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Optimization of an enzymatic cocktail for obtaining cellulose nanofibers.

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ABSTRACT

Earth's resources are getting exhausted, and thus, some alternatives are being contemplated to raw materials traditionally used; one of these alternatives are cellulose nanofibers. One of the pretreatments applied to cellulose fibers consists in enzymatic hydrolysis. This study expects to accomplish the optimization of an enzymatic cocktail with the aim of making the process more efficient, emphasizing the fact that it goes in tune with green chemistry.

The established objectives consist on determine the unknown components, obtaining them separately, and assay different combinations in order to optimize the enzymatic cocktail avoiding antagonisms and promoting synergisms.

In the present study, the laboratory has been able to know the two main enzymes of the cocktail, and nanofibers have been obtained applying different combinations and enzymatic proportions. Then, nanofibers have been characterized in terms of: nanofibrillation yield, transmittance, carboxyl content, cationic demand, specific surface and diameter. Moreover, it has been tried to produce hydrogels with nanofibers obtained applying the commercial treatment, since these nanofibers have shown greatest characteristics.
Therefore, the principal objective consisting on optimizing the enzymatic cocktail has not been accomplished.
Despite nanofibers obtained applying the commercial cocktail have been the ones that have stand out when it comes to characterization, they still are away from the values achieved when a TEMPO-mediated oxidation treatment is applied. Based on this fact, some hypotheses have arisen, these include the fact that maybe there is another key enzyme, or the possibility that the enzymatic proportions facilitated by the commercial company were distorted.

These unsatisfactory values have been reflected when producing hydrogels, since they rapidly disintegrated when were submerged in water. The explanation consequently generated, resides in the fact that enzymatic nanofibers present low carboxyl content, and thus, the crosslinking reaction with citric acid, that allows obtaining hydrogels, is not favored.

Therefore, it is seen that there is still a long way to go when it comes to enzymatic studies for nanofiber's obtaining and its applications. In this work, some possible lines for future studies are traced.

RESUM

Els recursos de la terra s'estan exhaurint, així doncs, es plantegen alternatives a les matèries primeres utilitzades tradicionalment; una d'aquestes són les nanofibres de cel·lulosa. Un dels pretractaments que es pot aplicar a les fibres és la hidròlisi enzimàtica. En aquest estudi es pretén optimitzar una mescla enzimàtica per tal de fer més eficient el procés d'hidròlisi, amb la intenció de desenvolupar nanofibres de cel·lulosa, posant especial èmfasi en el fet que combregui amb els principis de la química verda.

Els objectius establerts consisteixen en determinar els components desconeguts, obtenir-los per separat, i assajar diferents combinacions per tal d'optimitzar la mescla enzimàtica evitant antagonismes i afavorint sinèrgies.

En el present estudi, s'han conegut els dos principals enzims de la mescla i s'han obtingut nanofibres de cel·lulosa amb diferents combinacions i proporcions enzimàtiques que s'han caracteritzat i comparat amb les obtingudes aplicant la mescla comercial en termes de: rendiment de nanofibril·lació, transmitància, taxa de carboxils, demanda catiònica, superfície específica i diàmetre. A més, s'ha provat de produir hidrogels amb les nanofibres que han mostrat unes millors característiques, que han estat aquelles obtingudes amb el pretractament comercial.
Per tant, no s'ha acomplert l'objectiu principal d'aconseguir optimitzar la mescla enzimàtica objecte d'estudi.

Tot i això, els valors que mostren aquestes nanofibres encara disten molt dels que s'assoleixen quan s'aplica un pretractament del tipus *TEMPO-mediated oxidation*. Arran d'aquest fet, han sorgit diverses hipòtesis, aquestes inclouen el fet que hi hagi algun altre enzim clau encara que en menys proporció a part dels dos identificats, o la possibilitat que les proporcions facilitades per l'empresa fossin esbiaixades.

Aquests valors poc satisfactoris s'han vist reflectits a l'hora de produir els hidrogels, ja que ràpidament es desintegraven en ésser submergits en aigua. L'explicació generada en conseqüència, rau en el fet que les nanofibres enzimàtiques presenten taxes de carboxils molt minses, i per tant, la reacció de *crosslinking* amb l'àcid cítric, que permet obtenir hidrogels, no es veu afavorida.

Així, queda plasmat que encara hi ha molt de camí per recórrer pel que fa a estudis de tractament enzimàtic per a l'obtenció de nanofibres de cel·lulosa i les seves corresponents aplicacions. En aquest treball es tracen línies a seguir en futurs estudis.

RESUMEN

Los recursos de la tierra se están agotando, así pues, están surgiendo alternativas a las materias primas utilizadas tradicionalmente; una de esas alternativas son las nanofibras de celulosa. Uno de los pretratamientos que se aplica a las fibras de celulosa es la hidrólisis enzimática. Este estudio pretende optimizar una mezcla enzimática aplicada a la obtención de nanofibras de celulosa, enfatizando el hecho de que vaya en sintonía con la química verde.

Los objetivos consisten en determinar las enzimas desconocidas, obtenerlas por separado, y evaluar diferentes combinaciones para poder llevar a cabo una optimización de la mezcla evitando antagonismos y favoreciendo sinergismos.

En el presente estudio, el laboratorio ha podido conocer las dos enzimas mayoritarias de la mezcla, y diferentes nanofibras han sido producidas variando combinaciones y proporciones enzimáticas. Después, se han caracterizado en términos de: rendimiento de nanofibrilación, transmitancia, contenido de carboxilos, demanda catiónica, superficie específica y diámetro. Además, se ha intentado producir hidrogeles con las nanofibras que han mostrado unas mejores características, que fueron aquellas obtenidas aplicando el pretratamiento comercial.
Por consiguiente, no se ha cumplido el objetivo principal de optimizar la mezcla enzimática.

Aun así, los valores mostrados por esas nanofibras todavía se alejan mucho de aquellos conseguidos cuando se aplica un pretratamiento del tipo *TEMPO-mediated oxidation.* Así pues, han surgido diferentes hipótesis, incluyendo que haya alguna otra enzima en una menor proporción, o la posibilidad que las proporcionas facilitadas por la empresa sean sesgadas.

Estos valores poco satisfactorios se han visto reflejados a la hora de producir hidrogeles, ya que rápidamente se desintegraban al ser sumergidos en agua. La explicación generada reside en el hecho que las nanofibras enzimáticas presentan tasas carboxílicas muy bajas, y por tanto, la reacción de *crosslinking* con el ácido cítrico no se ve favorecida.

Se concluye que todavía hay mucho camino por recorrer en los estudios de tratamiento enzimático para producir nanofibras de celulosa. En este trabajo se trazan líneas a seguir en futuros estudios.

