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Enhancing the value of lignocellulosic
biomasses through the production of
bionanocomposites

Experimental degree thesis

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Abstract

The need of biodegradable plastics is nowadays a crucial topic to switch from a linear and old economic model to a circular one, as the fight against climate change is now at its maximum. On the other hand, agricultural wastes are constantly increasing, and various herbaceous biomass can be grown with negligible impact to the environment to act as a cellulose source. This cellulose can after be processed into nanocellulose. Nanocellulose (NC) can be utilized in many applications in distinctive areas (bionanocomposites, electronic devices, biomedical applications, etc). Yet, extracting cellulose, in a sustainable way, from herbaceous crops, and to produce NC from it, still represent a challenge. Therefore, the aim of this work was to optimize a methodology to extract cellulose and to produce NC, from different lignocellulosic biomasses (sorghum, *Sorghum bicolor* (L.) Moench and sunn hemp, *Crotalaria juncea* L.). These two biomasses were chosen for the negligible agronomical inputs required, and, furthermore, for the ecosystem services provided, e.g. the restoration of marginal and degraded land and the improvement of local biodiversity. In addition, the NC produced was tested as a reinforcing agent in chitosan (Ch) films, to understand its effects on the properties of this biopolymer.

The nanocellulose was obtained via alkaline pretreatments with NaOH, followed by a bleaching and acid hydrolysis. In the alkali pre-treatment process optimization, some parameters were tested, namely time of reaction and temperature, and taking into consideration a scale up of the process, the time of 3h and temperature of 60°C were chosen as optimum. Comparing the two biomasses, NC extracted from sunn hemp was easier to process mechanically and also presented a better nanocellulose/micro cellulose ratio. From sorghum, two different NC were prepared, from inner and outer layer, as the original stems were divided in these two parts which have been processed separately after chopping and grinding.

The nanoparticles obtained from sorghum and sunn hemp were incorporated in Ch films at a rate of 2.5% w/w of chitosan, and the resultant bionanocomposites (Sorghum NC films and sunn hemp NC films) were fully characterized in terms of their morphology, mechanical and optical properties, permeability (water vapor), water wettability, and FT-IR spectra analysis. Chitosan films reinforced with commercial nanocellulose at the same rate were tested for comparison, as well as pristine chitosan (control).

Bionanocomposites made from sorghum and sunn hemp NC were slightly more saturated and opaque than the pristine chitosan films, in particular outer sorghum NC films. Sunn hemp NC films also showed a slightly higher thickness than sorghum NC films and pristine chitosan films. Further, the results confirmed that sorghum NC, improved the strength and stiffness of the chitosan biopolymer and that sunn hemp NC improved the plasticity of the chitosan polymer. Hence, results indicate that those lignocellulosic crops may afford a source of NC for the production of bionanocomposites. Considering the application of those bionanocomposites by the food packaging industry, sorghum NC - chitosan films showed more promising results than sunn hemp NC – chitosan films.

Key Words: Nanocellulose, Sunn Hemp, Sorghum, Biocomposites

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1.

Introduction

The need for more ecological and biodegradable materials, and of renewable origin represents nowadays a key focus of the scientific community to fight the massive use of non-renewable and non-biodegradable resources, that are contributing to a high level of pollution. Lignocellulosic crops are rich in cellulose, hemicellulose, and lignin, representing, therefore, an important feedstock to multiple value-added products, able to replace the fossil feedstocks. Nanocellulose (NC) is a suitable example that has applications in many different areas, ranging from automotive manufacturing, medicine, food industry, and extending to the energetic field. In the food industry, the change from traditional fossil fuel-based plastics to biodegradable polymer materials present some challenges, namely the poor mechanical, thermal, and barrier properties, that restrict the opening of markets to biopolymers. The insertion of homogeneously nanoparticles, like nanocellulose, as a reinforcement agent, into the biopolymer matrix is seen as a promising possibility to surpass these shortcomings, generating bio nanocomposites. Therefore, the aim of the work was to optimize a methodology to extract cellulose and to produce NC, from different lignocellulosic biomasses (sorghum, *Sorghum bicolor* (L.) Moench and sunn hemp, *Crotalaria juncea* L.) and to evaluate its application as a reinforcing agent in chitosan biofilms.

An improved methodology was foreseen in this work to optimize the production of nanocellulose via alkaline pretreatments with NaOH, followed by a bleaching and acid hydrolysis, namely by optimizing certain parameters, such as temperature and reaction time. To evaluate the application of the different NC produced, bionanocomposites of

chitosan (with NC at 2.5% w/w over the mass of chitosan) and glycerol as a plasticizer agent were prepared and analysed. The biobased plastics were analyzed with FT-IR, absorbance at 600nm, solubility, swelling degree, color L* a* b*, thickness and mechanical properties (elastic modulus (EM), tensile strength (TS) and percentage of elongation at break (EAB)).

The first part of the thesis is a bibliographic research, aimed at deepening the knowledge about our society's needs to fulfill certain requirements in the next few years, nanocellulose state of art and current applications and methods utilized for its extraction and an overview about herbaceous lignocellulosic feedstocks, especially sorghum and sunn hemp.

The second part of the thesis is the experimental part, performed from January 2022 to July 2022 at FCT Nova in Lisbon, Portugal, in the CubicB Lab headed by Professor Ana Luisa Fernando, and with her supervision, and with co-workers João Pires and Leandro Gomes. This second part of the thesis addresses the experiments made, and the presentation and discussion of the results obtained.

1.1

The European Green Deal and How Biomass can tackle Climate Change

During the last years the world is facing several challenges regarding the worrying and inexorable Climate Change which is negatively impacting the earth with dramatic consequences. Governments from all over the world are now asked to limit the main Climate Change causes by limiting every kind of emissions, switch from a traditional economic linear path to a circular and sustainable one without depleting earth resources, improving the social and cultural wellbeing of the world by introducing several new laws and rules.

In 2019, the European Commission decided to create a set of policy initiatives to reach a carbon neutrality before 2050, the European Green Deal (EGD). It was a great initiative as for the first time an international institution recognizes the Climate Change as a problem and decided to work actively to limit it as soon as possible. Furthermore, the green deal aims to reduce regional and social inequalities in Europe. In addition, it outlines investments needed and financing tools available to fulfill its goals (European Commission, 2019).

What are the main causes which pushed Europe for the plan's creation? Just some of these are an enormous loss of biodiversity, ozone depletion, water pollution, urban stress, and waste production. Several worrying statistics highlighted the climate related issues in Europe, for example carbon dioxide levels are predicted to double by 2030 if nothing changes. Again, Europe is responsible for one third of world gas emissions (European Commission, 2019).

Other essential goals related with the European Green Deal initiative are: A circular economy plan, a review and possible revision of all relevant climate-related policy instruments, a Farm to Fork strategy, a revision of the Energy Taxation Directive, a sustainable and smart mobility strategy and an EU forest strategy that aims to support afforestation and forest preservation and restoration. In the framework of the European Green Deal topic, the following targets should be also achieved: reducing by 50% the use of pesticides by 2030, the use of fertilizers by 20% and to reduce nutrient loss by at least 50% (Keating et al 2020). Those targets are aligned with the implementation of dedicated biomass/industrial crops systems, as most of them require low inputs (i.e. pesticides, fertilizer) and furthermore they are able to recycle nutrients and to substitute fossil-based products (Abreu et al., 2022).

All these objectives lead to the necessity to implement a new economic model and new social approach. Therefore, implementing a Circular Economy Action Plan is highly relevant. Renaming wastes as by-products and reusing them in a processing unit, is certainly a contribution to reduce pollution (gaseous, liquid and solid) and to reduce the amount of resources needed (either renewable and non-renewable). At European level, the emphasis is on the production of more sustainable products, with a focus on resource-intensive sectors such as textiles, construction, electronics, and plastics. In this respect, the chemical industry has a huge role to play in the climate action as chemical products are essential for many low-carbon technologies.

The introduction of biomass in the resource-intensive industrial sectors, is definitely a contribution to the European Green Deal and to the targets aimed. Biomass is a renewable feedstock and when substituting non-renewable feedstocks represents a way to improve the sustainability of the value chains. Biomass applications include, among many, bio composite materials, pulp and paper, biomedical devices, cosmetic and textile materials, bioenergy and biofuels. For this reason a well stabilized biomass plan would positively impact a vast range of industries in Europe. Lignocellulosic biomass can meet the needs of many of the mentioned biobased industries and products. Lignocellulosic biomass is plentiful and renewable but a sub-utilized bio-resource. It is highly available and with the correct technology, this biomass provides many opportunities for bio-refineries, converting this resource into biomaterials and bioproducts (including

bioenergy), and contributing to reduce the use of fossil resources (Lee et al., 2014). Residues from agricultural crops (straw, bark, shells, leaves, bagasse); forest (hardwood and softwood); lignocellulosic crops; certain food and other industries wastes, are lignocellulosic resources that can be used. Interest in lignocellulosic crops has increased widely due to their diversity, abundance, high productivity, fast growing, contribution to reduce CO₂ emissions, low cost and potential to be grown productively on low quality soils (Gomes et al., 2022). Indeed, lignocellulosic biomass grown on non-arable, degraded or marginal lands present the benefit of reducing the competition of these crops with food crops, which represent an opportunity, not only economically and environmentally but also socially, ethically, and culturally (Fernando et al., 2018). That is why lignocellulosic biomass is a truly important actor in the climate change action, and its several applications can definitely help Europe to reach its target until 2050.

However, the biomass processing, needs to be well studied and well established and still, few problems have to be fixed in order to scale-up in the market. Issues regarding the biomass feedstock availability, transportation distances which need to be accurately defined, local topography, infrastructures, in addition to the processing of lignocellulosic material, is a challenge. Moreover, the processing of this type of biomass is also a defiance, as lignocellulose recalcitrance to chemical deconstruction is high, and there is a need to develop greener and more sustainable techniques to process this type of biomass (e.g. ionic liquids, deep eutectic solvents, enzyme cocktails, innovative mechanical and thermochemical processes) (Souza et al., 2020; Pires et al., 2019), in order to have positive outcomes in ecological and climate terms.

Therefore it is mandatory to ensure that biomass is a good feedstock for the biobased industries (e.g. for the production of nanocellulose), replacing fossil-resources and by consequence, leading to a reduction of carbon emissions.

It could be useful in order to go deeper in the biomass topic to perform a SWOT (Strengths, Weaknesses, Opportunities, Threats) analysis with a focus on nanocellulose production, once it is the aim of this work.

Starting from the strengths, one of the major advantages of biomass, and in particular lignocellulosic biomass, is its availability worldwide. In addition, there are several herbaceous biomass crops which can be grown in marginal lands where food crops

cannot be economically exploited. And furthermore, those herbaceous crops water and fertilizer requirements are in most cases lower than any other crop resulting on a reduction of water resources needs and also in lower nitrogen emissions (derived from the application of the N-fertilizers) and GHG (greenhouse gases) emissions, due to fossil fuel use to produce N-fertilizers (Fernando et al., 2015). The major strength for the use of biomass for nanocellulose production is the major and drastic decrease on CO₂ emissions due to the replacement of fossil-based resources as raw feedstock. And, as underlined in next chapters, the implementation of those herbaceous biomasses in marginal and degraded soils is also relevant through the ecosystem services provided, which includes the prospect of soil rehabilitation.

As weaknesses, the transportation costs and the issues regarding transportation distances as several biomasses could undergo biological degradation and its quality could be affected by several microbes which may cause biological damage. By consequence, the fiber quality could be degraded and the nanocellulose source could be highly damaged and not anymore suitable for this purpose, furthermore the cellulose yield could be highly affected. Again, the industrial switch from an old and obsolete system to a new one would require huge investments.

Opportunities are several. Nanocellulose has the potential to penetrate different markets as it will be shown in the next chapters, from biomedical applications to electrode material for supercapacitors, and perform a suitable alternative to non-renewable-based products. In addition, the use of nanocellulose in biobased industries, by substituting fossil based feedstocks presents also the benefit of contributing to produce a new product that is biodegradable, reducing the impacts of its release at the end of its lifetime (Souza and Fernando, 2016). Biomass use also decrease the CO₂ in the atmosphere trough photosynthesis, impacting also on the air quality.

