Transglutaminase Effect on the Gelatin-Films Properties

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Research Article

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Abstract

BACKGROUND: The development of biodegradable materials, especially those from renewable sources, is important to reduce the impact of plastic waste on the environment. On the other hand, protein-based films have high solubility in water, in addition to limited mechanical properties, which makes their application in high humidity environments a challenge. The enzyme transglutaminase (TGase) can reduce the interaction of gelatin films with water, improving these properties, while improving the mechanical properties. Therefore, this study aimed to determine the best condition for using the TGase enzyme, through variations in the gelatin and enzyme mass, in addition to the time that the enzyme acts on the gelatin, evaluating the properties tensile strength, elongation, and solubility in water. In a second set of experiments gelatin amount was kept fixed at 4%, and other proportions of an enzyme (1% and 5% w/w gelatin) were studied, evaluating beyond these properties the degradation in simulated soil through thermogravimetry analysis and Fourrier Transform Infrared Spectrometer (FTIR).

RESULTS: It was concluded that the higher concentration of TGase (5%) promoted a greater reduction in the solubility of the films, making the films more resistant to biodegradation, facilitating their application due to the increase in their useful life.

CONCLUSION: At the end of the degradation test, it was noticed that the films were degraded, presenting the potential for substitution of polymers of fossil origin, which could be an alternative to the problem of polymeric residues in the environment.

Highlights

- The addition of the enzyme decreased the solubility of the films;
- Films with the enzyme have increased resistance to biodegradation when compared to films without the enzyme.
- Gelatin films with and without enzyme have been completely degraded when expose to soil.

1. Introduction

Technological advances in agriculture, driven by increased demand for food, have led to the search for technologies that give greater control over the variables that interfere with crop productivity [1]. Several techniques stand out to optimize production capacity, including cultivation in a protected environment and mulching, making agriculture the sector responsible for around 3.4% of the global demand for plastics in 2019 [2]. To expand the use of these techniques, petroleum-based synthetic polymers are employed due to their low cost, ease of processing, and lightness [1]. However, these materials generate large amounts of waste that require proper handling [3]. As much as petroleum-based polymers are consolidated in industries, these environmental aspects, together with the growing consumer demand for safe and non-toxic materials, make this topic of great relevance for current and future research [4].

In this way, the development of biodegradable polymers, made from renewable natural resources is considered a viable solution to mitigate the consumption of the fossil fuel reserve and, at the same time, reduce the negative environmental effects caused by the disposal of waste-based materials. of non-biodegradable polymers [5].

Among the materials studied, gelatin stands out for being abundant and low cost, in addition to presenting promising film-forming properties [6]. In addition to having a high degree of biocompatibility and biodegradability, it is widely used in the food industry as a gelling agent and as a stabilizing ingredient for foams and emulsions [7]. It is the product of the denaturation and partial hydrolysis of collagen, composed mainly of carbon (50.5%), oxygen (25.2%), nitrogen (17.0%), and hydrogen (6.8%) [8;9].

Gelatin films have good mechanical properties, although they are sensitive to humidity and have high permeability to water vapor due to their hydrophilic character. One of the ways to reduce water vapor permeability and solubility in water of gelatin films is through cross-linking, which promotes the joining of two or more polymeric chains by covalent bonding [10].

Enzyme-mediated gelatin crosslinking, in which transglutaminase (TGase) can be used, can catalyze acyl group transfer reactions, forming intra and intermolecular cross-links in proteins, peptides, and various primary amines, mainly through covalent bonds between glutamine and lysine residues. This type of treatment, in addition to reducing the hydrophilic character of gelatin films, also changes the mechanical properties of the material [11;12].

In this context, the objective of the present work is to produce polymeric films using commercial gelatin and evaluate the effects of the addition of TGase on thickness, water vapor permeability, solubility in water, biodegradation, and mechanical properties of the film.

2. Materials And Methods

2.1 Materials

Commercial TGase (ACTIVA® YG - Brazil), commercial gelatin (Dr Oetker, SP), analytical grade glycerol (Cinética – Brazil), and chitosan (Sigma – Brazil) were employed for the elaboration of films.

