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Microscopic and Biochemical Analysis to Better Understand the Impact of Alkaline Pretreatments on Lignocellulosic Biomass

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ABSTRACT

Due to an increased demand for more sustainable energy sources, Anaerobic Digestion (AD) of solid organic wastes such as biomass and energy crops have been gaining attention. In this study, varieties of sorghum and miscanthus were chosen as lignocellulosic biomass to be used for biodigestion because of their promising features for energy production in the context of a French initiative called Biomass for the Future (BFF). In the process of AD, organic material such as cellulose is broken down, but in lignocellulosic substrates, lignin acts as an inhibitor for this process. Pretreatments can be applied to the substrate in order to break down lignin and expose cellulose before the digestion. In this study, alkali pretreatments were applied using CaO and NaOH at 10% of concentration according to the sample's Dry Mass value in different temperatures (26 and 55ºC) to evaluate its efficiency, quantifying both the removal of lignin and the availability of cellulose. Pretreatments were evaluated from 0,5 to 144 hours. Staining with FASGA and Phloroglucinol HCl solution were performed and a kinetics study was made. Phloroglucinol-HCl staining showed that temperature has a positive correlation with lignin removal. The most effective pretreatments on both sorghum and miscanthus samples was pretreatment using NaOH at 55ºC.

Keywords: Anaerobic digestion, lignocellulosic biomass, alkali pretreatment, FASGA, Phloroglucinol HCl.

RESUMO

Devido ao aumento da demanda por fontes de energia mais sustentáveis, a Digestão Anaeróbia (DA) de resíduos orgânicos sólidos, como biomassas e culturas energéticas, vem ganhando atenção. Neste estudo, variedades de sorgo e miscanthus foram escolhidas como biomassa lignocelulósica para biodigestão devido às suas características promissoras para a produção de energia no contexto da iniciativa francesa chamada Biomassa para o Futuro (BFF). No processo de DA, a matéria orgânica como a celulose é decomposta em substratos lignocelulósicos. A lignina atua como um inibidor desse processo. Pré-tratamentos podem ser aplicados ao substrato para quebrar a lignina e expor a celulose antes da digestão. Neste estudo, pré-tratamentos alcalinos foram aplicados com CaO e NaOH com 10% da concentração de acordo com o valor de massa seca das amostras em diferentes temperaturas (26 e 55 ºC) para avaliar sua eficácia, quantificando tanto a remoção de lignina quanto a disponibilidade de celulose. Os pré-tratamentos foram avaliados de 0,5 a 144 horas. A coloração com FASGA e solução de Phloroglucinol-HCl foi realizada e um estudo cinético foi feito. A coloração com Phloroglucinol-HCl mostrou que a temperatura tem uma correlação positiva com a remoção de lignina. O pré-tratamento mais eficaz em ambas as amostras de sorgo e miscanthus foi o pré-tratamento usando NaOH a 55ºC.

Palavras-chave: Digestão anaeróbia, biomassa lignocelulósica, pré-tratamento alcalino, FASGA, Phloroglucinol–HCl.

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AD	Aerobic Digestion
BFF	Biomass for the Future
BMP	Biochemical Methane Potential
CIRAD	Center for International Cooperation in Agricultural Research for Development
CO_2	Carbon Dioxide
densVBZ2	Density of Vascular Bundles in Zone 2
DM	Dry Matter
DP	Degree of Polymerization
EU	European Union
GHGs	Green House Gases
INRA	National Institute of Agronomic Research
MNT	Miscanthus Non-treated
MC	Moisture Content
MRI	Montpellier Imaging Resources
nbVBZ2	Number of Vascular Bundles in Zone 2
PerbluZ2	Percentage of Blue Tissue Area in Zone 2
PerSclZ1	Percentage of Sclerenchyma area in Zone 1
perZ1	Percentage of Zone 1 Area
perZ2	Percentage of Zone 2 Area
PH	Phloroglucinol–HCl
PHIV	Histology Platform and Plant cell Imaging
PT	Pretreatment
PT1	Pretreatment 1
PT2	Pretreatment 2
PT3	Pretreatment 3
PT4	Pretreatment 4
PT5	Pretreatment 5
PT6	Pretreatment 6
PT7	Pretreatment 7

PT8	Pretreatment 8
PW	Primary Wall
SNT	Sorghum Non-treated
SW	Secondary Wall
VFAs	Volatile Fatty Acids
Z1	Zone 1
Z2	Zone 2

SUMMARY

1 INTRODUCTION

Energy is the key subject when referring to sustainability. Renewable energy sources have been growing in the energy market, as their prices have been falling. As a consequence of population growth, the topic of sustainable energy production appears to be the only possible alternative to ensure the transition from a natural resource depletion scenario to a more sustainable future (KUMARI, 2015). Fossil resources are limited in quantity, time and cause an enormous environmental impact due to Greenhouse Gases (GHGs) emissions into the atmosphere (ENERGY INFORMATION ADMINISTRATION, 2013). GHGs emissions continue to increase, even though since 2014 the rate of carbon dioxide emissions from energy and industry has decreased, maintaining the rates stable mainly due to the rise of investments in renewable energy sources (UNITED NATIONS ENVIRONMENT PROGRAMME, 2014).

This challenge is achievable, but far from simple. The three main sources of GHGs emissions in the European Union (EU) are electricity generation, heat production and road transportation (TISEO, 2021). Reports show that in 2018, renewable energy (specifically biofuels) stood for 8% of the fuel used in road transport in the EU, increasing 2.8% from 2010 and lacking 10% for the 2020 goal. The EU aims to have 14% of renewable energy in transport by 2030, focusing at least 3.5% of that on ‘advanced biofuels’ and biogas (FRANKFURT SCHOOL-UNEP CENTRE, 2020).

Studies reveal that biomass is the most sustainable source of organic carbon on earth and the perfect equivalent to petroleum for the production of biofuels, biomolecules and biomaterials. In this context, lignocellulosic biomass, which is the most abundant and biorenewable biomass on earth, has a critical importance (ISIKGOR; BECER, 2015).

Biofuels are considered the most environmentally friendly energy source (NIGAM; SINGH, 2011) as they when burned produce no more CO_2 than they would have if the feedstock’s regular growth had been permitted, as opposed to fossil fuels that when burned release into the atmosphere gases that had been stored for millions of years (BÉDUÉ; WERTZ, 2013). Biofuels can be the solution to the world’s dependence on fossil fuels. As the interest in this subject rises, the key to reducing the world’s dependence on non-renewable energy sources is only a few steps away from being fully implemented throughout the world.

Four main energy vectors can be produced from lignocellulosic biomass: liquid biofuels, hydrogen, biogas, and synthesis gas. Amongst them, biogas is particularly interesting

as its production lies on Anaerobic Digestion (AD) process. This biological process has the advantage of accepting as feedstocks various organic substrates, of which most are organic waste and lignocellulosic biomass (SOLARTE-TORO et. al. 2018).

The replacement of fossil energy sources by biomethane (biogas after purification) contributes to controlling water, soil and air pollution, in addition contributing to reducing risks of potentially devastating accidents related to energy production. Research shows that 1 m^3 of biogas can replace about 0.6 L of heating oil. From an economic point of view, biogas is also seen as a tempting alternative due to its relatively low production cost in addition to the fact that biogas has a lower sale price when compared with diesel and oil (BHARATHIRAJA et al., 2018).

Countries such as Brazil and the United States of America (USA) already use 1st generation biofuels on commercial scale. Brazil is known for the large production of biofuels from sugar cane and the USA from corn maize (both lignocellulosic crops). First generation biofuels are synthesized from edible plants, whereas second generation biofuels come from non-edible biomasses of whole plant parts such as the steam, leaves and bark or from non-edible food crops. Therefore, 2nd generation biomass feedstock production does not compete for food supplies as 1st generation does (ULLAH et al. 2017).

Influenced by the recent advancements in environmentally friendly technologies, the EU created the goal of supplying at least 32% of the European energy demands from renewable energy sources by the year of 2030 (IEA, 2020). A large percentage of this renewable energy will originate from farming and forestry. At least 25% of all bioenergy in the future can originate from biogas (HOLM-NIELSEN; SEADI; OLESKOWICZ-POPIEL, 2009). In this context, as an option to produce both heat and electricity, biogas is a sustainable, relatively low coast, promising developing technology.

2 OBJECTIVES

This study has been developed within a project called Biomass for the Future (BFF), an initiative developed in France, aiming to approach the environmental and social issues surrounding the production and the use of biomass to ensure the future development of environmentally friendly industries. The project proposes a number of approaches regarding optimizing energy production, based on two types of biomass: Sorghum and miscanthus.

2.1 GENERAL OBJECTIVES

The main objective of this research is to participate on the value chain development of lignocellulosic biomass by studying the impacts of alkaline pretreatments in both sorghum and miscanthus using Sodium Hydroxide (NaOH) and Calcium Oxide or lime (CaO), at a concentration of 10% according to its Dry Matter content (DM). This study focuses not only on the pretreatment's actions in the large scale but also on trying to understand its impacts microscopically on the biomass tissues and anatomy. For this, activities such as sectioning, staining, microscopic rehearsals, and biochemical analysis have been performed.

2.2 SPECIFIC OBJECTIVES

a) Create and compare four alkaline pretreatment designs varying reagent and temperature;
b) Evaluate the cellulose/lignin ratio after alkaline pretreatment applications;
c) Understand the impact of temperature when applying alkaline pretreatments;
d) Study and compare the staining methods FASGA (developed at CIRAD), and Phloroglucinol–HCl (PH) and Congo Red;
e) Investigate if sorghum and miscanthus behave similarly under pretreatment action;
f) Microscopically analyze the anatomy of both samples of biomass before and after pretreatments.

3 BIBLIOGRAPHIC REVIEW

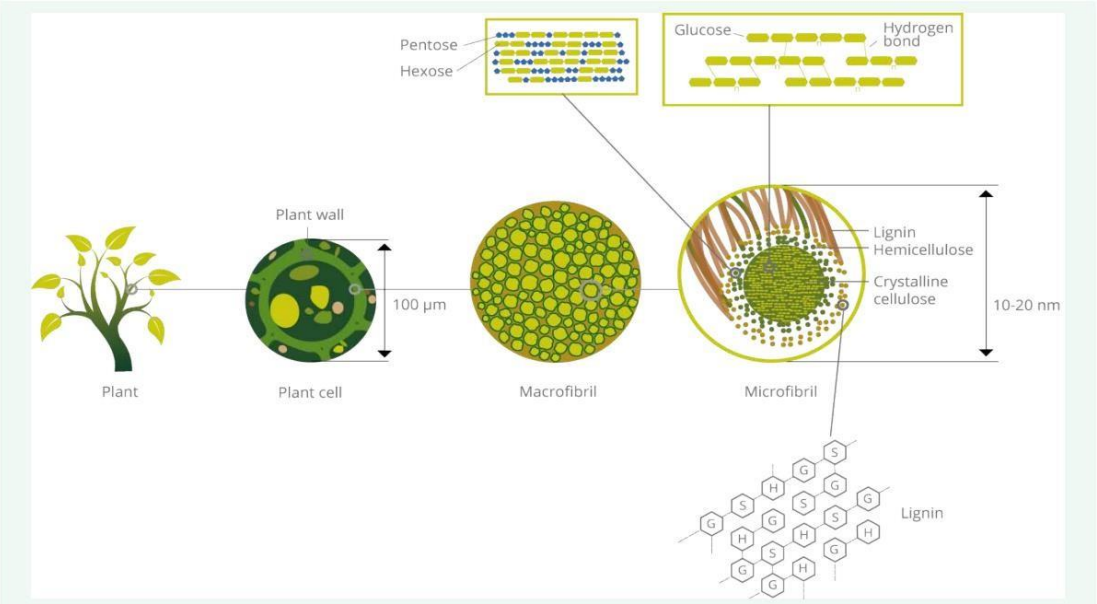
3.1 LIGNOCELLULOSIC BIOMASS

Amid the main categories of lignocellulosic biomass, there are: agricultural residues (corn stover, crop straws), herbaceous crops (alfalfa, switchgrass), short rotation woody crops, forestry residues and wastes (municipal and industrial). They are known for being abundant resources. Amongst these, two plants stand out in the European context of scientific research: Sorghum and miscanthus, both known for their resistance in dry climates and high potential for bioenergy production (HU et al., 2017).

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin (RAVINDRAN; JAISWAL, 2016). Both cellulose and hemicellulose fractions are polymers of sugars; therefore, a possible substrate for fermentation. Dry plants in general are composed of 40–50% cellulose, 15–25% hemicelluloses, 20–25% lignin and 5–10% other components, such as small amounts of pectin, nitrogenous compounds and ash components (HONGZHANG, 2014).

Cellulose polymers are well arranged; gathered into bundles, determining the framework of the cell wall. Fibers are filled with hemicellulose and lignin. There are different bounds among cellulose, hemicellulose, and lignin (Figure 1).

Figure 1 - Schematic structure of lignocellulose.



Source: STREFFER (2014)

3.1.1. Cellulose

Cellulose is the most abundant organic molecule on the planet, and it is formed when solar energy is absorbed in the process of photosynthesis (BÉDUÉ; WERTZ, 2013). It is also the major structural component of cell walls (HONGZHANG, 2014). Cellulose $(C_6H_{10}C_5)_n$ is a linear polymer of a D-anhydroglucopyranose unit. It consists of 10,000–15,000 D-glucose units linked by β(1-4) covalent bonds (SAWATDEENARUNAT et al., 2015). The number of glucose units that make up the molecule defines many of its proprieties, this is evaluated by an indicator called Degree of Polymerization (DP) (HONGZHANG, 2014). Hydrogen bonds oversee stabilizing its chains, where the hydroxylic groups can be found (BRODEUR et al., 2010).

Cellulose macromolecules have different orientations throughout its structure; therefore, they have several levels of crystallinity. For this reason, cellulose can be qualified into two different regions: the amorphous region (that presents lower crystallinity) and the crystalline region (that presents higher crystallinity). The higher the crystallinity, the more difficult is the biodegradation of cellulose. It is also important to notice that cellulose microfibrils are also attached to each other by hemicellulose and/or pectin and involved by lignin. These complicated connections explain why cellulose can be resistant to biological and chemical attacks (ZHENG; ZHAO; XU; LI, 2014) and it also explains why cellulose is insoluble in water, where it swells (MOOD et al., 2013).

The solubility of the polymer is strongly related to the degree of hydrolysis. High temperatures increase its solubility, due to the rupture of the hydrogen bonds that hold the crystalline structure of the molecule (ALCÁZAR-ALAY; MEIRELLES, 2015).

3.1.2. Hemicellulose

Hemicellulose consists in a complex structure of carbohydrates with different types of heteropolymers including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (ISIKGOR; BECER, 2015). Hemicellulose is considered as the second most common polysaccharide in nature. As opposed to cellulose, it is formed by combinations of pentoses and/or hexoses (PAUL; DUTTA, 2018). It has an amorphous structure; therefore, it

has side groups extending off the main hemicellulosic backbone linked by hydrogen bonds (SAWATDEENARUNAT et al., 2015).

Hemicelluloses are more amorphous, random, and branched than cellulose. Short and branched chains of hemicelluloses help build a network with cellulose microfibrils and interact with lignin, rendering the lignocellulosic matrix extremely rigid. The amorphous and branched properties make hemicelluloses more susceptible to biological, thermal, and chemical hydrolysis (ZHENG; ZHAO; XU; LI, 2014).

3.1.3. Lignin

Lignin the most complex natural polymer formed of phenylpropane units linked in a three-dimensional structure known to be difficult to biodegrade (HARMSEN et al., 2010). The higher the proportion of lignin in the cell wall, the higher the resistance to chemical and enzymatic degradation (TAHERZADEH; KARIMI, 2008).

Excluding cellulose, lignin is the most abundant macromolecule polymer component of the cell wall, consisting of three units: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units linked by aryl ether or C–C bonds. Commonly, lignin is detected by histochemical staining or by using ultraviolet light that excites the lignin's aromatic structure generating blue fluorescence (ZENG et al., 2014). This component gives strength to the plant's secondary cell walls, as it also gives its hydrophobic characteristics. Highly resistant to mechanical and enzymatic action, lignin is linked to both cellulose and hemicellulose, interfering with the hydrolysis of sugars during biodigestion (BÉDUÉ; WERTZ, 2013).

The lignin content is around 27–32% in woody plants and about 14–25% in herbaceous plants. The cell wall of protective tissues can contain cutin, suberin, wax, and other fatty substances. For instance, the cell wall surfaces of the epidermis cells are covered with cutin; the cell walls of cork cells in secondary protective tissue contain suberin and cutin, often combined with wax. The task of these elements is to reduce water loss from the plants (HONGZHANG, 2014).

SI *et. al.* (2015) demonstrated that lignin removal in miscanthus species induced by alkali and acid pretreatment lead to an increase in biomass enzymatic digestibility by effectively co-extracting hemicelluloses and lignin polymers, altering cellulose crystallinity.

The composition of lignocellulosic biomass depends on its source. There is a significant variation of the lignin and hemicellulose content depending on whether it is derived from hardwood, softwood, or grasses (HARMSEN et al., 2010).

3.1.4.Cell Wall Structure

The cell wall is a structural layer formed outside the cell membrane, and it is made up of primary and secondary cell walls. When one mother cell divides into two offspring cells, a primary cell wall forms between each two new cells. The cell wall is mostly made of cellulose, arranged into thin hair-like strands called microfibrils. The secondary cell wall is constructed between the plasma membrane and the primary cell wall after the cell is fully developed, composed of sclerenchyma and xylem vessels. This process occurs by laying down successive layers of cellulose microfibrils and lignin. Mature xylem cells are heavily lignified, as they make up the 'wood' of woody plants formed by a thick cross-linked matrix (RAVEN et al., 2005; CHRISTENSEN et. al., 2019).

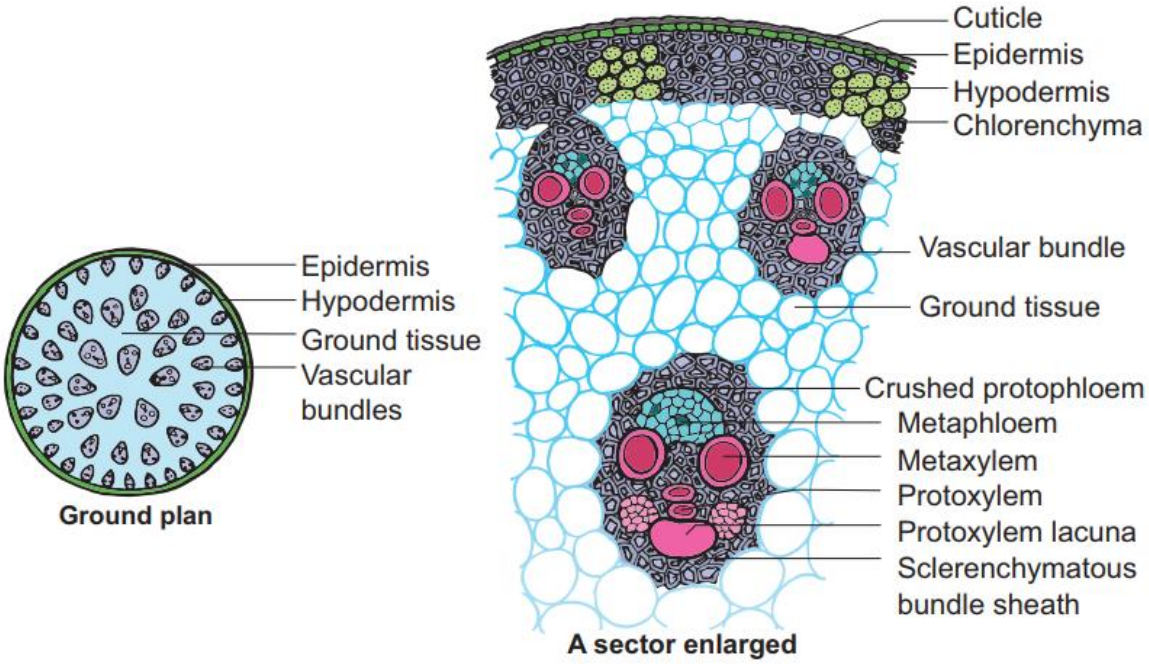
3.1.5.Vascular Plant Tissues

Plant tissues can be qualified into simple or complex, each of them containing specific cells that have unique functions. Parenchyma and collenchyma are simple tissues, composed of only one type of cell. However, xylem, phloem, and epidermis are considered to be complex tissues (BIOLOGY ONLINE, n.d.).

Tissues can be classified as ground tissues (composed of parenchyma, collenchyma and sclerenchyma), vascular tissues (xylem and phloem), or dermal tissues (epidermis). (PRATEEKSHA, n.d.).

Most of a plant’s body is made up of ground tissue, responsible for the mechanical support of the plant, it is also where photosynthesis occurs (Figure 2). Regarding leaves and stems, xylem tissues are usually located towards the interior of the vascular bundle as the phloem is located toward the exterior. Sclerenchyma cells support the plant and are also strongly lignified, they often appear as bundle cap fibers. Sclerenchyma cells are distinguished by the thickenings in their secondary walls. The vascular bundles composed of xylem and phloem tissues are scattered throughout the ground tissue (RYE et. al., 2016).

Figure 2 - Organization of stem in monocotyledons.



Source: SMRITI (2020)

3.2 SORGHUM

Sorghum is a type of grass with high potential for bioenergy production concerning lignocellulosic biomass. It presents high biomass and cell wall digestibility and elevated hydrolysis yield potential. It can be cultivated under low input agrosystems, enduring dry and marginal environments. This monocot can adapt and grow into temperate or tropical climates (THOMAS et al., 2018; TROUCHE et al., 2014).

Sorghum is a C4 annual plant presenting a high genotypic and phenotypic variability, and similarly to other C4 species, it is very efficient for biomass accumulation. Because of high water and nitrogen use efficiency, it also shows agronomic advantages when compared to other C4 crops such as maize and sugarcane when the targeted cropping conditions present limitations in water supply and/or when fertilizers inputs are reduced (TROUCHE et al., 2014).

