	5.03	0.00	0.00
Arabinan	3.61	2.09	2.10	0.00	0.00	0.00
Acetyl groups	2.15	2.13	2.15	1.49	0.00	0.00
Klason Lignin	25.63	38.41	42.92	58.50	60.98	61.81
Ash	2.84	0.92	0.84	0.45	0.39	0.38
Others	29.53	19.49	14.82	0.76	0.00	0.00

Table 4-2 contains the data obtained for the solid yield and the composition of the solid residues after autohydrolysis. As expected, the solid yield diminished with the severity. This trend is justified by hemicellulose removal and also by the removal of extractives, namely inorganics and other soluble compounds that decreased significantly already at mild severities, what is an advantageous trait if their recovery is required. Regarding the most noteworthy fraction, hemicellulose, its removal reaches 74.5% for the optimal condition for XOS production and comparing to the seed cake composition, is completely removed for the higher temperature treatments. Conversely, and as a consequence of these removals, the relative amounts of

glucan and lignin in the solid residues increased with the severity of the treatment. A complete recovery of glucan was observed, but lignin recovery typically exceeds 100%. This behavior has already been reported for several other lignocellulosic materials e.g. [25] and may be related to binding reactions, for instance between protein and lignin.[26,27]

These results showed that after autohydrolysis, the solid phases were enriched in glucan and, putatively, lignin (and protein), clearly indicating the process' high selectivity for hemicellulose removal and the solids' possible suitability for further processing e.g. lactic acid or ethanol production through simultaneous saccharification and fermentation. In fact, this is advantageous for subsequent cellulose hydrolysis, as it limits cellulase adsorption.[27] Furthermore, the presence of protein in the cellulose enriched solids can also be advantageous for fermentation (as a substitute/complement nitrogen source). Nevertheless, if deem relevant, it is also possible to recover protein from hydrolyzed materials.[28]

The assumptions above are confirmed by Scanning Electron Microscopy (SEM) imaging (Fig. 4-3). The lignocellulosic matrix, intact in figure 4-3a, is partly removed after autohydrolysis (Fig. 4-3b) and some cellulose fibers can be distinguished along with re-condensed lignin droplets. The removal of hemicellulose, revealing the cellulose fibers, is evident in less detailed photomicrographs (Fig 4-3c). The appearance of lignin droplets on the surface of plant cell walls has been attributed to lignin melting at high temperature and pressure, migration to the surface and subsequent condensation.[29-32] These lignin droplets are more clearly observed in higher magnifications (Fig. 4-3d).

4.3.2 Mathematical modeling of the autohydrolysis process

The hydrolysis of lignocellulosic materials can be described by an interdependent sequence of chemical reactions. Based on these reactions it is possible to establish a model which describes the kinetics of the autohydrolysis process, using a system of differential equations based on a

mass balance among the species involved.

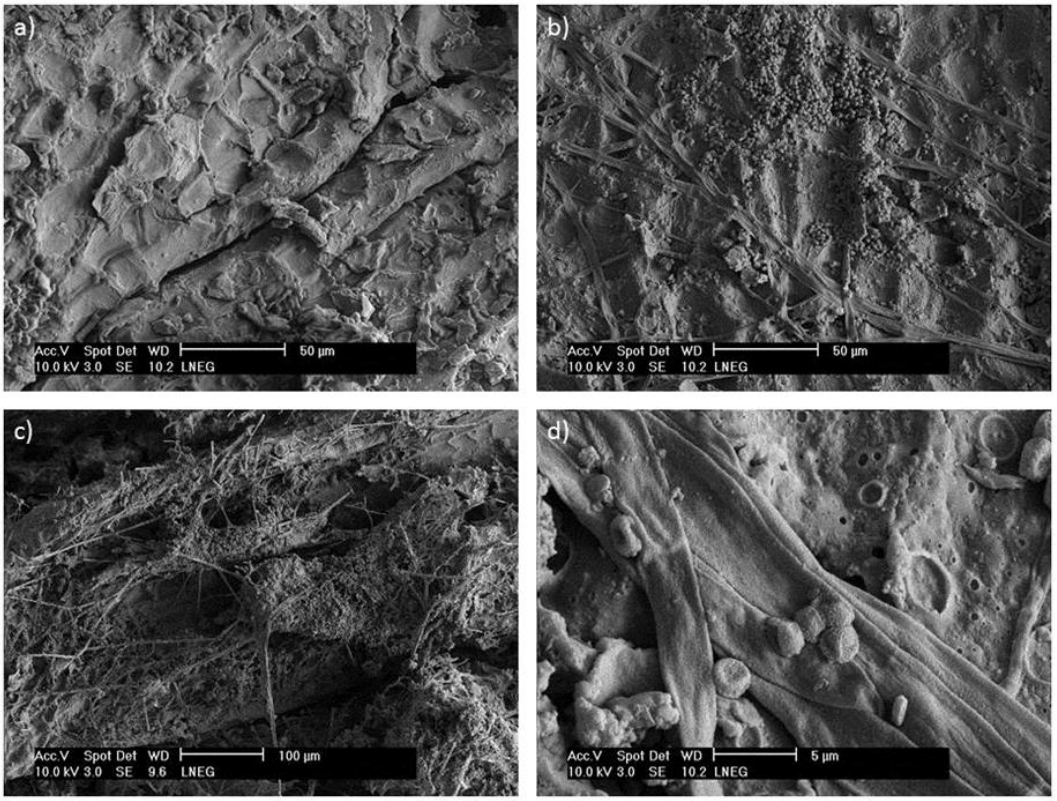

Figure 4-3 SEM photomicrographs a) before and b, c and d) after autohydrolysis at 190 ºC

Several specific kinetic models have been reported for the hydrolysis of the hemicellulosic fraction of raw materials.[6,12,33] In this work it is assumed that the OS are produced from the hemicellulose fraction, further hydrolyzed into monomers that are then degraded into diverse products, according to the following path (Fig. 4-4):

$$\text{Hemicellulose} \xrightarrow{k_1} \text{OS} \xrightarrow{k_2} \text{Monosaccharides} \xrightarrow{k_3} \text{Degradation products}$$

Figure 4-4 Pathway of *Ac*Ms' hemicellulose conversion during the autohydrolysis process

Nevertheless, the rigorous kinetic modeling to study hemicellulose autohydrolysis is a complex problem, especially if the model has to be adapted to describe hemicellulose hydrolysis under non-isothermal conditions and simplifications are assumed, as described in section 4-1. Specifically, in this chapter we assume that the kinetic constants (k1 to k3) dependence on temperature do follows Arrhenius' equation, all

hemicellulose is hydrolysable and for OS, only one fraction was considered, independently of their molecular weight.

As such, the hemicellulose degradation can be mathematically translated into the following equation:

$$\frac{dHemicel}{dt} = -k_1 \times Hemicel \qquad \textbf{(Equation 4-3)}$$

Taking in consideration the Arrhenius equation (Equation 4-2), equation 4-3 can then be rewritten as

$$\frac{dHemicel}{dt} = -A_1 \times e^{\left(-\frac{Ea_1}{RT(t)}\right)} \times Hemicel \qquad \textbf{(Equation 4-4)}$$

As the reaction takes place under non-isothermal conditions, and the cooling time is considered to be negligible as compared to the heating time, the temperature profile as a function of time was empirically described by a linear trend, fitting the experimental data for the heating period (Fig 4-5). Hence, the T(t) term can be substituted by the expression T = αt+ß, where α represents the heating rate (2.4 K.min^{-1}) and ß the temperature at the beginning of the treatment (*ca.* 298 K):

$$\frac{dHemicel}{dt} = -A_1 \times e^{\left(-\frac{Ea_1}{R(\alpha t+\beta)}\right)} \times Hemicel \qquad \textbf{(Equation 4-5)}$$

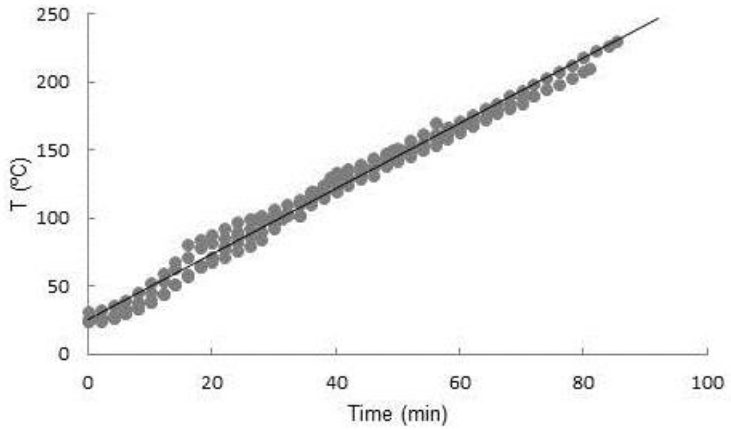

Figure 4-5 Heating profiles of the autohydrolysis treatments

In the same way, the following reaction step (Fig. 4-3), can be described by:

$$\frac{\mathrm{dOS}}{\mathrm{dt}} = \mathrm{A_1} \times \mathrm{e}^{\left(-\frac{\mathrm{Ea_1}}{\mathrm{R}(\alpha t+\beta)}\right)} \times \text{Hemicel-}\mathrm{A_2} \times \mathrm{e}^{\left(-\frac{\mathrm{Ea_2}}{\mathrm{R}(\alpha t+\beta)}\right)} \times \mathrm{OS} \qquad \textbf{(Equation 4-6)}$$

In a similar way, it is possible to develop the equations for monosaccharides and DP formation, equations 4-7 and 4-8, respectively:

$$\frac{dMonos}{dt} = A_2 \times e^{\left(-\frac{Ea_2}{R(\alpha t+\beta)}\right)} \times OS - A_3 \times e^{\left(-\frac{Ea_3}{R(\alpha t+\beta)}\right)} \times Monos \qquad \textbf{(Equation 4-7)}$$

$$\frac{dDP}{dt} = A_3 \times e^{\left(-\frac{Ea_3}{R(\alpha t+\beta)}\right)} \times Monos \qquad \textbf{(Equation 4-8)}$$

Due to the non-isothermal nature of the system, the system's analytical integration is not trivial, contrary to what happens in models for isothermal conditions.[25] Therefore, the system of non-linear equations that contains only six parameters (the Arrhenius parameters for each of the three reactions) has to be solved numerically and fitted to the full set of experimental data points. Based on the feedstock composition, corresponding to t=0, the molar concentrations of hemicellulose, OS, monosaccharides and DP can be calculated for $t=t_i$. For example, hemicellulose concentration at $t=t_i$ can be calculated according to the following expression:

$$Hemicel_{t_i} = Hemicel_{t_{i-1}} - Hemicel_{t_{i-1}} \times A_1 \times e^{\left(-\frac{Ea_1}{RT(t)}\right)} \times \Delta t \qquad \textbf{(Equation 4-9)}$$

The accuracy of these calculations is greater the smaller the increment Δt implemented in Microsoft Excel® 2007. The values determined for the pre-exponential factor ($\ln A_n$) and activation energies (Ea_n) for the three steps of the pathway proposed in figure 4-4 are shown in table 4-3.

Table 4-3 Determined parameters for the proposed model for the autohydrolysis of cherimoya seeds – pre-exponential factor ($\ln A_n$) [A_n, h^{-1}] and activation energies (Ea_n) [$kJmol^{-1}$]

	Ea_n	$\ln A_n$
k1	58.83	17.05
k2	25.53	8.39
k3	56.36	16.48

The reliability of the proposed model is assured by the good agreement between experimental results and model predictions (Figure 4-5). Furthermore, these results are in the order of magnitude as compared to data obtained for other residues,[11,33,34] but present lower levels than the reported values for more recalcitrant lignocellulosic materials, that justify the milder conditions found for optimal cherimoya seeds hydrolysis. In fact, the comparison based on the kinetic modeling, due to taking into account the composition of the materials and temperature influence more effectively, is a more useful tool than severity parameters for the comparison between different biomass feedstock.

