Isolation, characterization and applications of nanocellulose produced by ancestral enzymes

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Summary

Efficient, controlled and sustainable nanocellulose isolation is still a challenge with current methodologies. Enzyme hydrolysis shows up as a novel alternative, but yields are lower in comparison with chemical methods. To improve this process, we need new enzymes with higher performances. Here we propose the use of ancestral enzymes developed with ancestral sequence reconstruction (ASR); these had shown higher activity, stability and promiscuity than the extant ones, matching them ideally for biotechnology application. Here, we propose a method to produce high pure nanocellulose by ancestral endoglucanase hydrolysis. This method allows controlling nanocellulose size and maintains the native cellulose structure where the chemical or mechanical methods fail. This enzymatic nanocellulose shows higher crystallinity and thermostability than a commercial nanocellulose sample produced by acid sulfuric treatment.

The optimized protocol was used to isolate nanocellulose from lignocellulosic substrates. In this case we used treatments with addition of different ancestral enzymes as xylanase and lytic polysaccharide monooxygenase (LPMO), to help ancestral endoglucanase hydrolysis. We achieved nanocellulose isolation from two lignocellulosic pulps with different properties. Also, we observed how LPMO produced nanocellulose oxidation. Here, we propose LPMO as substitution of chemical oxidation of cellulose, demonstrating that the enzymatic method can substitute both nanocellulose isolation and modification by chemical methods.

The enzymatic nanocellulose can be used in high performance tailored materials. In this work we studied our nanocellulose in two different applications. The first one was as reinforcement for thermoplastic materials, in our case waterborne polyurethane (WBPU). As control we used commercial nanocellulose produced by sulfuric acid. We observed that small nanocellulose addition produced nanocomposites with higher thermal and mechanical properties, and nanocomposites with our nanocellulose had better properties than the ones prepared with the acid hydrolyzed nanocellulose. Moreover, we introduced the enzymatic nanocellulose to manufacture conductive nanopapers with graphene addition by two different strategies. We produced nanopapers with high thermal, mechanical and conductive properties by mixing enzymatic nanocellulose with different concentration of reduced graphene. Moreover, by graphene chemical vapor deposition (CVD) over a nanocellulose film we manufactured transparent conductive films, as substitution of plastic or metal substrates.

Resumen

El principal objetivo de esta tesis ha sido desarrollar y optimizar un método para el aislamiento de nanocelulosa basado en el uso de enzimas ancestrales y estudiar sus posibles aplicaciones. La nanocelulosa es un nuevo biomaterial que ha atraído la atención de la comunidad científica debido a sus extraordinarias cualidades como tamaño nanométrico, flexibilidad, o sus propiedades mecánicas, térmicas y eléctricas. Además es un material biocompatible y sostenible. Estas partículas se pueden organizar según su tamaño, cristalinidad y su origen: los nanocristales de celulosa (CNC) son pequeñas partículas cristalinas con longitudes entre 50 a 1000 nm. Las nanofibras de celulosa (CNFs) son fibras nanométricas de varias micras y contienen regiones amorfas y cristalinas en su estructura. Además, existe un grupo de bacterias capaces de secretar nanofibras de celulosa como ocurre con la celulosa bacteriana (BC).

Existen diferentes métodos para el aislamiento de nanocelulosa: mecánicos, químicos y enzimáticos. El proceso mecánico consiste en diferentes pasos de homogenización a alta presión que permiten la obtención de CNFs. Normalmente es combinado con tratamientos químicos o enzimático para mejorar el rendimiento y reducir el consumo energético. El método químico es el más utilizado, concretamente el tratamiento con ácido sulfúrico. Este ácido es capaz de degradar las regiones amorfas de la celulosa y mantener los dominios cristalinos, pero presenta algunos inconvenientes. Durante el proceso se generan grandes cantidades de residuos tóxicos como las aguas residuales generadas

durante los pasos de neutralización y diálisis. Se producen reacciones de esterificación en la superficie de los cristales sustituyendo los grupos hidroxilos por grupos sulfatos, modificando las propiedades fisicoquímicas y dificultando el secado del material por la gran hidrofilicidad de estos grupos.

Es por todo ello que se necesitan nuevos métodos que mejoren los actuales para producir nanocelulosa de manera eficiente, sostenible y controlada. Las enzimas lignocelulosicas, capaces de degradar la biomasa, son una de las alternativas más prometedoras, sin embargo para su implementación necesitamos enzimas con mayor actividad y promiscuidad. En esta tesis hemos propuesto el empleo de enzimas ancestrales desarrolladas con técnicas de reconstrucción de secuencias ancestrales (ASR). Estas enzimas han demostrado tener mayor actividad, promiscuidad y estabilidad que las enzimas actuales. En una tesis anterior desarrollada en el grupo de Nanobiomecánica (CIC nanoGune), realizamos la reconstrucción de una endoglucanasa, enzimas capaces de degradar celulosa, ancestral con 2.000 millones de años. Esta endoglucanasa ancestral (ANC EG) demostró mayor actividad y estabilidad en un amplio rango de pH y temperatura que las endoglucanasas modernas, además degradaban con mayor eficiencia sustratos como cartón. Esta ANC EG mostró las características ideales para su implementación en la producción de nanocelulosa.

En primer lugar nuestro objetivo fue la optimización del proceso de obtención de nanocelulosa y su posterior caracterización empleando la hidrólisis de la ANC EG sobre papel de filtro. Con la intención de mejorar la actividad catalítica en sustratos recalcitrantes, añadimos a la

ANC EG un dominio de unión a celulosa de la endoglucanasa de *Bacillus subtilis* (CBM), obteniendo la enzima quimérica ANC EG+CBM. En estos experimentos medimos una mayor conversión de nanocelulosa y azúcares reducidos durante la hidrólisis de ANC EG+CBM en comparación con la enzima con solo dominio catalítico, ANC EG. Además, ambas enzimas ancestrales demostraron mayor actividad que la endoglucanasa de *Thermotoga maritima* usada como control. Analizamos el tamaño y morfología de la nanocelulosa usando microscopía de fuerzas atómicas. Observamos que la hidrólisis a tiempos cortos producía fibras con morfología correspondientes a nanofibras. Al continuar la hidrólisis hasta 24 horas, el tamaño se reducía, apareciendo partículas similares a nanocristales. La población más homogénea de nanocristales se consiguió manteniendo la hidrólisis de ANC EG+CBM durante 24 horas.

Al comparar la morfología de los cristales producidos por hidrólisis enzimática (EnCNC) con una muestra comercial de nanocristales obtenidos por tratamiento con ácido sulfúrico (AcCNC) observamos diferencias. Los EnCNC mostraban aspecto de aguja, en cambio los AcCNC de cinta. Estas morfologías correspondían a diferentes polimorfos estructurales de celulosa. Los EnCNC mostraban la estructura nativa de celulosa, celulosa tipo I y AcCNC de la celulosa tipo II. Los EnCNC y AcCNC fueron caracterizados por diferentes técnicas fisicoquímicas. Con estos análisis confirmamos que EnCNC mantenían la estructura de celulosa tipo I, mientras que los AcCNC eran mezcla tipo I y tipo II, el tratamiento ácido transformaba parcialmente la estructura. La cristalinidad y estabilidad térmica de los EnCNC era mayor que los

AcCNC debido a la sustitución de los grupos hidróxilos por sulfatos en la superficie de los cristales y a que estos catalizan la degradación.

En la segunda parte de esta investigación decimos aislar nanocelulosa utilizando como sustrato dos materiales lignocelulosicos compuestos por todos los polímeros de la biomasa: celulosa, hemicelulosa y lignina. En este caso, realizamos tratamientos enzimáticos integrando otras enzimas ancestrales con diferentes actividades catalíticas, como xilanasas capaces de depolimerizar hemicelulosa, y monoxigenasas líticas de polisacáridos (LPMO) que degradan la celulosa por oxigenación. En estas hidrólisis se estudió el efecto de diferentes mezclas de estas enzimas, todas ellas incluyendo la ANC EG+CBM, en el rendimiento y las propiedades fisicoquímicas de las nanocelulosas obtenidas. Los rendimientos de nanocelulosa y azúcares reducidos aumentaban con la adición de xilanasa y LPMO debido a la actividad sinérgica de estas enzimas con ANC EG+CBM. La utilización de las tres enzimas en cocktail exhibió la mayor conversión de nanocelulosa en los dos sustratos. Además, comparamos la actividad de este cocktail ancestral con un coktail de enzimas actuales, obteniendo mejores resultados con las enzimas ancestrales. Las nanocelulosas producidas desde estos sustratos mostraban, en general, mayor cristalinidad con la adición de xilanasas y LPMO, pero eran menores que las obtenidas en la nanocelulosa de papel de filtro, debido a la baja cristalinidad de los sustratos de partida. Las nanocelulosas mantenían estabilidades térmicas similares y tamaños menores.