2.2 Production of gelatin films

The production of the films was carried out in two stages, with two different experimental plans. The first consists of an experimental design aimed at identifying the effect of the concentration of gelatin, TGase enzyme, and the time of cross-linking with the enzyme on the solubility in water properties and mechanical tensile strength and elongation (Table 1).

The films were produced by solubilizing the gelatin in 100 mL of distilled water, with the addition of the plasticizer glycerol (20% w/v over the gelatin mass), with heating at 60°C, under stirring for 30 min. In parallel, the TGase enzyme was solubilized in water and added to the filmogenic dispersion. The temperature of the dispersion was lowered to 37°C, the enzyme was added and agitation was maintained for the pre-defined times (Table 1). Chitosan was added at the end to a concentration of 1% v/v. The dispersion (90 mL) was poured into Petri dishes (19.5 x 2.5 cm) and dried in an environment with controlled conditions (23 ± 2°C and 50% humidity) for 48 h. For the control sample, films were produced without adding the enzyme.

From the results obtained in the first experimental design, new experiments were carried out, according to Table 2, the objective, in this case, was to identify the best concentration of enzyme to be applied in the production of the films. The obtained films were characterized in terms of solubility in water, water vapor permeability, thickness, elongation, tensile strength, and degradation in simulated soil. Degradation was evaluated through thermal analysis (thermogravimetry), structural properties (Fourrier Transform Infrared Spectrometer - FTIR), and photographic records.

2.3 Films characterization

2.3.1 Thickness determination

Thickness of the films was determined using a digital micrometer with a resolution of 0.001 mm (Mitutoyo, Japan). Ten measurements were taken at points located the extremities and at the center of the films.

2.3.2 Mechanical properties determination

Tensile tests of films were performed according to [13] in a universal testing machine (Model DL 2000, Emic, Brazil) to determine tensile strength and percentage of elongation at break. Two samples of 20 x 100 mm were tested for each film produced.

2.3.3 Determining permeability in water vapor

Water vapor permeability (WVP) was determined according to [14] Desiccant Method. The samples were sealed to the dish (2.5 cm of mouth diameter) in such a manner that it was possible to prevent the passage of vapor into, out of, or around the specimen edges. 10 g of silica (4–8 mm) were placed inside the dish and the dish assembly was placed inside a chamber with controlled humidity (75%, controlled with sodium chloride saturated solution). The assembly was weighted every 1.5 h for 11.5 h. The ratio between weight increase, and the area of the sample through which water permeated, multiplied by the time of the test, resulted in the water vapor transmission (WVT). When WVT is divided by vapor pressure difference (vapor pressure inside the chamber/outside the dish assembly minus vapor pressure inside the dish assembly), the water vapor permeability (WVP) is found. All weight measurements were done in an analytical balance (AUY 220, Shimadzu, Japan).

2.3.4 Determination of solubility in water

Solubility in water of the material was determined according to the method described by [15] with some adaptations 2 x 2 cm square samples were oven dried (A35ED, DeLeo, Brazil) at 70°C for 24 h and then weighed in analytical balance (AUY220, Shimadzu, Japan) to determine initial mass. The dried sample was immersed in 50 mL of distilled water and kept under stirring for 24 h, at 80 rpm and 25°C in a thermostatic bath (501/1D, New Era, Brazil). After this period, the samples were dried in an oven for 24 h at 70°C and weighed to obtain the final mass. The difference between initial and final mass corresponded to the solubility in water of the sample (measured in percentage).

2.3.5 Thermogravimetry

Thermogravimetry of the films was performed in a simultaneous thermal analyzer (Jupiter 449, Netzsch, Germany). 10 mg of each sample (previously kept in a desiccator for 5 days) were heated under a nitrogen atmosphere (50 mL/min) from 20 to 700°C at a heating rate of 10°C.min^{-1} in an alumina crucible.

2.3.6 Determination of structural properties (FTIR)

The FTIR assays were performed on a Fourrier Transform Infrared Spectrometer (Spectrum 400 Model, Perkin Elmer) using attenuated total reflection (ATR) and a diamond crystal. 32 scans were performed with a resolution of 2 cm $^{-1}$ in the wavelength range from 450 to 4000 cm^{-1}.