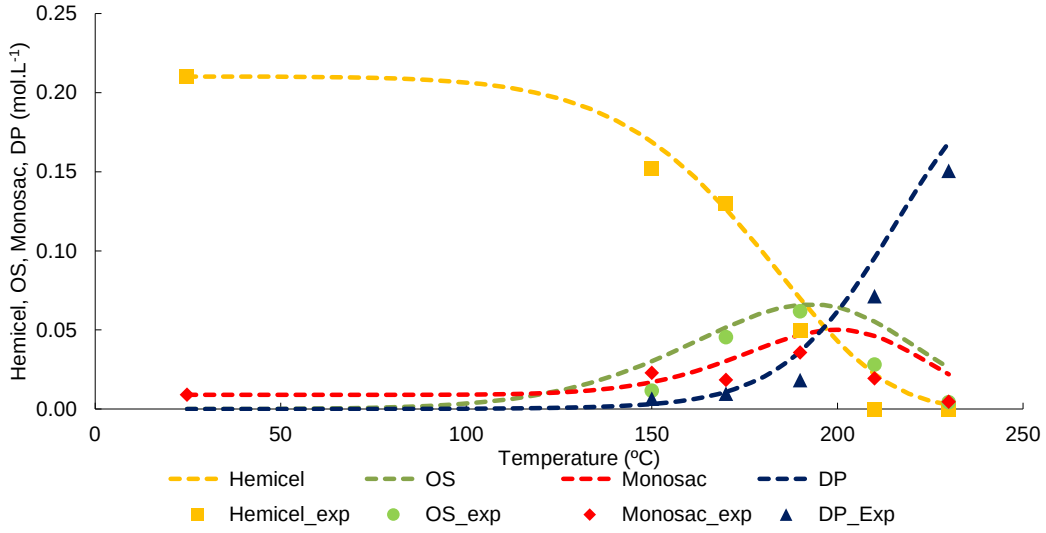

Figure 4-6 Experimental and calculated time courses for the non-isothermal autohydrolysis of cherimoya seed cake

It is important to stress that autohydrolysis modeling based on data obtained from experiments carried out under isothermal conditions would require far more extensive datasets than the ones presented in this work. These results confirm that the use of non-isothermal conditions is an effective experimental approach to support the kinetic modeling of the autohydrolysis process.

4.3.3 Hydrolyzate's shelf life evaluation

Considering a possible industrial application, the capability of the oligosaccharide-rich hydrolyzates to be stored for long times at room temperature was evaluated.

During the 3 weeks of the test, gluco-oligosaccharides' (GlcOS) and xylo-oligosaccharides' (XOS) concentrations presented no changes (Table 4-4). Also, no microbial growth was observed, even when, deliberately, no sterilization was made to avoid microbial contamination. The low pH value of the hydrolysate and the significant amounts of furfural and acetic acid, reported inhibitory for microbial growth,[35-37] may explain the microbial stability of the hydrolysate. Furthermore, the mild conditions during storage (as compared to the production conditions) may also justify the hydrolysate's chemical stability. As this is the first report regarding the evaluation of the storage stability, no data is available for comparison.

Table 4-4 Evolution of the OS' concentrations during the shelf life test

	Week			
	0	**1**	**2**	**3**
GlcOS ($g.L^{-1}$)	2.5	2.5	2.5	2.5
XOS ($g.L^{-1}$)	5.0	5.0	5.0	5.0

4.3.4 OS' stability

OS are mainly used in the food sector as prebiotic agents. For that, their source must be as free as possible from microbial growth inhibitors, such as furans and aliphatic acids. In this work this was easily achieved by using a simple evaporative concentration step that allowed a significant removal of furfural and acetic acid, without OS degradation.

Among the several OS with high dietary and economic value, the non-digestible oligosaccharides (NDOS) are of great importance as they can reach the intestine and be used to feed the bacteria in the gut flora, acting like prebiotic agents.[3,4] In this work two preliminary evaluative simple

screening tests are used to assess if the produced and partially purified OS are potential NDOS. These tests evaluate the stability at 100 ºC to appraise the possibility of the OS to be processed per se or in combination with foodstuffs and the stability at digestive pH and temperature conditions as a first indication that they will be able to pass the gastrointestinal tract to reach the large bowel.[38]

The stability profiles of both GlcOS and XOS at 100 ºC for 1 h as a function of the pH is shown on Figure 4-7. Both GlcOS and XOS are quite stable in the whole tested pH range (1.5% of XOS and 11.5% of GlcOS were degraded at pH 1). Moreover, XOS are highly stable at pH values between 2 and 11, as expected, since XOS are described to be more stable at 100 ºC than GlcOS.[38] This is a particularly interesting result as OS obtained from wheat bran or chicory have been reported not to be this stable at pH values of 3 and 11, mainly those derived from chicory.[38,39] The produced OS are thus deemed suitable for applications in the food industries and likely to be processed.

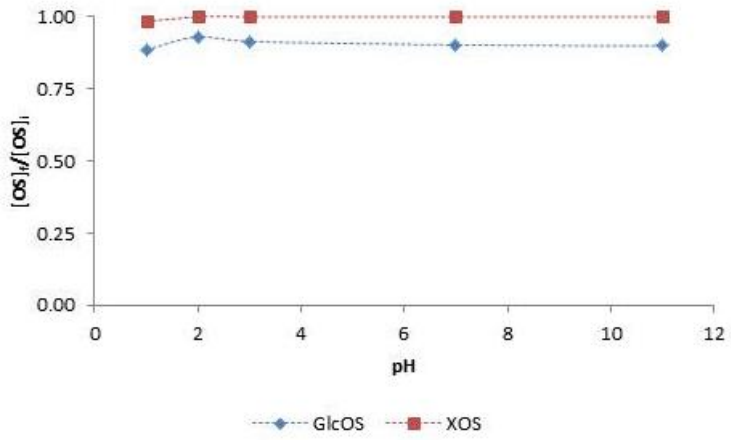

Figure 4-7 Stability profiles of the produced OS at 100 ºC (lines are for eye guidance only)

Consequently, these OS need to be evaluated according to the pH conditions of the human stomach. The digestive process in the stomach occurs at 37 ºC for about 3 hours and pH values between 1 and 3. The produced XOS are highly resistant to this simulation of the digestive process (Figure 4-8), as no degradation was observed, and only 8% degradation was observed for GlcOS. This is an expected result, according to the reported stability for

other similar compounds at 25, 37 and 50 ºC at pH values of 1 and 2 and a first assurance that their potential nutritional properties may be retained during digestion.[39,40]

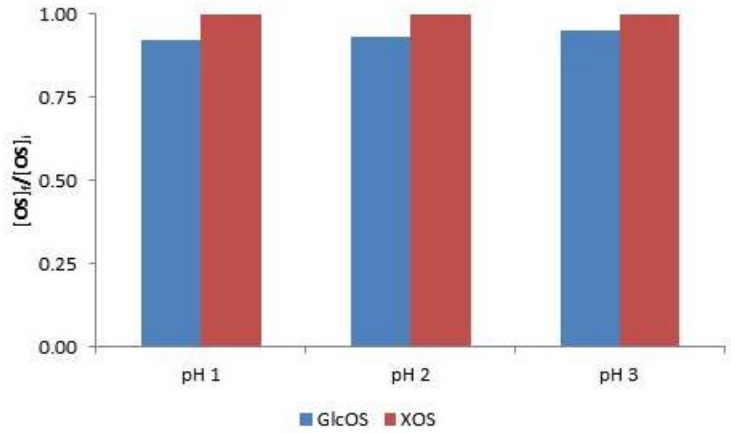

Figure 4-8 Stability of the produced OS in conditions similar to the human digestion process

In fact, the ability of these OS to be processed and the possibly to pass the stomach undigested makes them potential NDOS, but this must be study further, as this screening test is only a preliminary step before more complex (and expensive) in vivo testing to definitely classify these OS as prebiotic agents.

4.3.5 Enzymatic digestibility of the solid residues

After the autohydrolysis treatment the remaining biomass must also be valorized. In this sense, the lignin- (protein) and cellulose-rich solids were treated enzymatically to saccharify cellulose.

The enzymatic digestibility is clearly higher for the more severe treatments. Glucose concentration increased 2.3 times (Figure 4-9a) from the least severe treatment to the higher concentration obtained (4.77 $g.L^{-1}$) and the highest yield (Figure 4-9b) was 83% (1.6x higher than the yield obtained for the least severe treatment). Here it is important to stress that, as the amount of enzyme was kept constant, due to the glucose enrichment of the solids, less enzyme per glucan is present, which indicates that the digestibility is even higher, and that this value will translate into a significant glucose concentration when used under typical solid loadings

reported in literature, e.g. 20%. In fact, the reported digestibility is in line with values reported for similar pretreatment and enzymatic hydrolysis conditions for other materials, e.g. it is significantly higher than the glucose yield reported for eucalyptus residues (54%), and close to 82% for olive tree pruning, and only slightly lower for straws (88, 90 and 91 %, respectively for rice, corn straw and wheat straw),[41-43] what may be explained by the higher lignin content of the cherimola seed cake as compared to straws. Although, it is reported that the highest glucose yield can only be achieved after the complete removal of hemicellulose, lignin also plays a role on preventing cellulose hydrolysis that must not be neglected.[27]

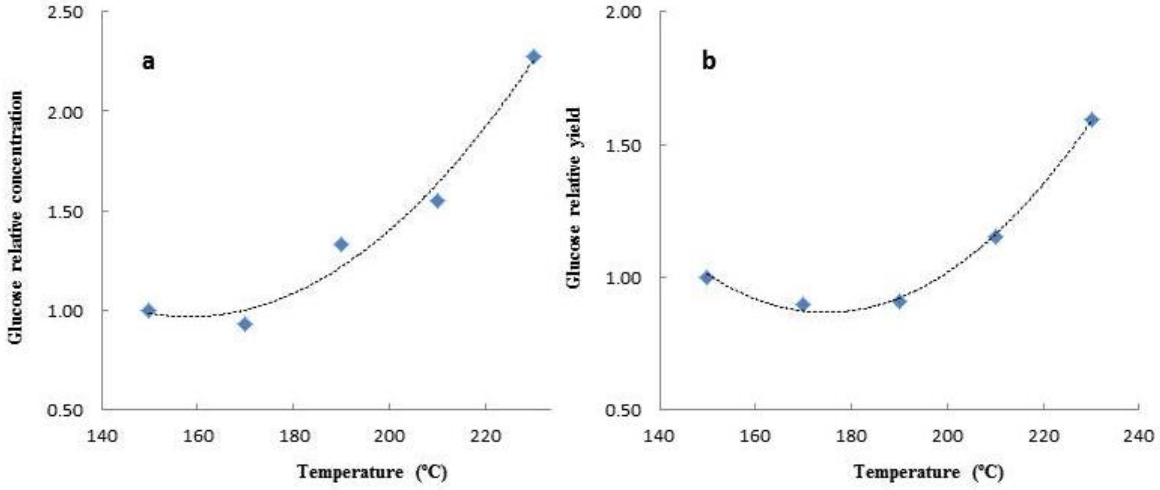

Figure 4-9 Enzymatic digestibility of treated cherimoya seeds in terms of **a** glucose relative concentration and **b** glucose relative yield (lines are for eye guidance only)

This behavior can be related to the extent of the autohydrolysis treatment. Increased severity of the autohydrolysis results in hemicellulose removal, a factor known to increase cellulose digestibility.[44] Nevertheless, this removal only partially explains the high increase in cellulose digestibility. As such, it is suggested that the further increase in cellulose digestibility after hemicellulose removal may be related to the decrease of cellulose crystallinity (observed by SEM imaging – Fig. 4-10) or to an increase of pore sizes obtained for more severe conditions that enhance cellulase penetration into biomass.[44]

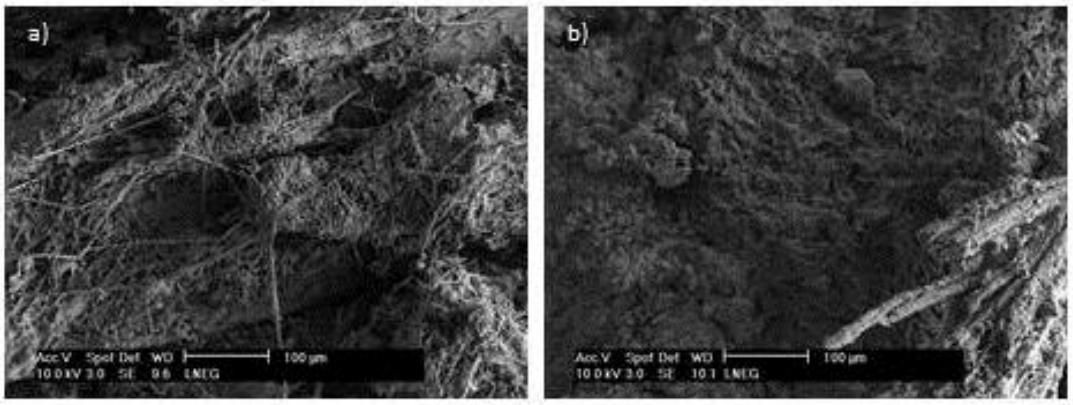

Figure 4-10 SEM photomicrographs after autohydrolysis at a) 190 ºC (where the crystalline cellulose rods can be clearly observed) and b) 230 ºC (where the "blurry" area corresponds to amorphous cellulose)

4.4 Conclusions

The lignocellulosic fraction of cherimoya seeds after oil extraction, seed cake, is a possible source of OS, yielding XOS under mild operational conditions that could be modeled by mathematical kinetic models based on the parameters of the Arrhenius equation. The developed model will be a useful tool to predict the behavior of the autohydrolysis process, namely on scale-up procedures, as the produced OS have been demonstrated to be chemically stable under relevant processing and digestive conditions. This enables to maintain their physicochemical properties, and hence their inherent potential nutritional/bioactive properties. In fact, the ability of these OS to be processed and the possibility to pass the stomach undigested, deem them as potential NDOS, a trait that must be further characterized and explored.

