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**Dipartimento di Chimica e Biologia "A.Zambelli"**

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## ISOLATION AND CHARACTERIZATION OF LIGNINS FROM WHEAT STRAW AND CARDOON BIOMASSES

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# Summary

# 1 Introduction

The use of residual biomasses to obtain bioenergy and green chemicals is a real possibility to contribute to sustainable development. These materials are composed mainly of polycarbohydrates (cellulose, hemicellulose) and phenolic polymers (lignin) and have great potential for use. Lignocelluloses are mainly composed of cellulose $(C_6H_{10}O_5)_n$, hemicellulose $(C_5H_8O_4)_m$, lignin $[C_9H_{10}O_3(OCH_3)_{0.9–1.7}]_x$, pectins, extractives, glycosylated proteins, inorganic salts. The cellulose, hemicelluloses, and lignin content of such biomasses fall in the range of 30–50%, 15–35%, and 10–20%, respectivley (Kaparaju et al., 2009, Pettersen et al., 1984, Girio et al., 2010). Cellulosic and hemicellulosic component of the biomasses are tightly linked to the lignin component through covalent and hydrogenic bonds that make the structure highly robust and resistant to any treatment.

Cellulose and hemicellulose could be hydrolyzed into monomeric sugars such as glucose, xylose, and arabinose, which could then be converted to biofuels such as bioethanol and methane. However, because the chemical components and physical structure of lignocellulosic biomass could protect cellulose from degradation, the bioconversion of these materials has remained challenging (Akimkulova et al. 2016). Typically, lignin and hemicellulose in the lignocellulosic materials need to be removed before the enzymatic hydrolysis of cellulose.

In concern with resolving the energy and environmental crises, the production of renewable and sustainable energy, such as liquid biofuels (ethanol, biodiesel, etc.) and gas biofuels (hydrogen, methane) from different raw materials is currently being developed. All biofuels are classified into four generations considering the different raw materials and a technological perspective (Soltanian et al., 2020); first-generation biofuels are made from food-based crops containing starch (e.g., corn, wheat, cassava, sugarcane) and therefore their production creates conflict with food or feed supply and arable lands. Interestingly, first-generation biofuels account for 96% of the total worldwide production of biofuels in 2020. Lignocellulosic-based biofuels referred to second generation biofuels, which could be a potential petrol substitute in the close future. Third- and fourth-generation biofuels are obtained

using algal biomass except that fourth-generation biofuels use genetically modified algae, which contain high lipid content.

Recalcitrant structure and complexity remains a major economic and technical obstacle to lignocellulosic based biofuel. As a result, only 20% of its theoretical maximum yield of glucose can be obtained by enzymatic hydrolysis unless it is pretreated properly (Akhtar et al., 2016). Properly conditioned lignocellulosic biomass is used as a fermentation substrate and reserve aromatic substances to produce biofuels or basic monomers. A fundamental phase of this transformation is the separation of the three macroconstituents, a stage called pretreatment that can be achieved in very varied modes. Of particular interest is the use of saturated steam at 200 °C, which is effective, economical and with a low environmental impact. A side effect of hydrothermal pretreatments is the production of unwanted molecules resulting from dehydration of certain sugars, which can affect subsequent stages. The success of the entire supply chain depends on the optimization of these early stages of biorefining. In the first phase of the process of pretreatment of biomass, the destructuring of lignocellulosic fibers is caused by chemical-physical means; this process separates lignin (polymer from complex three-dimensional structure containing phenylpropionic units), and cellulose from hemicellulose.

Lignin is one of the three main components of plant cells along with cellulose and hemicellulose. For many years, this macromolecule has been used almost exclusively, after isolation, for energy purposes in the pulp and paper industries. Today, lignin is considered a potential renewable precursor in the development of high-value applications such as controlled release devices, novel composites, nanoparticle applications, application devices in electronics, carbon fiber manufacturing, and absorbing and dispersing agents. Furthermore, among the various applications, lignin is currently being studied to be used in the future as a starting point for the preparation of adhesives for the production of green building panels, polyurethanes and coating surfaces. The study of lignin for these applications is still in a pioneering phase of experiments that hopefully will help resolve some negative aspects related to the use of fossil-based products.

Although this macromolecule is of great interest in the sustainable material production and sustainable chemistry field, it has considerable complexity with respect to the extraction from lignocellulosic biomass and subsequent precipitation for isolation pruposes.

## 1.1. Circular economy and bioeconomy

Today more than ever, with global warming, pollution and the problem of plastic waste, it is essential to rethink the economic cycle in terms of circular economy; it is therefore necessary to think about reuse, greater process efficiency and longer product life thanks to new technologies and materials. In Europe and the rest of the world, an economic system designed to be able to regenerate itself and therefore also guarantee its own eco-sustainability is becoming increasingly popular. This is a circular economy concept. A circular economy is a vision of a sustainable society, demanding greater responsibility for sustainable biomass production and the recovery of material value from products at end-of-life. To do this, fertilizer production must be uncoupled from mineral and fossil feedstocks, and a diverse renewable energy market established that utilizes waste biomass. Integrated waste management technologies will be essential to extract the maximum value from mixed wastes. By circular economy, the European Union specified in 2016, we mean a production and consumption model that provides for the sharing, loan, reuse, repair, reconditioning and recycling of existing materials and products for as long as possible.In practice, it is a zero-waste economy, where any product must be consumed and disposed of without a trace. Obviously, in the circular economy, renewable energy and the versatility and modularity of objects are very important, which can and must be used in various contexts in order to last as long as possible. So it is clear that the circular economy presupposes a systemic way of thinking, which does not end in the design of products intended for a single purpose; according to this model, not only is the environment protected by saving on production and management costs, but also profits are produced.

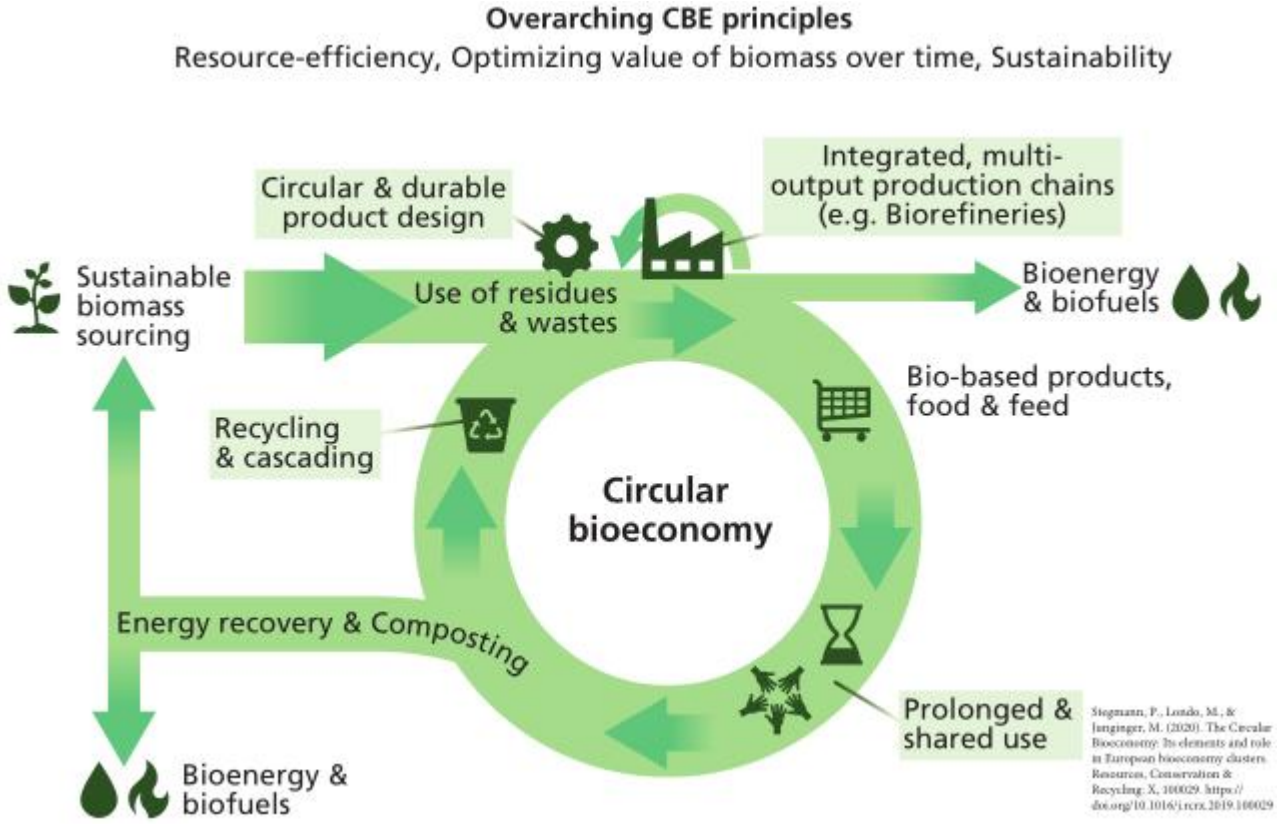


**Figure 1.** The circular bioeconomy principle and its elements (Stegmann et al., 2020)

So far, we read in the paper "Closing the loop-new circular economy package" of the European Parliament, the economy has worked with a "production, consumption, disposal" model linear where each product is inexorably destined to reach the "end of life". Precious materials are used to produce food, build houses and infrastructures, manufacture consumer goods or supply energy. When they have been fully exploited or are no longer needed, they are disposed of as waste. The increase in population and growing wealth, however, push the demand for (scarce) resources more than ever and lead to environmental degradation. In the European Union every year almost 15 tons of materials are used per person, while each EU citizen generates an average of over 4.5 tons of waste a year, of which almost half is disposed of in landfills. The linear economy, which relies exclusively on the exploitation of resources, is no longer a viable option (Tollefson, 2018).

To understand the importance of the circular economy, we must first consider some of the problems that humanity is facing right now. One of the most relevant issues of recent years is certainly the overheating of the environment caused by the $CO_2$ emitted mainly for energy production, for industrial activity and for transport. In particular, according to the latest study by the IPCC (Intergovernmental Panel on Climate Change) of October 2018 (Tollefson, 2018), to avoid the increase in global

temperature over 1.5 °C compared to the pre-industrial period (the maximum due to catastrophic effects on the global ecosystem) we have about 12 years to reduce $CO_2$ emissions by 50% and about 30 years to completely eliminate them. Otherwise, some of the effects that are already manifesting themselves at present will widen even more with a devastating impact of drought, fires and floods. These events have already caused approximately $ 320 billion in damage in 2017. In addition, the damage caused by air pollution, which causes about 9 million people to die a year, should be noted.

Another particularly relevant issue is plastic and the damage deriving from it. Only 15% of plastic is recycled worldwide (OECD 2018), (Chinaglia et al., 2018) 25% is subjected to energy recovery and the remaining 60% ends up in landfills. The circular economy is however something more than waste recycling: it involves the development of a real economy to be opposed to the linear one, from the production of a product to its becoming waste. On 2 December 2015, the European Commission adopted an ambitious circular economy package. It consists of an EU action plan with measures relating to the entire life cycle of products: from design, procurement, production and consumption to waste management and the secondary raw materials market. The rules aim to have a practical effect on the life of European citizens. The provision obliges member countries to recycle at least 70% of urban waste and 80% of packaging waste and prohibits the disposal of biodegradable and recyclable waste in landfills. The rules should come into force from 2030. MEPs will have to find a balance on the concepts of "waste" and "recycling" and harmonize a system that includes countries with different habits: Germany and Austria already recycle 66% of waste, the Czech Republic which does not reach 30%.

Nowadays, the “circular economy” is gaining significant interest in integrating the whole process for a cost-effective process. The circular economy approach utilizes the resources throughout their shelf-life towards maximally profitable usage, whereas in a linear economy, the resources are created, utilized and disposed of. Besides, the circular economy approach focuses on the recovery and regeneration of materials and resources at the end of each life cycle product. Lignin valorization aims to integrate the whole process that fits the circular bioeconomy principle (Fig. 1).

The circular economy is a production and consumption model that involves sharing, lending, reuse, reconditioning and recycling of existing materials and products for as long as possible.

This extends the life cycle of products, helping to reduce waste to a minimum. Once the product has finished its function, the materials it is made of are in fact reintroduced, where possible, into the economic cycle. Thus they can be continuously reused within the production cycle, generating additional value.

The principles of the circular economy contrast with the traditional linear economic model based on the typical "extract, produce, use and throw" scheme. The traditional economic model depends on the availability of large quantities of materials and energy that are readily available and at low prices.
The European Parliament calls for the adoption of measures also against the planned obsolescence of products, a strategy typical of the linear economic model.
In the European Union, more than 2.5 billion tons of waste are produced every year. The EU is updating waste management legislation to promote the transition to a circular economy, as an alternative to the current linear economic model.
In March 2020 the European Commission, under the European green deal, in line with the proposal for the new industrial strategy, the action plan for a new circular economy that includes proposals on the design of more sustainable products, on waste reduction and on give more power to citizens such as "the right to reparation". Resource-intensive sectors such as electronics and information and communication technologies, plastics, textiles and construction, enjoy specific attention. The European parliament voted for a new plan action for the circular economy, calling for additional measures to achieve a zero-carbon, environmentally sustainable, toxic-free and fully circular economy by 2050. Also included are stricter rules on recycling and targets binding by 2030 on the use and ecological footprint of materials.

The bioeconomy must be understood as the set of food, bioenergy and biomaterial production activities or all activities that transform biological resources. In some sectors such as forestry and the production of biomaterials, biotechnological processes are not involved but biophysical or biochemical processes. Others believe that focusing attention on the use of biomass flattens the relative importance of the various activities of the bioeconomy, in particular neglecting the role of quality food production and the multifunctionality of agriculture (Brunori, 2013 and Schmid et al., 2012).
A sustainable bioeconomy considers the production of high-quality food and the transformation into energy, the last step in a series of cycles of use and reuse, of primary importance. These principles require adequate technological paradigms, which shift the focus on second type productivity, centered on new products and new functions in the primary sector (Esposti, 2012).
The COVID-19 pandemic, the war in Ukraine and the economic sanctions against Russia have affected globalization processes and the bioeconomy. The Ukrainian war radically changed the contours of the global economy and brought about a change in the established world order (Roubini, 2022). The impact of the war in Ukraine, the scarcity of non-renewable energy sources and the increase in the prices of non-renewable energy could also be essential for the future analysis of the willingness to use bioenergy in neighboring countries and in the rest of the world (Liobikienė & Miceikienė, 2022). A

review of future strategies for food security starts from the need to give more weight to the responsibility that Europe has towards the rest of the world. In addition to guaranteeing access to sufficient, safe and nutritious food for all European citizens, Europe must in fact respect its commitment in international fora to promote the right to food as a global right. This means first of all taking into account the systemic implications of policies, environmental strategies, the development of technologies and setting up governance mechanisms capable of preventing unwanted effects.

## 1.2 Natural resources sustainability

The concept of bioeconomy highlights a close interdependence between the ecological system and the human system which is also at the basis of the dilemma between economics and ecology: how to optimize the performance of the economic system without eroding the ecological foundations of wealth. Managing natural resources in a sustainable way implies the creation of institutions capable of regulating access in the name of the collective interest and the provision of feedback mechanisms that report imminent risks and stimulate the necessary initiatives to prevent them (Young et al., 2006). These mechanisms should control both the supply and demand for resources. According to some scientists, humanity has already crossed some of the "boundaries" of a safe operating space with respect to the Earth system, increasing the possibility that some subsystems alter their state irreversibly (Rockstrom et al., 2009) drastically decreasing the ability to provide ecosystem services.

The scientific world today agrees on a series of general principles of intervention and research:

stop the expansions of agricultural areas to the detriment of forests and natural environments, reduce the waste (estimated at around one third of the entire production), (Foley et al., 2011).

The circular economy is a much discussed pathway towards sustainability. There are many definitions of the bioeconomy, as well as usage of similar terms, such as biobased economy and green economy. In practice, the bioeconomy has turned out to be a changing concept and adjustable for many purposes. In this thesis the definition from the Global Bioeconomy Summit 2015 is used: *'bioeconomy as the knowledge-based production and utilization of biological resources, innovative biological processes and principles to sustainably provide goods and services across all economic sectors'*. However, emphasis is on two key aspects:

• the transformational role of the bioeconomy in replacing fossil-based products (e.g., oil-based plastics or textiles), non-renewable materials (e.g., steel, concrete), or non-sustainable biological products (e.g., cotton in certain regions);

• the enhancement of the natural capital approach to economy, that is, better integration of the value of natural resources and life sustaining regulatory systems (e.g., biodiversity, fresh water supply) to economic development.

The first part is generally already well understood in bioeconomy strategies, the latter less so. The long-term sustainable production of natural capital relies on the key role of forests as the most important land-based biological infrastructure on the European continent. Forests provide the largest source of renewable biological resource not competing with food production. Finally, although not specifically addressed in this report, we are aware that combining digital technology with biology can offer significant advances for the bioeconomy in the future. The bioeconomy covers a wide variety of products and industrial sectors (and services), such as construction, bio-plastics, packaging materials, food ingredients, textiles, chemicals, pharmaceuticals, and bioenergy. It also includes the services related to bio-based products, such as intellectual property rights, consulting, R&D, marketing, sales, servicing of machinery, administration, etc. Ecosystem services such as recreation, tourism and water supply are also part of bioeconomy. Despite its sectoral importance, the bioeconomy should be seen in a holistic way, given its full potential to deliver broad social, economic and environmental benefits at the societal level. These include:

1. Inclusive economic growth and job creation. The use of biological resources provides better opportunities for sustainable, inclusive growth than fossil-based resources. Typically the oil assets and incomes generated by these are owned by relatively few. On the other hand, the EU has 16 million private forest owners and the Member States (citizens) own one-third of the forest area. The distribution, ownership and characteristics of forest biological resources offer high potential for inclusive economic development and jobs, also in rural areas. In cases where biological resources are owned by few, or there is a lack of well-functioning markets, there is a need to develop the institutional setting to allow inclusive growth.

2. The emergence of climate-friendly cities and industrial sectors Urban areas are home to half the world's population, and almost three-quarters of the EU28 population lived in an urban area in 2014 (EUROSTAT 2016). Cities account for more than 80% of global economic output, consume close to two-thirds of the world's energy, and account for more than 70% of global greenhouse gas emissions (World Bank).

The SDGs have a specific goal (no. 11) for sustainable cities and communities – "to make cities inclusive, safe, resilient and sustainable". A circular bioeconomy can be an important contributor to this. The biomass building blocks cellulose, hemicellulose, lignin and extractives are already available today, and can increasingly in future be the basis for materials in many sectors and products. The role of climate-resilient cities in a sustainable circular bioeconomy is vital because cities are the place where most consumers live, purchase, and consume. The choices related to commerce, infrastructures, health, mobility, and education significantly affect urban livelihoods and the environment, both through demand and supply. Urban agriculture will have a decisive role, especially about the provision of fresh vegetables, and urban forestry could become an essential nature-based component of rebuilding cities to provide healthy spaces while providing local feedstocks for the bioeconomy, and biodiversity gains (Acuto et al., 2020).

3. Europe's biological capital and environmental sustainability should be seen as the two sides of the same coin. Biodiversity should be recognised as a crucial part of the natural capital, and valued and managed as a priority. Biodiversity increases the productivity and resilience of ecosystems (Liang et al., 2016). Second, long-term investments in a bioeconomy can enhance biodiversity and adaptation to climate change (Nabuurs et al., 2015) . The existing linear fossil-based economy threatens biodiversity through its impacts on climate change, toxic wastes and other environmental aspects. Investing in biodiversity conservation should be a priority in a sustainable bioeconomy, with the aim of a positive coupling between economy and ecology and synergies with the energy and food nexus. The bioeconomy should ensure synergies with sustainable renewable energy production based on forest, non-food agro- and waste biomass. It should advance closed circular nutrient cycles - nutrients (mainly phosphorus and nitrogen) need to be recovered and nutrient leakages prevented. The negative impacts of biological production, such as the expansion of the agricultural frontier (deforestation, loss of valuable habitats) and emissions of nutrients and agrochemicals to soil, water bodies and the atmosphere must be avoided. A bioeconomy must ensure sustainable nutrient use, through more efficient fertiliser use and nutrient recycling. It can also help soil carbon restoration e.g. by putting $CO_2$ back in the soil. Regenerative agricultural practices can reduce atmospheric $CO_2$, while also boosting soil productivity and increasing resilience to floods and drought. Techniques include planting fields year-round in crops or other cover, and agro forestry that combines crops, trees, and animal husbandry. It is vital in Europe not only to stock $CO_2$ in soil, but also to improve soil fertility, reduce the impacts of drought and increase erosion resistance.

For the first time in human history, we face the emergence of a single, tightly coupled human socio-ecological system of planetary scope. The world and Europe are facing unprecedented interconnected

challenges which will even strengthen in the coming decades: increasing demand for food, water, materials and energy while mitigating and adapting to climate change and reversing environmental degradation, including biodiversity loss, nutrient emissions and land degradation. Addressing such grand challenges, while supporting social and economic prosperity for a growing population, requires a system change in our economic model. For 200 years we have had an industrial era built on a fossil-based, linear economy. We have seen the transformation of global societies as never before in human history. The industrial era has delivered economic and demographic growth as well as social and technological progress. Over the last 50 years the global economy has experienced a great acceleration, which has triggered significant global economic convergence and a significant reduction of both poverty and inequality between rich and poor countries. However, poverty and inequality are still an issue, even for developed countries. The industrial era and its economic acceleration have also resulted in an unprecedented rate of environmental degradation related to economic growth. This is clearly seen when comparing GDP growth with other indicators adjusted for natural capital destruction. The world has grown out of the planet. According to the Global Footprint Network, in 2015, we already used a full 1.6-times the sustainable level of resources in our planet. In two decades it will require two planets to sustain our current economic system. The context of global and European societies has changed. Now we need a new concept for the new context, a new economic paradigm that puts the basis for human prosperity within the planetary boundaries. The year 2016 was a turning point: the 2030 Agenda for Sustainable Development and its Sustainable development Goals (SDGs) were adopted, and the Paris Agreement on climate change came into effect. These sent out a global political message on the way forward to transform our economic system to end poverty, protect the planet, and ensure prosperity for all. This requires new concepts to realize these international agreements, and bring them to action. The circular biobased economic paradigm can be this – it builds on the synergies of the circular economy and bioeconomy concepts. These two concepts have so far been developed in parallel, but now need to be connected to reinforce each other. On 13 February 2012, the European Commission adopted a strategy for "Innovating for Sustainable Growth: A Bioeconomy for Europe". Many European and world countries have developed their own bioeconomy strategies in recent years. In 2017, the EU started to review the existing Bioeconomy Strategy to reflect on its future development. We believe the circular bioeconomy has great potential to catalyse an inclusive European economic, political and societal project that is urgently needed. A project in which economic prosperity is more equally distributed among citizens and placed sustainably within the renewable boundaries of the planet. The shift to a circular biobased economic paradigm should be a long-term strategy for decoupling economic growth from environmental degradation. It needs to be socially, economically and environmentally sustainable. The story of the first-generation biofuels in the

beginning of this century is a lesson from which we should all learn. Science and technology are laying the foundations for the bioeconomy age. Biobased products have emerged that can substitute fossil-based materials like plastics, chemicals, synthetic textiles, cement and many other materials. Now the big question is how do we take this scientific and technological success to a scale of economic paradigm shift. How can we ensure that longstanding industries such as the textile, petrochemical, construction and plastic sectors join and even lead this paradigm shift in a sustainable way? We welcome this report coordinated by the European Forest Institute (EFI). It reflects on the main needs to update existing bioeconomy strategies, connecting to the UN SDGs, the Paris Agreement and other recent developments including the circular economy. It provides strategic recommendations which should be considered when developing a new bioeconomy strategy for Europe, based on sustainability principles. It also provides science-based insights on the potential of forest resources, our main biological infrastructure, and on how forest-based solutions can help to develop the bioeconomy from niche to norm.

A circular economy is defined by two governing principles: maximize the service provided by the materials embedded in products; and minimise the loss of service with time (Clark et al., 2016). Unsustainable resource consumption, product redundancy, waste and pollution need to be avoided. Policies promoting renewable energy and bio-based products have elevated the importance of biomass feedstocks in the European Union (EU). This helps create the conditions that enable a circular economy through the use of renewable materials. China also has strong circular economy ambitions (albeit via different policies).

The incentivisation of “eco-parks” exemplifies the Chinese emphasis on industrial development (Matthews and Tan, 2016). This is a form of industrial symbiosis, where the waste of one operation (material or energy) is the input of another.

One fundamental principle is that the materials from which a product is made has an impact on suitable end-of-life waste management options (Fig. 2).

The term “circular bioeconomy” has been adopted to describe the integration of bioeconomy initiatives into the newer policy emphasis on circularity. An OECD paper highlights some confusion in policies that need to be resolved to fully integrate the two philosophies (Philipp et al., 2018).

For instance, the presumption that only low value waste is fit for biorefineries is impeding the development of a bio-based economy and unnecessarily restricting the potential for retrieving maximum value from wastes.

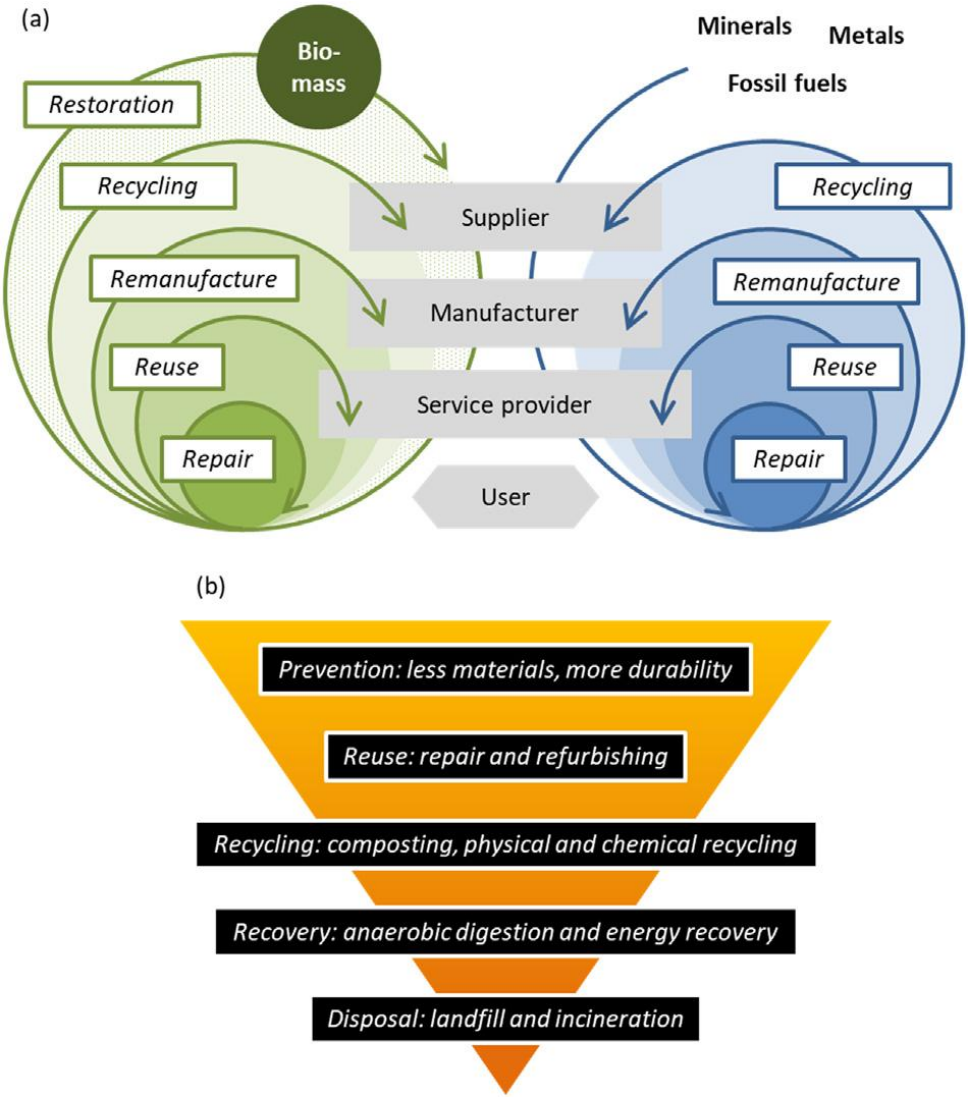


**Figure 2.** (a) Circular economy conceptual diagram (b) The waste hierarchy (Sherwood, 2020).

## 1.3 Renewable and sustainable resources

The choice of appropriate feedstocks is a particular concern if resources are small compared to demand often a product can be made from different materials and scarce resources avoided. Furniture can be made from wood, metal (steel or aluminium), natural or synthetic textiles, partially from glass, or a combination of these abundant materials; only a major re-evaluation and restructuring of the infrastructure surrounding the electronics industry (and many other sectors) will prevent the complete depletion of the economically viable reserves of many elements (Sherwood , 2020).

In addition to metals and other minerals, crude oil and natural gas are vitally important resources at present. Approximately 90% of petroleum stock is used as fuel, and the remainder is separated and processed into base chemicals, and materials like bitumen (asphalt) for construction (Morrison et al., 2015).

Base chemicals (syngas, olefinic and aromatic hydrocarbons) are needed to make the majority of the organic products that consumers and businesses demand. Thus, the substitution of petroleum must happen earlier than if it were only decided by its finite quantity. However, it is this non-renewable nature of coal, crude oil and natural gas that demands once utilised it remains in use virtually indefinitely to fulfill a circular economy. This is certainly not the case at present, especially for fuels.

Despite this, countries such as the UK, Germany, and France persist in subsidies for fossil fuels, although more money does go towards the provision of renewable energy. Petrochemicals at least have the possibility of being reused and recycled, given product design and waste management infrastructure are adequate. Figure 3 shows the quantity of feedstocks (left) and what products they are converted into (right), with the height of each section representative of the relative mass of each substance. Nitrogen fertiliser (275 million tonnes, MT) and plastics (222 MT) are the largest outputs. Accordingly, these markets require the greatest attention in a circular economy, not necessarily just for the risk of exhausting the feedstock but because of the scale of waste created.

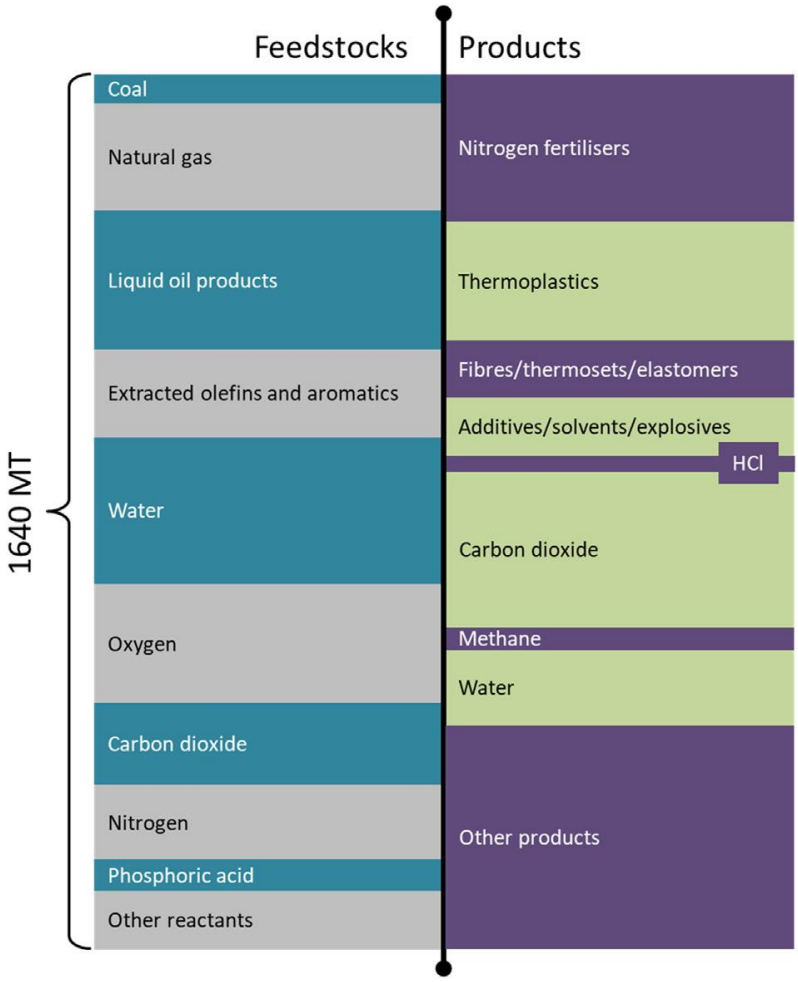


**Figure 3**. Proportional product streams in the petrochemical industry (globally 1640 million tonnes (MT) in 2013, excluding energy products) (Levi and Cullen, 2018).

Biomass is the alternative organic feedstock to crude oil and natural gas. Biomass is a general term applicable to all plant and animal derived materials. Plant-based biomass is cultivated to make food and animal feed, bio-based products, and burnt for renewable energy.

The major structural components of terrestrial biomass are cellulose, hemicellulose, and lignin. These are the inedible polymers that form wood and straw for example. Edible portions of plants can include starch, free sugars, protein, and vegetable oils (triglycerides). Some crops also provide a source of essential oils and other secondary metabolites of high value. The processes of separating and valorizing biomass are the function of a biorefinery (analogous to an oil refinery).

A waste-free biorefinery utilises all the available biomass components to make products and energy, consistent with the fundamental objective of a circular economy.

Demand for biomass feedstocks, like any other, can be managed by implementing efficient reuse and recycling strategies for the products made from them. This is how a circular bioeconomy paves the way for the substitution of unsustainable feedstocks with biomass.

Without extending the longevity of biomass thorough cascaded uses, it will be impossible to satisfy demand for both materials and energy in a sustainable way.

A circular bioeconomy requires sustainable biomass as a guarantee that the restoration cycle is completed and can be completed indefinitely.
Certification schemes are used to validate the sustainability of biomass, a requirement of the revised EU renewable energy directive, but which represents a small proportion of all biomass crops (i.e. 4% of all sugarcane cropland).
To enact a circular bioeconomy, sustainable biomass feedstocks must be produced in the necessary quantities for the foreseeable future, yet certification schemes do not demand enough of operators to ensure longevity to their biomass production. It is true that certification can be revoked if unsustainable practices are discovered, but accreditation is mostly based on current practices and not long terms goals. A stronger obligation for biomass producers to sustainably manage their land use for a much longer period of time is important in a circular bioeconomy.
A stronger obligation for biomass producers to sustainably manage their land use for a much longer period of time is important in a circular bioeconomy. A business model that maintains sustainable production is ultimately profitable for a longer time in the face of new legislative actions and the preservation of the environment necessary to continue producing high yielding biomass.
The actions stated in the EU bioeconomy strategy (updated in 2018) are more concerned with promoting, supporting and monitoring a bioeconomy than establishing the conditions to sustain it.

## 1.4 Fertiliser

Producing biomass incurs energy and material costs. Fertilisers based on nitrogen, phosphorus and potassium, and the energy needed to make them, are very important considerations in a circular economy because of the magnitude of this industry, and its vital importance to the bioeconomy in order to produce high yielding biomass crops. A significant 1% of global energy demand is consumed making nitrogen fertilisers (Dawson et al., 2011). Fertiliser demand increases at a greater rate than crop productivity which will accelerate resource depletion in the context of an increasing world population (Sattari et al., 2016) .
The abundance of potassium mined from mineral reserves means it causes the least concern over the supply security of the three primary fertiliser nutrients. Current annual extraction is 0.7% of reserves and an estimated 0.02% of the estimated total potash on Earth. Regarding nitrogen and phosphorus, the

situation is much more severe. Anthropogenic "interference with the nitrogen and phosphorus cycles" has exceeded sustainable levels (Rockstrom et al., 2009); there is also an inescapable link between fertiliser and fossil fuels that means the bioeconomy is dependent on the petrochemical industry. Even if we set energy arguments aside, nitrogen fertiliser production requires methane, used as a source of hydrogen. This methane is near-exclusively sourced from natural gas. The isolation of phosphorus from mineral ores requires sulphuric acid, made by oxidation of the sulphur isolated from natural gas exploration. Thus, natural gas is vital to the fertilizer industry and therefore biomass production. For biogas to be used to any meaningful extent to make nitrogen fertilisers the annual volumes will need to be huge: 120 MT for ammonia production 200 MT to serve the whole petrochemical market (Fig. 3), and 2800 MT including energy uses (BP, 2019). Current biogas production is 26 MT in natural gas energy equivalents by comparison (Levi et al., 2018).

In the context of substantial projected increases in biomass production (for food, energy, and bio-based products), nutrient recycling rates must be improved. Denitrification (the process of bioavailable nitrogen being converted to $N_2$) occurs naturally and is promoted in water treatments to nullify pollution. In both instances the nitrogen recirculation is at its longest and least efficient pathway, having to re-enter the Haber-Bosch process or biological nitrogen fixation as $N_2$ (Scarlat et al., 2018). Simplified annual global nitrogen fluxes (not total quantities) are represented as a Sankey diagram in Figure 4 (Canfield et al., 2010).

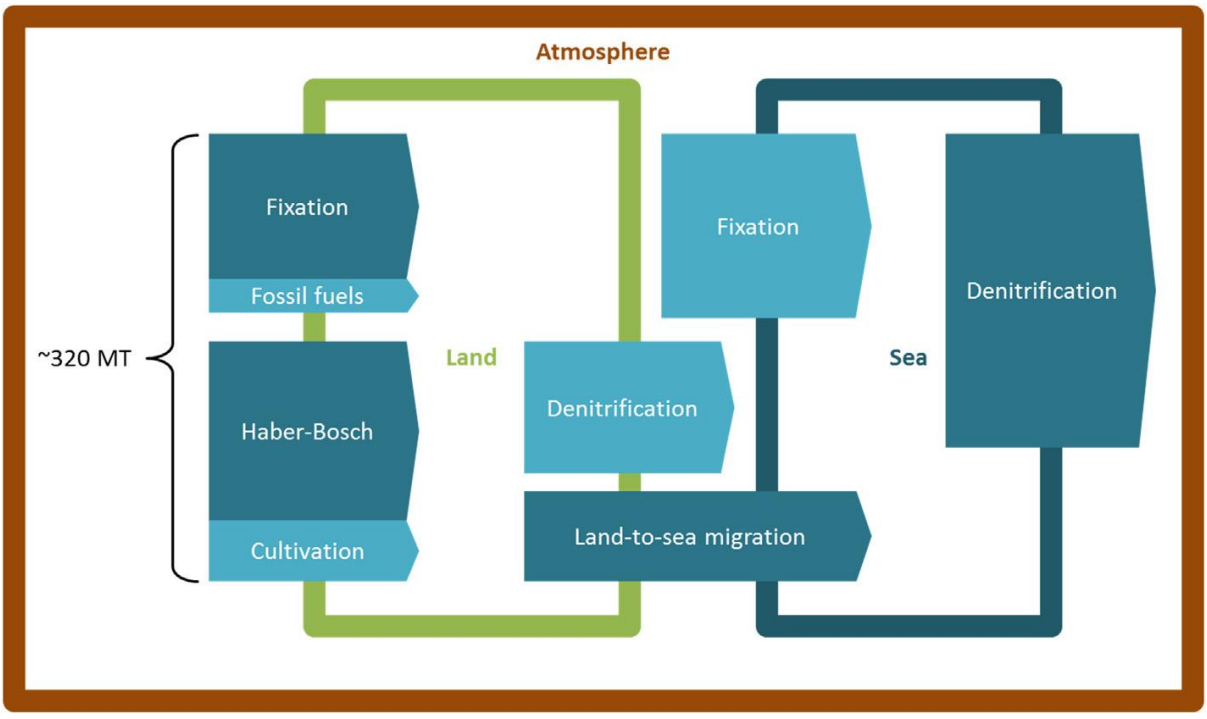


**Figure 4.** Annual nitrogen fluxes between atmospheric, land, and ocean domains (Canfield et al., 2010).

Figure 4 shows mass flows, with proportions reflected by the size of the arrows. The nitrogen flows occur between land, ocean, and atmospheric domains, as represented by the boxes. Nitrogen flows to air and water nearly cancel out anthropogenic efforts to replenish it. One solution being explored by synthetic biologists is to engineer cereals to fix nitrogen through the expression of nitrogenases(Rogers et al., 2014). Optimistic projections calculate that 60% of mineral phosphate demand could be eliminated by phosphorus recovered from wastewater, preventing food waste, and changing agricultural practices to eliminate overuse where there is no return for the fertiliser applied could be eliminated by phosphorus recovered from wastewater, preventing food waste, and changing agricultural practices to eliminate overuse where there is no return for the fertiliser applied (Koppelaar et al., 2013). Sources of annual losses of phosphorus are indicated in a Sankey diagram as Figure 5 (Cordell D. et al., 2009). Resource depletion is shown when the material flow arrows leave one of the value chain domains (processing, farm, consumption). The majority of phosphorus is lost due to erosion, underutilised manure, and the non edible parts of crops.

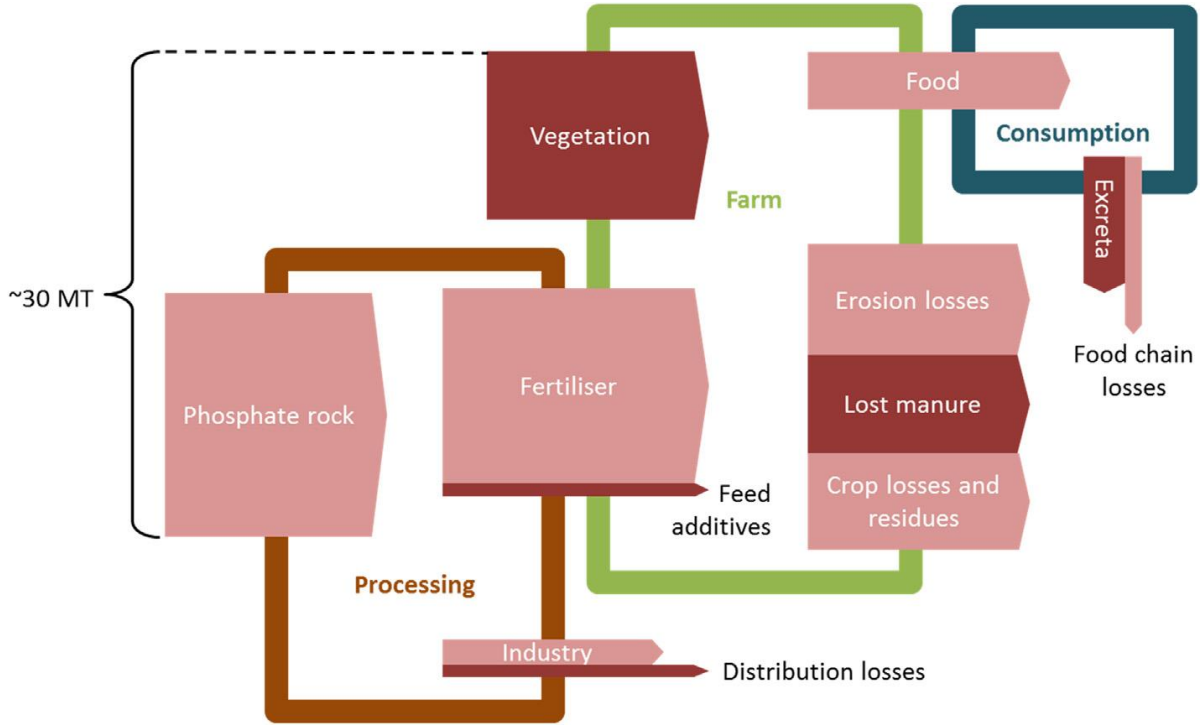


**Figure 5.** The material balance in the global phosphorus cycle (Cordell et al., 2009).

Research continues into a host of other materials as well as biological and chemical methods to improve nutrient removal from wastewater, but normally without demonstrating its application as a fertiliser. An alternative approach is to remove elements from wastewater that are uncomplimentary to

fertiliser applications (e.g. metal salts) and then use the water (still containing bioavailable nitrogen and phosphorus) in agriculture. Research showed, for example, that hydrogels can remove copper and chromium from wastewater and that the nutrient-containing water is an adequate fertilizer (Yuan et al., 2020).

This approach is helpful to recirculate nitrogen and phosphorus but also capture water for biomass production. The availability of water has been described as a "significant constraint" on supplying biomass for energy purposes (Shuster, 2018). Concerns about water use will only increase with growing demand for bio-based products and energy. Unfortunately, wastewater is a dilute source of minerals and so as a source of nutrients it is unlikely to become economically competitive with virgin minerals until reserves are all but depleted.

Phosphorus recovery from manure is seen more viable than recovery from wastewater, but the costs, energy and global warming potential of both these recycling approaches are greater than mining phosphate rock (Golroudbary et al., 2019) .

Ultimately a combination of different approaches will be needed to wean biomass production from its dependence on fertilisers derived from mineral reserves and natural gas.

## 1.5 Energy

The energy market is changing fast. The phasing out of pollution sources, notably coal for electricity generation and petroleum transport fuels, is dictated by policies to promote renewable energy. Energy is a critical factor that controls the socio-economic development of a country as per Global Status Report (GSR) on energy the large majority i.e. 78.4% is met by non-renewable fossil fuels such as petroleum, coal and natural gases and merely 19% from renewable resources such as solar, hydropower, wind, and biomass (Renewables Global Status Report, 2009).

Biomass is somewhat different to other renewable energy sources because it is a material combusted to produce heat (before it can be converted into electrical or mechanical power) or indeed used as the precursor to products as well. In that sense, it is more like coal, crude oil or natural gas than it is wind or solar energy. Biomass is increasingly being used as an energy source for power stations.