2.3.7 Degradation in soil

Microbial susceptibility of the films was evaluated by laboratory soil burial according to [16]. The soil was used after a reduction in tensile strength of more than 50% was recorded for a cotton sample after 5 days of exposure.

2.2.9 Data treatment and statistical analysis

The significance of the factors tested was evaluated through analysis of variance (ANOVA) using the software Statistica 12 (StatSoft Inc.). A confidence interval of 95% was used, which means that p-Values under 0.05 indicate a significant factor.

3. Results And Discussion

3.1 Determination of enzyme action time and concentration of gelatin and enzyme

The results for the properties of solubility, elongation, and tensile strength for the films obtained according to planning 1, are referenced in Table 1.

Table 1
Solubility in water, elongation, and tensile strength for films produced with different concentrations of gelatin and enzyme and enzyme action time

Gelatin (%) *	Enzyme (%) **	Time (min)	Solubility in water (%)	Elongation (%)	Tensile strength (MPa)
2	0	15	34.59	4.37	58.98
2	2	15	28	4.61	97.70
2	0	30	56.36	5.84	49.64
2	2	30	29.10	4	53.41
4	1	22.5	34.78	10.09	63.42
6	0	15	32.35	11.03	38.95
6	2	15	26.88	7.09	31.82
6	0	30	36.33	8.23	35.01
6	2	30	23.32	9.79	33.17
*Gelatin amount on filmogenic solution (100mL)					
**Enzyme amount by mass of gelatin					

All analyzes were performed in duplicate

The effects presented on the ordinate axis whose values are to the right of the line (p-value = 0.05) had significant effects on the variables under study, with 95% confidence (Fig. 1).

From the results presented in Fig. 1, it can be seen that all the controllable factors studied had a significant effect on the solubility in water property. It is desired that the solubility is as low as possible, for which the highest concentrations of gelatin and enzyme should be used. For the time of action of the enzyme, we chose to use 15 min. The results obtained can be justified by the action of the TGase enzyme that acted by forming cross-links between the lysine and glutamine residues present in the protein, reducing the intermolecular and intramolecular spaces, consequently, decreasing their solubility [11; 17; 18].

The gelatin concentration showed different effects between the properties analyzed, and when included in greater services the solubility in water and the tensile strength decreased. This result can be explained by taking into account that these properties are directly associated with both the distribution and the density of intermolecular and intramolecular interactions of the materials that determine the blend [20]. Thus, the greater interaction between the materials allowed to obtain a film with greater structural stability, making them less soluble and more rigid, and this stiffness impacted the tension drop at the break. For the elongation property at rupture, there was no significant effect of any of the controllable factors tested. Based on this, it was decided to use 4% gelatin and 15 min action time for the sequence of the experiments, and to carry out new tests with larger amounts of enzyme.

3.1 Characterization of gelatin films with different concentrations of TGase enzyme

In this planning, the gelatin concentration was fixed at 4% w/v and the time of action of the enzyme at 15 min. Due to the enzyme's action on solubility, enzyme concentrations of 1 and 5% were tested to analyze whether the properties of the films would be improved.

3.1.1 Solubility in water, water vapor permeability, thickness, and mechanical properties

Table 2 shows the results of the analysis of properties for thickness, solubility in water, water vapor permeability, and mechanical properties, in addition to the analysis of variance for the respective properties for the films developed in step 2.

Table 2
– Solubility in water, water vapor permeability, thickness, elongation, and tensile strength of gelatin films with different concentrations of TGase enzyme

Samples	Solubility in water (%)	Water vapor permeability (g.mm/m2.d.kPa)	Thickness (mm)	Elongation at break (%)	Tensile strength (MPa)
Gel	35.37 ± 7.26	0.83 ± 0.04	0.118 ± 0.08	20.41 ± 0.84	65.50 ± 12.09
Gel/T1	34.71 ± 0.01	0.80 ± 0.04	0.129 ± 0.01	10.09 ± 0.91	63.42 ± 0.43
Gel/T5	26.66 ± 0.82	0.82 ± 0.03	0.130 ± 0.01	32.18 ± 0.81	50.65 ± 1.8
p-value**	8.59×10^{-6}	0.237726	1	0.267913	0.001527
Gelatin films without enzyme; Gel / T1: Gelatin films with 1% TGase; Gel / T5: Gelatin films with 5% TGase.					
*Enzyme amount by mass of gelatin					
** p-value < 0.05 means that the effect of adding TGase was significant in the response variable.					

