J. Mater. Environ. Sci. 6 (9) (2015) 2542-2549
ISSN : 2028-2508
CODEN: JMESCN

Assessment of biodegradation of PLA/PCL and PLA/PEG biopolymers under aerobic and anaerobic conditions

T. Kossentini Kallel[1], H.Houichi[2], A. Sayari[3] and M. Kammoun[4]

[1] *Laboratoire de Chimie Industrielle, National School of Engineering BP1173-3038 Sfax, University of Sfax, Tunisia.*
[2]*Laboratoire d'Ingénierie des Matériaux Polymères (IMP - UMR CNRS 5223): - 69 622 Villeurbanne Cedex Université de Lyon 1, France*
[3] *Départment de Génie Biologique, National School of Engineering of Sfax BP1173-3038, University of Sfax, Tunisia.*
[4]*Laboratoire de Genie Enzymatique et de Microbiologie, National School of Engineering, BP1173-3038, University of Sfax, Tunisia.*

Received 04 Jun 2015, Revised 05 Sep 2015, Accepted 09 Sep 2015
**Corresponding author: tasnimkallel@yahoo.fr ; Tel: (+21697776922)*

Abstract
This paper concentrates on the biodegradation capability of poly (lactic acid) blends with poly(caprolactone) and polyethylene glycol in the solid state and in the liquid phase under aerobic and anaerobic conditions. To this end, blends were processed on a twin-screw extruder with a film die and to determine the efficiency of the biodegradation of polymers, quantitative (mass variations, BOD) and qualitative (DSC and SEM) analyses were made. All biopolymers were degraded in the liquid phase under aerobic conditions and the addition of 5 or 10 wt % PEG mainly increased their biodegradability with values of BOD_{15} about 38 and 52 mg of O_2/mg CO and a weight loss of 0.65 and 1.23 wt %, respectively. Similarly, in anaerobic conditions, biopolymers with 10 wt % PEG displayed the best outcome in terms of biodegradability and the weight loss was around 0.68 wt %. DSC and SEM analyses, on the other hand, showed that PLA/PEG blends were more biodegraded by microorganisms than PLA and PLA/PCL blends. Otherwise, various fungi grown on solid state and using different biopolymers (for their growth) as sole carbon source, were isolated and screened. Observation under UV light, on the other hand, demonstrated the presence of lipase activity.

Keywords: PLA, PCL, biopolymer blends, plasticizer, biodegradation

Introduction
There has been a growing interest over the past few years in the development of biopolymers partly because of their renewable, sustainable and biodegradable properties [1-5]. As a good example of such biopolymers, Poly (lactic acid) (PLA) has attracted much attention. It is thermoplastic polyester that can be produced from annually renewable resources [6-7]. It is also one of the typical biodegradable and compostable thermoplastic polymers with good optical properties, high strength and high modulus, analogous to petrolium-based polymers like polyethylene terephtalate (PET) and polypropylene (PP) in packaging applications [8-10]. These advantages, in parallel with the recent technological developments in PLA production, have rapidly expanded its applications as a competitive commodity polymer in a variety of processes.
However, because of its inherent brittle nature and low thermal stability, PLA needs to be modified to be suitable for use in various applications where mechanical properties are important [11-14]. There have been a considerable number of studies to toughen PLA with the goal of balancing and increasing tensile strength and modulus and impact strength while retaining the biocompatible and biodegradable nature of the polymer [15-16]. Several attempts to toughen PLA have included blending with other flexible polymers, such as poly (butylene succinate) (PBS), poly (3-hydroxybutyrate) (PHB), and polycaprolactone (PCL) [17]. PLA/PCL blends, for instance, offer an interesting characteristic because of their wide range of physical properties and biodegradability [18-21]. Actually, the glassy PLA with high degradation rate in such blends shows better tensile strength, while the rubbery PCL with much slower degradation rate shows better toughness. The complementarity between these two biocompatible polymers is very important to their blend materials because

the performance of the blend can be controlled by adjusting the blending ratio and the preparation conditions to meet various applications. Polyethylene glycol (PEG) is also an attractive polymer for biomedical applications because of its biocompatible nature. It has been found that PEG shows great promise as a plasticizing agent for PLA as it gives a large increase in ultimate elongation with the smallest decrease in tensile strength [22, 23].
The objective of the present work is to analyze the effect of PCL and PEG on the biodegradation properties of PLA and subsequently to improve the processability of this polymer in order to avoid its thermal degradation. This paper sheds light on the results of the aerobic and anaerobic biodegradation behavior of PLA, PLA/PCL and PLA/PEG blends in the solid state under aerobic conditions and in the liquid phase under both aerobic and anaerobic conditions. Otherwise, various fungi grown on solid state and using different biopolymers (for their growth) as sole carbon source were isolated and screened.

