A chemiluminescence study on degradation of gelatine
Biodegradation by bacteria and fungi isolated from cinematographic films

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Abstract

Chemiluminescence (CL) has become a sensitive tool for the study of polymer degradation, induced by exposure to various factors, such as heat, UV-light and oxygen. In this paper, the results obtained with this technique in the study of gelatine samples hydrolytically degraded under sterilisation conditions are presented. Also, photographic gelatine exposed to bacterial and fungal degradations, in water solution and under controlled conditions, have been study by the chemiluminescence emission of their corresponding films and the biodegradation extent was determined by viscosity. The bacteria and fungi employed in this work have been isolated from cinematographic films in a previous work.

The high intensities of chemiluminescence emission obtained for gelatines biodegraded by bacteria and fungi, in aqueous solution at 37 and 25 °C, respectively, are different to those obtained in the thermal degradation. The hydrolytic degradation mechanism is through a cleavage of the peptide bond of the protein without significant oxidation of the material. In contrast, biodegradation by bacteria and fungi at low temperatures decreases the molecular weight of the gelatine (viscosity) by the enzymatic activity but, also, produces an important oxidation in the material due to the reactive oxygen species (ROS) generated in the microbial metabolism.

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1. Introduction

From the XIXth century the application of gelatine revolutionised the photography, and nowadays, it is still the principal polymer employed as component in silver halide photographic films. The photographic gelatine is mainly type-B, obtained from collagen [1,2] of cattle bone by the liming process (alkaline pre-treatment). High molecular weight gelatine characterised by high gel strength or Bloom values are required due to the important properties [3] related with this top quality gelatine grade. Gelatine is important in the entire photographic process [4,5], covering the formation of the silver halide emulsions, through the manufacturing steps to the developing and printing operations.

In an earlier work, we studied the biodegradation of gelatine in aqueous solution by bacteria [6] and fungi [7] previously isolated and identified [8] from black and white cinematographic films stored in Spanish archives. Fourteen strains of bacteria were isolated. Five species of Staphylococcus, viz. S. epidermidis, S. homini, S. lentu, S. haemolyticu, and S. lugdunen i, and five species of Bacilli, viz. B. amyloliquefa, B. ubtili, B. megaterium, B. pichinotyi and B. pumilu, were identified, together with Sphingomon a paucimobili, Kocuria kri tinae and Pa trell a haemolytica. Also, 17 strains of filamentous fungi and 1 yeast, Cryptococcus albidu, were isolated and identified. The fungal strains present in the films consisted of four strains of A pergillu, viz. A. u tu, A. nidulan var. nidulan, A. ver icol, seven Penicillium chr o ogenum strains, as well as, Alternaria alternata, Clado portiun clado porioide, Mucor racemo u, Phoma glomerata and Trichoderma longibrachiatum.

Most of the microorganisms that were found colonizing the cinematographic films were resistant to adverse environmental conditions. The ability of these isolated microorganisms to degrade gelatine was checked [8]. In Table 1, the different species of microorganisms are listed.

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It is well known, that the degradation of a polymer is accompanied by formation of oxidative species, such as hydroperoxide (POOH), by the reaction of oxygen with radicals (P.*) originated in the macromolecular chain [9]. The chemiluminescence in polymers is due to the light emission that accompanies the thermal decomposition of those thermooxidative degradation products (hydroperoxides). This bimolecular reaction promotes ketone products to its lowest triplet state and the radiative deactivation gives chemiluminescence emission in the visible region.

The mentioned reactions are shown in Scheme 1.

The technique of chemiluminescence (CL) has become a sensitive tool for the study of polymer degradation, induced by exposure to various factors such as microorganism, heat, oxygen, UV-light, humidity and mechanical stress [10–15]. The emission of chemiluminescence from polymer samples in an inert gas, such as nitrogen, is proportional to the hydroperoxide content formed in the processing of the material; this being related to its thermal oxidation history. The intensity of chemiluminescence can be determined as the peak-top intensity $I_{CL-max}$, or area of the peak, $A_{CL-max}$, and these parameters could be used to evaluate degradation of polymers. In the presence of oxygen [16], the samples are highly oxidised in a diffusion-controlled reaction simultaneously to the emission, and the relative concentration of (POOH) enhances respect to that under nitrogen. The rate of oxidation $R$, increases under these conditions, and the bimolecular termination of peroxy radical will be large. Hence, the chemiluminescence intensity $I_{CL-max}$, $A_{CL-max}$ is significantly enhanced with respect to the emission produced under nitrogen.