Moreover, in a technological point of view, the autohydrolysis process enabled the recovery of hemicellulose as potentially marketable products, and to obtain cellulose-enriched pretreated solids presenting higher enzymatic saccharification yields, thus increasing the value of these solids as substrates for fermentative processes. Furthermore, it also does not threat the upgrade potential of the lignin and protein fractions, thus contributing to a zero waste approach on the valorization of the cherimoya seeds.

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CHAPTER 5 OPTIMIZATION AND MATHEMATICAL MODELING OF CHERIMOYA SEEDS' DILUTE ACID HYDROLYSIS FOR THE PRODUCTION OF HEMICELLULOSIC HYDROLYZATES

Summary

In this chapter, the influence of time (up to 180 min) and sulfuric acid concentration (up to 4%, w/w) were studied at 121 ºC, using a Doehlert experimental design. Empirical models describing the influence of these variables on sugars and by-products solubilization into the liquid fraction were validated for the entire domain and used for establishing the optimal operational conditions. The hydrolyzates obtained under optimized conditions mainly contained monosaccharides (xylose, arabinose and glucose; total monosaccharide concentration *ca.* 39 $g.L^{-1}$), with xylose as the major sugar present. Acetic acid, furfural, and HMF were the main potential microbial inhibitors found, but in relatively low concentrations.

The residual solid fraction was also characterized and the data for the solid fractions is further modelled based on the combined severity parameter.

5.1 Introduction

Cherimoya (*Annona cherimola* Mill.) seeds are the residues of the production of several agro-industrial products (*e.g.* cherimoya pulp), quite common in some South American countries such as Chile and Peru, but also in Europe, mainly Spain and Portugal (Madeira island). To the present there is no commercially relevant application for cherimoya seeds. They are rich in an oil (around 30% w/w), that can be used for FAME (biodiesel) production,[1] and the remaining lignocellulosic fraction has a high (hemi)cellulose content that can potentially be upgraded in the biorefinery framework.[2]

Pre-treatment processes are a necessary step for the use of lignocellulosic biomass within the biorefinery framework. These processes should effectively separate hemicelluloses and/or lignin to facilitate subsequent cellulose hydrolysis and should enable high recovery of the fractionated components, in order to increase the biorefinery economic viability by means of their upgrade. Promising biomass pre-treatments (mainly autohydrolysis, steam explosion, acid or acid alkaline hydrolysis) may produce a sugar-rich liquid stream selectivly derived from the hemicelluloses. Mild processes usually produce sugars in oligomeric form,[3] which can impose some upgrade problems as these oligomers are almost unusable by most industrial microbial catalysts that cannot directly metabolize them. In this sense, a post-hydrolysis step is usually mandatory to enable the upgrading of the hemicellulosic oligosaccharides.

Dilute acid hydrolysis pre-treatment still presents significant advantages due to its simplicity and selectivity towards hemicellulose, with the further advantage that solubilizes saccharides mainly in their monomeric form, typically with high yields. On the other hand, monosaccharide degradation reactions (degradation of pentoses to furfural, hexoses to 5-hydroxymethylfurfural – HMF – and both of these furans to aliphatic acids like formic or levulinic acids)[4] and partial lignin removal may occur, yielding

inhibitory compounds that can hinder the upgrade of both the liquid and solid fractions. Therefore, a careful optimization of the operational conditions is required to maximize monosaccharide recovery, while minimizing degradation products formation. Among the several parameters that influence acid treatments, catalyst concentration (typically up to 4%) and reaction time are usually the most studied.[5] The optimal conditions are, nevertheless, raw material dependent.

The Doehlert experimental design allows the study of several parameters simultaneously, carrying out a set of experiments chosen in a systematic way, in order to predict the optimal conditions and to evaluate the interactions between variables.[6] In this work, the effects of the catalyst (sulfuric acid) concentration and reaction time on cherimoya seeds dilute acid hydrolysis were studied, in order to obtain an hemicellulosic hydrolyzate to be used as microbial growth medium, maximizing monosaccharide content, while minimizing by-product formation.

5.2 Methods

5.2.1 Materials

Cherimoya seeds were taken from fresh fruits, washed and dried in an oven at 35 ºC to constant weight. Seeds were then roughly ground with a blender and the oil was extracted with commercial grade hexane. The remaining solid residue was dried in an oven and ground again, now with an IKA® Werke (Staufen, Germany) MF10 basic mill, to particles smaller than 1.5 mm, homogenized in a defined lot and stored in plastic containers at room temperature for further processing. The processed lot contained (w/w, dry weight basis) 21.2% cellulose (measured as glucan), 21.1% hemicellulose, 25.7% Klason lignin, 22.8% protein, 2.8% ash and 6.4% of extractives and others (calculated by difference).

Sulfuric acid (95-97%), D-(+)-xylose (≥99%), levulinic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma-

Aldrich (Steinheim, Germany), L-(+)-arabinose (≥99%) and acetic acid (>99.8%) were purchased from Merck (Darmstadt, Germany), D-(+)-glucose (>99.5%) was purchased from Duchefa Biochemie (Haarlem, The Netherlands) and formic acid (98%) was purchased from PANREAC (Barcelona, Spain).

5.2.2 Experimental Design

A Doehlert uniform design was used to establish the effects of H_2SO_4 concentration (X_1) between 0.1 and 4.0% (w/w) and reaction time (X_2) between 0 and 180 minutes.[7] Five levels were selected to study H_2SO_4 concentration and three levels for reaction time, which enables the estimation of curvature effects for each independent variable. The design results in seven combinations (Table 5-1). All assays were carried out at least in duplicate to provide a measure of the inherent experimental error.

Table 5-1 Codified matrix for the Doehlert experimental design for two variables and the corresponding experimental conditions

Trial	Variables				CS
	Coded		Real		
	X_1	X_2	H_2SO_4 (%)	Time (min)	
A	0.000	0.000	2.05	90	1.89
B	1.000	0.000	4.00	90	2.18
C	-1.000	0.000	0.10	90	0.58
D	0.500	0.866	3.03	168	2.33
E	-0.500	-0.866	1.08	12	0.74
F	0.500	-0.866	3.03	12	1.19
G	-0.500	0.866	1.08	168	1.88
Z	1.000	1.000	4.00	180	2.48

The model used to express the responses (concentrations of the specified chemical species) was a second order polynomial equation: [8]

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon \qquad \textbf{(Equation 5-1)}$$

where, Y is the response, X are the independent variables, and the subscripts 1 and 2 are related to H_2SO_4 concentration and time,

respectively. β_0 is the regression coefficient at center point; β_1 and β_2 are the linear coefficients of the variables 1 and 2, respectively; β_{12} is the second-order interaction coefficient between variables 1 and 2; and β_{11} and β_{22} are the quadratic coefficients for variables 1 and 2; and ε are independent random errors, assumed to be normally and independently distributed.[6]

The linear multiple regression of Equation 5-1 and its analysis of variance (ANOVA) were carried out using Microsoft® Excel 2007 regression tool pack, using all replicates. The best hydrolysis conditions were determined by using the Microsoft Excel® 2007 Solver tool based on the best-fit equations, using a constrained model ($-1 \leq X_1, X_2 \leq 1$). Coded representation of the variables was used for all calculation purposes. Data for regression and numerical optimization are presented in tables 5-1 (X_1 and X_2 values) and 5-2 (Y values).

5.2.3 Dilute acid hydrolysis

Dilute acid hydrolysis assays were performed in autoclave at 121 °C, in universal Schott flasks capped with red stoppers. The effects of H_2SO_4 concentration and reaction time (isothermal period) were studied according to the experimental design previously described. The solid (10 g) was mixed with H_2SO_4 aqueous solutions with the prescribed acid concentration to get a liquid-to-solid ratio (LSR) of 5. The reaction temperature was maintained for the whole reaction time and then the autoclave was rapidly cooled down to 100 °C (approximately 4 minutes). Heating time from 100 to 121 ºC was approximately 6 minutes. The liquor and the solid residue were separated using a manual French press.

The control conditions were defined by 4% H_2SO_4 and 180 minutes at 121 °C (condition Z, see Table 5-1), as the most extreme condition (*CS* = 2.48) in this experimental design. The combined severity parameter (*CS*) was calculated for the isothermal period according to Equation 5-2.[9]

$$CS = \text{Log}\, R_0 - \text{pH} \quad \textbf{(Equation 5-2)}$$

$$R_O = \int_0^t exp\left(\frac{T(t)-T_{ref}}{\omega}\right) \cdot dt \quad \textbf{(Equation 5-3)}$$

where T(t) is the maximum temperature, T_{ref} is the temperature up to which the hydrolysis process is considered to be negligible (usually 100 ºC) and ω is an empirical constant related to the influence of the temperature on the reaction (typically 14.75, meaning that the reaction rate is doubled for every increase in 10 ºC).[10.11]

5.2.4 Analytical Methods

Feedstock material and solid residues were characterized by quantitative acid hydrolysis according to standard NREL protocols and ISO standards as described in section 3.2.3.[12-16]

Total phenolic compounds content was assayed spectrophotometrically by the Folin-Ciolcateu method as described by Moore and Yu using a JASCO UV/Vis spectrophotometer model V-530 (Japan).[17] Gallic acid was used as calibration standard.

5.3 Results and discussion

5.3.1 Composition of hydrolysis liquor

The concentration of monosaccharides and by-products obtained after the different hydrolysis trials, those defined by the Doehlert experimental design (A–G) as well as the control trial (Z), are presented in Table 5-2. Xylose is typically the main sugar in the hydrolyzates, except for trial C (which corresponds to the lowest severity tested) where glucose concentration slightly overcomes xylose concentration. Anyway, the total amount of pentoses overcomes (slightly in this case) the total amount of hexoses, as in all other trials. The increase in pentoses concentration with increasing severity is markedly more significant than for hexoses, with xylose concentration increasing more than 6 times from the least severe

conditions to the highest concentration achieved and arabinose concentration increasing about 10 times its value; on the other hand, the increase in glucose concentration is less than 3 times the lowest value.

Table 5-2 Monosaccharides and by-products concentration (gL^{-1}) obtained for the different trials (A-G) and control conditions (Z)

	Trial							
	A	B	C	D	E	F	G	Z
Xylose	25.98±0.04	25.82±0.01	4.21±0.06	24.16±0.11	11.54±0.57	24.89±0.21	24.20±0.24	22.30±0.00
Glucose	11.00±0.33	11.66±0.11	4.55±0.08	11.56±0.12	5.46±0.35	8.56±0.08	9.69±0.23	12.53±0.24
Arabinose	3.44±0.05	3.44±0.05	0.36±0.00	3.28±0.03	3.26±0.20	3.30±0.01	3.19±0.06	3.13±0.05
Total Sugars (TS)	40.42±0.33	40.92±0.14	9.12±0.13	39.00±0.04	20.26±1.12	36.75±0.28	37.09±0.53	37.95±0.29
Formic acid	1.25±0.01	2.16±0.02	n.d.	2.32±0.03	0.02±0.01	0.20±0.01	0.68±0.18	2.84±0.01
Acetic acid	4.19±0.18	4.51±0.10	0.32±0.00	4.58±0.14	1.09±0.04	3.00±0.10	3.59±0.02	4.37±0.08
Levulinic acid	1.05±0.01	2.40±0.01	n.d.	2.69±0.03	0.03±0.01	0.26±0.02	0.38±0.01	3.53±0.05
HMF	1.62±0.07	0.67±0.02	n.d.	0.69±0.01	0.37±0.01	1.25±0.02	2.14±0.02	0.60±0.03
Furfural	0.64±0.04	1.57±0.04	0.02±0.00	2.02±0.12	0.02±0.00	0.09±0.00	0.54±0.09	2.46±0.03
Phenolics	2.25±0.03	2.98±0.12	0.49±0.04	2.82±0.02	0.87±0.01	1.39±0.20	2.05±0.06	2.98±0.05
Total inhibitors (TI)	11.00±0.19	14.29±0.03	0.83±0.04	15.13±0.27	2.38±0.01	6.19±0.06	9.38±0.15	16.78±0.10
TS-TI	29.42±0.53	26.63±0.17	8.30±0.18	23.87±0.31	17.88±1.13	30.57±0.33	27.71±0.38	21.17±0.20

mean±standard deviation
n.d. – not detected

In any case, total sugars concentration increases with increasing severity, reaching a top value of *ca.* 41 $g.L^{-1}$ for *CS*=2.18, point from which it decreases for more severe conditions. The explanation for this behavior is that from a certain point sugar degradation becomes predominant over hemicellulose removal from the lignocellulosic matrix and hydrolysis, leading to a slight decrease in monosaccharide content while inhibitors concentration continue to increase. Glucose has a different behavior though, never decreasing its concentration in the liquor, indicating that glucan hydrolysis may occur for more severe conditions. Furan derivatives produced during dilute acid hydrolysis follow different patterns: while furfural content is higher for harsher conditions, reaching *ca.* 2.5 $g.L^{-1}$ in trial Z, HMF reaches a maximum value in trial G (*CS*=1.88) and then its degradation to levulinic acid is predominant. Formic acid formation is parallel to furfural production, as expected, because the former is produced as a degradation product of the latter. Acetic acid, a structural constituent of hemicelluloses, is the main by-product/inhibitor present. Its concentration reaches a maximum value of *ca.* 4.5 $g.L^{-1}$, slightly above the limit reported for microbial inhibition.[18]

5.3.2 Dilute Acid Hydrolysis Modeling

Process modeling can be a useful tool to establish the conditions that maximize sugar recovery while minimizing by-product formation and also to minimize catalyst spending and energy requirements. An empirical model based on the relations between the relevant factors (catalyst concentration and reaction time) and the final hydrolyzate composition, in a straightforward manner, was used.

