Pretreatment of Hemp Fibers to Enhance Enzymatic Accessibility for Hemp Fibers

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Preface

The current thesis is submitted as a partial fulfilment of the requirements for obtaining the Master of Science degree in Biological Engineering at Technical University of Lisbon (IST), Portugal. The studies were conducted from the 16th of February 2015 to the 16th of July 2015, at the Center of Bioprocess Engineering (BioEng), Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), Denmark.

The carried out investigation was part of the plan of studies of PhD student Ming Liu and it is integrated in CelfiMat project, with the aim of using high quality cellulosic fibers as reinforcements in the production of strong biocomposite materials. The project counts with the partnership of the Departments of Wind Energy, Chemistry and Chemical Engineering of the Technical University of Denmark, the Department of Forest Products of the Swedish Agricultural University, the German company Bafa Neu GmbH and the French company Planète Chanvre.

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____________________________________________________

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Abstract

Hemp (Cannabis sativa) fibers have been considered as a sustainable biomaterial to replace man-made fibers in composite applications, due to their low cost and density, good mechanical properties and biodegradability. However, for high-grade composites, the cementing materials from middle lamella (ML) regions must be degraded to obtain individual fibers or small fiber bundles, in order to create a strong interface and reduce void space between fibers (or fiber and matrix). In this context, an enzymatic treatment is one of the most promising and ecological methods, but the process is not efficient because enzymes are too large to penetrate the well lignified ML region, and thus requires an auxiliary pretreatment.

Among the conducted experiments, a fungal pretreatment at controlled conditions for half a week, a hydrothermal pretreatment in an autoclave at 1 bar (121 °C) for 30 min and a chemical pretreatment with NaOH at 60°C for 4 h were shown to be the best solutions, by allowing partial degradation of pectin (decrease of 55, 41 and 67% in galacturonic acid content, respectively) and subsequently enhancing the accessibility of pectinases for said substrate, indicated by the final low content of GalA in final treated fibers. The direct combination of EDTA-2Na (0.5%) with endopolygalacturonase was also demonstrated as a time saving option.

However, significant negative effects (p<0.05) in the mechanical performance of pretreated hemp fibers were noted, except for hydrothermally pretreated, for which only strain significantly (p<0.05) decreased by 40% and no apparent effects on stiffness and Ultimate Tensile Strength were recorded.

Keywords: Hemp fibers; Enzyme; Hydrothermal pretreatment; Biological pretreatment; Chemical Pretreatment; Mechanical Properties
Resumo

As fibras de cânhamo (*Cannabis sativa*) têm sido reconhecidas como uma alternativa sustentável às fibras sintéticas para aplicações com materiais compósitos, devido ao seu baixo custo e densidade, boas propriedades mecânicas e biodegradabilidade. No entanto, para fabricação de compósitos de alta qualidade, os materiais de cimentação da lamela média (LM) devem ser degradados a fim de se obter fibras individuais ou pequenos feixes de fibras, criando uma interface forte e reduzindo o espaço vazio entre as fibras (ou entre fibras e matriz). Neste contexto, um tratamento enzimático é um dos métodos mais promissores e ecológicos, mas o processo não é eficiente porque as enzimas são demasiado grandes para penetrar a bem lignificada LM, e, portanto, exige-se um pré-tratamento auxiliar.

Entre as experiências realizadas, um pré-tratamento fúngico em condições controladas durante meia semana, um pré-tratamento hidrotérmico numa autoclave a 1 bar (121 °C) durante 30 min e um pré-tratamento químico com NaOH a 60 °C durante 4 h, mostraram ser as melhores soluções, permitindo a degradação parcial de pectina (diminuição de 55, 41 e 67% do teor de ácido galacturônico, respectivamente) e aumentando posteriormente a acessibilidade de pectinases para o referido substrato, resultando num baixo teor de ácido galacturônico nas fibras finais. A combinação direta de EDTA-2Na (0,5%) com endopoligalacturonase demonstrou ser também uma alternativa rápida.

No entanto, foram registados efeitos negativos significativos (p<0.05) no desempenho mecânico das fibras de cânhamo pré-tratadas, excepto quando pré-tratadas hidrotermicamente, para as quais apenas a deformação diminui significativamente (p< 0,05) em 40% e não foram registados efeitos aparentes sobre a rigidez e limite de resistência à tração.

**Palavras-chave:** Fibras de Cânhamo; Enzimas; Pretratamento Hidrotérmico; Pretratamento Biológico; Pretratamento Químico; Propriedades mecânicas.
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ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA – Analysis of Variance
Ara – Arabinose
BSA – Bovine serum albumin
EDTA – Ethylenediaminetetraacetic acid
Endo-PG – Endopolygalacturonase
EU – European Union
$f_{\text{anhydrous}}$ – Anhydrous correction factor for monosaccharides in HPLC
GalA – Galacturonic acid
Gal – Galactose
HG – Homogalacturonan
HM-pectin – High Methylated Pectin
HPAEC-PAD – High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
LM-pectin – Low methylated pectin
Man – Mannose
MW – Molecular weight
NFCs – Natural Fiber Composites
PAHBAH – 4-hydroxybenzoic acid hydrazide
PL – Pectin lyase
RS – Reducing Sugars
RGI – Rhamnogalacturonan I
RGII – Rhamnogalacturonan II
Rha – Rhamnose
SAH – Strong Acid Hydrolysis
SRS – Sugars Recovery Standards
UTS – Ultimate Tensile Strength
THC – 9-∆ tetrahydrocannabinol
Xyl – Xylose
YM – Young’s Modulus
1. Introduction

Sustainability, recycling, reduction, reuse, recovery, renewable.

These might be the most important words when it comes to define the 21st century. Their echo has been spreading across modern society, from economics to politics, which increasingly have been joining efforts to encourage a paradigm shift in the way man inhabits this unique planet. Throughout the world the developed countries have been implementing directives and goals, with regard to the reduction of the anthropological impact on the environment, and to the use of natural resources. The emergent investment in biofuels and the use of biomaterials to the detriment of synthetic materials are just two examples of the rising combat against greenhouse gases emissions and climate changes, mainly associated to the extensive use of fossil fuels.

In this context, natural fibers are becoming popular worldwide as an alternative to the broadly used synthetic fibers. These versatile, natural materials provide several sustainable solutions in a wide range of industries, from textiles and consumer products to the automotive and construction industries.

1.1 Natural fibers

There are two types of fibers: man-made fibers and natural fibers. The first are usually called synthetic fibers and, among others, includes glass, carbon, graphite, boron and aramid fibers, while natural fibers come directly from the nature.

Natural fibers are substances produced by plants and animals that can be spun into filaments, threads or ropes, which subsequently can give rise to woven, knitted, matted or bound. According to its source, they can be divided into three groups: animal fibers (protein fibers) as wool or silk; vegetable fibers (cellulosic fibers) obtained from different parts of several plants and mineral fibers (asbestos), as schematized in Figure 1.1.

Natural fibers have been used since the dawn of civilization. Their first sign dates back to the Prehistory, when the human being started to utilize fibers of contemporary species to produce clothes, cloth, cordage or even buildings, assuming a key role in human species survival. Since then, the use of natural fibers has been spread through all ages and civilizations, where flax, cotton, hemp, wool and silk are prominent.

In recent times, natural fibers have been used predominantly for textile applications. However, in the end of the 19th century, the first synthetic fiber was created and the world witnessed the expansion of the use of man-made fibers, which allowed the beginning of a new era for textile industry. In addition, man-made fibers like carbon and glass fibers, spread to other applications such as automotive and construction
industries, dominating the market in detriment of natural fibers. Nowadays, due to current concerns related with depletion of fossil resources and increase of oil prices and greenhouse gas emissions, the World has been encouraged to develop new biodegradable materials produced from renewable resources. Therefore natural fibers returned to the limelight.

In a study of natural fiber economics for the organization Discover Natural Fibers Initiative (DNFI), Townsend T. and Sette J. reported that in 2013, the world production of natural fibers was estimated at 33 million tons, from which 26 million tons are related to cotton, mainly due to its use in textile industry. However natural fiber production only counted for ca. 30% of the world’s production of fibers, reflecting the influence of man-made fibers in our daily lives. Figure 1.2 summarizes some data collected by the same organization in the previous year (2012), provided by different natural fiber organizations and by Food and Agriculture Organization (FAO) of the United Nations.

![Figure 1.1 – Different sources of natural fibers [1].](image)

**Figure 1.1** – Different sources of natural fibers [1].

![Figure 1.2 – World fiber production (in %) of man-made and natural fibers (left) and main produced natural fibers (right), in 2012. source: www.dnfi.org](image)

**Figure 1.2** – World fiber production (in %) of man-made and natural fibers (left) and main produced natural fibers (right), in 2012. source: www.dnfi.org
Scientific and technological development allowed to find more than 1000 cellulosic species, from which fibers might be extracted and used in several applications. Some of those natural fibers are currently under investigation throughout the world. Among them are hemp bast fibers, a class of cellulose-rich fibers with high potential of being used as reinforcements in composite materials, due to its low cost, low weight, high strength and stiffness, eco-friendliness and bio-degradability.

1.1.1 Hemp fibers

Hemp is commonly known by its scientific name, *Cannabis sativa*, and is traditionally cultivated for its long and strong bast fibers and seeds. Even so, the term “hemp” is used to describe the plant itself, its components and any products extracted and manufactured from the plant [2].

Hemp is one of the world’s oldest cultivated annual crops, sown in the spring and harvested in autumn, and capable of reaching 5 m of height (Figure 1.3). For centuries it has been grown mainly to produce ropes, cordage, fabrics, paper and sails from its fibers. As a matter of fact, the debris originated by those industries have been function as archeological evidence of hemp production and led to the hypothesis of the plant being native to central Asia and cultivated for more than 12000 years [3], [4]. Nevertheless, the plant has been grown in a wide variety of geographic zones, since it can adapt to most regions and climates throughout the world [5].

![Figure 1.3 – Hemp crop (left) and a close look of its stalks and leaves (right). source: www.hempfarm.co.nz](image)

However, hemp’s cultivation suffered a drawback in most western countries, when it was forbidden for decades due to several reasons, including direct competition with other feedstock’s (e.g. cotton) and synthetic fibers, high labor costs and the use of its leaves as narcotic. For the latter, it has to be highlighted that, industrial hemp has practically no 9-Δ tetrahydrocannabinol (THC), the narcotic resin extracted from the leaves. In fact, this was one of the arguments presented by the campaigners which allowed the overturn of the ban of hemp cultivation, now considered a legitimate crop [3]. Currently the
European Union together with China and Canada covers nearly two-thirds of worldwide production of hemp [5] and almost 50% of the world’s industrial hemp is supplied by China [2].

1.1.1.1 Hemp cultivars

Hemp plant and its fibers quality are highly dependent on several factors, including growing conditions (such as day length, temperature and types of climate and soil) and the kind of cultivar grown.

In Europe, modern breeding programmes have been established to develop hemp cultivars for cultivation, according to the end use (fiber hemp or seed hemp) and ensuring a low level of THC. These cultivars are classically developed for specific environments, seasons and crop conditions. In relation to hemp fiber cultivars, the breeding programmes were able to increase the fiber content of the plant from 12-15% to 25-33% [2]. A list of cultivars approved by the EU for cultivation, until 2013, is shown in Table 1.1.

<table>
<thead>
<tr>
<th>Armanca (RO)</th>
<th>Asso (IT)</th>
<th>Beniko (PL, NL)</th>
<th>Bialobrzeskie (AT, CZ, PL)</th>
<th>Cannakomp (HU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carma (IT)</td>
<td>Carmagnola (IT)</td>
<td>Chamaleon (NL)</td>
<td>Codimono (IT)</td>
<td>CS (IT)</td>
</tr>
<tr>
<td>Delta-405 (ES)</td>
<td>Delta-liosa (ES)</td>
<td>Denise (RO)</td>
<td>Diana (RO)</td>
<td></td>
</tr>
<tr>
<td>Dioica 88 (FR)</td>
<td>Epsilon 68 (FR)</td>
<td>Fedora 17 (FR, CH)</td>
<td>Felina 32 (FR)</td>
<td>Ferimon (FR, DE)</td>
</tr>
<tr>
<td>Fibranova (IT)</td>
<td>Fibrimor (IT)</td>
<td>Fibrol (HU)</td>
<td>Finola (FI)</td>
<td>Futura 75 (FR)</td>
</tr>
<tr>
<td>Ivory (NL)</td>
<td>KC Dora (HU)</td>
<td>KC Virtus (HU)</td>
<td>KC Zuzana (HU)</td>
<td>Kompolti (HU)</td>
</tr>
<tr>
<td>Kompolti hibrid TC (HU)</td>
<td>Lipko (HU)</td>
<td>Lovrin 110 (RO)</td>
<td>Marcello (NL)</td>
<td>Markant (NL)</td>
</tr>
<tr>
<td>Monoica (HU, CZ)</td>
<td>Santhica 23 (FR)</td>
<td>Santhica 27 (FR)</td>
<td>Santhica 70 (FR)</td>
<td>Secuieni Jubileu (RO)</td>
</tr>
<tr>
<td>Silvana (RO)</td>
<td>Szarvasi (HU)</td>
<td>Tiborszállási (HU, IT)</td>
<td>Tisza (HU)</td>
<td>Tygra (PL)</td>
</tr>
<tr>
<td>Uniko B (HU, CH)</td>
<td>Uso-31 (NL, CH)</td>
<td>Wielkopolskie (PL)</td>
<td>Wojko (PL)</td>
<td>Zenit (RO)</td>
</tr>
</tbody>
</table>

1.1.1.2 Hemp fibers quality issues

The major drawbacks of using natural fibers are related with their quality issues, namely variations in fiber quality and processing limitations. Among the factors affecting the quality of hemp fibers are genotype, sex, harvesting time, stem part, length and diameter. In addition, geographic location together with soil quality, climate and weather conditions, also affect hemp fibers growth and its final properties [3], [4].

After being harvested, there are also other sources of variability for the end-use fibers, including extraction methods, possible damage during handling and processing, differences in drying processes and further treatments, to which the fibers may have to be submitted [1]. Therefore, variations in the previously mentioned parameters will ultimately contribute to unwanted variations in morphology, structure and chemical composition of the fibers, affecting its final physical and mechanical properties [4].
1.1.1.3 Physical and mechanical properties of hemp fibers

Hemp fibers can be characterized as regards to key mechanical and physical properties, especially in order to be used as reinforcements in composite applications.

The mechanical properties of fiber reinforcements and/or composites are usually measured in terms of tensile strength, elastic modulus (or Young's Modulus) and strain. Tensile strength represents the maximum load that a material can withstand while being stretched, before breaking. Strain is a measure of deformation, in this case, the elongation of the fibers before breaking apart. The elastic modulus is a measure of stiffness (the higher the Young's Modulus the stiffer the material), representing the resistance of a material to deformation, when a force is applied to it. This parameter can be obtained in a typical stress-strain curve. The three properties are related with each other according to Hooke’s Law.

Regarding the physical properties, fiber reinforcements are usually compared in terms of density, diameter, water retention and water sorption, orientation and adhesion properties. The microfibril angle can be used as a measure of orientation, since it refers to the angle between the direction of the helical windings of cellulose microfibrils in the secondary cell wall of fibers and the long axis of the cell [6]. Atomic Force Microscopy or Scanning Electron Microscopy can be used to characterize the interfacial adhesion of fiber-matrix and ultimately the morphology of the fibers [7].

1.1.1.4 Hemp fibers vs other fibers

Different natural fibers present remarkable variations in chemical composition and physical properties, making them more or less suitable for a certain application. Ahmad et al. (2015) [1] recently compared the chemical composition and physical and mechanical properties of several natural fibers, by collecting data from different studies and sources, which are presented below in Tables 1.2 and 1.3.

To be used as reinforcements for composite materials, the mechanical properties of the fibers are crucial, and can be correlated with the glucose content. Hemp fibers are known as one of the strongest and stiffest available natural fibers (see Table 1.3), thus having a high potential for biocomposite manufacturing [4]. Flax, jute, ramie and kenaf also show good mechanical properties and have been investigated for the same application.

Hemp and flax fibers have similar properties and are difficult to distinguish. However, hemp has several advantages in comparison to flax and other natural fibers. In hemp’s breeding, the plant flourishes without the use of herbicides or pesticides. The plants are sown very close to each other to increase the yield of production, and therefore they grow tightly bunched, which together with its dense foliage prevents the growth of weeds and other plants. In addition, hemp growing does not require much chemical fertilizers and, consequently, enriches the soils for succeeding crops and helps them to retain moisture. Hemp has also a deep root system, thus it requires very little or no irrigation.
Table 1.2 – Chemical composition of main natural fibers [1].

<table>
<thead>
<tr>
<th>Natural Fiber</th>
<th>Cellulose (%)</th>
<th>Lignin (%)</th>
<th>Hemicellulose (%)</th>
<th>Pectin (%)</th>
<th>Wax (%)</th>
<th>Moisture (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abaca</td>
<td>56-63</td>
<td>7-9</td>
<td>20-25</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bamboo</td>
<td>26-43</td>
<td>1-31</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>9.16</td>
<td>-</td>
</tr>
<tr>
<td>Banana</td>
<td>83</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.71</td>
<td>-</td>
</tr>
<tr>
<td>Coir</td>
<td>37</td>
<td>42</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.36</td>
<td>-</td>
</tr>
<tr>
<td>Cotton</td>
<td>82.7-91</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>0.6</td>
<td>7.85-8.5</td>
<td>-</td>
</tr>
<tr>
<td>Curauá</td>
<td>73.6</td>
<td>7.5</td>
<td>9.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flax</td>
<td>64.1-71.9</td>
<td>2-2.2</td>
<td>-</td>
<td>1.8-2.3</td>
<td>1.7</td>
<td>0.8-1.2</td>
<td>-</td>
</tr>
<tr>
<td>Hemp</td>
<td>70.2-74.4</td>
<td>3.7-5.7</td>
<td>17.9-22.4</td>
<td>0.9</td>
<td>0.8</td>
<td>1.2-6.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Jute</td>
<td>61-71.5</td>
<td>12-13</td>
<td>17.9-22.4</td>
<td>0.2</td>
<td>0.5</td>
<td>12.5-13.7</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Kenaf</td>
<td>45-57</td>
<td>21.5</td>
<td>8-13</td>
<td>0.6</td>
<td>0.8</td>
<td>6.2-12</td>
<td>2-5</td>
</tr>
<tr>
<td>Ramie</td>
<td>68.6-91</td>
<td>0.4-0.7</td>
<td>5-14.7</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sisal</td>
<td>78</td>
<td>8</td>
<td>10</td>
<td>-</td>
<td>2</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Sea grass</td>
<td>57</td>
<td>5</td>
<td>38</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.3 – Physical and mechanical properties of main natural and synthetic fibers (*) [1].

<table>
<thead>
<tr>
<th>Fiber</th>
<th>Density (g/cm³)</th>
<th>Diameter (µm)</th>
<th>Tensile Strength (MPa)</th>
<th>Young Modulus (GPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abaca</td>
<td>1.5</td>
<td>-</td>
<td>400</td>
<td>12</td>
<td>3-10</td>
</tr>
<tr>
<td>Bamboo</td>
<td>1.1</td>
<td>240-330</td>
<td>500</td>
<td>35.91</td>
<td>1.40</td>
</tr>
<tr>
<td>Banana</td>
<td>1.35</td>
<td>50-250</td>
<td>600</td>
<td>17.85</td>
<td>3.36</td>
</tr>
<tr>
<td>Coir</td>
<td>1.2</td>
<td>-</td>
<td>175</td>
<td>4-6</td>
<td>30</td>
</tr>
<tr>
<td>Cotton</td>
<td>1.6</td>
<td>-</td>
<td>287-597</td>
<td>5.5-12.6</td>
<td>7-8</td>
</tr>
<tr>
<td>Curauá</td>
<td>1.4</td>
<td>170</td>
<td>158-729</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Flax</td>
<td>1.5</td>
<td>-</td>
<td>800-1500</td>
<td>27.8-80</td>
<td>1.2-3.2</td>
</tr>
<tr>
<td>Hemp</td>
<td>1.48</td>
<td>-</td>
<td>550-900</td>
<td>70</td>
<td>2-4</td>
</tr>
<tr>
<td>Jute</td>
<td>1.46</td>
<td>40-350</td>
<td>393-800</td>
<td>10-30</td>
<td>1.5-1.8</td>
</tr>
<tr>
<td>Kenaf</td>
<td>1.45</td>
<td>70-250</td>
<td>930</td>
<td>53</td>
<td>1.6</td>
</tr>
<tr>
<td>Ramie</td>
<td>1.5</td>
<td>50</td>
<td>220-938</td>
<td>44-128</td>
<td>2-3.8</td>
</tr>
<tr>
<td>Sisal</td>
<td>1.45</td>
<td>50-300</td>
<td>530-640</td>
<td>9.4-22</td>
<td>3-7</td>
</tr>
<tr>
<td>E-glass*</td>
<td>2.55</td>
<td>&lt;17</td>
<td>3400</td>
<td>73</td>
<td>3.4</td>
</tr>
<tr>
<td>S-glass*</td>
<td>2.5</td>
<td>-</td>
<td>4580</td>
<td>85</td>
<td>4.6</td>
</tr>
<tr>
<td>Aramid*</td>
<td>1.44</td>
<td>11.9</td>
<td>3000</td>
<td>124</td>
<td>2.5</td>
</tr>
<tr>
<td>HS Carbon*</td>
<td>1.82</td>
<td>8.2</td>
<td>2550</td>
<td>200</td>
<td>1.3</td>
</tr>
</tbody>
</table>

According to all of these factors, hemp is seen as a very economical and ecological crop. The fact that the plant does not require too much care until be harvested, represents a great advantage of hemp fibers production in relation to other competitors.

Hemp fibers have rounded ends, making them more suitable for textiles, without the itchiness of ramie. Also for textile industries, hemp fibers have an outstanding durability and are much finer than flax and...
ramie. Therefore, textiles with higher contents of hemp fibers are softer and more comfortable. Finally, in comparison to cotton hemp fibers are two times stronger.

In comparison to synthetic fibers, the data presented in Table 1.3 shows that glass fibers have better physical and mechanical properties than hemp fibers, especially in terms of tensile strength. However, considering the specific modulus (ratio between the Young’s modulus and fiber density), hemp has a higher value: 47.3 GPa against only 34 GPa and 28.63 GPa for S-glass and E-glass fibers, respectively. Therefore, due to its low density, some mechanical properties of hemp fibers are comparable to the widely used man-made glass fibers, making them a promising ecofriendly alternative in composite manufacturing.

1.2 Hemp fibers structure

Hemp fibers are a multi-celled structure bast fiber plants [4], which are organized in bundles as long as the stems and help to hold the plant erect. The fibers are present in the cortex of hemp stems and can be easily peeled off the xylem surface, either by hand or by using proper machines.

The hemp stem has a cylindrical shape, within which there is an empty space (in dried stems) called pith, and it is composed of a wood core (xylem) surrounded by an outer layer of bark. In turn, bark consists of three distinguishable layers: cambium, cortex and epidermis. Figure 1.4 gives a schematic diagram of a transverse section of the hemp stem, showing its different layers and the organization and location of single fibers.

![Schematic diagram of a transverse section of hemp stem showing the organization and morphology of a bast strip and single fiber (e.g. primary and secondary fibers) in the bast layer [8].](image)

Each layer of hemp stems has its own importance: xylem, the thickest layer (1-5 mm) of the structures, provides stiffness to the stem; cambium plays as a physical barrier, separating wooden and bast fiber layers; bast fibers offer tensile and flexural strength and epidermis protects the plant from parasites.
The cortex (100-300 µm of thickness) is composed by bundles of primary and secondary single fibers, which are individualized in two sublayers (see Figure 1.4). Primary fibers are nearby the stem surface and are larger (cell wall thickness of 7-13 µm and length of 20 mm) while secondary fibers are closer to the cambium layer and are smaller (cell wall thickness of 3-6 µm and length of 2 mm) [9].

The primary fiber cell wall itself is divided in two different layers (Figures 1.4 and 1.5): the primary cell wall and the secondary cell wall (known as L layer). The latter is further divided in another 3 sublayers of cellulose fibrils that are bound by lignin, designated by L1, L2 and L3 [7]. All of the mentioned layers are made of lignocellulosic materials, although in different proportions. The Layer L2 is the thickest layer, containing around 50% of the fiber’s cellulose content and thus it has the biggest contribution for fiber properties [4].

![Figure 1.5 – 3-Dimensional view of the structure of a single fiber and its different layers [7].](image)

The interstitial space that separates consecutive single fibers, is an adhesive extracellular layer called middle lamella, which is rich in pectin and lignin. A series of single fibers kept together by middle lamella is known as fiber bundle.

### 1.3 Chemical composition of hemp fibers

The composition of hemp fibers varies between cultivars and within different parts of the stem. Additionally, the area of production and its environmental conditions, and also the stage of maturity of the plant [8], influence its chemical composition. Even so, the magnitude of the values is quite similar, where lignocellulosic materials (cellulose, hemicellulose and lignin) comprise 80-90% of their dry weight. The other components include pectin, waxes and other extractives, minerals and water-soluble components.

In the next sections, the main components of hemp fibers will be described in detail.
1.3.1 Cellulose

Cellulose is a homogeneous linear polymer of glucan, i.e. D-glucose repeating units linked by glycosidic bonds (two adjacent glucose units are linked by elimination of one water molecule, between their hydroxyl groups at carbon atoms 1 and 4), as illustrated in Figure 1.6. Cellulose is the most abundant and largest organic polymer on Earth.

![Figure 1.6 - Parallel chains of cellulose, a polymer of glucose monomers](image)

The glucose monomers in cellulose form hydrogen bonds both within its own chain (intramolecular) forming fibrils, and with neighboring chains (intermolecular), forming microfibrils [9]. These extensive hydrogen bounds give cellulose a linear crystalline structure with high tensile strength in axial direction [11]. Therefore, cellulose acts as reinforcement material in the cell wall, providing strength to the plant.

The microfibrils contain ca. 40 cellulose chains and are composed of crystalline segments alternating with regions of amorphous cellulose (Figure 1.7). A common technique to determine cellulose crystallinity is X-Ray Diffraction (XRD), where diffraction peaks of cellulose crystals can be separated from diffraction peaks of amorphous cellulose, hemicellulose, lignin, pectin and minerals. Both crystallite length and diameter can also be determined based on the width of the peaks [12].

![Figure 1.7 - Detailed chemical structure of a microfibril with alternating crystalline and amorphous regions](image)
Due to its highly crystalline polysaccharide structure, cellulose is resistant to enzymatic hydrolysis and only the strongest acids and alkaline agents can act on crystalline cellulose. A good way to indirectly estimate the cellulose content in natural fibers is to determine the amount (% w/w) of glucose by Strong Acid Hydrolysis [14] followed by HPLC for monosaccharide determination [8], [15].

Cellulose is also characterized by its degree of polymerization, i.e. the number of glucose units in the chain. For hemp fibers, a value of 7000 was estimated by Thygesen A. (2006) [9].

1.3.2 Hemicellulose

Hemicellulose is a fully amorphous polysaccharide with a linear backbone linked by β-1-4 glycosidic bonds (Figure 1.8). When compared to cellulose, which is only composed by anhydrous glucose, hemicellulose is a shorter polymer (i.e. lower degree of polymerization) and it has a heterogeneous and heavily branched structure, consisting of different pentose sugars such as xylose and arabinose or even hexose sugars as mannose [11]. Another common substituents in hemicellulose structure are acetyl groups and sugar acids as glucuronic and galacturonic acid [9].

![Figure 1.8 – Example of a hemicellulose polymer branched (L-arabino-D-xylan) structure [16].](image)

Hemicellulose structure and composition vary extensively between different species and cell types [17]. In natural fibers, the most representative sugars are D-xylopyranose, L-arabinofuranose, D-mannopyranose, D-glucopyranose and D-galactopyranose, where xylose is the monosaccharide present in largest amount. Hemicellulose family includes xyloglucans, xylans, mannans and glucomannans, and β-glucans.

The main biological function of hemicelluloses is its contribution to strengthening the cell wall [17]. Hemicellulose’s random distribution throughout the fibers’ primary cell wall allows the formation of covalent bonds with lignin and hydrogen bonds and van der Walls forces with cellulose, creating a highly cohesive structure. Hence, by occupying the cavities between cellulose microfibrils, hemicellulose acts as a link between cellulose and lignin, permitting the effective transfer of shear stress between them.

Hemicellulose amorphous nature exposes its hydroxyl groups, mainly from acid residues substituents, making hemicellulose the major contributor to the hydrophilicity of plant fibers [11]. This characteristic makes it partially soluble in water, alkaline solutions and other solvents.

The presence of hemicellulose in hemp fibers structure is undesired for several applications. For instance, most of the polymer matrixes that interact with hemp fibers, when they are used as reinforcements in
composite materials, are non-polar. Therefore, the hidrophilicity of hemicellulose raises a problem of interaction between fibers and matrix, introducing a need of a pretreatment of hemp fibers to partially remove hemicellulose, as will be discussed in section 1.5.

1.3.3 Lignin

Lignin is an extremely complex three-dimensional polymer, consisting of aliphatic and aromatic components (Figure 1.9). About its location, lignin is distributed throughout the primary and secondary cell walls, and the highest concentration is found in the middle lamella. Thus, together with hemicellulose, lignin forms a matrix sheath around the cellulose microfibrils and fibers, resulting in a stiff structure with high compression strength.

![Figure 1.9 – Structure of lignin and its precursors H-, G-, and S-units [18].](image)

After cellulose, lignin is the most abundant organic polymer on Earth, and it is present in every vascular plant. Besides of being crucial for the plant structure, lignin plays an important safety role, by protecting the plant against pathogenic attacks and consumption by herbivores. The latter function is due to lignin’s phenolic components [11].

As hemicellulose, lignin is totally amorphous. However its physical and chemical structure and also the possible interactions of lignin with other compounds are still a challenge for scientific community, mainly due to its complexity. Of what is known, lignin’s three-dimensional structure is formed by radical coupling polymerization of three monolignol precursors: p-coumaryl, coniferyl, and sinapyl alcohols [19], whose structures were represented in Figure 1.9. These precursors give rise to the so-called p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units, which appear in different abundances for
different groups of vascular plants, as well as for different plant tissues and cell-wall layers [20]. The aromatic building units are linked with a variety of ether and carbon-carbon bonds. The most abundant one is known as β-O-4 ether linkage, consisting of approximately 40-60% of lignin linkages (see Figure 1.9).

Lignin is less hydrophilic than hemicellulose and cellulose, which combined with its complex structure, makes its dissolution/degradation difficult to fulfill. In the pulp and paper industries, delignification is commonly achieved by using sodium chlorite (Kraft process). Several fungi have also been identified to produce lignin hydrolytic enzymes as laccase and peroxidases.

### 1.3.4 Pectin

Pectin is a family of complex linear polysaccharides which is responsible for the structural integrity and cohesion of higher plant tissues [21]. It is also involved in other numerous functions as plant growth, development, morphogenesis, defense, signaling, cell expansion, etc. [22].

Pectic substrates are most abundant in the primary cell wall and middle lamella region. Their main component (ca. 70%) is the sugar D-galacturonic acid (GalA), an oxidized form of D-galactose that has an aldehyde group at C1 and a carboxylic acid group at C6.

#### 1.3.4.1 Pectic substrates

Within the pectin family, different polysaccharides have been identified and characterized. The most representative are homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), whose structures are represented in Figure 1.10.

