UNIVERSITÀ DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE

XXVIII CICLO

Hemp hurds biorefining for chemicals production

Chemical characterization, organosolv fractionation and enzymatic degradation for sugars exploitation

Docente guida: Prof. Loredano **Pollegioni**

Tutor esterno: Dott. Gianluca **Ottolina**

Tesi di dottorato di:
Stefano **Gandolfi**
Matricola 701257

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Abstract

Lignocellulosic biomass has been highlighted to be a sustainable and renewable feedstock for fossil source replacement. As in the petroleum refinery, the challenges are in making the cellulose fraction accessible to enzymes during enzymatic hydrolysis and maximizing the utilization of all the constituents within the feedstock. In a biorefinery facility the fermentation of the saccharidic fraction to liquid fuels or chemicals provide the gross value, whereas the valorization of the others constituents help to make the process economically viable. In this work the effective fractionation of hemp hurds (HH) into its three main components, cellulose, hemicellulose, and lignin, has been obtained by means of an organosolv pretreatment step followed by enzymatic hydrolysis of the pretreated HH. The effect of different process variables and the kinetic of enzymatic hydrolysis have been studied; the two steps were optimized to yield the highest amounts of fermentable monomeric xylose (from hemicellulose, C5) and glucose (from cellulose, C6). The aptness of C5 and C6 streams has been evaluated, in a fermentation process, by producing butanol and lactic acid, two of the most valuable platform chemicals for biorefinery. In particular, 42 g of polymer-grade lactic acid has been obtained from 100 g of raw HH. These results can be considered promising for HH valorization through the biorefinery concept.

Summary

Renewable production of chemicals can provide a great variety of benefits ranging from reducing environmental impact to development of a green economy, which bypass the use of fossil sources as feedstock. Research and innovation are needed along the entire development pipeline, beginning with the biomass sources that will serve as input, till the recognition of platform chemicals that could satisfy a sizable share of the market. Within this context, the development of green industrial processes for chemicals production is seen as a main pillar for bioeconomy growth.

Lignocellulose, the most abundant renewable biomass on earth, is composed mainly of cellulose, hemicellulose and lignin. Both the cellulose and hemicellulose fractions are polysaccharides and thereby a potential source of fermentable sugars. Among different lignocellulose residual, hemp hurds (HH) represents an overlooked by-product generated from hemp fiber extraction process. Compared to other lignocellulose source, HH is considered a promising feedstock for biorefinery because of its high carbohydrate and low ash content. Moreover, its monosaccharide composition is less heterogeneous than other biomasses; glucose and xylose accounted for 57 and 31% of total HH sugars, respectively. This is of great importance in fermentations of lignocellulose-derived sugar streams, as many microorganisms show carbon catabolite repression phenomena.

Due to the complexity and packed structure of lignocellulose material, hydrolysis of hemicellulose and cellulose into pentose and hexose (C5 and C6) has to be carried out prior to the fermentation. Different approaches to overcome lignocellulose recalcitrance have been studied; however, the release of sugars from the lignocellulosic feedstock is still the main

bottleneck of modern biorefineries. Among deconstruction technologies, the most efficient scheme includes a pretreatment step in which the cellulose polymers are made accessible, followed by an enzymatic step in which cellulose is hydrolysed to fermentable sugars using cellulase enzyme cocktails.

Pretreatment technologies are described based on the mechanisms involved and encompass: biological, mechanical, chemical methods and various combinations thereof. In this step hydrolysis of hemicellulose, as well as removal and separation of lignin, are the main objectives. Moreover, the easy isolation and high-yield recovery of fractionated components are also required to improve the economy of the process. Organosolv pretreatment (OS) provides a highly effective method for biomass fractionation as it allows the selective separation of lignin, cellulose and hemicellulose in three distinct streams. In order to extract the lignin and hydrolyse the hemicellulose, in the OS treatment biomass is heated in a hydrorganic solution in the presence of a low amount of acid acting as a catalyst. Temperature used for the process can be as high as 200 °C, whereas solvents include ethanol, methanol, acetone, and ethylene glycol. Sulfuric acid is the most frequently used catalyst for the OS process; however other mineral or organic acids such as hydrochloric acid, phosphoric acid, formic acid and oxalic acid are employed. The change in OS pretreatment severities (CS), by modifying process variable (i.e. temperature, catalyst, reaction time and solvent concentration), resulted in a group of pretreated HH with different cellulose hemicellulose and lignin content, which in turn affected the degree of enzymatic hydrolysis. Interestingly, the recovered amounts of solid pretreated HH and its components composition show a good correlation to

the CS applied. Similarly, the amount of solubilized hemicellulose and lignin in the process liquor well correlated with the pretreatment severity. Nevertheless, the catalyst concentration played the biggest effect on hemicellulose solubilization and on the production of sugars dehydration compounds (i.e. furfural and HMF). For most feedstocks, the cellulose-rich substrate produced during OS pretreatment results in high glucose yields after enzymatic hydrolysis. Compared to untreated HH, an eight-fold increase in enzymatic hydrolysis was appreciated for pretreated samples, which rises as a function of the CS.

However, main challenges of the enzymatic hydrolysis of cellulose include lower rate of hydrolysis, high cellulase loading and poor knowledge about the cellulase kinetics on lignocellulosic substrate. The time course of enzymatic hydrolysis of pure cellulose and pretreated HH exhibited a fractal-like kinetic behavior and the analysis of fractal parameters disclosed the positive effect of OS pretreatment. The increase in rate constant as a function of CS indicated an improved substrate accessibility towards cellulolytic enzymes.

Under optimized pretreatment and enzymatic hydrolysis optimized conditions (in terms of the highest sugars recovery) a yield of 0.29 g of glucose (C6-stream) and 0.17 g of xylose (C5-stream) per gram of raw HH was obtained. Moreover, the generated C5 sugar stream contains a low amount of microorganisms' inhibitory compounds.

Although fermentation of sugars to usefully platform chemicals is a relatively robust and efficient industrial process, the exploitation of lignocellulosic-derived ones is the achievement of this century. Fermentability of HH C6 and C5 sugars streams has been demonstrated by

producing *n*-butanol and lactic acid as platform molecules for fuel and bio-plastic production, respectively. In particular, the fermentative production of lactic acid by the *B. coagulans* strain seemed promising since high conversion yields and product titers were obtained from both C5 and C6 sugars streams. Moreover, the selected strain showed favorable features such as high substrate concentration tolerance, low nutritional requirements, thermostability and high L-lactic acid enantiomeric excess.

The results of this PhD project disclosed the potential of HH as suitable feedstock for biorefinery purposes. The developed biotechnological process for HH upgrading into valuable platform chemicals, provided useful information concerning the control of the lignocellulose fractionation process. Such information can be translated to other lignocellulose material and will help to improve the sustainable growth of the Green Economy.

Riassunto

L'odierno modello di sviluppo economico, caratterizzato dallo sfruttamento intensivo delle risorse fossili, ha generato serie problematiche sia a livello ambientale sia a livello economico. La crescente consapevolezza riguardo questa tematica ha portato allo sviluppo del concetto di "Green Economy" quale modello vincente per uno sviluppo sostenibile e in grado di preservare la qualità ambientale ed economica. Gli elementi centrali di tale modello sono sia l'utilizzo di risorse rinnovabili come materia prima, sia l'integrazione di processi biotecnologici nel settore energetico sia in quello chimico. Tra le fonti di energia rinnovabili, la biomassa di origine vegetale rappresenta la fonte più abbondante di carbono disponibile sul nostro pianeta ed è considerata neutrale ai fini dell'incremento delle emissioni di gas ad effetto serra.

La piattaforma biotecnologica grazie alla quale le biomasse sono trasformate in energia, combustibili, prodotti chimici di base, biopolimeri e bioplastiche è definita bioraffineria. Attraverso il processo di bioraffinazione gli zuccheri contenuti nella biomassa sono estratti e fermentati mediante l'impiego di un ampio spettro di processi biologici che comprendono l'utilizzo di ceppi microbici ed enzimi, a dare differenti prodotti d'interesse industriale. I principali bioprodotti derivati dalla fermentazione del glucosio comprendono: etanolo, butanolo e acidi organici (acetico, lattico, succinico, propionico, itaconico e glutammico), questi ultimi costituiscono le piattaforme per la sintesi di polimeri biodegradabili in grado di sostituire materiali plastici convenzionali, mentre l'etanolo viene ampliamente utilizzato come combustibile liquido. Tra le biomasse utilizzabili per i processi di bioraffinazione, è di notevole interesse, sia economico che

scientifico, l'impiego, come materia prima, degli scarti lignocellulosici provenienti da processi agricoli o industriali. Il principale ostacolo nel loro sfruttamento è la scarsa accessibilità della frazione polisaccaridica all'azione idrolitica enzimatica mediante cellulasi. Questo fenomeno (conosciuto con il termine di recalcitranza del materiale lignocellulosico) è superato mediante l'impiego di un pretrattamento, il cui scopo è quello di destrutturare la matrice lignocellulosica e rendere la componente polisaccaridica più suscettibile all'idrolisi enzimatica.

In questo progetto di Dottorato, uno scarto lignocellulosico, proveniente dal processo di estrazione della fibra dalla canapa, il canapulo (HH), è stato impiegato come materia prima per la produzione biotecnologica di acido lattico e butanolo, seguendo il concetto di bioraffineria. Partendo da una dettagliata caratterizzazione chimica del materiale, che ha evidenziato un elevato contenuto polisaccaridico, è seguito uno studio sistematico del processo di pretrattamento al fine di ottenere la massima resa di zuccheri fermentabili (glucosio e xilosio). A tale scopo è stato impiegato un pretrattamento basato sul processo organosolv in quanto, questo metodo, permette di separare le tre principali componenti del materiale lignocellulosico (cellulosa, lignina ed emicellulosa) in tre frazioni distinte. La differente resa di frazionamento di HH, ottenuta variando le condizioni di processo (temperatura, concentrazione di acido e tempo di reazione) è stata correlata alla severità del processo (CS), mentre la frazione solida residua, arricchita nella sua componente cellulosica, è risultata fino ad otto volte più suscettibile all'idrolisi enzimatica rispetto a HH. Anche in questo caso si è osservata una correlazione tra il grado d'idrolisi e il CS. La variazione del grado d'idrolisi è stata studiata mediante la cinetiche di reazione, utilizzando

come substrato campioni di HH diversamente pretrattati e cellulosa pura; a tal fine è stato applicato un modello frattale. L'analisi delle variabili del modello frattale (costante di velocità "*k*" ed esponente frattale "*h*") ha permesso di attribuire l'incremento d'idrolisi a un'aumentata accessibilità enzimatica al substrato. Questo fenomeno è evidenziato dall'incremento del valore della costante di velocità *k* e dalla diminuzione della costante *h* in funzione del contenuto di lignina presente nel campione.

La massima resa di zuccheri fermentabili, sia pentosi (C5) che esosi (C6), ottenuta ottimizzando le due fasi (pretrattamento e idrolisi enzimatica) ha permesso di ottenere 0.17 g di xilosio e 0.29 g di glucosio per grammo di HH. Inoltre, in queste condizioni, si sono ottenute basse concentrazioni di composti di degradazione da zuccheri e lignina nella frazione C5, quali: furfurale, HMF, acido levulinico, acido acetico e acido formico. La fermentabilità delle frazioni C5 e C6 è stata quindi valutata producendo *n*-butanolo come esempio di bio-carburante e acido lattico come composto d'interesse per il settore bioplastico. In particolare la produzione di acido lattico, ottenuta impiegando un ceppo selezionato di *B. coagulans*, ha mostrato sia elevate rese di conversione e produzione volumetrica sia elevati eccessi enantiomerici di prodotto (L-acido lattico), utilizzando entrambe le frazioni.

Concludendo, i risultati ottenuti durante questo progetto di Dottorato hanno dimostrato che il canapulo, grazie al suo elevato contenuto in polisaccaridi, è una biomassa adatta per la produzione biotecnologica di composti chimici di base per l'industria chimica. Lo studio sistematico delle condizioni di pretrattamento e idrolisi enzimatica ha permesso di ottenere importanti informazioni sul controllo del processo di destrutturazione, frazionamento e

idrolisi del canapulo. Questo modello potrebbe essere esteso ad altre fonti lignocellulosiche al fine di sviluppare filiere agroindustriali pienamente sostenibili, sia a livello ambientale che economico.

Contents

1 Introduction

1.1 Sustainability and Bioeconomy

Globalization, population growth, rise of emerging economies and increase of living standards in industrialized country have had resulted in the exploitation of natural resources to their limits and an ever increasing contamination of the environment. Thanks to the UN Stockholm Conference in 1972, together with the first oil crisis in 1973, a strong socio-political debate on how human activities were harming the environment and putting humans at risk has been started. Few years after, the World Commission on Environment and Development formulated the concept of "sustainable development" that meant to provide a long-term balance between the environment, the economy and the social well-being, creating a better quality of life for future generations [1]. This concept became the catalyst for global thinking processes about the relationship between man and nature and about future prospects of mankind in the potentially conflicting contexts of ethics, state policies and social, ecological and economical interests [2].

Nowadays the "sustainable development" vision has been embedded into the bioeconomy concept and green chemistry principles, which ultimately have to address the grand challenge being faced by society: food and energy insecurity, resource constrains and climate change [3].

Bioeconomy is seen as a set of economic activities relating to the invention, development, production and use of renewable products and environmental friendly processes. The bioeconomy encompasses the production of renewable biological resources and their conversion into food, feed, bio-based products and bioenergy *via* innovative and efficient technologies provided by industrial biotechnology. These are the key objectives of the Organization for Economic Co-operation and Development (OECD)

members, as set out in last report relative to economic growth and welfare [4]. In this report the emphasis is on: sustainable uses of natural resources, decouple economic growth from fossil feedstock, increases competitiveness and reduce CO2 emissions. As response, OECD members have put forward strategies for building a sustainable bio-based economy by national and international policies [5,6].

On the one hand, climate protection is one of the most relevant socio-political drivers globally for bioeconomy. According to The International Panel on Climate Change (IPCC) anthropogenic greenhouse gas emission (GHG), such as carbon dioxide (CO2), methane (CH4) and nitrous oxide (N2O), are increasing and there is clear scientific evidence that fossil oil refinery and combustion processes are the main GHG-cause. Over the past few years, the transportation sector has shown the highest growing rate in GHG and by 2030 emission from transport is predicted to be 80% higher than current levels [7]. On the other hand, fossil resource dependence, security and crude oil shortage, are the main political and economical drivers. Fossil feedstocks have served, and continues to serve mankind demands for energy, materials and synthetic substances in a un-ecofriendly and unsustainable fashion way. In 2014, crude oil consumption around the world was approximately 95 million barrels (mb) day^{-1} [8]. As emerging economies become more successful and industrialization increase, crude oil production is predicted to rise to approximately to 110 mb day^{-1} by 2040 [9]. Following this trend, production is expected to not be able to meet the demand. Therefore, considering the needs of population growth with the resulting impact on environment, the dependence on of fossil feedstock has to be redirected through renewable source.

