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Additional Information

Melt processability, characterization, and antibacterial activity of compression-molded green composite sheets made of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) reinforced with coconut fibers impregnated with oregano essential oil

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Abstract. This paper describes the preparation, characterization, and antibacterial activity of new green composites of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and coconut fibers (CFs) obtained by twin-screw extrusion (TSE) followed by compression-molding. The first part of the study was focused on the effect of varying the CF weight content, *i.e.* 1, 3, 5, and 10 wt.-%, and processing conditions, *i.e.* screw speed of 75, 150, and 225 rpm, on the aspect ratio and dispersion of the fibers in the green composites. In the second part, for the optimal processing condition, the thermal, mechanical, and barrier properties of compression-molded PHBV sheets were analyzed as a function of the CF content. In the last part, CFs were loaded with oregano essential oil (OEO) by an innovative spray coating methodology and then incorporated again into the PHBV sheets. The antimicrobial activity of the PHBV sheets with different amounts of OEO-containing CFs was tested against food-borne Gram-positive *Staphylococcus aureus* bacteria, showing bacteriostatic effect from contents as low as 3 wt.-%. The use of CFs as sustainable fillers in PHBV sheets shows a great deal of potential in active food packaging, particularly as vehicles to entrap natural extracts.

Keywords: PHA; Coir fibers; Essential oils; Plastics engineering; Active packaging; Waste valorization

1. Introduction

The use of agro-food residues for the preparation of polymer composites is gaining a significant attention because of their high availability and low price, being at the same time a highly sustainable strategy for waste valorization. Natural fibers (NFs), particularly those obtained from plants, represent an environmentally friendly and unique choice to reinforce bioplastic matrices due to their relative high strength and stiffness (Yang et al., 2006). The substitution of oil-derived polymers with bio-based polymers as the matrix component results in the term “green composites” (Zini and Scandola, 2011), which indicates that the composite as a whole, *i.e.* both matrix and reinforcement, originates from renewable resources. In this regard, the incorporation of NFs such as jute, sisal, flax, hemp, and bamboo fibers on biopolymers has been recently intensified (Bogoeva-Gaceva et al., 2007). Resultant green composites do not only offer environmental advantages over traditional polymer composites, such as reduced dependence on non-renewable energy/material sources, lower greenhouse gas and pollutant emissions, improved energy recovery, and end-of-life biodegradability of components (Joshi et al., 2004), but also a potential reduction of both product density and energy requirements for processing (Faruk et al., 2014).

Polyhydroxyalkanoates (PHAs) are a well-known family of biodegradable bacterial polyesters with the highest potential to replace polyolefins in a wide range of applications (Bugnicourt et al., 2014). Among PHAs, poly(3-hydroxybutyrate) (PHB) and its copolymer with 3-hydroxyvalerate (HV), *i.e.* poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), have received so far the greatest attention in terms of pathway characterization and industrial-scale production. The use of PHA copolymers presents certain advantages since they have a lower melting point and higher flexibility than homopolymers, which improves melt stability and broadens their processing window (Torres-Giner et al., 2016a). Furthermore, the introduction of comonomer units induces defects in the crystal lattice, reducing both the degree of crystallinity and crystallization rate (Kunioka et al., 1989).

So far, several plant-derived NFs such as regenerated and/or recycled cellulose, pineapple leaf fibers (PALF), wheat straw fibers, wood floor, jute fibers, flax fibers, banana, sisal, and coir fibers, hemp fibers, abaca fibers, bamboo fibers, sugarcane bagasse fibers, kenaf and lyocell fibers, wood powder, and pita (agave) fibers have been studied as a reinforcement to produce PHA-based composite materials (Torres-Giner et al., 2016b).

Recently, cellulose fibers from wheat straw and other by-products have been used in the European Projects ECOBIOCAP and SUSTAINPACK as a cheap source of fillers to reduce the cost of a PHB matrix for packaging applications, where up to 20% of cellulose fibers were allowed in the final composition. Previous research studies have also suggested that the incorporation of NFs can definitely strengthen the mechanical performance of both PHB and PHBV and, in some cases, also improve biodegradability (Avella et al., 2000; Barkoula et al., 2010; Teramoto et al., 2004). Additionally, due to the well-known limited processability of these materials, the most promising composite manufacturing techniques are extrusion and compression molding, given the lower shear-rates involved and associated thermo-mechanical stresses.

Coconut shells represent a good example of agro-industrial non-food feedstock that is still considered waste, for which important industrial new end uses are being currently pursued (Rosa et al., 2009). In particular, it is estimated that about 55 billion coconuts are produced annually worldwide; most husks are abandoned and create a waste of natural resources and a cause of environment pollution (Gu, 2009). Coconut fibers (CFs), also referred as coir when ground up, are plant-derived NFs of the coconut fruit mesocarp, which constitutes 30–35 wt.-% of the coconut obtained from the palm tree (*Cocos nucifera* L.) (Tomczak et al., 2007). Individual CFs show a length of 0.3–1.0 mm and a diameter of 0.01–0.02 mm, resulting in an average aspect ratio of approximately 35 (Hasan et al., 2015). They are also characterized by high toughness and durability and improved thermal stability due to their high lignin content (~40%) and relatively low cellulose content (~32%) (Rosa et al., 2010). These lignocellulosic fibers are not only abundant in tropical countries but also versatile, renewable, cheap, and biodegradable. Therefore, they could become potential substitutes for energy-intensive synthetic fibers in many applications where high strength and modulus are not required (Satyanarayana et al., 1982).

CFs have been tested as a reinforcement upon the formulation of different thermoplastic materials, such as low-density polyethylene (LDPE) (Choudhury et al., 2007; Owolabi and Czvikovszky, 1988) and linear low-density polyethylene (LLDPE) (Choudhury et al., 2007) polypropylene (PP) (Gu, 2009; Hasan et al., 2015; Mir et al., 2013; Owolabi and Czvikovszky, 1988; Wambua et al., 2003), poly(vinyl chloride) (PVC) (Owolabi and Czvikovszky, 1988), starch–gluten (Corradini et al., 2006), natural rubber (NR) (Geethamma et al., 1998), among others. These studies have demonstrated that the

incorporation of both untreated and chemically modified CFs into polymer formulations can represent a feasible route to produce low-cost composites with a moderate improvement of the mechanical properties. Interestingly, CFs are exceptionally hydrophilic as they contain strongly polarized hydroxyl groups on their surface (Westerlind and Berg, 1988). CFs are thus inherently capable to adsorb and hold moisture up to 7–9 times their weight, being proposed as biosorbents for water treatment (Bhatnagar et al., 2010). This outstanding capacity of CFs to retain water and other polar components certainly opens up attractive opportunities for potential uses as vehicle of functional substances.