Threats are linked with the implement of an industrial biomass system. The major threat is the supply chain. Indeed, along the entire value-chain, biomass and nanocellulose costs and quality can be affected and present high variability. Another important threat is the cheaper price of fossil feedstocks respect to biomass, and also the lower cost of the processes. For this reason the biobased industries, and in particular the nanocellulose production process, needs to be optimized to decrease the overall costs and threads and

enabling the production of an added value product that can be simultaneously, technically, economically, environmentally and socially feasible and attractive.

1.2 Nanocellulose - General Overview

1.2.1 Background and Chemical Structure

The constant depletion of non-renewable resources, such as natural gas, petroleum and coal (fig.1), is inevitably leading to high levels of pollution and earth resources exploitation.

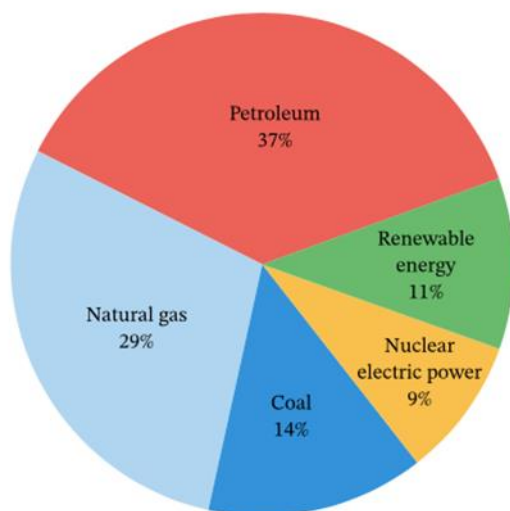


Figure 1 Renewable and non-renewable energy sources (Nagwa.com)

The world needs sustainable, renewable, and feasible alternatives and technologies to replace non-renewable materials consumption. Lignocellulosic biomass, rich in lignin, hemicellulose and cellulose, is one of the most promising renewable sources, and it can

hypothetically push to a switch from a linear traditional economy to a circular and sustainable one. The quantity of lignocellulosic material coming from industrial crops, food crops, agricultural and forestry activities is massive, and the amount of cellulose could be interesting in terms of yield. In fact, the three parts composing lignocellulosic material can be separated and isolated and cellulose could be fractionated again to produce a new and innovative bio-based material, nanocellulose (NC), that can respond to several nowadays challenges: it is abundant, renewable, costs aren't prohibitive and being a biomaterial, it is biodegradable. Nanocellulose is a natural material with at least one dimension in the nanoscale. And NC combine important cellulose properties with the features of nanomaterials, bringing new horizons for materials science and its applications. (Klemm et al., 2018)

From a chemical point of view the three parts consisting of the lignocellulosic material are structurally different. Cellulose is the most abundant biopolymer existing on earth and usually cover a variable content of lignocellulosic material which goes from 35% to 50% (Brandt et al., 2013). Chemically, cellulose $(C_6H_{10}O_5)_n$ is a polysaccharide made of linear chains of β (1 \rightarrow 4) D-Glucose units, which are aggregate via hydrogen bonds and Van der Waals forces, and unlike starch no branching is occurring in its structure (fig. 2).

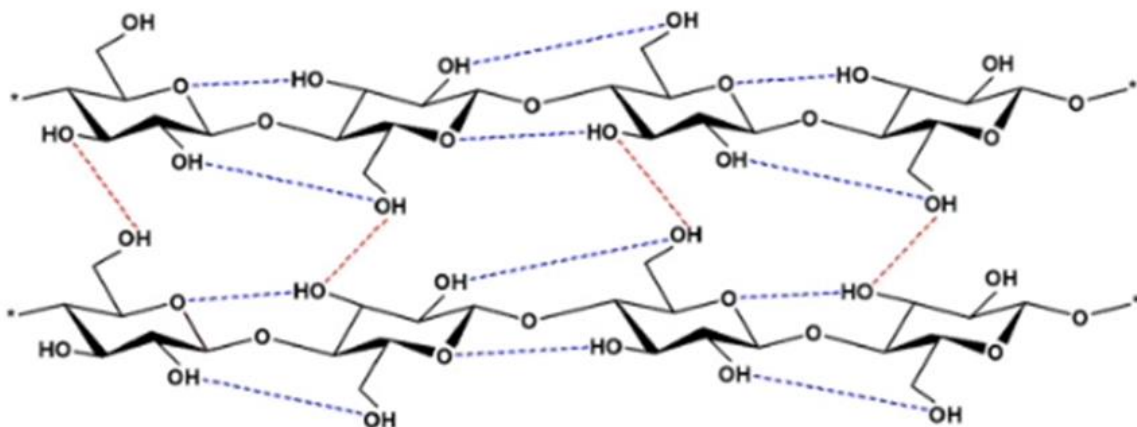


Figure 2 Cellulose structure (Phanthong et al, 2018)

While cellulose is crystalline, strong, and resistant to hydrolysis, on the other hand hemicelluloses have a random and amorphous structure resulting in less recalcitrant

molecules, being easy to remove them from the cellulosic materials in the lignocellulose fractionation to obtain first pure cellulose and then to produce nanocellulose as final product. Hemicelluloses are heterogeneous compounds composed of different sugars, pentoses and hexoses as xylose, arabinose, mannose, or galactose. They represent abundant carbohydrates and they consist of 25% to 35% of dry weight of wood material. Since hemicelluloses have side-branch groups their structure is non-crystalline and more accessible to be attacked by chemical reagents, such as acids or bases (Benaimeche et al., 2020). Hemicelluloses can react under mild alkaline conditions and are more easily degraded in an acid medium than cellulose (Chen et al., 2014).

Lignin is the most resistant fraction of the lignocellulosic material to deconstruction. And this characteristic is the biggest obstacle for the separation of highly crystalline cellulose from the lignocellulosic material. Lignin is an amorphous, aromatic and water insoluble polymer with a three-dimensional network composed mainly of phenylpropane units linked to each other by irregular coupling of C-C and C-O. Lignocellulose is then, a complex of cellulose, hemicelluloses and lignin, usually in the ratio of 4:3:3, and this complex is the main component of cell walls, in biomasses, and it gives structural properties to it (Chen et al., 2014). Extracting cellulose from this complex is a challenge and after the cellulose extracted can be processed to the nanometer dimension, NC.

Nanocellulose fibers usually contains less than 100nm in diameter and several micrometer length (fig. 3).



These nanofibers are biodegradable, with light weight, low density (1.6 g/cm^3) and they show impressive strength properties derived from cellulose ones (they can show a high tensile strength up to 10 GPa). These nano fibers are transparent, and the high amount of reactive hydroxyl groups in the surface, that can be functionalized, may be exploited to achieve different materials presenting different surfaces properties (Phanthong et al., 2018). Nanocellulose can be divided into three categories: cellulose nanofibrils (CNFs) - a mixture of amorphous and crystalline NC -, cellulose nanocrystals (CNCs) - crystalline NC -, and bacterial nanocellulose (BNC) – NC produced by bacteria. And the different types of NC reflect the different producing methods (Klemm et al., 2018).

1.2.2 Cellulose Extraction

The production of nanocellulose is a multiple step process. It starts with the extraction of cellulose, and for this, non-cellulosic components, as hemicelluloses and lignin, need to be removed. Then, once pure cellulose is obtained, nanocellulose is produced following different techniques.

Once the biomass is collected, as the first step we need a physical pretreatment. The biomass should be chopped, cut and grinded to obtain a fine powder. This process is essential to increase the surface area of the biomass and facilitate any following step to isolate cellulose from lignin and hemicelluloses. The increment of the accessible surface and pore size also decrease cellulose crystallinity, and this process has a disadvantage as it generally requires a high energy consumption (Pires et al., 2019).

The two classical methods for the removal of non-cellulosic materials are acidic and alkaline treatments, and those are typically applied on large scale processes. Acidic treatments are still considered the most effective and the cheapest ones, but they are not environmentally friendly, as the formation of toxic sub-products raises several environmental concerns. The acid treatment, also known as “delignification” or “bleaching” process is one of the most used by the pulp industries. In the process, most lignin and other undesired components are being removed of with a mixture of sodium chlorite and acetic acid at a high moderate temperatures (*ca.*70-80°C) for a certain

interval of time (4-12 hours). The alkaline treatment consists usually in the application of sodium hydroxide (4-20%) to remove hemicelluloses and lignin. After those processes, acidic or alkaline, almost the entire quantity of the final product is cellulose (Phantong et al., 2018) (fig. 4).



Figure 4 Cellulose powder(microcrystalline cellulose, biolla chemicals)

New cutting-edge and promising alternatives to acids exist already, but they still need to be developed and studied further. Moreover, as a handicap, some of these techniques require high investments and are way more expensive.

The use of ionic liquids, is a promising and green alternative to the classic methods. These ionic liquids are thermally stable organic salts typically composed of an asymmetric cation and a counterpart anion. The advantages are several: high polarity; great thermal stability; negligible volatility and non-flammability; low energy requirements, possibility to be recovered and reused (Zhao and Anderson, 2012), which is another central topic in the green chemistry and circular economy concepts. On the other hand, there are some disadvantages, for example the high cost of chemicals, or the need to upgrade the process to a commercial scale which is still a challenging task. Furthermore, the recovery of solubilized cellulose and hemicelluloses is a challenge to overcome.

Another technique that is gaining attention is steam explosion. The process relies on the explosive decomposition of biomass. Firstly, the action of the compressed steam compresses the biomass and, in a second time, the biomass is rapidly decompressed when the water steam is quickly evaporated. The main effect is that a physical breakdown of the lignin-carbohydrate complex is achieved. The hot steam will induce hydrolysis of the hemicellulose and degradation of the lignin, resulting in an increased accessibility to the cellulose. Compared to other mechanical procedures, steam explosion method demands a 70% lower energy consumption to get the same result, which is essential to reduce emissions derived from fossil fuel use in a chemical plant. For this reason, technologies like steam explosion are fundamental for the success of future bio-refineries. In addition, there is no need for chemical reagents, and therefore no costs associated to those, as well as no pollutant emissions related with the use of chemicals. As for the disadvantages, with the steam explosion, an excessive degradation of the physical and chemical properties of cellulose can be reached (Shrotri et al, 2017), there is a need for high pressure, and the destruction of lignin-carbohydrate complex can be incomplete.

Another promising method under consideration is the application of a biological pre-treatment. This method is based on the degradation of the lignocellulosic cell wall structure by hydrolytic enzymes and ligninolytic enzymes which are able to depolymerize lignin. This strategy could hypothetically enable us great advantages as it is carried out in mild conditions, so with low energy demand and without the release of toxic compounds. (Sharma et al., 2017). However, this technique is a very slow process, which is a disadvantage, and for this and several other reasons this process is still not very effective at a commercial stage. One way to reduce the operation time is to combine biological pre-treatments with other pre-treatments.

1.2.3 Nanocellulose Production

Once pure cellulose is obtained, nanocellulose can be produced. For this, several techniques have been developed in last years, being the most used the following: acid hydrolysis, enzymatic hydrolysis, and mechanical process.

Acid hydrolysis (being sulfuric acid the most used acid), is the main technique used for the production of nanocellulose from cellulose. The main controlling factors which affect the properties of nanocellulose are reaction time, acid concentration and temperature which need to be constantly monitored to have an efficient outcome of the reaction. In the process, nanocrystalline cellulose are isolated and the nanocellulose can stay dispersed as a stable colloid system due to the esterification of hydroxyl groups by sulfate ions (Das et al, 2009).

The enzymatic hydrolysis is a biological treatment in which enzymes are used to digest or modify the cellulose structure. Generally, mild conditions are used for this process, but it needs a long operational time. To overpass this problem, this process is always integrated with other effective processes. Mechanically, NC can be obtained through the application of high shear force to cleave the cellulose fibers. Most used mechanical processes are ball milling methods, ultrasonication and pressure homogenization (Phantong et al., 2018).

1.2.4 Nanocellulose Sources and Possible Applications

Table 1 shows some biomass feedstocks that can be used for the production of NC, and the composition in cellulose, hemicellulose and lignin.