The scale of biomass production needed to reach political renewable energy targets is achievable (Oliver and Khanna, 2017), but we cannot be confident biomass will get anywhere near to completely replacing the total demand for electricity and liquid transport fuels because of the aforementioned reasons. Contrary to plans to increase the contribution of biomass towards energy demand (Scarlat et al., 2015) wind, hydro, and solar power sources must make up the majority of the electricity market in

the absence of fossil fuels. Biomass can then primarily be converted into chemicals and materials once food and feed markets are satisfied. Waste biomass however remains an appropriate option for solid and liquid fuels. The use of waste cooking oil triglycerides to make renewable diesel is a commercial enterprise operated by Neste for road transportation (Ondrey, 2016). British company Bio-Bean (Atabani et al., 2019) produce solid fuel pellets from coffee grounds sourced from airports and other centralised waste locations. The calorific value of coffee pellets is actually greater than wood pellets but attention to their preparation is necessary (e.g. creating sufficiently high density) to meet the requirements of renewable energy accreditation schemes.

Should a combination of waste sources be needed to make an impact on the pellet energy market, then the careful selection of the fuel composition will be needed to ensure a stable power output.

## Lignin within the circular economy and sustainable society contexts

The industrial production of most of the fuels, plastics and many other materials commonly used is completely dependent on fossil resources. In recent years, the lignocellulosic biorefinery has shown enormous potential for the development of sustainable renewable resources. The valorisation of waste vegetable biomass, or specifically cultivated in marginal land in an integrated biorefinery context, will be the best approach to compete with fossil-based refineries. The role of the lignocellulosic biorefinery is not only to meet energy needs, but also to reduce environmental problems by replacing conventional oil sources. For a large-scale biorefinery, an economic, natural and renewable resource such as lignocellulosic feedstock is widely used for the production of biofuels / bioenergy and chemicals / value-added products that include sugars and their derivatives, such as alcohols (ethanol, butanediol) or microbial oils, 5-hydroxymethylfurfural (5-HMF), organic acids, such as levulinic acid, succinic acid, formic acid and phenolic compounds. In addition, these chemicals can be converted into a wide range of derivatives for various applications in the polymer, biofuel and solvent industries. For the conversion of lignocellulosic biomass into value-added products, various efficient technologies are used in which waste is fully exploited through a process aimed at the circular economy (Salem et al., 2021).

Lignin-based circular economy (Fig. 6) is a promising concept as the latter applications are restricted to energy sources and also expanded for pharmaceuticals production, biopolymers, development of biosensors for glucose estimation, and biocomposites, biosorbents, nanocomposites, hydrogels etc.

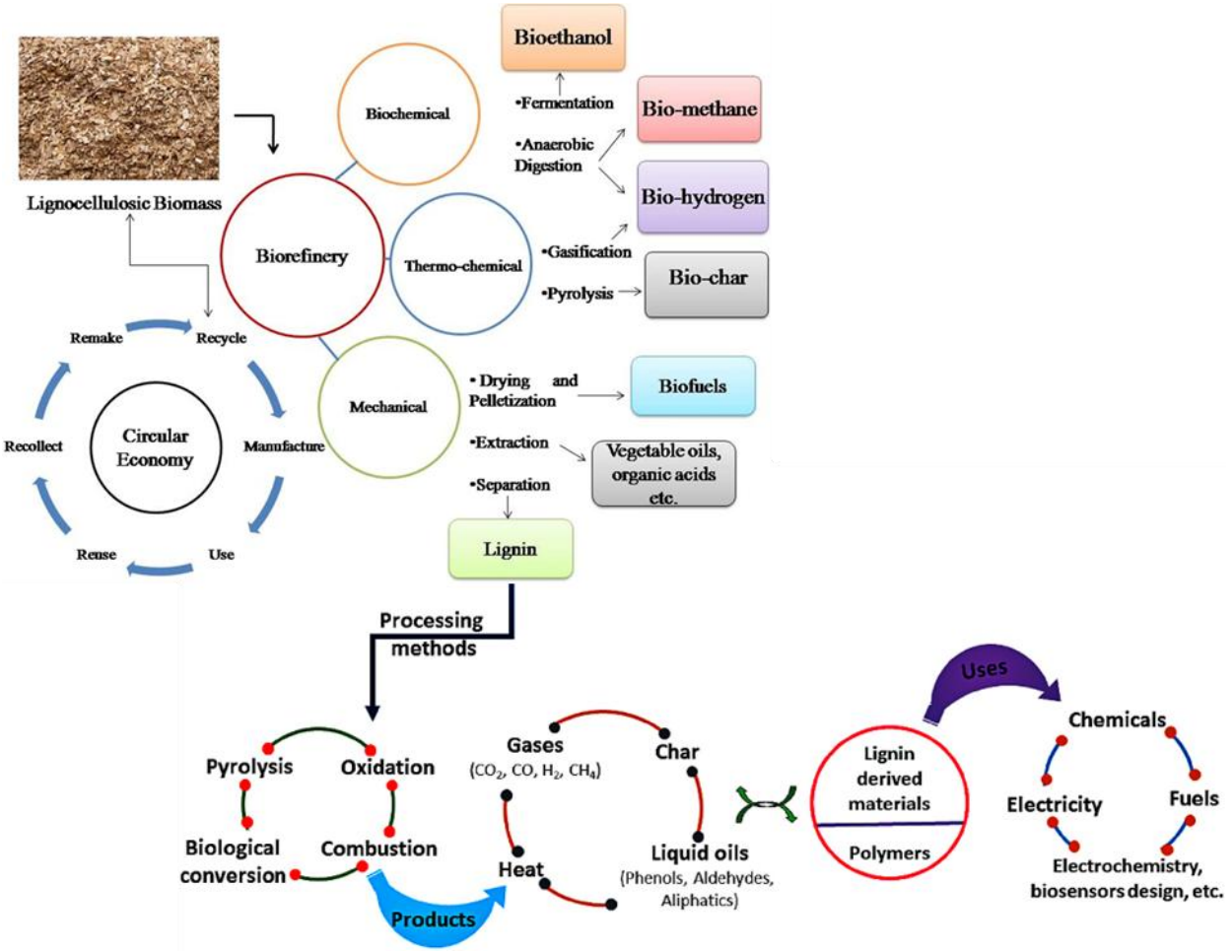


**Figure 6.** Lignin based circular economy (Devi et al., 2022, Garlapati et al., 2020).

Apart from technological backstopping, policy measures also play an essential role in promoting lignin-based biofuels and the role of biorefineries within the circular economy idea. Some measures such as incentivization of lignin-based chemicals and products, buy-back policy of lignocelluloses substrates from farmers, subsidized rates for the inception of lignin-based industries and commercialization of products, increased investment of carbon tax on lignin-based research, etc., would usher a new regime for sustainable and cost-effective technologies development (Garlapati et al., 2020).

## 2. Biorefinery

Fossil fuels are in finite amount and causes severe environmental pollution. Several attempts have been made to efficiently utilize the fossil fuel to increase its specific heat with less emission of environment pollutant. Microwave pretreatment of coal resulted in improved grind ability of high ash Indian coal and resulted in better combustion efficiency (Sahoo et al., 2011a).

However, there are several other limitations associated with the fossil fuels i.e. finite reserves which are located in politically unstable regions of the world, uncertain price fluctuations, not renewable, greenhouse gas emissions etc. (Asomaning et al., 2018) that limits the application of fossil fuels for meeting future energy and chemical needs. The fossil fuels are also a source of different chemicals and the depleting fossil fuels reserve is also an important concern for different chemical industries.

Biomass is most rational carbon-based feedstock that is obtained from plants, animals, and microorganisms. It has emerged as sustainable alternative renewable bio-resource of energy to the conventional non-renewable energy sources. The expected return is so huge that scientists, economists, and state policy decision-makers have coined a term of a parallel economy based on these bio-based products: bio-based economy, and, due to its renewable nature, also circular bio-economy (Mohan et al., 2016).

Among different biomasses, with an annual production of 200×109 tons per year the lignocellulosic feedstocks (LCF) are abundantly available. Interest in lignocellulosic bio-refining to produce biofuels and chemicals as an alternative and to supplement fossil fuels is growing in many countries (Chen et al., 2017, Limayem and Ricke, 2012). With depleting fossil fuels, there is a shift in focus to lignocellulosic biorefinery. These bio-refineries facilitate generation of biofuels and value-added products, e.g., sugar, bioethanol. Thus, in order to improve the sugar and subsequent biofuel yield, several pretreatment techniques have been investigated and have been categorized into physical, chemical, physicochemical and biological methods. The biorefineries were initially focused on the optimization of pre-treatments in order to enhance the holocellulosic component of the biomass, relegating the lignin to a waste role for purely energetic uses or as biomass impregnation after recovery process, as for example in the case of the production of Kraft lignin (Fig. 7). In recent times it is being understood that the phenolic constituents of lignin can be a great added value for a lignocellulosic biorefinery aimed to the sustainable production of green aromatic compounds. For this reason, the lignin molecules from the substrate of the lignocellulosic biomass are trying to isolate in the first instance and only subsequently enhance the polysaccharides (Paone et al., 2020). This process is called

lignin-first and one of the most promising ways of implementing this biorefinery involves for example organosolv processes (Fig. 8).

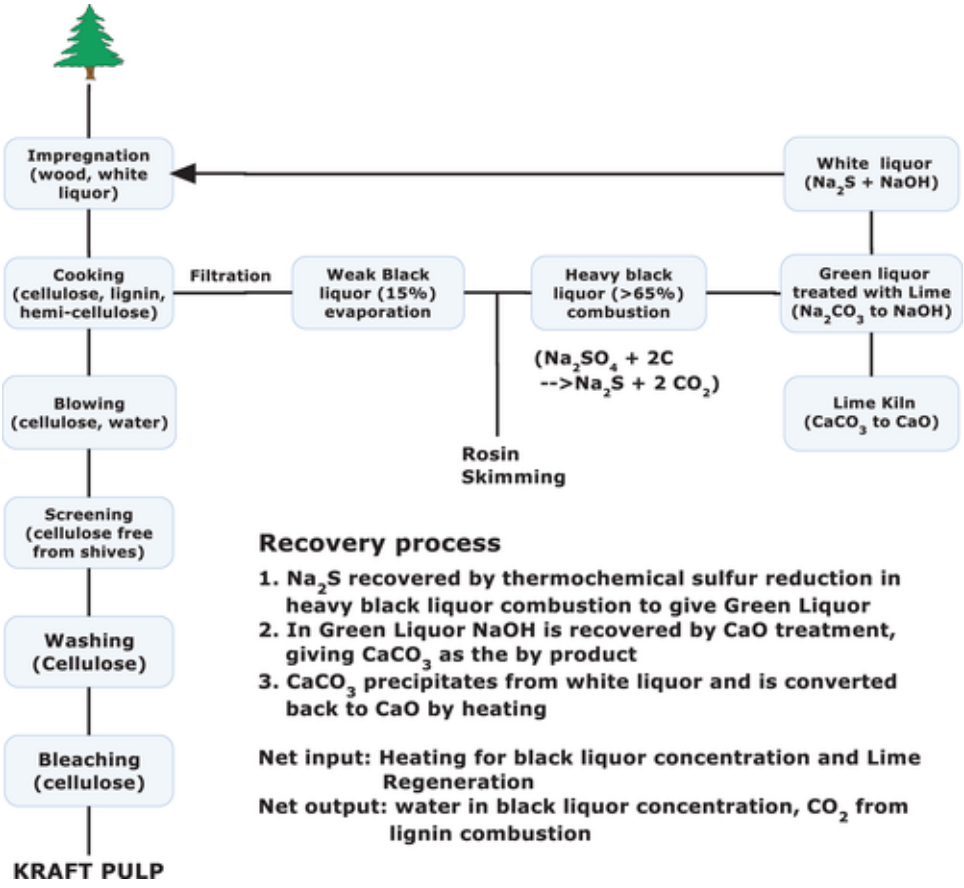


**Figure 7.** Kraft pulping and recovery process scheme (Takkellapati et al., 2019).

Organosolv pretreatment processes use organic or aqueous solvents to extract lignin from lignocellulose. In this process, lignocellulose is mixed with organic liquid and water and heated. A large number of organic or aqueous organic solvents at temperature ranging from 150 to 200°C can be used with or without addition of catalysts such as oxalic, salicylic, and acetylsalicylic acid that typically produce higher solubilization of hemicellulose. During organosolv acid delignification, the Acetosolv process (based on the utilization of HCl-catalyzed acetic acid media) and Formacell process (formic acid-catalyzed media) have proved to be promising process to achieve complete utilization of lignocellulosics without impact to environment. Both processes have ability to cause extensive removal of both lignin and hemicelluloses under mild conditions, with no significant cellulose degradation (Xu et al., 2006).

Organic solvents including alcohols with low boiling point (methanol and ethanol), alcohols with higher boiling point (ethylene glycol, glycerol, tetrahydrofurfuryl alcohol), esters, ketones, organic acids, phenols and ethers have been tested. The main advantage of using solvents is that they produce relatively pure, low molecular-weight lignin. However, the price and recovery of solvent is often a drawback (Sidiras et al., 2022)

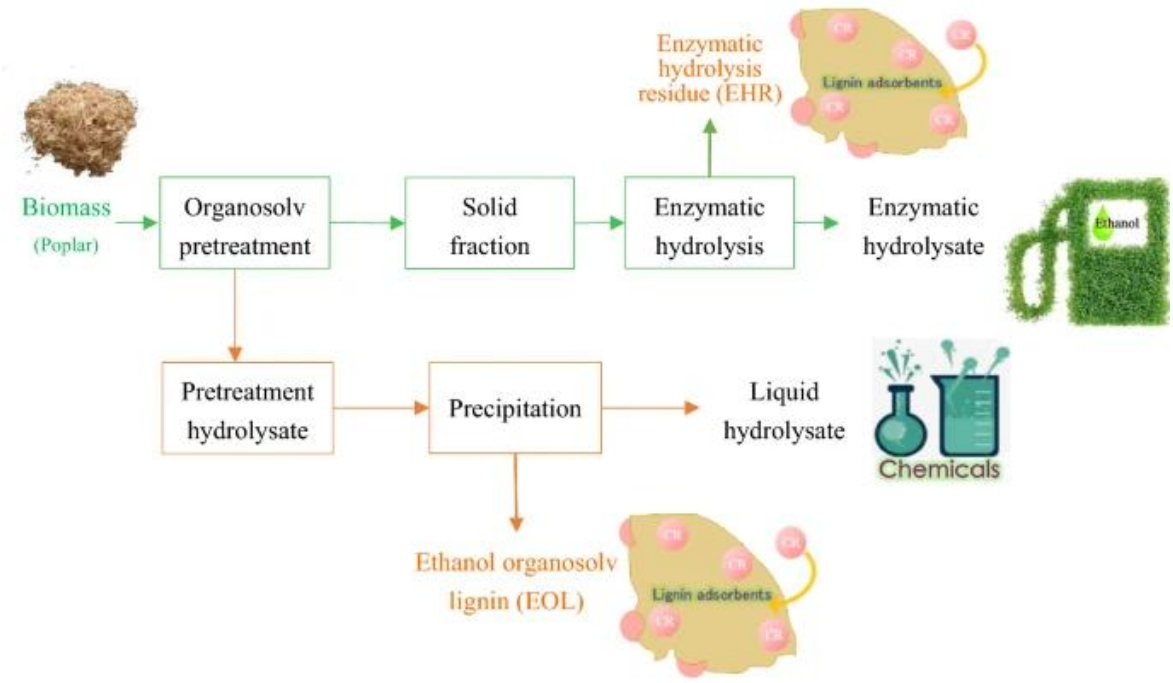


**Figure 8.** Scheme of lignin-first approach by ethanol organosolv pretreatment (Chu et al., 2021a).

The complex structure of lignocellulosic feedstocks (LCF) provides rigidity to the plant on the one hand, and recalcitrance towards enzymatic hydrolysis hindering the conversion of LCF to biofuels on the other hands. Pretreatment helps in overcoming the natural recalcitrance of LCF by breaking down into its components, including the loosening of the together of structural polymers. Thus, pretreatment helps in improving the accessibility of hydrolyzing enzymes to the cellulosic and hemicellulosic part that results in the generation of simple sugars which are later fermented to generate biofuels (Sun et al., 2016). The polyphenolic lignin and other hydrolysates are converted to value-added chemicals and materials as well as building blocks for economically important chemicals (Werpy et al., 2004).

Based on the type of biomass (processed and unprocessed) used for biofuel production, these are classified as primary and secondary biofuel and based on source/class of feedstock used the secondary biofuel are further subdivided in to different generations (Fig. 9):

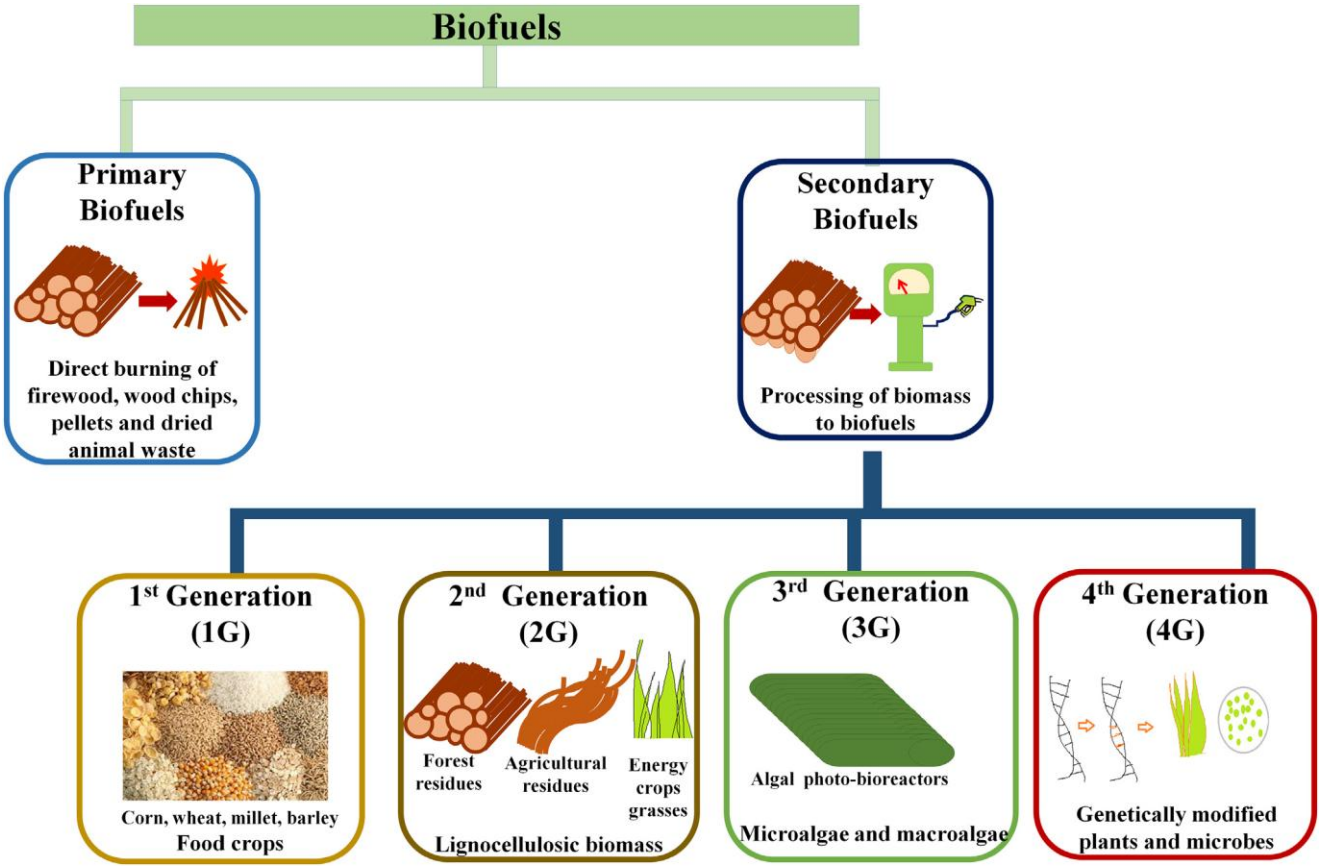


**Figure 9.** Different generations of biofuels based on primary substrate (Kumar et al., 2020).

Biofuels made up of parts of edible food crops such as starchy, sugary products, and plant oils, etc. are termed first generation biofuels. The edible parts are converted to bioethanol, biodiesel, biogas, and syngas. However, competition with food and feed availability was a great concern in 1G biofuel, and attention diverted towards 2G biofuels (Reddy et al., 2005).

The raw materials used for the 2G biofuels were non-food crops, as well as forest and agricultural residues. However, competition of growing non-food crops with food crops on total available arable land has limited the cultivation of non-food crops (Rabemanolontsoa et al., 2013).

Thus, in recent time researchers have focused on generating the food crops with higher cellulosic content in the non-edible parts of food crops (Sun et al., 2016 and Werpy et al., 2004). Thus in recent time researchers have shifted towards the 3G biofuel. The raw materials for the generation of 3G biofuels are mostly algae and seaweeds and are considered as low-cost and high energy alternative. The photo-bioreactors for industrially growing algae can be set up in areas unsuitable for the first and second-generation crops such as barren lands. This set up can use wastewaters and saltwater and therefore minimizes the competition with the freshwater and arable land use as in case of 1G and 2G

biofuels (Quinn et al., 2015 and Ullah et al., 2014). The drawbacks of 3G biofuel are high operational costs and required greater innovation for its efficient utilization (Kumar et al., 2020). In recent time a fourth category, i.e., 4G biofuel, emerged, which uses genetically modified plants and microorganism having high carbon capture capacity for generation of biofuels and bio-chemicals (Aro , 2016 and Ben-Iwo et al., 2016).

## 2.1 Biomasses in the biorefinery

In the biorefinery concept, the valorization of lignin with other biomass components, namely cellulose and hemicellulose, is equally essential. Thus lignin extraction is needed for holistic biomass processing in the initial step of the biorefinery concept (Pineda et al., 2016 and Wang et al., 2019). Unlike conventional petroleum refinery, biomass for lignocellulosic biorefinery is eventually locally available and comparably inexpensive. Moreover, biomass contains oxygen functionality, which makes it inherent in developing high value-added products (Bonini et al., 2008).

The concept of sustainability should be embedded throughout modern society as soon as possible, from the design of cities and methods of food production and transport, to the sourcing and recycling. Integral to these developments are new technologies able to deliver chemicals and fuels from renewable, non-fossil, resources, of which biomass has emerged as one of the most abundant and economically attractive feedstocks. Biomass as a replacement for fossil fuel feedstocks is problematic due to the high water and oxygen content of the former, the presence of a water-soluble fraction of alkaline and alogen elements, along with hazardous trace elements, and resultant low-energy density/heating value, pH and ash-fusion temperatures of biomass. The heterogeneous nature of biomass between different plants and microorganisms, and significant regional and seasonal variations even within the same species, further necessitate the development of versatile process for the chemical transformation of biomass.

However, there are strong political and financial drivers for renewable energy technologies, with the European Union mandating that a 20% of overall energy consumption must derive from renewable sources by 2020, rising to 27% by 2030. In regard of transportation fuels, 10% must originate from renewable resources, and provide reductions in greenhouse gas emissions of at least 35% as compared with fossil fuels (including a missions arising from cultivation, processing and transport). Policy directives are also being introduced to ensure that land designated for biomass production for bio-fuels

cannot have been previously used for carbon stock, such as wetlands or forest, or impact of regions with high biodiversity, such as primary forests or higly biodiverse grasslands (Pineda et al., 2016). However, lignocellulosic biomass was extremely stable against chemical and biochemical processing due to the rigid structure of polymeric composite and complicated interaction connecting the three main components (Negahdar et al., 2016 and Sanderson et al., 2011).

Lignocellulosic biomass is the most abundant organic materials in nature. It is a biomass feedstock mainly containing cellulose, hemicelluloses and lignin; usually including agricultural residues, woody crops, herbaceous energy crops and municipal solid wastes.

Lignocellulosic materials are then on-edible plant residues obtained from forests, agricultural farm sand savannas. They incorporate agricultural residues (e.g. corn stover, sugarcane bagasse, wheat straw, rice husk, rice hull, etc.); dedicated energy crops (e.g., switch grass, timothy grass, poplar, willow, etc.); wood residues (e.g. pine wood, spruce, etc.); sawmill residues; paper mill refuse; and municipal paper waste. Lignocellulosic feedstocksare an economical resource that are found aplenty and have the capability to support he sustain able production of liquid and gaseous biofuels (Nanda et al., 2013). With significant development in conversion technologies, a major proportion of the future energy supply could be contributed by energy crops, forest biomass and agricultural residues.

Increased industrialization and deforestation combined with enhanced price of conventional fuel has lead to depletion of feedstock such as sugar, starch, animal fats, and vegetable oil for first generation fuel. Worldwide, biomass represents an alternative and renewable energy resource to produce green fuel with the help of sustainable approach and technological advancement. Biomass is most abundant (worldwide 131010 MT annually), primary energy resource that can provide alternative transportation fuel such as bioethanol or biodiesel (Sun et al., 2002, Hamelinck et al., 2005, Sanchez et al., 2008).

Conversion of cellulosic biomass to biofuels and bio-based products has gained impetus due to the feasibility of an alternative processes available to convert the complex lignocelluloses to bioenergy. The Figure 10 summarizes the path for conversion of lignocellulosic biomass to biofuels:

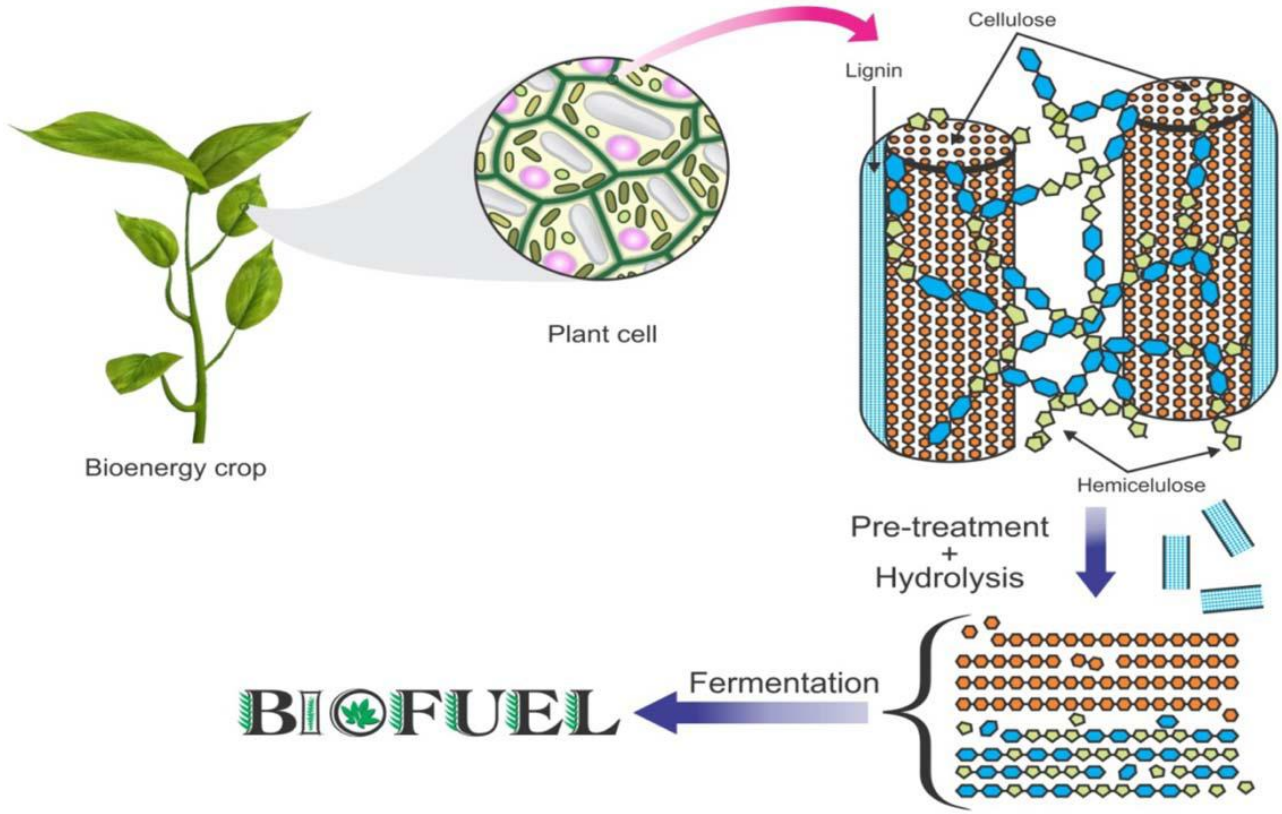


**Figure 10.** Production of second generation biofuel from bioenergy crop (Akhtar et al., 2016)

Over the last two decades, lignocellulosic feed stock such as wheat straw , rice straw , sugarcane bagasse , barley and timothy grass , woody raw materials , forest wastes such as sawdust, wood chips and slashes, dead trees branches , softwoods originating from gymnosperm and paper pulps have been tested for bioethanol production (Kaparaju et al., 2009, Ko et al., 2009, Rabelo et al., 2011, Naik et al., 2010, Zhu et al., 2010, Perlack et al., 2005, Hoadley et al., 2000).

The task of saccharification of lignocellulosic biomass is still technically problematic because of digestibility of cellulose which is hindered by many physico-chemical, structural and compositional factors (Palonen et al., 2004). The primary obstacle impeding the widespread production of bioenergy from different biomass feedstocks is the absence of low-cost technology for overcoming the recalcitrance of these materials due to presence of lignin (Tomas-Pejo et al., 2008). Production of ethanol from lignocellulosic biomass involves hydrolysis of cellulose and hemicellulose, fermentation of sugars, separation of lignin, and, finally, recovery and distillation of ethanol to meet fuel specifications (Tomas-Pejo et al., 2008, Alvira et al., 2010). The most important factors to reduce cost of ethanol production are: an efficient utilization of the raw material for high yields, high productivity, greater ethanol concentration in the distillation feed and process integration to reduce the energy demand (Tomas-Pejo et al., 2008, Galbe et al., 2007).

Biodiesel has also been identified as one of the notable option for complementing conventional fuels. It is primarily produced using sources such as vegetable oils and fats (Jain et al., 2010, Juan et al.,

2011, Aransiola et al., 2012) by esterification or transesterification process, and is considered a viable alternative of fuel because renewable energy sources are required for satisfying the energy demand; as an energy source, biodiesel can reduce the emission level of pollutants, is non-toxic and biodegradable, and it can be used along with conventional petroleum based fuels to create blends, considering the continuous increase in energy demand is contributing to petroleum-based oil depletion (Karmee et al., 2015).

Membrane and reactive distillation technologies are the recent advances applied in biodiesel production at reduced capital costs (Aransiola et al., 2014). Biodiesel is considered as safe, renewable, non-toxic, and biodegradable fuel, with negligible sulfur content and better lubrication property with respect to fossil-derived fuels.

Recently lignocellulosic biomasses have gained increasing research interests and special importance because of their renewable nature (Akhtar et al., 2016). Therefore, huge amount of lignocellulosic biomass can potentially be converted into different high value products including bio-fuels, value added fine chemicals, and cheap energy sources for microbial fermentation and enzyme production. Unlike trees, crop residues are characterized by a short harvest rotation that shows pronounce effect on production of bioethanol. In addition, biomass from switchgrass (Xu et al., 2010), Miscanthus giganteus (Brosse et al., 2009), Poplar sp. (Wang et al., 2012) and Eucalyptus sp. (Yanez et al., 2013) have proved themselves as potential candidate for bioethanol production. Among municipal and industrial wastes viz. food-processing by-products, processing papers and intermediates such as black liquors and pulps have also been used as raw material for biofuel production.

Cellulose, a form of biological carbon, forms structural component of a primary cell wall in green plants, many forms of algae, and some oomycetes. It is the most abundant organic compound on the earth consisting of β-1-4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose). Cellulose is more commonly considered as linear chain of several hundred to 10000s recurring D -glucose units, linked by β-1-4 glycosidic bonds as shown in Figure11a. Lignocellulosic material consists of both crystalline and amorphous forms of cellulose. Tightly bounded parallel arranged bundles of cellulosic chains formed by strong inter chain hydrogen bonding forms crystalline region, while it is less ordered and conspicuous in amorphous region. Cellulase readily hydrolyses the more accessible amorphous portion of cellulose as compared to less accessible crystalline portion and it is widely accepted that decreasing the crystallinity, increases the digestibility of lignocelluloses. On the contrary, more crystalline lignocelluloses have been reported to show more digestibility.

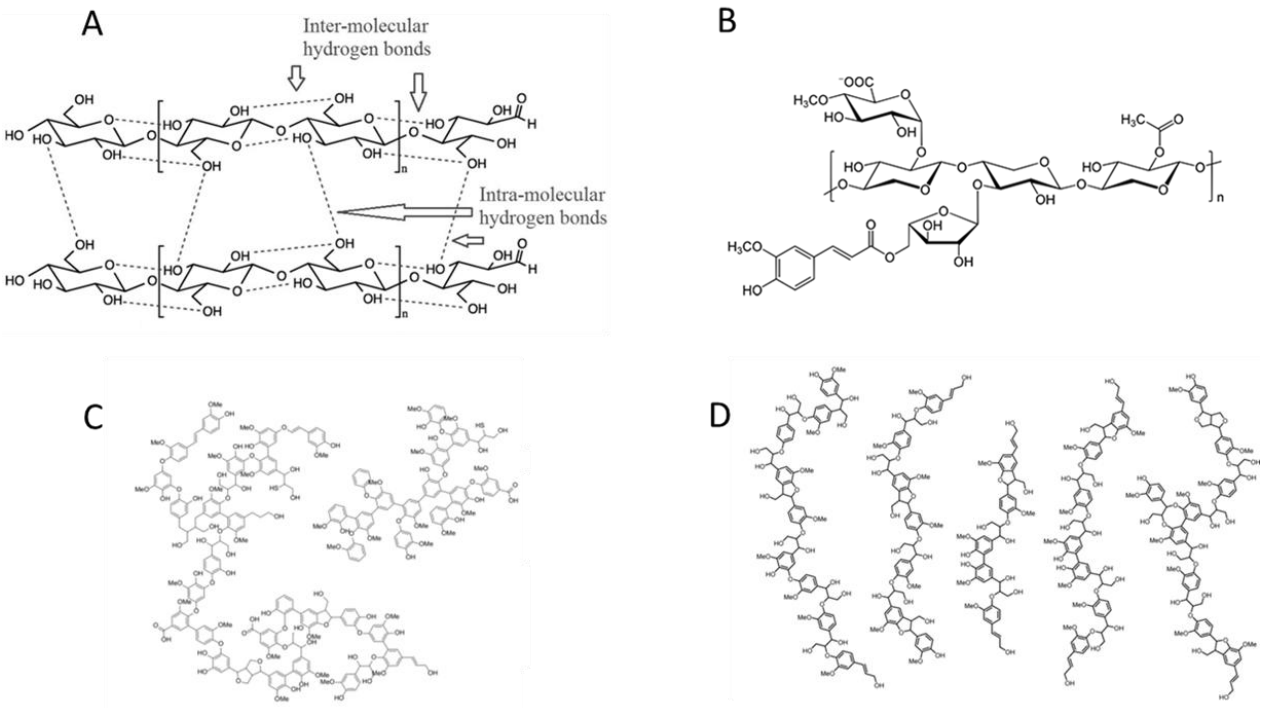


**Figure 11.** Schematic representation of (A) cellulose (Baghaei & Skrifvars 2020); (B) hemicellulose (Zhang et al., 2021); (C) proposed revised structure of Kraft lignin (Lange & Crestini, 2019) and (D) linear chains of oligomeric milled wood lignin (Lange et al., 2016a).

The third most abundant natural polymeric carbohydrate on the earth derived from forest biomasses is hemicellulose. Hemicellulose, a mixture of polymeric carbohydrate, including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose, and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose) and other heteropolymers. The structural component consists of pentoses (D-xylose, D-arabinose), methyl pentoses (L-rhamnose), hexoses (D-glucose, D-mannose, D-galactose), carboxylic acids (D-glucuronic acid, D-galacturonic acid, methyl glucuronic acid), which can be employed in bioconversion processes for the production of ethanol and other value added products. The molecular structure of hemicellulose is shown in Figure 11b. In hardwood, hemicelluloses are dominantly found as xylan, whereas in softwood, glucomannan are most common. Hemicelluloses have a random, amorphous, and branched structure with little resistance to hydrolysis, easily hydrolyzed by acids to their monomer components (Akhtar et al., 2016). Xylan, the most common type of polysaccharide in hemicellulose family consists of D-xylopyranose linked together by β-1,4-linkage with <30,000 molecular weight and up to 200 degree of polymerization. Though xylan is the major constituent of hemicelluloses, the later biomass fraction is actually a heteropolysaccharide with varying proportions of arabinose, mannose, and glucuronic acid. Hemicellulosic components of lignocellulosic complex are associated with cellulose molecule via

hydrogen bonds and physical interactions with lignin through covalent bonds. Acids can hydrolyze hemicellulose in lignocellulosic materials. When raw plant materials are treated using acid and high temperature to break strong chemical bonds and to release xylose directly hemicellulose could be broken down to expose cellulose, thereby directly resulting in further degradation by enzymes to produce monomeric sugars such as glucose (Pedersen et al., 2011).

Lignin, the second largest available biopolymer derived from forest biomasses, consists of phenyl propane units, namely *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin and carbohydrate moieties are chemically bound in native biomass forming a lignin–carbohydrate complex (LCC) that form complex with hemicellulose to encapsulate cellulose, making lignocellulosic biomass resistant toward chemical and enzymatic hydrolysis. Lignin is a complex aromatic heteropolymer whose biological role in plants is to increase cell wall integrity and resistance to attack by pathogens. Lignin is potentially suitable for producing value-added products, such as binders, dispersants, phenolic resins, polyurethane foams, and epoxy resins for printed circuit boards (Stewart, 2008). There are eight different types of lignin–carbohydrate bonds: benzyl ether, benzyl ester, glycosidic or phenyl glycosidic, hemiacetal or acetal linkages, and ferulate or diferulate esters that are linked to lignin at 4-OH and 4-O positions (Tarasov et al., 2018). The macromolecular polymeric structure of lignin is shown in figure 11c. Lignin, that generally have molecular weight of <20,000 Dalton, is found in greater amount in soft wood (pine, balsam, spruce, tamarack, fir) than hardwood (oak, walnut, maple, poplar, birch).

Not only the amount of lignin influences the subsequent enzymatic hydrolysis or fermentation processes. The type of lignin and the pretreatment can also cause inhibitions. During degradation process, biomass could produces furan compounds (furfural and hydroxymethyl-furfural) that could inhibit fermentation processes. Furthermore, lignin depolymerization and repolymerization reactions often occur simultaneously in several pretreatments including hydrothermal pretreatments, acid dilute processes and steam explosion. During this process reactive intermediates of the lignin are generated which can be further depolymerized into soluble phenolic compounds which can severely inhibit the cellulose hydrolysis of the entire slurry (Chu et al., 2021b).

### 2.1.1 Wheat straw

Wheat is one of the major crops grow around the world. Approximately 690 ktons of wheat were produced globally in 2009, later reaching 730 million tons in 2014 (Bakker et al., 2013). As the waste residue of wheat, large mass of wheat straw (WS) has been generated every year and become one of

the most abundant agricultural biomass worldwide (Baroi et al., 2017). Straw is an agricultural byproduct consisting of the dry stalks of cereal plants after the grain and chaff have been removed. It makes up about half of the yield of cereal crops such us oats, barley, rice, rye and wheat. It has a number of different uses, including livestock bedding and bedding, fuel, thatching and basket making. Straw is usually gathered and stored in a straw bale, which is a bale, or bundle, of straw tightly bound with twine, wire or string. Straw bales may be square rectangular or round and can be very large, depending on the type of baler used.

Result in generating issues of great impact on the economic waste and environmental pollution (Talebnia et al. 2010). WS is also a major source of renewable energy. As lignocellulosic biomass, WS is inexpensive and abundant, hence, has a great potential for production of bioenergy (Carrillo et al. 2005).

Wheat Straw (Fig. 12a) is a typical lignocellulosic biomass that mainly comprises cellulose, hemicellulose, and lignin. Cellulose and hemicellulose could be hydrolyzed into monomeric sugars such as glucose, xylose, and arabinose, which could then be converted to biofuels such as bioethanol and methane. However, because the chemical components and physical structure of lignocellulosic biomass could protect cellulose from degradation, the bioconversion of these materials has remained challenging (Akimkulova et al. 2016). Typically, lignin and hemicellulose in the lignocellulosic materials need to be removed before the enzymatic hydrolysis of cellulose. Various pretreatment approaches have been proposed and applied, including physical, chemical, and biological methods. Alkali, acid, steam explosion, and hot water pretreatments have been extensively performed in related fields (Liu et al., 2016).

Lignin functions in supporting plant structures to avoid microbial permeation and subsequently deterioration. Moreover, the amorphous heteropolymer is generally insoluble in water (Hendriks & Zeeman, 2009). These factors are the main obstacles to the efficient utilization of lignocellulosic biomass. Hence, to enhance enzymatic digestibility, it is necessary to remove lignin from the raw materials (Zheng et al., 2018). Alkali pretreatment can efficiently remove lignin in plant tissues, leading to high delignification (Wu et al., 2014).

In a biorefinery process, all the macro-constituents of the straw must be enhanced. The isolation of relatively pure lignin from wheat straw has led to slower progress in obtaining structural information about lignin. To improve the yield of ground straw lignin and to study a straw lignin sample more representative of the total lignin, toluene additions and dioxane extraction are carried out at elevated temperatures. Guaiacil units present in wheat straw lignin are thought to be a significant connector between lignin and carbohydrates. Even diferulates (mainly 5-5′) participate in the lignification of

wheat straw. Arabinoxylans were found to cross-link with lignins via ferulates via ether bond, via glucuronic acid via ester bond, and via arabinose / xylose via both ether and glycosidic bonds, respectively (Sun et al., 2005).


**Figure 12**. Wheat straw round bales (a) and *Cynara cardunculus* (b).

### 2.1.2 Cardoon lignocellulosic residue

Cardoon (*Cynara cardunculus* L.), (Fig. 12b) is one of the most promising raw materials for biorefinery in Mediterranean areas as it is a polyvalent perennial crop well adapted to marginal soils that are dry, with little rain and with little nutrients (Ciancolini et al., 2013; Pappalardo et al., 2020). The vegetable oil extracted from the seeds is rich in monounsaturated fatty acids useful for producing important intermediates such as azelaic acid or pelargonic acid, in great demand by the synthetic and cosmetic fertilizer industries all over the world (Caporusso et al., 2021). The remaining epigeal part represents an important source of lignocellulosic biomass potentially suitable for the production of intermediate compounds, such as bioethanol and Bio-butanediol, widely used for the production of biofuels and bioplastics (Petrone et al., 2017).

The hypogeal part constituted by the roots is also of great interest due to the presence of inulin, a hetero-oligosaccharide suitable for nutraceutical, pharmaceutical and other biorefining applications (Raccuia and Melilli, 2004).

Cardoon as a biomass feedstock has interesting potential for ethanol production in terms of high polysaccharides content and, with respect to other lignocellulosic residues (Giant reed, Poplar etc), lower lignin content (Cotana et al., 2015); previous studies investigated *C. cardunculus L.* applying a dilute acid pretreatment (Ballesteros et al., 2008).

The cardoon stalks have already been chemically characterized and comprised 5 to 11 % ash, 13 to 21 % extractives, 13 to 23 % total lignin, and around 53 % polysaccharides (Lourenco et al., 2015). For an appropriate valorization of cardoon as a raw material for the production of added-value products, the comprehensive characterization of their different components is of high interest, in particular the composition and the structure of the lignin polymer, since it is a potential source of aromatic chemicals and biofuels.

However, studies regarding the detailed structure of the lignin of cardoon stalks are still scarce. Recently, a study was made to characterize the lignin in cardoon stalks by pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS), after fractionation in stalks . The stalks presented around 21-24% of lignin and a syringyl to guaiacyl (S/G) ratio between 1.3 and 2.1 (Lourenço et al., 2015).

Unlike straw, cardoon is a multi-year plant that can be grown in soils with low nutrients and water. Cynara cardunculus is a plant species fully adapted to Mediterranean climatic conditions, capable of accumulating reserve substances in the roots, and of promoting vegetative reactivation after summer quiescence. Its outstanding growth and great adaptation to Mediterranean climates suggested it is a species which could be useful for biomass production (Kukić, 2008).

For industrial application, *C. cardunculus* is grown in the same way as in its natural growth pattern, that is, as a perennial field crop in dry farming. As an energy crop, the above ground biomass produced throughout the growth cycle is harvested once a year, in summertime. It is a species widely known throughout the Mediterranean basin for the use of the flower head inflorescence (artichoke) for food purposes and for its pharmaceutical properties; recently, interest in this species has increased as a source of substances with therapeutic properties: they derive mainly from the metabolism of phenylpropanoids and flavonoids (Kukić, 2008).

# 3 Lignocellulosic conversion processes

The estimated amount of bioethanol on the world market was about 110 billion litres in 2018 and is predicted to achieve 140 billion litres in 2022 . The majority of global bioethanol production belongs to U.S.A. (56%), Brazil (28%), European Union (5%), China (4%), and Canada (2%). It is expected that second-generation will increase its contribution in ethanol global market through growth in commercialization of cellulosic bioethanol from inexpensive wastes, particularly because, the U.S.A. and EU biofuel policies promote the progress of the generation of cellulose-based biofuels worldwide (Ko et al., 2020).