From the results shown in Table 2, the effect of the enzyme was not significant for the properties of water vapor permeability, thickness, and elongation at break.

The addition of different concentrations of the enzyme had a significant effect on solubility in water, and the greater the amount of enzyme used, the lower the solubility of the film, which corroborates the result of the previous one, where the addition of 1% (w / w of gelatin) of the enzyme had already reduced this property. The increase in TGase concentration formed a compact spatial network structure, by reducing the number of free amides and other hydrophilic groups, decreasing the binding capacity of gelatin to water, reducing the solubility of the films [11;20]. Thus, enzymatic treatment is seen as an effective approach to reduce the susceptibility of films to humidity [21]. The decrease in solubility in water can be an interesting indication of the greater stability of these materials to different environmental conditions, such as the application as mulching in agriculture.

With the increase in the concentration of the enzyme, the cross-links caused a reduction in the intermolecular space, making the blend more rigid / compact, causing a drop in tensile strength [19; 22].

3.1.2 Degradation on soil

It was not possible to determine the loss of mass of the films, as the soil adhered to the material in the first hours of exposure and, consequently, the washing step for subsequent weighing was not performed, but it was observed that the films were degraded after 24 hours of exposure to the soil, under the conditions specified in the standard [16].

Thus, through analysis, it can be said that the films produced are biodegradable.

The high biodegradability of the films is associated with the hygroscopic characteristics of gelatin, which easily absorbs humidity present in the soil, and from then on allows changes in the microstructure of the films through the activity of microorganisms, which alter the characteristics of the film (decrease the molecular weight and increases the degree of crystallinity), indicating the biodegradation of the polymer and metabolic activity of microorganisms [23;24].

Rapid degradation is a factor of great importance in the study of biodegradable polymers. These materials must be able to decompose completely, preferably before the next growing season [25]. It is suggested to apply the film as mulching for planting short cycle vegetables, such as arugula and lettuce, as the improvement in solubility properties may be related to greater resistance to humid environments. This suggestion corroborates the study by [22], where films made of commercial gelatin and TGase enzyme showed improvements in the interaction properties with water, and when subjected to accelerated aging in a controlled humidity environment, they presented better mechanical, thermal, and structural stability than when compared to another simulated aging in the study, such as exposure to ultraviolet light and thermo-oxidation. Reticulated films showed better interaction properties with water than non-reticulated films, which will allow them to better resist application conditions when used in high humidity situations, such as in agriculture [26]. Also, at the end of cultivation, the rapid degradation of the material allows it to return to the cycle as a source of humus. However, more research is mandatory in the development of composites based on compounds and/or additives that change and improvements the polymer matrix to allow its effective application [27].

Figure 2 shows the result of the thermogravimetric analysis for the films produced with the highest concentration of TGase (5% w / w) after the periods of 1, 2, and 8 h of exposure to the simulated soil. It was not possible to perform thermogravimetric tests for films produced without TGase or with 1%, as the soil adhered to the film.

The first loss of mass occurs at a temperature close to 100°C is related to the loss of low molecular weight compounds, such as water, glycerol, and oligomers [11; 28]. While the second, at approximately 220°C, is attributed to organic degradation and peptide bonds in the main gelatin chain. Degradation occurs by breaking covalent bonds (primary structure) or by chemical hydrolysis, which causes water molecules to enter between proteins, breaking down the protein structure. Therefore, there is a gradual unfolding of the protein molecule into peptones, polypeptides, and amino acids. These products continue to degrade, generating CO_2, NH_3, and organic acids [28;29].

