Orbital: The Electronic Journal of Chemistry
journal homepage: www.orbital.ufms.br
ISSN 1984-6428

| Vol 10 | | No. 1 | | Special Issue January 2018 |

Full Paper

Antimicrobial Analysis and Characterization of P(3HB) Films Containing Essential Oils

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Article history: Received: 02 June 2017; revised: 16 October 2017; accepted: 27 October 2017. Available online: 21 January 2018. DOI: http://dx.doi.org/10.17807/orbital.v10i1.1007

Abstract: Several studies have focused on the development of new materials for food packaging with the incorporation of antimicrobials such as essential oils. Thus, the present work aimed to develop biodegradable and antimicrobial films based on PHB incorporated with essential oils, named: melaleuca, cinnamon and citronella and also to characterize these films through the Fourier Transform Infrared Technique (FTIR). The films, containing 10, 20 and 30% (w/w) of essential oils, were microbiologically tested by agar-diffusion method against six species. The films containing melaleuca and citronella did not inhibit the microbial growth of any microorganisms tested. Unlike the films containing 20 and 30% of cinnamon that inhibited the growth of almost all microorganisms except *Salmonella* sp. The FTIR spectra of melaleuca and citronella overlap with those of PHB and it was not possible to observe the characteristic bands. The cinnamon spectrum shown characteristic bands of cinnamaldehyde, the main component of this oil. Through the FTIR technique the incorporation of the cinnamon oil into the polymer matrix can be verified. Microbiological analyzes showed that PHB film with 20 and 30% of cinnamon presented better antimicrobial activity.

Keywords: antimicrobial activity; essential oils; FTIR; PHB

1. INTRODUCTION

The polyhydroxyalkanoates (PHA's) are a class of aliphatic polymers widely studied once it is renewable, biodegradable [1] and synthesized by many microorganisms [2]. In addition, they have similar properties to polypropylene and polyethylene [3, 4].

Among the PHA's, the most widespread and currently studied, is polyhydroxybutyrate (PHB), synthesized within many Gram-positive and Gram-negative bacteria as a carbon and energy reserve [5]. PHB is a linear polyester of D(-)-3-hydroxybutyric acid, whose molecular weight differs from one organism to another, the growth conditions and the extraction method [6]. In addition, PHB is a renewable, biocompatible and linear thermoplastic [7] with low oxygen and water permeability and attractive barrier properties compared to other polyesters [8, 9]. The degree of crystallinity of PHB is in the range of 55-80%, with glass transition temperature (Tg) of approximately 5 ºC, melting temperature (Tm) at 175 ºC and decrease of molar mass at temperature above 170 ºC [10].

However, PHB is a highly crystalline and brittle polymer [11, 12] and its high cost of production prevents it from competing with conventional polymers [12]. For these reasons, the combination of PHB with other polymers (blends) or composites (with fibers) has been studied in order to improve its physical properties and decrease the cost of production.

Studies have also been devoted to the incorporation of natural antimicrobial agents into food packaging materials, due to increased consumer demands for natural foods, improved food safety, minimally processed products, "ready-to-eat" foods and longer shelf life [13]. Defined as a system incorporated in the packaging that releases or absorbs external or non-food or environmental substances, active packaging is intended to extend shelf life,

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maintain or improve the condition of the packaged food [14]. Among these agents, the essential oils present a future promissory, however the studies of incorporation of essential oils in PHB films and evaluation of their antimicrobial action are scarce. The mode of action that causes the microbial inhibition by essential oils and their chemical compounds involves several mechanisms that depend on the components present in the essential oil.

Cinnamon is obtained from the inner bark of trees of the genus *Cinnamomum* of the family Lauraceae and is a plant widely used by different cultures around the world. Among the almost 300 varieties that exist the most prominent are the true cinnamon or cinnamon from India (*Cinnamomum zeylanicum*) and the fake cinnamon or cinnamon from China (*Cinnamomum cassia*) [15]. The main component of bark and cinnamon leaf oil is cinnamaldehyde, which exhibits antimicrobial activity against a wide range of microorganisms.