Although for electricity and heat a variety of renewable alternative could be established (e.g. wind, solar, hydropower, geothermal and nuclear) these options do not produce liquid fuels and chemicals, which represent roughly 38% of the total global energy demand.

Within this context, an approach that has begun to receive much attention is using biomass as feedstock, in particular lignocellulosic are the most attractive renewable carbon source in terms of sustainability. Use of renewable rather than depletable feedstock is one of the green chemistry principles and biomasses are already contributing to an extent of 6% of the total liquid fuels production [3,10]. The replacement of fossil-based carbon with renewable carbon from biomass leads to the development of biorefinery facilities, where transportation biofuels, bioenergy, biochemicals, biomaterials, food and feed are efficiently co-produced [11].

Both bio-based fuel and chemicals generate expectations first, to access sustainable feedstock, second, to reduce the industrial carbon footprint, third, implement economically advantageous processing chains and fourth, start a bio-based innovation cycle. Today the development of new processes for fuels and chemicals from lignocellulosic feedstocks represents an extremely important field for research and development, and industrial innovation [12]. At the same time, venture capital and government funds are available and have been used by innovative companies working on biotech, biochemical, and thermochemical processes.

1.2 Biorefinery

A biorefinery can be considered to be an integral unit that can accept various biological feedstocks and convert them into a range of useful products including chemicals, energy, and materials. Among several definitions of biorefinery, the most exhaustive was formulated by International Energy Agency (IEA) Bioenergy Task 42: "*Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy*" [13].

The concept of producing products from biomass is not new. During the second half of the nineteenth century large-scale industrial conversion of biomass to chemicals and materials has been established to produce cellulose esters (nitrate and acetate), oxidised linseed oil (linoleum), furans (furfurol), levulinic acid and ethyl alcohol as well. Nevertheless, using biomass to produce multiple products through the integrated biorefinery concept is relatively new. An integrated, close-to-zero-waste system would exploit a sequential process of extraction followed by a combination of biochemical and thermal processing that includes internal recycling of energy and waste gases [14]. The challenge is to use green chemical technologies to ensure maximum conversion efficiencies and minimal waste to produce high-value low-volume (HVLV) and low-value high-volume (LVHV) products using a series of unit operations (Fig. 1) [15].

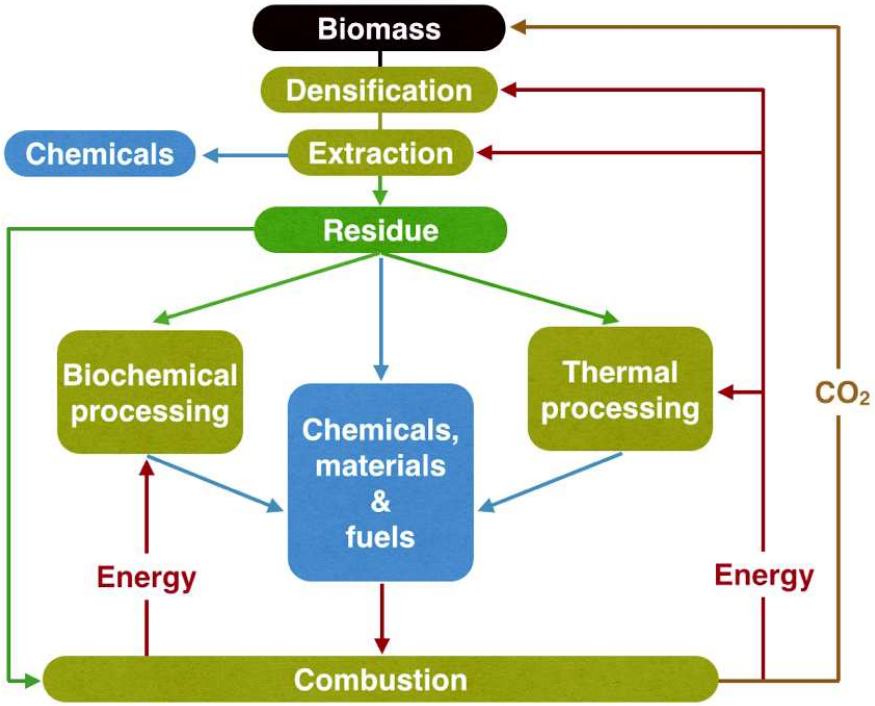

Figure 1. Scheme of an integrated zero-waste biorefinery system.

The identification of the most promising product portfolio with the most attractive economic potential is trivial as different processing option and combinations are possible. According to Cherubini et al., biorefining should be described as a conversion pathway from feedstock to product *via* platform and processes (individual system) [11]. In this way, the different network of individual system, which usually occurs, could be easily classified according to the main four features (feedstock, product, platform and process).

Examples of biorefinery classification include: C6 sugar biorefinery yielding ethanol and animal feed from starch crops, syngas biorefinery yielding FT-diesel and naphtha from lignocellulosic residues C6 and C6/C5 sugar and syngas biorefinery yielding ethanol, FT-diesel and furfural [11].

1.3 Biomass as feedstock

Biomass is the biological material derived from living, or recently living organisms. In the frame of its use as energy resource, most often biomass is referred to plant-based materials. Besides providing food and energy, biomasses were employed throughout recorded history to extract valuable products such as medicinal drugs and flavours and fragrances [16]. Nowadays biomass is still considered important as it is readily available in high quantities, is renewable and is cheap. Chemically speaking, biomass can be grouped into two wide categories: oleaginous feedstock and carbohydrates feedstock [17]. Both are of importance for biorefineries.

Carbohydrates, the most abundant component found in the plant biomass, are molecules formed of carbon, hydrogen and oxygen. As energy driver, carbohydrates are usually associated to the fermentative ethanol production. Mono and polysaccharides are the two groups in which carbohydrates are divided. The first one includes C6 sugars (e.g. glucose, galactose and mannose), and C5 sugars (e.g. xylose and arabinose) and is the less abundant in nature. Typical source of monosaccharide are sugarcane and sugar beet. Differently, polysaccharides are widespread in plants. This class includes starch, cellulose and hemicellulose. Starch is composed of α-glucose molecules linked through α-1,4 bondings with branches that takes place with α-1,6 bonds and is the most common carbohydrate in human diets. Starch has a semi-crystalline structure, which swell burst in hot water. This is of importance, as it could be easily saccharified into glucose.

Cellulose and hemicellulose, which are different polysaccharides, together with lignin are the main components of lignocellulosic material. Lignocellulose refers to plant dry matter and is the most abundantly

available raw source of carbon on the Earth. Large amounts of lignocellulosic biomass can be produced via dedicated crops like perennial herbaceous plant species, or short rotation woody crops. Other sources of lignocellulose biomass are wastes and residues, like straw from agriculture, wood waste from the pulp and paper industry and forestry residues. This material is recently awakening much interest as renewable and cost effective source of fermentable sugars and aromatics structures. However, components fractionation is not easily.

1.3.1 Lignocellulose structural features

More than 35 different cell types can be found in plant; however, all are characterized by the presence of a thick (0.1 to 10 μm) cell wall that provides rigidity [18]. The cell walls are composed of three layers, the middle lamella, primary cell wall, and secondary cell wall (Fig. 2). Nevertheless, the primary structural materials found are cellulose, hemicellulose and lignin. Others constituents that can be found are pectin, proteins, extractives and ash but usually they do not exceed 10% of total dry weight. All these components are present in different proportions. Outer wall (primary wall) is composed mainly of lignin while the inner (secondary wall) contains the majority of the carbohydrates. Additionally, chemical composition varies with the plant species, age, growth conditions and with certain parts of the plant [20].

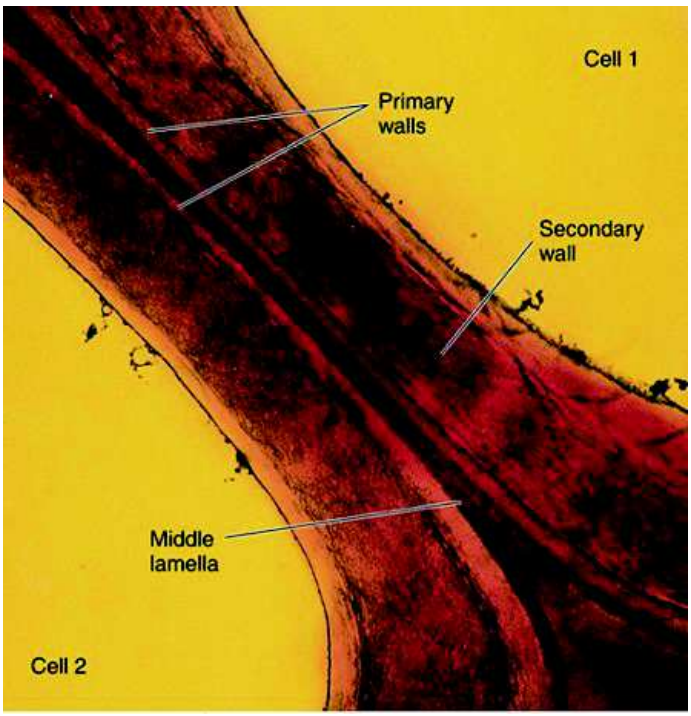

Figure 2. Plant cell walls structure [19].

1.3.1.1 Cellulose

Cellulose, the major structural component of plant cell wall consist of repeated cellobiose, an oligomer of two anhydrous D-glucose units joined together with β-1,4 glycosidic bond units. The glucose content in cellulose, which represents its degree of polymerisation DP, typically ranges between 100 to 10000 molecules [21]. Cellulose polymers are parallel linked together with hydrogen bonds and van der Waal's forces, forming the fibrils. The elementary fibrils are again attached to other plant cell wall components (e.g. hemicelluloses, pectin and covered with lignin). This compact form of cellulose bundles is referred to as cellulose microfibrils, and provides mechanical strength and chemical stability to the plants [22]. Several of cellulose microfibrils are often associated together in the form of macrofibrils (Fig. 3). The steric hindrance of cellulose fibers is responsible for the low saccharification rate of cellulose [23]. Cellulose is generally insoluble in water and common organic solvents due to its crystalline structure, but it also has some soluble amorphous, regions in which the molecules are less ordered [24]. These regions are less compact and more easily hydrolyzed by cellulases enzymes [25].

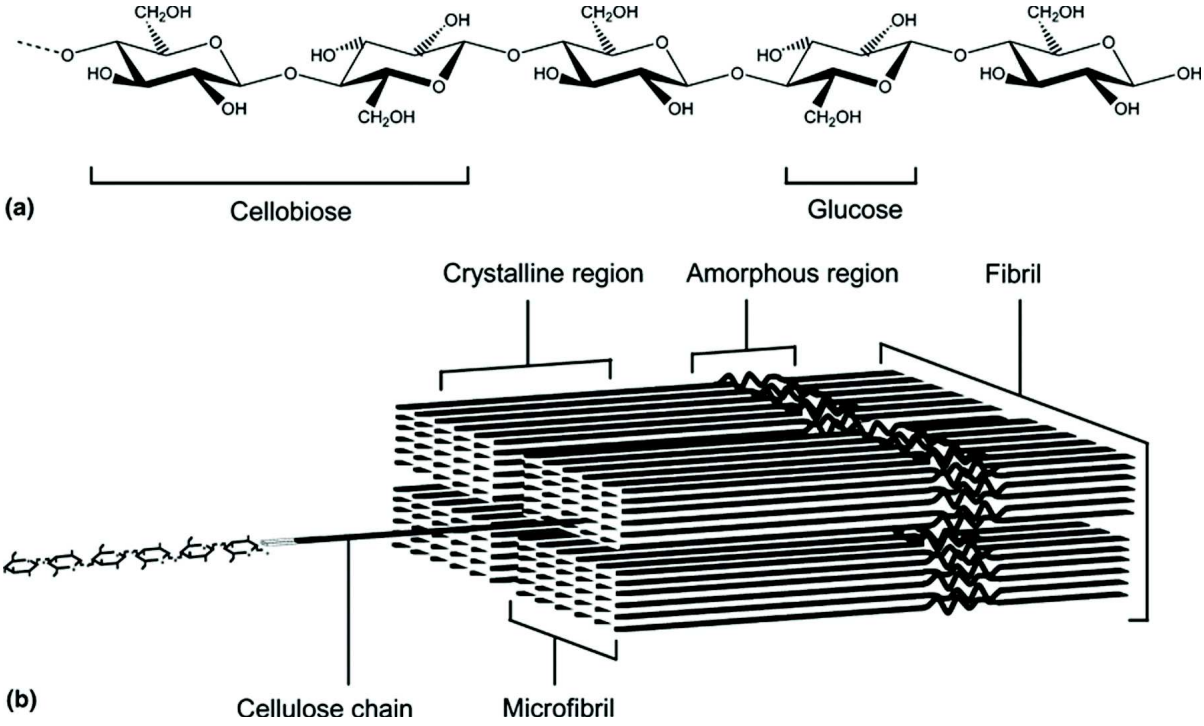

Figure 3. Primary structure of cellulose (a). Structure of a cellulose fibril (b). [26].

1.3.1.2 Hemicellulose

After cellulose, hemicellulose is the second major carbohydrate constituent of lignocelluloses [27]. Hemicellulose is composed of short-chain linear and branched heterogeneous sugar polymers, typically made up of five different pentose (L-arabinose and D-xylose) and hexose (D-galactose, D-glucose and D-mannose) sugars. Other sugars, such as L-rhamnose and L-fucose, organic acids such as acetic, 4-O-methyl glucuronic, galacturonic and ferulic acid, may also be present in small amounts. The hydroxyl groups of sugars can be partially substituted with acetyl groups [27]. These complex heteropolysaccharides can be classified into four structurally distinct classes: xylans (β-1,4-xylosyl backbone with arabinose, uronic acid, and acetyl side chains), mannans (β-1,4-mannosyl or glucosyl-

mannosyl backbones with galactose side chains), β-glucans with mixed linkages (*β*-1,3-1,4-glucosyl backbone), and xyloglucans (*β*-1,4-glucosyl backbone with xylose side chains) (Fig. 4) [28]. Unlike cellulose, hemicellulose composition and structure varies depending on their source. Moreover, hemicellulose DP consists between 70 and 200 thus being an amorphous polymer and easily degradable [25].

