BIODEGRADATION OF POLY (VINYLALCOHOL) WITH ENZYMATIC EXTRACTS OF *PHANEROCHAETE CHRYSOSPORIUM*

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SUMMARY: Commercial poly(vinylalcohol) samples (PVOH) of average molecular mass (Mw) 120,000 g/mol, were subjected to biodegradation with enzymatic extracts of the *Phanerochaete chrysosporium* fungus, in which the lignine peroxidase activity (LiP) was detected. The results of differential refractive index and ultraviolet (UV) absorption Gel Permeation Chromatography (GPC), Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography coupled to a Mass Detector (GC-MS) allow to deduce that in 15 days, under the conditions of this study, the average molar mass of the polymer decreases in 79.3 %. The benzaldehyde was detected as the main degradation product.

Words Key: Poly(vinylalcohol), *Phanerochaete chrysosporium*, lignine peroxidase, benzaldehyde.

Introduction

PVOH is a broadly used packing material for consumer products. It is used for food packing due to its excellent barrier properties to scents and oxygen and its inertia to fats [1)]. Its solubility in water and its biodegradability make this polymer an interesting material to produce copolymers with a biodegradable segment [2,3)]. Matsumura *et at* [4)] studied the biodegradation of high molecular weight sodium polycarboxylate containing vinyl alcohol groups and found degradation under microorganism exposure. Its biochemical demand of oxygen showed that the biodegradability increases with the vinyl alcohol content. On the other hand, Moritani *et al* [5,6,7,8)] have been interested in functional modifications of PVOH by copolymerization to produce different materials with improved physical and chemical properties. The biodegradability of these new materials is unknown.

PVOH is a synthetic polymer biodegradable by a limited number of microorganisms. This ability has been proved by the strain of symbiotic bacteria of *Pseudomonas pútida* VM15A, *Pseudomonas sp.* VM15C and *Pseudomonas O-3* [9)]; also by the strain of *Alcaligenes faecalis*

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KK314 and the denominated *PVA-IMX* which mainly contains the *Arthrobacter sp.* isolated from a river water, enriched with a specific culture where the PVOH polymer was the only carbon source [2]. However, further study, on the isolation and the effects of the individual strains, is required since the degradation of the PVOH with microorganisms is carried out in two steps: oxidation and hydrolysis and there are few microorganisms that can cause both reactions [2,10]. No all microorganisms cause degradation of the PVOH as shown by Kesel *et at* [11], who found that PVOH and its mixtures with polycaprolactone (PCL) were not biodegraded by a pure strain of microorganisms isolated from an industrial compost but the PCL films were totally degraded.

In the enzymatic extracts of the *Phanerochaete chrysosporium* fungus, besides lignine peroxidase (LiP), manganese peroxidase (MnP) and the lacasa enzymes, the system generates H_2O_2, veratryl alcohol and manganese [12]. These extracts constitute a powerful system able to oxidize a large amount of different phenolic and non-phenolic compounds.

So far, only limited research has been done on polymer biodegradation with this ligninolytic system [13,14,15,16], but none of these studies are related to the PVOH degradation. Among the important factors to be considered in the biodegradation are the enzymatic production conditions, the degradation rate as well as the degradation route which determines the kind of decomposition products obtained [3].

In this work, the study of the PVOH biodegradability with enzymatic extracts of the *Phanerochaete chrysosporium* fungus was attained. According with the decomposition products obtained a biodegradation mechanism was proposed.

Materials and Methods

Degradation assay: Commercial PVOH was kindly supplied by "gmp Productos Químicos S.A." with a nominal molar mass of 120,000 g/mol, a high degree of hydrolysis (88%) and denominated PVOH gmp. The expression fluid was prepared as described previously by López *et al* [17] and a solution of 1 w/v % of PVOH gmp in the expression fluid was prepared and filtered through 0,45 μm membranes, under sterile conditions. Four 75 ml aliquots of this solution were poured into 500 ml Erlenmeyer covered with cotton torundes. The spores of the *Phanerochaete chrysosporium* are inoculated until a final concentration of 15×10^4 spores/ml

was reached [18]. Orbital-shaking speed of 150 rpm and temperatures between 25°C and 28°C were used.