The high water-holding capacity of CFs can be explored to adsorb essential oils (EOs), *i.e.* aromatic and volatile oily liquids obtained from herbs and spices. Most EOs and their constituents are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. (López et al., 2007). Among EOs, oregano essential oil (OEO), extracted from *Origanum vulgare* L., is well recognized for its antioxidative and antimicrobial action in the food industry (Hosseini et al., 2013). The biocide performance of OEO has been mainly attributed to its rich composition in phenolic compounds, namely, carvacrol (up to 80%), thymol (up to 64%), and the monoterpene hydrocarbons γ -terpinene and p-cymene (both up to 52%) (Burt, 2004). The incorporation of OEO in plastic films to avoid microbial food spoilage represents an attractive option for packaging manufacturers. Several studies demonstrated its efficacy in inhibiting microbial development and synthesis of microbial metabolites, including pathogenic bacteria, yeasts, and molds (Benavides et al., 2012; Hosseini et al., 2015; Oussalah et al., 2004; Pelissari et al., 2009; Seydim and Sarikus, 2006; Zivanovic et al., 2005). Besides imparting antimicrobial characteristics to the films, OEO can change flavor, aroma, and odor. However, similarly with other EOs, OEO is volatile and easily evaporates and/or decomposes during processing and preparation of the antimicrobial films owing to direct exposure to heat, pressure, light or oxygen.

In the present study, OEO was entrapped into the CFs in order to overcome the partial loss and improve its stability during processing and even storage. The resultant OEO-containing CFs were subsequently incorporated into PHBV to develop green composites with antimicrobial properties for active packaging applications.

2. Experimental

2.1. Materials

Bacterial aliphatic copolyester PHBV was ENMAT™ Y1000P, produced by TianAn Biologic Materials (Ningbo, China) and distributed by NaturePlast (Iffs, France). The product was delivered as off-white pellets packaged in plastics bags. The biopolymer resin presents a true density of 1.23 g/cm³ and a bulk density of 0.74 g/cm³, as determined by ISO 1183 and ISO 60, respectively. The melt flow index (MFI) is 5–10 g/10 min (190 °C, 2.16 kg), as determined by ISO 1133. The molar fraction of HV in the copolymer is 2–3%, whereas the molecular weight (M_w) is approximately 2.8×10^5 g/mol. According to the manufacturer, this resin is suitable for injection molding, thermoforming, and extrusion.

CFs were kindly supplied by Amorim Isolamentos, SA (Mozelos, Portugal) in the form of a liner roll. The fibrous layers were obtained from ripe coconuts, which were manually de-husked from the hard shell by driving the fruit down onto a spike to split it. 100% pure OEO was purchased from Gran Velada SL (Zaragoza, Spain). Food-grade paraffinic oil (grade 9578) was obtained from Quimidroga SA (Barcelona, Spain). 2,2,2-trifluoroethanol (TFE) with 99% purity, *D*-limonene with 98% purity, Nile Red (9-diethylamino-5H-benzo- α -phenoxazine-5-one), and dimethyl sulfoxide (DMSO) anhydrous with purity $\geq 99.9\%$ were purchased from Sigma-Aldrich (Madrid, Spain). All chemical reagents were utilized as received.

2.2. Functionalization of CFs

CFs were initially separated from the roll and chopped to a length of *ca.* 5 mm in a Granulator 20-18/JM from Grindo SRL (Cologno Monzese, Italy). Chopped fibers were then sieved using a Test sieve model from Controls (Milano, Italy) with an aperture of 2 mm. The resultant bulk density of the CFs was 0.14 g/cm³, as determined by ISO 60.

Figure 1 shows the different stages for the preparation of the CFs.

Part of the obtained CFs was coated with OEO in order to provide them with antibacterial functionality. For this purpose, a 2:1 (vol./vol.) paraffinic oil/OEO solution was prepared, as this was the highest OEO content without creating phase separation. This solution, with a density of 0.902 g/mL, was added at 15 wt.-% to the CFs by means of an air spray gun from Nutool (Doncaster, UK) connected to a 24L 1.0HP PWB24S air compressor from PowerED® (Amiais de Baixo, Portugal) working at 2 bar. This way, the dried CFs

were sprayed every 5 seconds while rotating inside a drum attached to an ARTISAN 5KSM125EER household mixer from KitchenAid® (Michigan, USA) turning at 58 rpm.

2.3. Preparation of green composites

Prior to melt processing, the PHBV pellets and CFs were dried for 4 h at 80 °C in an oven to remove residual humidity. Then, different PHBV formulations were compounded varying the CF content, *i.e.* 0, 1, 3, 5, and 10 wt.-%, in a co-rotating intermeshing twin-screw extruder LSM 34 GL from Leistritz AG (Nuremberg, Germany). The screws have a diameter (D) of 34 mm and a length-to-diameter ratio (L/D) of 29. The modular barrel is equipped with 8 individual heating zones and is coupled to a strand die. The extruder layout and the screw configuration are presented in **Figure 2**. Basically, the screw contains two mixing zones, each consisting of a series of kneading disks staggered at 90° (in order to induce dispersion), that are separated by conveying sections that work mostly partially filled. The components are fed separately upstream of each mixing zone. PHBV pellets were fed into the main hopper with a volumetric DVM18-L feeder from Moretto S.p.A. (Massanzago, Italy) at a rate of 2 kg/h. The CFs were first pre-mixed in a zipped bag with 1 part per hundred resin (phr) of paraffinic oil and then introduced in the extruder after melting the polymer, at L/D=12, by a side feeder. As commonly faced with other low-density fibers, there were difficulties in guaranteeing a precise and constant feeding throughput. A venting port at L/D=23 coupled to a vacuum pump enabled the removal of both residual moisture and volatiles. Three different screw speeds were tested, *i.e.* 75, 150, and 225 rpm, and the following decreasing temperature profile was fixed from hopper to die: 195/190/185/180/175/170/165/160 °C. The extruded strand was cooled in a water bath, dried with an air-knife and then pelletized by a rotary cutter. The minimum residence time was estimated by measuring the time that a blue masterbatch pellet would take from being introduced directly into the extruder until exiting from the die.

The compounded pellets were shaped into sheets approximately 500- μ m thick by compression molding using a hydraulic hot press from Geo E. Moore & Son (Bham.) Ltd. (Birmingham, UK). About 10 g of material were placed in a hollow aluminum mold of 10 x 10 cm² and then introduced into the hot-press. Materials were initially preheated at 190 °C for 1 min without pressure and subsequently hot-pressed for 3 min at 4 tons. Then samples were cooled down to 25 °C by means of an internal water circulating system.

2.4. Characterization methods

2.4.1 Sheet thickness and samples conditioning

The thickness of the compression-molded sheets was measured with a digital micrometer series S00014 from Mitutoyo Corporation (Kawasaki, Japan), having ± 0.001 mm accuracy, at five random positions. Samples were aged for at least 15 days prior to any physical characterization in a desiccator at 25 °C and 0% relative humidity (RH).

2.4.2 Optical microscopy

Dispersion quality of CFs in the green composites was assessed with a light transmission optical microscope Olympus BH-2 from Olympus Optical Co., Ltd (Tokyo, Japan), coupled to a digital camera system Leica DFC280 from Meyer Instruments, Inc. (Houston, Texas, USA). Fiber attrition was investigated by monitoring fiber length distribution on composites with different CF contents and processing conditions. This was performed by immersing a small sample of each specimen in TFE at room temperature for 12 h. The resultant solution was centrifuged and filtered so that PHBV was removed and the recovered CFs were deposited onto microscopy glass slabs for analysis.