1. INTRODUCTION

1.1. Cellulose

Cellulose is one of the most abundant polymers on earth, occurring in wood, cotton, hemp and other plant-based materials and serving as the dominant reinforcing phase in plant structures. Despite its relative chemical simplicity, the physical and morphological structure of native cellulose in higher plants is complex and heterogeneous.[1]

Cellulose is constituted of fibers of monomers of β-D-glucopyranose that combined in an alternated way make a turn of 180º above its own horizontal axis; since this turn is produced, the repetitive structural unit is cellobiose; this turn provides cellulose a high symmetry because supplies the same number of hydroxyl grups to each side of the chain. Monomers are united between them by means of β-1,4-O-glycosidics linkages.
Its empiric formula it is $(C_6H_{10}O_5)n$, so that each chain presents n≥200.
Resulting chains are agglomerated in fibrils, located in parallel orientation between them, constituting microfibrils; these, in turn, gather forming cellulose fibers.[2]
The established hydrogen bonds inter-chain (2 per anhidroglucopyranose) and intra-chain (2-3 per anhidroglucopyranose) confer rigidity, stability and a high traction force to the supramolecular fibers.[2]

In **Figure 1** a cellulose chain with n+2 monomers is represented, it can be observed that thanks to the flip-flop structure, each side has the same number of OH groups. Inside square brackets the repetitive structural unit of cellobiose can be observed.

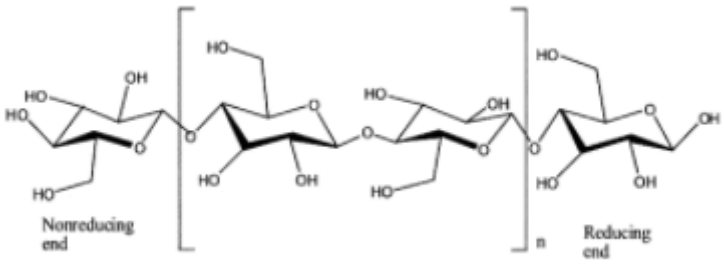


Figure 1. Representation of a cellulose chain.[2]

The alignment of these fibers shapes cellulose sheets, that in turn, stack up with more cellulose sheets, conferring tridimensional structure to the cellulose fibers.
Cellulose molecules are intimately associated with other polysaccharides and lignin in plant cell walls, resulting in even more complex morphologies.[1]
The bundles of elemental fibrils measure about 4-5 nm ant they are embedded to a hemicellulose matrix that measures about 7-30 nm.[2] Hemicellulose contains different polysaccharides as xylose, mannose and glucans.
The lignification process is carried out later, so that lignin is located fundamentally on the external part of microfibrils, covalently attached to hemicellulose.
Lignin is a complex polyphenolic structure.[3]

In **Figure 2** it can be observed the organization of cellulose, hemicellulose and lignin in a microfibril.

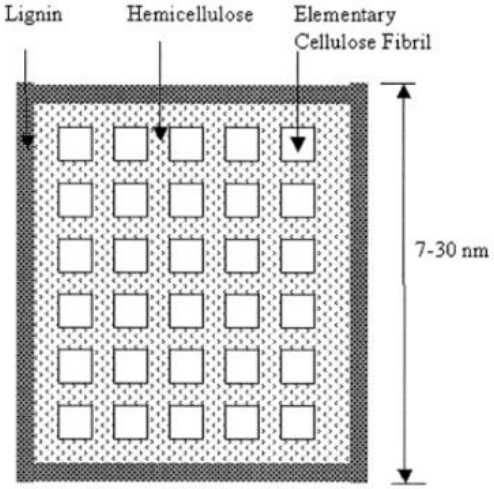


Figure 2. Representation of a microfibril.[2]

1.2. Nanocellulose

Native cellulose, *cellulose I*, has two different crystalline forms, I_α and I_β. I_α is dominant in bacteria and seaweed, while I_β is dominant in higher plants.
Initially, cellulose presents millimetric dimensions, while when it is submitted to treatments that disperse it, it is finally obtained cellulose of nanometric dimensions, which is called nanocellulose. Nanocellulose is between 5 and 6 nm of diameter, and between 10 and 20 nm of length, and presents mechanical properties that provide nanocellulose of a high added value.
Nanocellulose is a very light material, its density is 1,5 g/cm^3, it is even more resistant than Kevlar, and moreover, it presents a high specificity surface.[4]

The two main families of celluloses are cellulose nanofibers and cellulose nanocrystals. The concept cellulose nanofibers refers to suspensions that contain cellulose fibers of nanometric size.
Cellulose nanofibers are produced principally applying a strong mechanical shearing to make a disruption of the wall of the fibers and to release the cellulose fibrils in the shape of bounds of elementary fibrils.
Cellulose nanocrystals are extracted from fibers after a complete dissolution from the non-crystalline fractions.
As it has been mentioned above, cellulose comes from plants. This is its origin the majority of times, but sometimes it can also come from bacteria, then it is designated as bacterial cellulose.
Bacteria synthesize cellulose as a protecting wrapper, and this one stands out from the rest of celluloses because of its properties: high purity, resistance, adjustability and a high swollen retention capacity.[5]

1.3. Applications and advantages.

Cellulose nanofibers have a wide range of applications, among which stand out: paper addition, paper production (constituted only of nanofibers), aerogels and hydrogels.
The fact of adding nanofibers to the paper implies a significant improvement on its properties, increasing its specific surface, its useful life and the number of times that it can be recycled.
Moreover, when cellulose nanofibers are added to paper, its density and strength increase a lot, while on the other hand, porosity and opacity are reduced.
In order to produce paper with a good barrier property against water, alkyl-ketene-dimer is added to cellulose nanofibers.
When it comes to paper formed exclusively of cellulose nanofibers, it is a potential alternative to the use of plastics for the fabrication of food bags. It also have been used cellulose nanofibers in substitution of plastics for the fabrication of speakers for instance.[5]
Aerogels are colloids formed of a solid phase and a gas phase, where *ratio* solid-gas<1. In order to obtain the aerogels, cellulose nanofibers, that usually present a consistency lower than 5%, are submitted to a lyophilization process, in which the aqueous phase sublimates becoming a gas phase. Aerogels can be used to oil-removal when spills occur. In this case, like has been mentioned above, AKD (alkyl-ketene-dimer) is added, which confers hydrophobicity to the aerogel, so this one can tend to absorb oil, diminishing the amount of water absorbed. Moreover, these aerogels can be recycled up to three times by means of an organic solvent like toluene, which removes oil from the aerogel surface.[6]
Nanocellulose hydrogels are a network of nanofibers of cellulose with a high swelling capacity, meaning that they have a high capacity to absorb water. They can be obtained by adding other derivatives from cellulose, like sodium carboxymethylcellulose, which allow obtaining cohesion, and also adding other crosslinking agents, like citric acid.[7]
Hydrogels can be applied to agriculture and horticulture in arid zones, were water scarce. Functioning is as follows: when it rains, hydrogel located in the soil retains water, and then it is capable to release it gradually little by little. In the most sophisticated cases, the aerogel is capable of detect, by means of a sensor, the most optimum moment in which it is necessary to release water to the soil.[8]
Moreover, aerogels with methylcellulose indicate that could be used to repair defects in the brain, since methylcellulose is well suited as a biocompatible injectable scaffold.[9]
Applications based on nanofibers usually present the same or even better properties from those traditionally used, with the high added value of being biodegradable.[5]

1.4. Methodologies to obtain cellulose nanofibers.

To obtain cellulose nanofibers, cellulose fibers must be passed through a homogenizer at high pressures.
Even so, first, it is needed to do a pretreatment, in order to diminish its size, to avoid clogging and that not so many energy has to be spent when homogenizing. Following this line, it is necessary to do a previous digestion of the fibers.

