

## Research Article

# In Vitro Degradation of PHB/Bacterial Cellulose Biocomposite Scaffolds

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The present work reported the preparation of biocomposites based on poly(3-hydroxybutyrate) (PHB), plasticizer, and bacterial cellulose (BC) by melt processing and their testing by means of thermal properties (DSC), water absorption, and in vitro degradation. The surface of the biocomposites was analyzed via atomic force microscopy (AFM). In vitro degradation of the biocomposites was evaluated by weight loss and thermal properties (DSC) assessment after the immersion of the specimens in phosphate-buffered saline solution (PBS of pH 7.4) over 20 days. The results showed that the BC can reduce the PHB crystallinity and promote its degradation under PBS medium. Moreover, it was found that the water absorption increased as the percentage of BC increased.

## 1. Introduction

Poly(3-hydroxybutyrate) (PHB) is a bacterial polyester produced as an intracellular energy storage material under growth conditions characterized by a good biocompatibility and biodegradability. It has a similar degree of crystallinity, glass transition temperature, melting temperature, Young's modulus, and tensile strength as polypropylene (PP). In addition, PHB is not toxic for the cells [1], in part due to the fact that it degrades *in vivo* to 3-hydroxybutyric acid, which is a common metabolite that occurs in living organisms [2]. However, PHB alone does not fulfill all technical specifications for use as a biomaterial in medical and tissue-engineering applications and has low melt elasticity and a narrow thermal processing window [3], which is due to its high crystallinity and high melting temperature of

the crystalline domains. As a consequence, mixing PHB with other polymers and additives is a good strategy to obtain the desired properties. It was found that the reinforcement of PHA with cellulosic fibers gives the polymer composite good mechanical properties (stiffness, strength, and toughness) as well as a low cost [4–6].

Bacterial cellulose (BC) is mainly produced by Gram-negative bacteria of the *Gluconacetobacter* genus [7]. It is a highly flexible biomaterial used to treat chronic wounds and burns, such as artificial skin or wound dressings, to obtain artificial vessels and scaffolds for tissue engineering [8, 9]. BC differs from plant cellulose by high purity, crystallinity, degree of polymerization, tensile strength [10, 11], and high water-absorbing capacity [12]. In a previous paper, we reported the good biocompatibility of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

loaded with bacterial cellulose and microcrystalline cellulose via melt processing [13].

Conventional techniques to fabricate open porous scaffolds include solvent casting/salt leaching, phase separation, gel casting, precipitation, emulsion freeze-drying, and electrospinning methods. There are known scaffolds, mostly prepared by casting method, based on PHB blended with hydroxyapatite (HAP) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) using salt-leaching method [14], bacterial cellulose (BC)/polyethylene glycol (PEG) [15], polyacrylamide (PAm)/hydroxyapatite (HAP)/poly(vinyl alcohol) (PVA) [16], poly(lactic acid) (PLLA) [17], poly(hydroxyalkanoates) (PHA) [18–22], and polycaprolactone (PCL) [23]. The designed polymers for the production of scaffolds should have appropriate surface chemistry to favor cellular attachment, porous structure, favorable mechanical properties, a controlled degradation rate, preferably less than one year under physiological conditions, biodegradability or bioresorbability, and a good biocompatibility [24]. The degradation rates of polymers can be designed through the addition of various components to the polymeric composition, as well as selection of the chemical composition, molecular weight, and processing conditions. *In vitro* degradation of biodegradable polymers has been largely investigated and monitored by using various techniques to evaluate the changes of weight loss, water absorption, thermal properties, and chemical composition [25, 26].

This paper describes the synthesis of bacterial cellulose, preparing of PHB/BC formulations by melt processing and, subsequently, their characterization by surface examination, thermal analysis (DSC), water absorption technique, and *in vitro* degradation of scaffolds occurred after immersion of specimens in phosphate buffered saline (PBS, pH = 7.4) over 20 days.

