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Desenvolvimento de filmes poliméricos biodegradáveis com polissacarídeos da casca de castanha (Castanea sativa)

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POLITÉCNICO
DO PORTO

Desenvolvimento de filmes poliméricos biodegradáveis com polissacarídeos da casca de castanha (*Castanea sativa)*

Development of biodegradable polymer films from C*astanea sativa* shells polysaccharides

Grupo de Reação de Análises Químicas (GRAQ), ISEP

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Resumo

O amido tem ganho importância na área dos biomateriais e das embalagens alimentares por ser de baixo custo, biodegradável e de fácil processamento. No entanto, este polímero não apresenta boas propriedades mecânicas quando comparado com os materiais à base de petróleo comumente usados. De forma a melhorar estas propriedades, incorporam-se outros compostos, sintéticos ou naturais, como, por exemplo, nanopartículas ou fibras para reforçar a estrutura dos novos materiais. As fibras, em particular, podem ser obtidas a partir dos subprodutos da indústria das castanhas, sendo uma forma de promoção da sustentabilidade económica e ambiental deste sector industrial.

Este trabalho de investigação teve com objetivo desenvolver um filme de amido reforçado com fibras e lenhina, extraídos por tratamento alcalino dos resíduos da casca de castanha, com propriedades mecânicas melhoradas. A composição do filme foi otimizada por Metodologia de Superfície de Resposta, tendo sido avaliadas as propriedades mecânicas elongação, força de tensão e módulo de elasticidade.

A composição ótima do filme de amido foi obtida com 10% (m/m) de fibras de castanha e 50% (m/m) de glicerol. As respostas de elongação, força de tensão e módulo de elasticidade atingiram valores de, respetivamente, 34,19 %, 7,31 N e 4,15 N para o filme com fibra de castanha. Os valores de força de tensão e módulo de elasticidade foram aproximadamente 3,5 vezes superiores aos obtidos para o filme controlo (sem fibra e sem lenhina e com 50% (m/m) de glicerol).

O modelo otimizado permitiu concluir que a lenhina não é um componente de reforço das propriedades mecânicas do filme de amido. Recorrendo à análise por microscopia eletrónica de varrimento constatou-se que o Ultra-Turrax influencia a morfologia das fibras e também, que apesar de se observarem algumas diferenças entre as fibras branqueadas e não branqueadas, as propriedades mecânicas do filme não são afetadas por este aspeto. Comparando com a literatura, os resultados obtidos para as propriedades mecânicas dos filmes de amido reforçados com fibras têm uma evolução dentro do expectável, revelando que as fibras provenientes das castanhas podem ser usadas como reforço e que o branqueamento das mesmas não produz qualquer efeito significativo nas propriedades das fibras.

Para trabalhos futuros sugere-se o estudo de outras propriedades mecânicas, como a permeação ao oxigénio e à água, e propriedades físicas, como solubilidade e propriedades óticas. Estudos sobre a humidade relativa dos filmes e a interação deste fator com diferentes plastificantes também poderá ser abordada, visto a hipótese de ser um fator determinante nas propriedades mecânicas do filme. No campo das embalagens alimentares, a aplicação de extratos em filmes de amido, refletindo-se em atividades antioxidante e antimicrobiana, poderá ser avaliado. Ainda neste campo, a estabilidade das propriedades do filme ao longo do tempo também poderá ser avaliada de forma a garantir a viabilidade do uso de filmes de amido no embalamento de alimentos.

Palavras-chave: Subprodutos da castanha; Filmes de amido; Metodologia de Superfície de Resposta; Propriedades mecânicas;

Abstract

Starch has gained great importance in the biomaterials and food packaging fields due to it is low cost, biodegradable and easy to process. However, this polymer does not have good mechanical properties when compared to the commonly used petroleum-based materials. In order to improve these properties, fillers, synthetic or natural, such as, for example, nanoparticles or fibers, are used to reinforce the structure of new materials. The fibers can be obtained from the chestnut industry by-products, being a way of promoting the economic and environmental sustainability of this industrial sector.

This research work aimed to develop a starch-based film reinforced with fiber and lignin, extracted by alkaline treatment of chestnut residues, with improved mechanical properties. The composition of the film was optimized by Response Surface Methodology, the mechanical properties evaluated included the elongation, tensile strength and elasticity modulus.

The optimum composition of the starch-based film was obtained with 10% (w / w) brown fibers and 50% (w / w) glycerol. The elongation responses, tensile strength and modulus of elasticity reached values of 34.19%, 7.31 N and 4.15 N, respectively, for the film with chestnut fiber. The values of tension strength and modulus of elasticity were approximately 3.5 times higher than those obtained for the control film (without fiber and without lignin and with 50% (w / w) glycerol).

The optimized model showed that lignin from chestnut residues is not a good reinforcement component of the mechanical properties of the developed starch-based film. The scanning electron microscopy analysis showed that the application of Ultra-Turrax influences the fiber morphology. Also, some morphological differences between the bleached and unbleached fibers were observed, although this aspect did not affect the mechanical properties of the film. The results obtained for the mechanical properties of the starch-based films are comparable with literature, revealing that the fibers from the chestnuts can be used as reinforcements and that their bleaching treatment does not significantly affect the fiber properties.

For future work other mechanical properties, such as oxygen and water permeation, and other physical properties, namely solubility and optical properties, should be evaluated. Studies on the relative humidity of the films and the interaction of this factor with different plasticizers can also be addressed, given the hypothesis of being

a determining factor in the mechanical properties. In the field of food packaging, the addition of extracts with antioxidant and antimicrobial activities in the starch-based film composition can also be evaluated. Furthermore, the stability of the properties of the film over time can also be evaluated to guarantee the viability of using starch-based films for food packaging purposes.

Keywords: Chestnut by-products; Starch-based film; Responsive Surface Methodology; Mechanical properties.

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Abbreviations

AAR – Amylose : amylopectin ratio

BBD - Box-Behnken design

CF - Chestnut fiber

CL - Chestnut lignin

CS - Chestnut shell

db – dry basis

DCS - Differential scanning calorimetry

EC - European Comission

PEG – Poly (ethylene glycol)

PVA - Poly (vinyl alcohol)

r.s. - related to starch

RH - Relative humidity

RSM - Response surface methodology

SEM - Scanning electron microscopy

Tg - Transition temperature

1. Introduction

The last decades have been marked by an increase of petroleum-based plastics consumption [1]. According to the European Commission (EC), Europe annually produces 58 million tons of plastic, preferably made from petroleum sources [2]. The plastic is mostly used for packaging (40%) and consumer and household goods (22%) [2]. Among other advantages, petroleum-based plastics possess good mechanical and insulating properties, low cost, and high convenience for people, with several day-by-day applications [3].

According to the last *Plastics Europe (The Association of Plastics Manufacturers in Europe)* report (2019), Europe produces 25.8 million tons of plastic waste per year. Of this amount, 39% is incinerated, 31% is displaced in landfills, less than 30% is recycled and 5-4 % ends up on sea [1]. This last practice represents an enormous environmental and economic concern [1]. Due to several environmental and economic issues caused by an increase of plastic consumption and waste over the years, in 2017 the European Commission set some strategies to reduce the production and waste of this material [4]. The main strategies stablished by the EC were: *(i)* reduce the production of petroleum-based materials, *(ii)* decrease the greenhouse gas emissions and dependence of fossil fuels, and *(iii)* support the creation of innovative materials, more sustainable than non-renewable alternatives [4].

Bio-based materials derived from biological sources represents a renewable alternative to traditional plastics [5]. However, it is important to measure the sustainability of bio-based polymers when compared to petroleum-based polymers. For this purpose, aspects such as toxicity, land and water use, as well as carbon balance should be evaluated [5]. Also, the pollution generated during the production and recycle steps of these bio-based polymers are important parameters. In this sense, the concept of biodegradability is very important on polymers evaluation. Biodegradability reflects the ability of a material to be decomposed by microorganisms, releasing methane, carbon dioxide and inorganic compounds under a certain period of time [6]. Bio-based polymers, as bio-polyethylene terephthalate (bio-PET) and bio-polyethylene (bio-PE), are not biodegradable [5]. In contrast, petroleum-based polymers, such as polybutylene adipate terephthalate (PBAT) and polybutylene succinate (PBS), could be biodegradable [5].

Figure 1.1. presents several biodegradable polymers according to its source. Proteins, lipids and polysaccharides are biodegradable and bio-based polymer presents in obtained directly from biomass, without complex processes (e.g. fermentation). Starch, in particular, is a promising material due to its low cost, easy production, non-toxicity and edible form [8]. Indeed, starch is considered a thermoplastic, commonly named thermoplastic starch (TPS), which means that it could be shaped with application of heat. TPS is commonly employed in several applications and industries, for example, for drug delivery, cosmetic powders, adhesives production and biodegradable filler in plastics production [9].

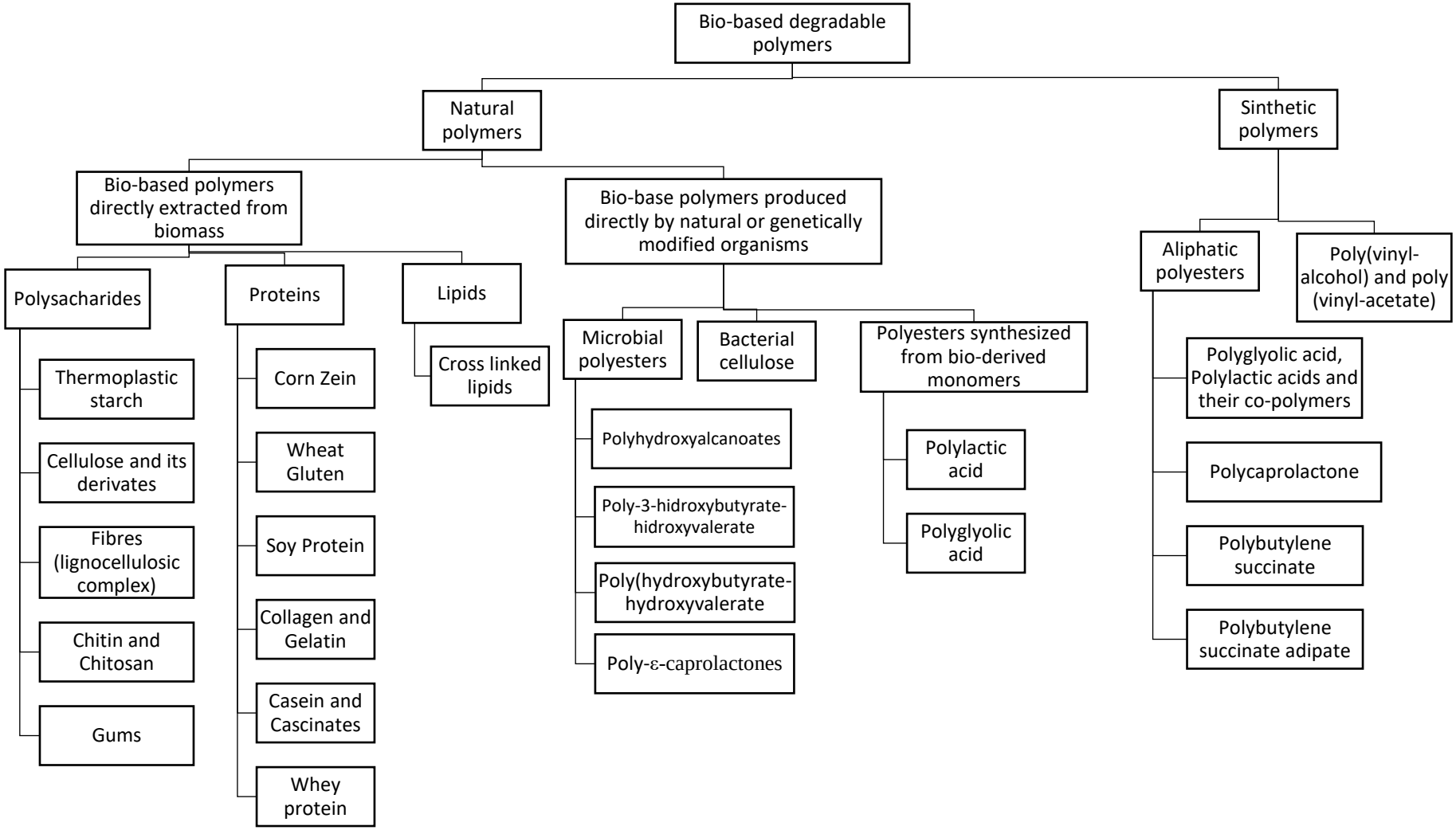

Figure 1.1.- Representation of biodegradable polymers according to its source [7].

