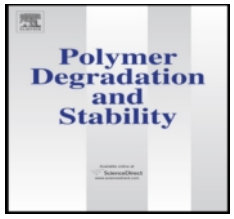

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Authors: Élida B. Hermida, O. Yashchuk, S.S. Miyazaki

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Changes in the mechanical properties of compression moulded samples of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) degraded by *Streptomyces omiyaensis* SSM 5670

Élida B. Hermida[1-3]*, O. Yashchuk [3,4] and S.S. Miyazaki[1, 4]

[1]*CONICET, Av.Rivadavia 1917, 1033 Buenos Aires, Argentina.*

[2]*Institute of Technology "Prof. Jorge A. Sabato", UNSAM-CNEA, Av.Gral Paz 1499, B1650KNA San Martin, Argentina.*

[3]*Department of Materials (CNEA), Av.Gral Paz 1499, B1650KNA San Martin, Argentina.*

[4]*Department of Applied Biology and Foods, Faculty of Agronomy, University of Buenos Aires, Av. San Martín 4453, 1417 Buenos Aires, Argentina.*

**Corresponding author.* Tel.: + 54 11 67727223; fax: +54 11 67727362

E-mail address: ehermida@cnea.gov.ar

Key words: biodegradation, tensile testing, surface erosion, poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate), *Streptomyces* sp.

Running title: Mechanical changes during PHBV biodegradation

Abstract

Streptomyces omiyaensis SSM 5670 was characterized by its ability to use compression moulded samples of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as its sole carbon source. Biodegradation of PHBV in liquid mineral salts medium was investigated using scanning electron microscopy, gravimetric measurements, capillary viscometry, tensile testing and wide angle X-ray spectroscopy. The biodegradation of PHBV proceeds via surface erosion mechanism, resulting in the formation of pits by microbial attack. PHBV specimens lost about 45% of their original weight after 45 days of exposure. During the degradation process the elastic modulus reduces less than 10%. The formation of pores and microcracks initiated at the degraded pits determines the reduction of the elongation and stress at break. However, the true stress at break is practically independent of the degradation time. No significant changes of PHBV molecular weight or crystallinity were observed during biodegradation. The polymer chain cleavage occurred only at the specimen surface and does not discriminate between crystalline and amorphous states.

Introduction

Polyhydroxyalkanoates (PHAs) are natural polyesters produced by numerous microorganisms under unbalanced growth conditions, that is, enough renewable carbon sources and depletion of an essential nutrient [1,2]. PHAs can be used in packaging, agricultural, medical, veterinary and marine applications [3] because they behave similarly to conventional thermoplastics with the advantage of complete biodegradation [4].

The chemical structure, depicted in Figure 1, corresponds to over a hundred different types of PHAs made from different monomers. The first PHA discovered in 1926 [5] was poly(3-hydroxybutyrate) (PHB), a extremely brittle polymer because of its high degree of crystallinity. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), a copolymer of PHB with monomers that include two carbons in the side chain, attracts the attention of many researchers because of its better mechanical performance and faster biodegradation. Thus, many studies have been primarily oriented to characterize the production, morphology, mechanical properties and biodegradation kinetics of PHBV [6-8].

Figure 1.

Biodegradability of PHBV in different environments has been evaluated not only regarding the weight loss, the kinetics and yield of intermediate products but also by monitoring changes in the morphology and mechanical properties [9-14].

Biodegradation tests of PHBV performed in natural environments, such as soils [15-16], composts [9,16], sludges [17,18] or natural water [10,13,19] were mainly oriented to determine how the degradation kinetics depends on the environmental factors. Biodegradation under laboratory conditions, however, has been useful to evaluate enzymatic or hydrolytic mechanisms [1,20]. Both mechanisms involve polymer chain cleavage and should result in molecular weight reduction. Doi et al. found, however, that the molecular weight of the sample does not change during enzymatic degradation because cleavage occurs only at the specimen surface while it decreases during hydrolytic degradation [21].

Similar results were achieved by Luo and Netravali [9], for bulk PHBV samples biodegraded in composting medium; they found that the molecular weight remained almost unchanged since degradation occurs by surface erosion. Furthermore, since the degree of crystallinity remained virtually unchanged they concluded that degradation during composting does not discriminate between crystalline and amorphous phases. Regarding the mechanical properties,

in agreement with previous tests [22], they found that the tensile modulus did not show much change with composting time but the ultimate tensile strength and the elongation at break reduces as the biodegradation proceeds. Since both the molecular weight and the degree of crystallinity do not change while degrading the sample, they presumed that surface defects should be responsible for the decrease in the tensile strength.