## 2. Materials and Methods

**2.1. Materials.** Poly(3-hydroxybutyrate) (PHB, T19 grade) with size dimensions in the range 0.5–50  $\mu$  was purchased from BIOMER, Germany. Bacterial cellulose (BC) was synthesized by The National Chemical-Pharmaceutical for Research and Development Institute (ICCF Bucharest, Romania). Tributyl citrate (TBC) supplied by Sigma-Aldrich was used as bioplasticizer. Sodium chloride (NaCl) with the size in the range 420–500  $\mu$ m was used as a porogen. Phosphate buffered saline (PBS) medium (pH 7.4) was prepared in laboratory.

**2.2. Preparation of PHB/BC Composite Scaffolds.** PHB/BC composite scaffolds were obtained following the salt leaching procedure [27]. PHB powder and BC powder containing 1% and 2% by weight, respectively, were melted in a Brabender Plastograph at 180°C, for 6 minutes and a rotor speed of 40 rpm, respectively. Then 3 wt% NaCl particles were introduced into the melted mixtures, and the process continued until 6 minutes. The plasticizer was added to each formulation at the beginning of mixing to provide 4 : 1 ratio between PHB and TBC. The samples were labeled PHB/BC1% and

TABLE 1: Characteristics for melt processing of PHB/TBC and PHB/BC samples.

Sample	$M_{1\min}$ (nm)	$M_{4\min}$ (nm)	$M_{6\min}$ (nm)	(nm/rpm)
PHB/TBC	29.5	20	11	0.275
PHB/BC 1%	36	22	12	0.300
PHB/BC 2%	34	23	12	0.300

PHB/BC2% according to their composition. PHB/TBC signifies the sample containing only PHB and plasticizer.

Before processing, both PHB and BC were oven dried at 60°C overnight and at 105°C, for 2 h, respectively. The melted biocomposites were pressed into thin films by compression molding using a laboratory press type POLYSTAT 200 for 5 minutes pressing time, at a temperature of 185°C and a pressure of 200 bars. The investigated methods were carried out on the films subjected to salt-leaching process to remove the NaCl.

### 2.3. Investigation Methods

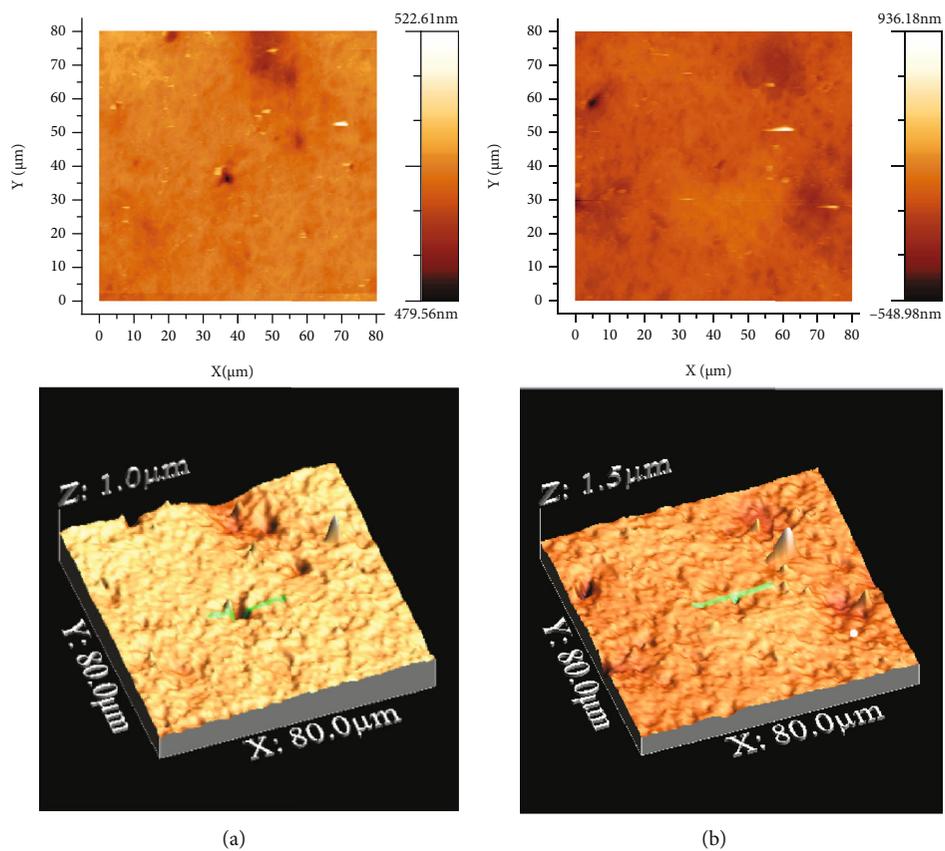
**2.3.1. Processing Behavior.** Processing behavior of plasticized PHB and PHB/BC biocomposites was evaluated by torque at different times up to 6 minutes and 40 rpm and melt viscosity obtained from torque-time Brabender diagrams.