1.1 Starch

Starch is the energy reserve of plants and is widely extracted from rice, cassava, corn, potato, barley, and other vegetables or fruits [3]. The production of starch is initiated with the conversion of carbon dioxide (CO_2) in the atmosphere into α-D-glucose (Glc). Starch is a mixture of two polymers: amylose and amylopectin. As shown in Figure 1.2., amylose is a glucose linear chain through α (1→4) glycosidic bonds, while amylopectin consists of a glucose chain with α (1→4) glycosidic bond and chain branches made by α (1→6) bonds, that occurs in each 24-30 glucose units [3]. Amylopectin chains are organized in a double helix, with hydrogen links between hydroxyl groups [8].

This polymer can be extracted by a simple process. Basically, the starch source is milled or shredded and mix with water; filtered, and the liquid fraction is dried to obtain a white solid fraction (starch). This procedure has been applied (with some modifications) by several authors [9-11].

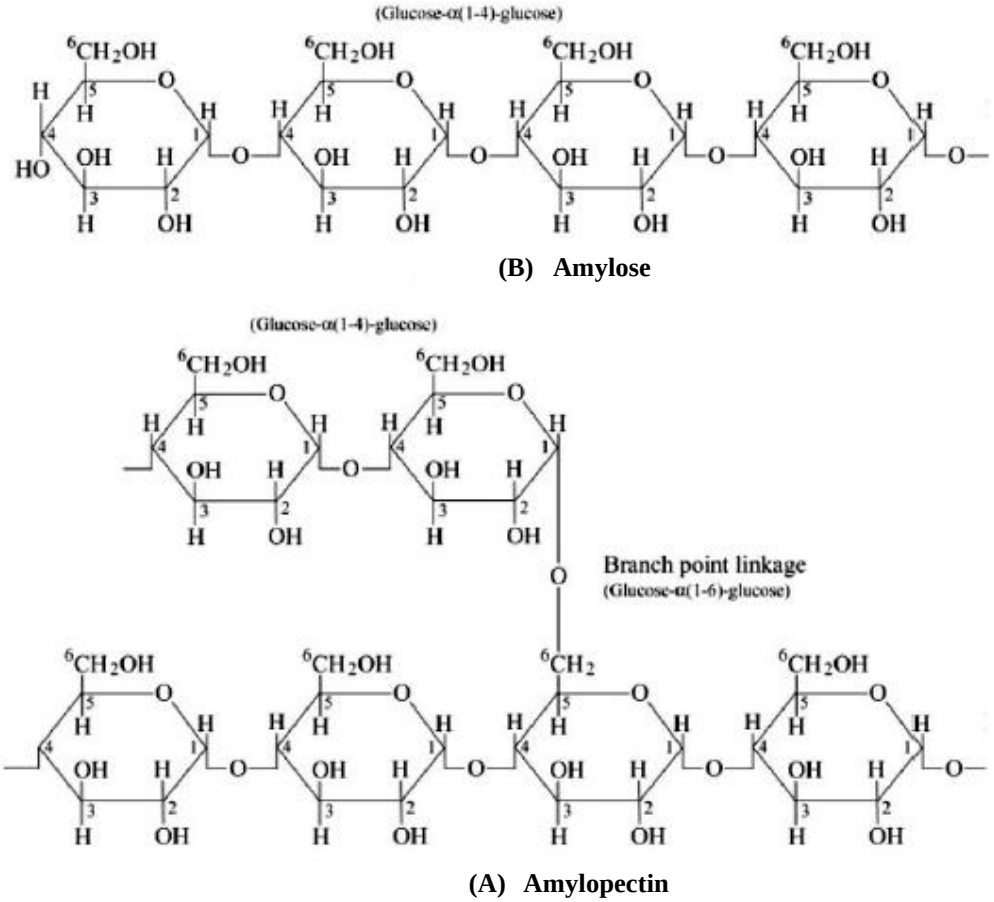

Figure 1.2.- Chemical structure of amylose **(A)** and amylopectin **(B)** [3].

The amylose: amylopectin ratio (AAR) depends on starch source and is influenced by crops conditions. Table 1.1. shows some reference values for AAR in different sources of starch. The low amylose content value (17%-35%) of starch has leading companies around the world to develop modified starch sources, increasing this value up to 48-77% [12]. The AAR can be calculated with determination of total amylose content of starch [9]. This colorimetric method consists in the interaction between iodine and amylose in a single left handed V-type helix, making starch dispersion solution exhibit a blue color [13]. Briefly, the collected starch is defatted and dispersed in dimethyl sulfoxide, then the absorbance of the solution is measured at 600 nm and the percentage of amylose is calculated from the standard curve [13].

Table 1.1.- Amylose: Amylopectin ratio (AAR) of different sources of starch.

Source	Amylopectin (%)	Amylose (%)	Ref
Corn	79	21	[12]
Wheat	78	22	[12]
Chestnut	80	20	[10]
Potato	78	22	[10]
Banana	79	21	[12]
Cassava	83	17	[14]
	78	22	[1]
Ulluco	65	35	[9]

The granules size and characteristics depends on the starch source [12]. The starch crystallinity is closely related to AAR, being amylose and branching zones responsible for the amorphous regions, while amylopectin is responsible for the crystalline structure. According to Ratnayake and Jackson [15], different amylopectin chains interact with themselves, creating crystalline lamellas, as can be observed in Figure 1.3

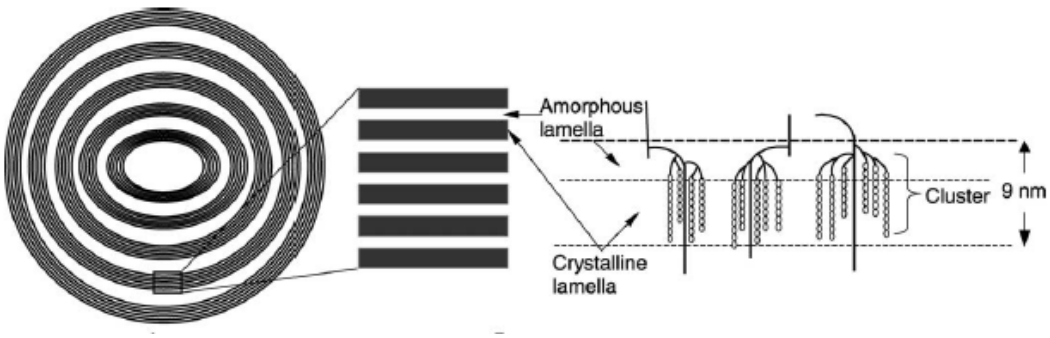

Figure 1.3.- Schematic representation of crystalline structures of starch granules [16].

Four allomorphic structures can be found in starch [15]**:**

- **A-type:** structure found in cereals produced in dry and warm conditions. The structure is made by a double helix conformation with six glucose units per turn. The helices are displaced in a monocyclic structure with 8 water molecules per unit cell (Figure 1.4.).
- **B-type:** structure find in tubers in starch with high amylose content. The structure is made by a left-handed double helix with six glucose units per turn in a hexagonal system containing 36 water molecules per unit cell (Figure 1.4.).
- **C-type:** structure mix A and B-type structures and commonly present in vegetables.

  V-type: structure synthesized in the presence of small molecules like iodine or fatty acids. The structure is made by a simple left helix with six glucose units per turn.

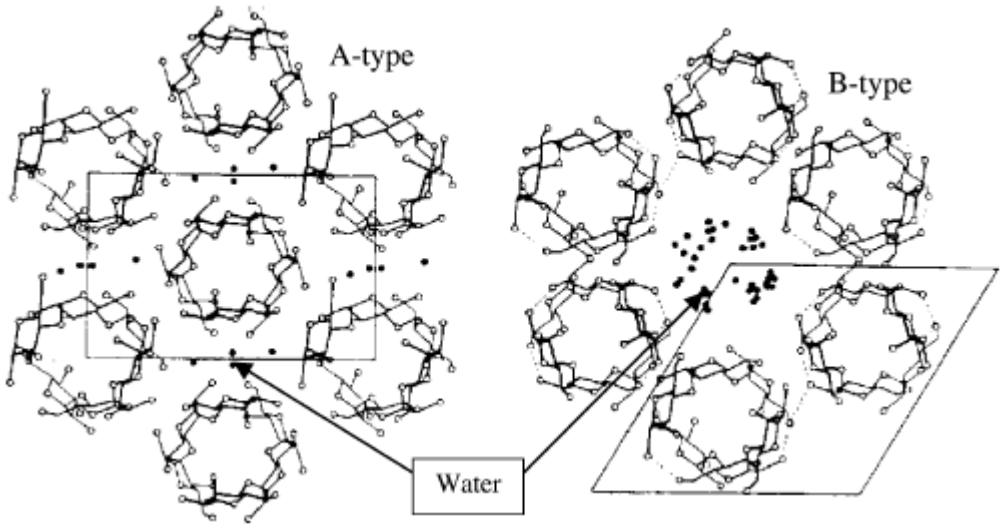

Figure 1.4.- A-type and B-type allomorphic structures found in starch [16].

The allomorphic structures of starch can be evaluated using X-Ray diffraction; each structure has a different pattern, as can be observed in Figure 1.5. [15].

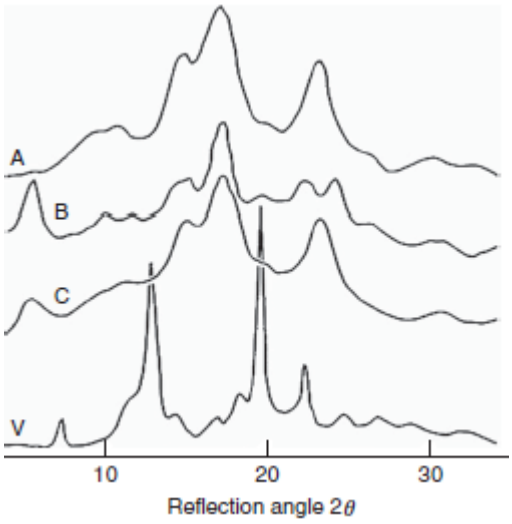

Figure 1.5.- X-ray diffraction of different allomorphic structures found in starch [15].

The gelatinization process occurs when starch is heated in water. This is an irreversible process of molecular reorganization, as shown in Figure 1.6.-(A). In the presence of water and heat, the hydrogen bond between starch chains is destroyed, leading to the disruption of starch granules, amylose diffusion and amylopectin collapse, resulting in amylose-amylopectin matrix [15]. In plasticized starch, it is observed the absence of crystalline structures and the predominance of amorphous parts [3]. Figure 1.6.-(B) shows the difference between the X-ray spectrum from starch before (native starch) and after heat treatment (plasticized starch). Due to crystallinity of native starch the spectrum shows a defined pattern with peaks. On the other hand, plasticized starch shows a flat spectrum without peaks, showing an amorphous nature.

