The Interaction Mechanism between Microorganisms and Substrate in the Biodegradation of Polycaprolactone

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ABSTRACT: The present work concerns the interplay of the degradation mechanism and the nature of the interaction between microorganisms and substrate. The biodegradation of polycaprolactone films by a pure strain of microorganisms isolated from an industrial composting unit for household refuse was studied in minimal medium with the polymer as sole carbon source. In conditions where the polymer surface is colonized and a biofilm is formed (under a low stirring rate), polymer weight loss is limited, whereas total degradation is observed when stirring conditions prevent biofilm formation. In the first case, holes are observed in the degraded film and a polysaccharide responsible for microorganism adhesion was identified by FTIR. SEM observation of the polymer surface as a function of the degradation time suggests that the crystalline and amorphous phase are degraded at about the same rate in the first case, whereas the amorphous phase is preferentially degraded in the latter. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 1334–1340, 2002

Key words: biodegradation; polycaprolactone; biofilm; FTIR; SEM

INTRODUCTION

To reduce the proportion of plastics in solid waste, biodegradable plastics were developed for specific applications. Polycaprolactone is well known for its biodegradability; it is produced industrially in blends with starch.[1] The biodegradability of polycaprolactone was observed in the presence of microorganisms in many environments. The main drawbacks of the exposure methods which were developed (landfill and compost simulations, soil burial, etc.) are their highly variable compositions in terms of both chemistry and microbial populations.[2] Several parameters, such as polymer molecular weight, crystalline morphology, film thickness, and degradation conditions, influence polycaprolactone biodegradation.[3–9] In our laboratory, we have undertaken the study of the degradation mechanism, working with a pure strain of microorganism from an industrial compost for household refuse selected for its ability to degrade polycaprolactone. This strain is able to grow in a minimal medium with polycaprolactone as the sole source of carbon. Depending on the stirring method and velocity, growth results in the formation of either a cell suspension in the medium or a biofilm at the surface of the polymer. The attached cells and

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their matrix are termed biofilm in this article, whereas biomass refers to cells free to move in the liquid medium. The present work concerns the interplay of the degradation mechanism and the nature of the interactions between microorganisms and substrate. Many bacteria associate with surfaces. Some of them adhere to these surfaces at first in a reversible association and eventually in an irreversible adhesion and initiate the development of adherent bacterial biofilms.[10,11] To our knowledge, the effect of the type of microbial growth on the yield and mechanism of biodegradation has never been studied. It has important practical meaning. Indeed, in industrial composting conditions, the degrading mass is at rest, whereas various types of stirring are used in composting simulating methods and in most experiments designed to study biodegradation.

The degradation mechanism in growth conditions leading to the formation of a biofilm and in conditions preventing it are compared. Gravimetric measurement of the residual substrate and the biomass produced was performed as a function of incubation time. The surface of the films was observed at various extents of degradation by scanning electron microscopy (SEM). The chemical structure of biofilms and biomass was characterized by Fourier transform infrared spectroscopy (FTIR). The net charge of the microorganism was evaluated by electrophoresis and related to the attachment mechanism of these microorganisms to the cell surface. The resulting effect on surface colonization and the mechanisms of biodegradation are discussed.

METHODS

Polymer

Polycaprolactone with a molecular weight of 37,000 was provided by Solvay Interox (Belgium). The polymer was pressed at 90° under a pressure of 120 kg/cm^2 as films about 80 μm thick. The films were cut and used as 1-cm^2 pieces at a concentration of 0.05% (w/v) in the culture medium. Films were sterilized by exposure to γ-irradiation (0.6 Mrd). Their crystallinity, determined by differential scanning calorimetry, is 61 wt %, using 135.6 J as the melting enthalpy of 100% crystalline polycaprolactone.[12] Spherulites of a few tenths microns diameter are clearly visible by scanning electron microscopy after partial biodegradation (Fig. 2).

Inoculum and Culture Media

A pure strain of bacteria isolated from an industrial compost for household refuse (strain 2.2) was used. The isolation method and the properties of this strain were published.[13] It is a strictly aerobic, gram-positive, nonsporulating rod. Colonies are orange, circular, and convex. The strain was propagated in rich medium, and biodegradation was assayed in a minimal medium as described in Dupret et al.[13,14] The composition of the minimal medium was as follows: KH_2PO_4 2.3 mM, Na_2HPO_4 4.2 mM, NH_4Cl 18 mM, $MgSO_4$ 4.2 mM, $CaCl_2$ 450 μM, $FeCl_3$ 31 μM (Merck, Darmstadt, Germany). All cultures were performed at 37°C.