The samples of 1 w/v % polymer in the expression fluid with spores were treated during 0, 5, 8 and 15 days and named gmp0, gmp5, gmp8 and gmp15, respectively. A gmpcontrol sample corresponds to the 1 w/v % polymer in the expression fluid without spores of the *Phanerochaete chrysosporium* fungus maintained at the same conditions than the samples mentioned above during 15 days. The experiments were carried out by triplicate. For the analyses 3 ml of each sample were taken every time and filtered by 0.45 μm membrane.

Activity of the enzyme LiP: The activity of the LiP enzyme was determined by the procedure described previously [17].

Gel permeation chromatography (GPC): 50 μl of 1 w/v % PVOH samples and diluted with water to a final concentration of 0.1 v/v % were injected for the determination of the molar mass by GPC. The concentration of 0.1 v/v % was used for the molar mass determination and non diluted samples were used for the UV detection.

The polymer is separated from the other components of the expression fluid by precipitation with methanol (procedure that will be described later), and the precipitated fractions were diluted in water to 0.1 w/v %. 50 μl of these samples also were analyzed by GPC.

GPC measurements were made using 600 pump, a linear column Ultrahydrogel Linear^{R}, mixed bed (10^{3} -10^{6}) and a second linear column Ultrahidrogel DP^{R} (10^{2}-10^{3}). Two detector were used: a 410 differential refractive index and 996 UV diodes array from Waters. Polyoxiethylene standards with molecular mass of 21,000; 45,000; 85,000; 160,000 and 270,000 g/mol diluted to 0.1 w/v % in water were utilized for the molar mass calibration. The mobile phase for the standards and the samples was an pH 7.4 buffer acetate-phosphate, at 35°C, the flow rate was 0.6 ml/min, as it was described by López *et al* 1998 [17], where the optimal conditions for the GPC and UV analyses were established to study the PVOH biodegradation in the expression fluid.

Fourier transform infrared analysis (FTIR): For each sample (gmp0, gmp5, gmp8, gmp15 and gmpControl), 2 ml were treated with 6 ml of methanol to precipitate the methanol insoluble fraction of PVOH and they were centrifuged by 30 minutes. The insoluble fraction

was treated two more times with the same amount of methanol and again centrifuged by 30 minutes. The residual solid was dried at 40°C during 72 hours. KBr disks with 3 w/w % of these solids and the original polymer sample denominated "initial" gmp were prepared and their infrared spectra ran in a FTIR Mattson model 5000.

Gas chromatography coupled to mass detector (GC-MS): The methanol soluble phases were analyzed after dried at 40 °C during 72 hours and redissolved in methanol. The GC-MS Perkin Elmer Q-Mass 910 with the 15 m x 0.18 mm x 0.25 μm, 20 M Carbowax capillary column was used. The conditions for the GC-MS analyses were: helium flow 40 ml/min, injector temperature 220°C, and oven temperature program which started at 50°C, hold at this temperature by 5 minutes, and then raised at heating rate of 10°C/min up to 150°C and then at 15°C/min up to 220°C.

Results

Results and analyses of the differential refractive index GPC chromatograms: Table 1 and Fig. 1 show the results of the molar mass distribution curves of the PVOH gmp0, gmp8, gmp15 and gmpControl samples, diluted to 0.1 v/v %. These results were obtained using the software Millenium Express from Waters.

The average molar mass (Mw) decreases substantially from 111,838 g/mol (gmp0) to 23,150 g/mol (gmp15), that is a total reduction of 79.3 % in Mw in 15 days. The gmpControl showed a reduction of only 1.7 % of Mw, that corresponds to the chemical effect of the fluid itself.

Table 1. Molar mass average Mw of 0.1 v/v % PVOH gmp samples.

Sample Name	gmp 0	gmp Control	gmp 5	gmp 8	gmp 15
Mw (g/mol)	111,838	109,989	85,863	66,964	23,150
% loss (Mw)	0	1.7	23.2	40.1	79.3

Figure 2 shows the differential refractive index GPC chromatograms of the 0.1 v/v % PVOH sample treated during 15 days (gmp15) and the methanol insoluble fraction of the same sample redissolved in water, at the same concentration. It is observed that the methanol insoluble fraction is the one with higher molar mass.