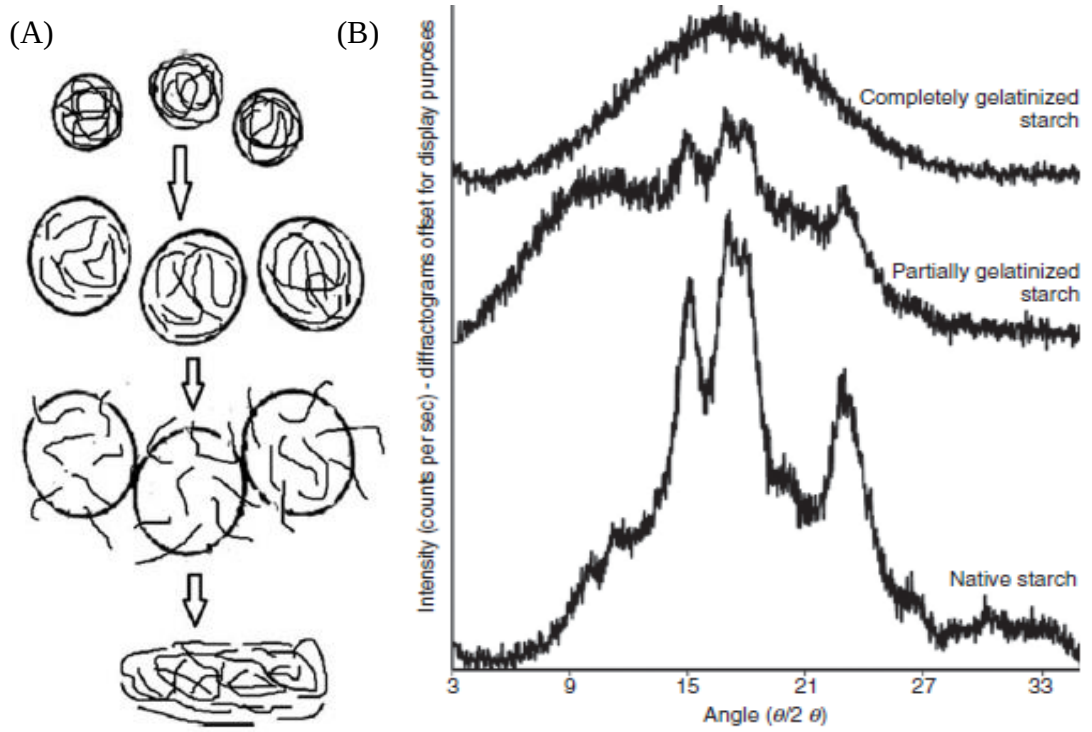

Figure 1.6.-**(A)** Gelatinization process of starch granules [3]; **(B)** X-ray diffraction profiles before, during, and after gelatinization process [15].

Retrogradation is a process of recrystallization of gelatinized starch when it is cooled. The decrease of temperature leads to an organization of starch chains and to the formation of new hydrogen bonds. This process could happen in several days, depending on the storage conditions [3,17]. The retrogradation process made films more brittle, which can be considered a limitation of starch-based films [3]. Soni *et al.* [18] integrated retrograded starch in starch-based films in order to improve the water resistance of the film. The retrograded starch was prepared after gelatinization (1 h, 90 ºC) and keep at room conditions (~22 ºC, 25% relative humidity (RH)) for 5 days. The starch films were produced by casting and using cellulose nanofiber as filler. Data showed an increase of water contact angle of retrograded starch-film increased (64.2º ± 3.2) in comparison to non-retrograded starch-film (55.3º ± 2.1). In addition, the hydrophobicity of the retrograded starch-based film also increased, which is important for the improvement of water resistance and the general mechanical properties of the film. Several water soluble polymers, such as poly(vinyl alcohol) (PVA) or *n*-propyl alcohol, could be added to gelatinized starch to limit retrogradation [3]. For instance, PVA can breakdown the starch units through hydrolytic attack, modifying polymer structure at molecular and morphological levels. Within this modification, PVA also improves the thermal and

mechanical properties of the final polymer [19]. Seligra *et al*. [20] suggested the use of citric acid (CA) in starch-glycerol films as cross linking agent. The authors reported that the addition of CA to starch-water-glycerol solution decreased the temperature of gelatinization around 5 ºC, when compared to the solution without CA. Apparently, several reactions between CA, glycerol and starch occurred, providing a decrease in water vapor permeation (WVP) of about 35% in the final film and making the film non-retrogradable.

1.2 Starch films production and properties

Starch-based films can be produced by extrusion (more suitable to industrial scale) or by casting solution [21]. In the extrusion method, the combination of heat and pressure in a single or twin-screw extruder become starch granules more thermoplastic [21]. This method enables the production of polymers with high viscosity degree and improves the process control [22]. However, chemical or physical modification of starch thermoplastic may occur, leading to air bubbles in the films [3]. An important condition of the extrusion process is the rotation speed of screws [23]. Seligra *et al.* [23] studied different screw's rotation speed in the extrusion of a mixture of cassava starch, glycerol and water. The results showed that rotation speed above 80 rounds per minute (rpm) produced homogeneous films, whereas rotation speed of 120 rpm led to a greater modulus and tensile strength, and a slower retrogradation [23]. According to Yan *et al*. [24], the extrusion process, prior to film formation, leads to an increase of elongation at break and reduce barrier properties when compared with films obtained through solution casting method. The solution casting is a simplest process, being normally used at laboratory scale [21]. The process consists in displaced an aqueous suspension of gelatinized starch in a mold and dry at 30-50 ºC with air circulation. The thickness and size of the resulting film depends on the mass and viscosity of the suspension putted into the mold [21].

Thakur *et al.* [22] made an extensive review about the influencing factors of starch-based films. According to the authors, the granule shape and size, AAR and chain lengths of starch affect de thickness of the films. Indeed, thickness affects permeability, optical and mechanical properties of the films. Regarding films solubilization, AAR had an important role; the presence of high amounts of amylopectin leads to a decrease of film solubilization and poor mechanical properties due to amylopectin aggregation. In

opposite, a high amylose content (> 70%) increase strength, flexibility, and gas barrier properties of starch films. Li *et al.* [25] reported that higher amounts of amylose decrease the processability of starch; however, the addition of water and the increase of temperature or screw speed may facilitate the processability of starch. Starches with high amylose content need more temperature and moisture due to the higher gelatinization temperature [25]. Other parameters, namely temperature, time of gelatinization, and plasticizers, affect the properties of casting films [22]. Different authors suggest different temperature and time conditions, which frequently are not correlated with the obtained properties [9,11,26]. The most frequent temperature used in gelatinization process is around 90 ºC. Nevertheless, the glass transition temperature (Tg), which represent the phase transition temperature, is not specific, varying with AAR, moisture content, ingredient concentration or chain length of starch source [22]. A higher Tg represents the necessity of a higher temperature of gelatinization. Liu *et al.* [27] studied the Tg for starches with different AAR. The authors analyzed four types of starch from a 0% amylose content with a crystallinity of 42.3% to an 80% amylose content with 28.3% of crystallinity. The results showed an increase of Tg from 52 ºC to 60 ºC with the increase of amylose content from 0 to 80 %.

Plasticizers are widely used to improve the mechanical properties and processability of starch films. The most common plasticizers for edible starch composites are glycerol and poly(ethylene glycol) (PEG) [22]. Plasticizers act like linking agents between starch chains, reducing the intermolecular force by decrease the internal hydrogen bonds and increasing the chain mobility by improving the intermolecular spacing [28,29]. Plasticizers also decrease Tg, improving ductility and extensibility [29]. Laohakunjit and Noomhorm [28] employed different amounts of PEG, sorbitol and glycerol to produced starch films from rice starch. The authors observed that PEG produced white and opaque films, while sorbitol and glycerol produced transparent films. On the other hand, the authors verified that higher amounts of glycerol (35%) and sorbitol (40%) made undetachable films, while films containing 6% and 9% of PEG become white, opaque and brittle, due to plasticizer concentration that exceed the compatibility limit of starch, resulting in physical exclusion of PEG [28]. The addition of plasticizers also affects other properties. For instance, higher amounts of plasticizers increase films

thickness and solubilization. Due to the higher hygroscopicity behavior, starch films with glycerol show higher solubilization than films with sorbitol.

Starch films have poor mechanical properties in comparison to petroleum-based polymers, which represents a limitation on its use [30]. The addition of fillers is commonly employed to increase the mechanical properties of starch film. In fact, several works have been emerged, suggesting fillers obtained from different sources, specifically from agro-industrial by-products [26,31-34]. Within this strategy, a minimum agro-food waste is achieved, leading to a major valorization of natural resources. Lignin and fibers have been studied as a suitable option for fillers, improving mechanical properties of starch films. Table 1.2. describes the increase or decrease of several properties in comparison to starch-based film without filler. In general, the authors suggest cellulose fibers/nanofibers as fillers [1,34,37-44] to achieve an increase of tensile strength, elastic modulus, Tg and water contact angle properties, and a decrease of elongation, water vapor permeation, oxygen permeation, moisture and water absorption. Miranda *et al*. [31] used piassava (*Attalea funifera Martius*) lignin to improve the mechanical properties of starch-based film. The authors reported an increase of tensile strength and elastic modulus, but a decrease of elongation.

In addition to mechanical and physicochemical properties, several authors added fillers to starch-based films to enhance their antioxidant and antimicrobial properties. Piñeros-Hernandez *et al.* [43] added a rosemary (*Salvia rosmarinus*) aqueous extract to cassava starch film formulation, leading to an increase of antioxidant activity and ultraviolet (UV) blocking properties. Tongdeesootorn *et al.* [44] studied the incorporation of quercetin and tertiary butylhydroquinone (TBHG) in cassava starch films. This addition modified the mechanical and physicochemical properties and retarded the oxidation of some fresh food products. Pinzon *et al.* [45] included *Aloe vera* gel in starch-based film; the results showed an increase of shelf life of strawberries up to 15 days. Regarding antimicrobial activity, pomegranate (*Punica granatum*) peel [48,49] and turmeric spent [48] were incorporated in starch-based films, having a positive effect against *Salmonella* strains, *Staphylococcus aureus* and *Escherichia coli*.

Table 1.2.- Effects of several fillers in the physicochemical and mechanical properties of starch-based films.

Filler	**Production method**	**Filler addition**	**Properties**		**Ref**
			Increase	**Decrease**	
Piassava lignin	Solution casting	Lignin up to 4:1 (lignin: glycerol, until a total of 50 % (w/w) starch mass)	E, σ	Ɛ	[31]
Coffee and rice husks fiber	Extrusion	Fiber up to 10 % (w/w) related to starch	E, σ	Ɛ, M, WVP, OP	[32]
Cellulose nanofibers	Extrusion	Nanofibers up to 1,5 % (w/w) total mass	Tg	WA	[35]
Cellulose nanofibers	Extrusion	Nanofiber up to 6% (w/w) related to starch	E, σ, WCA	Ɛ	[1]
Cassava peel	Solution Casting	Cassava peel up to 6% (w/w) of total mass	E, σ, M, BD	WS, Ɛ	[11]
Cassava starch nanocrystals	Solution casting	Nanocrystals up to 10% (w/w) of total mass	E, σ, M	WVP, Ɛ	[14]
Apricot and walnut shells	Solution casting	Milled shells up to 10% (w/w) of total mass	σ, WS	O, M, Ɛ	[26]
Cassava Fibers	Solution casting	Cassava fibers up to 3% (w/w) of total mass	σ, WS, UV	O, WVP, Ɛ	[36]
Cellulose and starch nanoparticles	Solution casting	1 % (w/w) related to starch	E, σ	WVP, Ɛ	[37]
Sugarcane fiber	Extrusion	10 % (w/w) related to starch	E, σ	Ɛ	[38]
Sugar palm nanocrystalline cellulose	Solution casting	0.5% (w/w) related to starch	WVP, BD		[39]
Cellulose nanofibers	Solution casting	Up to 0.4% (w/w) related to starch	σ, E	OP, WVP	[40]
Cellulose fibers	Extrusion	Up to 12% (w/w) of total mass	σ, E, WCA, Tg	Ɛ	[41]
Cassava fiber	Solution casting	1.5% w/w of total mass mixture	O, UV, WVP, σ, E, BD	M, Ɛ	[42]

Legend: Ɛ - Elongation; E - Elastic modulus; σ - tensile strength; WVP - Water vapor permeation; OP - Oxygen permeation; Tg - glass transition temperature; WCA - Water contact angle; WA - Water absorption; WS - Water solubility; UV- UV barrier; BD - biodegradability; M - moisture; O - Opacity.

1.3 Chestnut shells

Chestnut (*Castanea sativa* Mill) fruit is widely produced in Portugal. In 2016, the Portuguese chestnut industry produced (and processed) approximately 28 000 tons of chestnut [49]. During chestnut processing, high amounts of by-products are generated, namely low caliber chestnuts, rotten chestnuts and chestnut shells [50]. Low caliber fruits are used for animal's food, while the other by-products are burn or settled on a field as fertilizer. The released of by-products on fields leads to an increase of insects damaging crops. On the other hand, if by-products are burned, the emission of combustion gases, pesticides and/or heavy metals on ashes lead to environmental questions. Due to this negative impact on environment, several authors suggested new ways of using chestnut by-products [53,56].