Durante la caracterización de estas nanocelulosas, descubrimos que el empleo de LPMO resultaba en nanocelulosa oxidada. La nanocelulosa

oxidada generalmente es producida por tratamientos químicos y tiene una gran variedad de aplicaciones por su gran reactividad. La función de LPMO para la modificación de nanocelulosa no ha sido descrita previamente, ni su uso en la producción de nanocelulosa, siendo esta tesis uno de los primeros trabajos en demostrarlo. Además, demuestra que las enzimas ancestrales pueden sustituir los procedimientos químicos tanto en la producción como en la modificación de nanocelulosa.

Finalmente, quisimos estudiar distintas aplicaciones de nuestra EnCNC producida desde papel de filtro para demostrar su versatilidad y su implementación en aplicaciones de altas prestaciones. En primer lugar fue utilizada como refuerzo para materiales termoplásticos, concretamente poliuretanos en base de agua, y comparados a su vez con AcCNC. Observamos como pequeñas cantidades de nanocristales mejoraban las propiedades termomecánicas. Además, la adición de EnCNC aumentaba estas propiedades de manera más eficiente que los AcCNC en la misma concentración, manifestando como las propiedades fisicoquímicas de los nanocristales tienen un efecto en las propiedades del material final. En segundo lugar estudiamos la formación de nanopapeles conductores con EnCNC y grafeno, estos experimentos no pudieron compararse con AcCNC debido a la imposibilidad de éstos para formar nanopapeles estables. En este caso observamos como la adición de grafeno a la matriz de EnCNC resultaba en la fabricación de nanopapeles con elevadas propiedades mecánicas, conductoras y térmicas. Incluso, fabricamos nanopapeles conductores transparentes mediante deposición química en fase vapor de una mono capa de grafeno, proponiendo la nanocelulosa como sustrato en sustitución de materiales plásticos o metálico

Chapter I: Introduction

Cellulose is the most abundant renewable biopolymer on Earth and it is the main structural component of the lignocellulosic biomass. Cellulose is present not only in the cell wall of plants, but as a part of other organisms such as fungi [1], bacteria [2], algae [3] or animals like tunicates [4]. Cellulose is a linear homopolymer composed by D-glucose units bonded together by β-1,4-glycosidic bonds, where each unit is rotated 180º to the next one. The smallest unit that two D-glucose units form is named cellobiose, and it has a size of 1,03 nm (Figure 1.1). The polymerization degree of cellulose can oscillate depending on its source, in wood-derived cellulose is normally of 1000 glucose units, in cellulose from cotton is 10000 units, and in bacteria is around 500 units [5].

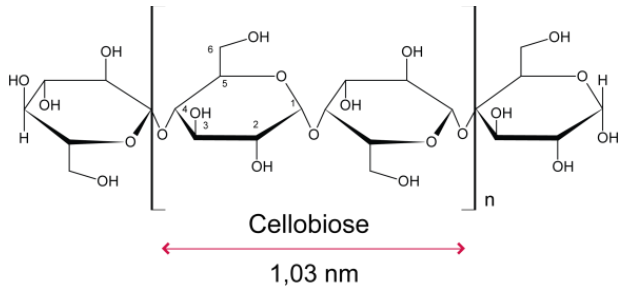

Figure 1.1. Cellulose chemical structure. Cellobiose is the repeating unit of the cellulose polymer and is formed by two D-glucose units bonded together by a β-1,4-glycosidic bond. Each unit is rotated 180º to the next and has three hydroxyl groups that made the polymer very reactive.

The D-glucose units have three hydroxyl groups that are responsible for some cellulose properties such as chirality, hydrophilicity, and biodegradability. The natural linear structure of cellulose and the number of hydroxyl groups help the formation of hydrogen bond that produces the ordered crystalline structure and provides the high mechanical properties to the fibers. The hydrogen bonds can be formed between different cellulose chains (intermolecular bond) or in the same chain (intramolecular bond) (Figure 1.2). The intramolecular bonding provides stiffness and the intermolecular bonding shapes the crystal structure. The high amount of hydrogen bonds and the crystallinity made the cellulose an insoluble material in water and in the majority of organic solvents [6].

Cellulose is a semi-crystalline polymer and is divided into crystalline and amorphous fractions. The crystalline domains are very packed together and are very difficult to degrade by chemical or enzymatic treatment. In the amorphous regions, the chains are disorganized and are easier to degrade [7]. The proportions between both domains depend on the cellulose source and the treatment used to extract the fibers [8].

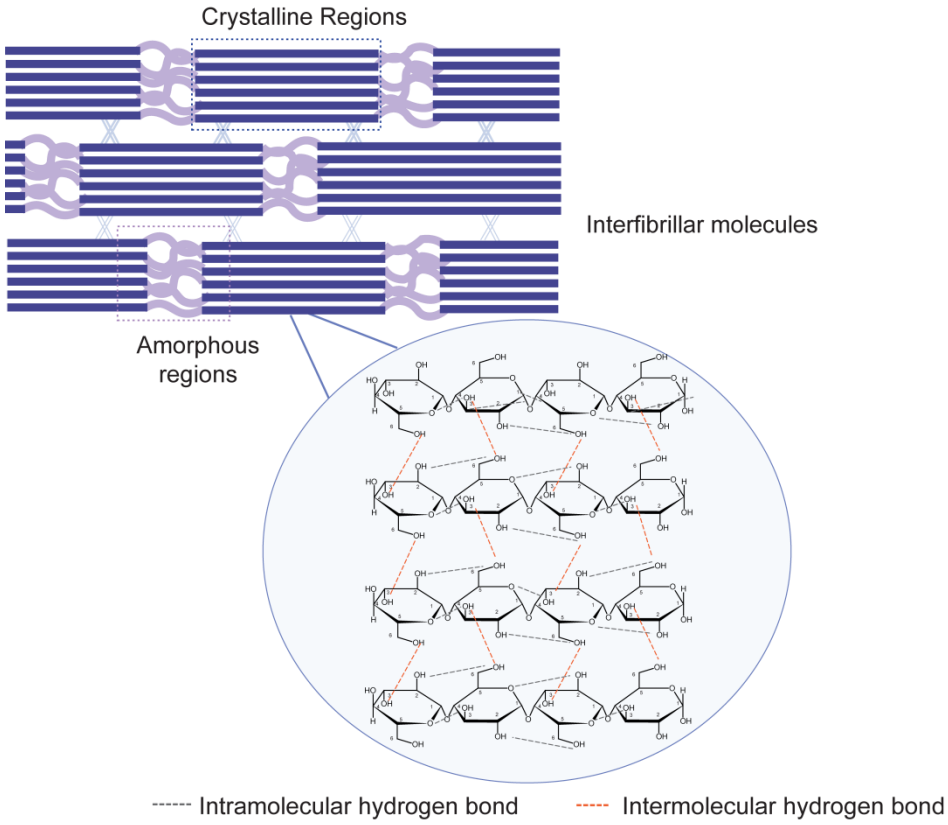

Figure 1.2. Cellulose semi-crystalline structure. Cellulose polymer is organized in crystalline regions, where the glucose chains are tied together by inter- and intramolecular hydrogen bonds producing a very recalcitrant structure, and an amorphous region where the chains are disorganized and are more accessible to degradation.

