PHB/cellulose Fibres Composites
Colonization and biodegradation behavior

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In this study, newly developed polymeric composites based on poly(3-hydroxybutyrate) (PHB), cellulose fibres (CF) and plasticizer (bis[2-(2-butoxyethoxy)ethyl] adipate) (DBEEA) were subjected both to microorganism's action in controlled conditions, and to soil's action by sample burial. The weight loss of PHB based composites at 60 days exposure were determined in comparison with neat PHB. The weight variation for PHB based composites buried in a natural soil at 45 days and 90 days was monitored. DSC-differential scanning calorimetry and SEM -scanning electron microscopy analysis were performed on the tested composites after 90 days of soil burial. It was found that the introduction of cellulose fibres into PHB leads to the increase of biodegradability of composites.

Keywords: poly(3-hydroxybutyrate); cellulose fibres; polymeric composites; colonization; fungi; biodegradability properties

Biodegradation has been defined as *a process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment,* according to the United States Environmental Protection Agency.

The degradation of renewable polymers depends on a number of factors such as environmental conditions (*p*H, humidity, temperature, salinity, etc.), shape of the polymers and the size of the exposure area, type of environment and also microorganism's availability. In addition, some chemical structures of polymers are more susceptible to microbial colonization and breakdown than others [1], depending on their composition, compatibility and miscibility between the components, composite surface roughness and of course, their structure phase (crystalline or amorphous) [2].

Biodegradation represents also the mineralization of materials as a result of the action of naturally-occurring microorganisms in the environment (fungi and bacteria). The biodegradation of polymers is caused, among other things, by microorganisms that colonize its surface forming biofilms that consist of cells enclosed in a polymeric matrix of their own origin, comprising proteins and polysaccharides [3]. Biofilms can also be seen as the aggregation of the microorganisms attached to the surface of an extracellular matrix [4].

Poly(hydroxybutyrate) (PHB) is the most common representative of poly(hydroxyalkanoate) family (PHA), and it has been proposed for short-term food packaging applications [5], given the fact that it has similar properties with polypropylene (PP), but with the advantage of being biodegradable and biocompatible and produced from renewable resources [6-8] or by a great variety of microorganisms, as an energy storage mechanism [9].

PHB can be processed like thermoplastic materials, is resistant to water and can thus be used to obtain similar materials to those derived from conventional plastic materials. PHB shows the melting temperature (T_m) in range of 174 - 180°C, a high degree of crystallinity (X_c) (60 - 70 %), and a glass transition temperature (T_g) about 5°C [10]. These thermal characteristics led to the narrow processing window of PHB materials [11] which limits their range of applications [12]. Furthemore, although poly(hydroxyalkanoates) are known to be good candidates for the replacement of petroleum polymers, their use in a wide field of applications is limited due to high cost price. The cost of the polyhydroxyalkanoates is about 15 times higher than that of conventional polymers, such as polypropylene [13]. Various mixtures of polyhydroxyalkanoates have been developed in order to reduce the price and to improve the PHA's performance. It is known that by mixing of polyhydroxyalkanoate and polylactic acid (PLA) with cellulose fibres, the composites show good mechanical properties (strength, stiffness, hardness) and low cost [14-16]. Also, renewable resources like lignin, clay, etc. represent a great alternative in the reduction of the final cost of the materials and to improve their properties, such as thermal, mechanical and barrier characteristics [17, 18].

Cellulose represents the major component in plants, being an inexhaustible renewable resource used as raw material [19]. Fibre reinforced polymeric materials represents a good alternative to metal or synthetic plastics used in various applications leading to the reduced final cost of the end material [20]. Besides this, cellulose fibres present high specific stiffness, low density, renewability and biodegradability [21]. In a previous paper [22] it was reported the polymeric materials based on PHB and cellulose fibres having good physical - mechanical characteristics, being suitable for packaging industry. Seggiani et al. [23] investigated the biodegradation of PHB/20% wood fibers composite in soil and compost and found the completed biodegradation after 78 days of composting (biodegradation of 95.1±8.3%) while after 195 days in soil the biodegradation was 60 %.

The aim of this work was to determine the biodegradation properties of some PHB/cellulose fibres composites and what role the cellulose fibres play in either acceleration or retarding PHB biodegradation.