Therefore, the aim of this work is to propose a model for the surface erosion that aids in explaining the reduction in the tensile strength of bulk PHBV samples as the degradation proceeds. In order to achieve this goal compressed moulded PHBV samples were degraded by *Streptomyces omiyaensis* SSM 5670 cultivated in liquid mineral salt medium (MSM). Few contributions have been devoted to analyze the morphological changes of PHBV films while degraded by *Streptomyces* sp. [23,24] so this work also provides new insights on the mechanical changes due to the PHBV degradation by this microorganism.

Experimental

Samples

Bacterial PHBV with 12 wt % of hydroxyvalerate, was purchased from PHB Industrial SA, Brazil. PHBV is sold as white granules with a weight average molecular weight 165 kg/mol. This material was used as received.

PHBV samples for the mechanical and morphological tests were prepared by hot compression moulding. The granules were put between two stainless steel plates separated by a metallic ring 200 μm thick, melted in a hydraulic hot press at 463 K for 10 min and then quenched at 269 K. In this way we got disks around 100 mm in diameter and 200 μm thick. Finally specimens for biodegradation followed by tensile tests were cut with a die according to the ASTM D 1708-84 standard [25]. The weight of each sample was (104± 6) mg.

Culture of microorganisms

Stock culture of *Streptomyces omiyaensis* SSM 5670 (Culture Collection SSM of AGRAL FAUBA) was maintained on at (30±1)°C on Actinomyces agar slants containing, in g/l distilled water: yeast extract 0.2; starch 1.0; agar 1.5, pH 7.2.

Analysis of biodegradation in liquid medium

150-ml Erlenmeyer flasks with 50 ml of the MSM, were inoculated with a 2% v/v fresh spore suspension of *Streptomyces omiyaensis* SSM 5670. Each flask contained one piece of surface sterilized (70%, v/v ethanol) compression moulded samples of PHBV polymer. The solution was agitated in a rotary shaker at 100 rpm and kept at (30±1)°C. Control experiments were carried out without the inoculation of the MSM. After 15 days a set of 5 samples was removed from the solution, washed with distilled water under the ultrasonic action and dried at 60°C to constant weight. This procedure was repeated after 30 and 45 days of the first immersion using inoculated solution.

PHBV biodegradation was measured in terms of the percentage weight loss, i.e. $\Delta w / w_0 \times 100\%$, where w_0 and Δw are the initial weight and weight loss of the PHBV sample, respectively.

Scanning electron microscope observation (SEM)

The aliphatic polyester films were retrieved directly from the culture broths, washed with distilled water under the ultrasonic action and dried at 60°C to constant weight. Then, changes in the morphology of the biodegraded surface of the polyester were observed with a Phillips PSEM 500 scanning electron microscope at various stages of cultivation.

Wide Angle X-ray Spectroscopy

Wide angle X-ray diffraction was used to determine possible changes in the crystallinity of biodegraded samples. A wide-angle X-ray diffractometer Phillips PW 3710 with a Ni filter to provide a Cu K_α radiation (λ = 0.154248 nm) was used. Crystalline peaks were well-defined and the degree of crystallinity X_c was obtained from the normalized X-ray diffractogram by the relationship: $X_c = [A_t - A_a] / A_t$, being A_t the integral of the whole diffraction pattern and A_a the area under the background associated to the halo produced by the amorphous structure.

Weight average molecular weight

The weight average molecular weight ($\overline{M}_w$) of the control and degraded PHBV specimens was calculated from viscosity measurements according to the equation $[\eta] = 1.18 \times 10^{-4} \ \overline{M}_w^{\ 0.78}$,

being [η] the intrinsic viscosity, defined by $[\eta] = \lim_{c \to 0} \left(\frac{\eta}{\eta_s} - 1 \right) \Big/ c$, where η_s is the viscosity of the solvent and η that of a solution with a concentration c of the polymer in a diluted solution [26]. Viscosity was measured at 30 °C using a glass capillary Ostwald viscometer. Several diluted solutions of PHBV in chloroform were prepared in the range from 0.01 to 0.2 g/dl in order to determine the limiting viscosity at c=0.

Mechanical properties

Tensile tests were performed according to ASTM D 1708 [25] in an Instron testing machine, model 1122 at a constant strain rate of 0.001 s^{-1} and at 25 °C. The characteristic parameters of the mechanical behaviour of PHBV - evaluated at different stages of the biodegradation process - were the tensile modulus (E, in GPa), that is, the slope at the beginning of the tensile curve, and the ultimate values: the tensile strength (σ_b, in MPa) and the elongation at break (λ_b).