Frequently, chemiluminescence based on the luminescent response of photoproteins that emit light after interaction with reactive oxygen species is often used to evaluate the potential of neutrophils to become activated after a direct contact with several biodegradable polymers. Recently [17], chemiluminescence has been used to study the degradation behaviour of alkaline treated poly(e-caprolactone) (PCL) films in two biological related media and in the presence of fibroblasts. It has been characterised the influence of the transitory oxidative stress previously observed in the seeded cells, on the PCL substrate. The momentary high levels of ROS species generated on the treated samples affects to the degradation behaviour and seems to be related with cell adhesion mechanisms. Chemiluminescence allowed discerning the different effect of these species on treated and non-treated membranes, resulting a tool of great interest since it allows to preview, before any defects become visible in the polymer or failure by fracture, physical or chemical changes in the early stage of the degradation.

In previous work [18], the chemiluminescence emission of type-B commercial gelatines films was studied under different conditions, and the influence of the gel strength resistance or bloom value of the gelatines on the characteristics of the emission evaluated. It was seen, that the differences observed in the chemiluminescence were sensitive to the molecular weight and gel resistance value. The higher bloom gelatine exhibited lower emission, under nitrogen and under oxygen, confirming its better quality and its higher resistance to oxidation, in comparison to the lower bloom gelatine. Hence, CL-emission could be used to study different processes that modify the gelatine structure.

The purpose of this research is to explore the possibilities of chemiluminescence technique to evaluate degradation of gelatines in the solid state. Hence, in this paper we will present the results obtained with this technique in the study of gelatine samples hydrolytically degraded under sterilisation conditions and, also, in samples exposed to bacterial and fungal degradations. The degradation of the gelatine was carried out in...
water solution and under controlled conditions of microorganism inoculum and temperature. The biodegradation extent was determined by viscosity and, with these characterised gelatines, polymer films were prepared for the proposed study.

2. Materials and methods

2.1. Material, film preparation and sterilisation treatment

Photographic grade gelatine used in this work (type-B and gel strength value/bloom value 225) was supplied by Aldrich Chemicals and used as received. The bloom value is a measure of the gelatine quality, since it is the value of the force in grams necessary to apply in a standard plunger to deform 4 mm the surface of the gelatine gel [19].

Sterilisation treatments carried out with gelatine and the employed glassware in all the experiments was done in autoclave at 15 pounds per square inch (PSI) with the steam getting to 121 °C (250 °F).

Films samples of approximately 60 μm of thickness were prepared using aqueous solutions (6.67 wt.%) of gelatine followed by solvent evaporation at 37 °C employing Petri-recipient cages of 10 cm of diameter. In the case of biodegraded materials, the gelatine solutions were sterilised by a cycle of 20 min under UV-germicidal lamp prior to film preparation to avoid microbial contaminations.

2.2. Bacteria and fungi present in cinematographic film

Bacteria and fungi present in cinematographic film sample were isolated and identified in a previous work [8] and their gelatinase activity was determined. The microorganism isolated and their hydrolysis activity to biodegrade gelatine are summarised in Table 1.

The code used for bacteria in this paper (Table 1) starts with an initial letter of the archive location from which the sample comes from (B-Barcelona, M-Madrid, GC-Gran Canaria), and in the case of fungal strains, a letter “H” is the first letter of the code. Only seven bacteria strain (six different species) were able to degrade gelatine (binder in photographic emulsion), in contrast to the fungal isolates, all of which hydrolysed gelatine, with the exception of the yeast C. albida (eight different species).

2.3. Biological procedure

In order to assess the rate and the extent of biodegradability of gelatine in solutions by bacteria and fungi, aerobic bioassays were conducted at 37 and 25 °C, respectively. The details of the preparation of bacterial and fungal suspensions for inoculums in saline solution and the general procedure followed in the biodegradation experiments of gelatine in solutions have been previously published [6,7]. Under the employed conditions, concentrations of 2.5 × 10⁷ cells/ml and 4.5 × 10⁵ spores/ml were used for bacteria and fungi, respectively. Only in the case of the genus Alternaria, the concentration was 10 times lower due to its larger conidia size. At different intervals of biodegradation time (0, 24, 48 and 72 h), viscosity of the solution was measured and gelatine films were prepared. In the case of fungal biodegradation, gelatine solutions were previously filtered by a sterile cotton tissue to remove residues of hyphae.