3.3 MISCANTHUS

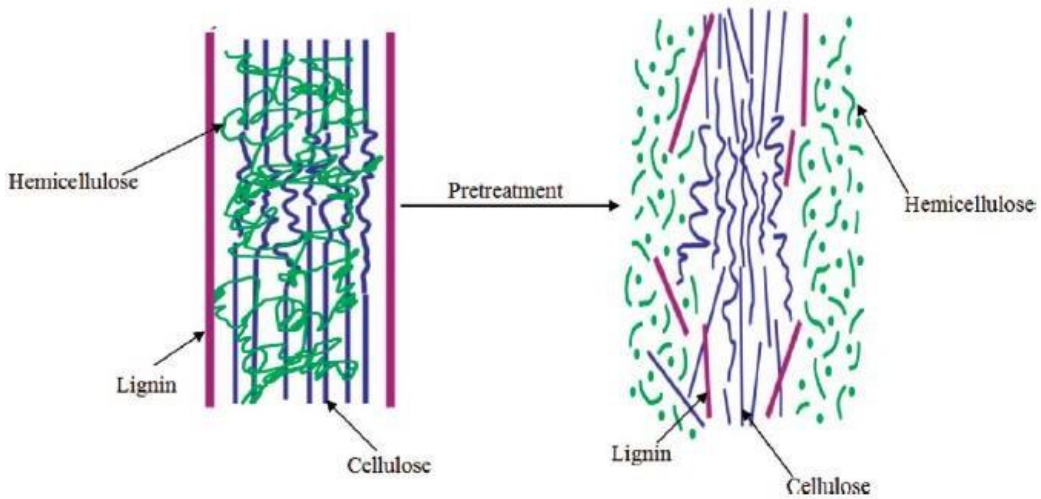
Miscanthus, a C4 grass native to East Asia, is a leading perennial biomass grass in Europe due to its high dry matter yield potential, resource use efficiency, and ability to grow

under a wide range of climatic conditions (LEWANDOWSKI et al., 2018). Research shows that miscanthus grows well even in acid sulphate soils (WATANABE et. al., 2006). For these reasons, miscanthus is considered as one of the best lignocellulosic energy crops. In addition to that, it presents rapid growth and low fertilizer and pesticide requirements. Miscanthus is very efficient at using the nutrients available because its rhizome system can recycle nutrients from soil and aboveground biomass for subsequent growing cycles (up to 20 years) and subsequent crops (MORANDI; PERRIN; ØSTERGÅRD, 2016). This crop can also grow on polluted soils (THOMAS et al., 2018).

3.4 PRETREATMENTS OF LIGNOCELLULOSIC BIOMASS

Pretreatments consist of procedures that intend to modify the biomass before industrial processes. They are particularly important upstream to biological processes because of biomass recalcitrance. A Pretreatment (PT) is set in place not only to break lignin, disarrange the crystalline structure of cellulose, and expose holocellulose (cellulose and hemicellulose), but also to assist in the process of formation of sugars directly by hydrolysis, avoiding losses or degradation of the sugars formed, limiting the production of inhibitory products and reducing energy demands meanwhile minimizing the cost of biofuel production, as demonstrated in Figure 3 (KUMARI; SINGH, 2018).

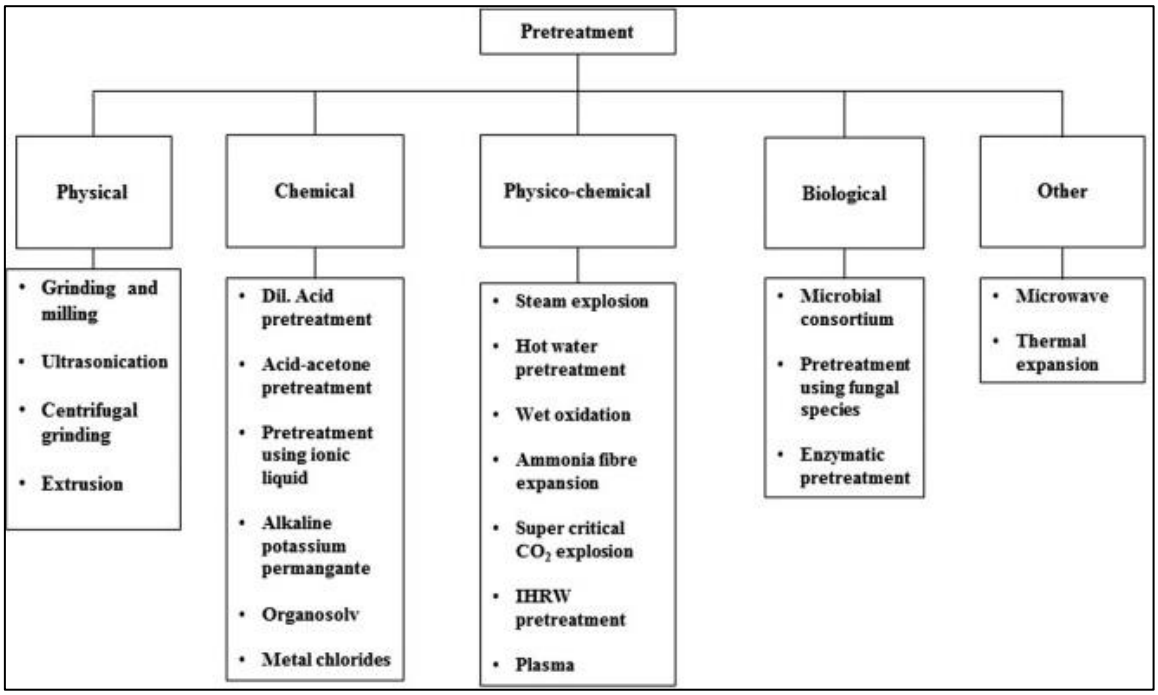
Figure 3 - Mechanism of Pretreatment's Action.



Source: KUMAR et al. (2009)

Different strategies have been applied to achieve these results, including mechanical, biological, and chemical pretreatments, and even a combination of all these, depending on the biomass feedstock used (TAHERZADEH; KARIMI, 2008). Several pretreatments are presented in literature as shown in Figure 4. As the type of lignocellulosic biomass varies, so do its properties and how it reacts to each type of pretreatment.

Figure 4 - Types of Pretreatments of Lignocellulosic biomass.



Source: RAVINDRAN; JAISWAL (2016)

3.4.1. Physical Pretreatments

The term physical pretreatment, represents, as the name suggests, a way to mechanically alter the form of the lignocellulosic feedstock in order to increase the area of contact of the material to the pretreatment applied, reducing crystallinity and improving hydrolysis. Some of these methods involve milling, chipping, grinding, freezing and radiation. Several of these pretreatment methods are not effective alone and are used in combination with other pretreatments (KUMARI; SINGH, 2018).

Physical pretreatments can improve methane yield of lignocellulosic biomass such as agricultural residuals (ZHENG; ZHAO; XU; LI, 2014), these methods are considered to be

expensive and sometimes the required energy to pretreat the material is higher than the theoretical energy content available in the biomass (BRODEUR et al., 2011).

3.4.2. Chemical and Physicochemical Pretreatments

Chemical and physicochemical pretreatments have been vastly studied; this segment is without a doubt, the focus of attention, when regarding pretreatment strains. There are several categories within this section.

3.4.2.1.Alkaline Pretreatments

Alkaline pretreatments use bases, such as such as sodium, potassium, calcium, and ammonium hydroxide, to remove lignin, and hemicellulose, making the lignocellulosic biomass more prone to fermentation. NaOH has been extensively used in the pulp and paper industry and it is the most common reagent used in alkaline pretreatments (ZENG et al., 2014).

The main results observed are the breakage of acetyl groups, lignin and other uronic acid substitutes, linked to the reduction of cellulose accessibility. The mechanism of action is to disrupt the cell wall by breaking down the linkage between polysaccharides and lignin, making cellulose and hemicelluloses more accessible to bacteria (MONLAU et al., 2012).

Chemical swelling of fibrous cellulose also takes place within the PT action, which includes solvation and saponification reactions causing the disruption of the crosslinks between hemicelluloses and other components resulting in an increase in the porosity of the biomass and of the internal surface area, decreasing the index of polymerization and crystallinity, also while disrupting the lignified structure. (KUMARI; SINGH, 2018; ZENG et al., 2014).

SAMBUSITI et. al (2013) applied alkali PT with NaOH to sorghum samples that led to a reduction of lignin (50-70%), hemicelluloses (18-35%), cellulose (16-45%) and galacturonic acids (up to 100%). The observed reduction of lignin content and solubilization of cellulose and hemicelluloses can accelerate the hydrolysis during anaerobic digestion, showing an increase in the first order kinetic constant. However, the alkali pretreatment applied on that research revealed no positive effect in enhancing methane yield, evaluated by Biochemical Methane Potential (BMP) tests.

THOMAS et. al (2018) studied CaO PT applied to miscanthus and obtained 14-37% improvement of BMP tests, and a 67-227% increase in the first-order kinetics constant of hydrolysis; a high contact time of PT was related to increased methane production. Although these researches are promising, further studies are required in order to validate these results.

3.4.2.2.Acid Pretreatments

Acid pretreatments are commonly divided into either under concentrated acid and low temperature (e.g. 40° C) or dilute acid and high temperature (e.g. 230 °C). It involves the use of both organic or inorganic acids such as sulfuric acid (H_2SO_4) (the most common), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4), acetic acid (CH_3COOH) and maleic acid ($C_4H_4O_4$).

The acidic pretreatment works solubilizing polysaccharides (such as hemicellulose) into monomers (ZENG et al., 2014). Although it has been proved that concentrated acid PT has positive outcomes on cellulose hydrolysis, it is also known to be toxic, corrosive, dangerous, and requires expensive materials, such as specialized non-metallic materials or alloys, for the assembling of the reactor. The inhibitors generated in this process are such as acetic acid, furfural and 5-hydroxymethyl furfural, inhibiting the growth of microbes. In addition, it is important to remark that the process of neutralization of pH, necessary for pretreated materials prior to anaerobic digestion, generates large amounts of gypsum resulting into disposal issues (KUMARI; SINGH, 2018).

While acid pretreatment is a common technology for the bioethanol production process, there are few studies exist about the impact of acid pretreatment on biogas production for AD of lignocellulosic biomass (ZENG et al., 2014).

3.4.2.3.Others

A common type of physico-chemical pretreatment is catalyzed steam-explosion, that use catalysts such as H_2SO_4, SO_2 (sulfur dioxide), and NaOH. There are not many studies relating this type of PT before AD. Moreover, the SO_2 used in the process is highly toxic and may present safety, health, and environmental issues. Catalyzed steam-explosion also generates inhibitors derived from the degradation of carbohydrates.

Still regarding physico-chemical PTs, wet oxidation PT uses water and oxidizing agents for promoting lignin solubilization, but is mainly used for bioethanol production, thus it has limited applications for biogas production. Other methods such as oxidative pretreatments (with ozone or with peroxides) are new upcoming technologies that still face challenges such as high cost and lack of toxicological data (ZHENG et al., 2014).

3.4.3. Biological Pretreatments

Biological pretreatments involve the use of microorganisms (such as fungi) to degrade lignin and hemicelluloses without altering cellulose. This type of PT needs mild conditions and are not of great cost; however, longer pretreatment durations are required when compared to other technologies. Both physical and biological processes are not cost competitive compared to the chemical and physicochemical pretreatments (BRODEUR et al., 2011).

Amongst all pretreatment methods here described, alkaline pretreatments were more extensively used for the pretreatment of lignocellulosic biomass due to optimal removal of lignin from the biomass (KUMARI; SINGH, 2018), therefore this study focuses on alkaline pretreatments applied to lignocellulosic biomass.

Previous studies have been made on sorghum and miscanthus in its milled form, and these results showed that PT with NaOH is more effective on these types of biomass than PT with CaO. These PTs, when applied to sorghum samples, had an influence in the kinetics of the digestion, as for the miscanthus it had a direct effect in its Biochemical Methane Potential (BMP) values, which improved significantly. Histological tests have been performed on the milled material, but the results were non-exploitable, therefore it would be interesting to repeat these studies (THOMAS et al., 2019).

3.5. ANAEROBIC DIGESTION

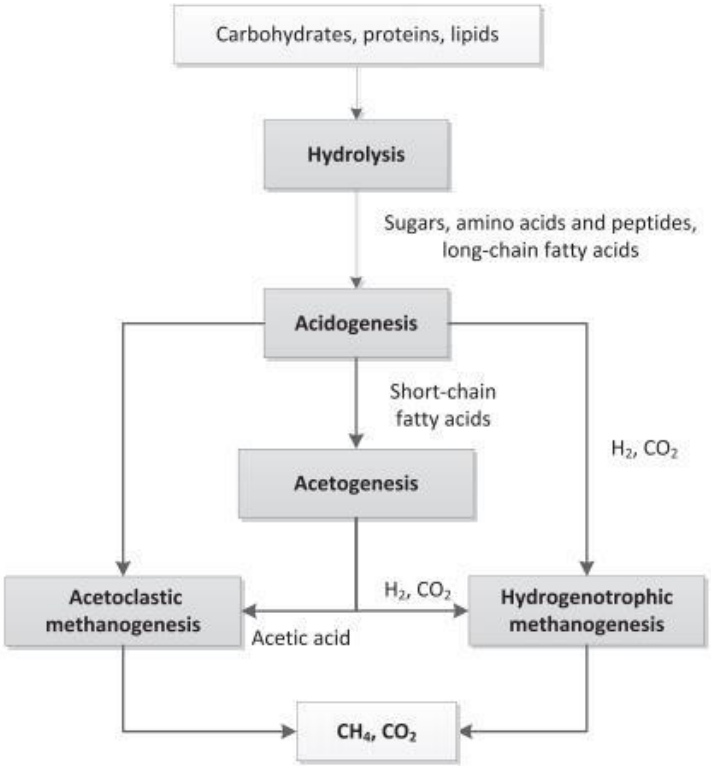
Anaerobic digestion (AD) is a biological process in which organic matter is decomposed by a consortium of microorganisms under oxygen free conditions and produces biogas as the final product (ZHANG; LOH; ZHANG, 2019). The process is divided into four main steps (Figure 5).

First, the hydrolysis occurs; this is when the complex biopolymers (such as cellulose

and proteins) are broken down into simple monomers and oligomers (sugars, amino acids, etc.). This step is followed by the acidogenesis, where these new small molecules are converted into a mixture of volatile fatty acids (VFAs) and other products. Alkaline PTs can help maintaining stable pH levels during this step, later optimizing methanogenesis and avoiding over acidification (MAO et al. 2015).

In the next stage, acetogenesis, these new products are converted into to acetate, carbon dioxide and hydrogen, creating direct substrates for the methanogenesis, the last step of the AD process, when methane is formed (BAJPAI, 2017). Regarding lignocellulosic biomass, the structural and compositional barriers caused by lignin make cellulose and hemicellulose hardly accessible. Pretreatments aim to break down these barriers, facilitate digestion and therefore increase the rate of biomass degradation and biogas production (ZHENG; ZHAO; XU; LI, 2014).

Figure 5 - Process of Anaerobic Digestion.



Source: ZHENG; ZHAO; XU; LI (2014)

Biogas consists primarily of methane (CH_4) and carbon dioxide (CO_2) with traces of hydrogen (H_2), nitrogen (N_2), hydrogen sulfide (H_2S), oxygen (O_2), water (H_2O) and saturated

hydrocarbons (i.e. ethane, propane) (BHARATHIRAJA et al., 2018).

Biogas has a high calorific value and it can be used as any other combustible gas in the generation of electric, mechanical or thermal energy, reducing production costs. Biogas can be recovered for various purposes, such as:

(i) Fuel in boilers, ovens and greenhouses in place of other types of fuels;

(ii) Generation of electricity for local use or for sale on the energy concessionaire's network;

(iii) Cogeneration of electricity and heat and

(iv) Alternative fuel for the injection into the natural gas line or the use as vehicular fuel after purification of biomethane (LOBATO, 2011).

The residue of anaerobic digestion, the digestate, consists of undegraded material and microorganisms. As the main part of minerals (ammonia, phosphorous) present in the substrate are found in the digestate, the latter can be used for land application as organic fertilizer.

4. MATERIALS AND METHODS

The varieties of sorghum and miscanthus developed by BFF present improved yield quality, high adaptation to different environments and limited negative environmental impacts. The project involved 24 partners: 9 public research institutes, 13 private partners and 2 local authorities. The project had a total investment of 27.5 million euros over 8 years. This research was performed within this project, in a partnership between 2 laboratories: INRA LBE and CIRAD PHIV platform (INRAE, 2018).

4.1.RAW MATERIAL

The sorghum used in this study is the variety B140 (*Sorghum bicolor*), harvested in the summer of 2015 at the CIRAD unit located in Montpellier, France (coordinates 43°39′02″ N 3°52′35″ E). The sorghum biomass was dried at 60°C for 72 hours before all experiments. As for the miscanthus, (*Miscanthus. x giganteus floridulus*) it came from the INRA experimental unit located in Estrées-Mons in the North of France (coordinates 49°53'00"N, 3°00’00” E) harvested in the winter of 2018 in its 8th year of life, and dried at 64 °C during four days in a ventilated oven.

For both sorghum and miscanthus samples, the internode was the part of the stem used for this study. The first step was to cut the harvested samples in around 1 m of length to be then sectioned transversally into 1 cm stems using an automatic saw.

4.2. DETERMINATION OF DRY MATTER (DM)

The determination of Dry Matter (DM) was essential to this experiment, because the quantity of reagent used for each pretreatment was based on the quantity of DM. DM represents the quantity of material remaining after removal of water. The DM content is determined according APHA (1998) methods. The determination of DM was carried out using a regular heating oven and ceramic pots. The pots were dried for 30 minutes in the oven at 105 ºC and weighed empty (tare t). Then, samples were selected, weighed (m) and repeated 3 times each.

Each pot containing the biomass was weighed in a high precision scale, and then put in the oven at 105 ºC for 24 hours. After drying, samples were cooled in a desiccator and weighed again (M_o). The formula used for the calculation of the DM percentage was:

$$DM\% = 100 \times (M_o - t) \div m \quad (1)$$

Where M_o represents the final weight of the dried biomass equal to around 2 grams, t stands for the empty pot's weight and m stands for the weight of the untouched samples.

4.3. SECTIONING

In order to make transversal sections of the 1 cm stems, it was first necessary to embed the raw dried material in an aqueous environment during 48 hours to hydrate the stems, making them softer to ease the sectioning procedure. For sectioning the hydrated material, a vibratome model MICROM HM650V was used.

The vibratome is a sectioning device that presents a simple interface: a tool's menu to be configured by the user, the metal tray where the plant material is to be glued, the knife holder and the blades (Figure 6). Speed, frequency and amplitude can be adjusted in the tool's menu. The disposable blade moves towards the specimen and cuts it, respecting the configurations settled before. The procedure is considered easy to master and it is fast, as is the sample preparation. Typically, the structure of the samples is well conserved (VERHERTBRUGGEN et. al., 2017).

The use of a vibratome offers consistency due to the fact that it generates similar sections of the same thickness, producing overall sharp images (MITRA; LOQUÉ, 2014). According to its manual, the vibratome MICROM HM650V can section samples in a range of 10-200 μm using disposable razor blades made of steel.

Figure 6 - Vibratome model HM650V.

Source: Taken by the author (2018)

For the pretreated and untreated miscanthus and sorghum samples the thickness was fixed at 115 µm for sorghum (due to their larger size, these were more difficult to cut) and 110 µm for miscanthus stems. These values of thickness were chosen because they showed to be easy to cut and still thin enough to properly observe the organization of the section. Once they were cut, the sections were collected and stored in an ethanol 70% solution, if stored for more than 48 hours, or simply in distilled water if stored for less time. Figure 7 shows the process of sectioning a sorghum stem glued to the metal tray of the vibratome.

Figure 7 - Sectioning process of sorghum stem.

Source: Taken by the author (2018)

4.4.STAINING

4.4.1. FASGA

After sectioning, staining was performed using the FASGA solution dissolved into water (1:7, v/v). FASGA is not a specific coloration of lignin or cellulose; however, when applied to the stem's sections, lignified tissues appear in red, whereas non-lignified or poorly lignified tissues appear in blue. FASGA is a staining solution composed of a mixture of Safranin solution, alcian blue Solution, Acetic acid, glycerin and distilled water. Safrananine is a basic colorant of the azine group. Alcian blue is a cationic colorant that binds to negatively charged macromolecules. The competition between the two colorants generates the double staining (TOLIVIA; TOLIVIA, 1987; LEGLAND et al., 2017). One of the advantages of using the FASGA staining is that it generates a double-colored section, producing interesting data for image processing. However, the results found using FASGA staining had to be confirmed using other staining methods that are specific to either lignin or cellulose.

Samples were soaked in the FASGA solution during twelve hours in the dark, afterwards rinsed in distilled water to be then assembled in microscope blades using a glycerol 50% solution and sealed for storage and digitalization with Vertex. The complete protocol developed by CIRAD can be found in ANNEX A.

This is the only solution in this study that is stable so it is the only one used on posterior quantification analysis.

4.4.2. Phloroglucinol-HCL

Phloroglucinol-HCl (PH) staining allows the detection of lignin. This coloration is specific to lignin and only stains it (POMAR et. al., 2002). To prepare the mixture, two solutions are required: one of ethanol saturated with phloroglucinol (Sigma, P3502-25G) at 2%, and another of hydrochloric acid (HCl) (Sigma, 07102_1L_D) at 18% (diluted in the water). This mixture is kept in the dark at 4 °C. The next step is to immerse the stem's sections in the phloroglucinol solution for 5 minutes. Following the immersion, the sample is placed on a glass slide and a few drops of the hydrochloric acid solution are poured into it, in order to cover the entire section. A red color appears immediately when there is the presence of lignin. As soon

as the color is visible, the sample must be assembled in between microscopic slides in an aqueous 50% glycerol solution and observed directly in white light. This coloration is not stable; therefore, the red color starts fading away after a few minutes. For this reason, the images were acquired using the LEICA microscope for this staining method. This staining method can be used to support FASGA staining results. The complete protocol developed by CIRAD can be found in ANNEX B.