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Experimental part

Materials and methods

In order to obtain the polymeric composites based on PHB and cellulose fibres, a commercial PHB type 319 E manufactured by BIOMER (Krailling, Germany), bis[2-(2-butoxyethoxy)ethyl] adipate (DBEEA), batch no. 201312170002 (PROVIRON Belgium) and cellulose fibres type EFC 1000 (CF) (Rettenmeier & Sohne AG, Germany) were used. The polymeric matrix has a density of 1.2197 g/cm^3 and melt flow index of 3.63 g/10 min. (180°C/2.16 kg). PHB pellets were previously dried in an oven at 60°C during 4 h while CF powder was dried at 80 °C for 4 h.

For *in vitro* microbiological biodegradation and colonization estimation, two minimal culture media were used: one with carbon source (coded M I) containing: KH_2PO_4 - 3 g•L^{-1}, K_2HPO_4 - 7 gL^{-1}, $(NH_4)_2SO_4$ - 7 gL^{-1}, peptone - 3 gL^{-1}, agar - 15 gL^{-1} and distilled water and one without carbon source (coded M II) containing: KH_2PO_4 - 3 gL^{-1}, K_2HPO_4 - 7 gL^{-1}, $(NH_4)_2SO_4$ - 7 gL^{-1}, agar - 15 gL^{-1} and distilled water. The specific fungal strains that are usually found in natural environment (water, soil) which were used are *Aspergillus brasiliensis* ATCC 16404 and *Paecilomyces variotii* ATCC 18502.

For the biodegradation assessment in soil conditions, it was used an active untreated soil, with the following characteristics: *p*H (1 g soil in 20 mL water) was 6.7; max 1.9 % of total N in dry matter; max. 0.5 % of P_2O_2 in dry matter; max. 0.9 % of K_2O in dry matter; min. 50 % content of burning substances; max. 1.2 mScm^{-1} electrical conductivity; max. 5 % of particles of more than 20 mm and max. 65 % humidity. Microbiological activity of the soil was determined by using the microbiological analysis - yeasts and moulds and total aerobic mesophilic count determination. The soil proved to be active from a microbiological point of view.

Composite preparation

The plastified polymeric composites based on PHB, plasticizer and cellulose fibres (CF) from 0, 2, 5 and 10 wt. % (coded: PHB/CF0; PHB/CF2; PHB/CF5 and PHB/CF10), were prepared by melting procedure, using a BRABENDER Plastograph, at a temperature of 180 ± 5 °C, a mixing time of 10 minutes, and the rotors speed of 40 rpm, as Tanase et al. [22] previously reported. There were obtained plates with a thickness of 1 mm (±0.001 mm) by the melt-pressed of material in the following conditions: temperature: 185 °C; preheated time: 10 min; pressing time: 5 min; cooling time: 40 min; and a pressure of 150 bars. All developed composites contain a ratio between PHB and plasticizer of 9:1.

In vitro biodegradability assessment

The method used was according to SR EN ISO 846:2000 [24]. The principle of this method consists of exposing the samples to microorganisms' action, for a certain period of time and constant temperature. Both minimal culture media (with and without carbon source) were prepared according to their recipes, sterilized at 121°C for 20 minutes, cooled at approximate 45°C and poured into Petri dishes. The specimens were cut from the melt-pressed plates in rectangular shapes of 6.0 x 1.5 x 0.1 cm dimension. *Aspergillus brasiliensis* ATCC 16404 and *Paecilomyces variotii* ATCC 18502 fungi were grown on Potato Dextrose Agar (PDA) medium in Petri dishes with 90 mm diameter for 7-9 days and stored at 25°C, before use. Fungal spore suspension (10^6 CFU) was obtained in aseptic conditions. Just before the solidifying of the culture media, the test samples were distributed into Petri dishes, and then each sample was inoculated in four points with the spore suspension using the method illustrated in figure 1. Two replicates were used for each sample. Neat PHB has been considered the reference material of the study.