With the aim of digesting the fibers, different methodologies can be used, including: TEMPO-mediated oxidation, enzymatic hydrolysis and mechanical shearing.[10]

TEMPO-mediated oxidation

TEMPO-mediated oxidation is a kind of chemical pretreatment. Chemical pretreatments are strategies very efficient that facilitate the disruption of the fiber networks generating ionic groups or groups susceptible of being ionized, in the inner structure of fibers.
In the case of TEMPO-mediated oxidation, cellulose is unstructured in microfibrils, thanks to the oxidation interceded with 2,2,6,6-tetramethylpiperidine-1-oxyl radical.
In this case, NaClO is the oxidizing agent used.
In this methodology, cellulose at a consistency between 1-2% is suspended in water that contains TEMPO and sodium bromide. Oxidation is performed adding different amounts of NaClO at room temperature and under agitation, pH is maintained at 10.5 with the addition of NaOH. When pH does not vary, it is assumed that reaction has finished and HCl is added to reach neutral pH, adjusting it to a value of 7. The obtained product is completely washed with water, filtered and stored at 4ºC. Carboxyl content in the solid product oxidated is determined using a method of valoration of electric conductivity. Aliquotes of each sample are again oxidated with $NaClO_2$ at a pH of 4-5, and the increase in carboxylic groups is considered to be the amount of aldehid groups presents in TEMPO-oxidized cellulose.[10]

Enzymatic hydrolysis

First of all, BKHP is dispersed at 1.5% in water in a pulper during 30 minutes at 3000 rpm. Then, fibers are filtrated. This process is carried out to let fibers absorb water, and thus, promote the activity of the enzymes. Enzyme dosages can be stablished at different values, typically ranging between 80-320 g/tone. The treatment time typically ranges between 2 and 4 hours. When it comes to pH, it is usually set up at 5 or 7. Then, suspension is heated at 50ºC under constant stirring in order to avoid local gradients. Up to this point, enzyme is added to the suspension. Enzymatic treatment can be stopped either heating the suspension or increasing pH for 15 minutes, producing enzyme denaturation. Pulp enzymatically hydrolyzed is washed with distilled water and it is stored at 4ºC.[10]

Mechanical shearing

Mechanical treatment for the preparation of cellulose nanofibers consists in refining the fibers mechanically. To achieve it, BKHP is dispersed at a 1.5% in water and unstructured in a pulper. Right after, suspension is filtered and refined at 20000 revolutions in a PFI mill.[10]

Fiber fibrillation

Treated suspensions are homogenized gradually in a homogenizer following the sequence of three passes at 300 bar, three passes at 600 bar and finally, three passes

at 900 bar. All suspensions follow this sequence to assure constant fibrillation, differing only in the kind of treatment received. Moreover, pressure is increased gradually to avoid clogging in pressure chambers.[10]

1.5. Enzymes applied for enzymatic hydrolysis

Endo-β-1,4-glucanases

Endo-β-1,4-glucanases are enzymes with a molecular mass of 42 KDa, that produce the scission of the monomers of β-D-glucopyranose by means of breaking the β-O-glycosidic bonds that are formed between carbon number 1 and 4 of contiguous monomers of β-D-glucopyranose. When these enzymes are applied to cellulose fibers, its length is diminished, since they act producing the scission parallel to the transverse section of fibers. It is expected that the diameter is the same because adjacent chains are united between them by hydrogen bonds and Van der Waals forces, which are not susceptible of being destabilized by enzymatic action.[2,11]

Xylanases

β-1,4-xylans are enzymes with a molecular mass of 40 KDa, mainly found in secondary walls of plant cells, particularly in vascular tissue. Xylanases are enzymes that attack internal xylosidic linkages on the backbone of xylose, they act breaking the β-1,4-D-O-glycosidic linkage and xylopyranose residues are released. It has been studied the combination of different xylanases for biotechnological exploitation, the judicious use of proper mixes of xylanolytic enzymes could result in cleaner reactions, higher yields, and lower consumption of enzyme and energy, parameters vital to the economic feasibility of industrial processes.
Xylanases also have attracted increasing attention in biotechnical research due to their potential applications in cellulose pulp bleaching.[12,13,14]

Mannanases

Mannanases are enzymes with a molecular mass of 65 KDa, that attack mannan endowise. In the literature, it is reported that when mannan reacts with β-1,4-mannosidic oligomers, the enzyme preferentially attacks β-1,4-mannosidic linkages situating at the third or fourth position from the non-reducing end.[15,16]

2. OBJECTIVES

In the present work, an enzymatic cocktail has been kindly supplied by the company Celodev®, it corresponds to 060B nomenclature and it has been applied to BKHP (Bleached Kraft Hardwood Pulp). It is known that it has an enzymatic content of 2%, of which 95% are endo-β-1,4-glucanases, while the 5% remaining are non-identified enzymes.

Taking all these into consideration, the next objectives have been outlined:

1. To determine the non-identified components.
2. The study of the effect* of every single component to the BKHP (mainly constituted of cellulose and hemicellulose).
3. The study of the effect* of all possible combinations of individual components to the fibrous support.
4. To identify the presence of synergies between components, in other words, if the presence of a particular combination has a better effect than the addition of the activities of every single enzyme that takes part on the combination on his own.
5. To identify the presence of antagonism between components, meaning if the presence of a particular combination has a worst effect than the addition of the activities of every single enzyme that takes part on the combination on his own.
6. Taking into account the action of all these combinations, it would be object of study, promoting synergisms and avoiding antagonisms, to determine which combination is the optimal one for the treatment of the fibrous support BKHP and develop applications of it.

*The study of the effect implies the study of the diminution of the length as well as the characterization of nanofibers in terms of: nanofibrillation yield, transmittance, carboxyl content, cationic demand, specific surface and diameter.

3. MATERIALS AND METHODS

3.1. Pretreatment of Bleached Kraft Hardwood Pulp

First of all, Bleached Kraft Hardwood Pulp is located in a pulper, which disjoints the fibers at 20000 rpm during 30 min. Right after, the fibers are moved to a dryer and kept there until it does not release water anymore. At this moment, consistence can be measured and the fibers are kept in a hermetic plastic bag and stored at 4 ºC until its use it is required. Consistency is defined as the weight of the fibers over the total weight, which includes the fibers and the swollen water.

Right after, some characteristics of the fibers can be measured by means of MorFi®. The parameters evaluated are the weighted length, the diameter and the percentage of fines.