It is noticed that the σ–λ plot is usually considered to represent the mechanical behaviour of elastomers with elongations higher than 100% and this is not the case of PHBV. However, this plot enables a more accurate determination of the tensile modulus from both the slope and the intercept to the origin of a line that approximates the initial portion of the tensile curve. In fact, at low deformations the tensile curve can be described by the linear equation $\sigma = \frac{F}{A_o} = E\varepsilon = E(\lambda - 1)$, where F is the applied force at time *t*, A_o the initial cross-sectional area, $\lambda = l / l_o$, the ratio between the length (*l*) of the sample at time *t,* and the initial length (l_o).

Results

Biodegradation ability of PHBV samples

The fresh suspension of *Streptomyces omiyaensis* SSM 5670 was grown in the liquid mineral salt medium with a (104± 6) mg sample of PHBV. The compression moulded PHBV specimens lost about 45% of their original weight after 45 days of exposure to the MSM as shown in Figure 2.

Figure 2.

The microorganism can excrete extracellular enzymes to solubilise the PHBV surface on which they are growing in homogenous form. The soluble degradation products are then absorbed through the cell wall and metabolized. This indicates that the bacteria use the copolymer as a carbon source for their growth and metabolism. The rate of weight loss, that is, the biodegradation speed, changes during the degradation. It is smaller at the beginning, increased up to three times of the initial value after 15 days and decreases after 30 days of degradation. These changes can be related to the cellular growth and the microbial activity of the *Streptomyces omiyaensis* SSM 5670. Furthermore the colony forming units (CFU) increased because of the increase in the effective surface area of the PHBV film. In fact, the action of *Streptomyces omiyaensis* SSM 5670 eroded the surface and increased its roughness.

On the other hand, Figure 2 illustrates that after 30 days of degradation the pH decreased from 7.0 to 5.8; this was due to the presence of degraded acid substances such as 3-hydroxybutyric acid, adipic acid, succinic acid and 3-valeric acid; this result has been previously shown by Calabia and Tokiwa for PHB degradation [24].

SEM observations of PHBV samples

Figure 3 presents the SEM micrographs showing changes in the surface topography of the samples before and after exposure to the MSM for various periods of time. The control specimen surface is smooth except for some scratches replicated from the mould (Figure 3a). After degradation for 15 days the surface becomes rougher and the SEM photo shows a significant number of irregular pits (Figure 3b). Mabrouk and Sabry observed the same feature on PHBV films degraded by a marine *Streptomyces* sp. SNG9 and remarked the presence of eroded areas separated by non eroded areas [23]. On increasing the biodegradation time up to 30 and 45 days (Figures 3c and 3d, respectively) the number of pits drastically increases, their size decreases and they spread over the whole specimen. This can be explained by considering that in the initial degradation stage there are few microbial colonies spread far apart. Later the microbial population multiplied and began to extend over other regions of the surface increasing the superficial area, where the micro-organisms can lodge to degrade the sample. This is consistent with observations by Luo and Netravali [9] on degradation of PHBV hot press films during composting.

Figure 3.

Furthermore, the microtopography of the biodegraded samples was analyzed using the Mountain Analysis Software® on SEM micrographs; this software computes the fractal dimension from a SEM image of the surface. This analysis shows that the original surface was not completely flat but has a roughness (replica of the mould), that can be characterized by a fractal dimension of 2.29. As the biodegradation proceeds, the fractal dimension of the eroded PHBV surface slightly increases and reaches an asymptotic value of 2.75, as shown in Table 1.

Changes of the tensile properties during biodegradation

Figure 4 shows that as degradation proceeds (45 days) the elastic modulus reduces only 10%. while the tensile strength σ_b diminishes to 64% of the initial value after 30 days of biodegradation in the MSM.

Figure 4.

This reduction of the tensile stress is only apparent because it is masked by the reduction of the cross sectional area of the sample from A_o before degradation to $A(t)$ after a degradation time t. Thus, to evaluate the *true stress at break* we should calculate the true stress at break, $\tilde{\sigma}_b = F_b / A(t)$, with F_b the force required to break the sample.