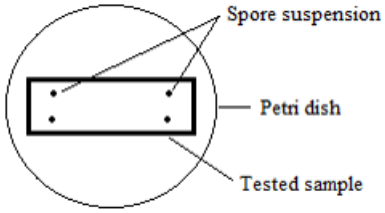

Fig. 1. Scheme of the working protocol for colonization assessment

- Colonization assessment

The colonization of the samples was monitored by visual observation every 20 days until 60 days of incubation in order to evaluate the degree of colonization, by quantifying the number of conidia and hyphae invading the samples.

Control samples (without fungi inoculum) were incubated in the same conditions as the inoculated ones. Five-grade scale of invasion ranging from 0 to 4 was established as a function of fungi observed on the surface of the films. Grade 0 indicates the absence of invasion; grade 1 is considered to correspond to a low attack with a maximum 25 % of the film surface covered with fungi, grade 2 indicates an expansion of moderate intensity with a maximum 50 % of the film covered with fungi, grade 3 is considered a high degree of colonization over 50 %, and grade 4 is denoted the growth of fungi occupying the whole surface of the specimen.

- Weight loss

After 60 days of exposure to microorganism's action, the specimens were washed with distilled water, submerged in ethylic alcohol for 5 minutes and thoroughly washed again and dried until a constant weight was obtained. Originally masses were noted for each test specimen with m_1, m_2, m_3, and after testing the masses were noted m_1^2, $m_2 2$, m_3^2. For each test specimen it was determined the weight change, $\Delta m = (m^2 - m)$ and then the arithmetic mean was calculated for each batch: Δm_0, Δm_i and Δm_s. The weight loss of PHB based composites by exposure to fungi using two minimal culture media was calculated using the following equation (1):

$$\Delta m_{biol.} = \frac{\Delta m_i - (\Delta m_s + \Delta m_0)}{m_e} \cdot 100, \% \quad (1)$$

where: Δm_i is the average mass of inoculated samples; Δm_s is the average mass of uninoculated control; Δm_0 is the average mass of control samples; m_e is the average mass of initial samples (uninoculated).

Biodegradability assessment using the samples burial in soil

The specimens cut in rectangular shape (6 x 1.5 x 0.1 cm) were buried in a natural soil, for 45 days and 90 days respectively. This method was adapted from SR EN ISO 846/2000 [24].

The glass recipients were filled with soil, and then the polymeric composites samples were buried vertically in soil in 3 replicates for each polymeric composite (fig. 2) and for each stage of removing the composites from soil (45 days and 90 days). To ensure the oxygen flow, the glass recipients were not sealed, the incubation being performed at a temperature of 25°C.

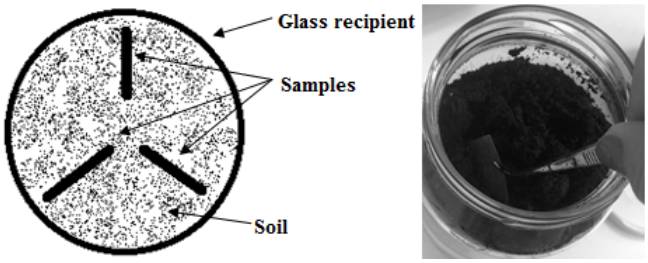

Fig. 2. Disposal of samples in soil

-Weight loss

At the end of each testing period (45 and 90 days respectively), the PHB composites were extracted from the soil, washed with distilled water in order to remove soil and dried on filter paper. The test samples were then stored in a desiccator for 48 h until they reached a constant weight, weighed, and the weight variation (biodegradation degree) was determined using the following equation (2):

$$Weight\ loss = \frac{\Delta M_i - \Delta M_f}{\Delta M_i} \cdot 100, \% \quad (2)$$

where: Weight loss represents the variation of samples weight (biodegradation degree), ΔM_i represents the average of samples initially weighed and ΔM_f represents the average of the samples finally weighed (at the end of the incubation period).

-Differential scanning calorimetry (DSC)

The extent of biodegradation of PHB composites has been deeply characterized by means of Differential Scanning Calorimetry (DSC) analysis using a DSC 823^e (Mettler Toledo, Switzerland) calibrated with indium standard. Test samples buried in soil for 90 days were heated from ambient temperature to 220°C at a heating rate of 10 °C/min. The melting temperature (T_m), melting enthalpy (ΔH_m) and degree of crystallinity (X_c) were extracted from the DSC curves. The degree of crystallinity (X_c) of PHB composite was calculated using equation (3):

$$X_c = \frac{\Delta H_m}{\Delta H_m^o} \cdot 100, \% \quad (3)$$

where: ΔH_m is the melting enthalpy of the test samples (Jg^{-1}) and ΔH_m^o is the enthalpy value for a theoretically 100 % crystalline PHB (146 Jg^{-1}) [25].