2. Materials and methods

2.1. Materials

PLA 2002D – with a molecular weight (Mw) of approximately 200000 g/mol and a melt index of about 4-8 g/10 min – was supplied by Cargill Dow LLC. The melting temperature is 143 °C and the glass transition temperature is about 58 °C. The enantiomeric form of the PLA contains 4,15 % D units. Poly ε-caprolacton (PCL), with a molecular weight (Mw) of about 50000 g/mol, was obtained from CAPA/SOLVAY (UK). The melting temperature is 60°C and the glass transition temperature is about -61°C. And polyethylene glycol (PEG) was purchased from SIGMA-ALDRICH. It has a molecular weight (Mw) of approximately 8000 g/mol and its melting temperature is 62 °C.

2.2. Film preparation

The polymers were dried for 10 hours at 40 °C in a vacuum oven before processing to remove eventual moisture. Then, they were extruded on a twin-screw extruder PTW 16/25 D (Thermo electron polylab system rheocord RC400p) with a film die, and then drawn by chilled rolls turning at variable speeds. The processing conditions were: die at 180 °C and 20 rpm. After storage for one month, the films obtained were carefully dried under vacuum at 40 °C for 10 hours to be analysed thereafter.
Several PLA/PCL blends with various percentages (100 % PLA= PLA0; 95/5= PCL5 and 90/10= PCL10) were prepared in addition to PLA/PEG blends (95/5= PEG5 and 90/10= PEG10).

2.3. Biodegradation study

At this stage, the objective of the project was to study the biodegradation of the different biopolymers, with the help of the microorganisms. To this end, three experiments were carried out in normalized environments.
For each test, several examples of the same polymer were tested (either three or five times) in order to improve the precision and the reliability of the results.
For each test, blank tests with microbial inoculums and without samples were also performed to check the activity of micro-organisms. The outcome was subtracted from the measured values to obtain exact assessment of the degradation activity. Thus, biodegradation studies were performed in the presence of micro-organisms.

2.3.a. Study of the aerobic degradation in liquid medium

The study of the biopolymers' biodegradation in the presence of oxygen was followed by measuring the biological oxygen demand for five days (BOD5) in a closed respirometer according to the standard ISO 14851 procedure [24] by using an electronic system of measurement (Oxi-Top : BSB-control Models 620T). Moreover, the aerobic degradation was evaluated by measuring the weight loss of biopolymers.
The pre-weight test material were therefore incubated in 500 ml bottles hermetically closed in the presence of 1,5 ml of microbial inoculums (extracted from an activated sludge from a municipal wastewater treatment plant) and 90 ml of mineral medium M1 whose composition is as follows: (g/l) 40 ml of solution A (KH_2PO_4:28.25; K_2HPO_4:146.08); 30 ml of solution B ($CaCl_2.2H_2O$:3.66; NH_4Cl:28.64); 30 ml of solution C ($MgSO_4.7H_2O$:3.6; $FeSO_4.7H_2O$: 0.7; $ZnSO_4$: 0.4) and 900 ml of distilled water. Biopolymers were used as sole carbon source. The pH of the medium was adjusted to 7, regularly stirred and placed in darkness.
The value of BOD_5 was measured once every 5 days during 15 days of incubation at 20 °C.

2.3.b. Study of the anaerobic degradation in liquid medium

The biodegradation of biopolymers under anaerobic conditions and according to the standard ISO 14853 [25] procedure, was monitored by measuring the weight loss.

The anaerobic medium was prepared by using the technique initiated by Hungate (1969) and developed by Miller and Wolin (1974). The previously weighed biopolymer samples were stored in 125 ml flasks in the presence of the mineral medium M2 whose composition is as follows: KH_2PO_4: 0,27 g/l ; $NaHPO_4.12H_2O$: 1,12 g/l ; NH_4Cl : 0,53 g/l ; $CaCl_2.2H_2O$: 0,075 g/l ; $MgCl_2.6H_2O$: 0,1g /l ; pH 7 [25]. Biopolymers were used as sole carbon source.
The test was prepared in an oxygen-free atmosphere (anaerobic conditions) under controlled nitrogen atmosphere. Samples were tested in five examples. They were placed to incubate at 37 °C during 35 days in darkness and were regularly stirred. A negative control was incubated without biopolymers in the same conditions.