The biochemical conversion of the lignocellulose wastes into bioethanol (e.g., fermentation) attracts more and more attention as a result of mild operating conditions compared to thermochemical methods (e.g., pyrolysis) which are capital-expensive processes. The bioethanol production via biotechnological methods from a research perspective deals with various aspects of main processing steps such as pretreatment, enzymatic hydrolysis, and fermentation bioethanol production performances are impeded by toxic by-products formation, and thereby detoxification strategies are introduced before fermentation to reduce their negative impact on microbial viability. Another issue in bioethanol production is non-productive cellulase binding to lignin, which can be alleviated by the application of non-ionic surfactants. Moreover, plenty of microorganisms have been developed using genetic engineering to improve the efficiency of cellulolytic enzymes, xylose and cellulose fermentation, and inhibitor resistance. In addition, many microbes have been immobilized on different kinds of support to enhance biosystem performance under stress factors, such as elevated temperatures, pH variations, or high substrate concentration (Siqueira et al., 2020, Robak et al., 2018).

Biofuel production, such as bioethanol and biobutanol, is being considered as a partial solution to the environmental problems caused by the indiscriminate use of fossil fuels. Bioethanol is produced using plant biomass, and it can be obtained either by the direct fermentation of simple sugars (called first-generation ethanol) or from saccharification of complex sugars, followed by fermentation (called second-generation ethanol). Second-generation bioethanol is costlier to produce than first-generation bioethanol because it has more steps and requires expensive material and industrial inputs; however, it can be produced using any cellulosic material. Utilization of agro-industrial residues for cellulosic bioethanol production aims to reduce the cost of feedstock, add value to agricultural residues and do not interfere with food production. Pineapple peel, banana pseudo stem, sugarcane bagasse, corncob, cashew apple bagasse, rice straw are just some examples of residues evaluated as cellulose sources for

bioethanol production. As well as ethanol, butanol is an alcohol that can be produced from lignocellulose feedstock and used as biofuel. It can be mixed, in some proportions, with gasoline and diesel, and because it has more oxygen in its structure than biodiesel, will reduce the release of soot into the atmosphere. In addition, butanol also needs lower temperatures for combustion than ethanol. The main difficulty with biobutanol use is its low production at industrial levels. Usually, the production under fermentation processes is carried out by bacteria, such as *Clostridium,* and may use lignocellulosic biomass, such as sugarcane bagasse and rice straw.

Lignocellulosic materials, such as forestry, agricultural and agroindustrial residues, are among the most important sources of biomass for the production of fuels, chemicals and materials. However there are physical and chemical barriers in the lignin carbohydrate supramolecular structure that render most plant cell wall components almost completely unavailable for conversion into commercial products. Thus successful conversion strategies must lead to the disruption of this structure and result in partial or total separation of the lignocellulosic components, increasing the accessibility of cellulose, hemicelluloses and lignins. It must also minimize the formation of by-products (Sun and Cheng, 2002).

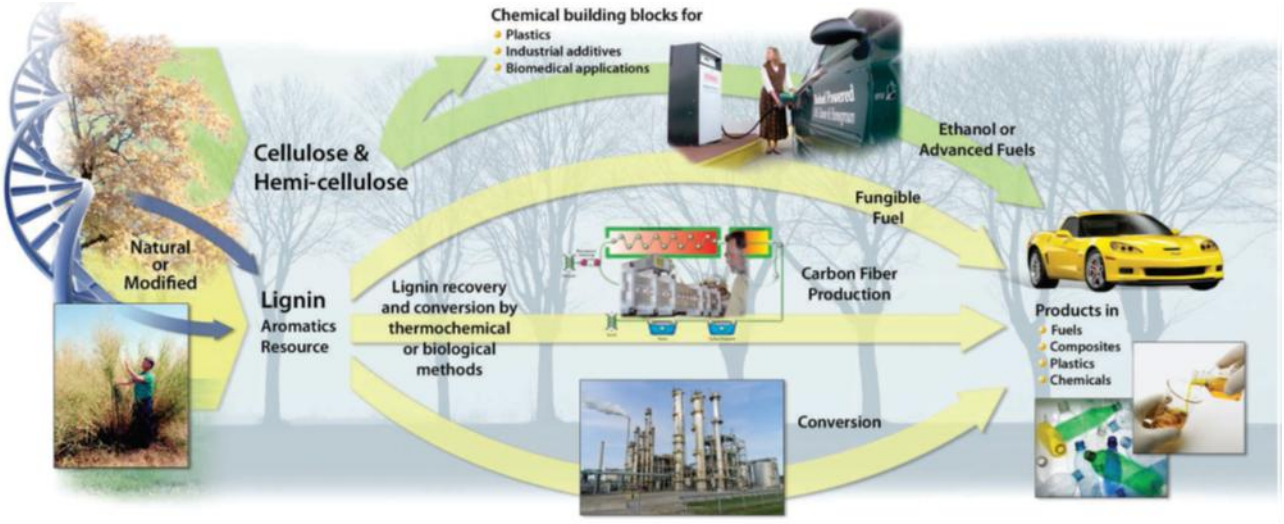


**Figure 13.** Production of biofuels from cellulosic biomass requires separation of large quantities of the aromatic polymer lignin (Ragauskas et al., 2014).

Lignocellulosic biomass - such as sugarcane bagasse and straw, corn stover, cotton stalks, wheat straw, rice straw, rice husk, and wood chips - are widely available at a relatively low cost and are good raw materials for the production of fuels, chemicals and materials because of their heterogeneous chemical composition (Ragauskas et al., 2014).

A consistent analysis of pretreatment methods - processes that are required to overcome the recalcitrance attributed to the structural characteristics of lignocellulosic materials - is fundamental. It must render polysaccharides easily accessible to chemicals and enzymes since this process determines the type and yield of the final products, the choice and efficiency of the subsequent transformation steps and other facts that make pretreatment responsible for the highest cost of the global project. Pretreatment processes generate chemical intermediates for several industrial sectors such as food, paper and timber and fibers (Seidl and Goulart, 2016):

-Liquid hydrocarbon fuels and chemicals like furfural, 5-hydroxymethylfurfural (HMF), 2,5-furandicarboxylic acid (FDCA), γ-valerolactone (GVL), polymers and organic acids from hemicellulose and cellulose carbohydrates;

-Thermal and electrical energy, carbon fiber, adhesives, additives, dispersants and aromatics from phenolic lignin compounds;

-Biogas.

Increasing energy demands are not only exploiting the fossil resources but, also depleting natural environment.

Biofuels from lignocellulosic biomass is a renewable, eco-friendly, sustainable and could be a promising alternative to fossil fuels. The lignocelluloses is considered as a potential feedstock for production of biofuels and other bioproducts including various chemicals, biofibers, biopulps, enzymes, etc. The lignocellulosic biofuel is renewable, cost efficient, eco-friendly and thus creating a global priority. However, the main hurdles in utilizing lignocellulosic materials lie in the crystalline nature of cellulose sheathed by hemicellulose, degree of polymerization, biomass particle size and recalcitrance of their bonding due to protective covering of lignin which allow very less surface area for enzymatic hydrolysis. Thus, to increases the digestibility of cellulose and hemicellulose, the removal or efficient breakdown of lignin from lignocellulosic biomass is usually a targeted step of pretreatment.

The physical pretreatment such as milling, grinding, chipping, ultrasonic, etc. and chemical pretreatment with acids, alkali or oxidative delignification can efficiently breakdown the recalcitrant bonding in a short time thus are being extensively used in several industries. However, it requires high energy and operational cost along with chances of high risk of chemical hazards on environment. The biological pretreatment on the other hand has its very wide application and gaining its popularity because it requires low energy, has no chemicals, less pollution and higher yield. The naturally occurring bacteria and fungi secret different lignocellulolytic enzymes for efficient breakdown of

biomass and help in formation of 5- and 6-carbon chain sugars. These sugars can be converted into biofuels and other various value added products (Sharma et al., 2019).

Currently, the chemical industry is largely petroleum based and although the number of ongoing large-scale biocatalytic processes is relatively low, a trend in growth is expected and the Organization for Economic Cooperation and Development (OECD) and other agencies aim to have 30% of the total chemical industry based on renewable sources by 2050. At present, a good number of bio-based products (bioethanol, acids such as lactic, succinic, itaconic, and others) are derived from corn syrup and other sugar sources; however, because of the food versus fuel controversy, new trends have been directed towards the production of bio-products/biofuels from lignocellulosic biomass – the most abundant and important renewable source for alternative petrol derivatives. Lignocellulosic biomass is the stored energy from sunlight in the form of chemical energy, and it refers to plant biomass that is composed of cellulose, hemicellulose and lignin and includes not only the green parts of vegetables, wood and straw, but also manure and the organic fraction of municipal solid wastes (MSW).
A major obstacle for the industrial-scale production of bioproducts/biofuels from lignocellulose by biological means, known as second-generation (2G) products, is the inefficient deconstruction of plant material due to the recalcitrant nature of the substrates and the success of 2G technology requires efficient pre-treatment (physical, chemical or physicochemical) of plant material to disorganize the fibres, which will be the target for the action of enzymatic cocktails that breakdown polysaccharides into their monomeric constituents. Enzymatic hydrolysis of lignocellulosic materials yields glucose, xylose and arabinose, and these sugars can be fermented to produce added-value chemicals such as alcohols (ethanol, butanol), acetone, aldehydes, aminoacids and other bioproducts. Liquid or gas bio-products/biofuels, derived from renewable resources, can only replace a fraction of the fossil fuels used in locomotion, as well as a number of chemicals that are currently derived from petroleum (Ramos et al., 2017).

Biomass pretreatment is a crucial step in the lignocellulosic conversion process. The overall function of pretreatment is to increase the accessibility of biomass-deconstructing enzymes to hemicellulose and cellulose to enable efficient depolymerization into fermentable sugars (Fig.14). Historically, pretreatment has been one of the most expensive unit operations within the biomass conversion regime, and, over the last two decades, many pretreatment techniques have been developed for biomass depolymerization and fractionation.

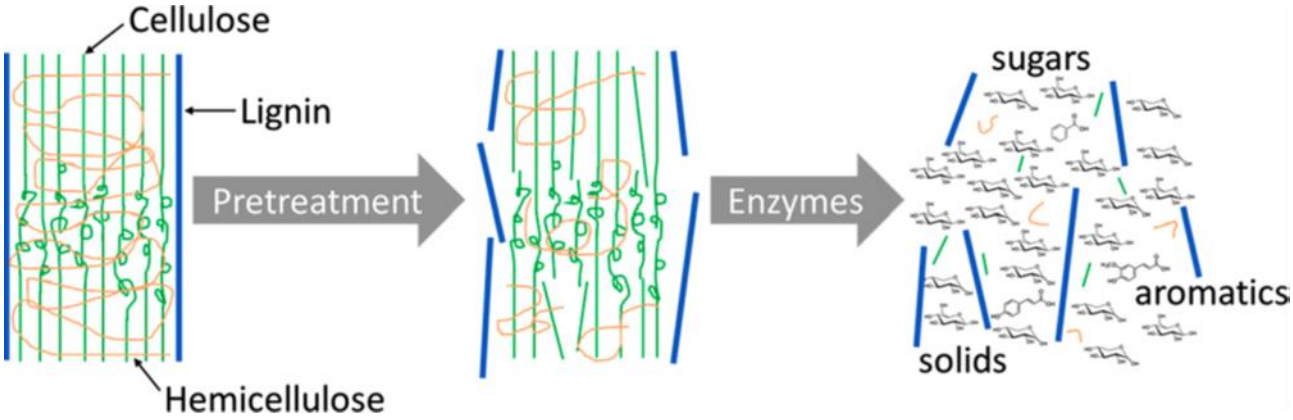


**Figure 14.** Schematic of pretreatment impact on lignocellulosic biomass (Baral et al., 2019).

Utilization of lignocellulosic material for biofuel and biochemical production could greatly reduce $CO_2$ emissions. Feedstock, in the context of the biofuel industry, represents any biomass that is used as a raw material for the production of biofuels. Lignin holds a great potential in different industries as a source for chemicals, fuels and other bioproducts. Lignin is a common byproduct derived from different industries, including paper and pulp industries. This industry alone creates 40–50 tons of lignin per year according to the International Lignin Institute, yet only 1.5% is currently used for purposes other than energy generation. Lignin is also the main waste product from bioethanol production. Besides its contribution to energy production, lignin is biodegradable, antimicrobial, an antioxidant, and $CO_2$ neutral (Doherty et al., 2011).

Biological conversion of cellulosic biomass to fuels and chemicals offers the high yields to products vital to economic success and the potential for very low costs. Enzymatic hydrolysis that converts lignocellulosic biomass to fermentable sugars may be the most complex step in this process due to substrate-related and enzyme-related effects and their interactions. Although enzymatic hydrolysis offers the potential for higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes, the mechanism of enzymatic hydrolysis and the relationship between the substrate structure and function of various glycosyl hydrolase components is not well understood . Consequently, limited success has been realized in maximizing sugar yields at very low cost (Upton et al., 2016).

Cellulases and hemicellulase (mainly xylanase) are carbo-hydrolases that catalyse the hydrolysis of cellulose and hemicellulose (xylan). Cellulase is a common name for exocellulase, endocellulase, and β- glucosidase, which hydrolyze cellulose and release glucose.

The (enzymatic) hydrolysis of lignocellulose is limited by several factors. The crystallinity of cellulose is just one of the factors. Other factors are degree of polymerization (DP), moisture content, available surface area and lignin content.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50 °C) and does not have a corrosion problem. Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase. During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol. The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters).

To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulase activity. Substrate concentration is one of the main factors that affects the yield and initial rate of enzymatic hydrolysis of cellulose. The susceptibility of cellulosic substrates to cellulases depends on the structural features of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. Therefore, removal of lignin can dramatically increase the hydrolysis rate (Upton et al., 2016).

Enzymatic hydrolysis can be defined as multi-step heterogeneous reaction in which insoluble cellulose is initially broken down at the solid–liquid interface via the synergistic action of endoglucanases and exoglucanases/cellobiohydrolases. This initial reaction is accompanied by further liquid-phase hydrolysis of soluble intermediates, that is, short celluloligosaccharides and cellobiose, which are catalytically cleaved to produce glucose by the action of β-glucosidase (Upton et al., 2016).

The saccharification process, which is conducted by enzymatic hydrolysis of cellulose, is a bottleneck in the process due to the high cost of these enzymes and long time required for hydrolysis. Hence, the focus on producing enzymes onsite, to be directly applied in the production line, as described in Figure 15. The figure depicts the main steps of enzyme production from lignocellulosic biomass and further enzymatic hydrolysis of the same substrate for its saccharification and conversion to biofuels. After cellulase production and formulation, the enzyme can be used in a single- or multiple-steps process, represented by Figure 15, routes A and B, respectively. In route A (Fig. 15), hydrolysis and fermentation occur in separate vessels, whereas, route B (Fig. 15) exemplifies a single vessel process with simultaneous saccharification and fermentation.

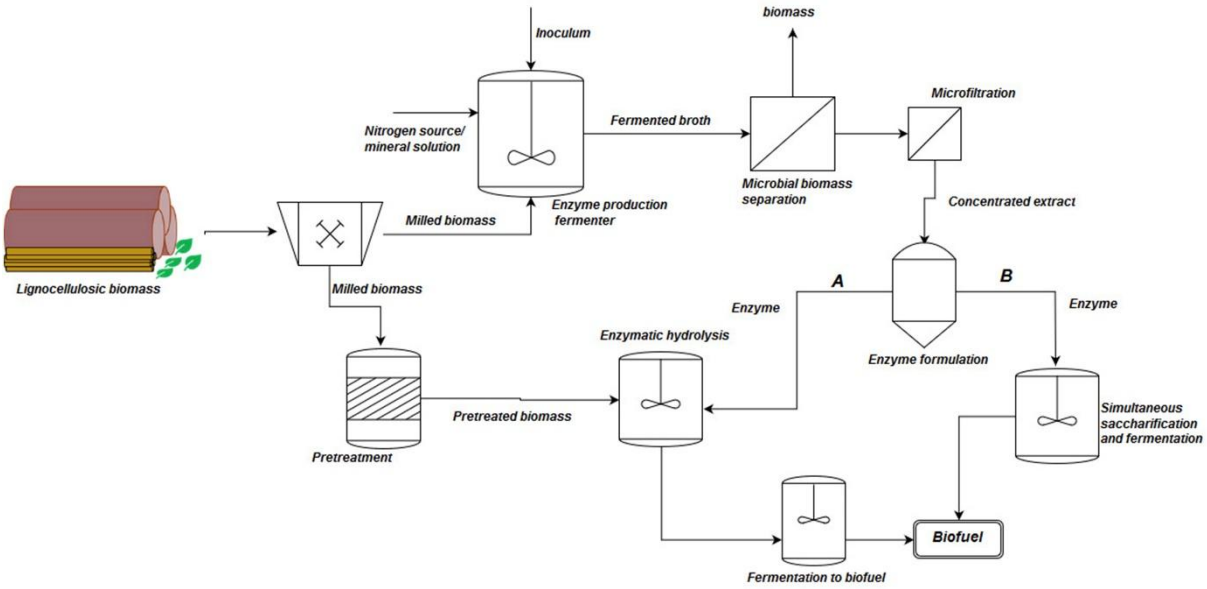


**Figure 15**.Schematic representation of lignocellulosic biomass bioconversion to enzymatic complex and fermentable sugars to be converted to biofuel (Siqueira et al., 2020).

Vegetable organic matter is produced as a result of the chlorophyll photosynthesis process, which thanks to the contribution of the sun's energy allows simple mineral elements to be transformed into complex organic molecules. Plant biomass absorbs $CO_2$ from the atmosphere during growth and returns it during combustion. The $CO_2$ balance of these processes is therefore zero, and therefore does not contribute to the greenhouse effect. For this reason, the energy produced from plant sources will probably become a significant portion of the total energy produced from renewable sources. Italy is committed to contributing to the reduction of $CO_2$ emissions into the atmosphere (-6.5% emissions in 2012 compared to those of 1990) by signing the well-known Kyoto agreement. The 26th United Nations Climate Change Conference (COP 26), which concluded in Glasgow on 13th November 2021,

was described by its organizers as "a huge step forward" in the fight against climate change. One of the great achievements of COP26 is the fact that 151 countries have submitted new or updated agreements. Among these we find, for example, Europe, which has included in its plan the recently adopted objective of reducing emissions by 55% by 2030 (compared to 1990 levels), but also the United States and China which have updated the goals (Climate Focus 2022).

The agreement, signed by nearly two hundred countries, expressly mentions for the first time the need to limit the use of fossil fuels.In this context, the production of energy from dedicated crops takes on a strategic role. Another aspect that can make the production of energy from vegetable biomass competitive is the reference regulatory framework, which if it is defined for the production of electricity, it is not the same for the use of biofuels (bio-oil, bio-alcohol, etc. ). The latter currently in order to be used in electrical or mechanical conversion systems are subject to taxation, which hinders their use. The ways to produce this energy go through different ways of conversion; the transformation processes of the energy included in plant biomass can be grouped into 3 large segments representing the three main types of conversions (Fig. 16):

. • thermochemical;

• biological;

• physical (and physicochemical including steam explosion).

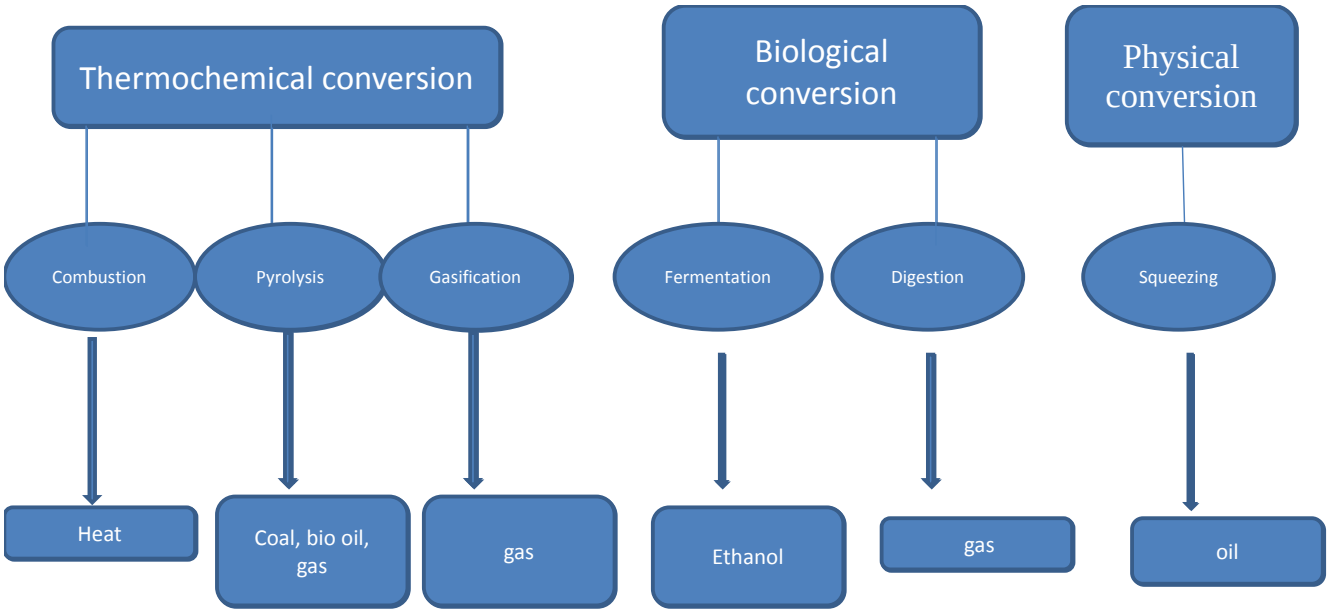


**Figure 16**. The energy contained in plant biomass can be converted by adopting thermochemical, biological or physical processes. The final result, apart from direct combustion, is a high energy density product, which can be used more easily and flexibly in subsequent energy conversion devices and processes (Candolo, 2005).

## 3.1 Thermochemical Conversion

The thermochemical conversion of the energy present in plant biomass can be obtained with various processes, such as combustion, pyrolysis and gasification. Combustion (Fig. 17) is the most traditional process, to be efficient it requires the reduction of the water content of the biomass, a reduction that is generally obtained by drying the products in the sun. Combustion is from a thermodynamic point of view, a process of converting the chemical energy of the fuel (biomass)into heat. Heat is generated thanks to the oxidation reaction of carbon in the presence of sufficient oxygen according to the reaction: $C + O_2 \rightarrow CO_2 + heat$

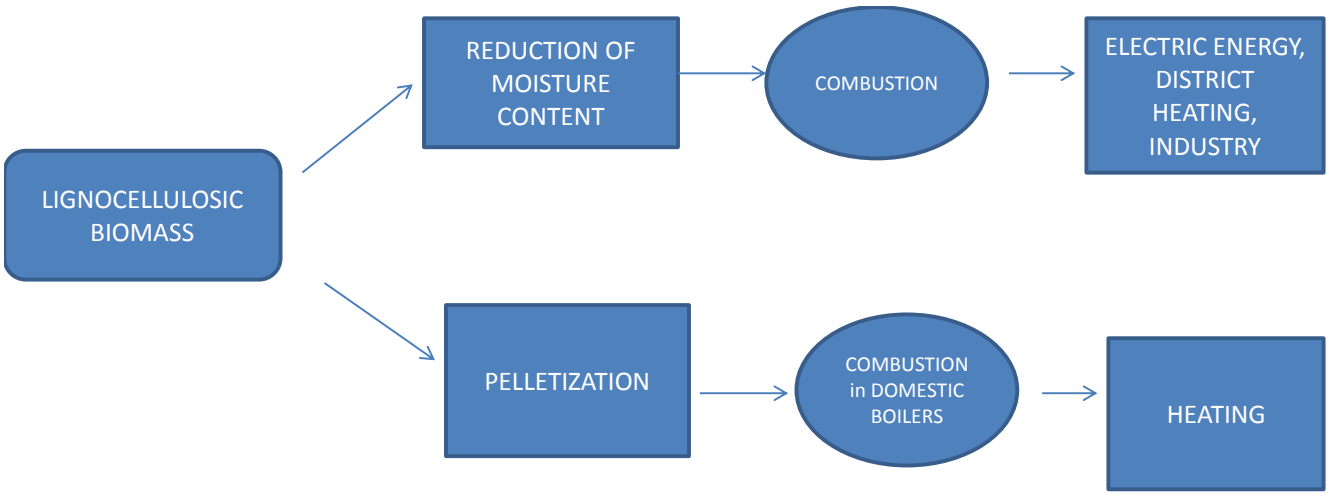


**Figure 17**. Combustion diagram (Candolo, 2005)

Pyrolysis (Fig. 18) is a thermochemical conversion process of organic matter, also called dry distillation, which is based on the transformation of biomass by heat in the practical absence of oxygen,. In practice, pyrolysis can be applied to any organic material as long as it has a low water content (<15%). The material is brought to temperatures between 200 °C and 700 °C, sometimes by introducing appropriate quantities of oxygen that allow the initiation of a partial combustion that leads to an increase in temperature. As a final product, gaseous, liquid and solid products are obtained in a percentage depending on the reaction parameters. Research on this has led to the development of three types of pyrolysis:

• slow pyrolysis, obtained with temperatures below 600 °C, and a long period of permanence at these temperatures; the main product obtained is a wood charcoal which represents about 30% of the initial dry substance;

• fast pyrolysis, obtained with temperatures between 500 °C and 650 °C: gaseous products are obtained that reach 80% of the initial weight;

• flash pyrolysis, carried out with temperatures around 650 °C and very short residence times at these temperatures, less than 1 second; allows to obtain 60% of liquid products.

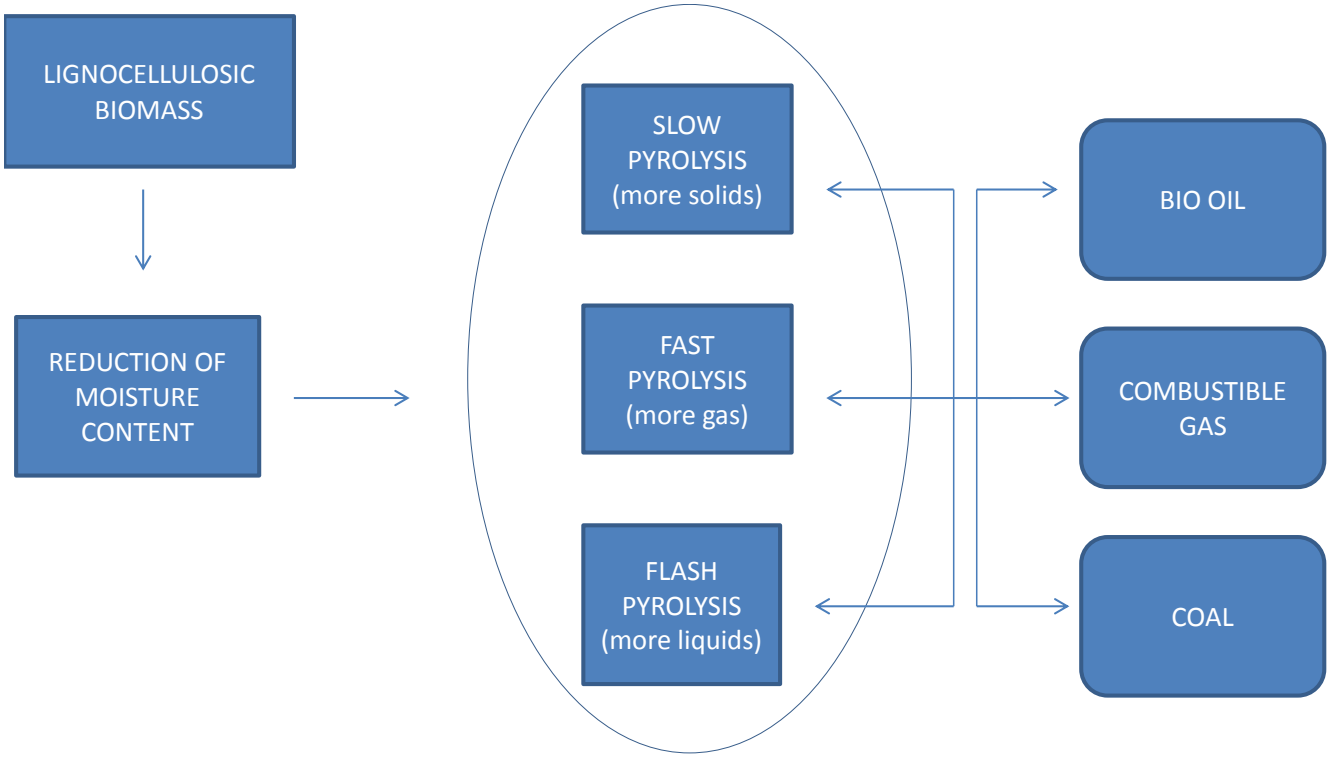


**Figure 18.** Pyrolysis scheme (Candolo , 2005)

Flash pyrolysis is the most promising process, as it allows biomass to be transformed into a liquid product called bio oil or raw tar, with a high energy content, easily transportable and storable for a long time without degradation problems. Huge resources are allocated to the study of pyrolysis all over the world, currently this process is still in an experimental phase. Gasification is a physical chemical process by which a solid fuel (wood, vegetable biomass in general) is transformed into a gaseous fuel. The process consists of an incomplete oxidation of the carbonaceous compounds brought to a high temperature (about 1000 °C) in an oxygen-deficient environment. The gas obtained, called syngas, can be used directly to power internal combustion engines that can be used for the production of electricity. Syngas is a mixture of nitrogen, methane, hydrogen, carbon monoxide and other gases.

The efficiency of gasifiers for the production of electricity is of the order of 30-35%, values significantly higher than combustion plants. The greater plant complexity, combined with some unresolved problems relating to the purification of syngas, relegates this process to the pilot plant phase. The gasification of woody biomass can also represent an extraordinary opportunity to obtain

hydrogen at relatively low costs, to be used, for example, in fuel cells. In Italy, experiments are being carried out on this, using a pilot plant that gasifies rice husks and wood residues (Candolo, 2005).

## 3.2 Biochemical Conversion

The conversion of plant biomass energy through biochemical processes is certainly the best known and tested way even in industrial energy transformation plants. Essentially, biological conversion methods can be divided into two processes:

• alcoholic fermentation;

• anaerobic digestion;

Alcoholic fermentation (Fig. 19) is the biochemical transformation process by which sugars are transformed into ethyl alcohol according to the reaction: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

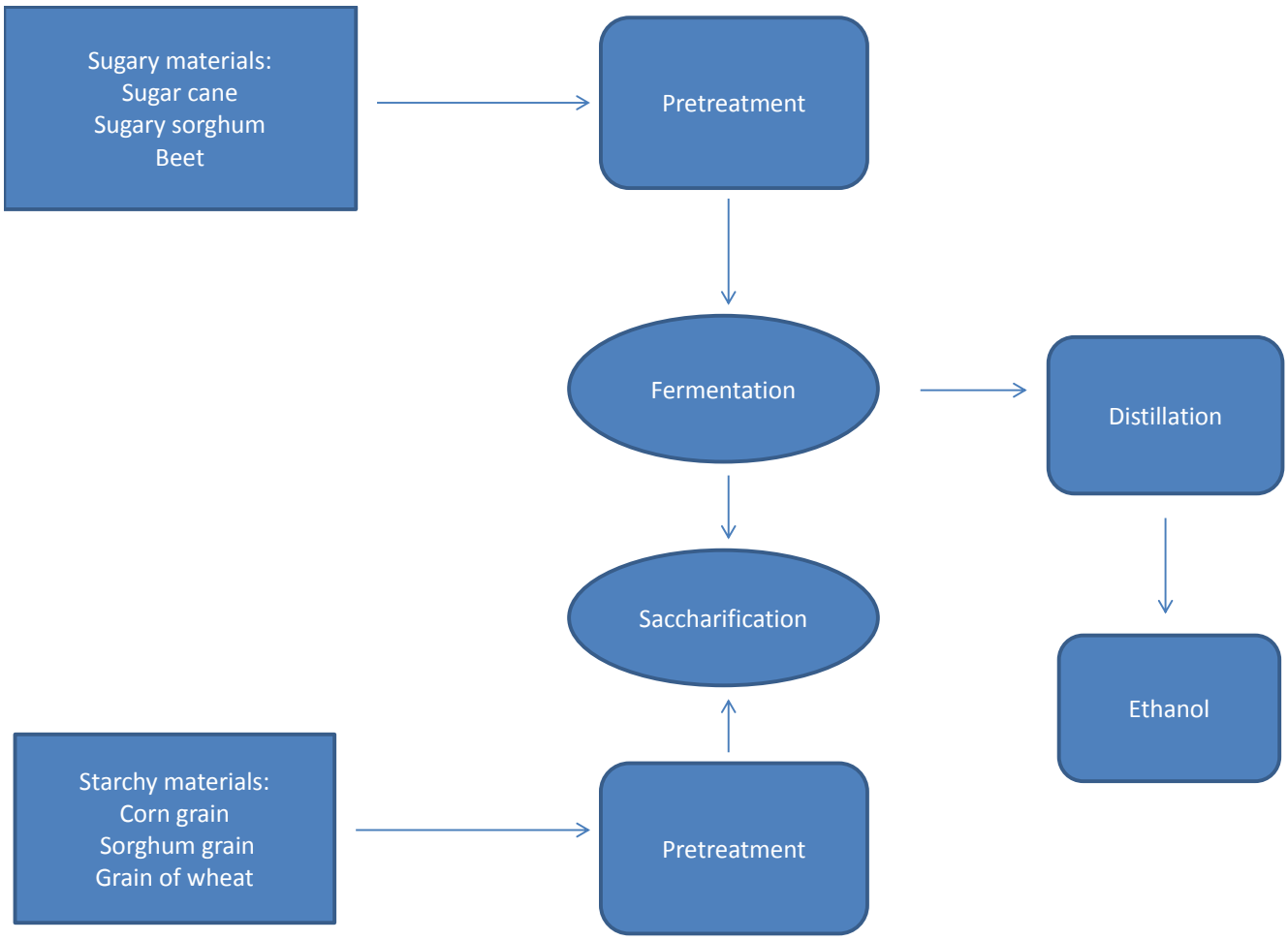


**Figure 19.** Fermentation scheme (Candolo, 2005)

The production of ethanol starting from biomass with a high sugar content is widely tested, in Brazil the fermentation of sugar cane allows to obtain ethanol at a cost competitive with that of gasoline. In Italy there have been experiences with the sugar beet, with uneconomic processing costs.

Anaerobic digestion (Fig. 20) is a conversion process operated by bacteria which, starting from biomass rich in cellulose, allows to obtain a biogas containing about 65% of methane. This gas is used to power an internal combustion engine connected to an electric generator. The electricity produced is directly fed into the distribution network, and sold at a profitable price (green certificate) as it is obtained from a renewable energy source. The most used biomass is corn silage, from 1 t of digested silage, about 10 $m^3$ of methane are obtained. The residual digested component can be used as fertilizer. Such plants are widespread in northern Europe. To optimize the efficiency of the system, it is convenient to have users of the heat produced, as for each electric kWh approximately 1 kWh is produced in the form of thermal energy. Approximately 300 hectares of corn silage are needed to power a biogas plant (Fig. 21) with 1 MW of electrical power. Such systems are relatively simple and do not require specialized personnel to ensure their operation (Candolo, 2005).

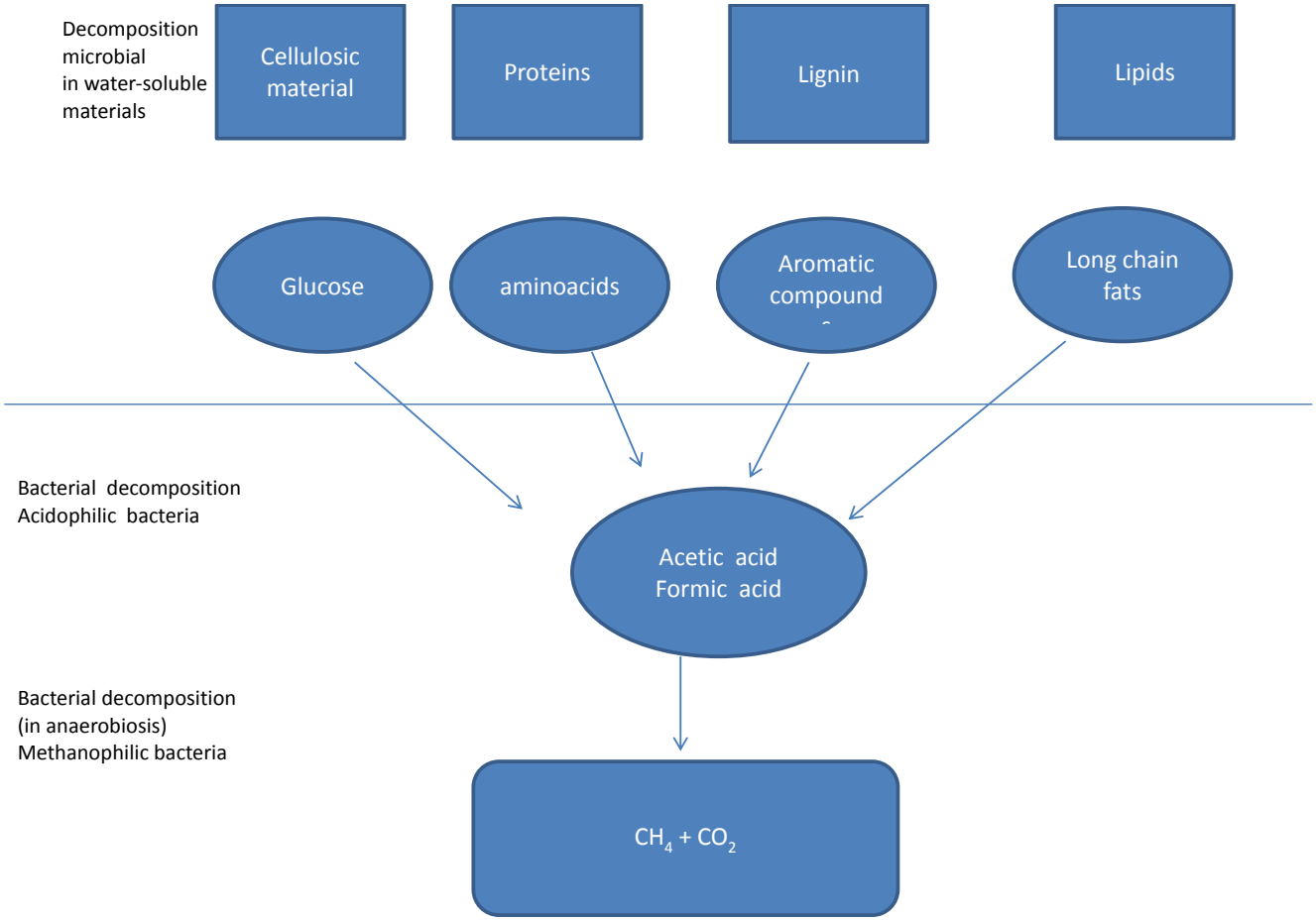


**Figure 20.** Anaerobic digestion scheme (Candolo, 2005)

**Figure 21.** Plant for the production of biogas

Although according to some authors the enzyme-based pretreatment using LiP, MnP and laccase has acquired incredible importance in lignocellulose conversion due to their unique properties including exquisite substrate specificity, lack of water requirement, optimal efficiency in delicate environmental conditions and the low risk of formation inhibitors (Bilal & Iqbal 2020), this actually applies to some types of enzyme tested on purified substrates and in optimal conditions of pH and temperature, which almost always do not coincide with the process conditions . In fact, currently no laccase has integrated the characteristics of thermostable, alkali-resistance, high redox potential and high activity when applied to the enhancement of lignin. The requirement of a specific degradation capacity of the lignin substrate could lead to modify the properties of the laccases and make them more efficient (Liu et al. 2020)

## 3.3 Physical conversion - Steam Explosion

The physicochemical pre-treatments of lignocellulosic biomass include ammonia fiber explosion (AFEX) which uses anhydrous ammonia to treat the material in a pressurized reactor at temperature between 60 and 140 ° C for a variable period time. Furthermore the liquid hot water (LHW) process is a hydrothermal treatment in which the pressure is applide to mantaine water in the liquid state at elevated temperatures. Supercritical fluids pretreatments (SCF) are compounds in a gaseous form (water, $CO_2$ etc) compressed at temperatures above their critical point to a liquid-like density. Finally, one of the most used methods to destroy lignocellulosics is the steam explosion.

Steam Explosion pretreatment is a method, which exposes raw materials to steam under high temperature and pressure for a specific time and subsequently reduces the pressure immediately to atmospheric pressure. Steam explosion technology is used to deconstruct materials with a lignocellulosic composition. Two actions contribute to the destructuring of the lignocellulosic matrix: instant decompression, carried out in the passage from the saturated vapor atmosphere of the reactor to normal conditions and a process of hydrolysis of the bonds by the action of high temperature water vapor. Steam explosion (SE) has been recognized as one of the most effective pretreatments for biomass fractionation and bioethanol production (Avellar and Glasser 1998). It uses saturated water steam at a high temperature (170-230°C) for a period of time followed by a sudden pressure release. Figure 22 shows a typical apparatus for steam explosion.

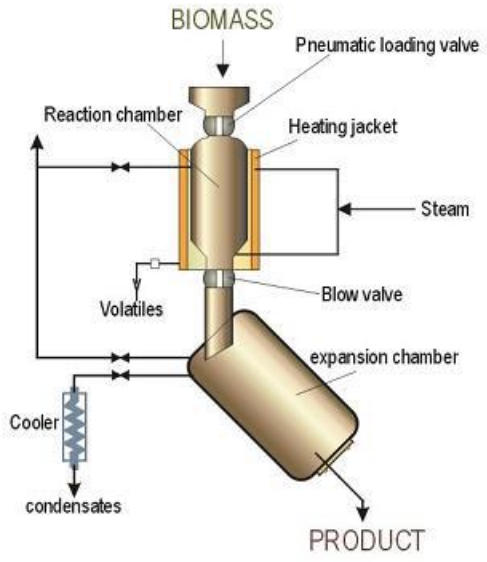


**Figure 22.** Steam explosion process

Steam explosion fractionates the biomass in two streams: a liquid fraction rich in monomeric and oligomeric sugars mainly from hemicelluloses solubilisation and a solid fraction of digestable cellulose and lignin (Tomas-Pejo et al. 2011).

The important variables in steam explosion pretreatment are residence time, particle size, and temperature. Steaming at high temperatures causes autohydrolysis conditions due to cleavage of the hemicellulose acetyl groups. The sudden pressure release provokes an explosive decompression that opens the densely packed cell wall in the biomass structure. As effect of these conditions, hemicellulose is partially solubilized and hydrolyzed and the inner surface area is increased, thus improving the accessibility to hydrolytic enzymes. An increase in temperature up to a certain level can effectively release hemicellulosic sugars. However, the loss of sugars steadily increases by further increasing the temperature, resulting in a decrease in total sugar recovery. Milder pretreatment conditions can minimize sugar degradation and generation of inhibitors (Mergner et al., 2013).

Some lignocellulosic biomass especially that with high lignin content, are often recalcitrant to steam explosion pretreatment, thus requiring severe process conditions that may help to overcome this drawback but, at the same time, reduce the sugars recovery.

The steam explosion process offers several attractive features such as the potential for significantly lower environmental impact, lower capital investment, increased energy efficiency, less hazardous process chemicals and conditions and high sugar recovery. Despite its versatility towards different biomass feedstocks, the main drawback of this method is the generation of toxic compounds derived from sugar degradation during pretreatment.

Most of the steam explosion pretreatments were carried out in small-size batch reactors. However, the continuous processing is of major interest for the industrial application. In particular the disruption and hydrolysis of cellulose fibers is more effective in the continuous reactor. On the other hand, the mechanical compression causes reduction of fiber accessibility to the enzyme at high pretreatment severity and lignin could recondense on the biomass pores (Mergner et al., 2013). Figure 23 shows conceptual design of a continuous steam explosion device.

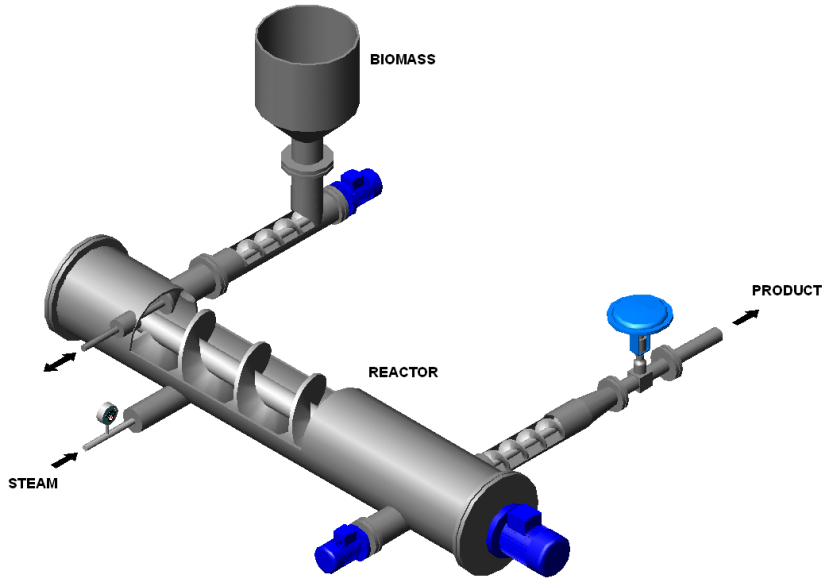


**Figure 23.** Conceptual design of continuous steam explosion device located at ENEA Trisaia centre (Basilicata, Italy)

Steam Explosion is a treatment to which any type of lignocellulosic biomass can be subjected, from which it is then possible to obtain a wide range of products. Steam explosion technology is used to deconstruct materials with a lignocellulosic composition. Two actions contribute to the destructuring of the lignocellulosic matrix: instant decompression, carried out in the passage from the saturated vapor atmosphere of the reactor to normal conditions and a process of hydrolysis of the bonds by the action of high temperature water vapor.