Since the length and density of the sample do not vary during degradation, it is easy to show that $A = A_o \frac{w}{w_o}$, where w_o and w are the weights of the sample before the degradation and after a degradation time t, respectively. Hence, the true tensile stress at break at time t should be

$$\tilde{\sigma}_b(t) = \frac{F}{A(t)} = \frac{F}{A_o}\frac{w_o}{w(t)} = \frac{\sigma_b}{1 - \text{weight loss}(t)} \qquad (1).$$

On doing this calculation we found that the true stress at break is practically independent of the degradation time, as shown in the last column of Table 1.

Table 1.

Molecular weight and crystallinity

The molecular weights of PHB and PHBV remained almost unchanged during the course of biodegradation [8, 14]. Nishida and Tokiwa reported that there was little change in crystallinity

during the enzymatic degradation of PHB [12]. The rate of enzymatic hydrolysis of PHB films decreases with increasing crystal size and that morphological changes affect the rate of enzymatic degradation for films where the degree of crystallinity is kept constant [27].

In Figure 5 we observed that molecular weight and crystallinity of PHBV specimens do not present significant changes during exposure in MSM for 45 days. These results suggest that the polymer chain cleavage occurred only at the specimen surface and does not discriminate between crystalline and amorphous states. According to these observations the biodegradation of polyester in this medium can be viewed as mostly enzymatic and produces superficial erosion.

Figure 5.

Discussion

It was shown that the degree of crystallinity and the mean molecular weight of compressed moulded samples of PHBV degraded by *Streptomyces omiyaensis* SSM 5670 do not change as the biodegradation proceeds. Then it makes sense to argue that the mechanical behaviour, mainly controlled by these two factors, should be independent of the biodegradation level. This statement agrees not only with the tensile modulus determined from the experimental data but also with the corrected tensile strength, $\tilde{\sigma}_b$. Furthermore, our statement is satisfied also by Luo and Netravali's data [9] since if we apply eq. (1) to their data we get a rather constant effective tensile strength of (31 ± 4) MPa, as shown in Table 2, except for the last point, where the sample exhibits deep erosion. The deviation (4MPa) agrees with the standard deviation of the experimental data reported in the original work.

Table 2.

Therefore, we claim that the surface erosion due to the biodegradation affects neither the elastic nor the plastic behaviour of the bulk. As the degradation proceeds, the size of the pores and microcracks at the surface increases until it achieves a critical size. At this size, the stress concentration factor around the crack is so high that a low stress promotes a catastrophic failure with practically no plastic deformation. Because of this highly degraded samples usually break while handling them even before the test.

Conclusions

From our data of the PHAs biodegradation in mineral salt media inoculated with *Streptomyces omiyaensis* SSM 5670 and the analysis of the mechanical properties, the SEM micrographs and the morphological observations we conclude that:

1. Changes in the rate of degradation of PHAs (measured by the relative weight loss) can be related to the cellular growth and the microbial activity of the *Streptomyces omiyaensis* SSM 5670.

2. The biodegradation of the compression moulded samples of PHBV proceeds via a surface erosion mechanism, resulting in the formation of pits by microbial attack.

3. As degradation proceeds, the elastic modulus shows only a slight change while the tensile strength diminishes meaningfully. However, the true tensile strength (calculated considering the cross-section reduction due to the surface erosion) is practically independent of the degradation time.

4. The crystallinity and molecular weight of PHBV specimens remain virtually constant after 45 days of degradation.

Acknowledgements

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Table 1

Tensile modulus (E), elongation and stress at break (λ_b and σ_b), true stress at break ($\tilde{\sigma}_b$) and fractal dimension of the eroded surface of PHBV specimens degraded in MSM after the indicated time.

Biodegradation (days)	E (GPa)	λ_b	σ_b (MPa)	$\tilde{\sigma}_b$ (MPa)	Fractal dimension
0	1.3	1.04	28	28	2.29
15	1.3	1.03	25	28	2.54
30	1.2	1.02	18	29	2.75
45	1.2	1.02	18	32	2.75

Table 2

Luo and Netravali's data [9] used to get the true tensile strength of PHBV during composting

Degradation time (days)	σ_b (MPa)	$\Delta w / w_0 \times 100\%$	$\tilde{\sigma}_b$ (MPa)
0	31.3	100	31.3
10	28.3	92	30.8
20	18.2	64	28.4
30	16.8	48	35
40	8.4	32	26.3
50	0.9	25	3.6

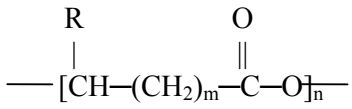

Figure 1

General molecular structure of PHAs. m=1, 2, 3, (m=1 is most common), n: from 100 to several thousands. R is variable. When m=1, R=CH_3, the monomer structure is 3-hydroxybutyrate, while m=1 and R=C_2H_5 is a 3-hydroxyvalerate monomer