**2.3.2. Microscopic Characterization.** The surface morphology of the scaffolds was evaluated with a 4000 SPM Multi-View/NSOM atomic force microscope system (AFM) (Nanonics Imaging LTD). SPM images were acquired in air, noncontact mode (at a distance between 0.1–10 nm of sample). Analyzed surface area was 80  $\mu$ m<sup>2</sup>, and the measurement speed used was 12 ms/point. The needle used for surface analysis showed a peak of Cr. Interpretation of the analysis was performed using the WSxM 4.0 software. Surface roughness of the PHB/BC biocomposites was expressed as the root mean square (RMS) of the vertical Z-dimension values within the examined areas, according to Selli et al. [28].

**2.3.3. Differential Scanning Calorimetry (DSC) Measurements.** Thermal properties of the biocomposites were determined directly after melting processing and after the degradation process using a calorimeter (Mettler Toledo, Model DSC 823e, Switzerland). The melting temperature ( $T_m$ ), melting enthalpy ( $\Delta H_m$ ), and the degree of crystallinity ( $\chi_c$ ) were investigated for all samples, from the first heating scan, with the aid of the software STARE 9.10 from Mettler Toledo. The degree of crystallinity ( $\chi_c$  (%)) of the PHB from the samples was determined according to the following equation:

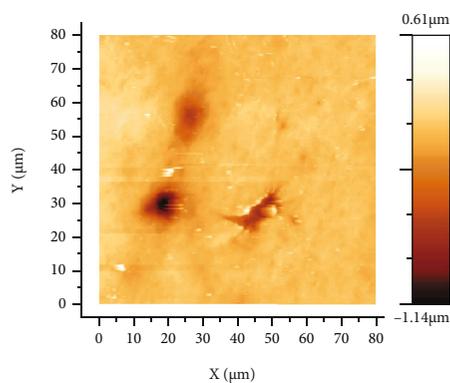
$$\chi_c = \frac{\Delta H_m}{\Delta H_{100\%,\text{PHB}} \cdot w} \times 100\%, \quad (1)$$

where  $\Delta H_m$  is the measured enthalpy of the melted blends (J/g),  $\Delta H_{100\%,\text{PHB}}$  is the theoretical heat of fusion for a fully crystalline PHB (146 J/g), and  $w$  is the weight fraction of



(a)

(b)



(c)

FIGURE 1: AFM topography images for the plasticized PHB and PHB biocomposites. (a) PHB/TBC, (b) PHB/BC1%, and (c) PHB/BC2%.

TABLE 2: Roughness (RMS) values for the samples calculated from AFM analysis.