The explosive decompression causes a rapid escape of liquids from the cell membrane and the destruction of the cell structures themselves.

The advantage of the Steam Explosion technology is certainly that of obtaining a highly hydrolysable substrate and the low energy requirement compared to mechanical comminution and no recycling or environmental costs; nevertheless, the disadvantage is that it generates degradation products that inhibit processes such as fermentation (useful in other areas such as the production of second generation bioethanol), and which, if present above a certain threshold, must be removed due to the high toxicity. Many of these substances are volatile, such as formic acid, acetic acid, 5-HMF or

hydroxymethylfurfural and 2-furfural. Other substances, such as benzaldehyde, are formed from the depolymerization of lignin and, being low volatile, have a relatively high boiling point. In addition, $CO_2$ and $H_20$ are also present as degradation products, and consequently it is difficult to recover all of these products (Zimbardi et al., 1999). These inhibitors can be removed by washing with water, taking advantage of the fact that they are water-soluble, but also removing the soluble oligomers from hemicelluloses (Ballesteros et al., 2000). Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes.

After the pretreatment two phases are obtained: the solid one consisting of cellulose and lignin and the liquid one consisting of hemicelluloses. Optimized steam explosion processes could have many advantages: good hemicellulose and lignin yield with low degradation byproducts, no use of chemicals (except hydrolysis catalysts in small quantities), mild reaction fluid pH which minimizes corrosion of equipment. However, if it is not optimized, the process can be conducted at too high a severity and therefore be energy-intensive. The final result is to make the cellulose and lignin polymers more accessible, increase the reactivity of these polymers and finally solubilize most of the hemicelluloses.

Figure 24 shows the two steam explosion plants in ENEA C.R. Trisaia: both the plant that works in batches capable of treating 1 Kg of biomass for each processing cycle and the plant that works continuously capable of treating up to 300 Kg/h of biomass.

**Figure 24.** Steam explosion plant at ENEA C.R. Trisaia.

Authors (Liu et al., 2014) stated that SE causes partial depolymerisation of hemicellulose and partial relocation of the lignin onto the biomass solid surface, which creates a large accessible surface area for enzymes. (Sun et al., 2020a) used green-like steam explosion followed by mild chemical pretreatments using $H_2SO_4$ and NaOH in different concentrations to enhance enzymatic hydrolysis of *Miscanthus straw* and concluded that steam explosion combined with 4% $H_2SO_4$ pretreatment of Miscanthus Mfl12 obtained hexose yields of 51%, however under steam explosion combined with 8% NaOH pretreatment achieved practically 100% saccharification yield.

The objective of a steam pretreatment/steam explosion is to solubilize the hemicellulose to make the cellulose better accessible for enzymatic hydrolysis and to avoid the formation of inhibitors.

In addition to the steam explosion there is also steam pretreatment; during steam pretreatment the biomass is put in a large vessel and steam with a high temperature (temperatures up to 240 °C) and pressure, is applied for a few minutes. After a set time, the steam is released and the biomass is quickly cooled down. The difference between 'steam' pretreatment and 'steam explosion' pretreatment is the quick depressurization at the end of the steam explosion pretreatment, which causes the water in the biomass to 'explode' by the explosion, on the digestibility is still doubted.

During steam pretreatment parts of the hemicellulose hydrolyze and form acids, which could catalyze the further hydrolysis of the hemicellulose. This process, in which acids formed in situ catalyze the process itself, is called "auto-cleave" steam pretreatment.

The role of the acids is probably however not to catalyze the solubilization of the hemicellulose, but to catalyze the hydrolysis of the soluble hemicellulose oligomers (Hendriks & Zeeman, 2009).

Although the technology to obtain sugars is mature, the study of lignin is in an embryonic and pioneering stage. Lignin is a natural aromatic compound that is suitable for producing value added-product, such as binders, dispersants, phenolic resins, polyurethane foams and epoxy resins for printed circuit board.

## 4. Lignin

The most abundant land-based biomass polymer is cellulose. It, together with lignin and hemicelluloses, are the principal components of plants. The principal function of lignin in plants is to assist in the movement of water; the lignin forms a barrier for evaporation and, thus, helps to channel water to critical areas of the plant. Lignin is present in plants for which water conduction is important. Of greatest interest is its presence in trees. Lignin structure can vary within the same plant, e.g., primary xylem, compression wood, early versus late wood, etc.

Lignin is a natural aromatic compound that is suitable for producing value added-product, such as binders, dispersants, phenolic resins, polyurethane foams and epoxy resins for printed circuit boards; makes up approximately 15–28% of lignocellulosic biomass; it is distinctly different from the other macromolecular components of lignocellulosic biomass. It is an amorphous, cross-linked and 3-D polyphenolic polymer that is synthesized by dehydrogenative polymerization of three types of phenyl propanoid units, including monolignols: coniferyl, sinapyl and p-coumaryl alcohol.

Lignin is one of the three major components constitutes the cell walls of natural lignocellulosic plants and the second most abundant plant polymers in our planet. Lignin accounts for approximately 30% of organic carbon in the biosphere and is the only scalable renewable feedstock consisting of aromatic moieties. It contributes to 20–25% of the mass of hardwood and about 30% of softwood. Besides the natural abundance, lignin is also a major by-product of pulp and paper industry, where only about 2% of the 70 million tons of generated lignin has been burnt to recover heat and substitute fossil materials in pulping mills. Instead of burning, lignin has been extensively sought after for producing high-value functional materials because of the nature properties such as biocompatibility, eco-friendliness, low toxicity, and sensitive to enzymatic degradation (Feofilova et al., 2016, Mattsson et al., 2016).

As one of the most abundant natural polymer in our world, lignin has drawn the attention of many scientists for several centuries. Due to its complexity, non uniformity, and conjunctive bonding to other substances, lignin has been difficult to isolate without modification and difficult to convert into useful consumer products, and its structure has been difficult to determine. It is necessary to distinguish the native lignin present in the plant with the technique obtainable through processing. Milled wood lignin is traditionally considered representative of native lignin and is isolated after extensive milling of biomass in a ball mill prior to extraction with dioxane–water (El Hage et al., 2009). Lignin is an amorphous polymer with very complex structure present in cell wall of plant cells. It’s a high molecular weight copolimer containing mainly three phenylpropane monomer units

(monolignols) and it's insoluble in water, in acidic environment and in a lot of organic solvents. It's soluble in concentrated basic solution and partially soluble in some organic solvents, including deep eutectic solvents, or in different organic solvents after derivatization of polar functional groups. The complexity of lignin and its challenging characterization are due to the extreme variability of the structure, which depends on numerous factors including the type of biomass and the pre-treatment used for isolation. Lignin is a heterogeneous, random, aromatic macromolecule that is cross-linked by carbon-oxygen (C-O) and carbon-carbon (C-C) bond networks and its exact structure is unknown (Meng et al., 2019).

Lignin is an amorphous and complex aromatic polymer providing terrestrial plants mechanical support, stress response, pathogen resistance, and water transport; natural lignin is a high molecular weight polymer consisting of phenyl propanol units. The backbone of lignin is comprised of three different phenyl-propane monomers, termed sinapyl alcohol, coniferyl alcohol, and ρ-coumaryl alcohol (Fig. 25).

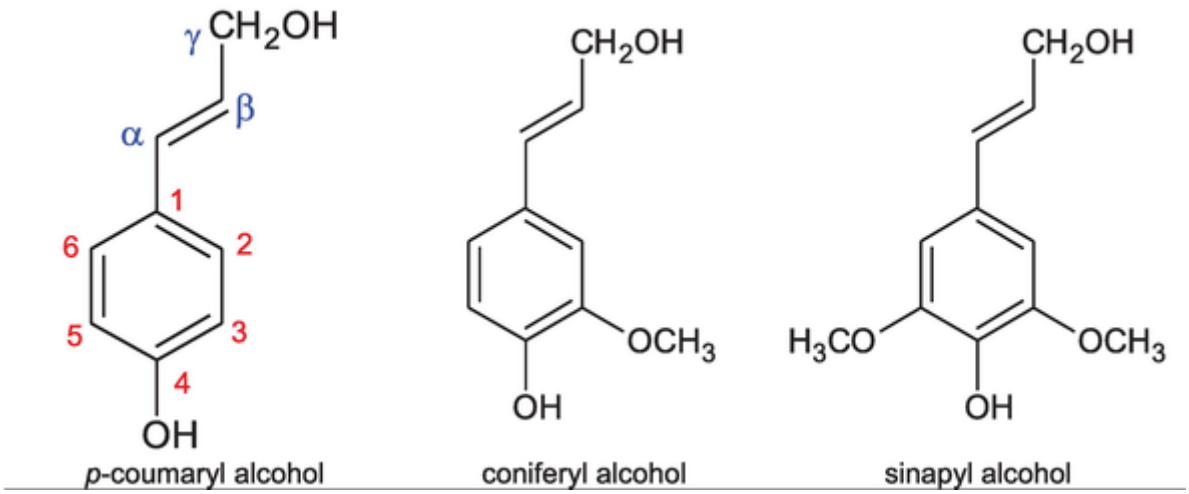


**Figure 25.** p-Coumaryl alcohol (H-unit), Conyferil alcohol (G-unit) and Synapyl alcohol (S-unit)

The proportions of the three monomers in lignin dictate the type of configuration in the lignin molecule, which in turn determine the degree of branching and the reactivity of lignin. The monomers transfer to three phenolic sub-structures, namely syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units. Depending on the natural source from which the lignin is isolated, and also depending on the method with which the lignin has been extracted from the wood and separated from the cellulose and hemicellulose wood components, the abundances of the different monomer types, as well as the nature and the distribution of the interunit bonding motifs via which they are linked differ. Three different general types of lignin are generally distinguishable on the basis of fundamental structural aspects: (i) softwood lignin, which is mainly comprised of G-type monomers and a small percentage of H-type

monomers; (ii) hardwood lignin, which contains mainly S- and G-units; and (iii) grass lignin, which contains all three monomer types (Lange & Crestini, 2019).

The lignin polymer can be initiated by coupling of two monomeric radicals, but more likely grows when monomer radicals couple with phenoxy radicals formed on the growing polymer. The phenoxy C–β position appears to be the most reactive, since the most abundant linkages in lignin involve this position (β–O–4, β–5, β–β), but other connections including diphenylethane dimers (β-1′), spirodienones, diaryl ethers (4-O-5′), dibenzodioxocines (5,5′-α'β-O-4′) and biphenyls (5–5′) (Fig. 26) were detected.

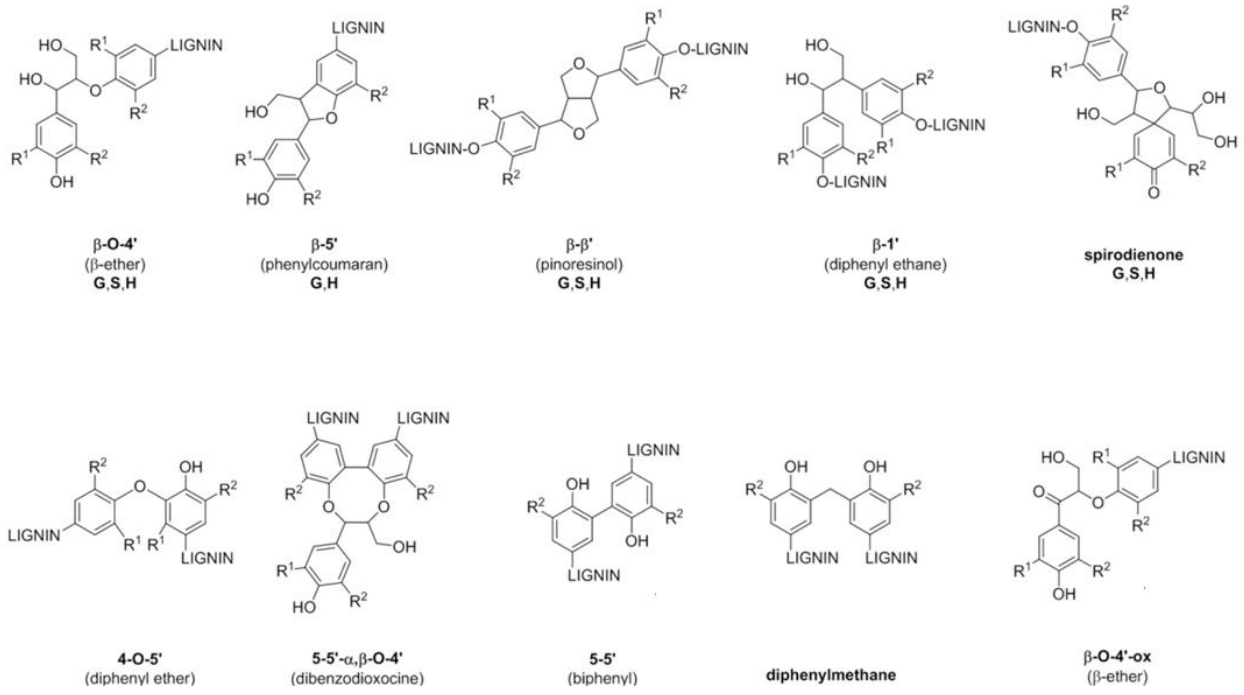


**Figure 26.** Lignin linkage types (Lange & Crestini, 2019).

These subunits contain many different functional groups, such as hydroxyls, carboxyls, carbonyls and methoxyls, which are active sites for further chemical modification and lignin utilization. Bioengineering to modify lignin structure and/or incorporate atypical components has shown potential toward facilitating recovery and chemical transformation of lignin under biorefinery conditions (Meng et al., 2019).

## 4.1 Properties and lignin applications

By far, the principal use for lignin is as a source of chemical energy in waste-to-energy processes, such as in the production of pulp used for paper and corrugated board. High-quality paper products require that the lignin be separated from the cellulose in wood. The pulping process produces a pulp rich in cellulose and a liquor rich in degraded lignin. The liquor is partially evaporated and burnt in a furnace. Inorganic pulping chemicals are recovered, and the energy generate is used in the pulp production.

Other mature industrial applications of lignin concern its use as a filler in composites, as a component in binders or coatings, or as a surfactant or dispersant. To date, its potential as a source of phenols for the production of high added value biopolymers has not been sufficiently developed. The main reason for the poor valorisation of lignin residues is their chemical heterogeneity. Lignins are in fact polyphenols characterized by a complex network formed by three main phenylpropanoid units linked together through a series of different intermonomeric bonds.

The lack of repetitive sequences of specific intermonomeric bonds and specific subunits makes the structural characterization and enhancement of lignins a major challenge for chemists.

Lignin as the second most abundant and the only poly aromatics-contained bio-polymer in plant has been most studied for various applications. In the past decade, the utilization of lignin for value-added materials has been extensively sought after since lignin valorization represents one of the main challenging issues of the paper industry and lignocellulosic biorefinery (Meng et al., 2019).

Lignin is hydrophobic compared with carbohydrate polymers in plant tissue; however, the number of hydroxyl groups attaching to lignin is sufficient to act as a reaction site for hydrogen bond formation with water molecules; lignosulfonate obtained as by-products of the sulfite pulping process, is an amphiphilic polymer and is water soluble (Hatekayama H. & Hatakeyama T., 2009).

Lignin could be an excellent candidate for chemical modifications and reactions due to its high functionality (i.e., rich in phenolic and aliphatic hydroxyl groups) for the development of new bio-based materials. Chemical modification of lignin has driven numerous efforts and researches with significant studies during the last decades. Technical lignins are obtained from different isolation processes and have different properties, differing in chemical structure, amount of ash, residual sugars, purity, molecular weight etc. The Kraft lignin is obtained from the process based on the treatment of biomass with sodium sulphide ($Na_2S$) and sodium hydroxide (NaOH) used in the paper industry. (Wang et al., 2016). During this process, the lignin becomes soluble and dissolves. Soda lignin is attained from the soda pulping process or soda-anthraquinone. Soda process has been known as a sulfur-free process, which is a major advantage with respect to the kraft process (Vishtal and Kraslawski, 2011). In this process, lignin is extracted from lignocellulosic biomass in the presence of

13–16 % sodium hydroxide. Sulfite process has been conducted by using a sulfite base (sodium, calcium, ammonium, or magnesium salts) and aqueous sulfur dioxide (pH range of 1–2) (Tribot et al., 2019). During the pulping, a large amount of sulfur is introduced onto lignin. Based on most biorefining concepts, wood can be treated with acid, base or enzyme to dissolve sugar (cellulose and a part of hemicelluloses) and use the sugar to produce materials such as ethanol. Meanwhile, lignin remains undissolved and usually used as fuel (Sameni, 2015). The steam explosion process is usually carried out by exposing the raw material to a moderate temperature (150−230 °C) and high pressure (around 3.5 MPa) for 1−20 min (Ahmad & Pant, 2018). In this process, the lignin structure breaks down reducing its β-O-4 content. Since fewer chemicals are used in this process, lignin undergoes a less bond cleavage. Also, the solubility of lignin produced in this process has been revealed to be enhanced in organic or alkaline solvents (Sameni, 2015).

The principle of organosolv process is based on the extraction of hemicellulose and lignin from biomass using organic solvents, such as methanol, acetone, ethanol, or mixtures of organic solvent and water at the temperature range of 100−250 °C (Zhao et al., 2009). In this process, β-ether linkages in lignin break down and result in lignin dissolution. To facilitate the extraction process, acid catalysts, such as sulfuric acid, phosphoric acid, hydrochloric acid, or organic acids, such as acetic acid or formic acid, could be added to the extraction process. In this process, the organosolv lignin is precipitated by solvents or distillation with water to recover the solvent (El Hage et al., 2009). Lignosulfonate, soda, and kraft lignin with higher hydroxy groups may be more suitable for water-soluble applications. In applications where the product purity is crucial, organosolv lignin may be a better option, but the commercial production of organosolv lignin is currently limited (Kazzaz & Fatehi, 2020).

The following Table 1 shows general characteristics of various lignins:

**Table 1.** General characteristics of various lignins

| Lignin type | Scale | Chemistry | Sulphur content | Ash content | Sugar content | Purity | Molecolar weight (g $mol^{-1}$) |
|---|---|---|---|---|---|---|---|
| **Kraft** | industrial | alkaline | moderate | Moderate/high | low | High | 1000-5000 |
| **Alkali** | industrial | alkaline | free | Moderate/high | moderate | Moderate/high | 2000-10000 |
| **Lignosulfonate** | industrial | acid | high | high | / | Moderate/low | 1000-50000 |
| **Organosolv** | Pilot/demo | acid | free | Free/low | low | Very high | 1000-6000 |
| **Enzymatic hydrolysis** | Industrial/pilot | acid | low | low | Free/low | Moderate/low | 2000-4500 |
| **Steam explosion** | Demo/pilot | acid | low | Moderate/high | moderate | Moderate/low | 3500-15000 |

Several projects aimed at developing technologies for the enhancement of lignin have recently been completed or are still in progress. The SWEETWOODS project aims to develop a state-of-the-art biofractioning plant in Estonia, the first of its kind, using mainly hardwood such as birch. The process combines innovative pretreatment technology with enzymatic solutions to provide sugar recovery at levels above 90% with exceptionally high quality lignin. Lignin is obtained through alkaline extraction. Dried solid lignin and depolymerized lignin are demonstrated in new applications, particularly in elastomeric foams for pipe insulation, rigid polyurethane foam panels for insulation and polymer compounds for injection molding. The process is at an exceptionally high TRL 8. The BIOFOREVER project (TRL 6-7) aims to demonstrate the commercial feasibility of converting lignocellulosic raw materials into chemical building blocks and high added value products from lignosulfonated lignin in order to produce carbon binders, FDCA and acid resins. The EUCALIVA project (TRL 6-7) is based on extracting high-purity soluble lignin from the kraft pulping process (black liquors) and to transform it through different lines, achieving a cost-efficient alternative to today's petroleum-based carbon fibre raw materials. The LIGNIOX project (TRL 6-7) is one of the most versatile processes as it can use kraft, organosolv or lignin derived from hydrolysis processes and aims to demonstrate the techno-economic viability of the unique alkali-O2 oxidation technology for the conversion of several lignin-rich side-streams into dispersants and plasticizers. The VALCHEM project (TRL 6-7) aims to demonstrate at a pilot scale the techno-economic viability of producing green chemical products, such as monopropylene glycol, bio-MPG, and lignin-based

performance chemicals, from wood-based raw materials. All the processes used have already been demonstrated at least at a pilot scale. Other projects with TRL 3-5 mainly involve Kraft lignin or alkalin lignin in order to produce carbon fibers, aromatic monomers, lignin oligomers, FDCA, polycarbonates, phenolic resins, polyols (Mastrolitti et al., 2021).

Novel approaches in hydrogel fabrication involved renewable materials, such as cellulose, hemicellulose, chitosan, starch, pectin and alginates. The hydrogels made from these natural polymers have advantages of inherent biocompatibility and biodegradability. Such bio-based renewable hydrogels in recent years have been broadly used in food, cosmetics, pharmaceuticals, and biomedical implants. Among the natural polymers, lignin is the only aromatic polymer, which makes lignin having a great potential to be used for functional hydrogels of a different type.

Due to the biocompatibility, biodegradability and biodegradation of lignin and the trait of hydrogel, lignin-based hydrogels have been widely used as biomaterials in many fields, such as industry for water treatment, agriculture for water retention and some smart biomaterials. Introducing lignin and its derivates into hydrogels represents a very promising approach to valorize this natural lignin. (The initial materials are not restricted to lignin or lignin derivates; other biopolymers like cellulose, hemicellulose, chitosan and alginate can be used to increase the active sites of the lignin-based hydrogels). In addition, other functional materials, such as graphene and carbon nanotubes have been used to promote the conductivity of the hydro-gels. On account of biocompatibility, biodegradability, low toxicity and eco-friendliness, lignin-based hydrogels can serve as absorbents for heavy metal ions, controlled release agent for controlled delivery and water retention, smart materials for stimuli response, and biosensors and electrodes. Among these applications, biosensors and electrodes are not well studied as others, which therefore still need more attentions to be paid from the researchers in future (Meng et al., 2019).

Micro- and nanosize lignin has recently gained interest due to improved properties compared to standard lignin available today. The nanoparticles offer a large surface area and, therefore, more functional and polyphenolic side chains on their surface. A large number of potential high- value applications fall within the field of view of micro- and nano-sized lignin particles (LPNs) (Schneider et al., 2021). LNPs have the advantages of being a “green”, biocompatible and potentially inexpensive nanomaterial; recently some applications of LNPs have been consolidated, such us their use in thermoplastics, nanocomposites, foams, bactericides, products that require protection from UV radiation and others and therefore lignin has been considered as a new potential feedstock for the production of eco-friendly nanoparticles. (Schneider et al., 2021).

A wide range of applications for lignin nanomaterials are reported in the literature. Several applications concern the biomedical field (Figueiredo et al. 2018), cancer drug delivery (Garg et al. 2022) environmental pollution remediation (Sajjadi et al. 2021).
Parameters affecting the properties of the final product include the source of lignin, the method of extracting the lignin from the lignocellulosic charge, the method of production / precipitation of the nanoparticles which affects the surface properties of the particles and, in the case of polymer mixtures, the method processing / mixing (Schneider et al. 2021).

## 4.2 Bio-adhesives

Not all lignocellulosic materials are suitable for the production of green building panels. Among the wood-type biomasses, in addition to cork, there are residues of spruce, pine, beech and poplar, although all of them require treatments to increase the adhesiveness of the fibers. The ideal biomasses have a low hemicellulose content which determines a better resistance to water but on the other hand a reduced binding strength of the fibers, the high cost and scarce availability of wood-type biomasses has pushed the research towards the identification of agro-industrial residues of non-woody lignocellulosic biomasses. Among these are reported in the scientific literature the mischantus, wheat straw, coconut waste, the residues from the processing of bamboo, kenaf, cotton, jute and hemp, vine pruning waste and lignocellulosic residues of thistle (Zhang et al., 2015). These biomasses can be an abundant and renewable source of raw material for a wide range of products such as paper and board (Ververis et al. 2004), medium density fibreboard (MDF), particle board and other bonded composites such as plywood. They are partly already used in the construction and furniture industry (Maloney 1996, Sellers 2001, Reddy & Yang 2005).

The sustainable use of these scraps for the construction of panels always implies the use as adhesives of important quantities of resins (the most used are those) mostly of fossil origin such as urea-formaldehyde and phenol-formaldehyde which involve also the risk of noxious emissions from manufactured articles mainly attributable to formaldehyde (Maloney, 1996, Sellers, 2001). The strict regulations on environmental safety and human health and the rising costs of raw materials have therefore prompted research to reduce the quantity of these components and replace them at least in part with more sustainable alternatives. The attention of panel producers to safety and environmental issues in recent decades has grown to such an extent that currently many manufacturing companies are able to almost completely satisfy the requests for panels using almost exclusively recycled wood. There is also the availability for the use (albeit in experimental tests) of other residual lignocellulosic materials such as those listed above. Last but not least, in the direction of sustainability, is the declared

commitment by many producers to use glues normally considered of fossil origin which, however, have been synthesized using basic chemical materials not obtained from oil but from other sources such as nitrogen and carbon dioxide. One of the options to increase the sustainability of biomass panels is to develop processes that increase the self-adhesion of the fibers by exploiting the natural ability to act as a glue of the lignin. In fact, one way to improve the adhesiveness of the fibers without adding glues is, for example, to pre-treat the lignocellulosic fibers with chemical-physical technologies based on the use of saturated steam, for example by steam explosion. An increase in adhesion can also be obtained through the use of cross-linking material added based on technical lignins derived from the paper industry. Activation of lignin for adhesion can also be carried out by oxidation with phenol-oxidizing enzymes (laccase and peroxidase) derived for example from white rot fungus (Widsten & Kandelbauer, 2008). The addition of technical lignin or mediator as a catalyst shows, in most studies, an acceleration and intensification of the radicalization process and this should lead to greater adhesiveness between the fibers themselves (Widstein & Kandelbauer 2008; Euring et al. 2016). Some mediators can be used during enzyme pretreatment. Mediators are molecules that catalyze the electron transfer reaction (Fig. 27). These include some types of waxes, some types of lignin and molecules such as caffeic acid and vanillic alcohol.

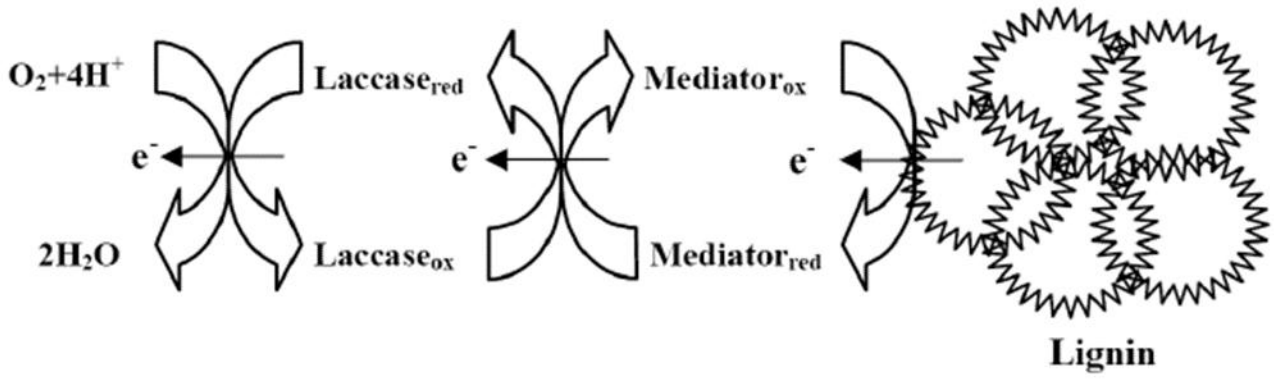


**Figure 27.** Role of the mediator in the mechanism of action.

Fiberboards are composites produced from lignocellulosic fibers with adhesives whose characteristics can be toned to be suitable for selected applications. The presence of adhesives is traditionally essential for fiberboard manufacture to keep proper physical and mechanical properties. Formaldehyde, one of the most common components in adhesives, has been widely employed in the

industry due to its low cost and desirable performance; the emission of formaldehyde from fiberboards gave rise to environmental and health concerns. However formaldehyde is toxic. For this formaldehyde-free adhesives made from natural resources, including lignin, wheat protein, starch and soy protein have been extensively researched. However high cost and relatively poor performance limited their application in the industry. Therefore, the production of fiberboards without adhesives addition is a promising strategy from economic and environmental perspectives (Zhang et al., 2015).
Lignin is a natural binder in wood and plays an important role in this kind of boards.
In recent decades an old process has been re-investigated. This process involves steam explosion of raw lignocellulosic material, thus hydrolyzing most of the hemicelluloses and plasticizing the lignin. The result of this pretreatment is a fiber that can be hot-pressed to produce fiberboard without the need for synthetic binders. Due to the important role of lignin in fiberboard manufacture, several studies have investigated the use of lignin as a natural adhesive and the possibility of replacing fibers with lignin.

There are studies in which are successfully produced medium density fiberboards (MDF) without the participation of synthetic resins. As a binder used is an enzymatic lignin (Yotov et al., 2017).
Steam explosion is one of the best ways of pretreating lignocellulosic materials for use in chemical fractionation, bioconversion, and the production of boards and composites because it preserves the fiber structure and separates the lignocellulosic material into its main components (cellulose, hemicelluloses and lignin). It has been claimed that steam explosion plasticizes the lignin and separates the fibers, thus improving the bonding capacity of the material. *Cynara cardunculus* is not the best material to produce binderless fiberboards compared with other materials studied previously such as *Miscanthus sinensis* and residual softwood. This is probably due to its lower lignin content and its higher ash content, but still it can be used to obtain fiberboards of good quality without adhesives of fossil origin (Mancera et al., 2008).
Medium density fiberboards (MDF) are produced mainly by urea-formaldehyde resins (UF) as binding agent, which are synthesized from finite fossil resources. Those boards may emit critical amounts of formaldehyde, which can influence the health of humans and animals. In recent times the wood panel board industry is looking for alternative glues which contain less or no formaldehyde (Euring et al., 2016).

The advantages of this type of boards as a building material and engineering possibilities are many, but there are drawbacks to natural wood. To improve the mechanical properties of the boards (MDF) wood pulp needs to be added adhesives such as emulsions. As adhesives are used water-emulsion

synthetic resins having thermosetting properties and strong adhesion to wood. In addition are observed some disadvantages - high value materials contamination of manufacturing equipment, emissions in the environment and toxicity.

Phenol-formaldehyde resins (PFR) compared to other resins have many advantages such as low cost, ease of use when incorporated in the wood mass, rapid gel time in hot pressing, low temperature curing, resistance to microorganisms and mostly water repellent ability and strength characteristics of the finished boards. MDF boards produced by the PFR are suitable for use in both internal and external conditions. One major drawback of the PFR is the given free formaldehyde into the environment when used as a component in the production of MDF.

Many products are manufactured based on Phenol-formaldehyde resin that vapors of free formaldehyde, which can cause health problems or illnesses in humans. Emissions of free formaldehyde most common causes: irritation of the eyes and upper airways, when the human body is exposed to the emission of formaldehyde in high doses there is a risk of severe poisoning, and prolonged exposure may result in chronic toxicity and even cancer.

For these reasons, worldwide continuously is conducted research to reduce and eliminate the release of free formaldehyde from wood-composite plates.

One of the effective methods to reduce the amount of free formaldehyde in fiberboard is the addition of lignosulfonate or technical hydrolysis lignin in the role of binders to appear partial or complete replacement of phenol-formaldehyde resins. On the other hand, it has globally experiments were performed in order to produce dry-formed fiberboards without a resin binder and they have generally been unsuccessful  a negative impact on the physical and mechanical properties of  the resulting boards (MDF).

The possible strategies for the enhancement of lignin are essentially focused in two directions:

- the first concerns the selective functionalization of the polymer to increase its compatibility and performance in composites;
- the second concerns the selective depolymerization of lignin in order to obtain monometric compounds, which can be used as feedstocks for the polymer industry as an alternative to petroleum derivatives (building blocks).

Thanks to the presence of a variety of functional groups including benzyl groups, alcohol groups on the aliphatic chains and phenolic groups that can be easily oxidized, which can be in recent years,

particular attention has been paid to studies on selective oxidation on lignin. In this context, a class of enzymes called laccases play a fundamental role in the oxidative processes of lignins. Laccases are promising biocatalysts with several bioremediation and biodegradation applications, food industries, producing cosmetics, nanobiotechnological productions, textile industries, woodworking industries, and pulp/paper processes. Laccases (Khatami et al., 2022) [1.10.3.2] are multi-branch oxidases that catalyze the reduction of oxygen to water. The enzyme contains four copper-containing centers, a type 1 Cu (T1), a type 2 Cu (T2) and a type 3 (T3) binuclear pair (Cole et al., 1990, Solomon and Lowery, 1993). The T2 and T3 sites form a trinuclear cluster of copper where oxygen is reduced of Cu T1 oxidizes the reduced substrate and transfers the electron to the copper atoms in the T2 and T3 centers. Laccases are catalysts capable of oxidizing phenolic systems through an electron-transfer process that generates a radical-cation, which, through a process of rapid deprotonation, generates a reactive phenoxy radical. The intermediate phenoxy radical that is formed during this process can further disproportionate and, consequently, start the degradation of the lignin. Laccases also show high thermal resistance (stable at about 60 °C), low substrate specificity and high oxidation rate, all characteristics that make these enzymes ideal candidates for the development of effective modification processes. of lignin. The redox potentials of laccases depend on the fungal species that have been used for their production, but in any case the oxidation of substrates in which the phenolic group is not free, as in the case of methoxybenzene, is prevented by their high redox potential and it therefore requires the presence of radical mediators such as 1-hydroxybenzotriazole (HBT) or 2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonate (ABTS). In fact, it has been shown that laccases in the presence of HBT generate the oxybenzotriazole radical which is able to oxidize both phenolic and non-phenolic model lignin compounds through the extraction of a hydrogen atom rather than through an electron process transfer. The treatment with laccase induces oxidation of the side chains, in particular the reduction of alcohol groups and the formation of condensed phenolic units due to the oxidative coupling process. Furthermore, the breaking of the aromatic rings was also observed.

The main disadvantage of laccases is due both to the need to recycle the enzymes and to their low stability. Several examples of laccase immobilization are reported in the literature to solve these problems (Crestini et al., 2011).

Typically, the effect of laccases on different types of lignin has been studied by evaluating oxygen consumption, the effect of modification on the chemical structure of the lignin and / or the physical properties of the lignin, or by studying the formation of radicals.

Traditional petroleum-based thermosetting adhesives have numerous advantages, such as excellent adhesion and water resistance properties, ease of handling, low curing temperature, short printing times, reasonable cost, etc. However, they have a serious drawback related to the dangerous release of

free formaldehyde and other volatile organic compounds from finished wood-based composites, associated with serious environmental problems and a number of significant risks to human health, such as irritation to the eyes, to the skin and the nervous system, skin sensitization, nausea and even cancer. There is therefore a growing interest in developing sustainable, bio-based, "green" adhesives from various renewable biomass raw materials, such as proteins, starch, tannins and lignin. The production of bio-based adhesives poses additional challenges; adhesives made from renewable natural materials often have low water resistance or are too expensive to effectively compete with fossil-derived polymers. The often large variations in properties due to different growing conditions, source shape, growth time, access to nutrients, climate and other factors, present another challenge when using bio-based polymers. The extraction and fractionation processes that are important for bio-based polymer isolation would also affect the final properties and costs of the adhesives. Therefore, the complete replacement of robust synthetic polymers, having well-known and reproducible characteristics, with completely based polymers and biology as adhesives for wood on an industrial scale is under study and development (Aristri et al. 2021).

The resins mainly used for the formation of wood panels are urea-formaldehyde and phenol formaldehyde. The reaction mechanisms that make bonding possible are shown in the Figure 28 (for urea-formaldehyde resins) and Figure 29 (for phenol-formaldehyde resins).

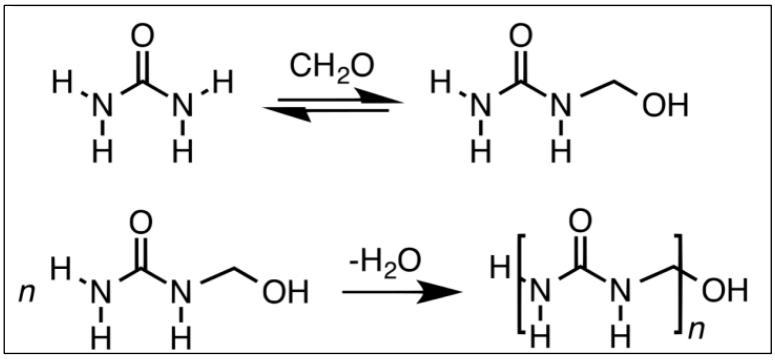


**Figure 28.** Urea-formaldehyde resin reaction mechanism of formation.

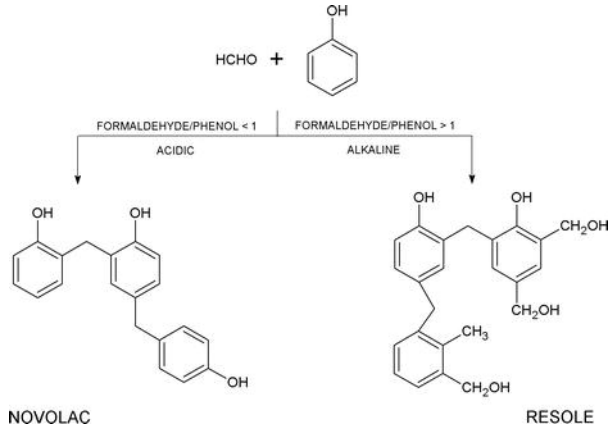


**Fig. 29.** Mechanism of phenol-formaldehyde resin reaction (Jamrozik et al., 2018)

Almost all the most marketed products (among these the most sold in the world are chipboard panels to be used in a dry place) are manufactured with urea-formaldehyde resins. The other type of resin is normally used for niche products that must have more marked resistance characteristics (especially to humidity).

Some of the major world producers of these two types of resins have introduced the use of raw materials (Urea, Phenol and Formaldehyde and their precursors) obtained from renewable sources.

The development of the resin market and in particular of products obtained with minimal quantities of products of "fossil" origin, is in continuous and inexorable expansion.

The global production of phenol-formaldehyde (PF) resins should have reached 10 Mt estimated at ca. $ 13.36 billion in 2019 [PR Newswire Europe]. Their main use (over 50%) is as adhesives for wood. A 2018 report on bioproducts (Fabbri et al. 2018) rates the bio-based resin market close to 1% of the global market size for these resins. The main constraints that influence the development of the market for this type of products are linked to lignin (difficult to obtain with a standardized price, in constant quality and in large quantities), to the costs for the conversion of existing plants to make them suitable for the production of resin and panels. low content of fossil products, intersectoral cooperation between stakeholders in order to increase the sustainability and cost-effectiveness associated with the use of fossil-free resins (bioindustrial poles, legal and economic constraints against the use of fossil resources, market promotion of bioproducts, etc.) (Lettner et Al, 2020). Lignin and tannins are two components of biomass with application in the production of biobased resins and adhesives. Lignin in biomass plays an important role as a natural glue of polysaccharides. This implies that the lignin could, opportunely activated, be used as an adhesive in the paneling. Lignin is one of the biomass components of great economic interest due to its high potential as a renewable aromatic raw material for high-value applications such as biopolymers, insulators, panels, foams and bitumen. However, the

rigidity and complexity of the lignin polymer, together with the variety of extraction and pretreatment processes and conditions, make its enhancement currently very challenging. The lignin, simply mixed with the components of the glues (in small quantities) does not affect their characteristics and manages to reduce formaldehyde emissions (Senyo et al., 1996). To increase the reactivity of lignin towards formaldehyde and therefore activate its properties as a component in adhesives in general, it is necessary to depolymerize it in shorter oligomers and make the phenolic sites more reactive.

In this context, chemical modification and activation strategies [methylolation (Goncalves, 2001), phenolation Batog, 2011)], enzymatic with laccase and oxidase are often reported (Cheng et al., 2013) and thermochemicals including hydrogenolysis under soft conditions (Beis et al., 2010) and pyrolysis (Zhang et al., 2012). The following Table 2 summarizes the most important results and the best technical solutions referable to various lignins used as starting material for various phenol-formaldehyde resins in which at least a part of the resin is not of fossil origin.

**Table 2.** Selection and main information on phenol-formaldehyde resins obtained from lignin

| Lignin | Percentage of phenolic sites of fossil origin replaced and reaction conditions | Technical properties of the product panel | ref |
|---|---|---|---|
| **Lignosulfonates** | Phenolic sites replaced:30%-50%.<br>Condensation in an alkaline environment with formaldehyde | Bonding strength (ply wood): 0.8-1.2 MPa;<br>At 30% the resin is more "workable" and resistant to temperatures. | [Alonso et al. 2004, Alonso et al. 2006] |
| **Kraft Lignin** | Up to 50% of phenolic sites replaced<br>Two process steps: (1) depolymerized lignin (MW 800-1700 g / mol) with phenol, NaOH sol., and methanol at 60 ° C;<br>2) addition of formaldehyde (solution in water at 37 vol.%) At 80 ° C | Wood chipboard panels stronger than commercial ones OSB panels have elasticity and breaking limits similar to commercial ones.<br>. | [Tejado et. al 2007, Lee et. al 2012] |
| **Lignin Organosolv** | Up to 30% replaceable phenolic sites; by activating this lignin the share can rise to 75% | With high replacement rates (50% -75%) the resin has adhesive properties and thermal stability comparable to those of fossil resins | [Goncalves 2001; , Batog & Przepiera 2011, Beis et al. 2010] |
| **Lignin from hydrolysis** | Phenolic sites replaced at 50% (F / P molar ratio of 1.5), necessary lignin activation reaction (phenolation) | With high substitution rates (up to 50%) the resin has adhesive properties comparable to those of fossil resins | [Jin et al. 2010, Qiao et. al. 2014] |
| **Sodalignin** | Phenolic sites replaced: 30% - 50%.<br>Activation of the phenolic sites of lignin through oxidation, and or reduction, and or acid hydrolysis (HCl 35%) is necessary | The gelation times and the production time are shorter. | [Khan & Ashraf 2005; Nada et al. 2003] |
| **Steam Exploded Lignin** | Complete replacement but provided that drastic conditions are used (temperature and pressure) | Unfortunately the products have properties that are close but not sufficient to meet EU standards | [Gravitis et al. 2010] |

In the literature there are numerous studies aimed at optimizing the production process of panels starting from lignocellulosic biomass through pretreatments. In our research group, the pre-treatment of steam explosion has been extensively studied for several purposes (De Bari et al. 2013). At the present state of the art, this technique is considered one of the best ways to pretreat the lignocellulosic biomass, the materials used for the fiber panels and the biocomposites. This is because the steam explosion can preserve the fiber structure and divide the lignocellulosic material into its main components (Anglès et al. 2001), with modifications of the polymeric structure of the lignin and consequently of the thermoplasticity. The thermoplasticity of the lignin is an important property in favoring the thermal adhesion of the fibers, from which the panel is formed by hot pressing (Ramos 2018). The performance improvement of a biomass panel following a steam explosion is mainly attributed to the high hydrophobicity of the exploded fibers. This hydrophobicity is due to the substantial reduction in the content of extractives and hemicellulose in the raw material, and to the fact that lignin is more available on the surface (Thakur et al., 2014), consequently improving the waterproofing of the panels (Ji et al., 2017). In fact, the non-polar hydrocarbon chains and the aromatic rings in the lignin molecule on the fiber surface are able to improve the water resistance of the fiber panels (Mancera et al. 2012, Rozman 2000). Thanks to the depolymerization process that allows the splitting of the different types of ethereal bonds that bind the phenylpropanoic units that characterize the lignin, the formation, within the same polymeric structure, of bonds of different nature is allowed, giving the lignin higher adhesive capacity. The depolymerized lignin fraction has a high content of phenolic hydroxyl groups (Mancera et al., 2008).

In addition to this, the steam explosion is responsible for a partial dehydration of the carbohydrates to furan compounds, which are responsible for the condensation reactions with the lignin polyphenols (Fig. 30) present in the biomass itself, favoring a natural adhesiveness of the products obtained (Sun et al., 2014).

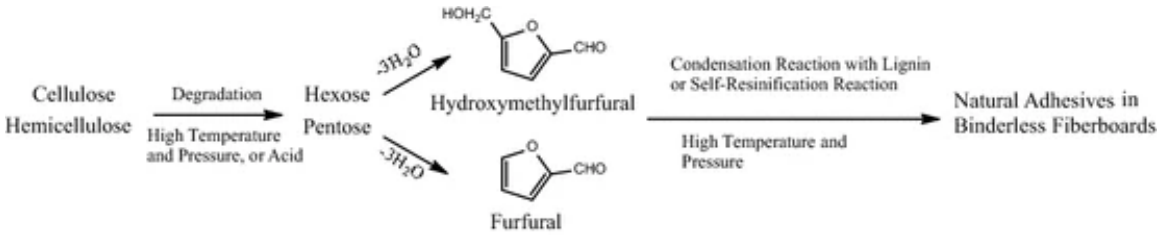


**Figure 30.** Potential self-bonding mechanism in the production of binderless fiberboards with steam pretreatment (Zhang et al., 2015).