Chestnut shells (CS) are one of most common by-products obtained from chestnut industry. Morphologically, CS can be divided in two "layers": integument, composed by the inner shell (IS) and pericarp, and the outer shell (OS) (Figure 1.7.). Several bioactive compounds can be obtained from CS, such as phenolics with antioxidant activity [52,56]. According to Rodrigues *et al.* [55], CS present in its constitution moisture (between 38.61 and 21.29 %), proteins (between 3.13 and 2.77%), fat (between 0.52 and 0.15) and carbohydrates (between 56.51 and 74.06%), having an ash content between 1.08 and 1.60 %. The hydroalcoholic extracts presented a Total phenolic content (TPC) up to 796.80 mg GAE (gallic acid equivalents) per g of dry basis (db) and a Total flavonoid content up to 43.33 mg CEQ (cathechin equivalents) per g db. Regarding the antioxidant activity, DPPH free radical scavenging was evaluated achieving values of EC_{50} up to 37.61 µg mL^{-1} and the FRAP (Ferric Reducing Antioxidant Power) values up to 8083.50 µmol of ferrous sulphate per g db. These values represent a significant antioxidant capacity of chestnut shell's extracts.

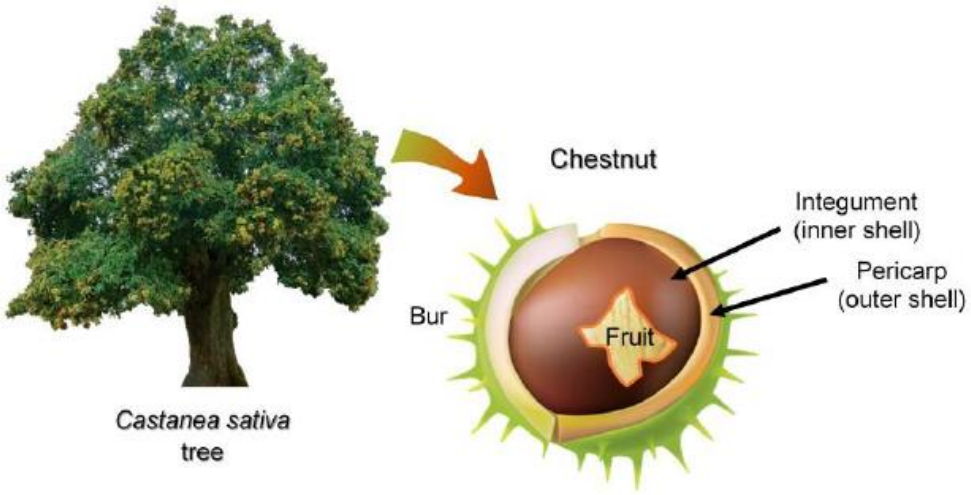

Figure 1.7.- Composition of chestnut [56].

Around 44% (w/w) of CS is lignin [51]. Chestnut lignin (CL) can be obtained through alkali treatment and acid precipitation and used as filler in starch-based films [31]. According to Miranda *et al.* [31], the addition of CL to films increase their mechanical properties (Table 1.2.). Chestnut fiber (CF) is other component of interest. Several authors studied the incorporation of fibers on starch films to reinforce their mechanical properties (Table 1.2.) [1,34,37-44]. The extraction of CL and CF from CS is preferable performed under alkali treatment (high pH) to disable the biological properties. Ramezani and Sain [57] studied the effect of time, pressure and temperature on the lignin extraction from wheat straw. Applying a Response Surface Methodology (RSM), the optimum conditions of extraction were 120 min at 200 ºC and 400 psi, achieving a lignin extraction yield of 90% [57]. Although this extraction process requires high energy consumption, maintains the biological properties of the extract, being a greener approach than the alkali treatment.

1.4 Response surface methodology (RSM)

The optimization of film formulation requires several experiments and time. RSM combines statistical and mathematical methods aiming the study of variables on specific responses with a minimum number of experiments [60,61]. Two main designs are usually observed in RSM: Central Composite Design (CCD) and Box-Behnken Design (BBD). In recent years, Face Central Composite Design (FCCD) and Central Composite Rotatable Design (CCRD) have been also applied [58]. According to Prabha *et al.* [60],

BBD is a spherical RSM design that provides efficient solutions compared to the three-level full-factorial design by reducing the number of required experiments, which become more significant as the number factors increases. Compared to CCD, BBD requires fewer runs, with three or four variables. Table 1.3. summarizes the application of RSM to optimize the formulation of several starch-based films.

Table 1.3.- RSM applied on starch-based film studies.

Design method	Starch-based films formulation variables	Responses	Number of runs	Ref
BBD	Starch, glycerol, Agar, Span80	T, WVP, WS, M, SC, Tr	29	[61]
BBD	Starch, carrageenan, sucrose esters, glycerol	T, WVP, WS, σ, Ɛ	27	[59]
BBD	Tapioca starch, glycerol, acetic acid	WVP, OP	17	[60]
CCD	PVA, starch, citric acid, glycerol	σ, SC, D	30	[62]
CCRD	Starch, carrageenan, glycerol	T, WVP, σ, WS	20	[63]
CCRD	Starch, glycerol, nanofibers	Ɛ, σ, WVP, WS, OP	17	[26]

Legend: Ɛ - Elongation; σ - tensile strength; WVP - Water vapor permeation; OP - Oxygen permeation; WS -Water solubility; M - moisture content; T - Thickness; D - In-vitro degradation; SC - Swelling capacity; Tr – Transparency.

1.5 Objectives

The present work aims to evaluate the best starch film composition reinforced with lignin and fiber obtained from CS. To achieve this goal, the following specific objectives were stablished:

1) Extraction of CF and CL from CS;
2) RSM optimization of starch film composition (glycerol, CF and CL contents);
3) Characterization of CF and starch films by Scanning Electron Microscopy (SEM) and thermal analyses;
4) Addition of an antioxidant CS extract to starch films formulation and assessment of the final antioxidant properties;
5) Evaluation of integrity and antioxidant activities of starch film during storage conditions.

Due to the preventive measures taken while the development of present work related to the Covid-19 pandemic situation, thermal analyses of objective 3) and objectives 4) and 5) were not possible to be made.

2. Experimental

2.1. Chemicals

Chestnut shells were provided by Sortegel, located in Sortes (Bragança, Portugal, latitude 41º 42'18.6''N and longitude 6º 48' 36.6''W). Potato starch and sodium chlorite ($NaClO_2$) were purchased from Merck. Acetic acid and glycerol (≥ 94%) were purchased from Sigma Aldrich. Sodium hydroxide (NaOH) was obtained from Lab Kem and sulfuric acid (96%) was purchased from Carlo Erba.

2.2. Chestnut Shells (CS) preparation

Figure 2.1. summarizes the experimental process applied in the present work for the development of a starch-based film reinforced with CS-lignin and -fiber.

Chestnut by-products were separated manually. The fruits were discarded while the CS were dehydrated (Excalibur Food Dehydrator equipment) at 42 ºC for 24 h, milled (Molinex equipment) and sieved in 500 µm and 100 µm mesh. The sieving process was effective in the separation of the outer shell solids from the inner shell solids.

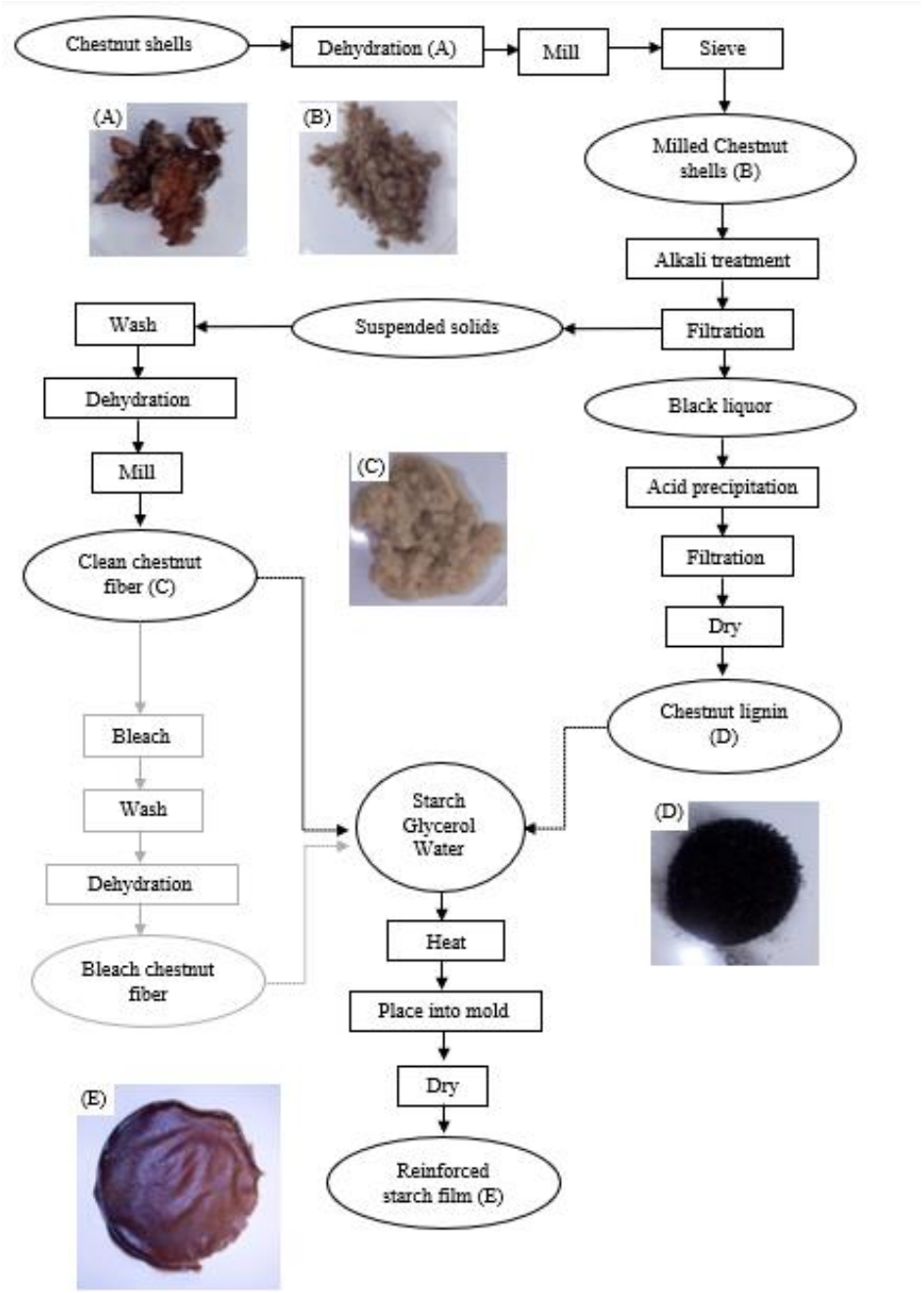

Figure 2.1. Methodology employed for the starch-based film reinforced with CS-lignin and -fiber. Legend: **(A)** dehydrated chestnut shells; **(B)** milled chestnut shells; **(C)** CF, clean chestnut fiber; **(D)** CL, chestnut lignin; **(E)** Starch film reinforced with chestnut fiber and chestnut lignin (glycerol-50 % (w/w), r.s.; CL-5 % (w/w),r.s.;CF-10 % (w/w),r.s.).

2.3. Chestnut Fiber (CF) extraction, cleaning, and bleaching

Chestnut fibers (CF) were extracted using the methodology described by Collazo-Bigliardi *et al.*, with minor modifications [32]. Sieved CS were alkali treated with 5 % (w/v) and 10 % (w/v) of NaOH in 1:15, 1:20 and 1:30 solid:liquid ratios for 3 hours at 80

ºC. This process was repeated 3 times. Then, the suspended chestnut fibers (CF) were washed with pure water until the complete remove of the alkali solution (brown). The black liquor was used for lignin extraction step. The bleaching treatment of CF was performed by adapting the Wu *et al.* protocol [64]. Briefly, CF was added to 1:1 mixture of 5 % (w/v) $NaClO_2$ aqueous solution and acetic acid in a solid:liquid ratio of 1:20, for 2 h, at 80 ºC. The bleach fibers were washed with pure water, dried for 24 h at 42 ºC and milled.