-Scanning electron microscopy (SEM) analysis

The morphology of the PHB/cellulose fibres composites was examined using a Carl Zeiss EVO LS 15 scanning electron microscope, at accelerating voltages between 0.2 and 0.5 kV, at different magnifications, in High Vacuum. Prior to analysis by SEM, the photographs of the composites were taken with a Carl Zeiss Stemi 2000-C stereo-microscope equipped with a Canon Power Shot A640 Digital Camera.

Results and discussions

In vitro degradation assessment

Degree of colonization

The degree of colonization of the tested samples with *Aspergillus brasiliensis* ATCC 16404 and *Paecilomyces variotii* ATCC 18502 is presented in table 1.

It can be observed that the colonization degree was between 1 and 4 at the end of the incubation period for both culture media tested, 1 being usually the value obtained by PHB [26]. Good results were given by the samples that were inoculated on the M II culture media (without any carbon source), especially for the samples containing CF (PHB/CF2, PHB/CF5, PHB/CF10), meaning that the fungi colonized the tested samples, using them as carbon sources for their development.

Samples colonization with both fungi registered a degree of colonization mainly of 3 and 4 at the end of the incubation period, without noticeable differences between the using of different culture media (M I or M II). Neat PHB obtained value 1 on both culture media used, while PHB/CF based composites obtained mainly the values 3 and 4. The obtained results led to conclusion that the newly developed composites can be considered great substrates for the

Sample	*Aspergillus brasiliensis* ATCC 16404						*Paecilomyces variotii* ATCC 18502					
	Incubation time (days)						Incubation time (days)					
	M I			M II			M I			M II		
	20	40	60	20	40	60	20	40	60	20	40	60
PHB	0	0	1	0	0	1	0	1	1	0	1	1
PHB/CF0	1	2	2	1	2	3	1	1	2	2	2	4
PHB/CF2	0	1	3	3	3	4	1	2	2	0	1	3
PHB/CF5	1	1	3	2	2	4	1	3	4	2	3	4
PHB/CF10	2	3	4	1	3	4	2	3	4	3	4	4

Table 1
THE DEGREE OF COLONIZATION OF THE TESTED SAMPLES USING *ASPERGILLUS BRASILIENSIS* ATCC 16404 AND *PAECILOMYCES VARIOTII* ATCC 18502 FUNGI

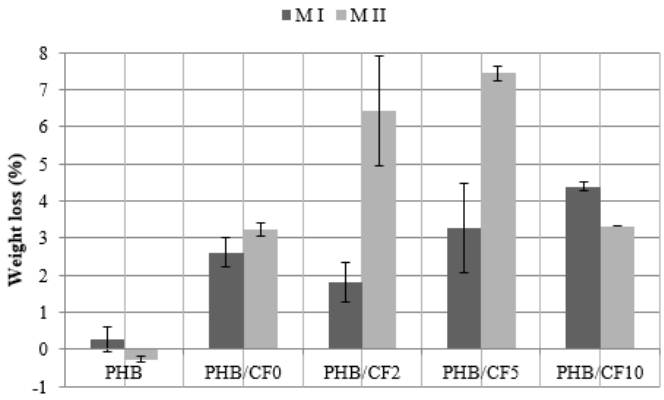

Fig.3. *Aspergillus brasiliensis* ATCC 16404 effect over PHB/CF based polymeric blends