2.4. Viscosity measurement

Water solution viscosity, often determined at 6.67 wt.% is a widely characteristic property of the gelatine used in the industry as an estimation of the relative molecular weight [20]. In this work, we have measured the viscosity of the gelatines at 37 °C in unbuffered water solution because these conditions favour the microbial growth and are interesting in the applied point of view in order to evaluate the biodegradation of gelatines. Temperature was maintained constant using a glass-panelled thermostatised bath of ±0.02 °C.

To measure the viscosity an Ubbelohde micro-viscometer (filling capacity of about 2.5 ml) from Schott, having a 0.40 mm capillary diameter was used. The viscosity of the gelatine solutions in centipoises (cP) can be calculated using the instrument constant by the equation: \( \eta (\text{cP}) = Kt \), were \( K \) is the instrument constant (K = 0.01 mm²/sec) and \( t \) is the flow time in seconds.

The viscometer was conveniently cleaned with suitable solvents and dried in oven after each run. When microorganisms were used, the viscometer was also treated in autoclave to avoid contaminations.

2.5. Chemiluminescence

Chemiluminescence spectra were obtained using a CL400 ChemiLUME analyser developed by Atlas Electric Devices Co. Samples are disposed in temperature controlled cells using specimen holders consisting of disposable aluminium dishes. Such cells are closed by optical lenses which focus the corresponding emission light of each sample in a photon counting photomultiplier (Hamamatsu R1527P), which is water-cooled at 17 °C. The photomultiplier was previously calibrated using a radioactive standard provided by Atlas. The instrument has an internal calibration for the photomultiplier tube of photon counts versus millivolts. Depending on the expected chemiluminescence emission of the samples and in order to prevent saturation of the photomultiplier tube (PMT) different sensitivity level of the PMT (gain setting) were selected. Hence, in the measurements under oxygen and nitrogen, low gain and high gain of the PMT were chosen, respectively.

Two different types of tests were performed—(A) isothermal: samples of material are preheated up to the test temperature under nitrogen, and the media is charged with oxygen and the temperature of the test maintained constant. (B) Dynamic: material samples are heated up with pre-test ramp (10 °C/min) under constant flow (50 ml/min) of gas, nitrogen or oxygen.

The acquisition and analysis of the data collected by the PMT were processed using the specific software supplied with the instrument. Samples for chemiluminescence measurements were prepared from the gelatine film (60 μm) by cutting circular specimens of 0.8 cm in diameter in case of biodegraded samples by bacteria and 0.4 cm in the case of materials biodegraded by fungi.
2.6. Hydroperoxide determination

The hydroperoxide concentration of polymer film samples was determined by a combination of the methods of Mair and Graupner [21] and Heaton and Uri [22]. Ten milligrams of film fragments was refluxed with 2.0 ml of a sodium iodide solution in isopropanol (200 g/l) after acidification with 7 ml of an acetic acid/isopropanol solution (1:10 ratio by volume). After 30 min of reflux, the solution was cooled, diluted with 10 ml of distilled water and the iodide, generated as I$_3^-$ by the reaction, ROOH + 3I$^-$ + 2H$^+$ $\rightarrow$ I$_3$ + H$_2$O + ROH, was determined spectrophotometrically at a wavelength of 360 nm using an absorption coefficient of 2.5 $\times$ 10$^4$ l mol$^{-1}$ cm$^{-1}$. In all the cases, reagent blanks were carried out on duplicate refluxes to avoid hydroperoxide impurities.

3. Results and discussion

3.1. Chemiluminescence of gelatine material degraded on sterilisation treatment

Gelatine is not stable in aqueous solvent systems and undergoes a progressive hydrolytic degradation lowering its molecular weight and consequently decreasing useful physical properties. Many studies have been carried out to determine the kinetics of the hydrolytic degradation reaction and the influence of different variables. Hydrolysis of the gelatine depends on the temperature, pH of the system and to a lesser extent on the nature of the other solutes that can be presents. The viscosity of the gelatine solution can be used as measure of the extent of hydrolytic degradation and also can be employed to determine the effect of different variables. Hence, the dependence of the degradation with the pH has been established from a long time [23] and it is well known that at neutral pH the hydrolytic degradation takes place at low rate.

