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# Banana rachis as a potential source of second generation ethanol

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**Dottorando**

Christian F. Larenas-Uría

*(firma)*

**Tutore**

Prof. Alessandro Medici

*(firma)*

**Co-tutore**

Prof. Paola Pedrini

*(firma)*


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# Chapter 1

# INTRODUCTION

Until 2025, global energy consumption is expected to increase by more than 50% in relation to the year 2000 [1]. At present, fossil resources provide about 86% of energy and 96% of organic chemicals [2] which, added to the constant fluctuation of oil prices, international agreements on environmental impact and the constant decrease from fossil natural resource sources, efforts are directed towards the search for renewable sources of fuels and biomolecules for synthesis. Initially, the production of technologies based on the use of renewable raw materials for the primary production of biofuels was promoted from crops of plants rich in fats such as sunflower, corn or soybeans and other vegetable sources rich in sugars such as Sugarcane, however, its large-scale use for fuel production is not sustainable considering that the raw materials used are primary sources for human consumption. Sustainable economic and industrial growth requires safe and sustainable energy resources, so second generation biofuels are presented as an efficient alternative thanks to the use of forest and agricultural waste, municipal waste and construction waste, contributing to the reduction of emissions net carbon and increasing energy efficiency. [3]. This is equivalent to designing a complete reorganization of the economy based on the use of biological raw materials with completely new approaches in research, development and production.

Lignocellulose is probably the main renewable raw material for the sustainable production of biofuels, however the difficulty of designing standardized processes and high yields has been the main impediment to generalize the use of lignocellulosic biomass as part of the commercial production of ethanol, concomitantly with the production of heat and electricity. [4]

To focus the problem is key to make a Green Chemistry approach, avoiding dangerous and polluting processes, using safe and compatible products with the environment to develop sustainable and ecological chemicals. To produce chemical products and organic materials must operate under agreed criteria and strict standards of use of widely available, fully renewable and low-cost carbon; the model necessarily involves the requirement of lignocellulosic biomass and the conversion of it into useful products carried out in facilities specially designed for this purpose called biorefineries. [5]

Biorefineries are productive complexes analogous to conventional refineries that generate a great variety of chemical products and fuels with maximum use of raw materials [6]. The concept of integrated biorefinery has associated complex conversion methods, both biochemical and thermochemical, to obtain a wide range of products [7]. The composition of

the selected biomass will determine the applicable transformation processes for biorefining. For lignocellulosic biomass matrices, the following processes[8] are considered:

- Hydrolysis of cellulose and hemicellulose to monosaccharides of five and six carbon atoms such as xylose and glucose.
- Conversion of glucose to intermediate chemical compounds such as ethanol, butanol and organic acids through biological (fermentative) processes and conventional chemical transformations.
- Conversion of xylose to products such as ethanol, xylitol and furfural.
- Valorization of lignin and other waste.

These stages can be combined among them optimizing the hydrolysis and fermentation and final production in a single process [9].

## Biomass

Biomass is all biological material from living organism product both of metabolism and of the tissues that constitute its vital structures [2]. There are several sources of biomass, plant origin being some of the most important considering its easy availability to come from natural environments or artificially cultivated or to be waste and / or by-products of different industrial processes. Biomass defined as such constitutes an important source of renewable materials and energy available in natural as well as anthropic contexts. The energy consumption needs of today's society, coupled with policies and laws in force with a strict environmental component, find in the exploitation of lignocellulosic biomass the opportunity to develop biotechnological and chemical processes with high value and industrial application [11].

### Lignocellulosic biomass and its chemical composition

The lignocellulosic biomass is composed mainly of three polymers: cellulose, hemicellulose and lignin together with small amounts of other components such as minerals and phenolic compounds [12]. The proportion of these components varies widely depending on the origin of the biomass, the factors of culture, humidity and other variables associated with their genetic and phenotypic characteristics of the plant species [13]. The purpose of the lignocellulosic structure (Figure 1) is to be a structural support tissue for the plants, as well as to constitute a

protective wall of great mechanical resistance against environmental factors, the attack of pathogens and animal predation [14].

These characteristics make lignocellulose a particularly complex material in terms of its diversity and structural characteristics, which in turn affects the difficulty of finding standardized processes of complete fractionation, being impossible not to degrade or modify any of its components. In general terms, lignocellulosic biomass, expressed as dry material, is characterized by an approximate content of: 20% -50% of cellulose, 15% -35% of hemicellulose and 10% -30% of lignin [15]. Other soluble minor components are proteins (3-10%), lipids (1-3%), phenolic compounds and simple sugars called extractive, in addition to a mineral fraction expressed as soluble ashes and insoluble in acid (10.5%) [16].

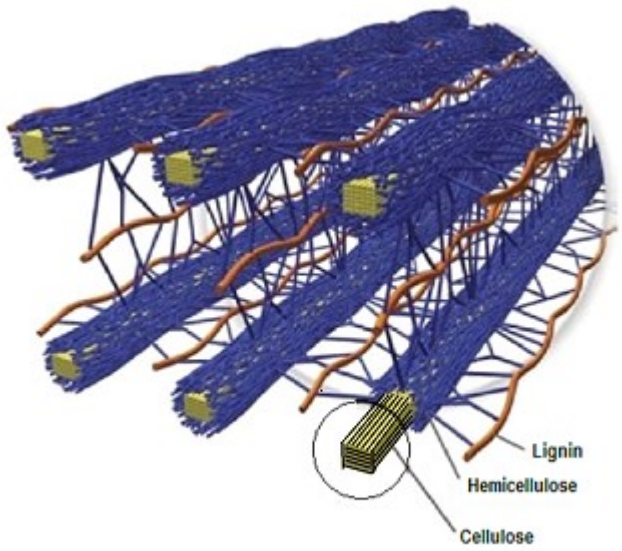



**Figure 1.** Lignocellulosic structure

**Cellulose**

Cellulose is the most abundant organic polymer on the planet, estimating that the total annual production of biomass is higher than 1500 trillion tons [17]. Being of natural biological origin, it can be considered as a renewable and practically inexhaustible source.

Structurally it was isolated and identified for the first time by Anselme Payen in 1838[18]. Later in 1922, Hermann Staudinger, described and characterized it as a polymer together with other macromolecules of natural origin [19]. Currently, cellulose is described as a linear polysaccharide of high molecular weight and high degree of polymerization (GP) constituted by monomeric units of glucose linked in Beta position by glycosidic bonds 1-4. The resulting

dimer, called cellobiose, is characterized in that the hemiacetalic -OH group of the carbon 1 anomeric carbon of a β-D-glucopyranose molecule reacts with the -OH group of carbon 4 of the other β-D-glucopyranose molecule [20]. This union gives rise to the structural characteristic of the cellulose chain where its ends are not equal; one end of the cellulose chain will have a reducing character by having an anomeric carbon that does not form a glycosidic bond, while at the other end of the chain, the anomeric carbon is protected by the glycosidic bond and does not have the possibility of oxidizing[21].

The polymeric cellulose chain is constituted as a series of glucose molecules in pyranose conformation in the form of a chair where the -CH2OH, -OH groups and the glycosidic bonds are in the equatorial position and the hydrogens in the axial position [22]. The equatorial position of the hydroxyl groups facilitates the intramolecular and intermolecular union between cellulose chains through the formation of hydrogen bonds, this configuration allows to form cellulose microfibers that give a crystalline character to the molecular conglomerate [23]. The degree of crystallinity or alternatively the presence of amorphous zones, give different characteristics to the macromolecule and give rise to the existence of seven known polymorphs of cellulose (Iα, Iβ, II, IIII, IIIII, IVI, IVII) [24]. The higher crystallinity of fibers will have better resistance to chemical attack and hydrophobicity, while the amorphous zones confer elasticity but are chemically more reactive and hydrophilic [25]. In nature only the Iα and Iβ forms are found and can be found together in the same microfiber in certain plant species. The Iα form predominates in cellular structures of algae and bacteria [26].

The degree of polymerization of cellulose is described as the number of repeating units of (β-D-glucopyranose) (Figure 2) and depends both on the origin of the plant material and on the extraction treatment given to cellulose[27].

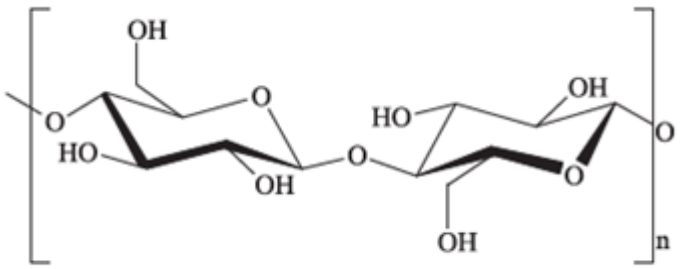



**Figure 2.** Cellobiose unit

The degree of polymerization is very variable andcan range from 200 to 7000 units or more, directly influencing the physicochemical characteristics of the polymer, with the longer chains also being more difficult to hydrolyze [28], which is directly related to the technology and performance of the processes to be used for the subsequent use of the resulting sugars.

**Hemicellulose**

Hemicellulose is the second most abundant polymer on the planet [17]. Unlike cellulose, hemicellulose has an amorphous and very random structure that is composed of several heteropolymers including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan [29]. This structure allows linking lignin with cellulose, directly influencing the water solubility and the rigidity of the cell wall.

Its typical degree of polymerization does not exceed 200 units [27]. It consists of glucose and other sugars, both hexose and pentose, which polymerize forming short and branched chains, giving rise to amorphous, flexible and labile molecular arrangements to hydrolysis [28]. There are five main monosaccharides existing in the hemicelluloses; three hexoses which are glucose, mannose, galactose and two pentoses, xylose and arabinose [29]. There are also uronic acids products of the oxidation of such sugars. The polymer will consist of β (1,4) glycosidic linkages and occasionally β (1,3) linkages. The characteristics of the main chain of heteropolymers allows to classify the hemicelluloses as xylans, xyloglucans, mannans and glucomannans [20], however there is no unified classification criterion, given the complexity and variability of the chemical structures (Figure 3).

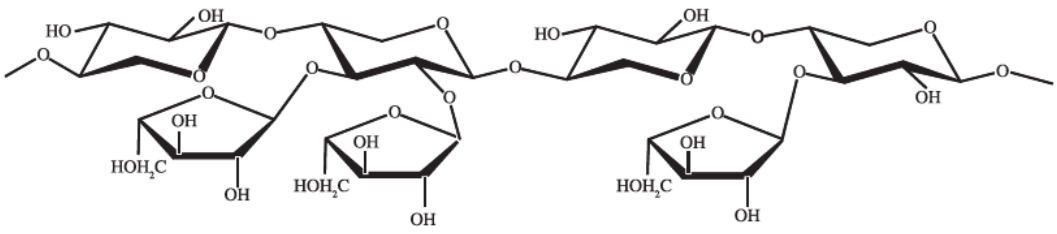


**Figure 3.** Hemicellulose structure

**Lignin**

Lignin is the most abundant aromatic polymer on Earth and the third most frequent polymer after cellulose and hemicellulose [17]. Lignin is present in all vascular plants, and like many other components of biomass, is formed by the reaction of photosynthesis. Lignin is considered

an affordable renewable resource of potential industrial use, whose annual production has been estimated in the range of $5.36x10^8$ tons [30]. Constitutes between 15 and 50% of the composition of lignocellulosic biomass, such variation depends on the origin of the plant material and the state of preservation of the tissues [31]. Lignin is a three-dimensional polymer whose repeating units are derivatives of phenylpropane [32]. Lignin works as a cellular glue next to the hemicellulose by packaging the cellulose microfibers and generating a structure that provides resistance and rigidity to the compression of the tissue, as well as resistance against insects and pathogens [14]. Lignin is very variable in its composition being mainly constituted by a branched non-crystalline chain of repetitive units derived from various types of aromatic alcohols such as p-coumaric alcohol, coniferyl alcohol and sinapyl alcohol, which differ by the number of methoxyl groups (-OMe) at positions 3 and 5 of the aromatic unit [33] (Figure 4).

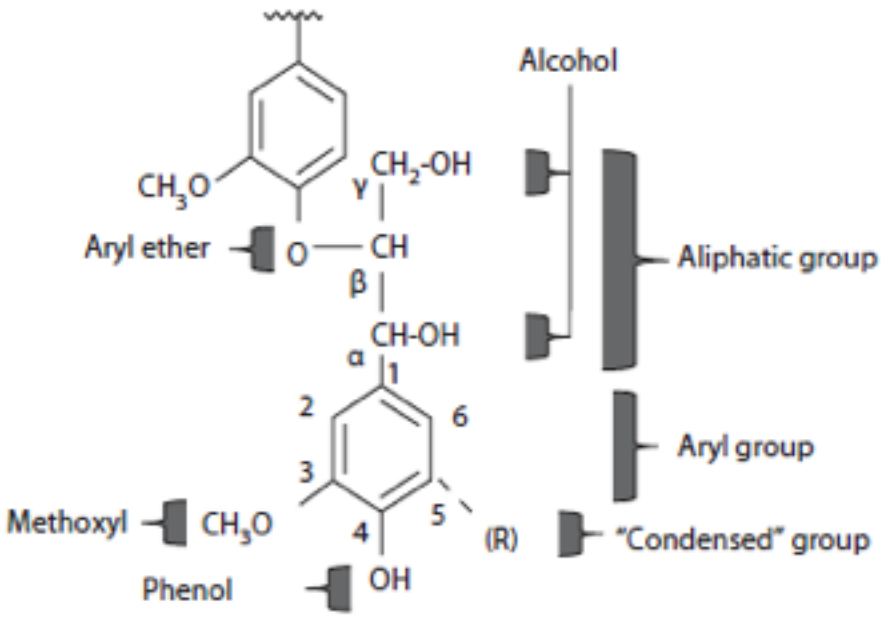



**Figure 4.** Lignin structural units

Depending of the chemical union and the monomers involved the structure of the polymer can be very diverse, characterizing the types of wood and vegetable fibers (Figure 5).

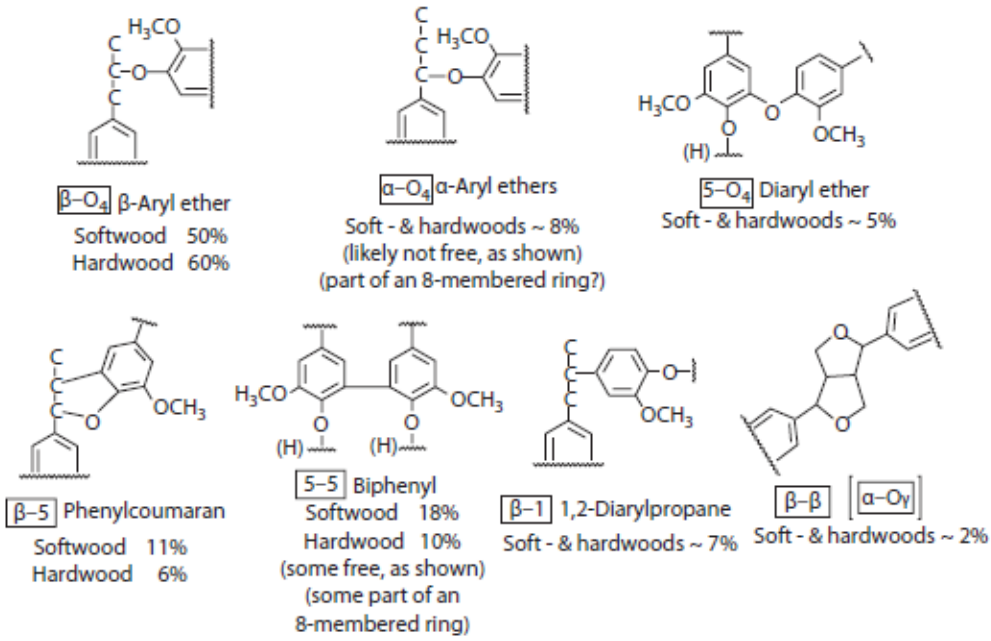



**Figure 5.** Lignin union monomers and different wood types

The union of these structures gives rise to the polymeric molecular lattice characteristic of lignin (Figure 6).

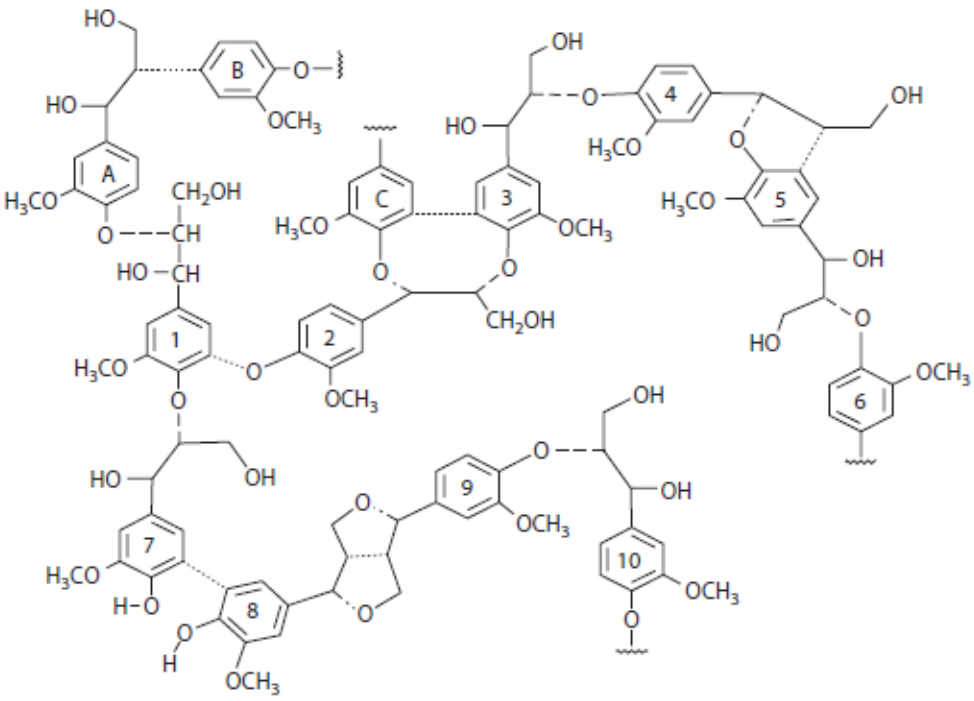


**Figure 6.** Lignin basic structure

**Other compounds**

The lignocellulosic material contains other chemical compounds in minority concentration; it is possible to find water-soluble organic compounds such as oxalates and inorganics such as carbonates, silica and metals [34]. It can also contain non-volatile liposoluble organic compounds such as fats, sterols and other such as volatile essential oils It is possible found other types of polysaccharides different than cellulose and hemicellulose, this molecules are rich in polygalacturonic acids and which are commonly referred as pectins(Figure 7) [35,36].