2.3.c. Biodegradation study in solid medium

The degradation study of biopolymers in solid medium was performed according to the conditions specified in ASTM G21-09 [26] and ASTM G22-76 [27].
M3 medium was prepared according to the instructions described in ASTM G22-76 [27] whose composition is as follows: (g/l) KH_2PO_4, 0.7; K_2HPO_4, 0.7; $MgSO_4.7H_2O$, 0.7; NH_4NO_3, 1; NaCl, 0.005; $FeSO_4.7H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; $MnSO_4.7H_2O$, 0.001; Agar agar, 15. The pH was adjusted to 6.5, and the medium was in its turn autoclaved at 121°C for 20 min.
Briefly, the Biopolymers (4 cm^2) immersed in a suspension of compost (1g soil in 10 ml of M3 medium) were fixed onto agar medium surface and incubated in darkness and regularly stirred at 30 °C for 45 days. Biopolymers were used as sole carbon source.
To determine the efficiency of the biodegradation of polymers, quantitative (mass variations, BOD) and qualitative (DSC and SEM studies) analyses were conducted. Otherwise, various fungi grown on solid state which used different biopolymers (as sole carbon source in order to ensure their growth) were isolated and purified.

2.4. Polymer characterization

2.4.a. Differential scanning calorimetry

A Mettler differential scanning calorimetry apparatus (DSC) TC10A TC1 was used for calorimetric investigations. About 10 mg of each sample was placed in an aluminum pan and heated at a rate of 5 °C/min. All DSC measurements were carried out under a nitrogen atmosphere at a flow rate of 50 ml/min.

2.4.b. SEM analysis

The biopolymers were directly removed from the medium and washed with distilled water. The samples were sonicated with a system equipped with a probe of 6 mm diameter (130-W series autotune High intensity ultrasonic sonicator) to remove the microorganisms trapped in the pores of the polymer and were then dried at 60 °C until they had a constant weight. The morphological changes brought to the surface of biopolymers were observed with a scanning electron microscope (Philips SEM 500) after gold coating.

2.5. Isolation of lipase fungi

The isolation was performed on an appropriate medium (malt extract, 20 g/l; agar, 15 g/l ampicillin 100 mg/ml; pH 5.5). The dishes were incubated at 30 °C for 3 to 5 days. In acidic medium, malt extract which was rich in carbohydrates provided all the nutrients needed for the metabolism of yeasts and molds. In addition, the acidity of the medium and ampicillin inhibited the growth of most contaminating microbes. Successive subcultures allowed the purification of the isolated strains.
In order to select the lipase-producing fungi, a surface inoculation was carried out on the isolation medium (malt extract, 20 g/l; agar, 15 g/l; ampicillin 100 mg/ml; pH 5,5) containing 1 ‰ Rhodamine-β and 1‰ olive oil [28]. The inoculated dishes were incubated at 30 °C for 3 to 5 days and the presence of a lipase activity manifested itself in the presence of fluorescent halo on UV.

2.6. Statistical analysis

All experiments were performed using at least three samples. The results are the averages and standard deviations that were calculated from these replicate measurements. Statistical analyses were conducted using Microsoft Excel Software. The significance level used was $P<0.05$.

3. Results and discussion

3.1.Biodegradation study

3.1.a. Aerobic biodegradation in liquid medium

The efficiency of the biodegradation of biopolymer films in aerobic liquid medium was detected by measuring the biological oxygen demand (BOD) and their mass variations before and after incubation.

After 15 days of incubation, the BOD values were measured and reported in Figure 1. Results show that, whatever the biopolymer used, there is a consumption of O_2, thus confirming the multiplication and growth of aerobic bacteria and their ability to assimilate biopolymers as only carbon source.

Moreover, it should be noted that after 10 days, consumption of O_2 was low and similar for all biopolymers (around 1 mg O_2/mg CO). This period of time could represent a period of adaptation in which, microorganisms assimilate biopolymers as carbon source by secreting the necessary enzymes. Then, the results show that the BOD values of PEG5 and PEG10 exponentially increased compared to other biopolymers.

On the other hand, weight loss values of all biopolymers were measured and presented in Figure 2. Results clearly show a decrease in weight of biopolymers, mainly for PEG10. These observations were in concordance with those obtained by the measurement of the BOD values, which indicates that PEG5 and PEG10 biopolymers were more hydrolyzed and assimilated by microorganisms than PLA0. Similar results were obtained by Stefani et al. [29] and Kammoun et al. [3] who demonstrated that the addition of PEG plasticizer increased the biodegradability of biopolymers. Otherwise, Figures 1 and 2 show that PCL blends have a slower degradation rate than PLA.

