Comparative Analysis of Bacterial and Microcrystalline Celluloses as Reinforcements for Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate)

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Cellulose and polyhydroxyalkanoates, polymers from renewable resources, have been intensively studied for the last decades and they are the main topic of this paper. Bacterial cellulose pellicles (BC) were synthesized in static culture fermentations and ultrasounds were used to disintegrate them into fibers (us-BC). Microcrystalline cellulose (MCC) and us-BC were characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray diffraction (XRD) and infrared spectroscopy (ATR-FTIR) with a view toward their use in fully biodegradable and biocompatible polymer composites. Mechanical properties of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) modified with the two types of celluloses were also investigated. AFM images showed that big aggregates of BC fibers disappeared after ultrasound treatment but small aggregates coexist with separated fibers in us-BC sample. Higher XRD crystallinity and crystallite dimension were obtained for us-BC as compared to MCC. Mechanical tests on poly(3-hydroxybutyrate-co-3-hydroxyvalerate), neat and modified with the two cellulosic fillers, showed that the highest values of tensile strength and modulus were obtained in the case of the composite containing us-BC.

Keywords: bacterial cellulose, polyhydroxyalkanoates, XRD, polymer composites, AFM

Polymers from renewable resources have become extremely interesting due to environmental concerns and petroleum resources mitigation [1]. Polyhydroxyalkanoates (PHAs) are linear polyesters composed of hydroxyl fatty acid monomers in which the carboxy group and the hydroxyl group of two different monomers form an ester bond [2]. They are produced by several bacteria such as *Rastonia eutropha* or *Bacillus megaterium* which have the capacity to convert renewable resources in PHA bioplastics. PHAs are insoluble in water and optically active containing the monomer units with only R configuration [3]. The molecular weight of PHAs ranges, in general, from 200,000 to 3,000,000 Daltons depending on the species of microorganism, the carbon source and the growth conditions [4]. Depending on their physical and chemical properties, PHAs can be used in different industrial fields: biodegradable plastics, medical implants or drug delivery. Due to their similar properties with several common polymers such as polyethylene or polystyrene, they can replace synthetic plastics in packaging and bottling or as medical and surgical materials [5]. PHAs are ideal materials for medical implants or devices, such as stents, orthopedic pins, cardiovascular patches, sutures, bone marrow scaffolds and tendon repair devices, due to their biocompatibility and biodegradation in human body, their removing after surgery being no longer necessary. The high cost of PHAs prevents for the present their widespread use, but several papers show that the use of waste vegetable oils or other cheap carbon sources for PHAs production could be the expected solution for reducing the costs [6]. Another barrier for the widespread use of PHAs is their high brittleness. Several papers have shown that the mechanical properties of PHAs could be improved by blending with other biodegradable polymers or with micro or nanofillers [7-8]. For example poly (3-hydroxybutirate) modified with 10 vol% hydroxyapatite showed improved mechanical properties [8].

Cellulose based materials have gained much attention in the last decades due to their availability, renewability, good mechanical properties and their ability to be used as both matrix or reinforcement in polymer composites [10-13]. Bacterial cellulose (BC) or microbial cellulose, an unbranched polysaccharide with long chains of (1→ 4)-linked β-D-glucopyranose is in this moment one of the most studied biopolymers [14-15]. BC is produced by several microorganisms as a carbon source for their growth and, despite the identical molecular structure with the cellulose from plants, its supramolecular structure as well as its physical and chemical properties are different from those of plant cellulose [16]. The most important differences are related to the high purity and crystallinity of BC as compared to plant cellulose, because it does not contain lignin or hemicelluloses. It is worth to mention that for dissolution and separation of cellulose from plants, laborious procedures involving polluting and hazardous chemicals must be applied [16].