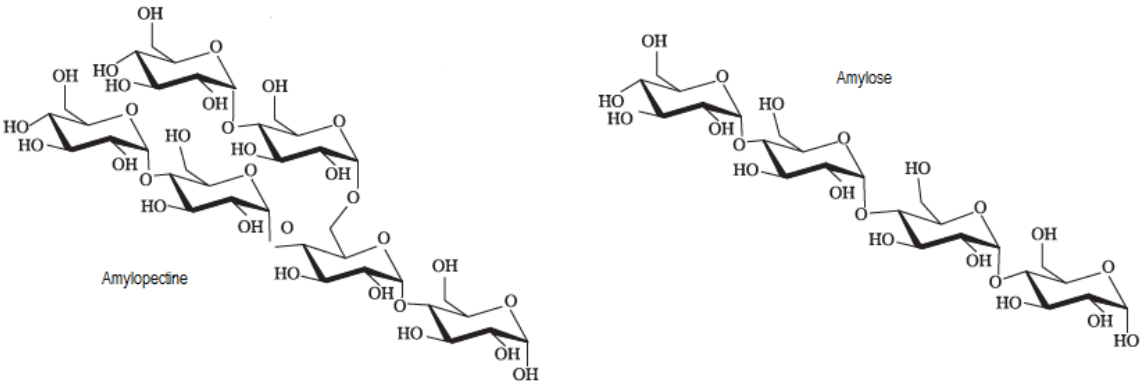



**Figure 7.** Other types of macromolecules in lignocellulosic materials

**Treatments of lignocellulosic biomass**

The lignocellulosic biomass requires a different physical-chemical process that optimize the conversion to the desired final products. In general, there are three common processes[37,38]:

- Drying and reduction of biomass size.
- Delignification and hydrolysis
- Fermentation and conversion to final products

All these processes must be efficient in all unit operations to maximize yields without negatively influencing the successive stages; it is particularly important to optimize the pretreatments to reduce the crystallinity of the cellulose, increase its porosity and improve the effectiveness of the reaction in that there is no significant degradation of the sugars. It is essential to minimize the production of toxic compounds that inhibit subsequent fermentation

processes. Of equal importance are the economic and environmental factors associated with each stage of the process; the technologies that are used for energy consumption, that use ingredients that are not expensive and in what can be associated with Green Chemistry procedures.

In summary, the optimal process for the treatment of lignocellulosic biomass should have the following characteristics [38]:

- Minimize waste production of treated solids.
- Maximize the content of sugars produced.
- Make all the processes involved compatible.
- Recover lignin.
- Maximize the performance of final products.
- Minimize energy consumption.
- Economically profitable.

**Methods for the degradation of lignocellulosic biomass.**

There are three general methods for the degradation of lignocellulosic biomass for conversion to biofuels and chemicals; Physical treatments, Chemical treatments and Biological treatments. All of them can operate in isolation or be part of a combined process [38].

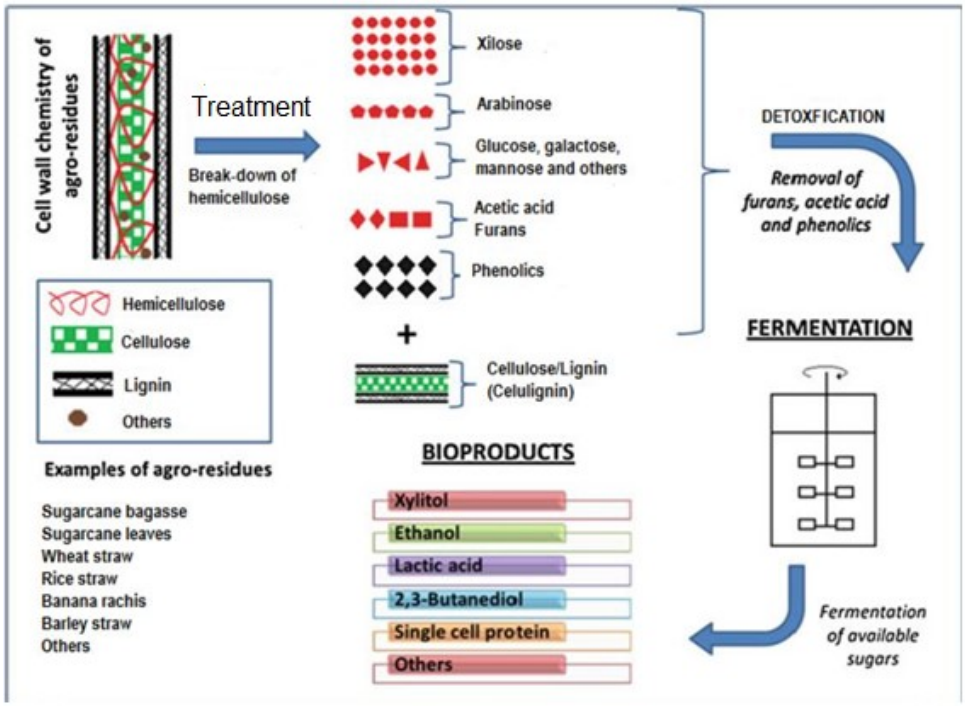



**Figure 8.** General process of lignocellulosic biomass processing

**Physical Treatments**

- **Mechanical fragmentation.**

The general purpose of mechanical fragmentation is to reduce the crystallinity of the cellulose present in the biomass. In the mechanical fragmentation can be used different grinding systems or more sophisticated processes such as gamma ray, electron beam or microwave bombardment, all necessary to reduce the particle size improving the mass transfer [37,38]. The great disadvantage of these methods is the very high energy cost; the amount of energy consumed is greater than the amount of energy that the biomass can generate [38].

- **Pyrolysis**

It is thermal processes that require high temperature and absence of air to generate the decomposition of the different lignocellulosic fractions [39]. During the treatment, different solid products, liquids and gases are generated depending on the pyrolytic system used. The fraction corresponding to the hemicellulose is degraded between 250-400 ºC, the cellulose fraction breaks up between 310-430 ºC and the lignin decomposes between 300-530 ºC [39]. At higher temperatures, the carbon-carbon and carbon-hydrogen bonds break down, generating compounds of lower molecular weight. The process carried out in this way is not profitable at this time [40].

- **Steam Explosion**

Steam explosion is a group of hydrothermal methods which work at a temperature between 160-260ºC and with corresponding pressures between 0.69 to 4.83 MPa, under which it is possible to degrade hemicellulose at a very profitable cost [41]. The process is given by a sudden decompression of the reactor generating a rapid vaporization of the water inside the lignocellulosic material, producing its degradation. They are autocatalysed methods because in situ the acid catalyst required for hydrolysis is generated, particularly acetic acid product of the ionization of water whose protons attack the acetyl bonds of hemicellulose [42].

The disadvantages of the method include the destruction of a portion of the xylan fraction, the incomplete breaking of the lignin and the generation of microorganism inhibitor compounds [40].

- **Ammonia Fiber Explosion**

It is a method where anhydrous liquid ammonia is used at high temperatures (60-100 ºC) and high pressures (1,7 - 2 MPa) very similar to the steam explosion [41]. It has the advantage that ammonia can be recovered almost in its entirety, therefore the cost of the treatment is profitable.

Other characteristics of the methods are that it increases the reaction surface and does not generate compounds that inhibit the growth of microorganisms [42]. It is not an adequate method for biomasses with a high lignin content [43].

- **Explosion with Supercritical Carbon Dioxide**

Similar to the methods of steam and ammonia explosion. The low temperature prevents the rupture of bonds in small molecules and the formation of toxic substances, however it is not an efficient method to treat biomass rich in lignin [44].

**Chemical Treatments**

- **Acid hydrolysis**

They are methods characterized by the use of dilute or concentrates mineral acids that hydrolyze the biomass generating fermentable sugars under very variable temperature conditions. The most common acids in these processes are $H_3PO_4$, $H_2SO_4$ and HCl[41]. The hydrolysis generates a liquid fraction rich in xylan and fermentable sugars from the hemicellulose, while the remaining solid fraction is composed of cellulose and lignin; the methods are not efficient in the total removal of the lignin fraction. The fractionation of lignin can generate toxic substances that inhibit the effectiveness of subsequent fermentative processes being one of the disadvantages of these methods that can also be highly corrosive and expensive on an industrial level.

- **Alkaline Hydrolysis**

These are treatments characterized by the use of basic solutions of NaOH, $NH_4OH$ and Ca $(OH)_2$ that hydrolyze the biomass at lower temperatures than acidic methods but with longer reaction times. They are efficient methods to reduce the crystallinity of cellulose by positively influencing the separation of lignin and hemicellulose fractions with little degradation of sugars. The main disadvantage of the alkaline treatments is the generation of salts that are incorporated into the matrix of the transformed biomass, the same ones that negatively affect the processes and subsequent fermentative treatments.

- **Organosolv**

They are treatments where mixtures of organic and aqueous solvents are used, in the presence or absence of acid catalysts, whose function is to fractionate the biomass, depolymerizing and solubilizing hemicellulose and lignin while the cellulose remains intact. As solvents more common are acetone, ethyl acetate, methanol, ethanol, ethylene glycol and tetrahydrofura. In addition, the mineral acids most commonly used as catalysts are HCl and $H_2SO_4$; organic acids

such as oxalic acid, maleic acid and succinic acid can also be used. Many solvents can be recovered and recycled, but they are still high cost processes.

**Oxidizing and delignifying treatments**

They are chemical treatments that use oxidizing reagents such as hydrogen peroxide, sodium hypochlorite, ozone and other oxidizing solutions to reduce the lignin content in the biomass. Normally the reaction conditions are at room temperature and pressure. The cellulose does not undergo any change, or its reduction is minimal, while a fraction of the hemicellulose can be hydrolyzed and lost. In general, oxidative treatments do not produce toxic residues that affect the following processes.

The amount of oxidizing reagents used can vary widely depending on the method used and the nature of the biomass. These methods can be expensive processes due to the amount of reagent used.

**Alternative chemical treatments**

- **Treatments with ionic liquids (LI).**

Ionic liquids are salts of cations and organic or inorganic anions that can dissolve the lignocellulosic fractions and have the capacity to regenerate cellulose after the elimination of lignin and hemicellulose. The reaction mechanisms are not yet clearly described, however they are methods that are in full development thanks to the fact that the LI have relevant properties such as virtual zero steam pressure, high thermal and chemical stability with great dissolving capacity of compounds of different polarity. They also act as catalysts and are easily recyclable.

- **Electro Chemically Activated solution (ECA)**

The ECA solution is produced by the electrolysis of distilled water containing dissolved sodium chloride (5g/L). The electrolysis produces a solution of hypochlorous acid and sodium hydroxide. The resulting solution is a very strong oxidant agent with "active chlorine" of about 1500 mg/L. The principal advantage of the ECA solution is to be an important and more "green" alternative for delignification than use of other oxidant agents like chlorine and hipochlorous salts in the cellulose production [45].

**Biological Treatments**

- **Enzymatic hydrolysis**

Biological hydrolysis are treatments that use living organisms such as fungi, yeasts or bacteria whose enzymes have the capacity to degrade different types of lignocellulosic matrices with varying degrees of selectivity. The great advantage of these techniques is associated to the biological nature of the processes, limiting or eliminating completely the use of chemical substances that are polluting or dangerous for the environment. They are methods of relative low energy consumption and with adequate yields. The enzymatic depolymerization of cellulose to glucose depends on the action of endoglucanases, exoglucanases and β-glucosidases, enzymes that belong to the family of glucoside hydrolases (GH) and that catalyze the hydrolysis of glycosidic bondsdepolymerizing cellulose into fermentable sugars (Figure 9) [46].

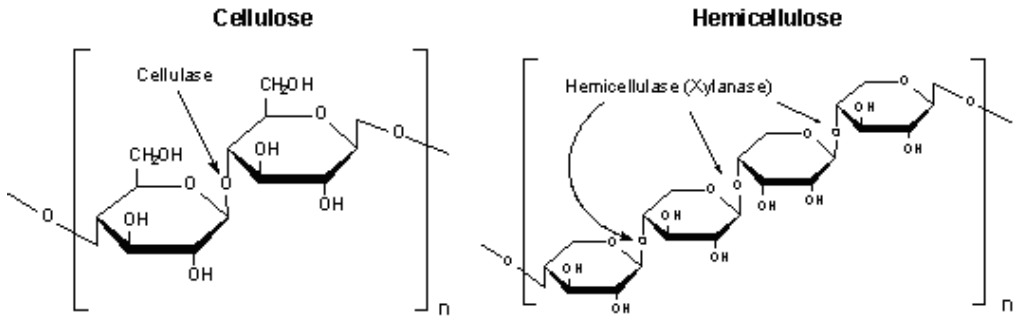



**Figure 9.** Enzimatic depolymerization examples

The fungi are the microorganisms of greater spectrum for the degradation of the lignocellulosic biomass given high delignification capacity. The species most commonly used today are the genera Phanerochaete, Ceriporia, Cyathus, Ceriporiopsis, Pycnoporus and Pleurotus. The use of bacteria is more limited to the degradation of cellulose and hemicellulose to final products due to its low enzymatic capacity to degrade lignins[46].

**Banana**

***Origin and cultivation***

Bananas and different species of the genus Musa are native of South Asia. Around the year 650 AD its cultivation began in the Mediterranean and from the 15th century it reached the Canary Islands, from where it was taken to America with Spanish colonization. Crops are currently grown in Central and South America, Africa and Asia. The Latin American countries producing the largest amount of bananas for export with around 10 million tons per year, which constitutes 90% of world production. The great demand for bananas and the different banana varieties respond to their important nutritional value, ease of consumption and great versatility for the preparation of food. In countries where bananas are traditionally grown, a great variety of dishes are prepared that are fundamental in the daily diet of its people.

***Taxonomic classification***

According to Tazán (2003), the taxonomic classification of green banana is as follows:

Kingdom: ……...Plantae

Class:……...Liliopsida

Order:……..Zingiberales

Family:…….Musaceae

Genus:……..Musa

Specie:…….AAB

Variety: .....Barraganete

***Botanical characteristics***

- *Morphology*

The different species of Musa are giant herbaceous plants lacking a true stem and with a short rhizome. Depending on the species and variety it can reach between 3.5 and 7.5 meters in height. The apparent stem (pseudostem) is the result of the union of conical leaf sheaths, which end in a crown of leaves. The rhizome also called a bulb is itself, a large underground stem with numerous meristems crowned with buds that once mature will generate inflorescence, has superficial roots that rarely exceed 40 cm in depth; most of them are concentrated around 17 cm.

The radicular system is constituted by roots initially soft and white that later will become yellowish and hard, that will be able to reach up to 3 m of lateral length and 1.5 m of depth.

The leaves are born from the terminal meristem, located in the upper part of the rhizome and emerge from inside of the psuedostem, rolled up like a cigar. They are characterized by being very large, 2 to 4 m long and up to 1.5 m wide, with a petiole 1 m or more in length and an elongated limbus. During flowering, a hanging cluster 1 to 2 m long and 5 to 6 cm in diameter is formed from the crown of leaves, from which a score of oval, red-purple elongated bracts are formed and covered with a white powder.

The flowers are large inflorescences, spike, hermaphrodite or unisexual that are born from the armpits of the bracts. The female flowers are born in lower rows while the masculine ones in the superior ones are yellowish, irregular and with six stamens, of which one is sterile. From each group of flowers, a fruit gathering is formed called "hand", which contains 3 to 20 fruits. The rachis traditionally refers to the part of the hanging cluster located between the male button and the last hand of the fruit. The set of hands is known as the "regime" of the plant and can generally have from 5 to 20 hands, depending on the cultivated variety.

The fruits are oblong-shaped berries that as they develop bend geotropically according to their weight, which in turn influences the shape of the bunch. Bananas are polymorphic fruits of greenish yellow, yellow, reddish-yellow or red color. Most of the fruits of the edible Musaceae family are sterile (Figure 10).

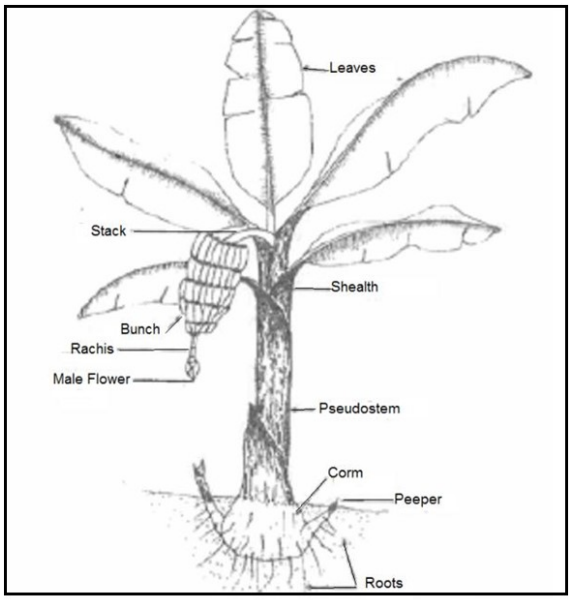



**Figure 10.** Banana plant

**Cultivation in Ecuador**

The cultivation of banana in Ecuador is a productive activity of great importance, currently reaching the second place as an exporter of this fruit worldwide. According to the Ecuadorian Institute of Statistics and Census (INEC) in 2011, a total of 144981 hectares cultivated plantains were reported in the country. The "Survey of Surface and Continuous Agricultural Production" reports in 2014 that banana production in its various varieties reached 6,907,376 metric tons. That's mean the banana production is an important source of work with around 400,000 direct places, which means that around 12% of the economically active population benefits from this activity in one way or another.

**Use of the non-fruit parts of banana**

The rachis and other parts of the banana plant are agro-industrial post-harvest residues that accumulate in the markets of agricultural products with little or no particular use. Some of the parts most commonly used at the domestic level include green or dry leaves, pseudostem and shells. Rhizomes are usually the least used parts. The most frequent uses are related to the production of compost, as building materials, in the manufacture of ropes for tying small animals and game items. In few places the yolks are used as food or medicine. Other uses include the extraction of fiber for the elaboration of works of art and crafts. The nutrient value of the pseudostem is high and could be promoted as animal feed. At the moment there is no large-scale use of the byproducts of the banana industry, focused on the production of materials with high added value through biotechnological processes in biorefinery to obtain second generation biofuels and biodegradable plastics.

**Rachis production.**

There are no official data or studies that indicate the amount of rachis produced in Ecuador, however, knowing the annual production of the different types of cultivated banana and considering that 10% of this amount corresponds to the rachis, can be approximated a production of around 690 thousand tons of this waste every year

# Chapter 2

# MATERIALS AND METHODS

**Collection and pretreatment of banana rachis**

The samples of banana rachis come from green banana hybrids of the species Musa AB, variety "Barraganete", which were provided by the "finca Calderón" family farm located in the city of Milagro, Guayas, Ecuador. The plantation is located at 12 msn and under organic farming system. The entire sample was processed by cutting it into cylindrical pieces (10 cm diameter and 3 cm height). Only fragments visually free of mold contamination were selected. Subsequently, the sample was dried in an oven at 104 °C for eight days until reaching constant weight. The sample was ground and sieved to # 200 mesh using all fractions above 75um.