Equation 5-1 was fitted to the different responses based on the data from the several experimental trials defined on the Doehlert experimental design (trials A-G). An extra trial (Z) was then carried out as a control to validate the proposed model.

Based on all data it was possible to estimate the regression coefficients for the polynomial model, which are presented in Table 5-3. The compounds (or sets of them) were all effectively correlated to the studied variables, using Equation 5-1, with statistically significant regressions at p value<0.01 and R^2 values generally above 0.95.

The high statistical significance, in a general way, for both the acid concentration linear and quadratic coefficients is an indicator of its importance on dilute acid hydrolysis. Acid concentration favored sugar recovery and increased acetic acid and furan derivatives production, but also lead to the formation of other inhibitors, as shown by the positive values of the linear coefficient. The high negative values determined for the quadratic coefficient mean that high acid concentrations, within the studied range, lead to increased sugar decomposition, thus decreasing sugar recovery. This effect is noticeably stronger for sugars (and acetic acid), but also, although in a minor extent, for all other compounds; the decomposition of hexoses to levulinic acid (via HMF formation) is favored for high acid concentrations, but the decomposition of pentoses to formic acid (via furfural) is not and furfural is the main product of pentose degradation.

Reaction time affected both sugar recovery and by-products formation in a positive way. In other words, longer reaction times lead to an increase in both monosaccharide and inhibitor contents. However, the low statistical significance of the reaction time quadratic coefficient indicates that under the studied range, longer reaction times do not favor by itself products (or by-products) recovery.

The interaction coefficient for acid concentration and reaction time is statistically significant for several relevant compounds. According to the estimated coefficients it can be stated that a synergistic effect between these variables occurs which leads to the degradation of hexoses into levulinic acid and to the degradation of pentoses into furfural. The same effect is observed for furfural and its further degradation into formic acid.

Table 5-3 Regression coefficients estimates for the polynomial model, for the different responses analyzed

	β_0	β_1	β_2	β_{12}	β_{11}	β_{22}	R^2
Xylose	**24.94±1.15 (0.00)**	**9.77±0.72 (0.00)**	**3.79±0.72 (0.00)**	**-6.34±1.26 (0.00)**	**-9.59±1.37 (0.00)**	-1.33±1.37 (0.35)	0.96
Glucose	**10.62±0.36 (0.00)**	**3.32±0.22 (0.00)**	**2.22±0.22 (0.00)**	-0.20±0.39 (0.62)	**-2.38±0.43 (0.00)**	**-1.43±0.43 (0.01)**	0.97
Arabinose	**3.46±0.34 (0.00)**	**1.04±0.21 (0.00)**	-0.03±0.21 (0.88)	0.00±0.38 (0.99)	**-1.57±0.41 (0.00)**	0.24±0.41 (0.56)	0.81
Total Sugars (TS)	**39.02±1.77 (0.00)**	**14.14±1.11 (0.00)**	**5.98±1.11 (0.00)**	**-6.54±1.95 (0.01)**	**-13.53±2.12 (0.00)**	-2.52±2.12 (0.26)	0.96
Formic acid	**1.33±0.07 (0.00)**	**1.00±0.05 (0.00)**	**0.78±0.05 (0.00)**	**0.73±0.08 (0.00)**	**-0.28±0.09 (0.01)**	**-0.64±0.09 (0.00)**	0.99
Acetic acid	**4.12±0.16 (0.00)**	**1.90±0.10 (0.00)**	**1.21±0.10 (0.00)**	**-0.44±0.18 (0.04)**	**-1.68±0.20 (0.00)**	**-0.81±0.20 (0.00)**	0.98
Levulinic acid	**1.17±0.08 (0.00)**	**1.18±0.05 (0.00)**	**0.76±0.05 (0.00)**	**1.04±0.08 (0.00)**	-0.01±0.09 (0.89)	**-0.50±0.09 (0.00)**	0.99
HMF	**1.32±0.23 (0.00)**	0.23±0.14 (0.14)	**0.45±0.14 (0.01)**	**-0.95±0.25 (0.00)**	**-0.89±0.27 (0.01)**	0.14±0.27 (0.61)	0.81
Furfural	**0.77±0.09 (0.00)**	**0.73±0.05 (0.00)**	**0.66±0.05 (0.00)**	**0.64±0.10 (0.00)**	-0.02±0.10 (0.85)	-0.19±0.10 (0.10)	0.99
Phenolics	**2.30±0.15 (0.00)**	**1.03±0.09 (0.00)**	**0.74±0.09 (0.00)**	0.07±0.16 (0.68)	**-0.58±0.18 (0.01)**	**-0.52±0.18 (0.02)**	0.96
Total inhibitors (TI)	**11.02±0.46 (0.00)**	**6.10±0.29 (0.00)**	**4.60±0.29 (0.00)**	1.09±0.51 (0.06)	**-3.50±0.55 (0.00)**	**-2.51±0.55 (0.00)**	0.99
TS-TI	**28.00±1.41 (0.00)**	**8.04±0.88 (0.00)**	1.38±0.88 (0.15)	**-7.63±1.55 (0.00)**	**-10.02±1.68 (0.00)**	-0.01±1.68 (0.99)	0.93

regression coefficient±standard deviation (*p* value)
Values in bold indicate the statistically significant coefficients

This modeling approach is a useful tool to identify and quantify the main features of the process and based on the above assumptions, it is now possible to define the optimal operational conditions regarding the desired purpose of the pre-treatment. Thus the optimal operational conditions can be adjusted according to the composition to be achieved for the hydrolyzate.

5.3.3 Numerical optimization

The desired criterion chosen for the best hydrolysis conditions was, among other possibilities, to maximize the direct difference between total sugars (TS) and total inhibitors (TI) concentration (TS-TI, total sugars minus total inhibitors). A constrained optimization model was implemented and no weighting was introduced to favor monosaccharide production or to minimize inhibitors. In the range defined by 0.515 (3.05% H_2SO_4 w/w)<X1<1.000 (4.00%) and a maximum of 15.30 min of reaction time (X2<-0.830) the value for TS-TI is over 32 $g.L^{-1}$, as shown in the contour plot for TS-TI related to acid concentration and reaction time (Fig. 5-1). This means that, regarding the most severe conditions used in this experimental design, it is possible to save up to 24% of sulfuric acid consumption. The short duration of the treatment also implies fewer energy spending and an advantage regarding continuous processing. However, a neutralization step might be needed in order to enable the upgrading of the hydrolyzate using microorganisms, which implies that alkali costs should also be considered.

5.3.4 Solid residue characterization

The solid residues obtained after the dilute acid hydrolysis trials were quantified in terms of solid yield and characterized by quantitative acid hydrolysis (Fig. 5-2). Regarding the structural components (glucan, xylan, arabinan, acetyl groups and Klason lignin), their behavior is in accordance with the results obtained for other lignocellulosic materials. Arabinan and acetyl groups' recovery in the liquid is complete under relatively mild conditions, while xylan is absent from the solid residues corresponding to

treatments with high *CS* values, as observed for other lignocellulosic materials.[19-21] Arabinan and acetyl groups are usually found in the sidechains of xylans, and are, therefore, more labile than the latter. On the other hand, glucan is not completely removed from the solid residues, and its content decreases to values below 20%, indicating either cellulose hydrolysis or that some glucose is present in the hemicellulose.

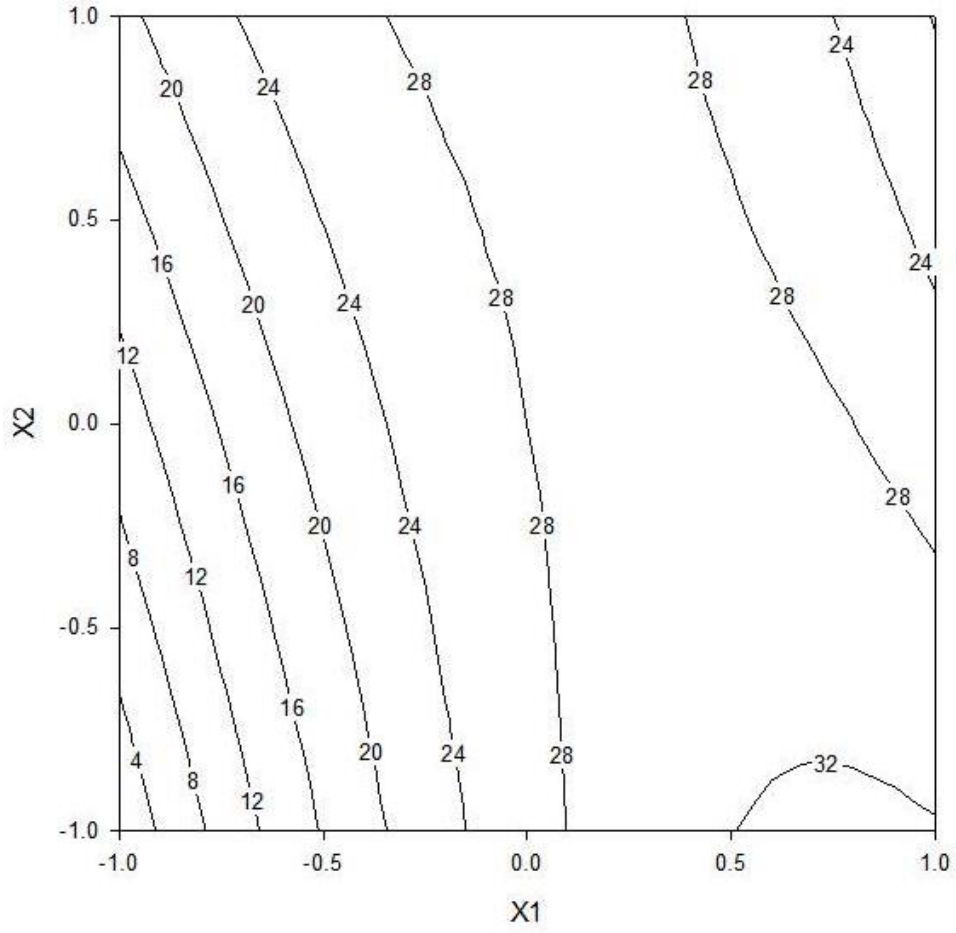

Figure 5-1 Contour plot for the concentration of total sugars ($g.L^{-1}$) minus total inhibitors (TS-TI) in relation to acid concentration (X1) and reaction time (X2)

Either way, these values are in accordance to those obtained for olive stones.[20] The high percentages of other compounds are probably due to monosaccharides present in the solid residues, as no washing step was performed after separating the hydrolizate from the solid residue. These free sugars were most certainly converted to degradation products during the quantitative acid hydrolysis procedure, which are then quantified as other components.