The same effect was observed by adding kraft lignin as an adhesive not derived from the fossil industry in fiber panels obtained from other agricultural waste (Domínguez-Robles, 2018). The presence of phenolic units of the lignin present in the fibers or added externally contributes to the antioxidant capacity of the fibers themselves and to the formation of stable free radicals. These radicals increase the reactivity of the samples and lead to the formation of covalent bonds (radical-radical reactions). These covalent bonds facilitate the self-adhesion of the fibers and thus improve the mechanical properties of the agglomerated panels (Alvarez-Lopez et al., 2015) and the high temperature saturated steam used during the steam-explosion has a plasticising effect on the lignin itself. Cardoon biomass has a lower amount of lignin than other lignocellulosic materials which do not make this raw material an ideal material for panels without external binders. A study on the use of cardoon residues as a fiber for the production of panels was carried out by (Mancera, 2008) through activation with steam-explosion. In the latter cited work, batch tests were carried out without catalysts, varying the temperature from 190 to 230 ° C and the time from 1 to 9 min (table 3). The liquid obtained composed of hemicellulose and other extractives has been removed because it is not convenient to use it in the process of obtaining the panels. On the solid exploded view, the compositions (cellulose, hemicellulose, lignin) were compared with the properties of the fibers (hygroscopicity, breakage, elasticity, etc.). The results obtained from this study did not guarantee a high quality of the properties of the resulting panel, due to the intrinsic characteristics of the lignocellulosic residue of the Cardoon.

The production of the binderless fiberboards was tested by Halvarsson et al. 2009 from pretreated wheat straw by acid catalysis hot water while other authors tested steam exploded mischantus without or with the addition of lignin external during the panels production (Velasquez et al. 2003a, Velasquez et al. 2003b). A combined study of the use of a laccase-mediator system (LMS) with addition of lignin (LLMS) was carried out by Euring et al. (2016). Two systems were tested in the pilot-scale production of MDF from spruce. The determination of the physical technological properties revealed that MDF treated with LLMS has higher stability than MDF treated with only LMS indicating a high potential of the procedure.

The following Table 3 summarizes some recent literature data relating to the use of chemical-physical pre-treatments and / or enzymes for the activation of lignin aimed at creating panels.

**Table 3.** Literature studies on the production parameters of panels following chemical-physical and / or enzymatic pre-treatment**.**

| | Fiber activation | | | | Fiberboard production parameters | | | |
|---|---|---|---|---|---|---|---|---|
| **feedstock** | **Enzyme** | **enzymatic pre-treatment** | **chemical-physical pre-treatment** | **Fiber additives** | **T (°C)** | **Time (min)** | **Pressure (MPa)** | **Ref.** |
| Plantain resiudes (*Musa L.)* | EC 1.10.3.2 from *Aspergillus oryzae* | 5% s/L; 30°C; pH=5.5; 1 hour | / | / | 200 | 1-7 | 7-14 | Alvarez et al., 2011 |
| Wheat straw (*Triticum aestivium L.)* | / | / | Sulphuric acid - hot water | Activation with Fenton's reagent(with variable $H_20_2$) | 200 | 1.5 | 0.5 | Halvarsson et al., 2009 |
| *Mischantus sinensis* | / | / | Steam explosion | / | 130-230 | 1,6-18 | 12 | Velazquez et al., 2003a |
| *Mischantus sinensis* | / | / | Steam explosion | External lignin 13-47% | 120-170 | 3-8 | 12 | Velazquez et al., 2003b |
| Banana Bunch *(Musacea)* | / | / | Steam explosion | / | 133-217 | 5 (two steps) | 4-14 | Quintana et al., 2009 |
| Cardoon *(Cynara cardunculus)* | / | / | Steam explosion | / | 190-230 | 1-9 | 4-20 | Mancera et al., 2008 |
| Beech wood residues (*Fagus. L*) | *Myceliophtera thermophila* | pH=7 50 °C rotary system | / | Waxes | 180-200 | 2.5 – 3.2 | 0.85 | Felby et al., 2002 |
| Spruce 80% (*Pìcea Mill.*) and fir 20% (*Abies Mill.*) | Novozym 51003 | 100 U/ml per 1 g of dry fibers | / | Caffeic acid or vanillic acid | 190 | 2 | nd | Euring et al., 2016 |

## 4.3 Lignin extraction

To study lignins it is first of all necessary to purify them from their matrix. Lignin extraction techniques involve the use of different solvents, traditional (alkaline; oxidative alkaline, ionic liquids) and innovative (DES, renewable solvents).

Furthermore establishing and selecting a method for isolation and recovery of lignin from non wood biomass such as bagasse is not an easy task (Al Arni, 2018).

### 4.3.1 Alkaline methods

Alkali treatment of lignocellulosic substances such us wheat straw disrupts the cell wall by dissolving hemicellulose, lignin and silica, by hydrolysing uronic and acetic acid esters and by swelling cellulose, decreasing the crystallinity of cellulose. This increases the biodegradability of the cell walls due cleavage of the bonds between lignin and hemicelluloses or lignin and cellulose (Al Arni, 2018). Delignification is a consequence of the saponification of the ester bonds between lignin and hemicellulose in herbs generally present as lignin-diferulate-hemicellulose complexes. Partial deconstruction of lignin is mainly caused by the cleavage of β-ether bonds (Zhao et al., 2019). The alkaline extraction process involves an extraction of the lignocellulosic biomass with highly concentrated Soda solutions (over 10%) at temperatures around 150 °C, generally obtaining low molecular weight lignins (Mw between 1000-6000 g $mol^{-1}$). It is preferable to use this method with non-woody biomass, including sugarcane bagasse, wheat straw and cardoon; some soda is generally applied to non-woody biomass (e.g. straw, sugar cane, bagasse). Other bases include $Ca(OH)_2$ and $Ba(OH)_2$. The lignin recovered following acid precipitation does not contain additives such as sulfur making it more suitable in applications where the final products are bioplastic or composites, but also for obtaining aromatic molecules following catalytic deconstruction.

### 4.3.2 Ionic liquids

Ionic liquids (ILs), organic salts that melt below 100 °C, are composed of cation/anion pairs . ILs are so-called designed solvents because their properties are tunable depending on the selection of anion and cation (Kim et al., 2011) .

Ionic liquids have received growing attention as green solvents for lignocellulose pretreatment ever since some of them were discovered to be able to dissolve cellulose and even intact wood. This approach has several advantages over conventional methods such as dilute acid pretreatment (Fu & Mazza, 2011).

It is more environmentally benign, has a significantly shorter processing time required to convert pretreated biomass to fermentable sugars, and there is less degradation of monosaccharides and consequently minimum formation of inhibitors that negatively affect downstream fermentation.

However it has some disadvantages: a large amount of expensive ionic liquids is required and recycling of pure ionic liquids, usually by evaporation or reverse osmosis, than is energy-intensive; finally, the solution becomes extremely viscous during pretreatment making it difficult to handle.

The goal of pretreatment is to enhance the digestibility of polysaccharides in lignocellulosic biomass without their substantive destruction and to maximize the release of fermentable sugars (Fu & Mazza, 2011).

Ionic liquids have been shown to be excellent pretreatment solvents irrespective of the type of biomass feedstock. Additionally, lignin can be entracte from lignocellulose via ionic liquid pretreatment without high energy consumption. To date, many ionic liquids have been synthesized and effectively used as cellulose solvents for pretreatment, although their toxicity as biorefinery solvents have yet to be assessed (Sathitsuksanoh et al., 2014).

Nowadays more selective, efficient, environmentally friendly, cost-effective and scalable processes to extract lignin from the lignocellulosic biomass are being actively investigated. One of the lignin isolation methods is organosolv pre-treatment.

In organosolv process, a mixture of an organic solvent and water is used for delignification. Lignin produced by this method has better quality and the extracted lignin is carbohydrates and sulphur free, therefore, it is suitable for industrial applications (Ramezani & Sain, 2018).

Organosolv has proved to be the most suitable among the others to fulfill these demands. This treatment is based on the dissolution of lignin with organic solvents such us formic acid, methanol or ethanol in the presence of a small amount of mineral acids, such as hydrochloric acid. Despite organosolv process provides a lignin structurally similar to the native one, it shows some major drawbacks such as high cost, poor recyclability, and in some cases health and environmental hazards.

### 4.3.3 Innovative green methods

A new generation of alternative solvents suitable as a substitute of conventional ionic liquids was presented at the beginning of this century. They are known as deep eutectic solvents (DES) and they are mixtures of substances, which have been arising from an interaction of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) in a specific molar ratio. DESs have great potential for use in pulp and paper and recycling industry. Researchers have shown that DES may be used for the dissolution and hydrolysis of certain components of lignocellulosic biomass (lignin) under mild conditions which prevent further degradation (Majová et al., 2017); DESs has immerged as a promising ionic liquid alternative for biomass fractionation since it not only overcomes the disadvantage of high viscosity of ionic liquid, but also is derived from renewable resources with low price and simple synthesis process.

The introduction of deep eutectic solvent in 2004 received a considerable amount of attention across different research fields, particularly in biomass processing (Loow et al., 2017). Deep eutectic solvents are relatively new topic in science. In the field of green chemistry is their study the great challenge. DESs are referred to as green solvents, mixed of two or more compounds;

they have the following properties: non-flammability, electrochemical and thermal stability, high conductively, biodegradability, recyclability explosion protection, non-toxicity, and negligible vapour pressure. They consist of mixtures of materials without chemical bonds. One agent is most often an organic or inorganic acid component (salt) and the second compound is a mono- or disaccharide alchol, a mono acid, di- or trialkanol or choline derivative (Škulcová et al., 2016).

Deep eutectic solvents are mixtures of two or more compounds with a freezing point well below the melting point for any of the original mixture components. For extraction, deep eutectic solvents that are liquid at room temperature are the most interesting. A large number of compounds have been used to prepare deep eutectic solvents (Fig. 31).

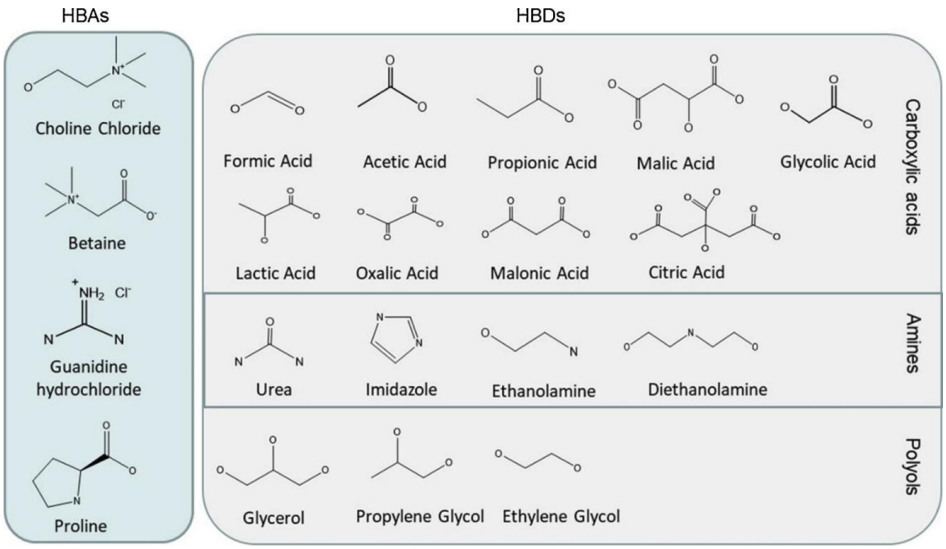


**Figure 31.** Deep eutectic solvents.

DES systems possess several attractive properties, such as low volatility, low toxicity, high thermal stability and are less expensive with respect to ionics liquid. On the other hand, DES need to be mixed at suitable molar ratios and could have low extraction yields. In the future extraction yields to improve.

The results of recent studies have shown that choline chloride and lactic acid based DES is also effective in extracting/removing lignin from woody and herbaceous plants biomass and the lignin obtained from the DES extraction has unique structural properties such as the absence of ether linkage and low molecular weight distribution (Lou et al., 2019).

Renewable solvents have been gaining a lot of interest in science, too. For example GVL is a promising renewable platform molecule that can be produced from lignocellulosic biomass by levulinic acid hydrogenation (Fig. 32).

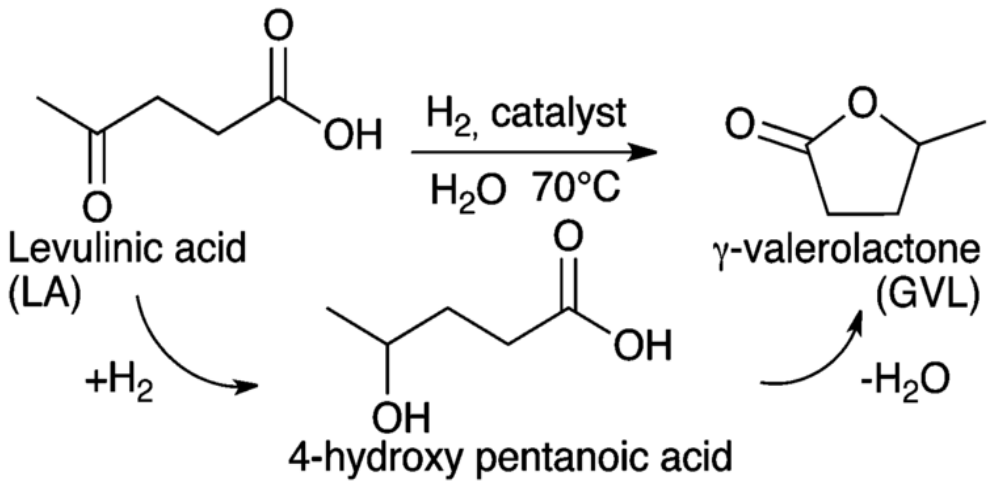


**Figure 32.** Gamma valerolactone (GVL) structure and it's renewable production from biobased molecules

GVL has attracted interest as a renewable platform molecule derived from lignocelluloses structural carbohydrates that can be used and recycled for the production of carbon-based chemicals,liquid fuels, additives or polymers. It is a solvent for green chemistry and it has low melting point (31°C), high boiling point (207°C) and no evidence toxicity. Furthermore it has high solubility in water to assist biodegradation (Fang and Sixta, 2015), GVL has been employed as a solvent in the processing of lignocellulosic biomass. Recently, microwave heating has been applied to the organosolv fractionation of lignocellulosic materials by adopting GVL as solvent (Angelini et al., 2016).

GVL can also be used in combination with $H_2O$ in different concentrations; $H_2O$ promoted the cleavage glycosidic bonds in hemicellulose, while the oxygen of GVL might interact with hydroxyl groups of xylose. GVL with $H_2O$ promoted the depolymerization of lignin to oligomers the effects of γ-valerolactone (GVL) and $H_2O$ on enhancing pubescens degradation via the cleavage of inter- and intramolecular linkages were studied. At 160 °C, $H_2O$ selectively promoted the cleavage of the intermolecular linkages by forming hydrogen bonds, making mainly contributions to hemicellulose dissolution, while GVL and $H_2O$ promoted lignin dissolution by forming hydrogen bonds with $-OCH_3$ group of lignin ( Luo et al., 2018). GVL is considered a green solvent as it regenerates from levulinic acid through hydrogenation, it is an exceptional solvent in the conversion of biomass and in traditional organic synthesis; it is also currently gaining momentum from a green chemistry point of view. It turns out to be an excellent means for the fractionation of lignocellulosic biomass because it is able to dissolve the lignin and facilitate the destructuring of cellulose.

## 4.4. Lignin characterization

The structural characterization of lignin can be divided into three strongly interconnected groups: the determination of functional groups, the determination of intermonomeric bonds and the determination of the molecular weight. These parameters are fundamental to establish the state of purity, the nature and the structural modifications induced in the polymer as well as being necessary to plan suitable structural modifications for the research and development of new materials.

These lignins obtained from lignocellulosic biomass were characterized with some of the techniques: GPC, TGA, $^{1}$H-NMR, $^{13}$C-NMR, $^{31}$P-NMR and FT-IR .

### 4.4.1 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) is a type of high-performance liquid chromatography (LC) used to understand and characterize the whole molecular weight distribution of lignins through the separation of their major oligomers on the basis of their size. The molecular weight of lignin is a fundamental property that influences the recalcitrance of biomass and the valorization of lignin (Lange et al., 2016b). The determination of the molecular weight of lignin in native biomass is dependent on the bioresources used and the isolation and purification procedures employed. Common characterization techniques for determining the molecular weight of lignin will be addressed, with an emphasis on gel permeation chromatography (GPC).

The number average molecular weight (Mn), weight average molecular weight (Mw) and polydispersity index (Đ) all vary in magnitude depending on the biomass source, pre-treatment conditions, and isolation method. The non-uniformity of the chain lengths of lignin precludes the characterization of a specific molecular weight.

Thus, it is necessary to characterize lignin in terms of average molecular weight. Two common averages used are number average molecular weight ($M_n$) and weight average molecular weight ($M_w$). The polydispersity index (Đ) represents the molecular weight distribution of the polymers. Here the index number, i represents the number of different molecular weights present in the lignin sample and N*i* is the total number of moles with the molar mass of $M_i$;

$$M_n = \sum M_i N_i / \sum N_i$$

$$M_w = \sum N_i M_i^2 / \sum N_i M_i$$

$$Đ = M_w / M_n$$

The narrower the distribution interval, the smaller the polydispersivity index. The molecular weight distribution can be monomodal (one peak only) or bimodal (two distinct peaks). For homogeneous polymers, the polydispersivity index is equal to one. Values between 1.05 and 1.2 indicate a very narrow molecular weight distribution. The principle on which this technique is based consists in the fact that the polymeric material to be fractionated is solubilized in an appropriate solvent. The single molecules take the form of a statistical ball (random coil) having its own characteristic hydrodynamic volume (depending on the extent to which intramolecular interactions are favored over those with solvent molecules). Larger macromolecules pass through the packed column faster because they cannot penetrate any of the pores on the gel particles. While the small particles diffuse through all the pores present in the gel particles, and take longer to pass through the column, so they are eluted last. Intermediate-sized macromolecules can only penetrate some pores; in this case there is a differential elution. Prior to analysis, derivatization is typically accomplished through methylation, acetylation, or silylation to enhance lignin's solubility in an organic solvent. During the acetylation process, all the hydroxyl functional groups are substituted by new acetyl groups (Tolbert et al., 2014).

GPC analysis are usually based on calibration using dissolved monodisperse polymer standards. The lignin sample mass distribution is then calculated from a calibration based on standards. However, as far as lignin is concerned, no specific standards are commercially available and other polymers are generally used as standard, making quantification inaccurate. Polystyrene is broadly used as GPC standard although other polymers like sodium polystyrene sulphonates or poly(methyl methacrylate) have also been reported (Liao et al., 2020).

The GPC data are highly dependent on the eluent, column type and experimental device used and only the relative molecular weight can be obtained. To overcome these drawbacks, a so-called "universal calibration" has been proposed. This calibration is not based on the molar mass of a polymer but on its the hydrodynamic volume, determined by viscometry. Usually, GPC of a lignin is performed by using organic solvents, usually THF, after derivatization through acetylation. The average molecular weights of lignins are highly variable and strongly influenced by the biomass type, by the method of isolation, by the sample preparative analysis and by the instrument setting used for analysis. The following Table 4 shows the ranges of average molecular weights and the polydispersivity index of some types of lignin.

**Table 4.** The average molecular weights (Mw) and dispersity indices (Đ, Mw/Mn) of different technical lignins (Mastrolitti et al., 2021)

| Lignin type | $M_w$ (g $mol^{-1}$) | Đ |
|---|---|---|
| Kraft lignin | 1000-5000 | 2.0 – 8.0 |
| Enzymatic or acid hydrolysis lignin | 2000-4500 | 1.5 – 3.2 |
| Alkali lignin | 2000-10000 | 2.0 – 5.2 |
| Organosolv lignin | 1100-5700 | 1.5 – 4.4 |
| Lignosulfonate | 1000-50000 | 2.5 -7.0 |
| Steam explosion lignin | 3500-15000 | 1.4 -7.0 |

In general there is a high variability of results, the organosolv, kraft and enzymatic processes tend to generate on average molecules with a lower average molecular weight than what has sometimes been shown with alkaline, lignosulfonate or steam explosion processes. The GPC analysis can be useful to compare different samples and to investigate the impact of delignification conditions on the chemical structure of the lignin isolated.

(Brosse et al., 2009) compared $M_w$, $M_n$ and PDI of miscanthus organosolv lignin and miscanthus Milled Wood lignin. Both lignin samples were analysed by GPC after acetylation in THF as eluent and using standard polystyrene samples. It was concluded from the elution curves that organosolv treatment degraded the macromolecular structure of lignin to a noticeable extent. Using the same GPC conditions, (El Hage et al., 2010) examined the impact of the conditions severity of the organosolv delignification on the elution curves of the resulting lignin in order to better understand the lignin break down mechanism. To overcome some variables such as derivatization, the use of neutral solvents for example as DMSO containing LiBr is gaining popularity. Addition of LiBr up to 0.5% w / v helped prevent sample aggregation through intermolecular associations and interactions with column packing (Sulaeva et al., 2017).

### 4.4.2 Thermo-Gravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the weight change that occurs as a sample is heated at a constant rate.

TGA were carried out in nitrogen and in air. In both cases the heating rate was 10 °C/min.

Thermogravimetry is a widely used thermal analysis technique in the case of polymeric materials. It is known how the thermal degradation mechanism of polymers can be influenced from the experimental conditions under which the heating is performed. Therefore, the reproducibility of the thermogravimetry of polymers requires checking as detailed as possible the operating conditions of the experiment, such as size and shape of the sample, rate of heating, type of atmosphere in which the sample is heated. The conditions of use of thermogravimetry depend on the information to be obtained.

However the first experiment that is usually performed to rapidly characterize the decomposition of a material with formation of volatile products, consists in a heating in inert gas stream with temperature rise of 10 °C/min. The results of the experiment are normally regulated and represented in terms of residual weight percentages of the sample, in operation of heating temperature.

One of the most frequent uses of TGA concerns the evaluation of the thermal stability of polymers, and therefore also of lignin, in relation the possibility of using a temperature above the room temperature, the maximum temperature to which a polymer can be heated before it undergoes physical modifications irreversible, with corresponding alteration of its properties. However, the identification of the reactions that often occur due to the effect of heating, cannot be carried out alone on the basis of the weight variations that they determine (complementary use of thermoanalytical techniques, DSC, etc.).

To describe the thermal properties of each lignin fraction, thermogravimetric analysis (TGA) and differential scanning calorimetric (DSC) were used to analyze the decomposition temperature (Td) and glass transition temperature (Tg), respectively. Information on the thermal properties of the lignin samples is particularly pertinent when lignin is considered for incorporation into moldable plastics.

The degradation of lignin takes place in a wide temperature range of 100°C-900 °C but, the major degradation takes place between 200°C and 700 °C (Sahoo et al., 2011b).

Basically, the TGA curves of lignins show two steps of degradation. At the first degradation step, it corresponds to the weight loss of water, carbon monoxide, carbon dioxide, and other pyrolysis products due to the breaking of the side chains around 30-120 °C. The second step of degradation is indicated to the weight loss of hemicellulose components that attached in the lignin structure at the range 80-350 °C as it is a byproduct during precipitation of lignin. Due to its complex structure, the

decomposition of lignin strongly depends on many variables intrinsic to the nature of the lignin itself, to the pretreatment method that generated it and to the analysis parameters, reaction temperature, heating rate and process atmosphere. TGA analyzes of lignin from different sources and / or from different pretreatment methods show that degradation processes and structure breakdown depend on the specific lignin treated (Tab. 5).

**Table 5.** Glass transition temperatures of different technical lignins determined

| Type of lignin | Glass transition temperature(°C) |
|---|---|
| Hardwood organosolv lignin | 95 |
| Alcell® (organosolv) lignin | 97 |
| Hardwood sodium-lignosulfonate | 127 |
| Softwood sodium-lignosulfonate | 138 |
| Wheat straw soda lignin | 150 |
| Rice straw soda lignin | 155 |
| Kraft lignin | 165 |

### 4.4.3 Nuclear Magnetic Resonance (NMR)

NMR has become an indispensable non-degradative technique for the structural elucidation of the carbon skeleton of lignin.

Lignin is one of the major polymers occurring in the plant kingdom. Despite extensive investigation, the complex and irregular structure of lignin is not completely understood. Analysis of lignin with different wet chemistry techniques and model studies on dehydrogenative polymerization of coniferyl alcohol has resulted in different models of lignin structure.

The advantage of spectroscopic methods over degradation techniques is the analysis of the whole lignin structure and direct detection of lignin moieties. The advantage of nuclear magnetic resonance (NMR) spectroscopy over other spectroscopic techniques, such as infrared (IR), ultraviolet-visible (UV), and Raman spectroscopy, is that NMR has a much higher resolution, enabling a larger amount of information to be obtained.

The development of quantitative $^{13}C$ NMR in lignin analysis was an important milestone in lignin chemistry. It is a spectroscopic technique well adapted to structural studies of lignins, despite the latter's very heterogeneous and complex chemical structures. It gives at the same time general and detailed information qualitative and quantitative, which are either new data that could not be obtained by another technique or which represent a valuable complement to data coming from another kind of

spectroscopic or chemical analysis. The NMR data, chemical shifts, coupling constants and relaxation times reflect with high accuracy the chemical structure, functionality and chemical environment of the carbon atoms.

Nevertheless $^{13}C$ NMR is used only for the estimation of some specific moieties; more recently, a quantitative two-dimensional (2D) NMR approach has been suggested. The application of heterocorrelated techniques allows to evaluate $^{1}H$-$^{13}C$ connectivity. Once the proton frequencies have been assigned, the use of HSQC or HMQC sequences allows the assignment of the carbons directly related to the individual protons.

However, due to the higher molecular weight, higher viscosity and lower solubility of polymers as a consequence of the short relaxation times and broadening of the signals, solid state NMR cannot currently provide sufficiently detailed information on the structure of a lignin. Using particular precautions, such as appropriate lignin isolation methodology, appropriate instrument sensitivity and appropriate acquisition parameters, NMR analysis of lignin in the solution state can provide interesting information (Mastrolitti et al., 2021). Quantitative $^{13}C$ NMR is still the most used NMR method for lignin characterization, being rather informative, reliable, and at the same time relatively feasible (Capanema, 2004).

$^{13}C$ NMR spectroscopy is an essential tool for the determination of lignin structures. 2D-HSQC NMR spectroscopy has been able to provide important structural information about lignin, such as the substructures (*β*-O-4, *β*-*β*, and *β*-5, etc.) and S/G ratios.

Recently a new analysis technique has been developed with $^{31}P$ particularly useful in the structural elucidation of lignins. This technique consists in the derivatization of all labile protons present in the lignins (alcoholic, phenolic and carboxylic protons) with a chlorodioxaphospholane to give the corresponding phosphorylated derivatives (Granata & Argyropoulos, 1995). The chemical shift of the phosphorus atoms thus introduced onto the lignin is dependent on the chemical environment and allows the different classes of functional groups to be distinguished. Furthermore, the sensitivity of the method is high thanks to the natural abundance of $^{31}P$ of 100%. $^{31}P$ NMR powerful tool for quantitative functional group determination, ratio of aromatic units and 2D HSQC NMR is nowadays established method to determine structural linkages, ratio of aromatic units. Solution $^{31}P$ NMR can be used to examine soluble lignin and carbohydrate samples after phosphitylation with 1,3,2 dioxaphospholanyl chloride. In addition, primary hydroxyls, carboxylic acids, and the two diastereomeric forms of arylglycerol-beta-aryl ether units (β-O-4 structures) present in lignin model compounds can also be determined from a single $^{31}P$ NMR experiment. When applied to carbohydrates, the technique gave characteristic signals for the alpha and beta anomers and the epimeric forms of monosaccharides (Argyropoulos, 1995).

Similarly, a technique was developed that allows the NMR analysis of carbonyl groups present on the lignin through the trifluoromethylation of the aldehyde, ketone and quinone groups followed by the $^{19}F$ NMR analysis of the sample (Ahvazi and Crestini, 1999).

### 4.4.4 Infrared spectroscopy (IR)

The detection and quantification of the functional groups of lignins is one of the primary objectives in the analysis of lignins.

Chemical structure analysis is also being studied through Fourier transform infrared spectroscopy (FT-IR). The absorption bands in the spectrum were associated with the characteristic functional groups of lignin, using the range between 4000-400 $cm^{-1}$ where there are transitions changes of vibrational energy states and rotational states in molecular structures.

Fourier transform infrared spectroscopy (FTIR) is a reliable technique used in order to determine the changes occurred in the functional groups of the lignin compounds. It has become a powerful instrument in order to determine the structure as well as provides a new interpretive and experimental framework for the study of complicated systems of natural polymers (Liao et al., 2020).

By studying the absorption peaks corresponding to specific frequencies, it is possible to obtain information regarding the functional groups present in the structure of the lignin; In the table 6 FTIR assignments of the hydroxyl, carbonyl, methoxyl, carboxyl, aromatic and aliphatic C- H groups present in this polymer are reported (Mastrolitti et al., 2021). The comparison of the S and G structural units of lignin using the FT-IR spectra is one indication of the key differences among the lignin sources. Understanding the differences, as well as similarities, resulting from the chemical structures and physical properties of lignins is critical to determining how these lignins can be fully implemented as new products.

**Table 6.** Assignments of Lignin IR Bands in FTIR Spectra of softwood and hardwood Milled and Wood Lignins (Williams et al., 2011; Mastrolitti et al. 2021)

| Softwood[a] ($cm^{-1}$) | | Hardwood[b] ($cm^{-1}$) | | Assignment |
|---|---|---|---|---|
| 3430 | vs | 3440 | Vs | O–H stretch, H-bonded |
| 2938 | m | 2942 | M | C–H stretch methyl and methylene groups |
| 2885 | sh | 2882 | Sh | C–H stretch in methyl and methylene groups |
| 2849 | sh | 2848 | Sh | C–H stretch O–CH3 group |
| 1717 | sh | 1737 | Vs | C]O stretch, unconjugated ketone, carboxyl, and ester groups |
| 1667 | sh | 1670 | Sh | Ring-conjugated C]O stretch of coniferaldehyde/sinapaldehyde |
| 1645 | sh | 1643 | Sh | Ring-conjugated C]C stretch of coniferyl/sinapyl alcohol |
| 1600 | s | 1596 | S | Aryl ring stretching, symmetric |
| 1513 | vs | 1506 | Vs | Aryl ring stretch, asymmetric |
| 1466 | s | 1464 | S | C–H deformation, asymmetric |
| 1458 | sh | 1425 | M | O–$CH_3$ C–H deformation, asymmetric |
| 1428 | m | 1379 | M | Aromatic skeletal vibration combined with C–H in plane deformation |
| 1375 | w | 1367 | Sh | O–$CH_3$ C–H deformation symmetric |
| 1331 | sh | 1330 | M | Aryl ring breathing with C–O stretch |
| 1270 | vs | 1252 | Vs | Aryl ring breathing with C]O stretch |
| 1226 | m | | | C–C, C–O, and C]O stretches |
| 1142 | s | 1159 | Sh | Aromatic C–H in plane deformation |
| | | 1127 | Vs | Aromatic C–H in plane deformation |
| 1085 | w | 1082 | Sh | C–O deformation, secondary alcohol, and aliphatic ether |
| 1035 | s | 1050 | Vs | Aromatic C–H in plane deformation |
| 914 | vw | 905 | W | C–H deformation of out of plane, aromatic ring |
| 878 | sh | / | / | C–H deformation of out of plane, aromatic ring |
| 863 | w | / | / | C–H deformation of out of plane, aromatic ring |
| 823 | w | / | / | C–H deformation of out of plane, aromatic ring |
| 748 | vw | / | / | CCH wag |
| 742 | vw | / | / | Skeletal deformation of aromatic rings, substituent side groups, side chains |

*[a]Black spruce milled wood lignin, guaiacyl lignin. bAspen milled wood lignin, guaiacyl and syringyl lignin.*

*vs very strong; s strong; m medium; w weak; vw very weak; sh shoulder; relative to other peaks in the spectrum.*

## 5. Aim of project

The goal of the project is to characterize and enhance one of the most important fractions of waste or residual plant biomass: lignin. This three-dimensional biopolymer which acts as a protector for plants and which shows great resistance to normal environmental aggressions must first of all be isolated from the various lignocellulosic biomasses; then the characteristics must be studied in depth and on the basis of this, the best transformation and enhancement strategies must be developed, although this fraction of plant biomass is the most difficult to transform due to its refractory to chemical-physical and biological environmental aggressions.

The research project concerns the structural analysis of lignins and their conversion into "BIO" products and raw materials for the green chemistry industry and it is oriented towards the use of lignocellulosic raw materials that are both waste and not in competition with the food chain to produce products to be re-inserted into the production cycle in partial replacement of the current products of fossil origin. All this is closely related to the Industry 4.0 model, or the development of a circular economy which provides for a constructive and balanced relationship between industry, of course, and all the other components of the economy.
Furthermore, the aim of the project is to develop processes for the synthesis of functional polymers starting from lignin and chemical modifications and synthesis for the production of bio-chemicals.

## 6. Materials and methods

Except where noted, all reagents and solvents listed in this section were purchased from Sigma-Aldrich (USA).

### 6.1 Feedstock and pretreatment

Wheat straw and cardoon pre-treatments were performed in Trisaia in batch reactor (Fig. 33A) able to treat 1 Kg of biomass for each processing cycle with saturated steam, varying the parameters of severity, namely temperature, reaction time and concentration of acid catalyst. The steam explosion parameters are indicated in the respective sections dedicated to the feedstock used.


**Figure 33.** Steam explosion technology in ENEA (Trisaia): batch plant (A) and continuos plant (B)

### 6.2 Chemical characterization of biomass

The composition of raw material was determined according the NREL method (Sluiter et al. 2008). The sample used for the determinations was ground to 50 mesh (8000 rpm 0.5 mm filter) and dried at 50 °C overnight. The primary hydrolysis of the carbohydrates present in the sample was performed with 72% w / w sulfuric acid. About 0.3 grams of sample were weighed in a Becker and added with 3 mL of sulfuric acid, then subjected to stirring for 1 h in an incubator at 30 °C. The acid hydrolysis was completed by diluting the sample with water to bring the sulfuric acid concentration to 3% w / w and boiling everything for another hour in an autoclave at T = 121 °C. The resulting suspension contains a insoluble part which is recovered by filtration and weighed: it constitutes the insoluble lignin. The

solution was instead brought to 1 L, and the determinations of soluble lignin were carried out on it, reading the absorbance at 205 nm by UV absorption spectrophotometry. The lignin content in solution is calculated using the formula:
lignin (g/l) = A/B x C
where: A = absorbance at 205 nm of the solution B = extinction coefficient 110 L / (g x cm) C = dilution factor.

The sugars present in the solution were determined by HIPC ion chromatography. The analysis was conducted using DIONEX chromatographic instrumentation, model DX 500. A concentration gradient NaOH solution (2-200 mM) was used as the eluent. As detector, electrochemical type with pulsed amperometry was used. The range of validity of the determinations is quite wide, but normally one operates in a concentration range of 0-150 ppm, in which the linearity of the detector response has been ascertained. The chromatographic column used consisted of a non-porous stationary phase in polystyrene-divinylbenzene (Carbopack PA1), activated with sulphonic groups for anion exchange. The ashes were determined following slow combustion of the biomass at 600 °C, in the muffle (ramp of 100 °C / h up to 600 °C, then 8 hours at 600 °C). The percentage of ash is indicative of the content of inorganic material present in a sample.
The organic compound content were determined using an ASE extractor (accelerated solvent extractor) through 3-7-minute cycles using ethanol as a solvent, capable of extracting oils, resins, waxes, fats and some soluble rubbers. The extracted material was recovered by removing the solvent with a rotavapor, then dried in an oven at 50 °C overnight and weighted .

### 6.2.1 Wheat straw

The raw feedstocks consisted of wheat straw deriving from agricultural waste from Basilicata, with a dry matter content of 91.9 ± 0.2%. The residues were divided into stocks of around 10 kg and stored indoors. After sampling, the wheat straw was ground to 50 mesh, air dried and analyzed for carbohydrate, extractives, lignin and ash content (Sluiter, et al., 2008; Sluiter et al., 2004). The steam explosion batch technology (Staketech, 10 L reactor) was used for the biomass pretreatment according to set-up conditions previously optimized for similar lignocellulosic feedstocks. In particular, prior to the pretreatment, biomass was crumbled to particles size in the range 1.7-5.6 mm and was soaked in a dilute $H_2SO_4$ solution (0.05 M) for 10 min (De Bari et al. 2013). The resulting acid load was 1.4% (w/w).

The steam explosion pretreatment was carried out at 203°C for 5 minutes based on temperature and treatment duration optimized by Ballesteros et al. 2006, for the ethanol production from wheat straw hydrolysates.

To separate the solids from the soluble fraction, containing mainly hemicellulose, the pretreated product was filtered.

The composition of the pretreated product, consisting of a solid fraction rich in cellulose and a liquid fraction containing soluble hemicellulose, was quantified according to standard procedure (Sluiter et al., 2008). The biomass after pretreatment had a high moisture content, due to steam condensation. It was then pressed to separate the solids and then remixed to obtain the target solids concentration for enzymatic hydrolysis.

### 6.2.2 Cardoon

Prior the pretreatment the cardoon biomass had a dry matter content of 91% and was analysed for carbohydrates and lignin content. The composition of the raw material is indicated in the table 7. After steam explosion process conducted at 180°C for 10 minutes, two fraction were obtained: a liquid fraction of hemicellulose which contained 2.4 g $L^{-1}$ glucose, 7.1 g $L^{-1}$ xylose, 0.5 arabinan g $L^{-1}$, 1.7 galattan g $L^{-1}$, 0.9 g $L^{-1}$ acid acetic and a solid fraction consisted mainly cellulose and lignin. The solid fraction was washed with water at 60 °C to eliminate the residual hemicellulose impregrated and analysed in its composition (Tab. 7).

**Table 7.** Composition of dry cardoon solids before and after the steam explosion process

| **Composition (w/w%)** | **Raw material** | **Washed solid pretreated fraction** |
|---|---|---|
| glucan | 35.0 ± 1.5 | 43.9 ± 1.6 |
| xylan | 14.0 ± 0.7 | 9.8 ± 0.2 |
| arabinan | 2.2 ± 0.1 | 0.8 ± 0.1 |
| galattan | 1.6 ± 0.1 | 1.1 ± 0.1 |
| acid insoluble lignin | 19.8 ± 1.3 | 23.8 ± 0.9 |
| Acid soluble lignin | 9.6 ± 1.1 | / |
| Ashes | 8.1 ± 0.9 | / |
| Extractives | 4.1 ± 0.5 | / |

Due to the partial solubilization of pentose sugars such as xylose in the hemicellulose, the pretreated material in steam explosion was enriched in cellulose and lignin. This material was used for the subsequent lignin isolation processes and subsequent enzymatic treatment with laccase.

## 6.3 Enzymatic hydrolysis

The wheat straw pretreated product was filter pressed in order to separate the solids from the soluble fraction mainly containing hemicellulose. The two fractions were then suitably mixed at the biomass consistency of 20 % for the enzymatic hydrolysis process.

The enzymatic hydrolysis of the pretreated wheat straw was carried out in a 2 L stirred bioreactor (Braun Biotech International, Germany) equipped with a helical impeller. The biomass consistency (insoluble fraction to liquid fraction ratio) used to produce the enzymatic hydrolysate was 20%. Commercial cellulases, Cellic™ CTec2, kindly provided by Novozymes A/S (Denmark), were added in dosage of 17.6 FPU per gram of insoluble glucan. The activity of the enzyme was determined by the Ghose method (Ghose, 1987) and found to be 190FPU per mL. The biomass slurry was hydrolysed

at pH =5, T=50 °C under stirring at 180 rpm for 72 hours. After each sampling, the temperature was then raised at 100°C for few minutes to denature the enzymatic proteins. The exhaust solids were then removed by filtration and sterilized through membrane filtration (0.22 µm). The liquid fraction was analysed in chromatography system for the sugar determinations. A cellulose conversion of 70% was obtained. The lignin residue was characterized in its chemical composition (including residual carbohydrates) according to the method developed by NREL (Sluiter et al., 2008).

### 6.3.1 Wheat straw alkaline treatment

Lignins were isolated from raw wheat straw before and after SE treatment, using NaOH as a solvent and $H_2SO_4$ in order to re-precipitate the biomass fractions.. In particular, 0.5 mm grounded biomass (50 g) were suspended into 1.5% sodium hydroxide solution (1:10 (w/v) solid to liquor ratio) at reflux temperature 90 °C for 30 min. After the alkali treatment process, the reaction mixture was filtered and centrifuged at 8000 rpm for 10 min to separate residual pulp biomass. The filtered liquid was adjusted to desired pH by adding $H_2SO_4$ (1 N) drop-wise to precipitate lignin. The obtained lignin samples were washed with deionized water, then dried in a hot air oven at 50 °C for 3 hours and stored for further process.

The percentage yield of lignin was calculated as follows:

$$\% \text{ lignin} = A/C \times 100$$

A = dry weight of lignin (g)

C= dry weight of the extracted sample (g).

### 6.3.2 Pretreatment of wheat straw with DES

All chemicals and materials were purchased from Sigma Aldrich. Three different DESs were tested: choline chloride with urea, lactic acid and oxalic acid. The solutions were stirred in an ultrasonic bath to form a homogeneous liquid. Straw residue (3.0 g. absolute dry weight) was added into each DES at a ratio of 1:20 (w/w). DESs, choline chloride and urea (1:2), choline chloride and lactic acid (1:2), choline chloride and oxalic acid (1:1), were used, and delignification was carried out for 8 hours in a drying oven with a preset temperature of 100 °C.

The extracted fraction was washed with an anti-solvent (ethanol) and after washing, the precipitated samples were filtered (whatman GFA) dried at 50 °C overnight. The set-up of DESs preparation are listed in Table 8 below:

**Table 8**. Set-up of DESs preparation

| DES (Molar ratio) | Biomass(g) | DES(g) | T (°C) | Time (h) | Stirring (rpm) |
|---|---|---|---|---|---|
| ChCl/OA (1:1) | 3 | 60 | 100 | 8 | 750 |
| ChCl/LA (1:2) | 3 | 60 | 100 | 8 | 750 |
| ChCl/Urea(1:2) | 3 | 60 | 100 | 8 | 750 |

### 6.3.3 Pretreatment of wheat straw with GVL

The experimental work was focused on the development of an experimental design aimed at optimizing the parameters related to the extraction of lignin from the wheat straw substrate. In particular, parameters tested were: GVL concentration, temperature and process time. The tests were carried out in an ultrasonic bath (J.P. Selecta ultrasons-H 40kHz - Spain) at different temperatures and ultrasound times. In particular, this ultrasonic bath allows you to control the temperature by means of an internal thermostat. In addition, the temperature was checked with an external mercury thermometer and the maximum variation was never more than 2°C with respect to the set one. After the extraction, the insoluble solid was separated by a filtering crucible from the GVL solutions containing dissolved lignin and other compounds. The solid fraction was washed, dried and weighed. The lignin in the liquid fraction was isolated from the GVL solution by precipitation with water. In particular, 40 ml of the GVL solution were treated with 400 ml of water (1:10 v/v). The sludge was magnetically stirred for 30 min and left to settle overnight. The precipitate was recovered by centrifugation, washed with water and dried in oven at 40°C overnight (Angelini et al., 2017).

Using the Design Expert10® software, a Box-Behnken factorial design was created, with three factors and three levels, including three replicas of the "center point" in order to create a second order response surface. The three independent variables ($X_1$, $X_2$ and $X_3$) and the respective three different levels (-1, 0, +1) are indicated in the Table 9:

**Tab 9.** Factors and levels used in the Box-Behnken factorial design (Design Expert10®)

| Factor | Level | | |
|---|---|---|---|
| | Inferior (-1) | Intermediate (0) | Superior (+1) |
| **GVL (%)** | 20 | 50 | 80 |
| **Temperature (°C)** | 30 | 45 | 60 |
| **Process time (hours)** | 3 | 6 | 9 |

The response variables were precipitation yields % and lignin removal % and were fitted with a second order model in order to relate it to the independent variables.
The general equation of the second degree polynomial equation is:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad \text{(Eq. 1)}$$

where Y is the expected response (precipitation %; lignin removal %), $\beta_0$ the intercept, $\beta_i$ the linear coefficient, $\beta_{ij}$ the quadratic coefficient, $\beta_{ii}$ is the linear interaction between the regression coefficients of $x_i$ and $x_j$ while $x_i$ and $x_j$ are the input variables that affect the response variable Y.

The accuracy and general capacity of the polynomial model described above (Eq. 1) was evaluated by means of the determination coefficient $R^2$, and the response surface regression procedure of the statistical analysis system was used to computationally estimate an optimized value of the response variable and the values of the independent variables associated with it. The optimized combination of parameters used will be described in the results and discussion paragraph.