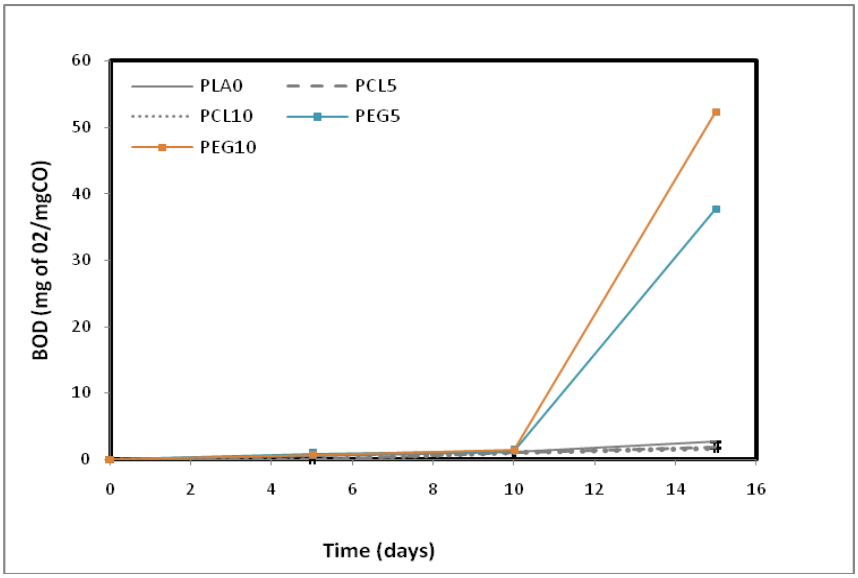

Figure 1: Biological oxygen demand (BOD) measurements (mg O_2/mg CO): (A) PLA/PCL blends (100 % PLA = PLA0; 95/5 = PCL5 and 90/10 = PCL10) and (B) PLA/PEG blends (100 % PLA = PLA0; 95/5 = PEG5 and 90/10 = PEG10).

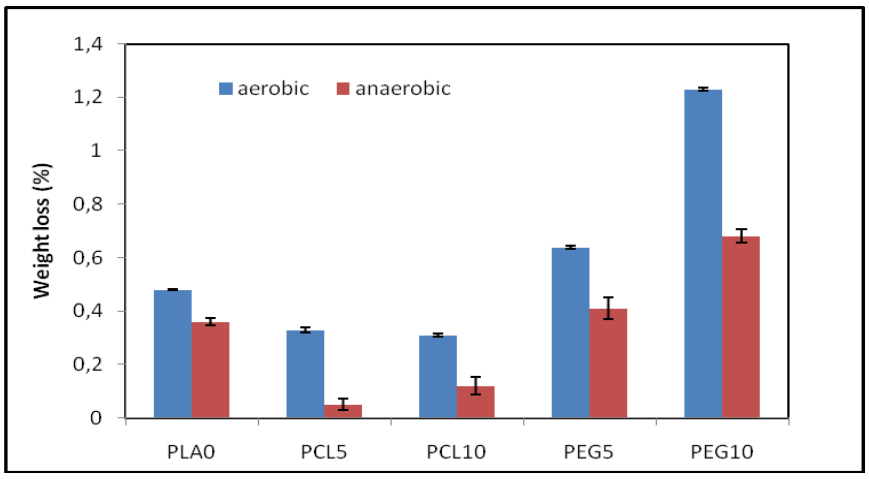

Figure 2: Percentages of weight loss of biopolymers under aerobic and anaerobic biodegradation.

3.1.b. Anaerobic degradation in liquid medium

The efficiency of anaerobic degradation in liquid medium was measured by the rate of weight loss.

Figure 2 illustrates the rate of weight loss (after incubation in anaerobic liquid medium for 35 days at 37 °C) in the histograms below. Actually, they reveal that there is an improvement in the degradation process as the PCL and PEG rate in the blends increases.

3.1.c. Hydrolysis in solid medium

Hydrolysis in solid medium was performed on M3 mineral medium in the presence of soil microorganisms for 45 days at 30 ° C under aerobic condition. The microorganisms that grew on the media containing the various samples were capable of degrading and using the biopolymers as sole carbon source (Figure 3).

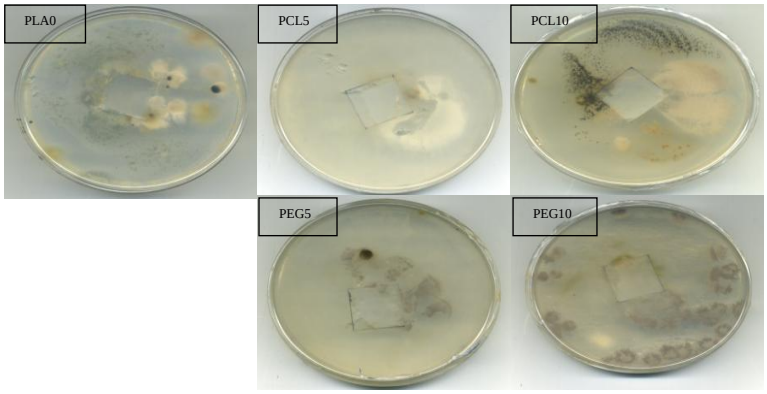

Figure: 3 Macroscopic evolution of the different biopolymers after 45 days incubation at 30 °C in solid medium.