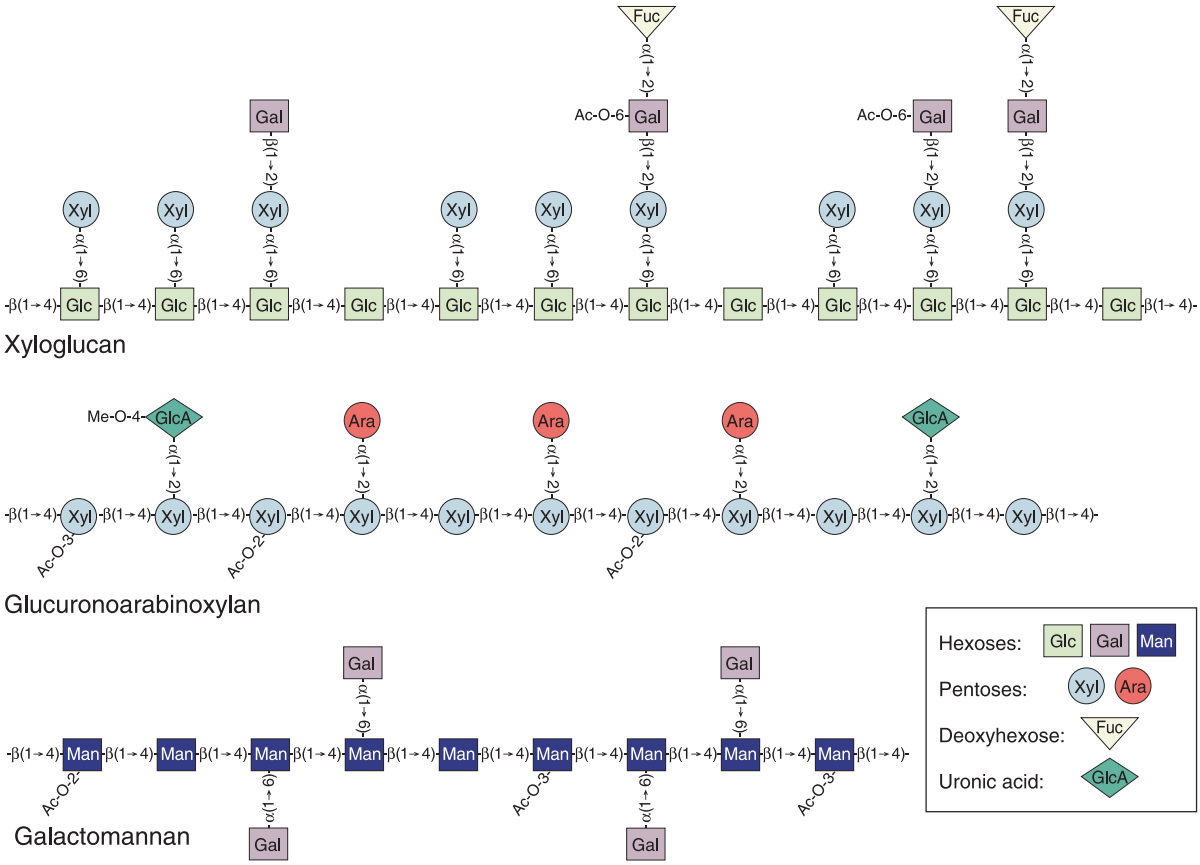

Figure 4. Schematic representation of hemicellulose structures in the plant cell walls [29].

1.3.1.3 Lignin

Lignin is by far the most abundant substance composed of aromatic moieties in nature and is one of the most abundant organic polymers in plants [30]. Its structure is amorphous and irregular. The combinatorial oxidative coupling of three main monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols, differing in their degrees of methoxylation, produces lignin. [31]. When incorporated into the lignin polymer, these monolignols produce differently linked *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units, respectively, generating a variety of structures within the polymer, including alkyl-β-aryl ethers, phenylcoumarans, resinols, spirodienones and dibenzodioxocins, among others [32]. About 70% of the monolignols linkage consist of phenol-ether bonds, alkyl-ether bonds, dialkyl bonds and diaryl ether bonds the are carbon-carbon couplings, which includes β-5, β-β, β-1, β-2 and 5-5 linkage [33]. The lignin composition varies between plants from different taxa and even between different tissues and cell-wall layers from the same plant. Generally, lignin from hardwoods is composed of S and G units in different ratios, whereas lignin from softwoods is composed essentially of G units with minor amounts of H units, and lignins from grasses contain the three units, with H-units still comparatively minor [31].

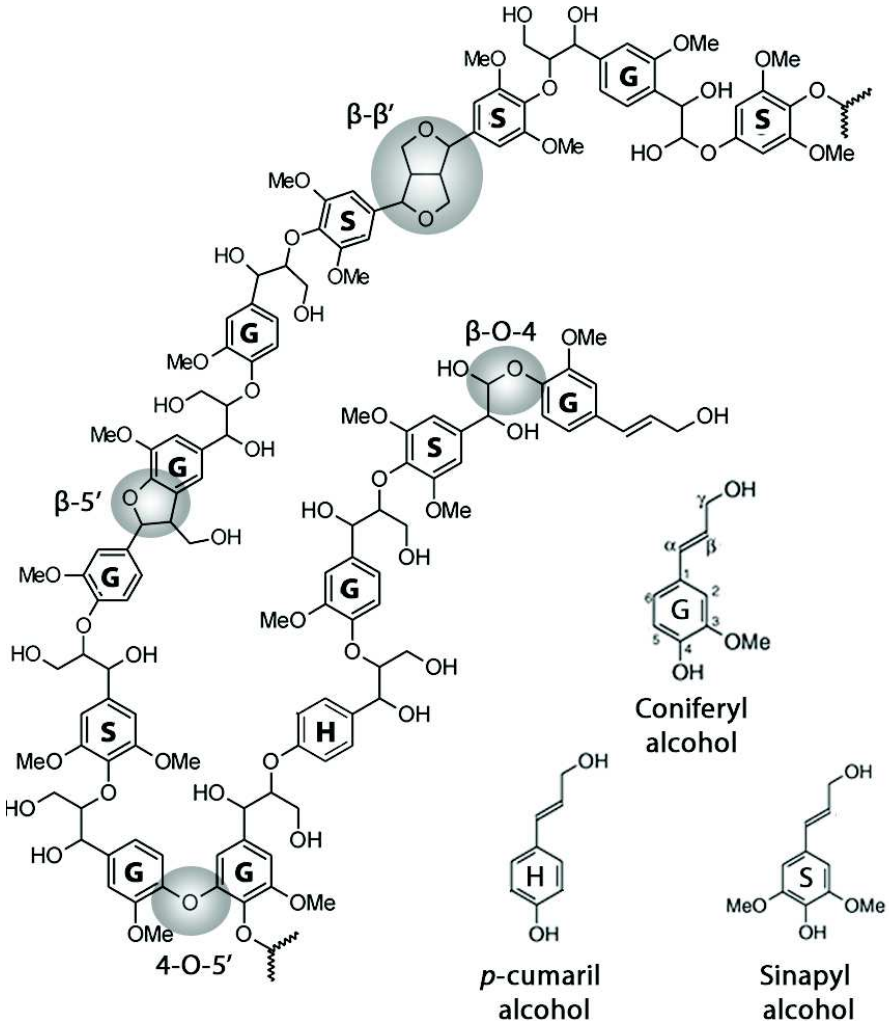

Figure 5. Schematic representation of plant cell walls lignin structure.

1.3.1.4 Other constituents

Lignocellulose cell wall also contain some other substances such as pectin, extractives e.g. terpenoids, steroids, fats, waxes, and phenolic constituents proteins, and ashes. Pectin, is composed of acidic sugar, usually galacturonic acid [38]. Pectin's are highly branched and complex heterogeneous polysaccharides composed of different subclasses: homogalacturonan, rhamnogalacturonan, and xylogalacturonan. They

functions in cell adhesion and wall hydration, and their crosslinking influences wall porosity and plant morphogenesis [39].

1.3.1.5 Chemical interaction between components

Although the chemical structures and compositions of plant cell wall polymers are well known, how these wall polymers form a three-dimensional network to provide mechanical strength to the wall is still poorly understood. As described earlier, in lignocellulose, cellulose acts as a skeleton of the structure. The current models for cell walls envision is cellulose microfibrils surrounded by a matrix of hemicellulose and lignin (Fig. 6) [23].

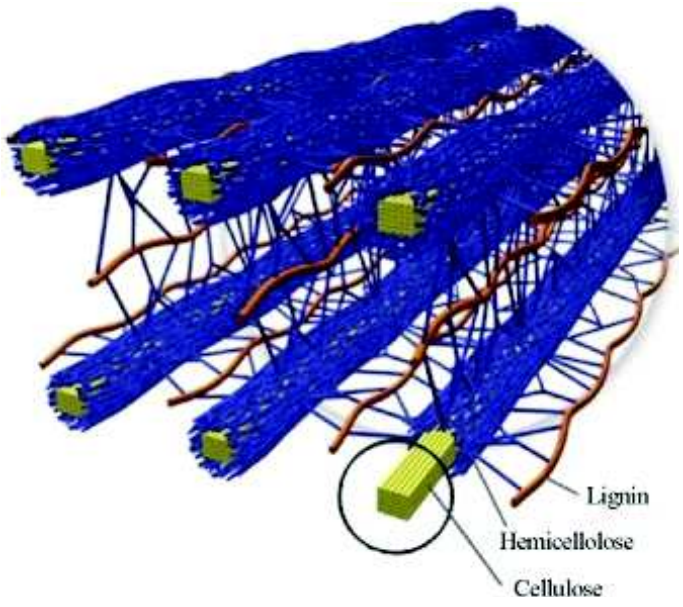

Figure 6. Representation of the network structure of lignocellulose [40].

Between these three components, intrapolymer linkages are identified. The most common are the cellulose-xyloglucan complex. Cellulose microfibrils are organized in successive lamellae, forming a web-like matrix, that are

separated by hemicellulose and pectin that control the overall wall porosity. Unbranched hemicellulose (xyloglucans, homoxylans, and mannans) forms hydrogen bonds with the surface of cellulose fibrils, whereas the side chains of the branched fraction e.g., uronic acids and arabinose units are covalently bonded to lignin to create enzyme-impenetrable cross-links, the lignin carbohydrate complexes (LCCs) [41,42]. Lignin and polysaccharide complexes (LCCs) are primarily composed of ether and ester bridges and lignin is connected to hemicellulose *via* ester bonds [43]. LCCs are thought to form inclusion complexes that exclude water and prevent chemical or enzyme-catalyzed deconstruction of cell walls [23].

1.3.2 Hemp

Hemp is one of the fastest-growing crops in the world and it comprises a number of varieties of *Cannabis sativa L.* that are traditionally grown for drugs, fibers and seeds. In Europe, has been the most important fibre crop from the 16th to the 18th century. However, nowadays hemp is increasingly comes to be seen as valuable crop thanks to its suitable agronomy feature. Among different crop hemp is a better biomass yielding species, it has less fertilizers and water requirements and is useful as rotational crop [44,45].

In technical hemp stalk consists of approximately ~30% bast fibers and 70% hurds. Fiber content has high cellulose and low lignin and hemicellulose values, whereas in hurds 40-50% α-cellulose is usually observed [46]. The global market for industrial hemp is potentially high and in Fig. 7 is show a summary of the obtainable products and of the possible uses of hemp [47].

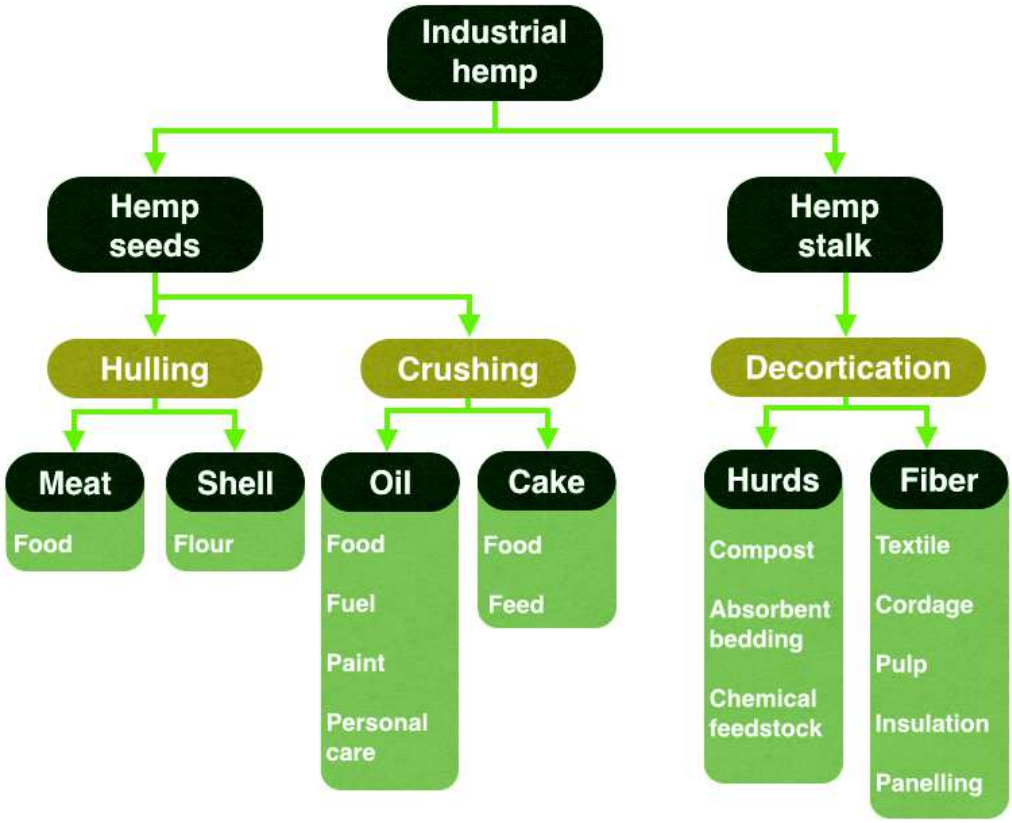

Figure 7. Flowchart of hemp products. Adapted from [47].

For high quality textile-production hemp plants are grown up to four meters, stalks are maintained in bundles during collection and, finally trough retting fibre are extracted. Two different methods can be applied; water retting is used to obtain textile quality fibers while dew retting allows obtaining low quality fibers [47]. After retting, the separation of the bast fibre is carried out through scrutching (breaking the woody core of the stalk into short pieces) and decortication.

Hemp hurds (Fig. 8) is the residual material obtained after fibre extraction and has only minor applications. Hemp hurds has high water-absorbing ability and thus is commonly used as such as animal bedding, garden mulch or in light-weight concrete [48]. However, different studies are now focused on its application as feedstock for biorefinery [46,49,50].

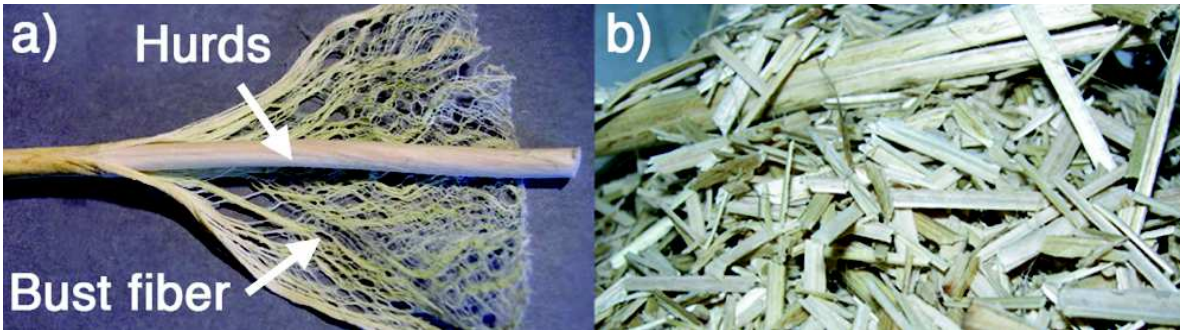

Figure 8. Hemp stalk (b). Hemp hurds (b).