2.4. Chestnut lignin (CL) extraction

The chestnut lignin (CL) extraction was performed according to Miranda *et al*. [31] procedure, wherein lignin is obtained by precipitation. For that, concentrated sulfuric acid was added to the black liquor obtained in the alkali treatment, the mixture was filtrated, and the solid lignin obtained was dried for 24 h at 50 ºC. Then, CF was submitted to Ultra-Turrax (T10, Ika, Wilmigton, USA) to facilitate de dispersion of fiber on films.

2.5. Starch film preparation and preliminary studies

Films were produced according to Miranda *et al.* protocol [31]. Briefly, starch (2.5 % (w/v), glycerol (25-100 % (w/w), relating to starch (r.s.)), CL (0-100 % (w/w), r.s.) and CF (0-20 % (w/w), r.s.) were mixed in water at 80 ºC for 30 minutes with continuous mechanical stirring. Then, 40 mL of mixture were transferred to a silicone mold and dried at 50 ºC for 12 h. After detaching, films were conserved in a desiccator for further analysis.

2.6. Box–Behnken design (BBD)

Based on the results of the preliminary experiments, a Box-Behnken design (BBD) was performed to optimize the best composition of the starch-based film reinforced with CS-lignin and -fiber. The independent variables were the concentrations of glycerol (X1, % (w/w), r.s.; in a range of 40-60 %), chestnut lignin (X2, CL, % (w/w), r.s., in a range of 0-5 %) and chestnut fiber (X3, CF, % (w/w), r.s., in a range of 0-10 %). The independent variables included the following mechanical properties: elastic modulus (Y1), tensile strength (Y2) and elongation (Y3); coded at three level, -1, 0 and +1 (Table 2.1.). In this design, 17 different trials were conducted with five replicates of the center point (Table 3.1) and each run was performed in duplicate. For the analysis (in triplicate),

two test pieces were randomly cut from each film, avoiding bubbles and other fragilizing structures.

Table 2.1. Correlation between variables and levels

Variables \ Levels	-1	0	1
X1: Glycerol (%)	40	50	60
X2: Fiber (%)	0	5	10
X3: Lignin (%)	0	2.5	5

2.7. Films and Fibers characterization

The tensile properties were evaluated using a texture analyser (Stable Micro Systems TA-XT2I, Goldalming, UK); the probe selected was the Miniature Tensile Grips for Tension analysis. Speed test was set to 0.10 mm/sec with trigger force of 0.049 N and a test distance of 100.00 mm. The equipment was previously calibrated for distance and force. The measure units were Newtons (N), millimeters (mm) and seconds (s), for force, distance, and time, respectively. The test piece dimensions of films for analysis were 30.0 x 10.0 mm. Results were collected using the Exponent Stable Micro Systems software (version 6.1.12.0). The test pieces thickness was measured with an Eletronic Micrometer (0.001 mm).

Scanning Electron Microscopy (SEM) images were obtained at the "Centro de Materiais da Universidade do Porto (CEMUP)", using a FEI Quanta 400FEG ESEM/EDAX Genesis X4M equipment. All samples were fixed on support stubs, previously covered with Carbon tape, and gold coated before the microscopy analysis. The obtained SEM images allow to observe the morphology, CF diameter, CF surface and CF-starch interaction.

2.8. Statistical analysis

Results were presented as mean ± standard deviation. The Design Expert trial version 7 (Stat-Ease Inc., Minneapolis, MN, USA) was used for the analysis of the response surface and contour plots and the statistical analysis of the BBD model. Analysis of variance (ANOVA) was performed, and regression coefficients of linear, quadratic and interaction terms were determined. Adequacy of the model was evaluated using model analysis, coefficient of determination (R^2) and lack-of-fit test. Significance of the

equation was determined by F value at a probability (p > F) <0.05. The t-test was conducted to compare the responses prepared under optimized conditions with those predicted by BBD model.

3. Results and discussion

3.1. Preliminary studies

- **Alkali treatment**

The milled and sieved CS were cleaner in 1:20 and 1:30 ratios of CS : NaOH solution (g: mL).

For the CF alkali treatment, two different concentrations of NaOH solution were tested, 5 and 10 % (w/v), at three solid:liquid ratios 1:15, 1:20 and 1:30. Figure 3.1. shows the visual differences of CF obtained under different conditions of alkali treatment.

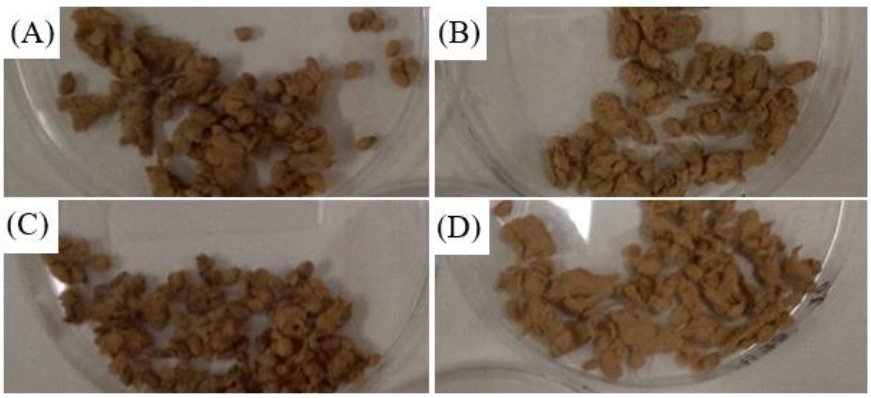

Figure 3.1.- CF obtained from alkali treatment.

Legend: **(A)** CF after 3 times treatment with NaOH 5 % (w/v) in 1:15 ratio; **(B)** CF after 3 times treatment with NaOH 5 % (w/v) in 1:20 and 1:30 ratio; **(C)** CF after 3 times treatment with NaOH 10 % (w/v) in 1:15 ratio; and **(D)** CF after 3 times treatment with NaOH 10 % (w/v) in 1:30 or 1:20 ratio.

CF obtained from equal solid: liquid ratios, but different concentrations of alkali solution (Figure 3.1. (A)(B) *versus* (C)(E)), were very similar, suggesting the absence of differences between 5 % (w/v) and 10 % (w/v) NaOH solution treatments. However, cleaner CF were obtained under higher solid:liquid ratio treatment conditions, i.e., 1:20 or 1:30 ratio of NaOH (Figure 3.1. (B) and (D) conditions). Based on these results, the best conditions stablished for the alkali treatment were 5 % (w/v) NaOH solution and a 1:20 solid:liquid ratio. Applying this cleaning conditions, the yield of cleaning fiber was 26.1 ± 1.2 % and the reagents consumption was minimized.

The reuse of alkali solution was tested. However, this procedure was inefficient to remove the lignin from the new milled CS.

In addition, the necessity of sieving CS after milling was evaluated. For this analysis, the same procedure for alkali treatment was adopted, without sieving milled CS. Adopting this practice, a black liquor with a high number of suspended solids and fibers and with a huge content of unwanted solids was obtained. Thus, this liquor is not clean enough for further extraction of lignin. For these reasons, sieving was considered an essential step of the process to facilitate de alkali treatment and cleaning of fibers.

- **Range of independent variables for BBD**

For films composition, the quantity of starch was fixed to 2.5 wt.% and the amounts of glycerol, lignin (CL) and fiber (CF) were variable. Preliminary studies were undertaken using single components or employing mixtures of two or three components, making sure that possible maximum and minimum points of RSM are achieved.

Results showed that glycerol concentrations higher than 75 % (w/w) produced films very sticky and scarcely detached from mold. Oppositely, glycerol concentrations below 25 % (w/w) resulted in a starch film extremely brittle. Considering these results, the content of glycerol was established between 40 and 60 % (w/w).

CL was tested up to 66 % (w/w). The results revealed that for a CL content >10% (w/w) the starch film was to brittle to detach (Figure 3.2. (A)). Also, when the amount of CL was above 30 % (w/w), the film easily cracks during the casting process (Figure 3.2.(B)). In this way, the composition of lignin to be tested was defined between 0 and 10 wt.% (w/w). The results obtained for CL are different from the ones reported by Miranda *et al.* [31]. This topic will be discussed in chapter *3.3. Comparison of the development film with literature*.

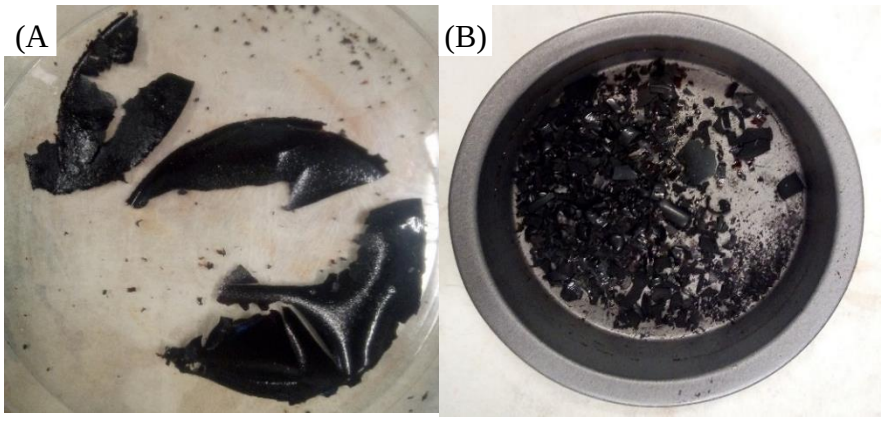

Figure 3.2.- Results of a casting solution containing starch: glycerol: CL.
Legend: **(A)**- Starch-based film containing CL 10 % (w/w), r.s.; **(B)**- Starch-based film containing CL 66 % (w/w), r.s..

The CF concentration was evaluated between 0 and 18% (w/w). The results showed that when CF was added in concentrations higher than 10 % (w/w), the CF was inefficiently mixed with starch, making empty spots among fibers. In opposite, adding CF in concentrations below 10% (w/w), CF was entirely included in starch, not showing weaker or empty spots. Based on these results, the range of CF was settled between 0 and 10% (w/w) in the BBD optimization.

Initially, the treatment of CF with Ultra-Turrax was not considered to be necessary. However, with the advance of experiences, this equipment proved to be highly effective in separating fibers agglomerates, allowing more homogeneous CF-starch-based films. A new experiment considering a CF treated with Ultra-Turrax in a concentration of 20% (w/w) produced a film with better visual and mechanical properties. In fact, Ultra-Turrax equipment may affect fibers, facilitating their inclusion on films. This hypothesis could be confirmed with characterization of both CF by SEM.

3.2. Experimental design (BBD)

The BBD applied for the starch film optimization comprises 17 runs, including five replicates of center point. Table 3.1. presents the independent variables under study and the experimental and predicted responses: Y1-elongation (%), Y2-tensile strength (N) and Y3-elastic modulus (N).

As observed in Table 3.1., the three responses are according to the expectations. The experimental Y1, Y2 and Y3 values for the control film (run 10: 50 % (w/w) of glycerol and 0% of CF and CL) were respectively 36.67%, 1.44 N and 0.71 N. Y1 ranged between 16.56 % (run 17) and 51.50 % (run 6); Y2 varied from 1.44 (run 10) to 8.11 N (run 15), and Y3 ranged between 0.71 (run 10) and 5.80 N (run 15).

In general, the results showed that higher amounts of fillers are related to higher values of Y2 and Y3. For instance, the run 15 (CF= 10 % (w/w) and CL= 0% (w/w)) resulted in Y2= 8.11 N, Y3= 5.80 N; and the run 16 (CF= 10 % (w/w) and CL= 5% (w/w)) resulted in Y2= 7.67 N, Y3= 4.40 N. These results suggest that the addition of CL resulted in worst mechanical properties of the starch-based film. Lower amounts of CF conducted to higher values of Y1 (run 5 - CF= 0 % (w/w); Y1= 46.07 %; run 6, CF= 0 % (w/w), Y1= 51.50 %).

Table 3.2. summarizes the ANOVA analysis of the BBD, which allows the evaluation of the model significance through Fisher's *F* test. According to the obtained results, independent variable X1 had no significant effect on any response, while independent variable X2 showed a significant effect on elongation (p <0.01), tensile strength (p <0.01) and elastic modulus (p <0.01). The independent variable X3 exhibit only a significant effect on elastic modulus (p <0.05). The quadratic term for X1 showed no significant effect on any response, while the quadratic term for X2 was significant for elongation (p < 0.05), tensile strength (p < 0.01) and elastic modulus (p < 0.01) responses. The quadratic term of X3 only showed significance on elastic modulus (p < 0.05).