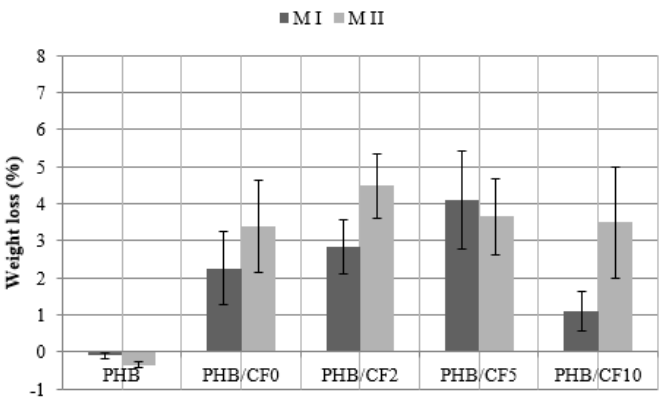

Fig. 4. *Paecilomyces variotii* ATCC 18502 effect over PHB/CF based polymeric blends

microorganism development. Similar results were obtained by Rapa et al. [26, 27], when tested the aptitude at colonization of *Trichoderma viride* and *Penicillium* spp. fungi on PHB loaded with wood fibres/cellulose fibres.

Weight loss

A great difference between the samples inoculated on M II minimal media (without any carbon source) and the ones incubated on M I (with carbon source), in case of both tested fungi (*Aspergillus brasiliensis* ATCC 16404 (fig. 3) and *Paecilomyces variotii* ATCC 18502 (fig. 4)) was observed.

The PHB composites incubated for 60 days on M II culture media presented a higher biodegradation rate, due to the process by which the microorganism is feeding on the tested materials, when no other carbon source is available. Thus, PHB/CF5 composite registered the higher biodegradation rate (7.3 %) when it was subjected to the action of *Aspergillus brasiliensis* ATCC 16404 fungus, compared to the other tested composites, while PHB/CF2 composite registered the higher biodegradation rate (4.3 %) when it was subjected to the action of *Paecilomyces variotii* ATCC 18502 fungus. Still, the polymeric composites based on PHB and CF presented a higher biodegradation rate compared to neat PHB and plasticized PHB, these results being in accordance with the colonization assessment (table 1).

Biodegradability assessment using the samples burial in soil

Weight loss

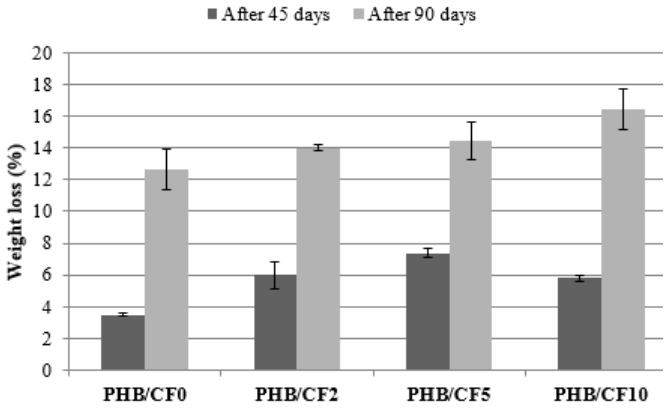

Fig. 5. The graphical representation of the weight loss values of the PHB/CF based polymeric blends buried in soil

The weight loss of PHB composites after 45 and 90 days of soil exposure is presented in figure 5.

During the soil incubation period, it was observed that PHB/CF based polymeric composites presented significant weight losses meaning that samples absorbed water, which facilitates the degradation process, according to the literature [28]. Thereby, at the end of the incubation period it can be observed an increasing of the biodegradation rate twice as much compared to the measurements performed after 45 days of incubation. After 45 days exposure in soil, from figure 5 it is observed that the PHB/CF 10 composite shows a lower weight loss than the composites containing 5 and 2% CF respectively, maybe due to the inhomogeneity of the cellulose fibres into polymeric matrix. Also, the biodegradation rate increases with the increasing amount of CF in the polymeric matrix, the highest value (16.1%) being obtained for PHB/CF10 composite at the end of the incubation period (90 days). These results took us to the conclusion that the developed polymeric composites based on PHB and CF can be easily degraded in soil.

Differential scanning calorimetry (DSC)

DSC curves for PHB composites initially and after degradation in soil are shown in figure 6. The DSC parameters for the PHB/CF composites before and after soil burial are evaluated from DSC curves and are summarized in table 2.