1.4 Biorefining of lignocellulosic biomass

In the near-term, lignocellulosic biomass likely to be the primary feedstock for deconstruction to reactive intermediates i.e. sugars and phenolics, which can be upgraded to fuels and chemicals. Lignocellulosic waste has crucial advantages over other biomass supplies because they are the non-edible portions of the plant and therefore they do not interfere with food production chain. Moreover, forestry, agricultural and agrindustrial lignocellulosic wastes are accumulated every year in large quantities [51]. From the economic point of view, lignocellulosic biomass can be produced quickly and at lower cost than other agriculturally feedstocks, such as cornstarch, soybeans and sugarcane. Relative to petroleum refining, lignocellulosic biomass conversion offers new logistic and scientific challenges. First, because lignocellulose has a lower density than lower crude oil (80-150 kg m-3 for herbaceous 150-200 kg m^{-3} for woody biomass and 800-900 kg m^{-3} for crude oil) [18]. So far, pelletization and briquetization are commonly used option; these methods also offer a solution for storage, loading, and transportation [52]. However, the correlated energy cost should be considered. Second lignocellulosic, in molar terms, have

much lower carbon, hydrogen and higher oxygen content than conventional crude oil (Fig. 9) [18]. Third lignocellulosic components fractionation is trivial due to its recalcitrant structure.

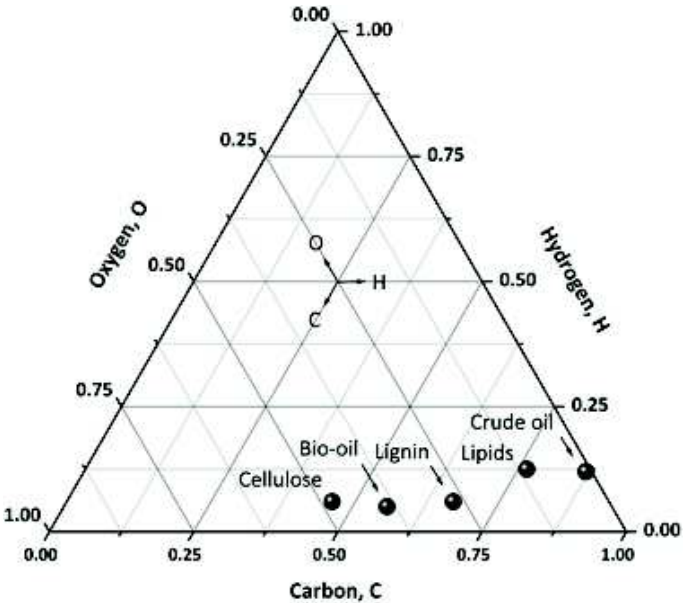

Figure 9. Ternary diagram showing the mass composition of usual biomass-derived raw materials [18].

Energy densification of lignocellulosic biomass can be achieved by thermochemical or by biochemical processes. Thermochemical conversion is typically delineated into two regimes based on the operating temperature of gasification and pyrolysis, which use heat and pressure produce synthesis gas and bio-oils, respectively [27]. The advantages of thermochemical conversion are low residence time and the ability to handle varied feedstock in a continuous manner. Different, biochemical conversion route uses low thermochemical treatment to weak down the cell wall structure and to obtain a more prone polysaccharide fraction to enzymatic attack. The process yields fermentable sugars, which could be upgraded into useful chemicals.

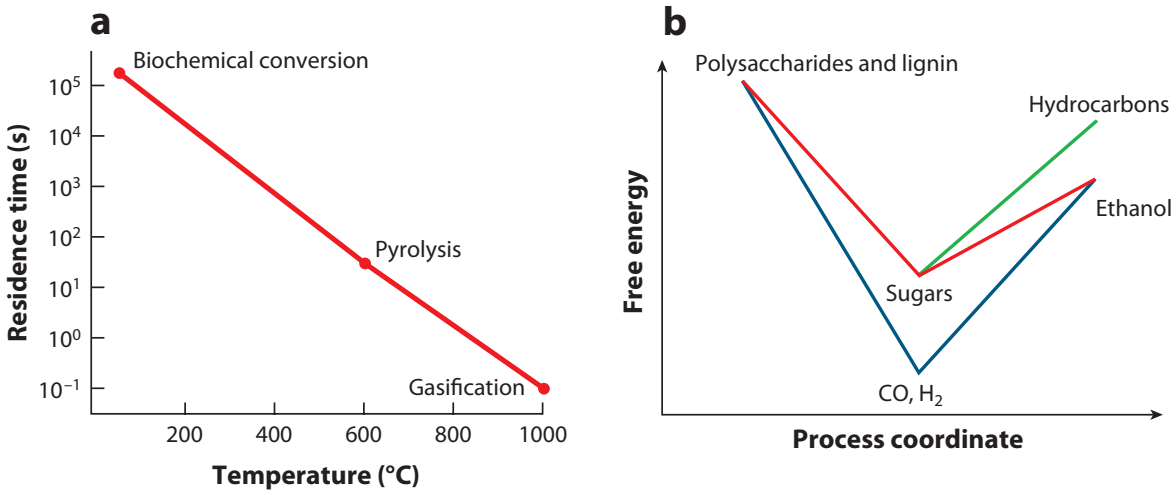

Figure 10. Kinetic (a) and thermodynamic (b) overview of biomass deconstruction by biochemical or thermochemical routes [27].

Energy analysis results demonstrated that both, thermochemical and biochemical process, are competitive in their energy conversion efficiencies [53]. Also, it has been shown that the overall economics are similar. Nevertheless, the comparative life cycle assessment suggests that the biochemical conversion would have better performance regarding GHG and energy balance [54]. However, each of these processes has limitations and a careful pairing of technologies is required for an effective biomass conversion [55]. For alcohols like products, biochemical conversion routes appear to be well suited, whereas for hydrocarbon like, the chosen production technologies tend to favor the thermochemical conversion routes [18].

1.4.1 Thermochemical process

Gasification, pyrolysis and hydrolysis are methods that are referred to as thermochemical conversion technologies of biomass. These can be used to produce gaseous (syngas) or liquid intermediates (bio-oils and hydrolysis liquor) that are further chemocatalytically upgraded to liquid fuels or chemicals (Fig. 11).

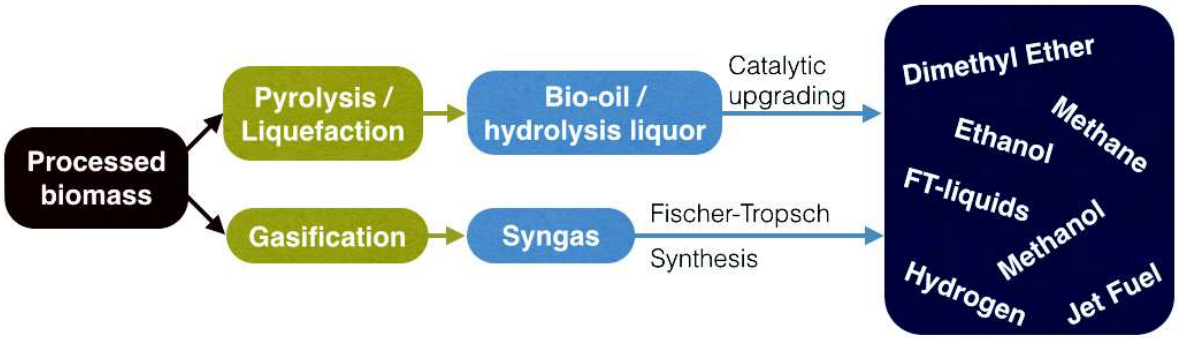

Figure 11. Thermochemical pathways for biomass upgrading.

The intermediate produced by thermochemical conversion of lignocellulose and their relative amounts typically depend on process conditions (e.g. temperature, pressure, feed rate, time of heating and particle size of biomass). Moreover, a number of technical issues, such as feedstock moisture, high energy input, cleaning of intermediate products and ash content have to be addressed. Gasification to afford syngas (a mixture of carbon monoxide and hydrogen) is analogous to syngas from coal gasification. The syngas can be converted to liquid fuels or platform chemicals *via* established technologies such as the Fischer-Tropsch process or methanol synthesis, respectively [56].

Pyrolysis is a densification technique where both the mass and energy density are increased by treating the raw biomass at temperature ranging

from 300 to 600°C and at short residence times producing bio-oil. In this way, an increase in the energy density by roughly a factor of 7-8 can be achieved [57]. More than 300 different compounds have been identified in bio-oil, where the specific composition of the product depends on the feed and process conditions used [57]. From a compositional point of view, bio-oils consist of two phases: an aqueous phase in which several low molecular weight oxygenated organics are dissolved and a non-aqueous phase composed of oxygen-containing structures and aromatic hydrocarbons. Yields can vary as a function of the lignin content in feedstock [58]. However, due to the presence of oxygenated compounds, bio-oils are generally immiscible with hydrocarbon fuels, are chemically unstable and and display low volatility, high viscosity and corrosiveness [18]. Nevertheless, bio-oil show suitable properties as fed for biorefinery. To this respect, different catalytic upgrading strategies have been reported to yield high-grade oil product equivalent to crude oil [59].

1.4.2 Bio-chemical process

Exploiting the original chemical structure and functionality, hence preserving high atom efficiency, is the smartest strategy for lignocellulosic biomass valorization [60]. Following the biochemical route lignocellulose is fractionated into its components (cellulose hemicellulose and lignin) by means of chemical and biochemical step. The obtained streams, mainly the saccharide fraction, are upgraded into chemicals usually by fermentation (Fig. 12). As previously discussed, lignocellulose recalcitrance is the major technical hurdle for lignocellulosic valorization, thus a pretreatment step is

usually required before the enzymatic hydrolysis. These last two steps are the most costly [61].

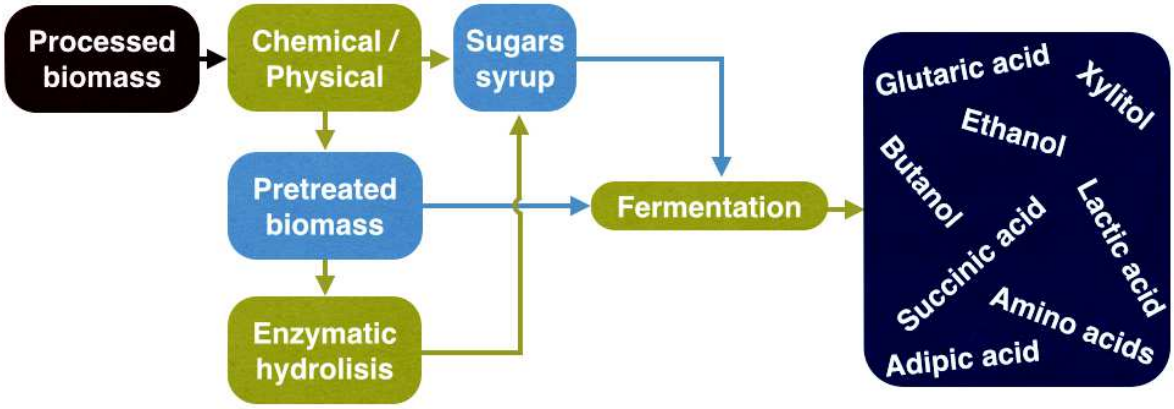

Figure 12. Biochemical pathways for biomass upgrading.

1.4.2.1 Pretreatment step

The discovery of *Trichoderma reesei* cellulases has had an enormous effect on fermentable sugars production from lignocellulose. Concentrated acids hydrolysis, which typically results in poor yields and extensive sugar degradation, is now substituted by mild pretreatment followed by enzymatic hydrolysis. In this respect, availability of aggressive enzymatic cocktails, also at industrial scale, has pushed the development of suitable pretreatments methods. Molecular scale integration in to the cellular/tissue one, has allowed the understanding of the pretreatment effect on enzymatic hydrolysis. For instance Li et al., correlated the reduced enzymatic hydrolysis to the lignin redeposition during dilute acid pretreatment of *Poplar wood* by means of scanning electron microscope and nuclear magnetic resonance [62]. Likewise, cellulase synergism has shown a strong correlation to disorganized cellulose surface, and the exoglucanase (Cel7A)

has been reported to be the major contributor to overall cellulose hydrolysis of different pretreated lignocellulosic substrates (Fig. 13) [63].

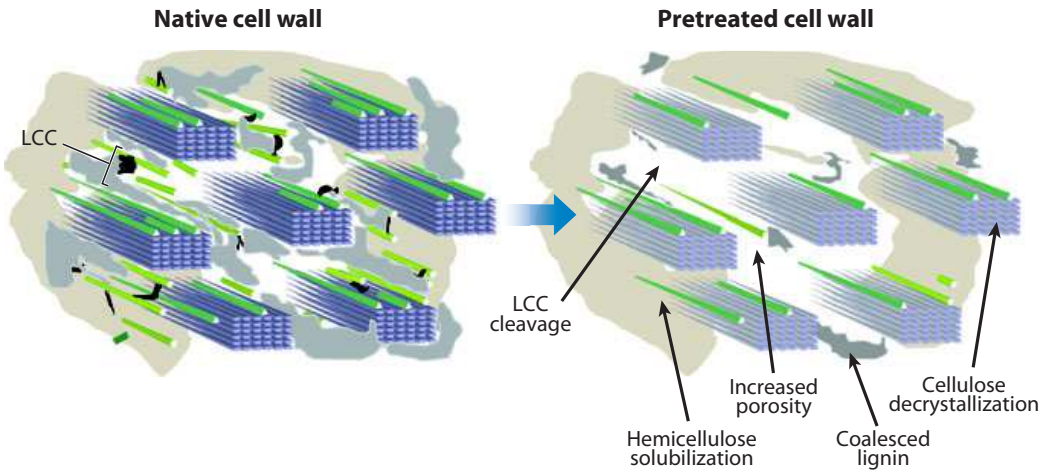

Figure 13. Ultrastructure model of native and pretreated cell wall [63].

A number of pretreatment options have been reported to be effective for cellulose, hemicellulose and lignin fractionation. Some of them include the utilization of special reactors or catalyst. Despite that pretreatments are generally classified into four categories: physical, chemical, biological and solvent-base. Among them, steam explosion and dilute acid hydrolysis are the most applied options, as they generate a suitable pretreated biomass and are cost effective. However such methods do not produce valuable hemicellulosic and lignin streams. Differently, organosolv allows obtaining high yield and high quality of all the fractionated components, thus increasing the economy of the process. Despite that, this method is more expensive [39].

