BIODEGRADATION IMPROVEMENT BY ENTOMOPATHOGENIC FUNGI OF POLY-(3-HYDROXY-BUTYRATE) FILMS MODIFIED WITH UV-ASSISTED SURFACE FUNCTIONALIZATION

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Abstract - Ultraviolet (UV)-assisted radiation method in the presence of oxygen was used to achieve controlled degradation of poly-(3-hydroxy-butyrate) (PHB) films by entomopathogenic fungi. Treated surfaces were investigated by WCA, FTIR-ATR, XPS, NEXAFS, OM and SEM. Film hydrophilicity increased with photolysis time in the presence of oxygen and grafting of new C-O-C and C=O functional groups at the polymer surface after the UV assisted treatments were observed. New carbonyl groups were detected by XPS and NEXAFS spectroscopy after irradiation of the PHB films. The higher hydrophilicity and concentration of oxygenated functional groups at the surface of the treated films compared to pristine PHB possibly improved the biodegradation of the films by Entomopathogenic fungi. After the UV treatments is was observed an increase in its growth. Our study shows how a simple methodology can be used to improve and control the degradation rate of PHB films that can be used in applications that require quick or controllable degradation.

Keywords: poly-(3-hydroxy-butyrate), biodegradation, UV-assisted treatment, entomopathogenic fungi, *Metarhizium anisopliae*

Introduction

Polyhydroxyalkanoates (PHAs) are naturally occurring polyesters produced as energy storage materials by many bacteria. The most common representative member of the PHA family is polyhydroxybutyrate (PHB). These microbial polyesters have unique physicochemical properties [1]. Besides their potential use as packaging materials, biodegradable polymers might play a major role in biomedicine due to various reasons [2]. They are natural biocompatible and more interesting, devices built of biodegradable polymers could be implanted in the human body [3,4]. Biodegradable polymers can be applied as biomedical in the fields of controlled drug delivery and tissue engineering [5-7]. They are also a suitable surface for cell attachment and growth.

A commonly used strategy to modify those natural polyesters is to introduce specific functional groups on the surface leaving the bulk properties intact. Contrary to other methodologies, UV-assisted treatment is mainly based on photo-induced chemical processes, where special photo-reactive moieties have a distinct, selective, and efficient reactivity. Depending on the chemistry of the photo-reactive group, controlled elimination or addition reactions are possible, including dimerization reactions leading to cross-linking of macromolecule chains [8] or destruction of alkyl chains and changes in the bond structure [9]. Additionally, UV-assisted treatment usually has a much simpler experimental set up and lower cost, based in a dry method that allows grafting of specific functional groups to the polymer surface without introduction of undesirable contamination.

In a recent study, injected samples of PHB were exposed to biodegradation in simulated soil and the obtained results suggested a possible layer-by-layer degradation process having a 3-month period [10]. In the last decade a greatest concern due to the accumulation of synthetic plastics in natural ecosystems and in landfills and underground has emerged. To overcome those problems a gradual replacement of synthetic polymers with new, biodegradable, materials is one of the solutions. Most of the research in this field is focused on developing biodegradable synthetic polymers and polymeric materials of natural origin together with the identification of polymer-degrading microorganisms. Biodiversity and occurrence of polymer-degrading microorganisms vary depending on the environment. [11-13] The biodegradation mechanisms of plastics can be applied to biomasses that are composed of polymeric materials (i.e., cellulose, lignin, chitin, etc.). Fungi are able to produce different enzymes to degrade or have activity on various polymer surfaces. [14] *Metarhizium anisopliae* is an entomopathogenic fungus wide spread in nature with an extremely versatile metabolism, enabling growth under various environmental conditions, with sparse nutrients and in the presence of compounds lethal to other fungi [6]. *Metarhizium spp*. are prolific producers of enzymes and diverse secondary metabolites with activities against insects, fungi, bacteria, viruses and cancer cells [6,15,16].

Based in our previous studies on surface functionalization of polymers using electromagnetic radiation [17-20], for the first time we used UV-assisted methodology under O_2 flow to modify and incorporate functionalities on the polymer surface trying to improve the biodegradability of PHB films by an entomopathogenic fungus – *M. anisopliae*.