The coefficient of determination (R^2) is an indicator of the proximation of real points to the prediction model. This value may range from 0 to 1; closer to 1 means a better fit of data on model [60]. According to the BBD presented above, the R^2 obtained for elongation, tensile strength and elastic modulus were 0.9215, 0.8597 and 0.9021, respectively, for the three independent variables (Table 3.2). The lack of fit for the three responses was not significant (p > 0.05). This statistic data shows the adequacy of the model to predict Y1, Y2 and Y3 responses.

Table 3.1.-Experimental design for evaluation of the effects of glycerol, chestnut fibers (CF) and chestnut lignin (CL) on tensile properties: elongation (%), tensile strength (N) and elastic modulus (N).

Point[a]	**Composition percentage optimization**			**Y1 Elongation (%)**		**Y2 Tensile Strength (N)**		**Y3 Elastic Modulus (N)**	
run	**X1 (Glycerol, % (w/w))**	**X2 (CF, % (w/w))**	**X3 (CL, % (w/w))**	**Exp**[b]	**Pred**[c]	**Exp**[b]	**Pred**[c]	**Exp**[b]	**Pred**[c]
1	40	0	2.5	35.45 ± 4.48	36.08	1.50 ± 0.14	1.52	0.98 ± 0.16	0.99
2	50	5	2.5	18.33 ± 1.11	20.11	3.18 ± 0.29	2.85	1.88 ± 0.28	1.73
3	50	5	2.5	17.68 ± 2.08	20.11	3.43 ± 0.33	2.85	1.99 ± 0.20	1.73
4	60	10	2.5	22.59 ± 1.01	21.96	5.48 ± 1.12	5.46	3.46 ± 0.91	3.45
5	50	0	5	46.07 ± 13.36	46.10	3.60 ± 0.74	3.96	2.51 ± 0.15	2.76
6	60	0	2.5	51.50 ± 5.78	47.49	2.64 ± 0.09	1.38	1.31 ± 0.09	0.57
7	40	5	5	20.79 ± 4.14	20.13	4.14 ± 0.38	3.76	3.43 ± 0.28	3.17
8	40	5	0	26.67 ± 3.37	22.69	3.17 ± 0.44	2.28	1.73 ± 0.17	1.24
9	50	5	2.5	20.55 ± 1.73	20.11	2.72 ± 0.33	2.85	2.06 ± 0.03	1.73
10	50	0	0	36.67 ± 3.27	40.02	1.44 ± 0.04	2.32	0.71 ± 0.05	1.19
11	60	5	0	26.95 ± 3.00	27.61	2.42 ± 0.26	2.80	1.25 ± 0.16	1.51
12	50	5	2.5	25.60 ± 2.50	20.11	3.10 ± 0.91	2.85	1.24 ± 0.16	1.73
13	50	5	2.5	18.38 ± 2.33	20.11	1.81 ± 0.19	2.85	1.50 ± 0.17	1.73
14	40	10	2.5	24.04 ± 1.21	28.05	4.45 ± 0.51	5.70	2.89 ± 0.54	3.63
15	50	10	5	21.79 ± 3.30	18.43	8.11 ± 0.86	7.23	5.80 ± 0.25	5.32
16	50	10	0	34.17 ± 11.47	34.14	7.67 ± 0.63	7.31	4.40 ± 0.42	4.16
17	60	5	5	16.56 ± 0.99	20.54	1.97 ± 0.10	2.87	1.80 ± 0.16	2.30

[a] Experiments were performed in a random order; [b] Average of triplicate determinations from different experiments; [c] Based on BBD evaluation.

Table 3.2.- Model summary and analysis of variance (ANOVA) for elongation (Y1), tensile strength (Y2) and elastic modulus (Y3).

Source	Sum of squares			Mean squares			*F* value			*p* value		
	Y1	**Y2**	**Y3**	**Y1**	**Y2**	**Y3**	**Y1**	**Y2**	**Y3**	**Y1**	**Y2**	**Y3**
Model	1531.54	51.63	25.91	170.17	5.74	2.88	9.13	4.76	7.17	0.0041*	0.0258**	0.0083*
X1. % Glycerol	14.20	0.07	0.18	14.20	0.07	0.18	0.76	0.06	0.45	0.4118	0.8152	0.5233
X2. % Fiber	562.96	34.12	15.24	562.96	34.12	15.24	30.19	28.34	37.97	0.0009*	0.0011*	0.0005*
X3. % Lignin	46.36	1.21	3.72	46.36	1.21	3.72	2.49	1.01	9.27	0.1588	0.3489	0.0187**
X1.X2	76.56	0.00	0.01	76.56	0.00	0.01	4.11	0.00	0.04	0.0823	0.9612	0.8558
X1.X3	5.08	0.50	0.37	5.08	0.50	0.33	0.27	0.42	0.81	0.6178	0.5399	0.3969
X2.X3	118.70	0.74	0.04	118.70	0.74	0.04	6.37	0.61	0.10	0.0396**	0.4589	0.7600
$X1^2$	1.93	2.73	0.80	1.93	2.73	0.80	0.10	2.27	2.00	0.7573	0.1757	0.1999
$X2^2$	669.33	9.14	3.14	669.33	9.14	3.14	35.90	7.59	7.83	0.0005*	0.0283**	0.0266**
$X3^2$	16.13	3.30	2.42	16.13	3.30	2.42	0.87	2.74	6.03	0.3832	0.1418	0.0437**
Residual	130.51	8.43	2.81	18.64	1.20	0.40						
Lack of fit	88.07	6.84	2.32	29.36	2.28	0.77	2.77	5.72	6.33	0.1753	0.0625	0.0533
Pure error	42.44	1.59	0.49	10.61	0.40	0.12						
Total	1662.05	60.06										

R^2 *pred* (Y1) = 0.9215; R^2 *adj* (Y1) = 0.8205; *Ratio* = 8.774

R^2 *pred* (Y2) = 0.8597; R^2 *adj* (Y2) = 0.6792; *Ratio* = 7.044

R^2 *pred* (Y3) = 0.9021; R^2 *adj* (Y3) = 0.7763; *Ratio* = 9.7699

Y1, Elongation (%); Y2, Tensile Strength (N); Y3, Elastic Modulus (N).

* $p < 0.01$; ** $p < 0.05$.

Using the collected data, several 3D contour plots were created, representing graphically the relations between the independent variables (Glycerol, CF, and CL) and the three responses (elongation, tensile stress, and elastic modulus). In Figure 3.3 are displaced 9 different plots: (A), (B) and (C) are related to elongation response; (D), (E) and (F) are associated with tensile strength while (G), (H) and (I) are associated to elastic modulus.

According to Figure 3.3. (A) and (B), higher elongation could be promoted with higher amount of glycerol (60 % (w/w)) and absence of CF or CL. Glycerol acts as plasticizer, allowing the film to be more elastic, while the addition of fillers compromises elongation, making the starch film stiffer. The plots of tensile strength and elastic modulus have identical shape when correlating the same independent variables. In plots (D) and (G), the highest point is observed for the higher amount of CF (10 % (w/w)) and middle amount of glycerol (50 % (w/w)). The same result is visible when correlating CL and glycerol (plot (E) and (H)), where a higher value of Y2 and Y3 is achieved by a higher amount of CL (5 % (w/w)) and an amount of glycerol between 45-50 % (w/w). According to plots (F) and (I), the highest point is achieved with 10 (w/w) % CF or CL with an amount of 0 or 5 % (w/w).

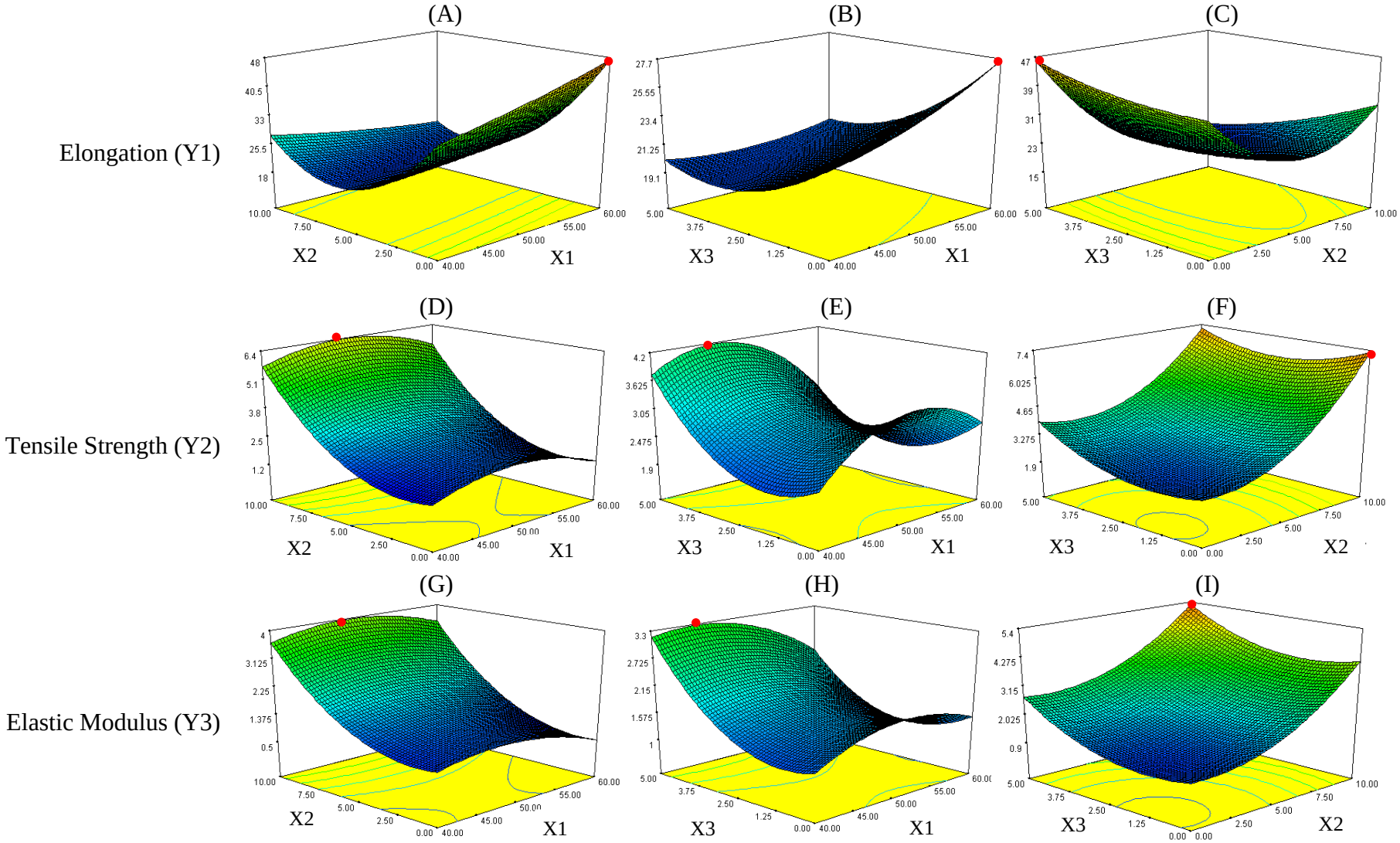

Figure 3.3.- Response surface 3D plots for BBD correlating independent variables to the three responses **(A)**, **(B)** and **(C)** - elongation; **(D), (E)** and **(F)** - tensile strength; **(G), (H)** and **(I)** - elastic modulus Independent variables: **X1**-Glycerol (% (w/w)), **X2**-CF (% (w/w) r.s.) and **X3**- CL (% (w/w) r.s.).

Table 3.3. presents the optimum composition of starch films to attain the maximum value of the three responses. With a desirability of 66.9 %, the use of 50 % (w/w) of glycerol and 10% (w/w) of CF in the starch-based film formulation enable an elongation of 34.19%, a tensile strength of 7.31 N and an elastic modulus of 4.15 N. The optimum conditions indicate that CL is not needed to achieve better tensile properties, being the extraction of lignin unnecessary.

Table 3.3.- Performance of BBD model in predicting the optimum composition to enhance the (**Y1)-** Elongation, (**Y2) -** Tensile Strength and (**Y3)-** Elastic Modulus of starch-based film.