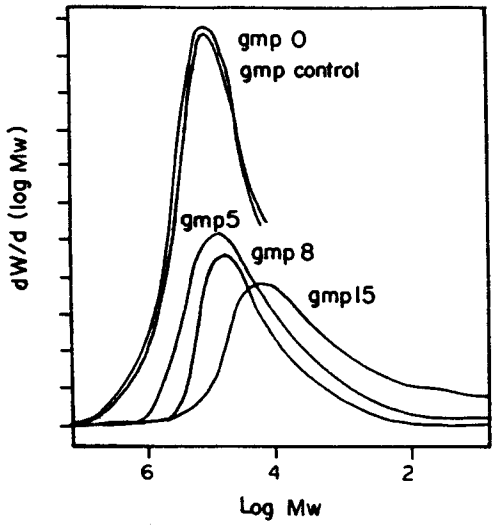


Figure. 1: Molar mass distribution curves of the PVOH gmp samples, diluted to 0.1% v/v in water.

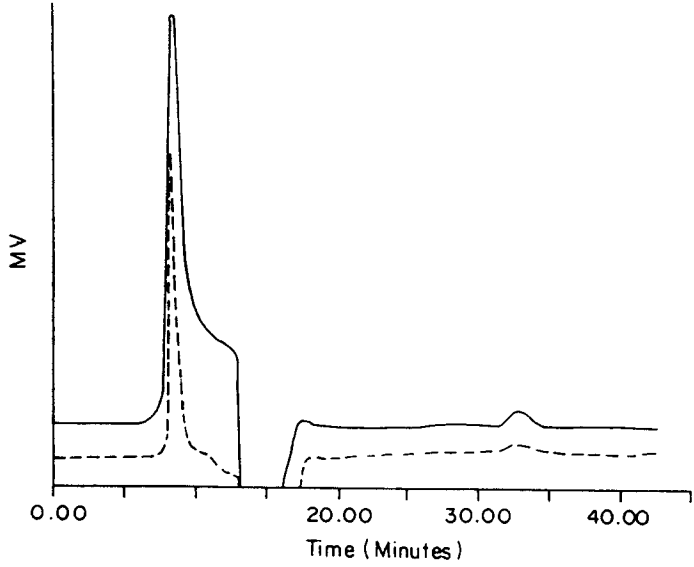


Figure. 2: Differential refractive index GPC chromatograms of the 0.1v/v % in water. gmp15 sample (——) and the methanol insoluble fraction of the same sample (- - - -).

Figure 3 shows the differential refractive index GPC chromatograms of the 1 w/v % PVOH samples treated during 0, 5, 8 and 15 days. The peaks that appear between 8.5 to 9.2 min correspond to the higher molar mass polymer and the small peaks between 9.2 to 12 min correspond to smaller molar mass polymer. The peaks between 20 min to 30 min, correspond to low molar mass degradation products (the peaks intensities change with degradation time).

Studies on separation mechanism of PVOH in biological fluid containing glucose, thiamin, veratrylalcohol, tween 80 and inorganics salts, showed that the retention times varies depending on the proportion of the culture medium added. With a low content, of 12 %, the PVOHs with different molar mass elute in a single peak that appears so early, probably due to an ionic exclusion effect. When the concentration of the medium is 25%, an intermediate ionic strength is achieved that allows some separation of the PVOHs but this is not good enough to differentiate them. With a higher concentration 88%, again all PVOHs eluted together but with a larger retention time, indicating that the excess of salts compensates the ionic sites of the stationary phase, allowing in this case an hydrophobic interaction between the aliphatic part of the stationary phase and the aliphatic part of the PVOH [18]. In order to obtain a pure size separation several mobile phases were used. They were selected considering the solubility of the polymer and trying to minimize the possible interaction of the polymer with the stationary phase. The best separation of the mixture was obtained using a phosphate-acetate buffer pH=7.4 [17].

Using phosphate-acetate buffer as mobile phase two types of separation mechanisms occurred for this system: the PVOH eluted by size exclusion and the other substrates of the fluid interact with the stationary phase [17]. The high retention that the veratryl alcohol and the thiamin present in this column that deviate from the general elution behavior predicted from their molecular weights, is due to their ionic aromatic character that let them to suffer a strong interaction with the stationary phase [19].

It is interesting to analyze in these chromatograms, the evolution that suffers the glucose concentration, which appears at 15.7 min. Its peak intensity decreases with degradation time and disappears completely at the eighth day (gmp8), which coincides with the day of maximum production of the LiP. The peaks that appear after 30 min correspond to the other fluid components (see Fig. N° 3).

