Universitat de Girona
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Major Project

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Study of the influence of different enzymatic treatments of flax on fiber composition

Thesis

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ABSTRACT

Dew retting is the traditional and still most used method to degrade flax stem non-cellulosic material in order to separate the fiber, but it has many disadvantages like weather dependence, inconsistent properties and long process time. Enzymatic retting is an attractive alternative which offers faster treatments and uniform properties. Enzymes are very selective and result in cleavage of specific molecules, but to successfully degrade the cementing matrix that glues the fiber to the stem one or more enzymes will be required. Hence, this project analysed the chemical composition (cellulose, hemicellulose and lignin) of flax fibers treated with different enzymes to evaluate the effect of the treatments on flax fibers. In view of the results, for the same operational conditions (0.6 %, 40 ºC and pH 5) flax fibers retted with Viscozyme presented a higher pureness when incubation time was increased from 6 to 24 hours. The activity of hemicellulases, pectin lyases, pectate lyases and pectin methyl esterases was improved when pH was increased from 5 to 6.5, while polygalacturonases activity was good in both pH levels. The hypothesis of an inactivation of pectate lyases with the presence of a chelator was investigated and results revealed that Scourzyme L (pectate lyase) is more efficient when a chelator is added. For the same operational conditions (0.3 % and 40 ºC) Pulpzyme (endoxylanase) completed a better retting than Xylanase from *Thermomyces lanuginosus* (XTl) at pH 6.5, but XTl achieved a better degradation of the matrix at pH 5. Hemicellulase from *Aspergillus niger,* Carezyme 1000L (cellulase) and Laccase showed poor performance when retting flax. Among the enzymes studied in this project Pulpzyme achieved the better degradation of the matrix, and thus, the most purified fiber. In general, hemicellulases and pectinases showed a good performance when retting flax fibers, and a mixture of these activities has a great potential to degrade the matrix to a greater extent. In addition the content of pectin of green flax and flax fiber was analysed by means of m-hydroxydiphenyl but results were not in accordance with literature and a validation of the method was initiated. The results of the validation suggested a poor hydrolysis of pectin substrates in the m-hydroxydiphenyl method.

Keywords: Flax fiber composition, cellulose, hemicellulose, lignin, pectin, enzymatic retting, flax fiber reinforced polymer composites.

1 LITERATURE STUDY

1.1 General introduction

Natural fiber reinforced composites (NFRC) are a novel material which have arose its popularity during the last four decades as a plausible substitute for glass fiber reinforced composites (GFRC). They are already being used for many specific applications like door panels in cars, panels in boats or interior panelling in aircrafts, but to become an alternative for glass fiber composites better treatments (i.e. enzymatic treatments to extract the fibers from the stem of the plants) and procedures (i.e. improved mechanical extraction methods) are needed.

Despite the fact that the properties of natural fibers are not good, they have desirable high specific Young's modulus and high tensile strength alongside low density, and for that reason, natural fibers became a competent substitute for many specific applications. Additionally, natural fibers have significant advantages, which will be explained in the following paragraphs.

The first advantage of natural fibers is being much more environmental friendly than glass fibers. The main reason is that natural fibers are plants, and thus, they are cultivated using solar energy to grow up. In contrast, glass fibers are man-made materials and their production process requires big amounts of fossil energy (Joshi *et al.*, 2004; Begum and Islam, 2013). A comparison of the non-renewable energy required in different steps between the production process of glass fiber and flax fiber, which is a natural fiber, is shown in Table 1-1.

Table 1-1: Non-renewable energy required for production of glass and flax fiber (Joshi *et al.*, 2004).

Glass fiber	MJ/kg	Flax fiber	MJ/kg
Raw materials	1.7	Seed production	0.05
Mixture of components	1.0	Fertilizers	1.0
Transport	1.6	Transport	0.9
Melting	21.5	Cultivation	2.0
Spinning	5.9	Fiber separation	2.7
Mat production	23.0	Mat production	2.9
Total	**54.7**	**Total**	**9.55**

The non-renewable energy required to obtain raw materials shows an important contrast in magnitude, though the big difference is consequence of the large quantity of fossil fuel required for melting and material production of glass fiber compared to the limited energy needed for flax fiber production. The energy required to produce flax fiber (9,55 MJ/kg) is approximately the 17% of the total energy required to produce glass fiber (54,7 MJ/kg) (Joshi *et al.*, 2004).

Moreover, at the end of its life, NFRC can be incinerated but GFRC cannot. Therefore, while glass fibers stay as a residue, natural fibers are incinerated (with energy recovery), and the carbon emitted to the atmosphere is a neutral contribution, since it had been absorbed by natural fibers plants during the cultivation. Nevertheless, this quality is closely restricted by the matrix, since not all of them can be incinerated. On top, natural fibers are biodegradable while glass fibers are not (Wambua, Ivens and Verpoest, 2003; Joshi *et al.*, 2004).

The second advantage of natural fibers is having a lower density than glass fibers. E-glass, one of the most common types of glass fiber used for GFRC, has a density of 2,5 g/cm^3, while natural fibers like flax, hemp, jute or ramie have a density of approximately 1,5 g/cm^3 i.e. about 40 % lighter than E-glass fiber (Pickering, Efendy and Le, 2016). This characteristic is a relevant feature for reinforced composites, since the fields with more potential application for NFRC are the automotive and transportation industry (Begum and Islam, 2013). If vehicles, planes and boats replace their components made of composites reinforced with glass fibers with composites reinforced with natural fibers their weight would notably decrease, and so will the fuel consumption. Thus, as they would be more fuel-efficient, the amount of emissions to the atmosphere would be greatly reduced as well (Joshi *et al.*, 2004).

Furthermore, our society is slowly turning its life style into a more environmentally friendly one, and adopting NFRC as a substitute for GFRC would be a great step forward towards a more sustainable and competitive industry.

Among natural fibers used for reinforcing composites, flax fiber is one of the most promising reinforcements due to its good mechanical properties, i.e. high specific tensile strength and Young's modulus. Table 1-2 shows the most important physic and mechanical properties for many different types of natural fibers as well as for E-glass, the most common glass fiber used for polymer composites materials.

On one hand, flax fiber specific tensile strength (230-1220 MPa/g·cm^{-3}) is significantly superior to other natural fibers such as hemp (370-740 MPa/g·cm^{-3}), jute

(300-610 MPa/g·cm^{-3}) or ramie (270-620 MPa/g·cm^{-3}), and furthermore, it is comparable to E-glass (800-1400 MPa/g·cm^{-3}) (Pickering, Efendy and Le, 2016).

Table 1-2: Properties of different *types of natural fibers and E-glass fiber* (Pickering, Efendy and Le, 2016).

Fiber type	Density (g/cm3)	Length (mm)	Failure strain[1] (%)	Tensile strength[2] (MPa)	Specific tensile strength[3] (MPa/g·cm^{-3})	Specific Young's modulus[4] (GPa/g·cm^{-3})
Flax	1.5	5-900	1.2-3.2	345-1830	230-1220	18-53
Hemp	1.5	5-55	1.6	550-1110	370-740	39-47
Jute	1.3-1.5	1,5-120	1.5-1.8	393-800	300-610	7.1-39
Coir	1.2	20-150	15-30	131-220	110-180	3.3-5
Cotton	1.5-1.6	10-60	3.0-10	287-800	190-530	3.7-8.4
Ramie	1.5	900-1200	2.0-3.8	400-938	270-620	29-85
E-glass	2.5	Continuous	2.5	2000-3000	800-1400	29

[1] Strain to failure gives the measure of how much the specimen is elongated to failure.
[2] Tensile strength is the capacity of a material to withstand loads tending to elongate.
[3] Specific tensile strength is the tensile strength divided by its density.
[4] Specific young's modulus is the young's modulus divided by its density. Young's modulus is a measure of the stiffness of a solid material.

On the other hand, flax fiber also has a better specific Young's modulus (18-53 GPa/g·cm^{-3}) than hemp (39-47 GPa/g·cm^{-3}) jute (7.1-39 GPa/g·cm^{-3}) and E-glass (29 GPa/g·cm^{-3}) (Pickering, Efendy and Le, 2016). Moreover, as stated before, in Table 1-2 can be observed that E-glass fibers have a considerable higher density than natural fibers.

Nevertheless, the principal drawback for using flax fibers in mass production for high quality fiber applications is its difficult extraction from the plants stem, since flax fibers are strongly glued to the core tissues. In order to achieve fibers extraction, two procedures are required; retting and mechanical extraction. Retting goal is to degrade the substances responsible for such strong adhesion e.g. pectin or hemicellulose. Afterwards, once the cementing substrates are degraded, a mechanical extraction to physically separate the fiber from the other tissues is required.

Traditionally two methods were used for retting: water retting and dew retting. Water retting consisted of submerging flax in water and produced uniform and high quality fiber. However, now is in disuse because, on one hand, it needed a large volume of water and a long time (7 to 14 days (Md. Tahir *et al.*, 2011)) to process the fibre and, on the other hand, it created pollution and putrid odours due to fermentation reactions.

Dew retting at the moment is the most used treatment for retting flax and consists of harvesting flax and spreading it on the open field for many weeks (Martin *et al.*, 2013). Weather and moisture provide micro-organisms like fungi and bacteria to treat flax and

degrade those cementing components. The quality of the fibers treated with dew retting is lower than those treated with water retting. Moreover, the weather dependence of dew retting causes a several decline and inconsistency on fiber properties and, on top of that, flax occupies a large area of land during the process (Sharma and Van Sumere, 1992; Meijer *et al.*, 1995; Van De Weyenberg, 2005; Martin *et al.*, 2013).

In the last decades, enzymatic treatments are being developed as an alternative for the traditional retting methods. These experimental treatments focus on enzymes capable of attacking different molecules e.g. pectin, lignin or hemicellulose in order to free the bast flax fibers. Their most attractive features are being fast, taking about 24 hours, and obtaining fiber with consistent properties. Moreover, they are not weather-dependent nor geographic restricted. Though many efforts and advances by Sharma & Van Sumere (1992) or Akin (2013), enzymatic treatments still need to be optimized in order to successfully ret fibers without affecting their properties.

Hence, it is crucial to keep doing research in enzymatic treatments and extraction procedures in order to overcome flax fiber principal drawback and thereby, make it a competent material attractive for high-volume production industries. Therefore, this project studies the effect of different enzymatic treatments on flax fibers by characterizing the chemical properties of the treated fibers. This includes the determination of the cellulose, hemicellulose and lignin content with a gravimetrical method. At the same time, a spectrophotometric method for the determination of pectin content is optimized.

The following chapters include an extensive literature review in order to fully understand this project purposes, experiments and conclusions. Although the theory contains a wide view of each subject it only focuses and extends those sections directly related to flax fiber reinforced polymer matrices. Firstly, there is an introduction to composites which explains their most important parts, matrices and reinforcements. Thermoset and thermoplastic polymer matrices have been described for they are the most used matrices with natural fibers. Regarding reinforcements, a general classification with the most common examples has been included. Secondly, there is an overview of flax focusing on its fibers. To begin with, a brief review of flax history and production is given, followed by a deep explanation of flax structure, flax fiber and their components. Finally, the traditional and novel retting treatments and extraction procedures of flax fibers are reviewed.

1.2 Composites

One of the most accurate definitions for a composite is: “Multifunctional material system that provides characteristics not obtainable from any discrete material. It is a cohesive structure made by physically combining two or more compatible materials, different in composition and characteristics” (Chandramohan and Marimuthu, 2011). Hence, the purpose of composites is to assemble two (or more) different materials with different but desired properties in order to create a new and improved material which aggregate the properties of its constituents (Campbell, 2010; Ashik and Sharma, 2015).

A composite is made out of at least two phases: a continuous phase called matrix and a discontinuous phase called reinforcement. The matrix is the basis of the composite, contains and wraps the discontinuous phase and its main function is to disperse the load towards the reinforcements. The reinforcement function is to give strength, stiffness and good mechanical properties to the composite as well as the ability to withstand loads (Chandramohan and Marimuthu, 2011; Chauhan and Chauhan, 2012).

1.2.1 Matrices

Composites are classified in three categories according to their matrix material: polymer (e.g. thermoset and thermoplastic), metal (e.g. aluminium) or ceramic (e.g. silicon carbide). Commonly polymer matrices are the most used ones, albeit their strength and stiffness is lower than those of metal and ceramic matrices because their processing do not require as much temperature nor pressure as metals and ceramics do. (Chandramohan and Marimuthu, 2011; Davim, 2014; Ding, 2014). The polymers used for polymer matrix composites (PMC) are either thermosets or thermoplastics. A comparison between both polymer matrices is summarized in Table 1-3.

Table 1-3: Comparison of the properties between thermoset and thermoplastic matrices (Congress, 1988).

Resin type	Process temperature	Process time	Use temperature	Solvent resistance	Toughness
Thermoset	Low	High	High	High	Low
Toughened thermoset	↑	↓	↑	↑	↓
Lightly crosslinked thermoplastic	↑	↓	↑	↑	↓
Thermoplastic	High	Low	Low	Low	High

Thermoset matrices are more suited for applications where resistance to heat or chemical attacks is desired, while thermoplastics may be better for those applications where being tougher, lighter or more resistant to cracking is desired. Moreover, it’s low production time make thermoplastics more attractive for high-volume industries production (Congress, 1988). The following paragraphs include further explanation on thermoset and thermoplastic matrices production and properties.

1.2.1.1 Thermoset polymer matrices

Thermoset polymers are produced by heating and melting polymer resins in order to create a cross-linked structure. During the process, their physical properties are altered. This process is called curing and is irreversible, which means that they cannot be melted again to be recycled or repaired. Once the polymer is cured, it has a solid and stable form (Congress, 1988; Budapest University, 2010; Leonardo, 2017). Polyester, vinylester and epoxy are examples of thermoset polymers commonly used for PMC.

Thermoset polymers have two remarkable advantages in comparison to thermoplastics. The first one is being cheaper than thermoplastics due to the fact that thermoset resins are liquid at room temperature, and thus the amount of energy required for melting thermoset resins is lower. The second advantage of thermosets is their higher melting temperature after the curing process, which allows working at higher temperature applications than thermoplastics (Budapest University, 2010; Leonardo, 2017). Moreover, thermosets also present a greater resistance to solvents (Congress, 1988).

1.2.1.2 Thermoplastic polymer matrices

Thermoplastic polymer matrices are produced by melting, moulding, and cooling (Leonardo, 2017). Their processing is easier and faster than thermosets, but more costly. Additionally, in contrast with thermosets, thermoplastics do not create a cross-linked molecular structure, but consist of a linear and long molecular chain (Budapest University, 2010). Therefore, the process is reversible, and thus they can be melted and reshaped or recycled. For this reason, thermoplastic matrices are said to be more eco-friendlier than thermosets (Congress, 1988; Budapest University, 2010; Leonardo, 2017). Polypropylene, polyethylene, polystyrene and nylon are examples of thermoplastic polymers commonly used for PMC.

Because of their linear structure, on one hand, thermoplastics are lighter, tougher and less brittle than thermosets. On the other hand, they need a remarkable energy income to reach the melting temperature (i.e. depending on the polymer the temperature required can vary from 260 º to 370 ºC (Congress, 1988)). Additionally, they are easily degraded (Congress, 1988; Holbery and Houston, 2006; Budapest University, 2010; Leonardo, 2017).

Since both matrices have pros and cons, the matrix type needs to be chosen in accordance the final application, and afterwards, it will be reinforced in order to achieve better properties.

1.2.2 Reinforcements

Figure 1-1 shows the two main types of reinforcement: particle and fiber, which will be discussed in next paragraphs (Chandramohan and Marimuthu, 2011).

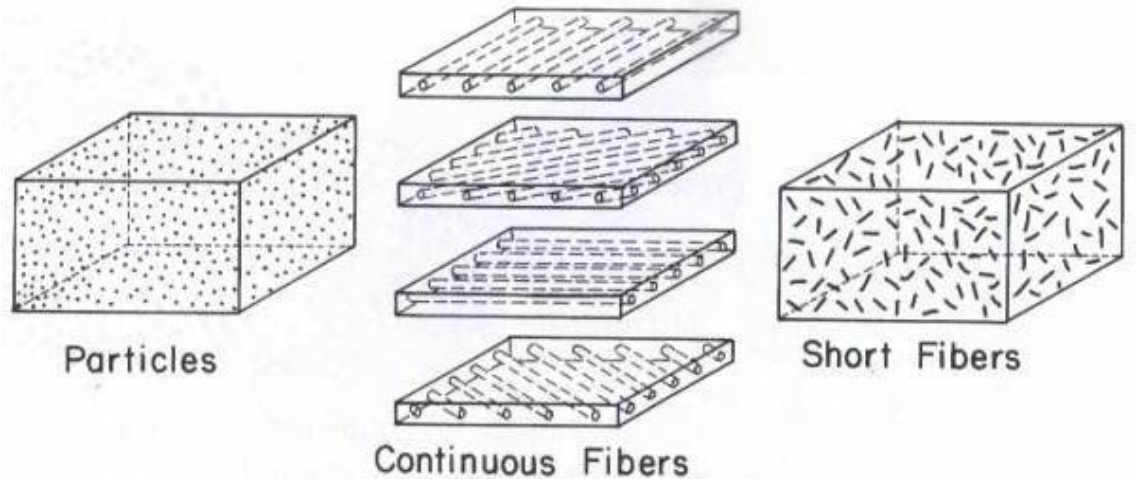


Figure 1-1: Different kind of reinforcement in composite materials. From left to right: particle reinforcement, continuous fiber reinforcement and short fiber reinforcement (Energi Norge, 2017).

1.2.2.1 Particle reinforcements

Particle reinforced polymers are typically made of ceramic, glass, metals or carbon. As shown in Figure 1-1, the particles are spread throughout the matrix. Improving the properties of the matrix and decreasing its ductility, as well as reducing costs, are the main interests for using particle reinforcement.

Particle reinforcements are normally cheaper than long fiber reinforcement. Additionally, they are easily produced. A common example for particle reinforcement composites is an automobile tire, which comprises a polymer matrix with carbon particles as reinforcement (Chandramohan and Marimuthu, 2011).

1.2.2.2 Fiber reinforcements

Fiber reinforcements have three characteristics responsible for its performance. The first one is the length of the fibers. There are continuous fiber reinforcements (long fibers) and discontinuous fiber reinforcements (short fibers). In view of the fact that the matrix transfers the loads to the fiber and it distributes the load along its length, the longer the fiber the better will be the composite's capability to resist loads (Campbell, 2010).

The second one is the orientation of the fiber. While long fibers are easily aligned, short fibers tend to be scattered randomly within the matrix. This aspect is relevant because the lack of orientation declines fiber reinforced composites properties. Hence, continuous fiber composites present a higher quality (Campbell, 2010). If the aspect

ratio value is too small, the interface adhesion may not be good enough to transfer the loads between the matrix and the fiber (Martin *et al.*, 2013).

Thirdly, the diameter of the fiber is commonly correlated to the length using a length/diameter ratio (also known as aspect ratio). A small diameter contributes to a higher strength, flexibility and amenability, but may increase the cost of the composite (Campbell, 2010).

In general, continuous reinforcements have better properties than discontinuous. Nonetheless, discontinuous reinforcements are cheaper than continuous. Therefore, the properties required for a target application and the budget will be decisive to choose the most appropriate reinforcement. Within fiber reinforcements, there are also two distinguished branches regarding their origin; man-made fibers and natural fibers, which will be explained in the following paragraphs.

1.2.2.2.1 Man-made fibers

Man-made fibers are produced by man and are subdivided in two groups regarding their origin: organic and inorganic. A schematic view of man-made fibers classification is shown in Figure 1-2.

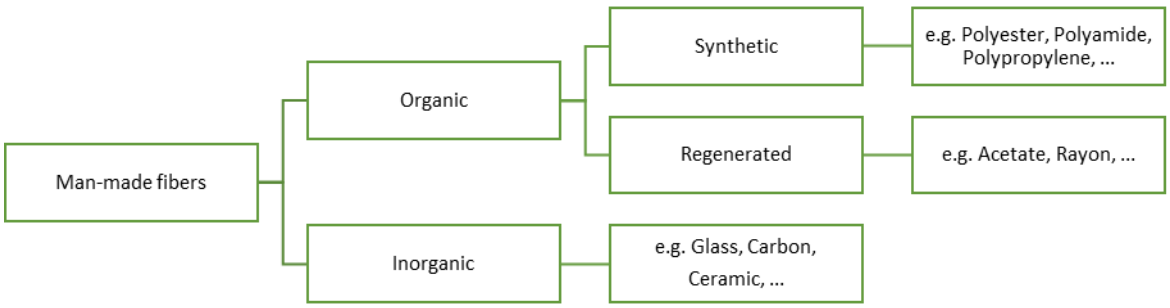


Figure 1-2: Classification and examples of man-made fibers (Needles, 1986; Fibre2fashion, 2017; Preston, 2017).

Man-made inorganic fibers include materials such as glass, carbon or ceramic, and are commonly used for polymer composites reinforcement. On the other hand, man-made organic fibers are subdivided in two categories, synthetic and regenerated.

Synthetic fibers, such as polyester, polyamide (e.g. nylon) or polypropylene, are the larger group within man-made fibers, and are made by chemical synthesis and, afterwards, are turned into fiber filaments. In contrast, there are the regenerated fibers, such as acetate or rayon, which are polymers being dissolved and regenerated in order to obtain the fibers (Needles, 1986; Fibre2fashion, 2017; Preston, 2017).

1.2.2.2.2 Natural fibers

In contrast with synthetic fibers, natural fibers are generated by nature. Despite being known for a long time, they did not captivate industry and research interests until 1980's (Westman *et al.*, 2010). They are classified according to their source into three categories: mineral, animal and plant (Chandramohan and Marimuthu, 2011). A schematic view of natural fibers classification is shown in Figure 1-3.