The essential oil of the leaves of *Melaleuca alternifolia* is rich in terpinen-4-ol (29.4 - 44.9%) effective against bacteria, fungi and viruses. This oil has stood out as the main antimicrobial or as a natural preservative in pharmaceuticals and cosmetics. In addition, it may be bactericidal or bacteriostatic, when used in low concentrations [16].

Cymbopogon winterianus, commonly known as citronella, is a plant of the family Poaceae, grown in India and Brazil. It presents antibacterial and antifungal activity, mainly, against *Candida albicans* [17].

Considering the above mentioned, the main objective of this study was to evaluate the incorporation of the essential oils of melaleuca, cinnamon and citronella into PHB films using Fourier Transform Infrared Spectroscopy (FTIR), which is used in the structural investigation of the polymer matrices and can detect small changes between amorphous and crystalline regions [18]. Moreover, the evaluation of the antimicrobial activity of the films using six patogenic microrganisms important for the food industry was carried out.

2. MATERIAL AND METHODS

Materials

PHB was supplied by Copersucar (Cooperative of Sugar, Sugar and Alcohol Producers of the State of São Paulo) with molecular weight (Mw) of approximately 221,000 g mol^{-1} and 99% purity.

Essential oils with 99% purity were obtained from a local company (Ferquima). According to manufacturer's specifications, *Cinnamomum cassia* oil is composed of aldehyde (81%), coumarin (3%), benzaldehyde (3%), cinnamic alcohol (3%) and styrene (3%). While *Cymbopogon winterianus* presents 38% citronellal, 12% citronellol and 22% geraniol. *Melaleuca alternifolia* is composed of terpinene-4-ol (42%), gamma terpinene (23%), alpha terpinene (11%) and cineol (2%).

All reagents used were from analytical grade.

Film Preparation

The PHB films were prepared by *casting*. The PHB polymer was dissolved in chloroform (Sigma-Aldrich) 5% (w/v) and left under stirring at medium speed on a magnetic stirrer until complete dissolution (about 24 h). Then this solution was filtered, and then the essential oils were added in different concentrations (10%, 20% and 30% w/w in relation to PHB), also pure PHB was elaborated as the control sample. After that, the solution was mixed for 15 minutes. After this period, the solutions were placed in Petri dishes and incubated under a saturated atmosphere of chloroform for 3 days, until the formation of a thin film.

Determination of antimicrobial activity of films containing essential oils

The technique used to determine the antimicrobial activity of PHB films with melaleuca, cinnamon and citronella was based on the NCCLS [19], with adaptations. The films, in all the elaborated concentrations, were cut in circles approximately 1 cm in diameter and tested, microbiologically, by means of the disk-diffusion method in agar. The action of the films was evaluated against the following species: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 27664, *Salmonella* sp. ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Aspergillus niger* ATCC 6275 and *Candida albicans* ATCC 90028.

The film disks were placed on the Petri dish containing Mueller Hinton agar after seeding the bacterial inoculum adjusted on the McFarland 0.5 scale. The plates were then incubated in a bacteriological oven for 24 hours at 35 °C.

Fourier Transform Infrared Spectroscopy (FTIR)

The absorption spectra in the infrared (IR) region were obtained in equipment of the Jasco brand, model FT-IR 4100 with ATR Pro 450-S accessory (Total Attenuated Reflectance) in the range of 4000 to 400 cm^{-1}, with resolution of 4 cm^{-1}. Data acquisition was performed using Spectra Manager software.

3. RESULTS AND DISCUSSION

Antimicrobial activity of PHB films incorporated with essential oils

The antimicrobial activity of films containing the essential oils of melaleuca, cinnamon and citronella are presented in Table 1. The films containing the essential oils of melaleuca and citronella did not present antimicrobial activity in relation to any of the microorganisms evaluated. Unlike cinnamon oil that did not show antimicrobial activity only against *Salmonella* sp., and at 20% concentration was not effective for *Staphylococcus aureus* and *Aspergillus niger*.