Sample	RMS (nm)
PHB/TBC	51.1
PHB/BC1%	67.3
PHB/BC2%	129.0

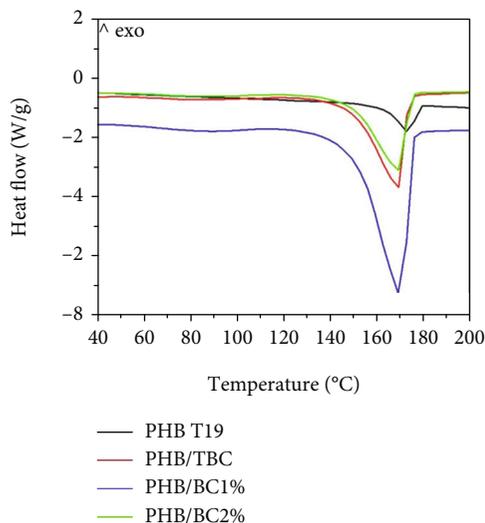


FIGURE 2: DSC thermograms for neat PHB, plasticized PHB, and PHB biocomposites (first heating scan).

PHB from samples. PHB powder was used as a control sample.

**2.3.4. Water Absorption.** The water absorption of each scaffold was measured after immersion of the round-shaped specimens in distilled water at 23°C for four different periods: 24 h, 168 h, 336 h, and 504 h, according to ISO 62. The test specimens were kept out in an oven at 50°C for 24 h, cooled, and weighed until the mass was constant ( $m_1$ ) with an accuracy of 0.1 mg. Then, the specimens were submerged into distilled water and, at target period, they were removed from water and reweighing ( $m_2$ ). The average content of absorbed water was calculated by using the appropriate formula:

$$\text{Water absorption} = \frac{m_2 - m_1}{m_1} \times 100\%, \quad (2)$$

where  $m_1$  is the mass of the test specimen in mg, after initial drying and before immersion;  $m_2$  is the mass of the test specimen in mg, after immersion.

**2.3.5. In Vitro Degradation Study.** *In vitro* degradation study was performed by immersion of specimens in phosphate-buffered saline medium (pH 7.4) at 37°C, to simulate the human physiological medium [29]. The dried scaffolds subjected to degradation were investigated by their weight loss, as follows: round pieces of 20 mm diameter were cut from samples, weighed ( $w_0$ ) and placed in sealed test tubes con-

TABLE 3: Thermal properties of PHB, PHB/TBC, and PHB/BC samples.

Sample	$\Delta H_m$ (J/g)	$T_m$ (°C)	$\chi_{c,PHB}$ (%)
Neat PHB	$90.3 \pm 0.6$	$174.0 \pm 2.1$	$61.8 \pm 0.6$
PHB/TBC	$61.0 \pm 0.9$	$170.8 \pm 1.7$	$52.2 \pm 0.9$
PHB/BC1%	$58.4 \pm 1.1$	$170.8 \pm 1.0$	$52.0 \pm 1.1$
PHB/BC2%	$57.1 \pm 1.1$	$169.5 \pm 0.8$	$51.4 \pm 1.1$

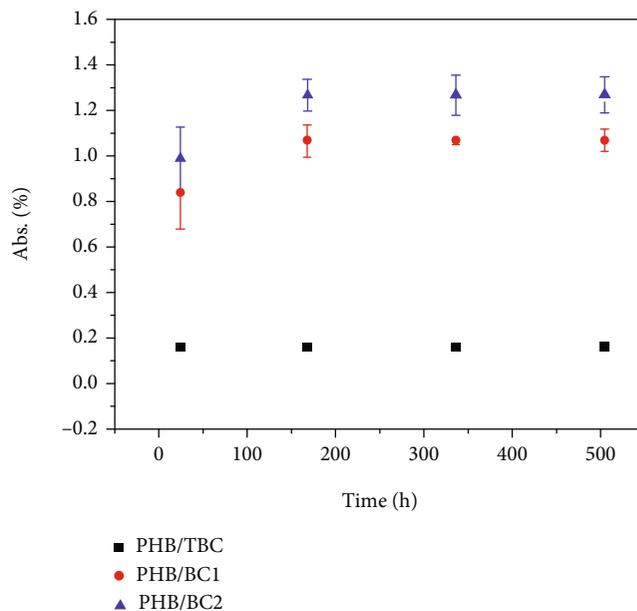


FIGURE 3: Water absorption for plasticized PHB/TBC sample and its biocomposites at different times.