Biomasses and biomass residues	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Bagasse	44	23	20
Peanut Shell	22.1	12.1	35.2
Rice hull	49.1	9.6	12.9
Sugar Cane Leaf and Stalk	40	29	13
Corn Cobs	45	35	15
Switchgrass	45	31	12

Softwood Stems	45-50	25-35	25-35
Sorted Refuse	60	20	20
Newspaper	40-55	25-40	18-30

Table 1 Cellulose, hemicellulose and lignin content of different Biomasses and biomass residues (% dry weight) (Nayak and Mukherjee, 2015)

Nanocellulose feedstocks can be primary sources, from the production of cellulose fibers as main product (fibers for textile or paper, industrial crops for ethanol or wood for building). Some vegetal natural fibers can be sorted in six groups: bast fibers (hemp or Kenaf), leaf fibers as pineapple, core fibers (hemp, jute, kenaf), seed fibers (coir, cotton, or kapok) or grass and reed fibers (wheat, corn, and rice) and other types as wood or roots (Faruk et al., 2012). But, NC feedstocks can be also retrieved from secondary sources, which are non-processed by-products from a processing unit or residues from either forestry or agricultural activities. Tertiary sources are wastes from the use, transformation, or conversion of cellulosic biomass, such as pulp or bagasse (Garcia et al., 2016).

As for Varun et al. (2020), wastepaper is still not utilized but a promising source for nanocellulose. Wastepaper contains a very high amount of cellulose, from 60% to 70%, which is higher than any other raw materials. Such composition makes it a suitable material for the production of nanocellulose in the future. Recovered paper, refused paper, scrap paper, secondary paper, discarded paper, used paper and paper stock frequently represented the wastepaper. With increased production of paper, a huge amount of wastepaper is generated annually: around 400 tons worldwide (Del Rio et al, 2022). Wastepaper is also one of the most recyclable cellulosic sources and usually can be recycled 3-4 times or 6, 7 times theoretically (Zhang et al., 2015). As per European Standard (CEN EN 643) grade list compiled by Confederation of European Paper Industries (CEPI), there are major five grades of wastepaper. Grade 1 means low quality, unsorted and sorted paper and board. Grade 2 means medium quality such as newspapers with and without flexographic printing. Grade 3 is high quality, mixed lightly colored wood and wood-free printer's shavings. Grade 4 is kraft quality as new shavings from

corrugated board, unused and used corrugated kraft for example. Grade 5 is special quality, mixed recovered paper and board, mixed packaging, liquid board packaging or wrapper kraft (CEN EN 643 list of European standards types for waste paper). Among them, only wastepaper of Grade 5 can be recycled using specific processes or that can cause some specific constraints to recycling. Yet, as for NC preparation, limited studies are reported from nanocellulose isolation from wastepaper. Anyway, the recyclability has a cost as wastepaper reuse decreases the fiber strength due to negative changes on fiber morphology, chemical composition, and surface conditions. This issue could represent somehow a weakness when using this wastes as a source of nanocellulose and by consequence the extraction of nanocellulose from industrial crops like sunn hemp and sorghum can ensure a higher quality product.

Once obtained, nanocellulose can have a huge number of possible applications.

Nanocellulose is of interest for photonic applications, for example both CNC and CNF can form optically transparent films. Nanocellulose is also interesting for sensing and biosensing purposes: it is being tailored and applied in (bio)sensing technology, with the goal to display analytical information related to various fields like medical diagnostics, environmental monitoring, food safety, physical/mechanical sensing, labeling and bioimaging applications (Golmohammadi et al, 2017).

Deeper studies on nanocellulose will enable to discover new ways to manipulate it and new opportunities are raising with the possibility to modify its surface. The hydrophobization of cellulose surface can be achieved via esterification reactions and the resulting material can be used for food packaging applications (Hofmann and Reid, 1929). Applications and end uses are possible thanks to surface modification, the large specific surface area, and the presence of a large number of hydroxyl groups within the nanocellulose structure. All these characteristics make these nanofibers a suitable platform for surface modification in order to achieve a desired surface functionality (Habibi, 2014).

Other application to NC is as a derived conductive material: Conductive materials usually contain metal particles, carbon materials, and conductive polymers. Nanocellulose can be combined with these conductive materials to produce conductive

composites with high mechanical strength, stiffness, foldability, and flexibility (Dias et al 2020). The fabrication of nanocellulose-based conductive composite has been achieved mostly by surface grafting and blending of conductive species and nanocellulose (Dias et al, 2020). Indeed, nanocellulose can be combined with conductive polymers or with conductive carbon materials. Carbon materials such as those made of graphene and carbon nanotube (CNT) have received wide attention due to their high surface area, excellent electrical conductivity, and ultralight weight. The combination of nanocellulose and carbon nanoparticles can improve the mechanical and conductive properties of the composite (Dias et al., 2020).

Besides these applications, nanocellulose has also been tested in various scientific areas and industries such as paper and food packaging , flexible electronics, energy harvesting, liquid crystals, biomedicine and cosmetics, catalysis, adsorption, separation, decontamination, filtration or even fire retardants (Pires et al., 2019).

1.2.5 Nanocellulose Impact on Climate Change

Besides the interest in nanocellulose, its production also raises the question: could nanocellulose applications impact positively the climate change by tackling it and helping Europe to reach the targets of the European Green Deal?

In the last 50 years 90% of the world's energy demands have been covered mainly by fossil fuels and 1.5×10^{10} Mg of CO₂ emissions have been released per year to the earth's atmosphere (Pires et al., 2019). Considering that lignocellulosic annual wastes are 1.3×10^{10} (Souza and Fernando, 2016), the reuse of those wastes to substitute fossil based products, will certainly impact positively on the CO₂ emissions reduction.

An evolution in science and technology is required to combat the environmental problems that affect our planet nowadays. The development of new sustainable materials and products is an important alternative to the traditional petroleum-based materials. Interest in herbaceous biomass is increasing and composes a significant potential to meet the future biobased industries and replace fossil based-resources with a valid and valuable alternative. The valorization of lignocellulosic biomass is then a sustainable approach (Pires et al, 2019).

To be effective for the climate action, the overall process must be not only feasible at an environmental level, but also it must be feasible socially and economically: the economics of making CNF materials is primarily dependent on the type of fiber pre-treatment. Similar to CNF, the cost of production of CNCs depends on the separation route, and currently the largest scale production uses the sulfuric acid hydrolysis process which is a low-cost solution (Klemm et al, 2018).

In the end, it is crystal-clear that nanocellulose could help reaching its target, considering also that the world population is expected to exceed 9 billion by 2050 that will most likely drive to an increase of demand of biomass and biobased products on the market. This exponential growth with its increasing consumerism trends and unprecedented levels of environmental impact is happening while the supply of natural resources is dramatically declining. This is asking us for deep changes in our lifestyle and a search for innovation by the industrial sector, looking for processes that are more efficient, effective, sustainable, and environmentally benign (Ramos et al, 2018). Fascinating biopolymers and sustainable raw materials have been widely studied to react to a global need for low cost and renewable material as a feedstock for next generation materials. The reinforcing effect of nanocellulose has been frequently utilized in less sophisticated products and materials for food applications such as packaging. The energy consumed during nanocellulose fabrication may cause it to be commercially not feasible in its utilization for certain applications. In addition, current studies reveal a trend toward renewable materials as starting materials for CDs (carbon dots), CNT (carbon nanotubes), and graphene production. This is particularly important to reduce the carbon footprint without affecting hybrid nanocomposite properties (Dias et al, 2020).

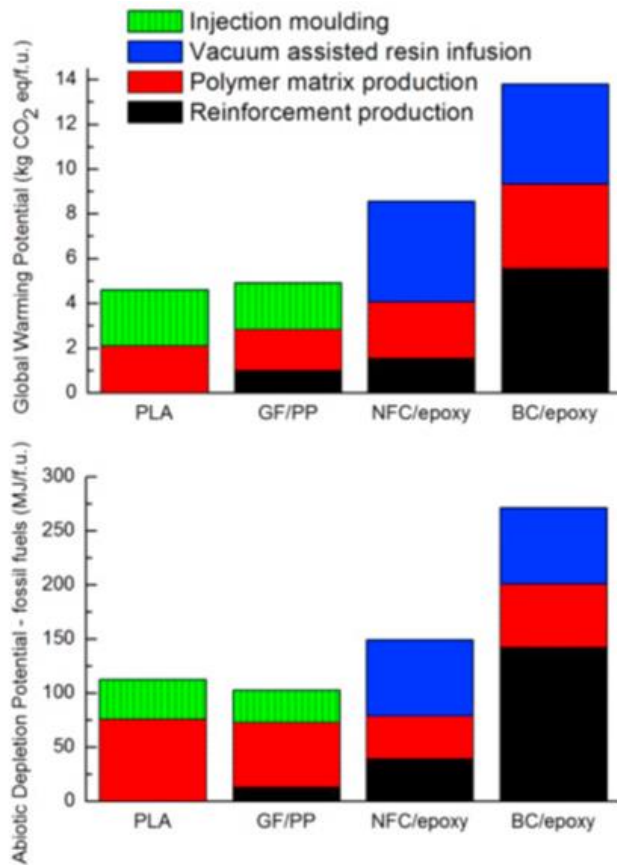


Figure 5 LCA comparison - Life cycle assessment of nanocellulose-reinforced advanced fibre composites (Hervy et al., 2015)

To be more accurate, an LCA (Life cycle assessment) (fig.5) approach could be taken in consideration, considering the environmental impacts of nanocellulose-reinforced polymers compared to other commercially available alternatives as PLA (Polylactic acid) and PP (Polypropylene).

In the study of Hervy et al., (2015), several impact categories have been considered, among them the Global Warming Potential GWP and the Abiotic Depletion Potential Adf. The results (fig.5) showed that if we are considering only a Cradle to Gate approach, nanocellulose-reinforced polymers are showing a much higher impact in both categories: the manufacturing of nanocellulose, which is through vacuum assisted resin infusion technique, seems to be the most impacting factor. On the other hand, if we consider the overall Cradle to Grave approach, we realize that PLA use phase is going to be the most impacting one and nanocellulose reinforced polymers are showing, in this category, better results. As it can be seen from this study, playing on a more sustainable technique for the extraction of nanocellulose is the most important factor to reduce the nanocellulose-reinforced polymer impacts. And, of course, the nanocellulose percentage

present in the polymer matrix is playing an important role (Henvy et al, 2015). So, optimization of NC production is a must, when considering the evolution of the biobased industries, and the utilization of NC as a feedstock.

Another interesting study compared different nanocellulose extracting/producing techniques: ENZHO (enzymatic pre-treatment followed by homogenization), TOSO (oxidative pre-treatment followed by sonification) and TOHO (oxidative pre-treatment followed by homogenization) for both virgin fibers and recycled materials. The study showed that ENZHO had extremely lower impacts compared to the other methods and, furthermore, showed a very little or negligible difference between virgin fibers and recycled material (Gallo Stampino et al 2021)(fig. 6). Indeed the enzymatic pre-treatment followed by a mechanical one appears to be the most effective method. But, still, search for enzymatic cocktails that can help achieving the scale up of processes to industry is a need when considering the exploitation of NC.

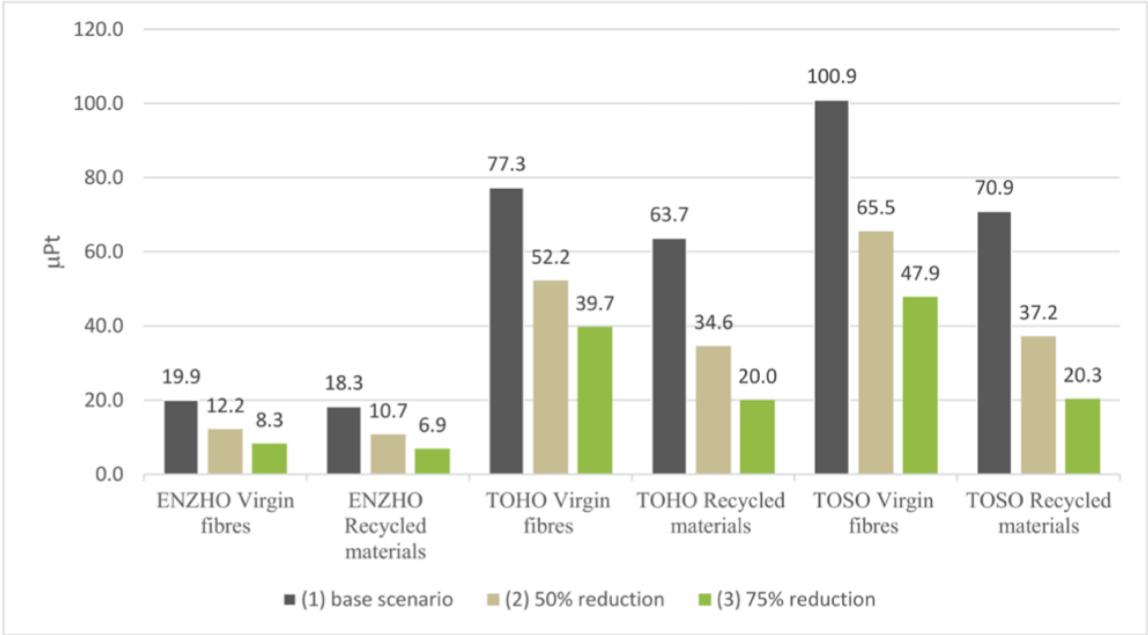


Figure 6 Impact scores of different pre-treatments comparison to produce nanocellulose (Gallo Stampino et al, 2021). The total energy consumption linked with NC was reduced by 50% (scenario 2) and 75% (scenario 3) with respect to the base scenario.