We can organize cellulose into polymorphs or allomorphs depending on the inter- and intramolecular hydrogen bonds and their molecular orientations. There are six polymorphs described in the literature: cellulose I, II, III_I, III_{II}, IV_I and IV_{II}. The native and most abundant form of cellulose is the type I, and all the polymorphs can be produced from it with different physicochemical treatments (Figure 1.3). Cellulose I has

the chains of the polymer in a parallel organization and it can be also organized in two polymorphs, cellulose Iα and Iβ [9]. These two type I polymorphs can be found together, and the ratio between then vary with the cellulose source. The cellulose Iα is abundant in algae and bacterial cellulose [10] and cellulose Iβ is more present in higher plants and tunicates [11].

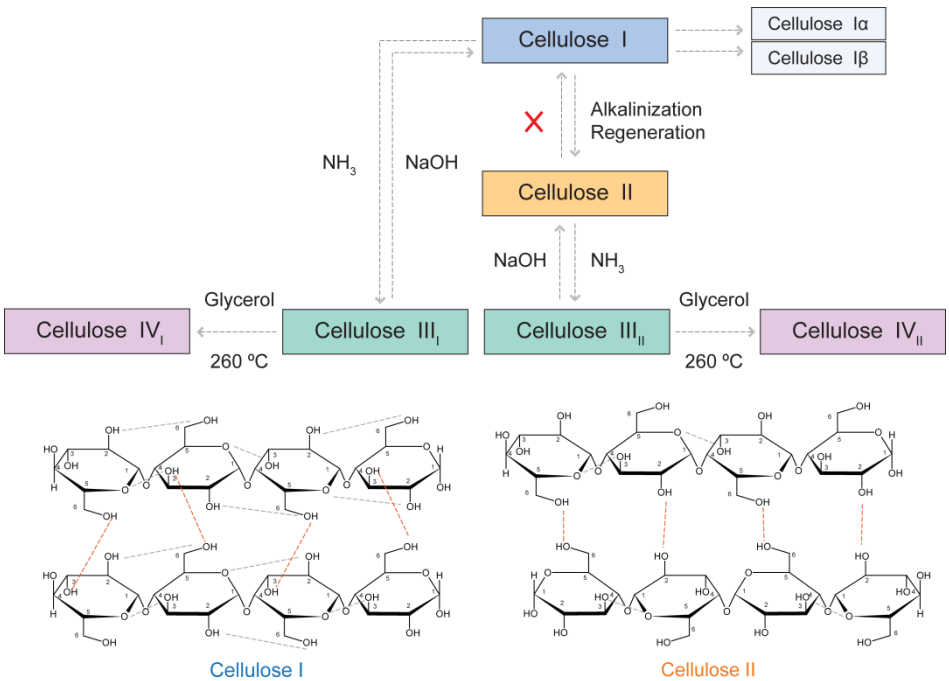

Figure 1.3. Cellulose polymorphs. We can differentiate six cellulose polymorphs depending on the inter- and intramolecular interactions of their hydrogen bonds. Cellulose I is the native and most abundant cellulose form and by chemical and physical treatments we can produce the other five.

Cellulose II is the second most abundant cellulose form; in this polymorph, the cellulose chains have an antiparallel organization. This distribution permits that the hydrogen bonds happen between the neighbor's hydroxyl groups and improved the interlayer attraction forces,

but there are less secondary hydrogen bonds [12]. These interactions made that cellulose II has added stability and produce the irreversibility to convert cellulose II into I. Also, cellulose II is more thermostable and weakly mechanically than cellulose I because of its different chain organization [13]. Cellulose II can be prepared from cellulose I by mercerization (alkaline treatment) [14] or regeneration [15]. From cellulose I and II we can produce the other polymorphs. Cellulose III is made from cellulose I or cellulose II by ammonia treatment that penetrates and degrades the cellulose crystal structure [16]. Cellulose IV is produced from cellulose III by glycerol and heat treatment at 260 ºC [17]. Cellulose III can be transformed into their previous polymorphs by alkaline treatment.

In nature, cellulose is produced as an individual long chain that during the biosynthesis process is organized in a hierarchical structure to form the fibers. The polymeric chains are packed together by hydrogen bonding forming the elementary fibrils, these have a diameter of 3-5 nm and a length around 2-20 μm depending on the source [18]. These tiny fibers are aggregated by van der Waals forces and more intra and intermolecular hydrogen bonds to compose the microfibrils that have a diameter around 30 nm and a length of several micrometers, depending on the source. The orientation and packing of these microfibrils are responsible for the crystalline and amorphous fractions. The microfibrils are gathered in larger structures called macrofibrils and they are further packed into the main cellulose fibers.

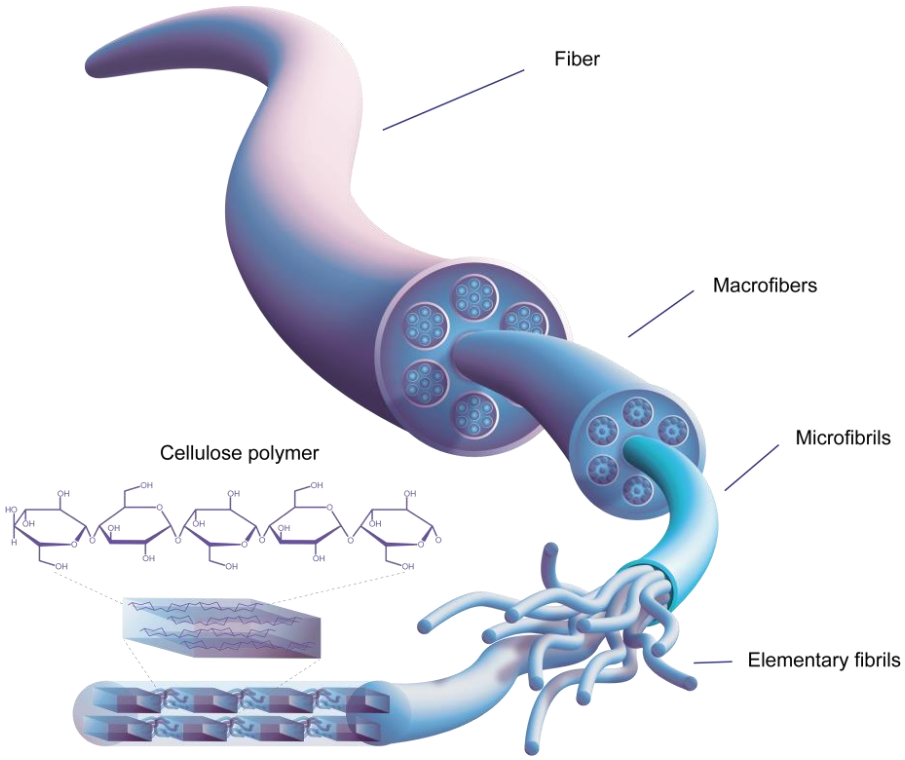

Figure 1.4. Hierarchical cellulose structure. Cellulose chains are packed together forming elementary fibrils. These tiny fibers are aggregated by hydrogen bonding and van der Waals forces into microfibrils and these are assembled in macrofibers; the macrofibers are the last unit that forms the main cellulose fibers.

This hierarchical structure of cellulose permits its degradation in nanoparticles, these particles are called Nanocellulose. Nanocellulose has gathered the attention of the research community and has been extensively studied since its discovering because of its extraordinary capabilities like its biocompatibility, renewability, it is sustainable, has nanometer size, large aspect ratio, and flexibility, good electrical,

thermal and mechanical features. Nanocellulose is organized in different types depending on their size, shape, crystallinity, and source: cellulose nanocrystals (CNCs) [19] have ribbon-like shape with very high crystallinity, normally have an average diameter between 2-30 nm and a length of 50 nm to 1 μm depending on the cellulose source and the isolation treatment (Table 1.1). Cellulose nanofibers (CNFs) [20] are long thin fibers with a length of several micrometers and diameters of nanometers, these fibers have both amorphous and crystalline domains. Bacterial Cellulose (BC) [21] is produced by a group of Bacteria that synthesize big thin nanofibers into the culture medium with high crystallinity, using glucose as a substrate.