After 1 h in simulated soil, the first loss of mass equal to 10%, the second loss of mass equal to 46%, and the third loss of mass equal to 23%. After 2 h in simulated soil, these mass losses were 11%, 47%, and 18%, respectively. Finally, after 8 h, the mass loss values were 9%, 29%, and 15%, respectively. Residual mass of 25% was observed after 1 h of exposure, 27% after 2 h of exposure, and 46% after 8 h of exposure. After 8 h in simulated soil, there is an increase in residual mass. This increase is related to the biodegradation process, as microorganisms break down organic matter and consume it. The same is not true of inorganic matter. Therefore, in percentage, the organic matter content increases due to the degradation of the organic matter, the ash content, and the residual mass of the films increases [30].

Figure 3 shows the FTIR spectra for gelatin films with and without added enzyme (1 and 5% w/w) after their exposure to the simulated soil for 1 and 3 hours.

According to the FTIR graphs, it is possible to observe the degradation of the films due to the reduction and displacement of the bands after 3 h of exposure to the ground.

The presence of gelatin is characterized by the vibration bands of the amide bonds, the Amida I and Amida II vibrations. The amide I vibration bands correspond to the elongation of the C = O (carbonyl) bond, while the amide II vibration bands correspond to the deformation or flexion of the N-H bond. The carbonyl and amide groups participate in hydrogen bonds responsible for maintaining the secondary structure of the protein. The band position of Amida II and I can be changed due to changes in the molecular structure of the protein. The O-H and C-H bond is common for gelatin [20;31].

The bands at 1650 cm^{-1}, 1545 cm^{-1}, and 1450 cm^{-1} correspond to the asymmetric angular deformation of the NH connection, the symmetrical angular deformation of the NH connection, and the asymmetric elongation of the C = O connection, respectively [32]. Typical amine gelatin bands and amide groups (secondary and tertiary) are present in the range from 1235 to 1541 cm^{-1}. Also important is the absorption band at 1541 cm^{-1}, often used to identify proteins [33].

The band around 3276–3292 cm^{-1} may be due to the OH groups of glycerol and OH groups of water in the films, which are hygroscopic. After 3 h of exposure, the film can be seen in simulated soil the film without enzymatic treatment. For the film with 1% and 3% TGase, it moved from 3,282 to 3,276 cm^{-1} and from 3,282 to 3,285 cm^{-1}, respectively, after 3 h on simulated soil (smaller displacements). These results indicate that TGase promoted the formation of hydrogen bonds between the groups -OH and –NH, demonstrating changes in the molecular level with the addition of TGase [11].

The band identified at 3,056 cm^{-1} for the film without enzyme moved to 2,927 cm^{-1} and 2,919 cm^{-1} after 1 and 3 h in the simulated soil, respectively. These bands are characteristic of the O-H bond of the carboxylic group present in the amino acids proline, hydroxyproline, and lysine, all of the gelatinous structure [34].

4. Conclusions

From the results presented in this work, it is possible to conclude that the films had their properties improved by the addition of TGase, which acted on the films, mainly, reducing the solubility in water. The degradation test in simulated soil proved that the films are biodegradable and that the addition of the TGase enzyme promoted the cross-linking of the polymer matrix, maintaining the integrity of the films for a longer time, thus improving their application.