To assess the effect of a pretreatment and to compare results from experiments carried out at different conditions, numbers of mathematical models have been developed [64]. Among them the most used is the

combined severity factor (CS) (Eq. 1) that is based on the equation proposed by Chum et al. [65].

$$R_0 = t \cdot \exp\left(\frac{T - 100}{14.75}\right)$$

$$\mathrm{CS} = R_0 \cdot [\mathrm{H}^+]$$

$$\mathrm{CS} = \log(R_0) - \mathrm{pH} \quad (1)$$

Equation 1. Combined Severity Factor (CS). *t* is the time and *T* is the temperature.

This equation, based on pseudo first order kinetics, give an indication of components recovery as a function of pretreatment harshness. It has been used in several studies to optimize pretreatment conditions, to improve enzymatic hydrolysis yield and components recovery.

Acid and alkaline pretreatments

Acid pretreatment is one of the most effective and traditionally used methods. Diluted mineral acids (H_2SO_4, HCl, H_3PO_4, and HNO_3) are generally used as catalysts [66], but organic acids such as fumaric acid or maleic acid can be used as alternatives [67]. H_2SO_4, is the most commercially used option and its effectiveness is well documented over a wide range of plant type. The overall pretreatment offers good performance but hemicellulosic sugars might be degraded to furfural and hydroxymethyl furfural, which are strong fermentative inhibitors. Furthermore, acids neutralization results in the formation of solid waste. Despite that, this method is suitable for biomass with low lignin content like straw and grasses.

Alkaline pretreatments, is obtained by soaking biomass in aqueous alkali solution also at room temperature. Ammonia, calcium or sodium hydroxide, are the most used base [66]. This method is effective in lignin removal, thus improving the reactivity of the polysaccharides. Some of the hemicelluloses are also hydrolysed during the process but the majorities are recovered as oligomers. Alkaline hydrolysis mechanism is based on saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin [68]. Other forms of pretreatment techniques include ammonia fiber explosion (AFEX) [69] and ammonia recycle percolation (ARP) processes [70].

Non-catalysed pretreatments

The most common non-catalysed pretreatments are: steam explosion and hydro-thermolysis [70,71]. These methods allow the breakdown of lignocellulose trough an auto-catalyzsed generation of organic acids and/or by the shearing forces due to the expansion of the moisture. The advantage of these pretreatments is the no need of chemicals and corrosion problems are avoided. However, the hemicelluloses being hydrolysed and dissolved in process waters are not completely converted into monomer and the lignin is poorly solubilized. Lignin redeposition can occur, affecting the enzymatic hydrolysis.

Solvent-base pretreatments

Organic or aqueous-organic solvent mixtures composed by low boiling alcohols or aliphatic acids and with or without acid catalyst are known as Organosolv process. These methods are effective in breaking the linkages

between lignin and carbohydrate polymers in lignocellulose, leading to an improved accessibility to the cellulose fibers [39]. Additionally, the problems related to cellulase enzymes absorption to lignin are minimized as lignin is solubilized and recovered from the organic phase. Recovered lignin has high quality. However, the pulp generated must be fully washed before saccharification, as the solvent may act as inhibitor to the enzymes and to the subsequent fermentation process. The design of efficient solvent and catalyst recovery process would give beneficial effect, both at environmental and economical and levels.

Oxidative pretreatments

Oxidative processes are obtained by treatment with oxidizing agents like hydrogen peroxide, ozone, oxygen or air. These methods involve delignification and structural disruption of lignocellulose by the breakdown of lignin into carboxylic acids. However, using oxidative agents the generation of furfural, from hemicellulose, is high [72].

Ionic liquid pretreatments

Ionic liquids (IL) are molten salts characterized by room temperature melting point. Thanks to their polarity and unique properties IL exhibits some interesting properties such as chemical inertness, low volatility, good thermal stability, and solvation abilities. Despite the potential this method have several uncertainties such as the ability to recover the IL used, the toxicity of the compounds, and the combination of water with IL [72].

Biological pretreatments

Biological pretreatment involves microorganisms as bio-catalyst. White, brown and soft rot-fungi are capable of degrading hemicellulose and lignin but leaves the cellulose intact, thus enhancing the feedstock digestibility [66]. This method take advantages of low energy requirement, mild operation conditions and avoids the usage of hazardous chemicals. However, the incubation required several days and the need to control microorganism growth conditions makes these treatments commercially less attractive.

1.4.2.2 Enzymatic saccharification step

The second step in biochemical conversion of lignocellulose is the enzymatic hydrolysis. The objective of this step is to depolymerize the cellulose to soluble sugars. Like the pretreatment step enzymatic hydrolysis is one of the major costly step for biorefineries. Highly integrated approach are required, as the chemistry and severity of pretreatment directly impacts the on the pretreated biomass susceptibility to enzymatic digestion, which in turn dictates the enzyme loadings and composition.

Most biomass-degrading organisms characterized so far, secrete “free” enzymes, which diffuse independently and contain single catalytic domains for deconstructing cellulose, hemicellulose, and in some cases lignin [73]. The soft-rot fungus *Trichoderma reesei* (*Hypocrea jecorina*) is the most well studied model organism that produces free cellulases. The cellulolytic enzyme system of *T. reesei* and similar organisms primarily comprises endoglucanases (EGs) cellobiohydrolases (CBHs) and β-glucosidase (BGs) [74].

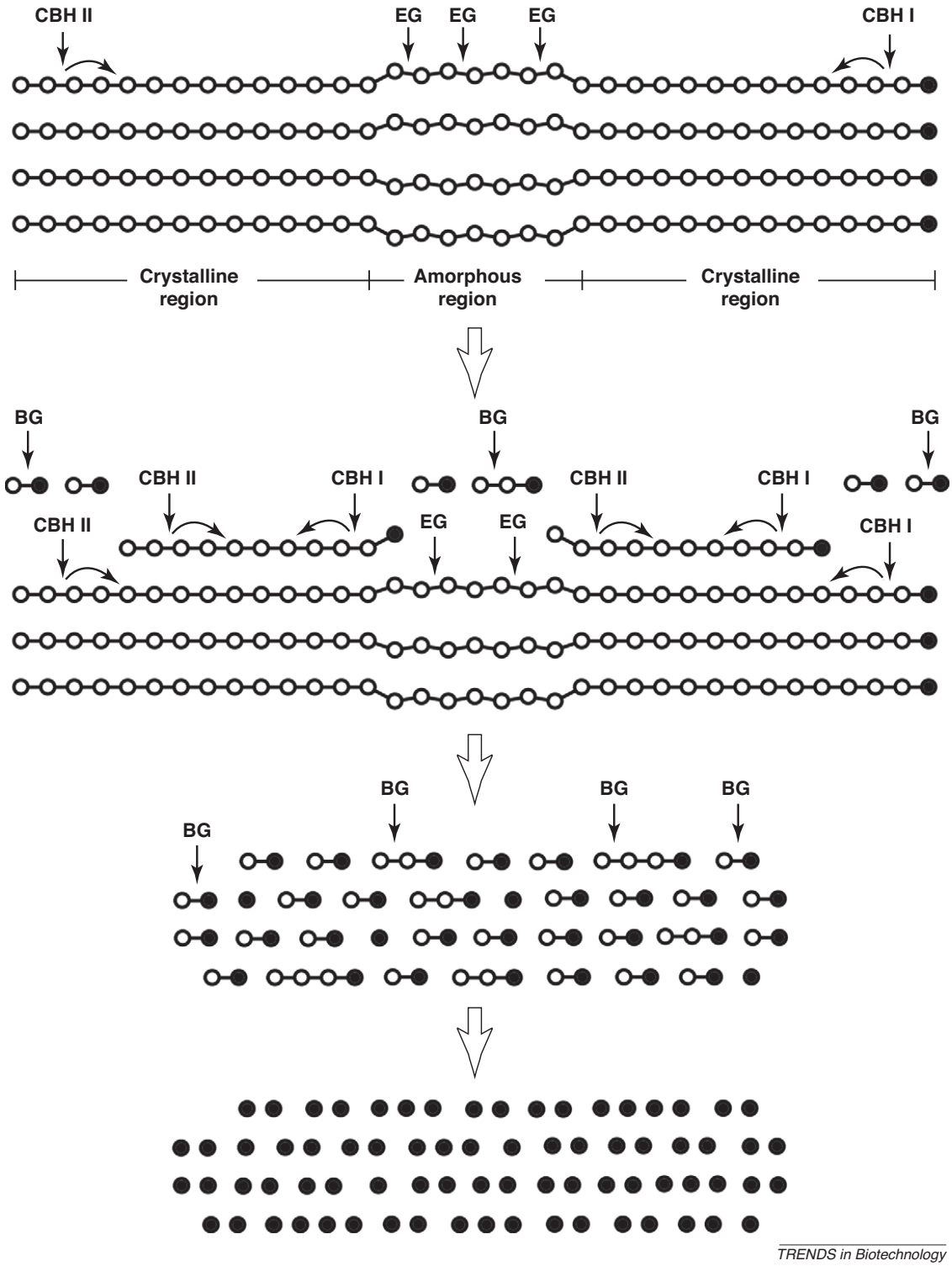

Figure 14. Simplified scheme of the free cellulase system for enzymatic hydrolysis of cellulose. The open circles represent anhydroglucose residues in cellulose and oligosaccharides; the solid circles represent reducing ends of cellulose and oligosaccharides or glucose [75].

In primary hydrolysis endoglucanases are thought to hydrolyze chains in amorphous regions of cellulose to create attachment and detachment points for both reducing and non-reducing end specific CBHs. These enzymes processively hydrolyze cellulose chains into crystalline regions without substrate decomplexation and detachment between hydrolytic events. This process takes place on the substrate solid surface and releases soluble sugars into the liquid phase. Secondary hydrolysis occurs in the liquid phase, primarily involving the hydrolysis cellobiose units into glucose molecules by BGs (Fig. 14) [27]. Moreover, oxidative enzymes, now termed lytic polysaccharide mono-oxygenases (LPMO), have recently been discovered and characterized. These enzymes are thought to perform endo-like cleavage of cellulose and hemicellulose chains in crystalline regions, thus complementing the activity of cellulase and hemicellulases [76].

In contrast to the free enzyme, another enzymatic system has found in some anaerobic organisms, like *Clostridium thermocellum* [27]. In this system enzymes are organized into large extracellular macromolecular complexes termed cellulosomes. Cellulosomes are composed of lignocellulose-degrading enzymes, noncovalently bound *via* cohesin-dockerin interactions to a scaffoldin protein either associated with the bacterial cell or free in solution [77]. Once assembled, cellulosomes can contain up to nine catalytic domains of glycoside hydrolases (GHs). The large multimodular complex contains multiple enzymatic specificities in close proximity.

Recently, the differences in the mechanism of free cellulases and cellulosomes have been visualized on larger more complex layered bundles of cellulose microfibrils. Specifically, cellulosomes exhibit superior performance relative to free enzyme cocktails when degrading model

cellulose, and the mixture of the two systems has a synergistic effect in performance. Conversely, on dilute acid-pretreated biomass that contains significant amounts of lignin, free cellulases exhibit superior performance compared to cellulosomes [78]. During hydrolysis, the substrate characteristics vary due to the combined actions of EGs and CBHs, which modify the cellulose surface behaviors over time, resulting in rapid changes in hydrolysis rates. However, CBHs dissociation rate is thought to be the limiting in cellulose depolymerization [27]. Other factors that influence the cellulose hydrolysis are the substrate concentration and the enzymatic loading. At industrial level, *T. reseei* cellulase system is the most used for cocktails formulation, this is due high titers of secrete proteins, more than 100 gL^{-1}, and its effectiveness on different pretreated biomasses [27]. Moreover, considerable research has been carried out recently on improving the properties of *T. reesei* cellulases, such as higher specific activity or thermostability, by means of directed evolution or protein engineering [79]. Modern commercial cellulase preparations from leading enzyme-producing companies, such as Novozymes (Cellic CTec series) [80] and Genencor (Accelerase series) [81], are based on genetically manipulated strains of *T. reesei*.

1.4.2.2.1 Kinetic of cellulase enzyme

Mathematical models are important tool to understand the mechanism of a complex reaction and the base for large-scale process development. Most of the experimental studies on enzymatic hydrolysis of cellulose showed that cellulase activities and thus rates fall precipitously as reaction proceeds [82]. Valuable information about catalytic and processive

mechanisms of cellulase has been obtained by studying isolated activities [83]. However, these models cannot capture synergism between multiple components as several factors related to both enzyme characteristics (adsorption, inhibition, synergism, activity) and substrate characteristics (degree of polymerization, crystallinity, accessible surface area, lignin content) are thought to affect the enzymatic kinetic [83]. Therefore, including all of them into a mathematical model, to describe the synergistic action of enzymes on lignocellulosic substrates, is one of the most challenging subjects in engineering for this process. While, models, which do so, would be more robust, they would require more variable and parameters.

The majority of the kinetic models are based on Michaelis-Menten equation (MM) implemented whit inhibition constant [84]. Some of them are extended to include Langmuir adsorption of cellulase onto the insoluble cellulose while the experimental facts indicate that partial cellulase binding does not comply with assumptions implicit in the Langmuir isotherm [84]. Fractal-like kinetic analysis provides a more detailed description of heterogeneous chemical reactions. In the basic fractal-like kinetic model the rate coefficient k_t, which corresponds to rate constant k, in the traditional MM kinetic (Eq. 2), is time dependent. The time dependence of k is determined by the fractal exponent h (Eq. 3) [85].

$$\frac{dC}{dt} = -kC \tag{2}$$

Equation 2. First order kinetic for cellulose enzymatic saccharification. C is the cellulose concentration, t is the time and k is the rate constant.

$$\frac{dC}{dt} = -k_t C$$

$$k_t = kt^{-h} \qquad \text{at } t \geq 1$$

$$k_t = k \qquad \text{at } 0 \leq t < 1 \tag{3}$$

Equation 3. Time dependence of k in the fractal-like kinetic. k_t is the time dependent rate coefficient, t is the time and h is the fractal exponent.

Based on Eq. 3 the substrate (cellulose) concentration can be expressed by Eq. 4. This describes the profile of enzymatic saccharification of cellulose with the two basic parameters k (rate constant) and h (fractal exponent) [85]. The effect of the k and h parameters in enzymatic cellulose hydrolysis is shown in Fig. 15.

$$C = C_0 \cdot \mathrm{EXP}\left(-k\left(1 + \frac{t^{(1-h)}-1}{1-h}\right)\right) \tag{4}$$

Equation 4. Expression developed to model cellulose concentration during cellulase hydrolysis. C cellulose concentration, C_0 initial cellulose concentration, t is the time, k is the rate constant and h is the fractal exponent.