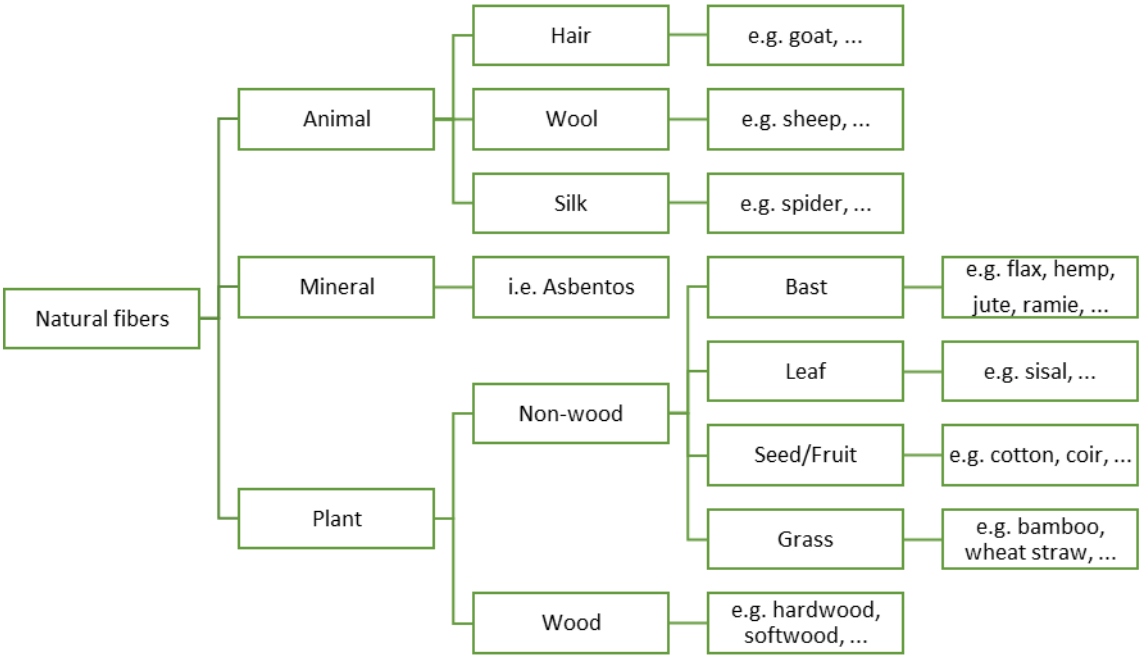


Figure 1-3: Classification and examples of natural fibers (Xu and Lewis, 1990; Van De Weyenberg, 2005; Chandramohan and Marimuthu, 2011).

Mineral fibers are created by nature or are faintly modified by man. The most common is asbestos, albeit its usage was restricted during the 90's for causing several health risks. Although not being as renowned as asbestos, there are also other types of mineral fibers such as zeolites (i.e. crystalline aluminosilicates) and silicates (Van De Weyenberg, 2005; Chandramohan and Marimuthu, 2011).

Animal fiber structures are made up of proteins. They are divided in three categories: hair (e.g. goat), wool (e.g. sheep and bison) and silk (e.g. spider fibers). Commonly they are woven for textile applications (Xu and Lewis, 1990; Van De Weyenberg, 2005; Chandramohan and Marimuthu, 2011).

Plant fiber structures are made up of cellulose, hemicellulose and lignin, though the content of each component depends on the source. The composition of different types of plant fibers will be further discussed in paragraph 1.3.4. Plant fibers are divided into wood and non-wood fibers depending on their origin and, in turn, non-wood fibers are

subdivided in bast, leaf, seed, fruit and grass, regarding which part of the plant they were extracted from (Van De Weyenberg, 2005). Most used natural fibers for polymer composites are flax, jute, ramie and sisal (Saheb and Jog, 2015).

For the reasons explained in paragraph 1.1, this project focuses on flax fibers enzymatically retted. Hence, the following paragraphs include an extensive review on flax. Firstly, flax structure and flax fiber are studied, and secondly, the retting treatments and dressing procedures are reviewed.

1.3 Flax

1.3.1 Introduction to flax

Flax (*Linum usitatissimum L.*) is one of the first natural fibers utilized by mankind (Akin, 2013). Even though it is not known when and where flax begun being cultivated, evidences point out to Switzerland at the stone age, about 10000 years ago. There are traces of flax in historic civilizations like in the ancient Egypt, where the emperors were entombed wrapped in linen clothes, or in the Roman empire, who used and traded flax spreading its use throughout the European continent (Sebesta and Bonfante, 1994; Van De Weyenberg, 2005; Akin, 2013).

Traditionally, flax had two main applications: linseed oil and fibers for textile (Baley, Bourmaud and Goudenhooft, 2017). Flax grown for linseed oil production has a more branched and shorter stem than textile flax (Van De Weyenberg, 2005), which requires high quality and long fibers. Linen apparels are renowned for their high standards among textiles. More recently, flax arose as a functional food due to its healthy properties, such as being a great source of two indispensable fatty acids (omega-3 and omega-6) and a diseases-preventive food (Jhala and Hall, 2010). Nevertheless, its relevance for food is minor compared to linseed oil and flax fiber for textile applications.

Furthermore, in the last decades a novel branch of application for flax fiber has arisen, named polymer composites. Natural fiber reinforced composites are a promising material and, among natural fibers, flax fiber is one of the most interesting due to its higher mechanical properties.

1.3.2 Production of flax

Flax is a plant cultivated worldwide and there are over 200 species of flax, but the most cultivated one to produce fibers is *L. usitatissimum* (Yan, Chouw and Jayaraman, 2014). Its optimal conditions for growing are wet climates like Canada or western Europe. In the latter region, flax is seeded at the end of March and harvested at beginning of July, taking about 100 days. At that time, the base of the plant will be turning yellow. If it is still green, the fiber will not be fully developed. Conversely, if flax turns brown, the fiber will start degrading and its properties will decline (Martin *et al.*, 2013; Yan, Chouw and Jayaraman, 2014; Dhirhi *et al.*, 2015).

Flanders (located in Belgium) begun producing flax in the 13th century. Its climate conditions were propitious for flax growing at its top qualities for textile applications. During the following centuries, its industry grew to the extent that it became considered as the capital of flax industry. However, flax production suffered a mighty decline in the

mid-20th century due to the arisen of the man-made fibers industry (Van De Weyenberg, 2005).

As Figure 1-4 shows, in 2014, western European countries (France and Belgium) were the principal producers of flax fiber for high quality textile and composite applications. Nevertheless, must be stated that FAO does not take into account Canada's data.

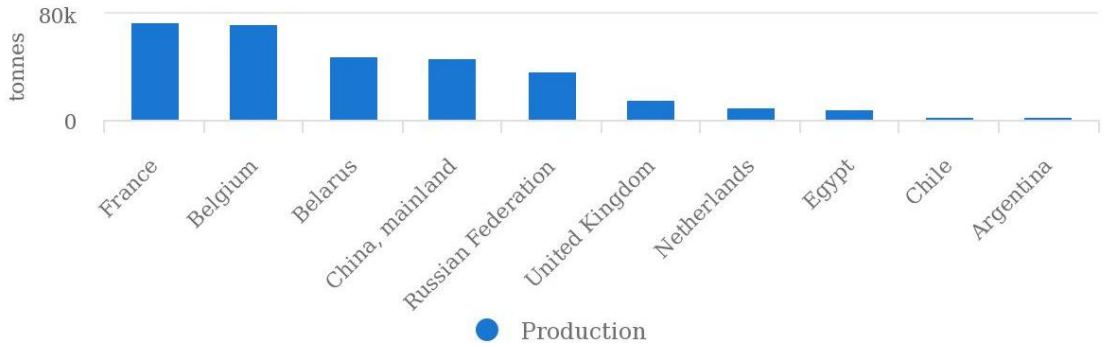


Figure 1-4: World top 10 producers of flax fiber and tow at 2014 (FAOSTAT, 2017).

Apart from western Europe, other regions like China, Russia or Egypt are growing flax, but their quality is not as good as flax produced in Belgium or France because their climate is not so propitious to cultivate flax. This lack of quality impedes its usage for high quality textile and composite applications (Akin, 2013).

Consecutively, the following paragraphs include an explanation of flax plan structure starting from the outer layer to the inner, and an emphasis is done in flax bast fibers due to its relevance in this project. Also, flax's more significant compounds are reviewed.

1.3.3 Structure of flax plants

Flax plants have thin stems with lengths of about 120 cm. Their stem has a layered structure comprised of three main sections: cuticle/epidermis, cortex region (contains bast fibers) and inner cells (Van De Weyenberg, 2005; Akin, 2013). Figure 1-6 represents a schematic drawing of the layered structure of flax plant.

The cuticle, together with the epidermis, forms the outer layer of the stem. Waxes and cutin comprise this layer, which represents between 13 to 24 % of the total weight of the bast fraction. It is a slender and impermeable coat that acts as a barrier to avoid water loss and penetration of microbial pathogens into the inner cells (Akin, 2013).

Following to the epidermis there is the cortex region, also known as phloem or bark (Van De Weyenberg, 2005). This region contains bast tissues and is in charge of

transporting nutrients from the leaves to the rest of the plant (Cambridge University Press, 2013).

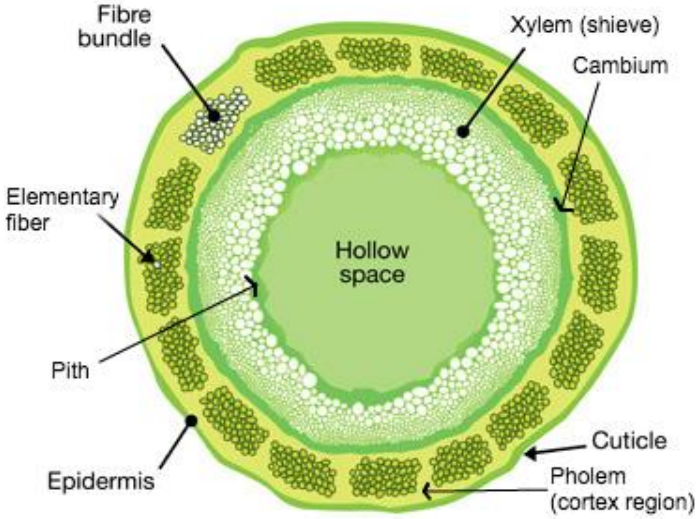


Figure 1-6: Cross section of flax stem. Adapted from (National research council of Canada, 2017)

Additionally, the cortex region includes bast fibers. The predominant compound of bast fibers is cellulose, which represents between 65 to 80 % of the total dry weight of the fiber, but they also contain hemicellulose, pectin and a small amount of lignin (Akin, 2013). They are spread out through the cortex area in 20 to 50 fiber bundles of 10 to 40 elementary fibers, and each elementary fiber is about 25 mm to 150 mm long and 15 to 20 µm thick (LaBat and Salusso, 2003; Van De Weyenberg, 2005; Akin, 2013). Figure 1-5 illustrates the layered structure of an elementary fiber.

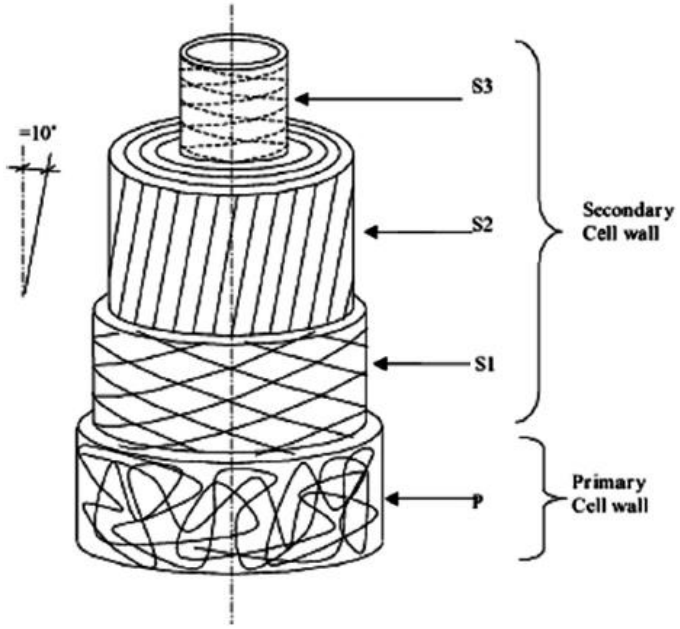


Figure 1-5: Schematic illustration of cell wall structure layers (Missouri University, 1998)

In its turn, elementary flax fibers have a layered and cylindrical cell wall structure with a lengthwise void in the centre called lumen. The cell wall is made up by the primary (P) and the secondary cell wall, and the latter, in its turn, is divided in three sublayers; S1, S2 and S3. Next, there is a matrix called middle lamella, which wraps the primary cell wall and separates the elementary fibers from each other (Van De Weyenberg, 2005; Md. Tahir *et al.*, 2011; Yan, Chouw and Jayaraman, 2014).

Cell walls comprise cellulose microfibrils, hemicellulose, lignin, pectin and waxes. Cellulose microfibrils are embedded by hemicellulose with small quantities of amorphous lignin and waxes while pectin, which generates cohesion, can be found in primary wall surfaces and cell junctions i.e. middle lamella (Morvan *et al.*, 2003; Van De Weyenberg, 2005).

The differences between the primary and secondary cell wall layers are caused by their different composition, thickness and orientation angle of the cellulose microfibrils. As Figure 1-5 shows, in the primary cell wall cellulose microfibers are arbitrarily oriented, while in the first (S1) and third (S3) layers of the secondary cell wall the cellulose microfibers are arranged in cross-parallel direction with an orientation angle between 30º to 60º (Van De Weyenberg, 2005; Yan, Chouw and Jayaraman, 2014; Thuault *et al.*, 2015). The second layer of the secondary wall (S2) is the thickest, representing about 75 % of the elementary fibre thickness (Van De Weyenberg, 2005), and its particular characteristic is the 10º angle orientation along the fiber axis of its cellulose microfibrils, which is responsible for flax fibers high specific tensile strength (Yan, Chouw and Jayaraman, 2014; Acera Fernández *et al.*, 2016).

Next to the cortex region there are the inner cells of the flax stem, of which some cell types can be distinguished. Just like with bast fibers; cellulose, hemicellulose, pectin and lignin comprise this layer, which represents between 65 to 75 % of the total dry weight of the stem. Almost all lignin of the stem is comprised in this region (Akin, 2013).

In the middle, there is a thin coat, known as cambium, which separates xylem tissues from phloem tissues (Van De Weyenberg, 2005). The first layer after cambium is comprised of woody tissue (shives), named xylem, which is in charge of carrying water and minerals from the roots to the leaves and provides structural support to the stem. Secondly, there is the pith, the inner layer of the stem formed by a soft, white liquid surrounding the lumen, a central hollow space throughout the stem (Cambridge Dictionary, 2013).

Next paragraphs include an explanation of the structures of the main compounds in flax, cellulose, hemicellulose, lignin and pectin, their functions and most relevant characteristics.

1.3.4 Chemical composition of flax fibers

The content of each component in flax fibers will strongly affect their properties and so, the properties of flax fiber composites (Akin, 2013). Nevertheless, each specimen of flax, even harvested in the same field at the same time, can present differences in their composition (Yan, Chouw and Jayaraman, 2014). Table 1-4 shows the concentration of cellulose, hemicellulose, lignin and pectin for different types of plant fibers.

Table 1-4: Composition of cellulose, hemicellulose, lignin and pectin for different types of plant fibers (Van De Weyenberg, 2005).

Fiber	Cellulose (w/w %)	Hemicellulose (w/w %)	Lignin (w/w %)	Pectin (w/w %)
Flax	65 - 85	10 - 18	1 - 4	1 - 3
Hemp	60 - 77	10 - 17	3 - 10	1 - 3
Jute	45 - 63	12 - 15	12 - 25	0.2 - 3
Kenaf	45 - 57	21.5	8 - 13	3 - 5
Sisal	50 - 64	10 - 14.2	± 10	± 1
Coir	± 30	± 1	40 - 45	3 - 4
Cotton	85 - 98	3 - 6	-	< 1
Wheat straw	38 - 41	-	12 - 16	< 1
Softwood	40 - 45	25 – 30	26 - 34	< 1
Hardwood	45 - 50	21 - 35	22 - 30	< 1

Cellulose is the major component in plant fibers, of which flax is one of those with most cellulose content. In contrast, lignin, which is a renowned structural component is rather marginal in flax. As will be explained in the following paragraphs, this high content of cellulose is the responsible for plant fibers strong structure.

1.3.4.1 Cellulose

Cellulose is a polysaccharide which comprises from 500 to 10000 glucose molecules linked together by β-1,4-glyosidic bonds forming a long, linear and unbranched chain. Figure 1-7 illustrates three glucoses, which form part of a longer cellulose chain, and their free hydroxyl groups. These groups interact with the hydroxyl groups of the other chains creating hydrogen bonds between them. When several chains of cellulose are aligned and bounded together by hydrogen bonds, they form highly ordered structures

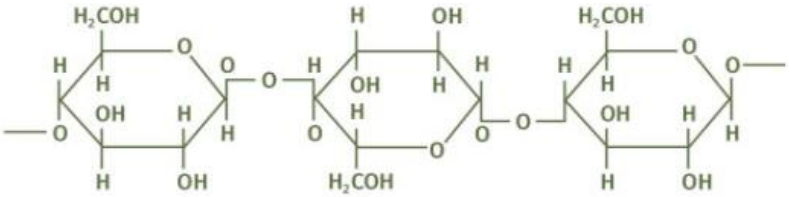


Figure 1-7: Schematic representation of the structure of a cellulose chain made of three glucose molecules (CelluForce, 2017)

known as cellulose microfibrils (Chloe and Block, 2010; Chen, 2014; Martin, 2016). Those regions with cellulose in this highly-ordered form are named crystalline regions, and those with non-crystalline structure are known as amorphous regions (John and Thomas, 2008). Cellulose is constituted of both regions intertwined.

Crystalline regions are characterised for being inaccessible due to its high amount of hydrogen bounds, high-density and stiff structure. Because of these hydrogen bounds it is a hydrophobic region well-shielded against enzymatic attacks (Taiz and Zeiger, 2006). In contrast, amorphous regions are lighter and easily reachable. There are free hydroxyl groups, which can interact with water molecules, and thus, can lead to water absorption (Van De Weyenberg, 2005; Chen, 2014).

1.3.4.2 Hemicellulose

Hemicellulose is a polysaccharide which comprises of 50 to 300 sugar molecules linked together forming a short amorphous branched chain. In contrast with cellulose, hemicellulose is made up of different types of sugars e.g. glucose, xylose, arabinose, mannose or galactose (John and Thomas, 2008; Parsons *et al.*, 2013).

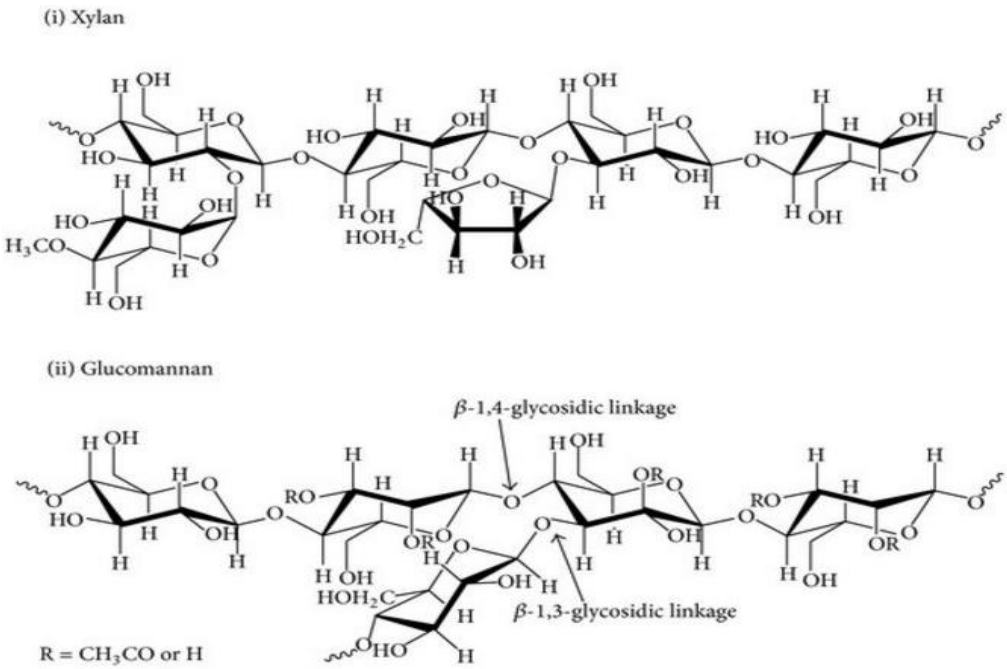


Figure 1-8: Chemical structure of two hemicelluloses: (i) xylan and (ii) glucomannan (Lee, Hamid and Zain, 2014).

Due to its amorphous and branched structure hemicellulose has a strong hydrophilic character, being the prime cause of water absorption problems of fibers. Moreover, hemicellulose is easily accessible and thus, vulnerable to chemical attacks (Van De Weyenberg, 2005). The chemical structure of two hemicelluloses, xylan and glucomannan, is shown in Figure 1-8, in which the branched chain structure can be observed.

Figure 1-8 shows a xylan (i) and a glucomannan (ii), which are the most abundant biopolymers. Hemicellulose is found amid the cellulose microfibrils, acting as a glue through hydrogen bounds to keep them together forming the cellulose/hemicellulose network (Kalia, Kaith and Inderjeet Kaur., 2011). This network is shown in Figure 1-9, which is a schematic model of how the principal components in the primary cell wall may be arranged.

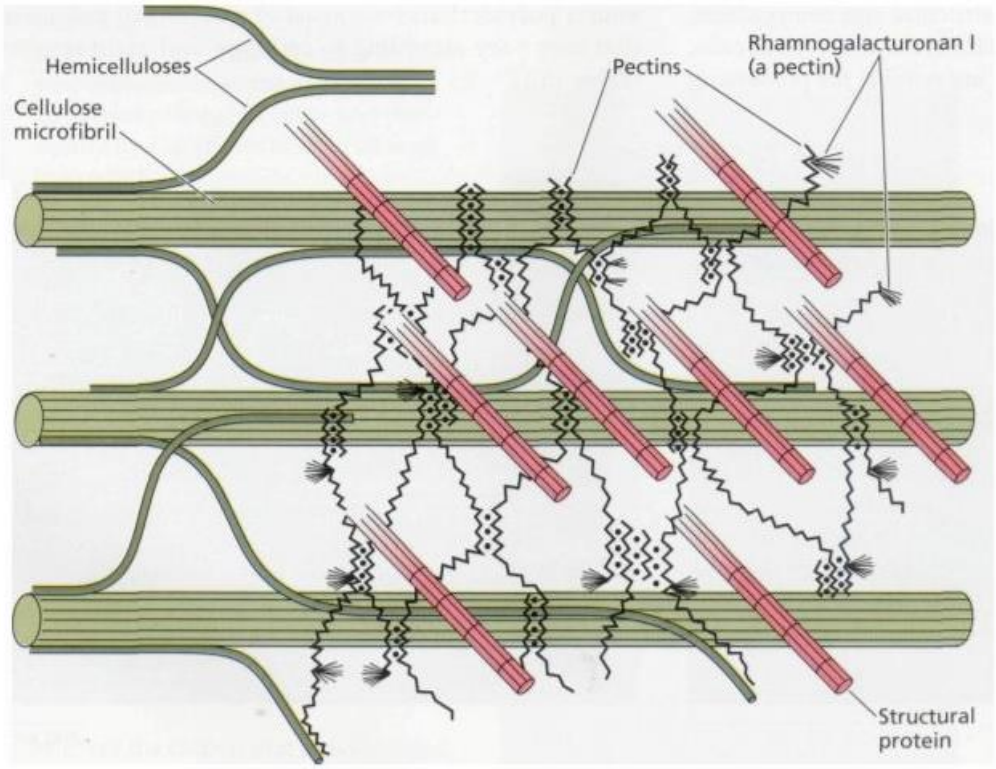


Figure 1-9: Schematic diagram of the principal structural components and their likely arrangement (Taiz and Zeiger, 2006).