Response	**Optimum conditions**			**Predicted value (desirability, %)**
	Glycerol (%)	**CF (%)**	**CL (%)**	
Y1, Elongation (%)				34.19 %
Y2, Tensile Strength (N)	50	10	0	7.31 N
Y3, Elastic Modulus (N)				4.15 N
				(66.9 %)

An important aspect of films is thickness, since it may affect several other properties, such as permeability and optical properties. The films average thickness was 0.1487 ± 0.0301 mm.

As shown in Table 3.4., the experimental value for the three responses are in accordance to the predicted values by the model ($p < 0.05$), showing the effectiveness of the BBD for the optimization of the mechanical properties of the starch-based films.

Table 3.4.- Mechanical properties for the optimal of CF-starch-based film (50% (w/w), glycerol, 10% (w/w) CF).

	Y1, Elongation (%)	**Y2, Tensile Strength (N)**	**Y3, Elastic Modulus (N)**
Experimental value[a]	28.19 ± 3.06	4.48 ± 1.48	3.44 ± 0.40
Predicted value	34.19	7.31	4.15
***p*[b]**	0.0769	0.0980	0.0918

[a] Results are expressed as mean ± standard deviation ($n = 3$). [b] indicates no significant differences ($p > 0.05$).

3.3. SEM analysis

Figure 3.4. presents the visual and SEM images of the optimum starch-based films reinforced with fibers and lignin from CS, developed in this work. As can be observed, the differences between surfaces of starch-based films with different formulations are clearly viewed with no ampliation and with a resolution of 250 x.

The control film (A) is translucent and clear, the starch-based film with CF 10% (w/w) r.s. (film B) is more yellow and less translucent, while the starch-based film with 10% (w/w) r.s. CF and 5% (w/w) r.s. CL (film C) has a brown color and an opaquer appearance due to the presence of the lignin. From SEM analysis it is observed that the surface of film A is clear and smooth (zoom 250 x), also presenting a cut clean (1 000 x) without any visible deformation.

The CFs components of film B are visible on the surface and on the cuts (pointed with black arrows at different zoom values). With a resolution of 5 000 x is clear the transactional cut of the fiber, as well as the adhesion between the CF and starch.

In film (C), CFs are visible on the surface, as well as some CLs components, pointed with black arrows at different zoom values, meaning that not all the lignin was dissolved in starch, possible creating some weaker spots. Indeed, the cut visible at 1 000 x resolution is less clear than the others, showing a different behavior of starch-based film reinforced with CL.

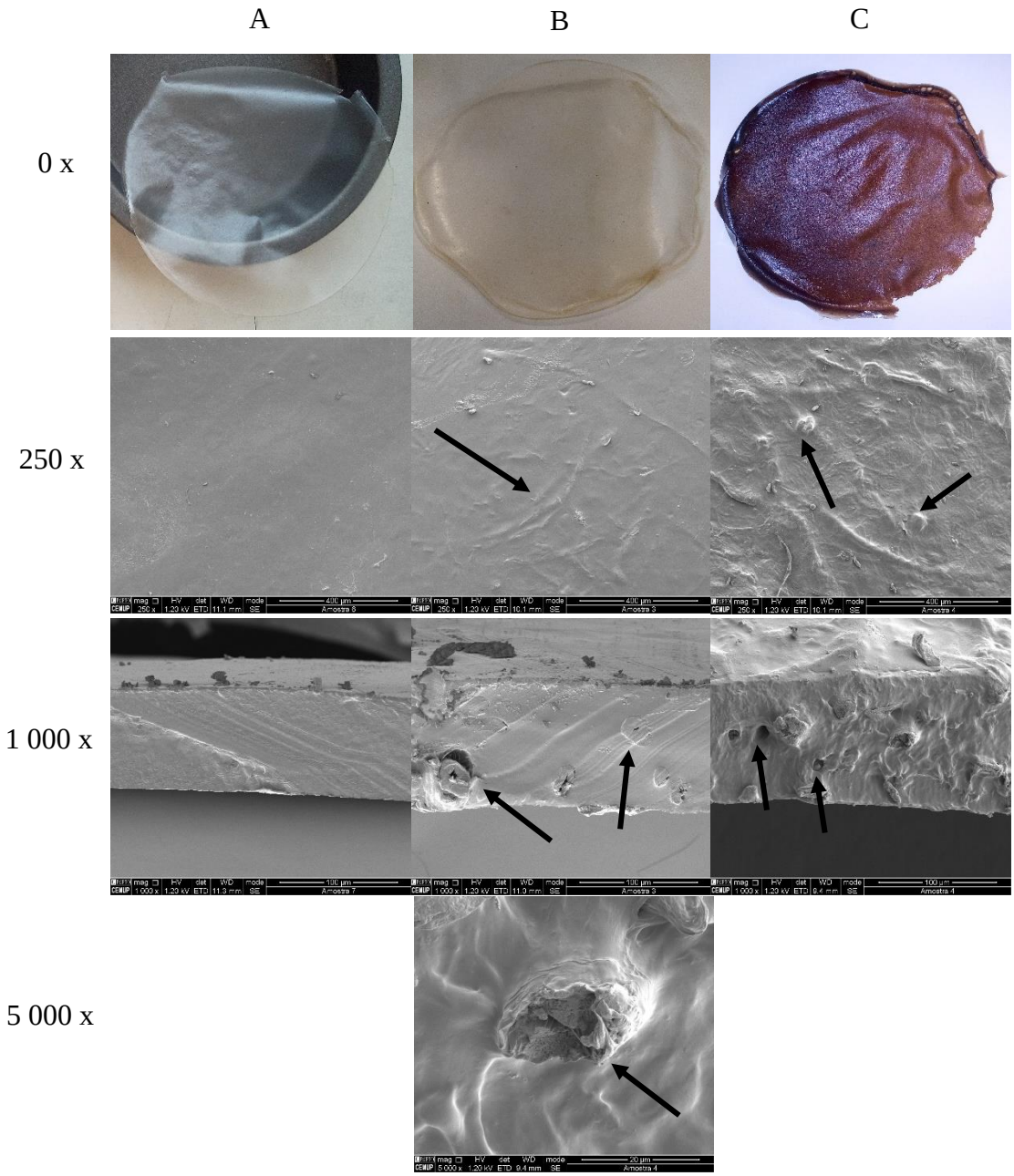

Figure 3.4.- Visual and SEM images of **(A)** control, i.e., starch-based film without CF and CL (run 10); **(B)** starch-based film with CF 10% (w/w) r.s. (run 16); **(C)** starch-based film with 10% (w/w) r.s. CF and 5% (w/w) r.s. CL (run 15).

Legend: the black arrows shown in **(B)** and **(C)** images indicate the respective CF and the CL components incorporated in the starch-based films.

3.4. Comparison of the developed film with literature

Table 3.5. compares the starch-based films developed in the present work with those reported in the literature. Due to differences on measurement units between the present work and the reported literature, the comparison of data will be adressed to the mechanical properties between the control and the reinforced film.

The lignin-starch-based films developed in this work followed the Miranda *et al.* [31] protocol. These authors varied the concentration of lignin and glycerol by a total of 50% (w/w), and the source of lignin was piassava. In the present work, it was not possible to obtain films with such composition, the incorporation of more than 5% (w/w) of CL made films brittle and easily crack during casting. Although the extraction methodology of lignin was similar, it is well known that different sources affect the composition and structures of lignin [65]. In fact, the preliminary results of this work showed a huge difference in the possible lignin composition of films (chapter *3.1. Preliminary studies*). For this reason, it is possible that CL have different interactions with starch, making a more brittle and hardener film. Further studies of CL characterization may provide information regarding this topic.

Concerning the tensile properties, Miranda *et al.* [31] reported that reducing glycerol by 10 % decreased the elongation and elastic modulus, but increased the tensile strength (B2 and C2, Table 3.5.). For both corn- and cassava starch-based films, the addition of 40% of lignin (respective B2 and C2, Table 3.5.) decreased the elongation but increased the other two tensile properties. In the present work, the effect of CL in the potato starch-based films can be observed from results present from A1 to A2 (Table 3.5.). As observed, the addition of CL up to 5% (w/w) provided a huge increase of tensile strength and elastic modulus (more than double) but a smaller increase of elongation. Regarding the addition of CF to reinforce the starch-based films, it is possible to observe that elongation decreases (with use up to 10% (w/w) r.s.), while tensile strength and elastic modulus increase 311% and 485% (A1, A3, Table 3.5.).

Collazo-Bigliardi *et al.* [32] employed the extrusion method to produce corn starch-based films reinforced with 10% (w/w) of bleach fibers from coffee/rice sources. According to these authors, the tensile strength and the elastic modulus showed a lower increase when 10% (w/w) of fiber was added (D1, D2 (146%) and D1, D3 (130%), Table 3.5.). This increase was significantly lower than the observed in the present work, which

can be explained by differences on the methodology applied. These authors used bleach fibers, while in the present work, the use of bleach fiber did not significantly affect the mechanical properties (results are not presented in this table). Bleaching treatment with sodium chlorite promotes oxidation of aromatic rings from lignin, resulting in cellulose white fibers [32].

Cheng *et al* [66] also showed an increase of tensile properties (E1, E2, Table 3.5.), closely to the ones reported in the present work. Indeed, an increase of 316% and 433% times for tensile strength and elastic modulus, respectively, could be observed. However, is important to refer that the reinforcement used was cellulose nanofibers and microfibrillated cellulose, different to the one employed in the present work.

Table 3.5.- Comparison of the tensile properties of starch-based films obtained in the present work with literature.

Starch	Code	Plasticizer and Reinforcement	Method	Elongation (%)	Tensile strength	Elastic Modulus	Ref
2.5% wt. Potato	A1	50%* glycerol + 0%* CL + 0%* CF	Casting	40.02 **	1.44 N **	0.71 N **	Present work
	A2	50%* glycerol + 5%* CL + 0%* CF		46.10 **	3.96 N**	2.76 N**	
	A3	50%* glycerol + 0%* CL + 10%* CF		28.19 ± 3.06***	4.48 ± 1.48***	3.44 ± 0.40***	
5% wt. Corn	B1	50%* glycerol	Casting	47.278 ± 2.994	1.717 ± 0.087 MPa	12.100 ± 0.944 MPa	[31]
	B2	10%* glycerol + 40%* piassava lignin		3.239 ± 0.293	14.293 ± 1.883 MPa	673.500 ± 53.203 MPa	
5% wt. Cassava	C1	50%* glycerol	Casting	50.697 ± 4.421	2.144 ± 0.193 MPa	21.600 ± 4.519 MPa	
	C2	10%* glycerol + 40%* piassava lignin		9.865 ± 2.844	20.153 ± 1.854 MPa	930.100 ± 96.479 MPa	
Corn starch	D1	30%* glycerol + 50%* water	Extrusion	12 ± 7	13.0 ± 1.5 MPa	260 ± 80 MPa	[32]
	D2	30%* glycerol + 50%* water + 10%* coffee bleach fibers		3 ± 1	19.0 ± 1.0 MPa	822 ±38 MPa	
	D3	30%* glycerol + 50%* water + 10%* rice bleach fibers		3 ± 1	17.0 ± 3.0 MPa	863 ± 2 MPa	
5% wt. Corn	E1	20%* glycerol + 0%* cellulose nanofibers	Casting	-	3.0 MPa	150 MPa	[66]
	E2	20%* glycerol + 10%* microfibrillated cellulose		-	9.5 MPa	650 MPa	

*(w/w), r.s.; ** according to the predicted model; *** according to the optimal conditions for the three responses.

3.5. Bleach fiber and the possibility of increasing the amount of fiber

In the method proposed by Collazo-Bigliardi *et al*. [32], rice and coffee fibers were alkali treated and bleached. In the present optimization study, the CF used to reinforce the starch-based films was only submitted to alkali treatment. Thus, an additional experiment was conducted to evaluate the need of bleaching CF. The effect of increasing the amount of CF by 20% on the performance of the starch-based film was also evaluated. Figure 3.5. summarizes the mechanical properties of three different starch-based films formulations: (A) film formulated under BBD optimized conditions, i.e., 50% (w/w) glycerol and 10 % (w/w r.s.) alkali treated CF; (B) film formulated with 50% (w/w) glycerol and 10 % (w/w r.s.) bleached CF; and (C) film formulated with 50% (w/w) glycerol and 20% (w/w r.s.) alkali treated CF.