The good thermo-mechanical properties of BC beside biocompatibility, biodegradability, hypoallergenity, bioconsistency, chemical stability and high water holding capacity make it a unique biomaterial more and more preferable for a wide range of applications in medicine, drug delivery systems, cosmetics, paper, textile, food industries, automotive and packaging industries [17]. BC is suitable for wound dressing, artificial skin, dental implants; vascular grafts; catheter covering dressing, dialysis membrane, cardiovascular and cranial stents, tissue engineering, vascular prosthetic devices, artificial blood vessels, controlled-drug release carriers to mention

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only biomedical potential applications [18]. Among all microorganisms, *Acetobacter xylinum* (*Gluconoacetobacter xylinum*), an aerobic, non-pathogenic, rod shaped and gram-negative bacteria, is the most efficient cellulose-producing specie [19]. The biosynthesis of BC which includes pathways involving enzymes and precursors has been extensively studied [15]. The relationship between the selected bacterial strain, type of substrate, cultivation conditions and additives and the supramolecular structure, physical and mechanical properties of BC has also been investigated [20-21].

The reinforcement of PHAs with cellulosic fillers for improving mechanical properties is a new trend in this field [22]. Biodegradable materials with improved barrier and mechanical properties were obtained from poly(3-hydroxybutyrate) and cellulose paper [23]. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) – PHBV reinforced with cellulose nanowhiskers displayed improved tensile strength and modulus and increase glass transition temperature [24]. PHAs reinforced with microcrystalline cellulose (MCC) have been studied only by Reinsch and Kelley [25] and, to the best of our knowledge, no work has been done on the influence of MCC and ultrasound treated bacterial cellulose (us-BC) on PHBV films.

An attempt to isolate BC fibers from pellicles by ultrasonication and to disperse them in organic solvents for preparing PHBV - BC composites by solution casting is presented in this paper. Both MCC and us-BC were studied as reinforcements for PHBV. The efficiency of BC disintegration into fibers was investigated by atomic force microscopy (AFM) and the morphostructural characteristics of MCC and BC were evaluated using scanning electron microscopy (SEM), AFM, X-ray diffraction (XRD) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Mechanical properties of PHBV modified with the two cellulosic fillers were determined and correlated with the degree of fillers dispersion.

Experimental part

Materials and methods

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with 2 wt% poly hydroxyvalerate and medium particle size of 300 μm, obtained by microbial synthesis, was purchased from Goodfellow Cambridge Limited, UK. Microcrystalline cellulose (MCC) with mean diameter 20 μm and bulk density 0.6 g/cm^3 was supplied by Sigma Aldrich (USA). Chloroform was purchased from Novachim Romania and used as received. Glucose, glycerol, citric acid monohydrate, sodium azide, ammonium sulfate and acetic acid were acquired from Scharlau Chemie.

Preparation of bacterial cellulose

BC was produced by Acetobacter xylinum using a method reported by Casarica et al [26]. The culture medium used for the fermentation of *Acetobacter xylinum* DSMZ-2004 (German Collection of Microorganisms and Cell Cultures) contained an extract from inadequate quality apples and 7.5 % glucose, 2 % glycerol, 0.2 % ammonium sulfate and 0.5% citric acid, the pH being adjusted to 5.5 by acetic acid. The culture media prepared in 500 mL Erlenmeyer flasks were inoculated with 5% (v/v) *A. xylinum* DSMZ-2004 inoculum media and were sterilized by autoclaving at 121°C, for 15 min. Incubation was performed under static conditions, at 30°C, for 14 days.

After incubation, BC pellicles grown on the surface of each liquid culture medium were collected and washed with water and then immersed in 1 N NaOH for 2 days at 30°C to dissolve the cells included in the pellicles. The pellicles were then immersed in water solution of NaN_3 (0.02%) to reduce microbial contamination and kept at 4°C, neutralized with 1% acetic acid and washed with distilled water. Several BC pellicles obtained as described above are shown in figure 1a. Two samples of BC pellicle were then dried on a glass plate to measure the weight.

Preparation of us-BC and composites

A microfibril suspension of bacterial cellulose (us-BC) was obtained using an ultrasonicator type Vibra Cell VC505 (500 W, 20 KHz), equipped with a sonication probe of 19 mm. The concentration of bacterial cellulose in distilled water was ~ 1%, ultrasonication power 80% and the overall time 30 min, cycles of 10 and 15 min breaks. For preventing the uncontrolled increase of temperature, the beaker with the cellulose suspension was put in a water bath.