The functionalized CFs were analyzed by fluorescence microscopy using an Olympus BX51 from Olympus Optical Co., Ltd. The samples were previously dyed with Nile Red, a lipophilic dye, in a 0.25 mg/ml solution of DMSO at 1:10 dye/sample (vol./vol.) and then covered in aluminum foil to protect them from light exposure and dried at 25 °C for at least 12 h prior to observation. The samples were analyzed at an emission wavelength between 470 and 490 nm.

2.4.3 Melt flow index

The melt flow index (MFI) of the PHBV and green composites pellets were measured at 185 °C with a load of 1.2 kg using a MFI Daventest LPF-002 from Lloyd Instruments Ltd. (Bognor Regis, UK). Higher temperatures and weights triggered excessive mass outputs that impaired measurement accuracy. Data were reported as the mean value and standard error computed from 10 tests.

2.4.4 Thermal analysis

Thermal transitions were analyzed by differential scanning calorimetry (DSC) on a DSC-7 analyzer from PerkinElmer, Inc. (Waltham, MA, USA), equipped with a cooling accessory Intracooler 2 also from PerkinElmer, Inc. A heating step from -30 to 200 °C was followed by a cooling step to -30 °C under nitrogen atmosphere with a flow-rate of 20 mL/min. The scanning rate was 10 °C/min and an empty aluminum pan was used as reference. Calibration was performed using an indium sample. All tests were carried out at least in triplicate. The cold crystallization temperature (T_{cc}), enthalpy of cold crystallization (ΔH_{cc}), melting temperature (T_m), and enthalpy of melting (ΔH_m) were obtained from the first heating scan, while the crystallization temperature from the melt (T_c) and enthalpy of crystallization (ΔH_c) were determined from the cooling scan. The percentage of crystallinity (X_c) was determined using the following equation:

$$X_c = \left[\frac{\Delta H_m - \Delta H_{cc}}{\Delta H_m^0 \cdot (1-w)} \right] \cdot 100 \quad (\text{Eq. 1})$$

where $\Delta H_m^0 = 146.6$ J/g is the enthalpy corresponding to the melting of a 100% crystalline PHB sample (Barham et al., 1984) while the term $1-w$ represents the polymer weight fraction in the composite.

Thermogravimetric analysis (TGA) was performed in a TG-STDA model TGA/STDA851e/LF/1600 thermobalance from Mettler-Toledo, LLC (Columbus, OH, USA). The samples, with a weight of about 15mg, were heated from 50 to 900 °C at a heating rate of 10 °C/min under a nitrogen flow-rate of 50 mL/min.

2.4.5 Tensile tests

Dumbbell 500 μm -thick samples were die-cut from the compression-molded sheets and conditioned to ambient conditions, *i.e.* 25 °C and 50% RH, for 24 h. Tensile tests were carried out at room temperature in a universal mechanical testing machine AGS-X 500N from Shimadzu Corp. (Kyoto, Japan) in accordance with ASTM D638 (Type IV) standard. This was equipped with a 1-kN load cell and the cross-head speed was 10 mm/min. A minimum of six specimens were measured for each sample.

2.4.6 Permeability tests

The water vapor permeability (WVP) and limonene permeability (LP) were determined according to the ASTM 2011 gravimetric method. In the case of WVP, 5 mL of distilled water were poured into a Payne permeability cup ($\phi = 3.5$ cm) from Elcometer Sprl

(Hermalle-sous-Argenteau, Belgium). The sheets were placed in the cups so that on one side they were exposed to 100% RH, avoiding direct contact with water. The cups containing the sheets were then secured with silicon rings and stored in a desiccator at 25 °C and 0% RH. Identical cups with aluminum foils were used as control samples to estimate water loss through the sealing. The cups were weighed periodically using an analytical balance with ± 0.0001 g accuracy. Water vapor permeation rate (WVPR), also called water permeance when corrected for permeant partial pressure, was determined from the steady-state permeation slope obtained from the regression analysis of weight loss data per unit area versus time, in which the weight loss was calculated as the total cell loss minus the loss through the sealing. WVP was obtained, in triplicate, by correcting the permeance by the average sheet thicknesses. For LP, similarly, 5 mL of D-limonene was placed inside the Payne permeability cups and the cups containing the sheets were stored under controlled conditions, *i.e.* 25 °C and 40% RH. Limonene permeation rate (LPR) was obtained from the steady-state permeation slopes and weight loss was calculated as the total cell loss minus the loss through the sealing. LP was calculated taking into account the average sheet thickness in each case, measuring three replicates per sample.

Oxygen permeability (OP) was obtained from the oxygen transmission rate (OTR) measurements recorded in duplicate, using an Oxygen Permeation Analyzer M8001 from Systech Illinois (Thame, UK) at 25 °C and 60% RH. The samples were previously purged with nitrogen in the humidity equilibrated samples and then exposed to an oxygen flow of 10 mL/min. The exposure area during the test was 5 cm². Sheet thickness and gas partial pressure were measured. Measurements were performed in duplicate.

2.4.7 Antibacterial assays

The antibacterial activity of the neat OEO, the OEO-containing CFs, and the green composite sheets with the OEO-containing CFs was evaluated against *Staphylococcus aureus* (*S. aureus*) ATCC 6538P. This bacterial strain was obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain) and it was cultivated at optimal growth conditions in tryptone soy broth (TSB) from Oxoid Thermo Scientific (Basingstoke, UK), having a concentration of 6.3×10^8 colony forming units (CFU)/ml. Previous to each study, a 100- μ l aliquot from the culture was transferred to TSB and grown at 37 °C to the mid-exponential phase of growth with an approximate count of 5 x

10^5 CFU/ml. To ensure sterilization, all materials were initially exposed to ultraviolet (UV) radiation for 30 min in a Biostar cabinet from Telstar S.A. (Madrid, Spain).

The effectiveness of the OEO and OEO-containing CFs against *S. aureus* was tested following the plate micro-dilution protocol, as described in the Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition (M07-A10) by the Clinical and Laboratory Standards Institute (CLSI). For this, a 96-well plate with an alpha numeric coordination system (columns 1-12 and rows A-H) were used, where 10 μ l of the tested samples were introduced in the wells with 90 μ l of the bacteria medium. In the wells corresponding to A, B, C, E, F, and G columns different concentrations of OEO, *i.e.* 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, and 40 μ l/ml, and of OEO-containing CFs, *i.e.* 0.315, 0.625, 1.25, 2.5, 5, 10, 25, 50, 100, and 250 μ g/ml were tested, in triplicate, from rows 1 to 10. Row 11 was taken as positive control, *i.e.* only TSB, and row 12 was used as negative control, *i.e.* *S. aureus* in TSB. The plates were incubated at 37 °C for 24 h. Thereafter, 10 μ l of resazurin, a metabolic indicator, was added to each well and incubated again at 37 °C for 2 h. Upon obtaining the resazurin change, the wells were read through color difference. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of OEO and OEO-containing CFs presenting growth inhibition.