The test was performed on solid medium in order to isolate microorganisms having a high lipase activity and to characterize biopolymers after incubation by SEM and DSC.

After incubation, it was observed that biopolymers exhibited changes in color and physical properties by becoming whitish and more rigid.

3.2. Polymer characterization:

3.2.a. Thermal analysis

The thermal behavior of biopolymers before and after incubation in a solid medium was determined by using the DSC analysis (Figure 4).

Figure 4 shows that before biodegradation, neat PLA (Figure 4A) exhibits an exothermic broad peak of cold crystallization Tcc centered at 107 °C. This peak decreased significantly with PCL and PEG addition and the Tcc of PLA/PCL 95/5 and PLA/PEG 95/5 blends were lower than of PLA by about 10 °C (Fig. 4B and 4C respectively). This result is considered to be directly related to the motion ability of the PLA chains. Indeed, the increase of PLA chain motion due to the plasticization effect of PEG and PCL addition allows an easier rearrangement of polyester chains to crystallize at lower temperature [17, 22, 30]. Moreover, the thermograms of PLA blends show that the crystallization is not homogeneous as evidenced by the presence of two melting peaks (Figure 4B and C).

After biodegradation for 45 days, the cold crystallization peak disappeared when it came to PLA (Figure 4A) and PLA / PEG biopolymers (Figure 4C). Yet, it went down to very lower level when it came to PLA/PCL blend (Figure 4B). Actually, the degradation of biopolymers by microorganisms led to less interaction between the molecular chains. It followed that the crystallinity of biopolymers was reduced [31]. The aforementioned factors led to the ~~diminution~~ decrease of the temperature and enthalpy of fusion after biodegradation for PLA/PEG blend [30], whereas an increase of these melting characteristics was observed in the case of PLA and PLA/PCL blend which could means that amorphous parts were preferentially degraded [6, 32]. These results are consistent with those observed in Figure 1 and 2 which indicates that biodegradation of PLA was highly increased by PEG addition.

3.2.b. SEM analysis:

The SEM photomicrographs highlight the surface texture of the different biopolymer films before and after incubation for 45 days on solid mineral medium (Figure 5).

Before incubation (Figure 5A_1, B_1 and C_1), the surfaces of different biopolymers were smooth except for some aliasing due to extrusion.

