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Bachelor's in Engineering of Micro and Nanotechnologies

Cork as a raw material for antibacterial membranes and fibers

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Abstract

Cork industry is one of the most profitable markets in Portugal with an annual production of 100000 tonnes. Yet, a large amount of cork waste is generated without commercial value. Currently, cork by-products are mostly used as burning fuel for energy production. Substantial valorisation can be attained if valuable components are extracted instead of burning to produce energy.

Cork is a remarkable biocomposite that combines a set of uniques and astonishing properties. Suberin, the main component of cork (~30-50% of its composition) is a hydrophobic and high thermal resistant biopolyester that plays a key role as a protective barrier between the plant and the environment. There are several depolymerisation approaches to isolate suberin from cork, such as alkaline methanolysis, cholinium hexanoate ionic liquids, or aqueous alkaline hydrolysis. Suberin films obtained from these processes show barrier properties similar to those of the suberin barrier in plants, including a potentially broad bactericidal effect.

Here, we report a simple, low energy demanding and "greener" process based on aqueous alkaline hydrolysis to explore the extraction of suberin, using cork stoppers as a raw material. The concentration of cork as well as the concentration and source of alkaline salt (LiOH vs. NaOH) is investigated throughout this work. ATR-FTIR analysis confirms the main presence of suberin peaks in the bottom pasty component, whereas only one peak appears in the top liquid phase. The suberin rich phase was selected for further study targeting antibacterial applications, while the liquid phase after drying has potential to be used as a resin-like electrolyte for electronic applications based on ionic response (iontronics).

The prepared solutions exhibit antibacterial properties against both the Gram-positive bacteria *Staphylococcus aureus* and the Gram- negative bacteria *Escherichia coli*. The antibacterial activity is enhanced with the increase of cork concentration, and it can be considerably improved with the addition of zinc-oxide nanoparticles in the aqueous alkaline hydrolysis process. Taking these results into account, PEO/suberin-based composite fibers were successfully prepared by solution blow spinning.
This work brings new insights in the field of recycling of cork wastes and top-down approaches to obtain suberin component from cork as a potential building-block for an innovative generation of biopolymers with high-value in the field of biomedicine.

Keywords: cork, depolymerisation, suberin, alkaline hydrolysis, antibacterial properties, solution blow spinning.

Resumo

A indústria da cortiça é um dos mercados mais rentáveis em Portugal, com uma produção anual de 100000 toneladas. No entanto, uma grande quantidade de resíduos de cortiça é gerada sem valor comercial. Atualmente, os subprodutos da cortiça são usados principalmente como combustível para produção de energia. Uma valorização substancial pode ser alcançada se componentes valiosos forem extraídos em vez de queimados para produção de energia.

A cortiça é um biocompósito notável que combina um conjunto de propriedades únicas e surpreendentes. A suberina, o principal componente da cortiça (~ 30-50% da sua composição) é um biopolímero hidrofóbico de alta resistência térmica que desempenha um papel fundamental como barreira protetora entre a planta e o meio ambiente. Existem várias abordagens de despolimerização para isolar a suberina da cortiça, como metanólise alcalina, *cholinium hexanoate ionic liquids* ou hidrólise alcalina aquosa. Os filmes de suberina obtidos a partir desses processos mostram propriedades estruturais semelhantes às da suberina nas plantas, incluindo um efeito antibacteriano extenso.

Aqui, relatamos um processo simples, de baixo consumo de energia e "mais verde", baseado em hidrólise alcalina aquosa para explorar a extração de suberina, usando rolhas de cortiça como matéria-prima. A concentração de cortiça, bem como a concentração e tipo de sal alcalino (LiOH vs. NaOH) é investigada ao longo deste trabalho. A análise ATR-FTIR confirma a presença de picos de suberina no componente pastoso, enquanto apenas um pico aparece na fase líquida após centrifugação. A fase rica em suberina foi selecionada para estudos posteriores visando aplicações antibacterianas, enquanto a fase líquida após a secagem tem potencial para ser usada como um eletrolítico semelhante a resina para aplicações eletrônicas baseadas na resposta iônica (iontronics).

As soluções preparadas exibem propriedades antibacterianas contra a bactéria Grampositiva *Staphylococcus aureus* e a bactéria Gram-negativa *Escherichia coli*. A atividade antibacteriana é ampliada com o aumento da concentração de cortiça e pode ser consideravelmente melhorada com a adição de nanopartículas de óxido de zinco no processo de hidrólise alcalina aquoso. Levando em consideração esses resultados, as fibras compostas à base de PEO / suberina foram preparadas com sucesso por *solution blow spinning*.

Este trabalho traz novas idéias no campo da reciclagem de resíduos de cortiça e abordagens de cima para baixo para obter o componente suberina da cortiça como um componente essencial para uma geração inovadora de biopolímeros com alto valor no campo da biomedicina.

Palavras-chave: cortiça, despolimerização, suberina, hidrólise alcalina, propriedades antibacterianas, *solution blow spinning.*

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Abreviations

ATRFTIR	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
SBS	Solution blow spinning
LP	Liquid phase
PP	Suberinic "pasty" phase
WPP	Washed suberinic "pasty" phase

1   Introduction

1.1 e-Waste

Electronic waste, known as e-waste, is an important subject matter since is growing at a fast rate (annual growth rate of 3–4%). The estimation regarding e-waste is concerning, since it will reach 52.2 million tonnes per annum by 2021 and currently, only 15% of e-waste is recycled [1].
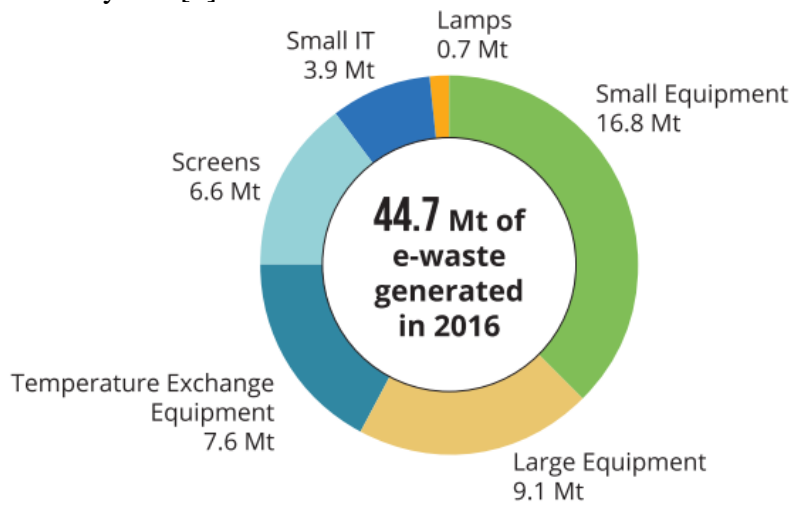
Figure 1.1 - Estimates of e-waste totals per category in 2016 [2]

The attributes of plastics worldwide, e.g. low price, ease of manufacturing and processing, extraordinary physical and chemical properties contributed to the continuous increase in the consumption at a global scale. One significant part of e-waste is plastic, accounting for up to 20% of the total amount. The presence of brominated flame retardants makes the e-waste plastic more complicated for recycling in comparison with plastic itself [1].