A modification of the Japanese Industrial Standard (JIS) Z 2801:2010 was performed to evaluate the bacterial efficiency on the sheets surface. This technique is useful to determine the antimicrobial activity of finished products, including polymer composite pieces (Torres-Giner et al., 2017). Briefly, a bacterial suspension of *S. aureus* of about 5×10^5 CFU/mL was spread uniformly on the surface of sheets with dimensions of 2×2 cm² and then covered by an inert 10- μ m LDPE film of 1.5×1.5 cm². After 24 h of incubation at 95% RH, bacteria were recovered with phosphate-buffered saline (PBS), inoculated onto tryptic soy agar (TSA) plates, and incubated at 37 °C for 24 h to quantify the number of viable bacteria. PHBV sheets with different CF contents were analyzed, using both sheets without fibers and with fibers without OEO as the negative controls. The antimicrobial activity was evaluated as synthesized, *i.e.* 1 day after preparation, and 15 days later. Surface reduction (R) was calculated using the next equation:

$$R = \left[\log\left(\frac{B}{A}\right) - \log\left(\frac{C}{A}\right) \right] = \log\left(\frac{B}{C}\right) \quad (\text{Eq. 2})$$

where A is the average of the number of viable bacteria on the control sample immediately after inoculation, B is the average of the number of viable bacteria on the control sample after 24 h, and C is the average of the number of viable bacteria on the test sample after 24 h. Three replicate experiments were performed for each sample and the antibacterial activity was evaluated with the following assessment: Nonsignificant ($R < 0.5$), slight ($R \geq 0.5$ and < 1), significant ($R \geq 1$ and < 3), and strong ($R \geq 3$).

3. Results and discussion

3.1. Effect of screw speed

Figure 3 shows the surface of the green composite sheets obtained with different CF contents and processing conditions. In all cases, the green composite sheets exhibit a smooth, defect-free, and uniform surface, in which CFs appeared randomly dispersed. Distribution was relatively uniform, albeit some small areas seem to contain lower CF concentration. As expected, CF content highly influenced the color intensity of the PHBV sheets. Sheets produced with the highest CF contents, *i.e.* 5 and 10 wt.-%, presented a wood-like visual feature, which could be aesthetically suitable to imitate solid wood parts for boards, lids or containers of interest in rigid packaging. In contrast, the processing conditions had a minor influence on both surface aspect and distribution of the CFs into the PHBV matrix. Only the sheets obtained from green composites compounded at 225 rpm presented a slight color intensification, which was probably caused by thermal degradation arising from the viscous dissipation associated to flow under higher shear-rates.

CFs in the green composite sheets were imaged by optical microscopy, after dissolving and filtrating PHBV. Representative pictures are displayed in **Figure 4**. Residence time and fiber dimensions were evaluated in order to select the optimal screw speed for which the set temperature and feed-rate were kept constant. **Table 1** reports both characteristics for each composition and screw speed. As expected, at a given feed-rate, residence time was gradually reduced with increasing screw speed, as flow along the screw was more effective and the number of fully filled channels decreased. For instance, for the neat PHBV formulation, residence time decreased from 131 s, at 75 rpm, to 97 s and 64 s, at 150 rpm and 225 rpm, respectively. The incorporation of CFs slightly increased the residence time due to their lower bulk density in relation to the PHBV pellets, *i.e.* the

total volume occupied by the material in the extruder increased though the output remained constant. For all processing conditions, CFs presented a similar diameter, varying in the range 100–400 μm and with a mean value of approximately 215 μm , whereas their length significantly decreased with increasing both the content and screw speed. Specifically, the average fiber length decreased from above 1900 μm , for the composite formulations with 1 wt.-% CFs, down to below 600 μm , for those containing 10 wt.-% CFs. This corresponds to a decrease of the fiber aspect ratio from approximately 9.0 to 2.5. Fiber lengths also decreased with increasing the screw speed, which is expected as stresses are larger for faster screw speeds. Interestingly, the green composite with 10 wt.-% CFs, presented the highest fiber length at 150 rpm, *i.e.* approximately 1179 μm , and the lowest at 75 rpm, *i.e.* approximately 542 μm .

In order to correlate fiber attrition to melt viscosity, MFI was evaluated on the PHBV samples processed at 150 rpm. As one can observe in **Figure 5**, the MFI values decreased with increasing the CFs content. This means that the melt viscosity of the composite formulations was higher than the neat PHBV formulation and, thus, the intensity of the thermomechanical stresses raised. This was mainly related to the intensification of fiber-to-fiber collisions. While fiber dispersion requires stresses to be larger than the cohesive strength of the aggregates, larger stresses also favor fibers rupture (Torres-Giner, 2016). As a result, composites formulated with more CFs presented higher viscosity and lower lengths. However, the lowest MFI value was observed for the composite formulation with 3 wt.-% CFs and then it increased for the larger CF contents. This viscosity increase at high CFs content suggests an enhanced biopolymer degradation or melt-shear thinning due to the presence of more fibers, which is known to occur in PHBV materials (Hilliou et al., 2016). Therefore, the complex interplay concerning reduced melt viscosity at higher CF contents, high residence times at low screw speeds, and thermal degradation of PHBV due to possible viscous heating, may explain the non-trivial evolution of fiber length.

From the above and within the experimental window tested herein, it can be concluded that the optimal screw speed to process the PHBV/CFs composites is 150 rpm. This processing condition delivered the most balanced fiber distribution, average fiber length, and intermediate residence times. In particular, the fiber aspect ratio remained in the range of approximately 5.5–9.0, decreasing with increasing the CF content, and the residence time is of the order of 100 s. It can be then considered that it could be beneficial to process both thermally sensitive PHBV and thermolabile additives.

3.2. Characterization of green composites sheets

The green composite formulations melt compounded at 150 rpm were thereafter compression-molded into sheets and these were characterized in terms of their thermal, mechanical, and barrier properties.

Table 2 summarizes the thermal properties determined by DSC and TGA. Within the experimental precision, all PHBV sheets presented similar values of T_g , T_c , and T_m (about $-3\text{ }^\circ\text{C}$, $121\text{ }^\circ\text{C}$, and $173\text{ }^\circ\text{C}$, respectively). Similar results have been reported in the literature for other PHBV materials, indicating that the HV content plays the major role in defining the thermal transitions (Shang et al., 2012). In addition, cold crystallization was not observed and all the samples crystallized from the melt in a single peak. Interestingly, the presence of CFs significantly affected the biopolymer degree of crystallization. While the neat PHBV sheet presented a X_c of approximately 54%, the addition of 1–5 wt.-% CFs content reduced it to 45–50%, whilst X_c increased to about 58% for the 10 wt.-% CF content. This result suggests that crystal growth in the green composites was controlled by two competing factors, namely, nucleation and confinement. As with other PHBV composites (Cunha et al., 2015), the presence of low contents of long fibers inhibited the chain-folding process of PHBV molecules, hindering molecular organization at the crystal growth front. Conversely, the existence of a high content of shorter fibers seems to have created a nucleating effect. The latter can be related to the degradation process triggered by the high CFs content, as explained above, by which PHBV molecules are shorter and thus easier to crystallize.

Concerning the TGA data, also listed in **Table 2**, one can observe that thermal decomposition of the PHBV composites started in the range of $270\text{--}280\text{ }^\circ\text{C}$, presenting a degradation temperature (T_{deg}) close to $300\text{ }^\circ\text{C}$ with a mass loss of approximately 70%. The incorporation of CFs slightly reduced the thermal stability of PHBV, but it also delayed the amount of mass loss during degradation. It is also worthy to note that the amount of residual mass increased, approximately from 3% to 6%, which can be related to the presence of inorganic impurities in the fibers. In general, the addition of CFs up to 10 wt.-% did not significantly alter the thermal degradation profile of PHBV, thus, positively not impairing its processing window.