The previous studies showed that, a continuous improvement in the production phase of nanocellulose could drastically decrease its impact and finally, compared with PLA, it will show lower scores in several impact categories. For this reason the focus of this

thesis aimed to optimize the process parameters, so that an actual positive impact on the final product could be achieved, relevant for next process improvements.

1.3 Lignocellulosic Biomasses An overview of Sorghum and Sunn Hemp as a source of nanocellulose

1.3.1 Introduction to Biomass

As has been said previously, the extraction of cellulose and the production of nanocellulose from herbaceous biomass will be investigated during this research.

Biomass has been defined as “the biodegradable fraction of products, waste and residues from agricultures, including plant and animal substances from land and sea, as well as the biodegradable fraction of industrial and household waste” (EU 2009 Directive). anyway, generically and in ecologic terms, biomass refers to any type of organic matter as for example wood, agricultural residues, or energy crops. Moreover, a focus on agricultural wastes and its re-use in biorefineries is also an important issue, as the global agricultural production system generates a huge amount of waste every year and an improper agri-waste management could cause environmental pollution, economic losses, and human-related problems (Sourisse, 2020).

Synthetic fibers in composites harm the environment raising the concern of exploring sustainable and natural materials. And in this field, biomass can play a decisive role to help Europe meet the targets preconized in the European Green Deal. Biomass can be a feedstock for different processes and end uses and can potentially replace fossil feedstocks in different areas, and in particular in the polymer area. For what concerns the bio-based films production, nanocellulose from biomass show outstanding features. Lignocellulosic material is renewable and recyclable, easily accessible worldwide and by consequence it is versatile for an extensive range of end products and for replacing fossil based plastics (Sugiarto et al, 2022). In addition, the plant mass on a global scale, although unevenly distributed, is extremely abundant. Another essential data to take in consideration is the CO₂ released in case of biomass products at their end of life which

is considered to be near a net 0 value, considering the amount of CO₂ captured from the atmosphere during the photosynthesis.

Once nanocellulose from biomass is obtained it can replace fossil based products in a vast range of areas which goes from nanofillers, food packaging applications to form edible food packaging material, energy applications as nanocellulose can be incorporated in electrodes materials as a spacer between graphene layers, and many others (Raghuwanshi et al 2022).

According to Schetter et al (2022) herbaceous crops are considered ideal for multiple reasons, starting from the yield potential (high yielding crops) to the high tolerance that these crops present when facing environmental stresses, such as low fertility and degraded soils, high salinity soils and drought conditions. In fact those crops show promising yields even under adverse conditions. Sunn hemp (fig. 7) and sorghum (fig. 8) are two herbaceous crops that have shown promising yields under different environmental stresses (Zegada-Lizarazu et al., 2022), and therefore, they were chosen as a biomass feedstock to conduct this study. The better knowledge on possible uses linked with these herbaceous crops will help the biorefinery market. Indeed, the use of biomass as alternative in fossil-based-processes does present advantages but it also present some disadvantages. Most of those are connected with the economic viability of the value chains, and with the risks of market shortages or imbalances, once the biomass supply need to be constant throughout the value chain.

1.3.2 Sorghum and Sunn Hemp

Fig 7 and 8 represents respectively Sorghum and Sunn Hemp, the biomass taken in consideration in the thesis.

Sorghum (fig.7) or *Sorghum bicolor* L. Moench has been primarily known as a forage crop or as a cereal but is nowadays increasing in attention as it is suitable for other purposes also thanks to its high cellulose concentration (Zegada Lizarazu et al., 2022).



Figure 7 Sorghum (PI@ntnet)

Sunn Hemp (fig.8) or *Crotalaria Juncea* L. belongs to the Fabaceae family, and it is considered to be the oldest fibre-yielding crop native to India (Bhatt et al, 2014) and according to Zegada Lizarazu et al. (2022) this crop shows negligible requirement for what concerns the agronomical management, an advantage shared with Sorghum. This is also due to the fact that Sunn Hemp is a N₂ fixing legume crop and doesn't require nitrogen fertilization. Traditionally it was used as a cover crop or fibre feedstock.



Figure 8 Sunn Hemp Crops (Alabama A&M & Auburn Universities Extension)

Both species shows high dry matter yields. According to Zegada Lizarazu et al. 2022, the average yield for sorghum is 22 Mg/ha while the average yield for sunn hemp is 14 Mg/ha.

Not only sunn hemp has some peculiarities regarding nitrogen use efficiency, but biomass sorghum is also known for its high nitrogen use efficiency at low nitrogen fertilization. In fact sorghum requires no or negligible fertilization (Maw et al., 2020). The low input needs of both crops show the benefits of these biomasses in the framework of a biorefinery. Moreover, the low input needs of nitrogen from both crops indicates that lower N emissions will come up. Nitrogen application is associated with acidification, greenhouse effect, ozone depletion, and ground and surface waters eutrophication (Fernando et al., 2010). Therefore, the lower the N input, the lower the risks associated with it.

Overall, these biomasses (sorghum and sunn hemp) are similar in terms of agronomical requirements, and it is a valuable fact that they require no or negligible fertilization which is an agronomical practice to keep under control in terms of reduction of environmental impacts. The production costs of sorghum seems to be cheaper and less impacting overall, comparing the euro per ton of dried matter harvested, 50.5 for sorghum and 65.8 for sunn hemp (Zegada Lizarazu et al. 2022).

Table 2 presents the composition of sunn hemp and sorghum. As shown in table 2, both sunn hemp and sorghum contain a low lignin content and high content in sugar polymers as cellulose which has great interest to be used as a lignocellulosic feedstock for the production of bioplastics.

In general terms sunn hemp shows higher content of cellulose, and lower content of hemicelluloses than sorghum. Lignin content is variable among both biomasses and references. According to references in table 2, sorghum and sunn hemp showed a composition of 30-38% and 35-41% of cellulose, respectively (table 2). In any case, both in sorghum and sunn hemp the lignin content is lower. This feature ensures that the recalcitrance to cellulose extraction of both biomasses can be overcome.

Table 2 Sunn Hemp and Sorghum typical composition

	Zegada Lizarazu et al. 2022		Kamireddi et al. 2013		Zegada-Lizarazu et al. 2021		Dong et al 2019; Srikavi and Mekala 2023	
	Sorghum *	Sunn Hemp *	Sorghum *	Sunn Hemp *	Sorghum *	Sunn Hemp *	Sorghum *	Sunn Hemp **
Ash %	4.5	5.6	3.3-4.2	5.2	4.9	5.1	-	-
Cellulose %	31	41	34	37	30	35	38	66
Hemicellulose %	27	14	21-23	21	26	14	28	19
Lignin %	6	8	14-16	14	5	8	21	15
N %	0.8	1.7	-	-	0.6	1.8	-	-
C%	47	47	-	-	-	-	-	-

*- results from biomass harvested; ** -results from extracted free biomasses (biomasses that were washed with water and ethanol to remove nitrites/nitrates, proteins, chlorophyll, and waxes)

Considering sunn hemp and sorghum, these two crops show a promising feedstock for the production of NC. Indeed, these two crops present interesting and low environmental impact features, when looking into the cultivation and harvesting phase, and they are rich in cellulose. In addition, scaling up the cultivation of these two crops is feasible in marginal soils due to their tolerance to this stress, which reduces the food versus food ethical issues, allowing an increased availability of biomass for NC production. These two crops are important as they're giving an ecosystem service too to restore marginal and degraded land and improve the air quality; also, sunn hemp, is a nitrogen fixing plant which help fixing N₂ from the atmosphere, and reducing the costs and the environmental burden associated with the application of N fertilizers. Yet, a major concern for the sorghum and sunn hemp supply chain is the seasonality of harvest. Stocks must be managed to last during non-harvest periods, which has yet to be fully overcome. This is important for a long term production as an appropriate logistic system should be installed preventing the degradation of the biomass and the quality of the cellulose.

2.

Objectives

The objective of this thesis is to optimize the production of nanocellulose from two lignocellulosic biomass crops (sorghum and sunn hemp) and to characterize the potential of those nanocelluloses obtained as reinforcement of biobased polymers.

The target of this study is to understand if nanocellulose reinforcement is actually positively impacting the bio-based polymer matrix by improving its mechanical characteristics among others. To this, the nanocellulose obtained from sunn hemp and sorghum will be used to reinforce chitosan and the characteristics of the biobased films will be evaluated and analysed. Ultimately, the aim is to identify if the reinforced films can be a promising alternative to fossil-based polymers.

In addition, the work aimed at optimizing the process of nanocellulose production from sunn hemp and sorghum by playing on critical parameters as temperature and reaction time, to reduce, on an industrial level the hypothetical costs of nanocellulose production.

3.

Materials and Methods

3.1 Materials and reagents

Sorghum and sunn hemp biomass were obtained from the harvest of pilot fields of these crops established in the Cadriano Experimental Farm of Bologna University, Bologna, Italy. The biomasses were collected in October 2021. The collected material was dried at 40°C in a vacuum oven, cut in small pieces and stored in darkness, in a dry place at room temperature until use. Sorghum biomass was divided into outer layer and inner layer for analysis and nanocellulose production. Outer layer was considered to be the epidermis of the stems while the inner layer the parenchyma and sclerenchyma cells. The separation between the outer and inner layer was performed as it was expected to have a higher yield of cellulose per g of dried biomass in the inner part.

Chitosan was used to produce the films, where NC will be tested as a reinforcement agent. Chitosan (Ch), is industrially produced in various quality grades from chitin, the second most abundant polysaccharide in nature. Chitin and chitosan are natural antimicrobial compounds against an extensive variety of microorganisms including bacteria, yeasts and moulds (Vu and Hollingsworth, 2011). In fact, biobased polymers with antimicrobial activity are of interest as edible films due to their ability for controlling foodborne microbial growth, especially in minimally processed fresh products (Yemenicioğlu et al, 2007). Chitosan is a non-toxic and biodegradable compound and has excellent performance in forming films. Its films have successfully been used as packaging materials for the preservation of food quality (Fernandes et al., 2009), which motivated us to select this carbohydrate polymer as a matrix context for preparing desirable edible films. Commercial high molecular weight (31–37 kDa) chitosan (poly(D-glucosamine)) with 75% of deacetylation, was purchased from Sigma Aldrich (Germany).

In the study, commercial NC was also used for comparison of its properties with sunn hemp NC and sorghum NC. Commercial Nanocellulose was kindly supplied by Nanocrystacell (Slovenia).

Ethanol, and sodium hydroxide (NaOH) were purchased from Sigma Aldrich (Germany). Glycerol, glacial acetic acid, calcium nitrate ($\text{Ca}(\text{NO}_3)_2$), sodium bromide (NaBr), potassium acetate (CH_3COOK , 99% purity), sulphuric acid 95-97% (H_2SO_4), and hydrogen peroxide (H_2O_2) were purchased from Alfa Aesar (Germany). All chemicals were of analytical reagent grade and were used as purchased. The water was purified employing the Milli-Q system (Millipore, USA).

3.2 Biomass Characterization

Sorghum and sunn hemp were chemically characterized. The analysis was performed prior to the treatment made to extract the nanocellulose. The biomass was characterized in terms of ash content, fiber composition, and nitrogen content.