The components of e-waste can be metals, ceramics, glass and plastics. The value of these raw materials was estimated to be 55 billion Euros. As depicted in Figure 1.2, adopting a *Circular Economy* model allow the increase in value of electrical and electronic equipment's when wasted, inspiring to close the loop for these new raw materials by reusing and recycling. Business opportunities can be created around these materials and at the same time the mitigation of environmental pollution [2].
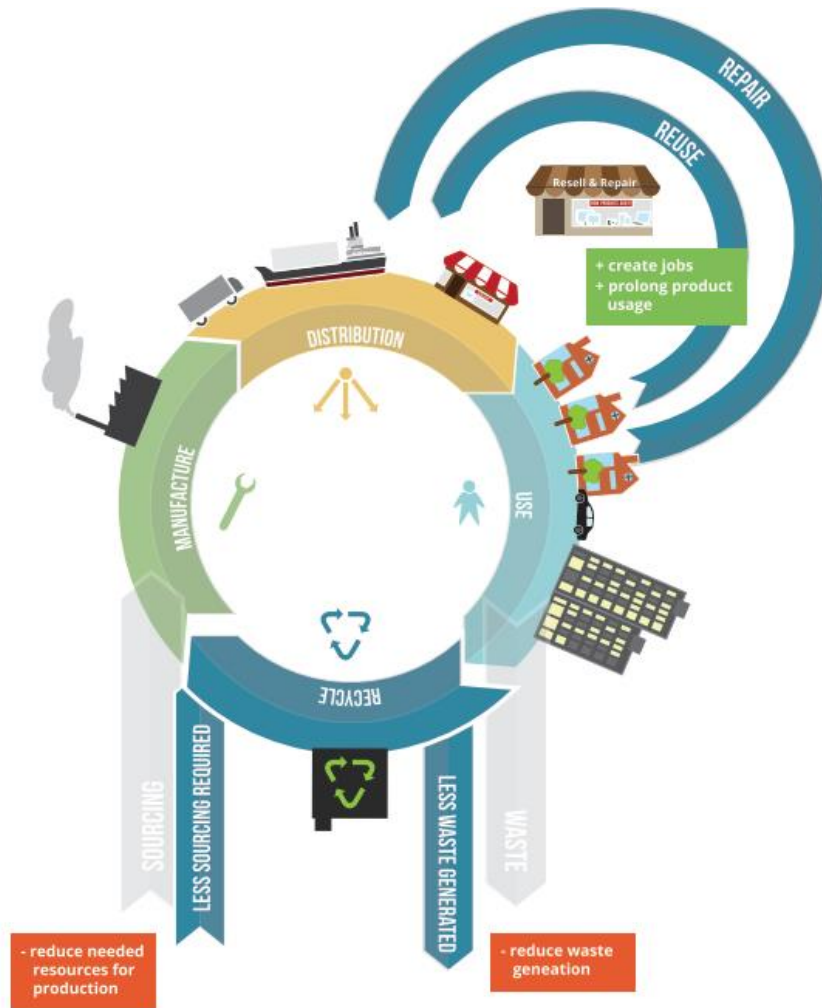
Figure 1.2 - Simplified model of the Circular Economy [2]

In order to develop more sustainable practices, biopolymers are an attractive alternative to typical fossil-based polymers as their nature origin combined with low-consumption manufacturing processes and recyclability / biodegradability. In this sense, cork is very interesting due to its outstanding properties and potential applications [3]. A detailed description of this material will be given in the following chapters.

1.2 Cork origin and commercial value

Cork oak forests are of crucial importance to the economy and ecology of several Mediterranean countries, where Portugal stands out as the world's leading producer of cork (~50% of world production) (Table 1.1)[4]. In Portugal, the cork oak forest are considered to be 'Europe's Amazon forests', supporting the greatest bio-diversity found in Europe [5].

Table 1.1 - Cork production by country [4]

Country	Annual production (Tonnes)	Percentage (%)
Portugal	100000	46.6
Spain	61504	30.5
Morocco	11686	5.8
Algeria	9915	4.9
Tunisia	6962	3.5
Italy	6161	3.1
France	5200	2.6
Total	201428	100

As shown in Figure 1.3, the evergreen oak bark (*Quercus suber L.*) is harvested every 9 to 13 years without harming the tree, which continues to live on as a carbon sink for up to 300 years. They have a vital role regarding ecological procedure, such as water retention, soil conservation and carbon storage. The cork oak trees are able to sequester carbon, with the purpose of regenerate their bark (a harvested cork oak tree absorbs up to five times more CO_2 than one that is left alone), which is a rare example where mankind's involvement helps the environment [5].
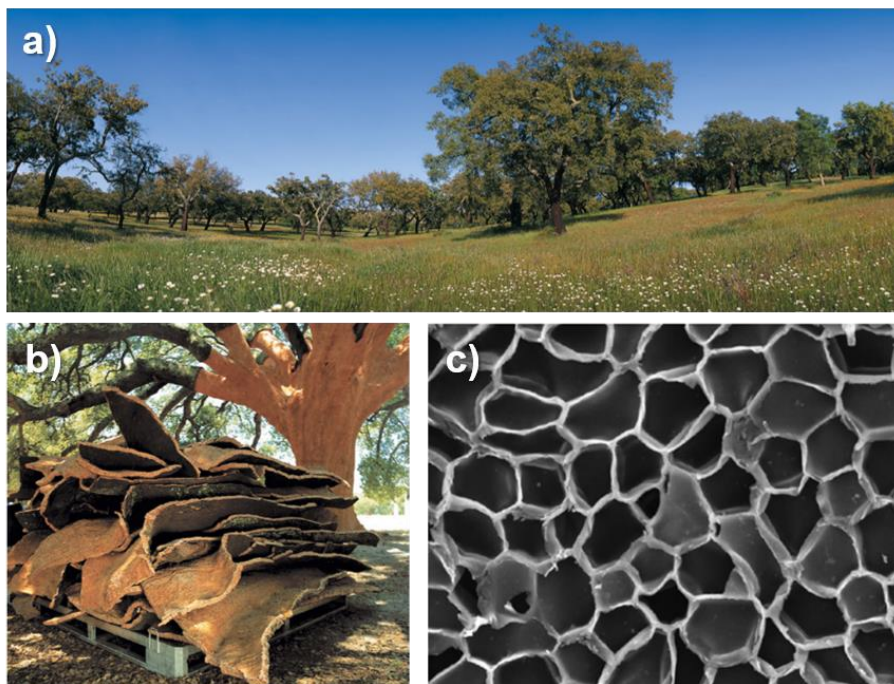
Figure 1.3 – a) Cork oak (*Quercus suber L.*) forests. b) Cork extraction. c) SEM micrographs of cork (adapted from reference [6]).

This plant composite presents an outstanding combination of properties, for example elasticity, low density (120 to 240 Kg m^{-3}), low permeability, and considerable chemical, thermal and microbial resistance [7]. The relatively compliant cell wall material with the low volume fraction of solid promotes its compressibility (since cork is about 15% solid, being the 85% air) [6].