Continuing with the pretreatment, enzymatic treatments are performed with the aim of shorting the length, and consequently, reducing the amount of energy that will be later needed to obtain the nanofibers. Consistency is measured again and the fibers are kept in a hermetic plastic bag and stored at 4 ºC until its use it is required.

All enzymatic treatments have been developed under same conditions: a concentration of 320 g/tone, stirring (in order to avoid local gradients) for 4 hours and at a pH and temperature conditions of 5 and 50ºC, respectively.

In a reunion that was carried out with Celodev® company, the laboratory has been able to know that endoglucanase and xylanase where the two major enzymes of the 060B cocktail. So apart from the enzymatic cocktail supplied by Celodev® above mentioned, xylanase and endoglucanase supplied by Sigma-Aldrich® with an activity factor of 2500 units/g were assayed as single components and as combinations of both.

Next, the same parameters evaluated before by means of MorFi® are evaluated again.

Obtaining of nanofibers

Once the pretreatment has been carried out, the fibers are passed through a high-pressure homogenizer Panda Plus (Gea Niro Soavi) that works until 2000 bars. The established protocol in the laboratory is to pass the fibers three times at three different pressures, which are 300, 600 and 900 bars, respectively. This gradually increase of pressure helps avoiding clogging in the pressure chambers. When lengths are larger than 300 nm, it is more susceptible clogging to happen. Right after, they are kept in a plastic bottle and stored at 4ºC.

Determination of consistency

First of all, the weight of a watch glass is measured. Then, the watch glass is tared and about 2 g of nanofibers are weighted, and the exact weight is written down. Next, the watch glass with the nanofibers is put in a heater overnight until there is no water, that is to say, until constant weight, and the weight is measured. Once the three weights are obtained, consistence can be measured following **Equation 1**:

$$Consistency = \left(\frac{DryWeight - Tare}{TotalWeight - Tare}\right) \times 100 \qquad \textbf{Equation 1}$$

Where:

$Consistency$: Weight of fiber per total weight (%)
$DryWeight$: Dry weight of fibers and watch glass (g)
$TotalWeight$: Total weight, including fibers, water and watch glass (g)
$Tare$: Weight of the watch glass (g)

3.2. Characterization of nanofibers

Nanofibrillation yield and Transmittance

Nanofibrillation yield is defined as the percentage of fibers that actually exhibit nanodimensions compound the solution once it has been passed through the homogenizer.

In order to determine the yield of nanofibrillation, a solution of 0.1% g dry is centrifuged at 4500 rpm for 20 minutes, so the supernatant contains the nanofibrillated fraction, while the non-nanofibrillated fraction remains on the precipitated. Right after, the supernatant fraction is discarded, while the sediment is recovered and dried until constant weight is achieved. The difference between the dry weight of the sample and the sediment is the dry weight of the supernatant, and then the nanofibrillation yield can be easily calculated by means of **Equation 2**:

$$NY = \left(\frac{DryWeightsn}{DryWeightsample}\right) \times 100 \qquad \textbf{Equation 2}$$

Where:

NY : Nanofibrillation yield (%)
$DryWeightsn$: Supernatant's dry weight (mg)
$DryWeightsample$: Sample's dry weight (mg)

Transmittance is an indirect way to corroborate the yield of nanofibrillation, it can be easily measured submitting the supernatant to a sweep from 400 to 800 nm wavelength by means of a Shimadzu UV-160A Recording Spectrophotometer. A higher value of transmittance will be traduced in a higher nanofibrillation yield.

Carboxylic content

Since it is required to determine the amount of mmoles of carboxylic acid per gram of cellulose, the methylene blue technique is used. It is performed in a pulp previously enzymatically hydrolyzed but not homogenized.

First of all, 10 dry mg of the pulp have to be weighted. Secondly, 25 mL of methylene blue have to be added (at a concentration of 300 mg per L). Then, 25 mL of a borate buffer at a pH of 8.5 are added (in order to obtain it, a solution 1 M of boric acid is prepared, and it is adjusted with NaOH until the pH desired is reached). Next, the sample must be submitted under gentle stirring for an hour, and then settling during another one. Continuing with the procedure, the sample must be centrifuged at 10000 rpm for 20 minutes, and if that is not enough, the time necessary until a precipitate it is formed. Right after, 2 mL of supernatant ought to be transfered to a 25 mL flask. 2.5 mL of HCl 0.1 M are added and make up to 25 mL volume with distilled water.

Before measuring the absorbance, it is required to prepare the patterns. 4 patterns of 25 mL must be prepared in flasks of 50 mL at increasing equidistant concentrations of methylene blue of 3, 6, 9 and 12 mg/L, respectively, dissolved in distilled water. Right after, 25 mL of borate buffer are added in each one, so the volume makes up to 50 mL.

Once the patterns and the sample have been prepared, the next step is to measure the absorbance at a steady wavelength of 664 nm (the value of the wavelength at which the absorption of methylene blue is maximum). To do the blank measure, distilled water was used; for each pattern and for the sample, three measures are developed.

The next step consists in building the straight line with the four points obtained that correlate the concentration of the patterns with its respective absorbance. As the concentration it is the independent variable, it will be represented in the abscissa's axis; and the absorbance, as is the dependent variable, will be represented in the ordinated axis.
At this point, the concentration associated with the absorbance obtained can be calculated, what leads to determine the mg of methylene blue that have not been absorbed, and finally, the number of carboxylic groups.

To determine the amount of carboxylic groups, **Equation 3** has been used:

$$COOH = \frac{(7.5 - X) \cdot 0.00313}{W}$$ **Equation 3**

Where:

$COOH$: Amount of carboxylic groups (mmol/g)
X: Amount of methylene blue that has not been adsorbed (mg)
W: Dry weight of the sample (mg).

In all cases, absorbance has been measured with a Recording Spectrophotometer Shimadzu UV-160A.

Cationic demand

Cationic demand it is used to determine the specific surface and it is based on a setback titration. First, 0.04 g dry of the nanocellulose fibers obtained have to be weighted, taking into account the consistency of them. Then, water is added until a weight of 30 g is reached. The next step consists in adding 50 mL of poly-DADMAC, which presents a concentration of 0.00107 N. The whole solution must undergo 5 minutes of gentle stirring, and then it has to be centrifuging at 10000 rpm for 20 minutes, or the time that is required until a sediment is formed. In the supernatant remains the fraction of poly-DADMAC that has not been adsorbed. 10 mL of this liquid are put in a Mütek PCD04 charger analyzer from BTG, S.L. (UK) and then, Pes-Na, which presents a concentration of 0.00104 N, is added little by little with the help of a micropipette until non-voltage is exhibit.

If even adding Pes-Na with drops of the smallest amount possible, the value shown passes from a little positive value to a little negative value, it would be appropriated to do a linear interpolation of both points taking the two little values with its respective volumes associated, and then substituting the value of the potential for zero, in order to obtain a volume of Pes-Na more exact.