Table 3 shows the tensile properties of PHBV and its green composite sheets. The neat PHBV sheet presented a tensile modulus of *ca.* 3.7 GPa, a tensile strength of about 34 MPa, and an elongation at break of about 1.2%. These properties clearly indicate that the PHBV sheets are rigid and brittle. As expected, the incorporation of CFs further increased, although not significantly, the tensile modulus and also reduced both tensile strength and elongation at break. The same response was reported for other compression-molded green composite sheets based on PHAs (Shibata et al., 2004; Torres-Giner et al., 2016b), which have been mainly related to a poor fiber-matrix adhesion. The 5 wt.-% CF-containing sheet presented the highest tensile modulus, *i.e.* approximately 4.1 GPa, with tensile strength and elongation-at-break values of approximately 29 MPa and 0.9%, respectively. It is also worthy to mention the low elastic modulus observed for the green composite sheet containing 10 wt.-% CFs, *i.e.* about 3.7 GPa, which can be related to the presence of fibers with lower mean fiber lengths, as previously shown in **Table 1**. The lowest values of tensile strength and elongation at break were also observed for the green composite sheet with 10 wt.-% CFs, *i.e.* approximately 25 MPa and 0.7%, respectively. Therefore, the CFs acted as an effective reinforcement of PHBV matrices when their length exceeds a critical threshold, in this case approximately 1500 μm , which corresponds to an aspect ratio of about 7. However, the exact mechanism for the reinforcement might go beyond the effect of fiber size, as both PHBV matrix thermal degradation and composite enhanced crystallinity in the sheet with 10 wt.-% CFs may add complexity to the mechanical-structural relationships.

Finally, the barrier properties against water vapor, limonene, and oxygen were determined and the permeability values are included in **Table 4**. The barrier performance is, in fact, one of the main parameters of application interest for food packaging. One can observe that the permeability to both water and limonene vapors slightly raised by increasing the CF content. Such increase was higher in the case of water vapor, reaching permeability values close to $4 \times 10^{-15} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$. Since water vapor is mainly a diffusivity-driven property due to moisture sorption in PHAs is relatively low (Razumovskii et al., 1994), this effect can be mainly related to heterogeneities in the sheets due to the inclusion of fibers. In addition, CFs are cellulosic fillers, being highly hydrophilic, and these can also certainly contribute to increase water solubility. To put these values into a more packaging context, WVP values of the here-developed green composite sheets are in the same order of magnitude as polyethylene terephthalate (PET)

films, *i.e.* $2.30 \times 10^{-15} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$ (Lagaron, 2011). Although the green composite sheets were still approximately 5.5 times more permeable than polypropylene (PP) films, *i.e.* $7.26 \times 10^{-16} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$, these were also over 5 times more water vapor barrier than films made of polyamide 6 (PA 6), *i.e.* $2.06 \times 10^{-14} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$ (Lagaron, 2011), which are both polymers widely employed in commercial plastics for flexible and rigid packaging applications.

Limonene transport properties are also important in packaging applications because this vapor is usually used as a standard system to test aroma barrier. The effect of fiber content on LP was relatively small and the neat PHBV and its green composite sheets with CFs showed values in the range of $1.1\text{--}1.5 \times 10^{-14} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$. In this sense, limonene, as opposed to moisture, is a strong plasticizing component for PHAs and, then, solubility plays a more important role in permeability than diffusion. For example, Sanchez-Garcia *et al.* (Sanchez-Garcia *et al.*, 2008) reported a limonene uptake of *ca.* 12.7 wt.-% for films made of PHBV with 12 mol.-% HV prepared by solvent casting, resulting in a LP value of $1.99 \times 10^{-13} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$. Interestingly, the here-prepared green composite sheets were 13–17 times more barrier to limonene, which can be ascribed to both the lower HV content of the copolyester and also to the preparation methodology. The observed increase in permeability when introducing CFs, though slight, can be related to the high sorption capacity of the fibers for organic components and/or to their poor adhesion to the PHBV matrix, as suggested by the previous mechanical analysis. In any case, the here-obtained PHBV-based sheets still presented LP values close to those also reported by Sanchez-Garcia *et al.* (Sanchez-Garcia *et al.*, 2007) for PHB films, *i.e.* $8.8 \times 10^{-15} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$, and approximately 8–10 times lower than PET films, *i.e.* $1.17 \times 10^{-13} \text{ kg}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$, both obtained by compression molding. In addition, due to the high thickness of the green composite sheets, these may still present low values of limonene permeance for being useful also in aroma barrier applications.

In relation to oxygen, the presence of CFs induced a slight reduction effect on permeability though the trend was not monotonic. Since this is a noncondensable small permeant, the presence of the cellulosic fibers can block oxygen diffusion while the heterogeneities within the composite can serve as preferential passage ways for the oxygen molecules. The highest barrier effect was observed for the PHBV/CFs 5 wt.-%, with a OP value of $1.73 \times 10^{-19} \text{ m}^3\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$, which is close to that of PET films, *i.e.* $1.35 \times \times 10^{-19} \text{ m}^3\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{Pa}^{-1}\cdot\text{s}^{-1}$. This suggests that at this fiber content the

contribution of the blocking effect over the heterogeneities was maximum. Overall, the here-obtained PHBV-based composite sheets presented OP values in the $0.7\text{--}0.8 \times 10^{-18} \text{ m}^3 \cdot \text{m} \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{s}^{-1}$ range, being slightly lower than those recently observed for electrospun homopolyester PHB films (Cherpinski et al., 2017) but higher than conventional 100- μm PHB films prepared by compression molding (Sanchez-Garcia et al., 2007).

3.3. Antibacterial activity of green composite sheets

In the last part of this study, the CFs were loaded by spraying different with weight contents of an oil–OEO mixture. **Figure 6** shows the fluorescence images taken by optical microscopy on the resultant OEO-containing fibers. One can observe that the oil–OEO mixture, shown as yellow-orange areas in the images, was homogeneously distributed along the fibers surface for contents up to 15 wt.-%. However, at the highest content, *i.e.* 45 wt.-%, the oily extract eventually saturated the fibers and accumulated on their outer surface. Based on this observation, the CFs containing 15 wt.-% oil–OEO mixture, which in turns provides a final content of 5 wt.-% OEO, were selected to further functionalize the green composites with antibacterial properties. As the CFs content was kept at 1–10 wt.-%, this resulted in a concentration range of 0.05–0.5 wt.-% OEO in the PHBV sheets.

The MIC values, defined as the lowest concentration of biocide substance that is capable of inhibiting bacterial growth, of both neat OEO and OEO-containing CFs were initially determined using the plate micro-dilution protocol. While the neat OEO presented a MIC value against *S. aureus* of 0.312 $\mu\text{l/ml}$, as expected, this value was significantly higher for the OEO-containing CFs, *i.e.* 25 $\mu\text{g/ml}$, which corresponds to a OEO concentration of 1.25 $\mu\text{g/ml}$. This reduction in antibacterial effectiveness, of about 4 times, can be related to partial losses during the spraying process, heterogeneous distribution of the OEO, and/or to in-depth entrapment of the antibacterial oil into fibers regions that were not available to interact with bacteria (*e.g.* pores). It is also worthy to indicate that the here-reported MIC values for OEO were relatively low, being for instance one order of magnitude lower than those reported by dos Santos Rodrigues *et al.* (dos Santos Rodrigues et al., 2017) against different planktonic and sessile cells of *S. aureus* isolates.