The use of cork dates to ancient Romans as their unique cellular structure allowed their application in stoppers and thermal/ sound insulation materials manufacturing [4]. Nowadays, higher potentialities are found in cork composites enabling a wider range of applications, such as bottle stoppers and gaskets to the soles of shoes, flooring, textiles, automobile components, and even heat-shields of spacecrafts (Figure 1.4). The reason that cork is a good source for gaskets is the same reason that it makes good bungs for bottles: the compressible, accommodating deformation, and its closed cells are impermeable to liquids. Cork is also a splendid flooring material, for the reason that it holds warmth, its comfortable to walk on and is does not become slippery, even when wet [5].
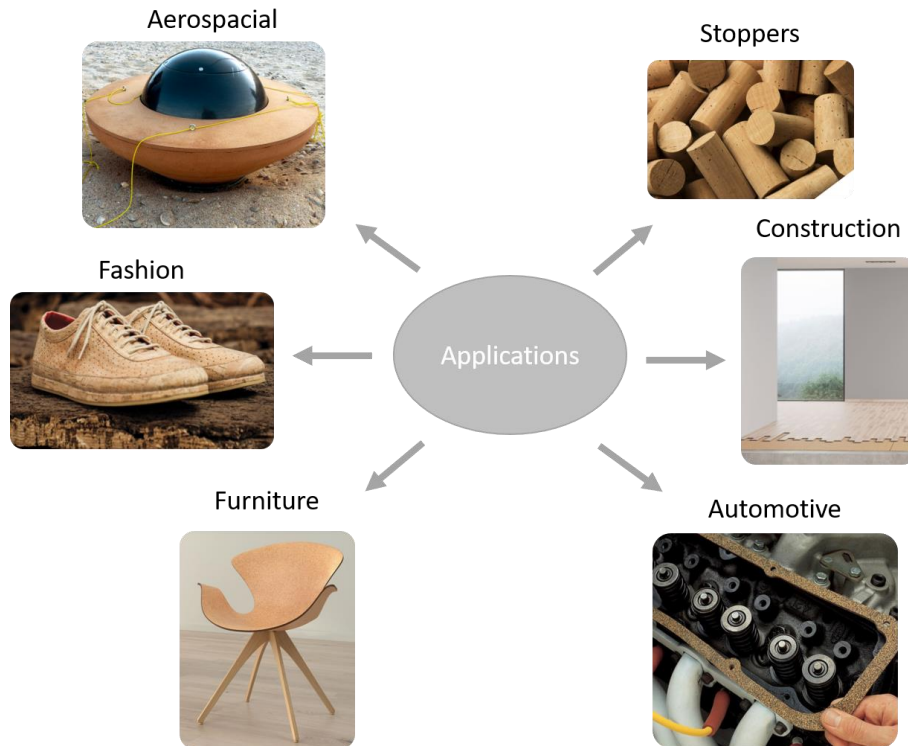
Figure 1.4 - Examples of cork applications (adapted from references [8], [9]).

Cork industry is facing a big challenge regarding the big amounts of cork wastes (~22 wt.% of total production per year) that are produced without commercial value, being usually burnt to generate energy [4]. Besides, cork is far to reach its full potential, as their properties are far to be fully understood and new processes have been recently reported to obtain a substantial valorisation of cork through extraction of valuable components from its composition, such as suberin, which is more appealing rather than burning cork wastes to produce energy.

1.3 Cork composition

Cork is a remarkable biocomposite that combines a set of unique and astonishing properties due to its composition: suberin, lignin, polysaccharides, and extractives, which corresponds to approximately 45, 20, 20, and 15 wt.%, respectively. With its specific cellular structure along with its chemical components, cork exhibits an outstanding barrier properties for polar liquids, heat and sound [10]. Suberin, the main component of cork, is a hydrophobic and high thermal resistant aromatic-aliphatic cross-linked biopolyester that plays a key role as a protective barrier between the plant and the environment [11]. Recently, new features have been found in this material, including waterproof, hydrophobicity, and antimicrobial properties. [12][13][14] The processes involved in the extraction of this material are specified in the following chapter.

Lignocellulosic constituents like lignin and polysaccharides are related to cellular structure and are regularly used as reinforcements to the green composite field. Lignin in particularly is a rigid polymer responsible for the strength and stiffness of the cork cell wall. Usually, lignin is used as a reinforcing filler of thermoplastics and elastomers [15]. Studies shown that lignin is biologically active and depending on the biomass, possess different antibacterial, antifungal or antiparasitic properties that can be applied to food stabilizers [16]. Polysaccharides is a cork constituent that includes cellulose and hemicelluloses. Due its tightly crystalline structures, cellulose owns outstanding mechanical properties [15].

1.4 Suberin depolymerisation process from cork

The big source of suberin in nature is the outer bark of higher plants and tuber periderms (Table B. 1). The content and composition in outer barks can be variable, because it depends on the wood species and the isolation method used. There are several depolymerisation approaches to isolate suberin from cork, such as alkaline methanolysis, cholinium hexanoate ionic liquids, or aqueous alkaline hydrolysis. In hardwoods of industrial relevance, suberin represents typically between 20% and 50% of the extractive-free bark weight [12], [17].

The domains of suberin are generally thought to be arranged in a lamellar-type structure, however the composition and native structural organization of suberin can generate controversial. However, the nature of the linking of suberin to the other cell wall domains remains uncertain.

Depolymerisation of in situ suberin and its simultaneous isolation from the plant composite is traditionally a laborious process requiring harsh chemical processes, which hinders the production method of suberin-based polyester to be applicable into real-world applications [7]. Most of the methods to isolate suberin from cork involve an alkaline methanolysis extraction using ethanol, water and dichloromethane followed by a method of isolation. This method has negative aspects as they are time consuming and require toxic chemicals for the procedure. Some other methods that rely on ionic liquids can be

more appealing, since they are less toxic, able to be recycled and reintroduced in the system, and allow the extraction of suberin with high yields (50~55%) [18]. Nevertheless, these ionic liquids take more time to produce and are expensive.

The depolymerised suberin samples are greatly affected by the method applied. For instance, variations can be observed regarding suberin extraction yields and suberin composition due to preferential removal of specific monomers and/or induced chemical modifications in some functional groups, such as in the epoxy moieties. Hence, the depolymerisation method should be carefully selected depending on the ultimate goal, i.e. suberin detailed chemical composition, monomers isolation or the study of suberin structure.

As already mention earlier, suberin is present in plant cell walls, where it serves as a barrier to keep water in and pathogens out. Despite its interest for many applications, suberin is a structural component of the cell walls and, as such, it cannot be extracted without destroying its chemical skeleton and consequently its inherent properties. Using the following technique it is possible to preserve the unique properties of suberin: waterproof, hydrophobic and bactericidal. [19]

Alkaline hydrolysis depolymerizes suberin structures by breaking ester bonds existing in aliphatic and aromatic chains. To optimize the yield a minimum stoichiometric mass of alkali salt is essential to increase the hydrolysis kinetics [15].

The production of new suberinic-based materials can bring a new purpose for already studied applications using suberin as a material. Its biocompatibility and antibacterial properties can be implemented (e.g. clinical environment) [14].

These properties can be explored implementing a technique like Solution Blow Spinning (SBS) for fiber production. This is a promising technique for commercial scale nanofiber production, with lower cost compared to electrospinning. The SBS process is compatible with a wider variety of solvents than electrospinning and eliminates the need to use high voltages. Another great advantage of SBS is that it is more portable as with commercial airbrush systems that facilitate depositing fibers on a broad range of collectors and surfaces. The applications for SBS mats include their use in sensors and biosensors, wound dressings, tissue sutures, drug delivery materials, filter membranes and adsorbents.[20]

Inspired in the work reported for dissolution of cellulose [21], lithium hydroxide (LiOH) together with urea and deionized water was the solvent system used for the extraction of the suberinic material from cork and simultaneous dissolution of cellulosic materials. This approach was already successfully explored in our group [19] targeting electronic applications based on ionic responses (iontronics). In this work, this method was explored to investigate the antibacterial activity of the extracted components from cork. Furthermore, sodium hydroxide (NaOH) was also investigated as an alternative to LiOH as it is the sixth most abundant element on Earth so is less expensive [22]. Besides

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that, the influence of the addition of zinc oxide (ZnO) nanoparticles was also investigated in order to enhance the antibacterial activity response [23].