4.4.3. Congo Red

This staining procedure colors cellulosic material in red, due to the strong interaction of polysaccharides with β(1-4) links. This coloration is specific to cellulose. The distance between the groups -N=N- to its immediately adjacent groups is 1.08 nm. The cellobiose pattern in cellulose is 1.03 nm. Thus, the Congo Red perfectly dyes the cellulose because its molecule adapts well to the support.

The procedure begins with the preparation of the sample. Sections can be made on a vibratome using a thickness of 80 µm to 150 µm. Afterwards, comes the staining process: it is sufficient to place the sample in the 0.1% Congo Red solution for 5 minutes, followed of a rinse with distilled water. Then, it is necessary to assemble the sample between the slide and the coverslip in a water / glycerol mixture (v/v). Observation is to be made in white light with the LEICA microscope. The complete protocol developed by CIRAD can be found in ANNEX C.

4.5. MICROSCOPY

For the acquisition of microscopic images, microscope LEICA DM4500 Optic was used based at the PHIV platform of CIRAD, Montpellier, as shown in Figure 8. This microscope possesses intelligent light and contrast adjustments. It operates with a mechanical Z-drive and a mechanical stage. The brightness of the image remains constant, and the contrast is brilliant when switching to a different magnification. The contrast and light are automatically adjusted by the microscope for best image quality. VOLOCITY is the software used for visualizing, exploring, analyzing, and saving the microscopic images (LEICA, 2018).

Figure 8 - LEICA DM4500.

Source: CLUZEL (2018)

4.6.MICROSCOPIC SLIDE SCANNER

In order to obtain good quality images for image processing, the blades were digitalized by a NanoZoomer. The Experimental Histology Network of Montpellier (RHEM) institute has a digitalization platform called Montpellier Imaging Resources (MRI) that holds a Hamamatsu NanoZoomer Digital Pathology (NDP) device, used in this research.

The results can be observed on the screen of a computer using a software called THE VIEWER, which reproduces the functions of a microscope. The NanoZoomer (Figure 9) is capable of scanning up to 210 standard slides in one batch. After scanning with magnification of 20x, the high-resolution images with a total size of approximately 2 GB each are saved and can be viewed using the viewer software from Hamamatsu. Images can then be compressed and converted into other formats. (UNIVERSITAT HEIDELBERG, 2018).

Figure 9 - Slide Scanner.

Source: UNIVERSITAT HEIDELBERG (2018)

The original images produced by the Nanozoomer come out in NDPI format, and due to the large file size and resolution it is usually only used for visual analysis. For image treatment, the pictures are compressed into the TIFF format using the software ImageJ (A SCHNEIDER, 2012).

4.7. PRETREATMENTS

After determining the DM, it is possible to calculate the quantity of reagent necessary for each case. The concentration of pretreatments was defined at 10% regarding the DM content of each substrate. The following formula was used:

$$Reagent\ (g) = 0{,}1 \times (DM \div 100) \times W1 \quad (2)$$

In which, 0,1 represents the concentration of 10%, the DM is Dry Matter content, and W1 the weight of the stems, each stem weighed around 0.2 grams, so it was necessary around 10 stems on one pot to reach 2 grams before drying was carried out.

Eight different experimental designs were created, variating biomass type, temperature and reagent, as shown in Table 1.

Table 1 - Pretreatment Designs.

Biomass	Pretreatment Name	Reagent	Concentration ($g.100g_{TS}^{-1}$)	Temperature (°C)
Sorghum	SNT	None	None	26 or 55
Sorghum	PT1	CaO	10	26
Sorghum	PT2	NaOH	10	26
Sorghum	PT3	CaO	10	55
Sorghum	PT4	NaOH	10	55
Miscanthus	MNT	None	None	26 or 55
Miscanthus	PT5	CaO	10	26
Miscanthus	PT6	NaOH	10	26
Miscanthus	PT7	CaO	10	55
Miscanthus	PT8	NaOH	10	55

Source: Made by the author (2018)

Control vials were employed (SNT stands for Sorghum Non-Treated and MNT for Miscanthus Non-Treated) where the samples underwent the same temperature and agitation as the PT observed, but no reagent was added to the distilled water.

4.7.1 Pretreatment – Stems of 1 cm

The idea of this experiment is to apply the pretreatments presented in Table 1 to the 1 cm length stems. The experiment was performed using 500 mL BMP flasks sealed shut with a rubber cap joined with a metal cap and a stirring oven (Figure 10). First, samples were weighed according to the specified quantity required (always using three repetitions); then, the reagent (g, according to Equation 2) and 100 mL of distilled water were added to the flasks containing the stems. The flasks were sealed shut and placed on the agitating oven, adjusted to the parameters according to each PT and were submitted to agitating at 90 RPM during 24 hours. Afterwards, the pretreated stems were sectioned and colored for microscopic analysis and finally BMP tests were carried out (section 4.7.5).

Figure 10 - PT applied to 1 cm length stems in the agitating oven.

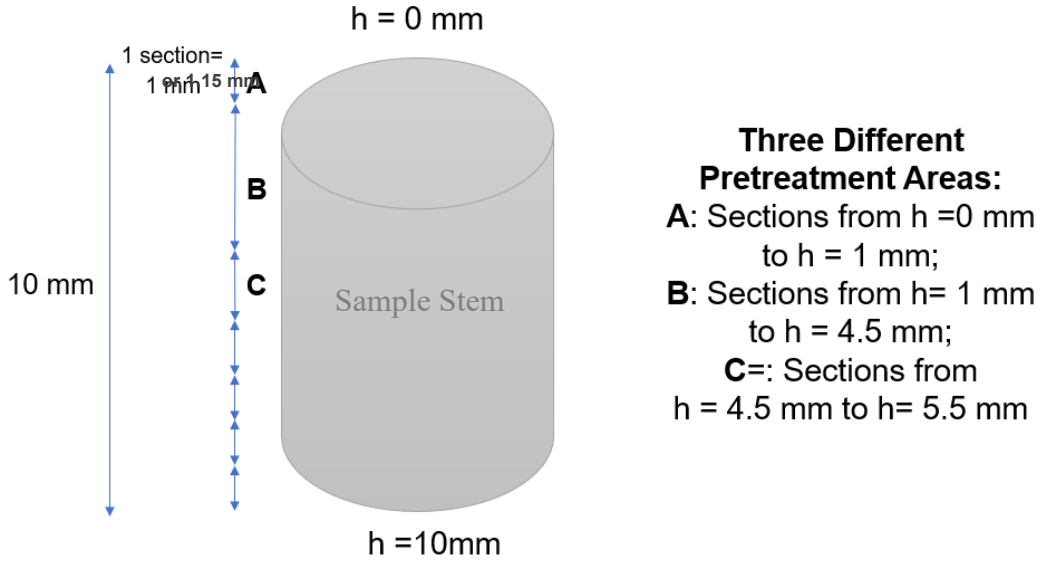
Source: Taken by the author (2018)

4.7.2 Study of Pretreatment Penetration

Another experiment was designed to ensure that the PT applied to the stems (presented in the previous section) was being equally absorbed by the entire stem in its length and volume.

Pretreated 1 cm stems were divided into 3 areas of sections at 110 μm of thickness for miscanthus and 115 μm of thickness for sorghum (Figure 11). The stem was divided into: Area A, which represents the initial part of the stem until 1 mm of length; Area B, which represents the sections made between 1 mm and 4.5 mm of length; and Area C, which represents the sections made between 4.5 mm and 5.5 mm of length.

Figure 11 - Pretreated 1 cm stem divided into 3 different areas.



Source: Made by the author (2018)

After sectioning, samples were stained with the FASGA solution, assembled into blades and scanned in the Microscopic Slide Scanner.

4.7.3 Dynamic Study of Pretreatment Using Sections

In this experimental design, each pretreatment was applied directly into the sectioned material at a thickness of 115 µm for sorghum and 110 µm for miscanthus samples. The following steps were the same as the PT applied to the 1 cm stems after sectioning.

- Sorghum

As the quantity of sorghum biomass available was more abundant than of miscanthus, PT was first performed on sorghum, using 23 vials of 30 mL, which represented the 22 different times during which the samples were collected, starting after 30 minutes of PT and lasting up to 144 hours (6 days) plus the control vial.

Each vial had four 115 µm thick sections of biomass and 10 mL of the alkaline solution.

The control vial had just water and the biomass that underwent the same conditions of temperature as the designed treatment during 24 hours. The quantity of reagent used for the solution was also calculated according to the DM content found for the sectioned material, using the procedure explained in Appendix A.

In this experiment, 88 sections were used. The quantity of water to be added to the reagent was calculated based on the equivalent quantity of water used by the PT performed on the 1 cm stems. The reagent was weighed on the day before, mixed with the water and left in agitation during a whole night to ensure that the reagent would properly dissolve in the distilled water. The pH was measured before and during the PT, in order to assess if there were any significant changes.

Samples for this experiment were taken in hours 0,5; 1; 1,5; 2; 2,5; 3; 4; 5; 6; 7; 8; 9; 10; 12; 14; 17;19, 21, 24; 48; 96 and 144. This experiment lasted longer than the experiment carried out on the 1 cm stems in order to more profoundly analyze the PTs action on the tissues over time.

- Miscanthus

Due to a lack of enough material, sampling was not made as often as for miscanthus as it was for sorghum. Here, 15 vials of 30 mL were used, representing the 14 different hours of sampling plus the control vial, totaling 40 sections. Some vials had 2 sections and others had 3. The control vial had only the biomass sample and water and underwent the same conditions of temperature and agitation during 24 hours.

To decide the amount of reagent to be used, the calculations were made using the same methodology as done for sorghum samples, but this time using 110 μm as the section's thickness and taking into account the fact that only 40 sections of miscanthus were used for this experiment.

The same reagent mass/volume ratio was used as the previous experimental design. pH values were also measured before and throughout the experiment.

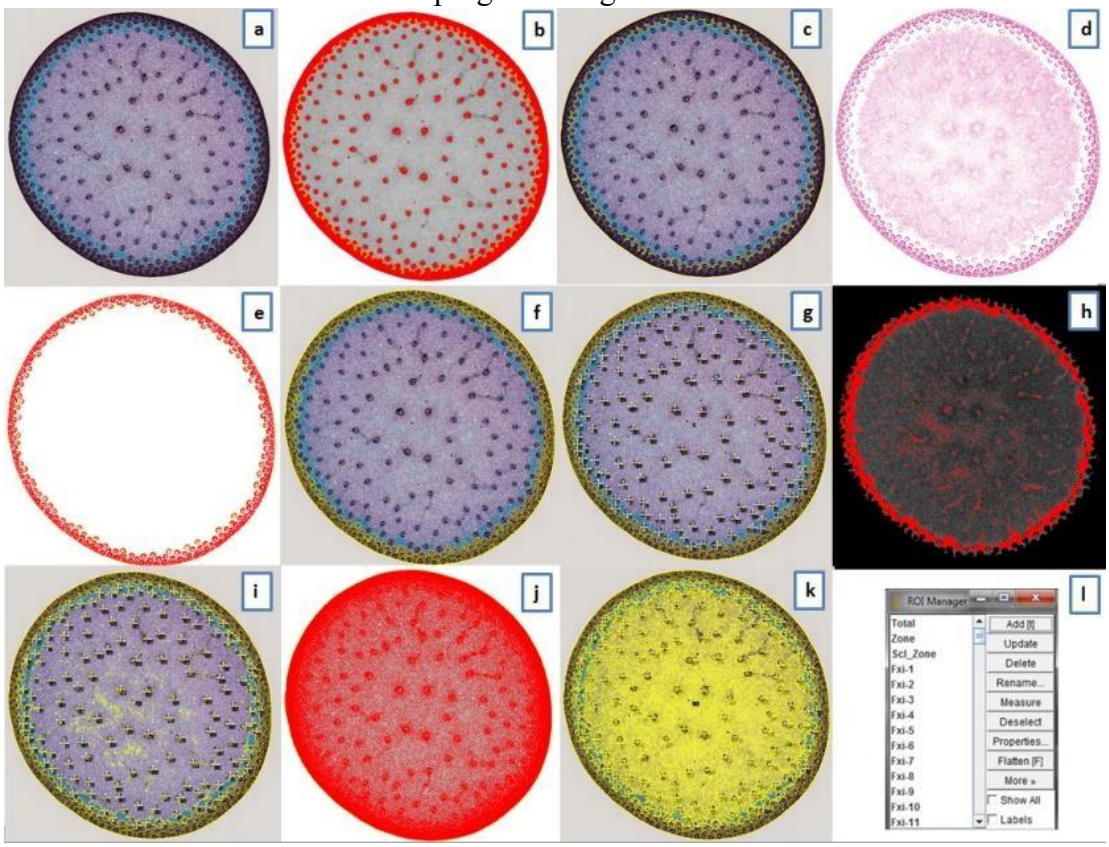
Samples were taken in hours: 0,5; 1; 2; 3; 6; 9; 12; 14; 19; 21; 24; 48; 96 and 144 after starting the pretreatment.

Then, for both miscanthus and sorghum, samples were stained, assembled into blades, digitalized and the resulting images were processed and analyzed.

4.7.4 Image Processing

ImageJ is a free software for image processing and analysis. An algorithm for this software was developed at CIRAD PHIV to be used on sorghum or other similar types of lignocellulosic biomass image treatment. The algorithm is called “Sorgho_BFF_Toolset”. The semi-automated algorithm creates an image segmentation of the input images into distinct tissue regions. The size and the fraction area of each tissue region can be quantified, as well as the average coloration within each region. A result table is presented at the final step of the image processing. The process is composed of the following steps, showed in Figures 12, 13 and 14.

Figure 12 - Process of image treating using the Algorithm “Sorgho_BFF_Toolset” and the program Image J.



Source: Made by the author (2018)

Where each letter represents:

a) Original Image;

b) Zone Selection;

c) Zones;

d) Gaussian Filter;

e) Sclerenchyma selection Zone 1 (Z1);

f) Sclerenchyma;

g) Vascular bundles count;

h) Filter and parenchyma selection;

i) Parenchyma;

j) Biomass selection;

k) All selections;

l) “Roi Manager”: Control panel.

The process works following these summarized steps:

1) It is necessary to first manually convert NDPI image into TIFF format (in this case, another algorithm was used called NDPI_TIFF_BFF_Conversion);

2) Image Selection: The next step is to open the image to be treated and duplicate it in order to preserve the original image (Step a, Figure 12).

3) Selection of complete or incomplete image: In this step, it is possible to decide whether the user wishes to work with the entire image or not, allowing the user to interact with only a part of the image if desired. In case of an incomplete section, the user can select what part of the section they would like to process, so the algorithm can statistically repeat the data found to make up for the entire area of the sectioned incomplete material. This is very interesting because, for instance, sometimes the images acquired by the Nanozoomer are not well focused on the entire section, or sometimes the section to be treated may be incomplete due to the strong pretreatment effect.

4) Zone definitions: This is where a delimitation of the two distinct areas in the stem occurs, dividing the section into external and internal zone. Zone 1 (Z1) is characterized as the external area where there is a significant larger density of conductor bundles and it has a strongly lignified sclerenchyma area than Zone 2 (Z2), the inner area. As a result of this process, two important values are generated, the Percentage of Zone 1 Area (perZ1) and the Percentage of Zone 2 Area (perZ2), as shown in Figure 12. A Gaussian filter is applied to facilitate the selections of both Z1 and Z2 (it attenuates the details). It is always possible to edit the filter configurations with a right click on the icon (Step b, Figure 12).

5) Sclerenchyma Zone definition: Here, the delimitation of sclerenchyma area in the external part of the section, Z1, occurs after adjusting a threshold. This step generates the values of percentage of sclerenchyma tissue in Z1 (percSclZ1) (Figure 13). A Gaussian filter is also applied in order to attenuate the details of the image. After, there is the “Pixels smoothing” process. This allows the user to edit the selection made by the filter by dilatation erosion of the selections. A color deconvolution filter is used in the form of a red layer. The quantity of sclerenchyma area can then be adjusted by the user (Steps d, e and f, Figure 12).

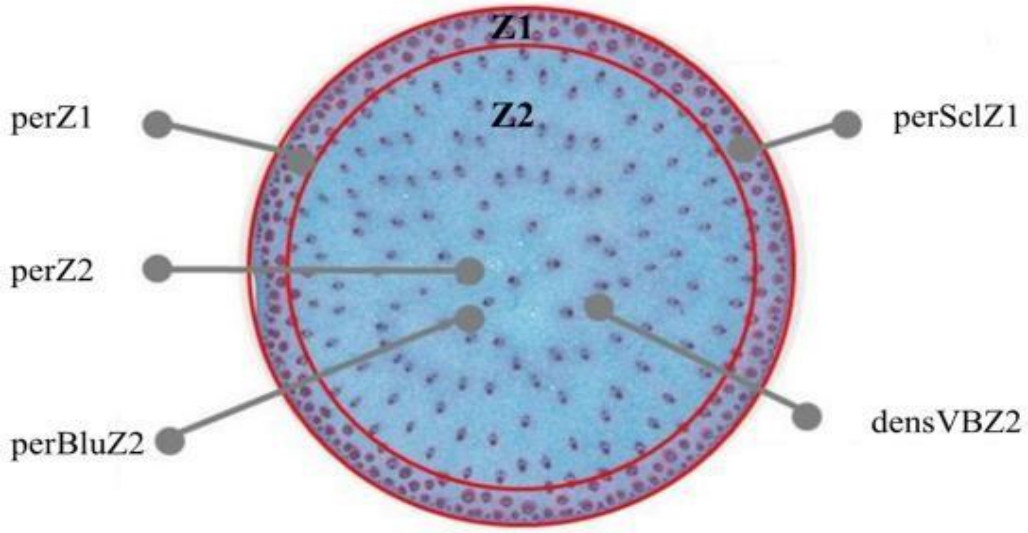
6) Bundles zone selection: In this stage, the program automatically counts the vascular bundles present in Z2 according to the settled parameters, later presented in the results as Number of Vascular Bundles in Zone 2 (nbVBZ2) and Density of Vascular Bundles in Zone 2 (densVBZ2). The process is very similar to the previous steps: initially, a Gaussian filter is applied, generating a smoother image. After that, the program searches for the peaks of intensity of the image: these will be the vascular bundles, which are darker after the first applied filter, if necessary, the user can add or delete a bundle selection (Step g, Figure 12).

7) Parenchyma selection: The next step is to select the parenchyma zone; for this, it is necessary to select the “blue zones” by defining a threshold using a Hue layer of HSB, a Gaussian filter and a pixel smoothing filter. (Steps h and i, Figure 12). This step is responsible for the results of percentage of blue area in Z2 (percbluZ2) representing the cellulosic material available in Z2 as the FASGA staining colors cellulose in blue.

8) Biomass selection: Finally, the amount of biomass is selected also using a filter, to help the thresholding process.

9) Results: The result is a table that presents all of the parameters presented in the previous steps and more. The table generated can be exported as an Excel file. All of the parameters involved in this step and its explanations can be found in Appendix B.

Figure 13 - Cross section of sorghum internode, with identification of an outer (Z1) and a central (Z2) zone.



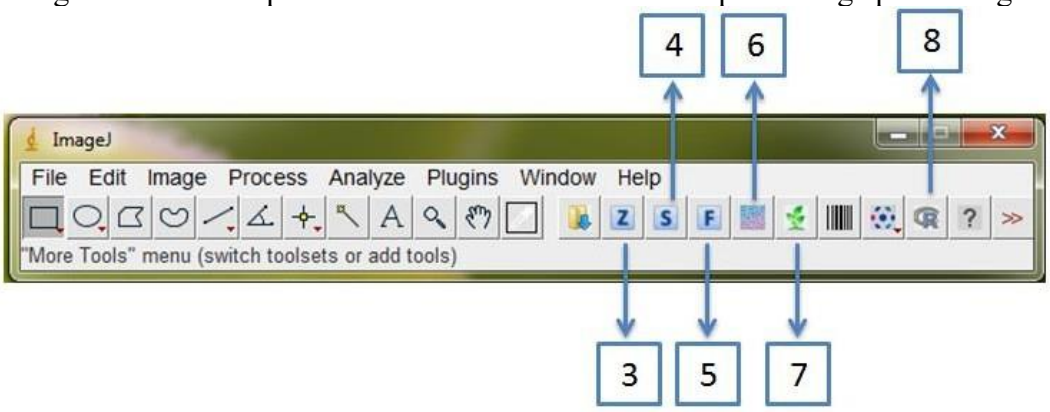
Source: PERRIER et al. (2017)

Where Zone 1 (Z1) is characterized by its area in percentage of internode section area and Zone 2 (Z2) is characterized by its area in percentage of internode section area.

The Percentage of Sclerenchyma area in Zone 1 (perSclZ1) represents the percentage of sclerenchyma tissue (red stained by FASGA solution representing lignin) in percent of Z1 area. The Percentage of Blue Tissue Area in Zone 2 (perBluZ2) represents the cellulosic share contained in Zone 2, the Density of Vascular Bundles in Zone 2 (densVBZ2) accounts for the number of vascular bundles per mm² of that area. The section represented in Figure 13 was stained by FASGA solution.

Throughout the entire process, there is a control panel called “Roi Manager”, in which all the steps can be selected individually or together, edited, or deleted. Figure 14 represents the icons of the program linked to the above-mentioned steps.

Figure 14 - Icon representation associated with the steps of image processing.



Source: Made by the author (2018)

4.7.5 BMP

The pretreated 1 cm stems were then digested using 500 mL flasks with a working volume of 400 mL. Bicarbonate buffer ($NaHCO_3$, 50 $g \cdot L^{-1}$), macroelement and oligoelement solutions, anaerobic sludge at 5 $gVS.L^{-1}$, and the substrate at 5 $gTS.L^{-1}$ were added. Degasification with nitrogen was carried out to ensure an anaerobic environment. The bottles were incubated at 35 °C for 45 days (THOMAS et al., 2018).

5. RESULTS AND DISCUSSION

This part is divided into two: First, the results of experiments applied to the 1 cm stems, and then the experiments carried out using 110 μm and 115 μm sections.