HG is a linear homopolymer of α-1,4-linked galacturonic acid, also known as pectin's smooth region. It is the simplest and most abundant polysaccharide in pectin's family and it can be acetylated and/or methyl esterified [15], [22]. Additionally, RGII and other less abundant pectin polysaccharides, such as xylogalacturonan (XGA) and apiogalacturonan (AP), are substituted HGs, meaning that they have a HG backbone with branched structures.

RGI represents 20 to 35% of pectic substrates and is composed of repeating diglycosyl rhamnose-galacturonic acid residues. These polysaccharides are associated to a high degree of variability, since different types and number of sugars and oligosaccharides can be attached to its backbone [22]. Among them, neutral sugars as galactose, arabinose and xylose are very common [21].

RGII represents 10% of pectic substrates and it is highly conserved among plant species. As the most structurally complex pectin, RGII consists of a polygalacturonic acid backbone with 4 long side chains (named from A to D), containing 12 different types of sugars in over 20 different linkages [22]. RG polysaccharides are commonly known as pectin's hairy region.
Figure 1.10 – Main pectic substrates and its composition: Homogalacturonan (HG), Rhamnogalacturonan I (RG-I), Rhamnogalacturonan II (RG-II), Xylogalacturonan (XG) and Arabinogalactan (AG) [23].

The way pectic substrates are linked to each other or to other polymers stills not entirely understood. However, due to the fact that pectin requires harsh chemical or enzymatic treatments to be digested and isolate HG, RG-I and RG-II, the most accepted idea assumes that pectic substrates are covalently cross-linked. There are also evidences that pectins may be covalently linked, or tightly associated with other types of wall polysaccharides including xyloglucans and xylans [22].

1.3.4.2 Egg-box model and gelation properties

The gelation mechanism in pectic substrates is entirely dependent on the degree of esterification (DE), which in turn determines the charge of pectin chain, given by the number of carboxylate anions (unmethylated C6 of GalA units). The lower the DE, the higher the charge density of the pectin chain.

In the presence of divalent salts such as calcium, and if more than 10 unmethyl-esterified GalA residues are coordinated, adjacent low methoxyl (LM) pectin polymers may cross-link through electrostatic and ionic bonding of carboxyl groups (calcium bridges), forming a stable gel [24], [25]. The most accepted mechanism of gel formation in LM-pectin is known as “egg-box” (Figure 1.11), where two pectic chains in a twofold helical conformation retain calcium ions between them, like eggs in an egg-box.

On the other hand, for high methoxyl (HM) pectins, there are not enough acidic groups to form a gel with calcium ions. In this case gel formation is caused by hydrogen bonding between pectin’s free carboxyl groups and the hydroxyl groups of neighboring molecules [22], in the presence of acid and sugars. When the pH value is higher than pKa, unesterified carboxyl groups of pectin exhibit a negative charge, which together with hydroxyl groups attract water molecules. The negative charge can be sufficiently strong to
prevent the formation of a pectin network. By adding acid (lowering the pH), part of the carboxyl ions are neutralized, decreasing the number of negative charges and leading to lower attraction between pectin and water molecules. Sugars are also used to compete with water molecules. These conditions allow hydrogen bonds and hydrophobic interactions to bind the individual HM-pectin chains together forming the well-known low water activity gels [25].

1.3.4.3 Pectin degradation

In hemp fibers, pectin degradation can provide separation of the fiber bundles from the xylem surface, while separation of the fiber bundles into single fibers requires both lignin and pectin degradation [9]. Pectin depolymerization can occur by chemical treatments with dilute acids or chelating agents [26] or by submitting the fibers to high pressures and/or temperatures [27], while water soluble pectin components can suffer spontaneous deesterification and depolymerization, depending on both pH and temperature.

Several enzymes have also been described to degrade specific linkages in pectin structure, causing depolymerization or deesterification. Those enzymes are widely known as pectinases and most of them cause HG degradation. For instance, endopolygalacturonase (Endo-PG) acts randomly on polygalacturonic acid, catalyzing the hydrolysis of its α-1,4-glycosidic linkages into D-galacturonate, while pectin lyase (PL) randomly cleaves glycosidic linkages through transelimination, giving rise to unsaturated products. The presence of calcium ions, although not essential, stimulates PL activity [21].

1.3.5 Waxes and ashes

The wax layer is located in the epidermis and works as the first barrier against insect attacks, protecting the plant. About its composition, waxes are mainly composed by alcohols and also esters and fatty acids (with long alkane chains). Cutin can be found embedded in wax and lipophilic amorphous polymer, which are held together by ester linkages [11]. Different chemicals can be used alone or in mixtures to extract waxes, among which are ethanol, acetone and toluene [8].

Figure 1.11 – Egg-box model: Calcium binding to polygalacturonic acid units [25].
In turn, the ash content is an indication of the mineral-uptake of the plant, thus it depends on the type of soil where the hemp plant is grown. The main mineral components of the fibers are potassium (K), phosphorus (P), magnesium (Mg) and calcium (Ca). As highlighted before, calcium assumes a very important role in fibers structure by stabilizing pectin in the form of calcium pectate. The ash content is usually determined by submitting the biomass source to high temperature (ca. 575 °C), where ashes represent the remaining material, i.e., the unburnt biomass.

1.4 Hemp fibers applications

Although hemp has been traditionally used in textiles, materials for construction and insulation and in pulp and paper industries, nowadays the plant is related to a wide range of products and markets, including agriculture, textile, biocomposites, paper-making, automotive, construction, biofuels, functional food, oil, cosmetics, personal care and pharmaceutical industry [5]. Figure 1.12 gives a perspective of the extensive range of applications for hemp plant.

![Figure 1.12 - Multiple applications of different parts of hemp plant [5].](image)

Among the best potential applications for the hemp plant are the use of hemp biomass as a renewable feedstock for energy production or second generation biofuels, and also the use of its fibers as reinforcement for biocomposite materials and concrete.

1.4.1 Hemp fibers as reinforcements for composites

All the solid materials composed of more than one component in separated phases are known as composite materials. In this class of materials are included fiber reinforced composites, which as its name
suggests, are composed of reinforcement fibers (discontinuous phase) dispersed in a polymer matrix (continuous phase) [2]. Currently, the use of fiber reinforcement composites is extended to automotive, wind power, aerospace, construction, consumer goods, marine and infrastructure markets.

While the reinforcement acts as load-carrying member, the matrix keeps the fibers in a desired location and orientation, protects them against environmental damage and transfers load to the reinforcement, through the fiber-matrix interface. In fact, the efficacy of reinforcing fibers is determined by interface, aspect ratio, distribution and orientation [2].

Natural Fiber Composites (NFCs) or biocomposites (Figure 1.13) are composite materials, in which at least the reinforcing fibers are derived from renewable and carbon dioxide neutral resources, such as wood or plants [2]. The possibility of using biopolymers, for instance epoxidized plant oils, to replace petroleum based matrixes, has also been considered, but it is still an expensive solution, and thus prohibitive to an industrialization process. Hence, the most manufactured NFCs result from the combination of natural fibers (mainly flax, hemp, jute and kenaf) with petrochemical matrixes.

![Door panel made of Natural Fiber Composites.](source: www.bioplastics.com)

Without excluding all the advantages inherent to natural fibers, one of its major drawbacks is related with the variability in its mechanical and physical properties, which in the end will introduce variability in the properties and dimensions of the final composites. Differences in quality also lead to fluctuation in fibers’ price. In contrast, synthetic fibers can be produced in a specific way and have a consistent quality [11].

For such application, natural fibers have also other disadvantages such as non-uniform and non-smooth surfaces, a restricted maximum processing temperature, low resistance to water absorption (which causes fiber swelling) [3] and lower durability and mechanical properties than synthetic fibers. Nevertheless, another challenging problem of natural fibers is their poor interfacial adhesion and compatibility with hydrophobic matrix resins.
1.5 Processing of hemp fibers

After harvesting, in order to get high-quality fibers for high-grade composites and textile industries, hemp stems must undergo a pretreatment to ease the separation of fiber bundles from the woody part of the stem. The ultimate goal is to degrade pectins and other cementing compounds (mainly lignin) in middle lamella region, which bind individual fibers together and keep them attached to non-fibril materials, and thereby obtain individual fibers and/or fiber bundles [28], [29]. On the other hand it is important to reduce the hidrophilicity of the fibers by removing its hydrophilic non-cellulosic components.

The most traditional retting methods to achieve defibrillation of hemp fibers are the natural processes field retting and water retting. However, due to some disadvantages of these approaches, in the past few years researchers have been focused on getting more efficient, controlled and reliable methods in order to improve fiber quality for further applications. Among those methods are chemical, mechanical and enzymatic pretreatments. Nevertheless the chosen process has to be determined by location and requirements of end use [2].

1.5.1 Field retting

In field retting (also known as dew retting) the stems are cut and left spread over the surface of the field, where they are soaked during the night by the dew [2]. The combination of the presence of moisture, warm temperatures and high relative humidity, allows the growth of natural bacteria (e.g. Bacillus, Clostridium) and fungi (e.g. Aspergillus, Penicillum). These microorganisms are basically fermentation entities that mainly produce hydrolytic enzymes which degrade pectic substrates (viz. pectinases) from middle lamella and allow hemp’s defibrillation into individual fibers and/or fiber bundles [4], [28]. Other secreted enzymes can also degrade proteins from protoplasm of plant cells, starches, fats and waxes [2].

The time of retting usually varies from 2 to 8 weeks, mainly depending on the location, the climate and the degree of retting required. A simple way to identify the degree of retting is based on checking the color of the stems, which turns to dark grey as a result of microorganism’s action. Another possibility is to manually inspect the stems and “measure” the ease of separation of fibers from woody core [2].

The main advantage of field retting is its low cost. The method consumes much less water than water retting and comparing to chemical or enzymatic pretreatments, it does not require any energy inputs, except for mechanical field operations. On the other hand, field retting has a great agricultural efficiency since it is fully mechanized and saves labor time.

However, field retting faces several problems such as high processing time and land use, increased dispersion of fiber properties, dependence of fiber supply for weather conditions and potential delays in the planting of subsequent crops [8]. The lack of control in the process and related sources of quality variability in final hemp fibers are definitely the major drawback of field retting. The retting process itself
varies both within and between crops, due to weather variation, climate patterns and soil quality, making it exclusive for specific geographic zones.

Liu et al. (2015) [8] reported differences on the chemical composition, morphology and mechanical properties on hemp fibers (cultivar USO-31) with harvesting time and field retting duration. For both early and late harvested hemp fibers (flowering and seed maturity stages, respectively), a decrease in the mechanical properties throughout field retting processing time was observed, along with a decrease in cellulose content, which is undesirable for hemp fibers application as reinforcement in composite materials. The rate of pectin degradation was also reported to decrease throughout the time of retting and to be possibly related with an enhancement in the accessibility of microorganisms for cellulose. A high degree of depectinization using field retting process was also shown previously by Meijer et al. (1995) [30] for flax fibers (cultivares Ariane and Belinka).

1.5.2 Water retting

In water retting hemp stems are soaked in water tanks at controlled temperature (usually between 30 and 40 ºC), allowing the growth of pectinolytic bacteria. In the first 40 hours of processing, Bacillus sp. are dominant, but when water tanks start to run out of oxygen, the anaerobic and spore-forming Clostridium sp. proliferates, seen as the major group of bacteria showing pectin-degrading activity [31].

In comparison with field retting, water retting is operated in a controlled environment which allows to obtain high quality fibers. The tanks can even be sealed or opened, as a way of adaptation to the climate. Since water retting is not dependent on weather conditions, it can be applied in locations that are not suitable for field retting. On the other hand, water retting does not compete for arable land and it is also a faster treatment, being applied in an hourly scale while field retting can last several weeks.

However, water retting has been gradually abandoned as pretreatment of natural fibers because of its high degree of contamination and consumption of fresh water [32], which ultimately increases the production costs and creates high volumes of fermentation waste. Even so, water retting is still being applied in some countries, where enzymes, microorganisms and chemicals are added to the process to offset part of the environmental impact and aid the retting process [2], [33], but obviously are even more expensive solutions.

1.5.3 Fungal treatment

Fungi play a crucial role in most ecosystems, acting in the nutrient cycling as decomposers. In this context, they are widely known for being able to hydrolyze woody materials. Among them, different white rot fungi have been identified to selectively degrade lignin, which promptly brought interest to the paper industry, for use in bleaching and pulping processes of different wood and non-wood raw materials [34].
White rot fungi (*Basidomycetes* class) are divided in two major groups, according to its degradation pattern: the group of fungi that can simultaneously degrade different lignocellulosic materials (viz. lignin, hemicellulose and cellulose) at approximately the same rate through hydrolytic and oxidative processes; and the group of fungi that is capable of performing selective delignification of biomass, by producing extracellular oxidases [35]. The most important lignin degrading enzymes are lignin peroxidases, manganese peroxidases and laccases, which together act on both phenolic and non-phenolic lignin.

Therefore, white rot fungi can be potentially used as agent to remove non-cellulosic components. In addition, a fungal pretreatment of hemp fibers is seen as low cost, efficient and environmentally friendly alternative [34]. However, the fungi to be used have to be meticulously chosen, since some of them might consume glucose from cellulose. The use of genetically modified fungus, for instance cellulase mutants, is an alternative that has been considered.

In the fungal pretreatment itself, different factors affecting fungi growth have to be taken in consideration to allow an extremely controlled process, including temperature, pH, moisture, oxygen and sources of carbon (energy source) and nitrogen (for protein, amino acids and nucleic acid synthesis). For instance, the white rot fungi can grow in a wide range of pH, but the optimal temperature range is limited (25-30 ºC).

Pickering *et al.* (2007) [34] showed the potential use of a two-weeks fungal pretreatment of hemp fibers, either alone or in combination with alkali treatment, to create better bonding characteristics in natural fiber reinforced polypropylene composites, by reporting an increase in composite strength of 22% (32% in combination with alkali treatment) using fungal treated fibers as reinforcement instead of untreated fibers.

In another study, Thygesen *et al.* (2013) [36] investigated the effect of fungal pretreatment with the white rot fungus *Ceriporiopsis subvermispora* and *Phlebia radiata* Cel 26 (cellulase mutant) on the chemical composition of hemp fibers. *P. radiata* Cel 26 showed the highest selectivity for pectin degradation (82%) along with 50% of delignification, 36% of hemicellulose removal and lowest content of cellulose degradation (14%). In addition, the use of Scanning Electron Microscopy allowed to conclude that hemp fiber reinforced composites manufactured with both fungal treated hemp fibers, revealed good epoxy impregnation, which did not happen with untreated or water retting treated fibers.

### 1.5.4 Chemical treatment

Another alternative to traditional natural retting methods is the use of different chemical agents to degrade non-cellulosic materials of natural fibers and improve fiber quality for end applications.

It was mentioned before that the inherent polar and hydrophilic nature of polysaccharides and the nonpolar characteristics of the majority of the matrixes worsen fiber-matrix bonding and negatively affect the final mechanical properties and efficiency of biocomposites [37]. The hidrophilicity of hemp fibers mainly comes from the hydroxyl groups present in its lignocellulusic materials. While the hydroxyl groups
from cellulose’s crystalline region are strongly linked and inaccessible, the ones from amorphous region are free to react with chemical agents, since they are weakly linked to the fiber structure [38].

Therefore, chemical agents have been applied as pretreatment of hemp fibers to "clean" their surface, i.e. to remove non-cellulosic materials, getting the most homogeneous surface made of glucose, and to optimize the adhesive strength in natural composites by improving the chemical bonding between fibers and polymer matrix in composite manufacturing [39]. In addition, pectin removal from middle lamella allows the separation of fiber bundles, increasing the surface area available for chemical bonding [7].

A list of different chemicals and strategies have been studied and applied, including alkalization, acetylation, silanization, bleaching, benzoylation and other treatments using peroxide, organic and inorganic acids, anhydrides, chelating agents, sodium sulfite (Na₂SO₃), sodium chlorite (NaClO₂), etc. [40]–[42]. Treatments using different chemicals will cause a variation in the degree of impurities and effluents generated and will have different effects on hemp fibers structure and chemical composition.

Although the chemical treatments can improve fiber quality, they are known to cause inevitable environmental problems due to the large amount of effluents produced, and are also associated to high energy inputs, resulting in an increase of production costs. Because of these reasons an attempt to use biological treatments has been followed. However, once hemp fibers contain approximately 30% of non-cellulosic materials, until now a total replacing of chemical processes by a single biological process cannot achieve the appropriate effects required in practical application, so chemical treatments, particularly alkali treatments, are still the most direct and efficient way of improving hemp fibers quality [43].

1.5.4.1 Alkali treatment

The alkali treatment is maybe the most popular applied chemical treatment on natural fibers. For several years it has been used to study natural fibers structure and properties [43], but nowadays it is well-known to selectively degrade non-cellulosic materials from fibers’ wall, mainly hemicellulose [40], [44], causing little effects on cellulose and increasing the number of cellulose reaction sites for matrix adhesion [38].

Sodium Hydroxide is the most renowned alkaline agent and it was reported to remove amorphous materials from the surface of hemp fibers [7], reducing the hydrophilic nature of the fiber (Equation 1).

\[ \text{Fiber} - \text{OH} + \text{NaOH} \rightarrow \text{Fiber} - \text{ONa} + \text{H}_2\text{O} \]  

Figure 1.14 shows the effect of sodium hydroxide treatment on fibers surface, demonstrated by Sawpan et al. (2011) [39], through Scanning Electron Microscopy. From these micrographs it can be seen that the cellulosic fiber surface is covered by non-cellulosic constituents (hemicellulose, lignin, waxes and impurities) – Figure 1.14A – and that alkalinated fibers show a very "clean" surface – Figure 1.14B.
Another studies to investigate the influence of an alkali treatment on hemp fibers’ physical properties reported improvements on adhesion force [7], a decrease in water retention power [44] and an increase in moisture sorption and electrical resistance [40] reported. For flax fibers, Van de Weyenberg et al. (2006) [45] reported a decrease in mechanical properties, but an increase in the mechanical performance of flax/epoxy composites, as a result of an improvement in fiber-matrix interaction.

1.5.4.2 **Chelation treatment**

Chelating agents are organic compounds capable of forming covalent bonds with metals through two or more of their atoms [26], including aminopolycarboxylic, phosphonic and polycarboxylic acids. Different chelating agents vary in terms of metal selectivity and activity, optimal temperature and pH and cost [46], among which Ethylene-diamine-tetraacetic acid (EDTA) is the most famous.

EDTA can be used to sequestrate calcium ions from pectin structure, which function as interstitial cementing material in the middle lamella by holding single fibers together in fiber bundles. As a result, pectin substrates become soluble in many liquids, aiding fibers defibrillation [47]. Therefore, chelation treatments represent a valuable solution as a pretreatment of hemp fibers.

In fact, chelating agents as EDTA, CDTA (cyclohexane-diamine-tetraacetic acid) and oxalate have also been used to extract pectin in plant cell wall materials, in structural studies [15]. Le Troëdec et al. (2011) [7] reported the presence of galactose, arabinose and galacturonic acid as main products solubilized in EDTA solutions after a chelation treatment of hemp fibers. Since all of them are part of pectin composition, it was concluded that the treatment mainly acts on the dissolution of weakly linked pectins.

However, due to its strong chelating properties, EDTA behaves as a persistent pollutant in the environment, enhancing the mobility and bioavailability of heavy metals. It is also resistant to biodegradation, since only a few natural aerobic gram negative bacteria (*e.g.* *Agrobacterium* sp.) have
shown the capacity of completely mineralize EDTA [48]. Ethylene-diamine-tetramethylene-phosphonic Acid (EDTMPA), a phosphanated analogue of EDTA, is an alternative with less environmental impact, since it can be easily removed from technical and natural systems [26].

1.5.4.3 Delignification treatment

Several methods have been used to achieve biomass delignification. The most famous is the Kraft process applied in pulp and paper industry. An usual alternative to be used at laboratory scale is the alkaline peroxide process, which on one hand can effectively remove both lignin and hemicellulose from biomass sources and on the other is considered to be an environmentally friendly process and easily to handle [41],[49]. However its use is limited since the peroxide radical can extensively degrade cellulose.

Sodium chlorite has been the most used chemical agent to achieve delignification of natural fibers, especially as a pretreatment of natural fibers to be used as reinforcement in composite materials [50]. After the treatment, fibers typically lose its color, a common consequence of bleaching.

Acid-chlorite delignification consists in soaking natural fibers in an aqueous solution of acetic acid and sodium chlorite. However it can also affect polysaccharides, including cellulose, as reported by Kumar et al. (2009) [51]. Cellulose degradation is most probably related with oxidative degradation of polysaccharides (chlorite effect) and/or acidic cleavage of glycosidic bonds (acid hydrolysis) [41] and it would negatively affect the tensile strength properties of treated fiber bundles and resulting composites. Nevertheless in bleached fiber composites, the matrix assumes the role of removed lignin, making the composite more hydrophobic, tougher and increased flexural strength [50].

An alkaline boiling process, which consists of a treatment with sodium chlorite and sodium hydroxide at boiling temperature, also reveals itself as an effective way to remove lignin and pectin from hemp fibers structure. This method was used by Wang et al. (2003) [52] where the authors reported that sodium chlorite and sodium hydroxide concentration are key parameters to achieve lignin removal. It was also shown a residual content of lignin after the tested treatments, proving that unlike pectin, lignin is very difficult to remove due to its cross-linking and presence of structural aromatic compounds.

1.5.5 Enzymatic treatment

The use of enzymes is currently one of the most promising pretreatments for hemp fibers, bridging the disadvantages of previously mentioned treatments. The ultimate goal is to produce high quality fibers, at least equivalent to those obtained by water retting, but without generating waste effluents [2].

The traditional retting and fungal treatments are time consuming solutions and can cause degradation of a fraction of the plant polysaccharides. With the enzymatic treatment the period of retting is reduced to a couple of hours. In comparison with the use of chemical agents, the enzymatic treatment is more specific,
since each enzyme works on a specific substrate. Thus it can be used to selectively remove hydrophilic hemicellulose and pectins, in theory without affecting other components. Moreover, enzymatic systems require lower energy inputs and can be recycled after use [53], while chemicals generate significant amounts of effluents that need further treatments.

The major drawbacks of the enzymatic treatment are the high cost associated to enzymes and equipment needed [2]. On the other hand the efficiency of the treatment can be lower than expected due to the enzyme difficulties of penetration in hemp fiber structure.

1.5.5.1 Pectinase

Essentially commercial pectinases have been used to degrade pectin from middle lamella of natural fibers, splitting the fiber bundles into finer units. The majority of them are acidic pectinases, with a pH optimal range of 4-6. However, commercial pectinases can exhibit side effects of residual cellulase activity, which in the end will cause a decrease in the mechanical properties of treated fibers [26], [54]. Since the optimal pH range of hydrolases varies between 3 and 6, the use of alkaline pectinases (pH at alkaline conditions) is a solution to be considered to eliminate the impact on cellulose [55].

Saleem et al. (2008) [54] studied the impact of pectinase treatment of hemp fibers in the mechanical properties of final reinforced thermoplastic composites. Despite of detecting a decrease in tensile strength of hemp fibers treated with a commercial pectinase (SIHA-Panzym® DF), the final thermoplastic composites showed improved tensile and flexural characteristics, due to separation of the fiber bundles in the enzymatic treatment (indexed by a significant decrease in the cross section area of treated fibers). In another study, Li & Pickering (2008) [26] used chelating agents alone or combined with enzymes, including pectinase, as pretreatment of hemp fibers. An increase in cellulose crystallinity was noted along with an improvement of 19% in tensile strength of the final composites.

1.5.5.2 Laccase

Lignin-oxidizing enzymes, including lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases, have been investigated to directly attack lignin from wood and non-wood plant feedstocks [56]. In pulp and paper industry, these oxidative enzyme are seen as an environmental friendly solution to improve the efficiency of Kraft pulp bleaching, the so called biobleaching [57].

Laccases are the most important lignin degrading enzymes, which oxidize its substituted phenols using molecular oxygen as final electron acceptor. However, laccases alone cannot depolymerize lignin, since phenolic compounds only count for a small percentage of the total polymer. Nevertheless, some synthetic compounds have been identified as laccase mediators, assisting an increase in the degree of delignification of a certain biomass source, allowing an expansion in laccase biotechnological applications.
In a laccase-mediator system, laccase oxidizes the mediator generating radicals, which then acts as electron carriers and diffuses into the structure to oxidize lignin [26]. The mediators HBT and ABTS are the most famous and possess the highest oxidation power [58]. As alternatives, lignin derived phenols identified in natural processing of lignin by white rot fungi have been investigated, and are considered a low cost and toxicity solution.

As an example of laccase-mediator treatment of biomass, Gutiérrez et al. (2012) [56] reported a degree of delignification of 48% and 32% in *Eucalyptus* and *Pennisetum*, respectively, using 50 U/g of laccase and 2.5% of HBT concentration. On the other hand, George et al. (2014) [53] reported the effect of a commercial laccase, on the surface of flax and hemp fibers, without using a mediator. If for flax 65% of lignin was removed, in the case of hemp fibers the treatment was ineffective, with only 5% of delignification, justifying the need of a mediator.

In another study, George et al. (2015) [59] investigated the impact of sodium hydroxide swelling of hemp fibers prior to enzymatic treatments in their physical properties, viz. surface roughness and adhesion forces. Both properties were not significantly affected after a solo laccase treatment, comparing to untreated hemp fibers. To explain such results, the authors pointed the complexity of hemp fibers and the fact that lignin is confined in the inner regions of its structure and thus inaccessible for laccase. In fact, when combined with a pre-sodium hydroxide treatment, an increase in adhesive force was achieved, which was associated to the action of the alkali agent in fibers networking, opening space for laccase to penetrate and act on lignin. As regards to the enzymatic treatments, the action of laccase was not clear, since only 1% of weight loss was obtained and lignin counts for ca. 5% of hemp fibers composition.

1.5.5.3 Xylanase

Xylanases are known to degrade hemicellulose, more precisely xylan, one of the main components of cellulosic fibers. They specifically cleave β-1-4-glycosidic bonds in xylan backbone, releasing monomers (as mannose and xylose) and opening cracks in fiber surfaces, exposing cellulose hydroxyl and carboxyl groups [53]. As an example of application, xylanases have been applied in pulp and paper industry to degrade xylan and then ease lignin extraction.

George et al. (2015) [59] reported that xylanase treatment of hemp fibers preceded by its exposure to sodium hydroxide resulted in an increase in disruption of the networking and bonding in hemp fibers structure, proving the importance of the alkali treatment as enhancer of xylanase accessibility to xylan backbone. For xylanase treatment alone or in combination with NaOH, SEM micrographs showed clear signs of defibrillation.
2. Aim of studies

Natural cellulosic fibers, such as hemp, are attractive as reinforcement agents in composite materials due to their low cost, low density, good mechanical properties and potential sustainability and biodegradability. For example, cellulose rich bast fibers from hemp exhibit high tensile strength (300-800 MPa), high stiffness (30-60 GPa) and a relative low density of 1.50 to 1.64 g/cm³.

In order to be used for high-grade biocomposites manufacturing, the degradation of middle lamella-fiber bonding is essential to obtain individual fibers and/or fiber bundles, increasing fiber-matrix contacting area. In addition, during fiber extraction, the removal of non-cellulosic components from hemp fibers structure is important on one hand to increase glucose content, which can be correlated with its tensile strength, and on the other to decrease fibers’ hydrophilicity, improving the interaction between hemp fibers (reinforcement) and polymer matrix (usually hydrophobic).

As regards to fiber extraction, traditional retting methods (field retting and water retting) are either time consuming and subjected to geographic regions or cause serious, ecological problems. Another alternative is the use of chemicals, but it is also associated to environmental problems, due to large amounts of chemical effluents produced, and usually it requires higher energy inputs, which ultimately increases the fiber production costs.

Therefore, the use of enzymes (viz. pectinases) is seen as a promising, ecological alternative for hemp fiber defibrillation. However, due to the complexity of lignified middle lamella, enzymes are too large to penetrate into hemp fiber structure, and the treatment is not as efficient as expected. Hence, a pretreatment is required prior to enzymatic treatment, in order to enhance penetration of enzyme formulations into middle lamella regions and, at the same time, reduce the dosage of enzymes needed.

This thesis will focus on the use of three different approaches, functioning as pretreatment of hemp fibers to enhance the accessibility of enzymes for pectins in middle lamella region. The first is a biological pretreatment either using a short-period field retting or a fungal retting with the cellulase mutant Phlebia radiata Cel 26. The second is a hydrothermal pretreatment at different temperatures/pressures. The last is a chemical pretreatment using chelating (EDTA), alkali (NaOH) and bleaching (NaClO₂) agents. The changes in morphology, chemical composition and mechanical properties of fibers were investigated on each one of mentioned pretreatments.
3. Materials and methods

3.1 Materials

Stems of hemp, variety USO-31, were used as raw material in the present study. The plants were sown in France at a rate of 45 kg/ha on May 5th 2013, by hemp cultivation companies Planète Chanvre and Bafa Neu GmbH, and then harvested at seed maturity developing stage (late harvested) on September 6th 2013.

The chemicals disodium EDTA (EDTANa₂·2H₂O), sodium chlorite (NaClO₂), toluene and acetone were purchased from Sigma-Aldrich (Saint Louis, MO, USA), while sodium hydroxide, sulfuric acid (96%), citric acid monohydrate and trisodium citrate dihydrate were acquired from Merck (Kenilworth, NJ, USA). The monosugars α-L-Rhamnose monohydrated, D-Xylose, D-Mannose, L-Arabinose, D-Galactose and D-Galacturonic acid monohydrated were also purchased from Sigma-Aldrich (Saint Louis, MO, USA) and D-Glucose from Merck (Kenilworth, NJ, USA).

Table 3.1 describes the specific enzymes used in the enzymatic treatments, as regards to its origin, activity and concentration.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Origin</th>
<th>Activity* (U/mL)</th>
<th>Protein Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endopolygalacturonase</td>
<td>Locally produced by fermentation with <em>Pichia Pastoris</em></td>
<td>945 (113)</td>
<td>56.4b</td>
</tr>
<tr>
<td>Laccase</td>
<td>From <em>Trimetes versicolor</em> (Sigma-Aldrich)</td>
<td>4.3 (0.3)</td>
<td>56</td>
</tr>
<tr>
<td>Pectin lyase</td>
<td>Locally produced by fermentation with <em>Pichia Pastoris</em></td>
<td>39.7 (0.7)</td>
<td>12.0b</td>
</tr>
<tr>
<td>β–1,4–Xylanase M4</td>
<td>Megazyme</td>
<td>0.7 (0.1)</td>
<td>13.1</td>
</tr>
<tr>
<td>Xyloglucanase</td>
<td>Locally produced by fermentation with <em>Pichia Pastoris</em></td>
<td>2906 (98)</td>
<td>59b</td>
</tr>
</tbody>
</table>

* Determined at 40 °C and pH 6.0 by respective enzymatic activity assay, as described in appendix 7.2. Values between parentheses represent the standard deviation.

b – Determined by BCA Pierce™ Method, as described in subsection 3.2.8

3.2 Methods

In this section a detailed description of all methods used in this study will be presented. Among them are the fungal, hydrothermal and chemical pretreatments (subsections 3.2.1 to 3.2.3) of hemp fibers and subsequent enzymatic treatments (subsection 3.2.4), the chemical composition analysis (subsection 3.2.5), the tensile strength testing (subsection 3.2.6), the water retention determination (subsection 3.2.7),
the total protein concentration determination (subsection 3.2.8) and the statistical analysis (subsection 3.2.9).