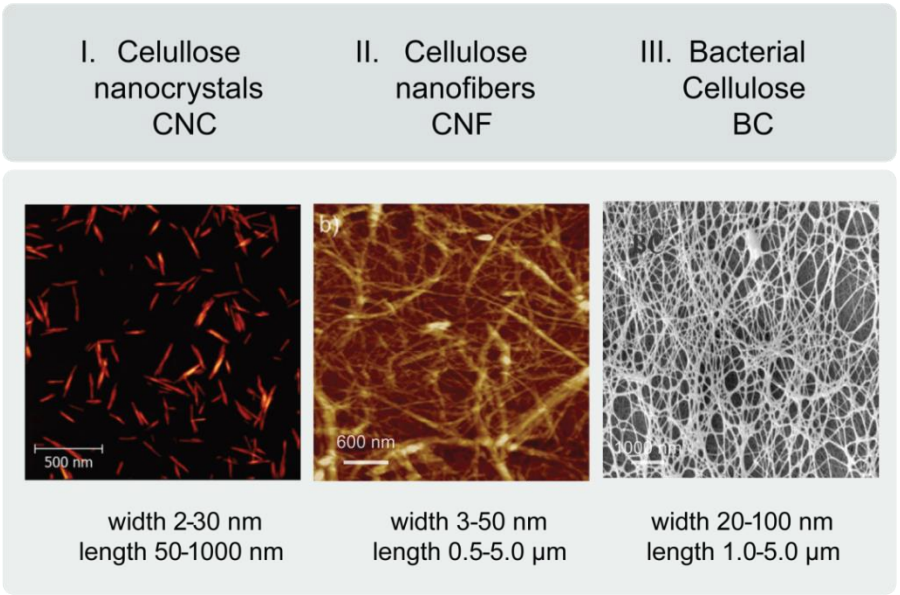

Figure 1.5. Nanocellulose types. (I) AFM image of cellulose nanocrystals (CNC) produced with sulfuric acid treatment [22], (II) AFM image of cellulose nanofibers (CNF) isolated with mechanical treatment [23], (III) SEM image of Bacterial Cellulose (BC) secreted into the culture medium and freeze-dried [24].

Due to CNCs higher crystallinity have higher mechanical properties in comparison with CNFs that have amorphous cellulose segments in its structure [25]. The CNC have also high surface area and tensile strength that can be compare to other nanomaterials used as reinforcement like kevlar or carbon nanotubes [26]. Furthermore, the morphology and degree of crystallinity on the nanocrystals affect their nanomechanical performance, a reduction on the diameter and crystallinity translate in a reduction of their strength.

Nanocellulose has several applications in different fields due to its extraordinary properties: for example, it is used as reinforcement for paper materials making strong nanopaper sheets for packaging [27, 28]. In photonics research is used to make transparent films [29], CNC can also produce iridescent films and chiral materials, and even can be modified to have other optical functionalities like UV-blocking [30] or fluorescence [31].

In biomedical research nanocellulose has a very widespread application because pure nanocellulose is relatively non-toxic and biocompatible [32], is used to made scaffolds for tissue engineering [24, 33], as a carrier for drugs [34, 35], in tissue regeneration [36], an even there are reports that nanocellulose helps fat absorption [37] in the intestine. Nanocellulose can be part of proteins composites, where nanocellulose act like reinforcement, for example to prolamin [38]. Nanocellulose films have been used for its gas barrier [39] and water absorption properties [40]. Nanocellulose can form foams and aerogels with good mechanical performances [41].

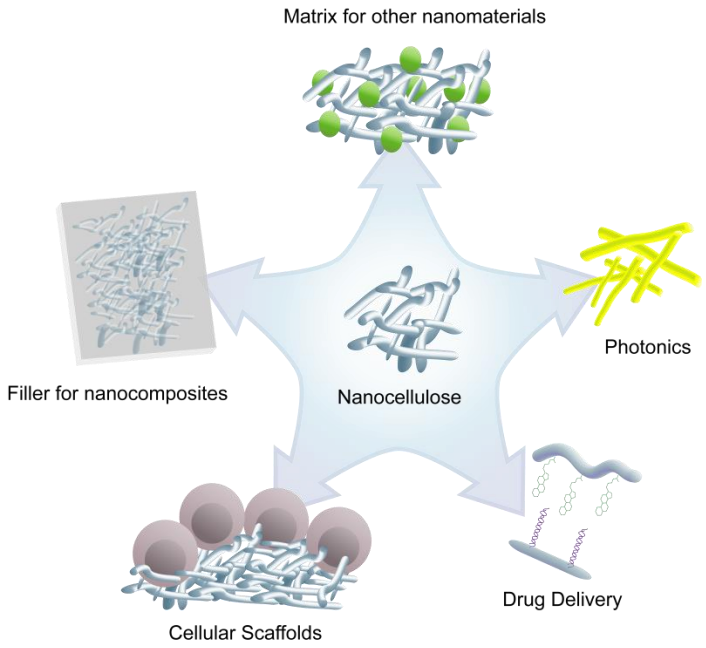

Figure 1.6. Nanocellulose application. There are several applications depending on the research field. Nanocellulose can be used as a matrix for other nanomaterials to produce nanocomposites with different properties. Nanocellulose can be used in photonic by making it fluorescent or iridescent films. In the biomedical field can be used in drug delivery systems or cellular scaffolds for tissue regeneration. One of the most common applications is as filler for nanocomposites to improve or change the mechanical and physicochemical properties of the matrix.

One of the focuses in nanocellulose application is as polymeric materials filler to manufacture a cost-effective, durable, and greener biomaterial. The physicochemical properties of nanocellulose can improve biomaterial performance. There are examples in polyvinyl alcohol (PVA) [42], polylactic acid (PLA) [43] and waterborne polyurethanes (WBPU) [44]. Due to climate change and to reduce the pollution produced by polymeric materials manufacturing, eco-friendly materials are attracting

the attention of researches, this is the case of WBPU. WBPU are capable to gather stable particles in water dispersion by addition of internal emulsifiers [45] and not using organic toxic solvent.

WBPU are block copolymers formed by two blocks or segments, the hard segment (HS) formed by urethane groups and soft segment (SS) composed by polyol [46]. These segments are thermodynamically incompatible and result in microphase separated phases or domains. The SS made the material flexible and the HS gives stiffness, but both can be ordered in amorphous or crystalline domains by hydrogen bonding (Figure 1.7)

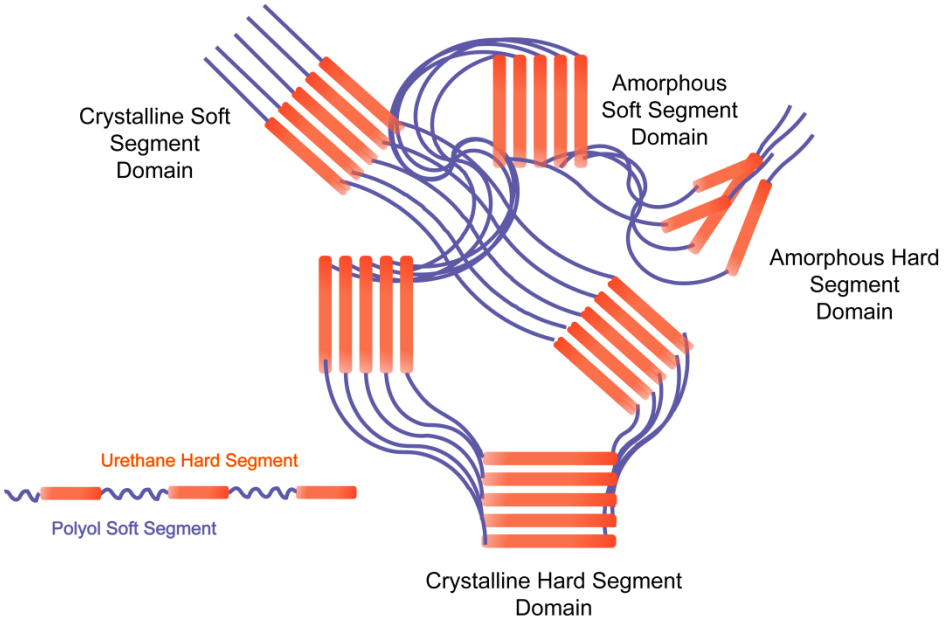

Figure 1.7. Polyurethane structure. The polyurethane is organized in two segments, the hard segment (HS) formed by the urethane group and the SS composed by the polyol. These two groups can be structured in crystalline and amorphous conformations by hydrogen bonding.