taining 2 mL PBS for up to 20 days. At predetermined degradation intervals (i.e., 1 day, 5 days, 10 days, 15 days, and 20 days), the specimens were taken out, washed with distilled water, and dried in an oven at 37°C for 72 h and then reweighed ( $w_1$ ). The degree of degradation was assessed by weight loss evaluation using the following equation [30]:

$$\text{Weight loss\%} = \frac{w_0 - w_1}{w_0} \times 100\%, \quad (3)$$

where  $w_0$  and  $w_1$  are the weights of the scaffolds before and after hydrolytic degradation, respectively.

The degraded scaffolds were also examined by DSC to find any detectable differences in their thermal profile.

### 3. Results and Discussions

**3.1. The Processing Behavior.** The processing behavior of the obtained blends was evaluated from the torque-time curves, and it is expressed as torque ( $M$ ) at 1 minute, 4 minutes, and 6 minutes, respectively, and melt viscosity ( $\eta$ ).

Table 1 shows that the samples can be easily processed by melt blending. As the BC agent was incorporated into

the plasticized PHB matrix, an increase in torque was obtained up to 4 minutes compared to that for the plasticized PHB. However, after 6 minutes of processing, there are no significant differences between the biocomposites and the plasticized PHB torque (i.e., values of 11-12 nm) and their melting viscosity (i.e., values of 0.27-0.3 nm/rpm). The advantages of this blending technique are its compatibility with the processing methods for conventional polymers and the fact that it does not use solvents.

**3.2. AFM Analysis.** AFM analysis reveals the rough surface of the PHB biocomposites (Figures 1(a)–1(c)) on which numerous holes and topographic features having relatively high depths and peaks ranging from 100 to 700 nm can be observed. The roughness of the samples characterized by AFM is listed in Table 2.

The roughness surface of PHB composites increased with the incorporation of BC into the plasticized matrix, due to the interaction between the polymeric matrix and cellulose. These results are in agreement with the melt viscosity reported in Table 1.

**3.3. Differential Scanning Calorimetry (DSC).** DSC curves for the neat PHB, plasticized PHB, and PHB biocomposites are shown in Figure 2.

The melting enthalpy ( $\Delta H_m$ ), melting temperature ( $T_m$ ), and degree of crystallinity ( $\chi_c$ ) of the samples evaluated from DSC curves are shown in Table 3. Data were expressed as mean values  $\pm$  standard deviation ( $n = 3$ ).

Data presented in Table 3 reveal that the melting temperature ( $T_m$ ) of PHB decreases with the addition of plasticizer and bacterial cellulose. Thus, the PHB/BC2% biocomposite shows a decrease of  $T_m$  to 4.5°C compared to the neat PHB. The reduction of  $T_m$  could be the effect of the introduction of plasticizer and the good dispersion of BC inside the polymeric matrix. Several studies showed that adding of plasticizer to the PHB matrix can lead to an increase in the thermal processing window [31–34]. Furthermore, the melting enthalpy ( $\Delta H_m$ ) was reduced from 90.3 J/g for neat PHB to 57.1 J/g for PHB/BC2%, and as a consequence, the crystallinity of PHB ( $\chi_c$ ) decreased from 61.8% to 51.4%. This can be explained by disruption of the regularity of PHB chains following the interactions with BC, which resulted in a decrease of the crystallinity. A similar mechanism was observed in green nanocomposites based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with various cellulose nanocrystals [35], PHB/BC composite membranes [36], and PHB blends [37].

**3.4. Water Absorption.** The water absorption capacity of scaffolds is an important measure of scaffold hydrophilicity, as it can affect the rate of scaffold degradation as well as the attachment of cells [38].