3.2.1 Fungal pretreatment

The fungal treatment was carried out with the white rot fungus *Phlebia radiata* Cel 26, which was kindly supplied by Professor Daniel, G., from the Swedish Agricultural University, Dept. Forest Products. The fungi was stored and pre-cultivated on 2% (w/v) malt agar plates at 20 ºC for 2 weeks (Figure 3.1).

![Malt agar plate showing white rot fungi *P. radiata* Cel 26.](image)

The mycelium suspension was applied on hemp stems according to Thygesen *et al.* (2013) [36]. The growth medium (NS medium) was prepared with 2.5 g/L KH₂PO₄, 2g/L K₂HPO₄, 1 g/L MgSO₄·7H₂O and 2.5 g/L glucose, all from Merck (Kenilworth, NJ, USA), and 1.5 g/L NH₄NO₃ from Sigma-Aldrich (Saint Louis, MO, USA). In turn, the mycelium suspension was prepared by homogenizing the fungi spores, grown on an agar plate, into 100 mL of miliQ water.

Before starting the treatment, 15 g of hemp stem pieces, randomly selected from the whole stem, were wetted for 30 min in 40 ºC miliQ H₂O, since the existence of moisture is essential for microbial growth. Afterwards, the stems were transferred into a 1 L Erlenmeyer flask, to which 100 mL of NS medium and 25 mL of mycelium suspension were added. The latter was omitted from control experiments, *i.e.* without *P. radiata* Cel 26 inoculation.

For the fungal pretreatments with pre-sterilization, both stems and growth medium were sterilized at 120 ºC for 60 min and after cooling to room temperature, the mycelium suspension was applied.

For the fungal treatment itself, the Erlenmeyer flasks were sealed with parafilm and placed in an incubator from Termaks (Bergen, Norway) at 28 ºC, for the retting time in study (0.5, 1 or 2 weeks). After the biological pretreatment, hemp fibers were manually peeled and washed 3 times with distilled water to
remove epidermal and fungal residues from the surface of the fibers. Then, samples were dried at 50 ºC for 12 hours, before being used in enzymatic treatments or chemical composition analysis.

The weight loss during fungal pretreatments was roughly determined using Equation 2, assuming that hemp fibers account for 40 % of the total weight of the stem. This assumption was based on a preliminary estimation.

\[
\text{weight loss} (\%) = \frac{0.4 \times W_1 - W_2}{0.4 \times W_1} \times 100
\]  

(2)

where \( W_1 \) is the initial dry weight of hemp stems and \( W_2 \) is the dry weight of recovered fibers after treatment and further peeling, washing and drying steps.

3.2.2 Hydrothermal pretreatment

The hydrothermal pretreatment was carried out in autoclaves from SANO clav GmbH (Bad Überkingen, Baden-Württemberg, Germany), model LaM-MCS-J.

Parts of hemp stems, randomly selected, have been submitted to an autoclaving pretreatment at different pressures, viz. 0.5 bar (112 ºC), 1 bar (121 ºC) and 2 bar (134 ºC), in 1 L Erlenmeyer flasks with 100 mL of miliQ water. After cooling to room temperature, the fibers were manually peeled, washed 3 times with miliQ water at 40 ºC to remove superficial contaminations, minerals and water soluble components and then dried in an oven at 50 ºC for 12 hours, before being used in enzymatic treatments or chemical composition analysis.

The weight loss of hemp fibers during the hydrothermal pretreatments was roughly determined using Equation 2.

3.2.3 Chemical pretreatment

After peeling, washing 3 times with miliQ water at 40 ºC and drying at 50 ºC for 12 hours, hemp fibers were submitted to different chemical pretreatments to sequentially remove non-cellulosic materials, viz. pectin, lignin and hemicellulose.

In this context, first the chelating agent EDTA-2Na.2H\(_2\)O was used alone at different concentrations (0.1, 0.5, 0.75, 1.0, 2.0 and 3.0% w/v) or combined with Endo-PG (0.2%, w/w) to remove pectin from raw hemp fibers. The treatments have been carried out for 4 hours, at 40 ºC and shaking speed of 100 rpm. The pH value of the EDTA solutions was adjusted to 6.0 with 5 M sodium hydroxide. In order to simplify the writing, from now on, EDTA-2Na.2H\(_2\)O will be referred as simply E\(_{\text{DTA}}\) or EDTA-2Na.

For hemp fibers pretreated with 1% EDTA-2Na, an additional treatment with sodium chlorite at different concentrations (0.7, 1.4, 2.1, 2.8 and 5.0%, w/v) has been carried out for 3 hours, at 40 ºC and shaking
speed of 100 rpm, to delignify the fibers. 20% (v/v) acetic acid was used to adjust the pH of sodium chlorite solutions to 5.0.

Finally, for hemp fibers sequentially pretreated with 1% EDTA-2Na and 0.7% sodium chlorite, a last treatment was done with sodium hydroxide at different concentrations (2.5, 5.0, 7.5, 10 and 15% w/v) to remove hemicellulose from hemp fibers. In another approach, a solo alkali treatment with sodium hydroxide, at the same concentrations, was done on raw hemp fibers. All this treatments have been performed for 4 hours, at 40 ºC and shaking speed of 100 rpm.

All mentioned chemical pretreatments were carried out in sealed plastic bags incubated in a water bath, using a fiber-to-liquid ratio of 1:40 (g/mL).

The weight loss of hemp fibers during each chemical pretreatment was determined through Equation 3, where $W_1$ is the initial dry weight of fibers and $W_2$ is the weight of recovered fibers after respective treatment and further washing and drying steps.

$$\text{weight loss (})\%\text{) = } \frac{W_1 - W_2}{W_1} \times 100$$ (3)

### 3.2.4 Enzymatic treatment

A pectinase treatment was conducted on biological, hydrothermal or chemically pretreated hemp fibers, in order to investigate the enzymatic accessibility of pectinases for pectin in middle lamella region, combining 0.1% (w/w) of pectin lyase with 0.2% (w/w) of Endo-PG, except for chemically pretreated fibers, for which only 0.2% (w/w) of Endo-PG was used.

A combined enzymatic treatment with xyloglucanase (0.2% w/w) and xylanase (0.05% w/w) was conducted on hemp fibers sequentially pretreated with the chemical agents EDTA-2Na (1%, w/w) and sodium chlorite (0.7%, w/w), to investigate the accessibility of said enzymes for hemicelluloses.

For both pectinase and xyloglucanase/xylanase treatments, 3 to 5 g of hemp fibers of interest were weighed and placed inside sealed plastic bags, and then submerged in the respective enzyme formulation (1:40 g/mL fiber-to-liquid ratio) at pH 6.0. The bags were then incubated at 40 ºC in a water bath, under a shaking speed of 100 rpm, during 4 hours, except for hydrothermally pretreated fibers, for which the treatment was followed throughout time, viz. 0 (t0), 30 (t1), 90 (t2), 150 (t3), 240 (t4) and 300 min (t5).

On the other hand, a laccase treatment (1% w/w, as George et al. 2015 [59]) was done, on chemically pretreated fibers with 1% EDTA-2Na, to investigate the accessibility of said enzyme for lignin. The treatment was performed for 3 hours at 40 ºC and pH 6.0 in 500 mL glass tubes, with an aeration rate of 100 L/min, a fiber-to-liquid ratio of 1:50 (g/mL) and using HBT (1.5%, w/w) as laccase mediator.
For all the enzymatic treatments, Buffer II (25 mM citric acid monohydrate and trisodium citrate dihydrate) at pH 6.0 was used to prepare the enzyme solutions. The exact volume of enzyme required was determined by the ratio between the mass of enzyme and the total protein concentration (see Table 3.1).

Enzymatically treated hemp fibers were washed 3 times with miliQ water to remove traces of enzyme and buffer. Afterwards all samples were dried at 50°C for 12 hours and stored in polyethylene bags for subsequent use in chemical composition analysis and mechanical properties characterization. The weight loss of hemp fibers during each enzymatic treatment was also determined through Equation 3.

3.2.5 Chemical composition analysis

In this study, the chemical composition of untreated and treated hemp fibers was determined in order to evaluate the effects of each tested treatment in hemp fibers composition. More precisely, it comprised the determination of the lignin content (Klason Lignin) and structural monocarbohydrates, particularly the most representative in the composition of lignocellulosic materials and pectin.

To fulfill the entire chemical composition analysis, a sequence of different methods was performed to hemp fibers samples as follows: milling, wax extraction, Strong Acid Hydrolysis and HPAEC-PAD.

3.2.5.1 Milling fibers

For subsequent chemical composition analysis, different treated samples of hemp fibers were ground in a microfine grinding miller from IKA® (Staufen, Baden-Württemberg, Germany), model MF 10, to a particle size of 1 mm. Milled samples were then kept in polyethylene bags until further use for wax extraction.

3.2.5.2 Wax extraction

Wax extraction was performed in a conventional Soxhlet extractor to remove the superficial waxes and extractives of hemp fibers samples [60]. A thermal plate from Gerhardt GmbH & Co. (Königswinter, Germany) was used as heat source. The basic setup is shown in Figure 3.2.

A glass filter containing one of the milled hemp fibers samples is placed inside the glass reservoir at the start of the procedure. Then the solvent mixture inside the distillation flask is heated up to boiling and the solvent vapor rises to the condenser where it condenses, due to cooling water flux. The droplets of solvent drip steadily into the filter containing hemp fibers powder and allow the extraction of its soluble components, viz. waxes and extractives [61]. In the meanwhile, the solvent fills the extractor and the siphon at the same time. When an overflow is reached in the siphon, the reservoir suddenly fluxes and the solvent (containing soluble extractives) returns to the distillation flask, at the bottom.
Figure 3.2 – Soxhlet apparatus consisting of a glass reservoir with a siphon tube on the side, placed between a condenser at the top and a distillation flask at the bottom, and heated by a hot plate [60].

The cycle is repeated over and over again, during the period of extraction, while waxes are being extracted. In this study, since there was no interest in determining the wax content of hemp fibers samples, the same solvent mixture was reused 3-4 times in other extractions, avoiding the expense of chemicals.

In the present study, 300 mL of a mixture of toluene, ethanol and acetone in a 4:1:1 liquid-to-liquid ratio was used as extracting solution, as Özmen et al. (2013) [62]. Each extraction lasted 5 hours, while the system was continually cooled with water at 5.5°C, by using a chiller from ScanCool, LaboGene™ (Lynge, Denmark), model ChillSafe. Microporous Carbon boiling chips from Sigma-Aldrich (Saint Louis, MO, USA) were used to avoid overheating.

After the extraction step, the samples were dried for 12 h at 50°C in an oven from Memmert GmbH (Schwabach, Germany), model ULE 600, transferred into polyethylene bags and placed in a desiccator until further use in Strong Acid Hydrolysis procedure.

3.2.5.3 Strong Acid Hydrolysis

The Klason lignin content and structural carbohydrates of hemp fibers samples were determined using one of the Laboratory Analytical Procedures (LAPs) provided by the National Renewable Energy Laboratory (NREL) [14].

Before the experiment, filtering crucibles were ashed for 3 hours at 550°C in a muffle furnace from Nabertherm (Lilienthal, Germany), model L9/06KM, and then immediately transferred into a desiccator to cool at room temperature. Afterwards the crucibles were weighed to the nearest 0.1 mg and then kept in the desiccator until further use.
For each replicate of hemp fibers of interest, 150-160 mg of wax extracted fibers were weighed and transferred into pressure tubes. Afterwards, to perform the Strong Acid Hydrolysis, a volume of 1.5 mL of 72% H₂SO₄ was pipetted into test tubes, which then were incubated in a water bath at 30°C for 30 minutes. In the meantime, at 10 and 20 minutes of incubation, a vortex was used to properly homogenize the acid-solid suspensions and favor the hydrolysis.

For the second step of hydrolysis (dilute acid hydrolysis), the sulfuric acid was diluted to 4% by adding an additional volume of 42 mL of miliQ H₂O with an automatic burette, performing a total volume of 43.5 mL. Additionally, a mother solution containing Sugars Recovery Standards (SRS) was prepared, according to Table 3.2. SRS were taken through the remaining hydrolysis and used to correct losses, due to destruction of sugars during dilute acid hydrolysis [14]. Thus, a volume of 1 mL of SRS mother solution was mixed with 1.5 mL of 72% H₂SO₄ in a pressure tube and then diluted with 41 mL of miliQ H₂O, performing the same total volume as test samples.

Table 3.2 – SRS mother solution as a sugar recovering factor in Strong Acid Hydrolysis procedure.

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>MW (g/mol)</th>
<th>Mother solution (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-L-Rhamnose monohydrated</td>
<td>182.20</td>
<td>1.319</td>
</tr>
<tr>
<td>2</td>
<td>L-Arabinose</td>
<td>150.13</td>
<td>2.205</td>
</tr>
<tr>
<td>3</td>
<td>D-Galactose</td>
<td>180.16</td>
<td>3.478</td>
</tr>
<tr>
<td>4</td>
<td>D-Glucose</td>
<td>180.16</td>
<td>126.2</td>
</tr>
<tr>
<td>5</td>
<td>D-Xylose</td>
<td>150.13</td>
<td>6.571</td>
</tr>
<tr>
<td>6</td>
<td>D-Mannose</td>
<td>180.16</td>
<td>6.572</td>
</tr>
<tr>
<td>7</td>
<td>D-Galacturonic acid monohydrated</td>
<td>212.16</td>
<td>6.538</td>
</tr>
</tbody>
</table>

All the tubes (test and SRS) were sealed and placed in appropriate autoclave safe racks to be autoclaved for 1 h at 121°C and the resulting hydrolysates were cooled at room temperature.

a) Klason Lignin determination

Each autoclaved hydrolysis suspension was vacuum filtered through one of the previously weighed filtering crucibles, while the filtrate was recovered in 50 mL falcon tubes for further use in HPAEC-PAD. Afterwards, miliQ water was used to transfer the residual solids in the pressure tubes into the crucibles, and then to rinse the residue until pH neutralization.

The crucibles with acid insoluble residue were placed at 105°C for 12 hours, in an oven from Memmert GmbH (Schwabach, Germany), model tv30u. After cooling in a desiccator, their weight was recorded to the nearest 0.1 mg (dried acid-insoluble lignin + acid-insoluble ash). Subsequently, the crucibles were ashed in a muffle furnace at 550 °C for 3 hours. Once again, after cooling in a desiccator, they were reweighed to determine acid-insoluble ash. Finally, acid insoluble lignin, also known as Klason Lignin, was
calculated through Equation 4, where the dry weight of the sample was obtained by multiplying sample's initial weight by its dry weight content, obtained by using a Halogen Moisture Analyzer from Mettler Toledo (Greifensee, Switzerland), model 18-22.

\[
\text{Klason Lignin (\%)} = \frac{\text{Weight}_{105^\circ C, dried crucible (g)} - \text{Weight}_{550^\circ C, ashed crucible (g)}}{\text{Dry Weight of sample (g)}} \times 100
\]  \hspace{1cm} (4)

### 3.2.5.4 Structural carbohydrates determination by HPAEC-PAD

The filtrates recovered from vacuum filtration of autoclaved hydrolysates during Strong Acid Hydrolysis procedure, were used to determine the content of structural monocarbohydrates, by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), as described by Arnous & Meyer (2008) [63], [64], in an apparatus from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Rhamnose, Arabinose, Galactose, Glucose, Xylose, Mannose and Galacturonic Acid were chosen to be quantified, since they are the main constituents of hemp fibers lignocellulosic materials and pectin. The separation and quantification of monosaccharides were performed using an ICS-3000 system consisting of a gradient pump (model DP-1), an electrochemical detector/chromatography module (model DC-1) and an autosampler, all from Dionex Corp. (Sunnyvale, CA, USA). Separation was accomplished using a CarboPacTM PA20 (3 mm × 150 mm) analytical column.

To start, the hydrolysates for HPLC analysis were rigorously diluted 100 times, and then transferred through a 0.2 µm filter into a properly sealed autosampler vial. A set of standards, containing all the monosaccharides of interest to be quantified, was also prepared from a standard mother solution, as described in Tables 3.3 and Table 3.4. It has to be noticed that the standards were prepared so that the concentration of each monosaccharide fell in the middle of the linear range of the calibration curve. Appendix 7.3 shows an example of a HPLC chromatograph, obtained for one of the standards.

**Table 3.3** – List of monosaccharides to be quantified by HPAEC-PAD and respective concentration in standard mother solution.

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Full name</th>
<th>MW (g/mol)</th>
<th>Retention time (min)</th>
<th>Mother solution (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rha</td>
<td>α-L-Rhamnose monohydrated</td>
<td>182.20</td>
<td>6.0</td>
<td>0.1136</td>
</tr>
<tr>
<td>2</td>
<td>Ara</td>
<td>L-Arabinose</td>
<td>150.13</td>
<td>6.4</td>
<td>0.1180</td>
</tr>
<tr>
<td>3</td>
<td>Gal</td>
<td>D-Galactose</td>
<td>180.16</td>
<td>8.2</td>
<td>0.4150</td>
</tr>
<tr>
<td>4</td>
<td>Glu</td>
<td>D-Glucose</td>
<td>180.16</td>
<td>9.5</td>
<td>3.5080</td>
</tr>
<tr>
<td>5</td>
<td>Xyl</td>
<td>D-Xylose</td>
<td>150.13</td>
<td>11.4</td>
<td>0.4000</td>
</tr>
<tr>
<td>6</td>
<td>Man</td>
<td>D-Mannose</td>
<td>180.16</td>
<td>12.0</td>
<td>0.8290</td>
</tr>
<tr>
<td>7</td>
<td>GalA</td>
<td>D-Galacturonic acid monohydrated</td>
<td>212.16</td>
<td>23.0</td>
<td>0.3935</td>
</tr>
</tbody>
</table>
As described by Arnous & Meyer (2008) [63] a two-eluent system comprising miliQ water and 0.5 M NaOH aqueous solution was used. The eluents were kept under a blanket of N₂ and the mobile phase was purged with N₂ to minimize carbonate contamination, which would affect the retention times of the monosaccharides. The elution step itself was done isocratically in two phases: 20 minutes of elution with 2.5 mM NaOH to elute natural monosaccharides and then 10 min with 0.5 M NaOH to elute acidic monosaccharides and wash the column at the same time. Each injection was preceded by a column reequilibration program, using 100 mM of NaOH for 5 minutes and then 2.5 mM of NaOH for the same period of time. The volume of sample’s injection was 10 µL, while the eluent rate was kept at 0.5 mL/min.

In the end, data was collected and analyzed on computers equipped with Chromeleon 6.80 Sp2 Build 1472 software (Dionex Corp., Sunnyvale, USA). As Liu et al. (2015) [8], for further interpretation of the results, it was assumed that arabinose, galactose, galacturonic acid and rhamnose are specific to pectin substrates, glucose to cellulose and xylose and mannose to hemicellulose. Moreover, the concentration of polymeric sugars was calculated from the concentration of the corresponding monomeric sugars, using anhydrous corrections \( f_{\text{anhydrous}} \), summarized in Table 3.5.

### Table 3.4 – Series of standards and respective dilution factors (in parenthesis) used in HPAEC-PAD, prepared from standard mother solution. The values presented are concentrations in g/L.

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Std 1 (x1000)</th>
<th>Std 2 (x667)</th>
<th>Std 3 (x500)</th>
<th>Std 4 (x250)</th>
<th>Std 5 (x167)</th>
<th>Std 6 (x125)</th>
<th>Std 7 (x111)</th>
<th>Std 8 (x100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rha</td>
<td>0.00011</td>
<td>0.00017</td>
<td>0.00023</td>
<td>0.00045</td>
<td>0.00068</td>
<td>0.00091</td>
<td>0.00102</td>
<td>0.00114</td>
</tr>
<tr>
<td>2</td>
<td>Ara</td>
<td>0.00012</td>
<td>0.00018</td>
<td>0.00024</td>
<td>0.00047</td>
<td>0.00071</td>
<td>0.00094</td>
<td>0.00106</td>
<td>0.00118</td>
</tr>
<tr>
<td>3</td>
<td>Gal</td>
<td>0.00042</td>
<td>0.00062</td>
<td>0.00083</td>
<td>0.00166</td>
<td>0.00249</td>
<td>0.00332</td>
<td>0.00374</td>
<td>0.00415</td>
</tr>
<tr>
<td>4</td>
<td>Glu</td>
<td>0.00351</td>
<td>0.00526</td>
<td>0.00702</td>
<td>0.01403</td>
<td>0.02105</td>
<td>0.02806</td>
<td>0.03157</td>
<td>0.03508</td>
</tr>
<tr>
<td>5</td>
<td>Xyl</td>
<td>0.00040</td>
<td>0.00060</td>
<td>0.00080</td>
<td>0.00160</td>
<td>0.00240</td>
<td>0.00320</td>
<td>0.00360</td>
<td>0.00400</td>
</tr>
<tr>
<td>6</td>
<td>Man</td>
<td>0.00083</td>
<td>0.00124</td>
<td>0.00166</td>
<td>0.00332</td>
<td>0.00497</td>
<td>0.00663</td>
<td>0.00746</td>
<td>0.00829</td>
</tr>
<tr>
<td>7</td>
<td>GalA</td>
<td>0.00039</td>
<td>0.00059</td>
<td>0.00079</td>
<td>0.00157</td>
<td>0.00236</td>
<td>0.00315</td>
<td>0.00354</td>
<td>0.00394</td>
</tr>
</tbody>
</table>

### Table 3.5 – List of anhydrous corrections to determine the concentration of polymeric sugars from corresponding monosaccharides.

<table>
<thead>
<tr>
<th>No.</th>
<th>Monosaccharide</th>
<th>Chemical Formula</th>
<th>Anhydrous Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-L-Rhamnose</td>
<td>C₆H₁₂O₅</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
<td>L-Arabinose</td>
<td>C₇H₁₀O₅</td>
<td>0.91</td>
</tr>
<tr>
<td>3</td>
<td>D-Galactose</td>
<td>C₆H₁₂O₆</td>
<td>0.90</td>
</tr>
<tr>
<td>4</td>
<td>D-Glucose</td>
<td>C₆H₁₂O₆</td>
<td>0.90</td>
</tr>
<tr>
<td>5</td>
<td>D-Xylose</td>
<td>C₅H₁₀O₅</td>
<td>0.88</td>
</tr>
<tr>
<td>6</td>
<td>D-Mannose</td>
<td>C₆H₁₂O₆</td>
<td>0.90</td>
</tr>
<tr>
<td>7</td>
<td>D-Galacturonic acid</td>
<td>C₆H₁₀O₇</td>
<td>0.91</td>
</tr>
</tbody>
</table>
a) Calculations

To start, the Sugar Recovering Standards (SRS) from Strong Acid Hydrolysis procedure were used to calculate the recovered amount of each sugar ($R_{\text{sugar}}$) after dilute acid hydrolysis, through Equation 5.

$$\% R_{\text{sugar}} = \frac{\text{Concentration}_{\text{sugar detected by HPLC (g/L)}}}{\text{Concentration}_{\text{sugar in SRS mother solution (g/L)}}} \times 100$$

(5)

A final value of $\% R_{\text{sugar}}$ for each monosaccharide of interest was determined as an average of 3 replicates. Then it was used to correct the corresponding monosaccharide concentration values, obtained by HPLC for each sample of interest, as presented in Equation 6, considering that all the samples have been diluted previously to HPLC analysis.

$$c_{\text{sugar}} (g/L) = \frac{\text{Concentration}_{\text{sugar detected by HPLC (g/L)}}}{\% R_{\text{sugar}}/100} \times \text{dilution factor}$$

(6)

After correction for loss during 4% acid hydrolysis ($c_{\text{sugar}}$), the concentration of each monosaccharide was corrected with correspondent anhydrous factor ($f_{\text{anhydrous}}$), previously presented in Table 3.5, as illustrated in Equation 7.

$$c^*_{\text{sugar}} (g/L) = c_{\text{sugar}} (g/L) \times f_{\text{anhydrous}}$$

(7)

Finally, the concentration of each monosaccharide in different tested samples is expressed as % (w/w) (or g of monosaccharide/ 100 g dry matter) through Equation 8, considering the volume of filtrate recovered ($V_{\text{filtrate}}$) and the initial mass of hemp fibers samples in Strong Acid Hydrolysis step.

$$\% \text{sugar} = \frac{c^*_{\text{sugar}} (g/L) \times V_{\text{filtrate}} (L)}{\text{dry weight of sample (g)}} \times 100$$

(8)

In the chemical composition analysis, the variation in the relative content of each monosaccharide, in a certain treatment, was determined through Equation 9, where $-\Delta X$ is the reduction in the relative content (in %) of the monosaccharide $X$; and $X_i$ and $X_f$ are the relative contents (in g/ 100 g dry matter) of the monosaccharide before and after the treatment, respectively.

$$-\Delta X (\%) = \frac{X_i - X_f}{X_i} \times 100$$

(9)

3.2.6 Tensile strength testing of fiber bundles

The tensile strength testing of fiber bundles was performed to hemp fibers from different tested treatments, according to Liu et al. (2015) [8] and it consisted in the determination of the following
mechanical properties: Ultimate Tensile Strength (UTS), strain (or elongation at break) and stiffness (measured as Young’s Modulus).

As regards to samples preparation, bast fiber strips were manually peeled with 60-80 mm long x 1 mm wide. A razor blade was used to guarantee a constant width along the entire length of the bast fiber and to remove eventual traces of epidermis and secondary fiber layers [8]. The tensile strength tests were carried out on 25 specimens for each sample of interest. The weight and length of each specimen were initially recorded to estimate its average cross section area (Equation 10), which is required for the calculation of tensile strength, as pressure per unit area. To this end, constant widths of specimens and hemp fibers density of 1.5 g/cm$^3$ were assumed.

\[
\text{Cross Section Area (m}^2\text{)} = \frac{\text{weight}_{\text{specimen}} (g)}{\text{density}_{\text{hemp fibers}} (g / m^3) \times \text{length}_{\text{specimen}} (m)} \tag{10}
\]

Each test specimen was prepared in a custom-made holder as schematically represented in Figure 3.3. First the fiber strips were straightly placed on the bottom tabs, aligned along the centerline and fixed at two adhesive points (see Figure 3.3). Afterwards both upper and bottom tabs were glued together by applying a drop of epoxy resin (DP 100) in the center of the bottom tab and then a cover-plate was used to clamp the tabs using screw nuts. It also has to be noticed that for all of the specimens, a Gauge Length of 10 mm was used, represented in Figure 3.3 as the space between consecutive pairs of tabs.

![Figure 3.3](image-url) – Scheme of the custom-holder containing a specimen for tensile strength testing [8].

The custom-holders were kept at 20 °C for 24 hours and then each specimen was removed from the older to be used for tensile testing. For that purpose an Instron (High Wycombe, Buckinghamshire, England) testing machine (Figure 3.4), model 2710-203, equipped with a 1 kN load cell, was applied at 25 °C and 50% of humidity, along with a tensile speed (elongation per minute) of 0.5 mm/min.

From the apparatus, the extension of fiber strip specimens (in mm) and applied load (in N) are recorded throughout time. The tensile strength or stress (in MPa) is given by the ratio between the applied load and the estimated cross section area (Equation 11). On the other hand, the strain (in %) is the percentage of elongation of the fiber strip over the Gauge Length (Equation 12).
Figure 3.4 – Instron testing machine used for tensile strength testing of hemp fibers.

\[
\text{stress (MPa)} = \frac{\text{Applied load (N)}}{\text{cross section area (m}^2\text{)}} \times 10^{-6}
\]

\[
\text{strain (\%)} = \frac{\text{elongation of fiber sample (mm)}}{\text{Gauge Length (mm)}} \times 100
\]

By plotting tensile strength against strain, a typical curve, as the one represented in Figure 3.5, is obtained, and all the mechanical properties of interest can be estimated for each specimen and ultimately for each sample, as the average of all of the 25 specimens.

As represented in the previous figure, the elongation (in \%) of the fibers increases along with the applied load until the fiber strip breaks. The ultimate tensile strength (UTS) of bast fiber strips is defined as the ratio of failure load (N) and the average cross-section area of the bast strip. Thus, in the curve, it is the tensile strength value followed by a sudden decrease (or the maximum tensile strength). The characteristic strain is the percentage of elongation correspondent to the UTS value. Finally the Young’s Modulus (in GPa) can be obtained from the slope of the linear regression between tensile strength and strain (\(\varepsilon\)), through Equation 13, based on the so called Hooke’s Law. To estimate the latter, linear regressions from 0 to 1.5\% of strain were considered, generally the range of strain values where the Hooke’s Law is applicable.

\[
\text{Young’s Modulus (GPa)} = \frac{\text{UTS (MPa)}}{10 \times \varepsilon \text{ (\%)}} = \frac{\text{slope(MPa/\%)} \times 10^3}{10^3} \times 100
\]
3.2.7 Water retention determination

The water retention power represents the quantity of water that is retained in the fibers after soaking in the water and a further centrifugation step [44]. In order to determine water retention capacity of hemp fibers, a standard method from ASTM International (ASTM D 2402-07, 2012) entitled “Centrifuge Method” [65] and suitable for natural fibers, was followed with some amendments.

For each tested specimen, ca. 0.5 g was weighed and transferred into a falcon tube. Afterwards, the fibers were immersed in 50 mL of miliQ water for 5 minutes and immediately transferred to customized centrifuge tubes (Figure 3.6). The hole in the base of centrifuge tubes allows the removal of remaining water from fibers surface, during the centrifugation step.

![Customized centrifuge tube with holes in the bottom to allow water drainage in the centrifugation step.](image_url)
The centrifugation lasted 5 minutes, including the acceleration time, at 2169 rpm (determined by using the radial distance of the fiber mass from the center of rotation). The specimens were then transferred into a pre-weighed crucible (C) and covered with a pre-weighed square of aluminum foil (AF). The crucibles were weighed ($W_1$) to know the mass of each moist specimen ($M$) and then completely dried (12h) in an oven at 105 °C. After drying, the crucibles were reweighed ($W_2$) to know the mass of dried specimen ($D$). The drying step differs from the cited standard method, where the specimens are only dried for 1.5 hours. Ultimately, the water retention value (WRV) of each specimen was determined by Equation 14. The final WRV was determined as an average of 3 replicates.

$$\text{WRV} \left( \frac{g}{g \ \text{dry fibers}} \right) = \frac{M - D}{D} = \frac{(W_1 - C - AF) - (W_2 - C)}{W_2 - C}$$

(14)

For this procedure, a centrifuge from Sigma Laboratory Centrifuges (Osterode am Harz, Niedersachsen, Germany), model 4-15, was used.

### 3.2.8 Total protein concentration determination

The present method was used in order to determine the total protein concentration of different enzymes used in the present project, namely endopolygalacturonase, pectin lyase, xyloglucanase and laccase. Knowing the concentration of said enzymes was crucial to calculate the volumes of enzymes to be used in different treatments.

Thereby, total protein concentrations were determined by spectrophotometry using Pierce™ BCA Protein Assay Kit from Thermo Scientific (Waltham, MA, USA), in which bicinchoninic acid (BCA) is used for colorimetric detection and quantification of total protein. Bovine Serum Albumin (BSA) was used in this study as standard protein.

The following procedure meets the instructions of Thermo Scientific for Pierce™ BCA Protein Assay. The reagents involved in this method, which were part of said Thermo Scientific Kit, are a solution of sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide (BCA reagent A) and 4% (w/v) cupric sulfate solution (BCA reagent B).