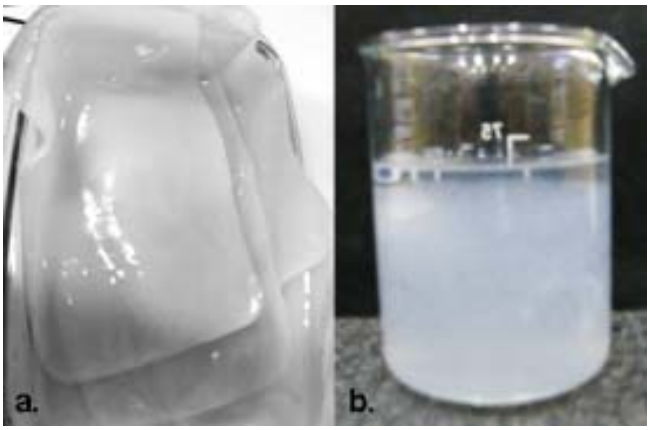


Fig. 1 a) BC pellicles as prepared by microbial synthesis; b) us-BC obtained from BC pellicles by ultrasonication

BC pellicles were disintegrated in BC microfibrils as shown in Fig. 1b. 1g (dry weight) of us-BC sample was dispersed in chloroform using a solvent exchange method from water through acetone into chloroform. The suspension of us-BC in water was centrifuged 15 min at 9000 rpm with a Universal 320R Ultracentrifuge to retain us-BC which was dispersed in acetone and homogenized at least 15 min using a magnetic stirrer. Us-BC was obtained from its dispersion in acetone by centrifugation in the same conditions as specified above. In the final stage, us-BC was re-dispersed in chloroform and mixed with the solution of PHBV in chloroform so that a final concentration of ~ 2 % us-BC in PHBV to be obtained. For MCC only this last dispersion of dry MCC (2 wt%) in chloroform solution of PHBV was performed. Composite films of ~ 30 μm from PHBV and MCC or us-BC were prepared using a solution casting method. A blank PHBV film was also prepared and served as reference.

Characterization methods

MCC was investigated by SEM using a Quanta Scanning Electron Microscope 200. BC before treatment and us-BC were investigated by atomic force microscopy using a Multimode 8 AFM from Bruker (USA) equipped with a Nanoscope V controller. AFM measurements were done at room temperature with 1 Hz scanning rate, using a silicon tip with nominal radius of 8 nm and a spring constant of 3 N/m.

XRD characterization of MCC and us-BC was performed using a DRON-UM diffractometer (horizontal goniometer Bragg-Brentano, reflection mode) with Co Kα radiation (wavelength λ = 1.79021A) as the radiation source. 2θ varied from 6 to 36° at a scanning rate of 0.05°/5 s. The