Aqueous solutions of photographic gelatine at pH 6.8–7.0 were treated under different sterilisation conditions. After treatments, gelatine films were prepared as described before and the chemiluminescence emission under nitrogen of the sterilised materials was compared. Results are shown in Fig. 1 and the data compiled in Table 2.

A cycle of autoclave sterilisation of gelatine solution in sealed ware, 20 min at 121 °C, decreases drastically the molecular weight of the gelatine as the viscosity values, initial (5.5 cP) and final (2.3 cP), put in evidence. Further cycles in autoclave destroyed the structure of the gelatine and the viscosity could not be measured in the viscometer. In contrast the viscosity remains constant after the sterilisation treatment under UV-germicidal lamp or a large drying treatment of the gelatine film at 60 °C during 21 days.

Autoclave acts like a pressure cooker allowing steam temperature to get above the boiling point of water and the protein structure suffers an efficient hydrolytic degradation giving raise low molecular gelatine due to the peptide bond hydrolysis.

The chemiluminescence of the film samples (Fig. 1) increases with the number of sterilisation cycles and the emission is about three times higher that the gelatine film prepared as control. This increase correlates with a higher hydroperoxide content in the film samples.

<table>
<thead>
<tr>
<th>Sterilisation treatment</th>
<th>$\eta^a$ (cP)</th>
<th>$I_{CL-max}$ (mV)</th>
<th>$A_{CL-1 h} \times 10^3$ (mV)</th>
<th>$I_{at2h}^b$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatine (as received)</td>
<td>5.5</td>
<td>170</td>
<td>470</td>
<td>200</td>
</tr>
<tr>
<td>1 Ster. Cycle$^c$</td>
<td>2.3</td>
<td>600</td>
<td>1543</td>
<td>650</td>
</tr>
<tr>
<td>2 Ster. Cycle</td>
<td>–</td>
<td>800</td>
<td>1600</td>
<td>600</td>
</tr>
<tr>
<td>3 Ster. Cycle</td>
<td>–</td>
<td>900</td>
<td>1940</td>
<td>1000</td>
</tr>
<tr>
<td>9 Ster. Cycle</td>
<td>–</td>
<td>980</td>
<td>2418</td>
<td>700</td>
</tr>
<tr>
<td>1 UV-cycle$^d$</td>
<td>5.5</td>
<td>380</td>
<td>958</td>
<td>350</td>
</tr>
<tr>
<td>21 days/60 °C</td>
<td>5.5</td>
<td>225</td>
<td>740</td>
<td>250</td>
</tr>
</tbody>
</table>

$^a$ Viscosity of the gelatine aqueous solution (6.67%, w/v, at 37 °C).

$^b$ CL-stationary intensity ($I_s$) after 2 h at 225 °C.

$^c$ Ster. Cycle—sterilisation cycle (steam–water 121 °C/15 PSI).

$^d$ UV-cycle—sterilisation under germicidal lamp for 20 min.
materials, measured by iodide titration, from $2.8 \times 10^{-6}$ mol/g in the initial gelatine film to $4.0 \times 10^{-6}$ mol/g after one sterilisation cycle. Here, the drastic reduction of molecular weight of gelatin after one cycle of autoclave is not proportional in magnitude to the little increase in hydroperoxides. This fact confirms that hydrolytic degradation mechanism is through a cleavage of the peptide bond of the protein without significant oxidation of the material, even that a high temperature, $121 \, ^\circ C$, is used. The UV-sterilisation cycle and the thermal treatment at $60 \, ^\circ C$ for 21 days slightly increase the CL-emission from the films. The data confirms that a little oxidation takes place in agreement with the employed low temperatures and the absence of protein degradation.