The neat PHB has an endothermic melting peak at 174 °C and the degree of crystallinity of 53 % [22, 29]. Adding of 10 % plasticizer into polymeric matrix led to a decreased melting temperature of 173.2 °C. Incorporation of CF into plas[illegible]. As a

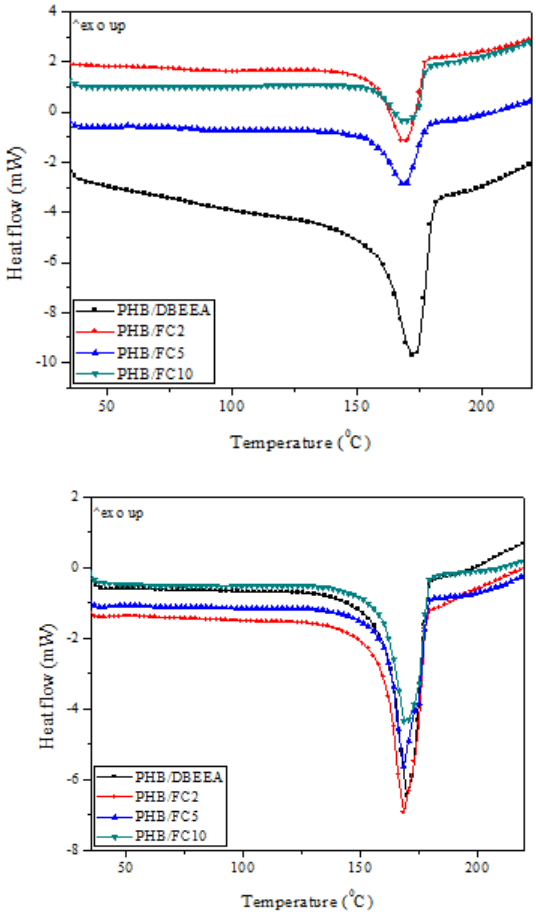

Fig. 6. DSC curves for PHB composites, first scan
a) Initial; **b)** After 90 days incubation in soil

Sample	Initial			After 90 days		
	ΔH_m ($J \cdot g^{-1}$)	T_m (°C)	X_c (%)	ΔH_m ($J \cdot g^{-1}$)	T_m (°C)	X_c (%)
PHB/CF0	94.7	173.2	64.8	80.6	170.2	55.2
PHB/CF2	52.5	168.9	35.9	95.9	168.8	65.6
PHB/CF5	59.0	168.7	40.4	82.4	168.9 174.9	56.4
PHB/CF10	62.8	173.1 168.4	43.0	78.6	169.4	53.8