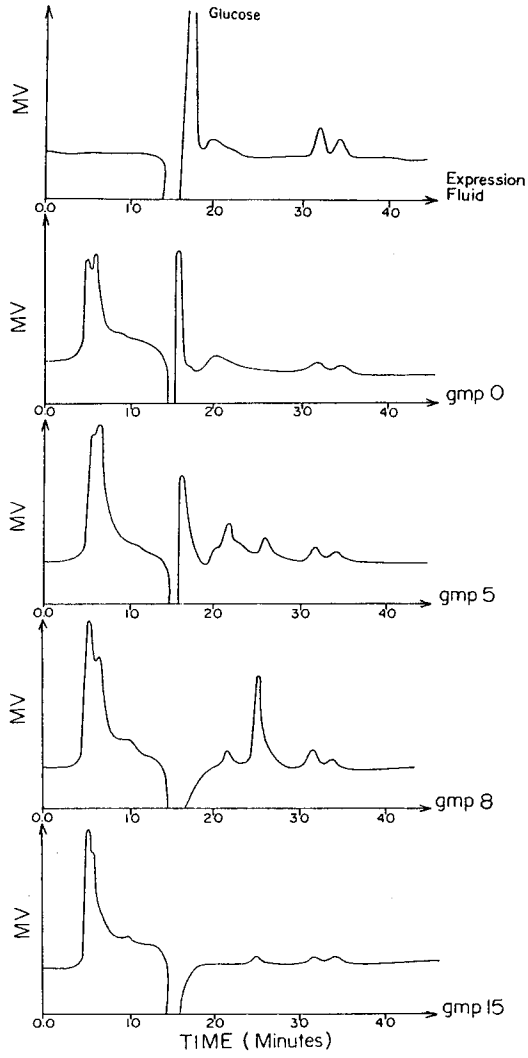


Figure. 3: Differential refractive index GPC chromatograms of the 1 w/v % PVOH gmp samples.

These 1 w/v % PVOH samples were analyzed by chromatography using UV detector in order to make the identification of the different components because this concentration allows enough sensibility for the UV detection of the degraded compounds.

Analyses of the UV spectra of the chromatographic peaks: Figure 4 shows the peaks of the 1 w/v % PVOH samples that present UV absorption. The peak in the differential refractive index GPC chromatograms of the high molar mass polymer (Fig. 3) between 8.5 to 9.2 min does not present absorption in the UV region and the first peak around 11 min (Fig. 4) corresponds to the polymeric fraction of low molar mass.

The UV spectra from 200 to 340 nm for each one of the chromatographic peaks are shown in Figure 5. It is observed that the UV spectrum of the compound that appears between 11 and 12 min changes with the degradation time. That is, sample gmp0 presents an absorption peak at 219 nm and a very small peak at 276 nm, that increases for samples gmp5 indicating a chemical transformation of the PVOH.

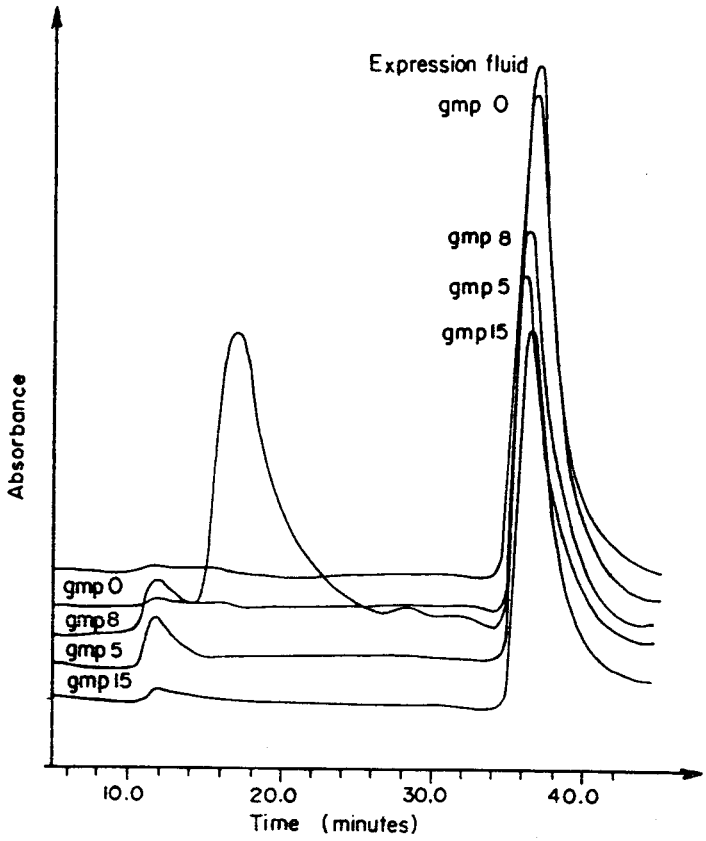


Figure 4: UV absorption chromatograms for the 1 w/v % PVOH gmp samples.