In the model illustrated above, cellulose microfibrils are aligned in parallel and an amorphous mixture of hemicelluloses, pectins and structural proteins form an adhesive to keep them tightly tied together.

1.3.4.3 Lignin

Lignin is a complex three-dimensional network polymer comprised of both aliphatic and aromatic compounds with an amorphous structure and high molecular weight (Van De

Weyenberg, 2005; Kalia, Kaith and Inderjeet Kaur., 2011; Chen, 2014). Lignin is not a specific molecule or structure, but a group of substances randomly linked together that varies regarding the plant, or even regarding the part of the plant (Chen, 2014).

Figure 1-10 shows a structural model of a section of lignin, albeit its real structure and linkages are still unknown. Lignin is a structural compound that gives rigidity to plants and possesses a hydrophobic character (Kalia, Kaith and Inderjeet Kaur., 2011; Faruk and Sain, 2015).

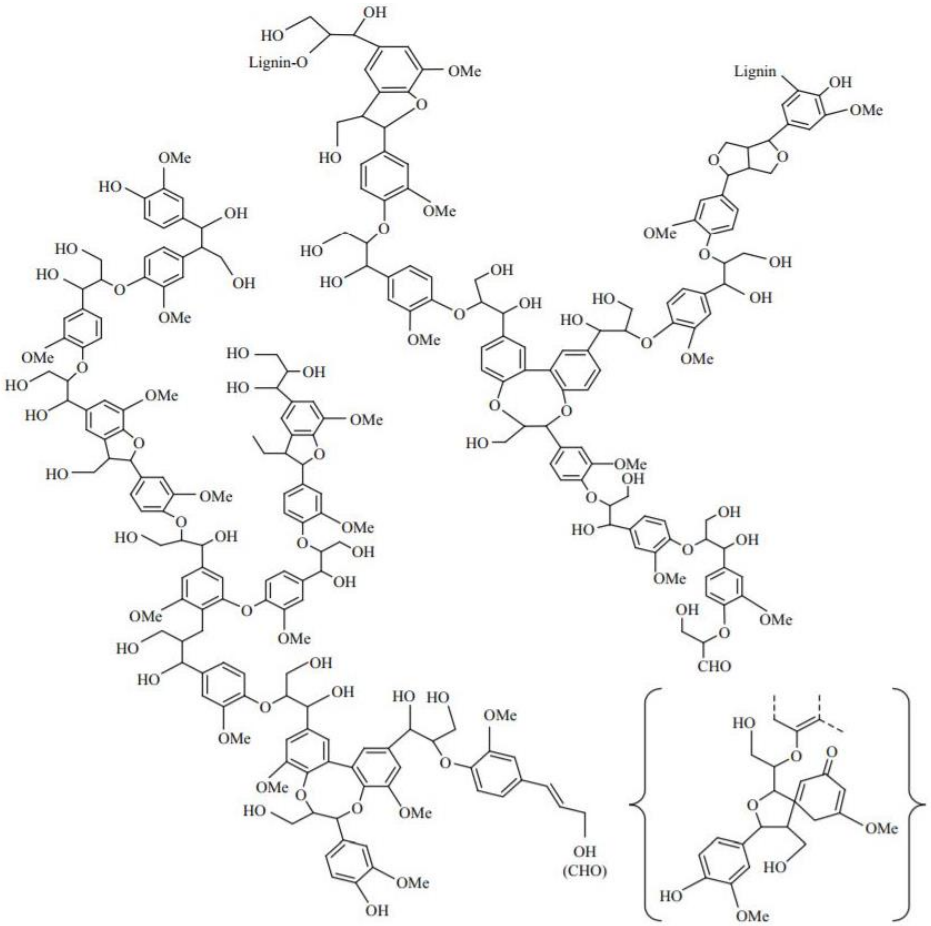


Figure 1-10: Structural model of a section of cork lignin (Chen, 2014).

Despite being present in important concentrations in many plant fiber types such as jute or coir (see Table 1-4) and having good mechanical properties though comparable to cellulose (Kalia, Kaith and Inderjeet Kaur., 2011), the content of lignin in flax fibers is rather small. This residual lignin can be found within the cellulose/hemicellulose matrix wrapping the cellulose microfibrils (Van De Weyenberg, 2005).

1.3.4.4 Pectin

Pectin substrates are a complex mixture of polysaccharides with a branched structure and high molecular weight (Van De Weyenberg, 2005; Pedrolli *et al.*, 2009). They are

soluble and can be extracted with hot water (Taiz and Zeiger, 2006). Pectin (used as a general name for pectic substances) comprises of acidic sugars like D-galacturonic acids α-1,4 linked to each other, which is the major constituent, often linked with β-1,2-bonds to rhamnose, a neutral sugar (Pedrolli *et al.*, 2009; Kalia, Kaith and Inderjeet Kaur., 2011). Pectin can be found in the middle lamella and in the primary cell wall contributing to the cell wall structure acting as a cement for the cellulose microfibrils (Walter and Taylor, 1992). Figure 1-11 shows a conventional model of a pectin with homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), the three most important polymers of pectin.

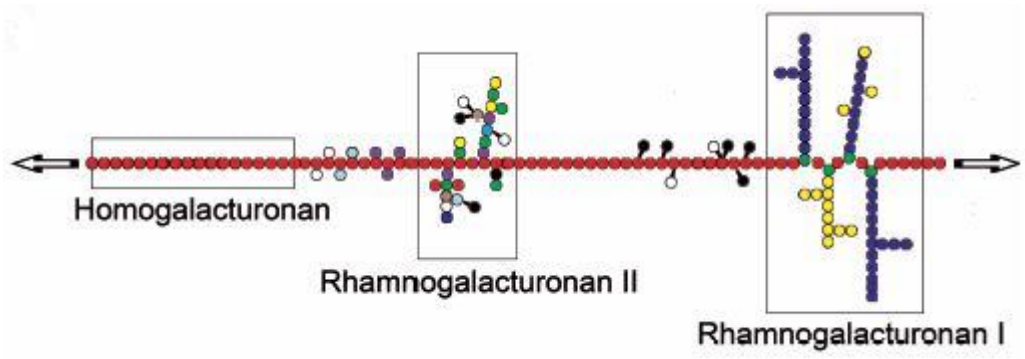


Figure 1-11: Possible model of a pectin structure (Pickering, Efendy and Le, 2016)

Homogalacturonan (HG) is a linear polymer constituted of D-galacturonic acid, which in turn can be acetylated and/or methyl esterified. When galacturonic acid is highly methyl esterified it is commonly known as pectin, if it has no methyl esterification it is called pectate or polygalacturonic acid (Pickering, Efendy and Le, 2016). Rhamnogalacturonan I (RGI) is formed by repeating disaccharide units of rhamnose and galacturonic acid. It can be acetylated and carry chains of neutral sugars. Rhamnogalacturonan II (RGII) is a homogalacturonan chain with complex side chains attached (Pedrolli *et al.*, 2009).

In conclusion, cellulose microfibrils are the components that give bast fiber its desired properties and they are glued to the cortex tissues by a cement mainly comprised of hemicellulose and pectin. Therefore, the following paragraphs explain the retting treatments, which degrade the cementing substrates, and the extraction procedures, which separate the bast fibers from the stem material.

1.3.5 Extraction process

There are different methodologies to extract bast fibers from the flax stem and each one of them affects the final quality of the fibers. Hence, each application will require a certain extraction method regarding the desired quality.

First of all, when harvested, flax is pulled out of the soil instead of being cut in order to maximise its length for fiber applications (Van De Weyenberg, 2005). Following, fiber needs to be extracted from its stem. Nevertheless, fiber is strongly fixed to the core tissues, and thus, it requires a previous treatment in order to degrade the adhesion between the tissues and the fiber bundles. This process is called retting (Sharma and Van Sumere, 1992). One of its challenges, no matter what method is used for retting, is determine its duration. If retting time is insufficient, fibers will be difficult to separate. In turn, if fibers are over-retted it may decrease its quality (Akin, 2013).

Traditionally two methods were used to free the bast fibers: water retting and dew retting. The water retting consisted of submerging stalks of flax in tanks, where anaerobic bacteria (e.g. *Clostridium felsinium*) degraded pectin and freed the bast fibers within 6-8 days. This method produced uniform and high quality fiber but now is in disuse because, on one hand, the process needed a large volume of water and a long time (i.e. 7 to 14 days (Md. Tahir *et al.*, 2011)) to process the fibre and, on the other hand, it created pollution and putrid odours due to fermentation reactions. Moreover, the maintenance of the water was an added cost to the process (Van sumere and Shekhar, 1991; Sharma and Van Sumere, 1992; Van De Weyenberg, 2005; Md. Tahir *et al.*, 2011).

Dew retting (also known as field-retting) consists of harvesting flax and spreading it on the open field for many weeks (i.e. between 3 to 6 weeks depending on the weather (Martin *et al.*, 2013)), where weather and moisture provide the micro-organisms (i.e. mainly fungi but also bacteria) which treat flax and degrade the non-cellulose components. The quality of the fibers treated with dew retting is lower than those treated with water retting because some of the agents (i.e. micro-organisms) not only degraded pectin and hemicellulose but also cellulose. Additionally, the weather-dependence of dew retting causes a several decline and inconsistency on fiber properties because it varies regarding the geographic zone and the year's climate. On top of that, dew retting takes a large area of land during the process and the fiber traded is slightly contaminated by soil (Sharma and Van Sumere, 1992; Meijer *et al.*, 1995; Van De Weyenberg, 2005; Martin *et al.*, 2013). Nevertheless, at the moment dew retting is the most used treatment for retting flax at flax fiber industry.

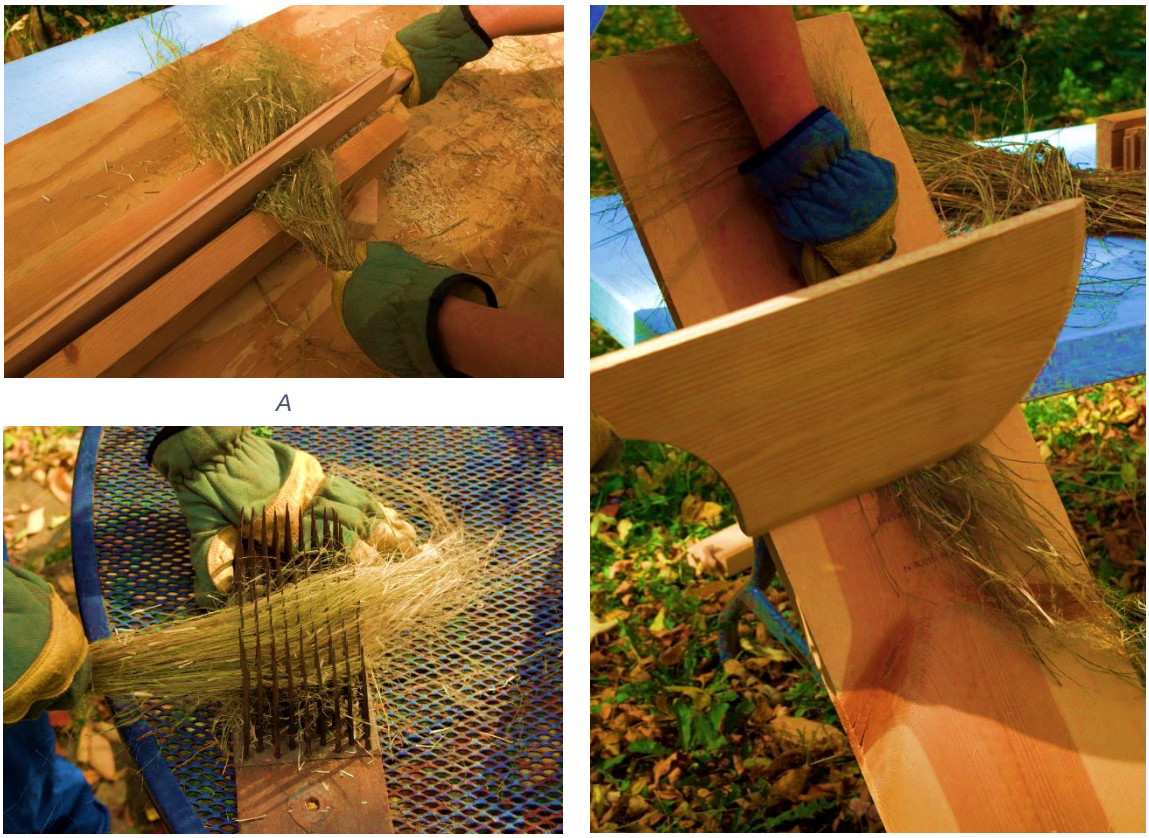
Enzymatic treatments are being investigated as an alternative treatment to degrade the non-cellulosic compounds responsible for bast fibers strong adhesion towards core tissues. Though having a higher cost than dew retting, enzymatic treatments can be carried within 24 hours (Van sumere and Shekhar, 1991; Md. Tahir *et al.*, 2011). In addition, they are not weather dependent and could provide a uniform and efficient

retting method to produce high quality fibers. Enzymatic treatments focus on enzymes capable of degrading non-cellulosic molecules such as pectin, hemicellulose or lignin in order to free the bast fibers. Although plant is attacked by many enzymes, pectic enzymes are the ones that initiate the degradation since pectin is the principal responsible for fibers' adhesion (Akin *et al.*, 2007; Md. Tahir *et al.*, 2011; Akin, 2013). Cellulases (enzymes that attack cellulose) sometimes can be beneficial to the degradation and improve the retting since there is also a lot of amorphous cellulose in the cement. Nonetheless, even in small quantities, cellulases may also provoke a decrease in fibers' strength.

Some commercial mixtures of enzymes have been developed for retting. For example, Flaxzyme, developed by Novo Nordisk (Denmark), included hemicellulases, cellulases and pectinases but is no longer available. Tests showed that fibers retted with Flaxzyme had qualities comparable to those water retted, but its strength was lower. Despite these good results, a commercial enzymatic method for industrial retting of fibers was never developed (Akin *et al.*, 2007; Akin, 2013). Other examples are Viscozyme L, which also includes cellulases, and BioPrep 3000L, which is a mixture created to be a cotton scouring agent purer in pectinases i.e. low or no cellulase content. Both were developed by Novozymes (North America). Akin *et al.* (2007) retted flax with both Viscozyme and Bioprep, and they were found to ret fibers properly, but fibers retted with Bioprep had a higher strength (Akin *et al.*, 2007). There must be stated that no matter which enzymatic mixture was tested for retting, all of them improved their efficiency adding the chelator ethylenediaminetetraacetic acid (EDTA) (Henriksson *et al.*, 1997; Akin, 2013).

Once the strong cementing between bast fibers and core tissues is degraded, fibers must be separated from the stem. This procedure is a mechanical extraction of the fiber, and consists of breaking, scutching and hackling (Dhirhi *et al.*, 2015). With the traditional retting methods, fibers require a rough mechanical treatment to be separated from the stem. Although the fact that with enzymatic retting a superior level of degradation can be achieved, fiber also requires mechanical extraction. Nevertheless, it may not be that intense. Figure 1-12 illustrates the traditional extraction of flax fiber.

First, the breaking process breaks up the straw flax to smaller bits, fracturing the body stick but without damaging the fiber (Das *et al.*, 2010; Dhirhi *et al.*, 2015). Figure 1-12 (A) shows how the traditionally breaking of flax was performed; bundles of straw flax were beaten till they got soft in the breaking machine, which consisted of a wooden blunt stick smash which was smashed till fibers got softer (Ash, 2012; Dhirhi *et al.*, 2015).



A

C

B

Figure 1-12: Traditional extraction of flax fiber: (A) Breaking; (B) Scutching; (C) Hackling (Ash, 2012)

Next, the scutching process removes the bigger traces of straw. As Figure 1-12 (B) shows, scutching was typically performed by hanging the fibers in a wooden board vertically positioned, and then, they were scratched with a wooden knife from the top to the bottom (Ash, 2012; Dhirhi *et al.*, 2015).

Finally, the hackling process consists of passing the flax over many combs of different sizes, starting by spacious combs to remove the straw, and then gradually decreasing to combs more thin in order to split and polish the fibers. Figure 1-12 (C) shows a traditional comb, made up of a wooden base and sharp nails (Ash, 2012; Dhirhi *et al.*, 2015).

Although existing machines capable of extracting flax, it is a complex process and the machines induce a certain damage to the fibers (Dhirhi *et al.*, 2015). Akin et al. (2005) extracted flax fibers retted by dew and enzymatic retting with a pilot plant for processing flax fiber. The pilot plant successfully processed the samples, but dew retted fibers were cleaner and stronger that those retted with an enzymatic treatment (Akin, Dodd and Foulk, 2005). Hence, to produce high quality fibers in high-volume production industries more research is required and a fully functional mechanized extracting process must be developed.

2 OBJECTIVES

This project aims to study the effect of different enzymatic treatments on flax fibers by characterizing the chemical properties of the treated fibers. This includes the determination of the cellulose, hemicellulose and lignin content through a gravimetrical method. The activities of the enzymes investigated included cellulases, hemicellulases, laccase and pectinases with the objective of degrading the pectin-hemicellulose matrix wrapping the fibers.

At the same time, a spectrophotometric method for the determination of pectin content is optimized.

3 MATERIALS AND METHODS

3.1 Gravimetrical analysis of chemical components

The main goal of this procedure is to determine the effect of different enzymes at diverse operational conditions on flax fiber by determining the content of cellulose, hemicellulose and lignin.

Material and equipment:

- Balance, Max. 120 g; d=0.1 mg (Sartorius)
- Balance, Max. 2200g; d=0.01g (Sartorius)
- Soxhlet equipment
- Oven (Heraeus)
- Bath, Max. 120 ºC (Jumo)
- Bath SW23 (Julabo)
- Autoclave (Fedegari)
- Filter paper (Whatman filter paper Grade 1)
- Glass fiber filter (Whatman)

Reagents:

- 99.9 % (v/v) Ethanol (Chem-lab)
- 99.9 % (v/v) Toluene (VWR)
- 100 % (v/v) Glacial acetic acid (Merck)
- NaCl (Suprasel)
- NaOH (VWR)
- 100 % (v/v) Aceton (VWR)
- 96 % (v/v) sulphuric acid (Acros)

Solutions:

- Ethanol-toluene mixture (1/3 ethanol with 2/3 toluene)
- 72 % (v/v) sulphuric acid (Acros): 75 ml 96 % (v/v) sulphuric acid was diluted in 100 ml distilled water.
- 18 % and 8.5 % (w/v) NaOH (VWR): dissolve 180 g or 85 g NaOH, respectively, in distilled water and dilute to 1 L
- 10 % (w/v) acetic acid (Merck): 95.238 ml acetic acid was diluted in 1 L distilled water

The following paragraphs explain the procedure followed to determine the composition of the fiber, which consists mainly of four subsequent steps: extraction, lignin determination, holocellulose determination and cellulose determination.

3.1.1 Extraction

Fibers were dried in the oven at 105 ºC for 24 hours. Filter holders with filter papers were dried in the oven at 105 ºC for at least 6 hours and were measured until constant weight.

The extraction was done with a Soxhlet equipment. A sample of about 1 g of dried fiber was measured in the extraction thimble in triplo. Some boiling chips and 190 ml ethanol-toluene mixture were added in the boiling flask. The extraction was carried during 5 hours with a solvent cycle of 6 times per hour. Afterwards, samples were filtered on Buchner funnel with the filter paper and washed with 100 ml ethanol and 400 ml hot water. The samples were dried in the filter holder overnight at 105 ºC and measured until constant weight.

3.1.2 Lignin determination

Crucibles were dried at 105 ºC for at least 6 hours. Subsequently, they were measured until constant weight.

A sample of about 80.0 mg of dried and extracted fiber were placed in a test tube with screw cap and 3.00 ml 72 % sulphuric acid was added. The samples were incubated during 1 hour at 30 ºC while stirring. After incubation, 84 ml of distilled water was used to transfer the solution into a 100 ml Schott flask, followed by autoclave treatment at 121 ºC for 1 hour. When cooled, fibers were filtered in a crucible and washed with 150 ml of distilled water. Crucibles were dried in the oven at 105 ºC overnight and were measured until constant weight.

3.1.3 Holocellulose determination

Filter holders with filter papers were dried in the oven at 105 ºC for at least 6 hours and were measured until constant weight.

A sample of about 0.900 g dried and extracted fiber and 160 ml distilled water, 0.5 ml 99.7 % glacial acetic acid (Merck) and 1.5 g NaCl were added in a 250 ml Schott flask. Flasks were incubated at 75 ºC during 1 hour while shaking at 120 rpm. After 1 hour, 0.5 ml 99.7 % glacial acetic acid and 2.5 g NaCl were added. Incubation during 1 hour and addition of 0.5 ml 99.7 % glacial acetic acid and 2.5 g NaCl were repeated twice. After incubation, flasks were cooled in an ice bath till flask temperature was below 10 ºC. Samples were then filtered in a Buchner funnel and were sequentially washed

with 25 ml acetone, 25 ml ethanol and 150 ml distilled water. Samples were dried in filter holder overnight at 105 ºC and were measured until constant weight.

3.1.4 Cellulose determination

Filter holders with glass fiber filters were dried in the oven at 105 ºC for at least 6 hours and were measured until constant weight.

