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Poly (Vinylalcohol-*Co*-Ethylene) Biodegradation on Semi Solid Fermentation by *Phanerochaete chrysosporium*

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SUMMARY. The biodegradation of the poly (vinylalcohol-*co*-ethylene) films (EVOH) was studied in semi-solid fermentation (SsF) with the *Phanerochaete chrysosporium* fungus, using as substrate a mixture of equal amounts of corncob and cornhusk. The initial humidity of the system was adjusted with basal medium between 70% and 90%. The EVOH films were analyzed by both infrared (IRTF) and differential scanning calorimetry (DSC), after 16 and 19 days of incubation. The results showed that in the system with higher humidity percent, the activity of lignin peroxidase (LiP) enzyme was more stable and the degradation of the chemical structure of the EVOH film was significant.

RESUMEN. "Biodegradación del Polietil-*Co*-Vinil Alcohol en Fermentación Semi-Sólida con *Phanerochaete chrysosporium*". Se estudió la biodegradación de películas del polímero etil-vinil-alcohol -EVOH-, en fermentación semi-sólida (FSS) con el hongo *Phanerochaete chrysosporium*, usando como sustrato la mezcla de iguales cantidades de capacho y tusa de maíz. La humedad inicial (HI) del sistema se ajustó con medio basal entre el 70% y el 90%, encontrando mayor actividad de la enzima lignino peroxidasa -LiP- en el sistema con HI del 90%. Las películas de EVOH fueron analizadas a los 16 y 19 días de inoculadas, por infrarrojo con transformada de fourier (IRTF) y calorimetría diferencial de barrido (DSC), encontrando una degradación significativa de la estructura química de la película de EVOH en el sistema de mayor HI.

INTRODUCTION

Plastics have many applications like packaging materials. But they cause an environmental problem, for the high volume of solid residues that they generate [1]. The norms imposed by the European Union's directives about waste of packaging and containers establish 45% recover and 15% recycle as a minimum objective (it includes regeneration and composting, not the incineration) for each material, goals that should be reached in the year 2001 [2]. These objectives are difficult to achieve for the plastic wastes from food containers, because their reutilization is not possible. Therefore the biodegradation alternative is important in order to increase the percent of recycled waste [3].

The EVOH is a copolymer made with polyvinylalcohol -PVOH- and polyethylene -PE-. The resins of EVOH have very good mechanical and gas barrier properties, as well as a good resistance to the permeability of scents and flavors [4], properties that favor their applications as food container [5]. Although the EVOH is a material that includes the PVOH biodegradable segment, its degradation only has been studied recently by Tomita [6] with a strain fungus of *Bacillus stearothermophillus* that requires temperatures of 60 °C which increases the cost and energy expenses for the composting processes starting from these waste. Recently, we have studied the biodegradation of the PVOH and the EVOH at room temperature, with the enzymatic extracts

KEY WORDS: Cornhusk, *Phanerochaete Chrysosporium*, Polymer biodegradation, Poly (vinylalcohol-*co*-ethylene) (EVOH), Semi-solid fermentation (SsF).
PALABRAS CLAVE: Biodegradación de polímeros, Capacho y tusa de maíz, Fermentación semi-sólida, *Phanerochaete chrysosporium*, Polietilenvinilalcohol.

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of the *Phanerochaete chrysosporium*, in submerged liquid mediums [7-9]. However, this liquid fermentation system is expensive and although the results obtained at laboratory level are important, these are difficult to scale, because it requires high volume of culture mediums that would make this process very expensive because of the cost of the chemical reagents.

At present time, the agricultural industries produce enormous quantities of waste, such as bean and banana peels, corncob, coffee pulp, sugar cane waste pulp, cereals, etc., which do not receive the appropriate treatment and in some cases they are thrown to the rivers and creeks causing an ecological damage. Some researchers are studying systems that catalyze or accelerate the degradation of these compounds [10] and to use them in the production of enzymes [11,12]. The agroindustrial wastes possess little biodegradability due to their high ligninocelulosic material content [13,14], but under appropriate conditions they can be an excellent support for the semi solid-state fermentation (SsF) [15].

The *Phanerochaete chrysosporium* is a basidiomicete classified as white root fungi due to the physical changes that cause in the wood. When it is cultivated under specific conditions the fungus produce extracellular enzymes. In 1983 the degradation of the lignin was related with these enzymes [16]. The fungus possesses a complex mechanism for the organic material degradation since he has several ligninolytic enzymes like lignine peroxidase (LiP), manganese peroxidase (MnP) and the poliphenoloxidase lacase. These enzymes have the capacity to oxidize a wide range of organic toxic compounds converting them to non-toxic metabolites or to CO_2 and H_2O [17].