1.5 Solution blow spinning

The advance of innovative biomaterials and their use in biomedicine has received much attention in recent years. Nanofibers are very interesting in this particular field, with applications extending from scaffolds for tissue regeneration to drug delivery systems. With a wide type of application, substantial advances in new technologies have been made to the development of polymeric biomaterials with controlled geometry and physico-chemical properties [24].

To obtain polymer nanofiber composite there is several methods. In this work the method used is solution blow spinning (SBS). This technique involves the feeding a polymer solution into a stream of pressurized air using a concentric nozzle. When the aerodynamic forces overcome the solution surface tension, a solution jet jettisons towards a collector. During the flight, the solvent is evaporated forming polymer fibers that are collected as non-woven mats. The SBS technique is known for its promising use for commercial scale nanofiber production, with inferior cost compared to electrospinning. One benefits of SBS technique is the fact that is compatible with an extensive variety of solvents when compared with electrospinning. Furthermore, the SBS technique rejects the necessity to use high voltages. Another benefit from SBS is its portability with commercial airbrush systems that facilitate depositing fibers on a broad range of collectors and surfaces [25].

2   Experimental Procedure

2.1 Cork pre-treatment

Used cork wine stoppers were collected from different sources and used as raw material. Following the procedure reported in the literature for the treatment of cork [14], cork stoppers were grinded using a domestic food blender (Becken BTB 2312) and then sieved with a 500μm mesh (Retsch Test Sieve), in order to increase the cork surface area and reduce the duration of the depolymerisation process. The obtained cork powder was sequentially washed using ethanol (96% vol. Aga N°CE: 200-578-6) and then deionized water (Milipore) to remove impurities. Firstly, the cork powder was washed twice in boiling ethanol at 80 °C for 2 h and then 1 h under stirring (800 rpm) to promote the collapse of its hexagonal honeycomb structure. The cork powder was then washed three times with boiling deionized water at 100°C for 30, 30 and 15 minutes, respectively. Between every washing step, the cork powder was collected through vacuum filtration.

2.2 Suberinic material extraction

The depolymerisation process used throughout this work is schematized in Figure 3.1. Following the process reported by Além [19], [21], the solvent system was prepared by mixing LiOH (98%, pure, anhydrous, CAS 1310-65-2, Acros Organics) or NaOH (LabKem, ≥ 98%), urea (99.5%, CAS: 57-13-6, Acros Organics) and deionized water, with a weight ratio of 4.6/ 15/ 80.4, respectively. Different proportions of LiOH/ urea/ water were also prepared to explore their influence on the suberinic material extraction. In order to study the influence of ZnO on the anti-bacterial activity of the cork-based extracted components, a solvent system was prepared with ZnO nanopowder (particles size: <100 nm, Sigma-Aldrich) in the following proportion: LiOH/ urea/ ZnO/ water – 4.6/ 15/ 0.5/ 79.9 wt%.

Cork was added to the solvent system with different concentrations (4, 6, 8, 10 and 15 wt. %) and stirred for 24 h, resulting in a homogeneous brownish solution. A neutralization procedure was performed with different acids (citric, acetic, ascorbic, phosphoric, lactic, tartaric, or poly(acrylic) acids) and the pH was controlled using pH test strips (Sharlau, pH indicator strips 0-14, Sharlab).The best results were obtained using acetic acid, thus further studies were performed using this acid as the resin-like membrane obtained after drying the resulting solution is free of dendrites.

A phase separation starts occurring in a short period of time (~4 h) after performing the neutralization step. Therefore, to speed up the separation of the two phases (liquid phase, LP, and "suberinic-based pasty phase", PP) a centrifugation step was carried out using Heraeus Multifuge X1R Centrifuge at 12000 rpm for 45 minutes and 24˚C.

Resin-like membranes were prepared from liquid phase solution by shear-casting on a glass plate and then dried at room temperature (23 ± 2 °C, ≈40 %RH) for 7 days and stored in air.

A small sample from PP was collected from each experiment and washed at least five times with deionised water to ensure the removal of remaining reagents. A centrifugation step at 12000 rpm for 45 minutes and 24˚C was carried out before each washing step to collect the "washed PP" (WPP). The final product was dried in vacuum at 60° C for 24 h.

2.3 Preparation of suberinic-based composite fibers using solution blow spinning technique

"Suberin"/PEO composite fibers were obtained by SBS technique. Firstly, W-SPP (15) sample was grinded in a mortar. Then a mixture of PEO (M_v 4000000, CAS: 25322-68-3, Sigma-Aldrich) and W-SPP (15) was prepared at a concentration of 0.6/0.4 % w/v in dichloromethane (M:84.93, CAS: 75-09-2, José Manuel Gomes dos Santos, LDA) by stirring at 1200 rpm for 24 h. The SBS apparatus was prepared as schematized in Figure 2.1, consisting of a concentric nozzle configuration where a polymer solution/suspension was fed through the inner nozzle and pressurized air flowed through the outer nozzle. The inner nozzle was positioned so it protruded 2 mm beyond the concentric outer nozzle. The glass syringe use for this method was (Arti Glass – 20ml). The parameters used for SBS process were 4 PSI of air pressure and a solution/suspension feed rate of 10 ml/hour. The feed rate was controlled with a (KDS 100 Legacy Syringe Pump). The collector was rotating and positioned at a working distance of 40 cm from the nozzle. The SBS process was performed at ambient temperature (24 °C) and 50 % relative humidity (RH).
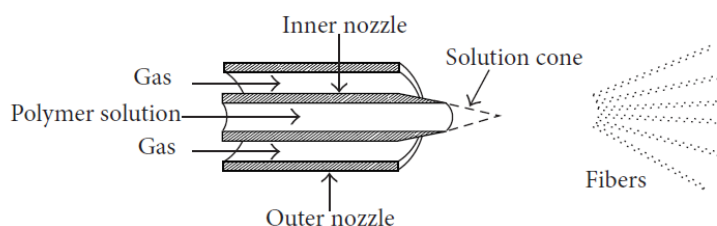

Figure 2.1 - Cutaway diagram of the concentric nozzle system used in solution blow spinning [26]**.**

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2.4 Characterization techniques

The Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATRFTIR) of the different stages of the process were collected by a Nicolet 6700 FTIR Thermo Electron Corporation device, using a SMART iTR adapter. The samples were collected at room temperature using a range from $4500 - 525$ cm^{-1} accumulating 32 scans with a resolution of 4 cm^{-1}.

Scanning Electron Microscopy (SEM) was used to visualise the morphology of the cork nanofibers obtained from SBS technique were observed. The results were obtained by a (Hitachi TM 3030Plus Tabletop).

2.5 Antibacterial study

Antibacterial activity of the prepared samples was evaluated for gram-positive (*Staphylococcus aureus* ATCC6538) and gram-negative (*Escherichia coli* ATCC8739) bacteria using the agar diffusion method [27]. All assays and procedures were performed under aseptic conditions (Steril Laminar Flow Chamber - VBH). Bacteria were inoculated in Petri dishes containing tryptone-soy agar medium (Biokar) from frozen cultures (-70 °C) in glycerol-containing culture medium (15% v/v). After inoculation, the plates were placed in an oven at 37 °C and incubated for 24 hours. The initial suspension for assaying was prepared from these plates by transferring isolated colonies to a tube containing 3.0 mL of saline (0.85% NaCl). The turbidity of the microorganism suspension was adjusted to 0.5 on the McFarland Scale (Densitometer Grant-Bio DEN-1B), which corresponds to a cell density of $1x10^8$ CFU/mL.