Experimental

Materials, Films Preparation and UV-surface modification of PHB films

Poly-(3-hydroxy-butyrate), PHB, (Mw 67,000) was purchased from Sigma-Aldrich (USA). Oxygen (O_2), 99.99%, from White Martins PRAXAIR INC, Brazil, was used as received. PHB films were obtained using the spin-coating technique

(2000 rpm) from a 10^{-4} M chloroform solution. Ten drops (ca 0.5 mL) were casted on ~ 15 mm in diameter glass substrates. For the UV irradiation was used a commercial medium-pressure mercury lamp (400 W) with a modified set up previously used [17]. In order to have a large number of samples for later statistical studies, 32 samples were set in a homemade reactor, composed by a disc made of stain steel that can rotate at a constant speed of 5 rpm. These reactor and set up allow each sample to be irradiated by the same amount of UV light while a constant flux of pure oxygen (5 $cm^3 s^{-1}$) flows by.

Surface characterization

WCAs of unmodified and UV-modified PHB films were measured using the sessile drop method. The values reported are averages of several measurements and the contact angles were calculated using the software package Surftens v. 3.0. FTIR-ATR analyses for the chemical composition of modified PHB films were carried out using a model Alpha-P instrument from Bruker with a diamond prism at 45º, and a spectral resolution of 4 cm^{-1}. X-ray Photoelectron Spectroscopy (XPS) and Near-edge X-ray Absorption Fine Structure (NEXAFS) analyses were obtained using the same experimental parameters of previously works. [17, 19]

Microorganisms and maintenance and Growth behavior evaluation

M. anisopliae was obtained from the Laboratory of Cellular and Molecular Biology of Filamentous Fungi collection, from UFRGS. This was maintained on agar slants in Cove's complete medium (MCc) at 4 ◦C [21]
Two culture media were used to evaluate the growth behavior in all the experiments: a minimal nutrient medium (MNM) containing (in w/v) glucose 1%, $NaNO_3$ 0.6%, and agar 1.5%; and a water-agar medium (WAM) containing agar 1.5% (w/v). Untreated and treated polymer samples in triplicate, were deposited on top of this medium in 100 ml Petri dish after being washed with 70% ethanol for sterilization. Around 10^7 spores of the previously grown fungus were inoculated on the center of the polymer surface. These samples were then incubated at 28 °C and were monitored for twenty days. Sampling was done every 10 days, in which samples of polymers were washed again with 70% ethanol to eliminate the fungus present on the surface. These polymers were then submerged in water and washed with assistance of ultrasound. Non inoculated Petri dish acted as control, while dishes with the culture medium and polymers samples were treated the same way as inoculated samples. Optical Microscopy (OM) and Scanning Electron Microscopy (SEM) were used for fungal growth evaluation. OM was performed with optical microscope (Zeiss – model Axioskop 4o) at magnification of x40. The SEM analyses were carried out with scanning electron microscope (JEOL – JSM 6060) at 10 kV with a magnification of x5000.

Results and Discussion

Chemical surface characterization of UV-assisted treated films

The WCA (Fig. 1_i) decreased with increasing photolysis time leading to almost superhydrophilic surfaces after 120 min of treatment. The decrease in the WCA can be explained by FTIR-ATR analyses which show the grafting of hydrophilic groups (carbonyl, carboxyl, and ester groups) on the surface of the treated PHB films (see Fig. 1_i).