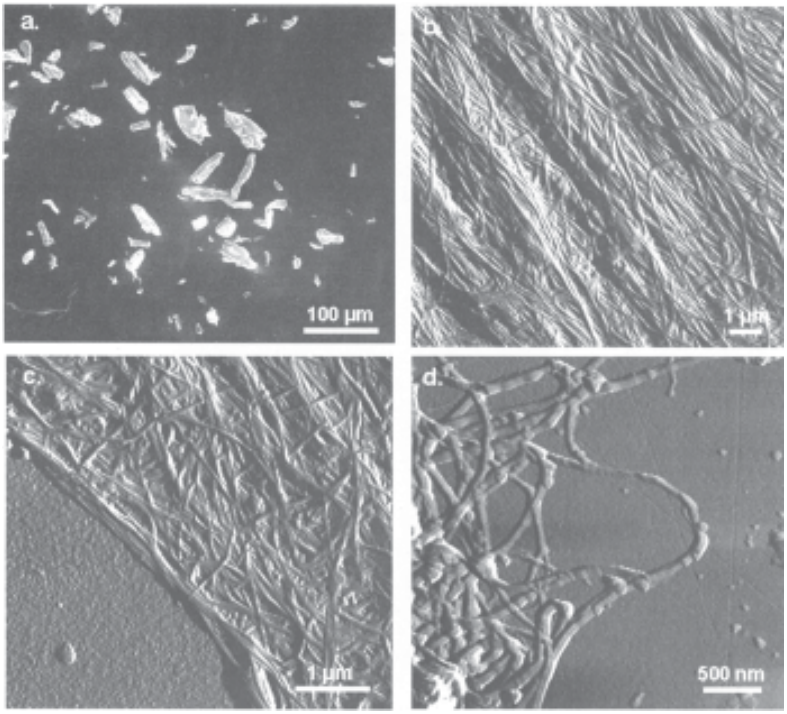


Fig. 2 a) SEM image of MCC; b) AFM image of original BC dried pellicle; c) and d) AFM images of us-BC after solvent exchange

crystallinity index (*C*) of samples was evaluated from the X-ray diffraction pattern as a ratio between the areas under the crystalline peaks (A_{cr}) and the total area (under the crystalline and amorphous peaks):

$$C(\%) = \frac{A_{cr}}{A_{cr} + A_{am}} \times 100 \quad (1)$$

where A_{am} is the area under amorphous halo.

Attenuated Total Reflectance Fourier Transform Infrared spectra of MCC and us-BC were recorded on a FTIR spectrometer TENSOR 37 from Bruker. Data were collected at room temperature from 4000 to 1000 cm^{-1} with 16 scans at a resolution of 4 cm^{-1}.

Tensile properties of PHBV film and composite films were determined at room temperature using Instron 3382 testing machine with a crosshead speed of 2 mm/min. Young's modulus was automated determined using the Bluehill 2 software of the Instron 3382. Five specimens were tested for each sample.

PHBV and composite films were also characterized using an optical microscope (MOTIC-DMW B1-223 ASC).

Results and discussions

Characterization of MCC and us-BC

SEM image of MCC is shown in figure 2a. The average width of MCC is 20 µm close to the size indicated by the supplier and the aspect ratio is between 2 and 4. AFM image of the original dried pellicle of BC (fig. 2b) shows a network of long cellulose fibers as obtained from microbial synthesis. It is worth noting that BC fibers length exceeds 20 µm, the largest length which can be observed by our AFM.

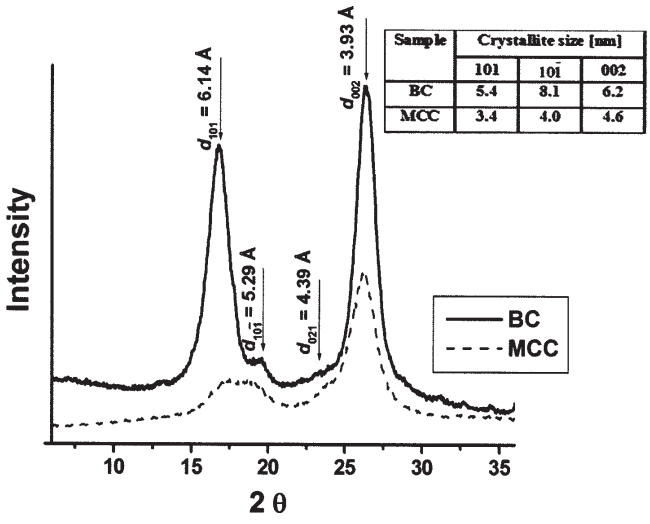
Highly rarefied network of BC fibers suspended in water was obtained after ultrasonication, as indicated in figure 1b. After solvent exchange, bacterial cellulose was investigated by AFM. AFM images of us-BC showed aggregated (frig. 2c) and almost separated fibers (fig. 2c) of ~ 100 nm width. It can be stated that big aggregates of BC fibers disappeared after ultrasound treatment but small aggregates coexist with separated BC fibers in us-BC sample after solvent exchange (fig 1c-d).

XRD pattern of BC is shown in figure 3. The diffraction peaks (16.8, 19.5, 23.5, 26.3°) as well as the corresponding *d*-spacing are characteristic to cellulose I and are similar to those mentioned in the literature [27], considering the wavelength of Co Kα radiation used in the test.