**Determination of humidity of banana rachis**

Using a triple beam balance, were weighed 250 g of fresh banana rachis cut into cylindrical pieces approximately 10 cm in diameter by 3 cm in height. Samples of approximately 10 g were weighed on the analytical balance, each sample was placed in previously calibrated porcelain capsules and kept in an oven at 104 °C until reaching constant weight. The test was performed in triplicate, reporting the result as the arithmetic mean with a confidence limit of 95%.
The% moisture was determined using following the equation:

$$\% \text{ Humidity} = \frac{Wo - Wf}{Wo} * 100 \textbf{(Eq. 1)}$$

Wo = Initial weight (g)
Wf = Final weight (g)

**Cellulose content**

The total cellulose content was determined by gravimetric analysis considering the initial weight of the untreated dry sample and the final weight of the dry fraction after the total oxidation of the dry rachis with 2% alkaline hydrogen peroxide solution in proportion of 4g / L. One gram of raw and dry rachis were added to 1000 mL Erlenmeyer flask. Subsequently, was added 250 mL of 2% (W / V) hydrogen peroxide solution alkalized with 0.5% NaOH (W / V) The mixture was stirred on a heating plate for a total time of 90 minutes and a temperature of 50 ° C. During the procedure, a significant amount of foam was generated, which meant constant monitoring was necessary to avoid spills.

After the reaction time, the solid fraction was recovered. A vacuum filtration system with Buchner funnel and glass fiber filters was used. The solid residue was washed with 500 mL of distilled water. The washing water was mixed with the liquid fraction of the filtrate and kept for the subsequent analysis of hemicellulose and other soluble biopolymers. The solid fraction was dried in an oven at 60 ° C for 24 hours. Finally, the resulting weight corresponding to the cellulose present in the spine was determined.

**Hemicellulose content**

The liquid fraction resulting from the preceding process was concentrated to a volume of 50 mL while maintaining stirring and boiling temperature at atmospheric pressure. During this process, the hydrogen peroxide residues are also completely eliminated. Once the mixture was cold, 50 mL of 70% Ethanol was added obtaining a powdery white precipitate. The mixture was filtered under vacuum on a Buchner funnel with glass fiber discs; the solid fraction was collected and dried at the oven (60 °C) until constant weight. The liquid fraction was re-concentrated again to the same conditions as those described. 50 mL of 70% Ethanol was added again. The process is repeated until solutions are obtained where precipitation with alcohol is no longer possible. All the solid fractions were weighed together, whose total mass corresponds to the sum of the soluble polymer fractions, salts from the addition of NaOH and products of the decomposition of the lignin.

**Ash content determination in banana rachis**

Using an analytical balance, 1 g of dry and ground rachis was weighed in triplicate. The sample was placed in a porcelain crucible and placed in a muffle furnace. The calcination was carried out for 12 hours at a temperature of 550 ° C until obtaining white ash corresponding to the mineral content of the sample.

The percentage of ashes was determined using the following equation:

$$\% \text{ Ash } = \frac{\text{Wa}}{\text{Wo}} * 100$$

Wo = Initial weight (g)

Wa = Ash weight (g)

The same procedure was used to determine the ash content in the delignified samples with the three selected treatments.

**Quantification of lignin by the Tappi Method (T 222 os-74)**

All the tests were carried out in duplicate. The rachis samples used for the various treatments tested were milled and sieved to a size of 75 μm, then dried in an oven at 104 °C until constant weight and kept in a desiccator with $CaCl_2$. On a 50 mL Erlenmeyer flask, using an analytical balance, approximately 0.5 g of the dried material was weighed and 10 mL of 72% $H_2SO_4$ (w/w) was added, constantly mixing the mixture and allowing it to react for twohours. After the whole content was quantitatively transferred to a 1000 mL distillation balloon, 380 mL of distilled water was added and it was boiled under reflux for four hours. At the end of the reaction time, the solution was filtered under vacuum using a tared micro-fiberglass filter (Whatman™ GF/B, 55 mm) on a Büchner ceramic funnel. The filtrate was dried in an oven at 104°C until constant weight was obtained; the amount obtained corresponds to Klason acid insoluble lignin (AIL).

$$\% AIL = \frac{Klasonlignin\ (g)}{Sample\ (g)} * 100 \quad \textbf{(Eq. 2)}$$

The liquid fraction of the filtrate was taken and measured in its volume to later determine the absorbance at 205 nm; The amount of lignin soluble in acid is determined using the following expression:

$$\text{Lignin}\ \left(\frac{g}{L}\right); B = \frac{\text{Absorbance}}{a \times b} \quad \textbf{(Eq. 3)}$$

$$\text{Lignin}\ \left(\frac{g}{L}\right); B = \frac{\text{Absorbance}}{110} \quad \textbf{(Eq. 4)}$$

Being the content of lignin soluble in acid (ASL):

$$\%ASL = \frac{B \times V \times 100}{1000 \times W} \quad \textbf{(Eq. 5)}$$

a (absorptivity coefficient) = 110 $cm^{-1}g^{-1}L$

b (optical path) = 1 cm

V = Volume of the filtrate

W = Dry sample weight

The total content of lignin was calculated with the following equation:

$$\%\, Totallignin = \%\, ASL + \%AIL \textbf{(Eq. 6)}$$

**Delignification methods**

The methods used to delignify the sample included pre-treatments with organosolv, water and with oxidizing substances.

***Pretreatment with organosolv***

The following solvents of different polarity and pH were used for the removal of polar and moderately polar molecules:

- Acetone pretreatment
- Ethyl Acetate pretreatment
- Ethanol 96% pretreatment
- Pretreatment with mixtures (W/W) Glacial Acetic Acid / Acetone / Water (5:95:5) ; (15:70:20) ; (10:50:30) ; (0:79:21)

In all of them, it worked with three proportions of biomass mixture with solvent volume: 1: 500; 2: 500; 3: 500. The mixture was placed in the 1L distillation balloons for 20 minutes under reflux. Later it was filtered. The insoluble material was dried and weighed. The liquid fraction was recovered for a subsequent UV spectral analysis between 190 and 400 nm.

***Pretreatment with distilled water***

In the semi-analytical balance, were weighed approximately 15 g of milled and dry rachis. Was added 250 ml of the selected liquid and refluxed for 2 hours. It was vacuum filtered on glass microfiber paper. The solid was dried and weighed. The liquid fraction was recovered for a subsequent UV spectral analysis between 190 and 400 nm.

***Pretreatment with oxidizing agents***

Approximately 4 g of milled, sieved and dry rachis were weighed on the semi-analytical balance. The sample was placed in a 2000 ml Erlenmeyer and 1L of oxidant solution was added. The system was kept under stirring for 15 minutes at room temperature. It was then filtered on

glass microfiber and washed until the oxidant reagent was completely removed. The treated sample was dried and weighed. The amount of lost material was determined. The treated and dried material was stored in a desiccator with $CaCl_2$ for the subsequent Tappi analysis. Was used Sodium Hypochlorite (5%) and ECA solution like oxidant solutions.

***Pretreatment with Alkaline Hydrogen Peroxide***

Approximately 4 g of milled, sieved and dry rachis were weighed on the semi-analytical balance. The sample was placed in a 2000 mL Erlenmeyer flask and was added 900 mL of $H_2O_2$ 2% (W/W) plus 100 mL of NaOH solution 5% (W/W). The final volume was completed up to 1000 mL. The system was kept under stirring for 90 minutes at room temperature. Subsequently, it was vacuum filtered in glass microfiber and washed until the hydrogen peroxide was completely removed. The treated sample was dried and weighed. The amount of lost material was determined. The treated and dried material was stored in a desiccator with CaCl2 for the subsequent Tappi analysis.

***Treatment with oxidizing solution ECA on pretreated rachis with organo-solvents.***

In the semi-analytical balance, approximately 4 g of samples pretreated with 96% Ethanol and Acetic acid / Acetone / Water mixture (10:50:30), sieved and dried, were weighed separately. Each sample was placed in a 2000 mL Erlenmeyer and 1L of an ECA oxidant solution was added. The system was kept under stirring for 15 minutes at room temperature. It was then filtered on glass microfiber and washed until the oxidant reagent was completely removed. The treated sample was dried and weighed in analitycal balance.

**Qualitative Analysis FT-IR**

Approximately 100 mg were taken of each one treated samples and untreated banana rachis previously milled and sieved. All the samples were dried at 104 ° C and kept in desiccator with silica gel. Separately was taken approximately 10 mg of each sample and mixed with 100 mg of KBr (spectroscopic quality). The mixture was homogenized in agate mortar. The mixture was placed in a stainless steel pressure mold with bolts; the sample was compressed until obtained a homogeneous and transparent pellet approximately 0.25 mm thick . The pellet was placed in the FT-IR spectrophotometer (Perkin Elmer Spectrum 1000). Through the Spectrum

10 Software, the corresponding Absorbance and Transmittance spectra were obtained in light beam transmission measurement mode, within the range of 4000 cm-1 to 600 cm1, with baseline correction, noise correction (Smooth) and identification of the wave numbers of the main peaks.

**Qualitative FT-NIR analysis of delignified samples**

One gram of the original sample of banana rachis and the samples treated with various organosolvs and oxidizing treatments were dried in an oven at 104°C. The dried and ground samples were placed in 9 cm petri dishes dispersing the material with a stainless-steel disc designed for this purpose. For the FT-NIR analysis, a NIRFLex N-500 spectrophotometer (Büchi, Switzerland) of diffuse reflectance was used. To minimize the effect of the eccentric rotating movement of the cup where the sample is placed, the scanning of each sample was performed in duplicate. The reflectance spectra were recorded using NIRWare 1.4 software (Büchi, Switzerland) in a spectral range of 8000 to 4000 cm-1, at 8 cm-1 intervals. The measurements were carried out with 2-4 scans / s with a resolution of wave number of ± 0.2cm-1 at a temperature of 25 ± 5 ° C. For each spectrum, 128 scans were generated, which were averaged computationally in a total measurement time of 30 s. Obtaining each spectrum was preceded by the acquisition of an internal reference to optimize the spectral baseline. In total, 98 spectra were collected.

For the mathematical pretreatment of the spectra, the net optical data were processed with the combination of Normal Normal Variation (SNV) and first spectral derivative (Savitzky-Golay 5 points).

All the chemometric analyzes, including the mathematical pre-treatments for calibration and validation were carried out with the software NIRCal5.4 (Büchi, Switzerland). The validation of the data used 32 points while the remaining 64 were used as a calibration set. The qualitative discriminant analysis was carried out through the use of cluster analysis based on principal component analysis (PCA).

**Quantitative FT-NIR analysis of delignified samples**

***Preparation of the lignocellulosic standard***

Using an analytical balance, were weighed 10 g of cinnamyl alcohol, 0.1 g of p-coumaric acid, 0.5 g of insoluble Klason lignin, 0.1 g of soluble lignin and 1.5 g of carboxymethylcellulose.In a 25 mL beaker was added the weighed amount of cinnamyl alcohol; using a water bath at 50°C, the reagent was melted, then all the previously weighed substances were added, mixed and homogenized. Subsequently, the mixture was allowed to solidify at room temperature.

***Inclusion of the lignocellulosic standard on the samples.***

Three pretreatments were selected for the quantitative analysis:

- pretreatment with 96% ethanol and subsequent oxidation with ECA solution;
- pretreatment with acetic acid / acetone / water mixture and subsequent oxidation with ECA solution;
- pretreatment with oxidizing solution ECA without using previous organolvents. In addition, the banana rachis sample was used without any previous treatment.

A total of 10 calibration points was prepared for each sample; each calibration point corresponds to 0.5 g of the selected dry sample plus a variable amount of lignocellulosic standard, included between 0 and 0.5 g and added in approximate intervals of 0.05 g. The total final weight of the sample plus the standard was 1.0 g; at the required calibration points, the weight was completed with the corresponding amount of cinnamyl alcohol. All the components were mixed and homogenized using a ceramic mortar. The mixtures were placed in glass bottles of 25 mL capacity with polyethylene cap and heated with an air dryer to melt the standard and homogenize it with the sample on the bottom of the container; all the glass jars with the samples were cooled and closed.

Following a procedure similar to that described in the previous section, we proceeded to obtain the respective spectral information, in order to obtain the linear equations corresponding to the calibration curves of soluble lignin, cellulose and insoluble lignin on each sample. Only equations with correlation coefficients greater than 0.95 were taken for the quantitative analysis. With the values of the slopes of each calibration curve was calculated the angle formed between them and the respective curve of the non-treated rachis. With the calculated angle was determined the corresponding area. Through the difference between areas was possible

calculate the relative percentage of variation in the amount of soluble lignin, cellulose and insoluble lignin respectively.

**UV-Visible Spectrophotometric Analysis**

The liquid fraction of the pretreatments with organosolv, oxidation solutions and water was used in the analysis. All samples were carried at a constant volume of 1000 ml. An ONDA UV20 single-beam spectrophotometer was used and the data was processed with the software M.Wave Professional 2.0 to obtain and process the second derived spectra within the spectral range between 190 and 400 nm, using quartz cells of standard length of 1 cm . The calibration was carried out with soluble lignin standards of 10, 40, 100, 140 and 180 ppm. Validation was carried out with standards of 20, 80, 110 and 120 ppm. The chemometric data were processed by PLS (Partial Least Squares) to determine the most relevant wavelengths and obtain a linear model to predict the concentration of soluble Lignin using a soluble Lignin standard (Sigma). Qualitatively, a cluster analysis was performed using PCA. All operations were carried out using the statistical package R version 3.1.3.

**Modeling the effect of pH and polarity of the organosolvent on the extraction of soluble materials.**

In five 500 mL Erlenmeyer flasks, were individually added 15 g of dried and milled banana rachis. For each flask was added separately 250 mL of acetic acid / acetone / water mixture in w / w ratios: 5: 95: 5; 15:70:20; 10:50:30; 0:79:21 and distilled water alone. The initial pH of each of the mixtures was measured, then maintained on reflux for two hours. At the end of the experimental time the mixtures were allowed to cool and the pH of each unit was measured one more time. Each of the remaining solids was filtered, dried and weighed. The test was carried in duplicate.

With the pH values obtained, knowing the theoretical polarity index and the weight of each insoluble fraction, a linear correlation was established between the average values of all the measured factors. Once determining the existence of a significant correlation ($p < 0.05$), a linear model was proposed that relates the average weight of the insoluble fraction with the variation

of pH and the polarity index. The linear model was evaluated with the usual parameters of statistical significance ($p < 0.05$), statistical significance of the ordinate ($p < 0.05$), linear correlation coefficient ($R \cong 1$) and variance inflation factors ($vif < 15$).

**Mineral acid hydrolysis**

Approximately 1 g of sample of the selected treatments (oxidation with ECA, alkaline $H_2O_2$ 2% and NaOCl 5%) was weighed in analytical balance. The sample was placed in 250 ml Erlenmeyer flasks and was added 100 ml of acid solution (5 M HCl or 0.25 M $H_2SO_4$). All the flasks were covered with hydrophobic cotton and aluminum foil and then autoclaved in three cycles of 20 minutes at 121 ° C. At the end of each 20 min cycle, with 3 ml glass pipette, 1.5 ml aliquots were taken and transferred in duplicate on Eppendorf tube, which were labeled, closed and freezing for a subsequent chromatographic analysis. At the finish of the reaction time (60 min), the material of each flask was filtered on previously calibrated fiberglass discs. The samples were dried at 104°C and subsequently the weight of the remaining solid was obtained. In the liquid fraction, the potentiometric measurement of the initial pH was made, and then it was brought to pH 5.5 with $NaHCO_3$ solution (20% W/V). All the samples were kept in freezing for use in the subsequent enzymatic hydrolysis.
A new hydrolysis was carried out with 0.25M HCl under the same conditions as those described. All the procedure was performed in duplicate.

**Maleic acid hydrolysis**

In triplicate, 1 g of each of the delignified samples was placed in 250 mL Erlenmeyer flasks. 50 mL of 2% maleic acid was added to each flask. All flasks were autoclaved for 3 cycles of 21 minutes each at 1.04 atm and 121 ° C. After each thermal cycle with a sterile pipette, 2 mL of supernatant fluid was taken for the subsequent HPLC analysis.

**Enzymatic hydrolysis**

All the chemically delignified material, both the soluble and the insoluble fraction, was adjusted to pH 5.5 with sodium citrate buffer in 250 mL Erlenmeyer flasks. To each suspension was added 0.3 g of *Aspergillus niger* cellulase (enzyme called 22178) and successively 50 µl of

Aspergillus sp cellulase (enzyme called 2605) (pH 7.5) used to degrade cellobiose oligomers into glucose are added for 48 hours at 45 ° C. The Erlenmeyer flasks were kept in orbital shaking (120 rpm, 45 ° C) to activate the enzymes. Every 24 hours, in the laminar flow cabinet 1.5 mL of each sample was taken and transferring to Eppendorf tubes. The tubes were kept frozen until analysis by HPLC.

**Fermentation Protocol.**

**Microorganism and culture conditions**

The *Saccharomyces cerevisiae* strain DSM 70449 was purchased in lyophilized form from the Leibniz Institute-DSMZ German Collection of Microorganisms and Cell Cultures and reproduced in laboratory conditions to ensure a Working Cell Stock (criovials with glycerol as a cryoprotective agent and stored at -20 °C).
*Saccharomyces cerevisiae* DSM 70449, grows optimally in the standard YPD Sigma-Aldrich medium.
The optimal growth conditions were maintained in the incubator SKI 4 (ARGO LAB) for 24 h, under mild stirring (120 rpm), at a temperature of 30 ° C. All microorganism transfer operations were carried out under sterile conditions under laminar flow hood (HerausHerasafe HS9). All the non-sterile material has been autoclaved.

**Preparation of the inoculum of *Saccaromyces cerevisiae***

*S. cerevisiae* stored in the fridge at 4° C inside slants with agarized YPD culture medium, is transferred, under sterile conditions, into 50 mL flasks containing 30 mL of culture broth. The liquid cultures are incubated at 30° C for 36-40h, under stirring at 120 rpm. In the case of tests in an Erlenmeyer flask (100 ml), the suspension obtained is entirely used as an inoculum, whereas for the tests in fermenter (500 ml), the suspension is used as a pre-inoculum for 150 mL of fresh YPD medium, which, grow in the same conditions, it is used as an inoculum.

**Small-scale tests (working volume, 100 mL)**

The fermentations were carried out using 100 mL of hydrolysed delimited rachis solution (after delignification and hydrolysis (acid, enzymatic) the solid part is separated by filtration or centrifugation from the liquid part), in bottles with a 250 mL screw cap and inoculated with 30

mL of YDP cell suspension, as described above. The hydrolyzed solution is added with a protein part as reported by Tamburini et al. 2011. After inoculation, the flasks are placed in an incubator at 30 ° C and 120 rpm. The pH had previously been brought to 6 with 30% NaOH.

The fermentation course is followed by taking culture samples (1 ml) under sterile conditions at time 0 and at the end of the process. Each sample is centrifuged and the supernatant analyzed to evaluate the consumption of sugars and the production of ethanol.

**Scaling-up in 1-liter fermenter (working volume, 500 mL)**

The fermenter (Millifors) consists of a glass jar with a total capacity of 1.5 L (working maximum volume, 1 L) and equipped with an automated system managed by a PC that allows monitoring through the probes and the control of the main chemical-physical parameters, such as temperature, pH, dissolved oxygen concentration. The sterile air flow is guaranteed by a system of inlet pipes connected to a flow meter, and to an output condenser. A 0.22 μm filter is inserted at the air inlet and one at the condenser outlet, so that the system maintains sterile air. The glass jar can be sterilized ex-situ in an autoclave.