3.2. Chemiluminescence of gelatine biodegraded by bacteria

Firstly, the chemiluminescence of bacteria biomass, obtained from their culture media, was examined in order to determine their possible emission which can affect the gelatin chemilumi-

Fig. 2. Chemiluminescence of gelatine films under nitrogen at $225 \, ^\circ C$. Biodegradation of gelatine by gelatinase-positive bacteria was carried out at $37 \, ^\circ C$ in aqueous solution during (■) 0 h, (●) 24 h, (▲) 48 h and (▼) 72 h. (a) *Bacillus amylo liquefaciens* (B3BA); (b) *Bacillus abilii* (B3BS); (c) *Staphylococcus hominis* (B4C); (d) *Bacillus megaterium* (B7); (e) *Bacillus pumilu* (M5); (f) *Bacillus pichinotyi* (M2).
Table 3
Chemiluminescence data obtained under nitrogen at 225 °C of gelatine film biodegraded by bacteria at 37 °C

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Biodegradation time (h)</th>
<th>A_{CL-4 h} (mV)</th>
<th>I_{CL-max} (cP)</th>
<th>Viscosity (cP)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>0</td>
<td>8.48 × 10^6</td>
<td>727</td>
<td>5.43</td>
</tr>
<tr>
<td>B3BA</td>
<td>24</td>
<td>7.47 × 10^6</td>
<td>544</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.38 × 10^7</td>
<td>3958</td>
<td>2.07</td>
</tr>
<tr>
<td>B3BS</td>
<td>24</td>
<td>1.17 × 10^7</td>
<td>1045</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.79 × 10^7</td>
<td>2583</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.59 × 10^7</td>
<td>3624</td>
<td>1.17</td>
</tr>
<tr>
<td>B4A b</td>
<td>48</td>
<td>9.19 × 10^6</td>
<td>568</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.15 × 10^7</td>
<td>787</td>
<td>5.01</td>
</tr>
<tr>
<td>B4C</td>
<td>24</td>
<td>7.80 × 10^6</td>
<td>486</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.10 × 10^7</td>
<td>855</td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2.63 × 10^7</td>
<td>2954</td>
<td>1.72</td>
</tr>
<tr>
<td>B7</td>
<td>24</td>
<td>9.98 × 10^6</td>
<td>806</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.16 × 10^7</td>
<td>925</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.45 × 10^7</td>
<td>3417</td>
<td>1.99</td>
</tr>
<tr>
<td>M2</td>
<td>24</td>
<td>7.81 × 10^6</td>
<td>638</td>
<td>4.66</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.00 × 10^7</td>
<td>809</td>
<td>3.70</td>
</tr>
<tr>
<td>M3 b</td>
<td>24</td>
<td>5.30 × 10^6</td>
<td>389</td>
<td>5.40</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.43 × 10^6</td>
<td>408</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>6.44 × 10^6</td>
<td>477</td>
<td>5.02</td>
</tr>
<tr>
<td>M5</td>
<td>24</td>
<td>7.47 × 10^6</td>
<td>581</td>
<td>4.90</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.69 × 10^6</td>
<td>438</td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.48 × 10^7</td>
<td>1920</td>
<td>3.13</td>
</tr>
</tbody>
</table>

a Solution of gelatine at 6.67% (w/v) in H2O.
b No degrading bacterium (gelatinase negative).

In all cases, no emission was detected; it would mean that results obtained by chemiluminescence analysis of gelatine are no altered.

With the gelatine-film samples previously biodegraded by bacteria after different bioassay times at 37 °C, chemiluminescence emission profiles were obtained under nitrogen at 225 °C. The obtained results with gelatinase-positive bacteria are plotted in Fig. 2 and the data compiled in Table 3 together with their corresponding characterisation by viscometry.

The measurement of chemiluminescence under an inert gas, such as nitrogen, gives information on the intrinsic oxidation state of the sample. The selected temperature, 225 °C, is above the gelatine glass transition and the loss of rigidity favours the emission reaction. The intensity of the chemiluminescence emission increases in all the films biodegraded by bacteria. In the case of the samples biodegraded by B. amyloliquefaciens (Fig. 2a) and B. pichinotyi (Fig. 2f) after 72 h, films were not possible to prepare due to the loss of material integrity. The intensity drastically increases after 48 h of biodegradation only in the biodegradation carried out by B. amyloliquefaciens, with the rest of the bacteria the high intensity of chemiluminescence is observed at 72 h. In hydrocarbon polymers the chemiluminescence emission is due to the decomposition of hydroperoxide (POOH) through the disproportion reaction shown in Scheme 1. Hence, CL-emission can be related to the hydroperoxide content of the sample [24] and being their proportional relationship generally accepted. In agreement with this correlation, a high increase of hydroperoxide, 4.8 × 10^-5 mol/g was found in the biodegraded film by B. subtilis (Fig. 2b) after 72 h, respect to the initial content, 2.8 × 10^-6 mol/g. It is important to remark that the hydroperoxide formation takes place by the metabolic action of bacteria at 37 °C, without any thermal contribution. The peak-top intensity I_{CL-max} and the integrated area of the peak at 4 h from the start of the CL-experiment, A_{CL-4 h} for the biodegraded films by bacteria are summarised in Table 3. The data put in evidence this clear increase and the viscosity decay with biodegradation.