## 6.4 Enzyme treatments of isolated lignin from cardoon

The lignin deriving from the alkaline extraction of cardoon biomass (pretreated at 180 °C for 10 minutes) was used for the enzymatic tests with innovative laccase. The most common commercial laccases have problems of specificity, thermostability and could inactivate at alkaline pH. For this reasons, a research was carried out on selective laccases, thermostable up to 70-80 °C and active even in alkaline condition. A company that met these selection criteria was MetGEN (Finland), with two

proposals with different thermo / alkaline properties. Lignin is one of the biomass components of great economic interest due to its high potential as a renewable aromatic raw material for high-value applications such as biopolymers, insulators, panels, foams and bitumen. However, the rigidity and complexity of the lignin polymer, together with the variety of extraction and pretreatment processes and conditions, make enhancement currently very challenging. Therefore, MetGen focuses on enzyme solutions that maximize lignin enhancement. Two types of MetZyme® PURECO ™ enzymatic mixtures (Pureco013 and Pureco038) were therefore purchased. Pureco enzymes are thermostable and / or alkalophilic laccases that allow a more efficient and specific oxidation of lignin, enhancing it under various aspects including depolymerization, degree of reactivity and potential functionalization of the final products without the aid of organic solvents or environmentally impacting processes. The reaction was conducted at 60 °C and pH= 6; caffeic acid was used as laccase mediator where specified.

The determination of the proteins was carried out by the Bradford method, using BSA as a standard to derive the calibration curve. The BSA and Metgen enzymes were diluted appropriately and the absorbance was measured at 595 nm. The MetGen Pureco 013 enzyme has a protein concentration of 0.44 mg / ml while the Pureco 038 of 1.18 mg / ml.

The extracellular activity of the laccase was determined spectrophometrically using ABTS 10 mM as substrate. The reaction mixture contained 800 µl of McIlvaine buffer at pH=4, 100 µl of ABTS and 100 µl of aqueous phase enzyme. The samples were incubated for 3 minutes. The formation of the cationic radical ABTS + was evaluated kinetically by the increase in absorbance at 420 nm. A unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of ABTS at 30 °C in 1 min.

## 6.5 Lignin characterization techniques

### 6.5.1 Gel Permeation Chromatography (GPC)

Before the analysis, the lignins obtained were acetylated in order to increase the solubility in the solvent used (THF) for elution in the chromatographic instrumentation. In particular, an acetobromination was performed as follows: 50 mg lignin was suspended in 10 mL glacial acetic acid/acetyl bromide (9:1 v/v) for 2 h. The solvent was then carefully fully removed in vacuo, and the residue was dissolved in THF (10 mL) and filtered over 0.45 µm syringe filter prior to injection (Lange et al., 2016b). Lignin solution was analyzed by Agilent HP 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with GPC columns SUPELCO TSKgel-G4000HHR, TSKgel-G3000HHR, and TSKgel-G2500HHR to determine the average molecular weights by coupling an Agilent 1260 ELSD detector setting evaporator temperature at 42°C, nebulizer temperature at 30°C and using nitrogen flow rate of 1.19. THF was used as eluent. Standard calibration was performed with polystyrene standards. The number-average molecular weight (Mn), the weight average molecular weight (Mw) and the polidispersivity (Đ ) were calculated as described in the materials & methods section.

Since the peaks deriving from the products analysis were not well resolved, a deconvolution process was carried out using the Origin PRO software. In particular, after exporting the data that generated each chromatogram, the straight line was subtracted, and through the "analysis" section of the aforementioned software the individual peaks were selected (fit multipick, gaussian).

### 6.5.2 Infrared Spettroscopy (FT-IR)

Infrared (IR) spectra were obtained utilizing a Bruker Alpha FT-IR spectrophotometer (Bruker Photonics, Billerica, MA) configured for attenuated total reflectance (ATR) at ambient temperature. Spectra from 64 scans were averaged in the range of 400 to 4000 $cm^{-1}$ with 1.0 $cm^{-1}$ resolution. The samples were acetylated and dissolved in chloroform before the acquisition.

### 6.5.3 Nuclear Magnetic Resonance (NMR)

As reported in the section 6.5.1, before the analysis, the lignins obtained were acetylated in order to increase the solubility in the solvent. The $^{1}H$ NMR characterization of the products were performed using Bruker 300 AM instrument spectrometers, (300 MHz for $^{1}H$). For NMR analysis, the samples were analyzed by dissolving 30 mg of sample in 0.4 mL of deuterated chloroform. Chemical shifts are reported in ppm referred to TMS as an internal standard. All the spectra were recorded in $CDCl_3$, the chemical shifts (δ) are reported in ppm relative to the residual peak of the solvent ($CHCl_3$: δ = 7.26 ppm). The chemical shifts values are reported in ppm.

Quantitative $^{31}P$ NMR analysis was performed as reported by Granata & Argyropoulos (1995). In particular, accurately weighed amount of lignin (about 30 mg) was phosphitylated using 2-chloro-4,4,5,5- tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP), and the spectra were measured on a Bruker 300 MHz spectrophotometer (256 scans at 20 °C). All chemical shifts reported are relative to the reaction product of water with Cl-TMDP, which gives a sharp signal in pyridine/ $CDCl_3$ at 132.2 ppm.

### 6.5.4 Thermogravimetrical Analysis (TGA)

About 3–4 mg of each sample was used for TGA performed with a Perkin Elmer TGA7 apparatus. The calibration of the apparatus was based on the Curie point of Nickel (354 °C) and Iron (970 °C). The reproducibility of the tests was on average within 0.5% of the weight at given temperature. The tests were carried out with variable heating rate to simulate the heating that biomass particles undergo in the updraft gasifier. Based on the average thermal profile measured inside the reactor during the gasification tests, the heating rate was set at 5.5 °C/min at the beginning of the run, from 60 °C (stable weight) up to 260 °C; then 6 °C/min up to 380 °C; 7.5 °C/min up to 510 °C; 7.5 °C/min up to 630 °C; 17 °C/min up to 900 °C. The gas flow in TGA was 20 ml/min and it was kept stationary at the inlet. The mixtures of gas were purchased in cylinders, the purity of $N_2$ and $O_2$ was 99.999%. From each run a set of 5500 couples of data was available, that is weight and temperature recorded at regular time intervals; the data were exported and worked out in Excel worksheet.The calibration of the apparatus was based on the Curie point of Nickel (354 °C) and Iron (970 °C). The reproducibility of the tests was on average within 0.5% of the weight at given temperature. The tests were carried out with variable heating rate to simulate the heating that biomass particles undergo in the updraft gasifier. Based on the average thermal profile measured inside the reactor during the gasification tests, the heating rate was set at 5.5 °C/min at the beginning of the run, from 60 °C (stable weight) up to 260 °C; then 6 °C/min

up to 380 °C; 7.5 °C/min up to 510 °C; 7.5 °C/min up to 630 °C; 17 °C/min up to 900 °C. The gas flow in TGA was 20 ml/min and it was kept stationary at the inlet. The mixtures of gas were purchased in cylinders, the purity of N2 and O2 was 99.999%. From each run a set of 5500 couples of data was available, that is weight and temperature recorded at regular time intervals; the data were exported and worked out in Excel worksheet.

### 6.5.5 Pyrolysis techniques (Py-GC/MS)

The relative molar abundances of compounds of lignin samples were analyzed using Py-GC/MS. Experiments were conducted on a pyrolyzer (SGE Pyrojector II) modified to work as a fixed-bed reactor. A small amount of each sample (8 mg) was loaded on the pyrolyzer and the alcohol was injected in single pulses of 0.2 μL, to maintain the catalyst in large excess. The operative temperature was set to 320 °C. Products were identified on GC/MS system Hewlett-Packard 5890 gas chromatograph connected to a 5972 Mass Selective Detector; a 25 m 0.32 mm internal diameter HP1 column was used. Products were quantitated from the areas of the eluted peaks using appropriate calibration factors.

# 7 Results and discussion

## 7.1 Determination of composition of biomasses investigated

The work of fractionation and valorising biomasses was focused on two raw materials, wheat straw and thistle. Wheat straw was supplied as a by-product of agriculture in the Basilicata region (southern Italy) as it is what remains of the cereals after threshing. The lignocellulosic cardoon residue is instead a by-product of the Novamont biorefinery, in which processes are optimized starting from oilseeds and roots rich in inulin. The compositions of the feedstocks were determined as reported in the materials and methods section, thus determining the content of inorganic components (ash), the lignin content, the content of organic substances by extraction in a suitable solvent (oils, waxes, etc.), pentose sugars (mainly arabinose and xylose) and hexose sugars (mainly galactose and glucose).

The percentage of glucan can be assimilated with good approximation to that of cellulose which constitutes 38.4% while the other sugars correspond to the hemicellulose content, mainly derived from xylan. In lower percentage there are other categories of organic substances such as waxes, acids, terpenes, resins, fats, ashes. Overall, after a comparison with the data in the literature, (Del Rio et al., 2012, Yue et al., 2020 and Zeng et al., 2013**)** the composition of the wheat straw is that typical of the species. Table 10 shows the characterization of the wheat straw before the steam explosion pretreatment; the composition is the average value of three replicates.

**Table 10.** Composition of wheat straw biomass before pretreatment (referred to dry weight)

| RM (Wheat straw) composition | % (m/m) |
|---|---|
| Glucan | 38.4 ± 3.2 |
| Xylan | 16.7 ± 1.1 |
| Organic extractives | 4.3 ± 0.3 |
| Insoluble lignin | 20.6 ± 1.1 |
| Ashes | 6.2 ± 0.1 |

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Table 11 shows the characterization of the cardoon biomass before the steam explosion pretreatment. The listed composition represents the average value of three replicates. Prior the pretreatment the biomass had a dry matter content of 91% and was analysed for carbohydrates and lignin content.

**Table 11.** Composition of cardoon biomass before pretreatment (referred to dry weight).

| RM (Cardoon) composition | % (m/m) |
|---|---|
| Glucan | 35.0 ± 1.5 |
| Xylan | 14.0 ± 0.7 |
| Organic extractives | 4.1 ± 0.5 |
| Insoluble lignin | 19.8 ± 1.3 |
| Ashes | 8.1 ± 0.9 |

As can be seen from the tables above, the percentage of glucan is higher in the wheat straw biomass while the percentage of xylan and organic extractives is in favor of the cardoon biomass; as regards the insoluble lignin, the percentages are very similar to each other in the two biomasses. Finally, in the biomass of cardoon there are more ashes than in the wheat straw but in any case the values are very similar to each other, demonstrating that both biomasses represent a good source for obtaining fermentable sugars and lignin.

## 7.2 Biomass pretreatment and fractionation

One of the objectives of the project was the study and development of a technology for the first step of the process for obtaining lignin: the pretreatment of lignocellulosic biomass. The final result of the pre-treatment is to make the cellulose and lignin polymers more accessible and reactive and to solubilize most of the hemicellulose in the form of pentosans, namely xylose, arabinose.

### 7.2.1 Wheat straw steam explosion pretreatment

The pre-treatment was performed at the C.R. ENEA Trisaia in batch reactor capable of treating 1 kg of biomass for each processing cycle with saturated steam, varying the severity parameters, i.e. temperature, reaction time and concentration of acid or base catalyst.

Other parameters that influence the steam explosion process are the biomass particle size and moisture content (Feofilova et al., 2016). It is very important to optimize the process and the conditions regarding the pretreatment phase, because during this process substances could be generated which act as enzymatic or fermentation inhibitors in the subsequent phases of the process of obtaining sugars and for the production of lignin both as an aspect quantitative than qualitative.

The biomass was impregnated with $H_2SO_4$ solution (0.5M) before the pretreatment. The resulting acid load was 1.4% (w/w). The steam explosion pretreatment was carried out at 203°C for 5 minutes based on temperature and treatment duration optimized by Ballesteros et al. 2006 for the ethanol production from wheat straw hydrolysates. Enzymatic hydrolysis tests were carried out on steam-exploded wheat straw to confirm the effectiveness of the pre-treatment used. On this pretreated product the hydrolysis yield of the cellulose was practically quantitative, indicating excellent destructuration of the biomass (data not shown). Furthermore, this pretreatment reduced the concentration of degradation products in the liquid fraction, solubilized the highest fraction of hemicellulose in monomeric form, and deconstructed the bonds between molecules of biomass in order to favor the recovery of lignin. After steam explosion pre-treatment, a solid fraction richer in cellulose and lignin and a liquid fraction richer in hemicellulose, soluble components of lignin, acetic acid and other possible inhibitors are obtained. The use of a high thermal severity or a significant use of acid catalyst could have the drawback of degrading the sugars too much and giving rise to the formation of molecules, such as furans, which can also be starting substrates for the formation of molecules with high added value, at high concentrations are inhibitors of biological processes following pretreatment, that is, enzymatic hydrolysis and fermentation. Subsequently by pressing each product was separated into its solid and liquid part by pressing and the fractions analyzed separately; the composition of the hemicellulose liquid fraction was: glucose 1.03±0.07 g $L^{-1}$; xylose 16.4±0.8 g $L^{-1}$; xylooligomers 1.1±0.1 g $L^{-1}$ ; acetyl groups 1.9±0.1 g $L^{-1}$ ; furan compounds 0.91±0.05 g $L^{-1}$.

As results of this pretreatment, the xylose was solubilized in the liquid fraction with a yield greater than 90% compared to the initial total xylan present in the raw material. Furthermore, almost all of this xylose is in monomeric form and the content of furan degradation compounds is relatively low. Following these observations, it can be stated that this hemicellulose is suitable for the recovery or conversion of pentose sugars into molecules with a higher varus added, for example with specific fermentation processes. The composition (average value of three replicates) of washed solid fraction obtained after steam explosion is shown in the table 12.

**Table 12**: composition of solid fraction after wheat straw pretreated at 203°C, 5 minutes, 1.4% $H_2SO_4$

| **Washed Solid composition after S.E.** | **% (m/m)** |
|---|---|
| Glucan | 53.2 ± 1.2 |
| Xylan | 2.1 ± 0.1 |
| Soluble lignin | 1.29 ± 0.01 |
| Insoluble lignin | 32.5 ± 0.9 |
| Ashes | 5.6 ± 0.2 |

As a result of the pre-treatment an excellent hemicellulose yield is obtained thanks to the fact that xylan passes from 16.7% to 2.1% in the exploded material. These reductions also apply to other soluble carbohydrates such as arabinan and galactan, albeit to a lesser extent and therefore not carried over. The percentage of hydrolyzable glucan increases from 38% to 53%. The overall hydrolysable sugar content in the solid residue after steam explosion was 55.3 g sugar/100 g starting, almost all composed of cellulose. A part of this residual solid material was treated with cellulolytic enzymes to obtain sugary syrups and a residue enriched in lignin, while another part was used directly to isolate the lignin through alkaline methods or using innovative solvents. It should be note that due to the almost complete solubilization of the pentose sugars in the hemicellulose, the relative internal content of insoluble lignin in the solid fiber increases by about one third compared to the raw material. Having a relative higher lignin content in a pretreated product than the raw material could makes the recovery and lignin qualitative analysis processes more advantageous . However, a relative percentage increase of insoluble lignin in the fiber does not necessarily correspond to higher quality lignin. During the

pretreatment processes, in particular acid catalyzed, the formation of multiple products, such as pseudo-lignin and humins, is inevitable. In particular, pseudo-lignin is formed under severe conditions through processes in which aromatic intermediates of lignin reaction have worked with molecules deriving from the thermal degradation of sugars (mainly furans) to generate pseudo-lignin which can be erroneously calculated quantitatively as acid insoluble lignin (Cheng et al., 2018).

### 7.2.2 Cardoon steam explosion pretreatment

The choice of the steam explosion parameters for this type of biomass was dictated by the activity needs of the green chemistry laboratory of ENEA Trisaia, which had the purpose of favoring the adhesiveness of the fibers by self-activation of the lignin following pretreatment. The aim was to reduce the use of phenol-formaldehyde based additives in the formation of binderless fiberboards. For this reason a preliminary literature search was done and the temperature factor was set at 180°C because it was included in the range tested for the production of binderless fiberboards from cardoon (Mancera et al., 2008). In addition, two acid-base additives were tested which act as catalysts to verify specific actions on the lignin and functionality. The pretreated products are indicated in the following table which shows the characterizations of the lignocellulosic components (Tab. 13):

**Table 13:** Lignocellulosic composition of solids derived from steam explosion pretreatments of cardoon. The composition is the average value of two replicates

| Conditions | Cellulose% | Xylan% | Insoluble lignin% |
|---|---|---|---|
| 180°C, 10 min | 43.9 ± 0.2 | 11.8 ± 0.8 | 23.8 ± 0.1 |
| 180° 10’ 1% $H_2SO_4$ | 55.2 ± 0.6 | 3.2 ± 0.2 | 35.5 ± 0.4 |
| 180° 10’ 2% NaOH | 48.1 ± 1.3 | 11.9 ± 0.4 | 22.4 ± 1.0 |

Figure 34 shows the comparison of insoluble lignins of the pretreated cardoon exploded with steam compared to the insoluble lignin of the raw material.

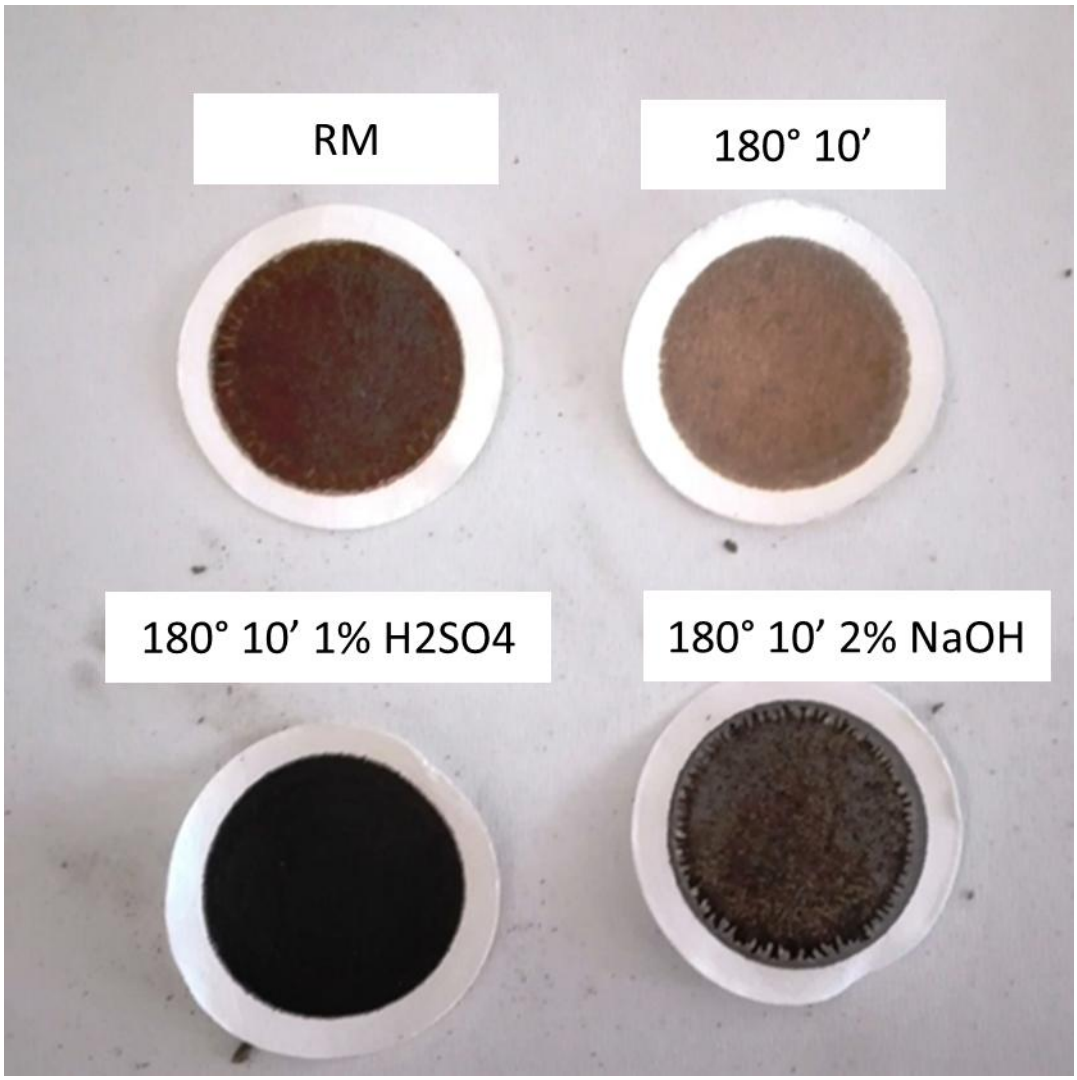


**Figure 34.** Insoluble lignins of pretreated steam-exploded cardoon compared with the insoluble lignin of raw material (RM)

The results in table 13 show that from a quantitative point of view only acid catalysis clearly changes the proportions between the three components since it favors the solubilization and therefore the removal of xylans in the liquid hemicellulose. As a result of this reduction, the insoluble lignin increases up to 35%. The basic catalysis returns a final product with a percentage composition of the macro-components similar to that of the raw material. In order to evaluate the use of cardoon lignin in different applications, including the production of panels without fossil binders, we focused on the isolation and enzymatic treatment of only one of these substrates, namely the lignin isolated from the exploded at 180 °C and 10 min without the use of catalyst.

## 7.3 Enzymatic hydrolysis of pretreated wheat straw

With a view to circular economy, the pretreatment, in addition to having the purpose of recovering lignin for the purposes of our work, must also allow to optimize the recovery of biomass carbohydrates with high recoveries of simple sugars. Therefore, in order to verify the effectiveness of the pretreatment, an enzymatic hydrolysis test of the wheat straw fibers was carried out. The enzyme blend, Cellic Ctec2, was kindly provided by Novozymes (Denmark). A 5% (w/w) solids load and an enzymatic load corresponding to 17.6 FPU per gram of glucan were used as set-up. The yield of cellulose hydrolysis after about 72 h of process was around 90% and this evidence indicates that both the substrate and the pre-treatment used have a high potential for use in biorefineries. The residue of the enzymatic hydrolysis (LWS7) was analyzed in its composition (Tab. 14) and was composed mainly of insoluble acid lignin (about 80%) but with significant presence of residual carbohydrates and ash. The material was ground to 0.5 mm for characterizations.

**Table 14.** Composition of solid fraction (LWS7) after the enzymatic hydrolysis of steam exploded wheat straw. The composition is the average value of three replicates.

| Enzymatic hydrolysis residue | % (m/m) |
|---|---|
| Glucan | 6.5 ± 0.3 |
| Xylan | 1.6 ± 0.4 |
| Soluble lignin | 4.3 ± 0.2 |
| Insoluble lignin | 80.1 ± 2.0 |
| Ashes | 3.3 ± 0.5 |

## 7.4 Lignin extraction

The techniques for the extraction of lignin from pretreated wheat straw involved the use of different solvents, traditional (alkaline) and innovative (DES, renewable solvents). The lignin from cardoon was first isolated from the pretreated biomass using alkali (soda) process and acid precipitation at pH 2. After this, enzymatic treatments were carried out on this substrate through the use of laccases. The following Tables 15-A and 15-B indicate the products obtained and the technique used for each biomass considered.

**Table 15-A**. Isolation process of lignins from acid catalyzed steam exploded (203°C 5 minutes) wheat straw. (LWS: Lignin obtained from Wheat Straw)

| Abbreviation | Isolation process |
|---|---|
| LWS1 | fractionated precipitation at pH7 after alkaline soda extraction (from pH11 to pH7) |
| LWS2 | fractionated precipitation at pH5 after alkaline soda extraction (from pH7 to pH5) |
| LWS3 | fractionated precipitation at pH4 after alkaline soda extraction (from pH5 to pH4) |
| LWS4 | fractionated precipitation at pH2 after alkaline soda extraction (from pH4 to pH2) |
| LWS5 | directly precipitation at pH2 after soda alkaline extraction (from pH11 to pH2) |
| LWS6 | directly precipitation at pH5 after soda alkaline extraction (from pH11 to pH5) |
| LWS7 | residue of enzymatic hydrolysis of steam exploded wheat straw |
| LWS8 | GVL (gamma-valerolactone) extraction |
| LWS9 | ChCl-OA (choline chloride/oxalic acid) extraction |
| LWS10 | ChCl-LA (choline chloride/lactic acid) extraction |
| LWS11 | ChCl-Urea (choline chloride/Urea) extraction |

**Table 15-B**. Laccase treatment of lignin derived from soda extraction of steam exploded cardoon at 180°C for 10 minutes. (LCY: Lignin obtained from Cynara cardunculus)

| Abbreviation | Lignin precipitation pH |
|---|---|
| LCY1 | substrate treated at pH6 without laccase (control process) |
| LCY2 | substrate treated at pH9 without laccase (control process) |
| LCY3 | substrate treated at pH6 with laccase MetGen® Pureco038 |
| LCY4 | substrate treated at pH9 with laccase MetGen® Pureco013 |
| LCY5 | substrate treated at pH6 with laccase MetGen® Pureco038 and caffeic acid (CA) |
| LCY6 | substrate treated at pH9 with laccase MetGen® Pureco013 and caffeic acid (CA) |

### 7.4.1 Lignin extraction from steam exploded wheat straw

An alkaline lignin extraction was carried out on part of the solid fraction from which six types of lignin were obtained, marked with the initials LWS1-LWS6, while extraction with innovative solvents produced the lignins labeled as LWS8 (GVL), LWS9 and LWS10 (Deep Eutectic Solvents). After extracting the residual hemicellulose from the exploded biomass by washing with hot water at T = 75 ° C, the residue was extracted twice with a 1.5% w /v NaOH solution, at an extraction temperature 90 ° C for 15 minutes. The alkaline solution obtained at pH 11 was recovered and the residual solid was further washed with hot water to neutral pH to remove excess soda. In these working conditions it is possible to reach the maximum possible extraction efficiency. The alkaline solution was slowly acidified using concentrated sulfuric acid (about 3.4 M) in order to precipitate the lignin. The precipitated solids were washed, dried and the obtained quantity was determined by weighing.

The characterization of the delignified residual solid showed that about 82% of lignin was removed from the raw material and dissolved in the alkaline solution. A fractional precipitation was realized at different pH values by filtering and separating the lignin residues after each passage: different amounts of lignin were recovered at different pHs; the major part of lignin was precipitated at pH higher or equal 4. In particular at pH = 7, 34% of lignin (LWS1) is obtained, at pH = 5, the 26 % of lignin is obtained (LWS2), at pH = 4, 33% of lignin (LWS3) is obtained and at pH = 2 only 7% of lignin (LWS4) is obtained .

Furthermore, from two aliquots of alkaline liquid fraction, a hot precipitation with 18% sulfuric acid was carried out separately at two different pH values: at pH = 2, 64% of lignin precipitated (LWS5) while at pH = 5 a value of 34% (LWS6) was reached. The percentages indicated refer to the initial lignin present in the dry substrate, neglecting however the possibility that other organic molecules,

such as oils or complexes between carbohydrates and lignins, are also precipitated, as is likely to happen. These values are therefore to be considered relative to get an idea of the weight aspects concerning the precipitation itself at different pHs.

These lignins were then characterized. FT-IR spectroscopy has several advantages including high sensivity and selectivity, high signal to noise ratio, accuracy, short time and small amount of sample required for the analysis (Yang et al., 2016). It is a rapid, economical and non-destructive technique that is very often used in polymer studies. The effects caused by the different pulping processes in the FT-IR spectra are presented in the Figure 35:

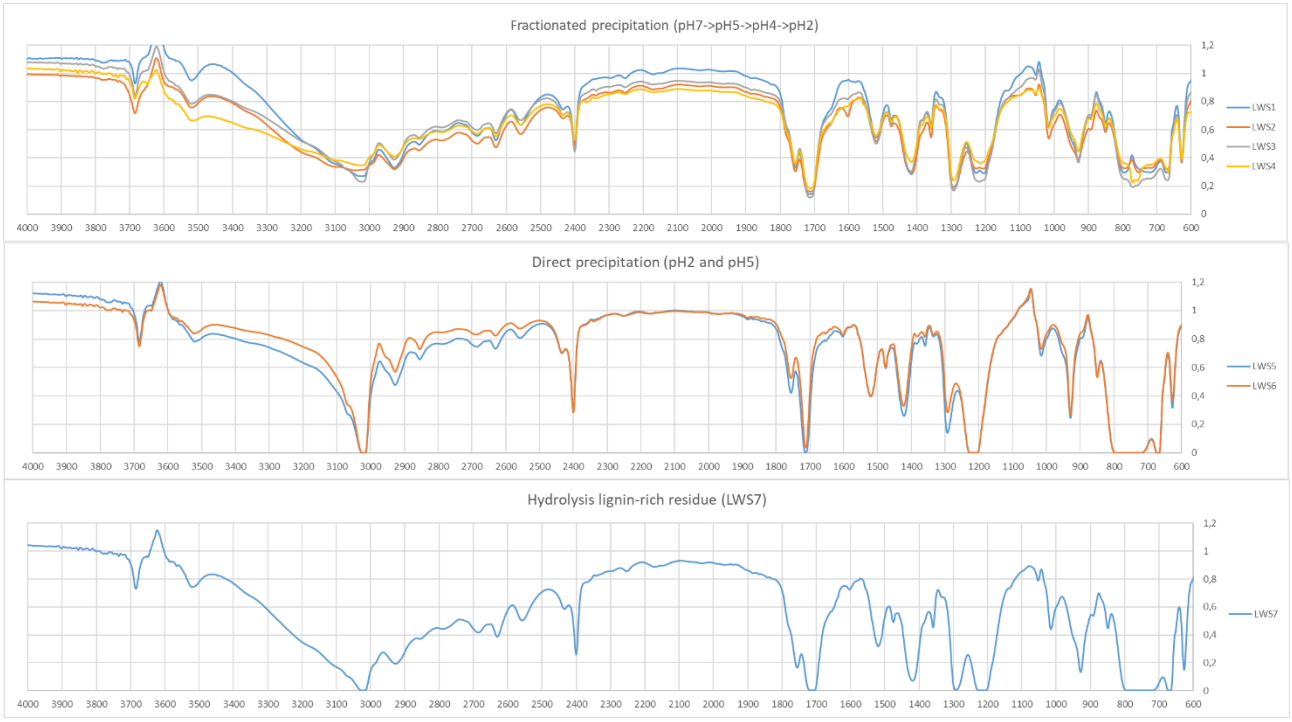


**Figure 35**. comparisons of FT-IR spectra of lignin precipitated at different pH (from pH7 to pH2), lignin precipitated directly at pH5 or pH2 and lignin rich residue derived from enzymatical hydrolysis of cellulose (LWS7)

The recorded spectra of lignin samples compared with the assignments found in other scientific papers (Ibrahim at al 2019, Ramezani & Sain, 2018, Sun et al., 2013). All lignins showed a strong wide band within the range 3700 $cm^{-1}$ assigned to hydroxyl groups in phenolic and aliphatic structures. However, they are usually much wider. Perhaps having acquired the spectra of the acetylated lignins, the OH groups have been reduced and transformed into O-CO-$CH_3$. Comparing with the data in the literature, the signals due to OH stretching resonate around 3420 $cm^{-1}$ with large peaks (Sun et al. 2013); the width varies according to whether they are alcohols or acids. In general the range is from

3600 to 3800 $cm^{-1}$. The lignins namely LWS1, LWS2, LWS3 and LWS4 which have been re-precipitated in a fractional manner at decreasing pH they strangely show a too similar profile. Different substances are expected to precipitate at different precipitation pHs. In these four types of lignins a peak around 1715 $cm^{-1}$ is evidently present due to the C = O stretching as evidenced by (Ramezani & Sain, 2018). There are peaks around 1300 $cm^{-1}$ and 1200 $cm^{-1}$ which should be respectively attributable to S- and G units. The C = O group resonates at 1712 $cm^{-1}$ for the aromatic carbonyl groups (Ramezani & Sain, 2018). The signals at 1607, 1515 and 1425 $cm^{-1}$ are related to the aromatic rings (Sun et al. 2013) while the C-H deformation due to carbohydrates resonates at 1371 $cm^{-1}$ (Ramezani & Sain, 2018). At 1328 $cm^{-1}$ syringyl ring vibration with C-O stretching was detected in according with other authors (Sun et al., 2013) while the stretching of the C-O bond is located around 1215 $cm^{-1}$. Finally at 1270 $cm^{-1}$ there is the guaiacyl ring vibration.

In the Table 16 FT-IR assignments of the hydroxyl, carbonyl, methoxyl, carboxyl, aromatic and aliphatic C-H groups present in this lignins are reported:

**Table 16.** Comparison of frequencies and assignment of bands in FT-IR spectra for the lignins derived from wheat straw (w: peak with weak intensity; m: peak with medium intensity; s: peak with stron intensity)

| Frequency (cm-1) | Assignment | LWS1 | LWS2 | LWS3 | LWS4 | LWS5 | LWS6 | LWS7 |
|---|---|---|---|---|---|---|---|---|
| 3030 | CH- insatured stretch | m | m | m | m | s | s | s |
| 2855 | CH- stretch (O-CH3 group) | w | w | w | w | w | w | w |
| 1735 | C=O stretch (ester) | m | m | m | m | m | m | m |
| 1715 | C=O stretch (generic) | m | m | m | m | s | s | s |
| 1515 | Aromatic ring stretch | m | m | m | m | m | m | m |
| 1440 | CH bend (ether) | w | w | w | w | w | w | w |
| 1270 | C-O stretch (alcohol) | m | m | m | m | m | m | s |
| 1220 | C-O(H) +C-O(Ar) (Phenolic OH and ether in syringyl and guaiacyl S and G units) | m | m | m | m | m | s | s |

Almost all the signals of the lignins obtained are in good agreement with the data present in the literature except for some signals such as the one related to the C-O bond where the values differ more (Ibrahim et al., 2019). Unsaturated C-H stretching at 3100 $cm^{-1}$ appears to be greater in lignins obtained by direct precipitation at pH2 and pH5 (LWS5, LWS6) than in fractionally precipitated ones (from LWS1 to LWS4). The same observation can be made for the generic C = O strech at 1715 $cm^{-1}$. The ratio between the peaks at 1290 and 1215 $cm^{-1}$ could give an indication of the ratios between syringil and guayacil units, in fact the intensities are similar for the lignins considered except for LWS6 and LWS7.

A deep understanding of the biopolymer chemical structure is necessary to optimize any downstream conversion to the final applications. About that the development of suitable analytical methods is necessary in order to gain knowledge of the biopolymer structure and composition. The most widely used analytical methods include: the thermogravimetric analysis (TGA) and the pyrolysis coupled to a GC-MS (py-GC/MS). Thermogravimetric analysis was used to study the pyrolitic thermal stability of the wheat straw. TGA curves reveal the percentage of weight loss of the polymer as a function of the increase in temperature to which it is subjected. Typically, lignin thermograms show a wide range of degradation temperatures from 100°C to 900°C due to the complex branched structures of these molecules. Figure 36 shows the thermogravimetric (TGA) weight loss curves for lignins samples . The volatiles content and residual char were determined from the TGA weight loss curve. In general, lignins such as ball milled wood lignin that are generally considered to resemble the native form of lignin in the plant more than technical lignins show a lower temperature at which the thermal degradation is at its maximum typical values around 316–330°C (Huijgen et al., 2014). In these lignins, the easily degradable β-O-4 intramolecular ether linkages are more abundant compared to technical lignins.The comparison between the lignin produced following enzymatic hydrolisis of cellulose and those produced through the acidic re-precipitation of the alkaline liquor show that the enzymatically treated lignin degrades more slowly, and presents a final plateau probably due to the greater presence of inorganic salts used for the preparation of the buffer solution for the enzymatic hydrolysis process.

What can be deduced from the thermogravimetric analyzes is that the lignin from enzymatic hydrolysis is more thermostable and contains more ash, due to the addition of the saline buffer to carry out the enzymatic hydrolysis itself, as already mentioned above. The TGA curves of lignins are shown in figure 36. Overall, the continuous pyrolysis of lignin was observed at broad temperature range 130 °C-780 °C. The TGA curves of all lignins sample presented similar three stage thermal decomposition processes despite their different characteristics. The first weight loss step corresponding to the remove of the moisture of wheat straw occurred from room temperature to 200 °C; the second, more pronounced, pyrolysis stage appeared between 150 °C and 480 °C, and it was likely ascribed to the degradation of carbohydrates, phenols, alcohols, and aldehydes into CO, $CO_2$, and $CH_4$. The third, and slower, pyrolysis stage occurred after 480 °C. Here, all lignins had an almost identical mass loss rate. This step corresponds to the pyrolysis of lignin and charring of the residue. All lignins resulted in a notable amount of ash residue (Yue et al., 2020). The result is in accordance with the literature.

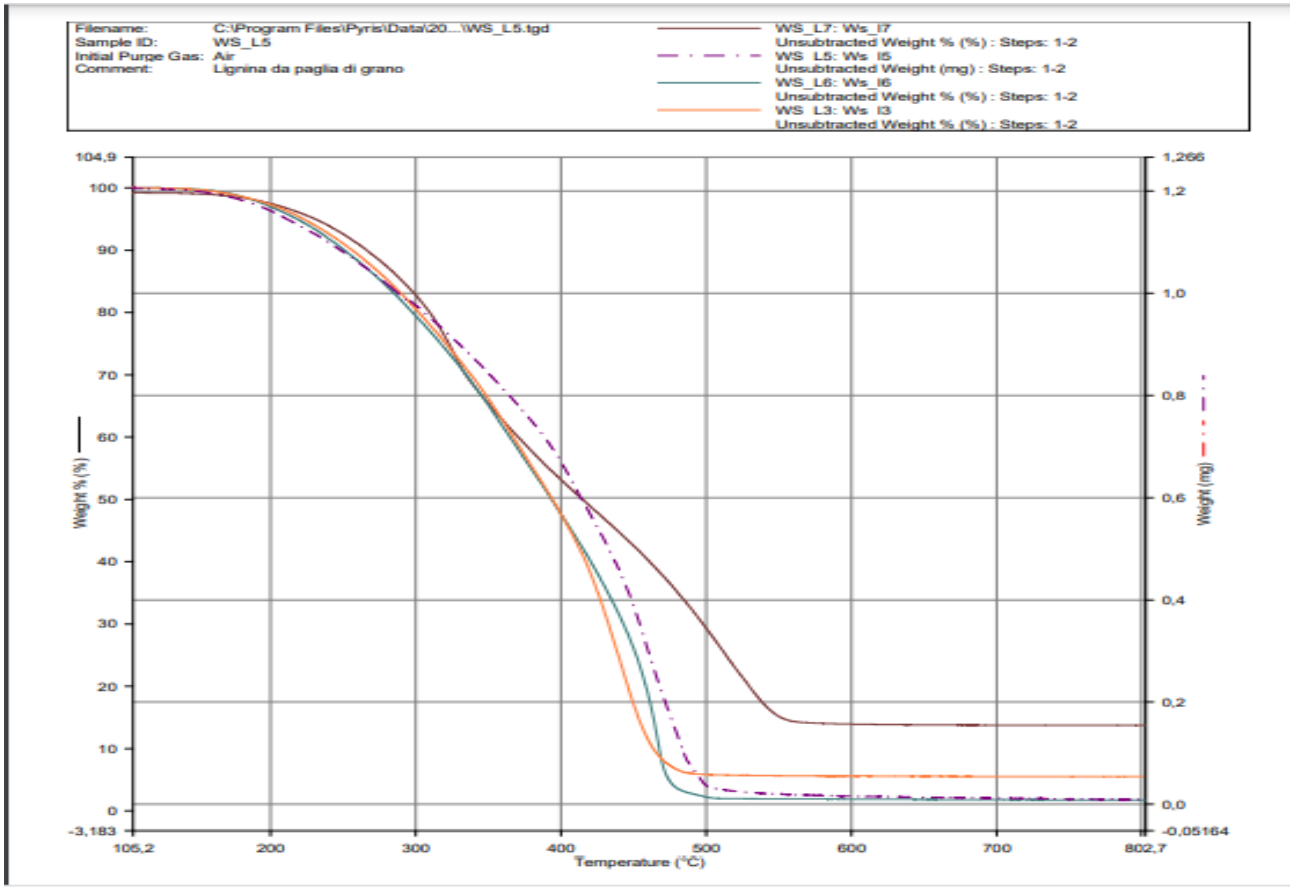


**Figure 36.** TGA curves of lignins: LWS3 (continue orange line); LWS5 (dashed violet line); LWS6 (continue teal line); LWS7 (continue brown line)

All lignin fractions had a great thermal stability end this behavior could be attribute to their chemical structure which can provide enhanced thermal properties useful in the fabrication of specific materials. The molar mass distribution of lignins is an important feature to rationalize lignins properties and reactivity and Gel Permeation Chromatography is one of the preferred methods. During the GPC analysis, the information on the molecular weight of the lignin polymers can be obtained by the average molecular weight (Mw), the average number molecular weight (Mn), and polydispersity index (PDI) (Mw/Mn). The values of the weight-average (Mw) and number-average (Mn) molecular weight were estimated from the GPC curves (relative values related to polystyrene standards). The Table 17 shows the average molecular masses of LWS1-LWS7 lignins and the polidispersivity index for each substrate.

**Table 17.** Weight-Average (Mw) and Number-Average(Mn) Molecular Weight and polidispersity index of isolated lignins

| Samples | Experimental conditions | Mn (g/mol) | Mw (g/mol) | PDI |
|---|---|---|---|---|
| LWS1 | Fractionated at pH=7 | 720 | 3040 | 4.20 |
| LWS2 | Fractionated at pH=5 | 680 | 1100 | 1.64 |
| LWS3 | Fractionated at pH=4 | 950 | 1060 | 1.12 |
| LWS4 | Fractionated at pH=2 | 850 | 950 | 1.13 |
| LWS5 | direct precipitation pH=2 | 850 | 2300 | 2.74 |
| LWS6 | direct precipitation pH=5 | 750 | 800 | 1.11 |
| LWS7 | resulting from residue of enzymatic hydrolysis | 3600 | 42000 | 11.66 |

The results of the analyzes were obtained through the analysis of a polynomial constructed with standard polystyrene solutions. Since the peaks were close together, a deconvolution was carried out using the Origin Pro® software. This process allowed us to more accurately identify the values of Mn and Mw and consequently the PDI. Looking carefully at the table above, it can be seen that the lignins with the most frequent molecular weights are those corresponding to number average molecular weights between 800 Dalton and 1200 Dalton. It could indicate that monomers, dimers or small oligomers of lignin can be obtained following the precipitation of the alkaline solution. There are other peaks corresponding to lignins with $M_w$ of 2000-3000 Dalton.

Lignins precipitated in a fractional way at pH = 2 corresponding to lignin LWS4 have a polydispersivity index (PDI) much lower than that precipitated directly (LWS5) at pH = 2. This evidence indicates that the fractional precipitation process helped to select lignin molecules with more similar macromolecular characteristics.

Lignin precipitated at pH = 7 (LWS1) has a low polymer quality considering a polydispersivity index over 4; the unpurified lignin deriving from enzymatic hydrolysis, *i.e.*, LWS7 has much higher molecular weights (greater peak corresponding to 5kDa) and also one that exceeds 1.2 MDa, which

could mean the presence of carbohydrates or complexes between lignin and carbohydrates; the latter lignin (LWS7) in fact has higher PDI than all the others.

### 7.4.2 Lignin extraction with innovative green solvents (GVL)

Organosolv processes have been used for a long time to extract high added value molecules from lignocellulosic substrates. This treatment is based on the dissolution of lignin with organic solvents such as acetic acid, formic acid, methanol or ethanol. These processes usually return a lignin that is believed to not differ much from the original structure *in planta*. On the other hand, the solvents used show some important disadvantages including high costs, poor recyclability and can have an impact on health and the environment. Recently, γ-valerolactone (GVL, Fig. 37) has gained much interest in science as a renewable platform molecule derived from lignocellulosic structural carbohydrates that can be used and recycled for the production of carbon-based chemicals, liquid fuels, additives or polymers. GVL can be produced from lignocellulosic biomass by hydrogenation of levulinic acid. Furthermore, it has interesting properties as a solvent for green chemistry, such as low melting point, high boiling point and no evidence of toxicity; for these reasons GVL has been recently used as a solvent in the processing of lignocellulosic biomass (Angelini et al, 2017). In any case, to produce GVL from green sources it is necessary to use homogeneous catalysts which are associated with some not negligible drawbacks. For example, the difficulty of recycling the catalyst, the low conversion yield and the difficulty of recovering and purifying the product make it currently unsuitable and expensive for commercial applications (Raj et al., 2021).

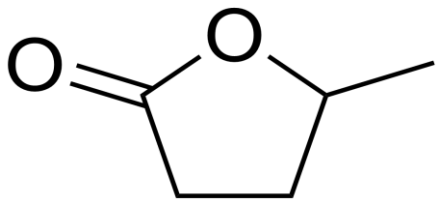


**Figure 37**. GVL structure

The extraction of lignin from the pretreated wheat straw substrate was tested by creating an experimental design aimed at optimizing the parameters related to the solubilization and reprecipitation process of the lignin itself from the starting feedstock. In particular, the percentage of GVL, the process temperature, and the process time were tested. The tests were carried out in an ultrasonic bath for a time of 3-9 hours, which could allow to obtain significant extraction yields without increasing the temperature, with possible advantages on the final process costs. The temperature was monitored and controlled by instrumental internal thermostat.

In particular, a Box-Behnken factorial design was created using the Design Expert10® software with three factors and three levels, including three replicas of the "center point" (condition in which all the variables assume their average value), in order to create a second order response surface. The three independent variables (GVL%, °C and hours) and the respective three different levels (-1, 0, +1) are indicated in the materials and methods, section 6.3.3.

A set of 15 experiments was performed as shown in the Table 18 determining two responses: the percentage of precipitate following the addition of anti-solvent and the percentage of lignin removed. The latter response was determined by characterizing (Sluiter et al., 2008) the solid residue after each treatment by comparing it with wheat straw not treated with GVL.