Fiber content was performed following the Van Soest methodology (Soest, 1963). In this method, hemicellulose, cellulose, and lignin were determined following a sequential extraction with neutral and acid detergents, a treatment with 72% sulfuric acid and, finally, an incineration at 550°C of the final residue. Ash content was determined following the adaptation of the ASTM E1755 - (Reapproved 2015). In this methodology, the ash content is the residue obtained after the incineration of the biomass carbonized at a temperature of 575 ± 25 °C. Determination of total nitrogen followed the Kjeldahl methodology described by Watts and Hallywell (1996). In the method, the samples are mineralized using H_2SO_4 , at 360°C, with a catalyzer, followed by distillation of the digested sample, and its titration with HCl 0.02N.

3.3 Optimization of Cellulose extraction and Nanocellulose production

The transformation of the raw material into cellulose and then its transformation into nanocellulose was performed in two steps. The first, passes through the pre-treatment of the raw material to obtain cellulosic fibers. In the second step the fibers are converted into nanosized cellulosic materials (Pires et al., 2019). The methodology implemented for NC isolation was accomplished by incorporating and adapting procedures reported

for different biomass sources (Collazo-Bigliardi et al., 2018; Ferreira et al., 2018; Xu et al., 2018). The process follows the application of alkali pre-treatment, bleaching, and acid hydrolysis.

The supplied raw material, in our case Sunn Hemp (*Crotalaria Juncea* L.) and Sorghum (*Sorghum bicolor* L.) has followed the same route of cellulose extraction and nanocellulose production, changing some parameters as temperature and reaction time in order to attest their impacts on the overall extraction yield.

3.3.1 Cellulose Extraction and Nanocellulose production from Sorghum

Starting from Sorghum, the biomass has been chopped and weighted to obtain 200 g of biomass, and was after sequentially cleaned with hot water and dried in the oven at 60°C for one night. The dried biomass has been grinded (fig.9) and sifted to obtain a fine powder until a 30 g sample was obtained.



Figure 9 Grindind machine

Before performing the pre-treatments, a washing was performed utilizing a solution with water and ethanol 50/50 heated at 80 °C degrees for 2 hours. The solution was filtered and dried as indicated previously in the oven at 60°C for one night. The resulting dried biomass in this step has been cleaned up from all the impurities.

Thereafter, every pre-treatment was performed to remove lignin and hemicellulose, and to obtain pure cellulose. In the optimization of the process NaOH was performed in this step. So, dried biomass, after the ethanol washing, was treated with NaOH 7% at 70 °C for approximately 3 hours, the biomass was then filtered, washed with water, and dried in the oven at 70°C degrees for one night. In order to optimize the methodology, the same procedure with NaOH 7% was tested at 60°C for the same time (3 hours). After recovering the biomass, it was understood that no significant differences among 60-70°C were perceived and it was decided to use only 60°C as an optimized procedure for the extraction of cellulose.

According to different properties and action principals of pulp bleaching agents, the bleaching can be divided into two types: one is oxidation bleaching; the other is reduction bleaching. Oxidation bleaching agent can further remove the residue of lignin and other impurities as well as coloured substances, which can not only improve the whiteness and purity of the pulp but also keep the whiteness being durable.

Bleaching was performed using 100ml of 7% (w/v) NaOH and 100ml of 24% (v/v) H₂O₂ for outer layer, 150ml of 7% (w/v) NaOH and 150ml of 24% (v/v) H₂O₂ for inner layer. the solution with biomass and NaOH was pre-heated in the range of 60-70 °C, and after that, H₂O₂ was slowly dropped in the solution. After the hydrogen peroxide has been totally dropped, the reaction time needed was approximately 3-4 hours. No significant differences were observed for the amount of sample obtained after 3 or 4 hours and subsequently it was decided that the reaction time could be only 3 hours. After the reaction time the sample was filtered, washed, and dried in the oven at 70 °C for one night.

After this process a second bleaching has been performed. In the bleaching process it was identified that there are inner layer biomass losses during the process. This lost is on the foam produced during the bleaching process. In fact, the foaming is one of the drawbacks of this operation, for this reason the hydrogen peroxide dropping needs to be

performed slowly. The second bleaching has been performed with the exact same amount of NaOH and H₂O₂ solutions. The temperature and the reaction time were the same of the previous bleaching. After the second bleaching, the samples were filtered, washed, and dried for the same time and temperature.

For practical reason this dried biomass has been grinded manually to obtain a fine powder to increase the surface exposition for the next step. At this point the sample is mostly cellulose (Pires et al., 2022). After the manual grinding, the samples have been weighted again: outer layer yield to initial biomass was 23% and inner layer yield to initial biomass was 31%, and the cellulose content is approximately 90-95%.

As last step, the cellulose needs to be broken down into nanocellulose, this is done utilizing sulphuric acid 64% (w/w) H₂SO₄. The sulphuric acid is pre-heated at 45 °C, and here the temperature must be maintained constant as much as possible to avoid sample destruction. As soon as the acid reach 45°C degrees, the samples were dropped manually and slowly into the stirred Erlenmeyer, and the reaction time was 45 minutes.

After the 45 minutes, the solution was quenched with 2 litres of freezing water to stop the reaction. After that, the biomass was stored in the fridge, and the nanocellulose started to accumulate in the bottom (fig 10). This sample was centrifuged, and dried. The yield of NC obtained after this process was 21% for sorghum outer layer and 27% for sorghum inner layer.

To disperse the NC in water, for subsequent use, different procedures were adopted. In a first attempt, 1 g of dried biomass was dispersed in water and the samples were homogenised with ultra turrax at 16 rpm x 1000 for 30 minutes, adding a mechanical method to process. After that, ultrasound bath at 50 Hz was applied during 15 minutes each, then the samples were filtrated with a 13 micrometres retention filter to be sure that the filtrated solution contained only micro and nanocellulose. This method didn't work as the entire sample was retained in the filter due to agglomeration of the nanocellulose. This was evaluated through a mass balance (amount of initial biomass was equal to the amount of biomass retained by the filter).

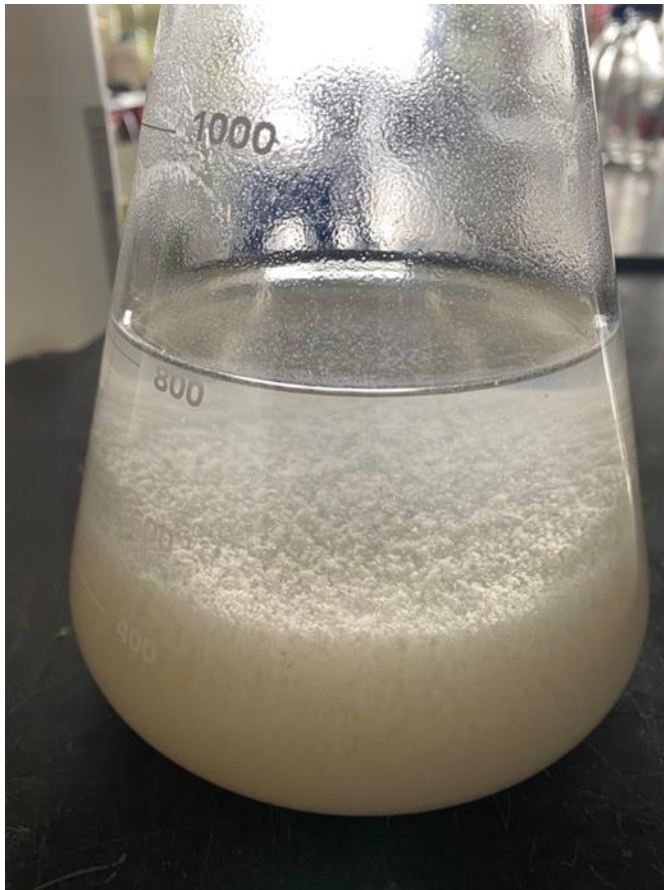


Figure 10 Nanocellulose in water

Another approach was then performed. The solution quenched was neutralized starting from a pH 2, utilizing NaOH 7% until pH 7-8. After, the solution was filtered, washed, and the biomass dried. After, the produced NC was mixed with 1L of water and a homogenization series was applied, 15 minutes each sample at 16 rpm x 1000 with ultraturrax. Ultrasounds were applied too twice per sample for 15 minutes (fig. 11). Then the solution was filtered. It was possible to identify that the amount of nanoparticles that crossed the filter were *ca.* 10%. This was observed through a mass balance, the amount of biomass retained by the filter was *ca.* 90% of the initial dispersed. At this point the amount of particles that crossed the filter and that are dispersed in the water are in the range of micro and nano meters, although still no turbidity in the solution was observed (fig 12).



Figure 11 Homogenization of the nanocellulose in the water solution



Figure 12 Nanocellulose dispersed in water solution

Aiming at reaching an improved yield of NC dispersed in the water solution, the same procedure was repeated and some parameters on the process were changed. A harsher ultrasound treatment was performed, at the same power but for longer time, around 30 minutes for two times with intervals where the samples were homogenized with ultra turrax. Results showed no major improvement in the process. The process was changed again and then homogenization and ultrasound were applied as previously but, in this

case, the homogenization was carried out at 16.4 rpm x 1000. This new procedure did not improve much the yields. In a tentative of optimization of the process, only homogenization was applied, at 16 rpm x 1000 and still no major improvements in yields were obtained. Therefore it was decided to keep the treatment of 15 minutes ultrasound and 15 minutes ultra turrax in 2 series. The process was done and repeated with the dried NC biomass, until 0.35 g NC in 1L solution was obtained. An amount of more than 0.30g per 1L of solution was a target for the production of the bionano-based films.

This improved procedure was applied to the outer layer of sorghum, to the bleached biomass, an acid hydrolysis treatment was applied and the solution neutralized, filtered and the biomass dried. After the biomass was dispersed in water and the treatment of 15 minutes ultrasound and 15 minutes ultra turrax (in two series) was applied. This treatment was quite effective (19% of NC was dispersed in the solution). A procedure applied again to a second gram of biomass to reach the right amount of NC dispersed in the solution, 0.32g in 1L of water.

3.3.2 Cellulose Extraction and nanocellulose production from Sunn Hemp

An optimization of the process to obtain cellulose and to produce nanocellulose from sunn hemp was also performed. Sunn Hemp was chopped and grinded to obtain *ca.* 30g sample (fig. 13). In fig. 13 the different grinded biomasses are presented, starting from the left: Sorghum inner layer, Sorghum outer layer and Sunn Hemp, and it is quite visible the color difference among the different samples. Sunn hemp biomass was cleaned with hot water, dried and after sequentially cleaned with a solution ethanol:water 25:75, for 2 hours at 70 °C (fig.14). Then the solution was filtrated and the biomass was dried. The first pre-treatment was performed with 7% NaOH at 60 °C degrees for 3 hours, then filtered, washed, and dried in the oven for the night.

After, the first bleaching was performed with 7% NaOH and H₂O₂, as it was done with sorghum, and 150ml of NaOH and 150ml of hydrogen peroxide were added to the biomass. The solution with biomass and NaOH was pre-heated at 70 °C, and after that, H₂O₂ 24% was slowly dropped in the solution. After the hydrogen peroxide has been totally dropped, the reaction time for the bleaching procedure endured 3 hours. After the

reaction time the sample was filtered, washed, and dried in the oven at 70 °C for one night (fig. 15).



Figure 13 Grinded biomasses (from the left: Sorghum inner layer, Sorghum outer layer and Sunn Hemp)



Figure14: Ethanol:water biomass washing

A second bleaching was performed even if the powder showed already with the first bleaching process a white colour meaning (signifying that pure cellulose was obtained). The yield obtained was 38% of bleached biomass (with more than 90% cellulose). Compared to sorghum inner and outer layer, at this point we have a higher yield of

bleached biomass cellulose for sunn hemp: 38% for sunn hemp, 23% for sorghum outer layer and 31% for sorghum inner layer.



Figure 13 Biomass drying in the oven

For the preparation of nanocellulose from the bleached biomass (almost pure cellulose), the following treatment was performed, as we did with sorghum. So, sulphuric acid 64% (w/w) H_2SO_4 was used. The sulphuric acid is pre-heated at 45 °C, and after the samples were dropped manually and slowly into the stirred Erlenmeyer, and the reaction time was 45 minutes.

After the 45 minutes, the solution was quenched with 2 litres of freezing water to stop the reaction. After that, the biomass was stored in the fridge overnight, and the nanocellulose started to accumulate in the bottom. The solution was neutralized to pH 7/8 with NaOH 7% and then filtrated, washed, and dried, and after manually grinded. The NC production from sunn hemp yielded 34%. The yield was higher compared with 21% for sorghum outer layer and 27% for sorghum inner layer.