Figure 3 shows the percentage of water absorption for PHB/TBC and its corresponding PHB/BC scaffolds measured at four time periods.

The PHB/TBC sample showed that the water absorption is very low ( $0.16\% \pm 0.003\%$ ) during 504 h, due to its initial high crystallinity. The PHB/BC1% and PHB/BC2% scaffolds

TABLE 4: Thermal properties of PHB/TBC and PHB/BC composite materials after *in vitro* degradation.

Sample	Period of exposure to PHB medium	$\Delta H_m$ (J/g)	$T_m$ (°C)	$\chi_c$ (%)
PHB/TBC	5 days	55.9 $\pm$ 0.3	168.9 $\pm$ 1.3	47.8 $\pm$ 0.3
	10 days	57.5 $\pm$ 0.3	166.9 $\pm$ 0.9	49.2 $\pm$ 0.2
	15 days	63.0 $\pm$ 0.4	168.1 $\pm$ 0.8	53.9 $\pm$ 0.4
	20 days	62.4 $\pm$ 0.3	168.0 $\pm$ 0.9	53.4 $\pm$ 0.3
PHB/BC1%	5 days	54.0 $\pm$ 0.3	167.6 $\pm$ 0.6	48.1 $\pm$ 0.3
	10 days	56.3 $\pm$ 0.2	168.3 $\pm$ 0.4	50.2 $\pm$ 0.2
	15 days	58.1 $\pm$ 0.3	168.9 $\pm$ 0.3	51.8 $\pm$ 0.3
	20 days	59.3 $\pm$ 0.4	167.1 $\pm$ 0.6	52.8 $\pm$ 0.4
PHB/BC2%	5 days	59.0 $\pm$ 0.3	168.3 $\pm$ 0.3	53.1 $\pm$ 0.3
	10 days	60.3 $\pm$ 0.3	167.6 $\pm$ 1.0	54.3 $\pm$ 0.3
	15 days	60.4 $\pm$ 0.2	168.2 $\pm$ 0.8	54.4 $\pm$ 0.2
	20 days	56.5 $\pm$ 0.3	168.9 $\pm$ 0.6	50.9 $\pm$ 0.3

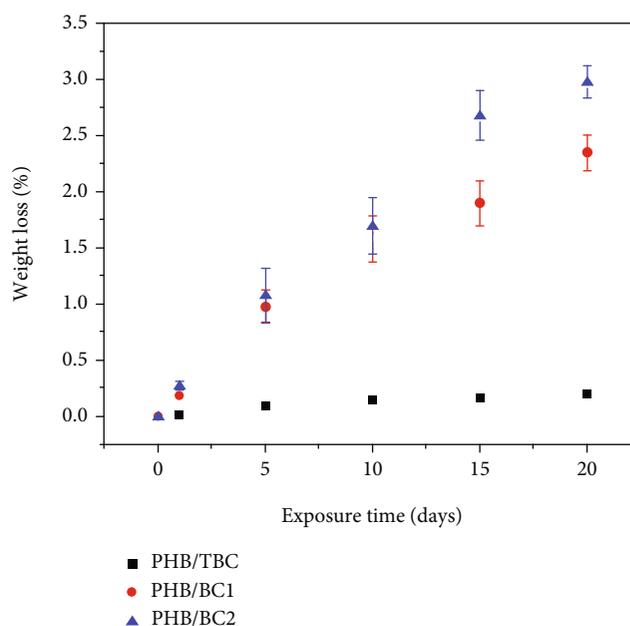


FIGURE 4: Weight loss of plasticized PHB sample and its biocomposites during 20 days of exposure to PBS medium.