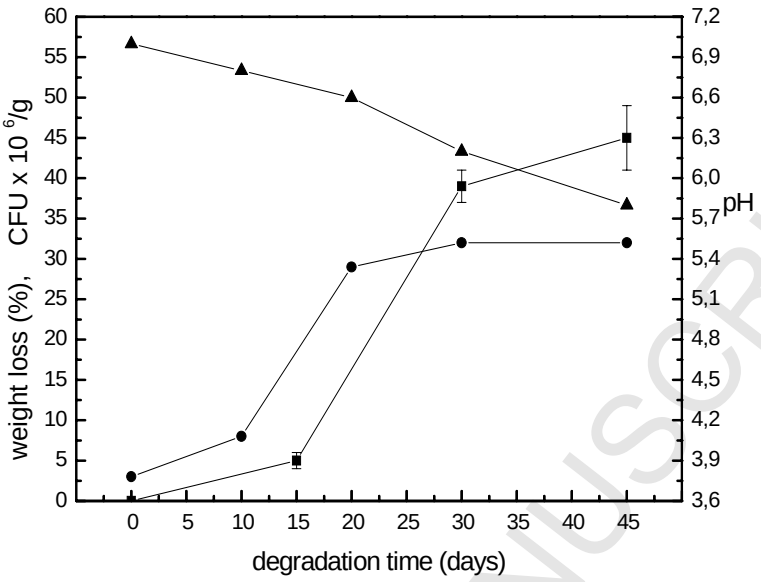

Figure 2

Relative weight loss of PHBV specimens (■), microbial growth (●) and change of pH (▲) in MSM as a function of degradation time