The temperature is set at 30 ° C and kept constant throughout the process thanks to a water-based thermoregulation system. The fermenter is equipped with an agitation system equipped with blades connected to a rotor.

The fermentation parameters are as follows:

• Temperature: set point between 29.9 to 30.5 ° C.

• Air: the air enters the system with a pressure of 0.1 L / min.

• Agitation: 90 rpm.

The fermenter is inoculated with 150 ml of S. cerevisiae suspension, prepared as previously described. The fermentation process is carried out for a time ranging from 48 to 144 h. After the inoculum samples are taken sterile, as described in the small-scale fermentation protocol and the samples analyzed to evaluate the consumption of sugars and the production of ethanol.

**Analysis of hydrolyzed sugars by HPLC-RID** (High performance liquid chromatography / Refractive index detector)

To this analysis was used a liquid chromatography module equipped with a binary pump (Jasco PU 4180), a manual valve injector (Rheodyne) with a loop of 20 uL, using a column for carbohydrates, with a stationary phase of Divinyl Benzene Sulfonated Styrene (Phenomenex, Rezex ROA-Organic Acid H + 8%) of 300 mm x 7.8 mm, with oven thermostat and refractive index detector (Jasco RI-4030). The detector's analytical signal is processed through the LC-NETII / ADC Interface BOX interface using the ChromNAV software. Milliq ultrapure water acidified with H2SO4 0.01M solution was used as the mobile phase, previously filtered in millipore of cellulose acetate of 0.45 um x 47 mm and degassing in ultrasound for 20 minutes in sonicator (Elmasonic S100).

Before to the chromatographic elution the column was washed for 12 hours at 80° C using ultrapure water with a flow of 0.2 ml / min. Subsequently the stationary phase will be conditioned with the acid mobile phase maintaining the temperature and flow conditions during the necessary time (4 to 16 hours) until stabilization of the baseline.

Isocratic elution of standard solutions and samples was done with a flow of 0.6 ml / min at 80° C for 20 minutes.

The calibration curve was made with four standard solutions with concentrations of 0.05%, 0.2%, 0.5% and 1% of glucose, xylose and arabinose, determining the characteristic retention times of each one and evaluating the calibration through Pearson's correlation coefficient of ($R2 \cong 1$).

Each sample was defrosted at room temperature and centrifuged at 5400 rpm for 20 min. The supernatant fluid was then taken with a syringe and filtered using 15 mm cellulose filters (Phenomenex) with a 0.2 mm porosity. Each sample was injected in triplicate. We identified the results obtained by comparing the retention times and the concentration was obtained automatically from the software by interpolation in the calibration curve.

**Quantitative analysis of total reducing sugars by the DNS method.**

The content of total reducing sugars was measured using the colorimetric method of 3.5 dinitrosalicylic acid (DNS) using a visible spectrophotometer, determining the absorbance of

the solutions obtained at 540 nm and interpolated in calibration curve of glucose standards between 0.5 g/L and 2.0 g/L.

- ***Preparation of 3,5 dinitrosalicylic acid reagent***

5 g of dinitrosalicylic acid, 150 g of Na-K tartrate and 8 g of NaOH were weighed. The NaOH is dissolved in 200 ml of distilled water and the Na-K tartrate is added slowly under stirring. It is completed with distilled water up to 400 ml, while 3,3-dinitrosalicylic acid is slowly added. The mixture is left stirring overnight, taken to a total volume of 500 ml and filtered.

- ***Development of the DNS reaction***

10 ml of sample and 0.5 ml of the DNS reagent were transferred onto 10 ml test tubes with cap. The tubes were placed in a water bath at 100 ° C for 5 min. Then they were cooled to room temperature by cold water bath. Finally, 5 ml of distilled water was added. The solution was homogenized and the Absorbance was read 540 nm.

**Morphological investigations under a Scanning Electron Microscope (SEM)**

For each sample, 10 images were taken, with enlargements between 50 and 800x. The micrographs are presented as a cross-comparison between the various treatments, at the same magnification. They are preceded by images of the rachis sample without treatment. All the micrographs are made with the secondary electron detector "LFD", 3.5µm spot, standard current at 25KV.

The digital processing and analysis of all the micrographs was done with the ImageJ 1.52a software, transforming the RGB images to 32 bit images and then obtaining the respective frequency analysis, which were then grouped according to the similarity measure of Bray-Curtis using NMDS classification algorithm and cluster analysis with the statistical software Past 2.17c. Another statistical test used was Kruskal-Wallis with the pairwise comparisons of Mann Whitney and the Bonferroni correction to establish the existence of statistical differences between treatments with a significance $p < 0.05$.

# Chapter 3

# RESULTS AND DISCUSSION

**Moisture content of banana rachis**

Table 1 shows the results of the moisture content of banana rachis obtained by gravimetric analysis.

**Table 1.** Moisture results

| | | | |
|---|---|---|---|
| Initial weight | 276.02 | 260.15 | 255.22 |
| Final weight | 18.02 | 16.25 | 16.51 |
| % Moisture | 93.47 | 93.75 | 93.53 |
| % Dry biomass | 6.53 | 6.25 | 6.47 |

Mean and standard deviation.

% Moisture: $93.58 \pm 0.37$

% Dry biomass: $6.42 \pm 0.38$

The results indicate that the composition of the banana rachis is mostly water, so all the methodologies associated with its use must optimize the maximum amount of biomass available to obtain the necessary sugars for the fermentation processes. Equally important will be to establish storage practices that minimize or avoid microbiological contamination of the rachis due to its high moisture content.

**Ash content**

The ash content of the untreated banana rachis is different from the values corresponding to the samples delignified by the three treatments used. All treatments used to delignify the rachis, significantly decrease ($p<0.05$) the values of the mineral content to be chemical reactions that favor the solubility of the main cations K, Ca, Mg and non-essential metals such as P and S, which will be removed during the oxidation process and the washing of the cellulose matrices.

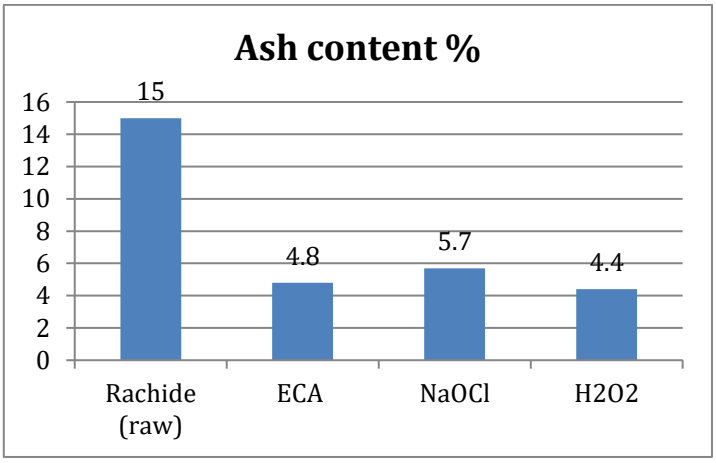



**Figure 1**

**Lignin content Quantification**

- ***Tappi method***

Table 2 shows the Tappi method results, comparing the total lignin content in banana rachis treated by three different delignification methods.

**Table 2.** Lignin content. Comparative oxidizing methods

| **Sample** | **% Total Lignin** | **Mass/Volume ratio** |
|---|---|---|
| Rachis (non treated) | $26.2 \pm 1.2^{a}$ | n.a |
| $H_2O_2$ 2% 90 min | $14.5 \pm 0.9^{b}$ | 4 g / 1000 mL |
| $H_2O_2$ 2% Alkaline 90 min | $12.6 \pm 1.1^{c}$ | 4 g / 250 mL |
| $H_2O_2$ 2% Alkaline 90 min | $12.3 \pm 1.2^{c}$ | 4 g / 1000 mL |
| ECA 15 min | $14.8 \pm 1.2^{b}$ | 4 g / 1000 mL |
| NaOCl 15 min | $15.7 \pm 0.9^{b}$ | 4 g / 1000 mL |
| NaOCl 30 min | $7.2 \pm 1.1^{d}$ | 4 g / 1000 mL |

Different letters mean statistically different results (alpha = 0.05)

The box plot (Figure1) resume the differences between oxidant treatments on banana rachis insoluble lignin content

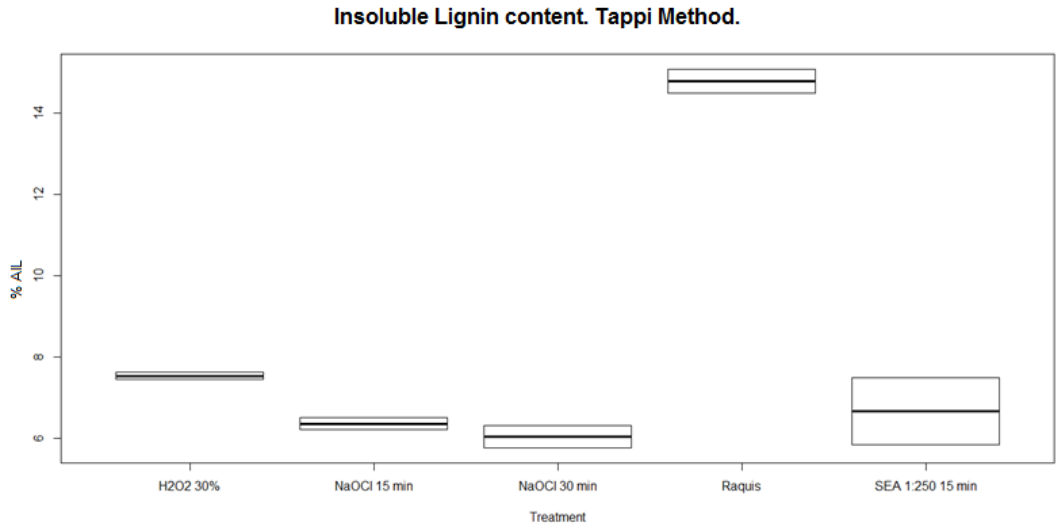



**Figure 2**

The results correspond to an oxidation time of 30 minutes with 5% sodium hypochlorite solution, producing the maximum loss of Klason lignin and soluble lignin. The treatment with the same solution for 15 minutes produces quantitatively equivalent delignification results for the oxidation with ECA solution and 2% hydrogen peroxide. The treatments with 2% hydrogen peroxide alkalized with 0.5% NaOH are quantitatively equivalent, however, it is appreciated that its oxidant activity is maintained even with a quarter of the equivalent volume of other treatments. This characteristic of hydroperoxides and peroxyacids is due to the fact that they act as nucleophiles or electrophiles during the delignification, in addition to being self-catalytic, which conduct the constant formation of oxidant reactive species. With both the ECA solution and the sodium hypochlorite solution there is an important effect of increasing the amount of biomass relative to the volume of the oxidant solution due to the limited reactive activity of the hypochlorous ion, subject to the narrow conditions of alkalinity (pH ≅ 9) to be effective as an oxidizing species; a lower volume ratio of the oxidant solution with respect to the mass of materials has an important negative effect on the delignification, by the reduction of the ideal pH by the subsequent formation of acidic substances.

**Proximate Analysis**

The dry banana rachis is constituted by 36.5% Cellulose, 22.3% Hemicellulose, 26.2% Lignin and 15% of ashes. All data were obtained in triplicate by gravimetric analysis of the individual fractions.

**Methods of delignification**

- ***Effect of the rachis weight versus volume and reaction time with ECA solution***

Figure 2 shows the effect of the rachis weight versus volume and reaction time with ECA solution.

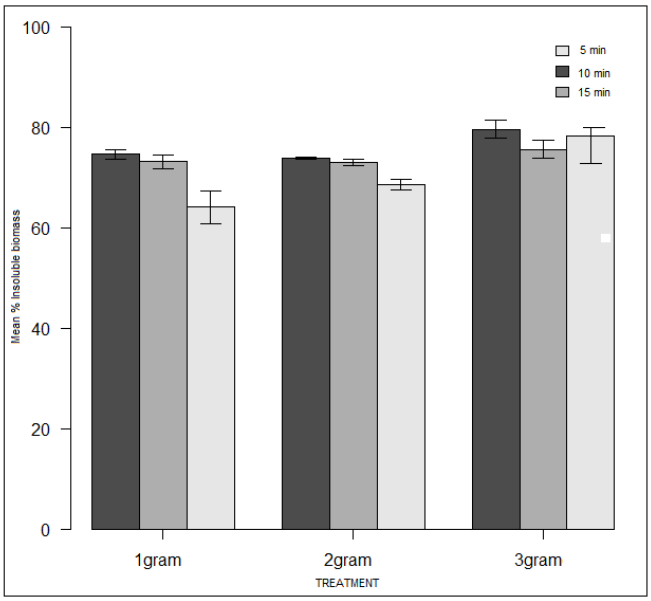



**Figure 3**

The delignification process is more effective in the 2:500 ratio than the corresponding 1:500 and 3:500; there are no significant statistical differences between the reaction times between 5 minutes, 10 minutes and 15 minutes ($p > 0.05$) (Figure 3). However, due to the combination of factors, quantity of biomass and reaction time, is very important keep the reaction time at not less than 10 minutes to maximize the removal of lignin and related molecules.

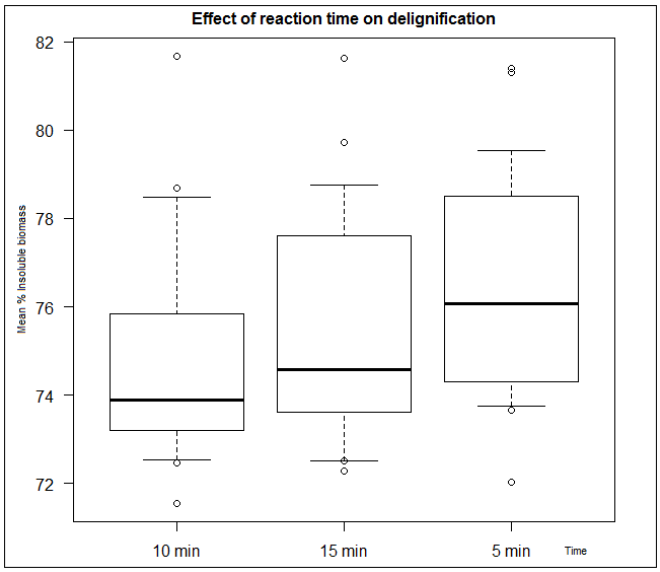



**Figure 4**

- ***Effect of pretreatment with Ethanol on the effectiveness of the treatment of the ECA solution***

The results indicate that the pretreatment of biomass with ethanol (96%) for subsequent oxidation with ECA solution does not produce a significant advantage over the absolute yield of the delignified biomass treated only with the oxidizing ECA solution. The oxidizing treatment with the ECA solution simultaneously removes a similar amount of soluble substances and lignin. It is observed that the result is maintained with respect to the ratio of biomass of 2 grams with 500 mL of oxidant solution as the proportion of highest yield of delignified biomass. The box plot (Figure 4) resumes the effect of the pretreatment.

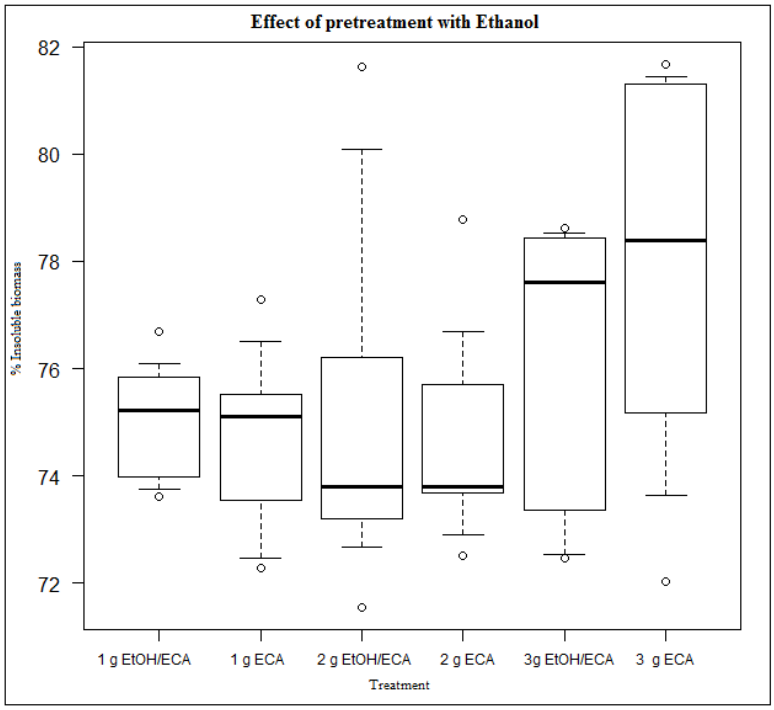



**Figure 5**

- ***Effect of the solvent on the elimination of soluble lignocellulosic materials***

There are significant differences in the amount of final biomass obtained after treatment with solvents of different polarities and pH. The box plot (Figure 5) shows the effect on rachis delignificated. The treatments with Acetic Acid / Acetone / Water of lower pH are those that generate greater removal of soluble material of the biomass, being the treatment of pH 5.43 which forms the lowest percentage of insoluble biomass. Treatments with Acetone, Ethanol, Ethyl Acetate and the Acetic Acid / Acetone / Water mixture pH 6.49 show quantitatively

equivalent removals of soluble substances from the biomass. The treatment with water produces the largest amount of insoluble biomass due to the lower solubilization of medium polar or apolar substances characteristic of the fraction associated with insoluble lignin. The resulting extracts present different color tonality product of the diverse composition and chemical nature of the extracted soluble substances, which will be discussed in the section corresponding to the quantitative analysis by UV spectrophotometry. Treatments that include acetic acid also remove part of the fraction of hemicellulose, due to the partial hydrolysis of the biomass, which is observed by the fermentation of the extracts after approximately 8 days of storage without prior sterilization of the containers and also it is verified in the subsequent analyzes by FTNIR and HPLC.