Biodegradation by bacteria induces hydroperoxide formation in the gelatine structure and the emission is very intense when the molecular weight (viscosity) of the gelatine is low, at 72 h of biodegradation. The intensity of CL does not correlate quantitatively with the viscosity decay indicating that the hydroperoxide formation depends on the metabolism of each bacterium.

In the case of the gelatinase-negative bacterial strains, S. paucimobilis, S. epidermidis, S. lentus, S. lugdunen i, S. haemolyticus, P. haemolytica, K. kristinae chemiluminescence emission of the gelatine films remained constant, after the bioassay procedure. This result confirms the absence of biodegradation. In Fig. 3, the CL-emission profiles, in the case of...
Staphylococcus haemolyticus and Sphingomonas paucimobilis, are plotted. Also, in Table 3 these two non-gelatinase active bacteria, M3 and B4A have been included for comparison.

The non-isothermal chemiluminescence emission under oxygen for the gelatine films biodegraded by B. subtilis is plotted in Fig. 4.

The CL-emissions obtained in oxygen with the linear increase of temperature are much more intense than that obtained under nitrogen due to the oxidation conditions. The different biodegraded films behave with the same order of stability commented before and the oxidation start at lower temperatures when the biodegradation time increase. The oxidation rates can be calculates from the slopes of the curves of Fig. 4, for the Bloom 225 gelatine a value of \( r_{\text{ox}} = 560 \text{ mV/h} \) was calculated and for the biodegraded gelatines: at 24 h, \( r_{\text{ox}} = 1370 \text{ mV/h} \); at 48 h, \( r_{\text{ox}} = 2929 \text{ mV/h} \) and at 72 h, \( r_{\text{ox}} = 3826 \text{ mV/h} \). As much biodegraded is the gelatine the oxidation takes places with a higher rate, in agreement with their lower rigidity and higher oxygen mobility in the materials.

In the isothermal chemiluminescence measurements on the biodegraded gelatines by bacteria under oxygen at 225°C, a drastic increase in the signal with biodegradation has been obtained. In all the cases, the emission of the film samples after 72 h of biodegradation was out of the photomultiplier scale because the signal was saturated with the employed size of the samples (disks of 0.8 cm of diameter). In Fig. 5, the emission of gelatine films biodegraded by B. subtilis are plotted.

The CL-intensity observed under oxygen on the biodegraded materials is highly intense and the stationary emission disappears after 24 h of biodegradation. At 48 h of biodegradation the material shows a sharp profile with a well-defined peak \((I_{\text{QL-max}} = 45,000 \text{ mV})\) indicating the destruction of the material by oxidation. The sample after 72 h of biodegradation gives an extremely high emission which saturates the photon counting detector.

### 3.3. Chemiluminesence of gelatine biodegraded by fungi

Firstly, the chemiluminescence of all the fungi employed in this work, from their culture media, was examined in order to determine their possible emission which can affect the gelatin chemiluminescence measurement. In all cases, no emission was detected.

The isothermal chemiluminescence analysis under nitrogen at 225°C was undertaken, for the film samples prepared from gelatines biodegraded by fungi, as function of biodegradation time. The first feature from this data is the observation that all fungi isolated from cinematographic films exhibited efficient chemiluminescence in accordance with their positive gelatinase activity (Table 1). The obtained chemiluminescence profiles versus time are plotted in Fig. 6, and data are summarised in Table 4.