5.1.STEMS

5.1.1. BMP

The values found for the BMP of the pretreated 1 cm stems were the following (Table 2 and 3):

Table 2 - BMP values for sorghum samples: Non-treated vs. Treated (PT1, PT2, PT3 and PT4).

$NmLCH_4/gVS$	SNT	PT1	PT2	PT3	PT4
Average value	245,7	235,3	241,6	191,7	214,9
Standard Deviation	20,4	29,7	30,3	43,4	7,5
Improvement (%)	0	-4,2	-1,7	-21,9	-12,5

Source: Made by the author (2018)

Noticeably, in sorghum samples, the PTs do not show improvement in BMP values, as the results for BMP were worse than before the pretreatment. These results are not unexpected, as previous studies have shown that alkali pretreatments applied to sorghum samples may not necessarily augment BMP values, but do act on accelerating hydrolysis (SAMBUSITI et. al. 2013).

Table 3 - BMP values for miscanthus samples: Non-treated vs. Treated (PT5, PT6, PT7 and PT8).

$NmLCH_4/gVS$	MNT	PT5	PT6	PT7	PT8
Average value	122,2	158,8	176,8	178,3	214,9
Standard Deviation	45	32,2	26,4	11,7	7,5
Improvement (%)	0	29,9	44,7	45,9	75,9

Source: Made by the author (2018)

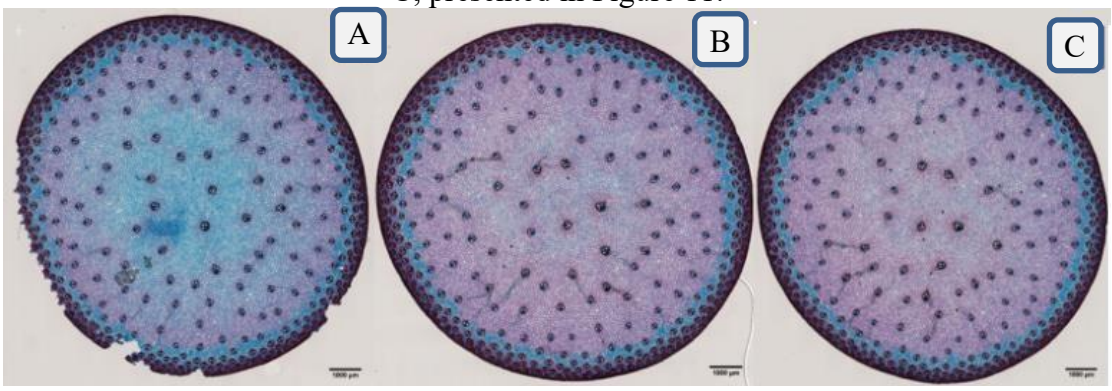
For miscanthus samples, all the BMP values for the pretreated samples have increased, suggesting that the PT can improve methane potential for miscanthus. Both pretreatments with NaOH showed good results, especially under high temperatures. PT8, also known as pretreatment at 55 °C with NaOH, showed the best value for miscanthus samples, indicating high potential for biodegradation and methane production.

As mentioned before, SAMBUSITI et. al. (2013) carried out experiments involving alkaline pretreatments on Sorghum bicolor and found similar results regarding BMP values after PT. Similarly, the studies carried out by THOMAS et. al. (2018) on miscanthus were coherent with these results.

5.1.2. Pretreatment Penetration

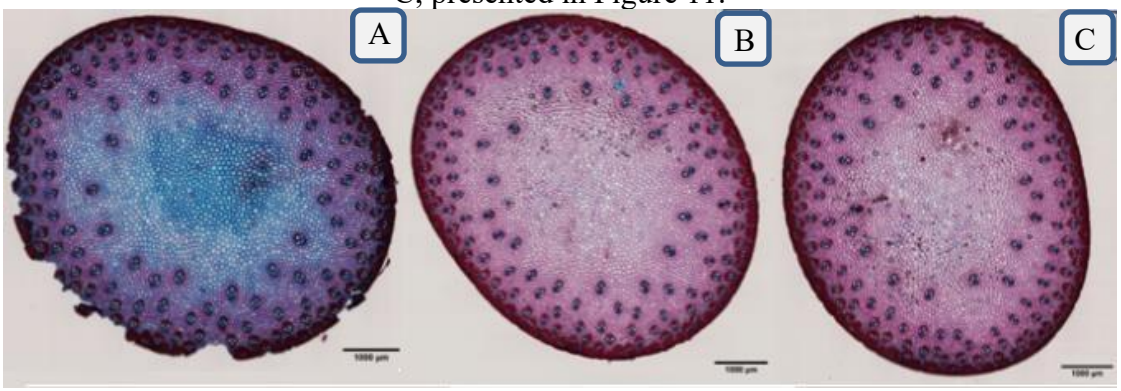
The DM content found for the dry stems were the following: 90% and 93% respectively for sorghum and miscanthus; therefore the calculation of reagent quantity was performed according to these values. The pretreated 1 cm stems were sectioned and stained, and the PT absorbency was studied. It was possible to confirm that there was a significant difference of pretreatment infiltration (therefore efficiency) comparing different heights of the 1 cm stem, as previously shown in Figure 11. Hence, the results obtained by pretreating this material could not be validated because the sections made do not represent the PTs actions on the tissues unless they were made at the tip of the stems (Figures 15 and 16).

Figure 15 – Pretreatment on Sorghum NaOH 26 °C, comparison in between Areas A, B and C, presented in Figure 11.



Source: Made by the author (2018)

Figure 16 - Pretreatment on Miscanthus NaOH 26 °C, comparison in between Areas A, B and C, presented in Figure 11.



Source: Made by the author (2018)

The sections made at the tip of the stems (Area A) present a larger area in blue than the sections made in areas B and C. This indicates that the pretreatment acted on Area A, exposing the cellulose, but, as the sections were made further bellow the tip of the stem, that is, Areas B and C, the material obtained was mostly colored in purple, thereby showing that the PT did not act, therefore did not infiltrate all the way to the center of the 1 cm stems.

The solution found for this situation was to change the approach and apply the pretreatment directly into the sectioned material (at 110 µm and 115 µm for sorghum and miscanthus respectively), avoiding any type of absorption problems by diminishing the height variable.

5.2. SECTIONS

5.2.1. Anatomic Differences Between Non-Pretreated Sorghum and Miscanthus

As a result of image processing, the following parameters were found for the untreated samples of sorghum and miscanthus colored by FASGA (Table 4):

Table 4 - Results of image processing for untreated samples.

Biomass	Area (mm²)	perZ1 (%)	perZ2 (%)	perSclZ1 (%)	nbVBZ2	densVBZ2 (n/mm²)	perbluZ2 (%)
Sorghum	68,2	20,8	79,2	49,7	93,6	1,7	1,8
Miscanthus	30,3	21,4	78,6	39,9	63	2,6	3,25

Source: Made by the author (2018)

The variables presented in Table 4 will be now explained: perZ1 and perZ2 indicate the percentage of outer (Z1) and central (Z2) area respectively according to the total area of the section. Both sorghum and miscanthus present a similar ratio of around 20% of Z1 and 80% of Z2 area.

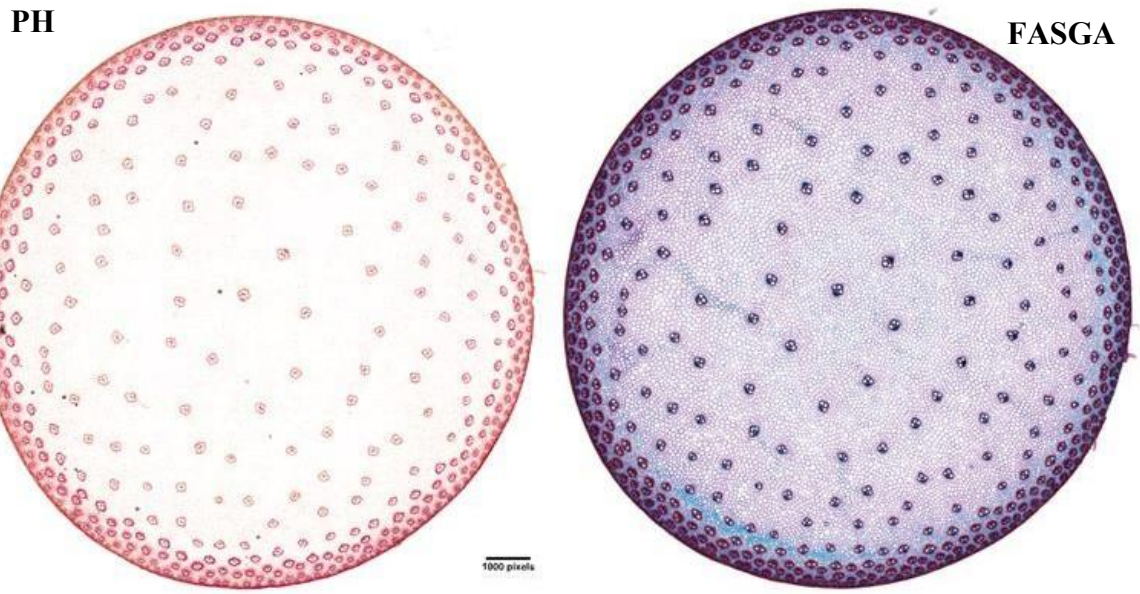
As for the perSclZ1 variable, it represents the percentage of sclerenchyma area in Zone 1. This shows that naturally sorghum is more abundant in sclerenchyma area, indicating larger lignified area in the outer zone of the transversal section (Z1) than miscanthus. This may indicate that the sorghum stems are naturally more lignified than miscanthus stems, and therefore more difficult to break during biodigestion. This may also be a consequence of the types of lignin (H, S and G) present in sorghum that could not necessarily be the same as in miscanthus.

The nbVBZ2 indicates the number of vascular bundles spread throughout the ground tissue, which are higher for sorghum samples; sorghum samples are nonetheless twice as big as miscanthus. Therefore, densVBZ2 indicates the density of vascular bundles, which showed to be higher in miscanthus mainly due to its smaller area.

Finally, perbluZ2 represents the amount of blue area in Z2, representing the main source of cellulose within the stems. Anatomically, both plants are very similar; nevertheless, there are a few outstanding differences, such as the area of the stem section, found to be more than twice as big in sorghum samples than in miscanthus samples. FASGA staining for non-treated samples were validated by PH staining, as shown in Figures 17 and 18. The red area in the left images (PH) are located in the same region as the purple stained area in the right images (FASGA). Miscanthus samples are characterized by being naturally shredded in the center.

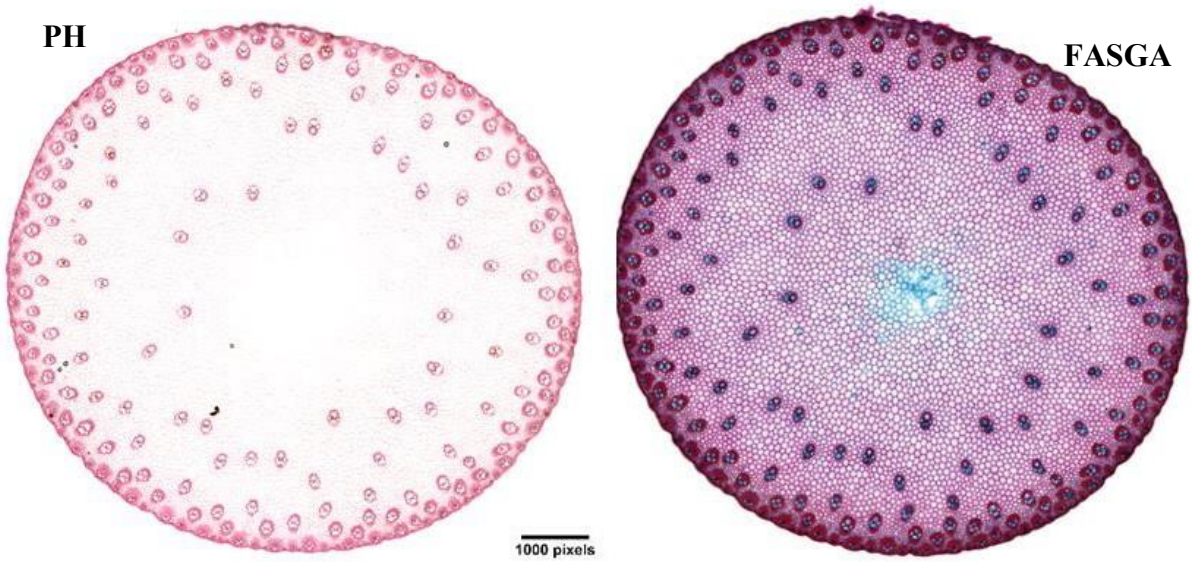
WANG et. al. (2017) presents that miscanthus is mainly composed of cellulose (40–60%), hemicelluloses (20–40%), and lignin (10–30%). THANAPIMMETHA et. al. (2011) shows that a similar variety of sorghum has around 58% cellulose, 25% hemicellulose and 14% lignin.

Figure 17 - Sorghum untreated section stained with PH and FASGA solutions.



Source: Made by the author (2018)

Figure 18 - Miscanthus untreated section stained with PH and FASGA solutions.



Source: Made by the author (2018)

5.2.2. Pretreatment in Sectioned Material

The DM content found for the hydrated sections was very low, a consequence of the hydration process (respectively 1,5% and 2,5% for sorghum and miscanthus). The calculation of reagent quantity was made applying these values to Equation 2, presented earlier. The amount of reagent, water and the pH value before the PTs can be found in Table 5:

Table 5 - Quantity of reagent, water and pH of each pretreatment

Pretreatment	Reagent type, quantity (g)	Water (mL)	pH
1 and 3	CaO, 0,4g	100	11,9
2 and 4	NaOH, 0,4g	100	12
5 and 7	CaO, 0,2g	100	12
6 and 8	NaOH, 0,2g	100	12,1

Source: Made by the author (2018)

In order to better understand the action of the PTs on both sorghum and miscanthus, the following results will be presented in two chapters. Firstly, a qualitative description of the observed results of the PT effects in the plant anatomy was made, comparing images from untreated with pretreated material using FASGA, Pholoroglucinol-HCl (PH) and Congo Red staining solutions. Secondly, a quantitative analysis was performed using the values found with the software ImageJ after image processing, comparing non-treated with pretreated samples. A junction of these two investigations is essential to integrate the results obtained, taking into account the largest number of variables possible. This thorough study of the structure of tissues and cells allows to better understand the real effects of the pretreatments in the plant and how it varies according to the environment, biomass, and reagent type.

5.2.3. Qualitative Analysis of the Anatomic Structure

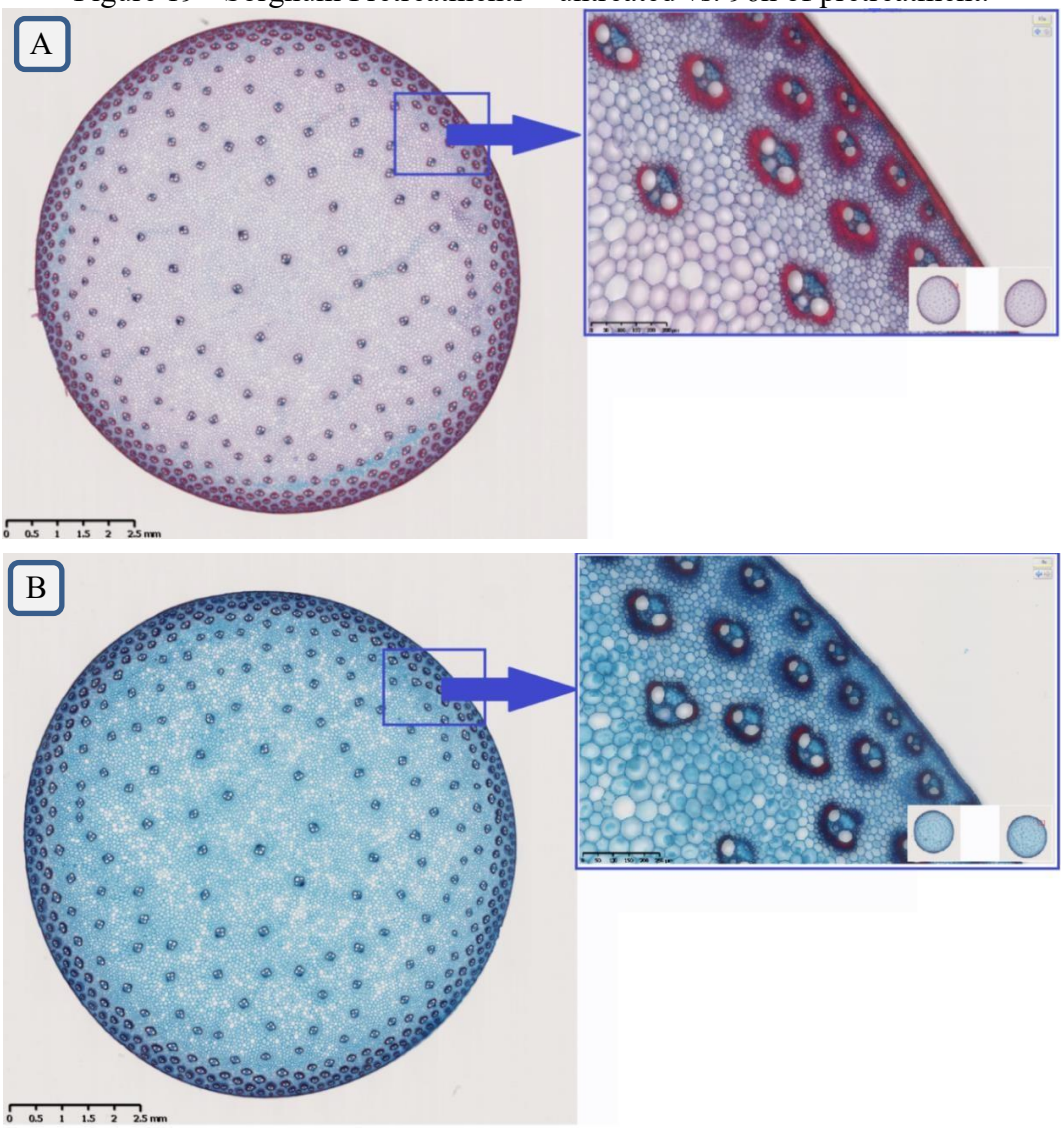
5.2.3.1.Sorghum

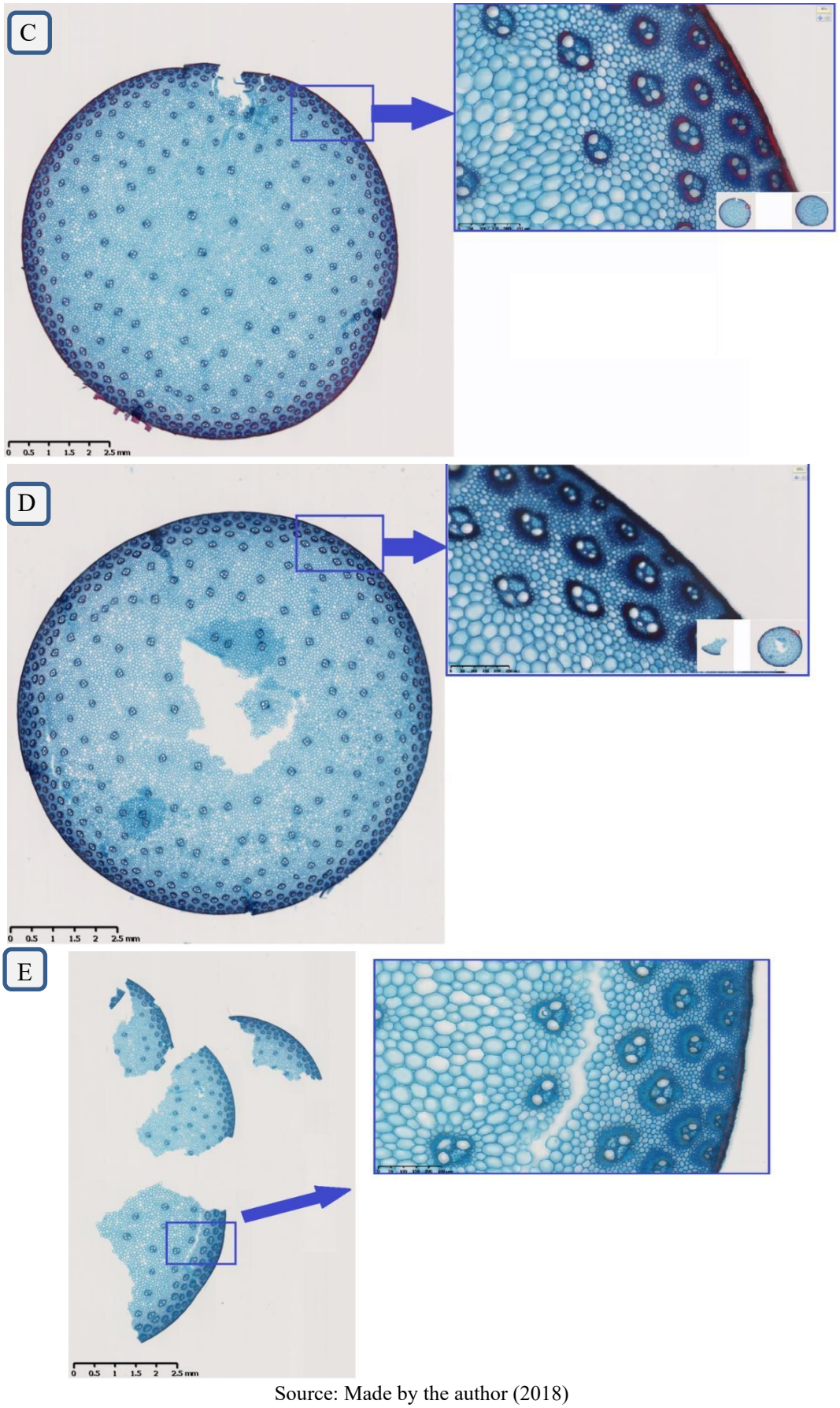
The stem of sorghum, anatomically similar to sugarcane and maize (BAKEER et al., 2012), is characterized by a lignified epidermis that surrounds the vascular bundles spread throughout the parenchyma or ground tissue.