WBPU have high strength and flexibility related with the hard and soft segment. They have different applications as elastomers [47], coatings, adhesives [48, 49], polymeric dyes [50] and even in biomedical applications due to its biocompatibility [51], like tissue regeneration [52] or wound dressing [53]. Production of WBPU can be made with bio-based raw materials [54], polyols from vegetable oils can be used, like castor oil [55] or soybean oil-based macrodiol [56]. Due to these properties, this material is perfect to be filled with nanocellulose to improve its physicochemical performance [44, 57-60].

Nanocellulose has been called the new graphene due to the high interest that has generated and its possible implementation in advances materials. Graphene is a two dimensional carbon base material with a thickness of one atom, where the carbon atoms are organized in a honeycomb network by sp^2 hybridization [61, 62]. Graphene poses perfect properties for manufacturing electronic materials: large specific surface, good thermal conductivity, high charge mobility (10000 cm^{-2} s^{-1}) and high strength and stiffness (1 TPa of Young´s Modulus) [63]. The extraordinary properties from both materials give the opportunity of manufacture new hybrids nanomaterials by their combination. A very interesting application is in flexible conductive papers that work as film transistors, energy storage and organic solar cells devices [64, 65]. This material has been built over polyethylene terephthalate (PET) [66], polycarbonate (PC) [67] or polyimide [68]. The problem of using plastic polymers are the high processing temperatures, low coefficient of thermal expansion (CTE) and they aren't renewable [69]. There are reports of films formed by graphene and cellulose [70, 71] or mixtures of

CNF and graphene oxide with conductive properties [72-75], but further investigation in the implementation of both materials are needed.

Regarding to nanocellulose isolation, different methods has been used: normally mechanical, chemical and, enzymatic treatments or a combination of two of them. The mechanical treatment consists of a high-pressure homogenization process that permits the CNF obtaining. Several mechanical processes have been used like refiners [76], cryo-crushing [77] or grinders [78]. This method needs numerous repeating steps and to reduce the energy and time consumption, chemicals like 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) [79] or enzymatic treatments [80] are usually used.

Table 1.1. Examples of length and diameter of CNC produced with different treatments from several sources.

Source	**Length (nm)**	**Diameter (nm)**	**Treatment**	**Ref.**
Bacterial	100 - 1000	10 - 50	Sulfuric acid	[81]
Microcrystalline cellulose	~500	10	Sulfuric acid	[82, 83]
Cotton	100 - 210	10 - 40	Sulfuric acid	[84]
Sisal	100 - 500	3 - 5	Sulfuric acid	[85]
Tunicate	100 - >1000	15 - 30	Different treatments	[86]
Wood	100 - 300	3 - 5	Sulfuric acid	[87]
Valonia	1000 - 2000	10 - 20	Sulfuric acid	[88]

The chemical treatment is the main process to manufacture CNCs and CNFs. The most common reactive used is sulfuric acid (H_2SO_4) that can swell the cellulose amorphous regions keeping the crystalline part. Depending on the time and concentration of the sulfuric acid solution, sulfate groups can be attached to the nanocrystal surface by esterification (conversion of -OH groups into $-OSO_3^-$) (Figure 1.8). The usual concentration of sulfate groups can vary between 0,5 to 2% [89] and helps to stabilize the nanocrystals in water suspension but has other physicochemical consequences. The sulfuric acid treatment is optimized by using 64 wt% acid solutions at 40-50 ºC for 45-60 min. The reaction is stopped by mixing the suspension with 10 fold water, centrifuged and dialyzed against water until neutral pH is reached. To achieve a good CNC dispersion sonication steps are needed during the process [90]. Other typical compounds for chemical treatment are hydrobromic acid [91], phosphoric acid [92] or TEMPO oxidation [93].

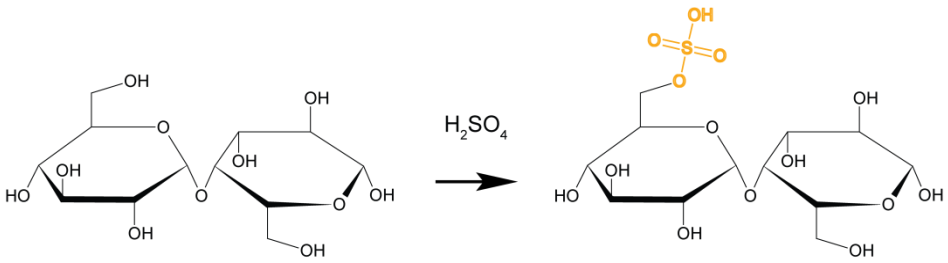

Figure 1.8. Esterification reaction in cellulose by sulfuric acid hydrolysis. Sulfuric acid treatment produces a transformation from hydroxyl groups on the nanocrystals to sulfate groups and charges negatively the surface of the particles.

The sulfuric acid treatment has some disadvantages: produces high amounts of toxic chemical residues that have a strong environmental impact. The high hydrophilicity of sulfate groups in nanocellulose made the drying process very expensive and time-consuming, and also a high volume of wastewater is produced during the washing process for neutralizing the pH after the hydrolysis [94].

We need new sustainable and efficient methods for nanocellulose isolation. The enzymatic treatment seems the best alternative because eliminates the toxic chemicals and requires less energy than mechanical production. There are some reports of nanocellulose isolation using enzymes in the literature in recent years. The first investigations used the combination of enzymatic and mechanical treatments to improve the cellulose microfibrillation [95]. Other reports from Filson at al. [96] explored the nanocellulose preparation using fungal endoglucanases and microwave heating treatments to Softwood Kraft pulp. These studies were followed by others where commercial endoglucanase were used in combinational protocols for nanocellulose production from different lignocellulose substrates like Bleached eucalyptus fibers [97, 98], old corrugated container fibers [99], Bleached Softwood Kraft pulp [100], Bleached Hardwood Kraft pulp [101], cotton [102], citrus waste [103], bacterial nanocellulose [104], microcrystalline cellulose [105] or sugarcane [106]. In 2017, Yarbrough et al. reported the use of the total exoproteome of the fungi *Trichoderma reesei* and the hyperthermophile bacteria *Caldicellulosiruptor bescii* on Bleached Kraft pulp achieving nanocellulose isolation.

To optimize the enzymatic treatment, we need to develop new enzymes with higher catalytic and promiscuous activity, and they need to work in different conditions. The more common protein engineering techniques for protein improvement are Rational Design and Directed Evolution. Rational Design [107, 108] consist on the mutagenesis of specific amino acids in the protein sequences. To achieve success with this technique it will require proteins with well-known structure and a studied mechanism that usually is not available. With Directed Evolution [109, 110] techniques changes can be produced in the amino acid sequences in an aleatory way. In this case, it's needed to produce and test a large library of mutants to obtain a protein with the features of interest, making this process very time and cost consuming.

A new approach in protein engineering is ancestral sequence reconstruction (ASR), normally used for evolution studies [111]. This technique permits us to bring back to life proteins from millions of years ago that were adapted to work in a totally different environment and had different properties in comparison with the nowadays enzymes. There are several reports in the bibliography that showed how the ancestral enzymes have more specific activities, are more promiscuous [112] and thermostable [113, 114] than extant enzymes. This extraordinary characteristic makes these ancestral enzymes suitable for biotechnology applications [115].

This technique consists of several bioinformatical and biomolecular steps. First, DNA or amino acids sequences from proteins of extant organisms are gathered from online databases, these sequences are then

analyzed by bioinformatics tools to calculate a phylogenetic tree that represents the evolutionary relationship between the proteins selected. By further informatics analysis, the sequence from the ancestors of each node of the tree can be inferred and the sequences are synthesized. By biomolecular tools we can produce these proteins in the laboratory. In this way, we can obtain several ancestral proteins, depending on the size of the tree, with different characteristic depending on the million years that they have and the node that we selected. This made this technique more efficient than the alternatives that need to produce a larger amount of proteins to achieve a desired one.