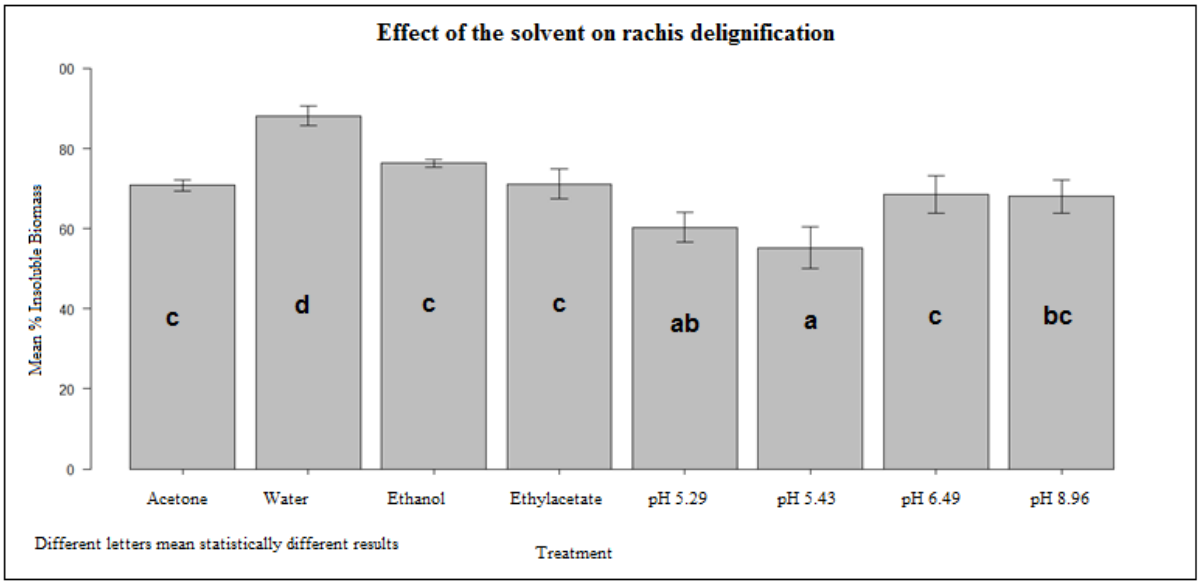



**Figure 6**

- ***Effect of the treatment of oxidizing solutions on delignification***

The results obtained with the ECA oxidant solution, shows that is the best treatment to removal of materials from the biomass (Figure 6), which is associated with the capacity of the ECA solution to remove not only the lignin but also part of the hemicellulose fraction, which will be verified with Subsequent analysis by HPLC. The treatments with acid of alkaline hydrogen in proportion 4: 250 and 1 : 250 are statistically equivalent to the treatment with solution of sodium hypochlorite (5%) during 30 minutes of reaction. Treatment with sodium hypochlorite (5%) for 15 minutes produces the minor removal of lignocellulosic biomass.

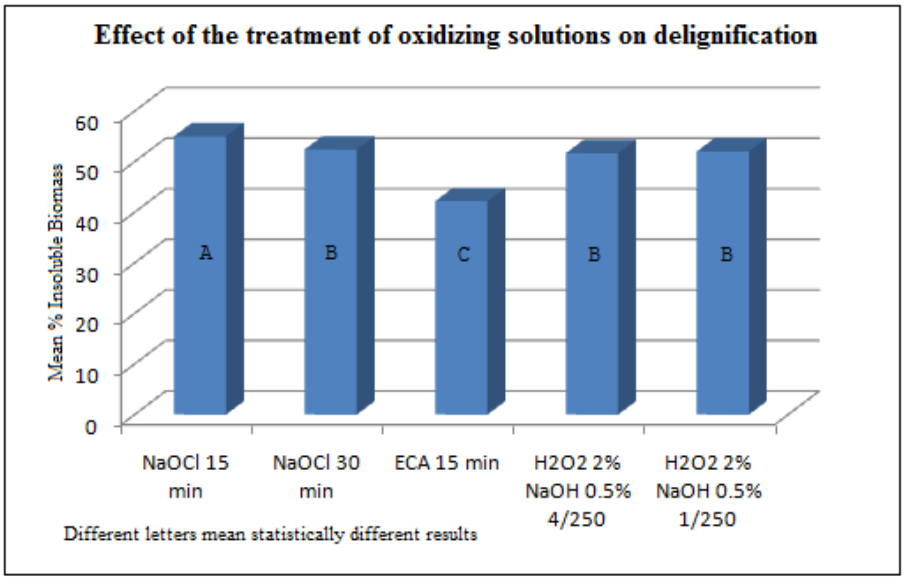



**Figure 7**

- ***Quantitative UV spectrophotometric Analysis***

The calibration and validation model with multivariate regression PLS using the second derivative UV spectra has allowed to quantify the amount of soluble lignin in the liquid filtered fraction obtained after treatment with oxidizing ECA solution. The results (Figure 7) allow to appreciate the decrease of the concentration of soluble lignin present in the filtrates due to the effect of the pretreatments with different organosolv and water compared with the treatments only with ECA solution. In general, approximately the previous treatments at pH 5.43 and 5.29 of the acetic acid / acetone / water mixtures remove approximately twice as much soluble lignin as the respective pretreatments with water and pH 8.96. The result obtained with the ECA solution clearly shows that its oxidizing action allows the extraction and / or formation of chemical species structurally similar to the soluble lignin used as a calibration standard, which is in agreement with the high concentration of the species found in the respective soluble fractions.

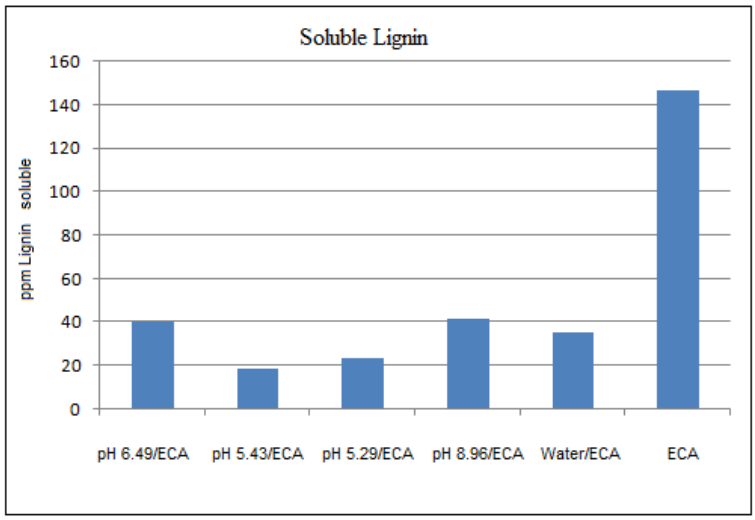



**Figure 8**

- ***Effect of Polarity and pH on delignification***

As mentioned in the previous paragraph, polarity and pH play an important role in the solubility of lignin and other molecules of medium polarity. According to the results of the amount of insoluble matter and the pH data taken at the beginning and end of the extractive process, with subsequent oxidation with ECA solution it is possible to establish a linear correlation model between these factors and the theoretical polarity index ϕ of the mixtures.

**Table 3.** Linear model coefficients

**log residue ~ ΔpH + logϕ**

**Coefficients:**

| | Estimate | Std. Error | t value | Pr(>\|t\|) | |
|---|---|---|---|---|---|
| (Intercept) | 2.318775 | 0.018967 | 122.25 | 0.00521 | ** |
| DeltapH | 0.035035 | 0.001579 | 22.18 | 0.02868 | * |
| log.Pol | -0.758045 | 0.023106 | -32.81 | 0.01940 | * |

**Residual standard error: 0.00213**
**Multiple R-squared: 0.9993**
**Adjusted R-squared: 0.9978**
**p-value: 0.02717**

**VIF**

| ΔpH | logϕ |
|---|---|
| 1.03 | 1.03 |

$$\log \text{residue} = 2.318 + 0.035\Delta pH - 0.758045\phi \qquad \text{Eq.1}$$

The model indicates a strong correlation between the amount of insoluble solids resulting with the pH variation during extraction and the initial polarity index of the solvents (Figure 8). A

greater amount of residue after oxidation with ECA solution indicates less removal of soluble substances and lignin. The mathematical signs of the coefficients of the obtained equation (Eq.1) indicate that there is an increase in delignification as the polarity increases while there is a decrease in the efficiency of the lignin removal process as it increases the variation of pH. According to this result, theoretically it should be expected that an extractive process with solvents of greater polarity that can maintain a minimum pH variation during the extraction will be more efficient for the removal of lignin and other soluble molecules, than less polar solvents. The validity of the model (Table 3) is verified by the values of the statistical significance ($p < 0.05$) for all the coefficients as well as for the global ANOVA model. Likewise, the value of the adjusted correlation coefficient $R^2$=0.9978 indicates a strong linearity without saturating the model, given the inflation coefficients of the variance (vif >> 15). Likewise, the standard error of the residuals is close to 0.2%.

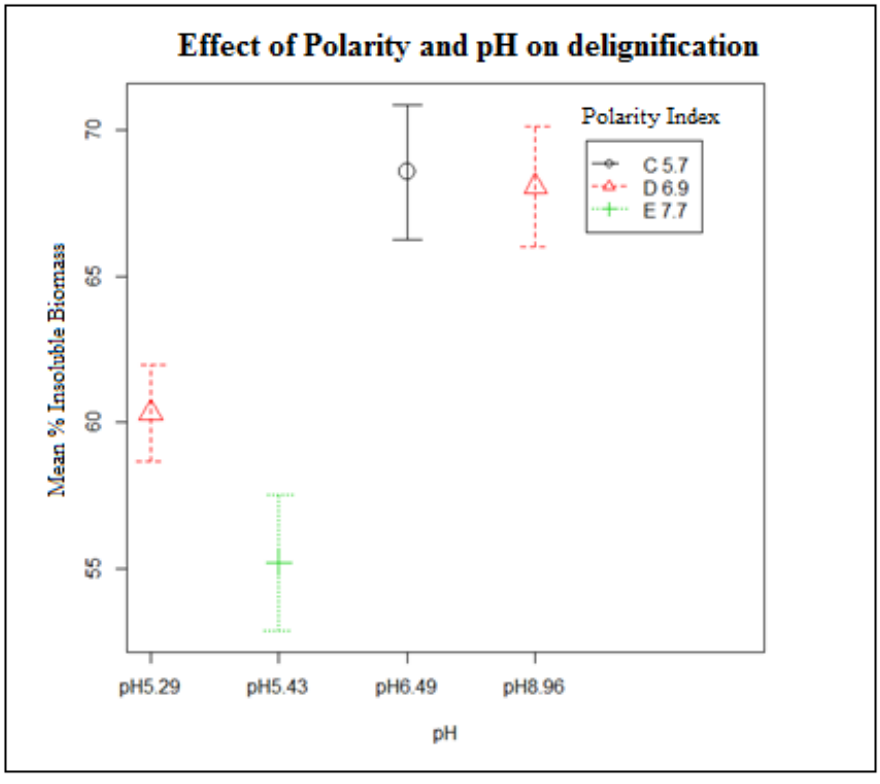



**Figure 9**

- ***Qualitative UV spectrophotometric analysis of extracts treated with organosolvs and water***

The multivariate analysis (PCA) (Figure 9) of the second derivative UV spectra obtained from the extracts with organosolvs at different pH values, in water and with ECA solution, denote the diverse chemical composition of the same and therefore the different composition of the resulting biomass of the treatment.

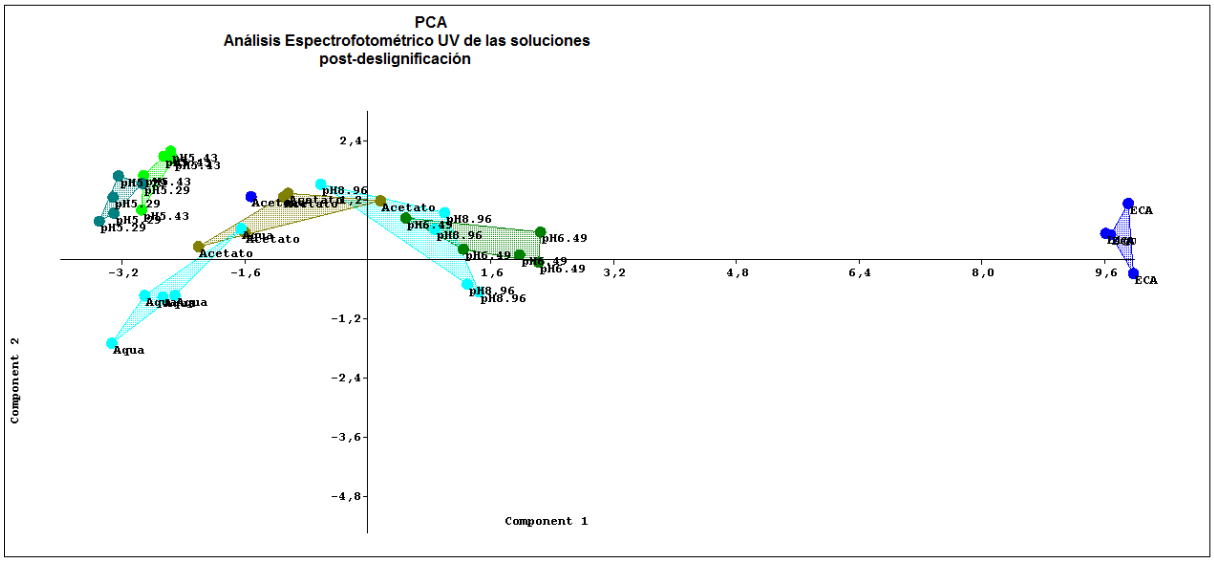



**Figure 10**

The analysis of the first component indicates a strong correlation with the properties and spectral complexity of the solutions resulting from the delignification of the biomass with the ECA solution. Very high and positive values of the first component may be associated with the presence of molecules extracted under very alkaline conditions. Negative or positive values of the first component close to zero, are associated with molecules extracted in acidic conditions or close to neutrality. The grouping of results of the extracts of pH 5.43 and 5.29 can be associated to spectral results product of the presence of molecules coming from the hydrolysis of hemicellulose in addition to the fractions corresponding to lignin and related. The negative values of the second main component correlate particularly with the spectral properties of the aqueous extracts; The same trend can be seen with the pH 8.96 extracts of the Acetone / Water mixture, which means that in the first case they will be substances of higher polarity extracted in acidic conditions, while for the Acetone/Water mixture an extraction of smaller molecules will correspond. polarity and in alkaline conditions.

## FTIR analysis

The results of the FTIR spectroscopy denote changes in the spectral region between 800 and 750 cm-1 for the treatments tested compared with the rachis without treatment. However, it is necessary to purify the spectral quality obtained by improving the sample preparation technique to obtain better results.

**Rachis non treated FT-IR Spectrum (KBr disc)**

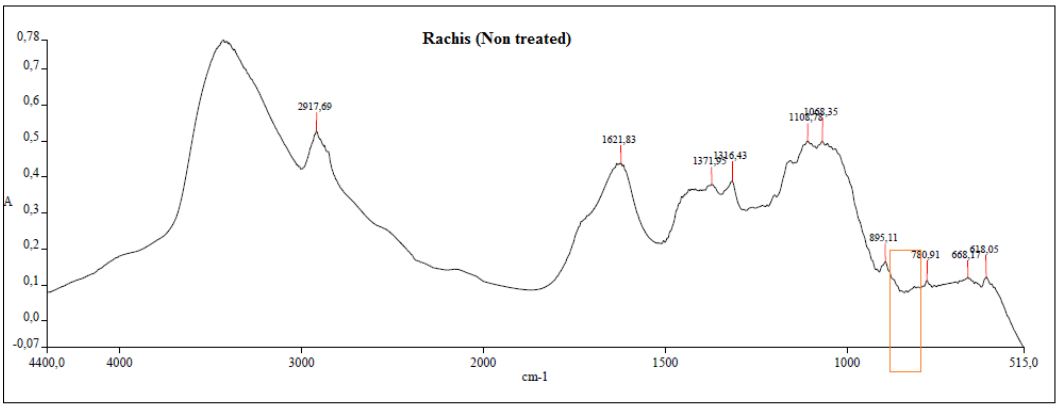



**Figure 11**

**Rachis Acid treatment pH 5.43 FT-IR Spectrum (KBr disc)**

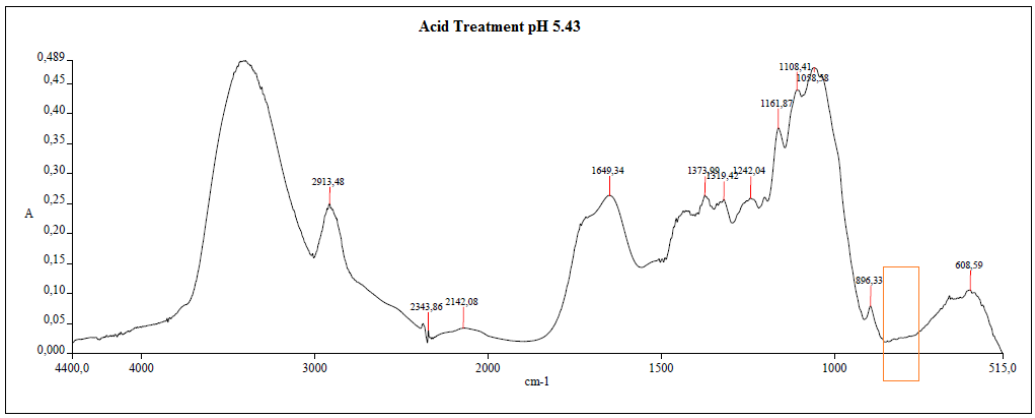



**Figure 12**

**Rachis ECA treatment FT-IR Spectrum (KBr disc)**

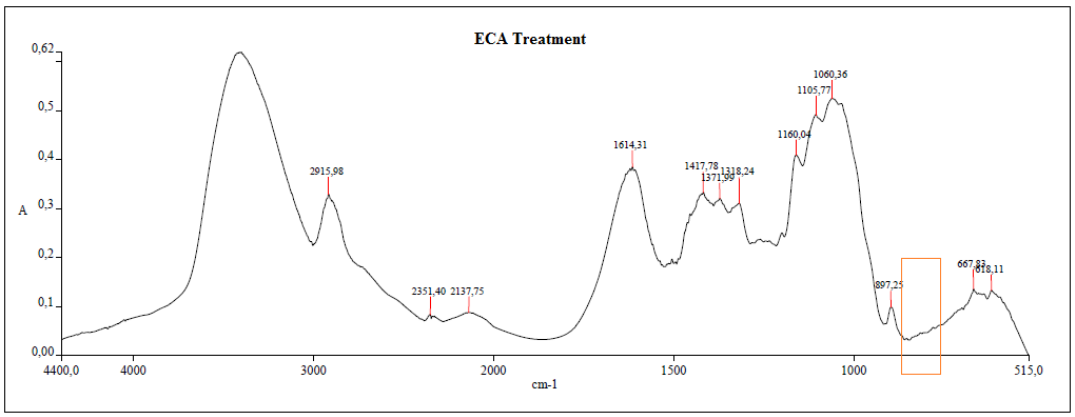



**Figure 13**

**Rachis Alkaline peroxide treatment FT-IR Spectrum (KBr disc)**

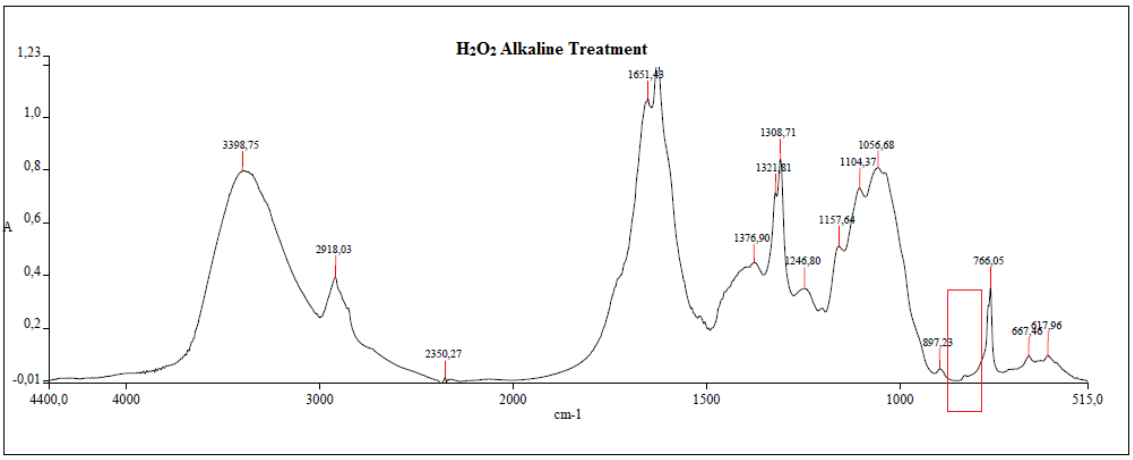
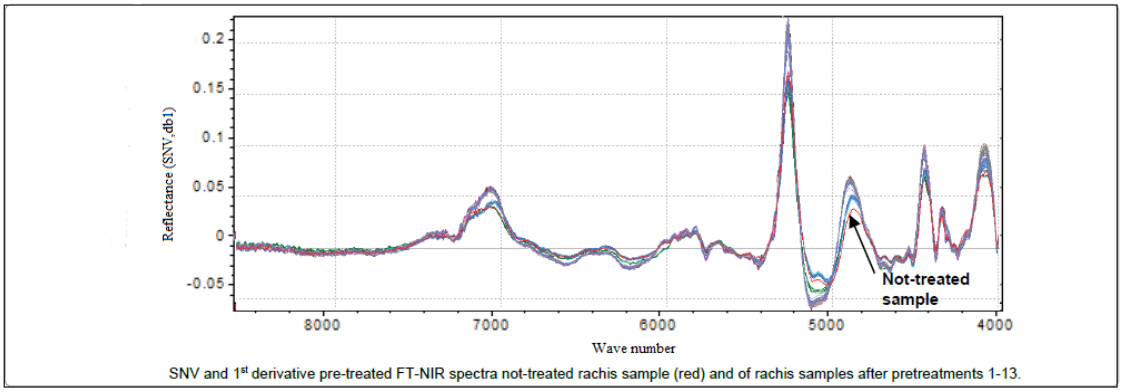