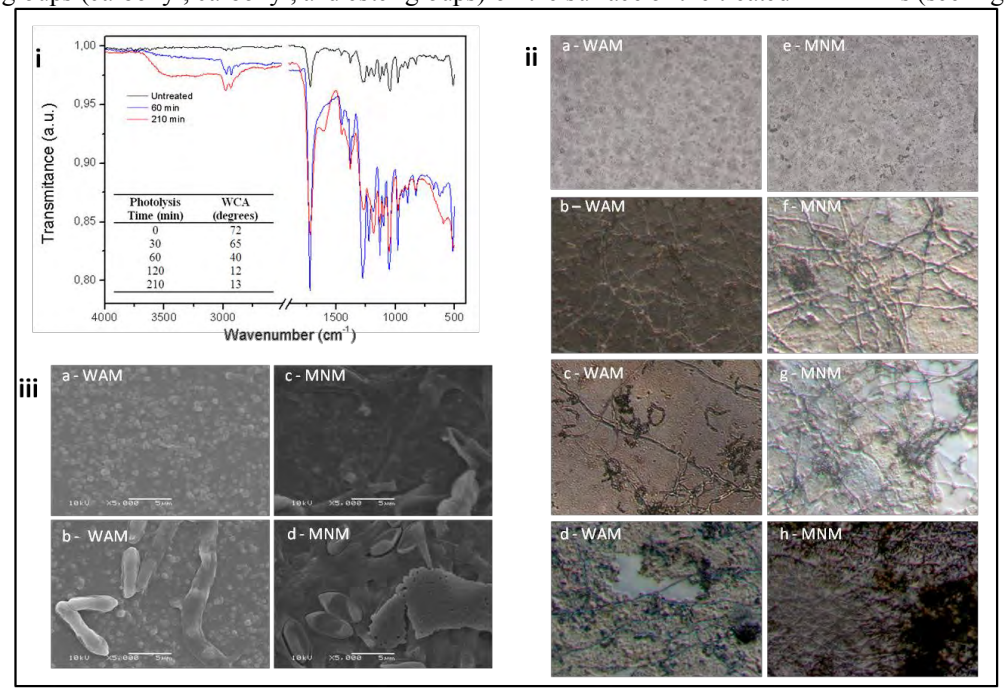

Figure 1. i) FTIR-ATR spectra and WCA values; **ii)** Optical Microscopic images of PHB films after 10 days of inoculation. Control samples (a-, e-), untreated PHB samples (b-, f-), PHB treated with UV radiation for 60 min in

presence of O_2 (c-, g-), and treated with UV radiation for 180 min in presence of O_2 (c-, g-). MNM = Minimal Nutrient Medium, WAN= Water-Agar Medium. **iii)** SEM images of samples after 10 days of incubation. Untreated PHB samples (a-, d-), PHB treated with UV radiation for 60 min in presence of O_2 (b-, e-), and treated with UV radiation for 180 min in presence of O_2 (c-,f-). MNM = Minimal Nutrient Medium, WAN= Water-Agar Medium.

XPS analyses of the untreated sample show a typical composition found in PHB films [10] (data not showed). When oxygen was present during the UV irradiation the spectra form treated samples show new functionalities having oxygen in its structure. The new functionality is shifted to lower energies indicating a less electronegative environment for this particular carbon atom. Peak-fitting of the C 1s and O 1s XPS spectrum of untreated PHB films showed the contributions of bonds corresponding to C=O and C–O–C groups. NEXAFS spectra (Fig. 2) show C K-edge before and after UV irradiation in the presence of O_2 for 30, 120, and 210 min UV irradiation. Due to untreated PHB films already have C=O functionalities, the presence of C1s→π*C=O signal is evident for all the untreated and treated samples. For the untreated sample the main components corresponds to the following transitions: C1s→π*C=O, C1s→σ*C-H, C1s→σ*C-C, and C1s→σ*C-O. After 30 min of photolysis in presence of O_2 there was a decrease on C1s→π*C=O and an increase on C1s→σ*C-O contribution which would mean that there are more ester groups on the PHB surface compared to untreated PHB films.. After 120 min of treatment the C1s→π*C=O and C1s→π*C=O/ σ*C-H signals dominate the spectrum and the intensity of C1s→σ*C-O and C1s→σ*C-C transitions decreased. Interesting our results did not show indication of C=C bonds formation. Even the NEXAFS spectra did not show any C=C signal which can indicate that Norrish Type II is suppressed during or by the treatments.

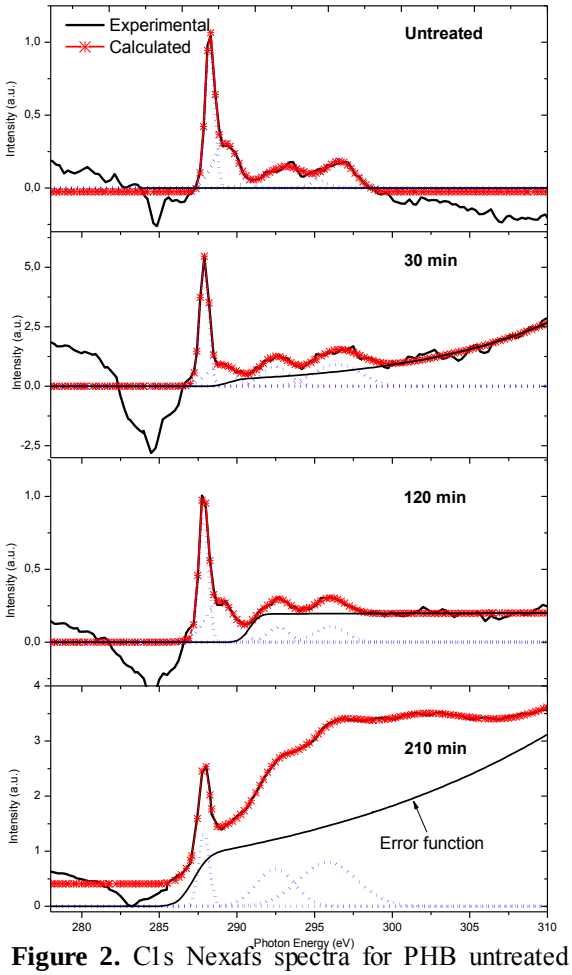

Figure 2. C1s Nexafs spectra for PHB untreated and treated with UV radiation in presence of O_2 for 30, 120 and 210 min.