Methods Used to Assess Biodegradation

Experiments were performed in liquid medium in two different conditions. A first series was incubated with gentle circular shaking in Erlenmeyer flasks at 150 rpm. Under these conditions, the culture medium was submitted to a low stirring rate. In the second series of experiments, cultures were submitted to a higher stirring rate (about 600 rpm) in closed bottles filled with air. The bottles were equipped with a sensor to measure oxygen consumption and an efficient magnetic stirrer, which induces important shearing forces and the formation of a cone in the medium. This method was used previously in our laboratory and was described.[13–15] In both series, polycaprolactone was the sole carbon source and was used at a concentration of 0.05% (w/v). Microorganisms grown in rich medium, harvested by centrifugation, washed with minimal medium, and finally put in suspension in the same minimum medium were used to inoculate the culture medium. The inoculum volume was calculated to obtain an initial concentration of 10^7 microorganisms per milliliter. After incubation, the culture flasks (Erlenmeyer flasks or bottles) were filtered through a weighed Teflon filter (0.22 μm) (Gelman Pall Life Sciences, Ann Arbor, MI). Biomass, biofilm, and residual substrate were recovered on the filter. Residual substrate was dried at constant weight and extracted with chloroform, a solvent of polycaprolactone. The filter supporting biomass was dried under vacuum and weighted. The carbon content of dry cells was obtained by using the general formula $CH_{1.8}O_{0.8}N_{0.2}$.[16] The global yield in biomass was given by the ratio of the carbon mass in the cells to the carbon mass in the utilized substrate. The percentage weight loss at time, t, was given by $100(W_0 - W)/W$, where W_0

and W are, respectively, the initial and residual polycaprolactone weight at time, t. The results reported here are the mean values of duplicates differing by less than 10%. Oxygen uptake was expressed as the ratio of the quantity of oxygen consumed to the quantity that would be consumed assuming quantitative transformation of the substrate into CO_2 and water. One hundred percent was of course never attained because part of the carbon was used for microbial growth.

FTIR

FTIR was used to study the biomass and biofilm composition. Pellets of biomass (or biofilm) were dried, ground with KBr (1 : 100 by weight) (Merck), and pressed for 20 min. Infrared absorption spectra were recorded by using a FTIR Bruker IFS-45 spectrometer.

SEM

SEM has proved invaluable for examining the surface of the residual polymer. Polycaprolactone samples were dried in vacuum at room temperature for 1 day. These samples were fastened on a copper plate with conducting tape. After Au coating, the samples were observed with a plane scanning at a voltage of 15 kV.

Electrophoretic Mobility

Electrophoretic mobilities were measured in a Rank Brothers Mark II apparatus. Following the procedure of Banerjee et al.,[17] measurements were carried out in the stationary level, in a capillary cylindrical cell (length, l = 8 cm) with an applied voltage of approximately 80 V (dc) at 25°C. The sign of the applied electric field was changed alternatively. The electrophoretic mobility of the bacteria, u_e, given by $dt/V/l$, where d, the bacterial displacement during time, t, was determined at least 10 times in each direction in the minimal buffer medium used in this work.

RESULTS AND DISCUSSION

Conditions Favoring the Formation of a Biofilm

Our polycaprolactone-degrading strain uses polycaprolactone as sole carbon source during growth in a minimal medium. With an identical medium composition and the same polycaprolactone film, growth can either occur in the medium, with cell division giving dispersed pellets, or as a biofilm adhering to the polymer. Typically, no agitation or moderate stirring on a rotary shaker of cultures in Erlenmeyer flasks resulted in biofilm development. Colonization of the polymer film by the bacterial cells could be readily observed. After 150 h of incubation, an orange film was apparent on the surface and holes were formed through the polymer films. When such films freshly taken out of the medium were examined by using phase-ontrast optical microscopy, they appeared as a tissue of microorganisms.

Vigorous agitation with a magnetic stirrer of cultures in cylindrical flasks prevented the formation of a biofilm. Colonization was never observed during degradation under those conditions. Samples from those bottles showed no sign of microorganism adhesion, even after 450 h incubation. Holes were never detected on such samples. Clearly, differences in the stirring method and velocity affecting the hydrodynamics of the culture are responsible for whether a biofilm is formed or not. It was shown by electrophoresis that the microorganism mobility, u_e, is lower than 0.05×10^{-8} m^2 s^{-1} V^{-1} in the experimental conditions used in this work. Typical values reported in the literature for bacteria at pH 7 lie between -3 and -0.5×10^{-8} m^2 s^{-1} V^{-1}.[18,19] This means that, in our case, the zeta potential, which is proportional to u_e, has a very low value, or also that the particle charge at the hydrodynamic plane of shear is very low. Electrostatic interactions with the substrate surface are thus poor. As a consequence, an efficient stirring rate could prevent close contact with the film.