The absorption at 276 nm decreases for sample gmp8, but this sample also showed other peak at 17 minutes (Fig. 4 and 5) that has an UV spectrum with absorption at 229, 276 and 309 nm, which seems to correspond to a degradation product of the PVOH as will be discussed later.

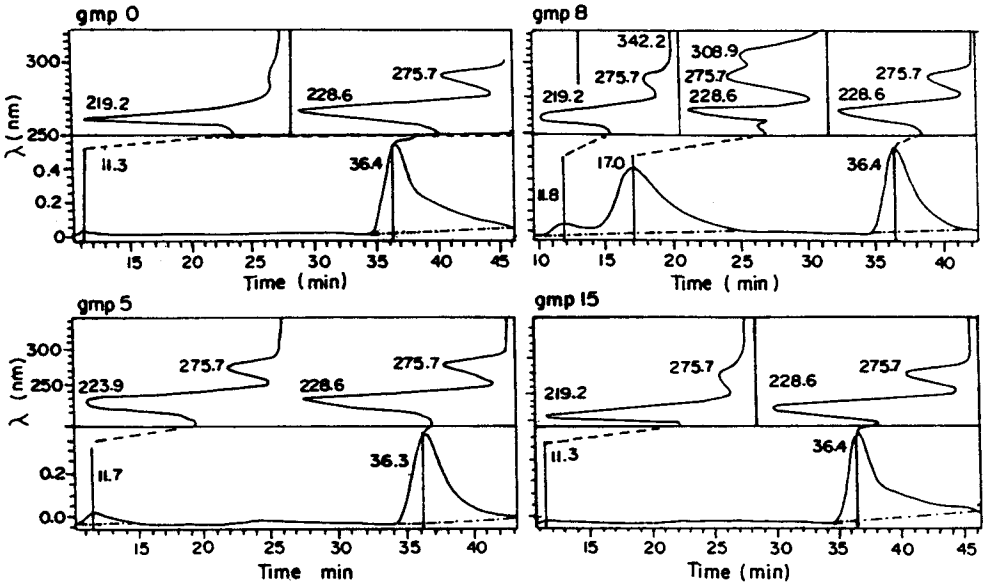


Figure 5: UV spectra for each one of the chromatogram peaks for the 1 w/v % PVOH gmp samples.

In samples gmp15, the elution peak at 17 min does not appear, this can be due to the transformation of this fraction into another compound or compounds that have an excessive retention in the column, this was deducted by the abnormal behavior of this sample during the elution causing pressure increases. It can be concluded that the low molar mass compounds that were formed after 15 days of degradation are completely retained in the stationary phase producing a flow blocking effect.

The peak at 36.5 min in the UV absorption chromatogram (Fig. 4) corresponds to veratryl alcohol, compound that is excessively retained in the column because of its chemical characteristics according to the study made by López *et al* [17]. This compound shows variation in the UV absorption with the degradation time. The absorbance is high before degradation

starts (initial sample, gmp0) and decreases as the degradation proceeds up the fifth day (gmp5), but shows an increase at the eighth day (gmp8), after which decreases again. This clearly indicates that this metabolite is initially consumed up in the biodegradation reactions, but the microorganism simultaneously produces it with the LiP, which presents its maximum activity at the eighth day [21]. Later on, it diminishes when the enzyme production already decays, and it is consumed during the disproportion reaction that takes place as the final biodegradation reaction.

Analyses for GC-MS chromatograms: Due to the abnormal behavior during elution of the sample gmp15 that causes pressure increases was necessary to identify the low molar mass products formed during degradation by GC-MS.

The analysis for GC-MS of the methanol soluble phase of the samples, give only benzaldehyde as relative volatile degradation products. The appearance of the benzaldheyde is explained for the occurrence of reactions showed in outline 1.

Analyses of the IR spectra results: The most representative vibration bands of the PVOH samples are listed in table 2.

In samples where the polymer is dissolved in the expression fluid, (samples gmp 0, 5, 8, 15 and control), the polymer is separated from the others components of the expression fluid, by precipitation in methanol. The commercial polymer in KBr disk is named as the initial gmp sample.