The suspensions were immediately applied with the aid of a swab on the surface of Petri dishes with Mueller-Hinton Agar (Biokar) medium, and then approximately 6 mm diameter equidistant wells were made in the various plates. The wells were then filled with 50 µL of each sample. The plates were refrigerated at 4 °C to fully diffuse the samples and finally incubated at 37 °C for 24 hours. After incubation time in the greenhouse, the inhibition zone diameters around each well were measured using a ruler, and the antibacterial activity was estimated from these same diameters.

3   Results and discussion

3.1   Suberin depolymerisation through alkaline hydrolysis

Using the method described above for suberinic material extraction and depicted in Figure 3.1, it was possible to perform suberin depolymerisation through alkaline hydrolysis. This method, besides being cheaper than its alternatives [14], accomplishes for a short period of time, and using low cost materials, good results in the suberin depolymerisation. Based on [19], this technique suffer improvement and it was possible to obtain a highest yield from the brownish dissolution. Consequently, the amount of pasty phase has been exponentially enhanced in order to be able to perform a superior number of post-testing.

Throughout this work several parameters were investigated, namely the concentration of cork, as well as the concentration as source of alkaline salt (LiOH vs NaOH), and the acids used for neutralization. Various acids were tested, including acetic, citric, tartaric, poly(acrylic), ascorbic, phosporic and lactic acids in neutralization using the LP samples, it was possible to obtain interesting results. A colour change was visualized when the neutralization was performed drop by drop.
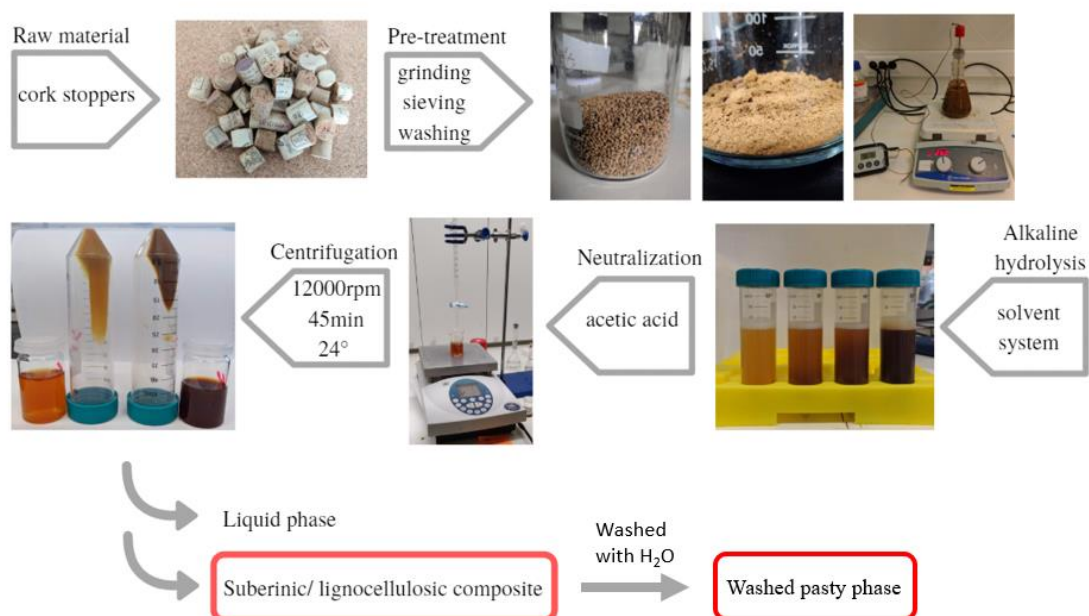
Figure 3.1 - Schematic representation of the depolymerisation process used throughout this work.

Through the shear casting technique, uniform films were created and as shown in Figure A. 1. The surfaces of the membranes were significantly different from each other. It was possible to detect a crystallization on most of the membranes excluding acetic and citric acid. Was observed that for weak acids crystallization on the membrane surface occurred and based on these results, the use of acid in the sample neutralization process was restricted using only acetic acid.

3.2 Compositional analysis of the extracted components from cork

As shown in the FTIR data (Figure 3.2), suberin, lignin and polysaccharides peaks were identified and compared between washed cork powder, alkaline hydrolysis solvent system, and after performing all the steps (alkaline hydrolysis, neutralization, and centrifugation) to extract suberinic materials from a starting solution of 8 wt.% washed cork powder.
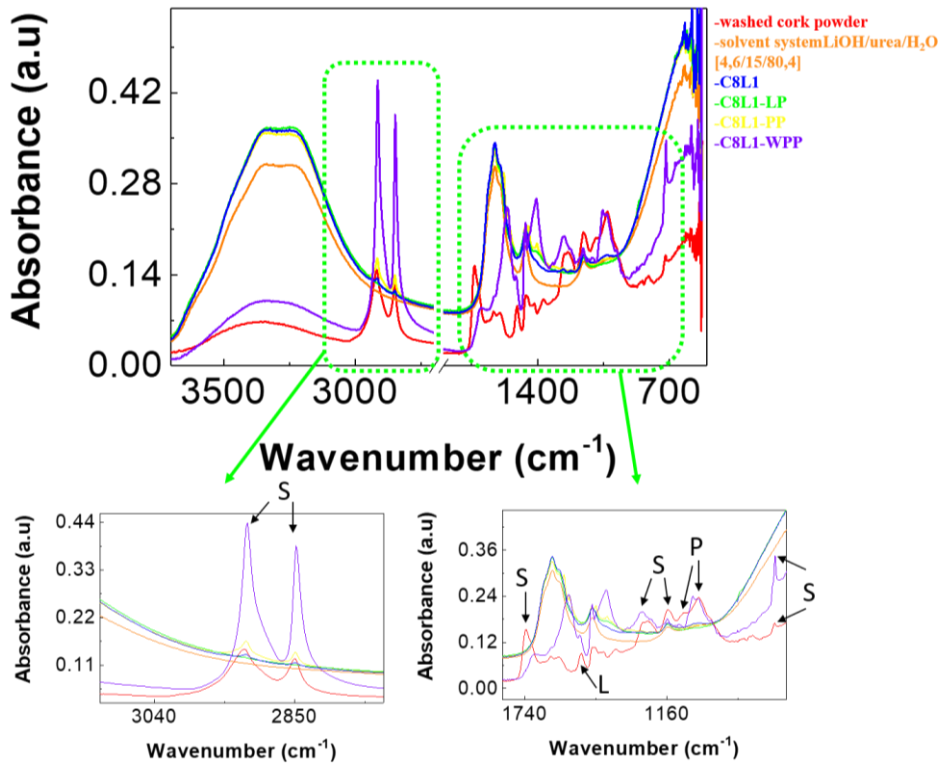
Figure 3.2 - Difference between peaks at all the stages of the process for a cork concentration of 8 wt.% (Table B. 2).

Two initial suberin peaks around 2850 and 2900 cm^{-1} are visible in the washed cork powder, C8L1-PP and with high absorbance at the C8L1-WPP, which are mainly attributed to the aliphatic chains of suberin, corresponding to symmetric C-H and asymmetric stretching vibrations, respectively. The remaining suberin peaks observed at 1740, 1160 and 700 cm^{-1} are also visible in the same samples, which are related to the presence of C=O stretching vibration from the ester groups, asymmetric C-O stretching, and C-H bend associated with vinyl groups, respectively.