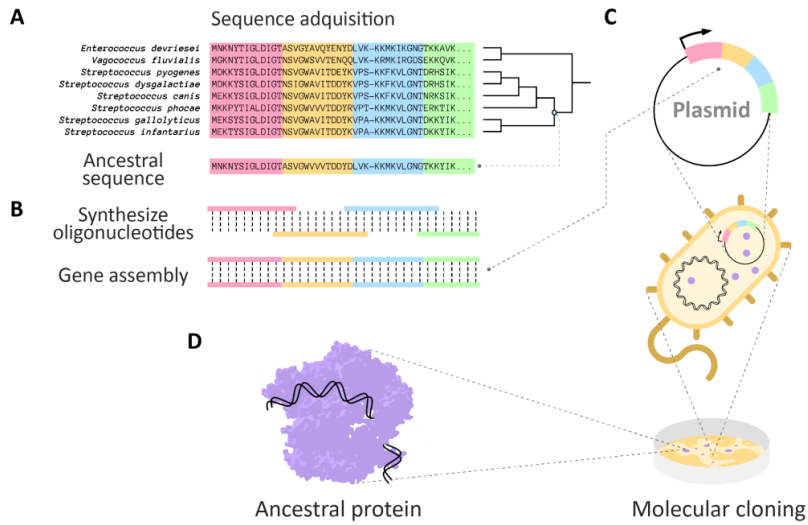

Figure 1.9. Ancestral sequence reconstruction. (A) Amino acids sequences from extant organisms are aligned and a phylogenetic tree is inferred with bioinformatical tool, (B) Sequences from the ancestral proteins are calculated from the nodes of the tree. (C) Expression plasmid with the gen from the ancestral protein and the bacterial host for protein expression. (D) Ancestral proteins expressed from the bacterial host.

Cellulases are enzymes produced by several organisms, the most common are bacteria and fungi, that degrade the cellulose polymer by breaking the β-1,4-glycosidic bonds. The total degradation of cellulose into glucose monomers needs three cellulases (Figure 1.10): Endoglucanase (EG) that degrades the cellulose chains in random locations to produce oligomers with reducing ends; preferably attack the amorphous regions of the fibers. Exoglucanase (CBH) that breaks down the crystalline cellulose and degrade the previous reducing oligomers into cellobiose units, working synergistically with EGs [116] and β-glucosidase (BG) that hydrolyzes cellobiose into glucose monomers [117].

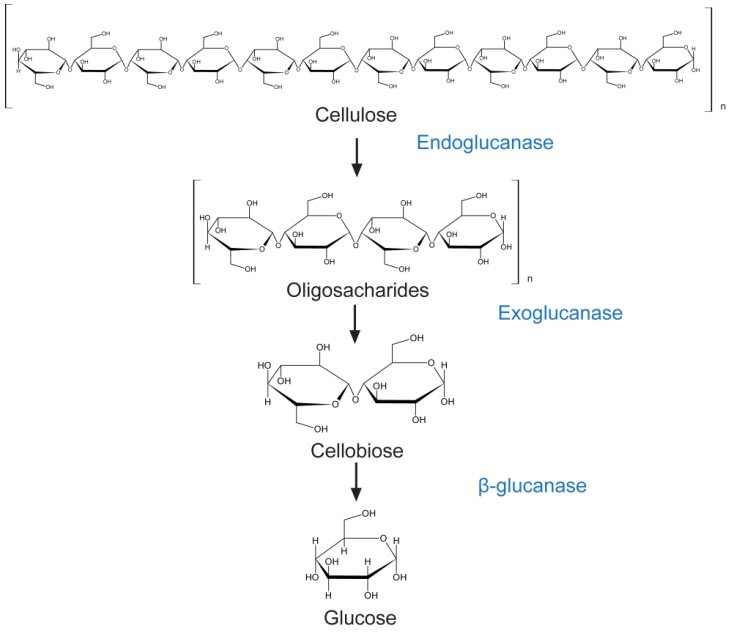

Figure 1.10. Enzymatic cellulose degradation by cellulases. Endoglucanase (EG) breaks down the cellulose polymer into oligosaccharides breaking the β-1,4-glucosidic bonds randomly. Exoglucanase (CBH) hydrolyzes oligosaccharides with reducing ends produced by EG into cellobiose units and β-glucosidase (BG) breaks the cellobiose unit into the glucose monomers.

Cellulases have different structures: they can have a single catalytic domain or be bound by a flexible linker to a Carbohydrate-Binding Module (CBM). CBMs help the attachment of the catalytic domain into the cellulose surface, improving its catalytic activity [118, 119]. The CBM also help the activity on insoluble and recalcitrant substrates as crystalline cellulose [120]. In nature, exists cellulases structured in a complex system called cellulosome that is produced by some anaerobic bacteria [121]. The structure is composed of non-catalytic proteins known as dockerins and cohesins that formed a protein scaffold [122], attached to this structure are several enzymes with different catalytic activities. This assemble has higher activity in an insoluble substrate than free enzymes [123]. There are several reports of designer chimeric cellulosome in the lab to improve the catalytic activity [124], but this big structure is hard to produce.

Cellulases are not the only enzymes that can attack the cellulose polymer; recently the discovery of the lytic polysaccharides monooxygenases (LPMO) has attracted the attention of the lignocellulosic research community. LPMOs are produced by fungi and bacteria [125], even is found in some virus [126]. LPMOs are copper-enzymes that produce oxidative cleave on the glycosidic bonds, first were studied their activity in crystalline chitin [127] and cellulose [128], but they showed activity on other polymers as hemicellulose [129] or starch [130]. LPMO oxidation is proposed to produce chain cleavage, the chain break is made on C_1 or C_4 carbon of glucose (Figure 1.11), even in C_6 in some cases [131]. C_1 oxidation produces soluble oligosaccharides with an aldonic acid in the reducing end and C_4 oxidation generates a

keltoaldose in the non-reducing end [128, 132]. There are LPMO that oxidate both C_1 and C_4 like the LPMO from *Streptomyces coelicol*or [133, 134] or oxidate selectively one of the C like the LPMO from *Myceliophthora thermophila* [135]. Since their discovery, several reports of using LPMO in combination with cellulases in biomass conversion has been published [136-138], the LPMO boost cellulases activity by making new cleavage in the chains helping the cellulases to attack the crystalline cellulose [139].

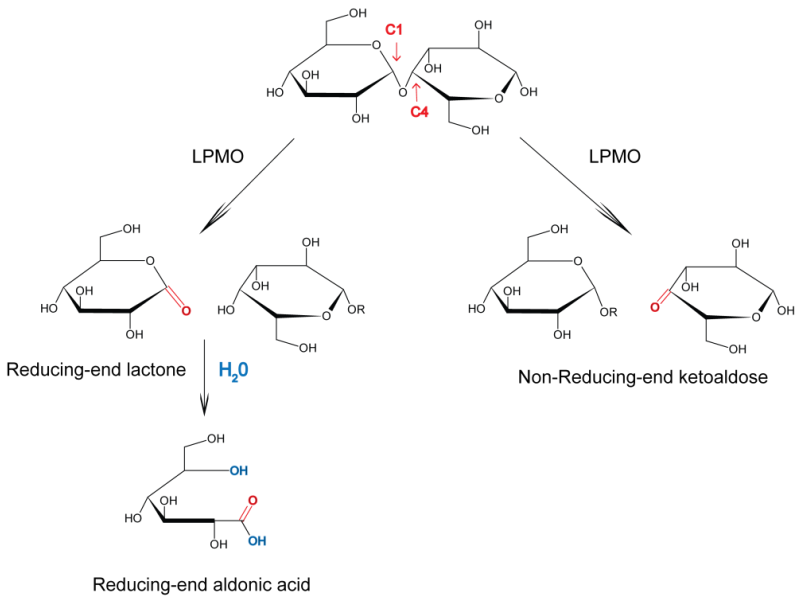

Figure 1.11. LPMO catalytic activity. LPMO can break the cellulose polymer by oxidation of C_1 or C_4 carbon in general. C_1 oxidation leads to soluble oligosaccharides with an aldonic acid in the reducing end and C_4 oxidation produces a ketoaldose in the non-reducing end.