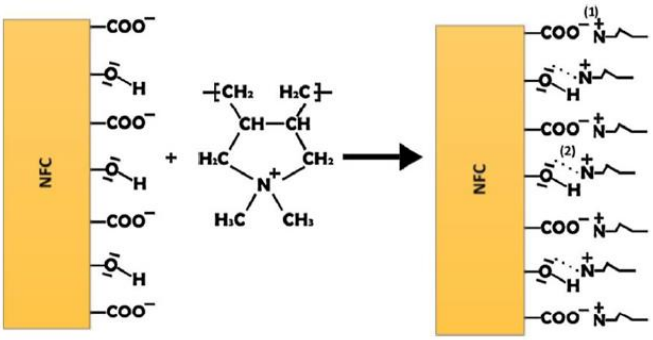
Once the amount of Pes-Na added is known, cationic demand can be calculated following the **Equation 4**:

$$CD = \frac{(V_{PolyD} \cdot C_{PolyD}) - (V_{Pes-Na} \cdot C_{Pes-Na})}{W_{sample}} \qquad \textbf{Equation 4}$$

Where:

CD: Cationic demand (µeq/L)
V_{PolyD}: Volume used of cationic polymer (mL)
C_{PolyD}: Cationic polymer concentration (g/L)
V_{Pes-Na}: Volume used of anionic polymer (mL)
C_{Pes-Na}: Anionic polymer concentration (eq/g)
W_{sample}: Sample's dry weight (g)

Scheme 1 shows two proposed mechanisms of interaction between LCNF and poly-DADMAC.



Scheme 1. Proposed mechanisms of interaction between LCNF and poly-DADMAC.[17]

3.3. Applications

Hydrogel's production

With the aim of producing hydrogels, cellulose nanofibers submitted to enzymatic hydrolysis with Celodev® commercial cocktail were used, following a methodology reported in literature[7] with some variations included.
In the paper consulted, CMCNa and HEC are used for the hydrogels preparation.
In the present work, a variation has been applied, consisting in applying CNF instead of HEC.
Citric acid (CA) has been the crosslinking agent used, since it does not present toxicity, and moreover, it is inexpensive.

Hydrogel's synthesis

Three samples were prepared adding CMCNa to CNF solutions submitted to gently stirring at room temperature during 24 h. The CMCNa:CNF mixtures were assayed at ratios of 3:1, 1:1 and 1:2, respectively, all of them established at a 2% polymer concentration by weight of water.

Once CMCNa was completely dissolved, CA was added and gently stirred at different concentrations by weight of polymer: 1.75%, 2.75%, 3.75%, 10%, 20% and 30% to obtain samples with various crosslinking degrees.

These solutions served to prepare 10-mm thick samples. Two cycles of drying of 24 h have been performed. The first, at 50ºC, with the aim of removing water; and the second one, at 80ºC, to let that crosslinking reaction took place. During the second cycle, intermediate controls were required to make sure that hydrogels were not withered.
Finally, hydrogels are submerged in distilled water to qualitatively assess the degree of swelling.

4. RESULTS AND DISCUSSION

4.1. Evaluation of pretreated fibers

At the beginning of this final project, a reunion was carried out with the company Celodev®. The aim of meeting with them was accomplishing the first objective of this project, that is to say, trying to discern which enzymes were present in the 060B cocktail supplied by them. The information provided was:

1. Endoglucanase, being the major enzyme, represents a 95% of the enzymatic content.
2. Considering the 5% remaining, xylanase is the major enzyme of this little but not less important percentage.
3. It was accorded that the company would provide the single components of the cocktail, so that different combinations and proportions of them could be studied.

In the end, after weeks of waiting, no single components were received, so the laboratory decided to order the two components that for sure were present in the cocktail, *id est*, endoglucanase and xylanase.

In **Tables 1-6**, the diameter, length and fine elements of BKHP fibers submitted to different enzymatic treatments have been evaluated.

Table 1. Evaluation of the BKHP fibers before going through any enzymatic treatment.

Sample	Diameter (µm)	Length (mm)	Fine elements (%)
1	15.8	0.766	35.1
2	16.2	0.772	41.0
3	17.1	0.719	30.2
4	17.4	0.711	12.6
$\bar{x}$	16.6	0.742	29.7
StDev	0.8	0.031	12.2

Table 2. Assessment of the BKHP fibers after being submitted to Celodev® 060B enzymatic treatment.

Sample	Diameter (μm)	Length (mm)	Fine elements (%)
1	17.5	0.230	55.5
2	18.3	0.228	70.5
3	18.3	0.228	65.7
4	18.8	0.231	63.0
$\bar{x}$	18.2	0.229	63.7
StDev	0.7	0.041	2.4

In figures, **Figure 3** and **Figure 4**, BKHP fibers can be appreciated and it can be compared how the length of the fibers is diminished before and after applying Celodev® enzymatic treatment. Both images have been obtained with polarized light.

Figure 3. Image of the BKHP fibers before any enzymatic treatment is developed.

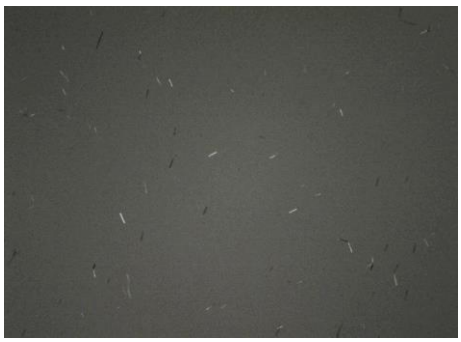


Figure 4. Image of the BKHP fibers after commercial enzymatic treatment is developed.

Table 3. Evaluation of the BKHP fibers after enzymatic treatment with exclusively endoglucanase.

Sample	Diameter (µm)	Length (mm)	Fine elements (%)
1	16.3	0.741	33.6
2	17.3	0.732	26.8
3	17.1	0.729	36.7
4	16.2	0.751	40.6
$\bar{x}$	16.7	0.738	34.4
StDev	0.6	0.010	2.4

Table 4. Analysis of the BKHP fibers after enzymatic treatment with xylanase. Note that the first row, highlighted in red, contains atypical values, and thus, it has not been taken into account to calculate the statistical parameters.

Sample	Diameter (µm)	Length (mm)	Fine elements (%)
1	16.8	0.733	28.4
2	17.9	0.580	69.6
3	19.7	0.511	55.9
4	20.5	0.504	38.3
$\bar{x}$	19.4	0.532	54.6
StDev	1.2	0.220	4.0

Table 5. Evaluation of the BKHP fibers after going through enzymatic treatment at an endoglucanase-xylanase ratio of 95 over 5, respectively.

Sample	Diameter (µm)	Length (mm)	Fine elements (%)
1	18.9	0.706	44.5
2	18.3	0.732	37.2
3	18.3	0.709	51.9
4	18.8	0.697	42.9
$\bar{x}$	18.6	0.711	44.1
StDev	0.6	0.118	6.1

Table 6. Testing of the BKHP fibers after being submitted to an enzymatic treatment at an endoglucanase-xylanase ratio of 50 over 50, respectively.