Peaks of lignin and polysaccharides are only observed in the washed cork powder and C8L1-WPP. Furthermore, these two samples also exhibit a very broad band between 3500 and 3000 cm^{-1}, which is associated with the O-H stretching vibration and can be attributed to carboxylic acids and alcohol groups. In comparison, the sample C8L1-PP does not exhibit peaks of lignin, and the peaks observed in the range of 3500 and 3000 cm^{-1} can be related to the solvent system used for depolymerisation. On the other hand, all the mentioned peaks are not existent in the liquid phase of sample C8L1-LP, excepting the peak associated with the C-H bend from vinyl groups of suberin and the broad peaks correspondent to the solvent system.[18]

Figure 3.3 shows the influence of the cork concentration from 4 to 15 wt.% in the resulting liquid phase, pasty phase, and washed pasty phases obtained from alkaline hydrolysis process using a solvent system consisting of LiOH/ urea/ H$_2$O at a fixed concentration of 4.6/ 15/ 80.4 wt.%.
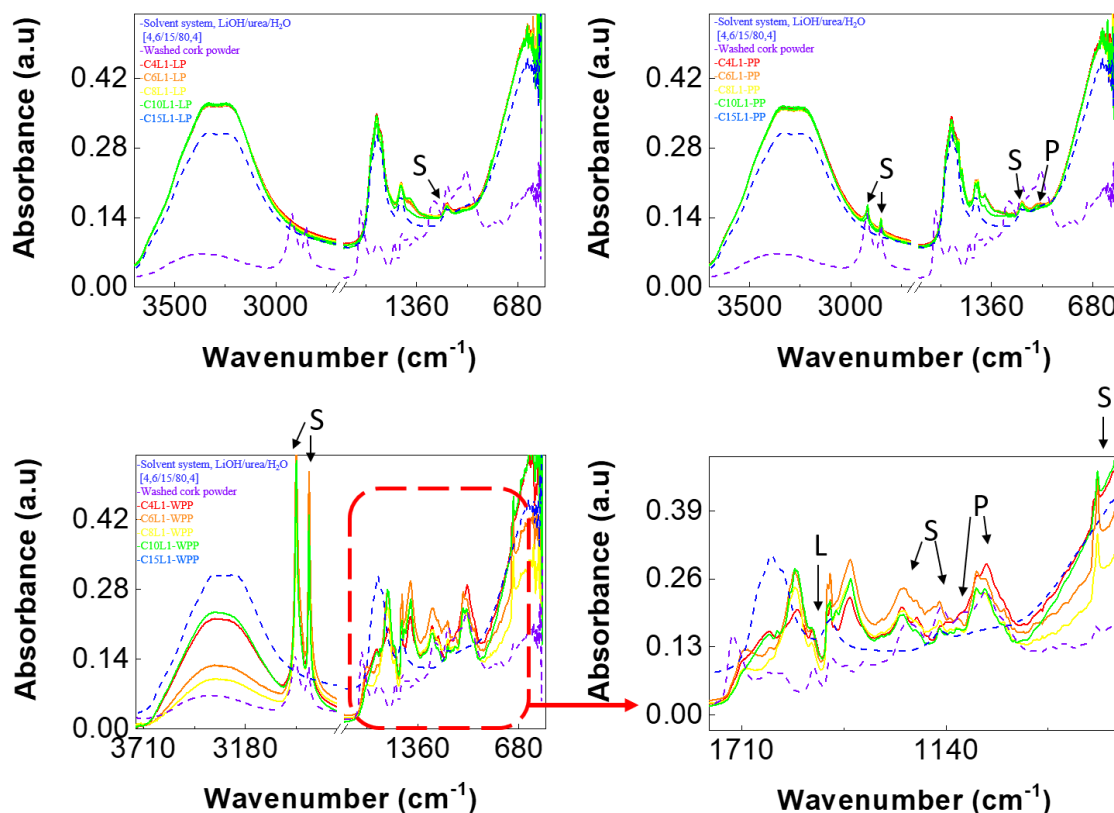
Figure 3.3 - Difference between peaks with the same solvent system for different cork concentrations through all procedures. Top left: difference between LP samples after centrifugation; Top right: difference between PP samples after centrifugation; Bottom left: difference between WPP samples after centrifugation and wash with deionised water; Bottom right: zoom from WPP samples between 1800 and 600 cm^{-1} (Table B. 2).

As previously observed in Figure 3.3, the results for the liquid phase or pasty phase are identical, which demonstrates that the concentration does not have any influence in the peaks observed. After washing the pasty phase in water to remove the remaining reagents alkaline hydrolysis process, the peaks correspondent to suberin are more intense, and it is clear the presence of lignin and polysaccharides. For all the tested concentrations, there is not any significant difference in the observed peaks.

Therefore, we can conclude that the resulting washed pasty phase consisted of a mixture of suberin-based material and lignocellulosic residue as reinforcement, which comprise lignin and cellulose structures obtained from the cork cell walls.

In order to optimize the extraction of the suberinic material, the proportion between the LiOH and urea was investigated for a fixed concentration of cork at 15 wt.% (Figure 3.4). A comparison between NaOH and LiOH was also performed, while keeping the cork concentration fixed at 15 wt.% and the alkaline salt/urea/H$_2$O solvent system at

4.6/15/80.4 wt.%. Note that the samples used for these studies were not centrifuged, and thus they are not stable overtime as the suberinic-based/ lignocellulosic material starts depositing in the flask after some hours, as already mentioned. Therefore, the solutions were tested right after vigorous stirring. The results demonstrate that there is some interference in the chemical composition of the extracted components depending on the proportion of the alkaline salt and urea used. The main differences are observed in the peaks at 1030 cm^{-1} and 1245 cm^{-1} correspondent to polysaccharides and symmetric C-O stretching from suberin, which are less evident in the solvent systems with high amounts of LiOH ($\geq$ 4.6 wt.%) as such concentrations promote a better dissolution of such materials, namely cellulose [21].

In addition, Figure 3.4 shows that there is no difference between LiOH and NaOH approaches, which demonstrates that NaOH is a suitable substitute of LiOH, adding extra benefits in the final formulation of resulting suberinic-lignocellulosic composite as it is low cost, less hazardous, and more abundant in comparison with scarce lithium sources.
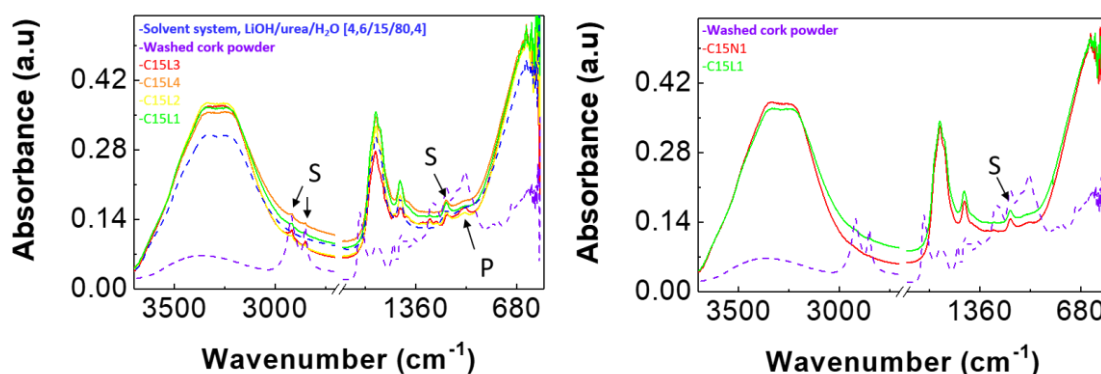
Figure 3.4 - Difference between peaks with different solvent systems for the same cork concentration 15 wt.%. Left: Suberin peaks evaluation based on various LiOH and urea proportions; Right: Suberin peaks evaluation for the difference between LiOH or NaOH (Table B. 2)

3.3 Antibacterial test

Antibacterial test is based on assessing the concentration of substance required to inhibit growth of a specific bacterial strain by measuring the growth inhibition halo created on the surface of an agar plate with a suitable culture medium and homogeneously inoculated with the bacteria under study. The size of the created halo may be affected by factors such as the diffusion capacity of the compound, the amount and concentration of the compound and the incubation time. pH is an important factor that must be controlled to ensure it has no major impact. As showed at Figure 3.5 reference samples were prepared for gram-negative (*E. coli)* or gram-positive (*S.aureus)* with the solvent system

used only to evaluate the antibacterial activity of the extracted components from cork using alkaline hydrolysis depolymerisation process.