**Figure 14**

- ***Analysis by FT-NIR spectroscopy***

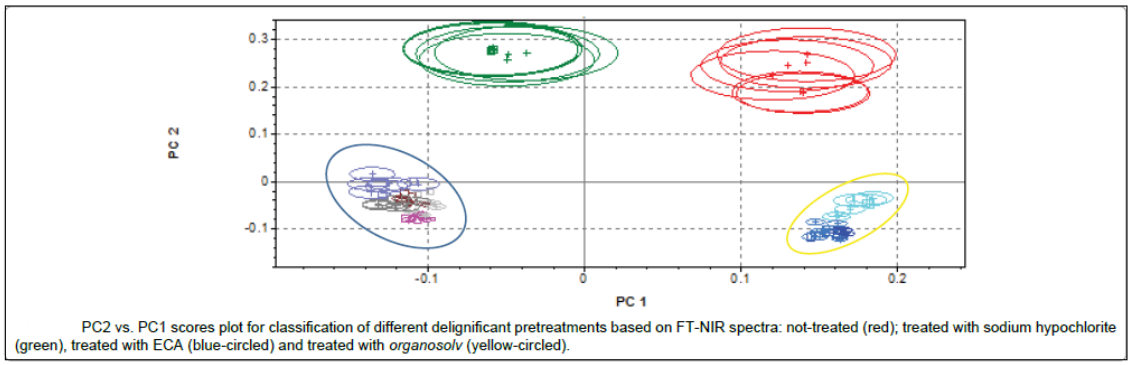
The technique has the ability to discriminate the effect of different treatments on the structure of the biomass resulting from delignification; Figure 14 shows the different spectral signals corresponding to each treatment.



SNV and 1st derivative pre-treated FT-NIR spectra not-treated rachis sample (red) and of rachis samples after pretreatments 1-13.

**Figure 15**

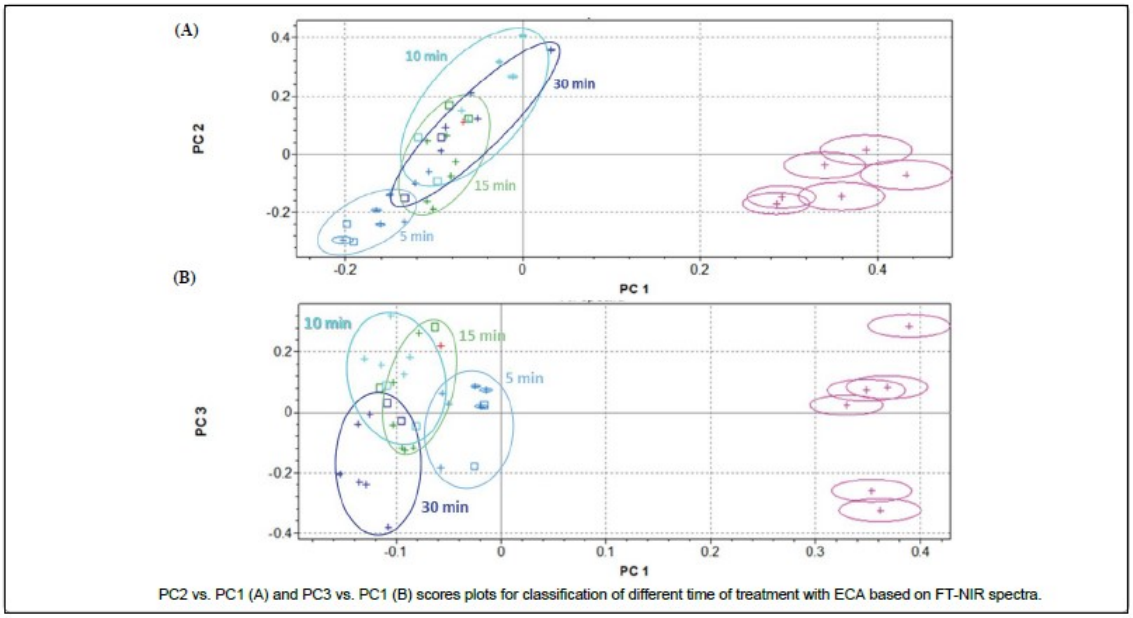
A first group of cluster analysis allows to clearly appreciate the difference between the biomass resulting from three treatments tested compared to the untreated banana rachis as shown Figure 15



PC2 vs. PC1 scores plot for classification of different delignificant pretreatments based on FT-NIR spectra: not-treated (red); treated with sodium hypochlorite (green), treated with ECA (blue-circled) and treated with *organosolv* (yellow-circled).

**Figure 16**

On the other hand, the effectiveness of the ECA solution is determined by evaluating the influence of the treatment time on rachis samples at 5,10,15 and 30 minutes, compared with the untreated sample. Conglomerate analysis allows a good separation to be drawn along PC1 between the untreated rachis and the oxidized samples. The analytical information provided by PC2 and PC1 is sufficient to clearly discriminate between treatment time with ECA solution (Figure 16). PC3 allows us to more clearly appreciate the variability in the NIR spectra and is partially able to solve clusters that overlap, improving the separation between 5 to 30 minutes of treatments.



PC2 vs. PC1 (A) and PC3 vs. PC1 (B) scores plots for classification of different time of treatment with ECA based on FT-NIR spectra.

**Figure 17**

FT-NIR spectroscopy has also made it possible to demonstrate the effect of the treatment with different solvents, although the residual lignin has almost the same content of carbonyl, carboxyl and hydroxyl groups, since the treatment with organosolvs does not imply significant changes in the main structure of the lignin.

The differences are probably attributed to a lignocellulosic matrix with a different three-dimensional arrangement that has been evidenced by PC4 and the improved cluster classification (Figure 19).

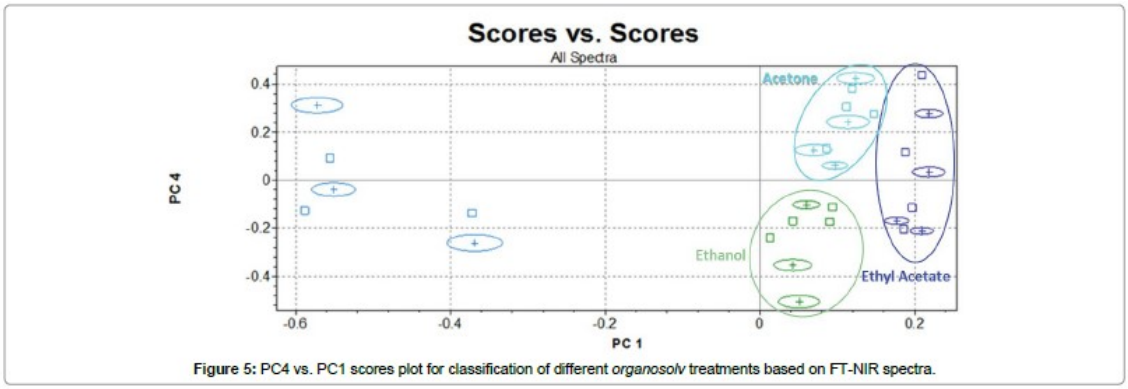
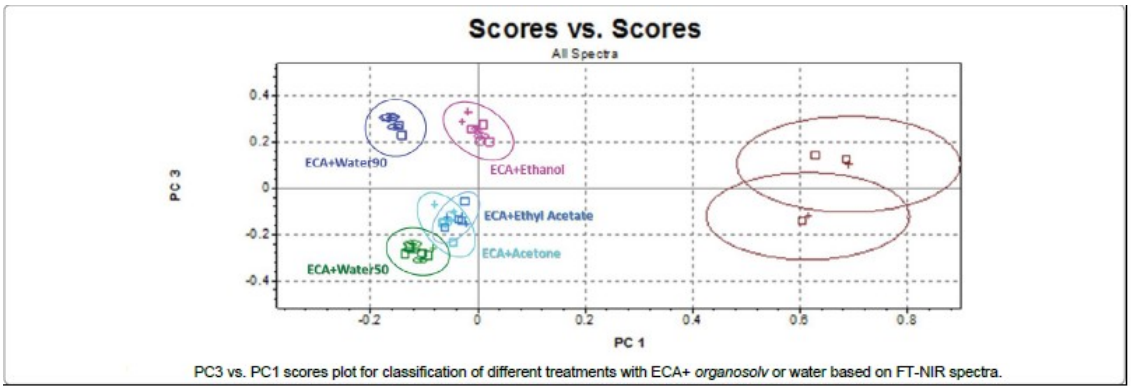


**Figure 5:** PC4 vs. PC1 scores plot for classification of different *organosolv* treatments based on FT-NIR spectra.

**Figure 18**

The results also show the ability of FT-NIR to group samples subjected to double treatment, ie with organo solvents and subsequent oxidation with ECA solution. The combination of

information from PC3 and PC1 significantly improves the grouping capacity of the model (Figure 19). There is a clear separation between RCT + water 50 ° C, RCT + water 90 ° C, ECA + Ethanol and the others could be correlated with the presence of OH groups derived from ethanol and water.



PC3 vs. PC1 scores plot for classification of different treatments with ECA+ *organosolv* or water based on FT-NIR spectra.

**Figure 19**

- ***Quantitative Analysis by FT-NIR Spectroscopy***

The results of the calibration and validation FT-NIR generate a group of 10 linear equations for each treatment from which an equation for the treatment has been selected based on its best linearity, verifying the value of $R^2$ coefficient (Table 4).

**Table 4.** Linear equations FT-NIR Analysis

| Treatment | Insoluble Lignin | | Soluble Lignin | | Cellulose | |
|---|---|---|---|---|---|---|
| RAQUIS | Y = 0,4267x+0,0058 | r2=0,9929 | Y = 0,4592x+0,0011 | r2=0,9883 | Y = 0,4232x+0,0176 | r2=0,9948 |
| ECA | Y = 0,9859x+0,0001 | r2= 0,9859 | Y =0,9850x+0,0000 | r2= 0,9850 | Y =0,9858x+0,0004 | r2= 0,9858 |
| H/A/W | Y = 0,9987x+0,0000 | r2=0,9987 | Y =0,9987x+0,0000 | r2= 0,9987 | Y =0,9987x+0,0000 | r2= 0,9987 |
| EtOH | Y = 0,9926x+0,0001 | r2= 0,9926 | Y =0,9916x+0,0000 | r2= 0,9916 | Y =0,9928x+0,0002 | r2= 0,9928 |

By obtaining the difference of angles of the slopes and areas found between the straight curves of calibration it is possible to establish the relative variation in the concentration of the three compounds analyzed: cellulose, insoluble lignin and soluble lignin, as shown in the table 5.

**Table 5.** Relative quantification of components in the biomass according to the treatment used

| % Removal relative to less effective treatment (x) | | | |
|---|---|---|---|
| Component | ECA | EtOH+ECA | H /A / W + ECA |
| Insoluble Lignin | x | 1.6 | 3.4 |
| Cellulose | 18.5 | 18.5 | x |
| Soluble Lignin | 5.1 | 6.1 | x |

**Variation in the content of Soluble Lignin**

For soluble lignin it is observed that there is a small difference between the extractive capacity, compared between the double treatment with 96% Ethanol with subsequent ECA treatment, simple treatment with ECA solution and double treatment with Acetic acid / Acetone / Water mixture of pH 5.43 and later oxidation with ECA (Figure 19). This last treatment produces the largest loss of soluble lignin compared to the other two. On the other hand, is clearly appreciated the different slope of the calibration curve corresponding to the untreated rachis  The graph allows to conclude that there is a relatively low content of lignin in the banana rachis, which is corroborated by the results of the Tappi method described above.

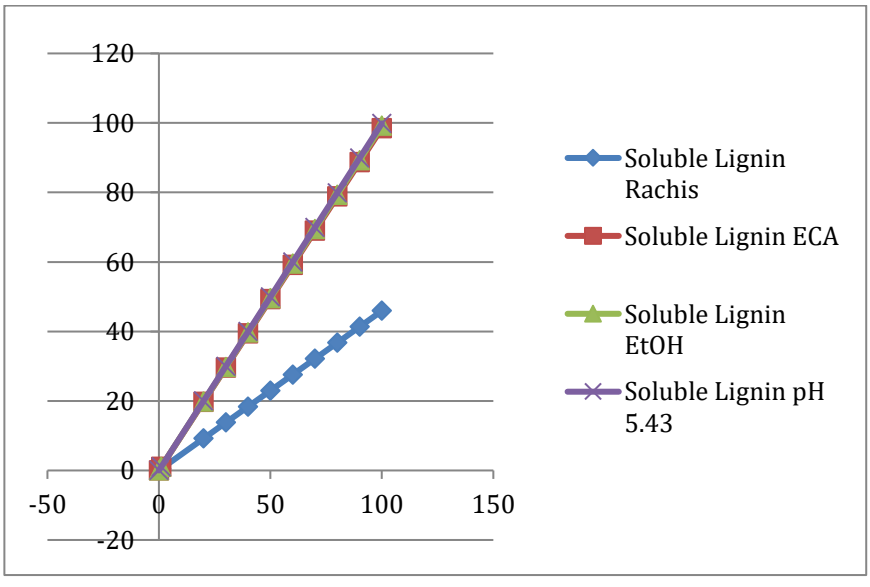



**Figure 20**

**Variation in cellulose content**

The evaluation of the cellulose content in the biomass resulting from the different treatments indicates that there is no significant difference between the samples treated with ECA solution and ECA plus Ethanol solution, however there is a variation in the cellulose content with the acid treatment with subsequent oxidation with ECA, which is expected given that there is a fraction of sugars with the possibility of hydrolyzing in an acid medium; according to the proposed model corresponds to 18.5% less compared to the other two treatments. It is interesting to conclude that the procedure described clearly shows the variation of the slopes (Figure 20), which in turn allows a relative quantification of the change in cellulose content according to the treatment used. Calibration involves methodological difficulties when working with Carboxi-Methylcellulose as an internal standard, which can generate problems when evaluating the effective cellulose concentration of the original sample.

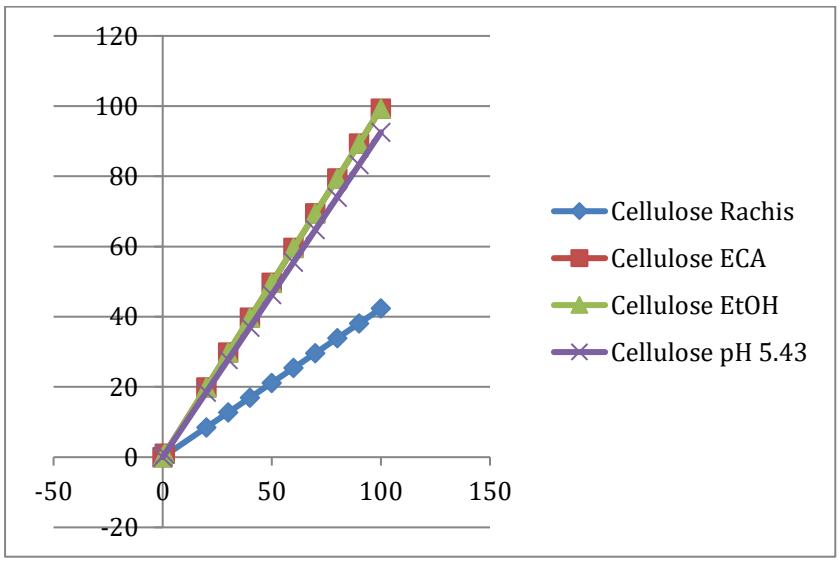



**Figure 21**

**Variation in Insoluble Lignin content**

The results obtained indicate that there is a minimum variation between the insoluble lignin content remaining in the biomass after the treatments used (Figure 21). Approximately there is a 3.4% reduction of insoluble lignin with the double treatment with acetic acid / acetone / water mixture of pH 5.43 and subsequent oxidation with ECA. The ethanol pretreatment represents a 1.6% decrease compared to treating the biomass only with ECA solution, therefore it can be

concluded that the previous organo-solv treatments do not represent a significant improvement for the extraction of insoluble lignin from the biomass treated only with solution ECA.

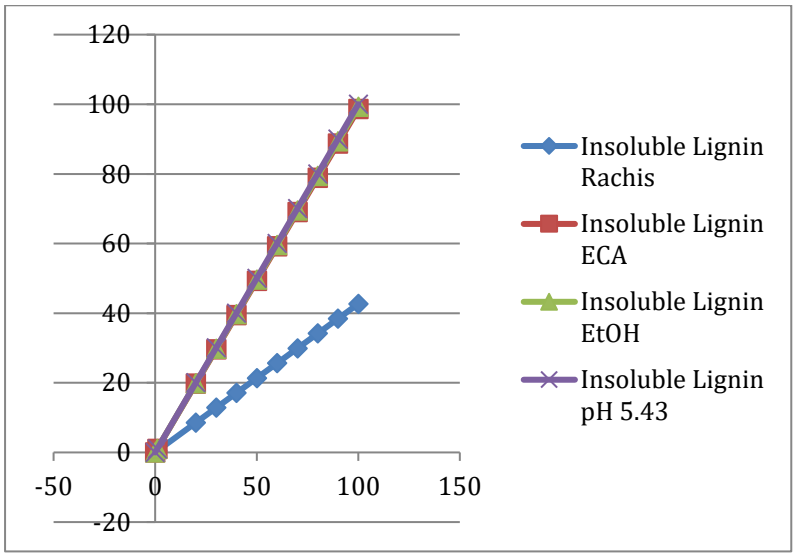



**Figure 22**

**Acid hydrolysis**

The results of the acid hydrolysis with 0.25M sulfuric acid evaluated by HPLC denote a high number of oligomers and a very low amount of monosaccharides in solution obtained with the three selected delignification treatments during the reaction time tested. However, it is possible to observe that there are important differences on the composition of the liquors of acid hydrolysis as a function of hydrolysis time and the treatment used for delignification.