A sample of about 0.800 g of fiber remaining from holocellulose determination and 70 ml 18 % (w/v) NaOH were added in a 250 ml Schott flask. Flasks were incubated during 30 minutes at 30 ºC while shaking at 120 rpm. Afterwards, 35 ml distilled water was added and flasks were incubated for another hour at 30 ºC while shaking was continued. After incubation, samples were filtered in Buchner funnel and were sequentially washed with 100 ml 8.5 % (w/v) NaOH, 200 ml distilled water, 15 ml 10 % (w/v) acetic acid and 200 ml distilled water. Samples were dried in filter holder overnight at 105 ºC and were measured until constant weight.

Finally, since holocellulose is comprised by cellulose and hemicellulose, the content of hemicellulose in the fiber was obtained by subtracting the content of holocellulose in the fiber minus the content of cellulose in the fiber.

3.2 Determination of pectin with m-hydroxydiphenyl (MHDP)

The main goal of this method is to determine the pectin content of flax fibers in order to determine the enzymatic effect by spectrophotometry.

Material and equipment:

- Spectrophotometer (Thermo fisher)
- Balance, Max. 120 g; d=0.1 mg (Sartorius)
- Soxhlet equipment
- Oven (Heraeus)
- Bath, Max. 120 ºC (Jumo)
- Filter paper (Whatman filter paper Grade 1)

Reagents:

- 90 % (w/w) Meta-hydroxydiphenyl (Acros)
- 6 M Hydrochloric acid (Chem-lab)
- 99.9 % (v/v) Ethanol (Chem-lab)
- ≥97 % (w/w) D-(+)-Galacturonic acid monohydrate (Fluka)
- NaOH (VWR Chemicals)
- 0.0125 M sodiumtetraborate decahydrate (Sigma-Aldrich)
- 96 % (v/v) Sulphuric acid (Acros).

Solutions:

- 70 % (v/v) Ethanol: 700 ml ethanol was diluted to 1 L with distilled water
- Galacturonic acid stock solution (75 µg/ml): 0.0410 g galacturonic acid was diluted to 500 ml distilled water in a volumetric flask.
- Galacturonic acid control solution (50 µg/ml): 0.0547 g galacturonic acid was dissolved to 100 ml distilled water in a volumetric flask and the solution was diluted 10 times.
- 0.5 % (w/v) NaOH = 0.125 M NaOH: 5 g NaOH was diluted to 1 L with distilled water
- Meta-hydroxydiphenyl solution (0.15 % (w/v) meta-hydroxydiphenyl in 0.5 % (w/v) NaOH): 150 mg MHDP was diluted to 100 ml with 0.125 M NaOH.
- 0.0125 M sodiumtetraborate decahydrate solution: 2.38 g sodiumtetraborate decahydrate was diluted to 500 ml with 96 % (v/v) sulphuric acid.

In a first trial, pectin content was determined of whole green flax and green flax fibers. The following paragraphs explain the procedure followed to determine the pectin content of flax fibers with m-hydroxydiphenyl, which consists of first extracting pectins with a soxhlet extraction, then hydrolyse them with HCl, and finally, measuring the content of pectin by means of spectrophotometry.

Flax and fibers were dried in the oven at 105 ºC for 24 hours. Filter holders with filters paper were dried in the oven at 105 ºC for at least 6 hours and were measured until constant weight.

The extraction of the pectin content was done with a Soxhlet equipment. A sample of about 600 mg of green flax and flax fiber was measured in the extraction thimble in duplo. Some boiling chips and 190 ml 70 % ethanol solution were added in the boiling flask. The extraction was carried during 5 hours with a solvent cycle of 6 times per hour. Afterwards, samples were filtered on Buchner funnel with the filter paper and washed with 300 ml hot water. The samples were dried in the filter holder overnight at 105 ºC and measured until constant weight.

A sample of about 200 mg of dried and extracted fiber and green flax and 10 ml 6 M HCl were added in a test tube with a screw cap in triplo. The samples were mixed with a vortex and incubated for 2 hours at 70 ºC. After incubation, samples were washed four times with 40 ml 40 ºC distilled water and diluted to 200 ml.

Next, seven standards of 75, 60, 45, 30, 15, 5 and 0 µg/ml galacturonic acid were prepared. Also a control of 50 µg/ml galacturonic acid was prepared. Then 0.4 ml

sample and standards with 2.4 ml 0.0125 M sodiumtetraborate decahydrate solution were added in a test tube in triplo. The samples were cooled down in ice water, shook with a vortex, warmed in a water bath at 100 ºC during 5 minutes and again cooled in ice water.

The samples were transferred into a cuvette and the absorbance was read at 520 nm with a spectrophotometer. After reading the absorbance, 40 µl meta-hydroxydiphenyl solution was added and the cuvette was mixed. Then, 30 seconds after the addition of the reagent the absorbance was read again at 520 nm. The correct value of absorbance is the second measurement minus the first measurement. Finally, the content of pectin of the samples was calculated by means of the equation of the calibration curve of the standards.

3.3 Bicinchoninic acid protein assay kit

The main objective of this method is to determinate the concentration of proteins in enzymes to, later on, prepare the most appropriate dilutions to carry out a SDS-PAGE study. A SDS-PAGE separates proteins depending on their sizes and its goal is to determinate the purity of the enzyme. The enzymes studied with this method are listed hereunder:

- Scourzyme L (Novozymes)
- NS59049 (Novozymes)
- Pulpzyme (Novozymes)
- Laccase NS 20621 (Novozymes)
- Rohapect CL (AB Enzymes)
- Rocksoft (Dyadic)
- Carezyme 1000 L (Novozymes)
- Hemicellulase from *Aspergillus niger* (Sigma-Aldrich)

Material and equipment:

- Balance, Max. 120 g; d= 0.1 mg (Sartorius)
- Microplate reader (BIORAD)
- Centrifuge (AWEL)

Reagents:

- 1.0 mg/ml BSA stock solution (Sigma-Aldrich)
- Reagent A, bicinchoninic acid solution (B9643, Sigma-Aldrich)
- Reagent B, Copper (II) sulphate pentahydrate (P0914, Sigma-Aldrich)
- 100 % (v/v) Glacial acetic acid (Merck)

Solutions:

- BCA working agent: 8.00 ml reagent A was mixed with 0.160 ml reagent B
- 0.05 M pH 5 Acetic acid : 0.143 ml 100 % (v/v) Glacial acetic acid was diluted to 50 ml with distilled water

The following paragraphs describe the procedure to determinate the concentration of proteins of the enzymes studied by means of bicinchoninic acid protein assay kit.

Dilutions performed to each enzyme are summarized in Table 3-1. For powder enzymes, 30 mg was diluted in 3 ml 0.05 M acetic acid, stirred during 30 minutes and centrifuged.

Table 3-1: Samples and dilutions used for bicinchoninic acid protein assay kit

Enzyme	Dilution 1	Dilution 2
Hemicellulase from *Aspergillus niger* *	Undiluted	5 x
Rocksoft *	Undiluted	5 x
Scourzyme L	20 x	50 x
Laccase	20 x	50 x
NS 26021	20 x	50 x
Pulpzyme	50x	100 x
Rohament CL	250 x	500 x
Cellulase from *Aspergillus niger*	20 x	50 x

* *Hemicellulase from Aspergillus niger and Rocksoft are powder enzymes.*

Standards of 800, 600, 400, 200 and 0 µg/ml BSA were prepared with the stock solution. 10 µL of the standards and samples were added in triplo to the 90-well microtitreplate with 200 µL BCA working reagent. The microtitreplate was shaken and incubated for exactly 2 hours at room temperature (25 ºC). After incubation, the absorbance was measured at 560 nm with the microplate reader. Finally, the content of protein of the samples was calculated by means of the equation of the calibration curve of the standards.

3.4 Ruthenium red staining

The objective of this method is to observe flax fibers structure through a microscope. To achieve this goal the unesterified groups of galacturonic acid present in the pectin substrates, which can be found between adjacent cells (see 1.3.4.4), are stained with ruthenium red. Hence, by means of ruthenium red staining acidic and methyl esterified pectins can be differentiated.

Material and equipment:

- Balance (max. 120 g; d= 0.1 mg)
- Light microscope (Olympus)

Reagents:

- 100 % (w/w) Ruthenium red (Sigma-Aldrich)
- Glycerol (VWR)

Solution:

- 0.02 % (w/v) ruthenium red solution: 5 mg ruthenium red was diluted to 25 ml with distilled water.

A sample of about 2 cm of fiber was brought into a 1.5 ml eppendorf cup and 1.5 ml 0.02 % (w/v) aqueous ruthenium red solution was added. After 15 minutes, samples were mounted in glycerol, placed on a viewing glass and observed under a light microscope.

4 RESULTS AND DISCUSSION

4.1 Gravimetrical analysis of chemical properties

By means of the gravimetrical characterization (see paragraph 3.1), cellulose, hemicellulose and lignin content of several flax fibers treated with different enzymes at diverse operational conditions have been analysed. Having knowledge of these contents is important to determine the effect of the enzymes on the chemical properties of fiber. The enzymes studied and their activities are summarized in Table 4-1.

Table 4-1: List of enzymes with which flax fibers have been treated and their activities.

Enzymes[1]	Supplier	Activities
Vicozyme (Vz)	Novozymes	Arabanase, cellulase, β-glucanase, hemicellulase, xylanase and pectinases
Scourzyme L (Sc)	Novozymes	Pure pectate lyase
NS59049 (Ns)	Novozymes	Pure pectin lyase
Pectinase from *Aspergillus niger* (PAn)	Sigma-Aldrich	Polygalacturonase
Rohapect MPE (RhMPE)	AB Enzymes	Pectin methyl esterase
Rohapect PTE (RhPTE)	AB Enzymes	Pectin lyase
Rohapect MA PLUS T PF (RhPF)	AB Enzymes	Pectin methyl esterase and polygalacturonase
Pulpzyme (Pz)	Novozymes	Endoxylanase
Xylanase from *Thermomyces lanuginosus* (XTl)	Novozymes	Purified endo-β-(1-4)-xylanase
Hemicellulase from *Aspergillus niger* (HAn)	Sigma-Aldrich	Xylanase, mananase and other activities
Carezyme 1000 L (CelAsp)	Novozymes	Cellulase
Laccase NS 20621 (LAC)	Novozymes	Laccase

[1] *In brackets are indicated the acronyms*

The discussion of the enzymatic effects on the fibers has been organized depending on their activities. First of all, the results of Viscozyme, a commercial mixture containing cellulase and hemicellulase activities, are reviewed. Then discussion will be based on the group of enzyme activities degrading pectin, hemicellulose, cellulose and lignin respectively. Concerning the pectinase activities, pectin lyases (NS59049 and Rohapect PTE), pectin methyl esterases (Rohapect MPE and Rohapect MA PLUS T PF), pectate lyases (Scourzyme L) and polygalacturonases (Pectinase from *Aspergillus niger* and Rohapect MA PLUS T PF) are studied. The investigated hemicellulose degrading enzymes are Hemicellulase from *Aspergillus niger*, Xylanase from *Thermomyces lanuginosus* and Pulpzyme. Finally, Carezyme 1000L contains cellulase activity while lignin degradation is analysed with the laccase NS 20621 enzyme.

In addition, different operational conditions have been studied, i.e. temperature of incubation, concentration of the enzyme, pH, presence of EDTA and duration of the treatment. Table 4-2 summarizes the enzymatic treatments and their operational conditions.

Table 4-2: Enzymes and operational conditions studied.

	Operational conditions				
Enzyme	**Temperature (ºC)**	**Concentration (%)**	**pH**	**Duration (h)**	**EDTA**
Mixture of activities					
Viscozyme*	40	0.60	5	6	Yes
	40	0.60	5	24	Yes
Activities concerning pectin					
Scourzyme L	40	0.30	5	24	Yes
	40	0.30	6.5	24	Yes
	40	0.30	6.5	24	No
	50	0.30	6.5	24	No
NS59049	40	0.30	5	24	Yes
	40	0.30	6.5	24	Yes
Pectinase from *Aspergillus niger**	40	0.60	5	24	Yes
	40	0.60	6.5	24	Yes
Rohapect MPE	40	0.30	5	24	Yes
	40	0.30	6.5	24	Yes
Rohapect PTE	40	0.30	5	24	Yes
Rohapect MA PLUS T PF	40	0.30	5	24	Yes
Activities concerning hemicellulose					
Pulpzyme	40	0.30	5	24	Yes
	40	0.30	6.5	24	Yes
	50	0.30	6.5	24	Yes
Xylanase from *Thermomyces lanuginosus*	40	0.30	5	24	Yes
	40	0.30	6.5	24	Yes
Hemicellulase from *Aspergillus niger*	40	3.00	5	48	Yes
Activities concerning cellulose					
Carezyme 1000 L	40	0.30	5	24	Yes
Activities concerning lignin					
Laccase NS 20621	40	0.30	5	24	Yes

** Pectinase from Aspergillus niger is a powder enzyme and thus its solubility is lower. Therefore, a concentration of 0.60 % is used to equalize the activity of other enzymes at 0.30 %.*

To have a better understanding of the effect of enzymatic treatments on the chemical composition of flax fibers, a comparison with the ground material has to be made. Figure 4-1 shows the composition of green flax fiber (not treated). This composition is a reference at which treated flax fiber compositions can be compared.

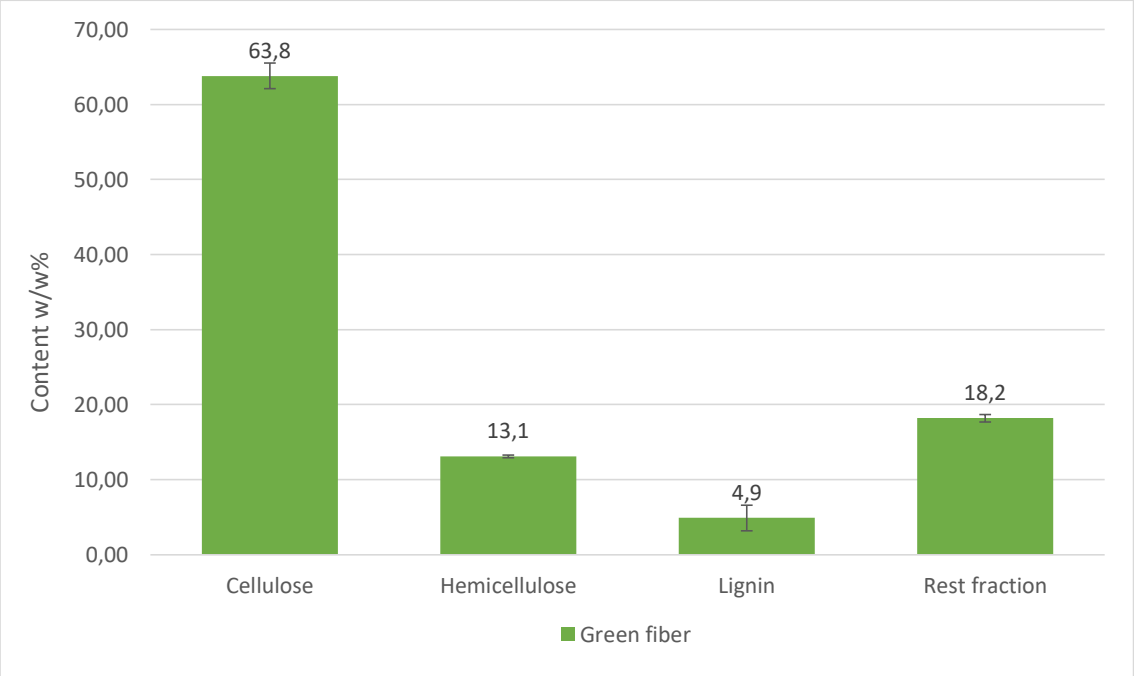


Figure 4-1: Composition of green flax fiber

Green fiber has a considerable content of hemicellulose and rest fraction. This is because the matrix was not degraded and thus it results in a lot of impure material (rest fraction) adhered to the fiber. The content of cellulose in w/w % is relatively low due to the important fraction of impure material present in the matrix.

Hence, the objective of the enzymatic treatments discussed in the following pages is to purify flax fiber by degrading the matrix.

4.1.1 Combined activities

Viscozyme is a commercial enzyme mixture of cellulases and hemicellulases[1], and thus, it is expected to degrade hemicellulose and cellulose. Figure 4-2 shows a comparison between the compositions of flax fibers treated with Viscozyme (Vz) at 0.60 % and pH 5 incubated during 6 and 24 hours at 40 ºC. Enzymatic treatment during 24 hours should have more effect than treatment during 6 hours.

[1] Though the supplier does not mention it, in house-made studies of Viscozyme showed pectinase activity.

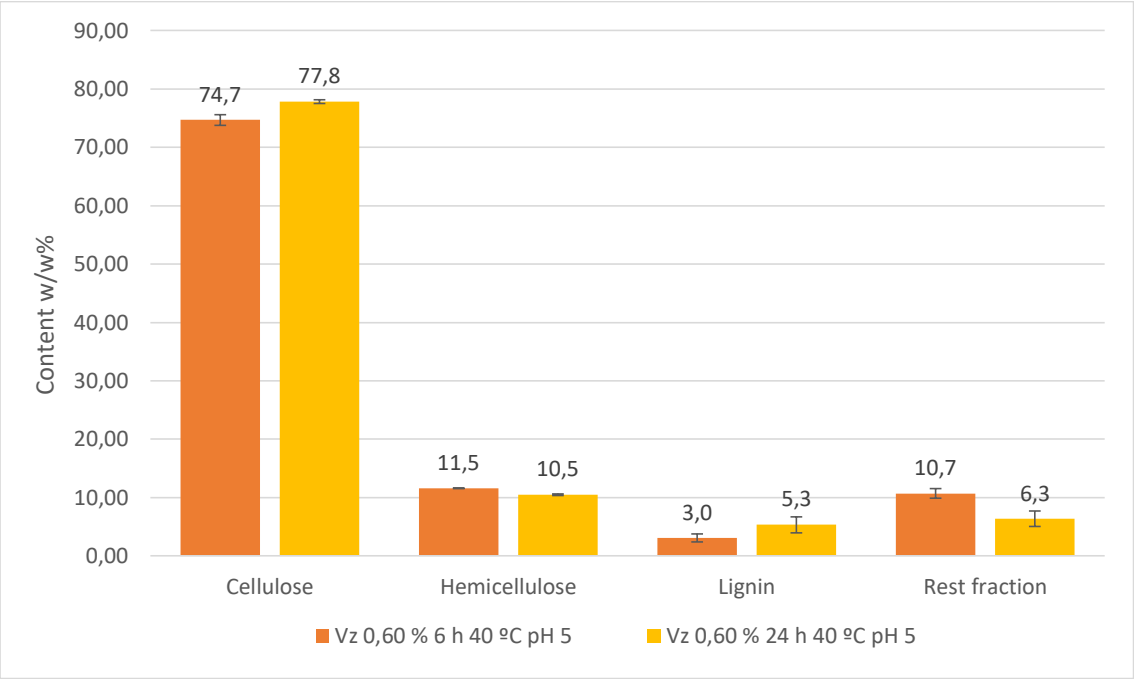


Figure 4-2: Composition of flax fibers treated with Viscozyme (Vz) at 0.60 % and pH 5 incubated during 6 and 24 hours at 40 ºC.

Hemicellulose content decreased with the 24 hours treatment, while cellulose and lignin content increased. With the longer incubation, Viscozyme was able to degrade the hemicellulose and amorphous cellulase present in the matrix to a better extent, and therefore, the fiber obtained was purer. With the better purification of the fiber the relative content of lignin and cellulase increased because they were not degraded while degrading the matrix.

4.1.2 Pectin degrading activities

Enzymatic treatments were performed with pectin lyase, pectin methyl esterase, pectate lyase and polygalacturonase activities. Pectin lyases are enzymes that degrade esterified polygalacturonic acid, while pectin methyl esterases deesterifies methyl groups on polygalacturonic acid. Pectate lyases and polygalacturonases are both capable of degrading polygalacturonic acid (Pickering, Efendy and Le, 2016).

First the results of NS59049 and Rohapect PTE, both pectin lyases, are analysed. Pectin lyases cleaves glycosidic linkages, preferentially those of high esterified pectins producing unsaturated methyloligogalacturonates (Pickering, Efendy and Le, 2016).

The effect of the pH in treatments with pectin lyases is studied in Figure 4-3 (A), where fibers were treated with NS59049 during 24 hours at 0.3 %, 40 ºC and pH 5 and 6.5 respectively. Then, in Figure 4-3 (B) the performance of two pectin lyases prepared by

different suppliers, NS59049 from Novozymes and Rohapect PTE from AB enzymes, at 40 ºC and pH 5 is compared.

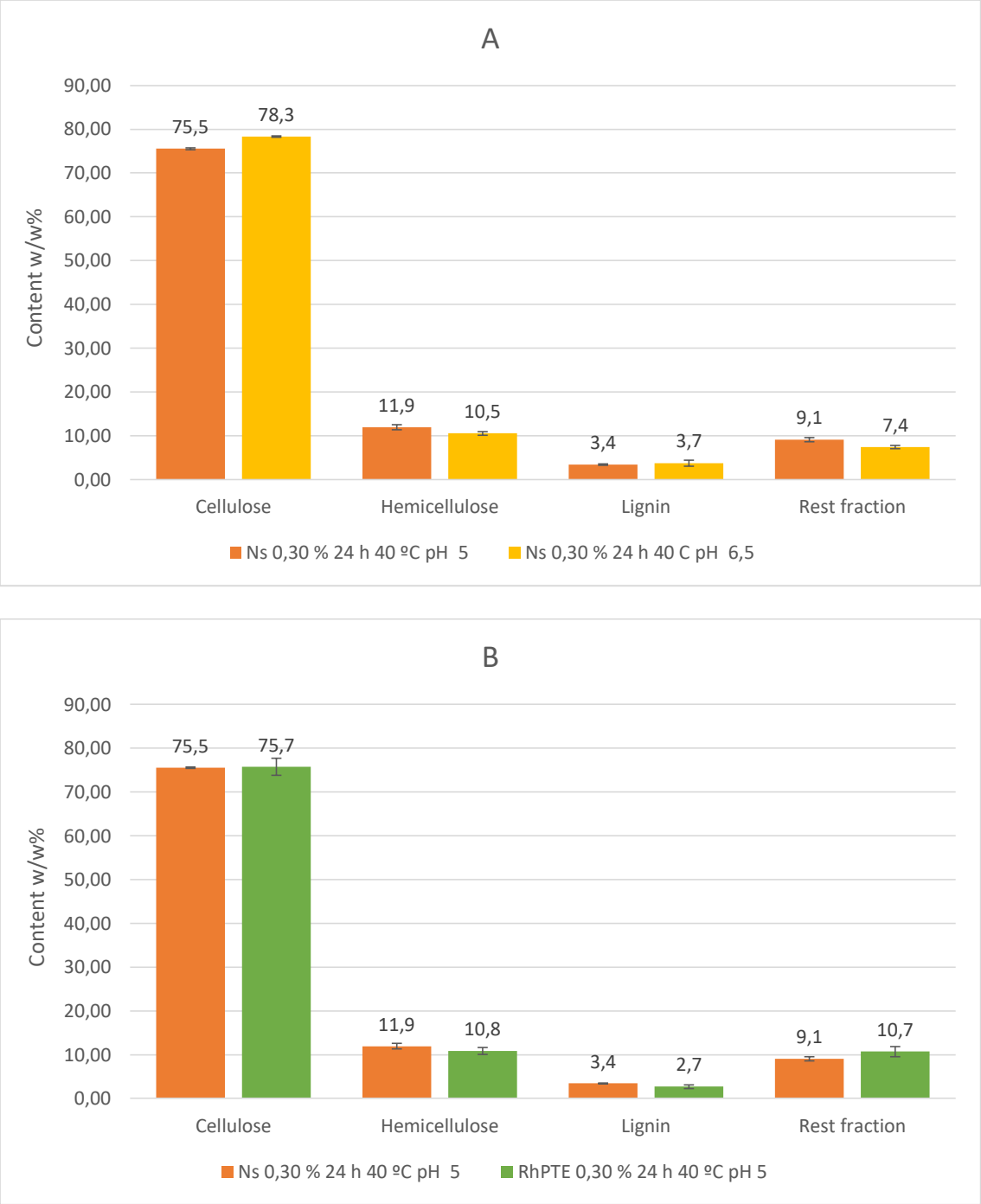


Figure 4-3: Composition of flax fibers treated with NS59049 at 0.30 % incubated during 24 hours at 40 ºC (A) at pH 5 and 6.5; (B) NS59049 at pH 5 and Rohapect PTE at 0.30 % incubated during 24 hours at 40 ºC and pH 5.