Table 2
THE DSC PARAMETERS FOR PHB/CF BASED BLENDS (FIRST HEATING RUN), INITIALLY AND AFTER 90 DAYS OF SOIL BURIAL

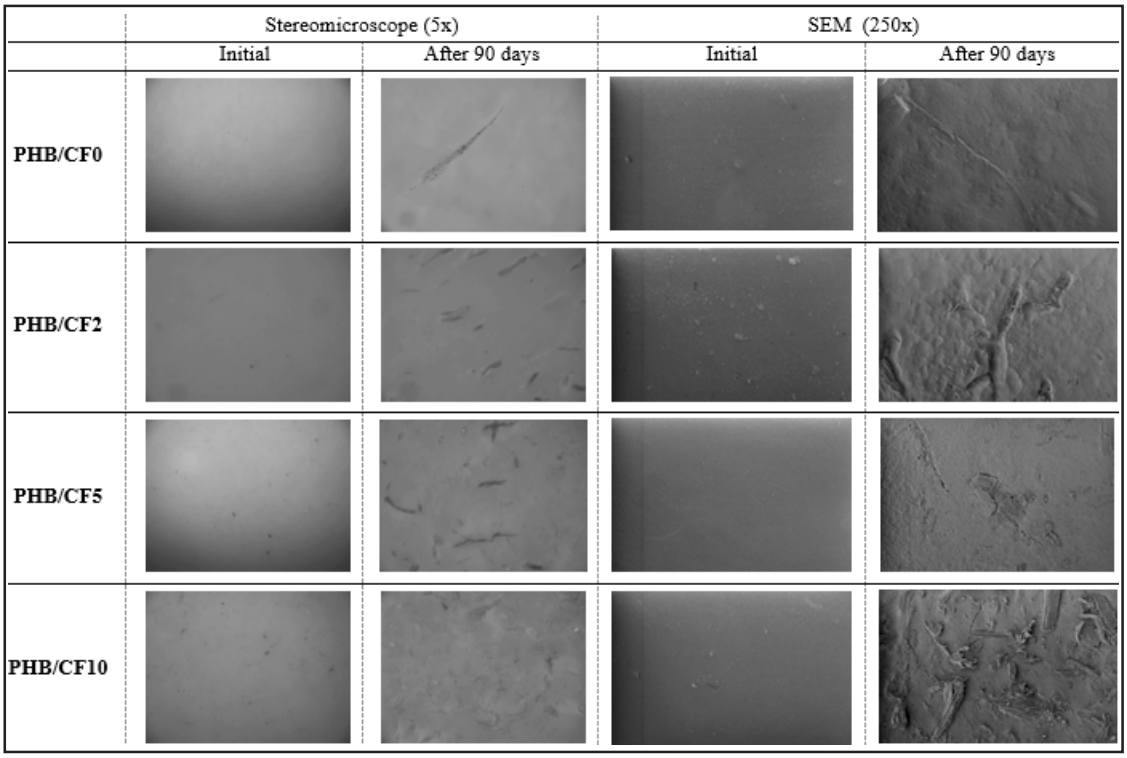

Table 3
STEREOMICROSCOPE AND SEM IMAGES OBTAINED FROM THE ANALYSIS OF THE PHB BASED POLYMER BLENDS BEFORE AND AFTER EXPOSURE TO THE SOIL ACTION

result, the processing temperature window was increased [31].

Analysing the data from table 2 it is observed that by adding of only 2% CF into plasticized PHB a more pronounced effect of perturbing the lattice structure of PHB than other contents of CF was registered. By increasing of CF, the crystallinity degree of PHB composites slowly increased than PHB/CF2 composite.

Generally, lower values of T_m were registered for the biodegraded composites compared to the initial values; these values may be associated with the breaking of the PHB chains [17]. Regarding the degree of crystallinity (X_c) and melting enthalpy (ΔH_m) of the composites, they increases after the exposure to soil compared to the initial values. The increasing of X_c for samples exposed to soil proved their degradability. After 90 days, it is observed that the PHB/CF2 composite shows the highest degree of crystallinity (65.6 %). Degradation becomes in the amorphous regions that are easy to attack and continues in the crystalline regions of composite that are less susceptible to attack due to their ordered structure [32]. This behavior at the soil action highlights the fact that a biodegradation process took place in the composites structure. Similar results were obtained also by Mousavioun et al. [2], who studied the degradation in environmental conditions of some composites based on PHB and lignin.

Microscopic evaluation

The surfaces of the tested polymeric composites were examined both by stereomicroscope and SEM analysis. The stereomicroscope micrographs show significant degradation, evident in holes around the cellulosic fibres (table 3), the changes being clearly observed when compared to the unexposed samples. These results confirm the gravimetric data presented in figure 5.

The SEM micrographs (table 3) show that the initial surface of the tested samples show a smooth aspect, the cellulose fibres could not be observed due to the homogeneous blends. After maintaining the analysed samples buried in soil for 90 days, cellulose fibres were revealed and they can be observed into the polymeric matrix, but also a rough surface of the tested material. This fact took us to the conclusion that the surfaces of the samples were modified as a result of the biodegradation process, which was more intense around the cellulose fibres from the polymeric matrix.

Conclusions

During this study it was observed that the colonization and the biodegradation process depended on the amount of cellulose fibres incorporated in the polymeric matrix. The results obtained after *in vitro* determination of the biodegradation rate using fungi on the developed materials showed that the materials could be easily colonized and biodegraded by *Aspergillus brasiliensis* ATCC 16404 and *Paecilomyces variotii* ATCC 18502. Furthermore, the biodegradation in soil revealed that the PHB/CF composites presented significant weight losses during the exposure period, the biodegradation rate after 90 days being more than twice as much compared to the measurements performed after 45 days of exposure. Overall, this study showed that the incorporation of CF to PHB matrix improved the biodegradability of the tested samples in soil conditions, fact that could lead to developing of environment-friendly materials especially used as packaging materials.

Acknowledgements: This paper was published under the frame of European Social Fund, Human Resources 335 Development Operational Programme 2007-2013, Project no. POSDRU/159/1.5/S/132765.

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Manuscript received: 16.11.2017