Declarations

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Figures

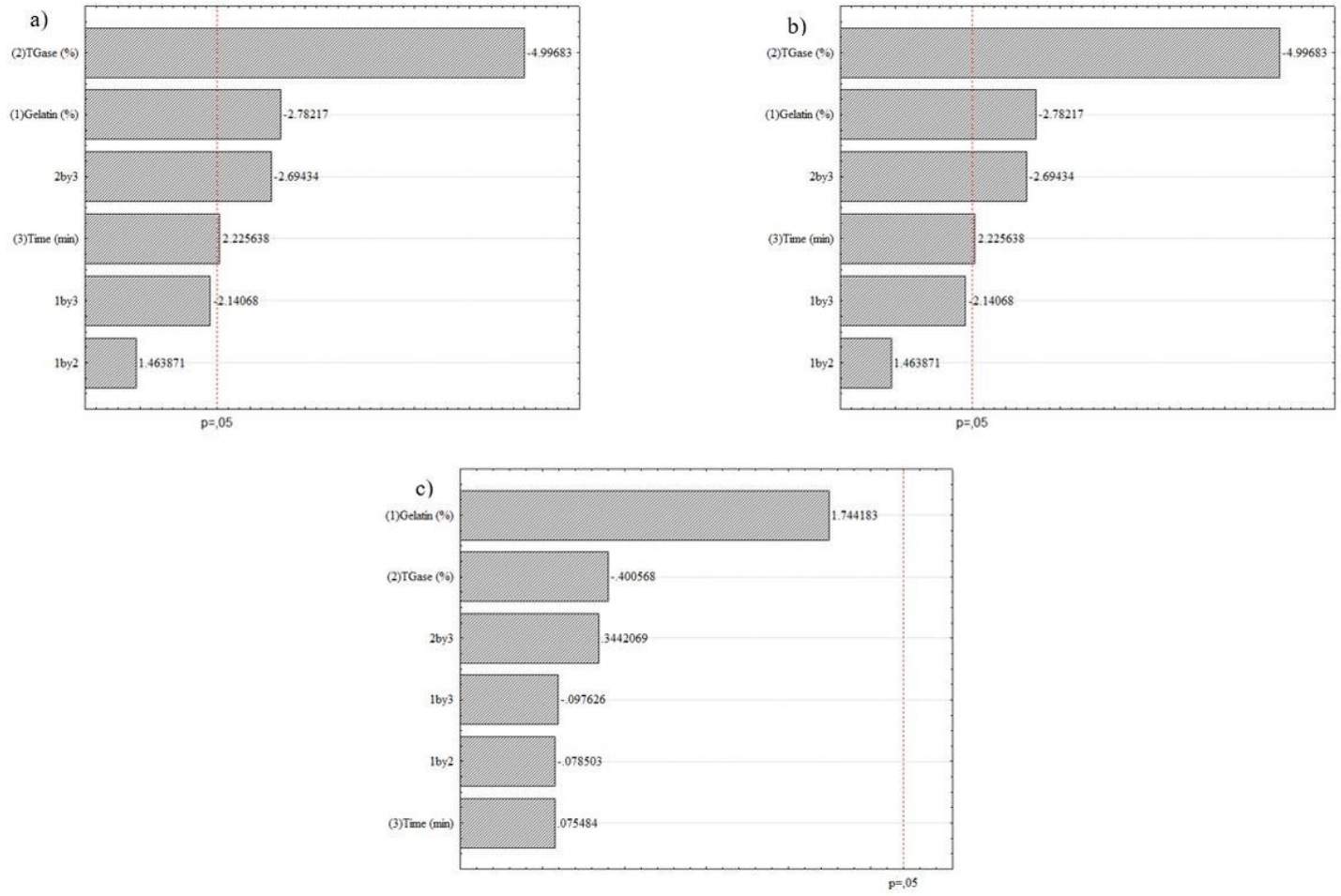

Figure 1

Pareto graph for water solubility (a), tensile strength (b), and elongation (c) of gelatin films with different enzyme concentrations