Cellulose is not alone in the lignocellulosic biomass, there are two more main components: hemicellulose, a heteropolymer composes by different sugars [140], and lignin, aromatic polymers formed by phenylpropanoid

precursors [141]. Cellulose composes around the 45% of the biomass dry weight, hemicellulose is the second most abundant with 25-30% and lignin is present around 20-10%. The three components are in different concentration depending on the source and the plant age [142]. For the proper cellulose isolation it is necessary to liberate it from the lignocellulosic matrix.

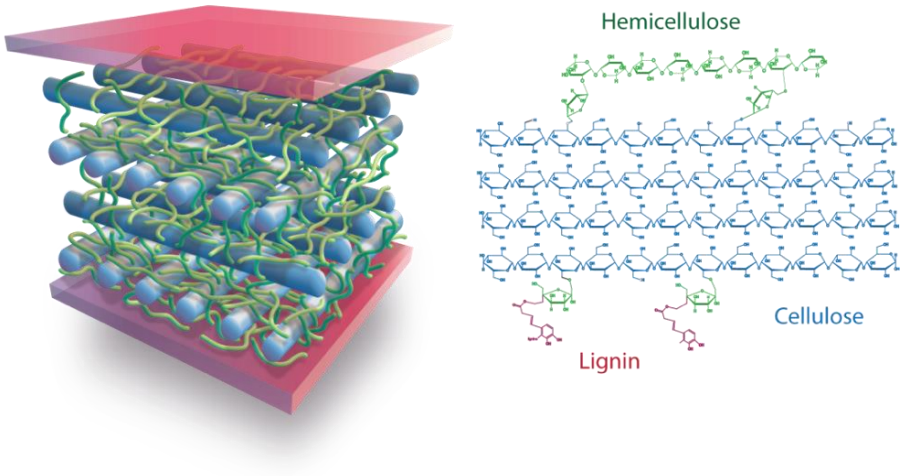

Figure 1.12. Lignocellulosic biomass. Cellulose fibers are located between a matrix of hemicellulose and lignin. Hemicellulose is composed by monosaccharides of 5 or 6 sugars binding together by β-glycosidic bond and lignin is form by phenylpropanoid precursors. Hemicellulose is bonded to cellulose and lignin by hydrogen and covalent bonding producing a very recalcitrant and stiff structure. This structure needs to be degraded in order to liberate cellulose fibers for further cellulose conversion into nanocellulose or sugars.

Hemicellulose structure consists of different carbohydrate polymers; the main polymer is xylan or glucomannan but has other sugars like xyloses,

arabinoses, glucose, galactose, mannose, and sugar acids. Hemicellulose is easier to degrade than cellulose due to the lower molecular weight and the short lateral branches that form the polymer [143]. Hemicellulose is linked to cellulose by hydrogen bonding and covalently to lignin, giving stiffness to the lignocellulose matrix [144, 145]. The hemicellulose can be extracted from biomass with sulfuric acid [146], alkaline pretreatment [147], steam explosion [148], mechanical treatments [149] but also can be removed by enzymatic hydrolysis. The most used enzyme is the Endo-1,4-β-xylanase that degrades the xylan polymer into oligosaccharides by breaking the β-glycosidic bonds between the monomers. Xylanase is produced by bacteria or fungi [150] and has been used in biomass degradation in process like paper pulp bleaching [151, 152].

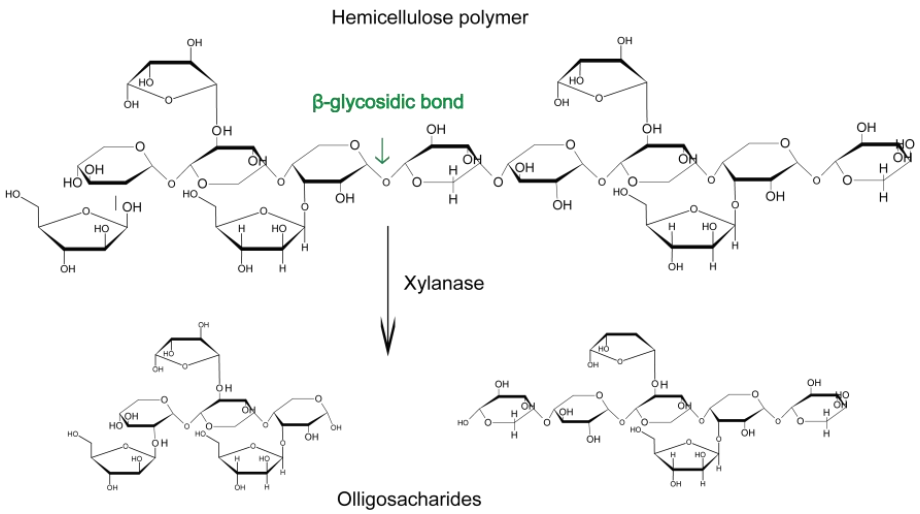

Figure 1.13. Xylanase catalytic reaction. Xylanase is able to break down the β-glycosidic bond between the xylan polymers and produces small oligosaccharides as product.

Lignin is a physical barrier that protects cellulose fibers due to its structural complexity, high molecular weight, and insolubility. The linkage between lignin, cellulose, and hemicellulose are believed that inhibit the enzyme activity [153]. Chemical and physicochemical treatment can be used to disrupt lignin structure [154, 155]. There are two main families of enzymes that can depolymerize lignin too: peroxidases and laccase, produced mainly by the lignolytic white-rot fungi [156] and higher plant, but also found in bacteria [157].

The principal objective of this thesis is to prove that ancestral reconstructed enzymes can have a potential industrial application in nanocellulose production as an alternative to the actual methods. At first, we focused in the nanocellulose isolation by ancestral endoglucanase (LFCA or ANC EG) that could improve the process where the extant enzyme fails due to its capability of degrading several substrates and its higher activity in different conditions than extant endoglucanases (Figure 1.14) from *Bacillus subtilis* or the extermophyllic *Thermotoga maritima* [158]. During the thesis, we also characterized with different physicochemical analysis the nanocellulose obtained by enzymatic treatment and found that they had different properties in comparison with commercial nanocellulose isolated with sulfuric acid treatment.

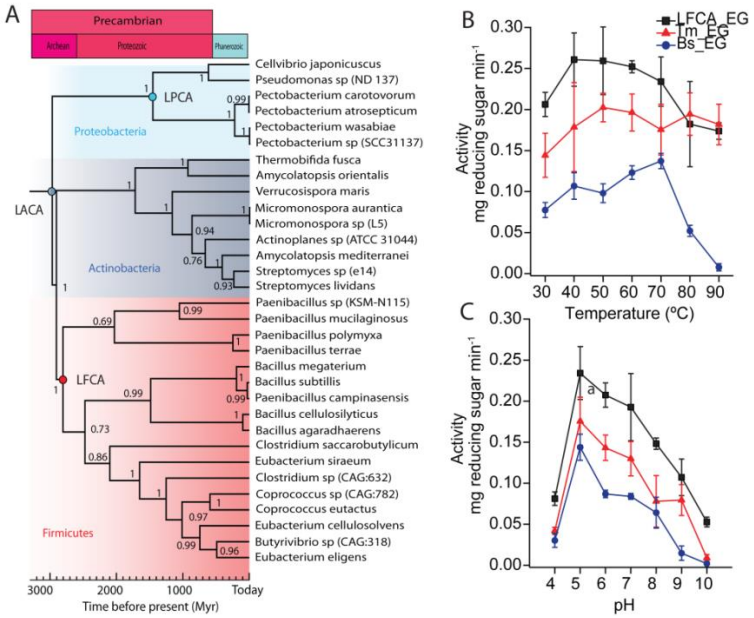

Figure 1.14. Ancestral endoglucanase reconstruction. (A) In previous work in the Nanobiomechanics group (CIC nanoGune), the ancestral reconstruction of bacterial endoglucanase was performed and the node of the last firmicute common ancestor (LFCA) of the tree was characterized. We observed that the ancestral EG has higher activity in a broad range of temperature (B) and pH (C) than extant EG from B. subtilis or the T. maritima.