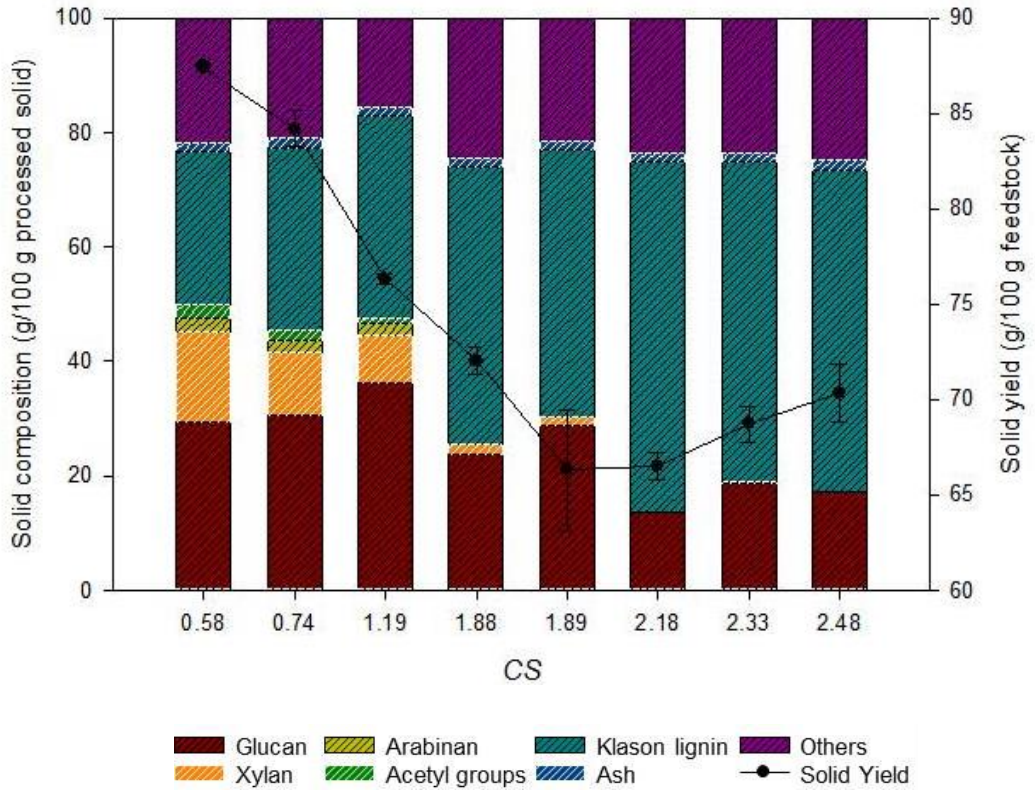

Figure 5-2 Effect of the combined severity parameter on the solid yields and composition of the solid residues after dilute acid hydrolysis

The solid yield follows the expected pattern. More structural components are solubilized with increased severity and, therefore, the solid yield lowers. Yet, for the more severe hydrolysis the pattern is inverted, and more solids are recovered after the treatment. A possible explanation is the occurrence of reactions between protein and lignin, which leads to an overrating of lignin. In fact, it has been reported that binding reactions between lignin and proteins can occur for more severe treatments,[2,3,22,23] leading to an miscalculation of lignin.

The polynomial function previously described (Eq. 5-1) was used to establish a correlation between the composition of the solid residues and the variables X1 and X2. Except for ash (whose percentage remained almost constant among the several trials), the relative amounts of the structural components can be correlated to the experimental variables (Table 5-4).

Table 5-4 Regression coefficients estimates for the polynomial model, for the different responses obtained on solid residue analysis

	β_0	β_1	β_2	β_{12}	β_{11}	β_{22}	R^2
Xylan	1.70±1.36 (0.24)	**-5.96±0.86 (0.00)**	**-5.04±0.86 (0.00)**	0.42±1.50 (0.79)	**6.18±1.63 (0.00)**	2.79±1.63 (0.12)	0.91
Glucan	**25.90±2.63 (0.00)**	**-4.22±1.65 (0.03)**	**-6.06±1.65 (0.00)**	-2.14±2.90 (0.48)	-3.53±3.14 (0.29)	4.38±3.14 (0.19)	0.79
Arabinan	0.05±0.28 (0.85)	**-0.89±0.18 (0.00)**	**-1.21±0.18 (0.00)**	0.02±0.31 (0.95)	**1.20±0.34 (0.01)**	**0.88±0.34 (0.03)**	0.89
Acetyl groups	0.08±0.20 (0.68)	**-0.89±0.12 (0.00)**	**-0.84±0.12 (0.00)**	0.27±0.22 (0.25)	**1.02±0.24 (0.00)**	0.44±0.24 (0.09)	0.92
Lignin	**48.76±3.07 (0.00)**	**12.52±1.93 (0.00)**	**9.97±1.93 (0.00)**	-0.63±3.38 (0.86)	-5.36±3.66 (0.17)	-6.94±3.66 (0.09)	0.89
Ash	**1.58±0.11 (0.00)**	0.03±0.06 (0.69)	-0.03±0.06 (0.69)	0.18±0.13 (0.19)	0.00±0.14 (0.98)	0.04±0.14 (0.77)	0.24
Other	**21.41±1.64 (0.00)**	-0.41±0.95 (0.67)	**3.38±0.95 (0.01)**	2.57±1.89 (0.21)	1.18±2.01 (0.57)	-0.90±2.01 (0.67)	0.67

regression coefficient±standard deviation (*p* value)

Values in bold indicate the statistically significant coefficients

5.4 Conclusions

The influence of the studied variables on the dilute acid hydrolysis process was identified and quantified. An empirical model, based on the data obtained from a Doehlert experimental design, was developed and a region was defined for the optimal operational conditions (sulfuric acid concentration between 3.05% – w/w – and 4.00%, and a maximum rection time of 15.3 minutes) to achieve a monosaccharide-rich hemicellulosic hydrolyzate. Within this region, it was possible to maximize hemicellulosic sugar recovery (while minimizing inhibitors formation) in a short-duration treatment. According to the developed model, a maximum of 32.73 gL^{-1} of TS-TI (total sugars minus total inhibitors) can be achieved at 121 ºC, in non-isothermal conditions, using 3.57% (w/w) H_2SO_4.

For an industrial application (dilute-acid hydrolysis is generally performed industrially in batch processes) this "flash" treatment can be a great improvement, as more quantities of feedstock can be processed and, consequently, higher amounts of hydrolyzate are produced, in a shorter time.

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CHAPTER 6

PRODUCTION OF XANTHAN GUM USING CHERIMOYA SEEDS' HEMICELLULOSIC HYDROLYZATE AS A CULTURE MEDIUM

Summary

In this chapter, several strains of *Xanthomonas campestris* were tested to evaluate their ability to produce xanthan gum using cherimoya seeds hydrolysate as a culture medium. No detoxification steps were performed and sugar consumption was almost complete after 24 hours.

Different behaviors were observed for the four tested strains. The results show that, in the tested conditions, some strains use the carbon source mainly for cell growth, while others use it for exopolysaccharide production. A maximum of 9.8 $g.L^{-1}$ of xanthan gum, corresponding to a productivity of nearly 0.2 $g.(Lh)^{-1}$, was obtained, while cell productivity reached 0.1 $g.(Lh)^{-1}$.

6.1 Introduction

Cherimoya seeds, a by-product from the production of juice, frozen pulp and alcoholic beverages, have high lipid content (≈30% w/w, in a dry weight basis) and can be considered as a potential biorefinery's feedstock in the Mediterranean and sub-tropical areas.[1] The lignocellulosic fraction recovered after lipid extraction has significant hemicellulose content, for which there is currently no definite upgrade solution.

Bacteria from the genus *Xanthomonas* are plant pathogens and affect important crops like rice and citrus plants. Particularly, *Xanthomonas campestris* is responsible for the "black rot" disease in cabbage, cauliflower or carrots. Besides being a phytopathogen, *X. campestris* is known as the producer of the exopolysaccharide xanthan.[2]

Xanthan is a heteropolysaccharide with a glucose backbone, in a cellulose-like structure, and trisaccharide side-chains (D-mannose-D-glucoronic acid-D-mannose) attached to every second glucose unit.[2] Moreover, approximately one-half of the terminal D-mannose contains a pyruvic acid residue linked in an acetal structure to positions 4 and 6 and the D-mannose units linked to the main chain contain an acetyl group at position O-6.[3]

Xanthan gum is used as a food additive and rheology modifier, mainly as thickening agent or as stabilizer.[4-7] It is estimated that its market capitalization will exceed US$ 400 million in 2015. Xanthan gum is usually produced from sterile aqueous carbohydrate solutions, but the search for alternative and cheaper culture media is a continuous endeavor. [5]

6.2 Methods

6.2.1 Materials

Annona cherimola Mill. seeds were provided frozen by J. Faria & Filhos Lda. (Funchal, Portugal). Upon collection seeds were washed and dried in to

constant weight. After drying, the seeds were stored at room temperature until further processing. The seeds were roughly ground with a blender and treated with commercial grade hexane for oil extraction, as described in chapter 3. After filtration the solid was dried in an oven at 55 ºC and then ground with an IKA® Werke (Staufen, Germany) MF10 basic mill to particles smaller than 1.5 mm, homogenized in a defined lot and stored in plastic containers at room temperature.

Sulfuric acid (95-97%), Iron(III) chloride hexahydrate (97%), D-(+)-xylose (≥99%), levulinic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma-Aldrich (Steinheim, Germany), hydrochloric acid (37%), ammonium sulfate (≥99.5%), citric acid monohydrate (99.5-100.5%), magnesium sulfate heptahydrate (≥99.5%), zinc oxide (99-100.5%), calcium carbonate (≥99%), calcium hydroxide (≥96%), L-(+)-arabinose (≥99%) and acetic acid (>99.8%) were purchased from Merck (Darmstadt, Germany), D-(+)-glucose (>99.5%) was purchased from Duchefa Biochemie (Haarlem, The Netherlands), boric acid (≥99.8%), ethanol (>99.8%) and glycerol (86-88%) were purchased from Riedel-de Haën (Seelze, Germany) and formic acid (98%), potassium dihydrogen fosfate (≥99%), yeast extract, malt extract and peptone (bacteriological) were purchased from PANREAC (Barcelona, Spain).

6.2.2 Dilute acid-hydrolysis

The solid was treated with 3.63% (w/v) sulfuric acid in an autoclave at 121 ºC for 1 minute (isothermal period) with a liquid-to-solid ratio (LSR) of 5:1, as previously optimized in chapter 5. Solid and liquid fractions were separated by pressing (up to 200 bar) with a manual oil press. The liquid fraction constitutes the hydrolyzate used for fermentation purposes.

6.2.3 Medium preparation

6.2.3.1 Pre-inoculum medium

A pre-inoculum medium containing cherimoya seeds' decoction water (100 ºC, 45 min in an autoclave) supplemented with peptone (5 $g.L^{-1}$), malt extract (3 $g.L^{-1}$) and yeast extract (3 $g.L^{-1}$) was prepared. The pH was adjusted to 7 with $Ca(OH)_2$ and the medium was filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters. Subsequent sterilization was performed using sterile 0.22 μm membranes.

6.2.3.2 Xanthan gum production medium

The hydrolyzate was supplemented with peptone (0.34 $g.L^{-1}$), yeast extract (0.75 $g.L^{-1}$), ammonium sulfate (3.3 $g.L^{-1}$), potassium dihydrogen fosfate (7.2 $g.L^{-1}$), citric acid (2.0 $g.L^{-1}$), magnesium sulfate (0.24 $g.L^{-1}$), calcium carbonate (0.03 $g.L^{-1}$), boric acid (0.007 $g.L^{-1}$), zinc oxide (0.006 $g.L^{-1}$), ferric chloride (0.0042 $g.L^{-1}$) and hydrochloric acid (0.16 $mL.L^{-1}$). The pH was adjusted to 7 with $Ca(OH)_2$ and the medium was filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters. Subsequent sterilization was performed using sterile 0.22 μm membranes.

6.2.4 *Xanthomonas campestris* fermentation

From slants of the four strains of *X. campestris* (NRRL B-1459 – further designated as 193F – and 3 other isolates – 302F, 304F and 353F – available in our lab) a portion was aseptically taken with an inoculation loop and inoculated in 85 mL of the pre-inoculum medium in 1 L baffled base Erlenmeyer culture flasks. The flasks were kept at 28 ºC in an Infors HT (Bottmingen, Switzerland) orbital shaker at 150 rpm. After 18 h the pre-inoculum was centrifuged in an Orto Alresa (Madrid, Spain) Digicen 21R centrifuge equipped with a RT 121 rotor at 5350 G for 30 min at 20 ºC.

The pellets of each strain (two) were re-suspended in 10 mL of the fermentation medium, homogenized and 5 mL transferred to 1 L baffled base Erlenmeyer culture flasks containing 60 mL of the fermentation medium and incubated under the same conditions. All fermentations were performed in duplicate.

6.2.5 Sampling

At determined intervals a 5 mL sample was taken aseptically for measuring the optical density and determining the dry weight.

6.2.6 Analytical methods

Hydrolyzate composition was determined according to standard NREL protocols as described in section 3.2.3.[8] Fermentation media consumption (monosaccharides, aliphatic acids, furan derivatives and glycerol) was monitored and quantified according to the same protocol.

Quantitation of the cells evolution was determined by gravimetric methods. An exact quantity (typically 4 mL) of the broth was filtered through previously dried and weighted Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters and dried to constant weight in an oven at 105 ºC.

Fermentation evolution was followed by measuring (at determined intervals) the optical density of the cell broth against water at 600 nm in a JASCO (Tokyo, Japan) V-530 spectrophotometer, using plastic cells with 1 cm of optic path. The fermentation broth was diluted with water 10 to 50 times, as required, in order to reach absorbance values between 0.1 and 0.8. Specific growth rates (μ_X) were calculated by determining the slope of the curves in the exponential phase of the growth.