The CL-profiles showed a similar trend to that observed for degradation by bacteria, and the intensity of chemiluminescence enhanced with biodegradation time. Although, a much higher CL-emission intensities were observed for samples biodegraded by fungi respect to those obtained for gelatine biodegraded by bacteria, and in consequence, the diameter of the specimens analysed was reduced to 0.4 cm. Since the emission is related with the content of oxidative species, this would indicate that gelatine was oxidised faster in presence of fungi than in presence of bacteria. The increase of chemiluminescence emission is gradually increased with biodegradation time.

Also, the chemiluminescence analysis under oxygen at 225°C was undertaken for those biodegraded samples. As expected, under oxygen conditions [25], a much higher intensity of chemiluminescence was observed to that obtained under a nitrogen atmosphere. Since in such conditions, the samples are highly oxidised in a diffusion-controlled reaction simultaneously to the emission. Macroradicals react with the oxygen to give peroxy radicals and its concentration will be large and the bimolecular termination reaction of two peroxy radicals to give ketone products will be enhanced. The obtained CL-profiles versus time for gelatines biodegraded by P. chryogenum and A. alternata, as example, are plotted in Fig. 7. As it was previously described for measurements under nitrogen, a
Fig. 6. Chemiluminescence curves vs. time at 225 °C under nitrogen of gelatine films. Biodegradation of gelatine by gelatinase-positive fungi was carried out at 25 °C in aqueous solution during (■) 0 h, (●) 24 h, (▲) 48 h and (▼) 72 h. (a) A. ustus (HB2); (b) C. cladosporioides (HB3A); (c) A. alternata (HB41N); (d) P. chrysogenum (HB7); (e) A. nidulans var. nidulans (HM3); (f) M. racemosus (HM4); (g) T. longibrachiatum (HGC2); (h) A. versicolor (HGCI).
higher activity was observed in samples biodegraded by fungi when compared with those biodegraded by bacteria; also taking into account the reduced surface of specimens analysed, 0.12 cm² instead of 0.5 cm² used on samples biodegraded by bacteria.

The high intensities of chemiluminescence emission obtained for gelatines biodegraded by fungi and bacteria, are pretty different to those previously obtained by thermal degradation. In the last case, molecular weight of the gelatine decreased with the time of degradation as a consequence of structure fragmenta-

![Fig. 7. Chemiluminescence of gelatine films under oxygen at 225 °C. Biodegradation assay at 25 °C in aqueous solution with Penicillium chrysogenum (HB7) and Alternaria alternata (HB41N) during 0 h, 24 h, 48 h and 72 h.](image)

...tion, and no significative increases of peroxides in the structure occurred, since the enhancement of chemiluminescence emission was negligible. However, biodegradation of gelatine by microorganism gives rise to the decreasing of molecular weight as result of enzymes activity, and also oxidation of gelatine with significant formation of oxidative species, hydroperoxide, responsible of the chemiluminescence observed. The scheme of the gelatine biodegradation is showed in Scheme 2.

In addition to enzymatic systems that give rise to selective breaking of bonds, the metabolic activity of microorganism
generates reactive oxygen species (ROS), as peroxide radical or hydroperoxides, hydrogen superoxide and radical anion superoxide. These species are intermediates in the microbial aerobic metabolism [26] and are frequent in physiological processes [27]. Some microorganisms can produce a high concentration of ROS, for example, in the case of likens [28] and some bacteria [29]. With the obtained results, it is clear that chemiluminescence is able to quantify the oxidation that is produced by microorganism in the structure of gelatine due to their metabolism.

4. Conclusions

Autoclave sterilisation of gelatine solution reduces drastically the viscosity of the gelatine. This fact confirms that hydrolytic degradation mechanism is through a cleavage of the peptide bond of the protein, since no significant oxidation of the gelatine is detected. In contrast biodegradation by bacteria and fungi, at low temperatures, decreases the viscosity of the gelatine via enzymatic degradation, but also the metabolic activity of microorganism generates reactive oxygen species, which induces an important oxidation in the gelatine structure. This oxidation was detected by the drastic increase in the chemiluminescence emission of the materials. In general, a much higher CL-emission intensities were observed for samples biodegraded by fungi respect to those obtained for gelatine biodegraded by bacteria.

The obtained results show the chemiluminescence as a technique able to quantify the oxidation that is produced by microorganism in the structure of gelatine due to their metabolism.

References