Once we optimized the process, the second objective that we had was to produce nanocellulose from lignocellulosic biomass. For that purpose we used an ancestral enzymatic cocktail with the addition of xylanase and LPMO in the treatment, these enzymes were reconstructed and characterized in a parallel work in the Nanobiomechanics group (CIC nanoGune). We found during these studies that we can modify nanocellulose by oxidation with an ancestral LPMO in a similar way that chemicals do.

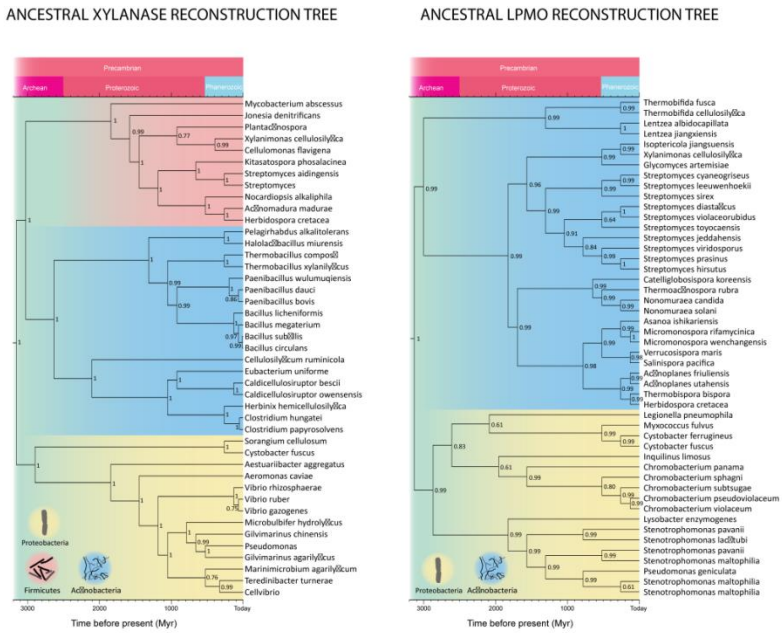

Figure 1.15. Ancestral xylanase and LPMO reconstruction. In a parallel thesis in the Nanobiomechanics group (CIC nanoGUNE); we reconstructed these two enzymes for an ancestral enzymatic cocktail production to achieve nanocellulose isolation from lignocellulosic substrates.

We studied two applications for nanocellulose isolated by enzymatic treatment (EnCNC), and also compared its performances respect to AcCNC. We found that our enzymatic nanocellulose used as WBPU filler have better mechanical and thermal performance than commercial nanocellulose produced by sulfuric acid. Also, we found that our nanocellulose can form films with nanopaper-like structure. This particular characteristic permitted us to made nanopapers with conductive properties mixing our nanocellulose with graphene, these nanopapers showed good thermal, mechanical and conductive properties. For conductive nanopapers manufacturing we even propose a new

strategy by using nanocellulose films as a substrate for Chemical Vapour Deposition of graphene, in order to substitute the plastic or metal substrates.

Objectives

The main objectives on this thesis were the optimization of a new method for nanocellulose isolation using ancestral enzymes from different sources, its characterization and applications. This work was carried out in three parts:

1. This part of the thesis involved the nanocellulose isolation by ancestral endoglucanase:
    - Development and optimization of a new method for nanocellulose isolation by ancestral endoglucanase from filter paper.
    - The study of the size and morphology of the nanocellulose produced at different hydrolysis time.
    - The physicochemical and morphological characterization of this enzymatically produced nanocellulose and its comparison with a commercial sample of cellulose nanocrystals isolated by sulfuric acid hydrolysis.
2. The second part was focused on nanocellulose isolation from two lignocellulosic substrates with the optimized method.
    - The addition of new ancestral enzymes as xylanase and lytic polysaccharide monooxygenase (LPMO) to help the ancestral endoglucanase activity.
    - The study of the effect of the complex substrates on the nanocellulose yield.
    - The physicochemical and morphological characterization of this new nanocellulose.

3. In the final part we studied two applications for the enzymatic nanocellulose and its comparison with the commercial sample in the same conditions.
    - The effect of nanocellulose as reinforcement in polymeric nanocomposites.
    - Preparation and final properties of nanocomposites based on polyurethanes and different content of nanocellulose isolated in this thesis by ancestral enzymes and commercial nanocellulose isolated by sulfuric acid treatment.
    - Produce conductive nanopapers by different strategies for graphene addition to enzymatic nanocellulose films.

This multidisciplinary work has been possible thanks to the collaboration of two research groups. The ancestral sequence reconstruction of the enzymes used in this thesis and the nanocellulose isolation was carried out in the Nanobiomechanics group from CIC nanoGune, specialist in ancestral protein reconstruction and its characterization. The characterization of the nanocellulose produced with the different enzymatic treatments and the materials fabricated in this thesis were carried out in the Materials + Technologies Group (GMT) of the Chemical and Environmental Engineering Department of the Basque Country University (UPV/EHU). This research group has the experience in nanocellulose characterization and its implementation in several applications. This work was funded by the Elkartek project from the Basque Country Government.

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List of Abbreviations

AcCNC: Nanocrystals prepared by sulfuric acid treatment

AFM: Atomic force microscopy

ANC EG: Ancestral Endoglucanase

ANC EG+CBM: Ancestral endoglucanase with Carbohydrate Binding Module attached

ANC LPMO: Ancestral lytic polysaccharide monooxygenase

ANC XLN: Ancestral xylanase

B. subtilis: Bacillus subtilis

BC: Bacterial cellulose

BG: β-glucosidase

BglS: *Bacillus subtilis* Endoglucanase

BKP: Bleached Kraft pulp

CBH: Exoglucanase

CBM: Carbohydrate-Binding Module

CI%: Crystallinity index

CNC: Cellulose nanocrystals

CNF: Cellulose nanofibers

CP/MAS ^{13}C NMR: Cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance

CTE: Coefficient of thermal expansion

CVD: Chemical vapour deposition

DBTDL: Dibutyltin dilaurate

DMA: Dynamic mechanical analysis

DMPA: 2,2-bis (hydroxymethyl) propionic acid

DNA: Deoxyribonucleic acid

DNS: 3,5-dinitrosalicylic acid

DSC: Differential scanning calorimetry

DTG: Derivative of thermogravimetric curve

E. coli: Escherichia coli

EDA: Ethylene diamine

EG: Endoglucanase

EnCNC: Enzymatic produced nanocrystals

FTIR: Fourier-transform infrared spectroscopy

GO: graphene oxide

HS: Hard segment

IPDI: Isophorone diisocyanate

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IR s-SNOM: Scattering-type scanning near-field optical microscopy

IR: infrared

KAPPA: Unbleached Kraft pulp

LPMO: Lytic polysaccharide monooxygenases

MW: Molecular weight

Nano-FTIR: Nanoscale-resolved Fourier transform infrared

OD: Optical density

PC: Polycarbonate

PET: Polyethylene terephthalate

PLA: Polylactic acid

PMMA: Poly (methyl methacrylate)

PVA: Polyvinyl alcohol

rGO: Reduced graphene oxide

S. viridosporus: Streptomyces viridosporus

SEM: Scanning electron microscopy

SS: Soft segment

T. maritima: Thermotoga maritima

TEA: Triethylamine

TEMPO: 2,2,6,6-Tetramethylpiperidine-1-oxyl

TGA: Thermogravimetric analysis

WBPU: Waterborne polyurethane

XLN: Xylanase

XRD: X-ray diffraction

List of Symbols

E′: Stage modulus

I_{200}: Intensity of crystalline peak

I_{am}: Intensity of amorphous peak

L/D: Length/diameter

q: Specific resistance

Rs: Sheet resistance

t: Thickness

Tanδ: Tangent of phase angle

Td: Maximum degradation temperature

Tg_{SS}: Glass transition temperature from the soft segment

T_{HS}: Hard segment short range order transition

Tm_{SS}: Melting temperature from the soft segment

To: Temperature of the loss of 5% of the weight of the total sample

β: Full-width at half maximum of the (200) peak

ΔH_{HS}: Enthalpy of hard segment short range order transition

θ: Angle of the (200) plane

θc: Contac angle

κ: Constant value from XRD

λ: Wavelength of the incident X-ray

τ: Crystallite size

ω: Weight of AcCNC in conductometry titratrion.