In this work, we evaluated the LiP activity in semi-solid fermentation (SsF) using corncob and cornhusk as substrate and we used this ligninolític system in the biodegradation of polymeric material specifically the EVOH. The fermentation in solid and semi-solid state simulated well the environmental and nutritional conditions that the fungus could have in a land where the agroindustrial wastes are deposited [18], so this system would be appropriate to perform the degradation of polymeric wastes. The objective of this work is to know the life cycle of the EVOH films and the aim is get the degradation at a low cost and to make this project technologically viable, contributing with the ecological balance.

MATERIALS AND METHODS

Poly (vinylalcohol-*co*-ethylene) films of 22 µm thickness with 29 mol percent ethylene content denominated EVOH (Elsatochem, Spain, donated by the Instituto Agroalimentario de Valencia) were used.

Solid substrate

The corncob and cornhusk were collected in the hill sidewalk located in the Carmen of Viboral, east of Antioquia at 1.800 meters over sea level, temperature of 18 °C. The corncob and cornhusk were dried off at 55 °C for 48 h and then were milled until particle size of 0.5 cm.

Fungus

Phanerochaete chrysosporum BKM-F-1767 (ATCC 24725) was cultivated and replied in YMPG medium. It was sterilized and incubated for 5 days at 37 °C. After that treatment it was kept at 4 °C. The fungus must not be more than 3 weeks old after its replication. The spores were rasped and dissolved in 0,5 p/v% tween 80, and completely mixed in a vortex shaker for 2 min. The quantity of spores was determined by the method described by Jiménez [19].

Variables studied

The influence of the initial humidity (IH) in the production of LiP enzyme and on the degradation of the EVOH films was studied.

SsF System

Substrate

For all the experiments 5 g of corncob and 5 g of cornhusk were weighed in 500 ml erlenmeyers, covered with sterile cotton torundes and then with aluminum paper and placed in autoclave at 120 °C for 15 minutes.

Basal medium

It consists in sterile solution of sodium tartrate buffer in distilled water, to pH 4.5, that also contains veratrylic alcohol 2.5 mM and Tween 80 to 0.05 % (p/v).

With the purpose of studying the effect of the humidity in the LiP activity, different amounts of basal medium were added to four groups of erlermeyers in order to obtain different initial humidities of 70%, 80%, and 90%, respectively. Then each erlenmeyer was inoculated with 5.4 x 10^6 spores by g of substrate in a laminar flow cabinet. Each group of erlermeyer was incubated for different time: 6, 9,12 and 14 days and then the LiP activity was measured.

In order to study the effect of the IH on the degradation of the EVOH films, other two erlermeyer groups, each one with three erlenmeyers at three different IH, were inoculated with 1 cm^2 EVOH film and one group was incubated for 16 days and the other for 19 days. The EVOH films were washed previously with 70% ethanol and dried at room temperature. The controls were prepared inoculating one EVOH film at different IH, without spores of the microorganism. All the experiments were made by duplicate. The erlenmeyers were kept at room temperature during the incubation time.

The complete content of each erlenmeyer was filtered using 0,45 µm membranes. The EVOH films were removed with pincers from the solid residue. They were rinsed with water and dried at constant weight at 50 °C. The activity of the LiP was determined in the filtrate.

Analysis of LiP enzyme activity

200 to 400 µl of extracellular fluid were poured in a 1 ml quartz cell and mixed with 400 to 600 µl of 0.15 M sodium tartrate buffer (pH = 3) until complete 800 µl and also 50 µl of VA 10 mM were added. The reaction begins with the addition of 50 µl of H_2O_2 (50 µl/50ml recently prepared) and the absorbance was measured vs time at λ = 310 nm [19].

Infrared analysis of EVOH films

The ATR-FTIR spectra were measured directly to EVOH films, with the same initial thickness in a Perkin Elmer, spectrum one Model.

Differential scanning calorimetry (DSC)

The samples were analyzed in a TA Instrument model 2920, under the following conditions: heating and cooling rate of 10 °C/min under nitrogen atmosphere at a flow of 40 ml/min; heating was raised from 25 to 220 °C; isotherm at 220°C during 2 min; cooling to 25 °C. Again, heating from 25 to 220 °C. The last run was analyzed.