BSA standard solutions were prepared from albumin standard ampules according to said protocol, using milliQ water as diluent. The working reagent was prepared by mixing BCA reagents A and B in a 50:1 liquid-to-liquid ratio, and the total volume needed was estimated taking into account the number of standards, samples and respective replicates in study.

With all the reagents ready, volumes of 25 µL of each standard/enzyme solution sample and 200 µL of working reagent were pipetted into microplate wells. Then the plate was mixed on a plate shaker for 30 seconds. Afterwards the plate was incubated at 37°C for 30 minutes of reaction. Finally the plate was...
cooled to room temperature and the absorbance of resulting reaction product was measured at 562 nm in a microplate reader from TECAN (Männedorf, Switzerland), model Infinite® M200 Pro.

A standard curve was designed, by plotting the blank-corrected $A_{562\text{nm}}$ of each BSA standard against its concentration (in µg/mL), from which the total protein concentration of each tested enzyme was estimated.

### 3.2.9 Statistical analysis

Analysis of variance (ANOVA) was performed on the results obtained through previously described methods, for each conducted study. Thus, for each monosaccharide in the chemical composition or physical (viz. water retention value) and mechanical property, the processing time or chemical concentration (when applied) were tested at a significance level of 5% on Minitab 17.

The differences between each factor of interest were evaluated by using Tukey multiple comparison test, at a level of significance of 5%. In this method, tested samples (factor) are divided in groups according to a particular property (response), which are distinguished by letters (starting at “a” and so on). Tested samples which share at least one letter are not statistically significant different; otherwise there is evidence, with a confidence level of 95%, that they are statistically significant different.
4. Pretreatment of hemp fibers to enhance enzyme accessibility for hemp fibers

This chapter is divided into sections, according to the different studied strategies of pretreatment for hemp fibers to enhance the accessibility of enzymes. Thus, section 4.1 is related with biological pretreatment, section 4.2 with hydrothermal pretreatment and section 4.3 with chemical pretreatment. In each one of the mentioned sections, the experimental design is presented, prior to the presentation and discussion of results. Finally in section 4.4, a comparison between different tested methods was attained.

4.1 Biological pretreatment of hemp fibers to enhance accessibility of pectinases

The goal of the present experiment was to evaluate and compare the effect of short-period field retting and fungal retting with Phlebia radiata Cel 26 on the accessibility of pectinases for pectins in middle lamella regions. To this end, changes in chemical composition and mechanical properties of hemp fibers were studied in each stage of the overall treatment.

4.1.1 Experimental design

Figure 4.1 schematizes the experimental design for the present study. Late harvested hemp stems were placed in the field for a short-period of dew-retting or were subjected to a fungal pretreatment with the cellulase mutant Phlebia radiata Cel 26 in a controlled environment, prior to an enzymatic treatment with pectinases (Endo-PG and pectin lyase), according to the methods described in chapter 3.

In general, fungal or bacterial treatments of natural fibers are preceded by a sterilization step to avoid the growth of contaminant microorganisms [36]. However, as will be addressed in section 4.2, an autoclave step can influence the chemical composition of the fibers. Thus, in order to “measure” the exclusive action of fungi on hemp fibers, two different fungal treatment approaches were tested, one preceded by autoclave sterilization and other without any sterilization step.

Subsequently, hemp fibers from different stages of the study were analyzed in terms of mechanical properties and chemical composition. A list of the resulting hemp fibers samples is summarized in Table 4.1, all of them obtained in triplicate. From now on, as a way to simplify writing, each sample will be called by its own code, also represented in Table 4.1.
Figure 4.1 – Overview of the study of biological pretreatment of hemp fibers as a way of enhancing pectinases accessibility for pectin substrates.

Table 4.1 – List of hemp fibers samples and respective codes, resulting from the biological pretreatment study, to be analyzed for chemical composition and mechanical properties.

<table>
<thead>
<tr>
<th>Hemp fibers samples</th>
<th>Code*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field Retted for x weeks</td>
<td>FR xw</td>
</tr>
<tr>
<td>Field Retted for x weeks + Pectinase Treatment</td>
<td>FR xw+Pec</td>
</tr>
<tr>
<td>Fungal Treated with <em>P. radiata</em> Cel 26 for x weeks</td>
<td>Pr xw</td>
</tr>
<tr>
<td>Fungal Treated without <em>P. radiata</em> Cel 26 for x weeks (Control)</td>
<td>Pr xw-CT</td>
</tr>
<tr>
<td>Fungal Treated with <em>P. radiata</em> Cel 26 for x weeks + Pectinase Treatment</td>
<td>Pr xw+Pec</td>
</tr>
<tr>
<td>Fungal Treated without <em>P. radiata</em> Cel 26 for x weeks (Control) + Pectinase Treatment</td>
<td>Pr xw-CT+Pec</td>
</tr>
<tr>
<td>Fungal Treated with <em>P. radiata</em> Cel 26 for x weeks with pre-sterilization step</td>
<td>A+Pr xw</td>
</tr>
<tr>
<td>Fungal Treated with <em>P. radiata</em> Cel 26 for x weeks with pre-sterilization step + Pectinase Treatment</td>
<td>A+Pr xw+Pec</td>
</tr>
</tbody>
</table>

* x represents the number of weeks: 0.5, 1 or 2.

4.1.1 Results and discussion

After the biological pretreatment, the field retted fibers showed its characteristic dark grey color and black spots, due to microorganisms’ activity. In turn, for fungal retted hemp stems for 1-2 weeks without pre-sterilization step (Figure 4.2.B-C), it was evident the presence of different wild fungi in both hemp stems and growth medium. The same did not occur with pre-sterilized hemp stems, proving sterilization efficacy. Even so, for non-pre-sterilized hemp stems retted for 0.5 weeks (Figure 4.2.A), there is also no evidence
of wild fungi growth, which allows to conclude that their growth rate is lower in the early days of retting. However the presence of wild microorganisms, especially bacteria, is predictable.

Figure 4.2 – Hemp stems after fungal retting: A1 – treated for 0.5 week without P. radiata Cel 26 inoculation (control); A2 – treated for 0.5 week with P. radiata Cel 26 inoculation; A3 – amplification of image A2, showing possible P. radiata Cel 26 spores; B1 – treated for 1 week without P. radiata Cel 26 inoculation (control); B2 – treated for 1 week with P. radiata Cel 26 inoculation; C1 – treated for 2 week with P. radiata Cel 26 inoculation.

For hemp stems that have been fungal retted for 0.5 and 1 week, without pre-sterilization step, test samples (with P. radiata Cel 26 inoculation) can be distinguished from controls (without P. radiata Cel 26 inoculation), since probable spores of P. radiata Cel 26 are identified on the stem’s surface by naked eye (Figure 4.2.A3-B2), similar to what had been observed on agar plates (see Figure 3.1).

Table 4.2 shows the weight loss of hemp fibers in the fungal pretreatment without pre-sterilization step. Firstly, it is evident a statistically significant increase (p<0.05) in the weight loss along with the retting time, from ca. 7% in Pr0.5w fibers to ca. 15 and 35% in Pr1w and Pr2w fibers, respectively. Furthermore, it has also to be highlighted that an increasing damage, during the washing step of fibers retted from 0.5 to 2 weeks, was observed. As a result of significant wild fungi activity on hemp stems retted for 2 weeks (Figure 4.2.C1), the fibers became softer and tiny fiber particles were lost during washing. For that reason, it is reasonable to admit that the accuracy of presented weight loss values decreases from Pr0.5w to Pr2w fibers. Thus, in this case, the weight loss is shown as a measure of harshness for fibers due to fungi
activity and not necessarily to express the exact loss of the main components of fibers (viz. cellulose, hemicellulose, lignin and pectin).

**Table 4.2** – Weight loss of hemp fibers after fungal pretreatment deprived of a pre-sterilization step and with or without inoculation of *P. radiata* Cel 26.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate</th>
<th>Initial mass of hemp stems (g)</th>
<th>Dry mass of recovered fibers (g)</th>
<th>Weight loss* ** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr0.5w</td>
<td>1</td>
<td>15.06</td>
<td>5.26</td>
<td>9.47</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.06</td>
<td>5.33</td>
<td>8.26</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.09</td>
<td>5.53</td>
<td>5.01</td>
</tr>
<tr>
<td>Pr0.5w-CT</td>
<td>1</td>
<td>15.09</td>
<td>5.52</td>
<td>5.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.96</td>
<td>5.43</td>
<td>5.91</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.04</td>
<td>5.37</td>
<td>7.45</td>
</tr>
<tr>
<td>Pr1w</td>
<td>1</td>
<td>15.08</td>
<td>4.82</td>
<td>17.15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.99</td>
<td>4.78</td>
<td>17.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.06</td>
<td>4.92</td>
<td>15.32</td>
</tr>
<tr>
<td>Pr1w-CT</td>
<td>1</td>
<td>15.01</td>
<td>4.97</td>
<td>14.17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.99</td>
<td>4.96</td>
<td>14.23</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.00</td>
<td>4.88</td>
<td>15.67</td>
</tr>
<tr>
<td>Pr2w</td>
<td>1</td>
<td>15.03</td>
<td>3.84</td>
<td>33.77</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.03</td>
<td>3.79</td>
<td>34.64</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.00</td>
<td>3.80</td>
<td>34.33</td>
</tr>
<tr>
<td>Pr2w-CT</td>
<td>1</td>
<td>15.08</td>
<td>3.21</td>
<td>44.82</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.04</td>
<td>3.78</td>
<td>34.85</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.06</td>
<td>4.21</td>
<td>27.54</td>
</tr>
</tbody>
</table>

* Dry matter content of raw fibers (untreated) and dried fibers is 95 and 98.5%, respectively; ** Differences between each retting time were evaluated using Tukey multiple comparison test with a level of significance of 5%. Treatments with different letters are significantly different (p<0.05).

### 4.1.1.1 Chemical composition analysis

The effects of tested biological pretreatments and further enzymatic treatment with Endo-PG and pectin lyase on the chemical composition of hemp fibers are shown in Table 4.3.

As regards to the biological pretreatment (colored in Table 4.3), the first thing to highlight is that all the tested pretreatments affected the content of non-cellulosic materials, viz. pectin, hemicellulose and lignin, although in different extents. Only the content of glucose is not statistically significant different (*F*-value: 0.72; *p*-value: 0.707) among all tested pretreatments, varying between 64 and 70%. Glucose is the most dominant component in the structure of hemp fibers, thus if there is a loss of other components in each pretreatment without damaging glucose, a relative increase in the glucose content would be expectable.
Table 4.3 – Anhydrous monosaccharides and Klason lignin content of biological pretreated hemp fibers before and after an enzymatic treatment with Endo-PG and pectin lyase.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>Klason Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw fibers</td>
<td>1.0</td>
<td>1.3</td>
<td>2.1</td>
<td>60.0</td>
<td>1.2</td>
<td>3.0</td>
<td>7.3</td>
<td>5.9</td>
</tr>
<tr>
<td>RMW*</td>
<td>0.7ab</td>
<td>1.2a</td>
<td>2.1a</td>
<td>68.0A</td>
<td>1.5a</td>
<td>4.5a</td>
<td>7.7A</td>
<td>5.3</td>
</tr>
<tr>
<td>RMW+Pec**</td>
<td>0.7</td>
<td>0.8</td>
<td>1.9</td>
<td>69.8</td>
<td>0.9</td>
<td>4.9</td>
<td>4.2</td>
<td>4.9</td>
</tr>
<tr>
<td>FR1w</td>
<td>0.6cd</td>
<td>0.5c</td>
<td>1.6bcd</td>
<td>63.9a</td>
<td>0.8cd</td>
<td>3.9bc</td>
<td>3.6c</td>
<td>4.4</td>
</tr>
<tr>
<td>FR1w+Pec</td>
<td>0.4</td>
<td>0.5</td>
<td>1.5</td>
<td>64.6</td>
<td>0.8</td>
<td>4.1</td>
<td>2.5</td>
<td>5.1</td>
</tr>
<tr>
<td>FR1w+Pec-CT</td>
<td>0.4</td>
<td>0.5</td>
<td>1.5</td>
<td>64.3</td>
<td>0.9</td>
<td>4.0</td>
<td>3.6</td>
<td>4.5</td>
</tr>
<tr>
<td>FR2w</td>
<td>0.5cd</td>
<td>0.3c</td>
<td>1.6bcd</td>
<td>67.3a</td>
<td>0.5d</td>
<td>3.6e</td>
<td>1.4f</td>
<td>3.0</td>
</tr>
<tr>
<td>FR2w+Pec</td>
<td>0.4</td>
<td>0.3</td>
<td>1.6</td>
<td>68.3</td>
<td>0.7</td>
<td>3.7</td>
<td>1.3</td>
<td>3.8</td>
</tr>
<tr>
<td>FR2w+Pec-CT</td>
<td>0.4</td>
<td>0.4</td>
<td>1.6</td>
<td>65.9</td>
<td>0.5</td>
<td>3.5</td>
<td>1.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Pr0.5w</td>
<td>0.6bcd</td>
<td>0.5bc</td>
<td>1.6bcd</td>
<td>67.2a</td>
<td>0.9bc</td>
<td>4.8b</td>
<td>3.4c</td>
<td>3.9</td>
</tr>
<tr>
<td>Pr0.5w+Pec</td>
<td>0.4</td>
<td>0.5</td>
<td>1.7</td>
<td>70.4</td>
<td>0.8</td>
<td>4.8</td>
<td>2.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Pr0.5w-CT</td>
<td>0.6abcd</td>
<td>0.4bc</td>
<td>1.7abcd</td>
<td>67.1a</td>
<td>0.5bc</td>
<td>4.8a</td>
<td>3.4c</td>
<td>4.3</td>
</tr>
<tr>
<td>Pr0.5w-CT+Pec</td>
<td>0.6</td>
<td>0.4</td>
<td>1.8</td>
<td>73.1</td>
<td>0.9</td>
<td>5.0</td>
<td>2.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Pr1w</td>
<td>0.4cde</td>
<td>0.5c</td>
<td>1.5cde</td>
<td>66.8a</td>
<td>0.7cd</td>
<td>4.5ab</td>
<td>2.2f</td>
<td>4.6ab</td>
</tr>
<tr>
<td>Pr1w+Pec</td>
<td>0.4</td>
<td>0.3</td>
<td>1.5</td>
<td>68.0</td>
<td>0.6</td>
<td>4.5</td>
<td>1.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Pr1w-CT</td>
<td>0.5cde</td>
<td>0.5bc</td>
<td>1.5bcde</td>
<td>66.2a</td>
<td>0.8cd</td>
<td>4.4bc</td>
<td>2.4cde</td>
<td>4.7</td>
</tr>
<tr>
<td>Pr1w-CT+Pec</td>
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<td>0.2</td>
<td>1.4</td>
<td>70.7</td>
<td>0.7</td>
<td>4.4</td>
<td>1.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Pr2w</td>
<td>0.4de</td>
<td>0.2c</td>
<td>1.4de</td>
<td>68.4a</td>
<td>0.8cd</td>
<td>3.9abc</td>
<td>1.6def</td>
<td>5.3</td>
</tr>
<tr>
<td>Pr2w+Pec</td>
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<td>0.2</td>
<td>1.3</td>
<td>68.1</td>
<td>0.6</td>
<td>4.0</td>
<td>1.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Pr2w-CT</td>
<td>0.3de</td>
<td>0.3c</td>
<td>1.4de</td>
<td>66.8a</td>
<td>0.8bc</td>
<td>4.7ab</td>
<td>1.7def</td>
<td>4.9</td>
</tr>
<tr>
<td>Pr2w-CT+Pec</td>
<td>0.4</td>
<td>0.3</td>
<td>1.4</td>
<td>68.1</td>
<td>0.5</td>
<td>4.0</td>
<td>1.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Pr0.5w+Pec-CT</td>
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<td>0.5</td>
<td>1.6</td>
<td>75.7</td>
<td>0.8</td>
<td>4.7</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Pr1.2w+Pec-CT</td>
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<td>0.4</td>
<td>1.5</td>
<td>73.5</td>
<td>0.8</td>
<td>4.7</td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>A ***</td>
<td>0.8a</td>
<td>0.8b</td>
<td>1.8a</td>
<td>68.2a</td>
<td>1.1b</td>
<td>4.7ab</td>
<td>4.3b</td>
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<tr>
<td>A+Pr1w</td>
<td>0.4cde</td>
<td>0.4c</td>
<td>1.3cd</td>
<td>66.4a</td>
<td>0.7cd</td>
<td>4.1abc</td>
<td>2.1def</td>
<td>3.9</td>
</tr>
<tr>
<td>A+Pr1w+Pec</td>
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<td>0.4</td>
<td>1.5</td>
<td>64.8</td>
<td>0.8</td>
<td>4.5</td>
<td>1.6</td>
<td>5.4</td>
</tr>
<tr>
<td>A+Pr1w+Pec-CT</td>
<td>0.2</td>
<td>0.4</td>
<td>1.2</td>
<td>67.2</td>
<td>0.7</td>
<td>4.0</td>
<td>1.7</td>
<td>5.4</td>
</tr>
<tr>
<td>A+Pr2w</td>
<td>0.2</td>
<td>0.2</td>
<td>1.2</td>
<td>72.2</td>
<td>0.7</td>
<td>4.5</td>
<td>0.7</td>
<td>5.8</td>
</tr>
<tr>
<td>A+Pr2w+Pec</td>
<td>0.2</td>
<td>0.2</td>
<td>1.2</td>
<td>72.3</td>
<td>0.8</td>
<td>4.6</td>
<td>1.0</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* Raw fibers after washing to remove contaminations, minerals and water-soluble components;
** Enzymatic treatment of raw fibers, after washing to remove water-soluble components;
*** Autoclaved fibers at 120 °C for 60 minutes;

Notes: Standard deviations of three replicates are represented in parentheses. In relation to biological pretreatments (colored), values in the whole column for the same monosaccharide and Klason lignin with different letters are statistically significant different at a confidence level of 95%.

Even so, just based on the chemical composition, it is not possible to conclude if glucose (i.e. cellulose) was affected due to the microbiological activity, considering the high standard deviations.
The data presented in Table 4.3 also shows that pectin was the most degraded component. This was expected since pectin is the most representative component in ML region and thus it is more accessible for microorganisms than hemicellulose, which is mainly found in the primary cell wall and is tightly bound to cellulose and lignin, by hydrogen and covalent bonds, respectively [20], [38]. The relative decrease in the content of pectin can be roughly estimated in terms of its major constituents, viz. rhamnose, galactose, arabinose and galacturonic acid (GalA). The latter is the major component in pectin structure and it is mainly found in homogalacturonan (which accounts for ca. 50% of pectin), as repeated unit of its polymeric structure. In turn, rhamnose-galacturonic acid is the repeated diglicosyl unit of rhamnogalacturonan I (RGI), while galactose and arabinose are part of the branched groups attached to RGI backbone [21], [24].

The highest degradation of pectin was achieved in fibers retted with *P. radiata* Cel 26 for 2 weeks, after a pre-sterilization step in an autoclave (A+Pr2w). In said pretreatment, there was a decrease in the relative content of rhamnose of ca. 69%, along with 80% of arabinose, 42% of galactose and 85% of galacturonic acid. In fact, it is evident in Table 4.3 that the content of rhamnose, arabinose, galactose and GaLA significantly decreased (p<0.05) with the retting time.

The effect on hemicellulose content, roughly measured in terms of xylose and mannose, was also significant in the fibers retted for 2 weeks. In comparison with non-pretreated fibers (RMW), a statistically significant decrease (p<0.05) in the xylose content was observed after each biological pretreatment, but its highest degradation was obtained in the field retting process carried out for 2 weeks (FR2w), for which there was a relative decrease of ca. 66%. As regards to mannose content, a statistically significant decrease (p<0.05) was only recorded for FR2w fibers, but only accounting for ca. 20%.

In the case of lignin, although the Klason lignin content of each biologically pretreated fibers decreased in comparison to RMW fibers, the value is only statistically significant different for FR2w and Pr0.5w fibers.

Table 4.4 summarizes the total relative reduction of pectin and hemicellulose contents, after each tested biological pretreatment. From Table 4.4 it is confirmed that the total pectin content decreased with the retting time. On the other hand, for hemicellulose, the same conclusion is only observed for field retting pretreatment. The lowest values of total pectin content reduction were observed in the fungal treatments carried out for half a week and in the FR1w, accounting for 47-48%. However, for the latter, the reduction in the relative content of total hemicellulose was 20% higher.

From Table 4.3, it is noteworthy that there was no statistically significant difference in the chemical composition of fungal retted fibers without a pre-sterilization step, whether the fungus *P. radiata* Cel 26 was inoculated or not (*i.e.* between test and control experiments). This fact is even more evident for pectin in Table 4.4, where its total reduction in content is presented. Therefore, it is clear that without a pre-sterilization step to prevent the growth of wild microorganisms, the action of *P. radiata* Cel 26 is omitted and, above all, its inoculation does not bring benefit to the treatment.
Table 4.4 – Estimation of the relative reduction in content of pectin and hemicellulose, after each biological pretreatment.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>GalA</th>
<th>Xyl</th>
<th>Man</th>
<th>Pectin</th>
<th>Hemicellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR1w</td>
<td>16.8</td>
<td>60.5</td>
<td>27.2</td>
<td>53.6</td>
<td>49.4</td>
<td>14.9</td>
<td>47.3</td>
<td>23.4</td>
</tr>
<tr>
<td>FR2w</td>
<td>36.3</td>
<td>73.1</td>
<td>25.5</td>
<td>81.2</td>
<td>65.5</td>
<td>20.5</td>
<td>67.6</td>
<td>31.6</td>
</tr>
<tr>
<td>Pr0.5w</td>
<td>15.4</td>
<td>60.9</td>
<td>23.2</td>
<td>55.8</td>
<td>37.1</td>
<td>-6.1</td>
<td>48.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Pr0.5w-CT</td>
<td>11.7</td>
<td>63.8</td>
<td>19.4</td>
<td>55.6</td>
<td>38.1</td>
<td>-6.1</td>
<td>47.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Pr1w</td>
<td>40.9</td>
<td>62.4</td>
<td>30.8</td>
<td>71.8</td>
<td>51.9</td>
<td>0.3</td>
<td>61.5</td>
<td>13.0</td>
</tr>
<tr>
<td>Pr1w-CT</td>
<td>36.2</td>
<td>59.6</td>
<td>29.3</td>
<td>68.5</td>
<td>48.1</td>
<td>4.1</td>
<td>58.5</td>
<td>15.0</td>
</tr>
<tr>
<td>Pr2w</td>
<td>48.1</td>
<td>80.2</td>
<td>36.6</td>
<td>78.7</td>
<td>49.2</td>
<td>13.8</td>
<td>69.4</td>
<td>22.5</td>
</tr>
<tr>
<td>Pr2w-CT</td>
<td>59.6</td>
<td>79.5</td>
<td>34.3</td>
<td>77.9</td>
<td>43.1</td>
<td>-4.3</td>
<td>69.1</td>
<td>7.4</td>
</tr>
<tr>
<td>A1B+Pr1w</td>
<td>45.7</td>
<td>66.3</td>
<td>37.5</td>
<td>72.3</td>
<td>52.4</td>
<td>9.0</td>
<td>63.8</td>
<td>19.7</td>
</tr>
<tr>
<td>A1B+Pr2w</td>
<td>68.6</td>
<td>80.1</td>
<td>42.1</td>
<td>85.1</td>
<td>49.0</td>
<td>-5.3</td>
<td>75.8</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Note: Negative values mean a relative increase in the content of the monosaccharide.

Now comparing the fungal treatments with *P. radiata* Cel 26 in the presence or absence of an autoclaving pre-sterilization step, there is no statically significant difference in the chemical composition of Pr1w and A+Pr1w fibers. The results demonstrated that, in terms of non-cellulosic components degradation, the action of *P. radiata* Cel 26 together with wild microorganisms (in Pr1w experiment) is comparable to the combination effect of autoclave sterilization and action of *P. radiata* Cel 26 (in A+Pr1w experiments). Therefore, it should be noted that the autoclave pre-sterilization alone had a great effect on the chemical composition of hemp fibers, by removing pectin, as indicated in Table 4.3 mainly through the decrease of GalA content from 7.3 g/100 g dry matter in non-pretreated fibers to 4.5 g/100 g dry matter in just autoclaved fibers (A). This fact has been demonstrated by Alix *et al.* (2014) [66] for flax fibers and the use of an autoclaving process as hydrothermal pretreatment of hemp fibers is the subject of chapter 4.2.

Another interesting conclusion taken from the results presented in Table 4.3 is that the effect of natural microorganisms on the chemical composition of hemp fibers, during 1 week of field retting (FR1w), is comparable with the effect of fungi in a controlled environment for half a week, without a pre-sterilization step (Pr0.5w), since there is no statistically significant difference in the chemical composition of fibers obtained after both treatments, except for mannose. In fact, it was expected that the fungi growth rate would be higher during fungal retting, since the treatment was carried out with a growth medium and optimal temperature, than in an open field where the variation of weather conditions (as indicated by the climate data reported in Liu *et al.* 2015 [8]) is just one of the reasons that justify the lower growth rate. Moreover, the problems associated to the field retting process, as longer processing time, the need for
land and mainly the dispersion of final fiber properties, due to lack of control and different variability sources, can be overcome with a short-period fungal retting, which reveals itself more advantageous.

All different biological pretreatments were used to partially degrade pectin, in order to increase the accessibility of pectinases for hemp fibers structure in a subsequent enzymatic treatment. The chemical composition of all biologically pretreated fibers after being subjected to an enzymatic treatment was previously presented in Table 4.3.

An enzymatic treatment of raw hemp fibers (without any pretreatment) with 0.2% w/w of Endo-PG and 0.1% w/w of pectin lyase, during 4 hours (RMW+Pec), was only shown to decrease the content of GalA from 7.7 to 4.2 g/100 g dry matter. In addition, as will be addressed in the next chapter, practically all the accessible GalA is degraded in the first 30 minutes of treatment and no significant decrease is observable afterwards, proving that the enzyme is not able to penetrate into the well lignified middle lamella region after consuming the accessible GalA.

For all biologically pretreated fibers, an additional amount of GalA was removed in the enzymatic treatment, except for FR2w fibers. This conclusion is supported by the fact that the relative content of GalA decreased after incubation with enzymes, while the same did not happen when biologically pretreated fibers were incubated in buffer, in the absence of the enzymes, i.e. controls for enzymatic treatment (see Table 4.3). Table 4.5 presents an estimation of the total relative reduction in content of GalA, by combining the different biological pretreatments with further enzymatic treatment with Endo-PG and pectin lyase.

From Table 4.5 it is clear that fibers which have been biologically pretreated prior to an enzymatic treatment allowed a significant increase in total pectin removal, comparing to the single enzymatic treatment (RMW), specifically from 46 to 67-91%. In addition, FR2w fibers were the only case where the enzymatic treatment was not effective, probably because the galacturonic acid was extensively degraded in the pretreatment stage and there was no more accessible substrate for the enzymes. For all of the other pretreatments, with more or less GalA available, the enzymatic treatment was effective in removing an extra portion of GalA, most likely due to an increase in middle lamella’s porosity during the pretreatment stage. Moreover, all the retting processes carried out for at least one week, combined with said enzymatic treatment, resulted in a total reduction of GalA content above 80%.

However, since the main purpose of this study was to find a suitable biological pretreatment that could partially remove pectin and, at the same time, increase the accessibility of pectinases for interstitial regions of middle lamella, only the fungal treatment for half a week (Pr0.5w) and the field retting for 1 week (Fr1w) are reasonable options to use as pretreatment. As shown in Table 4.5, for the remaining pretreatments, the GalA was widely degraded and the subsequent use of enzymes became unnecessary and inefficient. On the other hand, during Fr1w and Pr0.5w pretreatments, only half of the GalA was
Table 4.5 – Total relative reduction in content of galacturonic acid in hemp fibers, after combining a biological pretreatment with an enzymatic treatment with Endo-PG and pectin lyase.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment</th>
<th>Enzymatic Treatment</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMW</td>
<td></td>
<td>45,7</td>
<td>45,7</td>
</tr>
<tr>
<td>FR1w</td>
<td>53,6</td>
<td>13,5</td>
<td>67,1</td>
</tr>
<tr>
<td>FR2w</td>
<td>81,2</td>
<td>1,8</td>
<td>83,0</td>
</tr>
<tr>
<td>Pr0.5w</td>
<td>55,8</td>
<td>18,6</td>
<td>74,4</td>
</tr>
<tr>
<td>Pr0.5w-CT</td>
<td>55,6</td>
<td>15,9</td>
<td>71,5</td>
</tr>
<tr>
<td>Pr1w</td>
<td>71,8</td>
<td>8,8</td>
<td>80,6</td>
</tr>
<tr>
<td>Pr1w-CT</td>
<td>68,5</td>
<td>14,3</td>
<td>82,8</td>
</tr>
<tr>
<td>Pr2w</td>
<td>78,7</td>
<td>4,4</td>
<td>83,1</td>
</tr>
<tr>
<td>Pr2w-CT</td>
<td>77,9</td>
<td>4,8</td>
<td>82,7</td>
</tr>
<tr>
<td>A1B+Pr1w</td>
<td>72,3</td>
<td>6,4</td>
<td>78,7</td>
</tr>
<tr>
<td>A1B+Pr2w</td>
<td>85,1</td>
<td>5,6</td>
<td>90,7</td>
</tr>
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</table>

degraded, but an increase in the accessibility of pectinases allowed an extra efficient degradation of GalA in the subsequent enzymatic treatment, decreasing from 3.6 ± 0.1 to 2.5 ± 0.1 g/100 g dry matter and from 3.4 ± 0.1 to 2.0 ± 0.1 g/100 g dry matter, respectively (see Table 4.3).

In terms of accessibility, Pr0.5w was more efficient than Fr1w, since for both of them there was a similar decrease in the relative content of GalA, but in the enzymatic treatment of Pr0.5w fibers, more GalA has been removed (see Tables 4.3 and 4.5), most likely due to higher induction of porosity during the pretreatment. Additionally, by combining the Pr0.5w pretreatment with a subsequent enzymatic treatment, the total relative reduction of GalA content is comparable to other experiments. Also taking into consideration that it can save time and overcome some disadvantages of the field retting process, the fungal treatment for half a week in a controlled environment is a promising pretreatment for hemp fibers.

4.1.1.2 Mechanical properties

Figure 4.3 presents the mechanical properties of hemp fibers, viz. ultimate tensile strength (UTS), strain and stiffness (as Young’s Modulus), after each tested biological pretreatment.

According to UTS results (Figure 4.3-A), hemp fibers resulting from a field retting processing for one week (FR1w) showed the highest strength (832 ± 198 MPa), while for all the remaining pretreatments, the UTS decreased in comparison with non-pretreated fibers. However, for FR1w fibers the standard deviation is also one of the highest, which proves that even for a short period, the degree of variability inherent to field retting processing is significant. Even so, the analysis of variance (ANOVA) shows that hemp fibers pretreated by field retting for one or two weeks (Fr2w), by fungal retting for two weeks with *P. radiata*
Figure 4.3 – Ultimate Tensile Strength (A), strain (B) and stiffness (C) of biologically pretreated fibers by field retting (in green) or by fungal retting with (in orange) and without (in blue) a pre-sterilization step. Values with different uppercase letters within the same study (for the same color) are significantly different at a confidence level of 95%. For all samples, values with different lowercase letters are significantly different at a confidence level of 95%.
Cel 26 inoculation and a pre-sterilization step (A1B+Pr2w) and by fungal retting for half a week without \textit{P. radiata} Cel 26 inoculation and pre-sterilization (Pr0.5w-CT) are not statistically different (p<0.05) from untreated samples in terms of UTS, \textit{i.e.} the mentioned pretreatments do not significantly affect the strength of the fibers.