When comparing in Figure 6, the infrared spectra of initial and the gmp0 PVOH sample, it is observed that the methanol insoluble fraction keeps mainly the same functional groups, like the OH stretching band at 3970 cm^{-1} and the C-O extension bands at 1070 cm^{-1} A change is observed at 1727cm^{-1}, which has medium intensity in the initial gmp and decreases in the methanol insoluble fraction of gmp 0, indicating that the fraction of PVOH that contains carbonyl groups are methanol soluble, and for that is not showed in the IR [22]. The sample gmpControl after 15 days of degradation presents the same absorption that are observed in the PVOH gmp0, therefore, it can conclude that the fluid does not affect appreciably the chemical structure of the PVOH. The reference for the IR analysis of the biodegraded samples will be the polymer in expression fluid with degradation time 0 (gmp0).

Table 2. IR spectra of the methanol insoluble fractions of PVOH gmp samples.

Frequency (cm^{-1})	Relative Intensity				Interpretation according to the cited references
ν	gmp 0	gmp8	gmp15	gmp Control	References [10, 22, 23]
850	-	-	-	-	C-C stretching
906	-	M	W	-	Olefin CH flexion
1050-1100	S	M	S	S	C-O stretching
1120-1140	-	M	-	-	Crystallinity degree
1280	W	S	W	W	$-CH_2$ scissoring
1372-1390	M	-	M	M	Symmetrical flexion of
1400	-	M	-	-	$-CH_2$ scissoring
1566	-	M	-	-	Enroll form (α,β diketones)
1600-1624	M	-	S	M	C=C stretching
1715-1720	W	S	-	W	C=O stretching
2920	M	-	-	M	Saturated CH stretching
2969-2973	-	W	W	-	Olefin CH stretching
3268	-	S	-	-	OH stretching, strong
3400	S	-	S	S	OH stretching in polymers

- Band not observed

W = weak, M = Medium and S = strong.

Free or not-bonded OH groups usually absorb IR radiation at 3580-3650 cm^{-1}. Bands at lower frequencies indicate the formation of intermolecular bonds with the consequent decrease of the free OH groups. For polymers, the vibrations of OH groups forming hydrogen bonds are expected between 3400 and 3200 cm^{-1} [22, 23].

The infrared spectra of the methanol insoluble fractions of gmp5 and gmp8 are very similar and for that only the gmp8 is shown. Analyzing the infrared spectra of the gmp8 and gmp15 versus gmp0 samples (Fig. 7), it is observed that in gmp0, the wide and strong band, at 3397 cm^{-1}, characteristic of the OH stretching, shifts to 3268 cm^{-1} for the gmp8, and to 3400 cm^{-1} for gmp15. This indicates strong intermolecular interaction for sample gmp8 which can be corroborated with the appearance of absorption bands at 1128, at 1280 and 1407 cm^{-1}, which are related to an increase in the degree of Crystallinity of the PVOH [10,22]. These results suggest that degradation at the first stage is more oriented to the amorphous part of the polymer, leaving a more crystalline structure (gmp8), which is degraded later (gmp15).

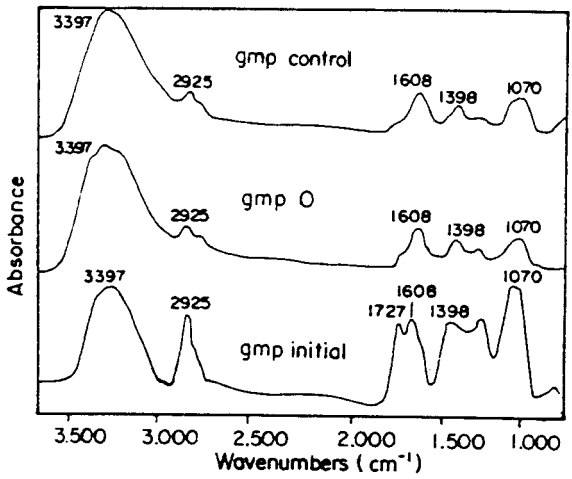


Figure 6: Infrared spectra of initial gmp and methanol insoluble fractions of the gmp 0 and gmp control samples.

The band of carbonyl groups at 1720 cm^{-1}, which is very weak in gmp0, becomes stronger for gmp8 and it disappears for gmp15. This indicates an increase of the carbonyl groups in the polymeric chain of the sample gmp8, due to oxidation of the OH groups. The carbonyl groups are not observed in the methanol insoluble fraction of the gmp15, because this sample has suffered a backbone chain break generating a polymer of smaller molecular weight, originating degradation products, which are soluble in methanol and contain C=O groups, such as benzaldehyde, as it was detected by GC-MS.