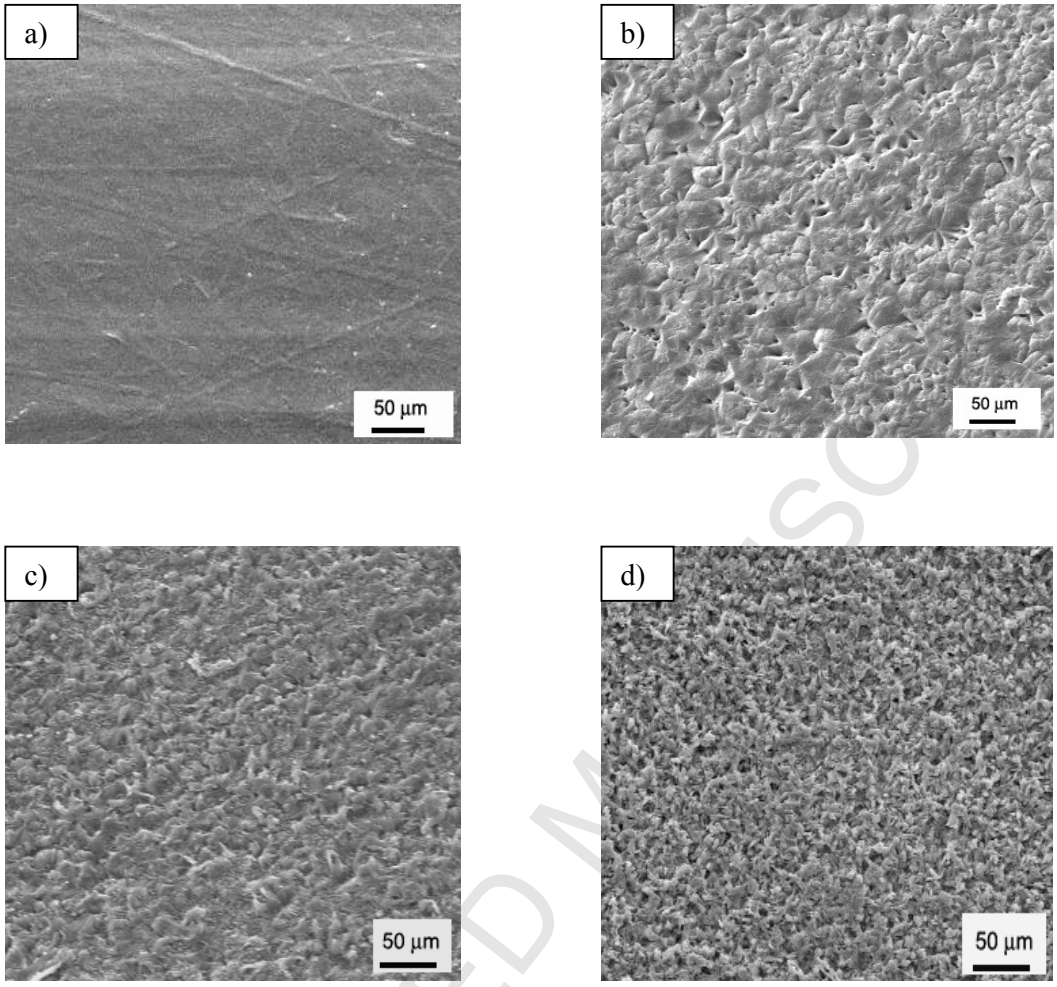

Figure 3

Scanning electron micrographs of the surface of PHBV specimens degraded in MSM at different degradation time: a) 0 days, (b) 15 days, (c) 30 days, (d) 45 days

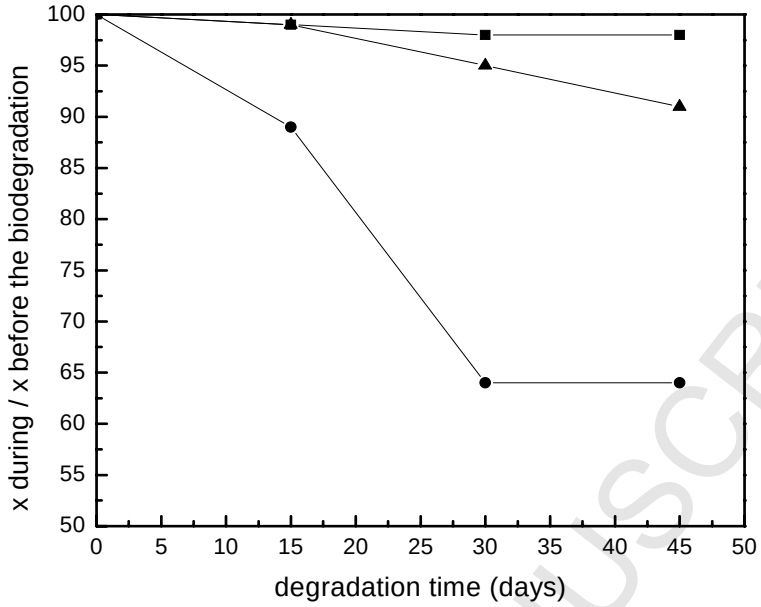

Figure 4

Relative deterioration of the mechanical properties of the PHBV specimens during the biodegradation in MSM by *Streptomyces omiyaensis* SSM 5670: (●) tensile stress; (▲) tensile modulus; (■) elongation at break

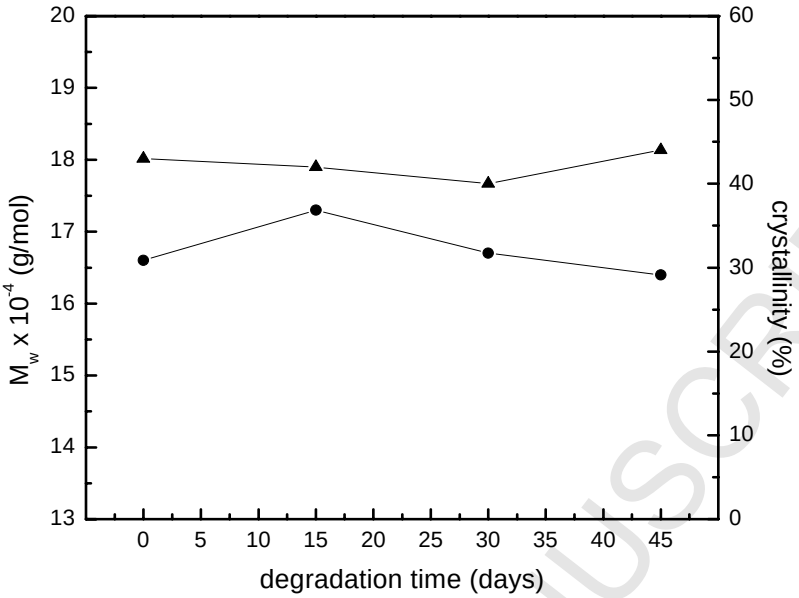

Figure 5

Weight-average molecular weight $\overline{M}_w$ (●) and crystallinity X_c (▲) vs degradation time of PHBV in MSM