As regards to strain (or elongation at break), only field retted fibers exhibit a comparable value in relation to untreated fibers. All of the remaining biologically pretreated fibers showed a significant decrease (p<0.05) in strain of approximately 50%. This reduction might be related with the degradation of non-cellulosic materials, especially pectin, and consequent loss of coherence in hemp fibers structure, which, as shown in the chemical composition of biologically pretreated fibers (see Table 4.3) was more extensive in fungal retting than field retting.

In addition, for all tested pretreatments and in comparison with untreated fibers, there is no statistically significant difference (p>0.05) in stiffness, proving that the said mechanical property is not greatly affected by microorganisms activity. The exception was A1B+Pr2w treated fibers, for which the stiffness surprisingly increased ca. 50%. Therefore, no or less damages were introduced by the cellulase mutant, which is characterized by high depectinization efficiency and no or little cellulase activity [36].

For field retted hemp fibers, the mechanical properties varied inversely with the retting time, although they are not statistically significant different (p>0.05) from untreated fibers. As shown previously in the chemical composition analysis (Table 4.3), there was a significant decrease in the relative content of pectin in field retted fibers, due to microorganisms' activity and inversely to the retting time, but not as significant as during fungal retting, for the same periods of retting. On the other hand no significant difference in the cellulose content was recorded. Thus, the fact that cellulose is not affected for such short periods of field retting and the lower degradation of pectin might explain why field retted fibers kept their mechanical performance. As a support example, it has been reported that cellulose content is correlated with tensile properties of hemp fibers [67].

Regarding the fungal pretreatment without pre-sterilization step, interesting conclusions can be drawn. As discussed before, the growth of wild microorganisms was inevitable and particularly extensive in fibers retted for 1 and 2 weeks (see Figure 4.2). The action of wild fungi may hid the action of \textit{P. radiata} Cel 26 in test samples, since no significant difference was found between tests (with \textit{P. radiata} inoculation) and respective controls (without \textit{P. radiata} inoculation), in terms of chemical composition and mechanical properties.

Nevertheless a significant decrease (p<0.05) in UTS throughout the time of retting (from 0 to 2 weeks) was observed, contrary to what happened in fungal treatments with pre-sterilization step, for which A1B+Pr2w fibers showed the best mechanical performance. Thus, even though the cellulose content remains unchanged (Table 4.3), it seems that the activity of wild microorganisms significantly affected the UTS of the fibers. The same can be observed for strain, which significantly decreased in Pr2w/Pr2w-CT
treated fibers. Only stiffness is not statistically significant different within fungal retted fibers and also in comparison with untreated fibers. Therefore, it is once again evident that microorganism’s activity does not affect the stiffness of the fibers.

Furthermore, there is no statistically significant difference (p<0.05) in the mechanical performance of A1B+Pr1w, Pr1w and Pr0.5w treated fibers. Three important conclusions can be taken from these results. Firstly, the autoclaving pre-sterilization step at 120 ºC may not significantly affect the mechanical properties of the fibers and since a partial decrease in pectin content was previously shown for just autoclaved samples (Table 4.3), it would be interesting to investigate the possibility of using an hydrothermal pretreatment of hemp fibers in an autoclave, in order to increase the accessibility of pectinases for pectins in middle lamella region (see chapter 4.2); Secondly, in terms of mechanical properties and also in agreement with the chemical composition analysis, in a fungal treatment perspective, there is no advantage on pre-sterilizing the hemp stems to avoid wild fungi growth in treatments carried out for less than one week. Moreover, since there is no statistically significant difference in the cellulose content (Table 4.3) and in the mechanical performance of Pr1w and Pr1w-CT fibers, a natural, controlled fungal treatment, i.e. without pre-sterilization and inoculation of a cellulose mutant microorganism, seems to be the best and cheapest option. Thirdly, a very short-period fungal retting (e.g. half a week), presents itself as a promising pretreatment of hemp fibers to enhance accessibility of enzymes. Despite of showing lower pectin degradation than A1B+Pr1w and Pr1w fibers (Tables 4.3 and 4.4), Pr0.5w fibers have slightly better mechanical properties and can duplicate the productivity of the pretreatment, by reducing the retting time twice.

In general, just considering the mechanical properties, short-period field retting was the best pretreatment, since none of the investigated properties was affected after 1 or 2 weeks of pretreatment. On the contrary, fibers that have been fungal retted for two weeks (Pr2w and Pr2w-CT), without pre-sterilization step, showed the worst mechanical properties, since the UTS and strain were significantly (p<0.05) lower than any other biological pretreatment.

### 4.2 Hydrothermal pretreatment of hemp fibers to enhance accessibility of pectinases

The goal of the present experiment was to evaluate the effect of a hydrothermal pretreatment of hemp fibers in the accessibility of pectinases for pectins. Different autoclaving pressures have been tested prior to an enzymatic treatment followed throughout time. The effectiveness of enzymatic treatment has been evaluated by determining the weight loss and the chemical composition and mechanical properties of treated fibers. The ultimate objective was to improve hemp fibers quality, combining the best mechanical properties with an acceptable extension of defibrillation, in order to be used as reinforcements in composite manufacturing.
De Roeck et al. (2009) [27] suggested that high pressures and/or temperatures can be used or combined with other chemical methods to allow pectin’s demethylation and/or depolymerization. In turn, Alix et al. (2014) [66] studied the impact of an autoclaving pretreatment on water sorption of flax fibers and reported an impact on pectic substrates, mainly for fibers autoclaved at 2 bar. The use of an autoclave processing as solo pretreatment of hemp fibers to study its impact on fibers’ chemical composition and mechanical properties has been reported by Korte, S. & Staiger, M.P. (2014) [68]. The authors showed that an enzymatic treatment combined with hydrothermal pretreatment resulted in extensive defibrillation and removal of non-cellulosic materials. However, the hydrothermal pretreatment was carried out at high temperature (170 ºC), which represents a significant cost in terms of energy spent. In the present study, lower temperatures will be tested.

As mentioned in the previous section, part of the motivation for this study was supported by the results obtained in the fungal pretreatment (section 4.1), where it was shown that a pre-sterilization step prior to a fungal retting process of hemp stems enabled the partial removal of galacturonic acid from the fiber structure, without significantly affecting the mechanical performance of the fibers.

### 4.2.1 Experimental design

Figure 4.4 illustrates the experimental design for the hydrothermal pretreatment study, consisting in an autoclaving pretreatment at different pressures, viz. 0.5 bar (112 ºC), 1 bar (121 ºC) and 2 bar (134 ºC), followed by an enzymatic treatment with pectinases along different periods of time, until 300 min (t5).

![Experimental design diagram](image)

**Figure 4.4** – Overview of the study of hydrothermal pretreatment of hemp fibers as a way of enhancing pectinases accessibility for hemp fibers.
To evaluate the effect of the hydrothermal pretreatment on the enzymatic accessibility for pectins, raw hemp fibers have also been peeled, washed, dried and directly submitted to the same enzymatic treatment, i.e. without autoclave pretreatment. Additionally, each enzymatic treatment in the experiment was associated to a control test, in which autoclaved fibers (or non-autoclaved fibers) were incubated in Buffer II, without the presence of enzymes, for the same periods of time.

All the experiments, which are part of the present study, are summarized in Table 4.6, all of them done in triplicate. From now on, as a way to simplify writing, each sample will be called by its own code, also represented in Table 4.6.

### Table 4.6 – Different tests in the study of hydrothermal pretreatment of hemp fibers and respective codes.

<table>
<thead>
<tr>
<th>Hemp fibers Samples</th>
<th>Code*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved at 1 bar (30 min) + Enz. Treatment with 0.1% PL and 0.2% Endo-PG for x min</td>
<td>A1B t x</td>
</tr>
<tr>
<td>Autoclaved at 0.5 bar (30 min) + Enz. Treatment with 0.1% PL and 0.2% Endo-PG for x min</td>
<td>A0.5B t x</td>
</tr>
<tr>
<td>Autoclaved at 2 bar (30 min) + Enz. Treatment with 0.1% PL and 0.2% Endo-PG for x min</td>
<td>A2B t x</td>
</tr>
<tr>
<td>Raw material (untreated) + Enz. Treatment with 0.1% PL and 0.2% Endo-PG for x min</td>
<td>UT t x</td>
</tr>
<tr>
<td>Autoclaved at 1 bar (30 min) + incubation without enzymes (Control) for x min</td>
<td>A1B-CT t x</td>
</tr>
<tr>
<td>Autoclaved at 0.5 bar (30 min) + incubation without enzymes (Control) for x min</td>
<td>A0.5B-CT t x</td>
</tr>
<tr>
<td>Autoclaved at 2 bar (30 min) + incubation without enzymes (Control) for x min</td>
<td>A2B-CT t x</td>
</tr>
<tr>
<td>Raw material (untreated) + incubation without enzymes (Control) for x min</td>
<td>UT-CT t x</td>
</tr>
</tbody>
</table>

* t x represents the duration of the enzymatic treatment in minutes: t0 – 0 min; t1 – 30 min; t2 – 90 min; t3 – 150 min; t4 – 240 min; t5 – 300 min.

### 4.2.2 Results and discussion

#### 4.2.2.1 Hydrothermal pretreatment

Tables 4.7 and 4.8 respectively show the weight loss of hemp fibers in the hydrothermal pretreatment stage and chemical composition of resulting fibers.

From Table 4.7, it is clear that there was a significant increase (p<0.05) in the weight loss of autoclaved fibers in comparison with non-pretreated fibers (UT). For the latter, around 10% of the initial dry weight was lost during the washing step with warm miliQ water (40 °C). One can assume that those 10% are essentially contaminations on the surface of raw fibers, water soluble minerals and a less amount of water soluble fiber components. In fact, comparing the chemical composition of raw hemp fibers and UT fibers (Table 4.8), it is evident a relative increase in the content of glucose, xylose, mannose and galacturonic acid, after the washing step. Moreover, the total sum of all presented monosaccharides increased approximately 10% from raw to UT fibers, confirming the recorded weight loss.
Table 4.7 – Dry matter weight loss of hemp fibers after each hydrothermal pretreatment, consisting in an autoclaving process at 0.5, 1 or 2 bar, and respective control (without pretreatment). Standard deviations are represented in parenthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate</th>
<th>Initial mass of fibers (g)</th>
<th>Dry mass of recovered fibers(g)**</th>
<th>Dry matter weight loss* ** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>1</td>
<td>10.00</td>
<td>8.70</td>
<td>10.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.03</td>
<td>8.71</td>
<td>10.42</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.01</td>
<td>8.70</td>
<td>10.34c(0.08)</td>
</tr>
<tr>
<td>A0.5B</td>
<td>1</td>
<td>6.49</td>
<td>5.69</td>
<td>12.33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.70</td>
<td>5.79</td>
<td>13.58</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.29</td>
<td>5.46</td>
<td>13.20</td>
</tr>
<tr>
<td>A1B</td>
<td>1</td>
<td>6.75</td>
<td>5.80</td>
<td>14.07</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.78</td>
<td>5.70</td>
<td>15.93</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.19</td>
<td>5.32</td>
<td>14.05</td>
</tr>
<tr>
<td>A2B</td>
<td>1</td>
<td>6.59</td>
<td>5.53</td>
<td>16.08</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.78</td>
<td>5.66</td>
<td>16.52</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.71</td>
<td>5.61</td>
<td>16.39</td>
</tr>
</tbody>
</table>

* Dry matter content of raw fibers (untreated) and dried fibers are 95 and 98%, respectively.
** Differences in weight loss between pretreatments were evaluated using Tukey multiple comparison test with a level of significance of 5%. Pretreatments with different letters are significantly different (p<0.05)
*** After washing 3 times with miliQ water and drying at 50 ºC for 12 h

Table 4.8 – Anhydrous monosaccharides of hydrothermal pretreated hemp fibers, at different pressures (0.5, 1 and 2 bar).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amount (g/100 g dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rha Ara Gal Glu Xyl Man GalA</td>
</tr>
<tr>
<td>Raw Fibers</td>
<td>1.0 (0.1) 1.3 (0.1) 2.1 (0.1) 60.0 (0.9) 1.2 (0.1) 3.0 (0.3) 7.3 (0.4)</td>
</tr>
<tr>
<td>UT</td>
<td>0.7 (0.1) 1.2 (0.1) 2.1 (0.1) 68.3a(2.7) 1.5 (0.3) 4.5 (0.2) 7.7a(0.8)</td>
</tr>
<tr>
<td>A0.5B</td>
<td>0.7 (0.1) 1.0 (0.1) 2.1 (0.1) 67.6a(0.7) 1.1 (0.0) 4.4 (0.1) 5.7b(0.4)</td>
</tr>
<tr>
<td>A1B</td>
<td>0.8 (0.0) 0.8 (0.1) 1.8 (0.1) 68.2a(1.3) 1.1 (0.1) 4.7 (0.2) 4.5b(0.3)</td>
</tr>
<tr>
<td>A2B</td>
<td>0.5 (0.0) 0.7 (0.2) 2.0 (0.1) 71.7a(2.0) 1.2 (0.1) 4.9 (0.1) 4.0b(0.2)</td>
</tr>
</tbody>
</table>

As regards to hydrothermally pretreated fibers, there was a statistically significant increase (p<0.05) in the weight loss of hemp fibers when the operating pressure of the autoclave increased from 0.5 to 2 bar (Table 4.7). Since after autoclaving, the fibers were also peeled, washed and dried, and considering that 10% of fibers would be lost even without pretreatment, one can assume that the exclusive weight loss in the hydrothermal pretreatment at 0.5, 1 and 2 bar was ca. 2.7, 4.4 and 6.0%, respectively. According to the chemical composition analysis (Table 4.8), this difference in the weight loss can be explained by the partial degradation of pectin substrates, since the content of rhamnose, arabinose and mainly galacturonic acid decreased from untreated fibers to fibers autoclaved at the highest pressure in study.
On the other hand, for all autoclaved and non-autoclaved (UT) fibers, no statistically significant difference \((F\text{-value}: 0.71; \ p\text{-value}: 0.573)\) was obtained relatively to the glucose content, from which one can foresee that the hydrothermal pretreatment had no significant impact on the cellulose microfibrils.

Among the pectin substrates, the homogalacturonan was clearly the most affected by the increase in the operating pressure/temperature of the autoclaving pretreatment, since a statistically significant decrease \((p<0.05)\) in the galacturonic acid content was recorded from A0.5B fibers to A1B fibers and then to A2B fibers, more precisely \(ca. 26, 42\) and \(48\%\) of reduction, respectively. In the same order, and related with pectin degradation, an increase in the degree of defibrillation is expected.

Figure 4.5 shows that there was a statistically significant increase \((p<0.05)\) in the water retention value (WRV) of hydrothermally pretreated fibers, comparing to UT fibers. The partial degradation of pectin from middle lamella presumably resulted in an increase in the water retention capacity of the fibers, due to an increase of void space \((i.e.\ porosity)\) in the structure of the fibers. However, a statistically significant decrease in the WRV is observable from A0.5B \((3.32 \pm 0.21 \ g \ H_2O/g\ dry\ matter)\) and A1B fibers \((3.24 \pm 0.10 \ g \ H_2O/g\ dry\ matter)\) to A2B fibers \((2.61 \pm 0.10 \ g \ H_2O/g\ dry\ matter)\), inversely to GalA degradation. To explain these results, the notion of capillarity has to be introduced.

The interaction of liquids with fibers may involve several physical phenomena. For instance the liquid that penetrates into the fiber can be taken up into the capillary space between fibers \((i.e.\ pores)\) or inside the fibers, where absorption takes place by hydrogen bonding formation. Thus, the liquid retention is governed by the pore structures as well as the wetting properties of the substrate [69].
Capillarity can be defined as the macroscopic movement of a fluid system under the influence of their own surface, pressure difference and interfacial forces [40]. This physical phenomenon is described by Laplace equation, which dictates that the capillary pressure varies inversely with the pore size. This means that the liquid advancement into a smaller pore is greater due to the higher capillarity pressure and dominates the equilibrium height, which happens when the capillarity action is balanced by gravity (the weight of raising fluid). Thus, when the fibers are soaked in water, the smaller pores will be firstly filled and need more time to reach the equilibrium height. Once they are filled, the liquid moves to larger pores [69].

Therefore, as pointed by Kostic et al. (2010) [40], the WRV has always to be considered together with the capillarity phenomenon, because fibers with higher retention capacity and shorter liquid advancement (i.e. bigger pore size) may have a similar WRV than fibers with lower water retention ability and longer liquid advancement (i.e. smaller pore size). In this context, considering that the water affinity (by absorption) and the water retention capacity of the fibers is similar after being autoclaved at different pressures, the decrease in the WRV from A0.5B to A2B fibers is most likely related with an increase in the pores size in the middle lamella, due to higher degradation of pectin, as previously shown in Table 4.8.

Figure 4.6 shows the impact of each hydrothermal pretreatment on the mechanical properties of the fibers, viz. UTS (A), strain (B) and stiffness (C).

![Mechanical properties of hemp fibers autoclaved at 0.5, 1 and 2 bar (A0.5B, A1B and A2B respectively), in terms of UTS (A), strain or elongation at break (B) and stiffness (C), measured as Young's Modulus.](image-url)
A slight downward trend is observable in the UTS and stiffness of hemp fibers that have been autoclaved, from the lowest to the highest operating pressure/temperature (Figure 4.6-A/C). For both properties, since the decrease was more significant in autoclaved fibers at 2 bar, it can be hypothesized that higher operating pressures/ temperatures may have some negative effect on cellulose microfibrils. However, what is noteworthy is that the UTS of hemp fibers was not apparently affected by hydrothermal pretreatments at 0.5 or 1 bar, since their UTS value (723 ± 83 and 686 ± 117 MPa, respectively) is not statistically significant different than the UTS of non-autoclaved (raw) fibers (771 ± 104 MPa). This conclusion also confirms the hypothesis suggested in section 4.1, when the UTS of fungal retted fibers with and without an autoclaving pre-sterilization step were compared: the tensile strength of hemp fibers is not affect by an autoclaving process, at least at moderate operating pressure.

Even so, a significant decrease of ca. 40% in the elongation at break was observed after the hydrothermal pretreatment of raw fibers, independently of the operating pressure/temperature (no statistical difference in the strain value of autoclaved fibers at different pressures). This may be due to a reduction in the adhesion and coherence of individual fibers, which get loosed after pectin removal.

4.2.2.1 Enzymatic accessibility test

Figure 4.7 shows the dry matter weight loss of hemp fibers in each enzymatic treatment of pre-autoclaved or non-autoclaved fibers (UT). It has to be emphasized that each presented point resulted from the subtraction of the real weight loss in the test treatment (with enzymes) by the respective control, i.e. fibers that have been incubated in buffer without enzymes, and also by the weight loss of t0 fibers, i.e. fibers that have been just washed, peeled and dried again without incubation, in order to normalize the curves for each pretreatment.

From Figure 4.7 it is evident that the weight loss of hemp fibers in the enzymatic treatment of hydrothermal pretreated fibers was significantly higher than for non-autoclaved fibers. Moreover, comparing the enzymatic treatments in which pre-autoclaved fibers at different pressures were used as substrate, an increase in the weight loss is observable from A0.5B to A2B fibers, for the same duration of enzymatic treatment.

In a statistical point of view, there is no significant difference between the weight loss of fibers in the enzymatic treatments carried out with A1B and A2B fibers, at least until 150 min (t3) of incubation. However, both are significantly different from the weight loss of enzymatic treatments carried out with A0.5B fibers.

On the other hand, it is noticeable that, for all pre-autoclaved fibers, the most significant increase in the weight loss occurred in the first 30 minutes of incubation (t1), more precisely 2.30 ± 0.15, 3.01 ± 0.33 and 3.81 ± 0.38% of weight loss for A0.5B-t1, A1B-t1 and A2B-t1 fibers, respectively. Then the weight loss rate decreases throughout time.
Figure 4.7 – Dry matter weight loss of fibers treated with Endo-PG and pectin lyase for different periods of time (0-300 min) after each tested hydrothermal pretreatment in an autoclave at different pressures (0.5, 1 and 2 bar) and respective control (without autoclave pretreatment). Values with different letters within the same time are significantly different at a confidence level of 95%.

From the illustrated curves, one can hypothesized that there was an increase in the accessibility of enzymes for hemp fibers previously submitted to a hydrothermal pretreatment and, furthermore, among hydrothermally pretreated fibers, the enzymatic accessibility increased in fibers pre-autoclaved from the lowest to the highest pressure/temperature.

The efficacy of each enzymatic treatment was effectively evaluated in terms of chemical composition analysis of the resulting fibers, which is shown in Table 4.9. According to the chemical composition analysis, and as expected, the action of enzymes resulted in a decrease in the galacturonic acid content, since both pectin lyase and Endo-PG are specific for homogalacturonan, a pectic substrate which has galacturonic acid as repeated unit of its polymeric structure [21]. No significant effect was observed for any other monosaccharide or lignin, throughout the incubation time. The variation in the glucose and galacturonic acid content during the incubation with enzymes, is presented in Figures 4.8 and 4.9, respectively.

From Table 4.9 and Figure 4.8, it is clear that for all the experiments, *i.e.* non-autoclaved (UT) and pre-autoclaved fibers at 0.5, 1 and 2 bar (A0.5B, A1B and A2B, respectively), there was an oscillation in the glucose content throughout the incubation time, but according to the ANOVA (Table 4.9), it is not statistically significant (*p* > 0.05). Therefore one can assume that there was no impact on cellulose during the enzymatic treatments. Then, for the same incubation time, there is also no statistically significant
Table 4.9 – Anhydrous monosaccharides and Klason lignin content of hemp fibers treated with Endo-PG and pectin lyase for different periods of time, after hydrothermal pretreatment in an autoclave at different pressures/temperatures.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>Klason Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-t0</td>
<td>0.7^a (0.1)</td>
<td>1.2^a (0.1)</td>
<td>2.1^a (0.1)</td>
<td>68.0^a (2.7)</td>
<td>1.5^a (0.3)</td>
<td>4.5^a (0.2)</td>
<td>7.7^a (0.8)</td>
<td>5.3^a (0.2)</td>
</tr>
<tr>
<td>UT-t1</td>
<td>0.7^a (0.1)</td>
<td>0.7^b (0.1)</td>
<td>2.0^ab (0.2)</td>
<td>71.4^a (3.1)</td>
<td>1.0^b (0.1)</td>
<td>5.6^a (0.6)</td>
<td>4.4^b (0.7)</td>
<td>4.7^b (0.7)</td>
</tr>
<tr>
<td>UT-t2</td>
<td>0.7^a (0.0)</td>
<td>0.7^b (0.0)</td>
<td>1.7^b (0.0)</td>
<td>71.8^a (0.8)</td>
<td>0.9^b (0.0)</td>
<td>5.6^a (0.1)</td>
<td>4.3^b (0.2)</td>
<td>4.6^b (0.3)</td>
</tr>
<tr>
<td>UT-t3</td>
<td>0.8^a (0.1)</td>
<td>0.9^b (0.1)</td>
<td>1.8^b (0.1)</td>
<td>70.4^a (0.4)</td>
<td>0.9^b (0.1)</td>
<td>5.7^a (0.6)</td>
<td>4.4^b (0.5)</td>
<td>4.8^a (0.4)</td>
</tr>
<tr>
<td>UT-t4</td>
<td>0.7^a (0.0)</td>
<td>0.8^b (0.1)</td>
<td>1.9^ab (0.1)</td>
<td>69.8^a (2.0)</td>
<td>0.9^b (0.1)</td>
<td>4.9^a (0.6)</td>
<td>4.2^b (0.6)</td>
<td>4.9^a (0.2)</td>
</tr>
<tr>
<td>UT-t5</td>
<td>0.7^a (0.0)</td>
<td>0.8^b (0.2)</td>
<td>1.9^ab (0.1)</td>
<td>71.6^a (0.7)</td>
<td>0.9^b (0.2)</td>
<td>5.3^a (0.2)</td>
<td>4.1^b (0.6)</td>
<td>4.4^b (0.2)</td>
</tr>
<tr>
<td>A0.5B-t0</td>
<td>0.7^a (0.1)</td>
<td>1.0^a (0.1)</td>
<td>2.1^a (0.1)</td>
<td>67.6^a (0.7)</td>
<td>1.1^a (0.0)</td>
<td>4.4^a (0.1)</td>
<td>5.5^a (0.3)</td>
<td>4.6^a (0.2)</td>
</tr>
<tr>
<td>A0.5B-t1</td>
<td>0.7^a (0.0)</td>
<td>0.8^a (0.0)</td>
<td>1.9^a (0.1)</td>
<td>66.8^a (0.6)</td>
<td>1.0^a (0.1)</td>
<td>4.6^a (0.0)</td>
<td>3.9^b (0.3)</td>
<td>5.0^a (0.1)</td>
</tr>
<tr>
<td>A0.5B-t2</td>
<td>0.7^a (0.0)</td>
<td>0.8^a (0.1)</td>
<td>1.9^a (0.1)</td>
<td>66.4^a (0.9)</td>
<td>1.1^a (0.0)</td>
<td>4.7^a (0.1)</td>
<td>3.9^b (0.3)</td>
<td>4.6^a (0.2)</td>
</tr>
<tr>
<td>A0.5B-t3</td>
<td>0.7^a (0.0)</td>
<td>0.8^a (0.0)</td>
<td>1.9^a (0.0)</td>
<td>69.3^a (1.2)</td>
<td>0.9^a (0.1)</td>
<td>5.0^a (0.1)</td>
<td>3.5^b (0.1)</td>
<td>4.6^a (0.3)</td>
</tr>
<tr>
<td>A0.5B-t4</td>
<td>0.6^b (0.0)</td>
<td>0.8^a (0.1)</td>
<td>2.0^a (0.0)</td>
<td>67.5^a (1.4)</td>
<td>0.9^a (0.1)</td>
<td>4.7^a (0.1)</td>
<td>3.5^b (0.2)</td>
<td>5.0^a (0.1)</td>
</tr>
<tr>
<td>A0.5B-t5</td>
<td>0.7^a (0.0)</td>
<td>0.8^a (0.0)</td>
<td>1.9^a (0.0)</td>
<td>66.2^a (0.5)</td>
<td>0.9^a (0.1)</td>
<td>4.5^a (0.1)</td>
<td>3.3^b (0.1)</td>
<td>4.8^a (0.1)</td>
</tr>
<tr>
<td>A1B-t0</td>
<td>0.8^a (0.0)</td>
<td>0.8^a (0.1)</td>
<td>1.8^a (0.1)</td>
<td>68.2^a (1.3)</td>
<td>1.1^a (0.1)</td>
<td>4.7^a (0.2)</td>
<td>4.5^a (0.3)</td>
<td>4.0^a (0.1)</td>
</tr>
<tr>
<td>A1B-t1</td>
<td>0.6^ab (0.1)</td>
<td>0.6^a (0.1)</td>
<td>1.6^a (0.1)</td>
<td>70.5^a (1.9)</td>
<td>0.8^b (0.1)</td>
<td>4.6^a (0.1)</td>
<td>3.1^b (0.2)</td>
<td>4.4^a (0.2)</td>
</tr>
<tr>
<td>A1B-t2</td>
<td>0.5^b (0.0)</td>
<td>0.7^a (0.1)</td>
<td>1.5^a (0.1)</td>
<td>69.5^a (3.9)</td>
<td>0.7^b (0.1)</td>
<td>4.2^a (0.3)</td>
<td>2.9^b (0.3)</td>
<td>4.2^a (0.3)</td>
</tr>
<tr>
<td>A1B-t3</td>
<td>0.5^b (0.1)</td>
<td>0.8^a (0.0)</td>
<td>1.5^a (0.0)</td>
<td>68.8^a (1.0)</td>
<td>0.8^b (0.1)</td>
<td>4.0^a (0.1)</td>
<td>2.8^b (0.1)</td>
<td>4.3^a (0.2)</td>
</tr>
<tr>
<td>A1B-t4</td>
<td>0.6^b (0.1)</td>
<td>0.7^a (0.1)</td>
<td>1.6^a (0.1)</td>
<td>67.1^a (3.2)</td>
<td>0.8^ab (0.1)</td>
<td>4.4^a (0.2)</td>
<td>2.7^b (0.2)</td>
<td>4.4^a (0.2)</td>
</tr>
<tr>
<td>A1B-t5</td>
<td>0.6^ab (0.0)</td>
<td>0.7^a (0.1)</td>
<td>1.6^a (0.0)</td>
<td>69.8^a (3.8)</td>
<td>1.0^ab (0.1)</td>
<td>4.8^a (0.2)</td>
<td>2.4^b (0.2)</td>
<td>4.3^a (0.3)</td>
</tr>
<tr>
<td>A2B-t0</td>
<td>0.5^a (0.0)</td>
<td>0.7^a (0.2)</td>
<td>2.0^a (0.1)</td>
<td>71.7^a (2.0)</td>
<td>1.2^a (0.1)</td>
<td>4.9^a (0.1)</td>
<td>4.0^a (0.2)</td>
<td>4.8^a (0.0)</td>
</tr>
<tr>
<td>A2B-t1</td>
<td>0.4^a (0.0)</td>
<td>0.6^a (0.1)</td>
<td>1.7^b (0.0)</td>
<td>69.1^a (0.8)</td>
<td>1.1^a (0.0)</td>
<td>5.1^a (0.1)</td>
<td>3.1^ab (0.0)</td>
<td>5.2^a (0.1)</td>
</tr>
<tr>
<td>A2B-t2</td>
<td>0.5^a (0.0)</td>
<td>0.6^a (0.1)</td>
<td>1.7^ab (0.1)</td>
<td>72.5^a (1.2)</td>
<td>1.0^a (0.1)</td>
<td>4.9^a (0.0)</td>
<td>2.5^b (0.1)</td>
<td>4.9^a (0.3)</td>
</tr>
<tr>
<td>A2B-t3</td>
<td>0.5^a (0.0)</td>
<td>0.6^a (0.0)</td>
<td>1.7^b (0.1)</td>
<td>71.6^a (0.6)</td>
<td>1.1^a (0.1)</td>
<td>5.2^a (0.1)</td>
<td>2.5^b (0.1)</td>
<td>5.3^a (0.2)</td>
</tr>
<tr>
<td>A2B-t4</td>
<td>0.5^a (0.0)</td>
<td>0.5^a (0.1)</td>
<td>1.6^b (0.0)</td>
<td>71.2^a (1.2)</td>
<td>1.0^a (0.1)</td>
<td>4.9^a (0.1)</td>
<td>2.2^b (0.1)</td>
<td>5.1^a (0.2)</td>
</tr>
<tr>
<td>A2B-t5</td>
<td>0.5^a (0.0)</td>
<td>0.5^a (0.1)</td>
<td>1.6^b (0.1)</td>
<td>72.6^a (2.1)</td>
<td>1.0^a (0.0)</td>
<td>5.1^a (0.1)</td>
<td>2.2^b (0.0)</td>
<td>4.7^a (0.2)</td>
</tr>
</tbody>
</table>

Notes: Standard deviations of three replicates are represented between parentheses. Values in the whole column for each monosaccharide and Klason lignin with different letters are statistically different at a confidence level of 95%.
Figure 4.8 – Glucan content of fibers treated with Endo-PG and pectin lyase for different periods of time (0-300 min) after each tested hydrothermal pretreatment in the autoclave at different pressures (0.5, 1 and 2 bar) and respective control (without autoclave pretreatment). For the same incubation time with said enzymes, values with different letters are significantly different at a confidence level of 95%, while the F-Test and p-value are presented for samples that are not significantly different.