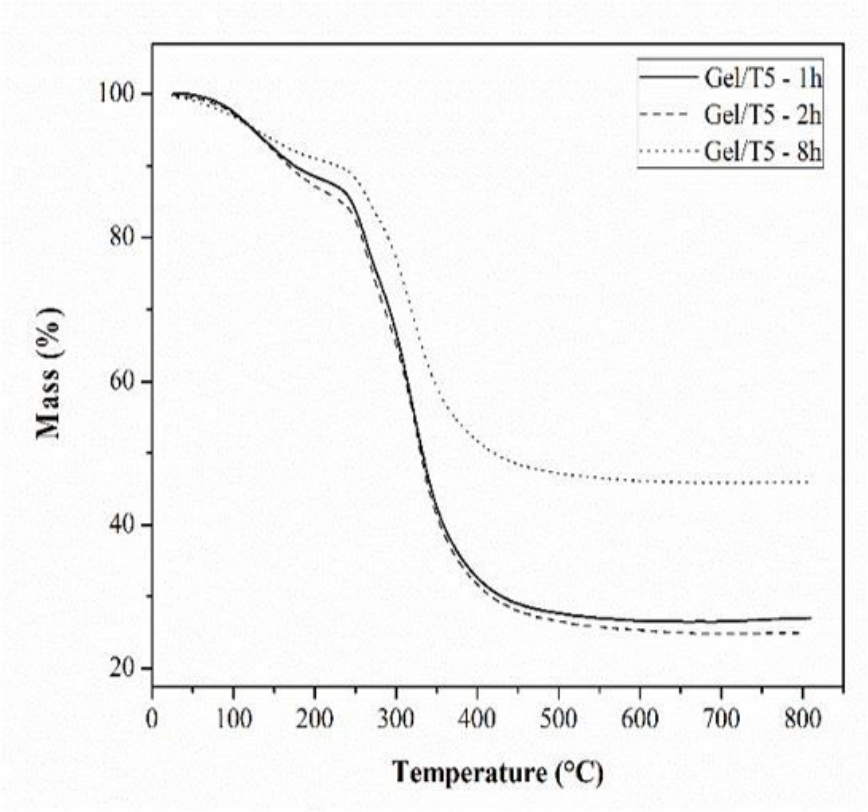

Figure 2

TG curve for commercial gelatin films with 5% TGase after 1, 2 and 8 h of exposure to simulated soil

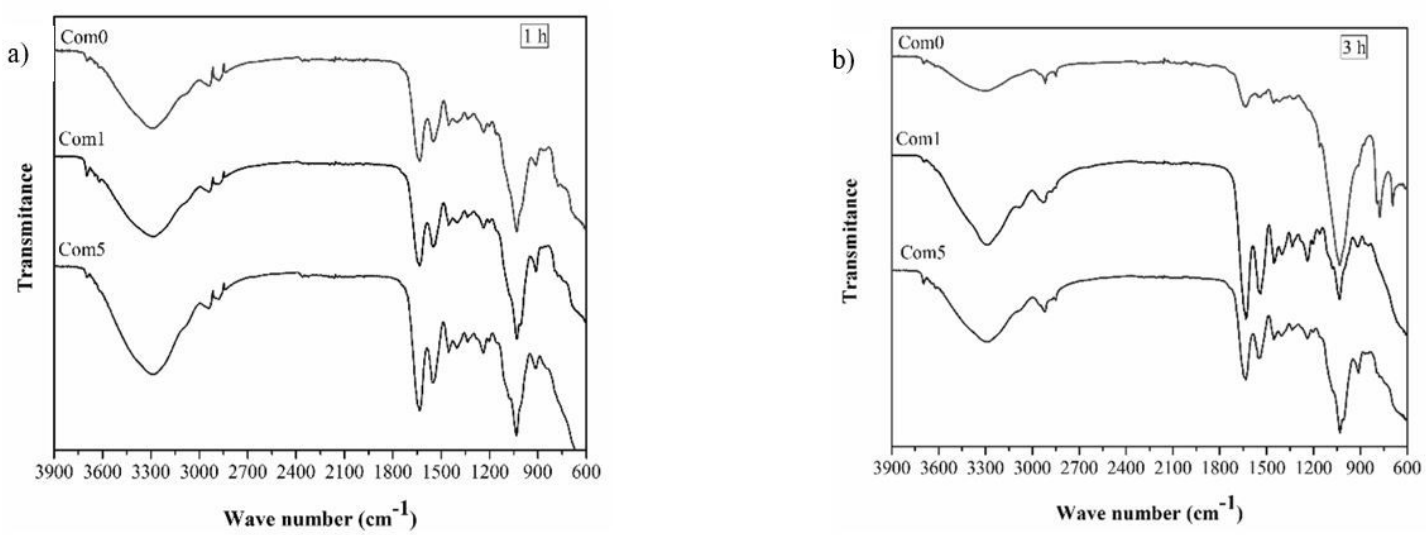

Figure 3

FTIR spectra for films produced with commercial gelatin (Gel: Gelatin films without enzyme; Gel / T1: Gelatin films with 1% TGase; Gel / T5: Gelatin films with 5% TGase) after 1 and 3 h of exposure simulated in soil

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.jpg
- GraphicalAbstract.png