In relation to the possible biological mechanism exerted by OEO on *S. aureus*, Dadalioglu and Evrendilek (Dadalioglu and Evrendilek, 2004) attributed this effect to two particular components: the phenolic compound carvacrol and the monoterpene p-cymene.

According to Zivanovic and Draughon,(Zivanovic et al., 2005) the proposed action of phenolic compounds is based on their attack on the phospholipid cell membrane, which causes increased permeability and leakage of cytoplasm, or on their interaction with enzymes located at the cell wall. Indeed, most studies investigating the antibacterial activity of OEO agree that, generally, this is slightly more active against Gram-positive than Gram-negative bacteria (Burt, 2004; Hosseini et al., 2015; Zivanovic et al., 2005). Not only the type of bacteria but also the source and concentration of the active plant extract compounds and the film composition have a key effect on the antimicrobial activity of OEO. For instance, whey protein isolated (WPI)-based films with 1 wt./vol.-% OEO prepared by Oussalah *et al.*(Oussalah et al., 2004) were effective against different bacterial colonies on the surface of beef. However, Seydim and Sarikus (Seydim and Sarikus, 2006) observed that a content of at least 2 wt.-% OEO in WPI films was needed to achieve the minimum inhibitory level against the same bacteria.

Finally, **Table 5** includes the antibacterial effect against *S. aureus* of the green composite sheets with varying functionalized CFs contents. It can be observed that both the unfilled PHBV sheet and the different green composite sheets containing CFs without OEO showed no inhibition effect on the bacterial growth ($R \leq 0.5$). In contrast, the incorporation of OEO-containing CFs into the PHVB sheets exhibited significant antibacterial activity. At the initial day, *i.e.* for the tests carried out 1 day after the green composite sheets production, the bacterial reduction on the sheets surface gradually increased with the OEO-containing CFs content. At its lowest content, *i.e.* 1 wt.-% OEO-containing CFs, the green composite sheet presented a slight antibacterial activity ($R = 0.97$). For higher contents, *i.e.* 3–10 wt.-% OEO-containing CFs, the sheets generated a significant surface reduction ($R \geq 1$ and < 3). Although none of the sheets produced a strong reduction ($R \geq 3$), contents as low as 3 wt.-% OEO-containing CFs successfully inhibited the bacterial growth. Indeed, materials with values of surface reduction in the range 1–2 are usually considered as bacteriostatic (Castro-Mayorga et al., 2017). Therefore, final OEO contents from only 0.15 wt.-% in the green composites, which corresponds to a OEO-containing CFs loading of 3 wt.-%, were able to provide a bacteriostatic effect against *S. aureus*. As also shown in the table, after 15 days, the green composite sheets still kept significant antibacterial activity. In particular, the sheets with OEO-containing CFs loadings of 5 and 10 wt.-% presented still significant ($R \geq 1$ and < 3) values of reduction while the sheets presented slight ($R \geq 0.5$ and < 1) and

nonsignificant ($R \leq 0.5$) values for loading of 3 and 1 wt.-%, respectively. This suggests that, though part of OEO was released from the sheets, the CFs were still able to retain a significant amount of the active oil.

Previous studies based on films loaded with OEO against food spoilage microorganisms have shown that a minimum concentration of approximately 1.0 wt.-% of active material was necessary to ensure antibacterial efficacy. For instance, Benavides *et al.* (Benavides *et al.*, 2012) incorporated OEO in alginate films prepared by solvent casting where active contents at a level of 1.0 wt./vol.-% provided antibacterial properties. In another study, Hosseini *et al.* (Hosseini *et al.*, 2015) prepared OEO-containing fish gelatin/chitosan films also by the casting method, showing antimicrobial performance at 1.2 wt./vol.-%. Pelissari *et al.* (Pelissari *et al.*, 2009) recently produced starch/chitosan films in which OEO was incorporated at 0.1–1.0 wt.-% by blow film extrusion. The antimicrobial activity against *S. aureus* was only evaluated qualitatively by the disk inhibition zone assay, producing halos between 13.26 and 30.81 mm. Therefore, the here-attained results suggest that antimicrobial efficiency of the green composites based on OEO-containing CFS is about 6–7 times higher than that observed in previous research works. However, these studies are certainly difficult to compare in a straight way, even for the same bacteria, due to differences in the film nature, the selected methodology of film preparation, the performed antimicrobial test, etc.

4. Conclusions

Incorporation of CFs into a PHBV matrix was successfully achieved by melt compounding at 150 rpm. This processing condition led to composite formulations with fibers of an aspect ratio varying from 5.5 to 9.0, which decreased with increasing the CF content, and a residence time of about 100 s. These green composite formulations were afterward processed by compression molding. Resultant green composite sheets were easily processable and presented several advantages, such as good thermal stability, high rigidity, improved oxygen barrier, and potentially low costs. Finally, in a last stage, the CFs were loaded by spraying different OEO in order to be applied as the vehicle to generate green composite sheets with bacteriostatic effect against *S. aureus*. Through this approach, it has been possible to achieve a great antibacterial efficiency due to the high capacity of CFs to entrap active substances, being able to resist the processing conditions of thermoplastic materials typically used in the packaging industry.

Interestingly, it was observed that OEO was released in slow manner to the green composite sheet surface, remaining at effective concentrations for a period of at least 15 days, which is usually considered the average shelf life of packaged food products. Resultant essential oil-containing green composite sheets can easily find applications as monolayers in active food packaging, for instance trays and lids, or even boards to control undesirable microorganisms on surfaces in contact with foodstuffs. Furthermore, the here-developed materials are highly sustainable because of their whole renewable profile and biodegradability.

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Figure 1. (a) As-received reel of coconut fibers (CFs); (b) Ground CFs; (c) Sieved CFs.

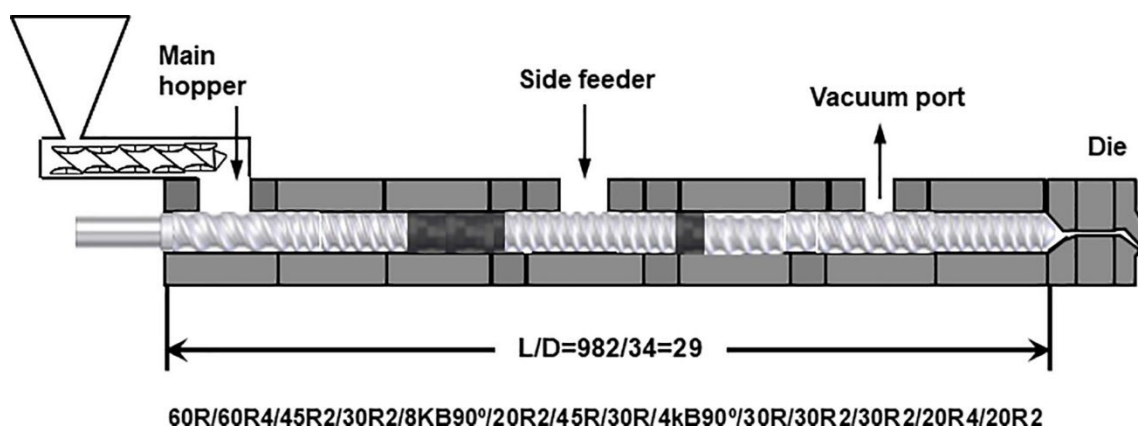


Figure 2. Detailed cylinder and screw configuration.