Figure 4.9 – Galacturonic acid content of fibers treated with Endo-PG and pectin lyase for different periods of time (0-300 min) after a hydrothermal pretreatment in the autoclave at different pressures (0.5, 1 and 2 bar) and respective control (without autoclave pretreatment). For the same incubation time with said enzymes, values with different letters are significantly different at a confidence level of 95%.
difference in the glucose content of pre-autoclaved and non-autoclaved fibers. The exception occurred for 300 minutes of incubation, due to a decrease in glucose content of A0.5B-t5 fibers, but it is reasonable to admit that it represents a false positive decrease.

As regards to Figure 4.9, one can conclude that the most significant decrease in the galacturonic acid content happened in the first 30 minutes of incubation with pectinases, which is in agreement with the results presented for the weight loss (Figure 4.7). More precisely, ca. 43, 29, 31 and 23% of the initial (t0) content of galacturonic acid was removed in UT, A0.5B, A1B and A2B fibers, respectively. It is true that the highest degradation rate was observed for non-autoclaved fibers, but that is because there was more substrate available for the enzymes than in pre-autoclaved fibers, for which part of galacturonic acid had been removed in the autoclaving process.

According to the statistical analysis (Table 4.9), for all the experiments there is no significant difference in the GalA content from 30 to 300 minutes of incubation (in Tukey multiple comparison test, there is at least one letter in common for all GalA contents in the same experiment, from t1 to t5). From Figure 4.9, it is mostly evident for non-autoclaved (UT) fibers, where a plateau is identified after 30 minutes of incubation, in the curve of GalA content vs. time. However, as regards to pre-autoclaved fibers (A0.5B, A1B and A2B), an exponential decrease throughout time in the GalA content is perceivable. From this fact, one can conclude that in the first 30 minutes of incubation (t1), the enzymes acted intensively on GalA, but afterwards, an accessibility limitation for the substrate is apparent for UT fibers. For pre-autoclaved fibers, although it is evident a continuous decrease in the GalA degradation rate, the substrate limitation most likely happened after 90 minutes of incubation (t2).

Nevertheless, the most noteworthy conclusion to retain, and actually the main purpose of this study, is related with the enzymatic accessibility of pectinases for hemp fibers structure. From Figure 4.9, it is evident that there was an increase in the accessibility of Endo-PG and pectin lyase for pectins in the middle lamella, for fibers that had been hydrothermally pretreated, which is explained by the total degradation of GalA. Otherwise, it would be expected that, independently of the initial amount of galacturonic acid (t0), the curves GalA content vs. time, would converge in the same plateau after a certain period of time.

Table 4.10 resumes the total reduction of galacturonic acid (in content) in each experiment, roughly determined through Equation 2 (see subsection 3.2.1), considering the average values of GalA previously presented in Table 4.9.

Comparing the enzymatic treatments using different hydrothermally pretreated fibers as substrate, A0.5B, A1B and A2B fibers show, respectively, 58, 69 and 72% of total reduction of GalA, against only 47% for UT fibers. Therefore, one can conclude that the increase in the enzymatic accessibility is correlated with an increase in the operating pressure/temperature at the hydrothermal pretreatment stage, most likely due to an increase in the porosity of middle lamella with the raising harshness of the autoclaving process.
Table 4.10 – Partial and total reduction in the content of galacturonic acid, after combination of a hydrothermal pretreatment at different pressures (0.5, 1 and 2 bar) with an enzymatic treatment with Endo-PG and pectin lyase.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment</th>
<th>Enzymatic treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min (t1)</td>
<td>90 min (t2)</td>
</tr>
<tr>
<td>UT</td>
<td>-</td>
<td>43.5</td>
</tr>
<tr>
<td>A0.5B</td>
<td>29.2</td>
<td>20.9</td>
</tr>
<tr>
<td>A1B</td>
<td>41.2</td>
<td>18.4</td>
</tr>
<tr>
<td>A2B</td>
<td>48.5</td>
<td>11.4</td>
</tr>
</tbody>
</table>

* Negative values indicate a relative increase in GalA content

However, it is interesting to highlight that, in terms of GalA content, no statistically significant difference was recorded between A1B and A2B fibers for different periods of incubation (see Figure 4.9). Thus, using an autoclaving pretreatment at 1 bar is advantageous, because it saves energy costs.

Furthermore, pre-autoclaved and non-autoclaved fibers treated for 90 minutes (t2) with Endo-PG and pectin lyase were selected to study the impact of the enzymatic treatment on the mechanical properties of hemp fibers, since, after said period of incubation, no significant differences were recorded in the chemical composition of hydrothermally pretreated fibers, particularly in glucose content. Table 4.11 presents the results in terms of UTS, strain and stiffness.

Table 4.11 – Mechanical properties of pre-autoclaved fibers at 0.5, 1 and 2 bar (A0.5B, A1B and A2B respectively) and non-autoclaved fibers (UT), before and after 90 minutes of incubation with Endo-PG and pectin lyase, in terms of UTS, strain and stiffness, measured as Young’s Modulus. For the same mechanical property and treatment stage, values with different letters are significantly different at a confidence level of 95%.
In comparison with the mechanical properties of the fibers after the hydrothermal pretreatment in the autoclave, one can conclude that the enzymatic treatment did not significantly affect the mechanical performance of pre-autoclaved fibers, since the UTS, strain and stiffness are comparable with UT fibers. The same conclusion was not reported by Korte S. et al (2008) [68], where an enzymatic treatment was shown to decrease the mechanical properties of the fibers, but in that study the treatment was carried out for longer periods of time.

On the other hand, for non-autoclaved fibers (UT) all the mechanical properties were affected after the enzymatic treatment: the UTS decreased from ca. 771 to 636 MPa; the strain decreased from ca. 5.1 to 3.8%; and stiffness decreased from ca. 29 to 18 GPa. The reason is most likely the same as previously enunciated for the hydrothermal pretreatments. After 90 minutes of incubation with enzymes, the individual fibers got loose with pectin’s removal from middle lamella, negatively affecting the mechanical performance of fiber bundles.

In the end, it is noteworthy that, after the enzymatic treatment, the fibers that had been pre-autoclaved show better mechanical performance than non-autoclaved fibers, in terms of UTS and stiffness, especially fibers pre-autoclaved at 1 bar. As regards to strain, the value decreases from UT to A2B fibers, along with the final GalA content (the lower the GalA content, the lower the coherence of fiber bundles, the lower the deformation resistance).

In short, combining the mechanical properties of resulting fibers, the pectin degradation rate (which can be correlated with defibrillation degree) and also the energy spent in the autoclaving processing, the hydrothermal pretreatment in an autoclave at 1 bar is assumed to be the best pretreatment in study, both to enhance the accessibility of enzymes, as to obtain the best fibers in terms of mechanical performance.

4.3 Chemical pretreatment of hemp fibers to enhance accessibility of enzymes

The main purpose of the current study was to investigate the influence of different chemical pretreatments of hemp fibers as a way to enhance the accessibility of enzymes for hemp fibers. The effectiveness of each pretreatment has also been evaluated in terms of chemical composition analysis and mechanical properties determination.

As a preliminary investigation, a study concerning the influence of the concentration of tested chemical agents in the chemical composition and physical and mechanical properties of hemp fibers was conducted. The experimental design and the correspondent results are described in appendix 7.1.

Based on the previous results, specific conditions for the chemical treatments have been selected, to be used as pretreatment in an enzymatic accessibility study, in which arises the hypothesis that a partial
removal of non-cellulosic materials can increase the accessibility of enzymes for hemp fibers structure. This hypothesis has been tested before by other authors. In the work of Li & Pickering (2008) [26], a pretreatment with the chelating agent (EDTMP) was used prior to a pectinase treatment, but the increase in enzymatic accessibility was not clear. Also George et al. (2015) [59] used NaOH, as fiber swelling agent, alone or combined with further enzymatic treatments with laccase, pectinase and xylanase. However, it was only shown an increase in xylanase accessibility based on weight loss and X-ray photoelectron spectroscopy results.

4.3.1 Experimental design

Figure 4.10 illustrates the experimental design for the chemical pretreatment study. Different combinations of chemical pretreatments have been tested prior to enzymatic treatments: EDTA-2Na was combined with Endo-PG to possibly enhance said enzyme accessibility for pectins in middle lamella; a pretreatment with 1% EDTA-2Na was used to remove pectin and possibly enhance the accessibility of laccase for lignin; and a sequential 2-stage chemical pretreatment with 1% EDTA-2Na and 0.7% sodium chlorite was used to respectively remove pectin and lignin, to possibly enhance xylanase and xyloglucanase accessibility for hemicellulose.

Figure 4.10 – Overview of the study to determine the impact of different chemical pretreatments in the accessibility of enzymes for hemp fibers structure, effectively “measured” in terms of chemical composition analysis and mechanical properties determination. Each treatment has a code associated, represented on the right part of the scheme.
According to the results obtained in the preliminary study described in appendix 7.1, an additional investigation was done, regarding the use of chemical pretreatments, more specifically with EDTA and/or NaOH, to enhance the accessibility of Endo-PG. Both sodium hydroxide and EDTA have also been used by other authors [32, 57, 64, 94], prior to an enzymatic treatment, with the purpose of getting a higher extension of defibrillation and removal of non-cellulosic components from hemp fibers.

In this context, both concentration of chemical agents and temperature (40 or 60 ºC) were combined prior to an enzymatic treatment with Endo-PG (0.2% w/w), as represented in Table 4.12. As control tests, an enzymatic treatment with Endo-PG was carried out on hemp fibers pre-incubated in miliQ water, i.e. without chemical pretreatment. The control tests are also represented in Table 4.12, as Test 0 and 7. The effect of each chemical pretreatment on Endo-PG accessibility to middle lamella was evaluated by chemical composition analysis and weight loss determination. The treatments have been carried out as described in subsections 3.2.3 and 3.2.4.

**Table 4.12** – Experimental design to investigate the use of chemical pretreatments with NaOH and/or EDTA at 40 ºC or 60 ºC as enzyme accessibility enhancers.

<table>
<thead>
<tr>
<th>Test No.</th>
<th>Step I – Chemical Pretreatment</th>
<th>Step II – Enzymatic Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDTA (%)</td>
<td>NaOH (%)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**4.3.2 Results and discussion**

**4.3.2.1 Chemical pretreatments to enhance enzyme accessibility for hemp fibers**

Table 4.13 shows the total weight loss of hemp fibers in all different experiments, previously illustrated in Figure 4.10. The enzymatic treatments carried out with laccase or a combination of xylanase and xyloglucanase (PLac and PL-XG/XN, respectively) were preceded by a pretreatment with chemical agents, while for Endo-PG both enzyme and chelating agent EDTA-2Na were mixed together in the same treatment (P0.5+EPG).

In comparison with the preliminary study about the effect of different chemical agents on hemp fibers, the laccase treatment has been used as alternative to sodium chlorite to delignify hemp fibers previously subjected to 1% EDTA-2Na treatment to partially degrade pectin (see appendix 7.1.2). The weight loss of
Table 4.13 – Total weight loss in different experiments using chemical agents as accessibility enhancers of Endo-PG, laccase and xylanase/xyloglucanase accessibility for hemp fibers structure.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment Code</th>
<th>Step I - Chemical Pretreatment</th>
<th>Weight loss (%)</th>
<th>Step II - Enzymatic Pretreatment</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-PG</td>
<td>P0.5+EPG</td>
<td>-</td>
<td>9.31 (0.30)</td>
<td>9.31 (0.30)</td>
<td></td>
</tr>
<tr>
<td>Laccase</td>
<td>PLac</td>
<td>1.48 (0.52)</td>
<td>0.67 (0.14)</td>
<td>2.15 (0.66)</td>
<td></td>
</tr>
<tr>
<td>Xyloglucanase &amp; Xylanase</td>
<td>PL-XG/XN</td>
<td>2.22 (0.66)</td>
<td>2.26 (0.50)</td>
<td>4.48 (1.16)</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** For presented weight loss values, a control has been subtracted, for which fibers have been soaked in water (step I) or in buffer (step II) in the same conditions as test studies (T, pH, shaking speed, etc.)

Hemp fibers for laccase treatment (0.67 ± 0.14%) is in the same magnitude as the chemical treatments carried out with 0.7 or 1.4% of NaClO₂, using the same substrate. George *et al.* (2015) [59] also reported 1.02 ± 0.19% of weight loss in a laccase treatment of hemp fibers, using the same concentration of enzyme.

In turn, the enzyme treatment with xyloglucanase and xylanase has been used as alternative to sodium hydroxide treatment to remove hemicellulose from hemp fibers that were previously submitted to a sequential pretreatment with 1% of EDTA-2Na and 0.7% of NaClO₂ (see appendix 7.1.3). In this case, the weight loss is in the same magnitude as PL fibers treated with 2.5% of sodium hydroxide.

As regards to the enzymatic treatment where 0.5% (w/v) of EDTA-2Na was mixed with 0.2% (w/w) of Endo-PG, the total weight loss was compared with solo enzymatic and EDTA-2Na treatments, as presented in Figure 4.11. From the results of weight loss, it is expected that the presence of EDTA-2Na significantly increased the accessibility of Endo-PG for pectin substrates in middle lamella, since the total weight loss (9.31 ± 0.30%) is ca. 50% higher than the maximum theoretical weight loss (6.19 ± 1.32%), obtained by summing the weight loss of single EDTA-2Na and Endo-PG treatments.

Table 4.14 shows the chemical composition analysis for the enzymatic treatments in study. In PLac treated samples the content of Klason lignin remains unchanged in comparison with just chemically pretreated fibers, *i.e.* non-enzymatically treated samples (P1). In the same way, for PL-XG/XN treated fibers, the relative content of xylose is statistically similar (p>0.05) to just chemically pretreated fibers (PL0.7).

Most part of lignin is allocated in the middle lamella and its complexity makes it very difficult to be degraded. As regards to xyloglucan, it is known that is tightly bound to cellulose microfibrils by hydrogen bonds [70]. Therefore, the most plausible reason for the inefficiency of said enzymatic treatments is that the degree of pectin degradation in the chemical pretreatment with 1% EDTA-2Na was not enough to improve the accessibility of the enzymes for inner regions of hemp fibers structure. On the other hand, and mainly for xyloglucanase and xylanase, the applied dosage of the enzymes may have been too low to
Bomogalacturonan

Figure 4.11 – Total dry matter weight loss of Endo-PG treatment in EDTA-2Na solution (A+B) vs. expected total weight loss (C*) as a sum of the weight loss of solo EDTA-2Na (A) and Endo-PG (B) treatments.

Table 4.14 – Anhydrous monosaccharides and Klason lignin content of enzymatically treated fibers with Endo-PG in 0.5% EDTA-2Na, laccase or xylanoglucanase combined with xylanase.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rha (g/100 g dry matter)</th>
<th>Ara (g/100 g dry matter)</th>
<th>Gal (g/100 g dry matter)</th>
<th>Glu (g/100 g dry matter)</th>
<th>Xyl (g/100 g dry matter)</th>
<th>Man (g/100 g dry matter)</th>
<th>GalA (g/100 g dry matter)</th>
<th>Klason Lignin (g/100 g dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw fibers</td>
<td>1.0 (0.1)</td>
<td>1.3 (0.1)</td>
<td>2.1 (0.1)</td>
<td>60.0 (0.9)</td>
<td>1.2 (0.1)</td>
<td>3.0 (0.3)</td>
<td>7.3 (0.4)</td>
<td>5.9 (0.2)</td>
</tr>
<tr>
<td>P0*</td>
<td>0.7 (0.1)</td>
<td>1.2 (0.1)</td>
<td>2.1 (0.1)</td>
<td>68.0 (2.7)</td>
<td>1.5 (0.3)</td>
<td>4.5 (0.2)</td>
<td>7.7 (0.3)</td>
<td>5.3 (0.3)</td>
</tr>
<tr>
<td>P0.5*</td>
<td>0.9 (0.1)</td>
<td>0.8 (0.1)</td>
<td>2.1 (0.1)</td>
<td>76.8 (1.3)</td>
<td>1.0 (0.1)</td>
<td>5.2 (0.1)</td>
<td>5.9 (0.3)</td>
<td>3.8 (0.1)</td>
</tr>
<tr>
<td>EPG*</td>
<td>0.6 (0.1)</td>
<td>0.9 (0.1)</td>
<td>1.9 (0.1)</td>
<td>70.9 (0.8)</td>
<td>1.1 (0.1)</td>
<td>5.0 (0.1)</td>
<td>4.8 (0.4)</td>
<td>4.8 (0.3)</td>
</tr>
<tr>
<td>P0.5+EPG</td>
<td>0.6 (0.1)</td>
<td>0.8 (0.1)</td>
<td>1.7 (0.3)</td>
<td>68.2 (4.3)</td>
<td>1.0 (0.1)</td>
<td>4.6 (0.4)</td>
<td>2.1 (0.3)</td>
<td>4.7 (0.1)</td>
</tr>
<tr>
<td>P1*</td>
<td>0.7 (0.1)</td>
<td>0.9 (0.0)</td>
<td>2.1 (0.1)</td>
<td>75.8 (2.1)</td>
<td>0.9 (0.1)</td>
<td>5.4 (0.1)</td>
<td>4.7 (0.2)</td>
<td>5.3 (0.3)</td>
</tr>
<tr>
<td>PLac</td>
<td>0.9 (0.0)</td>
<td>1.2 (0.0)</td>
<td>2.1 (0.1)</td>
<td>74.7 (4.4)</td>
<td>1.4 (0.0)</td>
<td>5.4 (0.4)</td>
<td>5.1 (0.1)</td>
<td>5.4 (0.3)</td>
</tr>
<tr>
<td>PL0.7*</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.0)</td>
<td>1.9 (0.1)</td>
<td>71.9 (1.1)</td>
<td>1.0 (0.1)</td>
<td>4.5 (0.1)</td>
<td>5.1 (0.1)</td>
<td>5.2 (0.3)</td>
</tr>
<tr>
<td>PL-XG/XN</td>
<td>0.7 (0.0)</td>
<td>0.7 (0.0)</td>
<td>1.9 (0.0)</td>
<td>71.8 (1.4)</td>
<td>1.0 (0.1)</td>
<td>5.0 (0.1)</td>
<td>4.3 (0.2)</td>
<td>4.9 (0.3)</td>
</tr>
</tbody>
</table>

Notes: Standard deviations of three replicates are represented between parentheses. Values in the whole column for the same monosaccharide or Klason lignin with different letters are statistically different at a confidence level of 95%.

*EPG – Treatment with 0.2% of Endo-PG in Buffer II (citric acid) at pH 6.0 and 40 °C for 4 h.
Px – Chemical treatment with x% of EDTA-2Na at pH 6.0 and 40 °C for 4 h.
PL0.7 – Sequential chemical treatment with 1% of EDTA-2Na at pH 6.0 and 40 °C for 4 h and 0.7% of sodium chlorite at pH 5.0 and 40 °C for 3 h

Effectively act on respective substrates.

On the contrary, for P0.5+EPG fibers, the chemical composition results confirm that the presence of EDTA-2Na allowed an increase in the accessibility of Endo-PG for homogalacturonan in middle lamella
region. As presented in Table 4.1, the galacturonic acid content decreased significantly (p<0.05) from 7.7 ± 0.8% (w/w) to 4.8 ± 0.4% (w/w) in a treatment carried out with 0.2% of Endo-PG, accounting for ca. 38% of reduction. However, in the presence of 0.5% of EDTA-2Na, the reduction in GalA content was more than two-fold higher (ca. 73%), with a statistically significant decrease (p<0.05) from 7.7 ± 0.8% (w/w) to 2.1 ± 0.3% (w/w).

The hypothesis that EDTA can destabilize and partially degrade pectin substrates, by sequestering calcium ions (which plays a crucial role in pectins structure) has been reported before [46], but a few authors investigated the possibility of enhancing the accessibility of enzymes for natural fibers. The work of Li & Pickering (2008) [26] is probably the most cited in this field, but in the reported results it is not clear if the chelating agent (EDTMP.Na5) increased the accessibility of the commercial pectinase. The pectin content of fibers, just treated with 0.5% of EDTMP.Na5 at pH 11 and 60 °C, is not much different from fibers treated with said chemical agent prior to an enzymatic treatment with pectinase (4.37 against 3.50%, respectively), and there is also a lack of a statistical analysis to clarify the results. However, the present study clearly shows the role of EDTA on Endo-PG accessibility.

The effect of the combined treatment with EDTA-2Na and Endo-PG on hemp fibers is now evaluated in terms of mechanical properties. According to the results presented in Figure 4.12, all the mechanical properties were negatively (p<0.05) affected in comparison with untreated fibers: the UTS decreased 28% from 771 ± 23 to 556 ± 99 MPa; the strain decreased 20% from 5.0 ± 0.9 to 4.1 ± 1.1%; and the Young’s Modulus decreased 47% from 28.5 ± 0.6 to 15.2 ± 4.0 GPa.

The fact that enzymatic treatment with pectinases can allow an extensive defibrillation, but ultimately affect the mechanical performance of the hemp fibers, especially in terms of tensile strength and stiffness, has also been reported by Korte & Staiger (2008) [68]. On the other hand Stuart et al. (2006) [47] only reported a decrease in tensile strength and no significant change in stiffness, after an enzymatic treatment alone or combined with EDTA. Even so, the results in terms of mechanical properties are highly dependent on the method used by each author to test the fibers.

From Figure 4.12, when comparing both single enzymatic and EDTA treatments of hemp fibers, it is also evident that the negative effect of the enzymes on fiber’s stiffness is more significant than the effect of EDTA, while for strain is the other way around. In terms of UTS, there is no statistically significant difference between fibers treated with 0.5% EDTA-2Na or 0.2% of Endo-PG, but when combined, the UTS is 15% lower. It is also noteworthy that fibers resulting from the combined treatment with EDTA and Endo-PG have more resistant to deformation than both solo treatments, as shown by strain values (elongation at break).
Figure 4.12 – Mechanical properties of hemp fibers, in terms of UTS (A), strain or elongation at break (B) and stiffness (C), after single or combined treatments with 0.5% of EDTA-2Na and 0.2%(w/w) of Endo-PG.

4.3.2.2 NaOH and/or EDTA treatment to enhance accessibility of Endo-PG

From the primary study about the effect of different chemical agents on hemp fibers (appendix 7.1), it was noticed that a chemical treatment with 0.5% EDTA-2Na does not significantly affect the tensile strength and stiffness of hemp fibers, under the experimental conditions. Moreover, it was previously shown that EDTA can be used to enhance the accessibility of Endo-PG in a combined enzymatic treatment.

In turn, in the study of the effect of NaOH on raw fibers (appendix 7.1), it was shown that the mechanical properties of treated fibers were affected independently of said alkali agent concentration. However, fibers treated with only 2.5% of NaOH showed less damage in the tensile strength, although less hemicellulose removal.

Therefore, the operating concentrations of said chemical agents have been selected according to the previous results and also taking into consideration that a lower concentration of chemicals will ultimately lead to less environmentally harmful treatments.

Table 4.15 shows the results of total weight loss in the combination tests, for which the experimental design was previously represented in Table 4.12. For each test, the enzymatic treatment (Stage II) has
been done immediately after the chemical pretreatment (Stage I) and thus, although the fibers were washed in between, there was no drying step to determine the weight loss of hemp fibers in each stage. Therefore the weight loss in the enzymatic treatment (Stage II) was estimated subtracting the total weight loss by the weight loss in the chemical pretreatment (Stage I), previously estimated.

**Table 4.15** – Total dry weight loss of hemp fibers in each accessibility test, after two stage treatments with chemical agents (Stage I) and Endo-PG (Stage II).

<table>
<thead>
<tr>
<th>Test</th>
<th>Stage I – Chemical Pretreatment</th>
<th>Stage II – Enzymatic treatment with Endo-PG</th>
<th>Total Weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDTA-2Na 0.5%</td>
<td>2.5% NaOH</td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>0</td>
<td>X</td>
<td>X</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>X</td>
<td>X</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>X</td>
<td>X</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>X</td>
<td>X</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>X</td>
<td>X</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td>X</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>X</td>
<td>X</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>X</td>
<td>X</td>
<td>60</td>
</tr>
</tbody>
</table>

**Note:** For the same operating temperature in the chemical pretreatment, values of weight loss in Stage II with different letters are statistically significant different at confidence level of 95%.

Using the estimated weight loss of control tests as base line (2.31 ± 0.43% and 2.79 ± 0.58, respectively for Tests 0 and 7), there was no statistically significant increase (p>0.05) in the estimated weight loss of enzymatic treatments after chemical pretreatment with EDTA-2Na at 40 and 60 °C (Test 1 and 4, respectively). However when NaOH was used alone (Test 3 and 6) or in combination with EDTA-2Na (Test 2 and 5) as pretreatment, there was a significant increase (p<0.05) in the estimated weight loss of the subsequent enzymatic treatment, suggesting that the mentioned tests might have increased the accessibility of Endo-PG for hemp fiber structures. On the other hand, the estimated weight losses were higher for hemp fibers that have been chemically pretreated at 60 °C, thus one can predict that temperature plays an important role on promoting an increase in the porosity of middle lamella.

In order to chemically analyze the effect of each presented test, Table 4.16 summarizes the chemical composition of hemp fibers after both chemical pretreatment (Stage I) and subsequent enzymatic treatment (Stage II). The values are organized in terms of pretreatment strategy, i.e. chemical pretreatment with EDTA-2Na (Tests 1 and 3), NaOH (Tests 3 and 6) or both combined (Tests 2 and 5);
and incubation in miliQ H2O (Tests 0 and 7). Pretreatments carried out at 40 °C and 60 °C are colored in blue and orange, respectively.

Table 4.16 – Anhydrous monosaccharides and Klason lignin content of hemp fibers after both chemical pretreatment (Stage I) and subsequent enzymatic treatment with Endo-PG (Stage II).

<table>
<thead>
<tr>
<th>Stage I</th>
<th>Samples</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>Klason Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount (g/100 g of dry matter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>RMW*</td>
<td>0.7 (0.1)</td>
<td>1.2 (0.1)</td>
<td>2.1 (0.1)</td>
<td>68.0 (2.7)</td>
<td>1.5 (0.3)</td>
<td>4.5 (0.2)</td>
<td>7.7 (0.8)</td>
<td>5.3 (0.2)</td>
</tr>
<tr>
<td></td>
<td>T0</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.1)</td>
<td>1.9 (0.1)</td>
<td>67.4 (3.0)</td>
<td>1.1 (0.4)</td>
<td>4.5 (0.2)</td>
<td>5.6 (0.7)</td>
<td>5.0 (0.1)</td>
</tr>
<tr>
<td></td>
<td>T0+EPG*</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.1)</td>
<td>1.9 (0.1)</td>
<td>69.8 (2.0)</td>
<td>0.9 (0.1)</td>
<td>4.9 (0.6)</td>
<td>4.2 (0.6)</td>
<td>4.9 (0.2)</td>
</tr>
<tr>
<td></td>
<td>T7</td>
<td>0.9 (0.0)</td>
<td>0.9 (0.2)</td>
<td>1.7 (0.0)</td>
<td>72.4 (3.6)</td>
<td>0.9 (0.2)</td>
<td>4.5 (0.2)</td>
<td>5.3 (0.3)</td>
<td>4.7 (0.3)</td>
</tr>
<tr>
<td></td>
<td>T7+EPG*</td>
<td>0.4 (0.0)</td>
<td>0.3 (0.0)</td>
<td>1.2 (0.0)</td>
<td>70.7 (2.2)</td>
<td>0.9 (0.1)</td>
<td>4.3 (0.1)</td>
<td>3.5 (0.3)</td>
<td>4.6 (0.1)</td>
</tr>
<tr>
<td>miliQ</td>
<td>T1</td>
<td>0.9 (0.1)</td>
<td>0.8 (0.1)</td>
<td>2.1 (0.1)</td>
<td>76.8 (1.3)</td>
<td>1.0 (0.1)</td>
<td>5.2 (0.1)</td>
<td>5.9 (0.3)</td>
<td>5.9 (0.3)</td>
</tr>
<tr>
<td>H2O</td>
<td>T1+EPG*</td>
<td>0.7 (0.0)</td>
<td>0.5 (0.0)</td>
<td>1.8 (0.0)</td>
<td>68.8 (2.0)</td>
<td>0.8 (0.0)</td>
<td>4.5 (0.3)</td>
<td>3.0 (0.0)</td>
<td>4.5 (0.2)</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>0.7 (0.0)</td>
<td>0.6 (0.1)</td>
<td>1.8 (0.0)</td>
<td>67.7 (2.6)</td>
<td>1.1 (0.0)</td>
<td>4.6 (0.1)</td>
<td>4.2 (0.4)</td>
<td>5.0 (0.2)</td>
</tr>
<tr>
<td></td>
<td>T4+EPG*</td>
<td>0.7 (0.0)</td>
<td>0.6 (0.1)</td>
<td>1.8 (0.0)</td>
<td>70.5 (5.9)</td>
<td>0.9 (0.1)</td>
<td>4.9 (0.5)</td>
<td>2.7 (0.1)</td>
<td>5.3 (0.4)</td>
</tr>
<tr>
<td>EDTA</td>
<td>T3</td>
<td>0.7 (0.0)</td>
<td>0.7 (0.0)</td>
<td>1.5 (0.0)</td>
<td>72.8 (1.5)</td>
<td>1.1 (0.0)</td>
<td>4.6 (0.1)</td>
<td>5.3 (0.4)</td>
<td>4.9 (0.1)</td>
</tr>
<tr>
<td></td>
<td>T3+EPG*</td>
<td>0.7 (0.0)</td>
<td>0.5 (0.0)</td>
<td>1.4 (0.1)</td>
<td>72.7 (1.4)</td>
<td>0.8 (0.0)</td>
<td>4.8 (0.5)</td>
<td>2.9 (0.3)</td>
<td>4.2 (0.3)</td>
</tr>
<tr>
<td></td>
<td>T6</td>
<td>0.6 (0.0)</td>
<td>0.5 (0.2)</td>
<td>1.9 (0.2)</td>
<td>74.3 (2.2)</td>
<td>1.0 (0.1)</td>
<td>4.7 (0.2)</td>
<td>3.4 (0.5)</td>
<td>5.4 (0.1)</td>
</tr>
<tr>
<td></td>
<td>T6+EPG*</td>
<td>0.5 (0.0)</td>
<td>0.3 (0.0)</td>
<td>1.1 (0.1)</td>
<td>84.0 (8.2)</td>
<td>0.8 (0.0)</td>
<td>4.9 (0.1)</td>
<td>1.1 (0.0)</td>
<td>3.6 (0.4)</td>
</tr>
<tr>
<td>NaOH</td>
<td>T2</td>
<td>0.7 (0.0)</td>
<td>0.5 (0.0)</td>
<td>1.8 (0.0)</td>
<td>75.1 (1.2)</td>
<td>0.9 (0.0)</td>
<td>5.0 (0.3)</td>
<td>3.7 (0.1)</td>
<td>5.4 (0.1)</td>
</tr>
<tr>
<td></td>
<td>T2+EPG*</td>
<td>0.7 (0.0)</td>
<td>0.5 (0.0)</td>
<td>2.2 (0.0)</td>
<td>73.7 (0.2)</td>
<td>0.9 (0.1)</td>
<td>5.1 (0.3)</td>
<td>2.4 (0.2)</td>
<td>4.1 (0.1)</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>0.5 (0.0)</td>
<td>0.6 (0.1)</td>
<td>1.9 (0.1)</td>
<td>78.5 (2.1)</td>
<td>0.9 (0.1)</td>
<td>4.9 (0.2)</td>
<td>3.6 (0.2)</td>
<td>5.8 (0.5)</td>
</tr>
<tr>
<td></td>
<td>T5+EPG*</td>
<td>0.4 (0.0)</td>
<td>0.3 (0.0)</td>
<td>1.6 (0.0)</td>
<td>81.9 (1.4)</td>
<td>0.8 (0.1)</td>
<td>4.8 (0.3)</td>
<td>1.2 (0.0)</td>
<td>4.1 (0.3)</td>
</tr>
</tbody>
</table>

* RMW – Raw fibers after washing 3 times with warm miliQ water (40 °C) and drying at 50 °C, for 12 h.
EPG – Treatment carried out with 0.2% of Endo-PG in Buffer II (citric acid) at pH 6.0 and 40 °C, for 4 h.