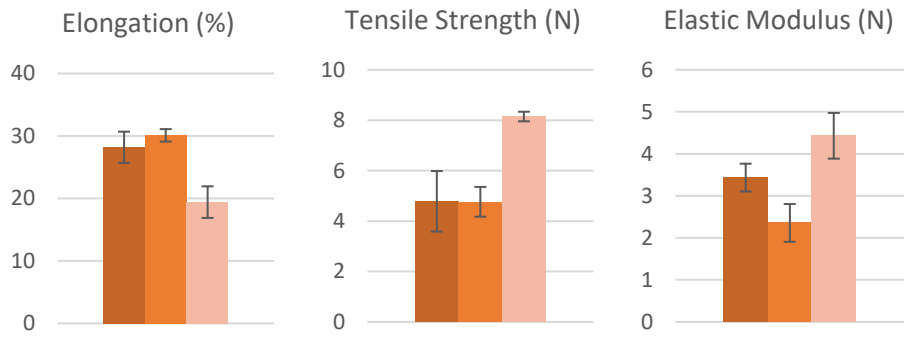

Figure 3.5.- Elongation (%), Tensile Strength (N) and Elastic Modulus (N) responses of starch-based films with different formulations.

Legend: **A**(■) Film formulated under BBD optimized conditions, i.e., 50% (w/w) glycerol and 10 % (w/w r.s.) ; **B**(■) Film formulated with 50% (w/w) glycerol and 10 % (w/w r.s.) bleached CF; **C** (■) Film formulated with 50% (w/w) glycerol and 20% (w/w r.s.) alkali treated CF.

Table 3.5. shows the statistical differences on the mechanical properties of the different starch-based films formulations. Plots A and B only differ in the type of fiber employed (film of plot A is composed by 10% (w/w) of alkali treated CF while film of plot B is composed by the same amount of bleached CF); plot C had an higher amount of alkali treated CF (20% (w/w)). In general, for the three mechanical properties evaluated, plots A and B had a similar effect, showing no significant ($p > 0.05$) differences for the three responses evaluated. Plot C reports the results of the fiber amount increase; and was significantly ($p < 0.05$) different from plots A and B regarding the tensile strength. These results are consistent with the reports on literature about the increasing of fillers' amount on starch-based films [32]. Still, these results suggest the possibility to load higher

amounts of CF (when compared to the preliminary results) without a negative effect on the starch-based film structure.

Table 3.5.- Statistical differences (*p*- value) on the mechanical properties of starch-based films with different formulations

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	***p*-value**		
	Elongation (%)	**Tensile Strength (N)**	**Elastic Modulus (N)**
A.B	0.284	0.983	0.173
B.C	0.050	0.021*	0.083
A.C	0.109	0.048*	0.151

*Indicates no significant differences ($p < 0.05$).

Legend: **A**- Film formulated under BBD optimized conditions, i.e., 50% (w/w) glycerol and 10 % (w/w r.s.); **B**- Film formulated with 50% (w/w) glycerol and 10 % (w/w r.s.) bleached CF; **C**- Film formulated with 50% (w/w) glycerol and 20% (w/w r.s.) alkali treated CF

Figure 3.6 represents the SEM analysis of CF with and without treatment with Ultra-Turrax. The fibers length was not measured but differences regarding morphology (100 x and 500 x resolution) were observed. Comparing Fig 3.6. (A and B), it is possible to see that CF became more folded and pointier, with some torsion spots. Likewise, some frays in fibers treated with Ultra-Turrax can be seen Fig.3.6. (B) and (C) with 500 x resolution), justifying the enhance of CF up to 20 %. The fibers morphology could benefit of the network structure and help in the cohesion between starch and fibers. Furthermore, the disentanglement of fibers agglomerates, due to the high-speed rotation of Ultra-Turrax, seems to facilitate a homogeneous mixing of fibers in starch solution. Cheng *et al.* [66] compared the addition of microfibrillated cellulose on corn-starch based films with the addition of smaller cellulose fillers (size not shown), reporting that the microfibrillated cellulose showed better results in mechanical tests due to the improved network structure. Nonetheless, to our best knowledge, literature never reported the possibility of increase the amount of fibers in films according to the fibers morphology or length.

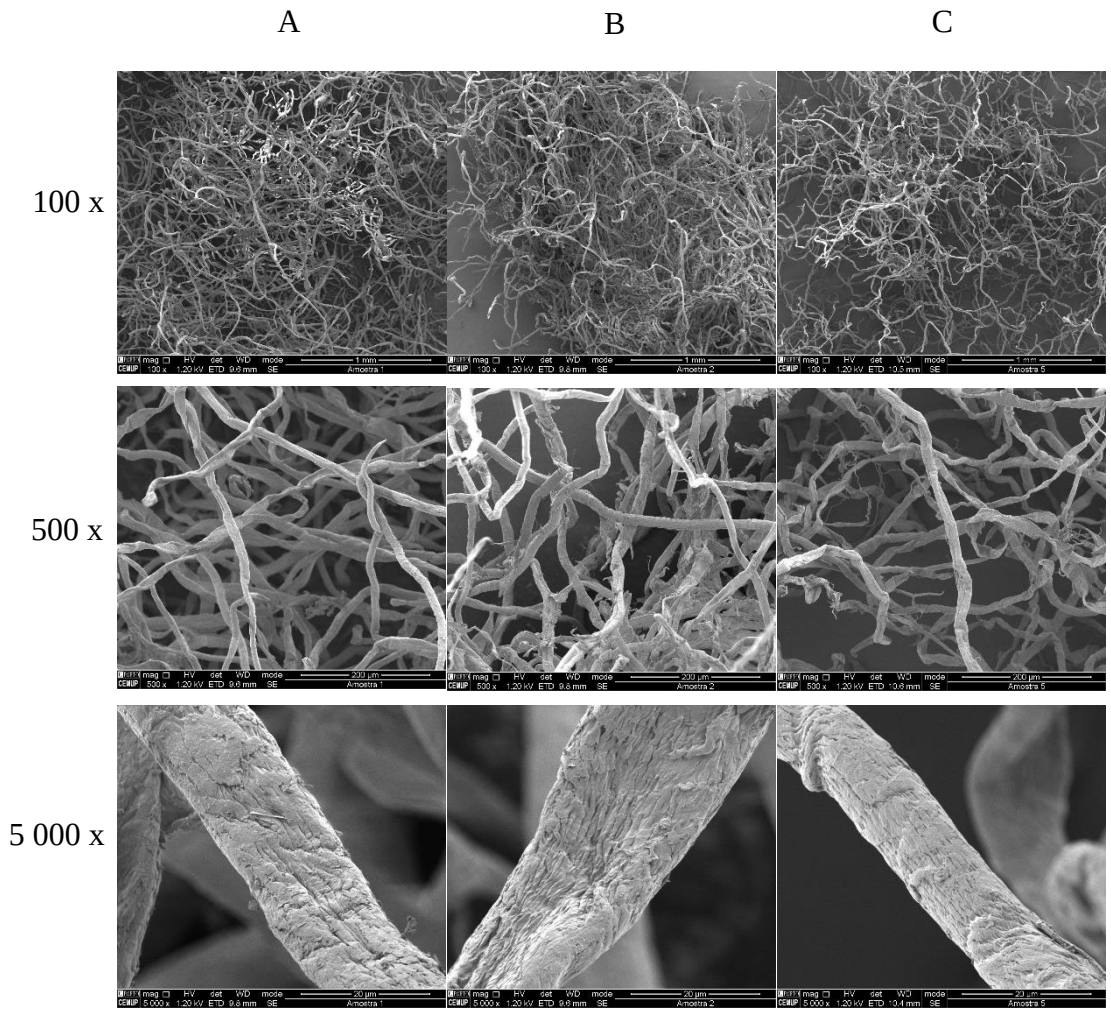

Figure 3.6.- SEM analysis of **(A)** CF alkali treated; **(B)** CF alkali treated and submitted to Ultra-Turrax; **(C)** Bleached CF treated with Ultra-Turrax.

Regarding the color of fibers, it was observed a variation from brown to white with the employment of bleach treatment, probably due to the removal of lignin compounds. This observation was also reported by Collazo-Bigliardi *et al.* [32]. Indeed, observing SEM images of CF (Figure 3.6. A and C, resolution of 5 000 x), it is possible to constate some differences. CF from figure 3.6.(A) has some residues on surface, while no residues were observed in Figure 3.6.(B), being the fibers more regular and smoother.

Figure 3.7. presents the SEM images of fibers (resolution of 500 x). The alkali treated CF (A) showed a mean diameter of 13.01 ± 2.54 µm; the same CF treated with Ultra-Turrax (B) presented a mean diameter of 16.72 ± 3.39 µm, while the bleached CF treated with Ultra-Turrax (C) showed a mean diameter of 13.22 ± 3.56 µm. Although the bleaching process had visible effects on color of CF, no significant effects ($p < 0.05$) on

CF diameter were reported. For this reason, it can be assumed that the bleaching procedure can be considered an unnecessary step to make starch-based films reinforced with CF. This aspect allows the use of less chemicals and water in the CF treatment process.

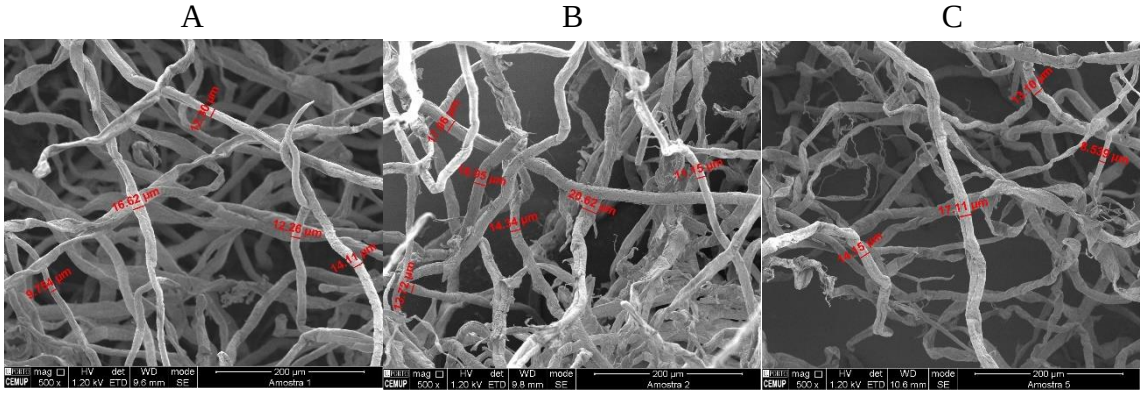

Figure 3.7.- SEM pictures (500x resolution) of **(A)** alkali treated CF; **(B)** alkali treated CF submitted to Ultra-Turrax; and **(C)** bleached CF submitted to Ultra-Turrax, with detail on the fiber diameter (µm).

4. Conclusion and future perspectives

This work evaluated the effect of lignin and fibers extracted from CS as reinforcement material of starch-based films. Applying a Box-Behnken design model, the optimum composition of starch films incorporating these compounds with higher elongation, tensile strength and elastic modulus properties was: 50% (w/w) glycerol and 10% (w/w) r.s. of CF. The results showed that CL was not a good filler to be added to starch films. The experimental results also revealed an improvement of tensile properties, comparable to ones obtained by other authors. Furthermore, the bleaching of fibers was considered an unnecessary step. In opposite, the employment of Ultra -Turrax allowed to achieve a maximum load of CF on starch-based films.

Despite the obtained results, further studies are necessary, including the evaluation of additional mechanical (water and oxygen permeation) and physicochemical (solubility, thermal analysis or optical properties, among others) properties. Moisture has significant interactions with starch and plasticizers, leading to weaker films. For this reason, it will be interesting to compare the tensile properties of starch films using two plasticizers, glycerol and PEG.

In the food packaging field, starch films could be doped with natural extracts to improve/ achieve antioxidant properties. In this way, further research could be done to evaluate the benefits of antioxidant extracts addition on antioxidant and mechanical properties of CF-starch-based films. The evaluation of starch reinforced films properties under storage conditions will be also interesting to guarantee a food packaging of excellence.

The predicted BBD model excluded the addition of lignin to reinforce starch-based films. However, to maximize the valorization of CS, further studies should be performed to characterize the lignin obtained from chestnut and to evaluate its potential use as polymer in other fields.

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