6.2.6.1 Calculations

Xanthomonas campestris production rate (Q_X, $g.(Lh)^{-1}$) was calculated according to the following expression:

$$Q_X = \frac{Dry\ weight_{final} - Dry\ weight_{initial}}{V_{filtered} \times duration\ of\ the\ fermentation}$$ **(Equation 6-1)**

Xanthomonas campestris yield (Y_X, g/g of consumed sugars) was calculated ccording to the following expression:

$$Y_X = \frac{Dry\ weight_{final} - Dry\ weight_{initial}}{Total\ consumed\ sugars}$$ **(Equation 6-2)**

6.2.7 Xanthan gum quantitation

At the end of the fermentation 25 mL of each flask was transferred into Falcon™ tubes and centrifuged in an Orto Alresa (Madrid, Spain) Digicen 21R centrifuge equipped with a RT 121 rotor at 5350 G for 30 min at 4 ºC. The supernatant was transferred to previously dried and weighted Falcon™ tubes, ethanol was added (2x the volume of supernatant) and the tubes were kept at 4 ºC for xanthan gum precipitation. After 24 h the content of the tubes was again centrifuged in the same conditions, the supernatant removed and the pellet was dried at 100 ºC overnight and weighted.

Xanthan gum productivity (Q_{Xant}, $g.(Lh)^{-1}$) was calculated according to the following expression:

$$Q_{Xant} = \frac{Xanthan\ gum\ weight}{V_{centrifuged} \times duration\ of\ the\ fermentation}$$ **(Equation 6-3)**

Xanthan gum yield (Y_{Xant}, g/g of consumed sugars) was calculated ccording to the following expression:

$$Y_{Xant} = \frac{Xanthan\ gum\ weight}{Total\ consumed\ sugars}$$ **(Equation 6-4)**

6.3 Results

6.3.1 Hydrolyzate composition

Sugars were the main compounds present (approximately 39 $g.L^{-1}$), xylose being the major sugar (Table 6-1). Among the aliphatic acids, only acetic

acid was detected in significant amounts. No formic or levulinic acids were detected. Furfural was the main furan derivative present (0.87 $g.L^{-1}$).

Table 6-1 Composition of the hydrolyzate obtained in optimized conditions

Compound	Concentration ($g.L^{-1}$)
Glucose	9.81
Xylose	26.20
Arabinose	2.87
Acetic acid	3.56
Furfural	0.87
5-HMF	0.15

At pH 7 most of the acetic acid is in its dissociated (less toxic) form, which corresponded to a concentration of 0.3 $mmol.L^{-1}$ for this hydrolyzate. According to the literature, no significant differences are observed in cell growth at this level of concentration, thus no acetic acid removal was considered necessary.[9]

6.3.2 *Xanthomonas campestris* fermentation profiles

The suitability of cherimoya seeds' dilute-acid hydrolyzate for xanthan gum fermentation was tested. Moreover, a performance comparison between four *X. campestris* strains was conducted.

Monosaccharide assimilation was also very different between the tested strains (Figure 6-1). During the 50 h of the experiment, whilst strain 304F consumed all available sugars (within 30 h), strains 193F and 302F consumed all glucose (Glc) and arabinose (Ara), and almost all available xylose (Xyl). Finally, strain 353F consumed all Glc and Ara but only 75% of the available xylose (and took longer time to assimilate the sugars).

Despite these differences, it is clear that Glc was the preferable monosaccharide for all strains. Additionally, there seems to be a preference to Xyl instead of Ara in a first stage (while there is still Glc to be assimilated). Yet, as soon as there was no more Glc left, Ara assimilation became predominant over Xyl assimilation. This is in accordance to previous studies where xanthan yields are better if Glc is used as the carbon source.

If sole Xyl is used as carbon source, the yield is the lowest of the three sugars, but in the presence of Glc its assimilation and xanthan production are stimulated.[10,11] Strain 353F had a different behavior though: no apparent preference to Xyl over Ara was observed when Glc was still available; then Xyl was the preferable sugar (in a first phase after Glc assimilation), to become second-rate sugar in the last phase of the fermentation.

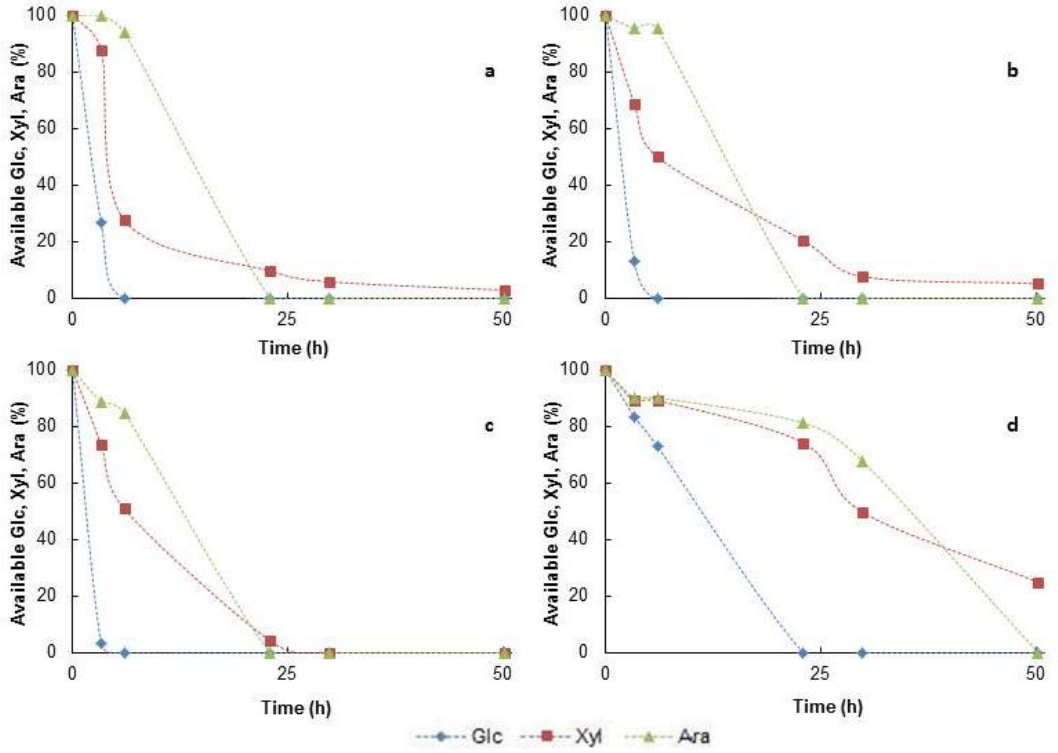

Figure 6-1 Monosaccharide assimilation pattern for the a) 193F, b) 302F, c) 304F and d) 353F strains of *X. campestris* (lines are used for eye guidance only)

The acetic acid evolution during the fermentation is presented in Figure 6-2. It showed an unusual behavior as, for all strains but 353F, it is first produced and then consumed. As acetic acid fermentation profiles for *X. campestris* are seldom presented in literature, it is not possible to compare results. It can be suggested that, similarly to what happens with *Escherichia coli*, acetic acid formation is due to an overflow-type metabolism, when sugar assimilation surpasses a critical rate.[12,13] In fact, high acetic acid production rates could be correlated to high sugar assimilation rates, especially for the initial phase of the fermentation. After glucose depletion, acetic acid production was either reduced or stopped, and

acetic acid was consumed, especially for strains 193F and 302F, but apparently not assimilated. This behavior was not so marked for strain 353F, that presented the lower sugar assimilation rates and for which acetic acid concentration remained fairly constant throughout the whole fermentation.

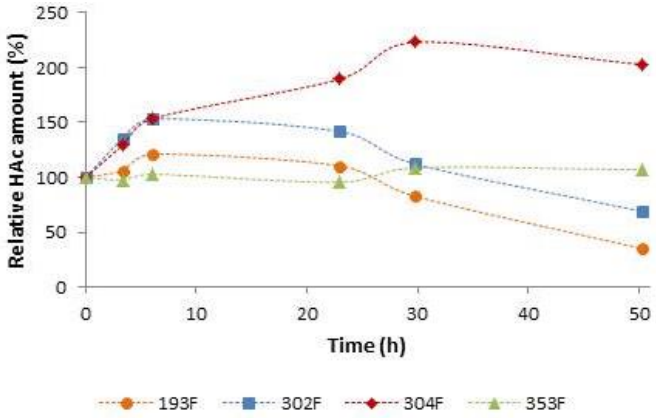

Figure 6-2 Acetic acid profiles during the fermentation

No adaptation period was observed (Figure 6-3) for any of the four strains as expected, probably due to the pre-inoculum step, even though the two media had different composition. However, they presented different behaviors. Strains 193F and 304F rapidly grew to reach steady state at about 24 h, strain 302F only reached steady state after 30 h and cell growth was quite linear for strain 353F after 3 h. Moreover, strain 304F seemed to have a new growth phase, after a period of adaptation, once there were no monosaccharides left to assimilate.

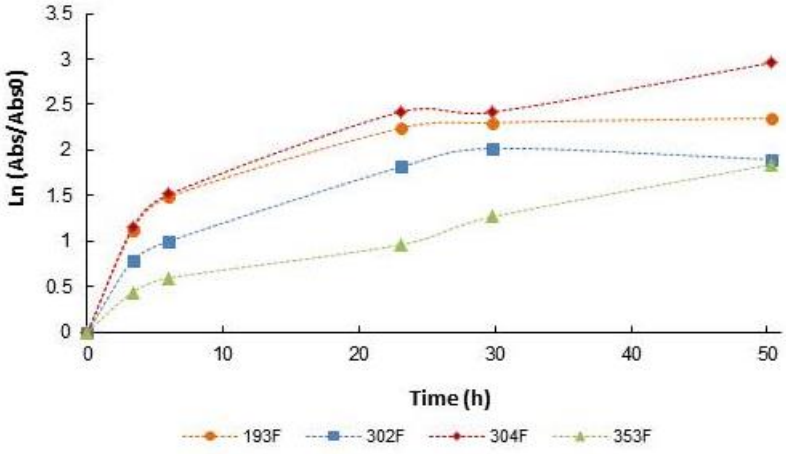

Figure 6-3 Growth profiles of the four tested strains of *X. campestris*

Specific growth rates were higher (Table 6-2) than those reported for *X. campestris* fermentations in carob extracts with strain 193F, but cell production rate is similar. Furthermore, for strains 193F and 304F μ values were even higher than the ones reported for strain 193F in other complex media.[14]

Table 6-2 Specific growth rates (μ_x), *X. campestris* production rate (Q_x) and *X. campestris* yield (Y_x) for the four strains of *X. campestris* in cherimoya seeds' dilute-acid hydrolyzate

	Strains			
	193F	**302F**	**304F**	**353F**
μ_x (h^{-1})	0.251	0.169	0.256	0.100
Q_x ($g.h^{-1}$)	0.073	0.072	0.102	0.056
Y_x	0.314	0.326	0.446	0.262

6.3.3 Xanthan gum production

Glucose is still considered the best option for the production of xanthan gum, but alternative media have been used and the search for novel ones is continuous.[5] The choice of the producing strain is also of great importance.

The productivity of the four strains (Table 6-3) did not present significant differences, even though there are differences in sugars assimilation. According to sugar assimilation curves and acetic acid and growth profiles, it was expectable that strain 353F presented a worse xanthan production. Instead, it was the best strain in terms of Y_{Xant}. On the other hand, strain 304F, being the sole strain that consumed all available sugars, presented the worst yield and productivity. These results *per se* can be erroneous, so they must be interpreted together with those from the fermentation profiles.

Table 6-3 Xanthan gum maximum concentration ($[Xant]_{max}$), productivity (Q_{Xant}) and yield (Y_{Xant}) obtained in the fermentations with the *X. campestris* strains

	Strains			
	193F	**302F**	**304F**	**353F**
$[Xant]_{max}$ ($g.L^{-1}$)	9.79	9.32	9.09	9.39
Q_{Xant} [$g.(L.h)^{-1}$]	0.195	0.185	0.181	0.187
Y_{Xant}	0.842	0.837	0.793	0.867

Apparently, strain 304F converted the carbon source preferably to produce cells, instead of exopolysaccharide (when compared to the other strains). The higher value for Y_x and lower for Y_{Xant} is an indicator of such assumption. Oppositely, strain 353F has a higher Y_{Xant} and a lower Y_x, indicating that xanthan formation was primary over cell production. This can also explain the almost constant acetic acid profile for this strain. As xanthan exhibits an acetylated mannose in its structure, no acetic acid seems to have been released to the broth. Instead, it was possibly used for xanthan synthesis. The highest xanthan concentration achieved was nearly 9.8 $g.L^{-1}$, for strain 193F, almost 50% higher than previous reports for batch fermentations of this strain in carob extract.[14]

Moreover, given that strain 304F consumes all available sugars within 30 h, xanthan production can be assumed to have ended at this point. Recalculating, xanthan productivity for strain 304F after 30 h becomes higher than 0.3 $g.(L.h)^{-1}$. Furthermore, cell growth did not stop and possibly some exopolysaccharide could have been used as a carbon source, resulting in a possible even higher productivity.