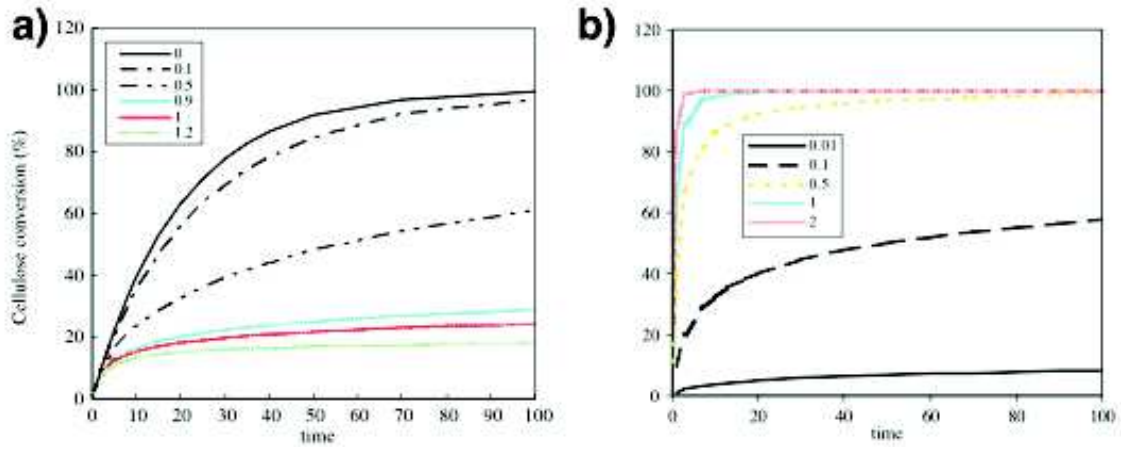

Figure 15. Effect of fractal kinetic parameters on the profile of enzymatic hydrolysis of cellulose. Effect of the fractal exponent h (at k fixed to 0.5) (a). Effect of the rate coefficient k (at h fixed to 0.8) (b). Adapted from [85].

The time course of enzymatic saccharification of different pretreated cellulose substrate, under different conditions, has been fitted with the fractal-like kinetic model by different authors and good fitness, between experimental data and simulation, has been observed [85–87]. Nguyen et al., have recently studied the time course enzymatic hydrolysis of differently pretreated corn stover samples. Analyzing the transient rate parameter k_t at high conversion regimes (60-100%) data suggest a "crowding effect", as theorized by Xu et al. [87,88]. Moreover, the relationship between lignin content and the fractal parameter h was observed as well, suggesting the effectiveness of fractal-like kinetic analysis in enzymatic hydrolysis of cellulose description.

1.4.2.3 Fermentation step

Modern biotechnology is focusing industry to the production of bulk chemicals from biomasses by taking advantage of new and abandoned fermentation process. Current research focuses mainly on the production of C2-C6 building blocks, as shown in Fig. 16. However, only a small number of chemicals are today produced in this way at relevant commercial scale levels. Among them ethanol, butanol and lactic acid are the main fermentative processes developed that compete with petrochemical routes [89]. In biorefinery, microorganism strain selection dictates the upstream process operations. Sugar uptake flexibility, termotollerance, high productivities and process compatibility with current industrial infrastructures are important issues that have to be considered. To this regard development of natural or engineered microorganisms and optimization of downstream processes will play important roles in reducing production costs

allowing bio-based processes to compete against the current petrochemical processes [90].

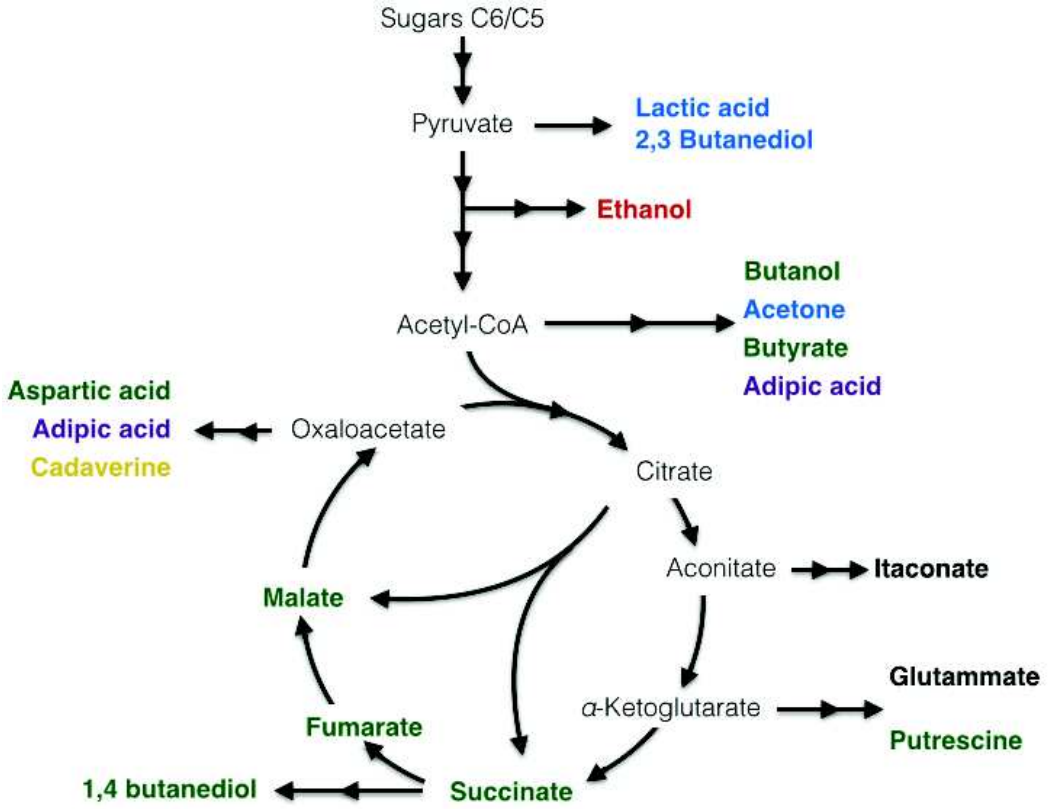

Figure 16. Biomass-based platform chemicals that could be obtained by fermentation. Red C2, blue C3, green C4, violet C5 and yellow C6 platforms.

Lignocellulosic biomass fermentation process can be performed separately from enzymatic hydrolysis (SHF) or in combination with enzymatic hydrolysis (SSF). The advantages of SSF are the reduced end-product inhibition of the enzymatic hydrolysis, and the reduced investment costs. Drawbacks are related to find favorable conditions (pH and temperature) for both enzyme and microorganism, the difficulty to recycle the enzymes and the need of pentose and hexose co-fermenting microorganisms [91]. While glucose fermentation is very rapid, xylose fermentation is usually much slower. The slow xylose fermentation has been related to the absence of

specialized xylose transporters and to the lack of efficient metabolic pathways for pentose utilization [92]. Another important issue for lignocellulose-derived sugars fermentation is the selection of microorganisms that tolerate inhibitors usually generated in the pretreatment step. Low molecular weight organic acids, furans, and aromatics are often found in hydrolysate and such compounds are considered potent inhibitors of microbial metabolism [93].

1.4.2.3.1 Acetone–butanol–ethanol (ABE)

Butanol is an industrial commodity considered to be a more promising gasoline substitute compared to ethanol. Renewed attention has been paid to butanol production from lignocellulose through the acetone–butanol–ethanol (ABE) fermentation process. Recently high-alcohols production has been received great interest from both small biofuel start-up and large oil and chemical companies.

There are number of wild strains ABE-producing bacteria; the most common are the clostridia. This genus has been classified into 4 species: *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, *and C. saccharoperbutylacetonicum* [94]. Some clostridia strains secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into monomers. This ability to utilize mixed sugars is of importance for fermentation because substrate is an important factor influencing the cost of butanol production [95]. For instance, in *C. acetobutylicum* 824 the presence of more than 90 genes encoding carbohydrate-degrading enzymes has been reported [96]. Successfully fermentations of lignocellulosic-derived sugars, such as wheat straw, barley straw, corn stover and switchgrass have been

reported [97–100]. Moreover, advances in fermentation and in situ product removal processes have resulted in reduced butanol toxicity to microorganisms, improved substrate utilization, and improved bioreactor yields and productivities [95].

1.4.2.3.2 Lactic acid

Fermentative lactic acid production is well established and its market size is over 4 mtons $year^{-1}$ [101]. Lactic acid is considered a versatile precursor for various chemicals and materials and ~40% of its production is used in manufacturing polylactic acid for bio-plastic synthesis [102]. Fermentative production of lactic acid has been reported from a wide spectrum of carbon sources including starchy materials, food industry by-products and agro-industrial residues [103]. Current processes uses optimized *Lactobacilli* strains and engineered yeast, whereby also other producers exhibit excellent performance. Recently, *Bacillus coagulans* species have received renewed interest thanks to their industrial superior fermentative performance. For instance, a newly isolated *Bacillus coagulans* C106, produced 215.7 g L^{-1} of L-lactic acid from xylose in fed-batch mode, with a 95% lactic acid yield and 99.6% optical purity [104]. The application of electrodialysis membrane to separate lactic acid from fermentation broths is considered one of the most promising options to reduce byproducts (e.g. gypsum) [105].

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2 Aim of the work

Driven by the need of sustainable growth, many countries recognize that energy security, environmental preservation and the development of alternative, cleaner sources of chemicals and materials for industries, is becoming imperative. Awareness in biomass conversion into useful products is growing, as it contains all the elements found in fossil resources, albeit in different combinations. Indeed, biotechnological upgrading of inexpensive lignocellulosic materials is attracting the industrial interest, as sustainable and ecofriendly alternative to petrochemical route for both the energy and chemical sectors. However, lignocellulose recalcitrance to deconstruction is the challenge for lignocellulosic-based biorefining processes commercialization. Recalcitrance is caused by the tight and complex network between the components of lignocellulose (i.e. lignin, hemicellulose and cellulose). Among deconstruction technologies, the most efficient scheme includes a pretreatment step in which the cellulose polymers are made accessible, followed by an enzymatic step in which cellulose is hydrolysed to fermentable sugars using cellulases enzymes.

This PhD project, embedded in the framework project "VeLiCa", is aimed at exploiting the hemp hurds (HH), an industrial lignocellulosic by-product, as feedstock for biorefinery. The setup of a organosolv (OS) pretreatment and of an enzymatic hydrolysis processes, allow to obtain the C5 and C6 sugars streams and to isolate the lignin fraction from HH. Fermentability of HH-derived sugars streams will be assessed through the production of *n*-butanol and polymer grade lactic, which are two of the most usefully platform chemicals for the fuel and plastic sectors, respectively.

Furthermore, the project is also aimed to study the OS process severity effect toward the HH enzymatic saccharification, hemicellulose hydrolysis and

delignification, for maximum overall sugars recovery by process variables optimization. Finally, a study of the kinetic behaviour of an industrial cellulases enzymes blend, on differently pretreated HH samples, will allow to gain information into the complex relationship between cellulases hydrolysis rate and substrate features. Such knowledge will contribute to the design of integrated processes for lignocellulosic biomass valorisation.

3 Results

3.1 Complete chemical analysis of Carmagnola hemp hurds and structural features of its components

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Complete Chemical Analysis of Carmagnola Hemp Hurds and Structural Features of Its Components

Stefano Gandolfi,[a] Gianluca Ottolina,[a] Sergio Riva,[a] Giuseppe Pedrocchi Fantoni,[b] and Ilabahen Patel [a,*]

As interest in lignocellulosic biomass as a feedstock for conversion into biofuels is steadily growing, analysis of its components becomes ever more important. The complete chemical composition of waste hemp hurds from the industrial variety "Carmagnola" has been determined to optimize its utilization as a raw material. The results from chemical analysis show that hemp hurds contain 44.0% alpha-cellulose, 25.0% hemicellulose, and 23.0% lignin as major components, along with 4.0% extractives (oil, proteins, amino acids, pectin) and 1.2% ash. Structural and physicochemical properties of hurds components were analysed by FTIR or GC/MS. The data revealed that isolated components are pure and comparable to standard components. Acetone extractives show higher total phenolic content and antioxidant capacity compared with lignin and dichloromethane extractives. Water extractive shows the presence of proteins (1.6%), free amino acids (0.02%), and pectin (0.6%). The degree of esterification of pectin was estimated to be 46.0% by FTIR and enzymatic hydrolysis. The results of this study show that Carmagnola hurds contain low amounts of ash and high amounts of carbohydrates compared with other varieties of hemp hurds; therefore they can be considered as a potential feedstock for biorefinery.

Keywords: Hemp hurds; Lignocellulosics; Biorefinery; Cellulose; Holocellulose

*Contact information: a: Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mario Bianco 9, 20131 Milano, Italy; b: Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mancinelli 7, 20131 Milano, Italy; *Corresponding author: ila.patel@icrm.cnr.it*

INTRODUCTION

Nowadays, the use of renewable biomass to replace non-renewable fossil fuels is becoming a priority in energy policy and management. The major production of biofuels originates from energy crops. These can be lignocellulosic materials, such as agricultural by-products, herbaceous crops, or forestry residues (Kim and Dale 2004). In a biorefinery, this biomass is converted into a variety of high value-added products and biofuels. Lignocellulosic materials, with a high content of carbohydrates, are abundant, inexpensive, and largely unused. The main chemical components of lignocellulosic materials are: cellulose, hemicelluloses, and lignin, with minor amounts of other compounds such as ash, proteins, lipids, waxes, and various extractives. Lignocellulose structure and composition vary greatly, according to plant species, plant parts, growth conditions, *etc*. (Ding and Himmel 2006; Zhang and Lynd 2004).

Hemp is one of the fastest-growing plants in the world and it comprises a number of varieties of *Cannabis sativa* L. that are traditionally grown for fibers and seeds.

Compared with other crops, industrial hemp is very high yielding in biomass (~30 tons/hectare) and requires a low level of irrigation and fertilizers after its establishment (Struik *et al*. 2000; Cappelletto *et al*. 2001; van der Werf 2004; Amaducci *et al*. 2008). Industrial hemp is characterized by low (less than 0.20%) tetrahydrocannabinol (THC) content, and many countries are represented on the list of approved cultivars. The European Union permits the cultivation of 54 different varieties of industrial hemp, and among them Carmagnola is one of the oldest approved varieties. Fibers are a valued product of hemp and are mainly used for textile applications. In a typical process, fibers are separated from the hemp stalk through retting and scutching. The residual biomass (containing mainly a woody core, dust, and small amounts of short fibers, known as core fibers) is considered a by-product of fiber production. These woody core parts constitute 70% of the stalk (Dang and Nguyen 2006) and have minor applications, such as for animal bedding (95%), garden mulch, or as a component of lightweight concrete (~5%).

The use of hemp hurds as a feedstock for a modern biorefinery facility could supply a variety of market sectors (*e*.*g*., chemistry, energy, transportation). The hemp biomass used in the present study is a by-product from the textile industry, and it is locally available as a waste material and considered a potential source of lignocellulose. As a prerequisite to add value to this waste biomass, an accurate compositional analysis is important in order to evaluate the conversion yields and the efficiency of the proposed process.