- Initial observations on Sorghum Pretreatments:

Figure 19 shows a comparison of cross sections of the internode between untreated material and the four pretreatments applied to sorghum for 96 hours, stained with the FASGA solution. The 96 hours duration PT was chosen for this analysis in order to better demonstrate the PT's action visually, as the time variable appeared to be directly related to PT effect on the sections.

Figure 19 - Sorghum Pretreatments – untreated vs. 96h of pretreatment.





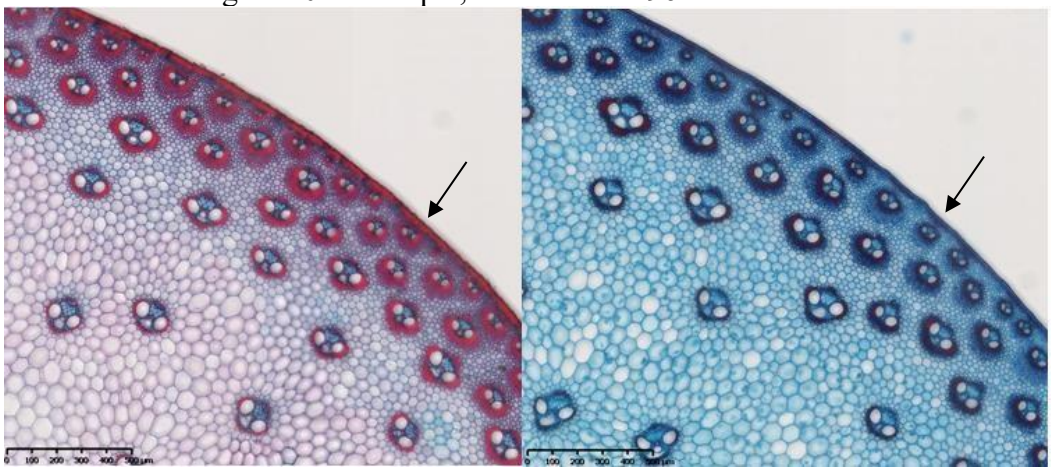
Source: Made by the author (2018)

Where each letter represents the following:

a) Untreated sectioned material;
b) Pretreatment 1: CaO 26°C;
c) Pretreatment 2: NaOH 26°C;
d) Pretreatment 3: CaO 55°C;
e) Pretreatment 4: NaOH 55°C.

From Figure 19 "a" (untreated sample) to “b”, “c” , “d” and “e” (pretreated samples) an important evolution of colors can be noticed: The purple in Zone 2 (Z2) turns blue as the red in Zone 1 (Z1) fades away. A more careful analysis shows that there is a significant decrease in the sclerenchyma area in Zone 1 when comparing the untreated sample with the pretreated samples. There is a great color difference in the parenchymal area in Zone 2 when comparing untreated and pretreated samples, as Z2 turns blue after PTs, showing that cellulose became more available due to the lignin degradation (Figure 20).

Figure 20 - Example, untreated vs. 96 hours of PT1.



Source: Made by the author (2018)

The sclerenchyma around the conductive bundles looks smaller after PT. Comparing between all the PTs, Figura 19 “e” (PT 4 representing NaOH at 55ºC) seems to have the most remarkable change in the reduction of the lignification portion of the sclerenchyma in Zone 1. Even without zooming in, a strong color difference can be noticed in this area when regarding other PTs, which are almost entirely blue.

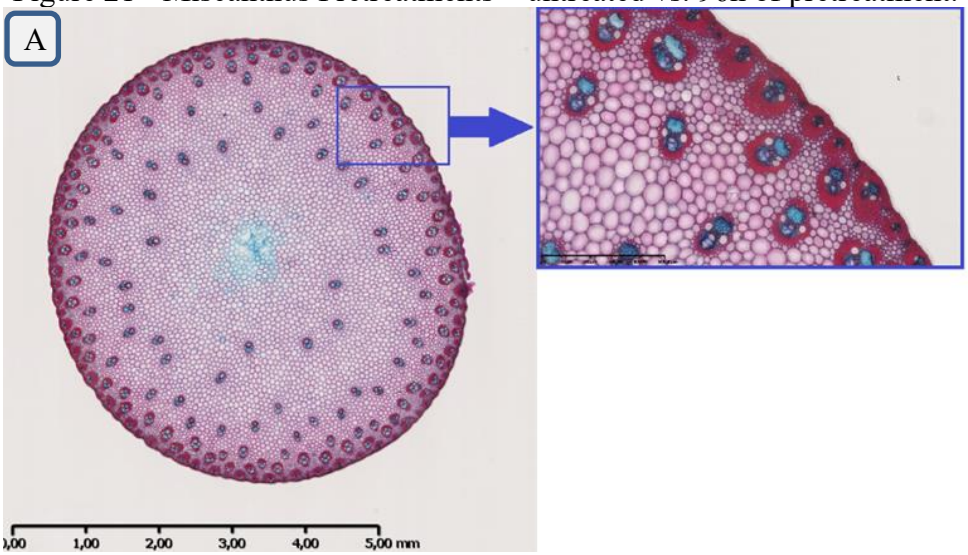
PT4 also showed signs of partition of the cell walls; this tendency of cells separation in the parenchyma generated a disruption on its surface. Moreover, as a result of the pretreatment, the increased fragility of the material made the process of sampling and assembling of the lame difficult, because the section would tear apart every time it was touched with the tweezers. A question is raised: can the strong pretreatment effect degrade cellulose as well as lignin causing the fragility of the section?

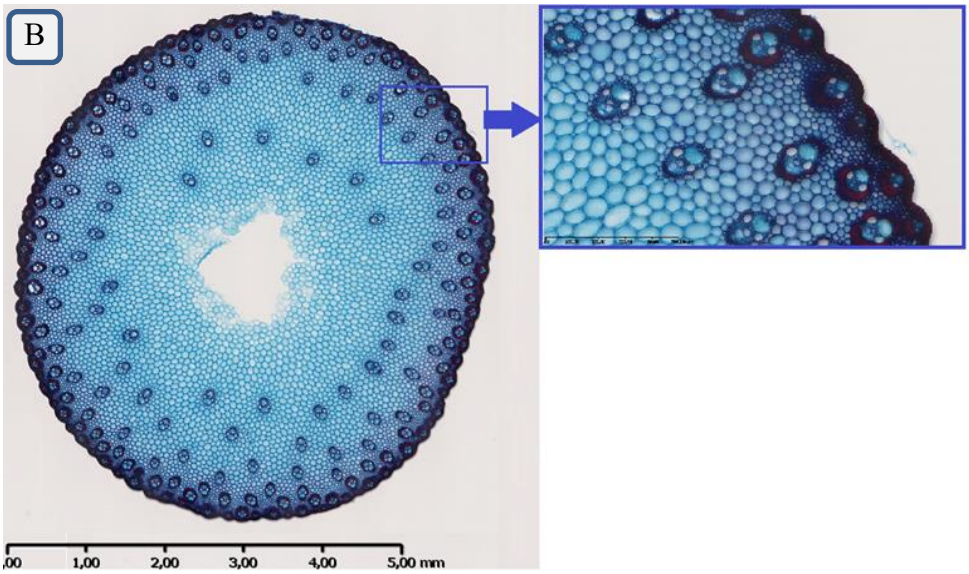
For all, the cellulose within the phloem inside the conductive bundles looks intact throughout the entire pretreatment and the cell walls look deformed on Z1 and inside the phloem.

5.2.3.2.Miscanthus

The anatomy of miscanthus is similar to the sorghum anatomy; therefore, there is no need to present another explanatory anatomy image. The main difference that can be noticed is on the distribution of the vascular bundles, since they are not as numerous in Z2 as in the sorghum samples. They are also arranged in concentric circles, but as they get closer to the center, they become scarcer. The diameter of the stem of miscanthus used for this study was about half of the size of sorghum. Figure 21 shows a comparison between untreated miscanthus samples and 96 hours of each PT.

Figure 21 - Miscanthus Pretreatments – untreated vs. 96h of pretreatment.




B
1,00
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3,00
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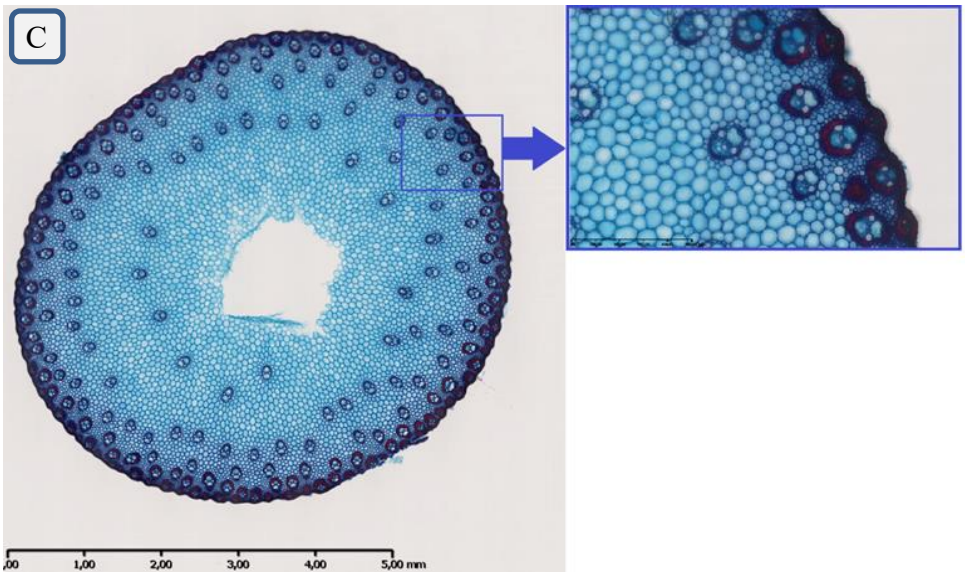
5,00 mm


C
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3,00
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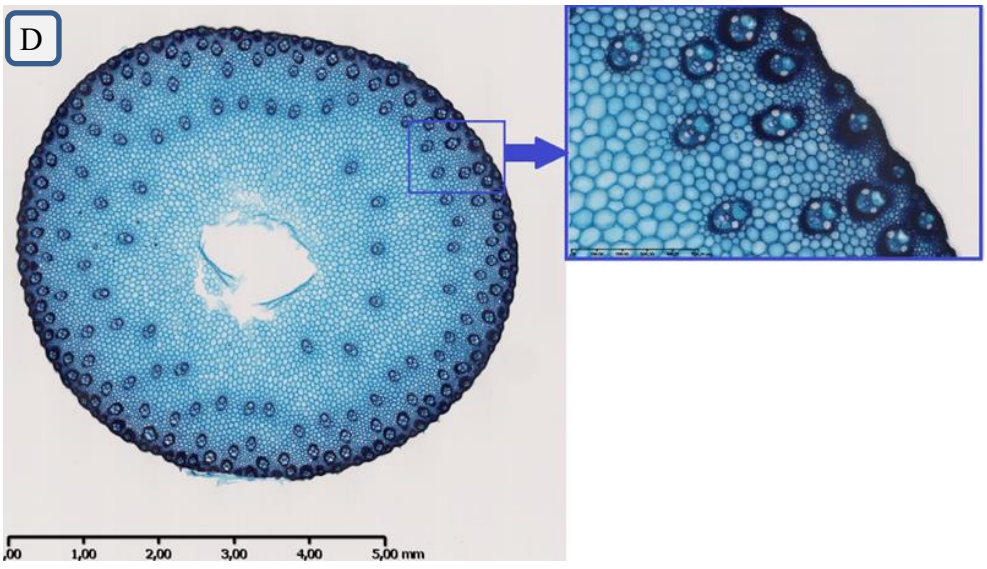
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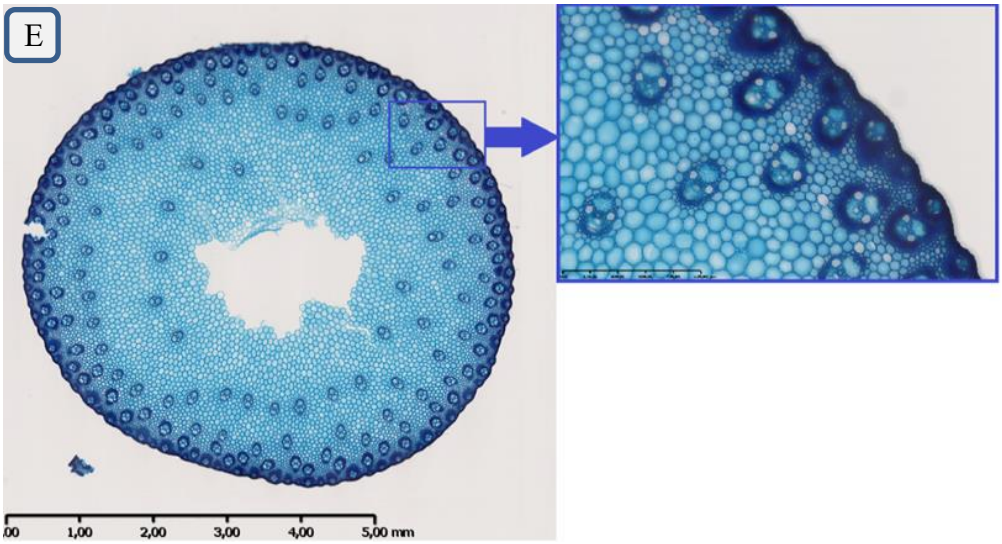


Source: Made by the author (2018)

Where each letter represents:

a) Untreated sectioned material;
b) Pretreatment 5: CaO 26°C;
c) Pretreatment 6: NaOH 26°C;
d) Pretreatment 7: CaO 55°C;
e) Pretreatment 8: NaOH 55°C.

As observed before for sorghum samples, the pretreatments appear to have a similar evolution. All PTs result in an increase in the availability of cellulose, as Z1 is blue on the pretreated samples and overall red ion the untreated sample. Visually, PTs 5, 6 and 7 seem to retain some of the red coloration in Z2, possibly because these treatments did not have such a strong effect on the lignified sclerenchyma area as PT8. Pretreatment 8 (NaOH at 55ºC) seems to be the most effective.

ZHENG et. al. (2018) studied the impact of three types of pretreatments on lignocellulosic biomass including miscanthus and found that amongst sulfuric acid (H2SO4), sodium hydroxide (NaOH), and hot water pretreatments, the highest cellulose conversion rate was obtained with the 4% NaOH pretreatment at high temperatures.

In all pretreated samples, the center is teared apart. This is a characteristic that presents itself sometimes also on the untreated miscanthus samples, but at its center is more fragile than that of the sorghum samples, pretreatment seems to attack the tissue increasing the hole in the center. For both sorghum and miscanthus samples, PT with NaOH at 55 ºC appears to have a stronger effect, when visually analyzed on the FASGA stained digitalized microscopic sections.

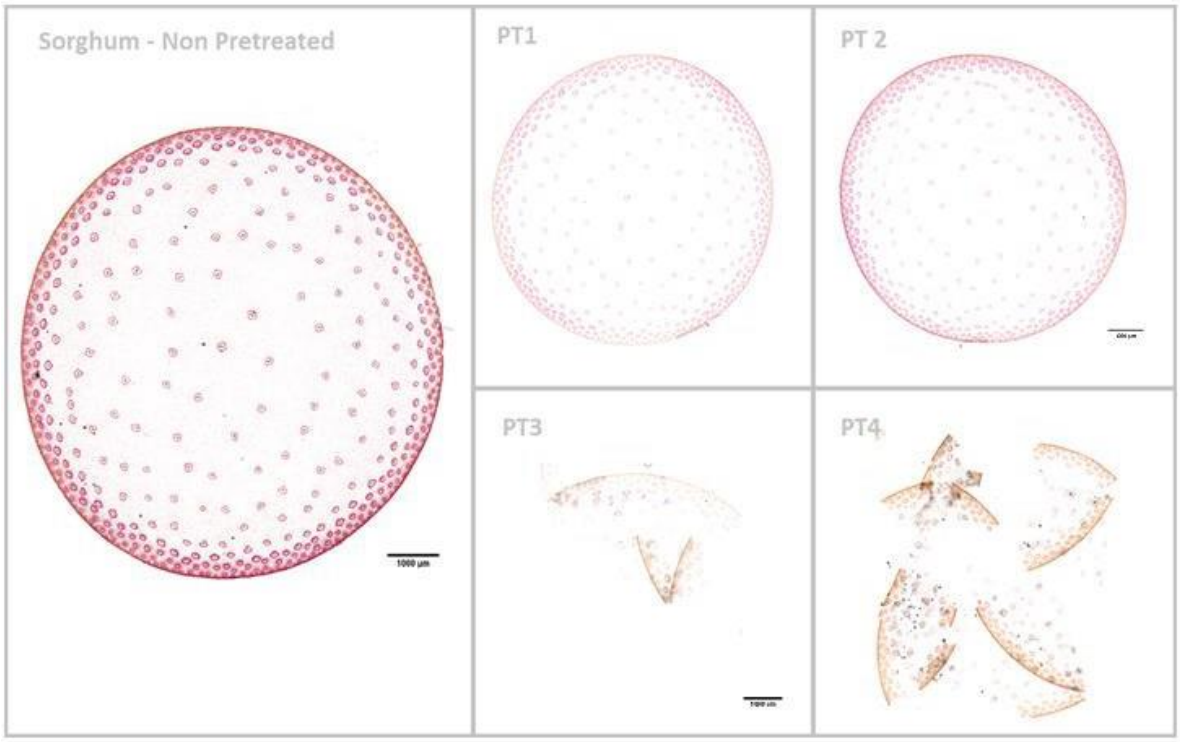
The epidermis and the perivascular sclerenchyma looked the least affected tissues by alkaline pretreatment. The parenchyma in Z2 was the most affected. Furthermore, these pretreatments did not affect only lignified structures as a thickening of cell walls was noticed.

5.2.3.3.Pholoroglucinol-HCl

This staining was performed in untreated samples and compared to 48 hours of PTs, in order to validate the results observed with the quantification of FASGA stained samples. For this representation, 48 hours of pretreatment were chosen because the effect the PTs was already evident within the pictures taken.

Figure 22 shows the untreated sorghum sample on the left, strongly lignified in red, whereas the treated samples on the right show red coloration that does not appear to be as strong, generating a light pink or sometimes orange. To recapitulate, PH staining colors lignified tissues in red. The more vibrant the red coloration, the heavily lignified it is. For both sorghum and miscanthus, it is clear that the pretreatment has a better performance in eliminating lignin when the temperature is higher, that is PT3, PT4, PT7 and PT8 (Figures 22 and 24).

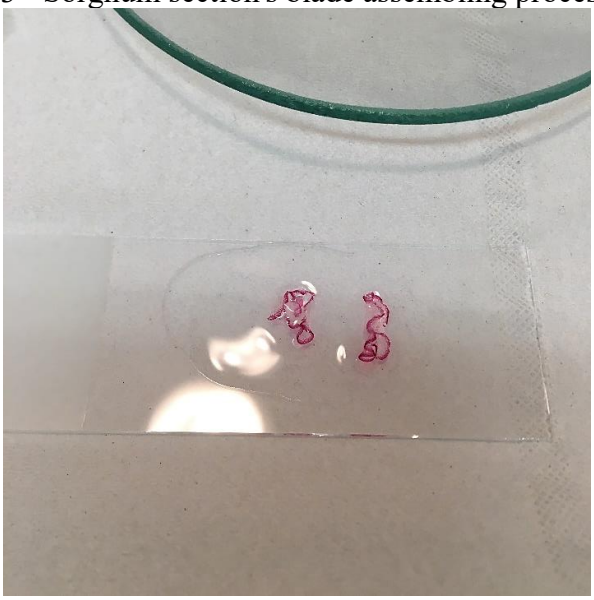
Figure 22 - Comparison between non-treated sorghum and Pretreatments 1 to 4 during 48 hours.



Source: Made by the author (2018)

In Figure 22, it is also possible to observe that the strong lignified sclerenchyma area around the conductive bundles on the untreated sample. As for the pretreated samples, these are not as strongly stained in red, due to the fact that the amount of lignin present was less significant after PT. For pretreatments in higher temperature, such as PT3 (CaO) and PT4 (NaOH), it was not possible to recover the entire section when the assembling of the blade was made, due to the fact that the PT weakened the tissues, which would break with a touch, as shown in Figure 23.

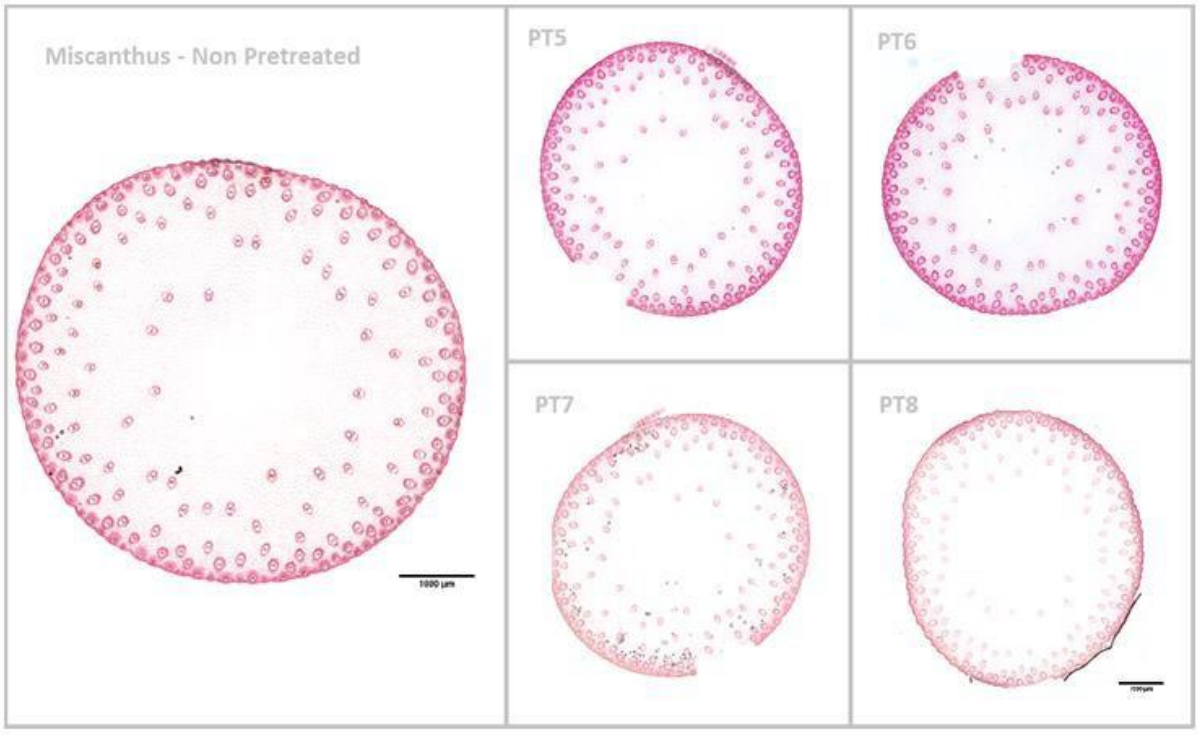
Figure 23 - Sorghum section's blade assembling process (PT4).