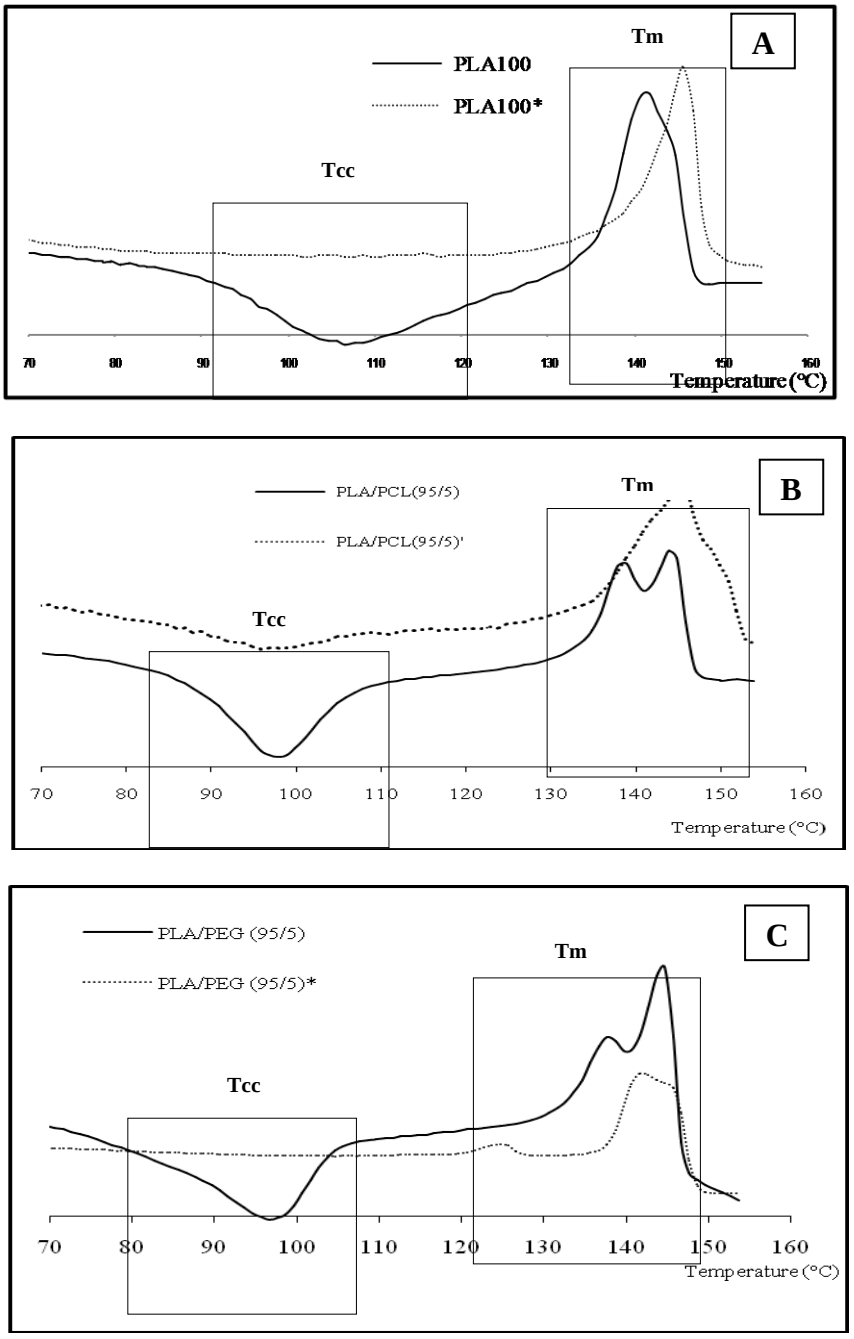

Figure 4: DSC thermograms of biopolymers before and after aerobic biodegradation. (A): PLA; (B): PCL5; (C): PEG5. * : refers to samples after biodegradation. Tm: melting temperature; Tcc: cold crystallization temperature.

After biodegradation (Figure 5 A_2, B_2 and C_2), the surfaces became rougher. The intensity of these changes depended on the blend composition and the types of microorganisms that insured biodegradation.
SEM observation showed that after degradation, there were many pinholes on the film surface of PLA, and partial degradation at eroded holes (Figure 5 A_2). These effects were prompted by the activity of microbial enzymes, thus indicating that microorganisms used PLA as sole carbon source for their growth [15, 32, 33]. As for PLA/PCL (95/5) blend, SEM observation showed that the surface smoothness disappeared after partial degradation (Figure 5 B_2). Concerning PLA / PEG (95/5) blend, (Figure 5 C_2) reveals that the film surface was totally eroded and its smoothness disappeared after partial degradation. This confirms that the addition of PEG facilitated the biodegradation of PLA whereas PCL has an adverse effect as previously presented in Figure 1 and 2.