**Table 18.** Experimental set-up and results of the extraction with GVL for each combination of parameters during Box-Behnken design.

| | | | | **Response 1** | **Response 2** |
|---|---|---|---|---|---|
| **Run** | GVL%(v/v) | Temp (°C) | Time (h) | Precipitation % | Lignin removal % |
| **1** | 80 | 45 | 3 | 13.9 | 8.08 |
| **2** | 20 | 60 | 6 | 5.1 | 3.5 |
| **3** | 20 | 45 | 9 | 5.05 | 2.69 |
| **4** | 80 | 45 | 9 | 14.95 | 7.75 |
| **5** | 50 | 60 | 9 | 11.35 | 6.58 |
| **6** | 50 | 30 | 9 | 11.7 | 6.47 |
| **7** | 50 | 45 | 6 | 10.7 | 7.26 |
| **8** | 20 | 45 | 3 | 4.85 | 3.13 |
| **9** | 50 | 60 | 3 | 10.25 | 7.49 |
| **10** | 80 | 30 | 6 | 11.15 | 7.15 |
| **11** | 50 | 30 | 3 | 9.95 | 5.79 |
| **12** | 20 | 30 | 6 | 6.65 | 4.36 |
| **13** | 80 | 60 | 6 | 15.15 | 9.15 |
| **14** | 50 | 45 | 6 | 11.55 | 7.39 |
| **15** | 50 | 45 | 6 | 12.7 | 7.52 |

All variables were analyzed in their mean value and in the arbitrarily defined maximum and minimum values.

The degree of precipitation with respect to the initial dry wheat straw is between about 5 and 15% while the percentage of lignin removed is in the range 2-9%. This evidence indicates that not all the precipitate is certainly composed of lignin but that on average an internal percentage between 50 and 70% of the recovered solid could be composed of the lignin removed from the initial substrate. The impurities that are evidently co-precipitated together with the lignin may be due to complexes between lignin and carbohydrates or more probably to the aforementioned pseudo-lignins. What seems evident at first sight is that the greater the percentage of GVL used, the greater the quantity of precipitate obtained but the greater the ratio between precipitate and insoluble acid lignin. This indicates that the% of impurities related to the precipitate increases in proportion to the use of GVL in the mixture.

The following Tables 19a and 19b indicate the results of the statistical analysis provided by the software following the response obtained in relation to the precipitation process.

**Table 19a.** Statistical analysis of the precipitation process (response 1)

| Source | Sum of Squares | df | Mean Square | F-value | p-value | significance |
|---|---|---|---|---|---|---|
| **Model** | 161.57 | 9 | 17.95 | 16.21 | 0.0034 | significant |
| **A-GVL** | 140.28 | 1 | 140.28 | 126.69 | < 0.0001 | significant |
| **B-temperature** | 0.7200 | 1 | 0.7200 | 0.6503 | 0.4566 | not significant |
| **C-time** | 2.10 | 1 | 2.10 | 1.90 | 0.2268 | not significant |
| **AB** | 7.70 | 1 | 7.70 | 6.95 | 0.0461 | significant |
| **AC** | 0.1806 | 1 | 0.1806 | 0.1631 | 0.7030 | not significant |
| **BC** | 0.1056 | 1 | 0.1056 | 0.0954 | 0.7699 | not significant |
| **$A^2$** | 9.83 | 1 | 9.83 | 8.87 | 0.0308 | significant |
| **$B^2$** | 0.9463 | 1 | 0.9463 | 0.8546 | 0.3977 | not significant |
| **$C^2$** | 0.4051 | 1 | 0.4051 | 0.3659 | 0.5716 | |
| **Residual** | 5.54 | 5 | 1.11 | | | |
| **Lack of Fit** | 3.52 | 3 | 1.17 | 1.17 | 0.4928 | not significant |
| **Pure Error** | 2.01 | 2 | 1.01 | | | |
| **Cor Total** | 167.11 | 14 | | | | |

The Model F-value of 16.21 implies the model is significant. There is only a 0.34% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, AB, $A^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The Lack of Fit F-value of 1.17 implies the Lack of Fit is not significant relative to the pure error. There is a 49.28% chance that a Lack of Fit F-value this large could occur due to noise.

The "p-values" are used as a tool to check the significance of each coefficient and also indicate the value of the interaction between each independent variable. The lower the p-value, the greater the significance of the corresponding coefficient (Liu et al., 2003).

**Table 19b**. Fit statistic data of the precipitation process (response 1)

| | | | |
|---|---|---|---|
| **Std. Dev.** | **1.05** | **$R^2$** | **0.9669** |
| **Mean** | 10.33 | **Adjusted $R^2$** | 0.9072 |
| **C.V. %** | 10.18 | | |

The calculated linear regression coefficient ($R^2$) is equal to 0.9669, indicating that the statistical model obtained can explain 96.69% of the variability of the response variable. The obtained $R^2$ value is not too far from the adjusted R2 value of 0.9072. This indicates a good coincidence between the experimental values and those predicted for the production of precipitate (Y). The adjusted $R^2$ value corrects the $R^2$ value for sample size and the number of model variables.

In the Figure 38 is shown a graph of the predicted response values versus the actual response values. It is clear that all of the values are predicted by the model.

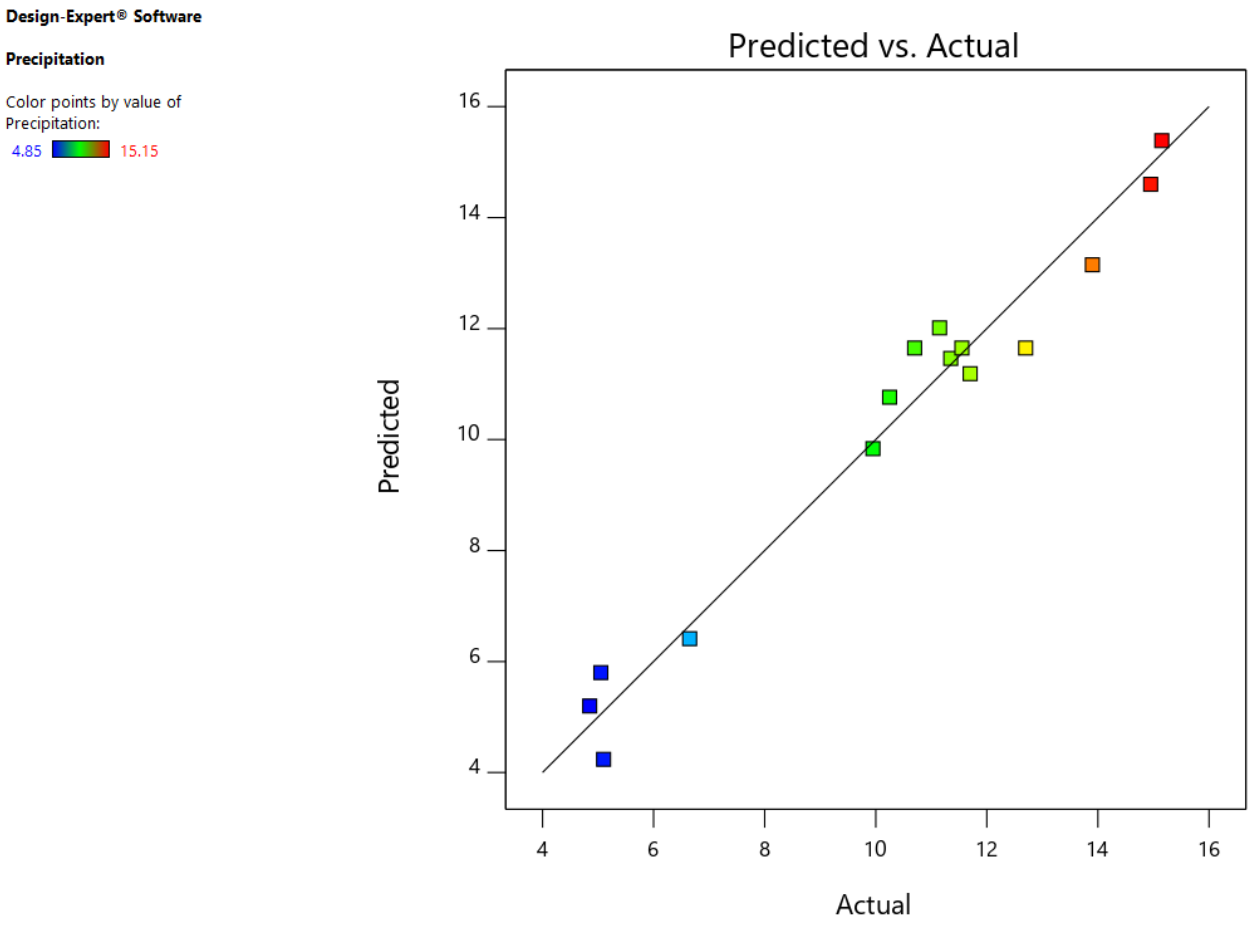


**Figure 38**. Predicted vs Actual plot (Response 1)

The following equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients:

Precipitation % = 11.65+4.19*A+0.3000*B+0.5125*C+1.39*AB+0.2125*AC-0.1625*BC-1.63*$A^2$-0.5063*$B^2$-0.3312*$C^2$

The coefficients of the three single parameters of the studied process are all positive, this indicates that both the percentage increase of GVL and the increase in the thermal severity of the process allow to improve the precipitation yield. In particular, in absolute value, the parameter associated with the coefficient A (GVL) is the one that most affects the precipitation results.

The 3D surface plots that graphically relate each pair of variables studied are shown below:

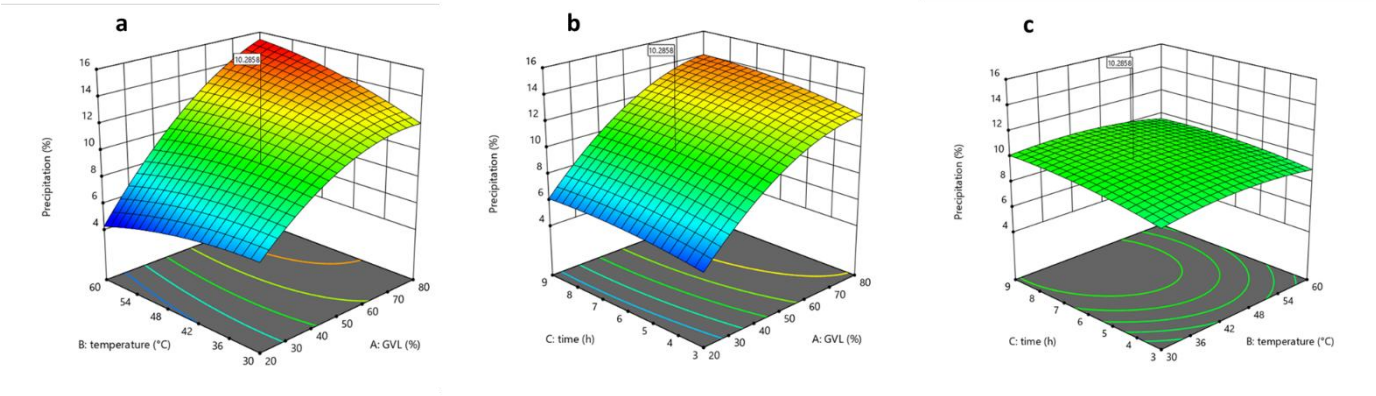


**Figure 39. V**ariation of the% of precipitation following the variation of the parameters (**a**: GVL%, temperatures; **b**: GVL%, time; **c**: temperature, time)

The following tables 20a and 20b indicate the results of the statistical analysis provided by the software following the response obtained in relation to the lignin removal:

**Table 20a.** Statistical analysis of the lignin removal (response 2)

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 53.75 | 9 | 5.97 | 68.72 | 0.0001 | significant |
| **A-GVL** | 42.55 | 1 | 42.55 | 489.68 | < 0.0001 | significant |
| **B-temperature** | 1.09 | 1 | 1.09 | 12.52 | 0.0166 | significant |
| **C-time** | 0.1250 | 1 | 0.1250 | 1.44 | 0.2841 | not significant |
| **AB** | 2.04 | 1 | 2.04 | 23.53 | 0.0047 | significant |
| **AC** | 0.0030 | 1 | 0.0030 | 0.0348 | 0.8593 | not significant |
| **BC** | 0.6320 | 1 | 0.6320 | 7.27 | 0.0429 | significant |
| **$A^2$** | 5.86 | 1 | 5.86 | 67.46 | 0.0004 | significant |
| **$B^2$** | 0.0299 | 1 | 0.0299 | 0.3442 | 0.5829 | not significant |
| **$C^2$** | 1.90 | 1 | 1.90 | 21.87 | 0.0054 | significant |
| **Residual** | 0.4345 | 5 | 0.0869 | | | |
| **Lack of Fit** | 0.4007 | 3 | 0.1336 | 7.90 | 0.1144 | not significant |
| **Pure Error** | 0.0338 | 2 | 0.0169 | | | |
| **Cor Total** | 54.18 | 14 | | | | |

The Model F-value of 68.72 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, BC, $A^2$, $C^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 7.90 implies the Lack of Fit is not significant relative to the pure error. There is a 11.44% chance that a Lack of Fit F-value this large could occur due to noise.

**Table 20b.** Fit statistic data of the lignin removal process (response 2)

| | | | |
|---|---|---|---|
| **Std. Dev.** | **0.2948** | **$R^2$** | **0.9920** |
| **Mean** | 6.29 | **Adjusted $R^2$** | 0.9775 |
| **C.V. %** | 4.69 | **Predicted $R^2$** | 0.8803 |
| | | **Adeq Precision** | 26.9074 |

The Predicted $R^2$ of 0.8803 is in reasonable agreement with the Adjusted $R^2$ of 0.9775; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 26.907 indicates an adequate signal. This model can be used to navigate the design space. In the figure 40 is shown the Predicted vs Actual plot:

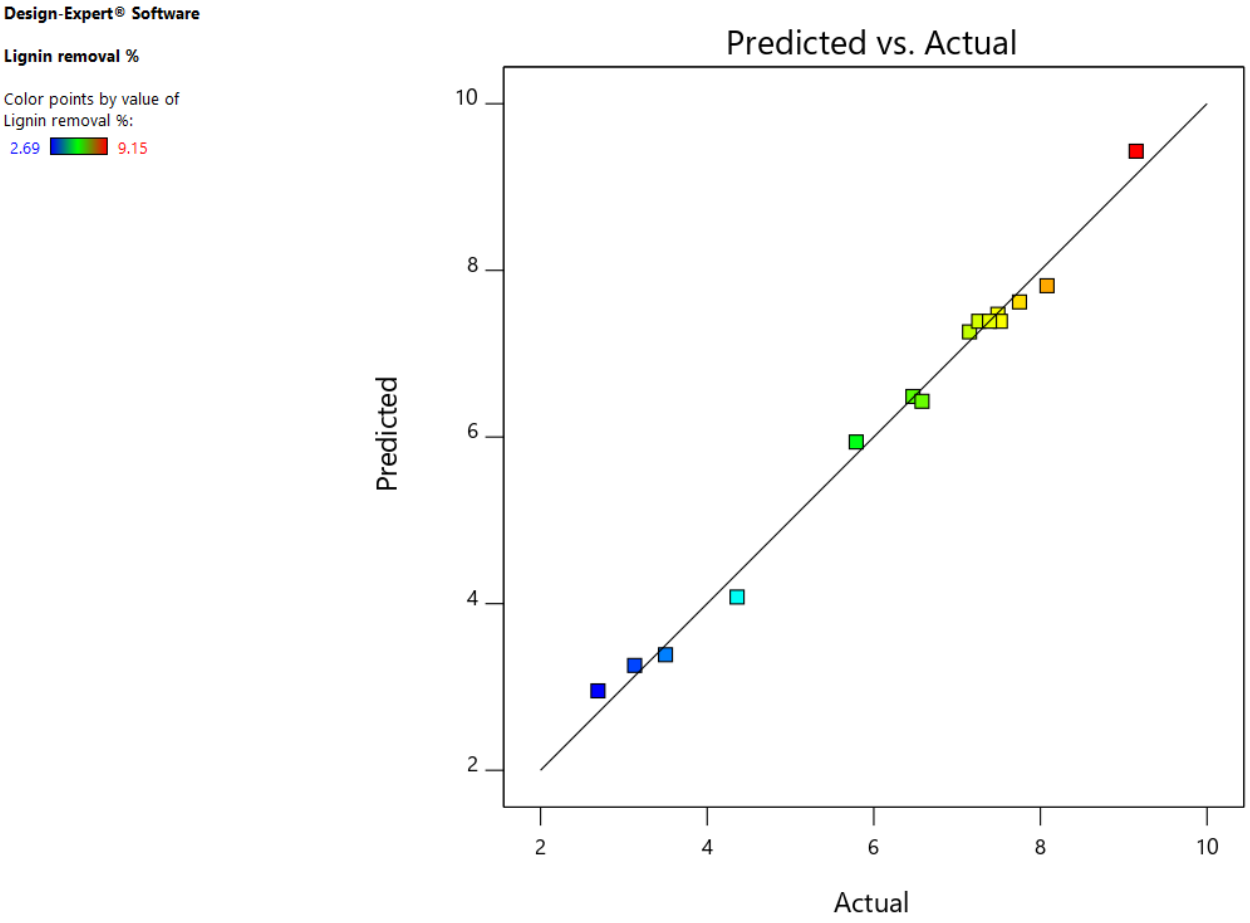


**Figure 40.** Predicted vs Actual plot of lignin removal determination

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients:

Lignin removal %= $7.39+2.31*A+0.3688*B-0.1250*C+0.7150*AB+0.0275*AC-0.975*BC-1.26*A^2-0.09*B^2-0.7175*C^2$

The coefficients of the equation indicate that the removal of lignin is facilitated by a higher concentration of GVL in the extraction mixture and that the temperature also plays an important role, although less prevalent. As suggested by Gaudino et al. 2018, the sonication time, even if extended beyond a certain limit, has no additional effect in terms of delignification.

The 3D surface plots that graphically relate each pair of variables studied with the lignin removal process are shown in the Figure 41 below:

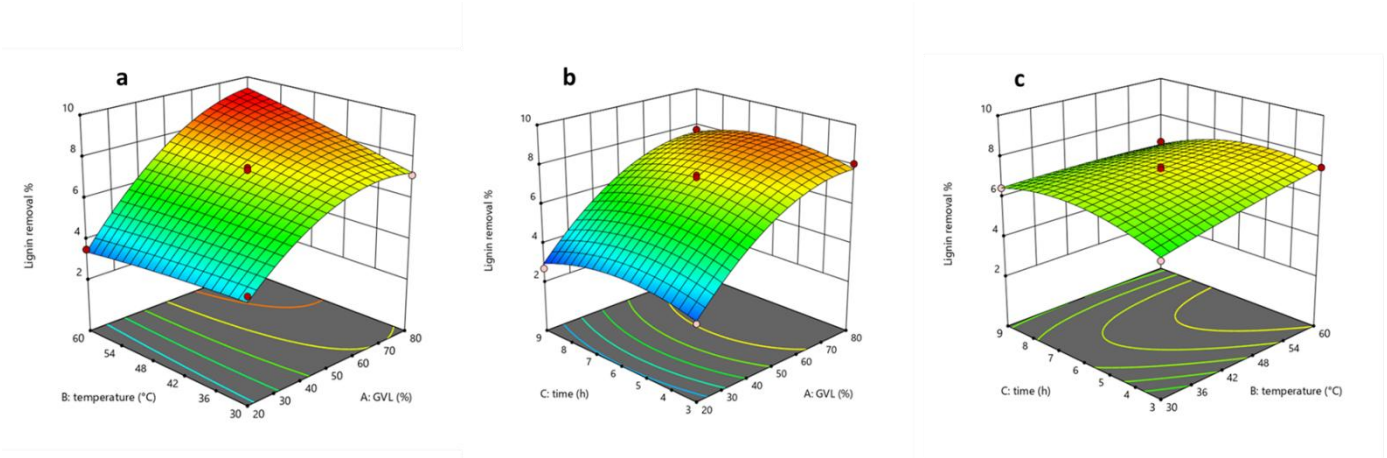


**Figure 41.** Variation of the% of precipitation following the variation of the indicated parameters (**a**: GVL%, temperatures; **b**: GVL%, time; **c**: temperature, time)

Since the highest precipitation yield (15.15%) was obtained with the combination of the parameters 80% GVL - 60 °C - 6 hours, this solid residue (LWS8) was the one selected for the subsequent characterization steps. This combination of values is also suggested by the Design expert® software optimization option to maximize the precipitation yield as the parameters vary in the intervals considered. The same combination of parameters was also tested on the non-pretreated starting substrate but with negligible precipitation results. This evidence suggests that the steam-explosion process as it was carried out by us, in addition to allowing a practically quantitative hydrolyzability of the cellulose, allows to remove part of the residual lignin by means of green solvents. The results in terms of precipitation yield from raw material (WS) and steam exploded wheat straw (SEWS) are reported in Table 21 below:

**Table 21.** Comparison between the precipitation yields on raw material wheat straw (WSRM) and steam exploded wheat straw (SEWS) under the selected ultrasound extraction conditions (80% GVL, 60°C, 6h)

| Biomass | Solvent | Volume (ml) | Ratio S/L % | T(°C) | T(h) | Precipitation yield % |
|---|---|---|---|---|---|---|
| WS | GVL/$H_2O$ 80:20 | 40 | 5 | 60 | 6 | <0.1 |
| SEWS | GVL/$H_2O$ 80:20 | 40 | 5 | 60 | 6 | 15.15 |

In the literature there are some studies that optimize the fractionation of lignocellulosic biomass using GVL as a green solvent, obtaining higher precipitation yields than those found in this work. The purpose of this experiment, however, was to test a process at a mild temperature while normally the processes studied by other authors involved the use of temperatures between 120 °C and 200 °C in a reactor or under microwave radiation (Angelini et al., 2017). The use of the ultrasound system normally implies the use of milder thermal conditions of the process for reasons intrinsic to the use of the instrument itself. This experiment was therefore conceived as a preliminary cognitive investigation of the use of ultrasound and green solvent potentials on the fractionation of lignocellulosic biomass pretreated in steam explosion and not as an absolute improvement of the process yields using more severe conditions. Furthermore, the advantage of using milder extraction conditions could result in lignins that can be obtained with linking distributions that better reflect natural lignin (Zijlstra et al. 2019).

### 7.4.3 Lignin extraction with Deep Eutectic Solvents (DES)

Deep eutectic solvents are mixtures of two or more compounds with a freezing point well below the melting point for any of the original mixture components. For extraction, deep eutectic solvents that are liquid at room temperature are the most interesting. A large number of compounds have been used to prepare deep eutectic solvents.

DES systems possess several attractive properties, such as low volatility, low toxicity, high thermal stability and are less expensive with respect to ionics liquid. On the other hand, DES need to be mixed at suitable molar ratios and could have low extraction yields. Most DES pre-treatments of herbaceous biomass have focused on delignification and biomass fractionation. Wheat straw biomass has a thinner cell wall and greater porosity than woody biomass facilitating the lignin extraction process. It has been

shown that the quantity of ethereal bonds decreases considerably with the increase of the treatment temperature. These features can facilitate lignin extraction and the removal of wheat straw components during DES treatment. (Lou et al., 2019). Several tests were carried out on both, exploded biomass and raw material with ChCl/LA, ChCl/OA and ChCl/Urea using the ultrasonic bath extraction technique at T = 60 °C . The comparison between the raw material and the steam-exploded biomass show that the pre-treatment is fundamental to lignin recovery.

Extraction tests were carried out after preparation of the DES solvents using the following conditions:

**Table 22.** Experimental conditions for the preparation of DES solvents (Hydrogen bond acceptor HBA: ChCl ; Hydrogen bond donator HBD: OA, LA or Urea)

| **DES** | **ChCl/OA** | **ChCl/LA** | **ChCl/Urea** |
|---|---|---|---|
| Molar ratio ($mol_{HBA}$:$mol_{HBD}$) | 1:1 | 1:2 | 1:2 |
| Weight ratio ($g_{HBA}$:$g_{HBD}$) | 1.55 : 1 | 0.78 : 1 | 1.16 : 1 |

In this experiment, the solid consists in the exploded wheat straw grounded at 0.5 mm and then subsequently dried at 105 °C.

The extraction tests were carried out in thermoblock, using a temperature of 100 ° C, a working time of 8 hours and an agitation of 750 rpm and with a solids content of 5%, suitable for reducing inhomogeneity phenomena due to rheology of the system.

The extraction set-up are datailed in the Table 23:

**Table 23.** Experimental conditions of extraction of wheat straw with ChCl/OA (1:1); ChCl/LA (1:2) and ChCl/Urea (1:2)

| DES | Abbreviation | Biomass(g) | DES(g) | T (°C) | Time (h) | Stirring (rpm) |
|---|---|---|---|---|---|---|
| ChCl/OA (1:1) | LWS9 | 3.0 | 60 | 100 | 8 | 750 |
| ChCl/LA (1:2) | LWS10 | 3.0 | 60 | 100 | 8 | 750 |
| ChCl/Urea(1:2) | LWS11 | 3.0 | 60 | 100 | 8 | 750 |

As for the precipitation, after DES treatment, anhydrous ethanol was added to the treated wood to separate the remaining solids from the DES and dissolved organics.

The ethanol soluble fraction containing dissolved lignin and emicelluloses was collected after filtration. Adding distillated water to this filtrate precipitated lignin while the emicellulose remained in solution; twice the volume of DES solvent was used, namely 60 ml of EtOH for 30 g of biomass. The precipitation was performed using an equal volume of $H_2O$ and then washing the filter with EtOH / $H_2O$ mixture.

As can be seen in Table 24, using an initial mass of 3 g. of lignocellulosic biomass if a ChCl / OA extraction mixture is used in a molar ratio of 1:1, 0.34 g of precipitated lignin is obtained with a recovery rate of 11.3% with respect to the initial dry biomass (LWS9). By keeping the mass of the substrate unchanged using ChCl/LA as an extraction mixture in a molar ratio of 1:2, it is possible to precipitate 0.43 g. of lignin, equal to 14.2% (LWS10). Finally, using a ChCl / Urea mixture in a 1: 2 molar ratio, always keeping the initial lignocellulosic biomass quantity unchanged, it is possible to precipitate a very small quantity of lignin, 0.096 grams equal to 3.2% (LWS11). From these results it is therefore clear that the DES ChCl/LA combination (molar ratio 1: 2) is the one that allows a higher percentage of precipitation and that is close to that obtained with GVL / $H_20$ as seen previously.

**Table 24.** Results of precipitation (mass and recovery %) by using three different combinations of DES

| DES | Abbreviation | Initial dry biomass (g) | Lignin precipitated (g) | % (m/m) recovery |
|---|---|---|---|---|
| ChCl/OA (1:1) | LWS9 | 3.0 | 0.34 | 11.3 |
| ChCl/LA (1:2) | LWS10 | 3.0 | 0.43 | 14.2 |
| ChCl/Urea (1:2) | LWS11 | 3.0 | 0.096 | 3.2 |

Then the lignins extracted with DES solvents from exploded wheat straw were subjected to GPC analysis. These extractions produce a "dirtier" residue with the presence of peaks around t = 45 min and quite high polydispersivity indices. Wheat straw extracted with ChCl-OA DES was labeled as LWS9 while wheat straw extracted with ChCl-LA mixture was labeled as LWS10. The Table 25 shows the results of the analyzes in detail.

**Table 25.** Weight-Average (Mw) and Number-Average(Mn) Molecular Weight and the Polydispersity (PDI) of lignins extracted from steam exploded wheat straw

| DES extraction | Abbreviation | Mn (Da) | Mw (Da) | PDI |
|---|---|---|---|---|
| ChCl/OA (1:1) | LWS9 | 700 | 17000 | 24.3 |
| ChCl/LA (1:2) | LWS10 | 600 | 8100 | 13.4 |

The molecular weight of a lignin is a very important property that affects the lignin valorization. The determination of the distribution of molecular weights of a lignin gives the possibility to know its chemical-physical properties, providing and determining several important parameters that affect many of the characteristics of this polymer. The data relating to the polydispersivity obtained indicate that the solids obtained through this treatment are rather inhomogeneous in their molecular weights, probably suggesting that pseudo-lignis or carbohydrate-lignin complexes are also obtained.

Other authors have also focused research on this process strategy. Jablonsky et al., (2015), studied the application of deep eutectic solvent for the processing of wheat straw; the goal was to find an efficient solvent for lignin isolation. Deep eutectic solvents represent promising alternative solvents systems for the treatment of biomass. The yield ranged from 59.1 % to 94.9 %. The amount of dissolved lignin is different depending on the components of individual DES. In particular Jablonsky et al. 2015 founded that a deep eutectic solvent composed of choline chloride and oxalic acid (1:1), was able to produce the best delignification results considering the decrease of lignin content (57.9%), by using mild temperature (60-80°C) in drying oven. In according with result showed in this thesis, the worst suitable eutectic mixture was found to be ChCl/urea (1:2) due to the lowest ratio of removed lignin (1.3%) founded by the authors. The main reason for this evidence was that the phenolic hydroxyl groups of lignin were deprotonated in presence of alkaline choline chloride-based DES. In fact, HBDs with extra amines/amides or hydroxyls can form more intermolecular H-bonds with HBAs, which reduces the number of free and active groups capable of interacting with lignocellulose and weakens the lignin production efficiency during DES pretreatment (Li et al., 2021). Therefore, in the present work, it was not characterized also because the material obtained was not sufficient for the purpose.

## 7.5 Lignin extraction from steam exploded cardoon

The exploded cardoon at 180°C and 10 minutes was used for this experiment. The lignin was obtained by alkaline extraction at 90°C in NaOH 1.5% solution stirred for 15 minutes and precipitated at pH 2; Table 26 shows in detail the quantities involved and the results obtained in terms of solids recovery.

**Table 26.** % recovery of lignins (exploded cardo) from alkaline extraction and precipitation at pH=2

| Dry biomass (g) | NaOH 1,5% (mL) | % S/L | Total lignin in Dry biomass (g) | Precipited residue at pH=2 (g) | % recovery |
|---|---|---|---|---|---|
| 75 | 1000 | 7.5 | 17.85 | 7.02 | 39.3 |

The process of isolating the lignin from the steam exploded cardoon at 180°C for 5 minutes produced about 7g of residue to be used for the characterization and for the enzymatic treatment tests with laccases. The percentage of precipitation with respect to all the initial dry biomass (75g) was 9.4%

while the yield relating to the recovery of the lignin is 39.3% if the residue obtained were free from other impurities.

### 7.5.1. Enzymatic treatments of lignin derived from steam exploded cardoon

Lignin modification is recognized as an important aspect of the success of lignocellulosic biomass within biorefineries. In particular, enzymatic processes are among the most attentive in the literature because they can modify the structure of lignin using oxygen as an oxidant. The enzymes used for this purpose are called laccases (EC 1.10.3.2) and can catalyze processes of polymerization or depolymerization of lignin, modify functional groups, make it more reactive or more recalcitrant, thus favoring its use as a bio-based molecule or use it as it is in other applications. One of the biggest problems of these processes relate to the low solubility of the lignin, especially at pH where the enzyme is usually most active (i.e. between 5 and 7). The most common commercial laccases have problems of specificity, thermostability and can become inactivated at alkaline pH. For this reason, a research was carried out on selective laccases, thermostable up to 70 °C-80 °C and active even at pH> 9 and the only company with these criteria was MetGEN (Finland), with two proposals having different properties thermo / alkaline. As part of the Cometa project, two commercial lacquers produced by METGEN were procured and tested. Two types of MetZyme® PURECO ™ enzymatic mixtures (013 and 038) were therefore purchased. MetGen Pureco enzymes are thermostable and / or alkalophilic laccases that allow a more efficient and specific oxidation of lignin, enhancing it under various aspects including depolymerization, degree of reactivity and potential functionalization of the final products without the aid of organic solvents or impacting processes from the point of view environmental. The enzymes were characterized in the ENEA laboratory. The determination of the proteins was carried out by the Bradford method, using BSA as a standard to derive the calibration curve. BSA and Metgen enzymes were diluted appropriately and absorbance was measured at 595 nm. The MetGen Pureco 013 enzyme appears to have a protein concentration of 0.44mg / mL while the Pureco 038 of 1.18mg / mL. The extracellular activity of the laccase was determined spectrophometrically using ABTS 10mM as substrate. The reaction mixture contained 800 µL of McIlvaine buffer at pH 4, 100 µl of ABTS and 100 µl of aqueous phase enzyme. The samples were incubated for 3 minutes. The formation of the cationic radical ABTS + was evaluated kinetically by the increase in absorbance at 420 nm. A unit of enzymatic activity (U) was defined as the amount of

enzyme which catalyzes the oxidation of 1µmol of ABTS at 30 ° C in 1 min. In Figure 42 below the absorbance values over time:

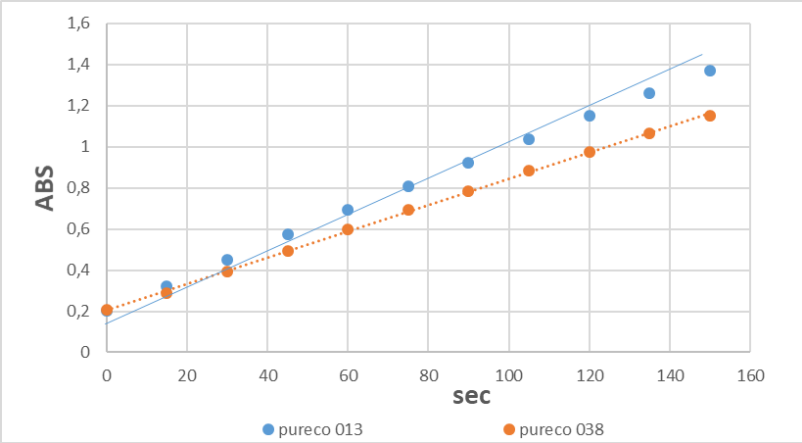


**Figure 42:** Activity kinetics of Metgen enzymes (in blue Pureco 013; in orange Pureco 038).

Through these kinetics the enzymatic activities were calculated, found to be in aqueous solution of 184 U/mL and 147 U/mL for Pureco013 and Pureco038 respectively.

The enzymatic activities at different pH were subsequently also calculated. The results are shown in the following Table 27:

**Table 27.** Enzymatic activities of Metgen enzymes in neutral aqueous solution and at various pHs.

| | **Enzymatic activity [U/mL]** | | | |
|---|---|---|---|---|
| | water | pH=4 | pH=6 | pH=9 |
| **pureco 013** | 184,0 | 2,0 | 245,8 | 131,6 |
| **pureco 038** | 147,3 | 29,8 | 337,2 | 58,9 |

Both enzymes appear to be much more active at pH = 6, and among them pureco 038 has an activity of about 337 U / mL. On the other hand, at pH = 4 there is a drastic lowering of enzymatic activity for both, while at a highly alkaline pH only pureco 013 maintains a moderate activity. The tests were carried out at room temperature. To avoid interference of carbohydrates and other organic molecules such as proteins, oils, etc, we worked on a lignin isolated from Cardoon pretreated at 180 °C for 10 minutes without acid or base catalysts. The set-up of the process involved the use of two enzymes at different pH with or without an oxidation-reduction mediator consisting of caffeic acid, a biobased molecule. Control tests without enzyme were carried out under the same conditions. The lignins obtained from these processes were characterized by different techniques. The criticalities are related to the difficulty of obtaining significant quantities of lignin and their poor ability to be solubilized even in organic solvents for a complete characterization. The IR spectrum showed that the laccase used at pH = 6 contributed to give a relative increase of the ester-type CO groups at 1735 $cm^{-1}$ compared to the generic CO stretch (Fig. 43).

The spectra of Figure 43a show that the treatment with laccase apparently does not involve great differences in the structures of the original lignins. However, a widening of the peak relative to LCY3 in the C-H stretch region (methyl or methylene groups) can be noted, which is strangely much less evident when the caffeic acid mediator (LCY5) was added. Furthermore, there are differences in relative peak areas in the region between 1200 and 1300 $cm^{-1}$, normally associated with ring vibration of syringil- and guayacil- groups or with alcoholic stretching C-O bonds, suggesting that laccase treatment, especially at pH9 (LCY6) can contribute to a significant modification of the structure (Fig. 43b).

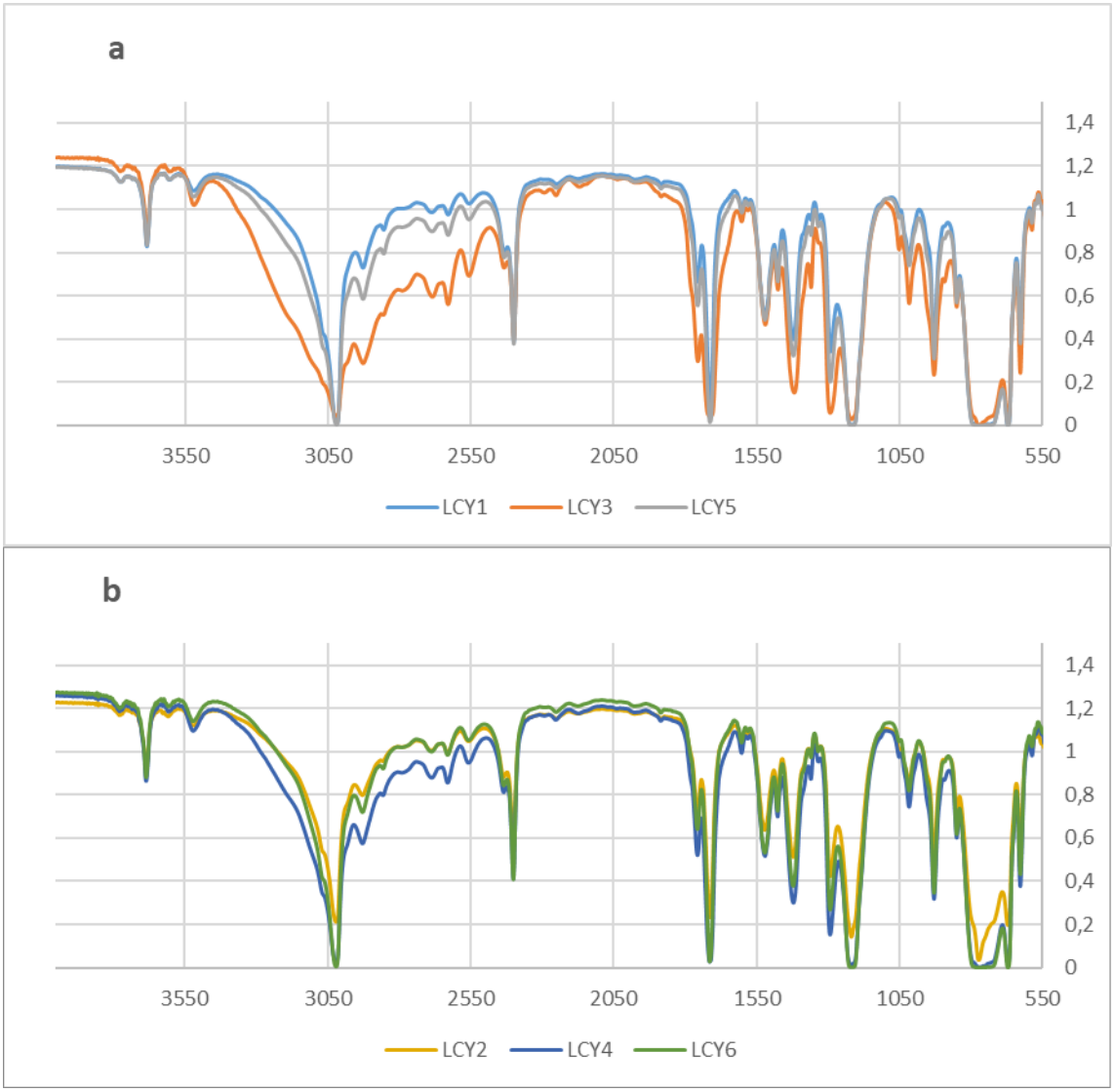


**Figure 43.** (**a**) IR spectra of lignin isolated from Cardoon (SE 180 ° 10min) treated with laccases at 60 ° C at pH = 6. Enzymes used: Pureco038 at pH6 (LCY1 = control process; LCY3 = enzymatic treatment; LCY5 = enzymatic treatment with added caffeic acid mediator); (**b**) Pureco013 at pH 9 (LCY2 = control process; LCY4 = enzymatic treatment; LCY6 = enzymatic treatment with added caffeic acid mediator). The spectrum were obtained by solubilizing the previously acetylated lignins in chloroform using a Bruker Alpha FT-IR spectrometer.

Gel permeation chromatography was used for the determination of the average molecular weights of the obtained lignins. The graphs are shown in Figure 44 while the results are shown in Table 28 and the calculated polydispersivity index are also shown in the same table. It appears that the process at pH6 induces greater variability indicating a more diverse lignin within it. It is probable that the process at pH9 favoring a greater solubilization, therefore favoring a more similar distribution.

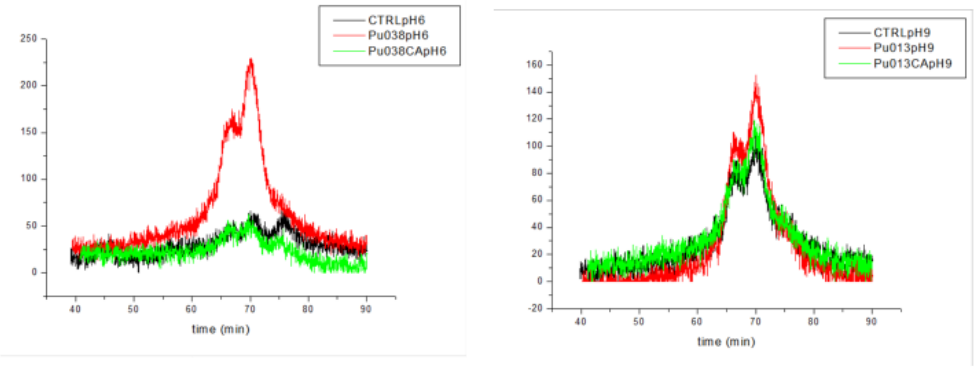


**Figure 44.** GPC spectrum of cardoon lignin treated with MetGen Pu038 and Pu013 enzymes and where indicated caffeic acid mediator (CA). Peak deconvolution was performed to identify mean molecular weights and polydispersibility index.

However, enormous noise was found in the spectra. Looking at the results obtained on the basis of these curves, it seems that the samples are not or are very poorly soluble.

**Table 28.** Determination of the average molecular weights, the number average molecular weight (Mn), the weight average molecular weight (Mw) and the polydispersivity index (iPD) of the lignins obtained after laccase treatments and compared with the control processes.

| | Control pH6 **LCY1** | Control pH9 **LCY2** | Enzyme pH6 **LCY3** | Enzyme pH9 **LCY4** | Enzyme + mediator pH6 **LCY5** | Enzyme + mediator pH9 **LCY6** |
|---|---|---|---|---|---|---|
| **MW1** | 583.61 | 620.81 | 844.24 | 503.43 | 653.16 | 747.87 |
| **MW2** | 455.08 | 514.98 | 606.91 | 609.20 | 281.94 | 606.15 |
| **MW3** | 271.44 | | | | | |
| **$M_n$** | 380.7 | 582.2 | 642.5 | 583.4 | 630.0 | 631.3 |
| **$M_w$** | 428.4 | 586.7 | 653.7 | 586.9 | 1302.9 | 631.9 |
| **iPD** | 1.12 | 1.01 | 1.02 | 1.01 | 2.07 | 1.01 |

It can be noted that at pH = 6 the areas of the signals attributed to the control process (black curve) and that relating to the process with the aid of the caffeic acid mediator (green curve) are much lower than the others. This observation in the control could be in agreement with a lower solubility of the lignin resulting from the treatment at a lower pH, while in the case of the addition of the mediator this could have favored the depolymerization and therefore the formation of soluble monomers not identifiable with this technique because it has too low a molecular weight. The use of the enzyme at pH = 6 substantially modifies the average molecular weights in the resulting residue and this determines a higher polydispersion index of the treated lignin. On the contrary, the more alkaline pH could have favored a greater solubilization of the lignin and therefore a more homogeneous process

with the obtaining of average molecular weights more similar to each other without a significant variation of the average molecular weights and of the polydispersibility index which remains low in all cases, suggesting that the enzyme did not change the internal proportion of molecular weights.

Finally we also evaluated the structure of the lignins obtained with a destructive method such as pyrolysis, which gives indications on the groups of molecules that form the lignin, in particular hydroxycoumaryl, guaiacyl and syringyl groups which differ from each other for the number of methoxyl groups in ortho position with respect to the phenolic group. With this method, a significant percentage of long-chain hydrocarbons (fatty acids and alkaloids) was found, highlighting the presence of "extractives", due to the precipitation of fatty acid molecules during acidification (the so-called Tall Oil); the chromatographic signal of these is on average predominant over the other signals, those corresponding to the characteristic peaks of the pyrolysis of a lignin.

Table 29 shows the quantitatively significant compounds in the chromatogram and the data relating to the yield of the pyrolysis process (i.e. how many % of molecules were actually pyrolyzed with respect to the initial dry substrate) and the ratio between the syringyl and guaicyl units determined.