Due to the incorporation in the PHB film, the concentration of the essential oil components in the films can decrease and, consequently, minimize the antimicrobial efficacy of the films tested by the agar diffusion technique [20-22]. In this test, non-halo formation of inhibition characterizes that the film has no antimicrobial effect [23]. In this way, Sanla-Ead et al. [24] points out that it is more reliable to use vapor diffusion to evaluate the antimicrobial effect of lipophilic films.

The essential oils are part of the film structure and interacts with the polymer and the plasticizer, and thus the diffusion of the antimicrobial compounds into the product can be reduced [25]. The release of antimicrobial agents from edible films, for example, depends on many factors, such as electrostatic interactions between the antimicrobial agent and the polymer chains, osmosis, structural changes induced by the presence of the antimicrobial, and environmental conditions [26].

Table 1. Antimicrobial activity of PHB films incorporated with essential oils.

Microorganisms	Average size of inhibition halos (mm)								
	Melaleuca			Cinnamon			Citronella		
	10%	20%	30%	10%	20%	30%	10%	20%	30%
Salmonella sp. ATCC 13076	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	2.00±0.00	2.00±0.00	-	-	-
Staphylococcus aureus ATCC 19093	-	-	-	-	-	6.67±2.88	-	-	-
Escherichia coli ATCC 25922	-	-	-	-	1.00±0.00	3.00±1.40	-	-	-
Aspergillus niger ATCC 6275	-	-	-	-	-	3.00±0.00	-	-	-
Cândida albicans ATCC 90028	-	-	-	-	3.00±0.00	10.00±0.00	-	-	-

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy has been employed to examine how chemical interactions between PHB and essential oils, seen as two or more substances, are combined physically and how chemical interactions are reflected by characteristic changes in spectra bands [27, 28].

Pure PHB spectra are observed to have dominant peaks in the region of 2975 cm^{-1} and 2935 cm^{-1} corresponding to C-H bonds, the 1054 cm^{-1} peak related to C-O bonds and the 1715 band indicating the C = O bonds. These values are very close to those reported by TĂNASE et al. (2015) [29] in which the main bands found in the PHB spectrum are attributed to C-C coupling with CH_3 stretch vibration, C-O-C (978 cm^{-1} and 895 cm^{-1}) vibration bands, C-O-C stretching (1182 cm^{-1}), elongation CO (1227 cm^{-1} and 1277 cm^{-1}), CH_3 (1379 cm^{-1}), asymmetric CH_3 (1456 cm^{-1}), crystalline C=O (1720 cm^{-1}) and elongation C-

H (2934 cm-1 and 2995 cm-1).

The incorporation of essential oils into the PHB matrix was analyzed through the ATR. The spectra of films incorporated with melaleuca are shown in Figure 1.

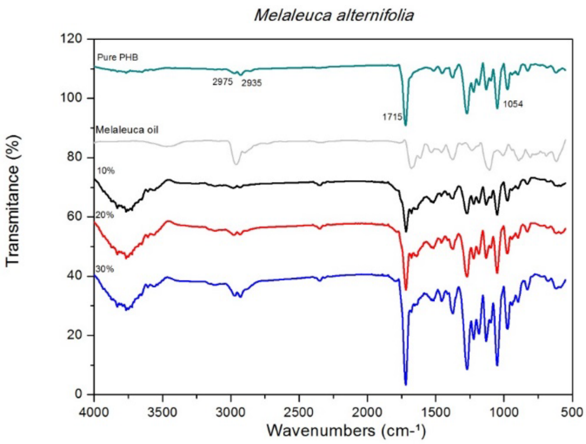

Figure 1. FTIR spectra of PHB films incorporated with melaleuca oil.