6.4 Conclusions

This is the first report of using a hemicellulosic hydrolyzate for xanthan gum production. Comparison with data from other complex media, using the same strain (193F), revealed that *Annona cherimola* Mill. seeds' dilute-acid hydrolyzate is suitable for xanthan production, even without detoxification.

The four tested strains of *Xanthomonas campestris* presented different behaviors. High xanthan gum productivity implied low cell productivity and vice-versa, indicating that carbon is either used mostly for exopolysaccharide production or for cellular growth. Maximum xanthan concentration and productivity (strain 193F, at the end of the experiment) did not correspond to the best xanthan yield. Yet, the lower xanthan

concentration and yield corresponded to the better productivity (for strain 304F, calculated at the time that all sugars were consumed).

Economically, the use of hemicellulosic hydrolyzates, cheaper than other media, can be a great improvement (especially if no detoxification step is needed). However, the supplementation used in this work is rather expensive and the need for such supplements must be evaluated. Moreover, when included within the biorefinery framework, in a whole-valorization/zero-waste approach, this approach can be a step towards industrial sustainability.

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CHAPTER 7

CHERIMOYA SEEDS HEMICELLULOSIC HYDROLYZATE AS A CULTURE MEDIUM FOR THE PRODUCTION OF KEFIR GRAINS

Summary

In this chapter, freeze-dried commercial Kefir inoculum was used for fermentation assays, using cherimoya seed hemicellulosic hydrolysates as culture media. The addition of supplements, as well as detoxification procedures, was found to be unnecessary. The growth of Kefir grains (kefiran and microbial biomass) was observed with a productivity of 0.07 $g.(Lh)^{-1}$ and the production of ethanol and lactic acid was detected by high performance liquid chromatography, following the consumption of monosaccharides. No lag phase was observed and stationary phase was reached within approximately one day.

7.1 Introduction

Cherimoya (*Annona cherimola* Mill.) belongs to the *Annonaceae* family, which accounts for *ca.* 75 genera and 2000 species. Nevertheless only the *Annona*, *Rollinia*, *Uvaria* and *Asimina* genera are used for human consumption, with *Annona* and *Rollinia* being the most important for commercial use, comprising around 60 species for the former and 20 for the latter. Besides naturally occurring species, hybrid species are also known. Cherimoya is mainly produced in Spain (33,000 tons/yr), Chile, Equador, Peru, Bolivia, Mexico and Portugal at approximately 50,000 tons/yr, worldwide.[1] Specifically, in Madeira Island (Portugal), its production has a considerable impact on regional agricultural economy. Cherimoya seeds can account for approximately 3% of the fruit weight and contain a very significant amount of oil (≈30% w/w, in a dry weight basis) that might be used for biodiesel production.[2] The lipid fraction composition also suggests its use in higher added-value applications, *e.g.* for cosmetics. The remaining lignocellulosic fraction can then be further upgraded.

Dilute-acid hydrolysis is a method commonly used to obtain hemicellulosic sugars, due to its simplicity and selectivity towards hemicellulose, with the further advantage that renders saccharides mainly in their monomeric form, typically with high yields.[3] On the other hand, monosaccharide degradation reactions (degradation of pentoses to furfural, hexoses to 5-hydroxymethylfurfural – HMF – and both of these furans to aliphatic acids like formic or levulinic acids) and partial lignin removal may occur, yielding inhibitory compounds that can hinder the upgrade of both the liquid and solid fractions.[4] Therefore, a careful optimization of the operational conditions is required to maximize monosaccharide recovery, while minimizing degradation products formation. Sulfuric acid is the most widely used, because it is cheap and effective, although hydrochloric, nitric, hydrofluoric and phosphoric acids are also used.[5]

Kefir is an acidic and mildly alcoholic fermented dairy product that is believed to have functional properties and high nutritional, biological and dietetic value.[6] Kefir grains are natural mixed cultures, containing a diverse spectrum of bacteria and yeasts, many with probiotic potential.[7] Many recent studies also indicate their potential novel applications in bread production, and fermented drinks,[8] but polysaccharide (kefiran) production may be the most attractive novel application. Kefiran is a polysaccharide composed of glucose and galactose (in a 1:1 ratio) presenting a hexasaccharide repeating unit.[9] Anti-carcinogenic activity has been attributed to Kefiran and positive changes in blood pressure and serum components were also observed.[10]

7.2 Methods

7.2.1 Materials

Annona cherimola Mill. seeds were provided frozen by J. Faria & Filhos Lda. (Funchal, Portugal). Upon collection seeds were washed and dried to constant weight. After drying, the seeds were stored at room temperature until further processing. The seeds were roughly ground with a blender and treated with commercial grade hexane for oil extraction, as decribed in chapter 3. After filtration the solid was dried in an oven at 55 ºC and then ground with an IKA® Werke (Staufen, Germany) MF10 basic mill to particles smaller than 1.5 mm, homogenized in a defined lot and stored in plastic containers at room temperature.

Sulfuric acid (95-97%), D-(+)-xylose (≥99%), levulinic acid (98%), DL-lactic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma-Aldrich (Steinheim, Germany), calcium hydroxide (≥96%), L-(+)-arabinose (≥99%) and acetic acid (>99.8%) were purchased from Merck (Darmstadt, Germany), D-(+)-glucose (>99.5%) was purchased from Duchefa Biochemie (Haarlem, The Netherlands), ethanol (>99.8%) and glycerol (86-88%) were purchased from Riedel-de Haën (Seelze, Germany)

and formic acid (98%) and yeast extract were purchased from PANREAC (Barcelona, Spain).

7.2.2 Dilute acid-hydrolysis

The solid was treated with 3.63% (w/v) sulfuric acid in an autoclave at 121 ºC for 1 minute (isothermal period) with a liquid-to-solid ratio (LSR) of 5:1, as previously optimized in chapter 5. Solid and liquid fractions were separated by pressing (up to 200 bar) with a manual oil press. The liquid fraction constitutes the hydrolyzate used for fermentation purposes.

7.2.3 Media preparation

7.2.3.1 Non-supplemented medium

The pH of the hydrolyzate was adjusted to 7 with $Ca(OH)_2$ and filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters. Subsequent sterilization was performed using sterile 0.22 μm membranes.

7.2.3.2 Supplemented medium

The pH of the hydrolyzate was adjusted to 7 with $Ca(OH)_2$ and then filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters. Yeast extract was added to achieve a concentration of 1 $g.L^{-1}$. Subsequent sterilization was performed using sterile 0.22 μm membranes.

7.2.3.3 Chemically defined medium (CDM)

A glucose solution (50 $g.L^{-1}$) was supplemented with yeast extract (1 $g.L^{-1}$) and the pH was adjusted to 7 with with $Ca(OH)_2$ and the medium was filtered with Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters. Subsequent sterilization was performed using sterile 0.22 μm membranes.

7.2.4 Kefir fermentation

Kefir inoculum was prepared in 50 ml of partially defatted milk using 0.5 g of freeze-dried inoculum (Ferments kefir, Lacto Labo, France). The culture was incubated at 28 ºC without agitation. After 48 h, the now solid broth (pre-inoculum) was homogenized and 1.5 mL of culture was used to seed 60 mL of each medium and incubated under the same conditions. All fermentations were performed in duplicate.

Subsequent fermentations were prepared in a similar way, seeding 1.5 mL of culture in 60 mL of the respective medium or milk.

7.2.5 Sampling

At determined intervals a 5 mL sample was taken aseptically for measuring the optical density and determining the dry weight.

7.2.6 Analytical methods

Hydrolyzate composition was determined according to standard NREL protocols as described in section 3.2.3.[11] Fermentation media consumption (monosaccharides, aliphatic acids, furan derivatives and glycerol) and products formation (ethanol and lactic acid) were monitored and quantified according to this protocol.

Quantitation of the kefiran-cells complex evolution was determined by gravimetric methods. An exact quantity (typically 4 mL) of the broth was filtered through previously dried and weighted Millipore® (Cork, Ireland) 0.45 μm cellulose acetate membrane filters and dried to constant weight in an oven at 105 ºC.

Fermentation evolution was followed by measuring the optical density of the cell broth against water at 600 nm in a JASCO (Tokyo, Japan) V-530 spectrophotometer, using plastic cells with 1 cm of optic path. The fermentation broth was diluted with water 5 to 10 times, as required, in

order to reach absorbance values between 0.1 and 0.8. Specific growth rates (μ_K) were calculated by determining the slope of the curves in the exponential phase of the growth.

7.2.6.1 Calculations

Kefiran-cells productivity (Q_K, g.(Lh)$^{-1}$) was calculated according to the following expression:

$$Q_K = \frac{Dry\ weight_{final} - Dry\ weight_{initial}}{V_{filtered} \times duration\ of\ the\ fermentation} \quad \textbf{(Equation 7-1)}$$

Kefiran-cells yield (Y_K, g/g of consumed sugars) was calculated according to the following expression:

$$Y_K = \frac{Dry\ weight_{fnal} - Dry\ weight_{initial}}{Total\ consumed\ sugars} \quad \textbf{(Equation 7-2)}$$

7.3 Results

7.3.1 Hydrolyzate composition

Sugars were the main compounds present (approximately 39 g.L^{-1}), being xylose the major sugar present (Table 7-1). Among the aliphatic acids, only acetic acid was detected in significant amounts. No formic or levulinic acids were detected. Furfural was the main furan derivative present (0.87 g.L^{-1}).

Table 7-1 Composition of the hydrolyzate obtained in optimized conditions

Compound	Concentration (g.L^{-1})
Glucose	9.81
Xylose	26.20
Arabinose	2.87
Acetic acid	3.56
Furfural	0.87
5-HMF	0.15

7.3.2 Kefiran production

Supplementation although favoring production and biomass yield should be avoided whenever possible in order to make the process economically competitive.[12] Based on this principle, the ability of kefir to grow in non-supplemented hydrolyzate was tested. Further, the adaptation of the strain to the hydrolyzate was also intended to be observed.

The production of kefiran was observed but it was not quantified as such: it was measured as kefiran-cells dry weight. The kefiran-cells productivity obtained in these assays for the supplemented and the non-supplemented hydrolyzates was 0.07 $g.(Lh)^{-1}$ and had a μ_K of 0.06 h^{-1}, whereas for the fermentation in CDM a μ_K of 0.03 h^{-1} and a Q_K of 0.01 $g.(Lh)^{-1}$ was observed. The fermentations using cherimoya hydrolyzate can be considered highly promising as the observed μ_K values are higher than the μ_{max} estimated in a previous study for the fermentation in milk, the traditional kefir production medium.[13] There was also a decrease in pH, namely due to lactic acid and ethanol production, whose presence was confirmed by HPLC analysis.

As it can be seen in figure 7-1, cell growth occurred, no significant lag phase was observed and stationary phase was reached in approximately 24h. While the two experiments using the hydrolyzate only slightly differ in terms of the adaptation period (the microorganisms took more time to adapt to the non-supplemented medium), a major difference was observed for the CDM. Initially, this fermentation followed the supplemented hydrolyzate pattern, but when the exponential phase started for the other media, after 3 hours, the growth simply kept the same pace.

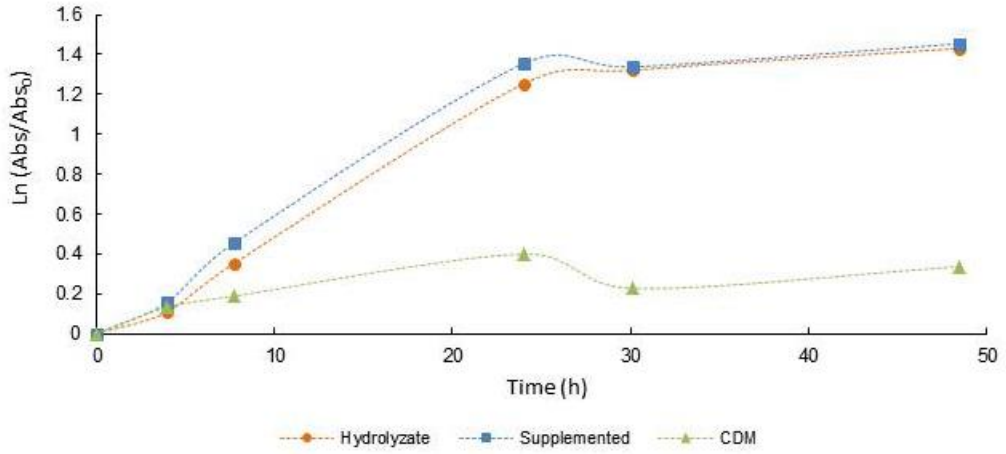

Figure 7-1 Growth profiles of Kefir grown in cherimoya seed hemicellulosic hydrolyzates and CDM (lines are used for eye guidance only)

Monosaccharide assimilation followed the pattern described in figure 7-2 for the fermentations in hydrolyzate media. Surprisingly, there seems to be a certain degree of preference to arabinose over xylose, even though xylose consumption is faster in an earlier stage. Almost no sugar consumption was observed in the fermentation in CDM, indicating that the slight growth observed in that fermentation was based on cellular carbon reserves. Kefiran-cells yield reached a value of 0.481 g/g of total consumed sugars.