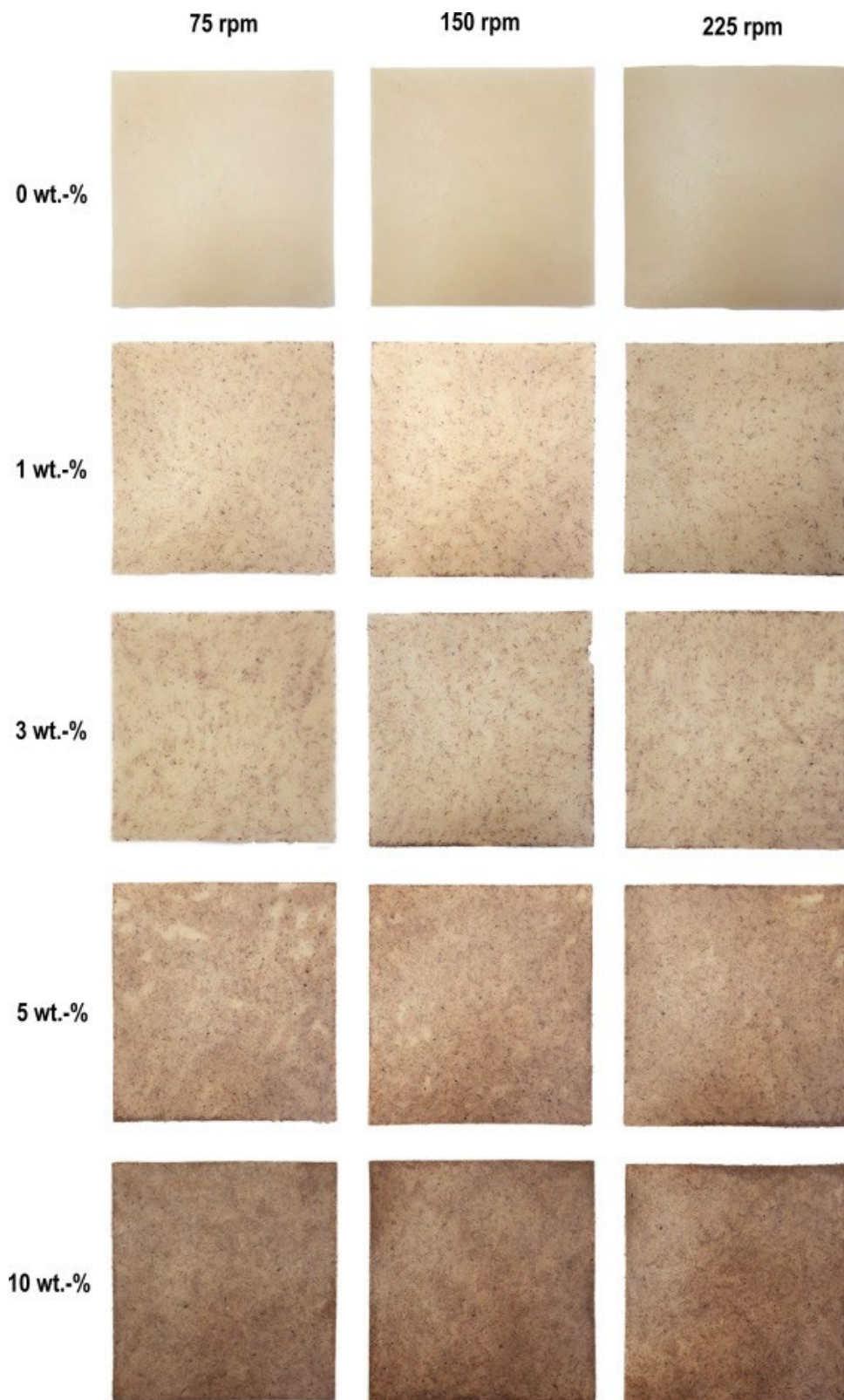


Figure 3. Surface appearance of the compression-molded sheets as a function of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and coconut fibers (CFs) weight content and screw speed.