Source: Made by the author (2018)

Pretreatments 3 and 4 (both performed at 55 ºC) showed better results in lignin removal than PT 1 and 2 (both performed at 26 ºC) by visual analysis under PH staining. Similar results were found for miscanthus samples (Figure 24).

Figure 24 - Comparison between non-treated sorghum and Pretreatments 5 to 8 during 48 hours.



Source: Made by the author (2018)

In Figure 24, an untreated miscanthus sample stands next to the 4 types of pretreatments. Similarly to the sorghum samples, higher temperatures seem to positively act on the pretreatment effect, showing that the pretreated 55 ºC samples are less lignified than the 26 ºC pretreated samples due to their lighter red color. Visually it is not possible to affirm whether the lignin in the sclerenchyma area has diminished in pretreatments 5 and 6 (CaO and NaOH at 26ºC respectively).

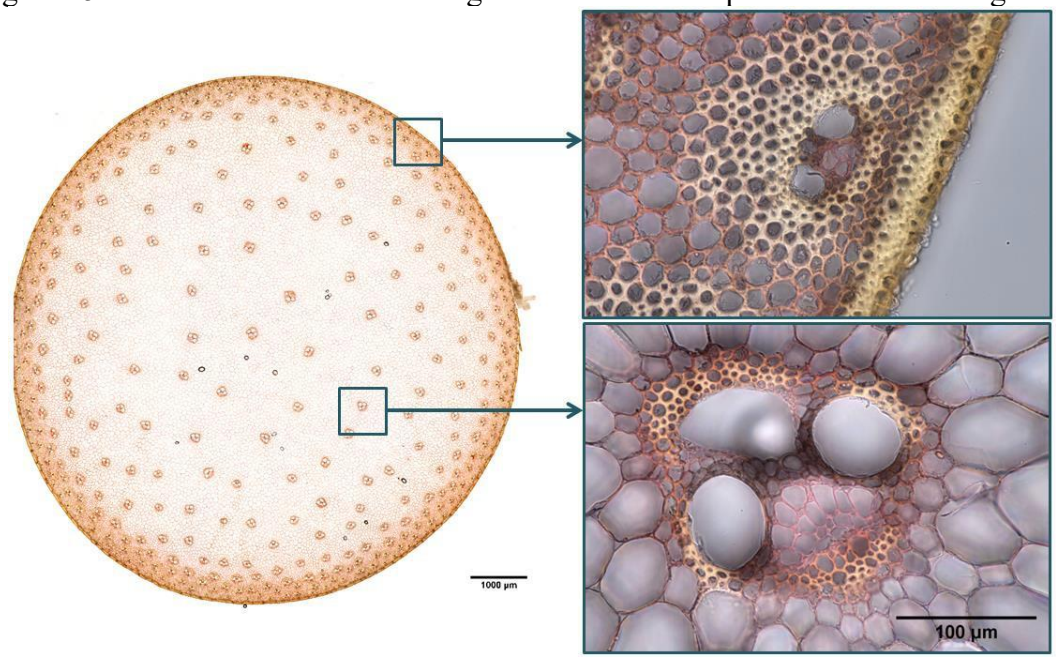
Alkali pretreatment acts on degradation of hemicellulose and lignin. During alkali pretreatment the acetate group from the hemicellulose will be removed, so the hydrolytic enzymes can more easily access the carbohydrates (BOCHMANN, 2019).

5.2.3.4.Congo Red

After the preliminary results, two pretreatments were chosen for Congo Red staining: PT4 and PT8, both using NaOH at 55ºC for sorghum and miscanthus samples respectively, comparing to its untreated samples in order to contrast with the PH staining results, and ensure that the PT also act on exposing cellulose.

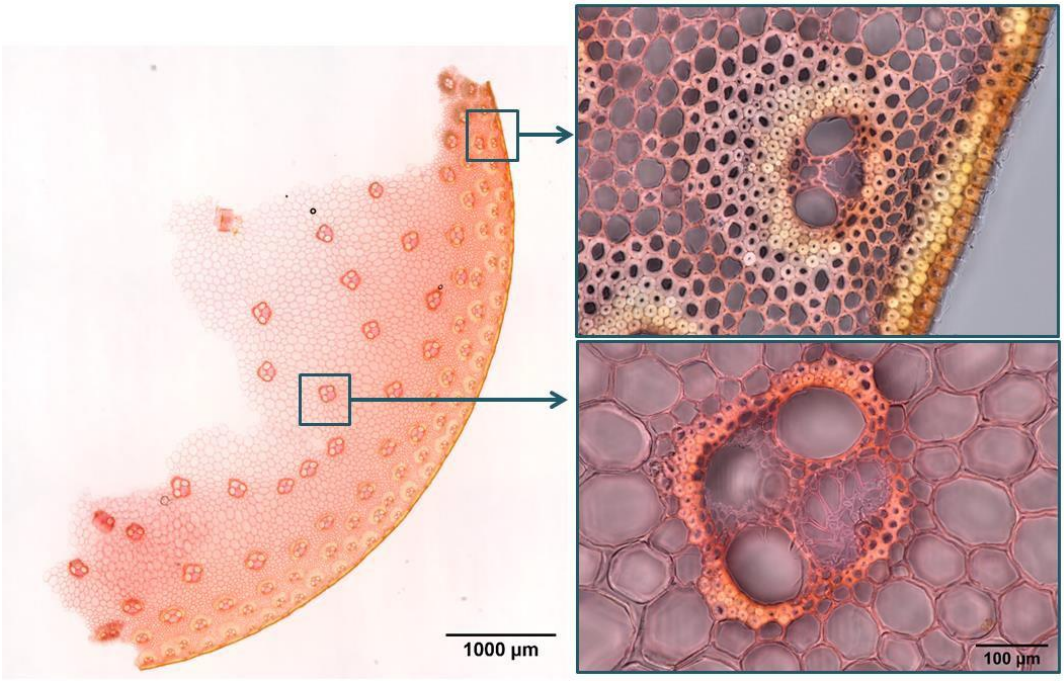
Images were taken from the entire cross section (when available) and from the conductive bundles, one located in Z2 and another located in Z1, with a zoom of x40. Red Congo colors cellulose in red/pink tones. For sorghum samples, it is possible to notice that the pretreated section shows a more red/pink color than when compared to the untreated sample (Figure 25 and 26).

Figure 25 - Transversal Section of Sorghum untreated sample stained with Congo Red.



Source: Made by the author (2018)

Figure 26 - Transversal Section of Pretreatment 4 stained with Congo Red.



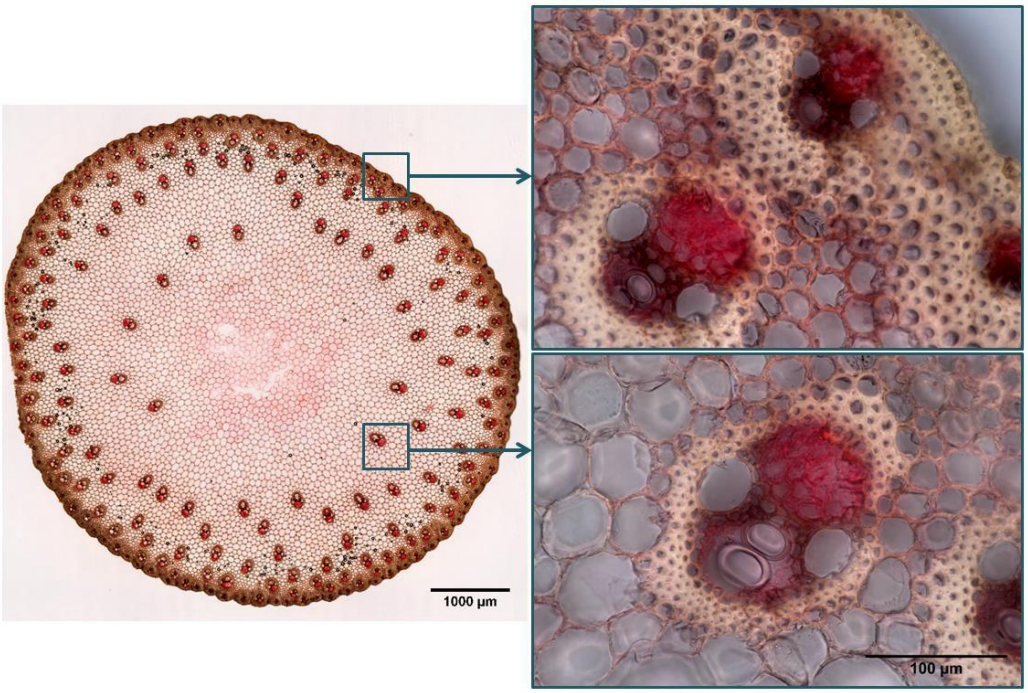
Source: Made by the author (2018)

The conductive bundle located in Z1 is more lignified on the untreated sample. As for the pretreated one, it is possible to see a gradient of color, as the bundle is less lignified in its

exterior. As for the conductive bundle located in Z2, for the untreated sample, its cell walls are overall yellow (lignified), but for the pretreated sample it is mostly red.

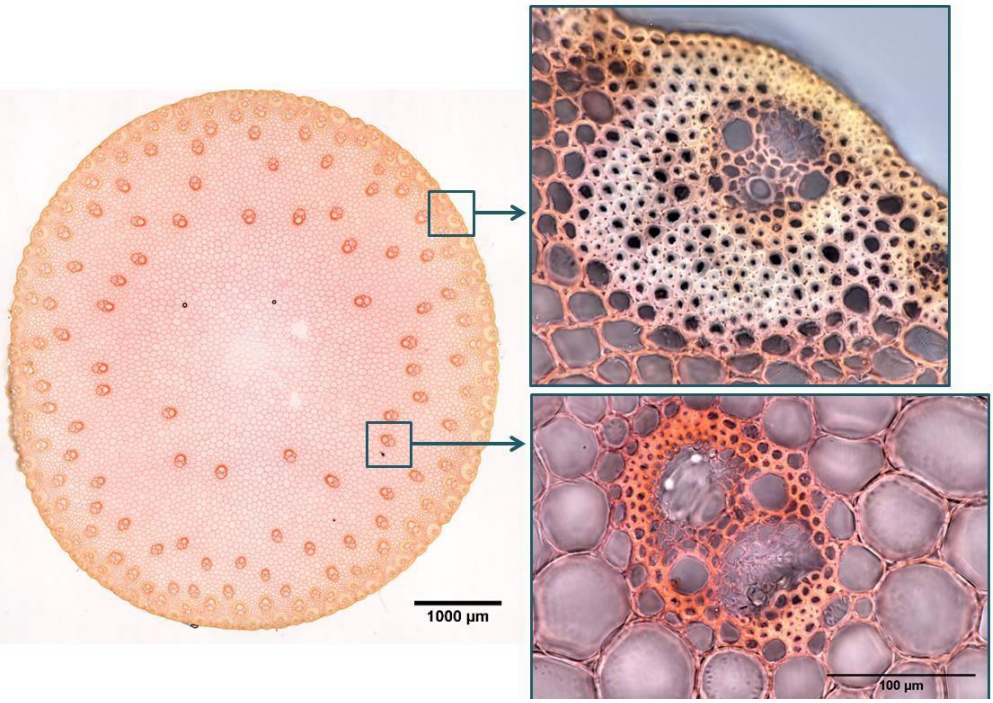
For the miscanthus samples the effect is similar. However, it is possible to notice that on the conductive bundles of the untreated sample the phloem is very red, but for the pretreated sample the phloem does not appear to have any more cellulose (Figures 27 and 28).

Figure 27 - Transversal Section of Miscanthus untreated sample stained with Congo Red.



Source: Made by the author (2018)

Figure 28 - Transversal section of Pretreatment 8 stained with Congo Red.



Source: Made by the author (2018)

This suggests that the PT might also be degrading cellulose within the conductive bundles of the miscanthus samples.

5.2.4. Quantitative Analysis

In addition to the visual analysis performed in the previous section, this section quantifies the amount of cellulosic and lignified area in the untreated and pretreated sections using FASGA staining and ImageJ software.

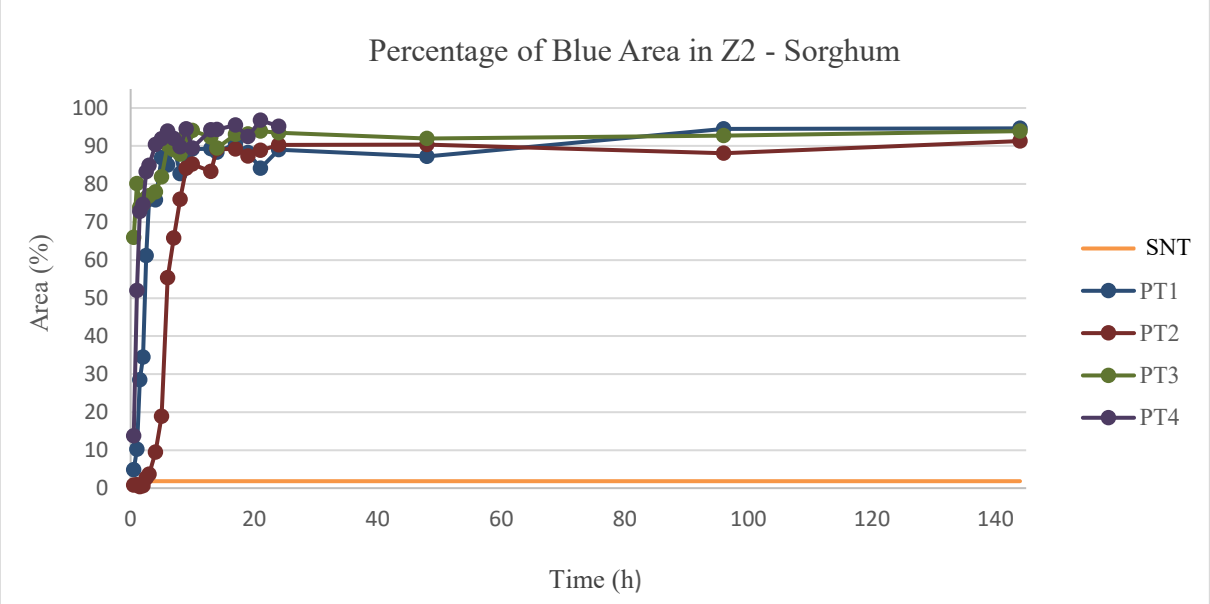
5.2.4.1.Kinetics

The results from the images obtained from the pretreatment applied to the sections were observed according to the quantity of blue area in Z2 (PerBluZ2) and the quantity of sclerenchyma area in Z1 (PerSclZ1). The straight orange line represents the not-treated material (MNT or SNT), in all graphs presented next. The pH values were monitored in all sampling hours, not showing any significant alterations; therefore, the pH results will not be exposed here.

- Sorghum

For the sorghum pretreatments, it was not always possible to recover enough of the pretreated section to quantify all parameters using the software ImageJ. Due to the PT's effectiveness, PT4 (NaOH at 55ºC) led to an extreme fragility of the samples that could not be collected without tearing apart, compromising the image treatment. Therefore, the graphs were plotted using points from 0 to 144 hours for pretreatments 1 to 3 and from 0 to 24 hours for pretreatment 4 (Graph 1 and 2).

Graph 1 - Percentage of blue area in Z2- Evolution in time during 144 hours for sorghum samples.



Source: Made by the author (2018)

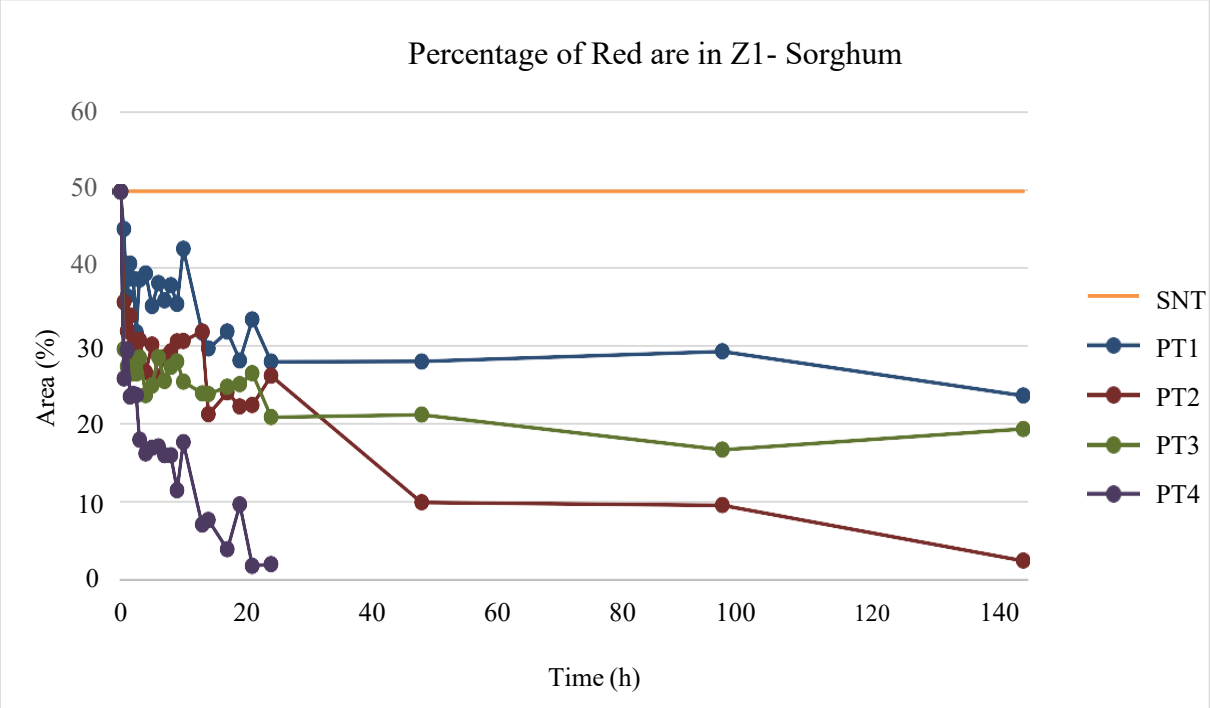
It is possible to remark that all pretreatments had good efficiency when analyzing the increase in the blue area of the section over time. PT 4 (NaOH at 55ºC) had better results than the others. The untreated sample presented 1.83% of blue area in Z2, compared to PT1 with 94.65%, PT2 at 91.3%, PT3 presented 93.89% for 144 hours and PT4 presented 95.15% in only 24 hours.

The most significant changes in Graph 1 happens within the first 20 hours of PT for all designs. This is extremely important when analyzing the cost benefit of pretreating lignocellulosic biomass, for this shows that longer PTs will result in unsignificant increase in

cellulose availability. There is also an influence of the thickness of the biomass. If other particle sizes are used, the pre-treatment time may be different.

Next, the kinetics of the sclerenchyma area reduction was plotted.

Graph 2 - Percentage of Sclerenchyma in Z1- Evolution in time during 144 hours for sorghum samples.



Source: Made by the author (2018)

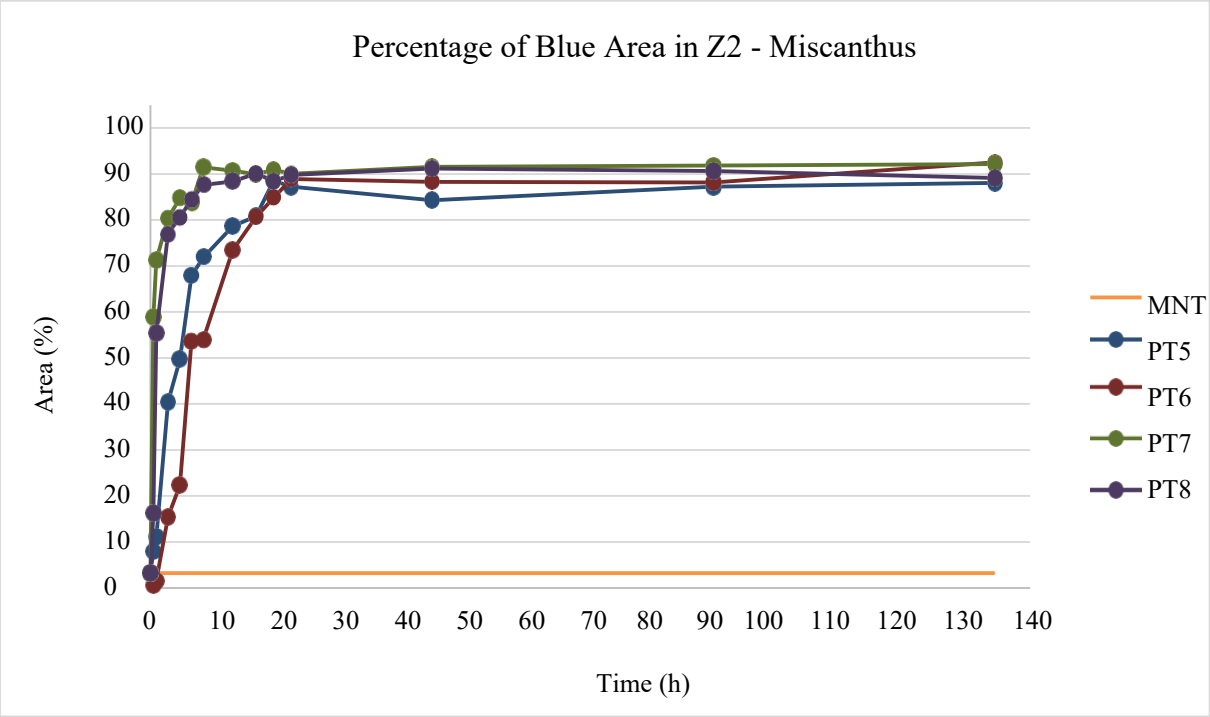
The untreated samples presented 49.68% of sclerenchyma area in Z1 as PT1 presented 23.42%, PT2 presented 2.41%, PT3 presented 19.25% for 144 hours and PT4 reaching the best value of 1.95% at 24 hours.