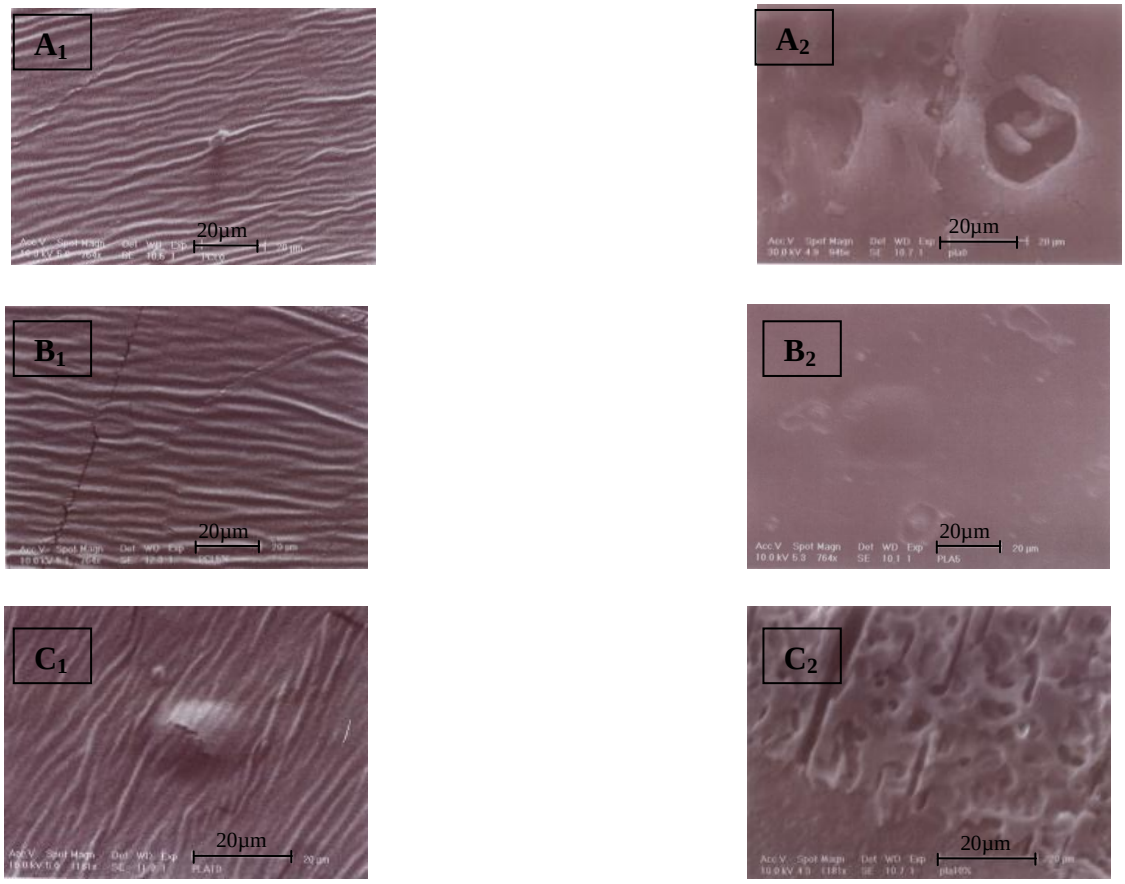

Figure 5: Morphologies of biopolymers observations by SEM before (A_1): PLA; (B_1): PCL5; (C_1): PEG5 and after (A_2): PLA; (B_2): PCL5; (C_2): PEG5 aerobic biodegradation.

3.3. Isolation of lipase fungi

During degradation on solid medium, fungi (which are responsible for the hydrolysis of biopolymers) were isolated and screened. Because of the presence of hydrolysable ester function in the structure of biopolymers (PLA0, PLA/PEG and PLA/PCL), we tested the ability of these microorganisms to secrete lipases activities. For this reason, surface inoculation was carried out on the isolation medium in the presence of Rhodamine-β and olive oil [28]. In doing so, the plates were observed under UV light.

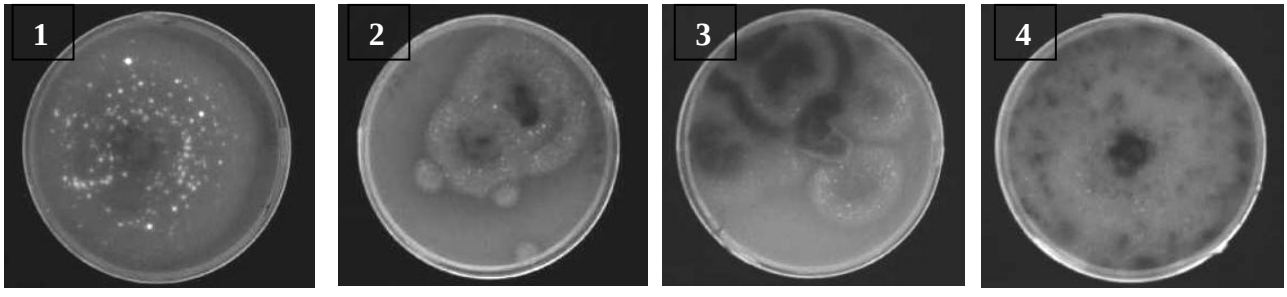

Figure 6: UV revelation of lipase activity of isolated fungi.

Results show that only 4 fungi possess and secrete lipase activity (Figure 6). Strain 1 displays strong fluorescence emission intensity which attests to the presence of lipase activity. Strains 2, 3 and 4 have lower fluorescence emission intensity than that observed in strain 1. The Fluorescence emission of Rhodamine-β confirms that the strains (which ensured the biodegradation of the different samples of biopolymers) produced lipases and this in turn confirms the hydrolysis of the ester linkages which constitute the biopolymer [34].

Conclusion

In this work, we have evaluated the effects of PCL and PEG addition on the biodegradation behavior of PLA biopolymers. The analysis of the biodegradation of biopolymers in liquid medium reveals that PLA/PEG blends were better hydrolyzed by microorganisms than PLA wheareas PLA/PCL blends have a much slower degradation rate than PLA. DSC analysis shows that the temperature and enthalpy of fusion of PLA/PEG blends were reduced after biodegradation in solid medium under aerobic conditions, which proves that microorganisms attacked even the crystalline phase. Moreover, SEM analysis of PLA/PEG (95/5) blend reveals that after degradation in solid medium the surface of biopolymers was totally eroded, which also confirms that the addition of PEG facilitated the biodegradation of PLA. Otherwise, The different fungi which used biopolymers as sole carbon source (and which grew on solid media) were isolated and purified and some of them demonstrated their ability to secrete a lipase activity under UV light Observation. We are currently working on the identification of isolated fungi and the biochemical and molecular characterization of produced lipases.