RESULTS

LiP activity

Figure 1 shows the average results of LiP activity for all the samples. The maximum LiP activity was 110 U/L at day 6 for the samples with 70% IH but decreased very fast getting the lowest value at day 16. Probably the stress caused by low humidity catalyzed the secondary metabolism of fungus, but it was not stable. The LiP activities obtained at 80% IH are the lowest during the total incubation time. The LiP activity for the samples with 90% IH get a maximum of 40 U/L and it showed appreciable activity values from day 7 to 19.

The LiP activities of 16 and 19 days correspond to LiP obtained in the samples with the EVOH films incubated during those days, without taking samples the previous days. The sample with 90% IH showed a slightly high activity. The most important aspect of the samples with 90%IH is that the secondary metabolism lasts during the 19 days, although with moderate LiP production levels.

Analysis of the FTIR spectra

The EVOH films were removed from the different media at the days 16 and 19. Their surfaces were covered with the fungus indicating that it was either growing supported on the films or depending of them, consuming part of their structure. The films were washed with enough water, until being free of the fungus and they were weighed to constant weight.

The infrared spectra of the 90% IH EVOH films: initial, the control and after 16 and 19 days of incubation are given in Figure 2. The peak at λ = 2920 cm^{-1}, which is correlated with the CH-stretching vibration [20], became markedly weaker in particular the day 19 of incubation. Also, the peak 1420 cm-1 characteristic of the flexion's CH_2/CH_3 decreased for the sample of day 19. The bands at 2920 and 1420 cm^{-1} pre-

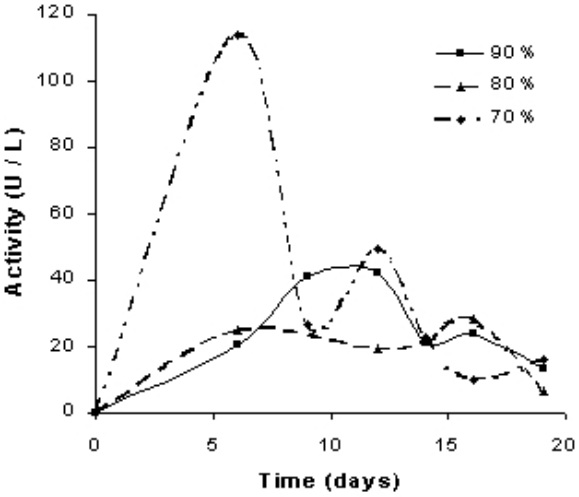

Figure 1. LiP activity average the samples with different % IH in the SsF system. The results until day 14 correspond to the samples without EVOH. The results of day 16 and 19 correspond to the samples with the EVOH films.

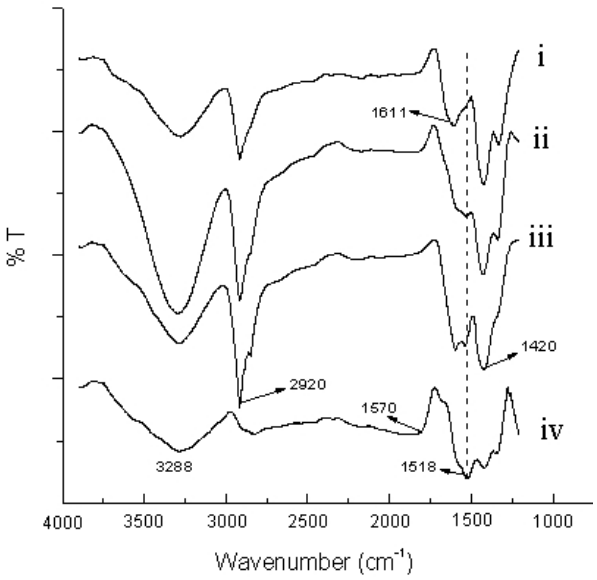

Figure 2. FTIR spectra of the EVOH films incubated in a 90% IH SsF system with Phanerochaete chrysosporium: **i**. Initial. *ii*. The control after 19 days of incubation. **iii**. After 16 days of incubation iv. After 19 days of incubation.