According to the higher content of cellulose and the lower content of hemicellulose of fibers treated with NS59049 at pH 6.5, pectin lyases have a better performance at pH 6.5 than at pH 5 and purer fibers were obtained. Thus, matrix was degraded during retting and the content of rest fraction decreased. At pH 5, NS59049 and Rohapect

PTE have a similar performance according to the cellulose content of the fibers after the treatment.

The following activities discussed are pectin methyl esterase and polygalacturonase. PAn contains polygalacturonase activity, which catalyses the hydrolysis of α-1,4-glycosidic linkages in polygalacturonic acid. Rohapect MPE deesterifies the methyl groups of pectin resulting in more substrate degradable by polygalacturonases. RhPF contains a mixture of pectin methyl esterase and polygalacturonases (Pickering, Efendy and Le, 2016).

Rohapect MPE was not expected to present great results since pectin methyl esterase activity does not break the pectin backbone, but only deesterifies the methoxyl groups. On the other hand, PAn can only attack non-esterified substrates. Combining these two activities could result in a synergy since the polygalacturonase enzyme will receive more substrate after pectin methyl esterase activity. A combination of these two enzymes is present in RhPF.

The influence of the pH on polygalacturonase activity treating flax fibers with PAn during 24 hours at 0.6 %, 40 ºC, pH 5 and 6.5 is compared in Figure 4-4. Then the effect of the pH is also studied for pectin methyl esterase activity in Figure 4-5 by comparing flax fibers treated with RhMPE.

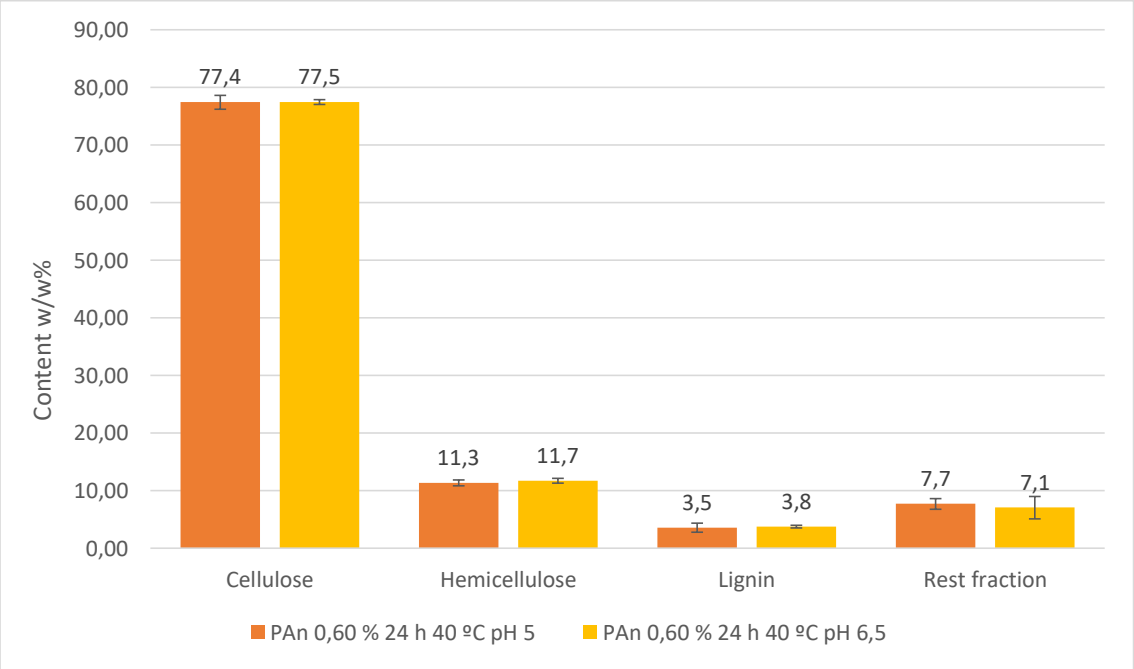


Figure 4-4: Composition of flax fibers treated with PAn at 0.60 % incubated during 24 hours at 40 ºC, pH 5 and 6.5

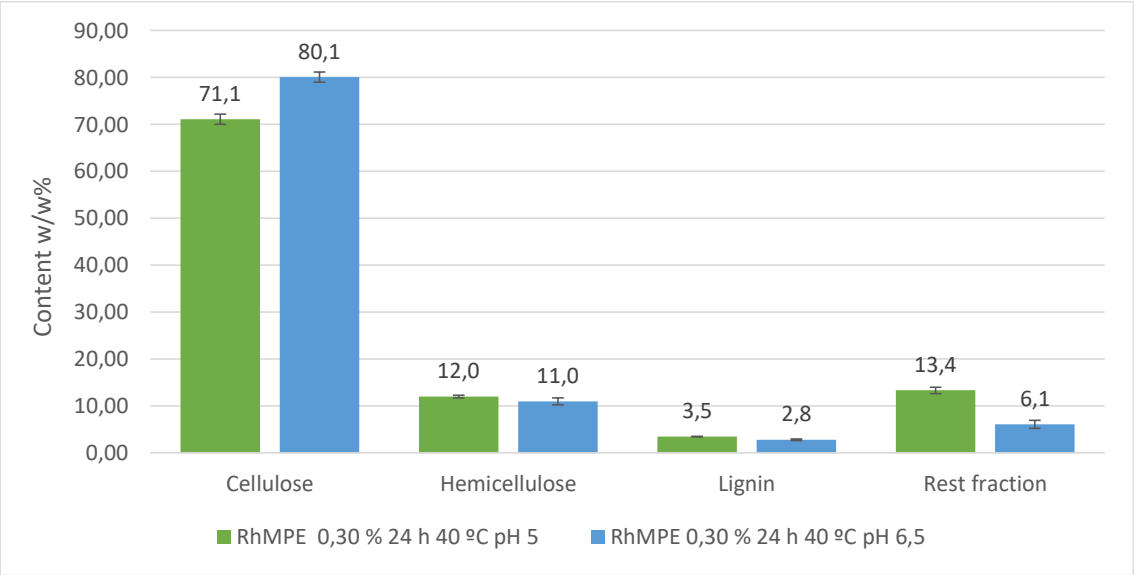


Figure 4-5: Composition of flax fibers treated with RhMPE at 0.30 % incubated during 24 hours at 40 ºC, pH 5 and 6.5.

The content of cellulose, hemicellulose, lignin and rest fraction are very similar and do not suggest any difference on polygalacturonase activity regarding the pH. On the other hand, the performance of pectin methyl esterase activity is enhanced by increasing the pH because the cellulose content is significantly higher at pH 6.5 while the content of hemicellulose and rest fraction decreased, indicating a good degradation of the matrix.

As explained in earlier paragraphs a synergy can be tailored mixing pectin methyl esterase and polygalacturonases. Hence, in Figure 4-6 the compositions of flax fibers treated with PAn (polygalacturonases), RhMPE (pectin methyl esterase) and RhPF (mixture of both activities) at 40 ºC and pH 5 during 24 hours are compared.

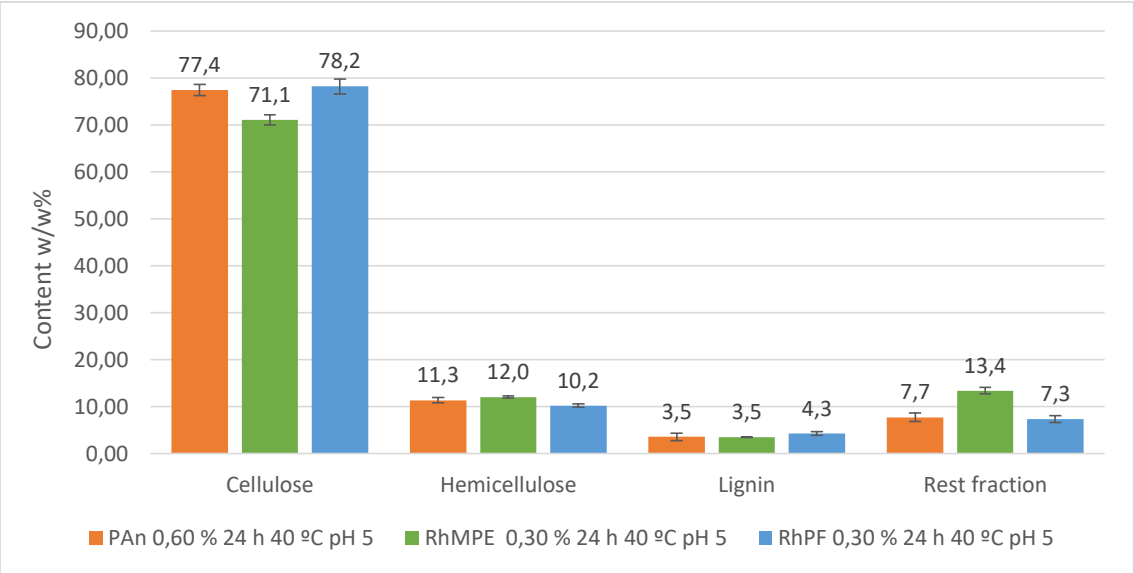


Figure 4-6: Composition of flax fibers treated with PAn at 0.60 %, RhMPE at 0.30 % and RhPF at 0.30 % during 24 hours at 40 ºC and pH 5.

Apparently RhPF is the treatment that purified flax fiber the most. This is because RhPF treated fibers have a higher content of cellulose and a lower content of hemicellulose than fibers after PAn and RhMPE treatments, indicating a better degradation of the matrix. Besides, the higher content of lignin, which is not degraded while degrading the matrix also suggests a better retting. Hence, these results suggest a possible synergy when combining pectin methyl esterase to deesterify pectin substrates and polygalacturonases to degrade pectate.

Finally, the last activity studied concerning pectin substrates are pectate lyases. Scourzyme L is a pure pectate lyase, resulting in cleavage of glycosidic linkages in polygalacturonic acid forming unsaturated products. Figure 4-7 shows a comparison between the compositions of flax fibers treated with Scourzyme L at different conditions. First the effect of pH retting with Scourzyme L treating fibers during 24 hours with EDTA at 40 ºC and pH 5 and 6.5 respectively was analysed. Then the effect of EDTA on Scourzyme L performance was studied by treating fibers with Scourzyme L without EDTA, at pH 6.5 and temperatures of 40 and 50 ºC respectively.

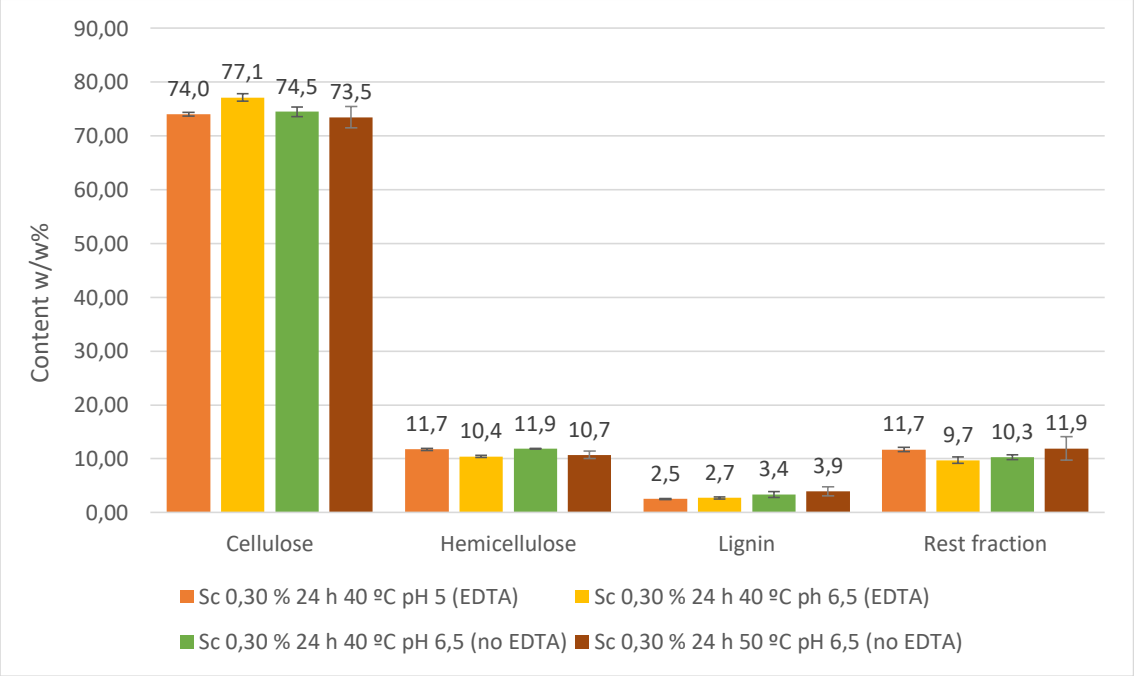


Figure 4-7: Composition of flax fibers treated with Scourzyme L at 0.30 % incubated during 24 hours with EDTA at 40 ºC and pH 5 and 6.5 respectively and without EDTA at pH 6.5 and temperatures of 40 and 50 ºC respectively.

The performance of Scourzyme L with EDTA at pH 5 and 6.5 is compared in Figure 4-7. Based on the higher content of cellulose and the lower content of hemicellulose, Scourzyme L treatment at pH 6.5 appears to have a higher performance for retting flax fibers.

Most enzymatic treatments were carried out with EDTA. If EDTA is present it forms a complex with calcium and extracts it from the solution. Calcium is a cation (positive charge) that interacts with the unesterified carboxyl groups (negative charge) of two polygalacturonic acid chains facilitating its aligning into more compact and ordered structures (Pickering, Efendy and Le, 2016). Normally enzymatic performance is enhanced by adding EDTA because when it “absorbs” calcium cations, polygalacturonic chains become more open and more accessible for enzyme activities. However, studies in literature mention a possible inactivation of pectate lyase when adding a chelator like EDTA (Akin *et al.*, 2007). Therefore, enzymatic treatments without EDTA were executed as well to investigate that hypothesis.

Comparing the composition of flax fibers treated at 40 ºC and pH 6.5 with and without EDTA, is concluded that Scourzyme L has a better performance with EDTA. This conclusion is indicated by the higher content of cellulose and the lower content of hemicellulose of flax fiber treated with EDTA, which indicate a better purification of the fiber. Thus, according to the results the hypothesis of inactivation of pectate lyases when adding a chelator should be refuted.

Furthermore, the effect of Scourzyme L without EDTA at pH 6.5 was studied at 40 and 50 ºC. The lower content of hemicellulose alongside the increment of lignin content and rest fraction of fibers treated at 50 ºC suggest a better retting, where matrix materials were degraded but not the lignin. Nevertheless, the content of cellulose should be higher, since its weight fraction would be more important. Hence, the performance of Scourzyme L without EDTA was not improved by increasing the temperature.

In summary, the pectin degrading activities studied were pectin lyase, pectin methyl esterase, pectate lyase and polygalacturonase. In order to determine the most effective pectinase activity, the treatment of each activity that resulted in the purer flax fibers is included in Figure 4-8. For polygalacturonases the purer fiber was obtained with PAn at 0.6 %, 40 ºC and pH 6.5 during 24 hours. The best results with pectin lyases, pectate lyases and pectin methyl esterases were obtained respectively with NS59049, Scourzyme L and Rohapect MPE with the same operational conditions as PAn but the concentration, which was 0.3 %.

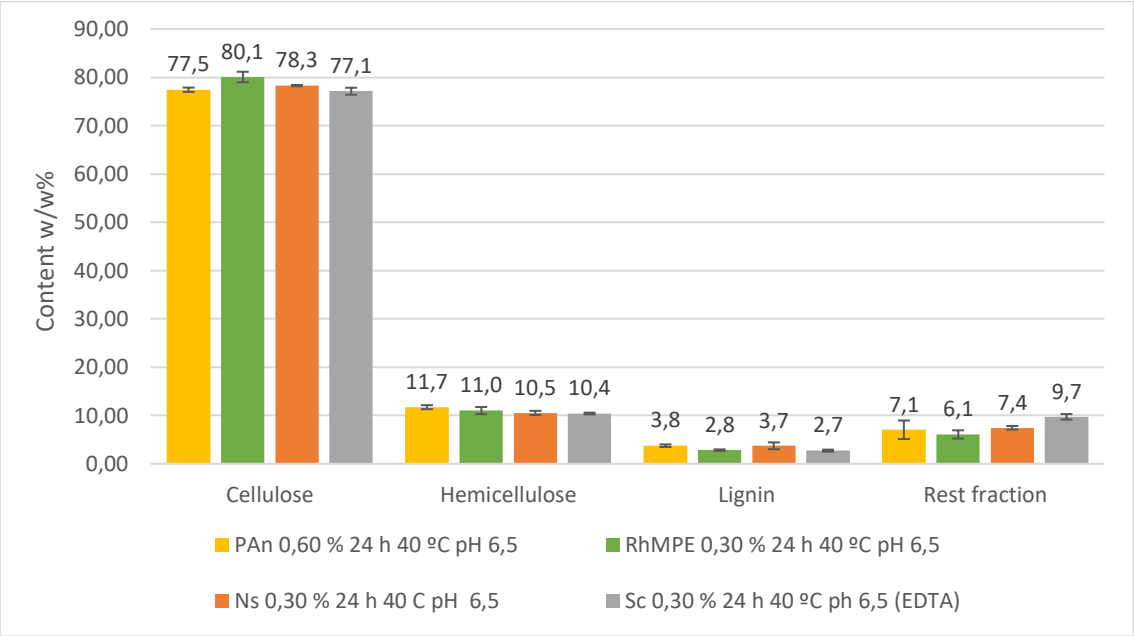


Figure 4-8: Composition of flax fibers treated during 24 hours with PAn, RhMPE, Ns and Sc at 0.3 %, 40 ºC, pH 6.5 but PAn, which was concentrated at 0.6 %.

In view of the results, the best pectinase activity for purifying flax fibers is pectin methyl esterase (RhMPE), since it has the highest content of cellulose alongside of the lowest content of rest fraction. This is an unexpected result since pectin methyl esterase does not degrade pectins, but deesterifies the methyl groups of esterified polygalacturonic acid. On the other hand, pectin lyases (Ns) also present a good performance with a higher content of cellulose than pectate lyases (Sc) and polygalacturonases (PAn), a lower content of hemicellulose than polygalacturonases (PAn), and a higher content of lignin than pectate lyases (Sc). Hence, pectin lyases appear to have better performance than pectate lyases and polygalacturonases, which have a similar performance since both degrade polygalacturonic acid.

Also must be denoted the good results shown by RhPF (mixture of polygalacturonase and pectin methyl esterase activities) in Figure 4-6. Despite the fact that the treatment with RhPF was performed at pH 5, the results obtained are comparable to those of pectin lyases, pectate lyases and polygalacturonases performed at pH 6.5. Moreover, since RhPF is a mixture of polygalacturonase and pectin methyl esterase activities, and pectin methyl esterase activity improved its performance when the pH was risen to 6.5 (Figure 4-5), is reasonable to expect also an improvement of the performance of RhPF if the treatment were carried at pH 6.5.

4.1.3 Hemicellulose degrading activities

Hemicellulose is present in the cementing matrix wrapping the cellulose microfibrils and thus its degradation is considered an important factor to achieve an efficient retting of flax fibers.

Pulpzyme, XTl and HAn are enzymes that degrade xylan, one of the most important polymers of hemicellulose, along with glucomannan (see paragraph 1.3.4.2). Apart of xylan, HAn also degrades other polymers present in hemicellulose. Since these enzymes degrade hemicellulose, flax fibers treated with them could be expected to present a low content of hemicellulose. Nevertheless, since pectin is not degraded, the pectin-hemicellulose matrix wrapping cellulose microfibrils may not be fully degraded, and so, they may be not able to reach all the hemicellulose content.

The chemical composition of flax fibers after being treated with Pulpzyme at different operational conditions are illustrated in Figure 4-9. Three treatments were performed with Pulpzyme at pH 5 and 40 °C, pH 6.5 and 40 °C and pH 6.5 and 50 °C, respectively, all with an enzyme concentrations of 0.30 % and an incubation time of 24 hours with the objective of studying the effect of the temperature and the pH.