The same procedure as for sorghum was then performed to disperse in water the NC produced: a gram of sample was added to 1L of water and then homogenized for 15 minutes and 15 of ultrasound treatment twice. The results showed better performance than sorghum: 19% of NC was effectively dispersed in the water solution. When the

second gram was added, after the same treatment, a total of 0.40g in 1L of water was effectively obtained.

Once solutions with a minimum of 0.30 g NC dispersed were used for the formulation of bio-based films with chitosan.

3.4 Nanobiobased films preparation

The films were prepared according to Souza et al., (2019). Film-forming dispersion (FFD) was prepared by dissolving 1.5% (w/v) of chitosan in 1% (v/v) of a glacial acetic acid solution under vigorous mechanical stirring for 24 h at room temperature. As mentioned before, chitosan was chosen as the biopolymer to be tested with the NC being produced from sunn hemp and sorghum, to test the ability of those NC's to reinforce biobased polymers. The chitosan is a linear polysaccharide that consists of glucosamine and N-acetyl- β -D- glucosamine units, obtained after deacetylation of chitin, which consist of β -(1-4)-2-amino D-glucose and β -(1-4)-2-acetamido-D-glucose linked through 1,4- β -glycosidic bonds (Younes and Rinaudo, 2015). It is a polycationic biopolymer with a unique structure, including amino groups (-NH₂) in its backbone, that grants it the high reactivity (Thakur and Voicu, 2016). Therefore, this biobased polymer has received great attention due to their physical, chemical, and antibacterial activity (Verlee et al., 2017). The use of antibacterial biofilms and coatings from food-grade biopolymers during processing can extend the shelf life of food and reduce health risks and economic losses of foodborne disease (Randazzo et al., 2018).

After, glycerol, as a plasticizer, was added at the percentage of 30% (w/w) of chitosan in all treatments. This amount of glycerol added (corresponding to only 0.45% w/v in the FFD) was chosen based on previous experiences with chitosan bionanocomposites (e.g. Souza et al., 2019), as it was found this amount is optimal for film formation, and to obtain improved barrier and mechanical properties.

To transform the bio-based films into biocomposites, the NC from sunn hemp, outer layer of sorghum, and inner layer of sorghum were tested and a rate of 2.5% w/w chitosan, and the nanoparticles were added to FFD (fig. 16). In this mixture we used the NC dispersed in solution. Thereafter, three agitation cycles of 5 min agitation with

ultraturrax (15.000 rpm) were pursued by 15 min degasification in an ultrasound bath (360 W) was performed. These three cycles were adopted to assure a suitable exfoliation and dispersion of the NC into the polymeric chain. The finished dispersion was then cast in glass molds (18 x 25 cm) and left to dry with the aid of a circulatory hot fan for approximately 24 h. When totally dried, the biocomposites were stored in a desiccator containing saturated calcium nitrate solution at 25°C and 50% relative humidity, monitored with a thermohygrometer, and protected from light until future evaluations. In addition, to study the reinforcing impact of mixing NC with chitosan (Ch), a pristine Ch film and a Ch film reinforced with commercial nanocellulose (at a rate of 2.5% also) were produced for comparison.



Figure 14 Biobased film formulation

The biobased films were produced in duplicate (fig17).



Figure 15 Dried Ch+NC biofilms

3.5 Nanobiobased films characterization

After optimizing the extraction and production of cellulose, and after producing the biobased nanocomposites, the films were characterized.

3.5.1 Thickness and mechanical properties

A Mitutoyo digital micrometer (Mitutoyo, Kawasaki, Japan), with a 0.001 mm precision, was used to measure the thickness of the biocomposites in ten random points of each sample. The mechanical properties were set according to ASTM D882-18 (ASTM International, 2018). Five strings of each sample (150 mm wide and 25.4 mm long) were mounted in the tensile grips with a 0.5 kN load cell (Autograph Shimadzu, Sydney, Australia), with a 50 mm initial gauge length and stretched at a cross-head speed of 50 mm/min until breakage. The tensile testing was performed and the values of Young's modulus also known as elastic modulus (EM), tensile strength (TS), and percentage of elongation at break (EAB) were calculated.

3.5.2 Optical properties

The optical properties of the biobased films were determined following the work of Souza et al. (2017). The color of each specimen was observed through the colorimeter CR410 (Minolta Co., Tokyo, Japan) with a 10 mm diameter window and D65 illuminant/10° observer, which exposes the CIE-L*a*b* coordinates (L* indicates black (0) to white (100); a* indicates red (+) to green (-) and b* indicates yellow (+) to blue (-)) useful to calculate the optical parameters chroma (c*) and Hue angle (hue). The measurements were taken on a white background pattern and the equations used are the following:

$$c^* = (a^{*2} + b^{*2})^{1/2}$$

(1)

$$hue = \arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi}, \text{ for } a^* > 0 \text{ and } b^* > 0 \quad (2)$$

$$hue = \arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi} + 180, \text{ for } a^* < 0 \quad (3)$$

$$hue = \arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi} + 360, \text{ for } a^* > 0 \text{ and } b^* < 0 \quad (4)$$

Likewise, the light absorption at 600 nm per mm of film thickness, which measure the light being absorbed due to the constituents of the films, were registered by direct absorbance reading of rectangular simples at 600 nm using a UV-vis spectrophotometer (Model Spekol 1500, Analytikjena, Germany) and calculated according to the Equation (5).

$$\frac{Abs_{600}}{mm} (\text{opacity, } mm^{-1}) = \frac{\text{absorbance } 600 \text{ nm}}{\text{sample thickness (mm)}} \quad (5)$$

Ultimately, the light transmittance was acquired from spectrum scans of each specimen (wavelengths between 190 to 900 nm) employing a UV-vis spectrophotometer. Air was used as a reference and the outcome expressed as a percentage of transmittance.

3.5.3 Solubility and swelling degree

In the first place, film samples were cut into a rectangle (2 x 2 cm) and weighted (precision 0.0001 g) in an analytical balance (Mettler Toledo AB204, Switzerland), obtaining the initial weight (M1); then the samples were placed to dry at 70°C for 24 h in a conventional oven, and were weighted to get the initial dry mass (M2). Later, to enable the swelling process, the films were placed in Petri dishes containing 30 mL of Milli-Q water and stored for 24 h at room temperature (25 ± 2°C). After this contact period, the samples were superficially dried with filter paper and weighted (M3) again. Finally, the remnant of each film was dried in oven at 70°C for 24 h to determine the final dry mass (M4). Two measurements from each film sample were taken, and the parameters were calculated according to Equations (6) and (7) (Souza et al., 2021).

$$\%Solubility \left(\frac{g}{100g} \text{ of film} \right) = \frac{(M2-M4)}{M2} \times 100$$

(6)

$$\%Swelling \text{ degree} \left(\frac{g}{100g} \text{ of film} \right) = \frac{(M3-M2)}{M2} \times 100 \quad (7)$$

3.5.4 Water vapor permeability

Water vapor permeability (WVP) was determined using a gravimetric method at 30°C as described by Ferreira et al. (2016). Briefly, the films were sealed on the top of 45 mm diameter glass cells containing 8 mL of saturated NaCl solution (relative humidity (RH) = 76.9%), then, these cells were placed inside a desiccator containing saturated potassium acetate solution (RH = 22.5%), and equipped with a fan to promote air circulation and keep a constant driving force. The temperature and relative humidity were monitored with a thermohygrometer (Vaisala, Finland). The water permeated through the film and absorbed by the desiccant was determined from the weight loss of the permeation cell (measured every 1 hour during 10 h), and WVP was calculated by the following Equation (8).

$$WVP = \frac{N_W \times \delta}{\Delta P_{w.eff}} \quad (8)$$

Where N_W ($\text{mol/m}^2 \cdot \text{s}$) is the water vapour flux, δ (m) is the film thickness and $\Delta P_{w.eff}$ (Pa) is the effective driving force. Results are the average \pm standard deviation of the three replicates analyzed.

3.5.5 Infrared absorption spectroscopy with Fourier transform

This analysis relies on the chemical bonds present in the sample that have specific vibrational frequencies. Therefore, the frequency of absorption of radiation in the infrared is particular for each type of bond, enabling the identification of interactions between the polymeric matrix and the compounds incorporated in the nanocomposites (Barbosa, 2007). Using this technique, it is expected to identify the interactions between the functional groups presents in the polymer chains and in the nanocellulose particles. The spectra were obtained using infrared spectrophotometer model PerkinElmer spectrum Two (Perkin Elmer, USA). Infrared absorption spectroscopy with Fourier transform (FT-IR) in ATR (“Attenuated total reflectance”) with 32 sweeps and 1 cm^{-1} resolution in the 4000 cm^{-1} to 500 cm^{-1} wavelength range was used.

4.

Results and Discussion

4.1 Analysis of Raw Materials

In this chapter the characterization of raw materials will be discussed and compared with data from literature. Ash content, cellulose, hemicellulose, lignin, total fiber and nitrogen content were evaluated. The analysis have been performed at FCT Nova, Universidade de Lisboa, Portugal.

Table 3 presents those results.

Table 3 Characterization of the samples (Sorghum Inner Layer, Sorghum Outer Layer and Sunn Hemp)

	Ash Content (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)	Fiber (%)	Nitrogen (%)
Sorghum Inner Layer	4.16 ± 0.07	38 ± 2	12 ± 3	36 ± 2	86 ± 4	0.51 ± 0.02
Sorghum Outer Layer	8.90 ± 1.90	38 ± 0	19 ± 4	26 ± 4	83 ± 3	0.94 ± 0.04
Sunn Hemp	8.92 ± 0.01	30 ± 1	10 ± 8	43 ± 9	83 ± 1	1.64 ± 0.03

The highest content in ash was observed in the outer layer of sorghum and in the sunn hemp samples.

Compared with data from other studies (e.g. Zegada-Lizarazu et al, 2021 and 2022), the data determined during this research confirm a higher level of ashes for sunn hemp respect to sorghum. This same trend was also observed in another reference, where sorghum ash content is approximately 4% and sunn hemp ash content is approximately

5.2% (Kamireddy et al, 2013). Data obtained for the inner layer of sorghum from references (Zegada-Lizarazu et al, 2021 and 2022 and Kamireddy et al, 2013) is in agreement with data from Table 3. Data from sunn hemp and outer layer of sorghum, compared to the ones found in the literature (Zegada-Lizarazu et al, 2021 and 2022), were higher. This higher amount of ashes derived, most probably, from the fact that this biomass used in this research, had been stored for a while, and was already partially degraded and part of the volatile matter was lost and mineral matter was concentrated. This was particularly noticed in the sorghum outer layer that was covering the sorghum inner layer. This outer layer was degraded protecting the inner layer, that presented a low ash content, similar to the one from literature.

Results from table 3 indicate that sorghum outer layer presented higher cellulose content and lower lignin content than sorghum inner layer and sunn hemp. All of those biomasses present a hi content in hemicellulose. The results presented in table 3 are not in agreement with results presented in literature (Zegada-Lizarazu et al, 2021 and 2022). Indeed, the results presented in Table 3 show a total fiber content in the samples that ranges 83-86% and in the data from Zegada-Lizarazu et al (2021 and 2022), total fiber was approximately 63-64%. Again this difference obtained might be attributed to some degradation of the biomass used in this study, that lead to some concentration in recalcitrant fibers and mineral matter. Also, the methodologies used for the determination of the cellulose, lignin and hemicellulose content was different from the one performed by Zegada-Lizarazu et al (2021 and 2022). The higher hemicelluloses obtained in this study and the lower cellulose content observed might be attributed to the process. In the process, in the extractions with acid detergent, some foams were also precipitated in the filtration which might have caused a higher amount of biomass fraction agglomerated in the filter, which caused a higher calculated hemicellulose content and a decrease in cellulose content. Yet, referring to the cellulose extraction process described in section 3.3, the amount of cellulose obtained is in agreement with the data from Zegada-Lizarazu et al (2021 and 2022). Indeed, sunn hemp cellulose production obtained was 38%, which agrees with data from Zegada-Lizarazu et al (2021 and 2022), in the range 35-41%. Also the amount of cellulose from sorghum inner layer was 31%, in agreement with the 30-31% presented by Zegada-Lizarazu et al (2021 and

2022). Only sorghum outer layer presented a lower amount of cellulose (only 23%), which was attributed to the degradation of this fraction of the sorghum biomass during the storage period.