Figure 3.5 – Wells filled with solvent system (LiOH/Urea/H2O – 4.6:15:80.4 wt. %) as a reference sample for both bacteria's (*S.aureus* at left and *E.coli* at right).

The liquid phases of samples C4L1-LP, C6L1-LP, C8L1-LP, C10L1-LP, and C15L1-LP were analysed in a culture of *S.aureus* or *E.coli*, as shown at Figure 3.6.

Figure 3.6 - Wells filled with cork LP samples (Table B. 2). Top row: Samples in a culture with *E. coli* bacteria; Bottom row: Samples in a culture with *S. aureus* bacteria.

As demonstrated in Table 3.1, by increasing cork concentration, it is possible to see an interference on the antibacterial activity in both cases through the blurred zones

around the wells. These halos also appear in the reference samples, suggesting that the solvent system has a key role in the interference on the growth of the antibacterial activity. However, a trend is observed with the increase of cork concentration, which indicates that cork will also influence the presented results. In particular, only the samples C8L1-LP and C15L1-LP exhibit an interference on the growth of the antibacterial activity of *S. aureus*. On the other hand, the addition of ZnO nanoparticles during the alkaline hydrolysis depolymerisation process is advantageous in the antibacterial activity in both cases. The sample C15L1Z-LP shows a completely transparent halo around the well, meaning complete inhibition and therefore antibacterial activity is confirmed due to the influence of the ZnO.

Table 3.1 - Results of antibacterial assays performed on liquid phase samples obtained from alkaline hydrolysis depolymerisation process.

Liquid samples	Growth Density Decrease Halo Diameter (mm) (lighter area around the well where the sample was placed)	
	E. coli	*S. aureus*
C4L1-LP	13 ± 1	-
C6L1-LP	12 ± 1	-
C8L1-LP	18 ± 1	20 ± 1
C10L1-LP	13 ± 1	-
C15L1-LP	20 ± 1	23 ± 1
Liquid samples	Growth Inhibition Halo Diameter (mm) (transparent zone around the well where the sample was placed)	
	E. coli	*S. aureus*
C4L1-LP	-	-
C6L1-LP	-	-
C8L1-LP	-	-
C10L1-LP	-	-
C15L1-LP	-	-
C15L1Z-LP	17 ± 1	13 ± 1

The samples correspondent to the pasty phase were also analysed after suspension in 500 µL of water (Figure 3.7). Only the samples obtained from concentrations higher than 8 wt.% showed antibacterial activity to gram-positive (*S. aureus*) due to the presence of the suberinic material. For lower concentrations, an interference in the growth of antibacterial activity, at the cost of blurred halo formations with lower diameters. For *E. Coli* the samples are slightly blurred, showing their poor interference in the growth of the antibacterial activity. Once again, the presence of ZnO nanoparticles in the composition of the extracted suberinic/lignocellulosic composite enhances the antibacterial activity for both cases.

It should be pointed out that the high viscosity of these samples directly affects the quality of the obtained results because it promotes migration issues, and thus the antibacterial agent cannot interact efficiently. To improve the results, a different method consisting of agar dilution method can considerably address such problem.

Figure 3.7 - Wells filled with cork PP samples (Table B. 2). Top row: Samples in a culture with *E. coli* bacteria; Bottom row: Samples in a culture with *S. aureus* bacteria.

Table 3.2 -Results of antibacterial assays performed on pasty phase samples obtained from alkaline hydrolysis depolymerisation process.

Pasty phase samples	Growth Inhibition Halo Diameter (mm) (transparent zone around the well where the sample was placed)	
	E. coli	*S. aureus*
C4L1-PP	-	-
C6L1-PP	-	-
C8L1-PP	-	7 ± 1
C10L1-PP	-	7 ± 1
C15L1-PP	-	9 ± 1
C15L1Z-PP	13 ± 1	13 ± 1

In a similar way, a poorer response is observed on the samples WPP due to superior viscosity in comparison with the latter case (Figure 3.8). Besides, it is possible to visualize the generation of a precipitate inside the well in the samples with higher concentration of cork, which is an indicator of non-migration of antibacterial agent. The sample with ZnO is the only that exhibits some antibacterial inhibition for the gram-positive *S. aureus*.

Figure 3.8 - Wells filled with cork WPP samples (Table B. 2). Top row: Samples in a culture with *E. coli* bacteria; Bottom row: Samples in a culture with *S. aureus* bacteria.

3.4 Suberinic/lignocellulosic-based composite fibers

Targeting the application of the extracted suberinic/lignocellulosic material from cork for medical applications, such as wound dressing, tissue sutures, filter membranes and adsorbents, composite fibers were prepared by SBS technique.

PEO together with dichloromethane, it is a good combination because the solvent doesn't need high temperatures to evaporate and a PEO matrix can promote the creation of uniform fibers [26]. It was possible to observe through the Figure 3.9 fibers had a consistent morphology with small variations in diameter with 5µm and lower. An explanation for this is that a balance must be achieved between solution viscosity and the fiber-forming forces that are derived from the pressurized air exiting the outer nozzle in order to produce fibers with regular cross-sections. Variations in air flow would then impart different degrees of stretching and shearing on fibers being formed. However, higher air flow was generally required because the viscosity of the polymer solution is high. For

higher viscosity polymer solutions, fiber stretching would become more difficult, less efficient, and unstable giving rise to broader fiber diameter distribution. Some beats are visible and come from unevaporated solvent.
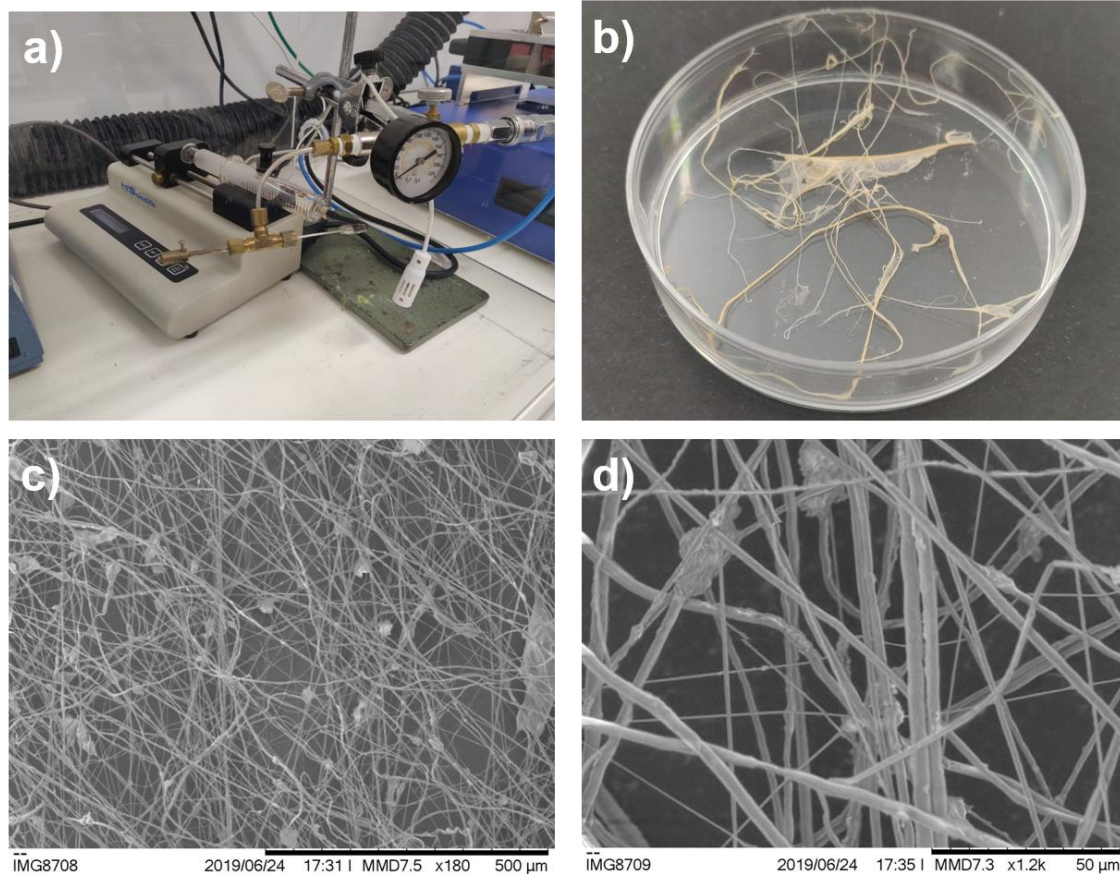