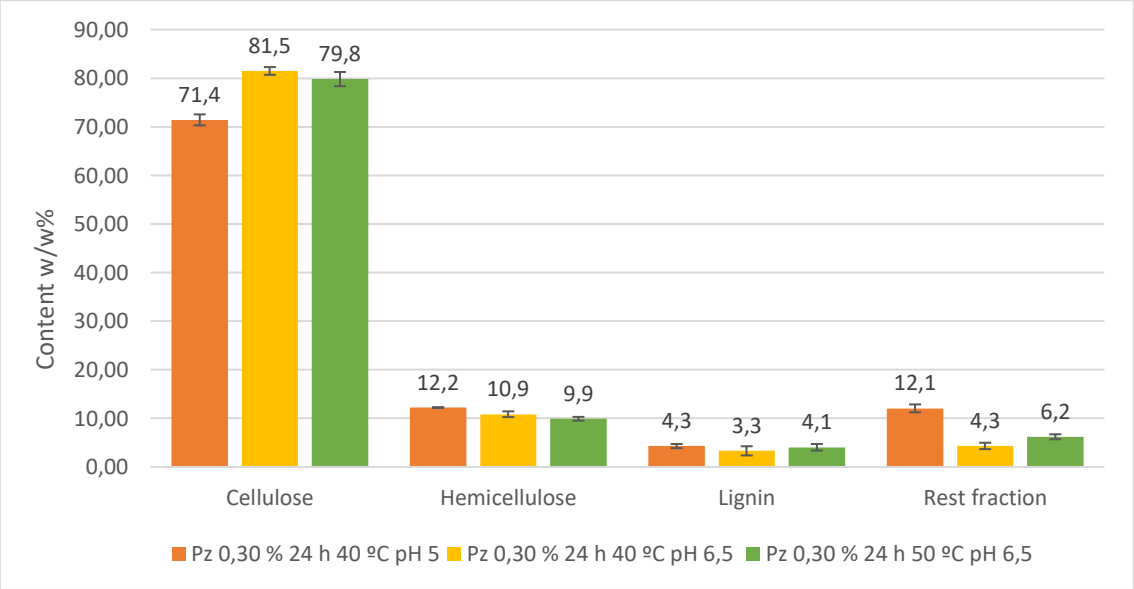


Figure 4-9: Composition of flax fibers treated with Pulpzyme during 24 hours at 0.30 % at 40 ºC and pH 5 and 6.5, and at 50 ºC and pH 6.5.

Among fibers treated with Pulpzyme at 40 ºC, those treated at pH 6.5 present a higher value of cellulose and a lower content of hemicellulose, and therefore, a more purer fiber was obtained. Besides, the high content of rest fraction of flax treated at pH 5 indicates a poor degradation of the matrix. Hence, enzymatic activity of Pulpzyme appears to increase at pH 6.5. However, increasing temperature to 50 °C at pH 6.5 does not lead to an improvement. Pulpzyme treatments thus appears to be optimal at 40 °C and pH 6.5.

The effect of the pH was also studied for Xylanase from *Thermomyces lanuginosus* (XTl) in Figure 4-10 comparing flax fiber compositions after treatments with XTl at 0.3 %, 40 ºC and pH 5 and 6.5 during 24 hours.

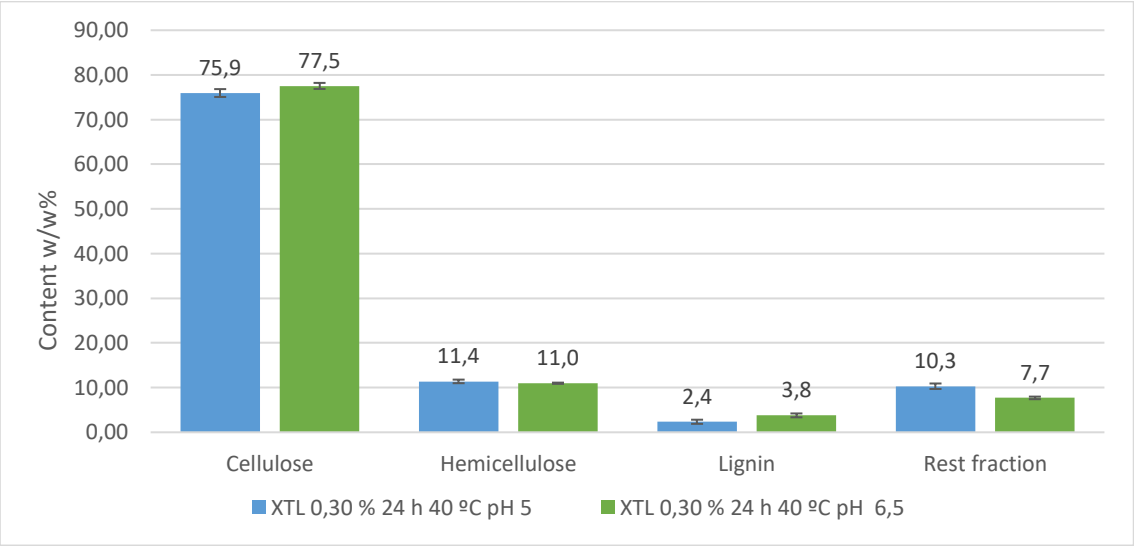


Figure 4-10: Composition of flax fibers treated during 24 hours at 0.30 % and 40 ºC with XTl at pH 5 and 6.5

As Figure 4-10 illustrates, the content of hemicellulose of flax fibers retted with XTl is almost identical for both treatments and the content of cellulose is slightly higher when the treatment was carried out at pH 6.5. Though the difference is quite small, the higher content of rest fraction at pH 5 and the increment of content of lignin at pH 6.5 point to a better enzymatic activity of XTl at pH 6.5.

The Pulpzyme treatments at 40 °C were compared with two treatments performed with xylanase from *Thermomyces lanuginosus* (XTl) at 40 ºC and pH 5 and 6.5 respectively, with the same enzyme concentration and incubation time as the Pulpzyme treatments. Compositions of flax fibers treated with XTl and Pulpzyme at different pH are compared in Figure 4-11.

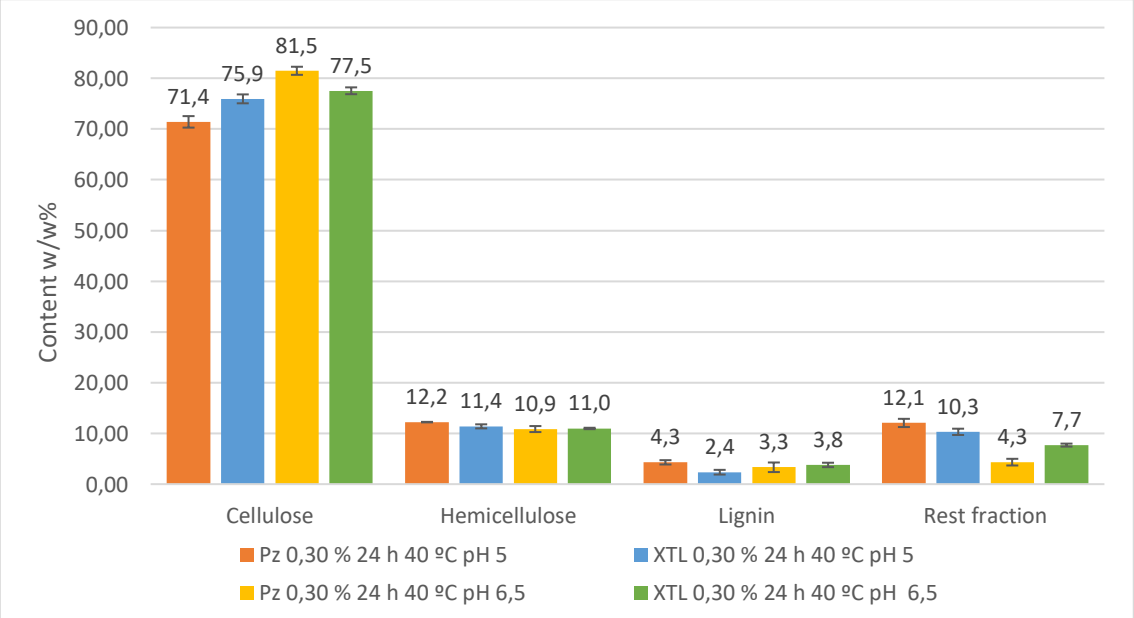


Figure 4-11: Composition of flax fibers treated during 24 hours at 0.30 % and 40 ºC with XTL and Pulpzyme at pH 5 and 6.5

Flax fibers treated with XTl at pH 5 got higher values of cellulose, and lower values of hemicellulose than those treated with Pulpzyme at the same ph. Hence, at pH 5 XTl appears to produce purer flax fibers. In contrast, at pH 6.5 Pulpzyme has a higher content of cellulose, a similar content of hemicellulose and a lower content of rest fraction. Though having close contents of hemicellulose, the higher content of rest fraction indicates that the matrix was not degraded successfully and thus there was a lot of impure material adhered to the fiber. This, together with the higher content of cellulose, denotes that at pH 6.5 Pulpzyme retted fibers more efficient than XTl.

Another more severe treatment performed with hemicellulase from *Aspergillus niger* (HAn) at 3.00 %, at 40 ºC and pH 5 during an incubation time of 48 hours is shown in Figure 4-12.

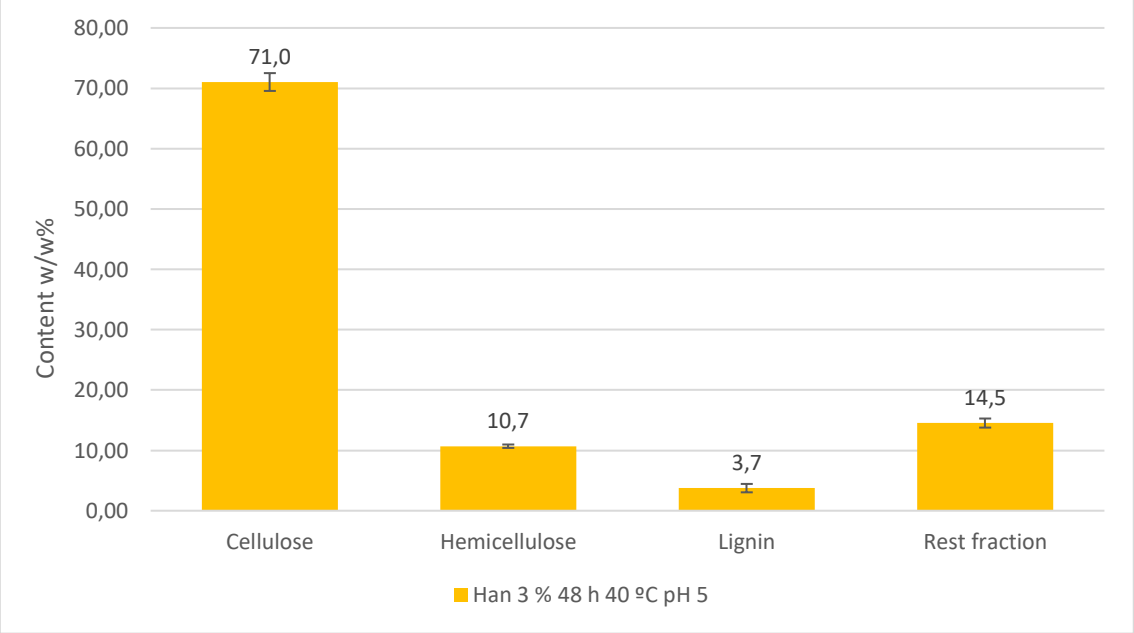


Figure 4-12: Composition of flax fibers treated with hemicellulase from Aspergillus niger at 3.00 %, at 40 ºC and pH 5 during an incubation time of 48 hours

HAn treatment was expected to present good results due to a wider enzyme activity, a higher enzyme concentration and a longer incubation time. Although having a lower content of hemicellulose than Pulpzyme and XTl at pH 5, it may be a result of the longer treatment or/and the higher concentration. In addition, the higher content of rest fraction and the lower content of cellulose in comparison to XTl and Pulpzyme at 40 ºC and pH 5 suggest a poor degradation of the fiber.

In view of the results from Figure 4-11 and Figure 4-12, among the enzymes degrading hemicellulose the best one purifying flax fibers was Pulpzyme at 40 ºC and pH 6.5, since it obtained the highest content of cellulose alongside a relative low content of hemicellulose and rest fraction.

4.1.4 Cellulose degrading activities

During the retting treatments cellulases are meant to degrade amorphous cellulose present in the matrix, but if the process is not properly regulated they may also start degrading cellulose microfibrils. Therefore, when using cellulases for retting the concentration and duration of the treatment must be exhaustively studied.

To study cellulase activity flax fibers were treated with Carezyme 1000 L (CelAsp) at 0.30 %, which is an enzyme containing cellulase activities. The treatment was carried at 40 ºC and pH 5 during 24 hours and the results are shown in Figure 4-13.

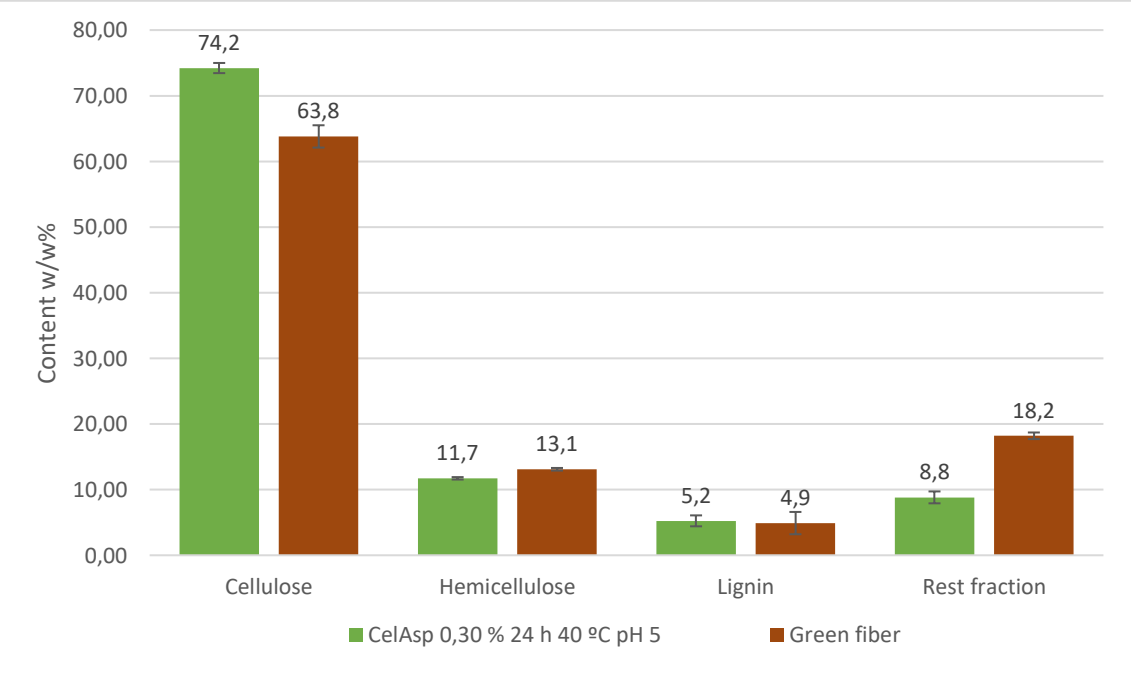


Figure 4-13: Composition of green fibers and flax fibers treated with Carezyme 1000 L (CelAsp) during 24 hours at 0.30 %, 40 ºC and pH 5.

Treatment with Carezyme 1000 L improved the purity of flax fibers in comparison to green flax fibers, indicating that cellulase activity degraded the matrix to a certain level. Nevertheless, Carezyme 1000 L performance was not as good as Viscozyme during 24 hours (Figure 4-2), which presented a higher content cellulose and a lower content of hemicellulose. This difference can be explained by a further degradation of the matrix by Viscozyme due to its hemicellulase activity.

4.1.5 Lignin degrading activities

Lignin is a structural compound present in many plants (see paragraph 1.3.4.3), but in flax fibers it is a residual component. Green flax was treated with Laccase NS 20621, an enzyme that degrades lignin, at 0.30 %, 40 ºC and pH 5 during 24 hours in order to study the effect of degrading lignin on flax fiber composition. The results of the treatment are shown in Figure 4-14.

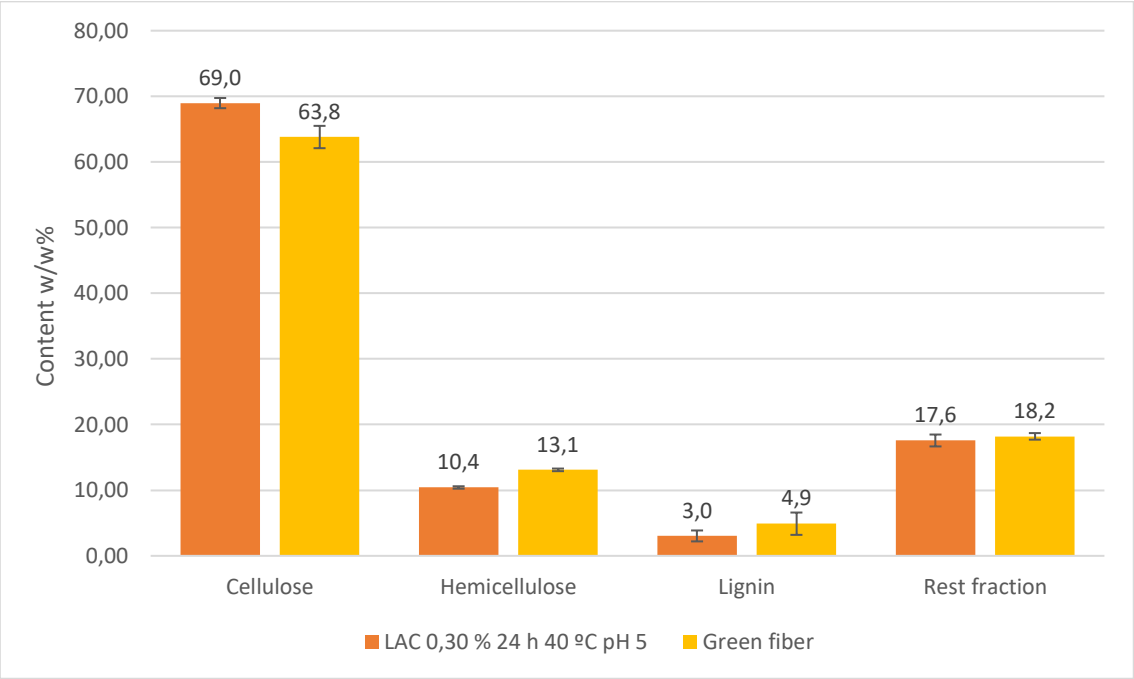


Figure 4-14: Composition of flax fiber treated with Laccase NS 20621 at 0.30 %, 40 ºC and pH 5 during 24 hours.

After Laccase treatment flax fibers present a lower content of lignin and a higher content of cellulose than green flax fibers. Besides, the high content of rest fraction after Laccase treatment is very similar to that of green fibers, pointing out to a poor degradation of the matrix, and thus, a poor retting. The cementing matrix is mainly comprised of pectin and hemicellulose, and therefore, lignin activities were not able to degrade the matrix resulting in high contents of rest fraction.

4.1.6 General conclusions of the gravimetrical analysis of chemical properties

The enzymatic activities with the best performance when retting flax fibers are those degrading hemicellulose and pectins. In particular, the purest fiber was obtained after the treatment with Pulpzyme (degrades hemicellulose) during 24 hours at 0.3 %, 40 ºC and pH 6.5. Also RhMPE (pectin methyl esterase), XTl (degrades hemicellulose), Ns (pectin lyase), Scourzyme L (pectate lyase) and PAn (polygalacturonase) at pH 6.5 and Viscozyme (cellulases and hemicellulases) at pH 5 presented a good performance when retting flax fibers. On the other hand, enzymes containing activities focused on degrading cellulose and lignin (Carezyme L and Laccase) resulted in a poor retting of flax fibers.

Hence, those enzymes containing hemicellulases and pectinases are more capable of degrading the matrix wrapping the cellulose microfibrils to a better extent. Therefore,

future efforts should be focused on studying mixtures of activities capable of degrading hemicellulose and pectin.

4.2 Determination of pectin with m-hydroxydiphenyl (MHDP)

The content of pectin of flax and flax fibers has been analysed by means of m-hydroxydiphenyl. The major content of pectin is found in the middle lamella, forming a matrix that glues the cellulose microfibrils together (see paragraph 1.3.4.4). In order to determine the effect of enzymes, it is necessary to characterize the pectin content in the fiber. However, this method still has to be optimised and checked for errors. For this reason, the method is tested on only green material as substrate, i.e. whole green flax and manually extracted green flax fibers. These results can subsequently be compared to the values of the pectin content of flax given in literature.

The calibration curve obtained with standards of 75, 60, 45, 30, 15, 5 and 0 µg/ml galacturonic acid is illustrated in Figure 4-15. Probably an analytical error was committed when standards of 60 and 45 µg/ml galacturonic acid were prepared since both presented a similar absorbance. Nonetheless, the coefficient of the curve obtained was 0.979.

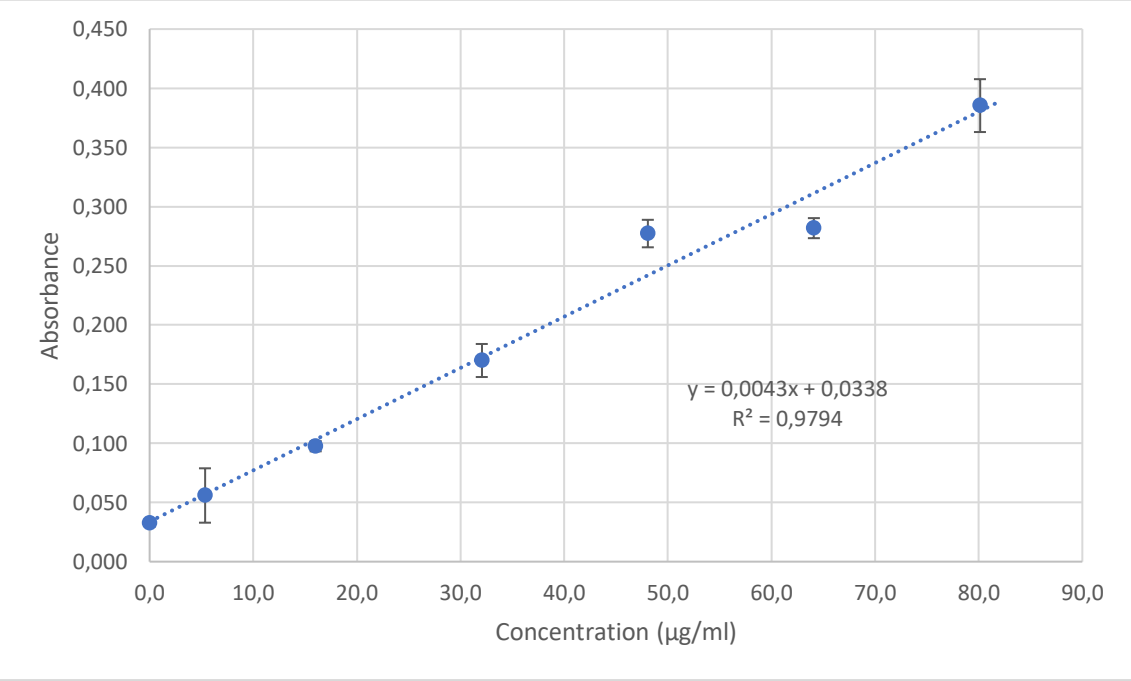


Figure 4-15: Calibration curve of the determination of pectin with m-hydroxydiphenyl.