Small differences regarding diffraction peaks (17.3, 18.9, 23.5 and 26.2°) and inter-planar spacing (5.99, 5.40, 4.39 and 3.95 Å) were observed in the case of MCC [28], showing the same characteristic features of cellulose I crystalline structure. The big difference between the two cellulose samples is the higher crystallinity of BC as compared to that of MCC, 84 % instead of 75 %. Moreover, much lower β values related to smaller peak widths were obtained in the case of BC as compared to MCC. This is an indication for higher crystallite dimension, as observed in the table attached to figure 3. All these features are in good agreement with the higher level of mechanical properties reported for bacterial cellulose [21].

FT-IR spectra of MCC and BC are shown in figure 4 and the significant absorption bands are discussed below.

A broad band centered on 3340 cm^{-1} corresponds to the -OH stretching vibrations of cellulose: free O_6-H and O_2-H as well as weakly absorbed water over 3550 cm^{-1}, O_2-H…..O_6 intramolecular hydrogen bond (3460 - 3400 cm^{-1}), O_3-H…..O_5 intramolecular hydrogen bond close to 3350



Sample	Crystallite size [nm]		
	101	10$\bar{1}$	002
BC	5.4	8.1	6.2
MCC	3.4	4.0	4.6

Fig. 3 XRD patterns of BC and MCC

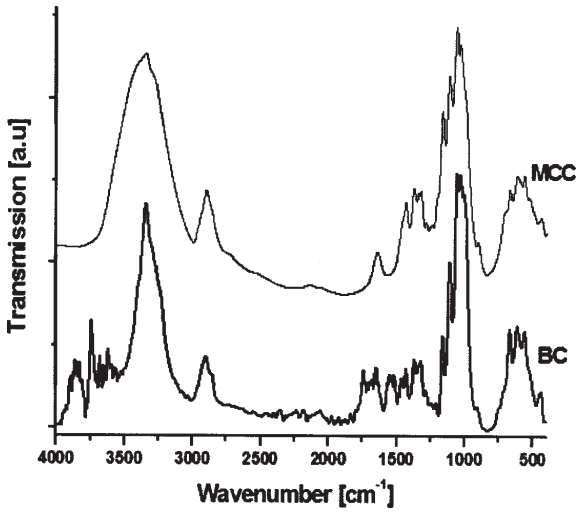


Fig. 4 FT-IR spectra of MCC and BC

Sample	Maximum tensile strength [MPa]	Young's modulus [MPa]
PHBV	6.4	550
PHBV-BC	7.1	710
PHBV-MCC	6.1	490