Biodegradation Tests. Fungal growth

Figure 1ii shows differences in the development of *M. anisopliae* 10 days after inoculation ((Fig. 1_ii (a) and (e) shows control samples). After 20 days the fungal culture on polymers present the same growth behavior but with more extension and thickness of hyphae structures in comparison with polymers with 10 days of fungi inoculation. The untreated samples (Fig. 1_ii (b) and (f)) showed less fungi hyphae attached to the polymer surface than the treated samples (Fig. 1_ii (c, d, g and h)). For 180 min of treatment (Fig. 1_ii (h)), there were verified a significant increase at the polymers areas colonized by the fungus including mucilage along hyphae. Also the appearance of surface topography changed after the fungal culture, that are the first sign of degradation [22,23]. On samples incubated with the fungus in WAM (Fig. 1_ii (b-d)) it was observed a reduction in the formation of *hyphae*, mainly due to the absence of phosphate, nutrient required for the initial germination of these microorganisms. When the polymer samples were inoculated with *M. anisopliae* in presence of MNM, the medium promotes initial germination of fungi by the use of simple carbon sources, and then it may use more complex sources such as the polymers. Fungi are involved in three major modes of hydrocarbon metabolism, each involving its own distinctive enzymatic mechanisms: (1) partial transformation reactions; (2) complete degradation of hydrocarbons in the presence of a second compatible substrate; and (3) independent utilization of hydrocarbons as a sole carbon source for growth [24]. When oxygenated groups were grafted on polymers surfaces there was observed an increase in the growth of this fungus. The filamentous fungi spores require higher oxygen concentration for growth [25]. The PHB film had a rough surface that when it is viewed under the SEM analyses (Fig. 1_iii) revealed discrete granules, before and after the treatments. These findings agreed with the description of Shah et al. [23] where roughening of the surface, formation of holes or cracks, de-fragmentation, changes in color, or formation of biofilms on the surface do not prove the presence of a biodegradation process in terms of metabolism, but the parameter of visual changes can be used as a first indication of any microbial attack. The granules revealed in SEM images correspond to crystalline polymeric domain of PHB. At higher magnification it was possible to observe the hyphae structure. The hyphae shows different structures in their extremities (Fig. 1_iii, b), which has similarity to the structure of an appressorium as shown by St. Leger et al.[26]. Marks and holes were observed on the polymer surface along the hyphae (Fig. 1_iii, b) showing signs of degradation [22,23].

The obtained results showed that fungi are able to grow in both medium WAM and MNM, but they grow with better efficiency in the medium where carbon sources are easily assimilated (MNM). Changes on the surface of the polymers may indicate degradation processes caused by the fungi. Filamentous fungi secrete a variety of enzymes such as lipases, esterases, ureases, depolymerases and hydrolases that could be acting in the degradation of these materials. The development on the growing-tips of their hyphae structures called appressorium, which are responsible for the secretion of many of those enzymes, may suggest the presence of biodegradation; however confirmation of this hypothesis needs further studies.

Conclusion

The improvement of biodegradation in PHB by *M. anisopliae* was achieved by previous modification of the surface of the films by UV radiation in the presence of oxygen atmosphere. The surface chemical changes of the PHB films after the photochemical treatment were carefully characterized by WCA, FTIR-ATR, XPS and NEXAFS. It was shown that efficient grafting of new functionalities containing oxygen was obtained by the UV treatments. The oxidizing atmosphere did not allow the formation of C=C bonds that are observed in traditional UV irradiation of polymers. NEXAFS spectra did not show any evidence of C=C signal indicating that Norrish Type II is suppressed during or by the treatments. The higher hydrophilicity and concentration of oxygenated functional groups at the surface of the treated films compared to pristine PHB improved the biodegradation of the films by *M. anisopliae*. It was observed a clear increase in the growth of this fungus when oxygenated groups were grafted on the polymers surfaces after the UV treatments. UV radiation did not reduce but improve the biodegradation rate in the PHB films. Tuning of photolysis time allows easy control of hydrophilicity and insertion of oxygen groups on the surface of polymeric materials. Because oxygen is extremely necessary for germination of the spores, along with other nutrients, the present development brings PHB more susceptible to biodegradation but without losing its properties.

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