As shown from table 3, the sunn hemp nitrogen content is much higher when compared to the Sorghum's ones. This is agreement with literature (Zegada-Lizarazu et al, 2021 and 2022). This result is explained from the fact that sunn hemp is a legume having the capacity to fix nitrogen from the atmosphere, accumulating this macroelement. The results obtained for sunn hemp is similar to the results presented in literature (Zegada-Lizarazu et al, 2021 and 2022), which was 1.7-1.8 % nitrogen. Results obtained for sorghum inner layer (0.5% nitrogen) are lower than the results presented by (Zegada-Lizarazu et al, 2021 and 2022), which is in the range 0.6-0.8% nitrogen, and results obtained for sorghum outer layer (0.9% nitrogen) are higher than the results presented by (Zegada-Lizarazu et al, 2021 and 2022). Those differences are attributed to the fractionation of the biomass in outer and inner layer. Mixing both fractions would results in an average of nitrogen content in sorghum of about 0.7% wich is in agreement with the results from (Zegada-Lizarazu et al, 2021 and 2022).

4.2 Production of nanocellulose

The yield of nanocellulose production with the different biomasses is presented in Table 4.

Table 4 Yields of nanocellulose production from d«sorghum (inner and outer layer) and sunn hemp.

	Yield (%)
Ch + Inner Sorghum	27%
Ch + Outer Sorghum	21 %
Ch + Sunn Hemp	34%

Results indicate that the optimization of the process to obtained NC, resulted in a yield of sunn hemp NC of 34% and a lower yield for both sorghum fractions, respectively,

27% for sorghum inner layer and 21% for sorghum outer layer. This means that comparing the two biomasses, NC extracted from sunn hemp was easier to process mechanically and also presented a better nanocellulose/micro cellulose ratio. Yet, and although sunn hemp yielded a higher amount of NC, it will be important to evaluate which of the NC produced present better properties when incorporated in the biobased films, which will be discussed in chapter 4.3.

4.3 Analysis of the biobased films

Some experiments have been driven to evaluate the biofilms and to attest the differences between the control and the nanocellulose-reinforced polymers.

4.3.1 Solubility and Swelling Degree of the biobased films

The solubility and swelling degree properties knowledge is indispensable to get some more knowledge on the film behavior when in contact with aqueous matrices (e.g. food). It also gives the knowledge to understand the behavior of the material when discarded in a marine environment (Souza et al., 2018). Table 5 shows the results obtained of the different reinforcing agents on the film's solubility and swelling degree.

Table 5 Solubility and Swelling Degree in the different chitosan films

	Solubility %	Swelling Degree %
Ch + Inner Sorghum NC	25.3% ± 2.2	85.1% ± 2.4
Ch + Outer Sorghum NC	25.3% ± 0.1	83.4% ± 0.01
Ch + Sunn Hemp NC	28.9% ± 2.5	87.5% ± 1.0
Ch + Commercial NC	20.3% ± 0.7	77.5% ± 1.5
Ch	26.5% ± 1.0	79.9% ± 0.01

In terms of solubility, no differences were observed among pristine films of chitosan and films reinforced with sunn hemp and sorghum NC. Commercial NC biobased films presented a lower solubility, thus indicating a higher hydrophobicity. The swelling degree of biobased films with commercial NC was also lower than the result obtained from pristine chitosan films, thus corroborating its higher hydrophobicity. Films reinforced with sorghum and sunn hemp NC presented a higher swelling degree than pristine chitosan films, and this indicates that the incorporation of those NC's in the chitosan matrix created a porous material with free -OH and free -NH₃ from chitosan, and also from the NC, where the water molecules can infiltrate. The results conflicts with the literature as showed by Pires et al (2022) where all the registered samples recorded a decrease in swelling capacity after the nanocellulose reinforcement, as it was observed also with commercial NC. However the impact of nanocellulose becomes even more negligible at +1.5% NC as lower value of NC reinforcement seems to show almost negligible impacts on both swelling and solubility. The higher swelling degree attributed to the incorporation of NC from sunn hemp and sorghum, may be interesting for the production of hydrogels, once the interaction between chitosan and the NC's was not hydrophobic. The higher water swelling may enhance the film biodegradability although it may limit its use in applications to food presenting high water content (Singh and Sharma, 2008). The presence of ions in the nanocellulose can act as crosslinker of the polymeric network enhancing absorbency and improving swelling rate and water-holding capacity (Ruiz-Hitzky et al., 2013), which is in agreement with the results of Lavorgna et al. (2010) that observed more water sorption of chitosan/nanocomposite compared to pristine chitosan film.

4.3.2 Water Vapor Permeability (WVP) of the biobased films

The capacity to prevent moisture and oxygen transfer between the inside of the packaging and the surrounding atmosphere is one of the most desirable features attributed to biodegradable films, thus, water vapor permeability (WVP) and oxygen permeability (OP) should remain as low as possible. Considering the central role of

water in food deterioration, the WVP is one of the most important properties of biofilms. Results obtained are presented in Figure 18.

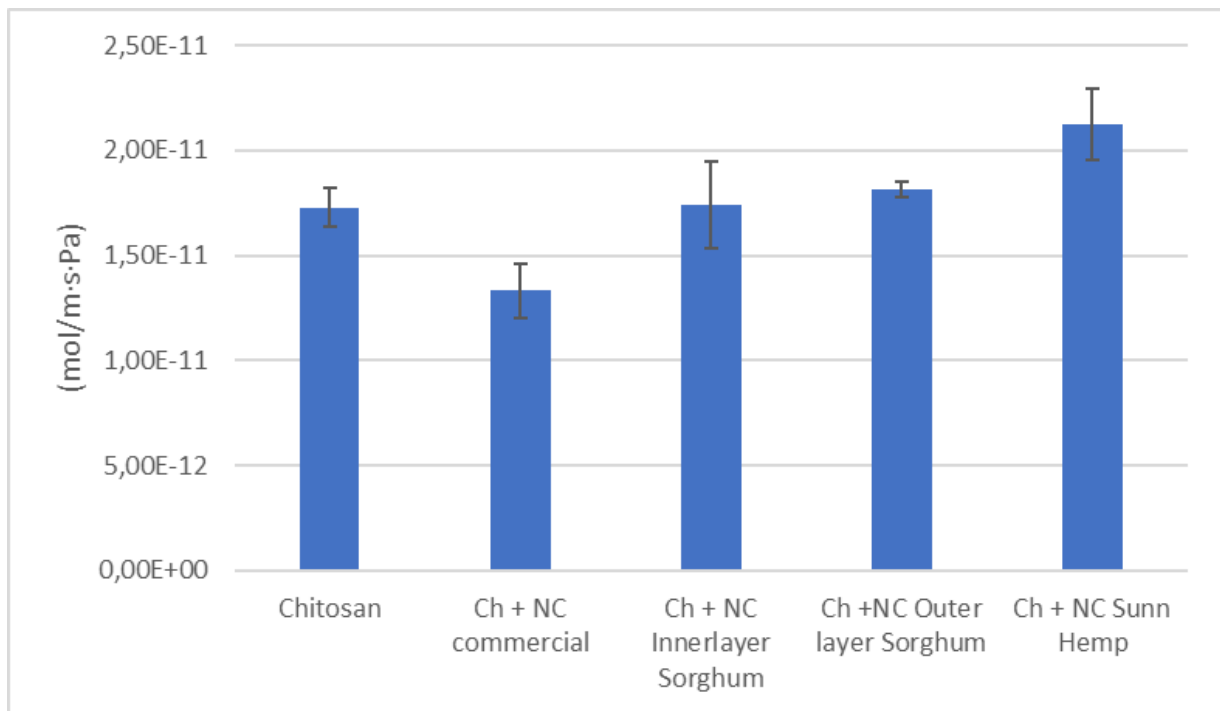


Figure 18 Water vapor permeability of the biobased films (mol/m·s·Pa)

Results presented in Figure 18 are in agreement with the data of solubility and swelling degree. Results show that the introduced nanocellulose (from sunn hemp and sorghum) in the chitosan films did not improve this parameter. The introduction of sorghum NC did not result in a significant change of the WVP, compared with pristine chitosan, and the insertion of sunn hemp NC increased the WVP. Those results obtained with sunn hemp NC and sorghum NC, can be explained by the hydrophilicity of the NC and of the bionanocomposites produced with those NC's, as it was seen in sub-chapter 4.3.1, where solubility of those bionanocomposites was similar to pristine chitosan films and swelling degree was higher. This hydrophilicity thus help the transport of water molecules through the film (Nouri et al., 2017), which probably explains the results found. The results obtained with sunn hemp and sorghum NC's are contrasting with the ones obtained from Pires et al (2022), and the results obtained with commercial NC, where WVP follow a descendent trend when NC was incorporated. Indeed, the film barrier properties depend on a vast variety of factors intrinsic to its composition, like the

biopolymer chemical structure, polarity, degree of crystallinity, density, and molecular weight, as well as the presence of other plasticizers or crosslinkers. These factors will determine the film affinity for the different gas molecules (Zhang et al., 2021). According to the Mujtaba et al., (2017), in bionanocomposites reinforced with NC, the most substantial factor affecting the WVP may be the nanocellulose crystallinity, enhancing the adsorption percentage in a negative or positive way. When NC crystallinity is small, they are not enough crystalline zones to capture water molecules, increasing the diffusion through the amorphous parts, in the contrary, for higher crystalline rates, the permeability is restricted since the crystalline parts are able to trap the water molecules.

4.3.3 Opacity of the biobased films

The opacity (mm^{-1}) was registered for every sample, and this parameter gives an idea of the quantity of light absorbed by the biofilm at 600nm. Results are presented in Table 6.

Table 6 Opacity (mm^{-1}) of the different chitosan films

	Opacity (mm^{-1})
Ch + Sorghum Inner Layer NC	0.64 ± 0.05
Ch + Sorghum Outer Layer NC	0.78 ± 0.07
Ch + Sunn Hemp NC	0.60 ± 0.04
Ch + Commercial NC	0.84 ± 0.09
Ch	0.56 ± 0.09

The results obtained show that the NC reinforced films presented a higher opacity than pristine chitosan films. And sorghum outer layer NC produced a film with the higher opacity, almost as high as the film reinforced with commercial NC. This indicates that those reinforced films absorbed light more than pristine chitosan films. Therefore, it indicates that NC improved effectively the light barrier's properties of pristine chitosan.

And once light can affect the properties of food being protected by biobased polymers, the higher interception of light will reduce its effects on the food. The increment in this parameter with the incorporation of NC is probably due to the insertion of the nanoparticles between the polymer chains, interrupting the continuous matrix. Furthermore, the interaction between NC and water molecules (already observed in the parameters discussed in 4.3.1 and 4.3.2) modifies the refractive index of chitosan and affects the material's transparency and consequently its opacity (Sánchez-González et al., 2011). The results obtained were also observed by the research from Pires et al (2022), where the insertion of different NC's also increased the opacity of chitosan films. Yet, and although a higher opacity is important because it shows an improved light barrier's property of biobased films, it may also represent a drawback related to the acceptance level due to the reduction of the film's transparency.

4.3.4 Thickness of the biobased films

The biobased films were also characterized in terms of thickness (Table 7).

Table 7 - Thickness of the different chitosan films (mm)

	Thickness (mm)
Ch + Sorghum Inner Layer NC	0.077 ± 0.013
Ch + Sorghum Outer Layer NC	0.073 ± 0.014
Ch + Sunn Hemp NC	0.094 ± 0.012
Ch + Commercial NC	0.110 ± 0.009
Ch	0.076 ± 0.013

Results indicate that the incorporation of sorghum NC did not influenced the thickness of the films. Possibly, this was due to a good dispersion of the NC in the chitosan matrix, and to a formation of strong interactions between NC and biopolymeric chains and the penetration of chitosan chains into NC gallery space, as it was observed with other bionanocomposites (Beigzadeh Ghelejlou et al., 2016). This is in agreement with results

of the parameters discussed in 4.3.1 and 4.3.2 where no differences or minor differences were observed among sorghum NC biobased films and pristine chitosan films. Yet, sunn hemp NC biobased films presented a significant increment in thickness, as also the commercial NC biobased films. In both cases, the addition of a new solid component to the matrix resulted in a thickness increment. In the case of sunn hemp NC biobased films, a possible explanation is that the interaction of NC with chitosan reduces the ordered alignment and increase spatial distance within chitosan matrix and decrease in matrix compactness, increasing thickness (which corroborates the results obtained for this film in 4.3.1 and 4.3.2). The results of sunn hemp NC biobased films and commercial NC biobased films are in good agreement with what was previously reported for other films also reinforced with NC. The works of Pires et al (2022), Costa et al (2021) and Dehnad et al (2014), also show that the nanocellulose-reinforced polymers presented an overall higher thickness.