These results indicate that for sorghum samples, pretreatment with NaOH at 55 ºC had faster and better results at removing lignin and increasing cellulose availability.

- Miscanthus

For the miscanthus samples, it was possible to recover at least one entire section for all pretreatments; therefore, the following graphs are complete, showing values taken from hours 0 to 144 (Graph 3 and 4):

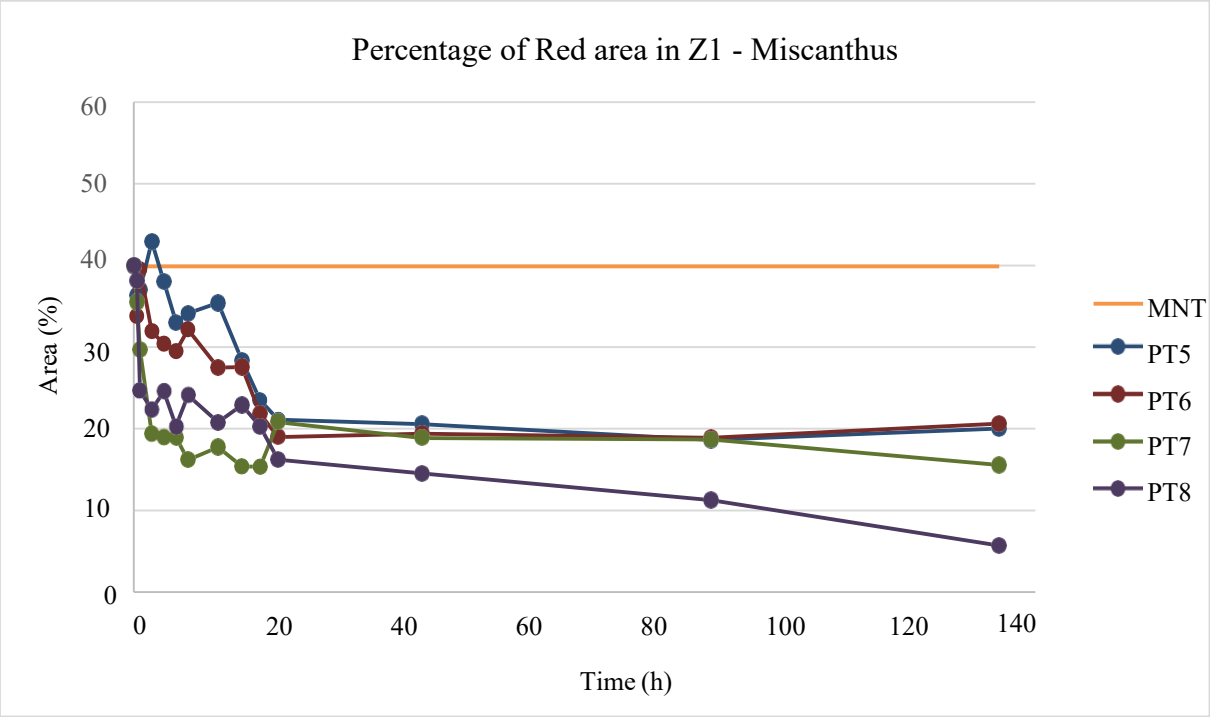
Graph 3 - Percentage of blue area in Z2- Evolution in time during 144 hours for miscanthus samples.



Source: Made by the author (2018)

The values found for the untreated sample were of 3.3% of blue area in Z2, as for PT5 the value was 88.1%, for PT6 the value was 92.54%, PT7 presented 92.13% and finally PT8 presented 89.14%. All values are very close to each other, but it is possible to notice in Graph 3 that pretreatments 7 and 8 work faster than pretreatments 5 and 6. Graph 4 presents the evolution in the quantity of the lignified portion in Z1 and its evolution in time.

Graph 4 - Percentage of Sclerenchyma in Z1- Evolution in time during 144h for miscanthus samples.



Source: Made by the author (2018)

As for the removal of lignin in Z1, PT8 was clearly the most effective pretreatment, presenting 5.7% of sclerenchyma area, followed by PT7 that resulted in 15.55%, after PT5 that showed 19.99% and PT6 at 20.63% when compared to the untreated sample at 39.9% after 144 hours of PT duration.

These results indicate that for miscanthus samples, PT7 (CaO at 55 ºC) and PT8 (NaOH at 55 ºC) are nearly equally efficient on increasing cellulose availability and PT8 is more efficient at reducing lignified area in Z1 after 144 hours.

Table 6 and Table 7 respectively present the consolidated results for all PTs regarding cellulosic area in Z2 and sclerenchyma area in Z1, starting at 9 hours of sampling.

Table 6 - Consolidated Results (perBluZ2).

SAMPLE	PT name	PT reagent	PT Temperature (°C)	% Blue Area in Z2					
				9h	12h	24h	48h	96h	144h
Soghum	SNT	Untreated Raw Material	26 and 55	1.8	1.8	1.8	1.8	1.8	1.8
Soghum	PT1	CaO	26	88.1	89.3	89.0	87.2	94.5	94.6
Soghum	PT2	NaOH	26	84.1	83.3	90.3	90.4	88.1	91.3
Soghum	PT3	CaO	55	92.8	92.3	93.5	92.0	92.7	93.9
Soghum	PT4	NaOH	55	94.5	94.2	95.1	-	-	-
Micanthus	MNT	Untreated Raw Material	26 and 55	3.3	3.3	3.3	3.3	3.3	3.3
Micanthus	PT5	CaO	26	71.9	75.3	87.2	84.3	87.3	88.1
Micanthus	PT6	NaOH	26	53.7	53.9	84.9	88.9	88.3	88.2
Micanthus	PT7	CaO	55	83.7	87.6	90.9	90.0	91.6	91.9
Micanthus	PT8	NaOH	55	84.5	86.1	88.5	89.8	91.2	90.6

Source: Made by the author (2018)

Table 7 - Consolidated Results (perSclZ1).

SAMPLE	PT name	PT reagent	PT Temperature (°C)	% Red Area in Z1					
				9h	12h	24h	48h	96h	144h
Soghum	SNT	Untreated Raw Material	26 and 55	49.7	49.7	49.7	49.7	49.7	49.7
Soghum	PT1	CaO	26	35.3	31.6	27.9	27.9	29.2	23.4
Soghum	PT2	NaOH	26	30.5	31.7	26.1	9.9	9.5	2.4
Soghum	PT3	CaO	55	27.9	23.8	20.8	21.1	16.6	19.3
Soghum	PT4	NaOH	55	11.4	7.03	1.95	-	-	-
Micanthus	MNT	Untreated Raw Material	26 and 55	39.9	39.9	39.9	39.9	39.9	39.9
Micanthus	PT5	CaO	26	34.1	34.7	21.1	20.6	18.6	20.0
Micanthus	PT6	NaOH	26	29.5	30.8	21.8	19.0	19.4	18.9
Micanthus	PT7	CaO	55	18.9	17.5	15.3	20.8	18.9	18.7
Micanthus	PT8	NaOH	55	20.3	22.2	20.3	16.2	14.5	5.7

Source: Made by the author (2018)

Considering all parameters presented, including the qualitative analysis (Chapter 5.2.3.), it is possible to affirm by these results that pretreatments using NaOH at higher temperatures have a stronger effect than the other types of PTs analyzed in this study. The lack of data for the PT4 also indicates strong PT action. Overall, as both time and temperature increase, so does the PT effect when NaOH is the reagent applied.

5.2.4.2.Statistical Analysis

To better understand the trend of each type of PT when regarding the quantity of lignified area in Z1 and the quantity of cellulosic area in Z2, the following model was proposed. Equation 3 represents a general model, and Equation 4 represents the personalized model proposed applied to the specific studied variables.

$$Yijk = \mu + beta\_0\ x\ Log(Hi) + Tj + beta\_j\ x\ Log(Hi) + \text{rijk} \quad (3)$$

Where, *Yijk* stands for the observed variable (PercSclZ1or PercBlueZ2); μ is the general mean; *beta_0* represents the mean regression slope; *Log(Hi)* is the log of pretreatment length; *Tj* is the Effect of treatment j; *Beta_ij* stands for deviation of slope due to pretreatment j and finally *Rijk* is the unexplained residual error, as shown next.

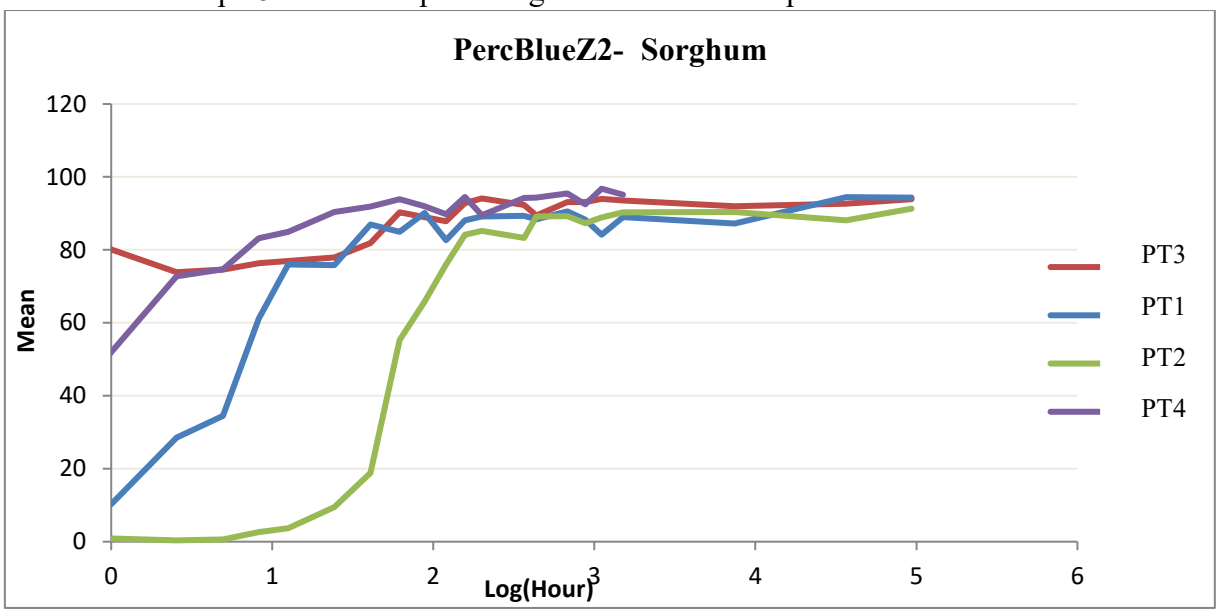
$$PercSclZ1\ or\ PercBlueZ2 = LogHour * Reagent * \text{Temperature} \quad (4)$$

Where *PercSlcZ1* is the percentage of red area in Z1; *PercBlueZ2* is the percentage of blue area in Z2,; *LogHour* is the logarithm of the length of pretreatment in hours*; Reagent*Temperature* represents the pretreatment type (PT1, PT2, PT3, etc).

The main parameters obtained by this statistical analysis using this model can be seen in Appendix C. These parameters validate the model proposed because the R^2 found was on average 0.98, showing that this model attends very well to what is proposed. The coefficient of variation in all cases was found to be low, therefore showing that the model's level of control is very good.

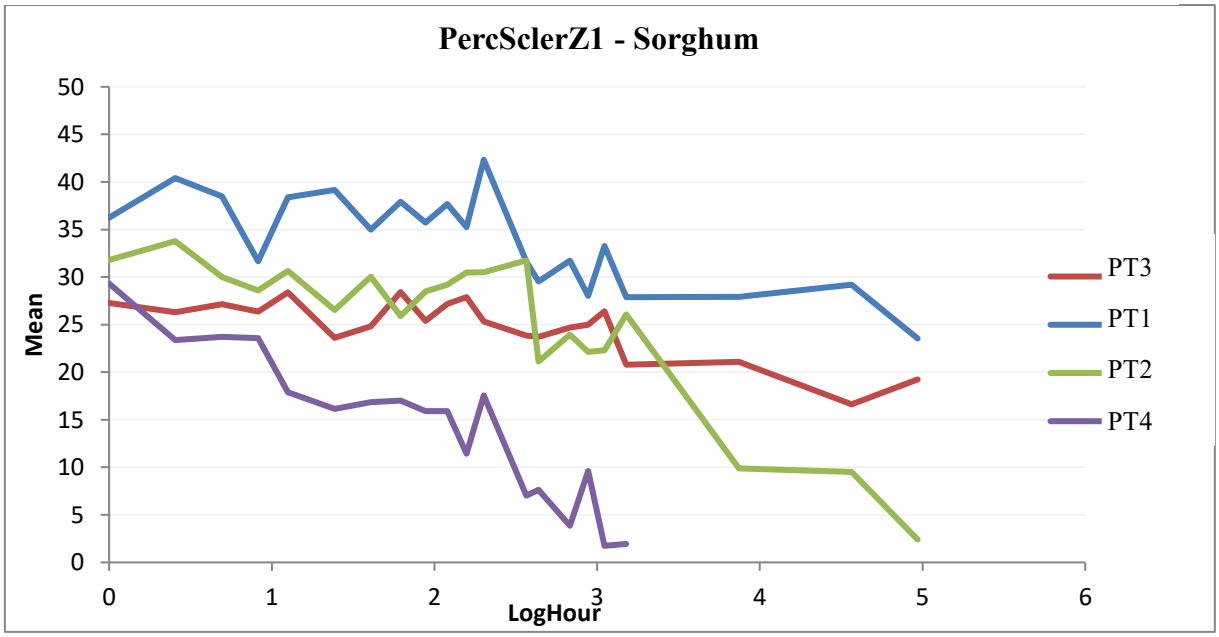
A strong effect of pretreatment length and pretreatment type was found on both variables of interest (PercBlueZ2 and PercScleZ1). In fact, there is a mean trend (mean regression slope beta_0) of the duration of treatment, but also a trend specific to each treatment j (beta_ij). The following graphs were plotted using the mean value found by the model proposed by the logarithm of the length of the pretreatment (in hours) for reasons of better adaptation to the visualization than the non-logarithmic function (Graph 5, 6, 7 and 8).

Graph 5 - Trend of percentage of blue in Z2 for pretreatments 1 to 4.



Source: Made by the author (2018)

Graph 6 - Trend of red area in Z1 for pretreatments 1 to 4.



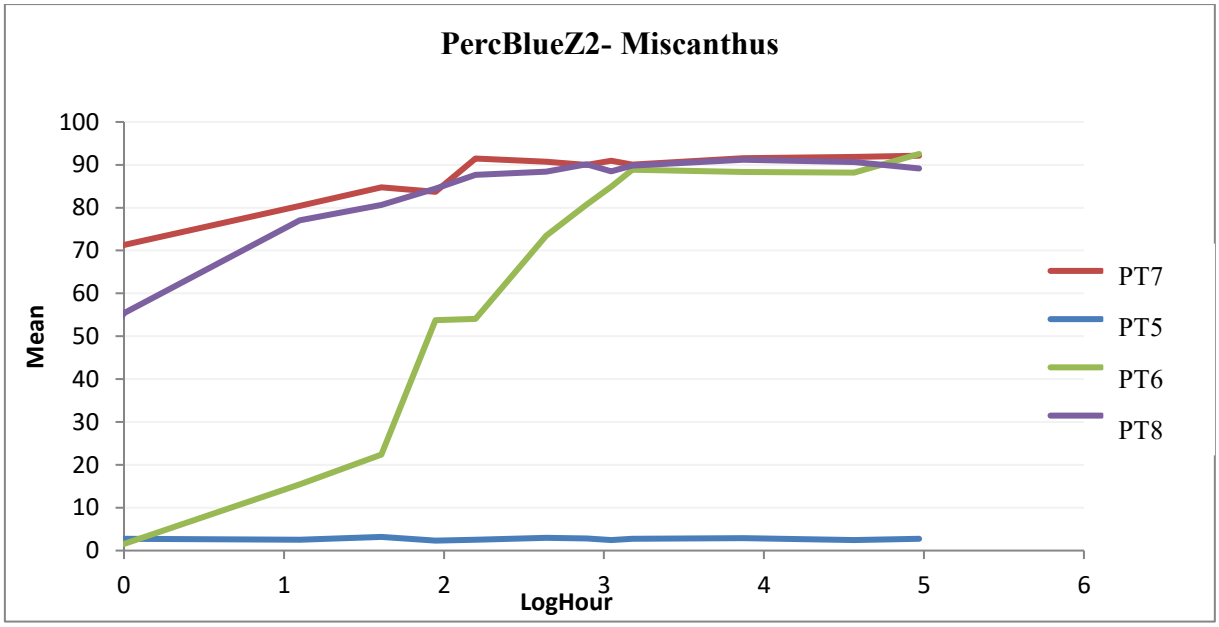
Source: Made by the author (2018)

Notably, higher temperatures (55 °C) accelerates both cellulose availability and lignin removal on sorghum samples. Pretreatments at 26 °C have a significantly lower speed of action regarding both parameters.

The percentage of lignified sclerenchyma in Z1 (red color) (PercSclZ1) decreased rapidly until 24 h and then decreased at a slower rate. In the same way as PercBluZ2, the kinetics

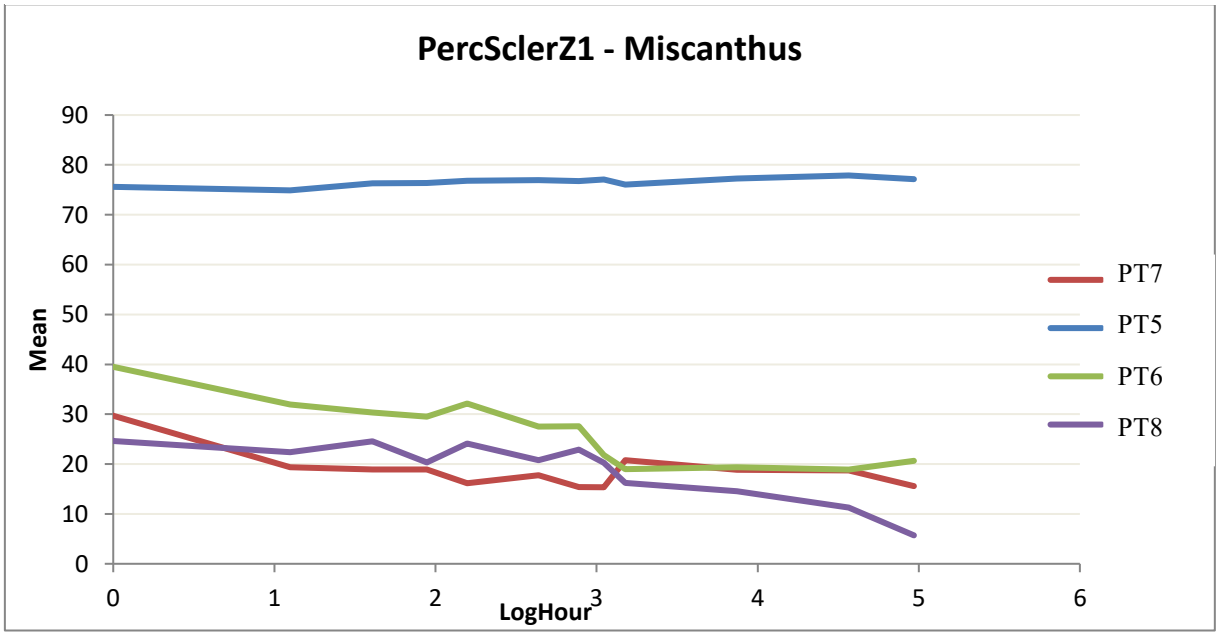
of the decrease of PercSclZ1 was faster at 55°C than at 26°C and was faster with NaOH than with CaO. A large part of the sclerenchyma was degraded in the first 24 hours. The pretreatment with NaOH at 55°C seemed more efficient on sorghum.

Graph 7 - Trend of percentage of blue in Z2 for pretreatments 5 to 8.



Source: Made by the author (2018)

Graph 8 - Trend of red area in Z1 for pretreatments 5 to 8.



Source: Made by the author (2018)

For miscanthus samples, pretreatment using CaO at 26 °C does not appear to be very effective, and it shows a tendency to stagnate over time. Pretreatments involving higher temperatures have a stronger effect in cellulose availability regardless of the reagent used, followed by NaOH at 26°C. For lignin removal in Z1, higher temperature can also be associated with faster elimination of lignin.

Overall, it is possible to affirm that the main parameters to be observed in both samples are first the temperature (55°C is more effective than 26°C), followed by reagent type (NaOH in general presented better results than CaO), and finally length of pretreatment, which shows the most significant changes during its first 24 hours (or around 3 in the graphs using LogHour).

6. CONCLUSION

This work was important to show that it is possible to quantify the effectiveness of a pretreatment. Nevertheless, these results can only be validated if completed by a descriptive analysis made by a thorough examination of the innumerous microscopic images obtained using different staining procedures that complement each other.