Sample	Diameter (µm)	Length (mm)	Fine elements (%)
1	18.1	0.635	31.1
2	17.5	0.622	55.7
3	18.0	0.599	63.4
4	19.1	0.590	58.3
$\bar{x}$	18.2	0.612	52.1
StDev	0.8	0.018	3.7

Since endoglucanase enzymes produce their cut parallel to the transverse section of the fibers, no decrease of the diameter was to be expected, so it can be considered

that providing the value of the diameter at every different treatment, in this case, serves as a control to corroborate what is being analyzed. As observed, there is not a decrease on it at any of the different treatments that have been carried out. As it was also expected, fine elements increased inversely to the length of the fibers.

When it comes to the length of the fibers, it was expected that it was shortened with the enzymatic treatment for the same reason above. Actually, when the commercial treatment was applied, the length obtained was about a third of the initial one. In the two treatments where the xylanase content was the major enzyme, the length went down to a seventh of the former size. Despite these results, in the two cases where endoglucanase was the main enzyme, no reduction of the fibers was observed, which suggests that endoglucanase maybe requires assistant enzymes other than xylanase to develop its function appropriately.

4.2. Characterization of cellulose nanofibers

Since the length practically does not decrease with any of the combinations tested, it is neither expected to achieve great values when it comes to characterization of the obtained cellulose nanofibers.

In **Table 7**, it can be seen the characterization of the obtained nanofibers in terms of: yield of nanofibrillation (NY), carboxyl content (CC), cationic demand (CD) and transmittance (T) at a wavelength of 800 nm.

In each row, the following nomenclature is used:

- CNF-E:X; where CNF is the acronym of cellulose nanofibers and E:X is the percentage of endoglucanase and xylanase, respectively.

Note that in the first row there is not a given value to the amount of xylanase, this is because the amount of xylanase in the commercial cocktail is unknown; it is only known that there is a 5% of this single component at the most.

Table 7. Characterization of the nanofibers that have been obtained with different endoglucanase-xylanase ratios. First row is highlighted in green with the aim of facilitating the comparison with the other enzymatic combinations.

Sample	NY (%)	CC (mmol/g)	CD (μeq/g)	T at λ=800 nm (%)
CNF-95:X	19.6	0.052	234	30.7
CNF-100:0	1.7	0.045	102	24.2
CNF-0:100	3.1	0.048	128	26.6
CNF-95:5	2.1	0.053	109	23.9
CNF-50:50	2.9	0.048	116	24.7

Nanofibrillation yield

Focusing on the yield of nanofibrillation, the best result is obtained with the commercial cocktail, which is 19.6% and stands out clearly comparing it with the other yields achieved. Looking at the other samples, as the amount of xylanase is increased,

the nanofibrillation yield is also slightly increased, achieving the highest value when the whole enzyme dosage is composed of exclusively xylanase. Nevertheless, in the best-case scenario, the nanofibrillation yield is 3.1%, which represents only a sixth part of the one achieved with the commercial cocktail. Moreover, when pressure was applied with the aim of obtaining nanofibers, clogging of the homogenizer was constantly produced because lengths were further larger than 300 nm.

All yields of nanofibrillation are in line with the values of large lengths obtained before, because if the enzymatic pretreatment gives a shorter length, it is understood that it will be a bigger amount of nanofibers achieved.

Transmittance

When it comes to transmittance, the highest value corresponds to 30.7% and it is reached with the cellulose nanofibers obtained with the commercial cocktail, it makes sense because it also corresponds to the greatest nanofibrillation yield value. For the rest of the values, transmittance decreases according to the decrease of the percentage of nanofibrillation; what was not expected was that values decreased as little. Maybe this small diminution could be attributed to a precipitation of the fibers while doing the measure, what led to a higher transmittance value than actually was.

Carboxyl content

Regarding at carboxyl content, it is about 0.050 mmol/g for all samples. These values were expected not to change between samples because when fibers are submitted to an enzymatic treatment, there is no oxidation, differently from what happens with nanofibers obtained after being submitted to a TEMPO-oxidation pretreatment. So in this case, it can be considered that measuring carboxyl content, has also another function, that is serving as a control measurement, since it was expected not to change with any enzymatic treatment applied.

Cationic demand

The highest value of cationic demand is 234 μeq/g, and it is achieved when commercial cocktail is used. This value is quite similar to other values reported in the literature for cationic demand of cellulose nanofibers obtained with enzymatic treatment. Looking at the rest of the samples, all of them take values much lower than usual, since are slightly higher than 100 μeq/g, but seem to be quite reasonable since the cationic demand value decreases as the amount of xylanase applied and yield also does. The best result is achieved when the whole enzymatic content is compounded of xylanase, but anyway, it represents only a half of the value obtained with the commercial cocktail. However, these low values seem to be reasonable as its correspondent yields are also very low. As reported in the literature, cationic demand is closely related to specific surface, and both parameters have a directly proportional relationship, so it is expected that cellulose nanofibers that have been obtained with commercial cocktail showed the greatest values for specific surface, while those obtained with the other combinations would show worst values for specific surface, as the value of cationic demand decreases.

Determination of specific surface and diameter

Specific surface can be determined by means of **Equation 5**:

$$\sigma_{CNF} = (CD - CC) \cdot \sigma_{DADMAC} \qquad \textbf{Equation 5}$$

Where:

σ_{CNF}: Specific surface area of 1 g of CNF (m^2/g)
CD: Cationic demand of the sample (μeq/L)
CC: Carboxylic content of the sample (μeq/L)
σ_{DADMAC}: Specific surface area of polydadmac (m^2/μeq)

Right after, diameter can be calculated using **Equation 6**:

$$d_{CNF} = \frac{4}{\sigma_{CNF} m^2/g \cdot 1600 \cdot 10^3 g/m^3} \qquad \textbf{Equation 6}$$

Where:

d_{CNF}: Average diameter of a single CNF fibre (m)
σ_{CNF}: Specific surface area of 1 g of CNF (m^2/g)

In **Table 8**, it can be seen the characterization of the obtained nanofibers in terms of: specific surface (σ_{CNF}) and diameter (d_{CNF}).

Table 8. Calculation of the intrinsic properties of nanofibers that have been obtained with different endoglucanase-xylanase ratios. First row is highlighted in green with the aim of facilitating the comparison between commercial cocktail and the other enzymatic combinations.

Sample	σ_{CNF}(m^2/g)	d_{CNF}(nm)
CNF-95:X	88.63	28.21
CNF-100:0	27.76	90.06
CNF-0:100	38.96	64.17
CNF-95:5	27.27	91.67
CNF-50:50	33.12	75.49

Since the calculation of specific surface and diameter has been carried out as a function of cationic demand and carboxyl content, it was expected, as it has happened, that these values went in tune between them. Like above, the greatest results for both parameters have been achieved for commercial treatment. Specific surface is about two and three times higher than the other combinations tested, while diameter is between two and three times smaller; since both parameters keep an inversely proportional relationship.