Industrial interest in hemp is increasing because it is eco-friendly and due to its possible applications such as in pulp and paper (González-García *et al*. 2010), bio-composite (Boutin *et al*. 2006; Carus *et al*. 2008; Magnani 2010), and as raw material for biofuel production (Sipos *et al*. 2010; Kreuger *et al*. 2011). For instance, an accurate measurement of biomass carbohydrate content is essential because it is directly related to ethanol yield in biochemical conversion processes (Aden *et al*. 2002). Furthermore, the minor components of a biomass can include proteins, ash, organic acids, and other nonstructural materials.

Although these individual components may make up only a small fraction of the feedstock, their presence can have a significant effect on the running of an industrial-scale biorefinery. Therefore, the objectives of this study are to quantify both the major (*e*.*g*., cellulose, hemicellulose, lignin) and minor components of Carmagnola hemp hurds, with the aim to utilize them as raw materials for biorefinery.

EXPERIMENTAL

Materials

The residual biomass of Carmagnola hemp was supplied by Assocanapa-Coordinamento Nazionale per la Canapicoltura (Carmagnola, Italy) as chopped pieces with a length of 5 cm or less.

Three morphological portions of the biomass—woody cores, short fibres, and dust—were separated using a sieve (screen size 2 mm). Sieving was used to separate the dust from woody cores and short fibres. The latter were manually separated from woody cores.

The recombinant enzyme pectate lyase from *Aspergillus* sp. (EC 4.2.2.2) was obtained from Megazyme (E-PCLYAN2). All chemicals used in this study were commercially available authentic samples and purchased from Sigma-Aldrich.

Methods

Sample preparation

For chemical analysis, woody cores, called hurds, were disintegrated into powder by using an IKA MF 10 knife mill and sieve (screen size 0.5mm). The moisture content of the milled samples was analysed according to TAPPI T 264 cm-97. Figure 1 shows the scheme used for the chemical analysis.

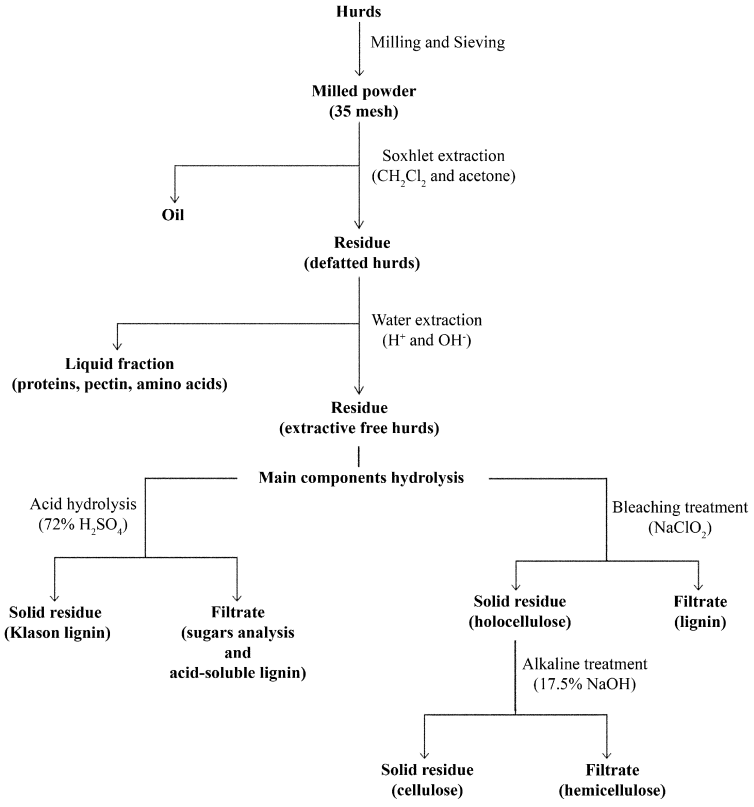

Fig. 1. Scheme for chemical analysis of hurds

Extractive analyses

A milled and oven-dried sample was used for isolation of extractives (solvent and water extractives). The oil was extracted with CH_2Cl_2 and with acetone by using a Soxhlet apparatus for 8 h at 90 ºC. The defatted hurds were used for further analysis. Proteins were quantified in a dry milled sample by total nitrogen determination using the Kjeldahl method (AOAC 1999). Isolation of water extractives was performed under basic and acidic conditions. Basic conditions were applied to obtain free amino acids and proteins. The extraction was done under continuous stirring, at pH 10 for 24 h at 40 °C. The solid/liquid ratio was 1:20. The liquid portion collected by filtration was adjusted to pH 7 and centrifuged in order to separate insoluble protein aggregates from free amino acids. The protein fraction was analysed by SDS-PAGE, while the free amino acids compositions were analysed by HPLC using the DABS-Cl pre-column derivatization method. Pectin extraction was performed under acid conditions and continuous stirring at pH 1.7 for 24 h at 85 °C using a solid/liquid ratio of 1:20. The pH of the filtrate portion was adjusted to 3.5, and pectins were precipitated by adding 3 volumes of 2-PrOH at 4 °C. The resulting gel was recovered by centrifugation, washed three times with 2-PrOH, and dried at 50 °C. Pectin identification was performed according to the JECFA method (Hansen *et al*. 2001).

Ash and lignin content analyses

Total ash content was measured according to TAPPI T 211 om-02. The content of acid-insoluble (Klason) and acid-soluble lignin (ASL) was determined from biomass samples according to TAPPI T 222 om-06 and TAPPI UM250, respectively. Isolation of ASL was also done by liquid–liquid extraction using $CHCl_3$.

Isolation of holocellulose and cellulose

The preparation of holocellulose and α-cellulose was carried out according to the literature (Yokoyama *et al*. 2002).

Chemical characterization of extractives

The oil (solvent extractive) was analysed by using GC/FID or GC/MS. Injector and detector temperatures were set at 300 °C and 350 ºC, respectively. Hydrogen was used as a gas carrier for GC analysis and helium for GC/MS analysis. The capillary column used was Agilent Technology DB-5HT (15 m × 0.1 mm × 0.25 mm film) for FID and DB-5MS (30 m × 0.25 mm × 0.25 mm film) for MS.

The quantification of galacturonic acid present in the pectin fraction was determined in accordance to the meta-hydroxydiphenyl colorimetric assay (Filisetti-Cozzi and Carpita 1991) using known concentrations of galacturonate as standard (in a range from 0 to 200 mg/L).

The degree of esterification (DE) of pectin was determined by Fourier transfer Infrared (FTIR) spectrometry (Gnanasambandam and Proctor 2000) and enzymatically using pectate lyase. For the enzymatic determination of DE, pectin samples or standards (from citrus fruit, Sigma P9561, P9436, P9311; esterification ≥85%, 55-70%, 20-34% respectively) were solubilized (2 mg/mL) in 50 mM Tris-HCl buffer, pH 8, and the pectin solutions were mixed with 790 µL of 50 mM Tris-HCl buffer, 1 mM $CaCl_2$, pH 8, and 10

µL of enzyme (0.01 U in 50 mM Tris-HCl buffer, 1 mM $CaCl_2$, pH 8). The reaction and blanks were conducted at 40 °C for 30 min (end point) and monitored at 235 nm. The amount of product (unsaturated oligogalacturonides) was calculated using the $\varepsilon 235$ = 4600 M cm^{-1} (Hansen *et al*. 2001). The degree of esterification was calculated from the calibration curve of the pectins standards (end points *vs*. DE). All measurements were performed in triplicate.

Free amino acid composition from basic water extraction was performed using HPLC on an Agilent Eclipse XDB-C18 5 µm (4.6 × 150 mm) column with the UV-Vis detector at 436 nm. The mobile phase consisted of two eluents: 50 mM acetate buffer (pH 4.1) (solvent A) and acetonitrile (solvent B), and the gradient was from 20% A to 70% B in 25 min (linear). The flow rate was 1.3 mL/min.

Spectroscopic characterization (FTIR)

FTIR spectra were obtained using a KBr disc containing 1% finely ground samples. Thirty-two scans were taken for each sample recorded from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

Total phenol content and antioxidant capacity

The total phenol content in Klason lignin and in solvent extractives was determined by the Folin-Ciocalteau colorimetric method (Vázquez *et al*. 2008) using gallic acid as a standard phenolic compound. The antioxidant capacity of the same samples was determined by the radical scavenging activity method using ABTS radical (Re *et al*. 1999). This method was modified as follows: the ABTS·+ solution was diluted with ethanol to an absorbance of 0.70 (±0.05) at 734 nm (ϵ = 1.6 × 104 $mol^{-1}L\ cm^{-1}$). The reaction was performed by addition of 1.0 mL of $ABTS^{\cdot+}$ solution to 100 µL of sample, or standard (Trolox). The mixture was stirred for 30 s and the absorbance was recorded until the end point (~30 min) at 30 ºC.

HPLC analysis of monosaccharides

The composition of monosaccharides from hydrolyzed liquid fractions of hurds was performed by derivatization of sugars with PMP (1-phenyl-3-methyl-5-pyrazolone) according to Dai *et al*. (2010) and analysed by HPLC on an Agilent Eclipse XDB-C18 5 µm (4.6 × 150 mm) column with the UV-Vis detector at 245 nm. The mobile phase was 0.1 M phosphate buffer (pH 6.7) and acetonitile (83:17 v/v, %) at a flow of 1 mL/min.

Nitrobenzene oxidation

Nitrobenzene oxidation of hurds for syringylpropane to guaiacylpropane units (S/G) ratio determination was performed according to Sun *et al*. (1995). The major components were identified by addition of authentic samples to the reaction mixture.

RESULTS AND DISCUSSION

The purpose of this work was to study the chemical composition of Carmagnola hemp hurds to optimize its utilization in high-value applications, such as the production

of biodegradable products, chemicals, and biofuels. Results of the hemp hurds characterization are reported in Table 1. The main components of this biomass are: cellulose, hemicellulose, lignin, lipids, proteins, pectin, water, and ash.

Extractives Yield

Extractive components (oil, waxes, pectin, proteins, and tannin) were isolated by increasing solvent polarity. For the extraction of oil and waxes, the well-known Soxhelt method was used with CH_2Cl_2 and acetone (instead of the usual hazardous extraction solvent, an ethanol–benzene mixture). The yield of total extractives was ~4.0% (Table 1), of which the oil and waxes content, estimated to be ~1.8% (sum of CH_2Cl_2, 1.1%, and acetone, 0.8%), was low, but comparable with those of other hemp varieties (Vignon *et al*. 1995). Water extraction was performed either under acidic or basic conditions, to obtain pectin (0.6%) and proteins (1.6%, including free amino acids ~0.02%). Extractives were removed before sample hydrolysis to avoid incorrect determination of Klason lignin. Extractive-free samples were used for all chemical analysis.

Table 1. Chemical Composition of Carmagnola Hemp Hurds Weight Percentage on a Dry Basis and Comparison with Other Referenced Values

Components	This Work [a]	Published [b]
Moisture	7.0 ± 0.1	
Extractives (solvent and water)		
Oil – CH_2Cl_2	1.1 ± 0.1	
Oil – Acetone	0.8 ± 0.1	
Pectin – Acidic water	0.6 ± 0.1	1
Protein and amino acid – Basic water	1.6 ± 0.1	
Ash		
Acid-insoluble ash	1.0 ± 0.1	2-4
Total ash	1.2 ± 0.1	
Lignin		
Klason lignin	21.0 ± 1.0 [c]	
Acid-soluble lignin (by UV)	2.4 ± 0.1	
Acid-soluble lignin (by extraction)	3.2 ± 0.1	
Total lignin	23.0 ± 1.0	16-23
Carbohydrates		
Holocellulose	75.0 ± 1.0	
α-Cellulose	44.0 ± 1.0	39-49
Hemicellulose	25.0 ± 1.0	16-23

[a] Standard deviations were calculated from triplicates
[b] Vignon *et al*. 1995; Hurter 2006; Barta *et al*. 2010
[c] After correction of acid-insoluble ash

Ash and Lignin Content

Ash constitutes an extensively studied component of biomass, which is nevertheless poorly understood. Ash is defined as the inorganic and the mineral matter of a biomass. For industrial biomass application, it is important to know the amount of ash that is present. The ash content of the sample was 1.2%, a very low amount when

compared with other varieties of hemp (Vignon *et al*. 1995), a feature that can be considered a positive point.

Lignin isolation was carried out by using a strong acid hydrolysis treatment (72% H_2SO_4): The solid residue, called acid-insoluble or Klason lignin (22%), contains 1.0% of acid-insoluble ash (Table 1). The acid-insoluble lignin content of hemp hurds is in line with that reported by Barta *et al*. (2010). During hurds hydrolysis, a portion of lignin was solubilized and called acid-soluble lignin (ASL, 2% to 3%). In this study, two different methods were used to define the percentage of ASL, namely the commonly used TAPPI method, by measuring the absorbance at 205 nm with a spectrophotometer, or by extraction with chloroform, to isolate ASL from the aqueous solution. This extraction method gives a slightly higher value compared with the UV measurement, probably due to the presence of lignin carbohydrate complexes (LCC).

Holocellulose and Cellulose Yield

The major component of hurds is holocellulose, a polysaccharide obtained by a bleaching process with sodium chlorite. The yield of holocellulose was 75% (Table 1), which is a little higher than reported by Barberà *et al*. (2011), but comparable with values obtained with hardwoods. To obtain α-cellulose from holocellulose, a 17.5% sodium hydroxide solution was used as the reagent. The α-cellulose content was 44% of the dry biomass, which is in good agreement with values reported for other varieties of hemp (Vignon *et al*. 1995). The value of hemicellulose (~25%) was calculated by subtraction of α- and β-cellulose from holocellulose.

Characterization of Extractives

The total lipid extractives (with CH_2Cl_2 and acetone) of Carmagnola hemp hurds accounted for 1.7% of the starting material. They were analyzed by GC and GC/MS. The chromatogram reported in Fig. 2 (A-CH_2Cl_2, B-acetone) shows the lipid extractive composition, which consists mainly of fatty acids, alkanes, aldehydes, and sterols; among them phenols, clionasterol, phytosterol, and coumarin were identified. Results from hurds oil were similar, except for waxes, to the composition of fibers oil (Gutiérrez *et al*. 2006). The protein content of defatted hurds isolated from basic water extraction was 1.6% (Table 1). The characterization of the isolated proteins was carried out by SDS-PAGE analysis. The results did not show the presence of predominant proteins, in contrast to what was observed in the hemp seeds' isolated protein profile (Tang *et al*. 2006).