Observing the results regarding the glucose content, it is appreciated that they are considerably high with the ECA treatment, while the content of xylose and arabinose are significantly higher with the treatments with alkaline hydrogen peroxide and sodium hypochlorite. These results, added to those explained in the part concerning the effect of the delignifying treatment on the amount of biomass obtained, can indicate that indeed the treatment with ECA solution removes part of the hemicellulose contained in the banana rachis.

The results of hydrolysis with 20% sulfuric acid solution (W/V) improve the amount of sugars obtained, however, it does not exceed 10% of glucose in relation to the total of dry rachis treated.

The hydrolysis with 2% maleic acid solution (W/V) presents the most remarkable results, reaching up to 0.51 g of glucose / g of dry rachis pretreated with hydrogen peroxide solution during the delignification stage. The pretreatment with ECA solution produces the lowest glucose yields per gram of rachis, however they are not statistically different to those obtained with the treatment of 5% NaClO used for the delignification.

**Mixed hydrolysis**

All matrices hydrolyzed with maleic acid were consecutively hydrolyzed with cellulase enzymes (a) and B-glucanases (b), obtaining a significant improvement in glucose production. Again the best results were obtained with the pre-treatment of delignification using alkaline hydrogen peroxide. The figures 23 and 24 summarizes the results obtained.

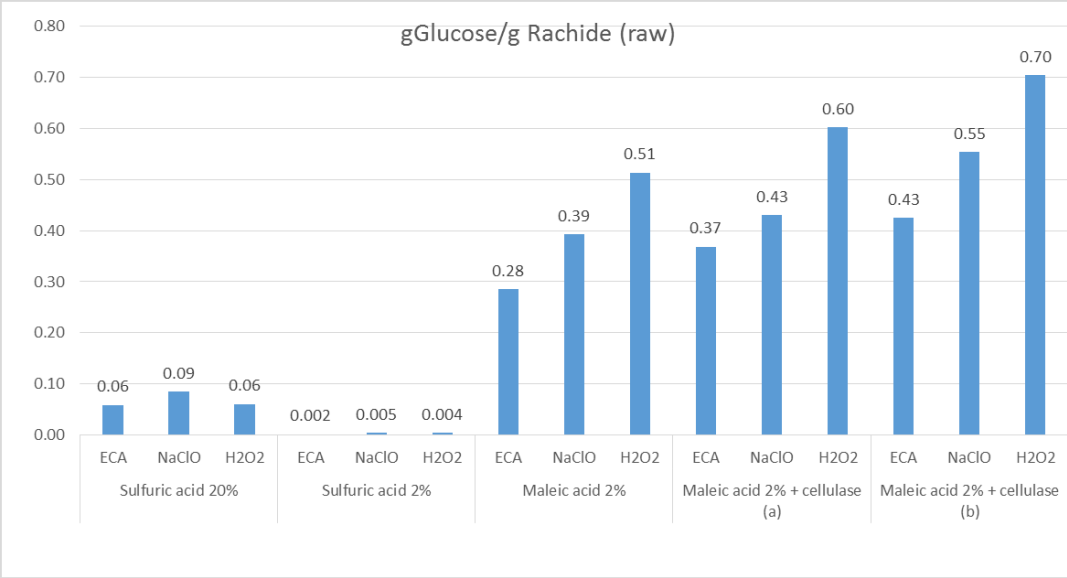



**Figure 23**

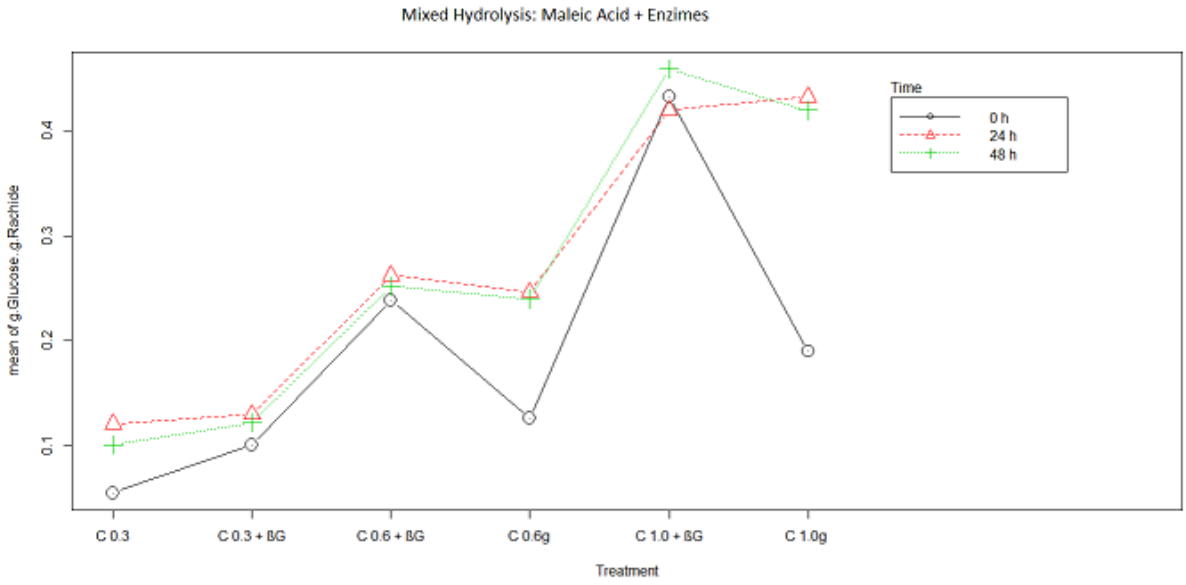



**Figure 24**

**Enzymatic hydrolysis**

The results of enzymatic hydrolysis are shown in Figure 25. All the tests were performed on the matrix delignified with hydrogen peroxide, given the best results with combined acid and enzymatic hydrolysis. The highest concentration of glucose per gram of treated rachis was obtained using 1 g of enzyme cellulases. There are no statistical differences with the addition of B-glucosidases. The results are summarized in the table 6.

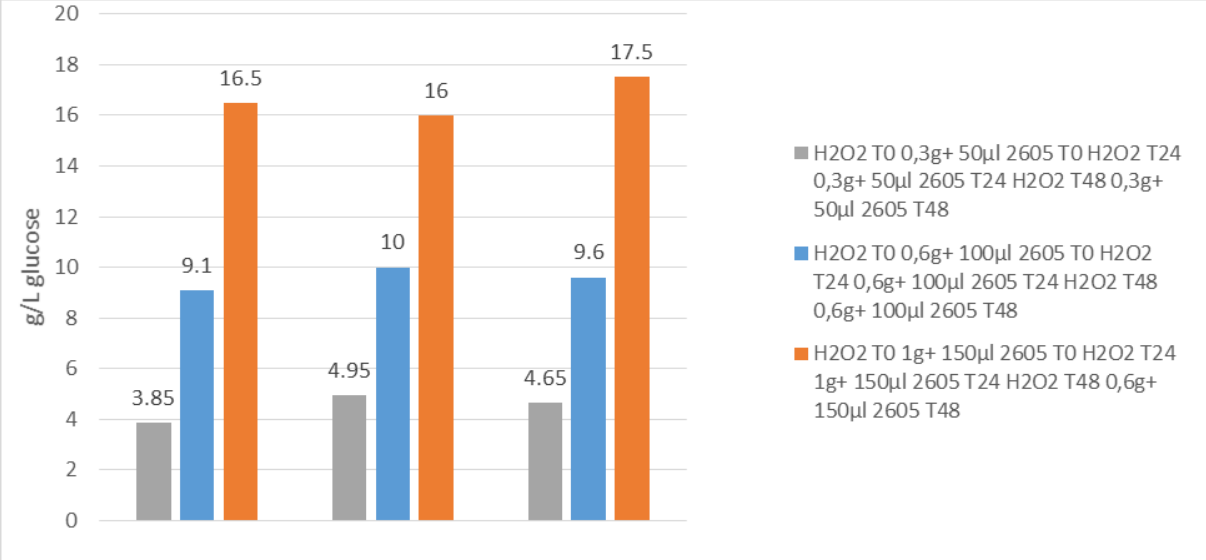



**Figure 25**

**Table 6.** Glucose production (g Glucose/g Rachis raw)

| Delignification treatment: H2O2 | Hydrolysis | Result |
|---|---|---|
| **Enzime** | Time(hours) | g Glucose/g Rachide |
| Cellulase (0.3g) | 0 | 0.06 |
| | 24 | 0.12 |
| | 48 | 0.10 |
| Cellulase (0.6g) | 0 | 0.126 |
| | 24 | 0.246 |
| | 48 | 0.240 |
| Cellulase (1.0g) | 0 | 0.19 |
| | 24 | 0.43 |
| | 48 | 0.42 |
| Cellulase (0.3g) + β (glucosidase) | 0 | 0.10 |
| | 24 | 0.13 |
| | 48 | 0.12 |
| Cellulase (0.6g) + β (glucosidase) | 0 | 0.24 |
| | 24 | 0.26 |
| | 48 | 0.25 |
| Cellulase (1.0g) + β (glucosidase) | 0 | 0.43 |
| | 24 | 0.42 |
| | 48 | 0.46 |

- ***Fermentation***

The results of fermentation in the delignified and hydrolyzed rachis flask with 30% inoculum (0.150 OD; diluted 1:30) are presented in the figures 26 and 27.

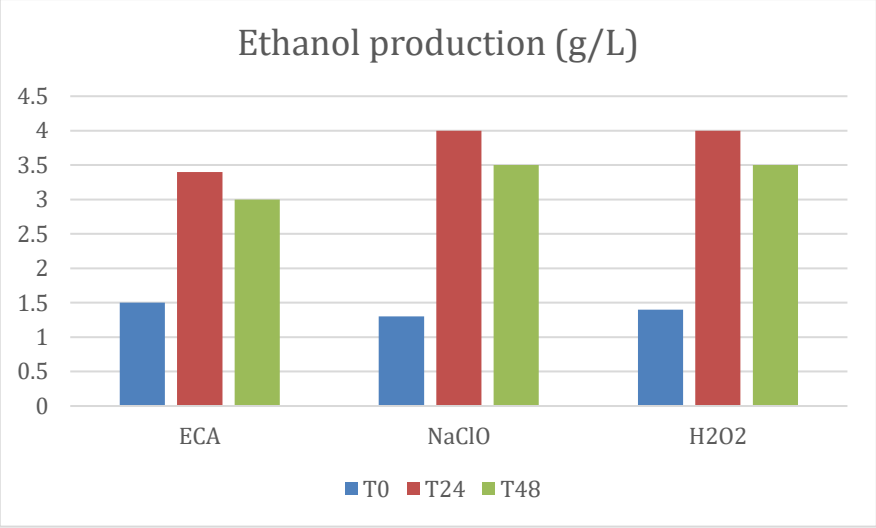



**Figure 26**

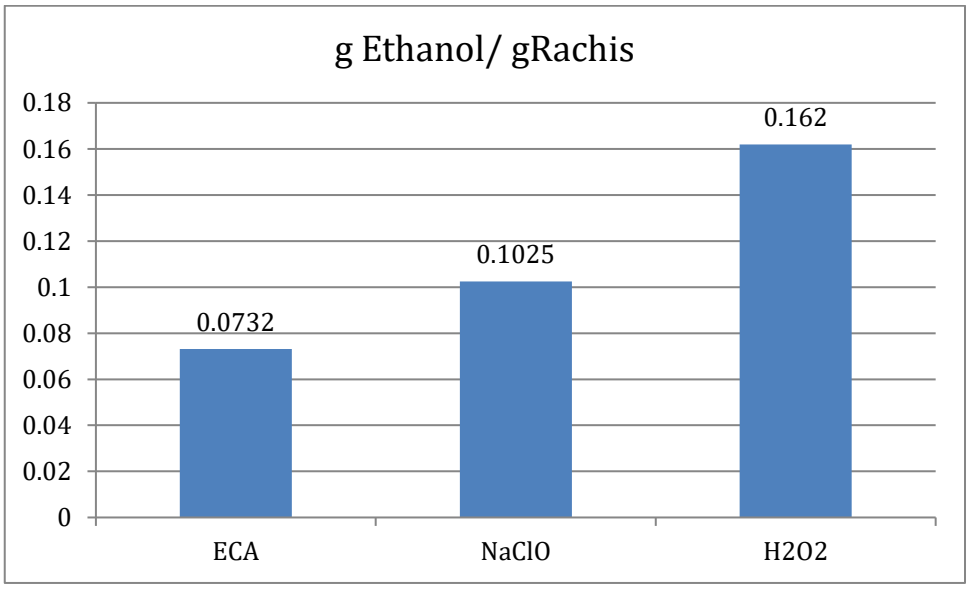



**Figure 27**

The best results (working volume, 500 mL were obtained with the matrix delignified with hydrogen peroxide. The results obtained with the samples treated with an oxidizing ECA solution show the lowest yield of ethanol produced.

The variation of the glucose decrease with the consequent increase in the concentration of ethanol as a function of the fermentation time is presented in the figure 28.

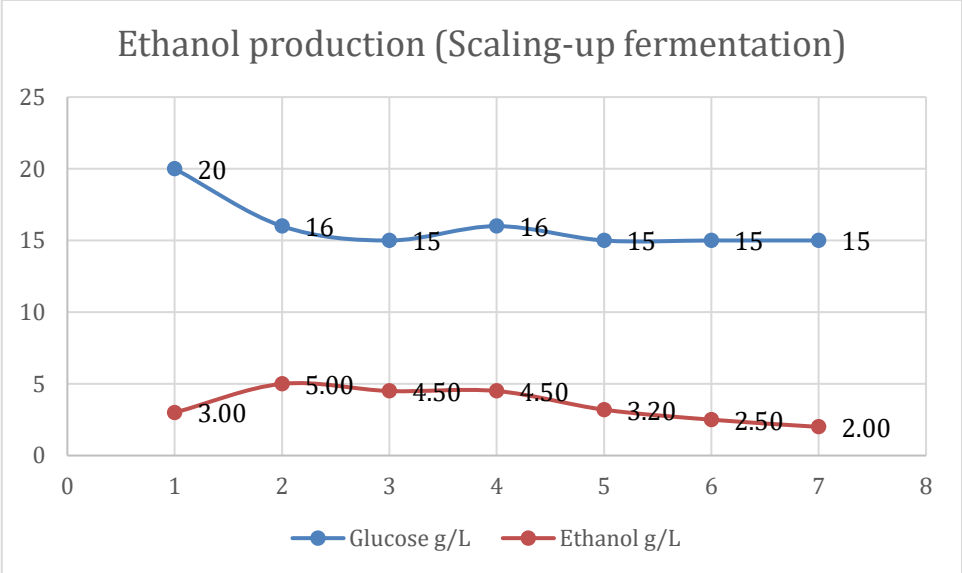



**Figure 28**

**SEM analysis of delignification methods**

The micrographs obtained in SEM do not present apparent differences at first sight. See figures 27, 28. The digital processing of the images helps to conclude that SEM microscopy allows to differentiate the samples obtained by different treatments by grouping statistically the frequency histograms obtained for each sample according to the magnification used. It is indicated by the color codes: ECA (green); Hydrogen peroxide (light blue); sodium hypochlorite (magenta) and rachis without treatment (red). Figures 29, 30.