Figure 3.9 - a) montage used at SBS technique; b) suberinic nanofibers produced; c) & d) SEM images from the suberinic fibers at 500µm and 50µm.

4 Conclusion

The formulation for suberin antibacterial activity was improved. Changing LiOH for NaOH we can obtain the same results and have a greener protocol to work on. Numerous acids were tested and can be concluded that a weak acid is not appropriate for the neutralization process. Depending on the application this choice can be an important fact to have in account.

Overall the antibacterial result gives critical information regarding suberin antibacterial activity. It was possible to concluded as shown from FTIR peaks that, Suberinic/lignocellulosic-based samples, mostly on PP samples give positive results despite migration problems that can be explored in future work. Despite WPP samples have the highest suberin peaks, the method used was not the most accurate. Using an alternative antibacterial method we can improve and get better result in these study.

The suberin-based lignocellulosic fibers resulting from SBS were a little tricky to produce. Although the simplicity of the technique, the parameters were not optimized, and the result was a reduced density of fibers. The use of the PEO/ dichloromethane combination was in fact a good choice because in the first try it was possible to produce these fibers at a room temperature.

5 Future Perspectives

Regarding the suberinic/ lignocellulosic-based composite, these formulations can be improved even further. As shown previously, it is possible to reduce the proportion of LiOH and urea without affecting the suberin peaks. Also, LiOH can be replace for NaOH to investigate the extraction of suberinic material comparably with the suberinic/ lignocellulosic-based composite that was produced.

The dissolution method used for the antibacterial assay, was not the most accurate. More than half the samples present migration problems and some even precipitate inside the well. The alternative is called agar dilution and it is one of the most typically used techniques to determine the minimal inhibitory concentration. This technique can determine the lowest concentration of the assayed antimicrobial agent that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. This technique is used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents.[28]

Ideally a PEO /suberinic-based lignocellulosic nanofiber blanket would have been obtained but since this formulation was never tested, adjustments to the parameters and formulation require some attention. SBS technique has the advantage of ease use allowing the production of nano or microfibres and the direct fibre deposition on any surface. Yet, in spite of these advantages, very little is known about the influence of such fibres on biological functions such as immune response and cell migration. As mentioned previously the study of antibacterial activity in this fibers is a must, so we can apply them in areas such as medical and electronic devices.

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Annex A

Figure A. 1 - Membrane obtain by shear casting/ doctor blade after neutralization with numerous acids.

Anexo B

Table B. 1 - Relative abundance of aliphatic suberin in the extractive-free outer bark of some higher plants and periderm of Solanum tuberosum ([a] Preliminary sequential boiling solvent extraction: (1) $CHCl_3$+MeOH; (2) CH_2Cl_2+EtOH+water; (3) no solvent extraction; (4) acetone; (5) CH_2Cl_2+EtOH+water+MeOH) [17].

Species	Percentage of extracted suberin (%)	Extraction method[a]
Laburnum anagyroides	61.7	0.5M MeONa in MeOH [1]
Fagus sylvatica	48.3	
Castanea sativa	43.2	
Quercus robur	39.7	
Populus tremula	37.9	
Cupressus leylandii	27.5	
Acer pseudoplatanus	26.6	
Acer griseum	26.1	
Quercus ilex	24.9	
Fraxinus excelsior	22.1	
Sambucus nigra	21.7	
Ribes nigrum	21.1	
Euonymus alatus	8.0	
Pseudotuga menziesii	53.0	0.02–0.03M MeONa in MeOH [2]
Betula pendula	58.6	0.5M MeONa in MeOH[1]
	51.0	1.3M MeONa in MeOH[1]
	32.2	0.5M KOH in EtOH/H2O(9:1, v/v)[3]
	49.3	0.5M KOH in EtOH/H2O (9:1, v/v)[3]
	46.0	96% H2SO4 in MeOH (1/9, v/v)[4]
Quercus suber	43.3	0.5M NaOMe in MeOH[1]
	37.8-41.2	3% MeONa in MeOH[2]
	37.0	0.1M NaOH in MeOH[2]
	60.0	0.02–0.03M MeONa in MeOH[2]
	62.0	3% MeONa in MeOH
	40.0-45.0	3% MeONa in MeOH[2]
	54.0-56.0	1–3% MeONa in MeOH
Solanum tuberosum	12.1	0.5M NaOMe in MeOH[1]
	25	0.0012M NaOMe in MeOH[5]

Table B. 2 - Nomenclature attributed for all the prepared samples, depending on the concentration of cork, solvent system used for the alkaline hydrolysis depolymerisation process and obtained phase separation (liquid phase vs. "suberinic" pasty phase vs. Washed "suberinic" pasty phase).

Nomenclature	Cork concentration (wt.%)	Solvent system (wt.%)	Phase separation
C4L1	4		
C6L1	6		
C8L1	8		NO*
C10L1	10		
C15L1	15		
C4L1-LP	4		
C6L1-LP	6		
C8L1-LP	8		Liquid phase
C10L1-LP	10		
C15L1-LP	15	LiOH/urea/H_2O: 4.6/15/80.4	
C4L1-PP	4		
C6L1-PP	6		
C8L1-PP	8		Pasty phase
C10L1-PP	10		
C15L1-PP	15		
C4L1-WPP	4		
C6L1-WPP	6		
C8L1-WPP	8		Pasty phase**
C10L1-WPP	10		
C15L1-WPP	15		
C15L2		LiOH/urea/H_2O: 2.3/15/82.7	
C15L3		LiOH/urea/H_2O: 2.3/7.5/90.2	
C15L4	15	LiOH/urea/H_2O: 7/15/78	NO*
C15N1		NaOH/urea/H_2O: 4.6/15/80.4	
C15L1Z		LiOH/urea/ZnO/H_2O: 4.6/15/0.5/79.9	NO*
C15L1Z-LP			Liquid phase
C15L1Z-PP			Pasty phase
C15L1Z-WPP	15	LiOH/urea/ZnO/H_2O: 4.6/15/0.5/79.9	Pasty**

* Solutions prepared without centrifugation step after neutralization with acetic acid.

** Washing with water of the obtained "suberinic" pasty phase