Pretreatments using NaOH resulted in better lignin removal and in an increase of cellulose availability other than pretreatment using CaO. As shown by qualitative and quantitative analysis, higher temperatures also positively affect the results, accelerating the effects of the PTs by exposing cellulose.

Congo Red staining indicates that pretreatment with NaOH at 55 °C may also degrade cellulose within the conductive bundles for miscanthus samples. This parameter must be better analyzed before applying PT in large scale.

FASGA qualitative results demonstrate that the impact of NaOH and CaO pretreatments affected parenchyma and sclerenchyma and did not affect the epidermis. PTs for miscanthus only increased the Biomethane Potential (BMP) test results. The most significant results observed by the PT's action happen within the first 24 hours of pretreatment duration.

Statistical analysis results show that the main parameters to be observed in both samples are first the temperature (higher temperatures works better), followed by reagent type (NaOH in general presented better results than CaO), and then pretreatment duration.

It is important to remark that the key to succeed in this type of experiment is to be able to select which of the numerous images are to be treated and analyzed, therefore optimizing the results and the collection of data.

Previous works have shown that the large amount of data produced can a be troubling factor for processing and analysis (Legland et al., 2017). Future studies can confirm these results by using other staining methods, such as fluorescence methods and Klason Lignin measurements.

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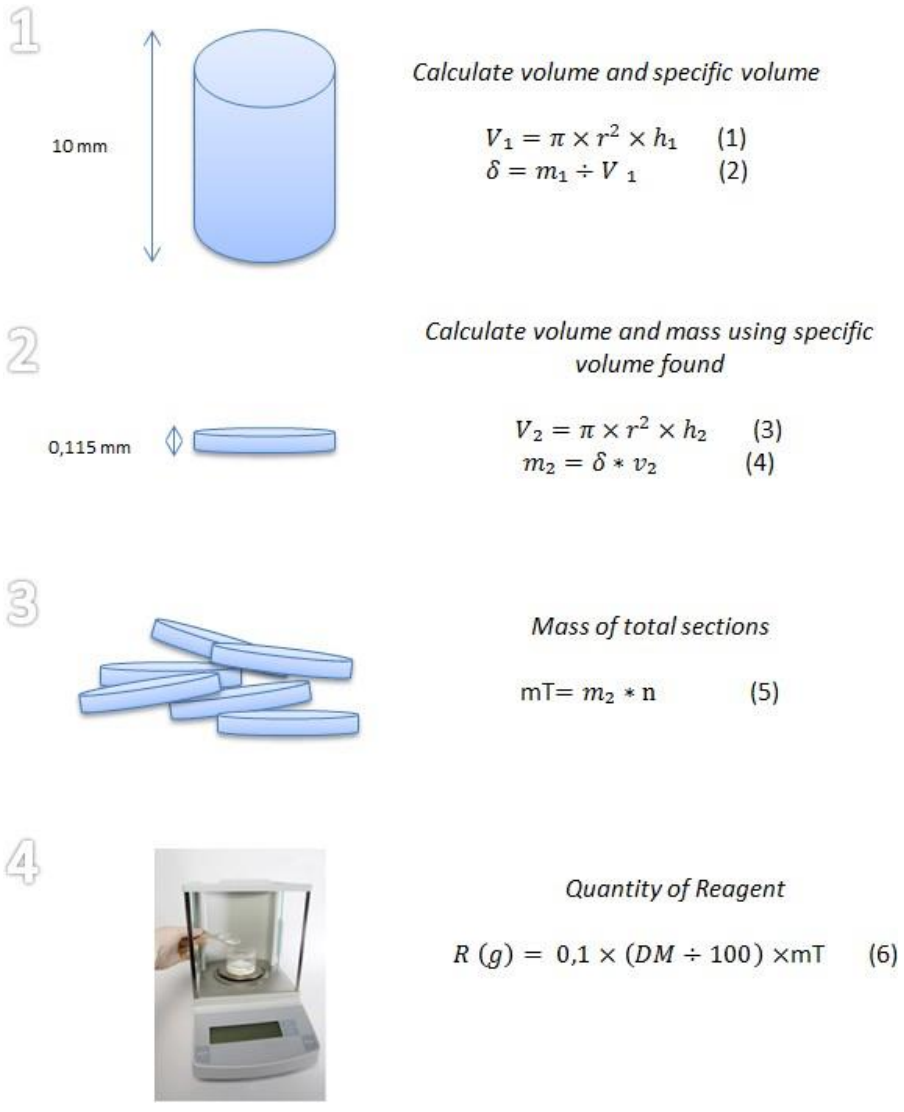
APPENDIX A – REAGENT CALCULATION

The amount of reagent used in the solution was also calculated according to the DM content found for the sectioned material, using the same formula presented before (Formula 1). Nevertheless, unlike the pretreatment applied to the 1cm stems, it was not possible to weigh the sections due to their low weight. It was therefore necessary to first calculate the total weight of the sections and their DM. The calculation was made according to the steps shown in the next figure.



These are the steps for calculating the necessary amount of reagent. (V (volume), r

(radius), h(height) and m(mass)). Step 1: Calculate cylindrical volume (Formula 1) and specific volume of 1cm stem (Formula 2). Step 2: Repeat the same procedure for volume calculation, but this time using the height of the section (in this case 115µm), obtaining the volume of one section (v2). To know the weight of one section, it was necessary to simply use formula 2, isolating the mass (m) and replacing the specific weight by the value calculated before and the volume by the value v2. Step 3: After having the weight of one section, it was necessary to multiply the number of sections (n) needed by the weight found, resulting in the total mass of sections (mT). Step 4: Formula 6 could be applied using the DM value found for the sections and the total mass of sections.

APPENDIX B – IMAGE J PROCESSING STEPS

Parameters of Image Processing	
Name	Name of each sample, donated by the user at time of acquisition
etat	Condition of the section (Entire, Incomplete or torn)
INdiameter	Diameter of section (mm)
surftotale	Total area of section (mm²)
surfportion	Portion of area to be ignored, when section if incomplete or torn
angle	angle of area to be ignored, when section if incomplete or torn
surfzone1	Area of Z1 (mm²)
surfscler	Area of sclerenchyma in red in Z1 (mm²)
nbrefxz2	Number of conductive bundles
surfbluez2	Area in blue in Z2 (mm²)
surfmanque	Missing Area (In the case of incomplete or torn section, mm²)
facteur_c	Correction factor (In the case of incomplete or torn section)
totalArea	Total area after correction factor (mm²)
percZ1	Percentage of Zone 1
percZ2	Percentage of Zone 2
percSclZ1	Percentage of Red area in Zone 1
nbVBZ2	Number of conductive bundles after correction factor
densVBZ2	Density of conductive bundles
percbluZ2	Percentage of blue in Zone 2
percbiomas	Percentage of biomass available

APPENDIX C - STATISTICAL ANALYSIS RESULT TABLE

Biomass	Dependent	RSquare	CV	RootMSE	DepMean	Source	DF	SS	MS	FValue	ProbF
Sorghum	percSclZ1	**0,946659**	**12,38925**	3,406206	27,49324						
						Model	86	19561,449	227,4587	19,6	<.0001
						Error	95	1102,2124	11,6022		
						Corrected Total	181	20663,662			
						LogHour	21	4499,7687	214,2747	18,47	**<.0001**
						Reagent*Temperature	5	9757,171	1951,4342	168,19	**<.0001**
						LogHou*Reagen*Temper	60	1958,7785	32,6463	2,81	**<.0001**
Sorghum	percbluZ2	**0,993836**	**5,2615**	3,62228	68,845						
						Model	86	200980,76	2336,9856	178,11	<.0001
						Error	95	1246,4866	13,1209		
						Corrected Total	181	202227,25			
						LogHour	21	79402,665	3781,0793	288,17	**<.0001**
						Reagent*Temperature	5	28763,441	5752,6881	438,44	**<.0001**
						LogHou*Reagen*Temper	60	34952,165	582,5361	44,4	**<.0001**
Miscant	percSclZ1	**0,994212**	**7,24522**	2,663758	36,76574						
						Model	53	57286,569	1080,8787	152,33	<.0001
						Error	47	333,4935	7,0956		
						Corrected Total	100	57620,062			
						LogHour	12	1662,4025	138,5335	19,52	**<.0001**
						Reagent*Temperature	5	52105,551	10421,11	1468,67	**<.0001**
						LogHou*Reagen*Temper	36	1551,1585	43,0877	6,07	**<.0001**
Miscant	percbluZ2	**0,99785**	**4,88065**	2,660982	54,52101						
						Model	53	147884,97	2790,2824	394,06	<.0001
						Error	45	318,6371	7,0808		
						Corrected Total	98	148203,61			
						LogHour	12	22121,865	1843,4888	260,35	**<.0001**
						Reagent*Temperature	5	90543,323	18108,665	2557,42	**<.0001**
						LogHou*Reagen*Temper	36	18240,557	506,6821	71,56	**<.0001**

ANNEX A – FASGA PROCEDURE

Coloration au FASGA

Ref : MO-MET-HCL-003
Version :2
Date de création : 03/06/2009
Page 1 sur 3

OBJECTIF : Mettre en évidence sur coupes de tissus végétaux des zones cellulosiques et des zones lignifiées.

MATERIEL : matériel frais ou fixé, non inclus ou inclus. Coupes réalisées au microtome ou vibratome.

REACTIFS :

Solution de Safranine à 1%		Bleu Alcian 0,5%		Solution mère FASGA	
Safranine	1 g	Bleu Alcian 8GX	0,5 g	Solution de Safranine	2 ml
Acétate de sodium	1 g	Ethanol 90%	100 ml	Solution de Bleu Alcian	14 ml
Formaldéhyde	2 ml			Acide Acétique	1 ml
Ethanol absolu	75 ml			Glycérine	30 ml
Eau distillée	25 ml			Eau distillée	19,5 ml

- Agiter la solution mère et filtrer
- Diluer la solution au 1/7eme avant utilisation

SECURITE :

Safranine
Toxicité XI
Phrase de risque 36 38
Acétate de sodium
Toxicité ND
Phrase de risque
Formaldéhyde
Toxicité T CMR
Phrase de risque 23 24 25 34 40 43 10
Ethanol absolu
Toxicité F
Phrase de risque 11
Bleu Alcian 8GX
Toxicité ND
Phrase de risque
Glycérine
Toxicité ND
Phrase de risque
Acide Acétique
Toxicité C
Phrase de risque 10 34

Rédacteur	Approbateur Scientifique	Vérificateur AQR
Frédéric Gatineau	Jean-Luc Verdeil	Christine Sanier

PROTOCOLE :

1- Si les coupes ont été conservées dans de l'eau distillée : remplacer l'eau par la solution de Fasga. (sous la hotte aspirante car présence de formaldéhyde)
- Si les coupes ont été stockées dans de l'éthanol 70% : Remplacer l'éthanol par de l'eau distillé par 2 fois, puis par la solution de Fasga (sous hotte).

Si les coupes ont été stockées dans de l'éthanol 70%, il est important de rincer à l'eau distillée afin qu'aucune trace d'éthanol ne subsiste, car le colorant est en phase aqueuse.

2- Colorer les coupes dans la solution de FASGA toute la nuit, à température ambiante, à l'obscurité.
3- Remplacer la solution de Fasga par de l'eau distillée pendant quelques minutes. (une légère décoloration peut survenir)
4- Monter entre lame et lamelle avec une solution glycérine/eau distillée (v / v),
5- Lutter la lamelle à la lame avec du vernis à ongle.
6- Après séchage du vernis, la lame peut être observée et conservée.

Après plusieurs jours de stockage des lames, une coloration du milieu de montage apparait. Cela n'altère pas la qualité de la coloration de la coupe. Il est toutefois possible de remonter la coupe dans un milieu de montage neuf afin d'améliorer la qualité de prise de vue. C'est pourquoi il est important d'observer rapidement (sous 24heures) les lames.

INTERPRETATION :

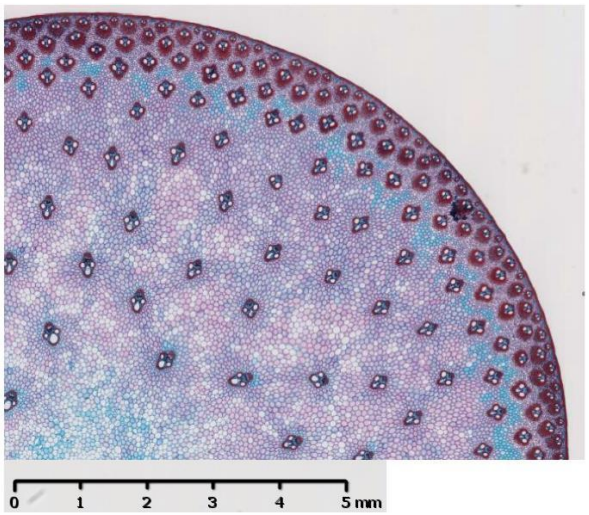
Le Fasga n'est pas un colorant spécifique de la lignine ou de la cellulose. La safranine est un colorant basique du groupe des azines. Le bleu d'Alcian est un colorant cationique qui se fixe sur les macromolécules chargées négativement..
C'est la compétition entre les deux colorants (Safranine et Bleu Alcian) qui donne une coloration:
- plutôt dans les tons rose / rouge pour les parois lignifiées.
- plutôt dans les tons bleus pour les parois cellulosiques.

REFERENCES BIBLIOGRAPHIQUES :

Tolivia, D. and J. Tolivia, 1987, Fasga: a new polychromatic method for simultaneous and differential staining of plant tissues Journal of microscopy (1): 113-117

EXEMPLE D'IMAGES :

Coupe transversale de Sorgho colorée au Fasga.



Rédacteur	Approbateur Scientifique	Vérificateur AQR
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ANNEX B – PHLOROGLUCINOL-HCL PROCEDURE

Ref : MO-MET-HCL-028
Version :1
Date de création : 29/1/14
Page 1 sur 3

Mise en évidence des lignines de type guaiacyle (G) et syringyle (S) par le phloroglucinol-HCl

OBJECTIF : mise en évidence des lignines de type guaiacyle (G) et de type syringyle (S)
MATERIEL : matériel végétal frais ou fixé coupé au vibratome ou à main levée
REACTIFS : Phloroglucinol : Ethanol Acide acétique HCl à 18 % dans H_2O NaOCl en solution commerciale (Sigma) : 6-14 de chlore actif H_20 distillée 100 ml
SECURITE **Phloroglucinol : ND** **Ethanol :** Toxicité: **Facilement inflammable.** Phrases de risques: **11** **Acide acétique :** Toxicité : **10, 35,** Phrases de risques : **1/2, 23, 26, 45,** **HCl :** Toxicité: **Corrosif** Phrases de risques: **23 35** **NaOCl :** Toxicité : **31, 34, 50,** Phrases de risques : **(1/2), 28, 45, 50, 61,**
PROTOCOLE : 1- Préparation : Préparer la solution d'éthanol saturée en phloroglucinol (2 %) en protégeant de la lumière. Ethanol : 100 ml Phloroglucinol : 2 g Préparer une solution d'acide acétique à 5% dans de l'eau distillée. Préparer une solution d'acide chlorhydrique à 18 % dans de l'eau distillée. 2- Blanchiment des tissus : Cette étape est facultative. Tremper l'échantillon dans la solution de NaOCl pendant 5 minutes. Rincer à l'eau distillée. Tremper l'échantillon dans la solution d'acide acétique 5% pendant 10 minutes. Rincer dans l'eau distillée 2 x 5 minutes. Coloration : Tremper l'échantillon dans la solution d'éthanol saturée en phloroglucinol (2%) pendant 5 minutes. Déposer l'échantillon sur une lame de verre.

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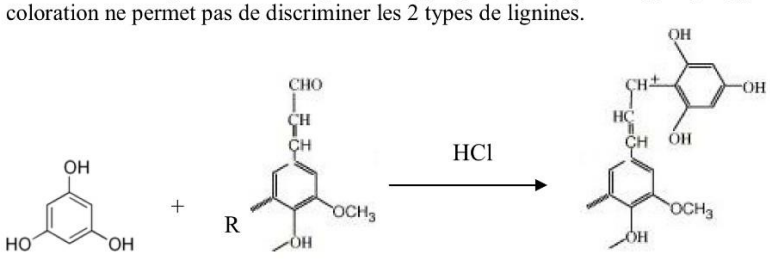
Mise en évidence des lignines de type guaiacyle (G) et syringyle (S) par le phloroglucinol-HCl

Recouvrir l'échantillon avec de la solution d'HCl à 18 %.
Attendre l'apparition de la couleur rouge.
Monter entre lame et lamelle dans un mélange eau/glycérol (50 -50). La coloration rouge est fugace. Observer immédiatement en lumière blanche.

INTERPRETATION :

Coloration en rouge brillant des lignines de type guaiacyle (G) et de type syringyle (S). Cette coloration ne permet pas de discriminer les 2 types de lignines.



phloroglucinol — coloration rouge

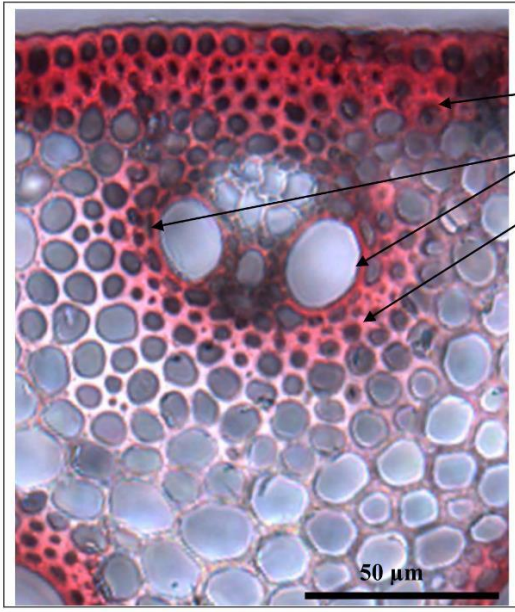
R = H : aldehyde coniferylique à l'origine de la lignine de type G (guaiacyle)

R = OCH3 : aldehyde sinapylique à l'origine de la lignine de type S (siryngyle)

REFERENCES BIBLIOGRAPHIQUES : Pomar, F., Merino, F. et al. (2002). "O-4-Linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction." Protoplasma **220** : 17-28.

EXEMPLE D'IMAGES : Sclérenchyme sous épidermique et faisceau vasculaire de tige de sorgho colorée en rouge par la phloroglucine-HCl traduisant la présence de lignine de type guaiacyle (tissu de conduction) et siryngyle (tissus de soutien).

Mise en évidence des lignines de type guaiacyle (G) et syringyle (S) par le phloroglucinol-HCl



Sclérenchyme sous épidermique

Xylème d'un faisceau vasculaire

sclérenchyme péri fasciculaire

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ANNEX C – CONGO RED PROCEDURE

Mise en évidence de la cellulose par le rouge congo

Ref : MO-MET-HCL-023
Version :1
Date de création : 06/02/14
Page 1 sur 2

OBJECTIF : Mise en évidence de la cellulose

MATERIEL : Coupes vibratome ou main levée de matériel fixé ou frais

REACTIFS :
Rouge congo

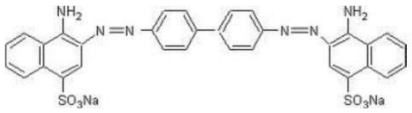
SECURITE
Rouge congo :
Toxicité : **T, CMR**
Phrase de risque : **45, 63, 11**

PROTOCOLE :

1 - Préparation de l'échantillon. Les coupes sont faites soit :
- au vibratome épaisseur 80 µm à 150 µm
- à main levée.

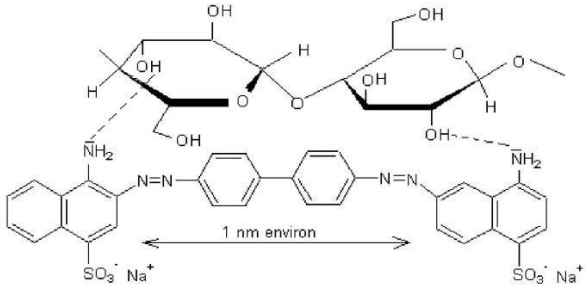
2 – Coloration
Placer l'échantillon dans la solution de rouge congo à 0.1 % 5 minutes.
Rincer à l'eau distillée.
Monter entre lame et lamelle dans un mélange eau/glycérol (v/v).
Observation en lumière blanche

INTERPRETATION :



Molécule de rouge congo

Forte interaction avec les polysaccharides présentant des liaisons β-(1-4) colorés en rouge.
La distance des groupes -N=N- dans les bis-azoïques à groupes immédiatement voisins est de 1,08 nm. Le motif correspondant au cellobiose dans la cellulose mesure 1,03 nm.
Ainsi le rouge congo teint parfaitement la cellulose car sa molécule s'adapte bien au support comme le montre le schéma ci-dessous où apparaissent les liaisons hydrogène entre le rouge congo et la cellulose.



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Mise en évidence de la cellulose par le rouge congo

Ref : MO-MET-HCL-023
Version :1
Date de création : 06/02/14
Page 2 sur 2

Liaisons hydrogène entre la molécule de rouge congo et la cellulose.

REFERENCES BIBLIOGRAPHIQUES :

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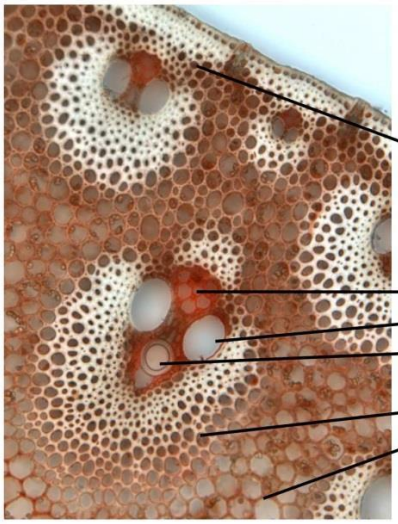
EXEMPLE D'IMAGES :



Détection de la cellulose sur une coupe transversale de tige de sorgho par le rouge congo.

Sclérenchyme sous épidermique

Faisceau vasculaire :
Phloeme
Métaxylème
Protoxylème

Sclérenchyme périfasciculaire

parenchyme

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