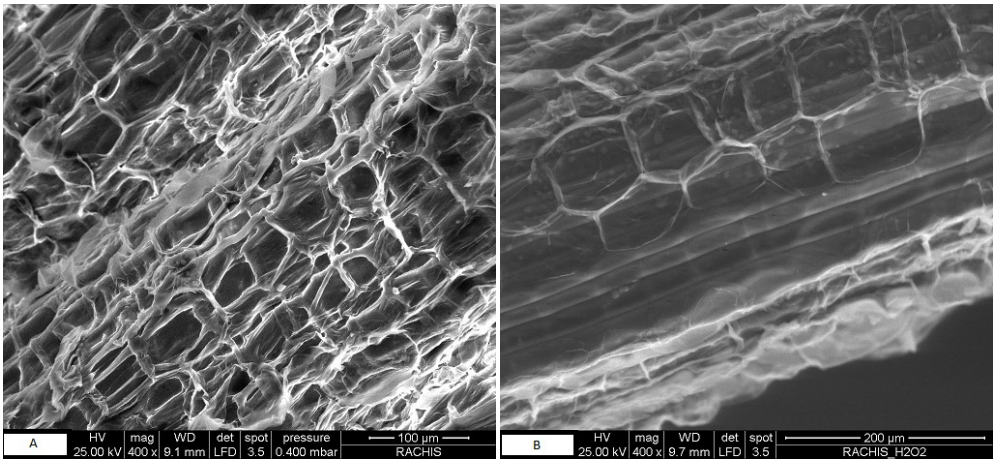



**Figure 27**

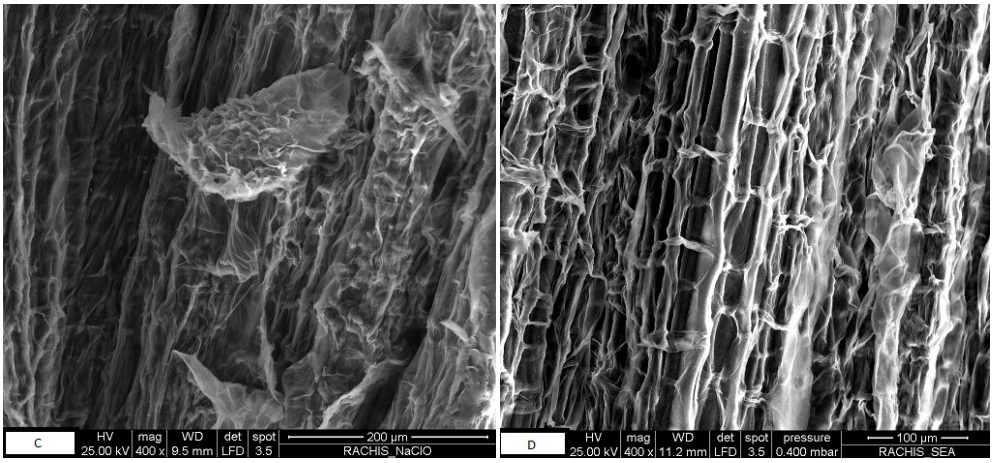



**Figure 28**

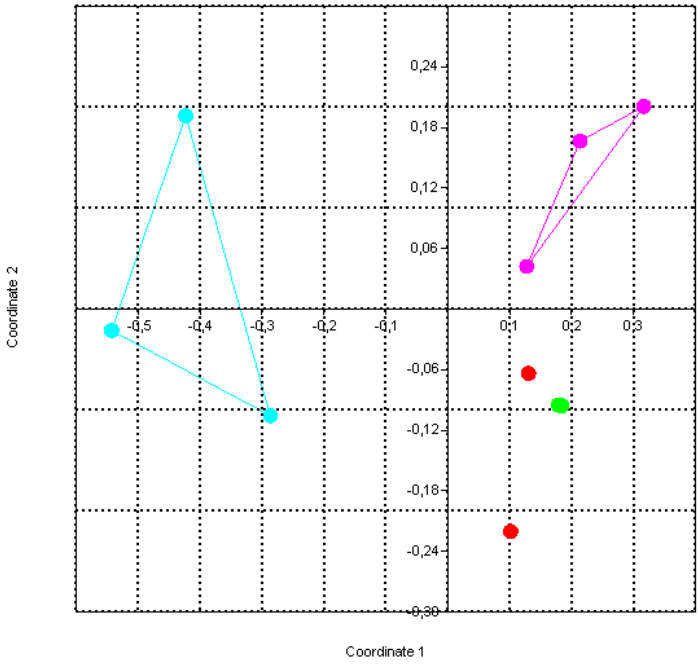



**Figure 29**

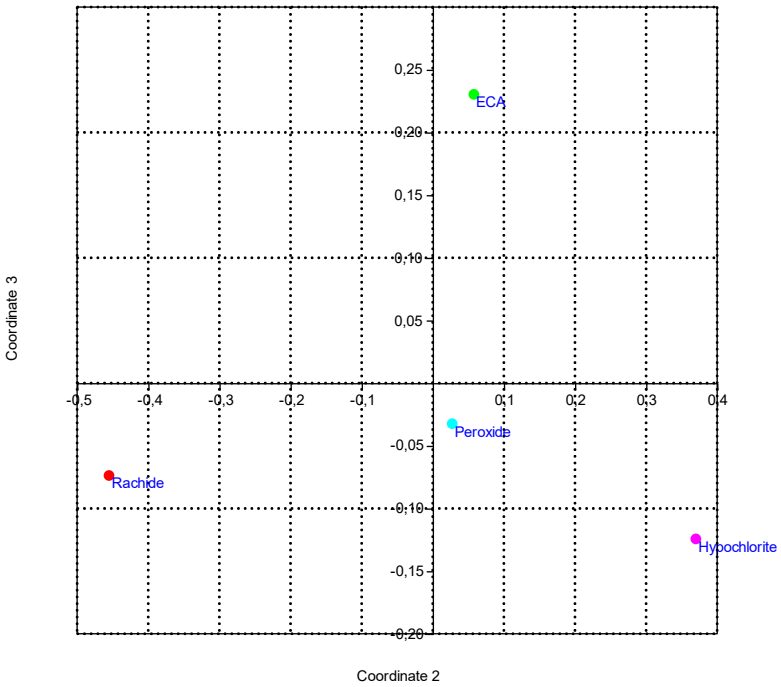



**Figure 30**

Through the Kruskal-Wallis test it was possible to establish that the frequency distribution of the micrographs corresponding to the four different treatments are significantly different ($p < 0.001$). By cluster analysis with Ward´s method (Figure 31) it can be concluded that the SEM images of the ECA and hypochlorite treatments are more similar to each other and statistically different to the corresponding ones of the peroxide treatment and the original rachis.

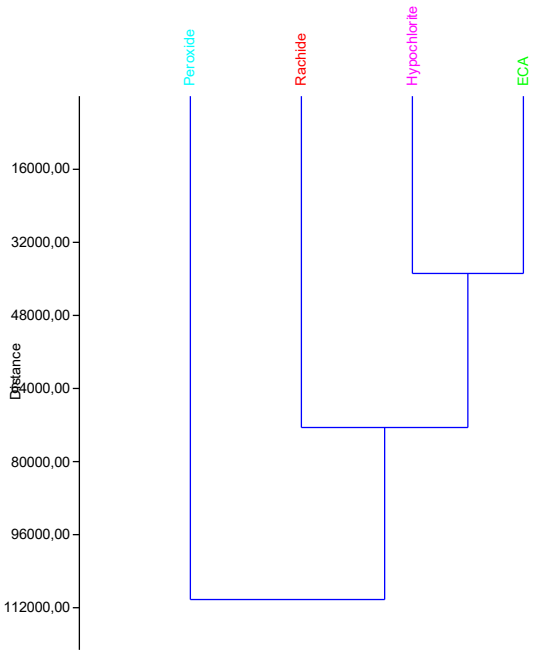



**Figure 31**

This observation agrees with the results obtained through the other analytical techniques presented in this work in conjunction with the expected chemical behavior of the ECA oxidant solution similar to the sodium hypochlorite used in the delignification of the banana rachis.

# Chapter 4

# CONCLUSIONS

The present research has two main objectives, one linked to the need to give a commercial value to the waste coming from the banana industry in Ecuador and the other as a response to the current demands of energy to replace fossil fuels, according to the trend global focused on developing green and environmentally friendly technologies that can maximize the reuse of agroindustrial, municipal and domestic organic waste.

The large amount of rachis produced by the Ecuadorian banana industry represents a valuable opportunity for research, development and innovation of Green Chemistry and Biotechnology processes that allow obtaining materials of high commercial value from the use of lignin and fractions of cellulose and hemicellulose existing in the biomass of the rachis.

The methods used for the processing of the banana rachis have allowed to evaluate and compare different options of delignification, hydrolysis and fermentation of the biomass depending on the type of solvent used, the oxidation method and the variables associated with the process such as the reaction time, the concentration of the reactants and the mixing ratios between biomass and solvent volume.

The composition of the biomass resulting from the different treatments presents a very diverse chemical composition, which could be evaluated through the FTNIR Spectroscopy method developed, results of which were published by Tamburini, Larenas-Uría et al, in the article "Potential of Near Infrared" Spectroscopy for Classification of Different Delignificant Pre-Treatments on Banana Rachis ", from the Journal of Analytical & Bioanalytical Techniques, 2016.

The test of different experimental conditions on the selected treatments has allowed to evaluate the results from new methodological approaches, especially those associated with the Chemometric analysis methods. In this way, an alternative method to quantify soluble lignin extracted by different organo solvents and different operating conditions has been found through second-order UV spectrophotometry and multivariate PLS calibration. Likewise, a quantitative method has been developed to determine the relative amount of cellulose, soluble lignin and insoluble lignin present in the solid matrices resulting from the different treatments applied to the biomass using the FTNIR Spectroscopy based on the measurement of the angle between the calibration curves corresponding to the different matrices and the subsequent measurement of the variation of the areas obtained. The method requires the addition of an internal calibration standard for lignin kraft, soluble lignin and cellulose which was specifically designed for lignocellulosic matrices and whose application is characterized by being operationally simple and easy to incorporate into a solid matrix, without being significantly

costly. Is particularly relevant the ability of FTNIR Spectroscopy as an adequate analytical technique to discriminate the composition of the biomass treated under various reaction conditions.

The statistical analysis of the digitized images from electronic microscopy (SEM) allows to corroborate the existence of structural differences between rachis matrices obtained by various chemical treatments.

The evaluation of the data obtained from the different procedures used to transform rachis, lets to conclude that the method followed to delignify the biomass using the ECA oxidant solution produces remarkable results thanks to its extractive properties of polar and moderately polar substances, avoiding the use of pretreatments with organo-solvents such as ethanol, acetone and ethyl acetate. However, the other proposed alternative method using 2% hydrogen peroxide with NaOH (0.5%) produces results with superior performance in the subsequent processes of delignification and hydrolysis.

The results of the hydrolysis indicate that the lignocellulosic matrix of the banana rachis is particularly resistant to the action of diluted mineral acids, while it is very sensitive to degradation by the effect of concentrated mineral acids, so the production of fermentable sugars is not significant under the usual reaction conditions. The use of organic acids, characterized by behaving as weak acids represents an interesting alternative for traditional hydrolysis methods by significantly increasing the concentration of glucose and other fermentable sugars. For the particular case with the use of maleic acid (2% W/V), the acid hydrolysis reached an increase of up to 20% of glucose compared to the process with sulfuric acid (20% W/V) and 60% when combined with enzymatic hydrolysis. On the other hand, it is important to reduce the volume of alkaline solutions used for the neutralization of the reaction medium and subsequent enzymatic treatment. In the same way, the joint use of hydrolysis with maleic acid and B-glucanase enzymes produces an increase of up to 24% of glucose with respect to enzymatic hydrolysis. It should be noted that maleic acid can be considered a reagent of green chemistry, being currently produced at the industrial level through biotechnological processes, being simpler the management of waste thanks to its ease of organic degradation and little environmental toxicity.

The results of the fermentation indicate that the average yield for obtaining second generation ethanol is 0.16 g / g of banana rachis. The amount indicates that it is approximately half of that obtained from other agro industrial waste and residues, mainly those coming from the processing of wheat straw and other herbaceous materials. For Ecuador, the potential

production of ethanol by this method amounts to 15 million liters per year, which can be an interesting opportunity for farmers in the productive areas.

The proposed process that starts from the use of banana rachis treated with 2% (W/V) alkaline hydrogen peroxide with 0.5% NaOH (W/V) for the corresponding delignification, 2% maleic acid (W/V) and cellulases from *Aspergillus niger* in the subsequent stages of hydrolysis to obtain glucose and the final stage of fermentation with *Saccharomyces cerevisiae* represents a valid alternative for obtaining second generation ethanol. All the proposed reagents and processes respond to an environmentally friendly production approach; however it is recommended to study the production costs at the industrial level to evaluate the feasibility of its use for commercial purposes. On the other hand, it is recommended the study and analysis of the composition of the soluble polymeric fractions present in the banana rachis given its unique characteristics that indicate a possible high concentration of polyuronides that can be exploited from the same productive process.

# Chapter 5

# BIBLIOGRAPHY

1. Arifin, Y., Tanudjaja, E., Dimyati, A., & Pinontoan, R. (2014). A second generation biofuel from cellulosic agricultural by-product fermentation using clostridium species for electricity generation. *Energy Procedia*, *47*, 310–315.

2. Pedram F. (2017). Production of Biofuels from Cellulose of Woody Biomass*, Cellulose and Biomass Conversion INTECH,3,*45-46

3. Kamm, B., & Kamm, M. (2004). Principles of biorefineries. *Applied Microbiology and Biotechnology*, *64*(2), 137–145.

4.Brosse, N., Mohamad Ibrahim, M. N., & Abdul Rahim, A. (2011). Biomass to Bioethanol: Initiatives of the Future for Lignin. *ISRN Materials Science*, *2011*, 1–10.

5. Cotana, F., Cavalaglio, G., Nicolini, A., Gelosia, M., Coccia, V., Petrozzi, A., & Brinchi, L. (2014). Lignin as co-product of second generation bioethanol production from ligno-cellulosic biomass. *Energy Procedia*, *45*(0), 52–60.

6. Ingram, T., Wörmeyer, K., Lima, J. C. I., Bockemühl, V., Antranikian, G., Brunner, G., & Smirnova, I. (2011). Comparison of different pretreatment methods for lignocellulosic materials. Part I: Conversion of rye straw to valuable products. *Bioresource Technology*, *102*(8), 5221–5228.

7. Wörmeyer, K., Ingram, T., Saake, B., Brunner, G., & Smirnova, I. (2011). Comparison of different pretreatment methods for lignocellulosic materials. Part II: Influence of pretreatment on the properties of rye straw lignin. *Bioresource Technology*, *102*(5), 4157–4164

8. Menon, V., & Rao, M. (2012). Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, *38*(4), 522–550

9. Brethauer, S., & Wyman, C. E. (2010). Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. *Bioresource Technology*, *101*(13)

10. Sahoo, S., Seydibeyoğlu, M. Ö., Mohanty, A. K., & Misra, M. (2011). Characterization of industrial lignins for their utilization in future value added applications. *Biomass and Bioenergy*, *35*(10), 4230–4237.

11. Conde-Mejía, C., Jiménez-Gutiérrez, A., & El-Halwagi, M. (2012). A comparison of pretreatment methods for bioethanol production from lignocellulosic materials. *Process Safety and Environmental Protection*

12. Stewart, D. (2008). Lignin as a base material for materials applications: Chemistry, application and economics. *Industrial Crops and Products*, *27*(2), 202–207.

13. Vanholme, R., Demedts, B., Morreel, K., Ralph, J., & Boerjan, W. (2010). Lignin Biosynthesis and Structure. *Plant Physiology*.

14. Higuchi, T. (1990). Lignin biochemistry: Biosynthesis and biodegradation. *Wood Science and Technology*, *24*(1), 23–63

15.Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polym. Chem.*, *6*(25), 4497–4559.

16. Zhang, L., You, T., Zhang, L., Li, M., & Xu, F. (2014). Comprehensive utilization of waste hemicelluloses during ethanol production to increase lactic acid yield: from pretreatment to fermentation. *Biotechnology for Biofuels*.

17. Nuruddin, M., Chowdhury, a., Haque, S. a., Rahman, M., Farhad, S. F., Jahan, M. S., & Quaiyyum, a. (2011). Extraction and Characterization of Cellulose Microfibrils From Agricultural Wastes in an Integrated Biorefinery Initiative. *Cellulose Chem. Technol.*, *45*(5–6), 347–354.

18.Hon D., (1994). Cellulose: a random walk along its historical path, *Cellulose* 1-25

19. Huang, Y.-B., & Fu, Y. (2013). Hydrolysis of cellulose to glucose by solid acid catalysts. Green Chemistry, 15(5), 1095.20. J.H. Clark, F.E.I. Deswarte, T.J. Farmer(2009), The integration of green chemistry into future biorefineries, Biofuels, Bioprod. Bioref., 3 72-90.

20. J. W. S. Hearle. (1958). structure and properties of Hemicellulose. Journal of Polymer Chemistry, 28(117), 432–435.

21. Murat Sen, S., Henao, C. A., Braden, D. J., Dumesic, J. A., & Maravelias, C. T. (2012). Catalytic conversion of lignocellulosic biomass to fuels: Process development and technoeconomic evaluation. *Chemical Engineering Science*, *67*(1), 57–67.

22. Axelsson, L., Franzén, M., Ostwald, M., Berndes, G., Lakshmi, G., & Ravindranath, N. H. (2012). Perspective: Jatropha cultivation in southern India: Assessing farmers' experiences. *Biofuels, Bioproducts and Biorefining*, *6*(3), 246–256.

23. Pérez, J., Muñoz-Dorado, J., De La Rubia, T., & Martínez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An overview. *International Microbiology*, *5*(2), 53–63.

24. Rinaldi, R., & Schüth, F. (2009). Acid hydrolysis of cellulose as the entry point into biorefinery schemes. *ChemSusChem*, *2*(12), 1096–1107.

25. Wada, M., Chanzy, H., Nishiyama, Y., & Langan, P. (2004). Cellulose IIII Crystal Structure and Hydrogen Bonding by Synchrotron X-ray and Neutron Fiber Diffraction. *Macromolecules*, *37*(23), 8548–8555.

26. Hallac B. ,Ragauskas A. (2011). Analyzing cellulose degreeof polymerization and its relevancy to cellulosic ethanol, *Biofuels, Bioprod. Bioref*. 5:215–225

27. Serrano-Ruiz, J. C., Braden, D. J., West, R. M., & Dumesic, J. A. (2010). Conversion of

cellulose to hydrocarbon fuels by progressive removal of oxygen. *Applied Catalysis B: Environmental*, *100*(1–2), 184–189

28. Kontturi, E. (2015). Hemicellulose: structure, characterization, dissolution, modification, (November).

29. Muszyński, A., O'Neill, M. A., Ramasamy, E., Pattathil, S., Avci, U., Peña, M. J., … Carlson, R. W. (2015). Xyloglucan, galactomannan, glucuronoxylan, and rhamnogalacturonan I do not have identical structures in soybean root and root hair cell walls. *Planta*, *242*(5), 1123–1138.

30. Chávez-Sifontes, M., & Domine, M. E. (2013). lignina, estructura y aplicaciones: métodos de despolimerización para la obtención de derivados aromáticos de interés industrial lignin, structure and applications:, *4*(4), 15–46.

31. Sorek, N., Yeats, T. H., Szemenyei, H., Youngs, H., & Somerville, C. R. (2014). The implications of lignocellulosic biomass chemical composition for the production of advanced biofuels. *BioScience*, *64*(3), 192–201.

32. Iiyama, K., Kasuya, N., Lam, T. B. T., & Stone, B. A. (1991). Lignin in wheat internodes: Linkages between phenylpropane units. *Journal of the Science of Food and Agriculture*, *56*(4), 551–560.

34. Watkins, D., Nuruddin, M., Hosur, M., Tcherbi-Narteh, A., & Jeelani, S. (2015). Extraction and characterization of lignin from different biomass resources. *Journal of Materials Research and Technology*, *4*(1), 26–32.

35.Alemdar, A., & Sain, M. (2008). Isolation and characterization of nanofibers from agricultural residues - Wheat straw and soy hulls. *Bioresource Technology*, *99*(6),

36. Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research*, *48*(8), 3713–3729.

37. Chen, X., Kuhn, E., Wang, W., Park, S., Flanegan, K., Trass, O., … Tucker, M. (2013). Comparison of different mechanical refining technologies on the enzymatic digestibility of low severity acid pretreated corn stover. Bioresource Technology, 147, 401–408.

38. Chung, B. Y., Lee, J. T., Bai, H. W., Kim, U. J., Bae, H. J., Gon Wi, S., & Cho, J. Y. (2012). Enhanced enzymatic hydrolysis of poplar bark by combined use of gamma ray and dilute acid for bioethanol production. Radiation Physics and Chemistry, 81(8), 1003–1007

39. Yang, H., Yan, R., Chen, H., Lee, D. H., & Zheng, C. (2007). Characteristics of hemicellulose, cellulose and lignin pyrolysis. Fuel.

40. Laurichesse, S., & Avérous, L. (2014). Chemical modification of lignins: Towards

biobased polymers. Progress in Polymer Science.

41. Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production : a review q. Bioresource Technology, 83(1), 1–11.

42. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96(6), 673–686.

43. Holtzapple, M. T., Lundeen, J. E., & Dale, B. E. (1991). Ammonia Fiber Explosion (AFEX) Pretreatment of Municipal Solid Waste. Abstracts of Papers of the American Chemical Society, 202, 16 BIOT.

44. Santos, A. L. F., Kawase, K. Y. F., & Coelho, G. L. V. (2011). Enzymatic saccharification of lignocellulosic materials after treatment with supercritical carbon dioxide. The Journal of Supercritical Fluids, 56(3), 277–282.

45. Tamburini E., Bernardi T.,Castaldelli G, (2011), Green electrochemical approach for delignification of wheat straw in second-generation bioethanol production, *Energy Environ. Sci.,* 4, 551

46. Zhang, Y. H. P., & Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnology and Bioengineering, 88(7), 797–824.