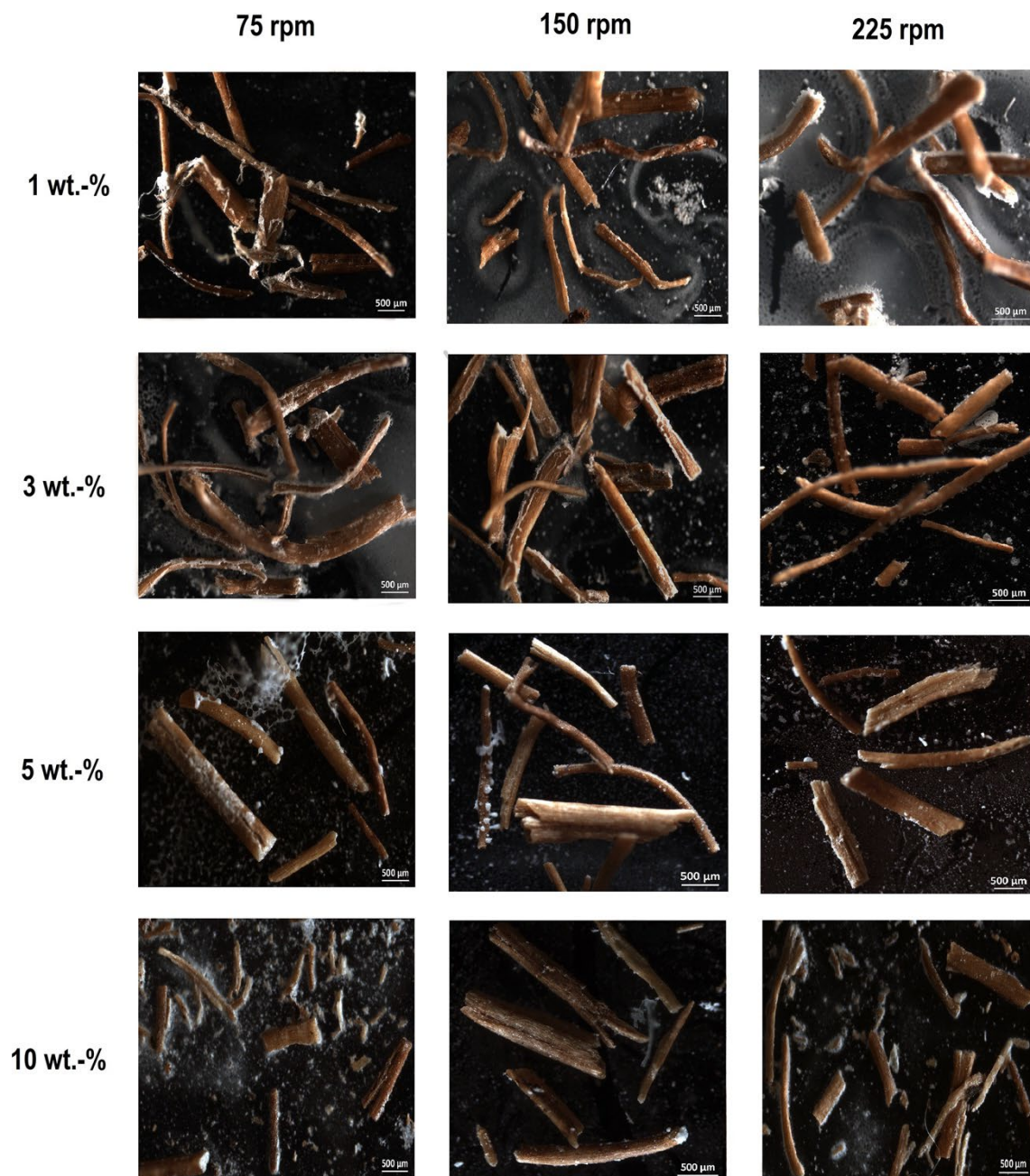


Figure 4. Melt flow index (MFI) of the green composite pellets as function of the coconut fibers (CFs) content. Test was performed at 185 °C with a load of 1.2 kg.

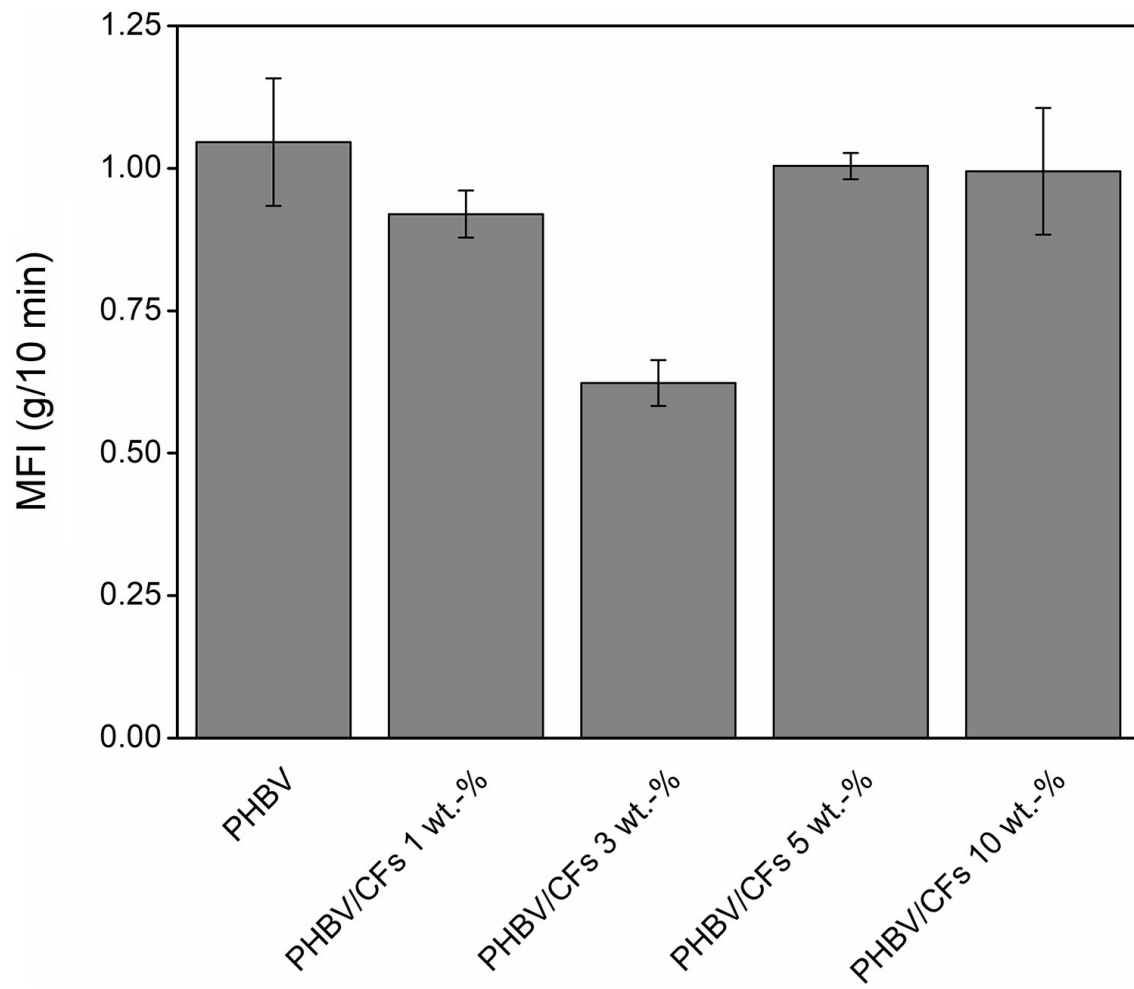


Figure 5. Optical microscopy images of the coconut fibers (CFs) extracted from the green composite sheets at different weight contents and screw speed. Scale markers of 500 μm .

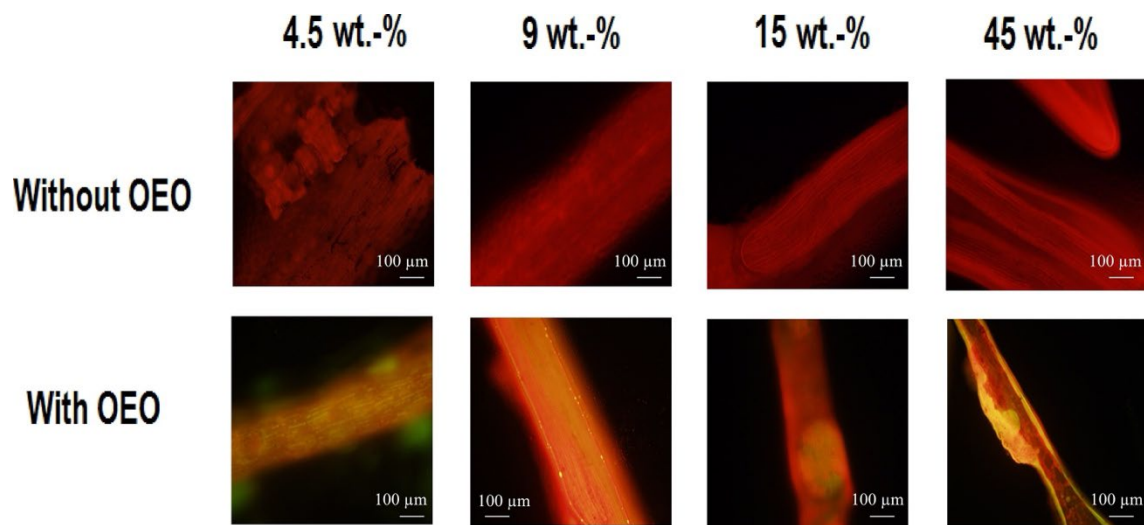


Figure 6. Fluorescence microscopy images of the coconut fibers (CFs) loaded with different weight contents of oil–oregano essential oil (OEO) mixture. Images were taken with an emission wavelength between 470 and 490 nm and scale markers of 100 μm .