Notes:
- Standard deviations of three replicates are represented in parentheses;
- Pretreatments carried out at 40 °C and 60 °C are colored in blue and orange, respectively;
- Values of GalA or glucose contents for the same experiment (i.e. miliQ H2O, EDTA, NaOH or EDTA+NaOH) with different letters are statistically different at a confidence level of 95%.

As regards to the pretreatment stage, the relative content of pectin, decreased in all of the tested experiments, although in different extents. This is easily observable in terms of GalA content, as shown in Table 4.16. Furthermore, it is noteworthy that chemical pretreatments at 60 °C seem to affect more significantly rhamnose, arabinose and GalA, since said monosaccharides content is lower than for the equivalent pretreatments at 40 °C. Thus, it is proven that temperature plays an important role on pectin’s removal from hemp fibers structure, as had already been discussed in section 4.2. On the other hand, the
glucose content is not statistically significant different (p<0.05) in the chemical pretreatments carried out at 40 or 60 ºC.

Afterwards, during the enzymatic treatment, an extra portion of GalA has been removed from hemp fibers (Table 4.16), as expected, since Endo-PG has GalA as specific substrate. The evolution of the GalA relative content of hemp fibers during different stages of the experiments, are illustrated in Figures 4.13 and 4.14, respectively for experiments where the pretreatment was carried out at 40 and 60 ºC.

![Figure 4.13](image)

**Figure 4.13** – Relative content of galacturonic acid in hemp fibers after chemical pretreatment at 40 ºC (Stage I) and subsequent enzymatic treatment with Endo-PG (Stage II). Stage 0 corresponds to raw hemp fibers after washing with warm miliQ water (40 ºC) and drying at 50 ºC for 12h. For the same stage and in comparison with stage 0, contents of GalA with different letters are significantly different at a confidence level of 95%.

Just considering the chemical pretreatment, it is clear that, for both operational temperatures, the highest decrease in the relative content of GalA (and generally pectin) occurred in the tests were both NaOH and EDTA were mixed together in the same treatment (Tests 2 and 5). However, if on one hand the GalA content of fibers treated with EDTA-2Na and NaOH at 40 ºC (Test 2) was statistically significant different than in fibers obtained from other pretreatments (Figure 4.13), on the other, at 60 ºC, there was no statistically significant difference in the GalA content of fibers treated with EDTA-2Na, NaOH or both combined (Figure 4.14).

Moreover, in pretreatments at 40 ºC, the decrease in the relative content of GalA in fibers treated with EDTA-2Na (Test 1) or NaOH (Test 3), is not statistically significant different (p>0.05) than in fibers incubated in miliQ water (Test 0). Thus no apparent effect of said chemical agents on the degradation of pectin is predicted at said temperature, when used separately. Nevertheless, NaOH seems to have a bigger effect on GalA removal than EDTA-2Na, since at 60 ºC, the extent of reduction in GalA content increased from fibers incubated in miliQ water (Test 7) to fibers treated with EDTA-2Na (Test 4) and even
Figure 4.14 – Relative content of galacturonic acid in hemp fibers after chemical pretreatment at 60 °C (Stage I) and subsequent enzymatic treatment with Endo-PG (Stage II). Stage 0 corresponds to raw hemp fibers after washing with warm miliQ (40°C) water and drying at 50 °C for 12h. For the same stage and in comparison with stage 0, contents of GalA with different letters are significantly different at a confidence level of 95%.

more to fibers treated with NaOH (Test 6).

Now looking for all experiments as a whole, and considering the control test (Tests 0 and 7) as base line, it is evident that all the chemical pretreatments led to an increase in total pectin removal in the final enzymatic treatment, shown by the evolution of the relative content of GalA in treated fibers (Figures 4.13 and 4.14). In other words, after the chemical pretreatment, the enzyme was able to reach deeper zones in the middle lamella that were not accessible when the fibers were just soaked in miliQ water. Moreover based on the results previously shown in section 4.2, relatively to the enzymatic treatment with pectinases throughout time, one can admit that, in 4 hours of each conducted experiment, all the accessible GalA has been degraded by the enzyme.

Compared to the control test, for the study at 40 °C, the extension of GalA reduction (in content) after the enzymatic treatment increased ca. 15, 17 and 23%, respectively when EDTA-2Na, NaOH or both chemical agents mixed together, were used in the pretreatment. On the other hand, for the study at 60 °C, and in the same order of experiments, the extension of GalA reduction (in content) after the enzymatic treatment increased ca. 10, 31 and 29%.

It is also noteworthy that when 0.5% EDTA-2Na was used as pretreatment at 40 °C prior to the enzymatic treatment with Endo-PG (Test 1), the total decrease in the GalA content was 61%, which is lower than when both EDTA-2Na and said enzyme were mixed together in the same treatment (73%). Thus, it can be concluded that in the presence of EDTA, the enzyme can access more GalA, probably due to its
destabilizing effect on pectin structure. On the other hand, the treatment for which both EDTA-2Na and Endo-PG were mixed together is much advantageous, since it saves time and spent energy.

In sum, these results prove that EDTA and NaOH, either together or alone, can be used as pretreatment to increase the accessibility of Endo-PG for inner regions of middle lamella, most probably due to an increase in porosity. The highest total decrease in GalA content was shown when NaOH was used in a pretreatment at 60 °C, alone or combined with EDTA-2Na, from 7.7 ± 0.8% to 1.1 ± 0.0% and 1.2 ± 0.0%, respectively (Table 4.16). However, to conclude which of the pretreatments is the best, the degree of defibrillation and also the mechanical properties of resulting fibers should be compared.

### 4.4 A comparison of different pretreatments

By way of summary, Table 4.17 summarizes the best pretreatments of hemp fibers prior to an enzymatic treatment with pectinases, according to what was shown in this chapter.

**Table 4.17** – Summary of the mechanical properties and total reduction in content of galacturonic acid of hemp fibers resulting from different experiments tested in the present project.

<table>
<thead>
<tr>
<th>Code</th>
<th>Pretreatment</th>
<th>-ΔGalA* (%)</th>
<th>UTS (MPa)</th>
<th>YM (GPa)</th>
<th>Strain (%)</th>
<th>Time of pretreatment (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pr0.5w</strong></td>
<td>Biological</td>
<td>73</td>
<td>646 (73)</td>
<td>29.4 (4.3)</td>
<td>2.8 (0.7)</td>
<td>84</td>
</tr>
<tr>
<td><strong>FR1w</strong></td>
<td>Biological</td>
<td>67</td>
<td>832 (198)</td>
<td>32.5 (4.8)</td>
<td>4.8 (1.8)</td>
<td>168</td>
</tr>
<tr>
<td><strong>A1B</strong></td>
<td>Hydrothermal</td>
<td>65</td>
<td>686 (117)</td>
<td>31.0 (6.1)</td>
<td>2.9 (0.7)</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>P0.5+EPG</strong></td>
<td>Chemical</td>
<td>73</td>
<td>556 (99)</td>
<td>15.2 (4.0)</td>
<td>4.1 (1.1)</td>
<td>0</td>
</tr>
<tr>
<td><strong>EDTA+NaOH 40</strong></td>
<td>Chemical</td>
<td>69</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4</td>
</tr>
<tr>
<td><strong>NaOH 60</strong></td>
<td>Chemical</td>
<td>86</td>
<td>563 (87)</td>
<td>18.1 (4.1)</td>
<td>3.7 (0.8)</td>
<td>4</td>
</tr>
</tbody>
</table>

**Notes:**
1 – Only for P0.5+EPG it was considered the effect of subsequent enzymatic treatment on the mechanical properties; 2 – The mechanical properties presented for NaOH 60 are correspondent to the treatment at 40 °C; 3 – After both chemical and biological pretreatments, both Endo-PG and pectin lyase were used in the enzymatic treatment, while only the first was used after chemical pretreatments.

* Decrease in the relative content of galacturonic acid after both pretreatment and subsequent enzymatic treatment

n.d. – not determined

Pr0.5w – Natural fungal treatment for half a week, in a controlled environment
FR1w – Field retting for one week
A1B – Autoclaving process for 30 minutes at 1 bar (121 °C)
P0.5+EPG – 0.5% EDTA-2Na directly mixed with 0.2% Endo-PG at 40 °C
EDTA+NaOH 40 – 0.5% EDTA-2Na mixed with 2.5% of NaOH at 40 °C
NaOH 60 – 2.5% NaOH at 60 °C
Comparing the total reduction in the relative content of GalA in the resulting hemp fibers, after pretreatment and subsequent enzymatic treatment, the highest decrease was recorded when fibers were pretreated with 2.5% of NaOH at 60 ºC (ca. 86%), followed by both fungal retting for half a week and combination of 0.5% of EDTA-2Na with Endo-PG (ca. 73%). However, the values are quite similar, and most likely the degree of defibrillation in all these final hemp fibers would be comparable.

In terms of mechanical performance, the best pretreatment was the field retting for 1 week, for which no significant difference was found in the mechanical properties of resulting fibers, in comparison with raw fibers (shown previously in section 4.1). For both hydrothermal pretreatment in an autoclave at 1 bar and fungal retting for half a week, only strain (or elongation at break) was significantly affected. In turn, when chemical agents were used as pretreatment, a decrease in all the mechanical properties was recorded.

As regards to the total time required to conclude the whole treatment, the experiment where both EDTA-2Na and Endo-PG where combined in the same treatment is clearly advantageous over used separately, since it saves the time needed for pretreatment. Then, the productivity of the hydrothermal pretreatment is 8 times higher than the chemical pretreatment, since only 30 minutes were used to subject the fibers to steam at 121 ºC. The biological pretreatments are clearly time consuming, even so the fungal retting is considered to be a better option than field retting, since it is faster and both present a similar total reduction of GalA content.

To project all presented treatments of hemp fibers to an industrial scale, environmental as well as economic factors must be carefully considered.

From an ecological point of view, the hydrothermal pretreatment has the lowest environmental impact, representing a clean process in terms of effluents. However, this conclusion would be dependent on the energy source used to produce steam. In the same way, for fungal retting, there wouldn’t be harmful effluents being generated, but an extra-sterilization step would be required to decontaminate the growth medium, before discharge. As regard to field retting, the process brings the advantage of not requiring water or any energy input, but, as previously highlighted, several problems associated to this method created the necessity for alternatives. The chemical pretreatments are the less ecological option, since, although the chemical agents were used in relatively low concentrations, there is always the need of effluents treatment, for instance neutralization in the case of sodium hydroxide.

From an economic perspective, excluding field retting, which does not require energy inputs, except for mechanical field operations, and thus is considered a low cost option, it is difficult to clearly conclude what would be the best option. Higher temperatures require higher inputs of energy. Thus, theoretically, both hydrothermal and fungal pretreatments would be more expensive than chemical pretreatments. Both would require steam production at 121 ºC to use as pretreatment or to sterilize the growth medium, respectively. However, this cost could be less effective if there was the possibility of process integration (also known as Pitch analysis), a technique for designing a process in order to minimize the energy
consumption and maximize heat recovery. On the other hand, although the processing temperature was lower for chemical pretreatments, the additional cost of chemicals and effluents treatment must be considered.

Taking into account all the factors analyzed above, the hydrothermal pretreatment is considered to be the best pretreatment of hemp fibers prior to an enzymatic treatment, resulting in a good balance between economics, ecology, performance of the treatment and mechanical properties of resulting fibers.
5. Conclusions and future prospects

In this project it was successfully demonstrated that different approaches can be used as pretreatment of hemp fibers, in order to enhance the accessibility of enzymes in a subsequent enzymatic treatment, improving its efficiency. More precisely, a hydrothermal pretreatment in an autoclave at 1 bar for 30 minutes, a natural short-period fungal treatment in a controlled environment, an alkali treatment with 2.5% of sodium hydroxide at 60 °C or the direct combination of 0.5% of EDTA-2Na with pectinases, were shown to be promising solutions for a future industrial application.

As regards to the different methods applied in this project, the combination of both weight loss determination and chemical composition analysis by HPAEC-PAD was found to be an effective way to investigate the effect of different pretreatments on hemp fibers and ultimately characterize the enhanced accessibility of enzymes for substrates. In addition, the method of tensile strength testing, used for the determination of the mechanical properties, was found to be very useful since it allowed to evaluate the effect of each treatment on the physical properties of hemp fibers due to the removal of non-cellulosic materials and/or modifications in cellulosic fibers. In turn, the Water Retention Value determination was proven to give useful and correlated information in regard to non-cellulosic components removal from hemp fibers. However, the water retention capacity of the fibers is dependent of different physical phenomena, thus, in order to easily interpret the results, other complementary parameters, as capillarity and water sorption, must be considered.

In section 4.1, combining the results of total reduction in the content of galacturonic acid with the mechanical properties of hemp fibers, after each tested pretreatment, a fungal retting process carried out for half a week was shown to be the best solution, since the pretreatment allowed to partially remove pectin, without affecting significantly the mechanical properties of the fibers, and at the same time enhanced the accessibility of pectinases in the subsequent enzymatic treatment. In general, the retting time was shown to be a crucial factor in this experiment. Treatments carried for more than 1 week revealed an extensive removal of pectin, turning the subsequent enzymatic treatment unnecessary and inefficient, and also resulted in higher damage in the mechanical properties of the fibers. Therefore, since the best treatment was the one carried out in the shortest period of time, it would be interesting to test natural fungal treatments with even shorter periods of retting, and also evaluate the degree of pectin removal and the effect on mechanical properties. Although the pretreatment was shown to be effective in relation to proposed objectives, it is, however, a time consuming option.

In section 4.2, a hydrothermal pretreatment proved to be effective in partial removal of pectin, with more galacturonic acid being removed from the lowest to the highest operating pressure (0.5 to 2 bar). In addition, no significant effect was observed in the mechanical properties of pretreated fibers, but both tensile strength and stiffness slightly decreased from lower to higher operating pressures, suggesting a
negative effect of the operating pressure in the mechanical performance of the fibers. The subsequent enzymatic treatment with pectinases was followed throughout time and allowed to conclude that said enzymes substantially degraded all the accessible substrate in the first 90 minutes of treatment, showing that longer treatments are inefficient. Looking for the treatment as a whole, the enzymatic pretreatment preceded by a hydrothermal pretreatment at 1 bar showed the best balance between mechanical performance, pectin removal and enhancement of enzymatic accessibility for pectins in middle lamella. In addition, despite the necessity for a higher energy input, the mentioned pretreatment of hemp fibers is notably distinguished as an ecological and time saving solution.

In section 4.3, pretreatments with 0.5% EDTA, 2.5% NaOH or the combination of both chemical agents were shown to increase Endo-PG accessibility either at 40 or 60 °C. Nevertheless, the pretreatments carried out at 60 °C showed higher degree of depectinization, once again reinforcing the idea that temperature plays an important role in pectin’s removal. Comparing the use of both chemical agents, the pretreatment with NaOH showed the higher selectivity for pectin and enhancement in the accessibility of Endo-PG, especially at 60 °C. In addition, EDTA used in combination with endopolygalacturonase on raw fibers was shown to be much advantageous over that used separately, which was demonstrated by the lowest content of remaining galacturonic acid. The latter treatment represents a promising solution for the proposed objective, saving energy and the time needed for the pretreatment. However the mechanical performance of the resulting fibers was negatively affected after the treatment. In general, the use of chemical agents as pretreatments of hemp fibers for subsequent enzymatic treatment is seen as a low cost option, but it is less ecological and involves extra care and costs with the generated effluents.

Combining the mechanical performance of resulting fibers, the degree of pectin removal (shown by a significant relative decrease in galacturonic acid content), together with economic and environmental issues, the hydrothermal pretreatment in the autoclave at 1 bar for 30 minutes was assumed to be the best pretreatment among the conducted experiments.

In short, this project contributed positively to the search for viable and ecological pretreatments of hemp fibers, in order to improve their quality, and with the ultimate goal of being applied as reinforcements in natural fiber composites. However, different supplementary experiments could be considered in a future work to complement the study presented in this thesis, such as the use of Scanning Electron Microscope to analyze the changes in the surface of treated fibers and possibly compare the different degrees of defibrillation or the use of X-Ray Diffraction to analyze and compare the crystalline structure of cellulose.

In addition, the next level of this study would go through the use of all differently treated fibers as reinforcements for composite manufacturing and compare its final performance, especially in terms of mechanical properties.

For all the conducted experiments, the conditions were selected based on a wide range of scientific publications. Once proved that some studied pretreatments have potential to increase the accessibility of
enzymes in following enzymatic treatments, optimization studies could be carried out to find the best combination of different treatment conditions, as operational temperature (or pressure), time of treatment, pH value, enzyme dosage, etc.
6. Bibliography


7. Appendixes

7.1 Effect of different chemical agents on hemp fibers

As a preliminary investigation, a study concerning the influence of the concentration of different chemical agents on the chemical composition and physical and mechanical properties of hemp fibers was conducted at mild conditions. For this purpose, two different approaches were done: on one hand, sequential chemical treatments were tested to remove the main non-cellulosic components from hemp fibers structure; on the other the impact of a solo alkali treatment was studied.

The use of chemical agents as pretreatment for natural fibers has been widely used by different authors, especially to investigate its impact on physical and mechanical properties of fibers, and also with the ultimate goal of using chemical treated fibers as reinforcements in biocomposites manufacturing [7], [37]–[40], [42], [44], [71]–[73]. However, the authors usually select specific treatment conditions, including the concentration of respective chemical agent and, most of the times, harsh conditions are applied, as boiling temperature. Therefore, this was the first study about the effect of chemical agents concentration on chemical composition and physical and mechanical properties of hemp fibers at mild conditions.

Figure 7.1 illustrates an overview scheme of the entire investigation. For all mentioned treatments, the effect on chemical composition and mechanical properties of the fibers was evaluated. More specifically for studies involving different concentrations of chemical agents to investigate its effect on hemp fibers, the weight loss in the treatment and the water retention value of treated fiber samples were used as complementary data. Table 7.1 systematizes all tested treatments and respective codes.

7.1.1 Effect of EDTA on hemp fibers

Figure 7.2 shows a representative sample of hemp fibers treated with different concentrations of EDTA-2Na. By comparing all the samples, there were no apparent changes in the morphology of treated fibers, even in relation to untreated fibers. Nevertheless, by analyzing the weight loss of fibers in each treatment (Figure 7.3) it is clear that there was a significant increase (p<0.05) in the severity of the treatment with an increase in EDTA-2Na concentration. From 0.1 to 1% of EDTA-2Na in solution, the weight loss in the fibers slightly increased from 0.82 ± 0.14 to 3.74 ± 0.48%. However, an abrupt increase in the weight loss was registered between treatments with 1 and 2% of EDTA-2Na. Furthermore, in terms of weight loss, there was no significant difference (p<0.05) in the treatments carried out with 2 and 3% of EDTA-2Na, suggesting that there is no change in the effect of said chelating agent on fibers above 2%.

Figure 7.4 shows the water retention value (WVR) for fibers treated with different concentrations of EDTA-2Na. For fibers treated with lower concentrations of EDTA-2Na, viz. 0.1, 0.5 and 0.75%, there is no
**Figure 7.1** – Overview of the preliminary study to determine the impact of sequential chemical pretreatments on the chemical composition and mechanical properties of hemp fibers. Each treatment has a code associated, represented on the right part of the scheme.

**Table 7.1** – List of different treatments relative to the present section, either using just chemical agents or combining chemical with enzymatic treatments.

<table>
<thead>
<tr>
<th>Treatment description</th>
<th>Code*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA treatment of raw hemp fibers for pectin degradation</td>
<td>P$x$*</td>
</tr>
<tr>
<td>NaClO$_2$ treatment of hemp fibers previously treated with 1% EDTA, for lignin degradation</td>
<td>PL$y$*</td>
</tr>
<tr>
<td>NaOH treatment of hemp fibers previously treated with 1% EDTA and 0.7% NaClO$_2$, for hemicellulose removal</td>
<td>PLHz$z$*</td>
</tr>
<tr>
<td>NaOH treatment of raw hemp fibers for hemicellulose removal</td>
<td>Hz$z$*</td>
</tr>
</tbody>
</table>

*x, y and z are the concentrations (in% w/v) of the chemical agents

statistically significant difference (p>0.05) in the WRV, comparing to untreated fibers (P0). However, there is a significant increase (p<0.05) in the WRV for treatments carried out at higher concentrations, viz. 1, 2 and 3%. As reported by Pejic et al. (2008) [44], the WRV includes all water absorbing and holding surfaces, cracks and cavities. Thus, an increase in the total water holding capacity of fibers treated with 1, 2 and 3% of EDTA-2NA suggests that some structural compounds have been partially removed, creating space for the water be retained.
In order to determine the effect of EDTA on hemp fibers, the chemical composition and the mechanical properties of treated fibers have been determined, which are presented in Table 7.2 and Figure 7.5, respectively. At this point, it has to be highlighted that after being subjected to a pre-washing step, the contaminants in hemp fibers surface, together with water-soluble minerals and components were practically removed. Thus it is expected, as shown in Table 7.2, a relative increase in the content of remaining fiber’s constituents (from raw fibers to P0).

From the chemical composition analysis, it is clear that the galacturonic acid content, which is the main component in pectin substrates, significantly decreased (p<0.05) from fibers treated with lower to higher concentrations of EDTA-2Na. For treatments carried out with 0.1 and 0.5% of said chelating agent, ca.
20% of galacturonic acid has been removed, against ca. 40% for the remaining treatments, among which there is no statistically significant difference in the final galacturonic acid content. As regards to the content of other monosaccharides that are assumed to be part of pectin, viz. rhamnose, arabinose and galactose, no relevant differences are detected within all the experiments, except the content of rhamnose in P2 and P3 treated fibers, which is statistically significant lower than in P0.1 and P0.5 treated samples.

Therefore, the results presented in Table 7.2 suggest that, under the applied experimental conditions, an EDTA treatment can partially remove pectin from fibers structure, more extensively for high concentrations. The hypothesis that chelating agents can sequester calcium ions from pectin in plant cell

![Figure 7.4](image-url) – Water retention value (in g H2O/g dry matter) of fibers treated with different concentrations of EDTA-2Na (Px, where x is the concentration of EDTA-2Na).

Table 7.2 – Anhydrous monosaccharides of hemp fibers treated with different concentrations of EDTA-2Na.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw fibers</td>
<td>1.0a(0.1)</td>
<td>1.3a(0.1)</td>
<td>2.1a(0.1)</td>
<td>60.0b(0.9)</td>
<td>1.2(0.1)</td>
<td>3.0 (0.3)</td>
<td>7.3ab(0.4)</td>
</tr>
<tr>
<td>P0 (Control)</td>
<td>0.7bc(0.1)</td>
<td>1.2ab(0.1)</td>
<td>2.1a(0.1)</td>
<td>68.0ab(2.7)</td>
<td>1.5 (0.3)</td>
<td>4.5 (0.2)</td>
<td>7.7a(0.8)</td>
</tr>
<tr>
<td>P0.1</td>
<td>0.9ab(0.1)</td>
<td>0.8bc(0.1)</td>
<td>2.1a(0.1)</td>
<td>70.4ab(0.5)</td>
<td>1.1 (0.1)</td>
<td>5.2 (0.1)</td>
<td>6.2bc(0.3)</td>
</tr>
<tr>
<td>P0.5</td>
<td>0.9ab(0.1)</td>
<td>0.8c(0.1)</td>
<td>2.1a(0.1)</td>
<td>76.8a(1.3)</td>
<td>1.0 (0.1)</td>
<td>5.2 (0.1)</td>
<td>5.9c(0.3)</td>
</tr>
<tr>
<td>P0.75</td>
<td>0.8bc(0.0)</td>
<td>1.0bc(0.0)</td>
<td>2.0a(0.1)</td>
<td>74.2a(0.8)</td>
<td>1.3 (0.0)</td>
<td>4.9 (0.1)</td>
<td>4.8cd(0.2)</td>
</tr>
<tr>
<td>P1</td>
<td>0.7bc(0.1)</td>
<td>0.9c(0.0)</td>
<td>2.1a(0.1)</td>
<td>75.8a(2.1)</td>
<td>0.9 (0.1)</td>
<td>5.4 (0.1)</td>
<td>4.7d(0.2)</td>
</tr>
<tr>
<td>P2</td>
<td>0.7c(0.0)</td>
<td>1.0c(0.1)</td>
<td>2.0a(0.0)</td>
<td>70.0ab(1.5)</td>
<td>1.0 (0.1)</td>
<td>5.0 (0.1)</td>
<td>4.6d(0.4)</td>
</tr>
<tr>
<td>P3</td>
<td>0.7c(0.0)</td>
<td>1.0c(0.1)</td>
<td>2.1a(0.1)</td>
<td>70.0ab(1.9)</td>
<td>1.3 (0.1)</td>
<td>5.0 (0.1)</td>
<td>4.4d(0.2)</td>
</tr>
</tbody>
</table>

Notes: Standard deviations of three replicates are represented between parentheses. Values in the whole column for the same monosaccharide with different letters are statistically different at a confidence level of 95%.

Therefore, the results presented in Table 7.2 suggest that, under the applied experimental conditions, an EDTA treatment can partially remove pectin from fibers structure, more extensively for high concentrations. The hypothesis that chelating agents can sequester calcium ions from pectin in plant cell.
Figure 7.5 – Mechanical properties of hemp fibers treated with different concentrations of EDTA-2Na, in terms of UTS (A), strain or elongation at break (B) and stiffness (C), measured as Young’s Modulus.

Walls, turning it soluble in many liquids, has been already reported by several authors [26], [47].

Li & Pickering (2008) [26] reported a pectin degradation of ca. 70% in hemp fibers treated for 6 hours with 1% of EDTPM-5Na (an ecological alternative to EDTA), at 60 °C and pH 11.0, which is significantly higher than the obtained in the current study. Thus it is assumed that both pH value and temperature may play a crucial role in pectin removal during EDTA treatment, specially temperature, since it has been reported by Florence et al. (1995) [74] that EDTA at boiling temperatures can be effectively used to remove pectin from flax fibers and Adamsen A. et al (2002) [46] showed that EDTA has a similar calcium binding ability at pH value of 6.0 and 11.0.

The mechanical properties of hemp fibers were affected after chemical treatment with EDTA-2Na (Figure 7.5). From lower to higher concentrations of said chelating agent, the UTS significantly (p<0.05) decreased from ca. 770 MPa in raw fibers to ca. 350 MPa in P3 treated fibers. The damage in UTS is mainly clear and severe for samples treated with 2 and 3% of EDTA-2Na, while for the lowest concentrations (0.1 and 0.5%) a minor decrease was recorded. Therefore, the decrease in UTS is most
probably related with the degradation of pectins found in the interstitial region of ML, which cement individual fibers together in fiber bundles [47].

As regards to strain (or elongation at break) there is a significant decrease from 5 to ca. 3%, which is common for all concentrations of EDTA-2Na tested. Thus, one can conclude that the amount of EDTA in solution does not affect strain differently, since there is no statistically significant difference (p>0.05) among strain values for EDTA treated samples. The decrease in strain may be due to the dissolution of water soluble components [8] or most probably due to a decrease in fibers ductility, as a result of pectin removal. About stiffness, the Young’s Modulus of hemp fibers slightly decreased from raw to treated fibers with 1% of EDTA-2Na. However a significant decrease in stiffness is shown for treatments with 2 and 3% of EDTA-2Na. In Figure 7.5-A, a decrease in UTS was shown from fibers treated with lower to higher concentrations of EDTA-2Na, while the strain remained unchangeable. Therefore, the decreasing trend of stiffness (as Young’s Modulus), inversely to EDTA-2Na concentration, is predictable by Hooke’s Law.

In general, hemp fibers treated with 2 and 3% of EDTA-2Na were harshly damaged. Table 7.2, shows that the relative content of cellulose content decreased from 76% in P1 treated fibers to 70% in P2 and P3 treated fibers, although the difference is not statistically different. In addition, the mechanical properties, including UTS and stiffness, were clearly affected by these treatments. Moreover, Figure 7.3 showed an abrupt increase in the weight loss of hemp fibers from P1 to P2 and P3 treated samples. Everything together suggests that high concentrations of EDTA (above 1%) may affect the structure of cellulose and this is the most likely reason for the loss of mechanical performance of P2 and P3 treated fibers. As a matter of fact, a decrease in stiffness is usually an indicative of degradation of cellulosic components of the fibers [47].

7.1.2 Effect of acid-chlorite on hemp fibers

Figure 7.6 shows an illustrative sample of hemp fibers treated with different concentrations of sodium chlorite, after being pretreated with 1% of EDTA-2Na (PL0). The evidence of the bleaching effect is noticeable in fibers treated from lower to higher concentrations of NaClO$_2$, due to the loss of color.

The dry matter weight loss of hemp fibers in each acid-chlorite treatment and the WRV for treated fibers are presented in Figures 7.7 and 7.8, respectively. From the results for both weight loss and WRV determination, it is anticipated that the degree of delignification was not that significant. Even so, one must highlight a statistically significant two-fold increase (p<0.05) in the weight loss of hemp fibers from treatments carried out with lower concentrations of sodium chlorite (0.7 and 1.4%) to higher concentrations (2.8% and 5%). As regards to the WRV, fibers treated with 0.7, 1.4 and 2.1% of sodium chlorite are not statistically different from untreated fibers (in this case, fibers pre-treated with 1% of EDTA-2Na). A significant increase (p<0.05) in the WRV was just recorded for fibers treated with 2.8% of NaClO$_2$. According to Pejic et al. (2008) [44], fibers with progressively removed lignin are capable to retain
more water. Therefore it is expected that the sodium chlorite treatments at lower concentrations were not effective on removing lignin.

Under the experimental conditions, the effect of sodium chlorite on hemp fibers was also evaluated in terms of chemical composition and mechanical properties, which are presented in Table 7.3 and Figure 7.9, respectively.
Figure 7.8 – Water retention value (in g H₂O/g dry matter) of fibers treated with different concentrations of sodium chlorite (PLₓ, where x is the concentration of NaClO₂) after pretreatment with 1% of EDTA-2Na (P1).