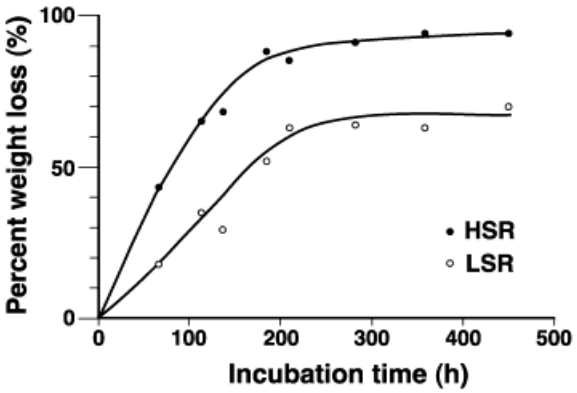

Figure 1 Percentage weight loss as a function of incubation time for the low (LSR) and high (HSR) stirring rates experiments.

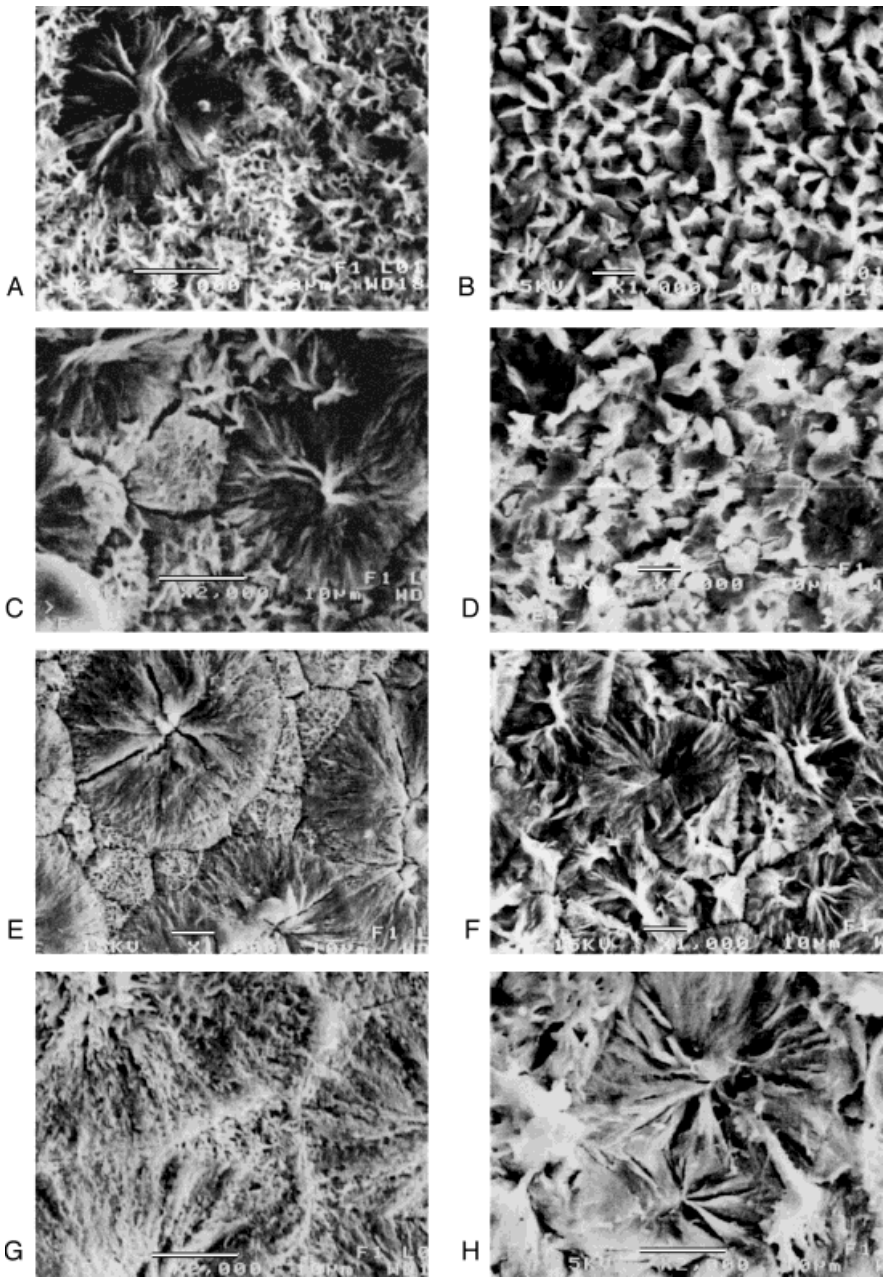

Figure 2 SEM micrographs of polycaprolactone films. Left: experiments under high stirring rate (HSR); right: experiments under low stirring rate (LSR), corresponding to the following incubation times and weight losses: (A and B) 3 days (43 and 18%); (C and D) 5 days (65 and 35%); (E and F) 8 days (88 and 52%); (G and H) 15 days (94 and 63%). The white bar represents 10 μm.

Effect of the Formation of a Biofilm on Weight Loss and Microbial Yield

A series of experiments were performed simultaneously, each time with one series in the conditions favoring growth in the medium and precluding the formation of the biofilm, and one series in Erlenmeyer flasks under moderate stirring, under which conditions a biofilm is formed. One experiment of each series was stopped after various predetermined incubation times. The weight loss of the degraded film surfaces was followed and compared as a function of time. Figure 1

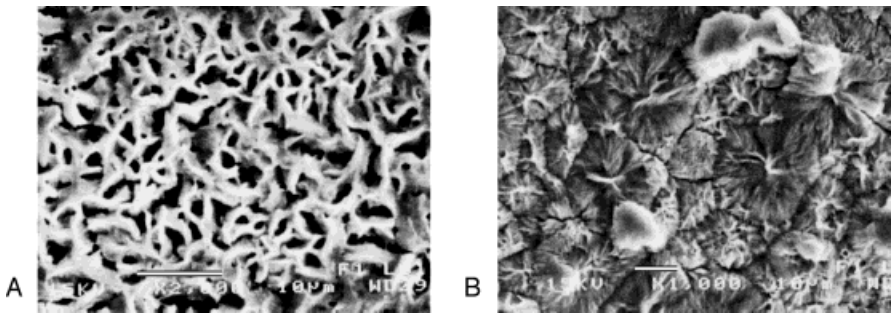

Figure 3 SEM micrographs of polycaprolactone films incubated for short times [(A) 2 days; (B) 3 days] under high stirring rate, with low weight losses [(A) 4%; (B) 23%]. The white bar represents 10 μm.

shows the evolution of this parameter for both types of experiments as a function of incubation time. In both cases, weight loss increased with time and tended to a plateau. Yet, at any incubation time, loss was always higher under vigorous stirring rate, in the absence of biofilm formation, as shown in Figure 1. Under those conditions, the limiting weight loss reached 95% after about 200 h. In the conditions where a biofilm is formed, partial degradation was observed and a weight loss of only 65% was