**Table 29.** GC-MS determination of the main monolignols following pyrolysis of the lignins pretreated with MetGen enzymes at pH=6 (LCY3 and LCY5) and pH=9 (LCY4 and LCY6). Comparison with the control substrate without enzymatic treatment (LCY1 and LCY2)

| | control pH6 | control pH9 | enz pH6 | enz pH9 | enz+CA pH6 | enz+CA pH9 |
|---|---|---|---|---|---|---|
| | LCY1 | LCY2 | LCY3 | LCY4 | LCY5 | LCY6 |
| **1,2- pentadiene** | 5.7 | nd | 0 | nd | 0 | nd |
| **octanoic acid** | 1.8 | nd | 0 | nd | 0 | nd |
| **1-penten-3-ethyl** | 1.4 | 2.6 | 0 | 0 | 0.4 | 0 |
| **decanoic acid** | 1 | nd | 0 | nd | 0,7 | nd |
| **dodecanoic acid** | 0 | 0 | 0.2 | 5.6 | 0 | 0 |
| **2-pentadecannone-6,10,14-trimethyl** | 0 | nd | 0,4 | nd | 0 | nd |
| **tetradecanoic acid** | 1.4 | nd | 0 | nd | 1.2 | nd |
| **palmitic acid** | 24.5 | 36.3 | 2.6 | 56 | 21 | 11.5 |
| **linoleic acid** | 5.7 | 3.9 | 2.5 | 8.8 | 9.7 | 11.2 |
| **octadecanoic acid** | 8.9 | 5.4 | 2 | 11 | 9.6 | 8.3 |
| **hexacosane** | 2.3 | 1.5 | 1.6 | 5 | 5.3 | 16.6 |
| **1-eicosanol** | 2 | 3.4 | 2.7 | 0 | 3 | 7.2 |
| **heptacosane** | 2.6 | nd | 7.1 | nd | 5 | nd |
| **squalene** | 4.2 | nd | 12 | nd | 0 | nd |
| **tetracosane** | 10.5 | nd | 0 | nd | 0 | nd |
| **nonanale** | nd | 2.5 | nd | 0 | nd | 0 |
| **4-hexen-2-one** | nd | 0.8 | nd | 0 | nd | 0 |
| **cyclotetradecane** | nd | 3.1 | nd | 3 | nd | 0 |
| **hentriacontane** | nd | 12 | nd | 0 | nd | 12.5 |
| **2-methoxy-4-vinylphenol** | 1.1 | 0.2 | 0.35 | 2.1 | 1.3 | 4.5 |
| **vanillin** | 0.8 | 0 | 0.3 | 1 | 0 | 0 |
| **ethanone-1-(4-hydroxy-3,5-dimethoxyphenyl)** | nd | 2.6 | nd | 3.2 | nd | 0 |
| **1,2 benzendicarboxylic acid diethyl ester** | nd | 6.4 | nd | 0 | nd | 0 |
| **1-ethenyl-1,3-benzendiol** | nd | 0 | nd | 0 | nd | 5.6 |
| **cyclopentanol-2-methyl** | nd | 1 | nd | 0 | nd | 0 |
| **% yield of pyrolysis** | **50** | **25** | **25** | **18** | **25** | **20** |
| **S / G ratio** | **0.4** | **0.7** | **0.3** | **0.2** | **0.2** | **0.1** |

The reported S / G values refer to molecules extrapolated from the chromatogram but not all reported in the table because they are quantitatively insignificant. In particular, with the use of laccase we highlight a decrease in the ratio between S / G and therefore a relative increase in group G to which,

from previous literature studies, a plasticizer role is associated during the hot pressing operation of panel formation. Figures 45a and 45b below graphically show the main derivatives of lignin obtained as a result of pyrolysis divided by type of enzymatic treatment.

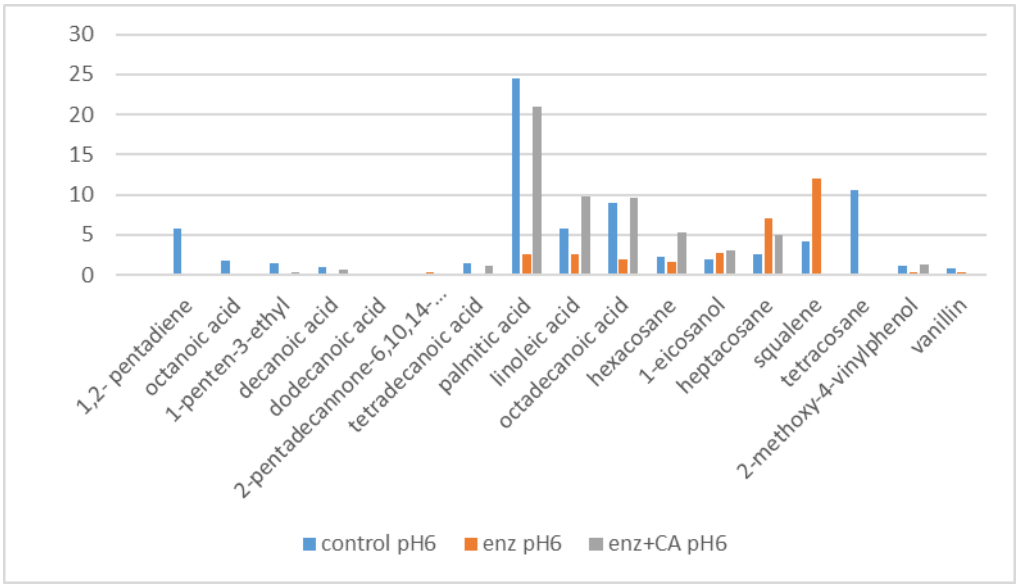


**Fig. 45a:** GC-MS determination of main monolignols following pyrolysis of lignins pretreated with Pureco038 enzyme

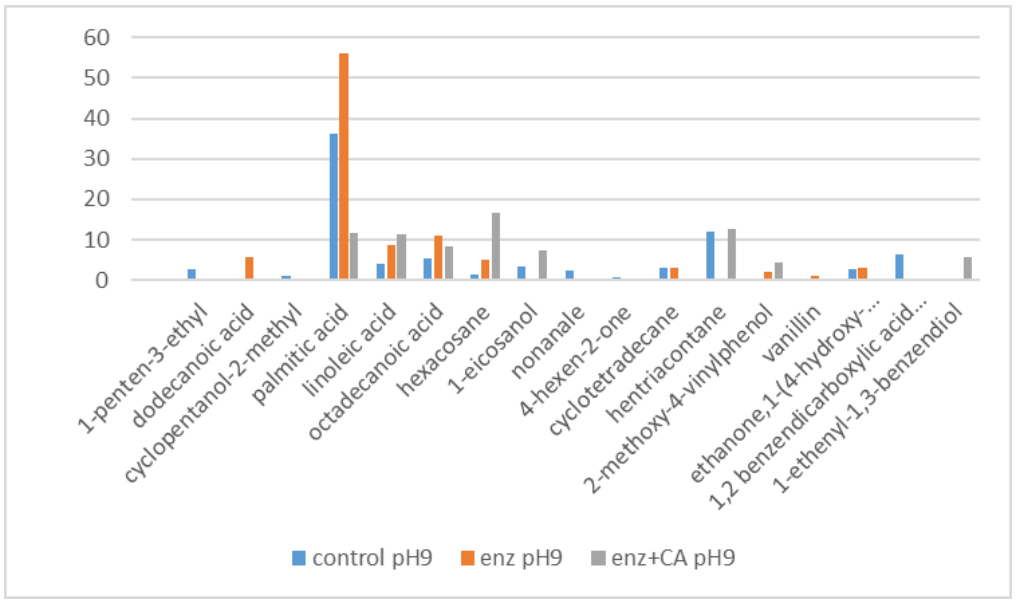


**Fig. 45b**: GC-MS determination of main monolignols following pyrolysis of lignins pretreated with Pureco013 enzyme

The use of laccases and / or mediators in general seems to imply a decrease in the S / G ratio with therefore a relative increase in the -G group which is associated with a role of plasticizer during hot pressing. Furthermore, acid and base catalyzed SE tests have already been carried out with severity factors similar to those used up to now to evaluate the goodness of these pretreatments on the final panel.

## 7.6 NMR Characterization Of Lignin

One-dimensional 1-D NMR methods, including $^{1}H$, and $^{31}P$ NMR, the latter only after derivatization, now became important techniques for the characterization of a lignin, and could provide an analysis of the distribution of functional groups and their amounts and H / G / S units as well as other components and characteristic bonds of lignin can be determined qualitatively and quantitatively, although for these purposes it is preferable to use 2-D NMR. The chemical shifts of functional groups in a lignin in the $^{1}H$ $^{13}C$ and $^{31}P$ NMR spectra have been well established. Neverthless, 1-D NMR is not exempt from drawbacks, and excessive spectral overlap deserves especially to be mentioned here. 2-D NMR provides additional signal dispersion and is therefore far more versatile in terms of unambiguous signal assignment. $^{1}H$-$^{13}C$ HSQC NMR is commonly applied for lignin characterization in order to identify interunit linkage motifs; applying dedicated pulse-sequences, direct quantification is possible.
Initially, $^{1}H$ NMR tests were done in DMSO-$d_6$ but all spectra were not significant, and therefore not shown. Subsequently, deuterated chloroform was used, and although many solubility problems were not overcome, some spectrum were detected.

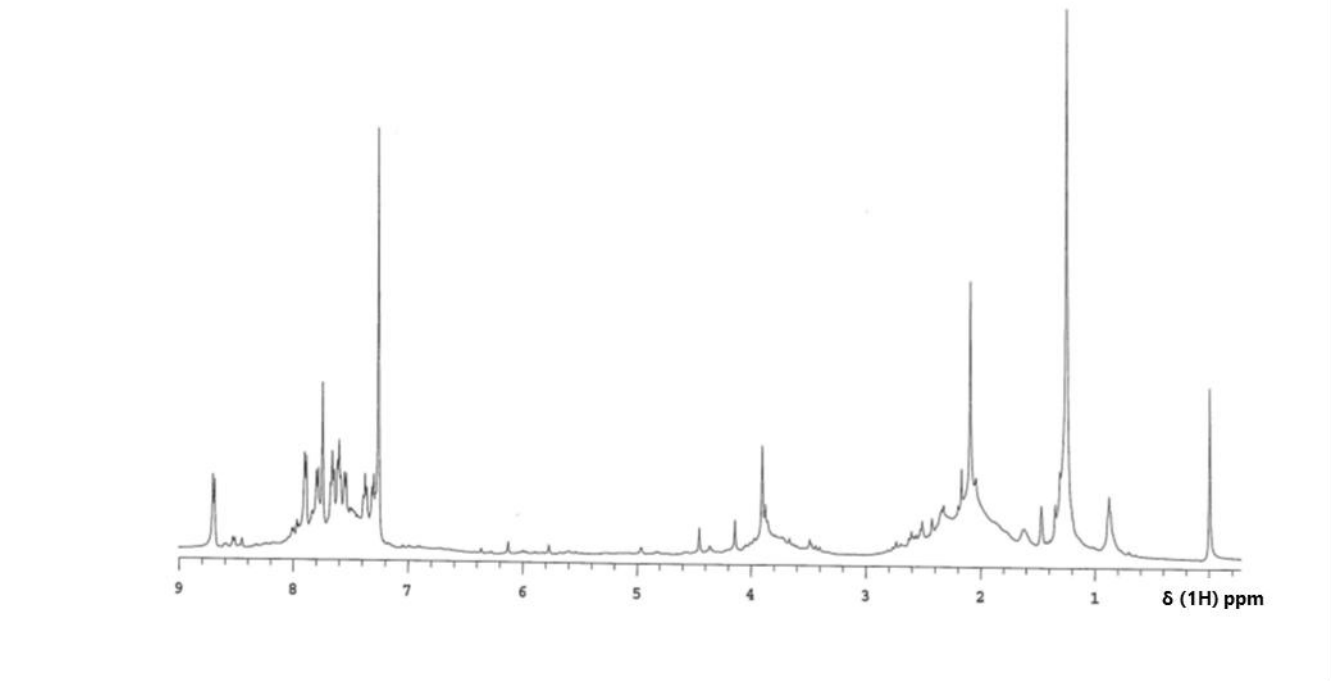


**Figure 46.** $^{1}$H NMR spectra of lignin obtained at pH7 after fractionated precipitation (LWS1).

Figure 46 shows the spectrum of LWS1 lignin (fractionated precipitation pH 7); making a comparison with the data present in the literature (Liao et al., 2020) it is possible to see signals in the aromatic zone between 6.0 ppm and 8.0 ppm. The signal intensity peak for aromatic proton in S and G units of lignin structure observe at the range between 6.2 to 8.0 ppm. Between 3.4 ppm and 4.2 ppm there are singlets due to the presence of methoxy groups O-$CH_3$; in the region between 1.2-2.8 acetyl group peaks and aliphatic moieties attribute at region 0.8-1.5 ppm were found. The peak at 7.26 for $CDCl_3$ is huge considering the concentration of substrate used (30 mg in 0,4 ml of solvent). This confirms that there are great problems of solubility of the obtained substrate.

The spectrum of LWS2, LWS3, LWS4 and LWS8 (fractional precipitation at pH5, pH4, pH2 and lignin dissolved in GVL) were not shown because the quality of the spectra technically does not allow for any meaningful discussion most likely due to poor substrate solubility.

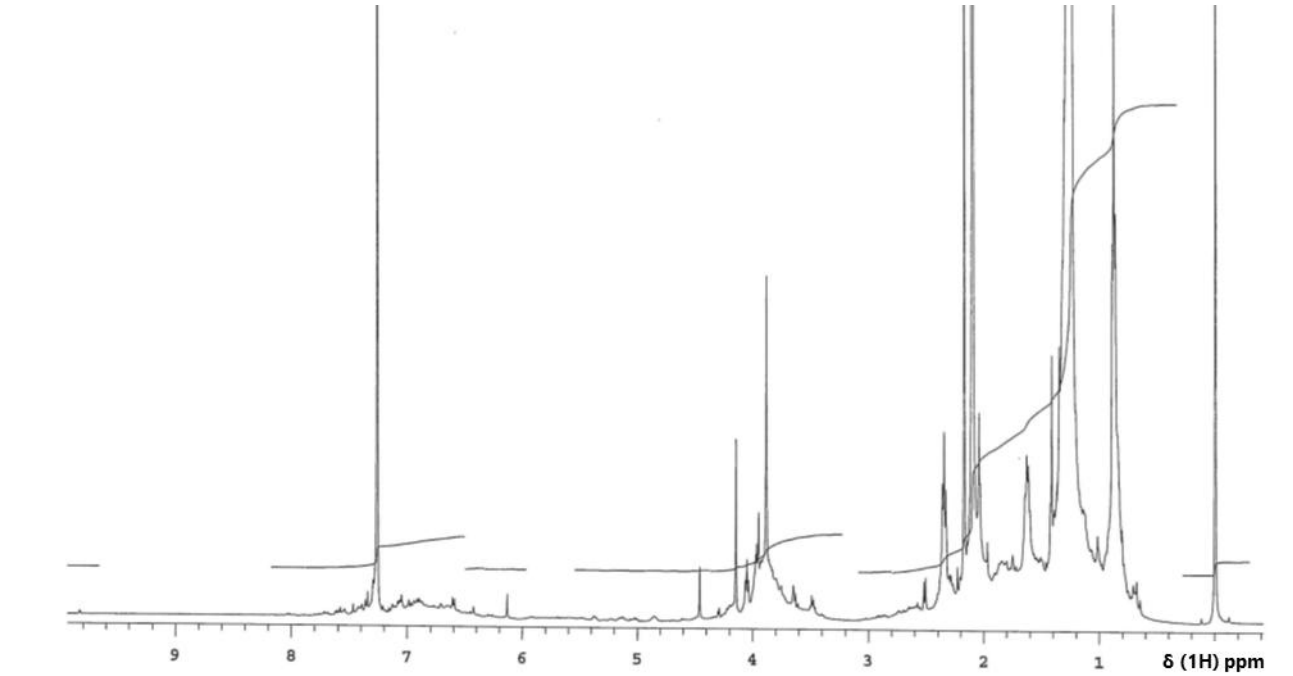


**Figure 47.** $^{1}$H NMR spectra of lignin obtained after precipitation at pH2 (LWS5).

Figure 47 shows the spectrum of LWS5, lignin precipitated directly at pH 2; making a comparison with the data present in the literature (Liao et al., 2020) it is possible to see signals in the aromatic zone between 6.0 ppm and 8.0 ppm. In particular, the presence of signals 6.2 ppm, 6.4 ppm and 6.6 ppm can be noted, the signal at 7.26 is that relating solvent to dissolve the lignin samples; unlike the previous spectra, three signals are noted instead of one relating to peaks 6.2 and 6.4 ppm and a doublet at 6.6 ppm; these signals can be attributed to the aromatic protons of the lignin structure together with signals up to 8 ppm. Between 3.5 ppm and 4.2 ppm there are singlets due to the presence of methoxy groups $O\text{-}CH_3$ found in wheat straw, in particular at 3.8 ppm. In the region between 1.2-2.5 acetyl group peaks are present while the aliphatic fractions are included in the region 0.8-1.6 ppm. However, the intensity of the aliphatic peaks with respect to the peaks relating to the methoxyl groups and especially to the aromatic zone indicates that most of the solubilized substrate is not composed of lignin but of other precipitated components, such as carbohydrates or their derivatives.

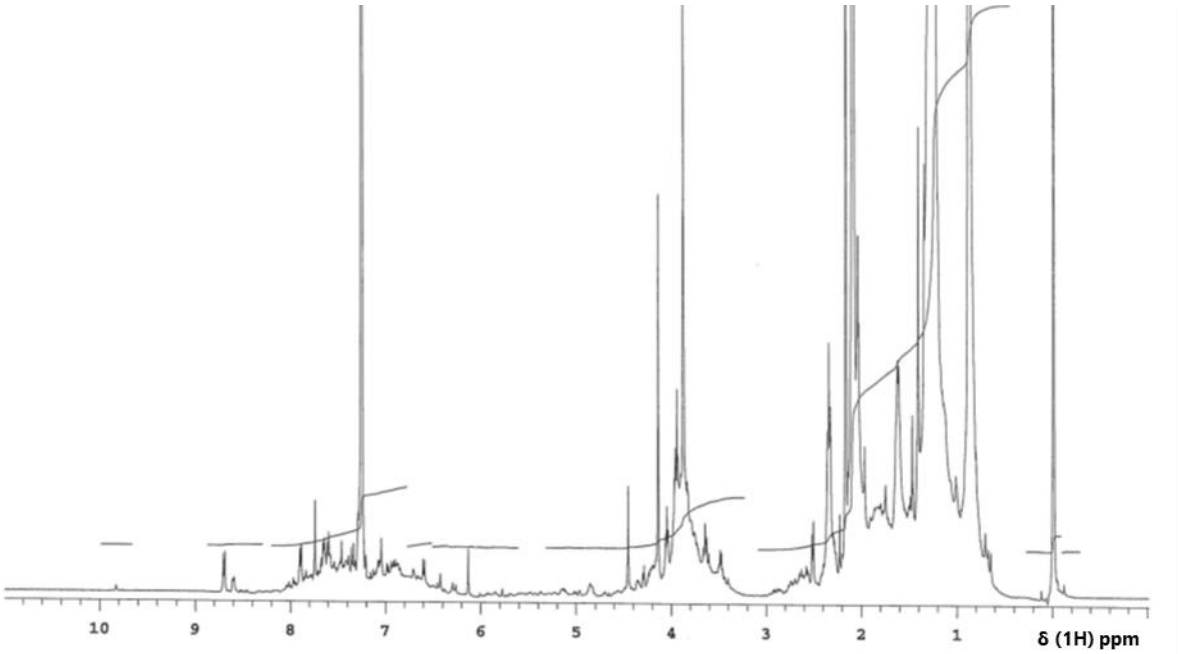


**Figure 48.** $^{1}$H NMR spectra of lignin obtained after precipitation at pH5 (LWS6).

Figure 48 shows the spectrum of LWS6 lignin precipitated directly at pH 5; in this case numerous signals can be seen in the aromatic zone between 7 and 8 ppm, a singlet at 9.9 ppm due to the presence of carboxylic groups, between 8.5 ppm and 8.8 ppm, probably further aromatic signals. Multiple signals are observed around 4 ppm, the most relevant around 3.8 ppm due to methoxy groups. Between 4 and 6.2 ppm there are signals due to the presence of hydrogens and the hydroxyl group in the β-O-4 structure. In the region between 1.2-2.8 acetyl group peaks are present while the aliphatic fractions are included in the region 0.8-1.8 ppm. This is probably the most significant spectrum presented so far in $^{1}$H NMR as the intensity of the aromatic peaks is significant. A direct precipitation at pH5 therefore seems to favor the dissolution of the substrate in deuterated chloroform with higher relative percentages of lignin with respect to the previous results.

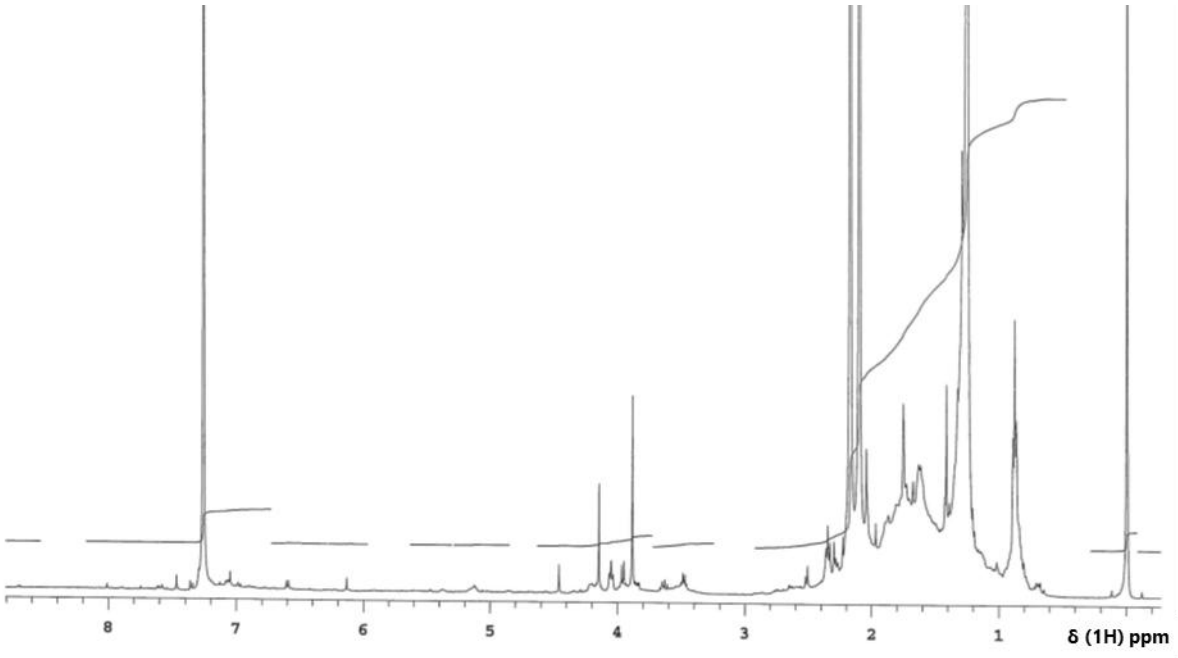


**Figure 49.** $^{1}$H NMR spectra of lignin obtained after enzymatical hydrolysis process (LWS7).

Figure 49 shows the spectrum of LWS7 lignin (enzymatically hydrolyzed residue). Due to the un-hydrolyzed cellulose and other carbohydrate residues, unfortunally, also this substrate was very poor soluble. The spectrum, showing the soluble part of the material in $CDCl_3$, represents sugars and extractives. Only minor part is lignin. At 4 ppm there are singlet due to the presence of methoxy groups O-$CH_3$ found in wheat straw. Overall, this spectrum is not to be considered significant to comment on a lignin-based structure.

Another characterization that can give useful indications of the morphology of lignin, the presence of carbohydrates and other information is the $^{31}$P NMR technique. $^{31}$P NMR spectroscopy is a promising technique for the determination of different hydroxyl groups in different lignin fractions. In current biorefineries, evaluating the chemical reactivity of lignin is indeed very important, which is closely related to the efficiency and effect of chemical modification. In addition, $^{31}$P NMR spectroscopy has a unique advantage in the determination of hydroxyl groups in lignin, high signal resolution, and high accuracy. Instead of determining the aromatic hydroxyl groups by other, complicated methods, $^{31}$P NMR spectroscopy allows the recognition of phenolic hydroxyl groups in the S, G, and H units, aliphatic hydroxyl groups, and carboxyl groups in a convenient approach. More importantly, these functional groups are significant for the chemical modification of lignin with the aim of developing lignin-based functional polymers (Sun et al., 2020b).

The $^{31}$P-NMR spectra relating to the substrates obtained from wheat straw are shown below in figures 50-53. Unfortunately, due to the absence or poor solubility, not all the spectra obtained are representative and have thus not been shown.

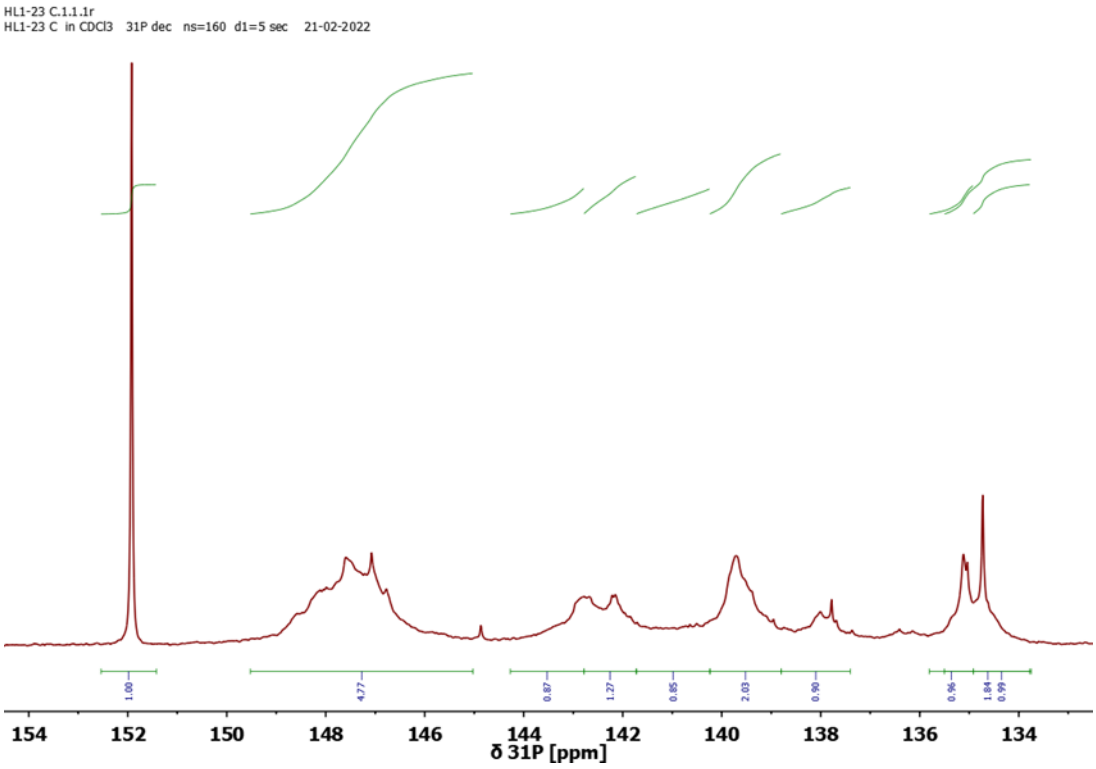


**Figure 50.** $^{31}$P-NMR spectra of lignin obtained at pH4 after fractionated precipitation at pH4 (LWS3)

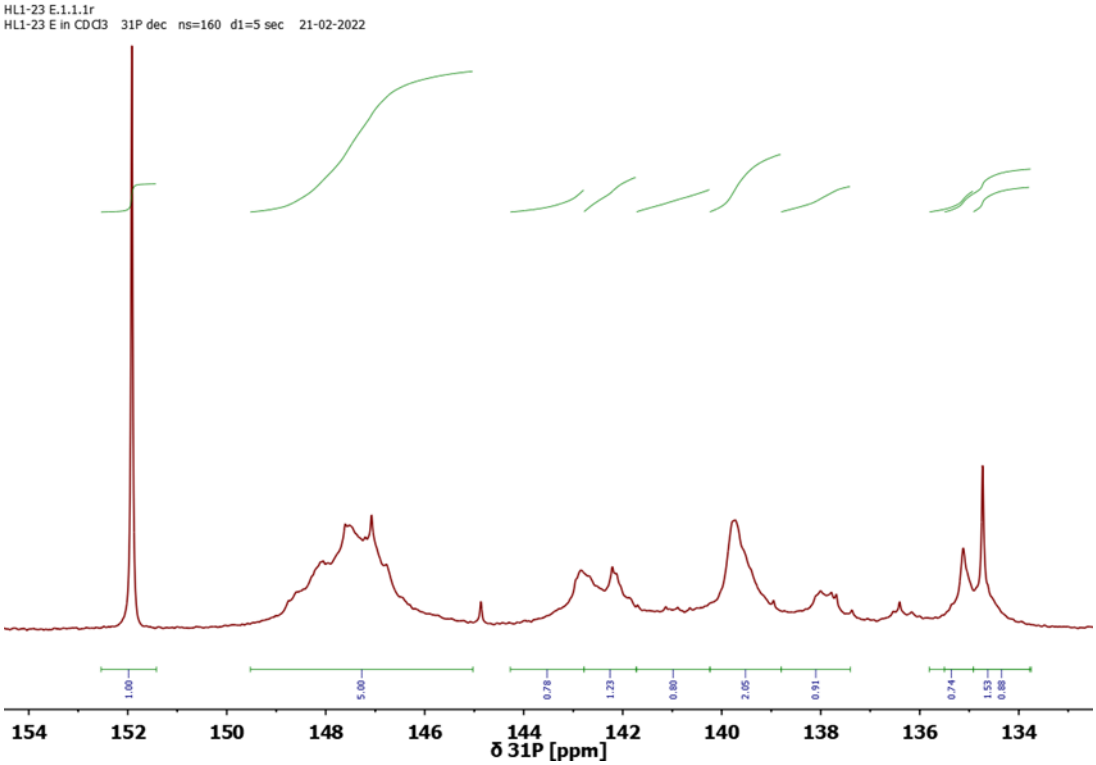


**Figure 51.** $^{31}$P-NMR spectra of lignin obtained after precipitation at pH2 (LWS5)

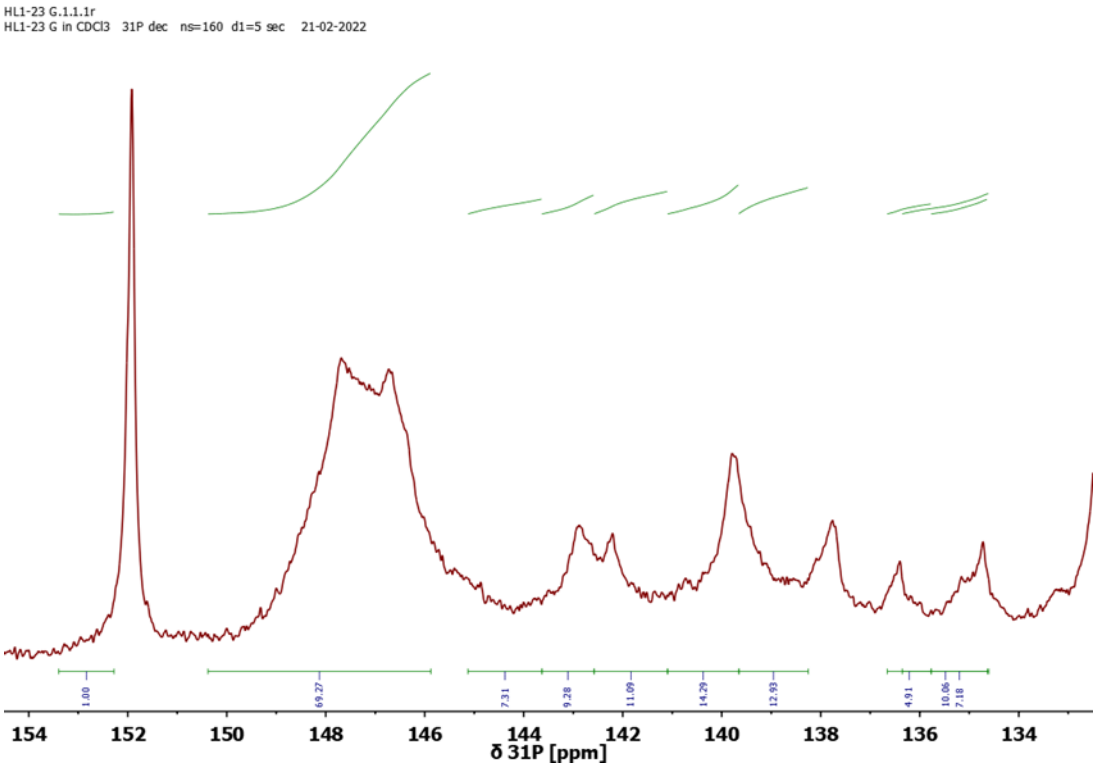


**Figure 52.** $^{31}$P-NMR spectra of lignin obtained from enzymatical cellulose hydrolys of wheat straw (LWS7)

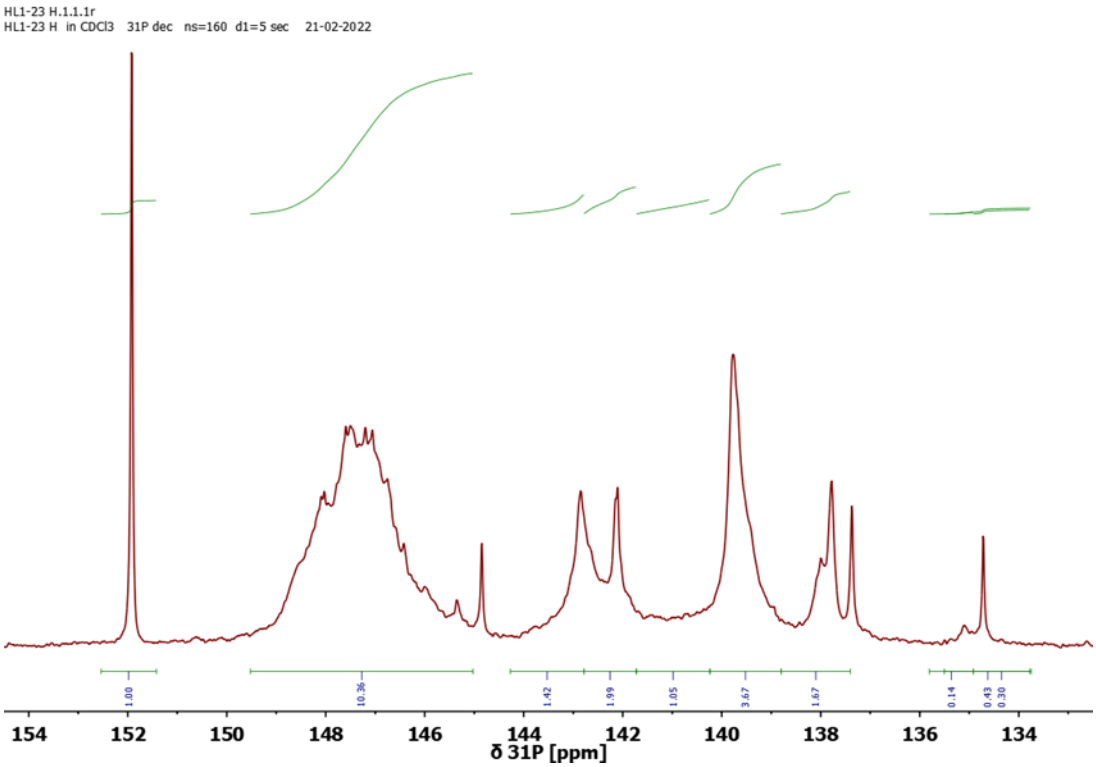


**Figure 53.** $^{31}$P-NMR spectra of lignin obtained from GVL extraction (LWS8)

In all the spectra showed, the internal standard used for the analysis is observed at 152 ppm. Between 148 and 146 ppm there are signals attributable to aliphatic OH groups (main indication of the presence of carbohydrates), phenolic groups are detectable in the range 144.27-140.27 ppm for condensates, 140.24-138.8 ppm for G- type, 138.8-137.4 ppm for H-type, while in the range 135.5-134.0 ppm there are signals relating to aliphatic and aromatic carboxylic groups (which can derive for example from ferulic acid or from residual fatty acids or other impurities). Table 30 summarizes the data obtained from the analysis of the spectra.

**Table 30.** Attribution and quantification of the signals derived from the spectra obtained by $^{31}$P-NMR on wheat straw substrates. (*data related to the LWS1 and LWS4 lignins are to be considered not representative due to the poor solubility of the substrate)

| sample | Aliphatic -OH [mmol/g] | Aromatic -OH [mmol/g] | | | | Acidic -OH [mmol/g] |
|---|---|---|---|---|---|---|
| | | condensed | G-type | H-type | total | |
| LWS1* | 0.69 | 0.35 | 0.26 | 0.13 | 0.73 | 0.25 |
| LWS3 | 1.83 | 1.14 | 0.78 | 0.34 | 2.27 | 0.70 |
| LWS4* | 0.71 | 0.55 | 0.40 | 0.20 | 1.15 | 0.59 |
| LWS5 | 1.93 | 1.08 | 0.79 | 0.35 | 2.22 | 0.59 |
| LWS7 | 6.56 | 2.24 | 1.28 | 0.96 | 4.49 | 0.81 |
| LWS8 | 3.93 | 1.69 | 1.39 | 0.63 | 3.72 | 0.16 |

In particular, higher ratio between aromatic and aliphatic phenolic groups in lignins derived from alkaline extraction (LWS3, LWS5) was observed with respect to the substrates obtained from enzymatic hydrolysis or extraction with GVL (LWS7, LWS8). This could indicate a greater presence of carbohydrates in the latter substrates, and this evidence indicates that the solvent GVL does not appear to have acted selectively as expected in agreement with the poor solubility highlighted during the acquisition in $^{1}$H NMR.

The following figure 54 shows the $^{31}$P NMR spectra of lignins treated with laccase enzymes (LCY3, LCY4) and mediators (LCY5, LCY6) compared with the control substrate while Table 31 indicates the attribution of quantified signals.

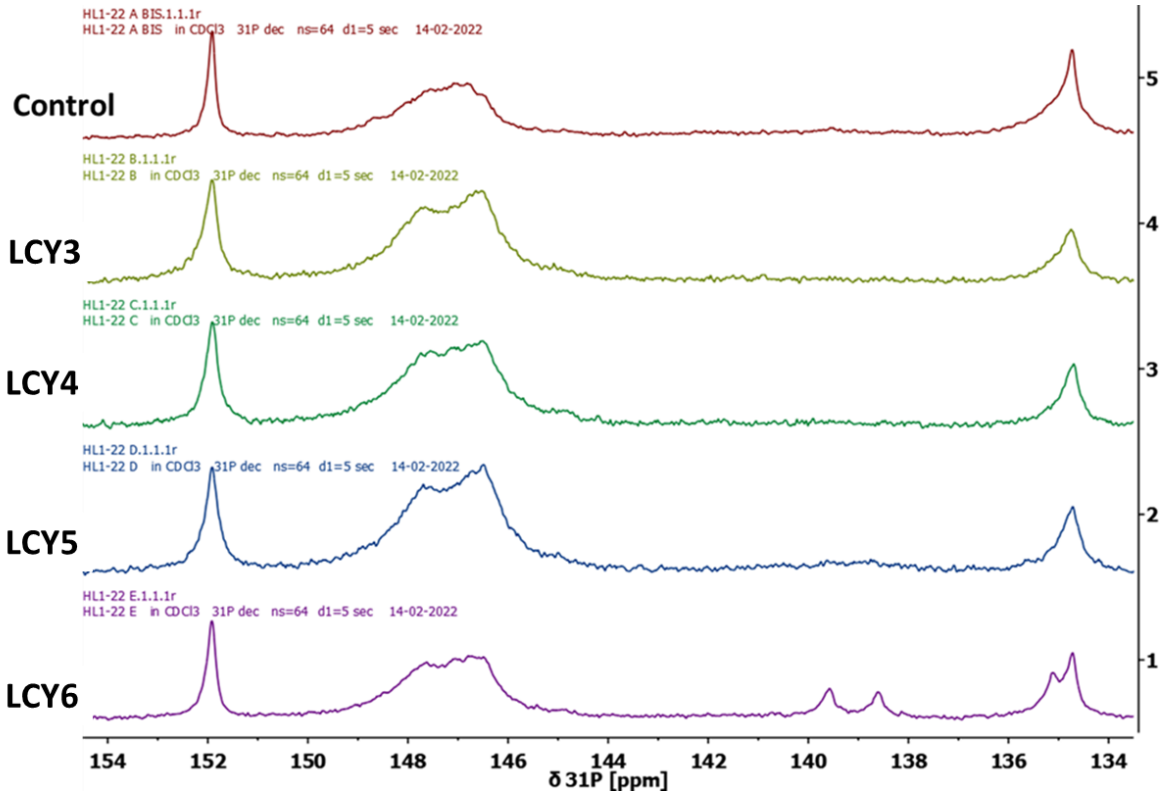


**Figure 54.** $^{31}$P-NMR spectra of lignin obtained from cardoon (Control: lignin precipitated at pH2 after alkaline treatment of cardoon steam exploded biomass; LCY3: laccase treatment at pH6; LCY4: laccase treatment at pH9; LCY5: laccase treatment at pH6 with caffeic acid as mediator; LCY6: laccase treatment at pH9 with caffeic acid as mediator)

**Table 31.** Attribution and quantification of the signals derived from the spectra obtained by $^{31}$P-NMR on cardoon substrates.

| **sample** | **Aliphatic -OH [mmol/g]** | **Aromatic -OH [mmol/g]** | | | | **Acidic -OH [mmol/g]** |
|---|---|---|---|---|---|---|
| | | **condensed** | **G-type** | **H-type** | **total** | |
| LCY1 (control) | 1.63 | 0.24 | 0.13 | 0.10 | 0.47 | 0.74 |
| LCY3 | 1.82 | 0.15 | 0.04 | 0.04 | 0.24 | 0.30 |
| LCY4 | 1.90 | 0.21 | 0.08 | 0.06 | 0.34 | 0.35 |
| LCY5 | 2.07 | 0.21 | 0.12 | 0.10 | 0.43 | 0.36 |
| LCY6 | 1.89 | 0.21 | 0.23 | 0.15 | 0.59 | 0.54 |

The spectra in figure 54 shows a high ratio between the internal standard and the phenolic peaks, which is typically an indication of low solubility, while table 31 shoes that all the samples, including the control, are rich in impurities probably due to the control substrate rich in aliphatic components. The analysis carried out in this paragraph indicate that some lignins deriving from wheat straw have higher purity with respect to the substrates derived from cardoon, which seems to be intrinsically very insoluble and rich in impurities.

# 8 Conclusions

This work allowed to investigate some aspects concerning the fractionation of two lignocellulosic biomasses, in particular the wheat straw and the residual biomass of cardoon. In particular, the study was focused on the isolation and characterization of the lignins. In order to deconstruct the biomass, an acid catalyzed steam explosion pre-treatment was used in a batch reactor located inside the ENEA Trisaia research center in Basilicata (Italy). The lignin deriving from wheat straw was extracted with different techniques, from the most traditional to the most innovative. In particular, after its alkaline solubilization and the consequent formation of a soda liquor, the lignins were precipitated with sulphuric acid. A classical precipitation involved the recovery of the lignins after lowering the pH directly to pH2 or pH5, while on other aliquots of black liquor a fractional precipitation from pH7 to pH2 was tested, separating the precipitate obtained at each pH. The higher precipitation yields were obtained at pH5 and pH4 (26% and 33% respectively) while only 7% was obtained acidifying up to pH2. FT-IR spectroscopy of these lignins revealed strong wide band within the range 3700 $cm^{-1}$ assigned to hydroxyl groups in phenolic and aliphatic structures. Several picks highlighted the presence of typical groups of lignins extracted with the alkaline method from wheat straw, and in particular attributable to the S- and G units groups. The thermogravimetric analyzes showed that the lignin from enzymatic hydrolysis is more thermostable but contains a lot of ashes. The analyses in GPC showed that the average weight average molecular weights of these lignins were between 700 and 2000 Dalton, with the exception of the substrate produced through enzymatic hydrolysis in which molecular weights even 40 times higher were identified and this was attributed to large quantity of residual carbohydrates or complex between lignin and carbohydrate which inevitably produced a substrate with a high polydispersivity index compared to the other lignins obtained. Another extraction strategy was adopted with renewable solvents, such as gamma-valerolactone (GVL) and some mixtures of deep eutectic solvents. The extraction with the GVL was optimized using a statistical approach to study in detail temperatures, process times and the percentage of solvent itself. The best combination of parameters in the considered range precipitated about 15% of material. This result is lower than those found in the literature. Furthermore, the characterization of the substrate showed that low quality lignin was obtained as there was co-precipitation of carbohydrates and other impurities. This experimentation can provide data to improve the extraction process with this type of so-called green solvents.

The study of cardoon biomass was instead dedicated to the modifications of specific enzymes on the structure of lignin. The starting lignin was produced through acidification of a liquor soda obtained

after the steam explosion of the residual cardoon biomass. The enzymatically treated lignins did not show large variations in terms of average molecular weight compared to the original lignin. On the other hand, pyrolysis, has shown that the enzymatic treatment, especially if mediated by redox catalysts, is able to decrease the S/G ratio of the lignins. The relative increase in guayacil groups is usually associated with an increase in the adhesiveness properties of lignin and this evidence may suggest the use of these substrates in the formation of binderless fiberboards. Some studies have found that during pelletizing these molecules migrate towards the surface, further favoring the mechanical properties of the product obtained from pressing.

# 9 Bibliography


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