Table 7.3 – Anhydrous monosaccharides and Klason lignin content of hemp fibers treated with different concentrations of sodium chlorite.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>Klason Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL0 (Control)</td>
<td>0.7 (0.1)</td>
<td>0.9 (0.0)</td>
<td>2.1 (0.1)</td>
<td>75.8ᵃ(2.1)</td>
<td>0.9 (0.1)</td>
<td>5.4 (0.1)</td>
<td>4.7 (0.2)</td>
<td>5.3ᵃ(0.2)</td>
</tr>
<tr>
<td>PL0.7</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.0)</td>
<td>1.9 (0.1)</td>
<td>71.9ᵃ(1.1)</td>
<td>1.0 (0.1)</td>
<td>4.5 (0.1)</td>
<td>5.1 (0.1)</td>
<td>5.2ᵃ(0.3)</td>
</tr>
<tr>
<td>PL1.4</td>
<td>0.7 (0.0)</td>
<td>0.9 (0.0)</td>
<td>1.8 (0.1)</td>
<td>70.2ᵃ(0.8)</td>
<td>1.1 (0.1)</td>
<td>4.9 (0.1)</td>
<td>5.3 (0.1)</td>
<td>5.1ᵃ(0.4)</td>
</tr>
<tr>
<td>PL2.1</td>
<td>0.8 (0.0)</td>
<td>1.1 (0.2)</td>
<td>1.9 (0.0)</td>
<td>72.5ᵃ(2.9)</td>
<td>1.0 (0.0)</td>
<td>4.8 (0.1)</td>
<td>5.2 (0.0)</td>
<td>4.4ᵇ(0.2)</td>
</tr>
<tr>
<td>PL2.8</td>
<td>0.8 (0.1)</td>
<td>0.9 (0.1)</td>
<td>1.8 (0.1)</td>
<td>74.0ᵃ(0.8)</td>
<td>1.0 (0.1)</td>
<td>4.8 (0.3)</td>
<td>5.2 (0.2)</td>
<td>4.2ᵇ(0.3)</td>
</tr>
</tbody>
</table>

Notes: Standard deviations of three replicates are represented between parentheses. Values in the whole column for glucose and Klason lignin with different letters are statistically different at a confidence level of 95%.

The chemical composition analysis (Table 7.3) proves what was anticipated from the previous results: the degree of delignification in the conducted treatments was not extensive. It is shown for treatments carried out with 0.7 and 1.4% of sodium chlorite, that there is no statistically significant difference (p>0.05) in the Klason lignin value, comparing to corresponding control (PL0). Only for PL 2.1 and PL 2.8 treated fibers, the Klason Lignin is significantly (p<0.05) different from PL0, but it can be estimated that only 20% of lignin has been removed. As regards to the relative composition in terms of different monosaccharides, all of the components remained unchangeable, after treatments with different concentrations of NaClO₂.

The mechanical properties of PL treated fibers are in agreement with chemical composition analysis. Mohamed et al. (2013) [75] reported a significant adverse effect on UTS of kenaf fibers treated with
sodium chlorite, with an increase in the delignification degree, but in the present study, since no significant effect has been done to fiber composition, there is no significant difference in the UTS of different treatments carried out with sodium chlorite (Figure 7.9-A). However, opposite effects on strain and stiffness have been recorded: while there was a statistically significant increase in strain from ca. 2.8 ± 0.9% for PL0.7 and PL0 fibers to ca. 3.5 ± 0.9% for the remaining PL treated fibers (Figure 7.9-B), a statistically significant decrease to ca. 16 ± 0.9 GPa in fiber's stiffness was observed for the latter, against 24 ± 0.9 GPa for PL0.7 and untreated (PL0) fibers. In theory, this phenomenon can be explained by Hooke’s Law, since for the same value of UTS, strain and Young’s Modulus are inversely proportional.

Chemically, the decrease in stiffness might be related with the partial removal of lignin from interstitial parts of middle lamella. Lignin has mainly a structural function, forming a matrix sheath around the cellulose microfibrils and fibers, resulting in a strong adhesion between fibers. Thus it is expected that lignin removal can negatively affect the stiffness of the resulting fibers.

As mentioned in section 1.3, lignin has a complex structure and establishes complex links with other fiber components. It is widely known that lignin is randomly distributed throughout the fiber structure and that it

Figure 7.9 – Mechanical properties of hemp fibers treated with different concentrations of sodium chlorite, in terms of UTS (A), strain or elongation at break (B) and stiffness (C), measured as Young’s Modulus.
is less hydrophilic than hemicellulose and cellulose. Therefore lignin’s dissolution/degradation is very difficult to fulfill. Eriksson et al. (1991) [76] reported the importance of lignin softening in delignification and consequent water sorption properties on pulps, which, according to the authors, occurs above 70-75 ºC. Even with temperatures that allow lignin to soften, the maximum delignification presented for pulps was ca. 50%. For hemp fibers, Kostic et al. (2010) [40] also reported around 50% of delignification in a treatment carried out at boiling temperature with 0.7% of sodium chlorite.

Therefore, since in this study the sodium chlorite treatments were performed well below lignin’s softening temperature (70-75 ºC), a maximum delignification of 20% is reasonable to present. Nevertheless, it has to be underlined that the main goal of this study was to investigate the effect of sodium chlorite concentration in the delignification of fibers, and in this regard, it was clear an increase in the delignification rate from lower to higher concentrations of said chemical agent. Thus, the use of mild conditions (in terms of temperature) was the best way to exclusively study NaClO₂ effect on fibers, since it has been proved that temperatures above 70 ºC play an important role in the process.

### 7.1.3 Effect of sodium hydroxide on hemp fibers

The effect of sodium hydroxide has been tested on both raw fibers (H) and sequentially pretreated fibers with 1% of EDTA-2Na and 0.7% of NaClO₂ (PLH). Figure 7.10 shows the appearance of the fibers after treatments at different concentrations of NaOH for both mentioned studies. It is perceived that the highest concentrations of sodium hydroxide (10 and 15%) affected the morphology of the fibers, by bending them. It was also noticed that, after the fibers being soaked for 4 hours, the respective colorless solution of sodium hydroxide became bright yellow, and consequently the fibers gained some color.

Figure 7.10 – Hemp fibers samples after the 3-stage sequential chemical treatment with 1% of EDTA-2Na, 0.7% of NaClO₂ and different concentrations of sodium hydroxide (PLHₓ, where x is the concentration of sodium hydroxide) – left – and raw hemp fibers directly treated with sodium hydroxide at different concentrations (Hₓ, where x is the concentration of sodium hydroxide) – right.
As regards to the effect of sodium hydroxide on PL0.7 samples, Figures 7.11 and 7.12, show the weight loss and water retention value of resulting fibers, after treatments conducted at different concentrations of sodium hydroxide. A surprisingly linear, significant increase (p<0.05) in the weight loss is observed from treatments with 2.5 to 10% of NaOH, where the value increase from 2.92 ± 0.67% to 10.75 ± 0.48%. Then, there is no statistically significant difference PL0.7 fibers treated with 10 and 15% of NaOH. In short, just by looking at the weight loss results, it is expected an increasing effect on hemp fibers structure with an increase of NaOH concentration.

Figure 7.11 – Dry matter weight loss of hemp fibers during alkali treatments at different concentrations of sodium hydroxide (from 2.5 to 15% w/v), after being subjected to a sequential chemical treatment with 1% of EDTA-2Na and 0.7% of NaClO₂. Treatments with different letters are significantly different at a confidence level of 95%.

Concerning the water retention results, an abrupt significant increase in the WRV was recorded after the treatment with 2.5% NaOH, in comparison to untreated fibers (PL0.7), from 1.96 ± 0.05 to 2.43 ± 0.10 g H₂O/ g of dry matter. On the other hand there is no statistical difference in the WRV of fibers treated with 5% of NaOH (PLH5) and untreated fibers. In fact, as will be shown later in the chemical composition analysis (see Table 7.4), under the experimental conditions, the chemical treatments until 5% of NaOH did not introduce any significant difference in the composition of fibers. Therefore, even assuming a small increase of void space in the structure (shown by the weight loss of fibers in Figure 7.11), it wouldn't be enough to explain such an increase in the water retention capacity of PLH2.5 fibers, which then has to be interpreted as a false positive increase.

For treatments carried out with higher concentrations of NaOH (7.5, 10 and 15. %) there was an increase in the WRV. This can be explained by the partial removal of hemicellulose (see Table 7.4) and consequent increase of void space. Since hemicellulose is tightly linked to cellulose by hydrogen bonds, after its partial removal, the surface of the fibers looks cleaner as shown by different authors [7], [38], [40], [59] using a scanning electron microscope.
Now for sodium hydroxide treatments of raw hemp fibers at different concentrations, Figures 7.13 and 6.14 also show, respectively, the weight loss of hemp fibers in each treatment and the WRV of resulting samples. As respect to the weight loss results, there is a statistically significant increase from 2.77 ± 0.27 to 6.10 ± 0.70% in treatments carried out with 2.5 and 5% of NaOH, respectively. However, from 5 to 15% of NaOH, although there is a slight increase in the weight loss of fibers, the values are not statistically significant different (p>0.05), i.e. there is no significant effect of NaOH concentration on the weight loss of fibers, above 5%.

In respect to the water retention determination, although not statistically significant, there was an increase in the WRV after treating raw fibers with 2.5% of NaOH (H2.5) from 1.52 ± 0.17 to 1.78 ± 0.09 g H2O/ g of dry matter, which is most likely due to the removal of water-soluble components, since any significant differences have been found in the chemical composition of both fibers (later represented in Table 7.5).

From H2.5 to fibers treated with higher concentrations of NaOH, there was a significant increase in hemicellulose removal (see Table 7.5), which can be associated to an increase of void space and porosity in the fibers structure. In fact, the WRV increased to 2.10 ± 0.12 g H2O/ g of dry matter in fibers treated with 5% of NaOH (H5). From H5 to H15, it is evident a decreasing trend in the WRV, which might be associated with the capillarity phenomenon, previously mentioned in section 4.2. One can assume that along with hemicellulose removal there was an increase in the porosity of the fibers, but bigger pores contribute negatively to the water retention of fibers [44]. So, in this case, the capillarity effect might be dominant relatively to the increase in void space.
Figure 7.13 – Dry matter weight loss of raw hemp fibers during alkali treatment at different concentrations of sodium hydroxide (from 2.5 to 15% w/v). Treatments with different letters are significantly different at a confidence level of 95%.

Figure 7.14 – Water retention value (in g H₂O/g dry matter) of raw fibers treated with different concentrations of sodium hydroxide (Hₓ, where x is the concentration of NaOH).

The effect of sodium hydroxide on both PL0.7 hemp fibers and raw fibers was substantially evaluated in terms of chemical composition analysis (Tables 7.4 and 7.5, respectively) and mechanical properties of treated fibers.

As regards to chemical composition analysis, the effect of the amount of sodium hydroxide in conducted treatments was evaluated in terms of the variation in the relative content of hemicellulose, which in this case was roughly measured as xylose and mannose. In fact, only the content of these monosaccharides
was significantly affected by sodium hydroxide, as highlighted in Tables 7.4 and 7.5.

Comparing to treatments carried out with 2.5% of NaOH (PLH2.5 and H2.5) and untreated fibers (PLH0 and H0), there was a statistically significant decrease in the relative content of xylose in both treatments carried out with 10 (PLH10 and H10) and 15% (PLH15 and H15) of said alkali agent. On the other hand a statistically significant decrease (p<0.05) in mannose was recorded in treatments carried out from to higher concentrations of NaOH, for both PL fibers and raw fibers, as shown in Figure 7.15.

In Figure 7.15 it is also clear the same the same effect of sodium hydroxide concentration on mannose, whether the fibers have been subjected to a pectin removal pre-step or not. For both studies a maximum
Figure 7.15 – Mannan content (in g/100 g dry matter) of PL0.7 fibers and raw fibers after alkali treatments with different concentrations of sodium hydroxide.

decrease in the relative content of mannose of ca. 42% has been fulfilled in alkali treatments with 15% of NaOH. For the same concentration, and roughly considering hemicellulose as the sum of xylose and mannose contents, ca. 43% of hemicellulose has been removed. The decreasing rates in the hemicellulose relative content, obtained in alkali treatments at 40 ºC during 4 hours, are comparable to that reported by Kabir et al. (2013) [38] after treatments carried out with 0-10% of NaOH for 3h, at room temperature. Even so, the hemicellulose degradation with 15% of NaOH is much lower than 69%, reported by Pejic et al. (2008) [44], where 17.5% of NaOH has been used in a treatment at room temperature for 30 min.

In addition for both studies, no statistically significant effect (p>0.05) has been detected on glucose and Klason lignin contents, among the fibers treated with different concentrations of NaOH, suggesting no apparent effect of NaOH on cellulose and lignin. In fact, after the alkali treatments, an increase in the relative content of glucose in the fibers is perceivable. On the other hand, it seems that by using NaOH on raw fibers, especially at higher concentrations (10 and 15%), a small portion of pectin can be removed, as represented in Table 7.5 in terms of rhamnose, arabinose, galactose and GalA.

Figures 7.16 and 7.17 respectively show the mechanical properties of PL fibers and raw fibers after treatments with different concentrations of sodium hydroxide, for which different results have been achieved, since distinct substrates have been used.

For hemp fibers previously subjected to a chemical sequential treatment with 1% of EDTA-2Na and 0.7% of NaClO₂ (PL fibers), the UTS slightly decreased from hemp fibers treated with lower to higher concentrations of NaOH (Figure 7.16-A). However, comparing to untreated fibers (PL0.7 fibers), the
Figure 7.16 – Mechanical properties in terms of UTS (A), strain or elongation at break (B) and stiffness (C) of hemp fibers treated with different concentrations of NaOH, after previous sequential, chemical treatments with 1% of EDTA-2Na and 0.7% of sodium chlorite.

decrease in UTS is only statistically significant (p<0.05) for fibers treated with the highest concentrations of NaOH (10 and 15%). For example, in PLH15 treated fibers, the UTS decreased ca. 27%, from 557 ± 114 MPa (in untreated fibers) to 409 ± 98 MPa. In fact, for these fibers, it was shown a change in morphology after the treatment (Figure 7.10). Probably some disorientation of the microfibrils occurred after hemicellulose removal, which can explain the decrease in the UTS.

In respect to strain (Figure 7.16-B), there was a statistically significant increase (p<0.05) in said property for all fibers treated with sodium hydroxide, from 2.4% (in untreated fibers) to 3.3-4.0%. The highest value of strain (or elongation at break) was obtained for PL fibers treated with 7.5% of NaOH. However it is also the value with the highest standard deviation, thus it is expected that NaOH caused an increase in strain, independently of its concentration. As regards to stiffness (Figure 7.16-C), for all treatments, the Young’s Modulus significantly decreased (p<0.05) in the same magnitude from 24.2 GPa (in untreated fibers) to 13.8-15.1 GPa (ca. 40% decrease), i.e. also independently of NaOH concentration, with the exception of PLH5 treated fibers, which surprisingly show a Young’s Modulus in between (19.6 ± 2.4 GPa).
Figure 7.17 – Mechanical properties in terms of UTS (A), strain or elongation at break (B) and stiffness (C) of raw hemp fibers treated with different concentrations of NaOH.

As regards to the study of NaOH effect on raw fibers, all the mechanical properties have been negatively affected after treatments with NaOH. The UTS (Figure 7.17-A) was severely affected (p<0.05) from untreated fibers to fibers treated with 5% of NaOH, decreasing from 772 ± 104 MPa to 480 ± 54 MPa (ca. 38% of decrease). From fibers treated with 5% of NaOH to higher concentrations, no statistically significant difference was recorded in UTS, i.e. no additional effect of NaOH concentration was observed.

The stiffness (Figure 7.17-C) of raw fibers was also severely damaged in NaOH treatments, decreasing from 28.5 to 16-18 GPa (ca. 40% of decrease), independently of its concentration, since no statistically significant difference (p>0.05) between NaOH treated fibers (H) was recorded. In terms of strain, a statistically significant decrease (p<0.05) was also observed, but not as drastic as in other mechanical properties. In this case, the strain decreased from 5.1 ± 0.9% (in raw fibers) to 3.7 ± 0.8% in H2.5 treated fibers (ca. 27% of decrease). Although the ANOVA shows that there is no statistically significant difference in the strain value of fibers treated with different concentrations of sodium hydroxide (from 2.5 to 10%). Then raw fibers treated with 15% of NaOH (H15) kept its strain value, since it is not statistically significantly different from untreated fibers.
The deterioration of tensile properties of fibers subjected to higher concentrations of NaOH has been suggested by Shahzad A. (2012) [77] to be related with the break of hydrogen bonds within the fibers. That might explain the significant decrease in UTS of raw fibers treated with 5-15% of NaOH. Van de Weyenberg et al. (2006) [45] also reported a significant decrease in tensile properties of flax fibers treated with 1, 2 or 3% of NaOH. Even so, the use of those fibers as reinforcements in composites led to an improvement of its final mechanical performance, mainly because of the enhancement of interaction between treated flax fibers and the epoxy matrix. This same conclusion has been reported by other authors [37], [71]. Therefore, it has to be highlighted that although the mechanical properties of individual fibers might have been affected during an alkali treatment, the hemicellulose removal is a key point to reduce the hidrophilicity of hemp fibers, which in the end will improve the mechanical properties of fiber reinforcement composites.

With the removal of hemicellulose in alkali treatments, the interfibrillar region is likely to be less dense and rigid, what can explain the decrease in stiffness. On the other hand the removal of hemicelluloses is also accompanied by swelling and shrinkage of ultimate cells, which result in some disorientation of the fibrils and texturing of hemp fibers [40]. Thus this might explain the decrease in fibers resistance to deformation (i.e. decrease in strain) and also the change in morphology of fibers treated with 10 and 15% of NaOH.

7.1.4 Conclusion

In this preliminary investigation, the effect of the concentration of different chemical agents on the chemical composition and mechanical performance of hemp fibers, was investigated in treatments at mild conditions (40 °C and pH 6.0).

Excepting sodium chlorite, both sodium hydroxide and EDTA were shown to negatively affect the physical and mechanical properties of hemp fibers when used at higher concentrations (above 7.5 and 1%, respectively).

In terms of non-cellulosic components removal, for sodium chlorite and EDTA treatments, the degree of pectin removal and delignification, respectively, was not shown to be extensive, even at higher concentrations of said chemical agents. For both treatments, it is expected that the operating temperature plays a crucial role in the removal of said non-cellulosic components. On the other hand, higher concentrations of sodium hydroxide showed an increase in hemicellulose removal, but higher concentrations should be avoided in an alkali treatment of hemp fibers, due to said damage on their physical properties.
7.2 Enzymatic activity assays

Different enzymatic activity assays were performed to measure the activity of all the enzymes used in the present project, namely endopolygalacturonase, pectin lyase, xylanase, xylglucanase and laccase. This section presents the procedures used to measure the activity of said enzymes.

7.2.1 Endopolygalacturonase activity assay

The activity of endopolygalacturonase was determined at pH 6.0 and temperature of 40 °C, through an enzymatic activity assay from Sigma-Aldrich (Saint Louis, MO, USA), based on the work of Kertesz, Z.I. (1955) [83], using polygalacturonic acid as substrate. It's a titrimetric procedure, where one considers that 1 unit of enzyme liberates 1.0 µmol of galacturonic acid from polygalacturonic acid per minute, under experimental conditions.

The activity determination of endopolygalacturonase is based on the hydrolysis reaction of polygalacturonic acid, in which the enzyme acts as a catalyzer (Equation 15). By knowing the amount of galacturonic acid produced in the reaction, the activity of said enzymes can be estimated. For that, two auxiliary reactions (Equations 16 and 17) are used in a process called iodometry.

\[
\text{Polygalacturonic acid} + \text{H}_2\text{O} \xrightarrow{\text{Endo-PG}} \text{Galacturonic acid} \\
\text{I}_2 + \text{Galacturonic acid} \rightarrow \text{Oxidation Products} \\
\text{I}_2 + 2 \text{Na}_2\text{S}_2\text{O}_6 \rightarrow 2 \text{NaI} + \text{Na}_2\text{S}_2\text{O}_6 \quad \text{(at low pH)}
\]

Iodine will oxidize galacturonic acid (Equation 16), which is the limiting reagent in this 1:1 reaction. Then, if one knows the amount of iodine that reacts, the amount of galacturonic acid produced in the previous reaction can be estimated. Even though, an excess of iodine will remains. In turn, the molar quantity of excess of iodine can be determined by titration (known as iodometry), as described in Equation 17. The sodium thiosulfate added (titrant) will react with the remaining iodine. While the iodine is being consumed the color of solution turns lighter. The end of reaction can be observed by using starch indicator.

In short, by measuring the volume of titrant, the molar quantity of sodium thiosulfate that reacted can be determined and then, by stoichiometry, the molar quantity of excess iodine is estimated. Once the initial molar quantity of iodine is known from its concentration in solution, the iodine that reacted with galacturonic acid (Equation 16) can be calculated by subtraction. Finally the molar quantity of produced galacturonic acid is known, from which the activity of Endo-PG can be determined.

As regards to the procedure, 4.90 mL (5.00 mL for blank test) of 0.5% (w/v) Polygalacturonic acid (substrate) in Buffer II (25 mM citric acid monohydrated with 25 mM trisodium citrate dehydrated at pH
were pipetted into a 50 mL Erlenmeyer flask and then incubated in a water bath at 40°C for several minutes. Then, a volume of 0.1 mL of the Endo-PG solution was added into test Erlenmeyer flasks. The reaction (Equation 15) took place for 5 minutes at 40°C and adequate agitation.

After 5 minutes of incubation, 1 mL of 1 M Sodium Carbonate and 5 mL of a 50 mM Iodine and Potassium Iodine solution were added to each Erlenmeyer flask and then swirled and kept in the dark for 20 minutes (Equation 16). Before final titration (Equation 17), 2 mL of 1 M sulfuric acid were pipetted into the Erlenmeyer flasks. Thus, both test and blank solutions were titrated with 100 mM Sodium Thiosulfate pentahydrated. Once a light yellow color was obtained, 1 or 2 drops of 1.0% (w/v) Starch Indicator were added to titrated solution and titration continued until solutions were colorless.

The total added volume of titrant was recorded and the experiment was done in duplicate. According to what was explained before, the activity of Endo-PG can be determined through Equation 18, where $V_{Na_2S_2O_3}$ is the added volume of sodium thiosulfate in titration (in mL); $\mu$eq$_{GA}/\mu$eq$_{I_2}$ is the molar quantity of galacturonic acid oxidized by 1 microequivalent of iodine (see equation 18); $\mu$eq$_{S_2O_3}/mL$ Reagent E is the molar quantity (in microequivalents) of thiosulfate per mL of sodium thiosulfate; $df$ is the dilution factor; $t$ is the time of enzymatic reaction (in minutes); and $V_{enz}$ is the volume of enzyme used (in mL).

$$\text{Act (U/mL)} = \left( \frac{[V_{Na_2S_2O_3}]_{\text{Blank}} - [V_{Na_2S_2O_3}]_{\text{Test}}}{V_{enz} \times \mu\text{eq}_{S_2O_3}/\mu\text{eq}_{I_2} \times t} \right) \times \left( \frac{[\mu\text{eq}_{GA}/\mu\text{eq}_{I_2}] \times [\mu\text{eq}_{S_2O_3}/mL\text{ Reagent E}] \times df}{V_{Na_2S_2O_3}} \right)$$  \hspace{1cm} (18)

### 7.2.2 Pectin lyase activity assay

The activity of pectin lyase was determined through another type of enzymatic activity assay, also from Sigma-Aldrich (Saint Louis, MO, USA) and developed by Albersheim, P. (1966) \cite{84}. In this case, a pectin solution from citrus peel was used as substrate, in a continuous spectrophotometric rate determination, where 1 unit of enzyme was considered to be responsible to cause a variation in absorbance ($A_{235\text{nm}}$) of 1.0/min at 40°C and pH 6.0, due to the release of unsaturated products from pectin.

The activity measurement of pectin lyase is centered in the hydrolysis reaction of pectin, in which this enzyme acts as a catalyzer (Equation 19). The resultant unsaturated products have a maximal absorbance at 235 nm, so there’s the possibility of following the reaction by measuring the absorbance of the solution (substrate + enzyme) throughout time.

$$\text{Pectin + H}_2\text{O} \xrightarrow{\text{pectin lyase}} \text{unsaturated products}$$ \hspace{1cm} (19)

A volume of 180 μL of 0.5% (w/v) Pectin solution in Buffer II and 20 μL of Buffer II were pipetted into a well of the microplate. Once equilibrated at 40°C in the microplate reader, 50 μL of the enzyme solution (properly diluted) was added to test solution wells and 50 μL of enzyme diluent (0.1%, w/v BSA in Buffer
Il) to the blank wells. Then the absorbance at 235 nm was monitored during the course of the reaction at 40 °C. Each experiment was done in triplicate.

Finally the activity of pectin lyase was determined through Equation 20, where $\Delta A_{235\text{ nm}}/\text{min}$ is the variation of absorbance (235 nm) per minute of reaction; $V_T$ is the total volume of reaction (in mL); $df$ is the dilution factor and $V_{enz}$ is the volume of enzyme used (in mL).

$$\text{Act (U/mL)} = \frac{([\Delta A_{235\text{ nm}}/\text{min}]_{\text{Test}} - [\Delta A_{235\text{ nm}}/\text{min}]_{\text{Blank}}) \times V_T \times df}{V_{enz}}$$  \hspace{1cm} (20)

### 7.2.3 Xylanase activity assay

The activity of xylanase was determined spectrophotometrically according to a standard protocol from Sigma-Aldrich (Saint Louis, MO, USA) based on a procedure designed by Lever, M. (1972) [78], a colorimetric method centered in reducing sugars determination. Thereby one unit of xylanase was considered to be responsible for liberate 1.0 mg/min of reducing sugars from xylan substrate (measured as glucose equivalents), under the experimental conditions.

PAHBAH was selected to measure the amount of reducing sugars released as consequence of the enzyme’s activity. This agent reacts with reducing carbohydrates in alkaline solution, forming an intensely yellow calcium chelate that can be used in a colorimetric assay procedure to determine the amount of reducing sugars in solution [79]. On the other hand, glucose was used for standards.

In the beginning, a volume of 3 mL of Buffer II and 1 mL of 2.5% (w/v) Xylan Solution in Buffer II (substrate) were pipetted to test tubes. For Blank tests, an extra volume of 1 mL of miliQ water was added. The tubes were mixed by swirling, and then incubated in a water bath at 40°C. Afterwards, 1 mL of xylanase solution (properly diluted) was added to test tubes and the reaction lasted 80 minutes at 40°C and 100 rpm of shaking speed.

To arrest the reducing sugars released in the reaction mixture, the hydrolysates and blank solutions were then mixed (30 µL) with 9 mL of 0.5% (w/v) PAHBAH solution in 0.5 M NaOH and the volume was completed with miliQ water until 10 mL. Regarding the glucose standards, 1 mL of each was mixed with the same volume of PAHBAH solution. The tubes were then placed in boiling water for 5 minutes. Hereafter the tubes were cooled at room temperature, and lastly, the reducing sugars were determined by measuring the absorbance at 410 nm of the final solutions in a spectrophotometer. All test or blank samples, along with glucose standards, were done in triplicate.

Finally, the activity of xylanase was determined through Equation 21, where $m_{\text{glucose}}$ is the mass of glucose liberated in the enzymatic reaction; $t$ is the time of reaction and $V_{enz}$ is the volume of enzyme used.
\[
\text{Act} (U/mL) = \frac{m_{\text{glucose eq.}} (mg)}{V_{\text{enz}} (mL) \times t \ (\text{min})}
\] (21)

7.2.4 Xyloglucanase activity assay

As seen for xylanase, xyloglucanase activity was also determined by the spectrophotometer method developed by Lever, M. (1972) [78], based on the estimation of reducing sugars released as a result of said enzyme activity, after a certain period of incubation with xyloglucan. However, in this case, one unit of xyloglucanase was considered to be responsible for releasing 1.0 \( \mu \text{mol} \) of reducing sugars (measured as glucose equivalents) per minute, at 40°C and pH 6.0.

As regards to the procedure, xyloglucan was dissolved in Buffer II (25 mM citric acid monohydrated with 25 mM trisodium citrate dehydrated at pH 6.0) within several hours in a water bath at 60 °C. Then for xyloglucanase activity assay, a volume of 1.8 mL of 1.0% (w/v) Xyloglucan (Tamarind) in Buffer II (substrate) was mixed with 0.2 mL of properly diluted enzyme solution (0.2 mL of Buffer II for control) into 2 mL Eppendorf tubes, as done by Benko et al. (2008) [80]. The reaction lasted 5 minutes in a thermomixer at 40 °C and 300 rpm. Afterwards, in order to determine the reducing sugars concentration in the reaction mixture, 0.1 mL of hydrolysates or control test solutions were mixed with 0.9 mL of 0.5% (w/v) PAHBAH solution in 0.5 M NaOH into 1.5 mL Eppendorf tubes, performing a 1:9 ratio as Fursova et al. (2012) [81]. Then the tubes were placed in boiling water for 5 minutes. Glucose standards from 0 to 2 mM were used for calibration.

Finally, after cooling the tubes at room temperature, the reducing sugars in each test/control/standard tube were determined by measuring the absorbance at 410 nm in the microplate reader. All test and controls, along with glucose standards, were made in triplicate. In the end, the obtained standard curve was used to calculate the concentration of reducing sugars (\( C_{RS} \)) in each test or control.

The activity of xyloglucanase was determined through Equation 22, where \( C_{RS} \) is the concentration of reducing sugars released in the enzymatic reaction; \( V_{assay} \) is the total volume of reaction used; \( df \) is the dilution factor applied in the preparation of enzyme solution; \( t \) is the time of reaction and \( V_{enz} \) is the volume of enzyme used.

\[
\text{Act} (U/mL) = \frac{C_{RS} \ (mM) \times V_{assay} (L) \times df}{V_{enz} (mL) \times t \ (\text{min})}
\] (22)

7.2.5 Laccase activity assay

The activity of laccase was determined in the oxidation of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), shortly known as ABTS, according to the procedure developed by Bourbonnais, R. et al
(1995) [82], but adapted to a microplate assay. One unit of laccase was considered to be responsible of forming 1 µmol of oxidized ABTS per minute, at 40°C and pH 6.0.

The absorbance at 420 nm was measured in the microplate reader at 40 °C and with moderate agitation. A volume of 180 µL of 1 mM ABTS (190 µL for Blank samples) and 10 µL of Buffer II were pipetted into the microplate. Once equilibrated at 40°C, 10 µL of laccase solution (properly diluted) was added to test wells. Then the A_{420nm} throughout the reaction time. Each experiment, and respective blank samples, was done in triplicate.

Finally the activity of laccase was determined through Equation 23, where ΔAbs_{420 nm/min} is the variation of absorbance (420 nm) per minute of reaction, ε_{max} is the maximum absorptivity of ABTS (36 mM^{-1}.cm^{-1}); L is the height of liquid (in cm) in each microplate well crossed by radiation; df is the dilution factor and V_{assay} is the total volume of reaction (in mL).

\[
Act (U/mL) = \frac{([\Delta Abs_{420 nm/min}]_{test} - [\Delta Abs_{420 nm/min}]_{blank}) \times V_{assay} \times df}{\varepsilon_{max} \times L \times V_{enz}}
\] (23)

Assuming a cylinder form for microplate wells, the height of liquid crossed by radiation can be estimated using Equation 24, where D is the average diameter (between the top and the base) of the well (in cm).

\[
L = \frac{4V}{\pi D^2}
\] (24)
7.3 Example of HPLC chromatograph for standard mother solution

Figure 7.18 illustrates an example of a HPLC chromatograph obtained for one of the standards from standard mother solution.

Figure 7.18 – Example of a chromatograph obtained by HPAEC-PAD for one of the standards. From left to right (i.e. increase of retention time), monosaccharides are identified as follows: 1 – Fucose; 2 – Rhamnose; 3 – Arabinose; 4 – Galactose; 5 – Glucose; 6 – Xylose; 7 – Mannose; 8 – Galacturonic Acid.