Global analysis

Taking a global analysis, for any of the parameters tested, none of the combinations has been as greatest as the commercial one. This was previously expected since it contains more compounds that are unknown; what was not expected was that the values exhibited for length and characterization differed that much from the commercial cocktail.
Moreover, different from what was expected, the cellulose nanofibers that have offered values of characterization relatively closer to those obtained with the commercial cocktail have been those obtained applying exclusively xylanase.
What was expected was that the combination showing greatest values when characterization was developed was the one with a ratio of a 95% of endoglucanase over a 5% of xylanase, since it is the more similar to the commercial one, according to the information provided by Celodev®.

What could be quite expected was that when only endoglucanase was applied, no effect was observed. This could be explained because endoglucanase degrades cellulose; since hemicellulose is in the external part of the fiber, could be that until it is not degraded, no degradation of cellulose could be performed.

In the two cases where endoglucanase was the main enzyme, no reduction of the fibers was observed, which suggests that endoglucanase maybe requires assistant enzymes other than xylanase to develop its function appropriately.
This also leads to think that maybe xylanase requires another enzyme to be functional when it is present at low dosages.

Hypotheses

It is surprising that if in the commercial cocktail it is supposed to be a concentration of xylanase smaller than a 5%, when a 5% of xylanase is applied, no effect can be appreciated on the length of the fibers, what leads to think two possible hypotheses to give an explanation:

1. It could be that actually the amount of xylanase in the cocktail was higher than a 5%; and thus, the percentage of endoglucanase would be lower than 95%.
2. If the percentage was lower than a 5%, it could be that the unknown compounds in the cocktail established a synergy with xylanase, letting it having a visible effect despite being in a lower concentration.

Focusing on endoglucanase, when it is applied as a single component, no effect is observed. But what surprises the most is that the effect of applying endoglucanase with xylanase gives the same results of applying only xylanase, so endoglucanase seems to not be working. This leads to a third hypothesis:

3. Endoglucanase maybe requires assistant enzymes other than xylanase to develop its function appropriately.

Suggestions for future studies

To face the first hypothesis, a determination (including identification and quantification) of xylanase and endoglucanase present in the cocktail could be carried out. The information reported in literature about purification of these enzymes refers to isolating them once they have been produced on a bacterial strain, what is quite different of having them in an enzymatic cocktail.

Nevertheless, the protocol could be followed, assuming the risk that the unknown enzymes present in the mix might have similar properties. For instance, it could be that an unknown enzyme precipitated at the same range of salt than endoglucanase or xylanase. To verify the purity of the enzyme, an SDS-page electrophoresis would be necessary.[14,18]

For hypotheses number 2 and 3 it is purposed to submit BKHP to an enzymatic treatment that contains mannanase apart from endoglucanase and xylanase.

Mannanase has been chosen as a candidate to develop further studies for the following reasons:

1. It degrades mannose, which is a component of hemicellulose.
2. Some other Celodev® cocktails contain this enzyme in major percentages, so maybe it is also part of this cocktail.

Table 9 shows the different combinations proposed to study the behavior of these three enzymes. Since in this case there is one more enzyme, a bigger number of combinations will be performed. If some combination showed results equal or even better to the commercial one, it is contemplated the possibility of studying intermediate proportions in order to optimize the enzymatic cocktail.

The methodology followed to obtain and characterize the nanofibers would be the same explained above, and the percentages tested would be:

Table 9. Samples of cellulose nanofibers that would be obtained at different endoglucanase-xylanase-mannanase percentages. First row, highlighted in green, refers to the combination of the commercial cocktail.

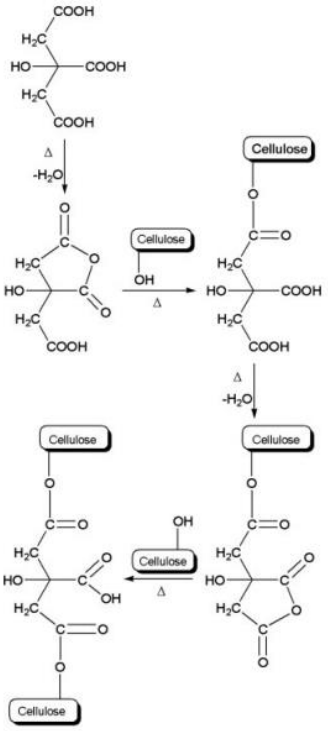
Sample
CNF-95:X:M
CNF-100:0:0
CNF-0:100:0
CNF-0:0:100
CNF-50:50:0
CNF-50:0:50
CNF-0:50:50
CNF-33:33:33
CNF-95:5:0
CNF-95:0:5
CNF-95:2,5:2,5

In line with the nomenclature used before:

- CNF-E:X:M; where CNF is the acronym of cellulose nanofibers and following the nomenclature above mentioned E:X:M is the percentage of endoglucanase, xylanase, and mannanase, respectively.

4.3. Applications

In **Scheme 2**, the proposed mechanism shows that each molecule of citric acid stoichiometrically reacts with two carboxylic cellulosic groups. It can be seen that when CA is heated, it dehydrates to yield the cyclic anhydride that reacts with cellulose; successively another cyclic anhydride function can be achieved into CA structure through the other two non-reacted carboxylic groups allowing the attachment of another hydroxyl cellulosic group.



Scheme 2. Proposed mechanism of reaction between citric acid and cellulose[7]

Produced aerogels have not exhibited good stability. Once submerged in water, they disintegrated rapidly, especially those at lower CA dosages. It is thought that this happens because of their low reticulation degree and carboxylic content.

The nanofibers used performed a carboxylic content of 0.052 mmol COOH/g. If we take as an example the test where the ratio of CMCNa:CNF was 3:1 and CA was 20%, and to facilitate calculations we assume that we use 3 g of CMCNa, then 1 g of CNF is required to achieve the stablished proportion. So we are working with 4 g of polymer and 0.8 g

of CA. 1 g of CNF that exhibits 0.052 mmol COOH/g, contains $3.13 \cdot 10^{19}$ COOH groups, while 0.8 g of CA contain $5.02 \cdot 10^{21}$ COOH groups.

This means that CA contains about 160 more times COOH groups than the used nanofibers, so only a 0.62% of COOH groups of CA are interacting with CNF.

It seems that nanofibers used were not the greatest ones, at least in terms of carboxylic content, in order to carry out the crosslinking reaction. Moreover, the fact of increasing CA content does not make much sense, because if even only a little amount of CA is added, the limiting reactant will still be carboxylic groups of CNF used. For further studies of crosslinking between CA with CNF it is proposed to use TEMPO-CNF since their carboxylic content is much higher, and in this way it could be determined if the disintegration of hydrogels was actually due to this hypothesis proposed.

Nanopapers prepared from enzymatic hydrolysis

Moreover, in the research group, another application using enzymatic hydrolysis was developed.

In **Figure 5** and **Figure 6**, nanopapers formed of exclusively nanofibers are shown. Those nanofibers have been pre-treated with enzymatic hydrolysis, and images have been taken by means of FE-SEM (field emission scanning electron microscopy) technique.