The content of pectin measured by means of m-hydroxydiphenyl was 6.84 ± 0.43 w/w % in whole green flax and 5.51 ± 0.52 w/w % in extracted green flax fibers. Literature differs on the content of pectin probably due to its difficult determination. According to

Meijer *et al.* (1995) the content of pectin is 2.5-3 w/w % in green flax and 0.7-1 w/w % in flax fibers dew or water retted.

Since the experimental results from the lab are not in accordance with pectin content illustrated in literature, the method was optimized by checking the optimal time to measure absorbance after the colouring reaction was initiated. Further validation of the method has been realised by determining the recovery when using the substrates polygalacturonic acid and pectin from apples.

The optimization of the measuring time after the addition of the reagent meta-hydroxydiphenyl was studied by measuring the absorbance as function of time.

In order to determine the optimal time of measuring, at which the colour intensity of the samples was stabilized, standard 4 from the determination of pectin with m-hydroxydiphenyl was prepared in quadruple, since it is the most representative standard of the calibration curve. However, instead of being analysed 30 seconds after the addition of the reagent meta-hydroxydiphenyl, absorbance measurements were taken every 15 seconds to obtain a curve of the colour intensity of the samples. The time required to achieve a constant absorbance measurement will be the optimum time for measuring the samples. The resulting curves obtained for standard 4 are represented in Figure 4-16.

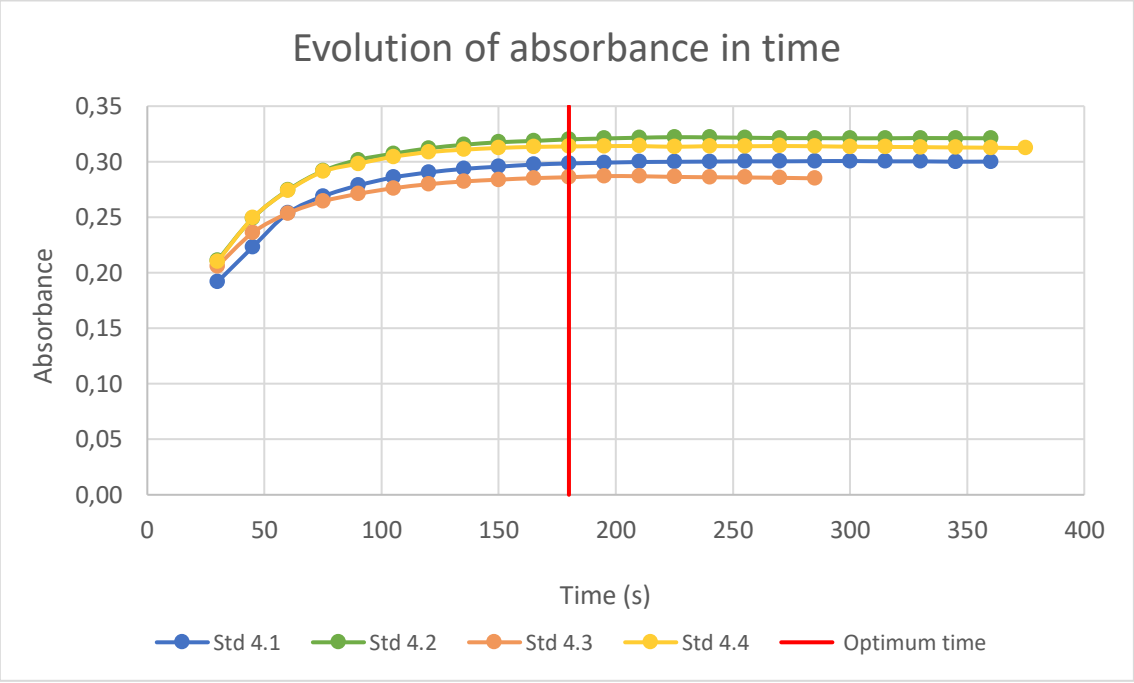


Figure 4-16: Curves of absorbance as function of time of standards 4 to determinate the optimal time of measuring. The red line indicates the optimal time of measuring.

The results indicate that the optimal time for measuring the absorbance is 180 seconds after the addition of the reagent meta-hydroxydiphenyl, since curves stabilizes at the top at that time. Hence, for further experiments was decided to measure absorbance not any time within 5 minutes as was indicated by Blumenkrantz and Asboe-Hansen (1973) but exactly three minutes after adding the colouring reagent.

Validation of the method was realised by analysing polygalacturonic acid (Sigma-Aldrich) and pectin from apples (Fluka) as a substrate and calculate the recovery. In order to do this, procedure somewhat differs from the original procedure.

In contrast with the initial determination of pectin with m-hydroxydiphenyl procedure described (see 3.2), the Soxhlet extraction was not done since there was no need to extract the pectin. To carry out the hydrolysis, about 200 mg of both polygalacturonic acid and pectin from apples and 10 ml 6 M HCl were added in a test tube with a screw cap in triplo. The samples were mixed with a vortex and incubated for 2 hours at 70 ºC. After incubation, samples were centrifuged and the liquid was decanted and diluted to 200 ml with distilled water. Dilutions of 10 and 20 were made. The procedure was then followed as described in 3.2, with the adjusted absorbance measurement time after 180 seconds instead of 30 seconds after adding the m-hydroxydiphenyl reagent.

The calibration curve obtained in the validation with standards of 75, 60, 45, 30, 15, 5 and 0 µg/ml galacturonic acid is shown in Figure 4-17. The coefficient of the calibration curve was 0.994.

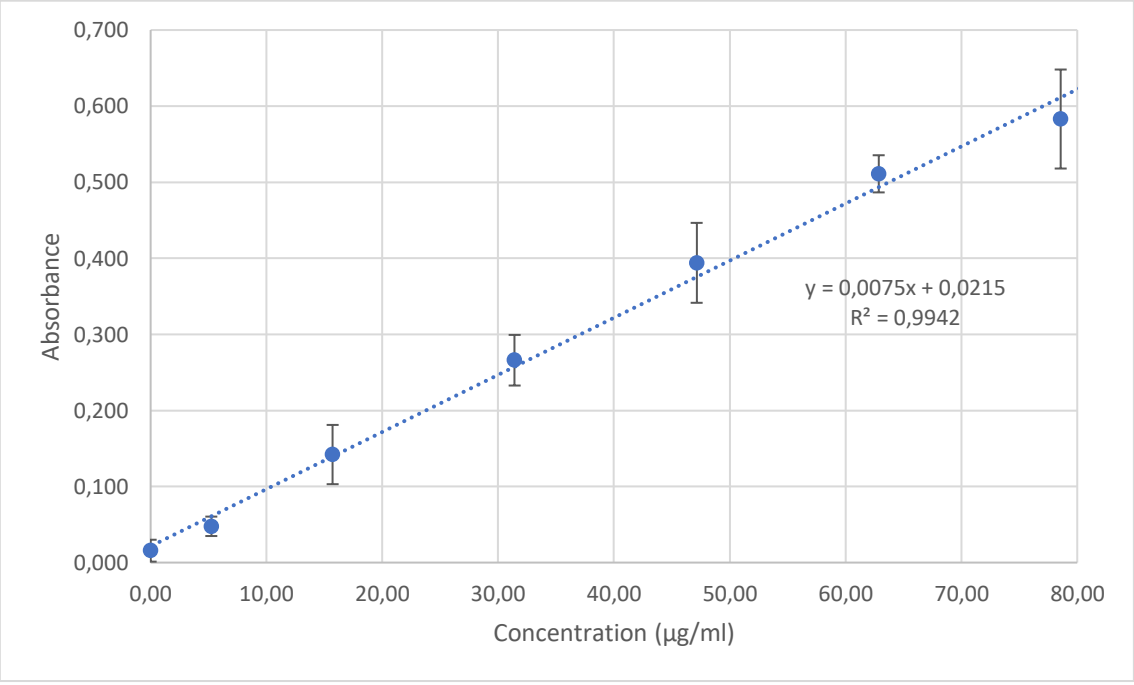


Figure 4-17: Calibration curve of the validation method

In order to validate the m-hydroxydiphenyl method the recovery of the samples is calculated. The recovery is expressed in w/w %, and is the quantity of pectin substrates measured of the total quantity of pectin substrates present. If the method is accurate and detects all the pectin substrates the recovery will be 100 w/w %. If the recovery is not 100 w/w % means that the method is not measuring all the pectin substrates present in the sample. The recovery values of pectin substrates in polygalacturonic acid and pectin from apples determined by means of the validation method are shown in Table 4-3.

Table 4-3: Recovery values of polygalacturonic acid and pectin from apples analysed in triplo.

Substrate	Total mass of pectin substrates	Experimental mass of pectin substrates	Recuperation in w/w %
Polygalacturonic acid 1	0.1778 g	0.0230 g	12.93
Polygalacturonic acid 2	0.1799 g	0.0187 g	10.41
Polygalacturonic acid 3	0.1795 g	0.0201 g	11.18
Pectin from apples 1	0.2008 g	0.0357 g	17.76
Pectin from apples 2	0.2002 g	0.0578 g	28.87
Pectin from apples 3	0.2001 g	0.0651 g	32.55

On one hand, all three samples of polygalacturonic acid show considerable low recovery but with an important accuracy. A possible explanation could be that the hydrolysis of the polygalacturonic acid was not completed. In this case, a significant part of the initial sample would have been lost when decanting after the centrifuge, and thus, the recovery resulted very poor.

On the other hand, results of pectin from apples show higher recoveries than those of polygalacturonic, but are still very far from being considered successful recoveries. However, pectin from apples is lacking accuracy since one of its values is notably lower. Is possible that the first value is deviated and the two other ones are more representative, but more experiments are needed to confirm this hypothesis.

Hence, there are two feasible explanations for these results. The first possible explanation is that pectin substrates were not fully hydrolysed because of some unexpected problem, e.g. insufficient contact time or low ratio substrate/HCl. In this case, the error of the first sample of pectin from apples would be attributed to an

analytical error. The second scenario[2] is when the method did not measure pectin substrates, and therefore, the m-hydroxydiphenyl method would not be a valid methodology to determinate pectin.

In view of the results, further experiments are needed to evaluate if m-hydroxydiphenyl is a valid method to determinate pectin substrates. For example, the hypothesis of a poor hydrolysis could be studied repeating the validation method of pectin determination with m-hydroxydiphenyl with a different contact time between the samples and the extractor agent (HCl).

4.3 Bicinchoninic acid protein assay kit

The main goal of this method is to determinate the concentration of proteins of several enzymes. The concentration of proteins is necessary to know what dilution factor must be utilized when those enzymes are investigated through a SDS-PAGE analysis. The SDS-PAGE separates enzymes depending on their size, and this allows to determinate the purity of the enzymes.

Table 4-4 shows the protein concentration of the enzymes analysed by means of this method developed as explained in paragraph 3.3.

Table 4-4: Concentration of proteins of the enzymes analysed

Enzyme	Concentration (µg/µl)
Hemicellulose from *Aspergillus niger* *	0.01 ± 0.005
Rocksoft *	0.425
Scourzyme L	7.56 ± 0.74
Laccase	21.00 ± 2.06
NS59049	17.02 ± 1.26
Pulpzyme	44.47 ± 4.03
Rohament CL	152.57 ± 18.26
Cellulase from *Aspergillus niger*	20.27 ± 2.51

** Hemicellulase from Aspergillus niger and Rocksoft are powder enzymes and their concentrations are expressed in µg/mg.*

Proper dilutions were performed according to the values obtained in this experiment in order to investigate enzyme's purity by means of a SDS-PAGE analysis. Though SDS-PAGE analysis was not performed during this project, the results are shown in Figure 4-18.

[2] In this scenario the value of the first sample of pectin from apples is still considered an error.

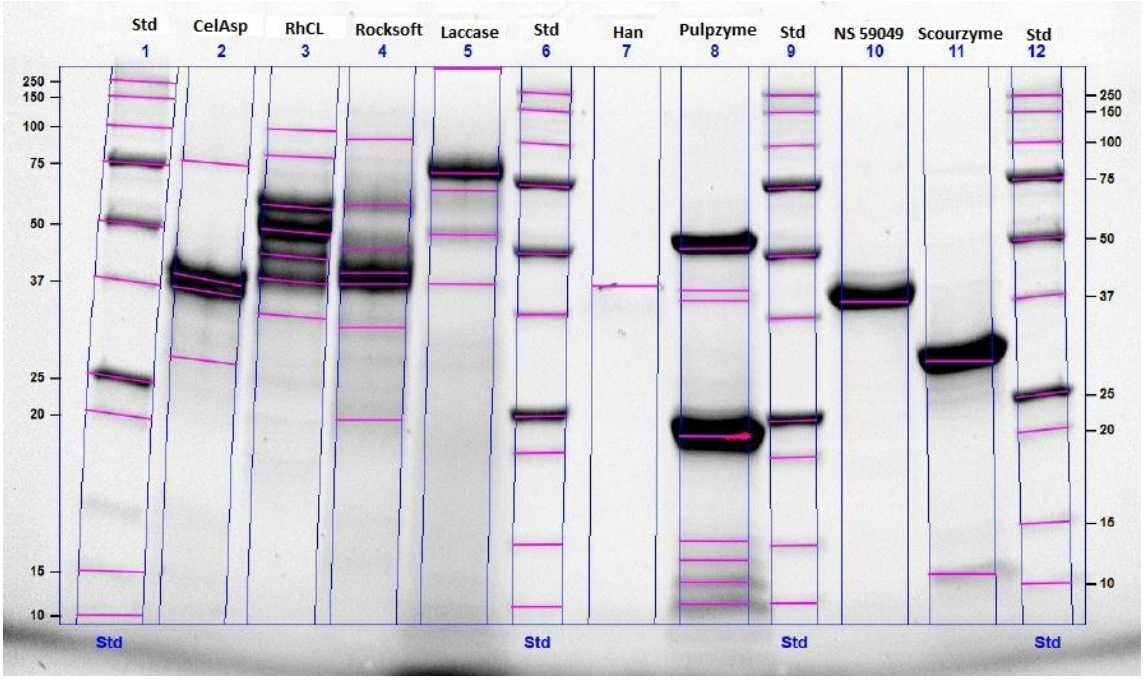


Figure 4-18: SDS-PAGE. Lanes 1, 6, 9 and 12 are standards, 2: Carezyme L (CelAsp), 3: Rohament CL (RhCL), 4: Rocksoft, 5: Laccase, 7: Hemicellulase from Aspergillus niger (HAn), 8: Pulpzyme, 10: NS59049, 11: Scourzyme L.

As explained in earlier paragraphs, SDS-PAGE separates the proteins of the enzymes depending on their size. Hence, if an enzyme is pure it will show only one type of protein. If the enzyme is a mixture then two or more sizes of protein will appear. In view of the results of the SDS-PAGE Hemicellulose from *Aspergillus niger* (Lane 7) and NS59049 (Lane 10) are pure enzymes, since only one type of protein was found. Laccase (Lane 5) and Scourzyme L (Lane 11) are enzymes formed by one major type of protein. On the other hand, Carezyme L (Lane 2), Rohament CL (Lane 3), Rocksoft (Lane 4) and Pulpzyme (Lane 8) have two or more protein types indicating that they have different activities.

4.4 Ruthenium red staining

The main goal of the ruthenium red staining method is to observe flax fiber structure through a microscope. This is possible by staining the unesterified groups of polygalacturonic acid present in pectin substrates with ruthenium red dye. Enzymatic treatments for retting flax focus on degrading the matrix comprised mainly of hemicellulose and pectin. Since most pectin substrates are found in the matrix, some differences were expected in fibers treated with different enzymes when examined with microscopy. Figures 4-19, 4-20, 4-21, 4-22, 4-23, 4-24 and 4-25 are flax fibers treated with different enzymes augmented 40 times observed through a microscope.

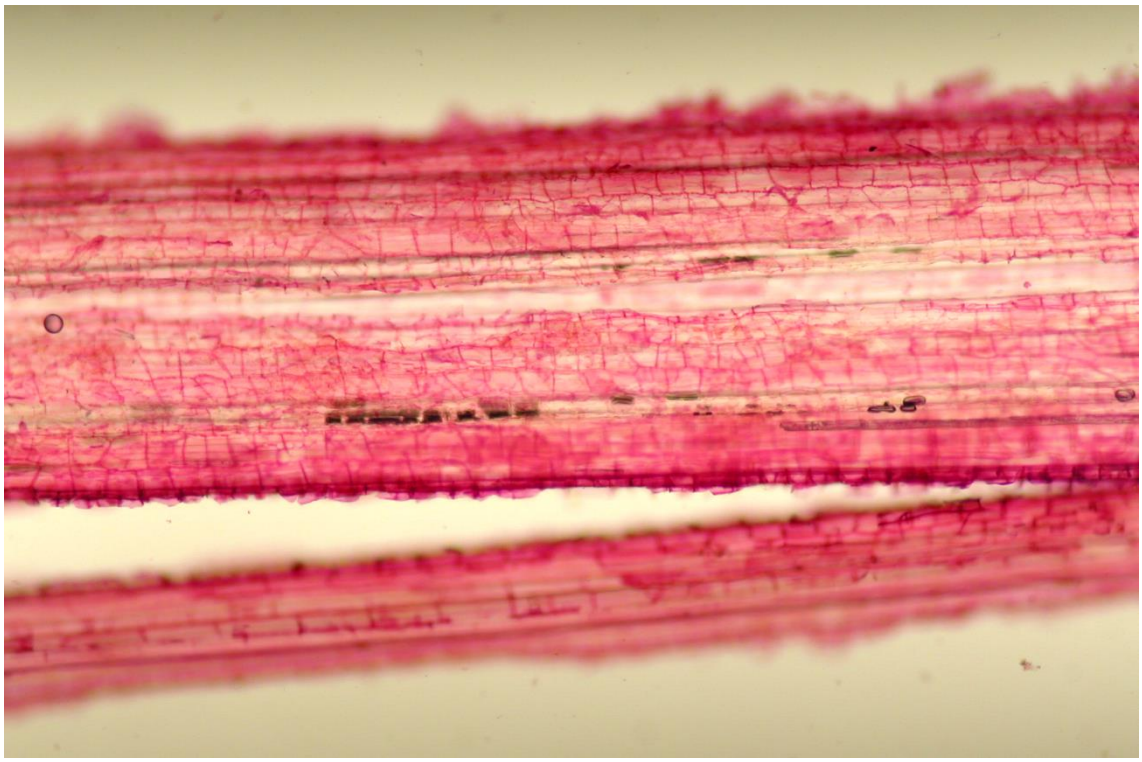


Figure 4-19: Green flax fiber augmented 40 times observed through a microscope.

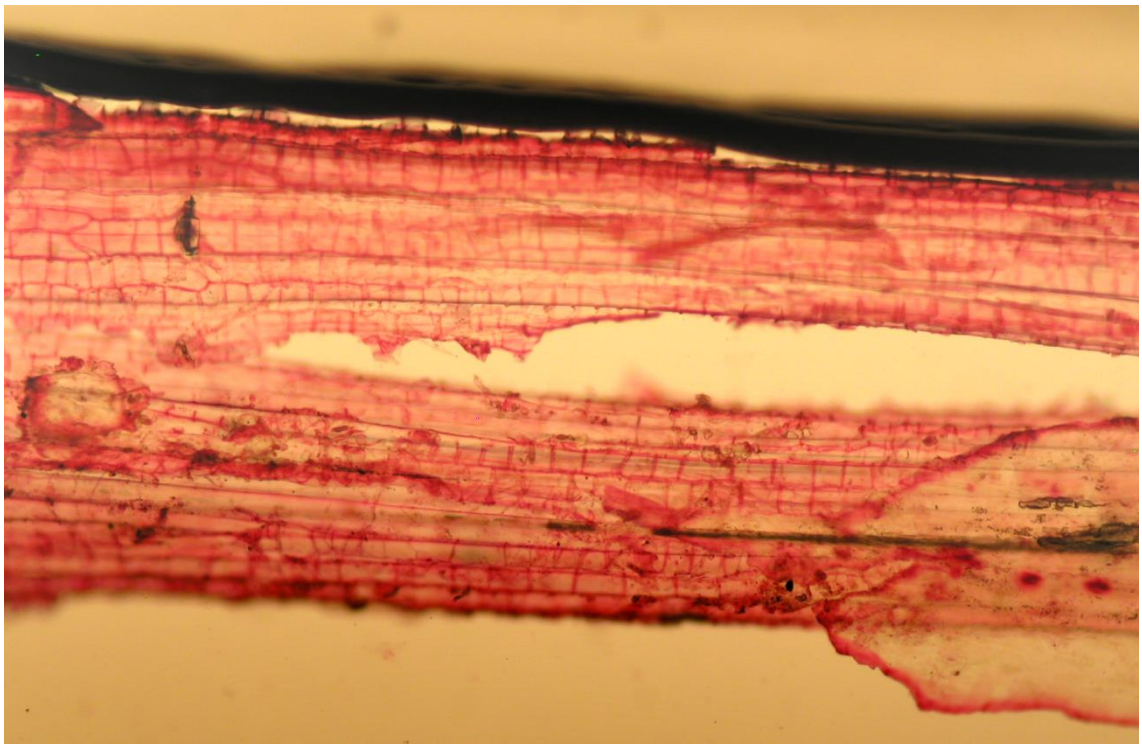


Figure 4-20: Flax fiber treated with Laccase at 0.3 %, 40 ºC and pH 5 during 24 hours augmented 40 times observed through a microscope.