Another band that changes significantly is the corresponding to C=C stretching that has medium intensity in the gmp0 at 1608 cm^{-1} in sample gmp8, this band is moved to 1565 cm^{-1} due to the possible formation of β-diketones. In the sample gmp15, the C=C stretching band appears at 1615 cm^{-1} and it is stronger than in gmp0, which means higher degree of C=C bonding. The sample gmp15, polymer after 15 days of biodegradation, has a very similar structure to the polymer at time zero of biodegradation (gmp0), but with a smaller molecular size, as it was detected by GPC.

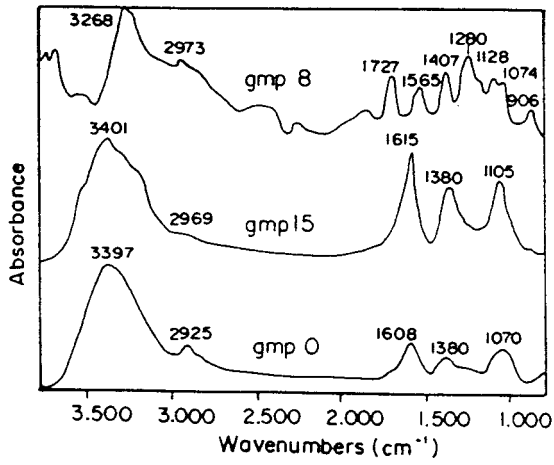


Figure 7: Infrared spectra of methanol insoluble fractions of PVOH gmp samples

Discussion

The ligninolytic enzyme (LiP) from the *Phanerochaete chrysosporium* in the culture medium, causes initial reactions along the main chain of the PVOH, forming carboxyl groups as well double bonds, increasing its unsaturation [17] as deduced by UV and IR spectra. Later, the chain is ruptured near to unsaturated sites giving a final polymeric structure with lower amount of carbonyl groups, generating low weight degradation products and a change in its molar mass.

Normally in the LiP catalyzed process, the substrate is transformed to cation radical that might be responsible of the initial degradation, as reported by several researchers who had proposed the following sequence of reactions [24]:

Ferric enzyme + H_2O_2 ⟶ Compound I (Oxoiron-IV porphyrin)$^{\cdot +}$

Compound I + VA ⟶ Compound II (Oxoiron-IV porphyrin) Complex + VAD

Compound II + Substrate ⟶ Ferric enzyme + cation radical substrate

Compound I is stabilized by veratryl alcohol (VA), which then is transformed to veratryl aldehyde (VAD). Compound II oxidizes the substrate and reestablishes the enzyme. The oxidized product is a cation-free radical [25].

According to this type of catalytic reactions, the rupture mechanism of the polymeric chain could involve initially formation of alkyl radicals as intermediate products [26, 27] (See outline 1).

The radical formation is usually the slowest reaction but its propagation is the fastest, therefore the radicals do not recombine again to form the original molecule but rather they are intermediate reagents that continue the chain reaction with other polymer molecules. Hundred of cycles can happen in the propagation stage before the termination takes place. The termination reaction is usually slow, through a complex series of reactions where the radicals combine each other by disproportion reactions, to produce compounds with a wide range of molar mass. The oxidation products can act as stabilization compounds and it is very probable that they can contribute to finish or to desacelerate the oxidation.

PVOH is a polymer that generally presents UV absorption at 225-230 nm, showing a weak band at 280 nm due to non conjugated carbonyl with a molar absortivity of $\varepsilon_{280} = 2$ m^2/mol (transition n-π^*) [26]. The carbonyl groups, coming from the polymerization, are residual groups at the end of the chain, just as they are observed in the gmp0 sample.

When the polymer increases the insaturations in the chain, the following groups, that present transition π-π^*, are formed: -CH=CHCO- with absorption at λ=225 nm, $(CH=CH)_2CO$ with amore intense band at λ = 280 nm (ε_{280}=2200 m^2/mol), and the structure $-(CH=CH)_3CO$ - that absorbs at λ=310 [26].