4.2.5 Fourier-transform infrared (FTIR) spectroscopy analysis of the biobased films

Figure 19 show the results obtained of the FTIR (Fourier-transform infrared spectroscopy) analysis. FTIR identifies the structural relation established between chitosan and nanocellulose and it was performed with FT-IR in ATR mode. This technique is widely used to identify the chemical composition of materials as well as the intermolecular bonds. Interestingly, all the samples followed approximately the same patterns with slightly differences in intensities.

All spectra showed major characteristic bands related to chitosan at 3325 cm^{-1} (-OH); 3265 cm^{-1} (-NH asymmetric stretching); 2900 cm^{-1} (C-H bonding of -NHC(O)CH₃ methyl group); 1637 cm^{-1} (amide I); 1550 cm^{-1} (amide II); $1375\text{-}1408\text{ cm}^{-1}$ (-CH₂ bending); 1342 cm^{-1} (skeleton vibration involving C-N stretching of amide III); 1134 cm^{-1} (asymmetric stretching of the C-O-C bridge); $906\text{-}1024\text{ cm}^{-1}$ (skeletal vibrations involving the C-O stretching) (Costa et al, 2021).

The FTIR spectrum (fig.19) seemed to show some NC typical bands, namely the bands located in the region of $3200\text{-}3400\text{ cm}^{-1}$ and the one at 2900 cm^{-1} , which correspond to stretching vibrations of the O-H groups of cellulose and the C-H stretching, respectively.

Yet, the chemical composition of cellulose and chitosan is practically identical and the NC amount introduced was small, and so it is difficult to find differences among the different films. Only differences in intensity. According to (Costa et al, 2021) successfully NC incorporation into chitosan matrix may become more visible with 10 wt% of NC and the bands are more pronounced accordingly with NC amount.

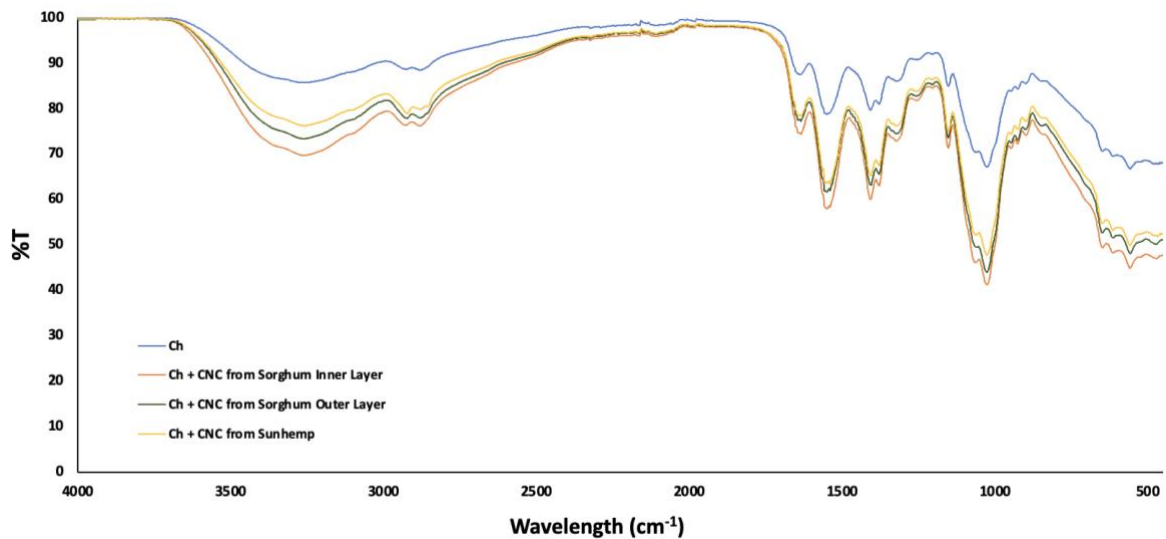


Figure 19 FT-IR of Ch biofilm and Ch + NC biofilms from Sorghum and Sunn Hemp (% transmittance versus wavelength, cm^{-1})

This analysis finds similar results in literature, as for (Pires et al, 2022), where the bio composites spectra were similar to the spectra of the Ch control film, with no big differences spotted in the Ch+NC samples. This indicates that the NC is completely integrated and homogeneously distributed in the biofilms but also, that at the low amount of NC incorporated, bands were more intense but differences among spectra are not visible.

4.3.6 Optical properties

The optical properties analysis is crucial when the biobased films are to be used as a packaging system because these may interfere with consumer acceptance. Table 8 presents the color of the biobased films, reflected by the Hue angle, the chroma and the luminosity.

Table 8 – Optical properties of the different chitosan films

	L*	Chroma	Hue Angle
Ch+Inner Layer Sorghum	90.7 ± 0.3	13.24	99.5
Ch+Outer Layer Sorghum	90.7 ± 0.3	12.76	99.3
Ch+Sunn Hemp NC	90.1 ± 0.1	13.80	99.2
Ch+Commercial NC	90.0 ± 0.3	12.68	98.1
Ch	90.8 ± 0.5	12.19	98.7

Results show that the hue angle of the NC reinforced films were slightly higher than pristine chitosan films which can be explained by the yellowish colour of the biofilms with NC according to Pires et al (2022). In addition, also the luminosity L* remained almost the same with no or negligible changes in the parameter. This could be explained by the low quantity of NC used to reinforce the films, an hypothesis supported by the analysis performed by Pires et al (2022) as the luminosity start to have significant changes and will decrease with higher nanocellulose percentages.

4.3.7 Mechanical analysis of the biobased films

Table 9 shows the mechanical response to the biobased films. Tensile Strength, Elastic Modulus and Elongation at break were analyzed. As we see in Table 9, the incorporation of NC increased the tensile strength of the biobased films, except in the case of sunn hemp where the NC did not resulted in a higher strength. The elastic modulus (EM) increased in the samples containing nanocellulose, especially in the sorghum one (but not in the films with sunn hemp NC).

Table 9 - Mechanical properties obtained for different chitosan films

	Tensile Strength (MPa)	Elastic Modulus (MPa)	Elongation at Break (%)
Chitosan + Inner Sorghum	35.1 ± 1.8	1722 ± 88	17.1 ± 1.9
Chitosan + Outer Sorghum	36.7 ± 3.1	1565 ± 110	19.1 ± 3.7
Chitosan + Sunn Hemp	32.2 ± 1.8	1356 ± 121	29.9 ± 3.2
Chitosan + NC commercial	37.2 ± 2.3	2035 ± 107	10.9 ± 2.2
Chitosan	34.6 ± 3.7	1415 ± 94	27.9 ± 1.6

The Elongation at break describes the material's capacity to support shape modifications without fracture development. Contrary to the increase presented by EM, it was noticed that the introduction of NC made the films less elastic, thus, for greater concentrations, lower will be the film's ductility, except in the case of films with sunn hemp NC. Therefore, the results confirmed that sorghum NC, improved the strength and stiffness of the chitosan biopolymer and that sunn hemp NC improved the plasticity of the chitosan polymer. Results obtained with sorghum NC were also obtained by Pires et al. (2022) and the improved strength is attributed to the presence of nanoparticles that fabricated a dense layout with increased continuities within the poly-saccharide network. Sunn hemp NC caused a more plastic biofilms which may not be suited for food packaging but for other applications where the plasticity is of importance.

5.

Conclusions

Utilizing nanocellulose has multiple benefits compared to other materials when it comes to sustainability and effects on the environment. Therefore, its potential to replace plastic made from fossil fuels is interesting. However, to be able to review their overall sustainability, a high number of factors need to be considered. To produce plastics from nanocellulose multiple processes are involved. Not only the production process in a plant, but also the cultivation of biomasses such as Sorghum and Sunn Hemp, their transport and processes involved. In the next part the difference to conventional plastics regarding these factors will be discussed. First, unlike conventional plastic, plastic made from nanocellulose is biodegradable. Usually, it takes around one year for the material to degrade. As a consequence, by using biodegradable plastic, nature will be less polluted by redundant plastic packaging. This can contribute to, among others, driving down the plastic wastes in the ocean. Thus, in this manner it does not only reduce the amount of plastic polluting the environment, but it also creates the opportunity for crops that are able to grow in marginal and contaminated soils. Therefore, plastic from nanocellulose can be seen as a truly circular product. Going back to the research, starting from the mechanical processes as chopping and grinding, Sunn hemp overall required less time and less efforts to reach 30 g of biomass powder, in addition, the grinder was run for less time respect to sorghum, also, the separation between outer and inner layer was required, thus increasing the processing time for sorghum. Overall, Sunn Hemp, after the optimization, yield the highest cellulose percentage among all the samples. Again, the pretreatments with sunn hemp were performed with slightly lower temperatures: while for sorghum the reaction temperature was kept in a range around 70 °C, sunn hemp was processed with slightly lower temperatures around 60 °C. That didn't show any negative effects as the overall sunn hemp cellulose content was higher at the end of the pre-treatments.

Also, the grinding performed before the acid treatment resulted to be easier with sunn hemp. Overall, the sunn hemp has been processed both chemically and mechanically in a lower time respect to sorghum.

When it comes to biofilms, we can notice that the difference between the control samples and the nanocellulose-reinforced biofilms were relevant, anyway, we need to consider that the amount of nanocellulose reinforcement per L was 0.30 g, despite that, we saw that the nanocellulose-reinforcement, even if low, had some impacts on the biofilms, thus showing us that playing on nanocellulose percentage on the biofilm formulation would be interesting for further studies. First of all, the nanocellulose reinforcement seems to highly improve the light barrier properties especially in the sunn hemp's case as it is shown in the absorbance parameters.

In addition, the average thickness showed again higher results with sunn hemp reinforcements, even if this can be given by an uneven distribution and drying of the biofilm, however, all the 10 thickness measurements showed higher results meaning that sunn hemp is somehow impacting the thickness increasing it in a relevant way.

Looking at the solubility, the highest one is still the one of sunn hemp reinforced films, even if this can be interpreted as a negative or positive value depending on the end uses, while, all the other nanocellulose-reinforce biofilms showed similar results, slightly decreased solubility of inner and outer layer of sorghum compared to the control samples.

Nanocellulose seems to give instead a higher impact on swelling degree as for the Ch samples it was registered to be 80% while all the nanocellulose-reinforced films (both Sorghum and Sunn Hemp) showed values from 83% and higher.

Looking at the other analysis performed, the WVP showed no changes for Sorghum NC while it showed an increase with the Sunn Hemp one allowing overall an higher water permeability in the biobased film.

Going to the most interesting results, which are the mechanical ones, we can notice that the nanocellulose reinforcement has no impact on Tensile Strength while it reduced the Elongation At Break and the Elastic Modulus. Meaning that Sorghum NC reinforcement improved the strength and the stiffness while Sunn Hemp reinforcement improved the plasticity of the chitosan polymer.

The sunn hemp behavior has been showing interesting values during these trials and deserve attention, as we saw nanocellulose even in low quantities impacted some parameters without impacting the overall transparency and color of the biofilms. It could be also important to mention that the biofilm preparation method dispersing chitosan directly into the water-nanocellulose matrix, followed by homogenizations and ultrasound bath treatments was successful as the biofilms showed a very good homogeneity and chitosan was homogeneously dispersed creating no lumps in the biofilms.

This research proved that nanocellulose has an impact on the biofilm, but this is not the only purpose of the project: hypothetically. this biofilm can be produced from 100% renewable sources as we can obtain glycerol and chitosan from wastes too. The biofilms are in that case completely composed from natural renewable resources and theoretically biodegradable and compostable even if the composability has not been assessed in this study, however, the high solubility for sunn hemp could show an overall better biodegradability. Again, these are not the only advantages, as we saw previously, the sunn hemp and sorghum would not only give us a valuable alternative to fossil fuel-based product, but they would also deliver us several other services, without competing with food crops which is another important factor to take in consideration.

6.

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