The absence of characteristic peaks of melaleuca oil may be related to the overlapping of these peaks by PHB characteristic peaks. However, it can be observed that films incorporated with melaleuca present a more intense band at 2975 and 2935 cm-1, as well as at 1715 cm-1 as the concentration of the oil increases. This band is more intense because they appear in both melaleuca oil and PHB matrix. A similar result was found by Pires [30] when evaluating the incorporation of melaleuca in sodium alginate.

Figure 2 shows the FTIR spectrum of cinnamon PHB films. A characteristic peak for the identification of cinnamon oil incorporation in the polymer film is in the 1676 cm-1 region and can be correlated to the C = O bonds of the cinnamaldehyde present in the cinnamon oil composition.

Similarly, to melaleuca oil, the spectrum of PHB/citronella films (Figure 3) did not show specific peaks for this oil, also characterized by the overlapping of peaks with PHB peaks.

However, the incorporation of essential oils caused the appearance of a new band in the spectra of the films in approximately 2343 cm-1. This peak is characteristic of several main components of essential oils, such as: limonene [31], main constituent of lemon oil [30], thymol, linalool, citral [32], terpinem-4-ol, and diterpenes and sesquiterpenes, constituents of copaiba oil [30].

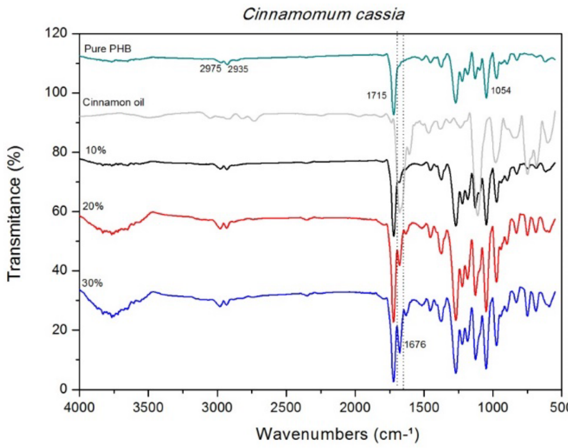

Figure 2. FTIR spectra of PHB films incorporated with cinnamon oil.

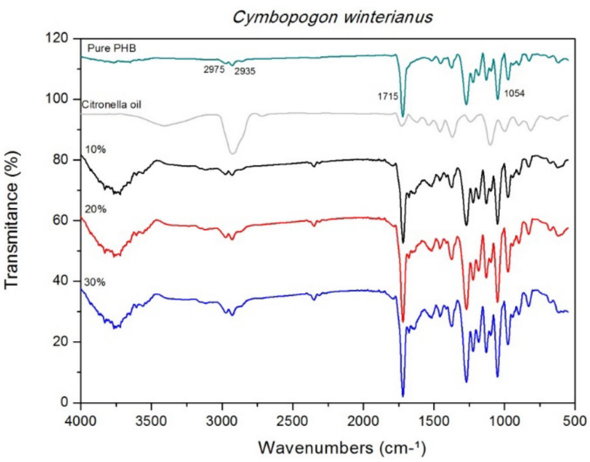

Figure 3. FTIR spectra of PHB films incorporated with citronella oil.

4. CONCLUSION

It was possible to elaborate PHB films incorporated with essential oils of melaleuca, cinnamon and citronella. As the films incorporated with cinnamon presented antimicrobial activity against fungi and bacteria, different from the others that did not present any antimicrobial activity for the studied microorganisms.

The most visible changes in the FTIR spectra were in the films incorporated with cinnamon, whereas melaleuca and citronella the peaks of the oils overlapped those of PHB and it was not possible to observe significant changes. It is believed that in the same way the incorporation occurred, since the films presented strong odor and characteristic smell of the incorporated oils.

5. ACKNOWLEDGMENTS

The authors would like to thank Copersucar (Cooperative of Sugar, Sugar and Alcohol Producers of the State of São Paulo) for assigning the PHB.

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