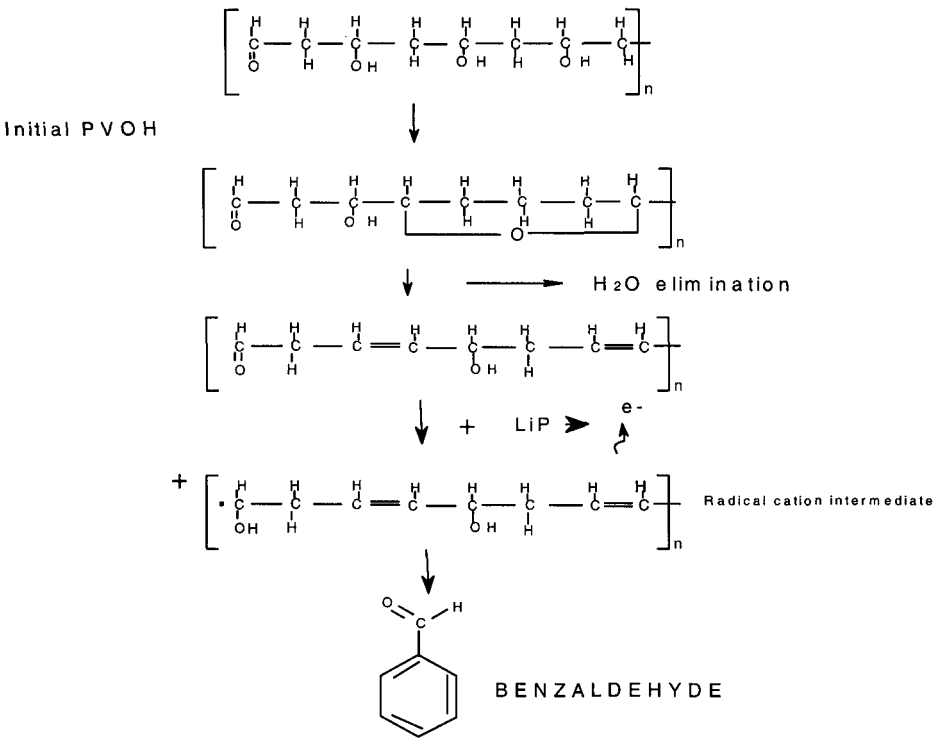
These polar groups and unsaturated sequences are observed clearly in the UV spectra at the fifth day (gmp5) and they increase much more at the eighth day (gmp8). The chemical interaction between the polar groups produces a more crystalline structure. At 15th day of degradation (gmp15), the polymeric structure substantially diminishes its C=O absorption because the polymer breaks in positions near to the groups C=O. The C-C bonds adjacent to carbonyl, carboxyl or other groups with unpaired electrons are easily attack by radicals; this causes loss of Crystallinity.

These changes in the functional groups of the polymer are detected also by the IR spectrum where it is observed that with degradation time, the polymer losses C=O groups, the unsaturations increase and the degree of Crystallinity decreases. It can be deduced that the

first degraded part is the amorphous one, followed by the crystalline part that degrades until being transformed into a physically broken film.

Benzaldehyde is not observed in IR spectra because is not present in the methanol insoluble phase that was analyzed by FTIR, since it did not eluted from the column either could not be observed by UV.

Outline 1: Rupture reactions of the polymer and formation of degradation products.



Conclusions

The enzymatic extract of the *Phanerochaete chrysosporium* causes to the PVOH, a molar weight loss of 79.3 % in 15 days, which means a decrease in the average molecular mass from. 111,838 g/mol to 23,150 g/mol. The "control" polymer, which was dissolved in the

same expression fluid but without the *Phanerochaete chrysosporium* spores, did not show these changes, only 1.7 % loss of Mw, that corresponds to the chemical effect of the fluid itself. In the enzymatic extract of *Phanerochaete chrysosporium* only the activity of the enzyme LiP was detected [17], which was maximum when the glucose was consumed completely. Parallel increase of the veratryl alcohol was observed, which has been reported as being an essential cofactor in the in vitro catalysis of the degradation reactions of the lignin, benzopyrenes, azo dyes, etc [21]. This confirms that these nutrients regulate the production of the LiP and at the same time the biodegradation of the PVOH is related with these changes in the ligninolytic system. The main degradation products obtained, is the benzaldehyde. The benzaldehyde has a great value and importance in the food industry as a flavoring product [28]. This has special interest, since the biodegradation of this polymer, not only decreases the pollution that produces the plastic waste but also can be useful for the production of a raw material of great commercial value using clean technology and at low costs [29]. The best conditions to improve the yield in the production of the benzaldehyde from the biodegradation of the PVOH and its purification are under study.

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