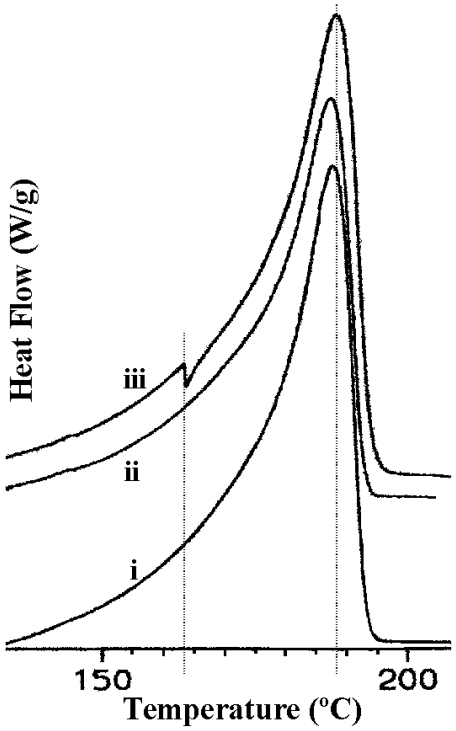

Figure 3. Results of the DSC EVOH films incubated in a 90% IH SsF system with *Phanerochaete chrysosporium:* **i**. Initial **ii**. The control after 19 days of incubation **iii**. After 19 days of incubation.

sented a relative less intensity in the sample EVOH 19 compared with the sample EVOH initial, which indicated a less number of saturated CH groups that could be attributed to a relative less presence of the PE in the EVOH structure. The peak at 1611 cm^{-1}, characteristic of the to isolated C=C group, is present in the EVOH initial, control and day 16 samples, but it was not resolved in the sample of day 19. New bands appeared for the sample at day 19, at 1518 cm^{-1} and 1710 cm^{-1}, that could due to the presence of α,β-diketones with the new C=O groups, as it can be deduced by its appearance, confirming the oxidation of the polymer [7].

The results of the spectra obtained with 70 and 80% IH are not shown, because although the changes were similar, they were less significant than those showed with the 90% HI.

Analyses of the DSC Thermograms

The results of the DSC 90% IH EVOH films: initial, the control and after 19 days of incubation, are given in Figure 3. All the EVOH films present the melting temperature (Tm) at of 189-189,5 °C, which is the value reported for 29% ethylene EVOH [20]. The 19 day sample presented other small endotherm at Tm = 163 °C, corresponding to two different crystalline sequences. The peak at higher temperature did not considerably change its position with the degradation time, indicating that some part of the copolymer was not degraded, and therefore it was attributed to the remaining copolymer. The peak at smaller Tm corresponds to a less crystalline structure, which could be assigned to the LiP oxidized chain, that contains, α,β-diketones and new C=O groups, according to that observed in the IR spectra.

The results of the thermograms obtained with the 70 and 80% IH the are not shown, because were similar to those obtained with the 90% HI.

DISCUSSION AND CONCLUSIONS

Phanerochaete chysosporium grow up well in the solid substrate (corncob and cornhusk). This indicates that this medium supplied the necessary nutrition to the fungus, specifically the carbon, nitrogen and minerals sources that were not given with the basal medium, favoring the degradation of the lignino-celulosic material, as well as the production of important secondary metabolites like the ligninoperoxidase enzyme. The humidity is one of the parameters that should be optimized since it causes evident changes in the evolution of the microorganism

and inside enzymatic system. With the 90% HI, the secondary metabolism lasted during the 19 days, while with a 70% HI, it was obtained very fast but it was not stable, since the humidity is fundamental for the growth and enzymatic activity of any microorganism.

It is important to emphasize that this semisolid fermentation system, where the substrate is impregnated with liquid medium, presents the additional advantage of being able to control the humidity by adding the basal liquid with smaller quantity of nutritious of those that are used in submerged cultivations. The other advantage is the fact that extracelullar medium can be extracted to evaluate the enzymatic activity and analyze the total content of the substrate in the specified biodegradation time without the use of enormous quantities of organic solvents, as pointed out by Barrios [18].

The remarkable differences in FTIR spectrum among the samples EVOH initial and 19 day sample, as well as the differences among the DSC thermograms let us conclude that some part of the EVOH structure was degraded [22,23].

It can be deduced that the degradation of the EVOH is not complete because there is a remaining part of EVOH that conserves its crystalline structure. Hence the higher melting point is unaffected with the degradation time. The EVOH that undergo oxidation present lower aliphatic chain creating a new less organized crystalline sequence that melts at lower temperature. Similar results were obtained in the degradation of the EVOH in submerged systems of the *Phanerochaete chrysosporium* [7].

These changes observed in EVOH film at 19 days of incubation, let us to conclude that the structure of the EVOH is degraded during the incubation time, and that the fungus is able to grow and to continue its metabolism at the expense of the polymer, causing ruptures to the chain. This can be concluded from the higher amount of EVOH pieces needed to obtain 10 mg sample for the DSC analysis of the EVOH sample at the 19 days of degradation.

In this system, the utilization of the agroindustrial wastes that causes contamination to produce the secondary metabolites that can be used to degrade highly recalcitrant polymeric material is a very promissory work, because the very low cost of the process.

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