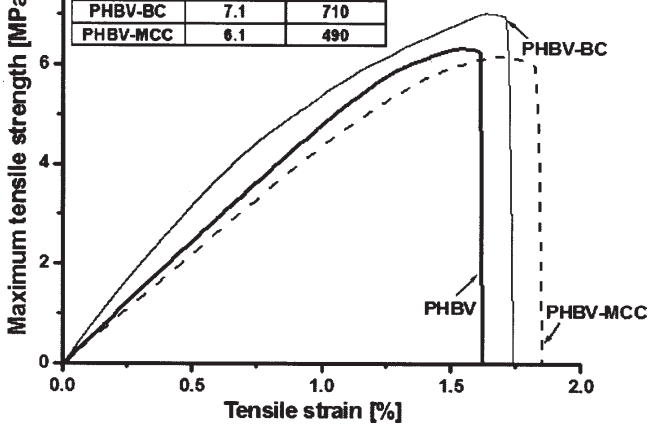


Fig. 5 Strain – stress curves of PHBV and composites

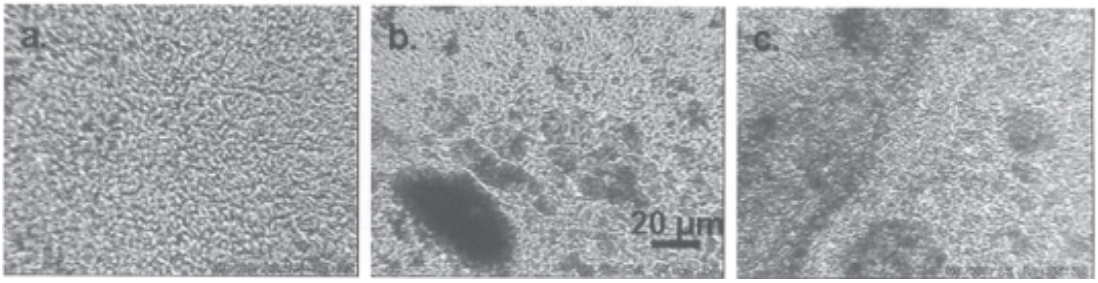


Fig. 6 Microscope images of PHBV (a), PHBV-MCC (b) and PHBV-BC (c)

cm^{-1}, O_6-H…..O_3 intramolecular hydrogen bond (3310 - 3230 cm^{-1}) in accordance with other reported data [25-26]. The peak which appears around 1640 cm^{-1} is assigned to the H-OH bending vibration of adsorbed water molecules. The region under 1200 cm^{-1}, the fingerprint region of native cellulose, shows several characteristic bands: C-C asymmetric stretching vibration mode of cellulosic ring at 1161 cm^{-1} and glycosidic C-O stretching vibration at 1107 cm^{-1} and also the C-OH stretching vibration of alcohols from cellulose molecule [29-30]. All these absorption bands are common to BC and MCC and appear at approximately the same frequency range in both samples. Besides these absorption bands characteristic to cellulose, bands that are characteristic to different additives and impurities from the culture medium, which have not been removed by the treatment, still appear in FT-IR spectrum of BC. For example, the low intensity peaks located at 1744 cm^{-1} and 1238 cm^{-1} are attributed to the C=O stretching of carbonyl and C–O stretching of acetyl groups, respectively and confirmed a very low degree of acetylation of BC [31]. Contribution from N-H stretching vibration of amide groups due to the biological impurities from bacterial cellulose should also be considered. The absorption bands around 1545 cm^{-1} could be related to amide II vibration from the proteins. It is worth noting that additional purification step of BC pellicles may lead to diminishing these bands.

Characterization of PHBV composites containing MCC or us-BC

Strain – stress curves of PHBV and composites containing 2 % us-BC or MCC are shown in figure 5.

Small values of elongation at break, between 1.62 and 1.85 % were obtained for all the films (neat PHBV and composites), showing increased brittleness. Small difference between the maximum tensile strength of PHBV and its composites can be seen in figure 5 and in the table attached to this figure. The reinforcing efficiency of BC in contrast to MCC is obvious from the values of Young's modulus shown in the table attached to figure 5. It is worth to note that PHBV modified with us-BC showed the highest values of tensile strength and modulus from all the samples. The efficiency of BC as reinforcement in PHBV as opposite to MCC can be due to both the higher aspect ratio and nanosize dimensions of us-BC. The reinforcing efficiency is limited by the dispersion level of cellulosic fillers. Optical microscope images (fig. 6a-d) showed poor dispersion of MCC in PHBV (fig. 6b) and areas with good dispersion (fig. 6c - right) and less good dispersion (fig. 6c - left) of us-BC in PHBV.

In the future works improved disintegration methods will be studied for obtaining separated BC fibers, considered very efficient reinforcing agents for PHAs by the authors.

Conclusions

Bacterial cellulose pellicles were synthesized in static culture fermentations and ultrasounds were used to disintegrate them into fibers (us-BC). AFM images showed that big aggregates of BC fibers disappeared after ultrasound treatment but small aggregates coexist with separated fibers in us-BC sample. XRD results showed the same features of cellulose I crystalline structure for both MCC and BC but different crystallinity. Higher XRD crystallinity was obtained for us-BC as compared to MCC, 84 % instead of 75 %. Mechanical tests on PHBV, neat and modified with the two cellulosic fillers, showed a higher reinforcing capacity for us-BC. The degree of cellulosic fillers dispersion in PHBV, observed by optical microscopy, correlated well with the mechanical properties of composites.

Acknowledgment: This work was supported by a grant of the Romanian National Authority for Scientific Research, project number PN-II-PT 158/2012.

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Manuscript received: 12.09.2013