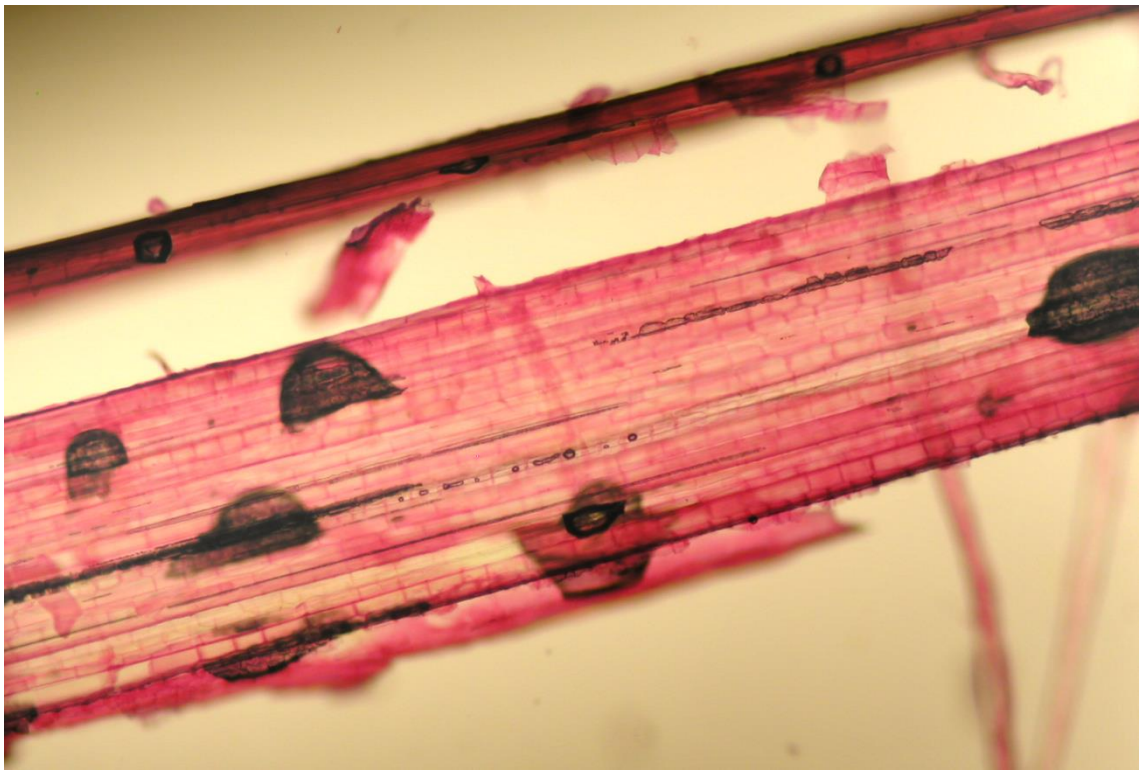


Figure 4-21: Flax fiber treated with NS59049 at 0.3 %, 40 ºC and pH 6.5 during 24 hours augmented 40 times observed through a microscope.

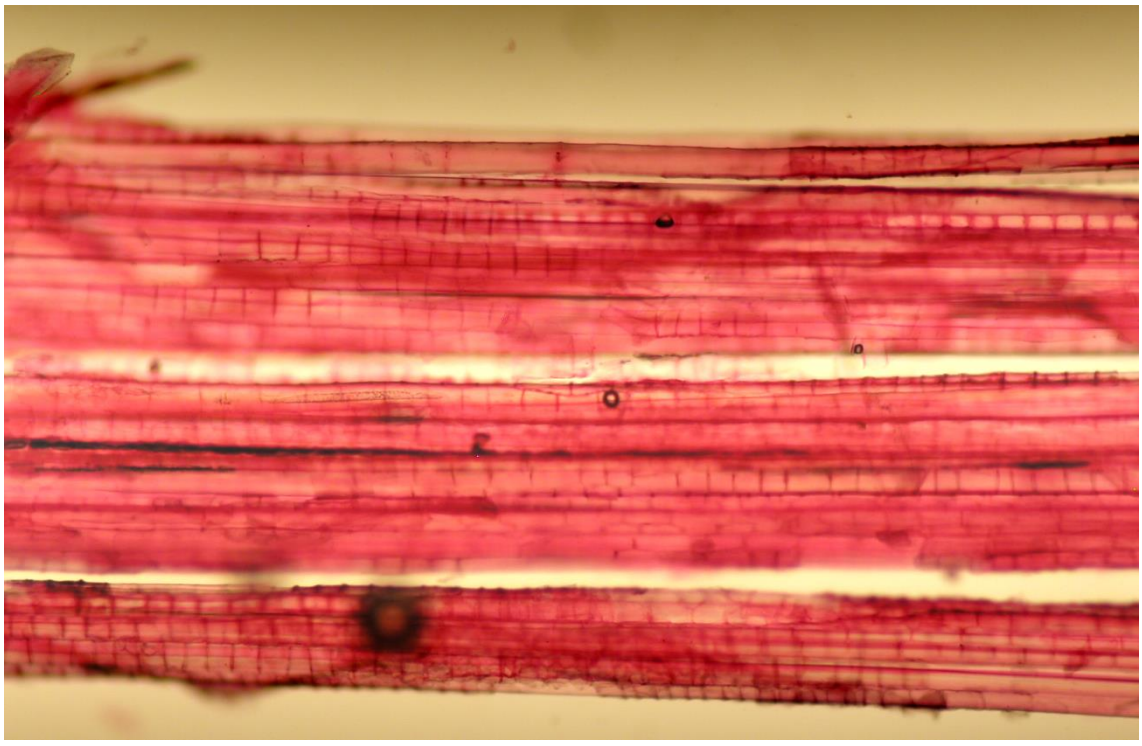


Figure 4-22: Flax fiber treated with PAn at 0.6 %, 40 ºC and pH 6.5 during 24 hours augmented 40 times observed through a microscope.

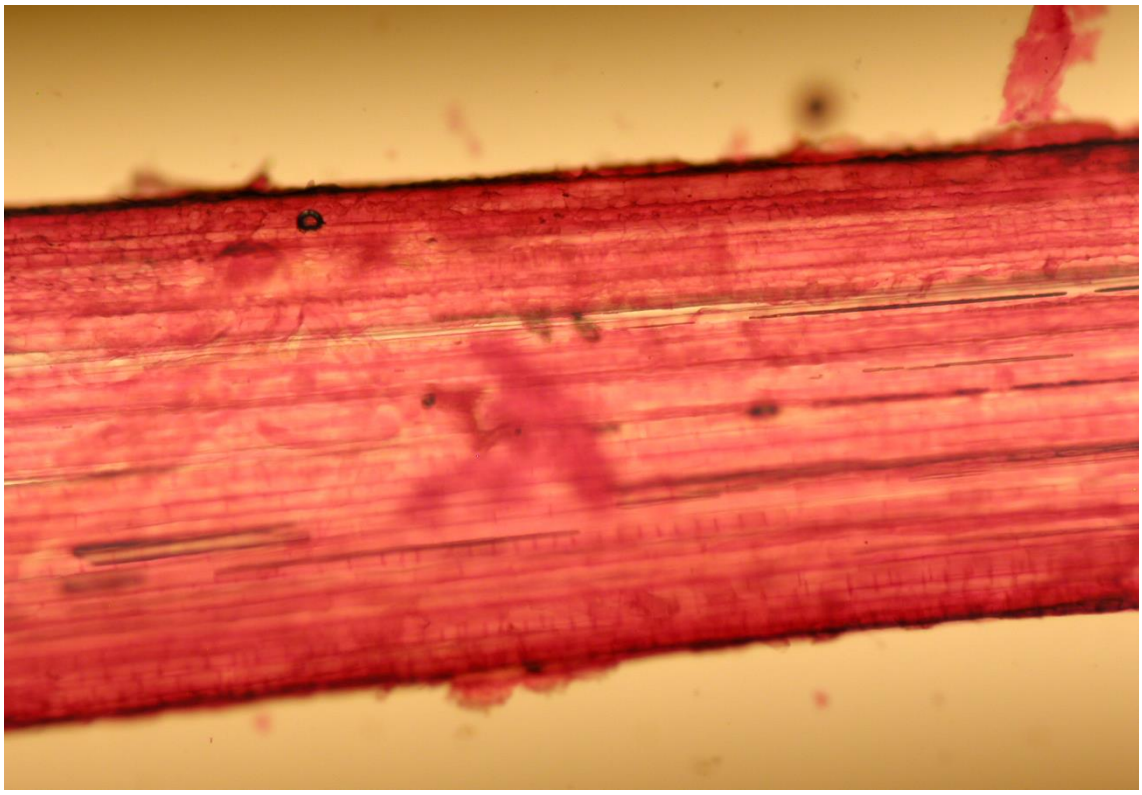


Figure 4-23: Flax fiber treated with RhMPE at 0.3 %, 40 ºC and pH 6.5 during 24 hours augmented 40 times observed through a microscope.

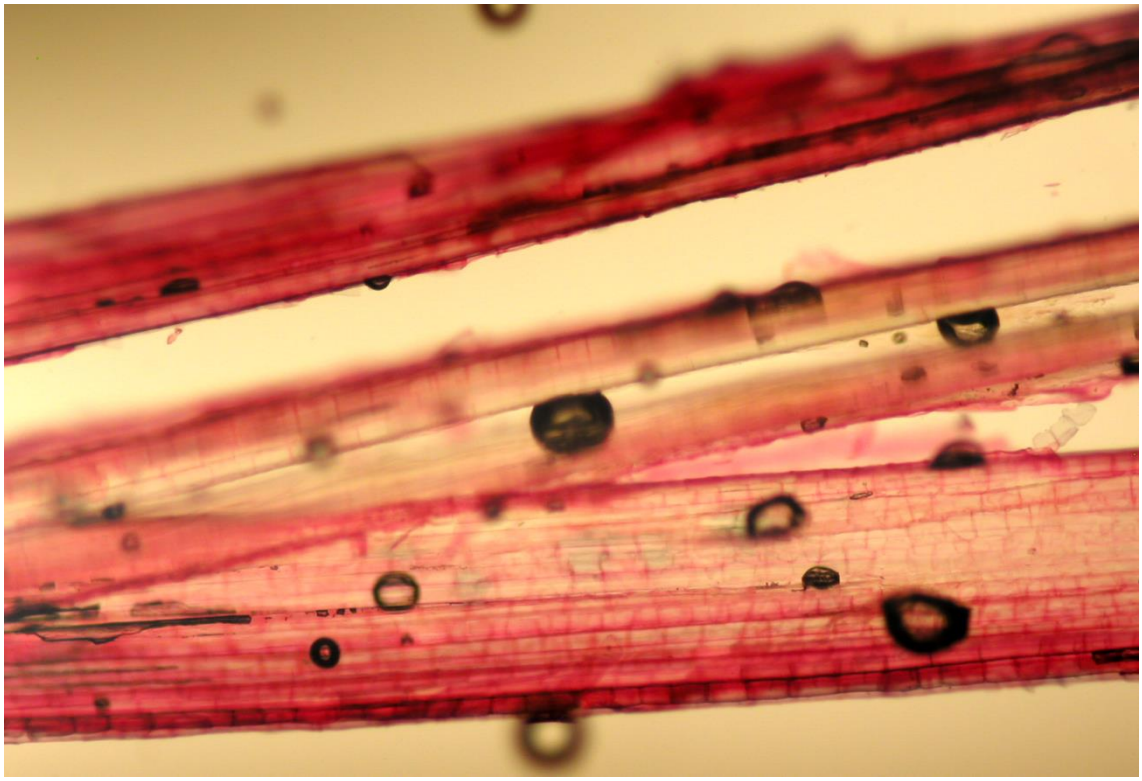


Figure 4-24: Flax fiber treated with XTl at 0.3 %, 40 ºC and pH 5 during 24 hours augmented 40 times observed through a microscope.

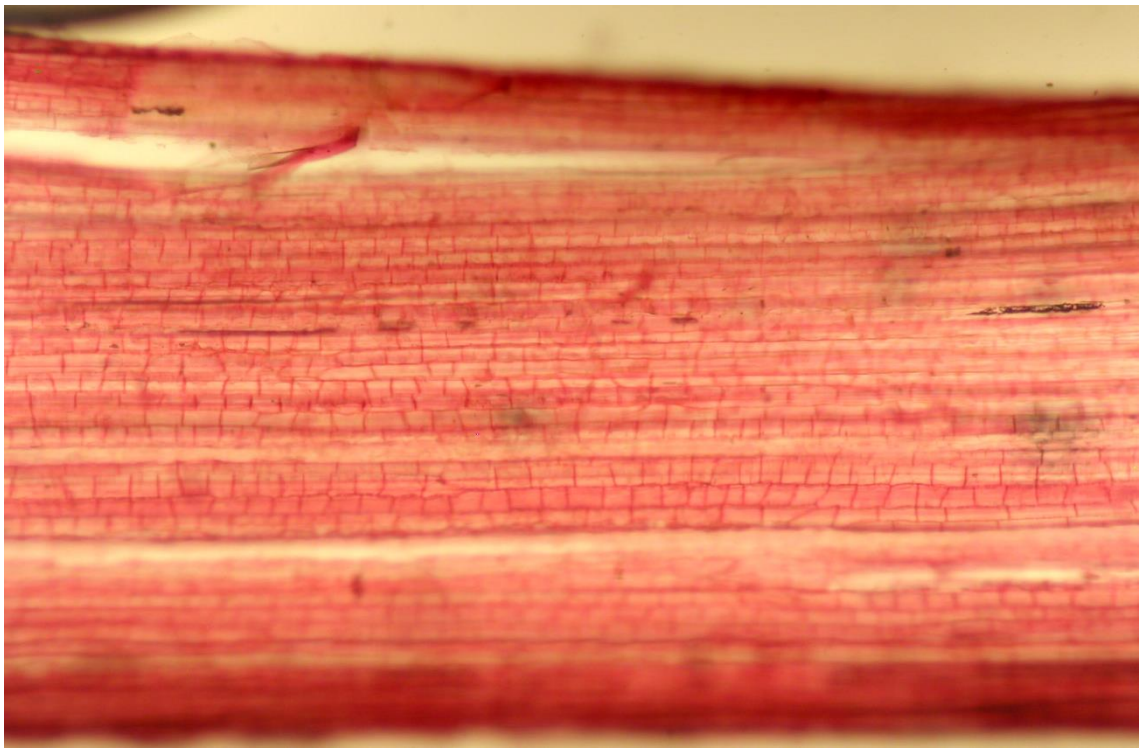


Figure 4-25: Flax fiber treated with XTI at 0.3 %, 40 ºC and pH 5 during 24 hours augmented 40 times observed through a microscope.

No matter what treatment was carried to the fibers, through microscopy were observed transparent and rectangular fiber cells surrounded by pectin substrates stained in red. The small differences of darkness or red intensity that can be appreciated in the pictures are attributed to the utilization of different light intensities during the observation, and also, to the variable thickness of the fibers. Hence, no differences caused by the effect of the enzymatic treatments were observed through microscopy.

5 CONCLUSIONS

In this project the effect of different enzymatic treatments on flax fiber composition was studied. The contents of cellulose, hemicellulose, lignin and rest fraction were determined my means of the gravimetrical characterization of fibers (see paragraph 3.1). Based on the chemical composition of the fibers the effectiveness of the treatments was assessed.

In view of the results, Viscozyme achieved a higher degree of pureness of the fiber when the incubation time was incremented from 6 to 24 hours. Hence, with the longer treatment Viscozyme was able to degrade the hemicellulose and amorphous cellulose present in the matrix to a better extent.

According to the results achieved with NS59049, the activity of pectin lyases increased when the operational pH of the treatment was increased from 5 to 6.5. Therefore, this could indicate that pH 6.5 is closer to the optimal pH for pectin lyases activity. Moreover, pectin lyases from Novozymes (NS59049) and AB enzymes (Rohapect PTE) were compared for the same operational conditions (40 ºC and pH 5) and any significant difference was found in their performance. Following the effect of the pH on the performance of polygalacturonases (Pectinase from *Aspergillus niger)*, pectate lyases (Scourzyme L) and pectin methyl esterases (Rohapect MPE) was studied. In accordance to the results, polygalacturonases did not present significant differences on the level of degradation of the matrix by incrementing the pH, which suggested that both pH 5 and 6.5 are in the optimal range of work for polygalacturonases activity. In contrast, pectate lyases and pectin methyl esterases achieved a superior level of the degradation of the matrix when the pH was incremented to 6.5.

Also a synergy consisting of deesterify the methoxyl groups linked to pectin substrates and degrading the unesterified pectins with polygalacturonases was studied. The results were favourable since the degradation of the matrix was enhanced when polygalacturonases and pectin methyl esterases were mixed.

Furthermore, in literature (Akin *et al.*, 2007) a hypothesis of inactivation of pectate lyases was stated when a chelator like EDTA was added. This hypothesis was investigated with Scourzyme L and in accordance to the results the hypothesis should be refuted, since a better degradation of the matrix was achieved in presence of EDTA.

In addition the effect of the pH and the temperature on hemicellulase activities was investigated with Pulpzyme. Experiments presented an enhanced degradation of the matrix when pH was increased from 5 to 6.5, suggesting that pH 6.5 is closer to the

optimal pH working range for Pulpzyme. However, the results obtained when temperature was incremented did not show a clear improvement. Besides, the effect of the pH was also studied with Xylanase from *Thermomyces lanuginosus* (XTl) and results also suggested a better degradation of the matrix when pH was increased to 6.5. Later on the performance of Pulpzyme and XTl (enzymes containing hemicellulases) was compared at pH 5 and 6.5. In view of the results, Pulpzyme achieved a greater purification of the fiber at pH 6.5, but XTl performance was better at pH 5.

Additionally the effect of hemicellulase from *Aspergillus niger*, another enzyme containing hemicellulase activity, was investigated. Although the treatment conditions were more severe the results showed a poor degradation of the matrix.

Furthermore, the effectiveness of the treatments with Carezyme 1000 L (cellulase activity) and Laccase NS 20621 (degrades lignin) was shown to be poor in comparison to the others. These results are attributed to their inefficiency to degrade the pectin-hemicellulose matrix wrapping the fibers.

In general, the enzymes that retted flax fibers the best were those degrading hemicellulose and pectins. In particular, the purest fiber was achieved by Pulpzyme L, which degrades hemicellulose. Hence, hemicellulases and pectinases showed the greatest potential for retting flax fibers and mixtures of those activities should be studied in future work.

On the other hand, the results of the determination of pectin of green flax and flax fiber with m-hydroxydiphenyl (see paragraph 3.2) were not in accordance with the values found in literature, and therefore, a validation method was developed. This validation method consisted in determining the content of pectin of two substrates (polygalacturonic acid and pectin from apples) and calculating the recovery obtained by means of m-hydroxydiphenyl. The recovery values were notably low and the results suggested a poor hydrolysis of pectin substrates while carrying the determination of pectin with m-hydroxydiphenyl. Also, the optimal time of measuring after the addition of the m-hydroxydiphenyl reagent was determined. Literature suggested to measure the absorbance within 5 minutes of the addition of m-hydroxydiphenyl but the experiments stated an optimal time of 180 seconds.

Finally, by means of ruthenium red staining method (see paragraph 3.4) flax fibers treated with different enzymes were observed trough the microscope but no differences were appreciated.

In conclusion, this project studied the performance of several enzymatic activities and revealed that those degrading hemicellulose and pectin purify flax fibers the most. Therefore, future efforts should focus on studying mixtures of enzymatic activities capable of degrading hemicellulose and pectin. Furthermore, in view of the good results showed by the synergy studied between pectin methyl esterases and polygalacturonases at pH 5, could be interesting to study its performance at pH 6.5.

Moreover, this study optimised the determination of pectin with m-hydroxydiphenyl determining the optimal time for measuring the absorbance after the addition of the m-hydroxydiphenyl reagent. Nevertheless, the content of pectin determined with the m-hydroxydiphenyl method are not in accordance with those in literature. A validation method was proposed without success. Therefore, more efforts are needed to evaluate if m-hydroxydiphenyl is a valid method to determinate pectin substrates. Future work could study the hydrolysis of the substrates by varying the contact time between the substrates and the hydrochloride acid.

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7 ANNEX A

7.1 Data from gravimetrical analysis of chemical components

Table 7-1: Content of cellulose, hemicellulose and lignin in flax fiber determined in the gravimetrical analysis.

Sample	Cellulose (w/w %)	Hemicellulose (w/w %)	Lignin (w/w %)	Rest fraction (w/w %)
Green fiber	63,80	13,10	4,90	18,20
Vz 0,60 % 6 h 40 ºC pH 5	74,70	11,55	3,04	10,70
Vz 0,60 % 24 h 40 ºC pH 5	77,82	10,49	5,34	6,35
Sc 0,30 % 24 h 40 ºC pH 5 (EDTA)	74,04	11,72	2,52	11,72
Sc 0,30 % 24 h 40 ºC pH 6,5 (EDTA)	77,14	10,41	2,72	9,72
Sc 0,30 % 24 h 40 ºC pH 6,5 (no EDTA)	74,48	11,87	3,38	10,27
Sc 0,30 % 24 h 50 ºC pH 6,5 (no EDTA)	73,48	10,70	3,92	11,90
Ns 0,30 % 24 h 40 ºC pH 5	75,54	11,94	3,43	9,09
Ns 0,30 % 24 h 40 C pH 6,5	78,29	10,53	3,72	7,44
Pz 0,30 % 24 h 40 ºC pH 5	71,41	12,22	4,30	12,06
Pz 0,30 % 24 h 40 ºC pH 6,5	81,48	10,86	3,32	4,34
Pz 0,30 % 24 h 50 ºC pH 6,5	79,81	9,92	4,05	6,21
PAn 0,60 % 24 h 40 ºC pH 5	77,42	11,34	3,54	7,69
PAn 0,60 % 24 h 40 ºC pH 6,5	77,45	11,72	3,75	7,05
RhMPE 0,30 % 24 h 40 ºC pH 5	71,14	12,03	3,47	13,36
RhMPE 0,30 % 24 h 40 ºC pH 6,5	80,09	11,02	2,82	6,08
XTL 0,30 % 24 h 40 ºC pH 5	75,95	11,39	2,35	10,31
XTL 0,30 % 24 h 40 ºC pH 6,5	77,54	10,98	3,79	7,70
LAC 0,30 % 24 h 40 ºC pH 5	68,97	10,42	3,04	17,58
RhPTE 0,30 % 24 h 40 ºC pH 5	75,74	10,83	2,72	10,71
RhPF 0,30 % 24 h 40 ºC pH 5	78,21	10,21	4,25	7,33
CelAsp 0,30 % 24 h 40 ºC pH 5	74,22	11,72	5,24	8,82
Han 3 % 48 h 40 ºC pH 5	71,04	10,69	3,75	14,53