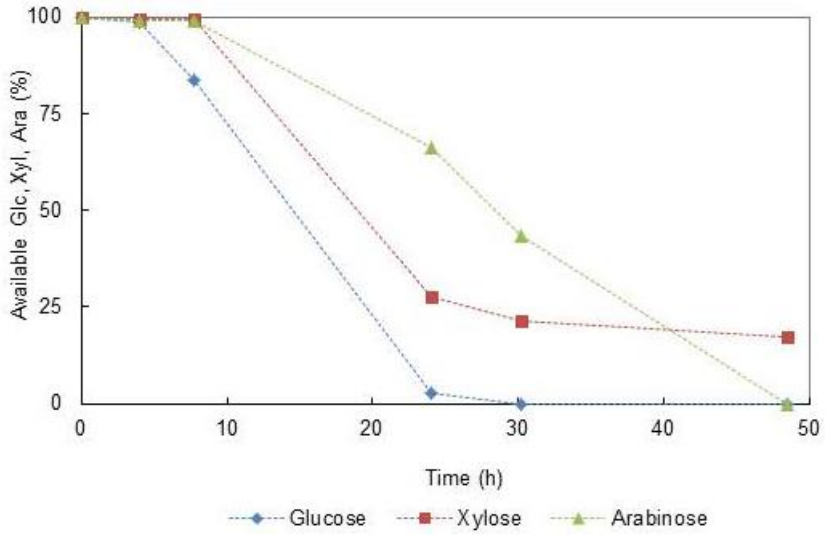

Figure 7-2 Monosaccharide assimilation pattern for Kefir grown in non-supplemented cherimoya seed hemicellulosic hydrolysate (lines are used for eye guidance only)

After reaching stationary phase, the culture was used to seed similar media and the same behavior was observed, with production of kefiran, ethanol

and lactic acid (except for CDM). Furthermore, the seeding of the culture in milk resulted in a solid broth similar to the pre-inoculum, a clear demonstration that the microbial consortium continues active.

7.4 Conclusions

These results are a proof of concept that kefir inoculum can be produced using hemicellulosic hydrolyzates.

Cell growth and kefiran production in cherimoya seed hemicellulosic hydrolyzate was found not to require any supplementation. Nevertheless, the impact of no supplementation on recurrent growth should be further studied, as the inoculum used in this work was produced in milk. Kefiran separation and characterization should also be studied; nevertheless, it should be kept in mind that the kefir grain (kefiran and microbial cells) is a product with many potential applications *per se*.

Cell viability was maintained (new inocula could be prepared from the previous fermentation after stationary phase without loss of productivity), promising its probiotic potential.

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CHAPTER 8

FINAL CONSIDERATIONS AND PROSPECTS

Summary

The global conclusions of the thesis are presented in this chapter, as well as some insights on possible strategies to proceed from this stage of the research.

The findings on the seed oil and its possible applications, and some breakthroughs on the aqueous treatments of the remaining cake are addressed. The lipid fraction and the lignocellulosic fraction are the main components of the seeds that can find further industrial applications.

Also, the results obtained for the use of the dilute-acid hydrolyzates as fermentation media are referred.

Some considerations on the contribution of this thesis to a broader picture are also made, as *A. cherimola* seeds can be one of the many biorefinery feedstocks available in Madeira.

The main goal of this work was to evaluate the potential *Annona cherimola* Mill. seeds as a feedstock, focused on waste valorization within a biorefinery facility. To include cherimoya wastes in an industrial installation requires the knowledge of the composition of the feedstock, which fractions can be used and what technological barriers must be overcome.

Cherimoya seeds, the most quantitatively significant waste from *A. cherimola* industrial processing, have very significant oil content. The results showed some variability between lots, not only in terms of oil quantity, but also in terms of its composition. Oil retrieval is, nevertheless, the first step to an integrated valorization. The optimization of the oil extraction procedure (chapter 3) is then a key factor, not only to obtain this product, but also to enable further processing of the remaining lignocellulosic fraction. It was possible to establish an optimal method to maximize oil extraction at laboratory scale and also to forecast the scale-up of the procedure taking into account some common industrial practices (*e.g.* solvent purity).

The obtained *A. cherimola* seed oil may have added-value applications, *e.g.* in the cosmetic industry, when used *per se* (which should be evaluated in future work). Nevertheless, its use in biodiesel production (as it is not considered as edible) should be addressed, considering the European Union (EU) directives on biofuels incorporation in automotive fuels. In this sense, cherimoya seed oil was characterized and converted in biodiesel, which was also characterized according to the European requirements for biodiesel quality (chapter 2). Most of the tested parameters were according to the requirements, except for oxidation stability, which can be overcome with antioxidant additives.

The quantity of biodiesel that can be produced from cherimoya seed oil is, however, reduced, due to the limited amounts of available feedstock. To overcome this limitation, other feedstocks for biodiesel production should be included in the biodiesel stream of the biorefinery.

After oil removal, a substantial lignocellulosic fraction (*ca.* 70%) of the seed remains available for further valorization. Several strategies can be followed from this point. Chemical hydrolysis of the hemicellulosic fraction is one of possible strategies.

Autohydrolysis renders a hemicellulosic oligosaccharides-rich liquor. These oligosaccharides can be recovered and their market demand has been increasing. The optimization of the autohydrolysis process and its kinetic modeling (regarding scale-up) was conducted (chapter 4). Furthermore, the hydrolyzate's shelf-life and oligosaccharides' stability (simulating the human digestive process or industrial processing) were evaluated, indicating the ability of the hydrolyzate to be stored and the possibility of processing the oligosaccharides. These oligosaccharides were also found to possibly be non-digestible, thus prone to be used as prebiotic agents. The confirmation of the prebiotic activity is therefore mandatory for classifying these oligosaccharides as prebiotic agents regarding their possible integration in food products.

The remaining cellulose-rich solid was saccharified and the obtained glucose yields indicate that the hydrolysis process is an adequate pre-treatment for further processing, namely to use as substrate for fermentative ethanol production or other bulk products (*e.g.* lactic acid). After saccharification, a lignin-rich solid is obtained and its characterization should be done in future work, as lignin can be a valuable source of bioactive compounds (namely phenolics).

Dilute-acid hydrolysis renders a monosaccharide-rich liquor, which can be used for fermentative processes. Based on an experimental design, the reaction time and acid concentration were optimized (chapter 5). The goal to maximize monosaccharides recovery, while minimizing microbial inhibitors formation, was achieved in as short-duration treatment. Reaction temperature effect should also be calculated to complete the study on this possibility of processing.

Several possible applications for the upgrade of the hemicellulosic monosaccharide-rich liquor are available. Among these, it's preferable to choose the ones that imply less processing (*e.g.* detoxification procedures) or supplementation, as much as possible.

Xanthan, an exopolysaccharide produced by the *Xanthomonas campestris* bacterium is widely used in various industrial sectors, including the food industries. Kefir, a microbial consortium used to produce a carbonated fermented beverage with the same name, is also used in the food sector, mainly in Eastern Europe. The dilute-acid hydrolyzate of cherimoya seeds was used as culture medium for these microorganisms (chapters 6 and 7, respectively for the former and the latter).

The four tested strains of *X. campestris* produced xanthan in appreciable amounts, whose composition is still to be unveiled, without any detoxification step. In this case, as the supplementation used is quite expensive and this issue must be assessed in order to make the process as cheap as possible. As so, the need for supplementation can also be the subject of further studies.

On the other hand, kefir fermentation occurred without any supplementation (and also without detoxification). Kefir grains were produced and cell viability was maintained. However, there is still room for improving the fermentation performance and issues like supplementation must be further addressed, taking into account economic viability, so that the supplementation costs do not surpass the economic gain.

It was possible to establish an integrated valorization strategy to upgrade one of Madeira's regional wastes (in a conceptual biorefinery), as it is forcibly necessary to fully understand a specific feedstock prior to its integration in a real upgrade solution. *Annona cherimola* Mill. is also produced in other areas of the globe, so this knowledge can be transferred to outside the regional sphere. This study can also be the starting point for the

development of a flexible industrial facility in the island, based on local natural resources and, most desirably, in regional wastes and byproducts.

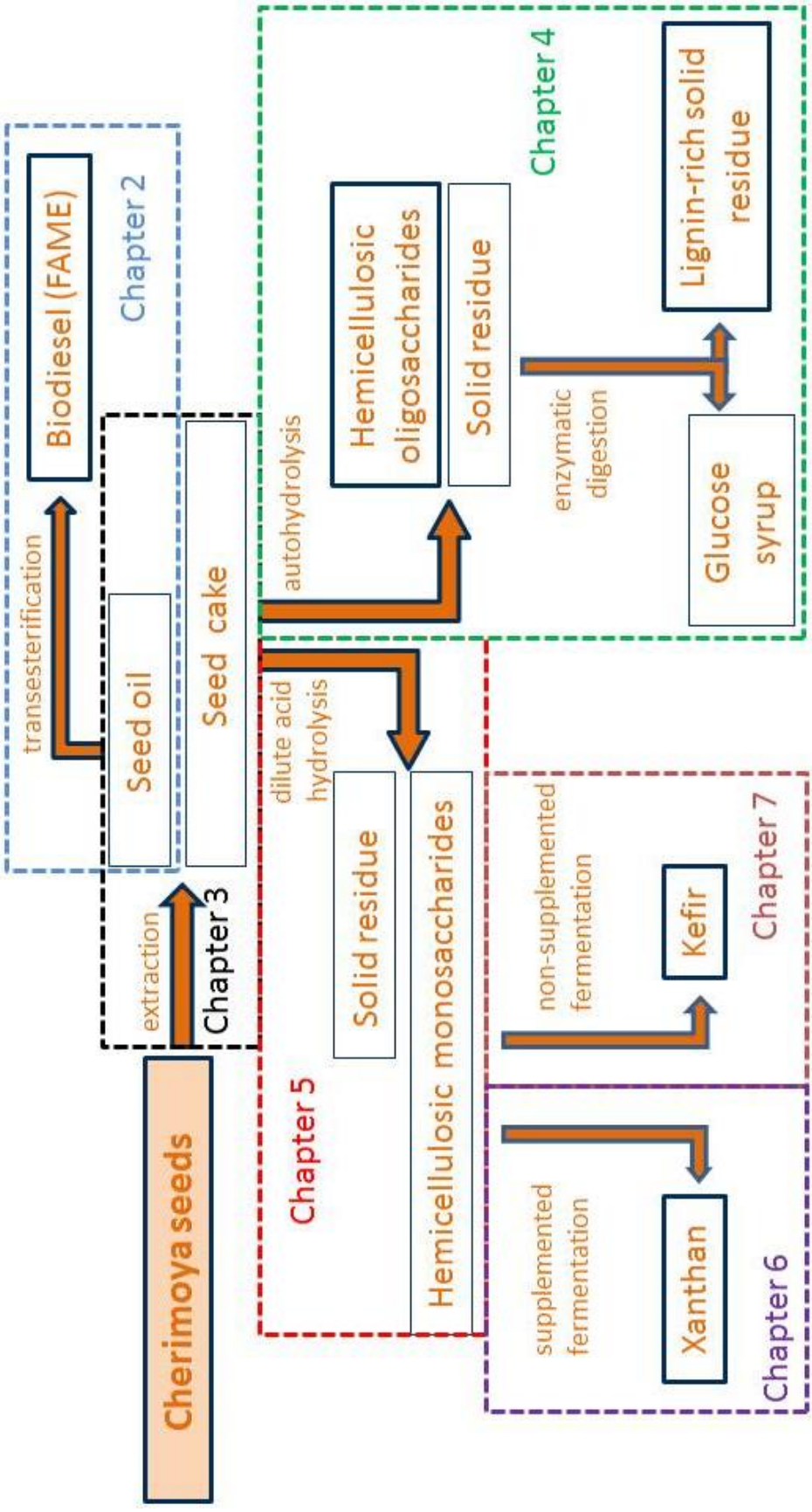

Figure 8-1 Proposed integrated valorization strategy for the upgrade of *A. cherimola* seeds