exhibited higher water absorption than that of PHB/TBC sample. Introduction of BC in the polymer scaffolds significantly enhanced the water absorption content. Thus, the PHB/BC2% scaffold shows the maximum hydration ( $1.27\% \pm 0.07\%$ ) at 168 h. This may be attributed to the addition of BC in the polymeric matrix that the decreased crystallinity of PHB (decreased  $\Delta H_m$  from Table 4) allowing the mobility of PHB chains and as consequence the production of more hydrophilic hydroxyl and carboxyl end groups due to ester bond cleavage [25, 37]. It is worth noting that after 168 h immersion in distilled water, the scaffolds

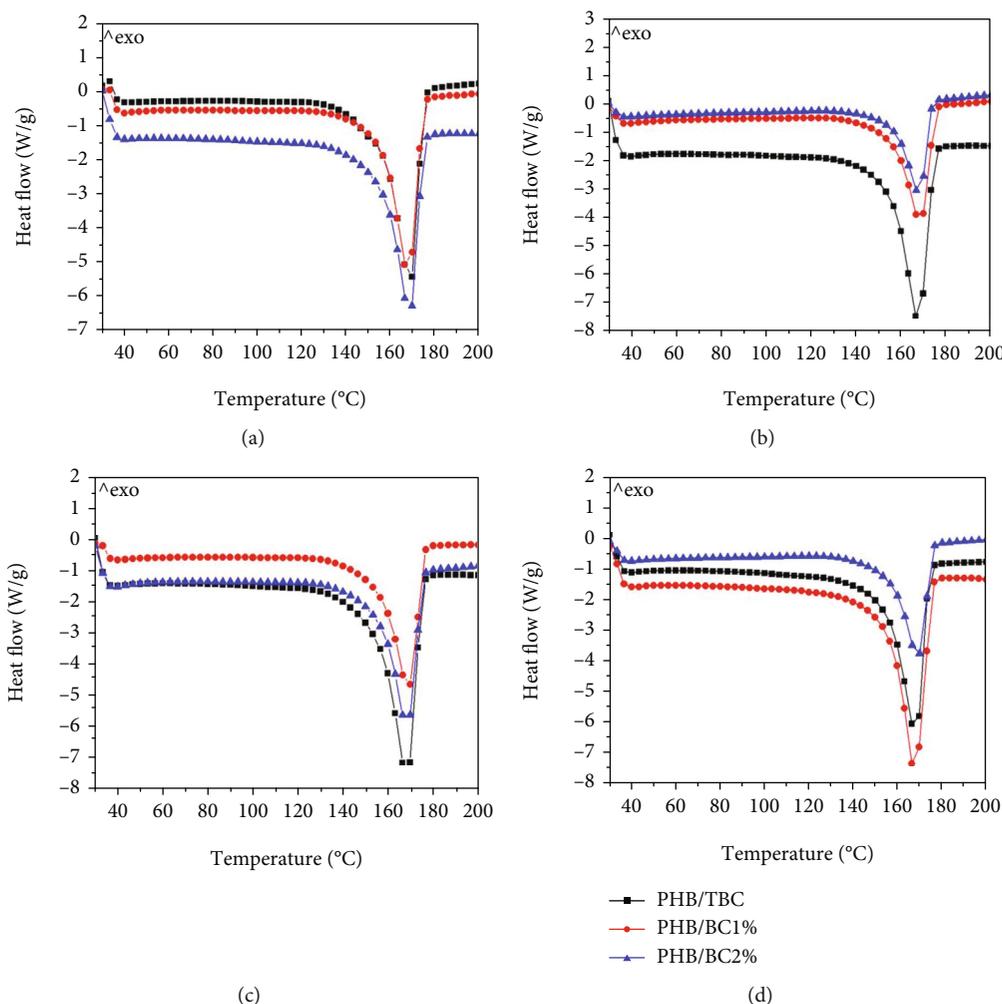


FIGURE 5: DSC thermograms for plasticized PHB sample and its biocomposites subjected in PBS medium at different time periods: (a) 5 days, (b) 10 days, (c) 15 days, and (d) 20 days.

reached the highest values for water absorption, which remained constant during 504 hours of testing.