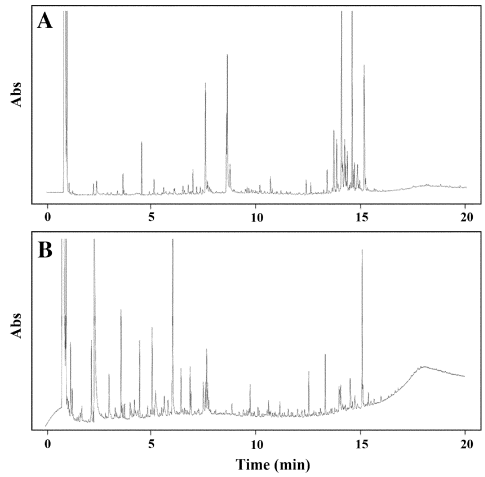

Fig. 2. GC/MS chromatograms of the lipid extracts from hemp hurds (A-CH_2Cl_2, B-acetone). Peak eluted between 4 and 6 min, fatty acids; 7–12 min, aldehydes; 13–15 min, aldehydes and sterols

After removal of proteins from the liquid fraction, the free amino acids content was evaluated to be 0.02%. In order to identify and quantify the free amino acids composition, HPLC analysis was carried out (Fig. 3A). The chromatogram shows the presence of at least nine different free amino acids; four of them were essential amino acids. The more abundant amino acids from the liquid fraction were proline and valine (24 and 18%, respectively). Pectin extraction from different sources may give different yields, according to process parameters (pH, time, temperature) and sample features. The yield of isolated pectin from hemp hurds was 0.6% on a dry matter basis, a lower value compared with those reported from major sources of pectic substances such as citrus fruits and even to what was reported for hemp straw (Vignon *et al*. 1995), probably due to the retting process to which the starting material was subjected. Galacturonic acid is the main component of pectin and was found to be 70% in the samples. The degree of esterification (DE) is an important industrial parameter for the gelling propriety of pectin. The DE of extracted pectin was determined using the enzyme pectate lyase. This enzyme splits the glycosidic bonds of a galacturonic chain, with a preference for glycosidic bond next to a free carboxyl group, by trans-elimination of hydrogen from the 4 and 5 carbon position of the galacturonosyl moiety to form a double bond, thus giving an increase in absorbance at 235 nm. Taking advantage of this peculiarity, the enzymatic hydrolysis of pectin standard (with different DE) and polygalacturonic acid were tested, showing a good linear response as a function of the DE (Tardy *et al*. 1997). By this approach, the DE of the pectin sample was estimated to be 46%, a result in accordance with the data obtained by FTIR.

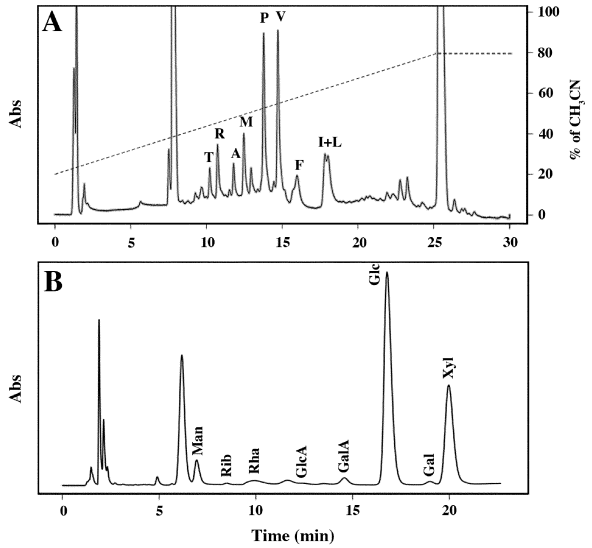

Fig. 3. HPLC chromatograms of the mixture of extracted free amino acids (A) and the monosaccharides mixture (B) obtained by acid hydrolysis of hurds. Dotted line: gradient of acetonitrile. Abbreviations used: T, threonine; R, arginine; A, alanine; M, methionine; P, proline; V, valine; F, phenylalanine; I, isoleucine; L, leucine; Man, mannose; Rib, ribose; Rha, rhamnose, GlcA, glucoronic acid; GalA, galacturonic acid; Glc, glucose; Gal, galactose, Xyl, xylose.

FTIR Spectra Analysis

FTIR spectroscopy was used as a simple technique to obtain rapid information regarding the structure and physicochemical properties of hurds and their components (*i.e.*, cellulose, lignin, holocellulose, and pectin) in comparison with standard materials. FTIR spectra of all samples are shown in Fig. 4. All samples were found to have different absorption in the range 3400 to 2900 cm^{-1}, a strong hydrogen bond O-H stretching absorption around 3400 cm^{-1}, and a prominent C-H stretching absorption around 2900 cm^{-1}. The area between 1800 to 900 cm^{-1}, called the finger print area of spectra, has many sharp and discrete absorption bands due to the various functional groups present in each component. Based on previous literature data, the bands at around 1740 cm^{-1} (hemicellulose), 1500 cm^{-1} (lignin), and 897 cm^{-1} (cellulose) are typical for characterization of pure samples. Spectra from hurds samples, following removal of extractives, show no difference compared with the starting material (data not shown).

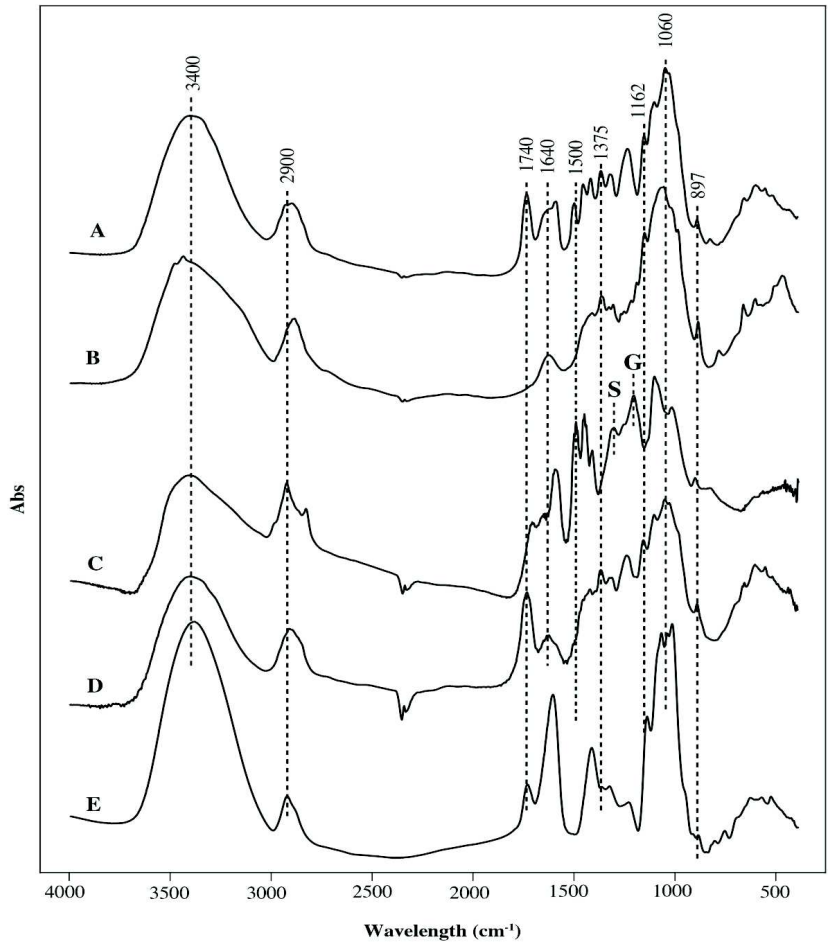

Fig. 4. FTIR spectra of hurds (A), and of cellulose (B), lignin: S, syringyl and G, guaiacyl units (C), holocellulose (D), and pectin (E) isolated from hurds

The absorption bands at 1462, 1423, 1311, 1214, and 1112 cm^{-1} arise mostly from lignin, while the bands around 1376, 1162, 1060, and 897 cm^{-1} are mainly due to carbohydrates and have no significant contributions from lignin (Pandey 1999, Pandy and Pitman 2003; Sun *et al*. 2004; Peng *et al*. 2009). Significant changes have been observed in the fingerprint region of the IR spectra due to various vibration modes in all samples. In two spectra (spectrum B and spectrum D), the absorbance around 1640, 1375, 1060,

and 897 cm^{-1} are attributed to native cellulose. The bands at 1740, 1245, and 1162 cm^{-1} present in spectrum D are due to hemicellulose in holocellulose samples. The band intensity at 1740 cm^{-1} was observed to be higher in the spectra of holocellulose compared with the hurds spectrum because of the C=O stretching vibration of carboxyl groups due to the acetyl moiety presence in hemicellulose (xyloglucan) (Popescu *et al*. 2011). The absence of the band at 1740 cm^{-1}, for a carbonyl group in spectrum B, suggests that the cellulose isolated from hurds with 17.5% NaOH is free of acetyl groups. The band at 1640 cm^{-1} is associated with the bending mode of absorbed water. The higher absorbance at 1375 cm^{-1} arises from C-H symmetric deformation in cellulose and holocellulose. The two bands at 1162 and 985 cm^{-1} are typical of arabinoxylans (Peng *et al*. 2009). The presence of arabinosyl side chains is suggested by weak shoulders at 1162 cm^{-1} (spectrum D). The change of intensity for this band suggests a contribution from arabinosyl substituents. The C-O-C pyranose ring skeletal vibration gives a prominent band around 1060 cm^{-1} in spectra B, D, and E. The region between 950 and 700 cm^{-1}, called the anomeric region, has bands at 897 cm^{-1} in spectra A, B, D, and E and not C, because of the C-1 group frequency or ring frequency, which is indicative of β-glycosidic linkages. The absence of this band in spectrum C reveals that isolated lignin was almost pure without sugar moieties.

The band around 1500 cm^{-1} is assigned to benzene ring vibration and can be used as an internal standard for the lignin sample. Hemp hurd lignin, called guaiacyl–syringyl (hardwood) lignin, is composed of coniferyl and sinapyl-alcohol–derived units, where guaiacyl-type lignin has a weak 1267 cm^{-1} band and a strong band at 1214 cm^{-1}, while syringyl-type lignin has a band near 1315 cm^{-1}. In the samples, a 1267 cm^{-1} band (Pandey 1999) was not detected. The band at 1460 cm^{-1} arises from methyl and methylene deformation, with very high intensity in lignin samples compared with hurds (spectrum A and C). The absorption band at 1715 cm^{-1} for C-O stretching shows the presence of hydroxycinnamates, such as p-coumarate and ferulate (Sun *et al*. 2000). The intensity of this band increases in spectrum C, indicating a higher content of hydroxyl-cinnamates in the isolated lignin sample.

In the case of a pectin sample (spectrum E), absorption in the O-H region is due to the inter- and intra-molecular hydrogen bonding of the galacturonic acid. Bands around 2950 cm^{-1} include CH, CH_2, and CH_3 stretching bending vibrations. Bands occurring at 1740 cm^{-1} and 1615 cm^{-1} indicate an ester carbonyl (C=O) group and carboxylate ion stretching band (COO-), respectively. A carboxylate group shows two bands, an asymmetrical stretching band near 1615 cm^{-1}, and a weaker symmetric stretching band near 1421 cm^{-1}. Bands at 1740 and 1615 cm^{-1} are important for the identification and quantification of the degree of esterification (DE) in pectin samples (Gnanasambandam and Proctor 2000). Three standard pectins with known DE were used to find the linear relationship between the area of the ester carbonyl band and the DE values (R=0.98, n=3), giving a ~46% of esterification for the sample.

Data from FTIR analysis revealed that isolated components are structurally comparable to the standard commercial samples (data not shown).

Phenol Content and Antioxidant Capacity of Solvent Extractives and Lignin

Total phenol content is expressed as gallic acid equivalent (GAE, g/100 g of sample). Acetone extracts showed the highest value of about 6.5 GAE, while Klason lignin and CH_2Cl_2 extracts gave a value of 4.0 and 3.4 GAE, respectively. The highest value of phenol content was obtained from acetone extracts due to the presence of tannins.

To test the radical scavenging ability of solvent extractives (CH_2Cl_2 and acetone) and Klason lignin from hurds, an ABTS test was chosen. The results, reported as Trolox equivalent antioxidant capacity (TEAC), gave 4%, 4%, and 3% for Klason lignin, acetone, and CH_2Cl_2 extracts, respectively.

HPLC Analysis of Monosaccharides

The sugar composition from the hydrolyzed liquid fraction of hurds was obtained by HPLC analysis. The HPLC profile of PMP-sugars (Fig. 3B) shows the presence of eight different monosaccharides, and among them glucose (56.7%), xylose (31.2%), and mannose (4.9%) were the most abundant. Minor amounts of rhamnose (2.1%), galactose (0.9%), and a trace amount of ribose (0.3%), but an absence of arabinose were observed in the samples. Uronic acid, including glucuronic acid (0.2%) and galacturonic acid (2.0%), also appeared in minor quantities. Since xylose and mannose were found in good percentage, we suggest that the hemicellulose fraction would be composed mainly of glucuronoxylan and glucomannan. This agrees with the classification of hemp as a hardwood. Glucose accounted for ~57% of monosaccharides, which correspond to 51% of glucan, this is in good agreement with cellulose found from isolation with NaOH solution. The percentage of glucan found in Carmagnola hemp hurds is higher than reported for other varieties (Moxley *et al*. 2008; Barta *et al*. 2010).

Nitrobenzene Oxidation of Hurds

The eight phenolic components obtained by alkaline nitrobenzene oxidation of hurds were identified by HPLC in comparison with authentic samples. Major components were found to be vanillin (45.1%) and syringaldehyde (35.1%). Minor amounts of gallic acid (0.5%), *p*-hydroxybenzaldehyde (8.5%), vanillic acid (0.9%), syringic acid (6.3%), *p*-coumaric acid (2.9%), and acetosyringone (0.6%) were also identified. The syringylpropane to guaiacylpropane units (S/G ratio) was estimated to be 1.42 and determined according to the method described by Santos *et al*. (2012). The monolignols composition is in accordance with FTIR spectra since the intensity of the syringylpropane units is rather weak, compared to the guaiacylpropane units (see Fig. 4).

CONCLUSIONS

1. The chemical analysis of hemp hurds from the industrial variety "Carmagnola" was performed using standard methods, and the isolated components were fully characterized in order to obtain the chemical composition and the main structural features of its components.

2. Cellulose, holocellulose, and lignin were assessed by hydrolysis. Polysaccharides (cellulose, 44.0%, and hemicelluloses, 25.0%) were the most abundant components of hurds followed by lignin (23.0%), extractives (oil, proteins, amino acids, pectin *etc*., in total 4%), and ash (1.2%).
3. Compared to other hemp varieties, Carmagnola hurds contain very small amounts of ash with high amounts of polysaccharides underlying potential benefits for biofuels production. Nitrobenzene oxidation and FTIR analysis confirmed the presence of guaiacyl and syringyl units in hemp hurds lignin. High yield of vanillin was observed suggesting high availability of guaiacyl units.
4. This study is useful as base line data for agro-economic evaluation of the Carmagnola hemp as a feedstock for an integrated biorefinery, because the valorization of hemp hurds is still overlooked and not fully exploited.
5. To fully valorize the hemp hurds, further investigation on the optimization of pretreatment technique is required.

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