Acknowledgments- We wish to thank Mr Wassim Kammoun (Faculty of Letters and Humanities of Sfax, Tunisia) for proofreading this paper and giving feedback as to the use of the English language.

References

1. G.Swift, Degradable polymers (2nd ed.). In G. Scott (Ed.), Netherlands, 2002.
2. Yu L., Dean K., Li L., *Prog. Polym. Sci.* 31 (2006) 576.
3. Kammoun M., Haddar M., Kossentini. Kallel T., Dammak M., Sayari A., *Int.J.Biol.Macromol*, 62 (2013) 433.
4. Xu J., Guo B-H., *Biotechnol.J*, 5 (2010)1149.
5. Rajan A., Prasad V.S., Abraham E., *Int.J. Biol. Macromol*. 39 (2006) 265.
6. Massardier-Nageotte V., Pestre C., Cruard-Pradet T., Bayard R., *Polymer. Degrada. Stab*. 91 (2006) 620.
7. Torres A., Li S., Roussos S., Vert M., *J. Appl. Polym. Sci*. 62 (1996) 2295.
8. Yang J., Qin Y., Yuan M., Xue J., Cao J., *Int.J. Biol. Macromol*. 62 (2013) 411.
9. Li J., Kong M., Cheng X.J., Li J.J, Liu W.F, Chen X.G., *Int.J. Biol. Macromol*. 49 (2011) 1016.
10. Cadar O., Paul M., Roman C., Miclean M., Majdik C., *Polymer. Degrada. Stab*. 97 (2012) 354.
11. Chen C-C., Chueh J-Y, Tseng H., Huang H-M., Lee S-Y., *Biomaterials*. 24 (2003)1167.
12. Hashima K., Nishitsuji S., Inoue T., *Polymer*. 51 (2010) 3934.
13. Rathi S., Chen X., Coughlin E., Hsu S., Golub C., Tzivanis M., *Polymer*. 52(2011) 4184.
14. Rasal R-M., Janorkar A-V., Hirt D-E., *Prog Polym Sci*. 35 (2010) 338.
15. Weng X-Y, Yang L., Zhang M., Wang X-L, Wang Y-Z, *Polym Test*. 32 (2013) 60.
16. Soares F., Yamashita F., Muller C, Pires A., *Polym Test*. 32 (2013) 94.
17. Wu D., Lin D., Zhang J., Zhou W., Zhang M., Zhang Y., *Macromol Chem Phys.* 212 (2011) 613.
18. Wu D., Zhang Y., Yuan Y., Zhang M., Zhou W., *J Polym Sci Pol Phys.* 48(2010)756.
19. Tsuji H., Ishizaka T., *Int. J. Biolo. Macromol*. 29 (2001) 83.
20. Liu L., Li S., Garreau H., Vert M., *Biomacromolecules*. 1 (2000) 350.
21. Gan Z., Yu D., Zhong Z., Liang Q., Jing X., *Polymer*. 40 (1999) 2859.
22. Ronneberger B., Kao W.J., Anderson J.M., Kissel T., *J. Biomed. Mater. Res*. 30 (1996) 31.
23. Bittner B., Witt C., Mader K., Kissel T., *J. Controlled Release*. 60 (1999) 297.
24. I.T. 61/SC 5/WG 22, ISO 14851:1999, Determination of the ultimate aerobicbiodegradability of plastic materials in an aqueous medium -- Method by measuring the oxygen demand in a closed respirometer, Multiple. Distributed through American National Standards Institute (ANSI), 2007.
25. International Standardisation Organisation. Determination of the ultimate anaerobic biodegradability of plastic materials in an aqueous system e method by measurement of biogas production. ISO 14853 (1999).
26. G03 Committee, Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi, ASTM International, 2009.
27. G03 Committee, Practice for Determining Resistance of Plastics to Bacteria, ASTM International, 1996.
28. Kouker G., Jaeger K.E., *Appl. Environ. Microbiol*. 53 (1987) 211.
29. Stefani M., Coudane J., Vert M., *Polym. Degrad. Stab*. 91 (2006) 2554.
30. Hassouna F., Raquez J-M., Addiego F., Dubois Ph., *Euro Polym J* .47 (2011) 2134.
31. Iovino R., Zullo R., Rao M.A, Kassar L., Gianfreda L., *Polymer. Degrada. Stab*. 93 (2008) 147.
32. Singh R.P., Pandey J.K, Rutot D., Degée Ph., Dubois Ph., *Carbohydrate Research* 338 (2003) 1759.
33. Cho H.S., Moon H.S., Kim M., Nam K., Kim J.Y., *Waste Manag*. 31 (2011) 475.
34. Kotogan A., Nemeth B., Vágvölgyi C., Papp T., Takó M., *Food Technol Biotechnol*. 52 (2014) 73.