### 3.5. In Vitro Degradation

**3.5.1. Weight Loss.** Weight loss profiles of PHB/TBC and PHB/BC scaffolds as a function of the degradation time are shown in Figure 4.

Plasticized PHB blend presented a negligible weight loss during the whole 20-day degradation period due to its high crystallinity (Table 3). The weight loss was 0.02% at one day exposure to PBS medium, and it slowly increased until 0.2% at the end of exposure time. It is well recognized the low degradation of PHB under physiological conditions, for example, PHB films had lost approximately 3.5% of their initial weight after 105 days incubation in PBS [37].

Data from Figure 4 clearly show that the increase of the BC content in the biocomposite samples leads to the increase of their degradation. Thus, the PHB/BC2% scaffold exhibits a higher weight loss ( $2.98 \pm 0.14\%$ ) than the PHB/BC1% scaffold ( $2.35 \pm 0.16\%$ ) at 20 days. This behavior is in agree-

ment with the water absorption test (Figure 3) and the decrease in crystallinity (Table 3).

**3.5.2. DSC Analysis for the Degraded Samples.** The DSC technique was used to evaluate the change of thermal parameters for the PHB/TBC and PHB/BC samples after *in vitro* degradation in PBS solution at 37°C and targeted periods (Figures 5(a)–5(d)).

The melting enthalpy ( $\Delta H_m$ ), melting temperature ( $T_m$ ), and degree of crystallinity of PHB ( $\chi_c$ ) evaluated from DSC curves could be observed in Table 4. Data were expressed as mean values  $\pm$  standard deviation ( $n = 3$ ).

Table 4 shows a general trend of the  $T_m$  to decrease for blends after subjected to PBS solution in comparison with the unexposed blends. A decrease of melting temperature ( $T_m$ ) from 170.8°C to 168.0°C was recorded for the PHB/TBC blend during the degradation with the lowest value at 10 days of exposure in PBS (166.9°C). The PHB/BC1% and PHB/BC 2% scaffolds showed a decrease of  $T_m$  from 170.8°C to 167.1°C after exposure in PBS.

The degree of crystallinity ( $\chi_c$ ) of PHB shows an increased trend up to 20 days, being more pronounced for

the PHB/TBC blend, which became more rigid, maybe due to the loss of plasticizer. While the amorphous regions of the samples were attacked, the degradation continues in the crystalline regions of the material, which are less susceptible to attack because of their orderly structure [29]. As a consequence, the crystallinity of all blends exposed to PBS medium increased, which points to their degradability. Indeed, up to 20 days, an increase of  $\chi_c$  is detected in the case of PHB/TBC (from 41.8% to 47.6%), PHB/BC1% (from 40% to 46.9%), and PHB/BC2% samples (from 39% to 45.6%), respectively. These data are well correlated with the weight loss of PHB blends (Figure 4). The burial degradation of PHB reinforced with cellulose fibers also showed an increased degree of crystallinity (65%) after 90 days of exposure [39].

#### 4. Conclusions

The present work investigated the effect of bacterial cellulose on thermal properties, water absorption, and *in vitro* degradation of scaffolds based on poly(3-hydroxybutyrate) (PHB) prepared by melt processing using salt-leaching technique.

It was found that the prepared scaffolds show a good processing and increased roughness of the surfaces.

The *in vitro* degradation study of biocomposites in PBS medium for twenty days was performed, and the occurring changes in comparison with unexposed samples were investigated by evaluation of weight loss and thermal properties. The obtained results revealed that all samples showed the increased degree of crystallinity after 20-day immersion in PBS, in good agreement with weight loss, which denoted their degradation under PBS medium.

Further interdisciplinary research is needed regarding the *in vitro* biocompatibility and clinical studies of the PHB/BC composites together with the production of bacterial cellulose at a large scale.

#### Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

#### Conflicts of Interest

The authors declare no conflict of interest.

#### Acknowledgments

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