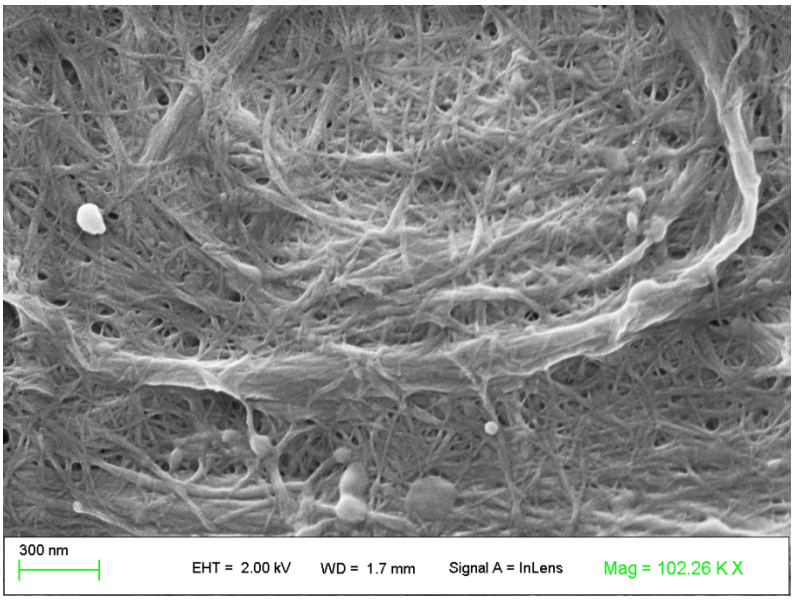


Figure 5. Image of a nanopaper at 300 nm scale.

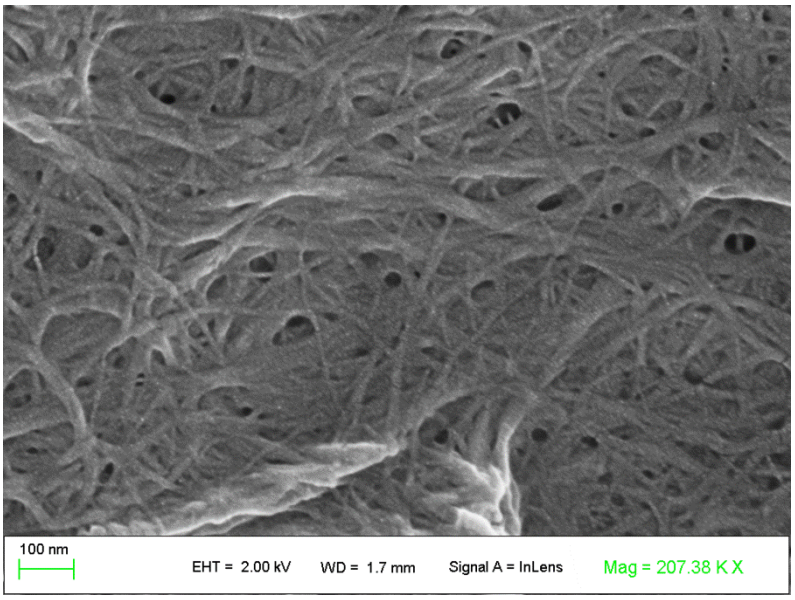


Figure 6. Image of a nanopaper at a 100 nm scale.

As it is possible to see, the obtained CNF presented diameters in the nanodomain, as estimated above from cationic demand and carboxyl content. Due to the huge amount of hydroxyl groups that CNF have on their surface, the interaction thereof is assured. However, in accordance to the yield of fibrillation, one can see some CNF bundles which lead to structures with higher diameter that are in the microscale instead of in the nano.

5. CONCLUSIONS

Attending to the objectives planned at the beginning of the project, next conclusions can be drawn:

1. The laboratory has been able to receive the information regarding to which are the two major components of the cocktail, that is to say, endoglucanase and xylanase.
2. It is not yet known which ones are the minority enzymes that are also taking part of the enzymatic cocktail.
3. None of the two single enzymes and neither the combination of them have shown great values when it comes to the length of the fibers and characterization of the corresponding cellulose nanofibers.
4. Despite not achieving the expected results, in the two cases were xylanase was in a bigger proportion, the results were slightly greater, achieving shorter lengths of fibers and bigger values for nanofibrillation yield, cationic demand and transmittance.
5. It has not been detected a synergistic effect since the values obtained when the two enzymes were combined were even worse than when only xylanase was applied.

6. It has not been detected an antagonistic effect since the values obtained when the two enzymes were combined were even better than when only endoglucanase was applied.

Taking a global vision, it would be necessary to develop further studies in order to discern the percentage of endoglucanase and xylanase present in the cocktail and also perform studies adding mannanase with the aim of studying possible synergies and antagonisms, and obtaining an enzymatic cocktail equal or even better than the commercial avoiding antagonisms and promoting synergies.

In the present work, cellulose nanofibers were obtained by means of a pretreatment with enzymatic hydrolysis, that is to say, using a biotechnological process. Regarding at the properties of the obtained nanofibers, in the best case, where a commercial enzymatic cocktail was used, values of 88,63 m^2/g and 28,21 nm for specific surface and diameter were achieved, respectively. Comparing it with those cellulose nanofibers obtained applying a TEMPO-mediated oxidation pretreatment reported in literature[19], values for specific surface are doubled and even quadruplicated, and values for diameter are between twice and up to four times diminished; since specific surface and diameter keep an inversely proportional relationship. This greater values for specific surface and diameter are due to the oxidation that is involved in the TEMPO-mediated oxidation process, which adds carboxyl groups to cellulose; while any kind of oxidation is developed when an enzymatic hydrolysis is applied, leading to worse values for both properties. On the other side, if a look is taken at the commercial prices, TEMPO-mediated oxidation costs about 10 € per Kg of nanofibers, while for the same amount pretreated with an enzymatic hydrolysis, the commercial price ranges between 1 and 2 €; it might does not seem much expensive at first sight, but if they are extrapolated at an industrial scale, lots of money could be earned if enzymatic treatment was optimized and, consequently, applied. Even clearly achieving worse results when enzymatic pretreatment is performed, it is important to point out that continuing with an study of the enzymes involved in the process and the optimization of cocktails would be of main importance, apart from the low-cost production, for developing a methodology that goes in tune with the principles of green chemistry, and thus, promoting a more environmentally friendly process.

Ethics & Sustainability Statement:

Raw materials traditionally used, *exempli gratia*, petroleum, usually are not respectful with environment and they are getting exhausted. For both reasons, some alternatives to these sources are being set out, making a special emphasis to the fact that they agree with the principles of green chemistry.
Cellulose, structural polysaccharide of plants, being the most abundant polymer on earth, has become a suitable candidate.
Moreover, in the present work, the pretreatment applied to the fibers has been enzymatic, so when it is extrapolate to a bigger scale, this means a lot of energy-earning in comparison with mechanical treatments.

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