measured. It will be shown in the next sections that the mechanism of degradation is quite different in both cases, justifying these differences in weight loss. Weight loss is negligible in the same growth medium in abiotic conditions. Oxygen consumption as a function of time could be measured (data not shown) in the growth conditions, precluding the formation of a biofilm (magnetic stirrer). Before the growth plateau was attained, weight loss was proportional to oxygen consumption, in agreement with the generally accepted mechanism for biodegradation by bacteria, when the substrate is transformed into CO_2 and biomass.[13,14] Measurement of oxygen consumption is experimentally not possible in the Erlenmeyer flasks where biofilms are formed. The yields in biomass were measured at the plateau in both growth conditions. They were, respectively, 60 and 50%.

SEM Observations

SEM observations of the degraded films surface as a function of incubation time and of weight loss suggest that the relative rate of degradation of the amorphous and crystalline components is different whether biofilm is formed or not. The micrographs are given in Figure 2. The initial polycaprolactone films appear completely smooth and homogenous at the same magnification. Figure 3 shows micrographs of polycaprolactone films degraded under vigorous stirring with no formation of biofilm. Incubation times are shorter than in Figure 2, resulting in lower weight loss. Figures 2(B) and 3(A) show that the formation of nonspherulitic structures always precedes the biodegradation of spherulites. We propose that these nonspherulitic structures result from partial attack of the amorphous phase. They are also present between the spherulites at higher weight loss. When no biofilm is formed, the spherulites are already observed at 23% weight loss [Fig. 3(B)], whereas they only appear at 52% weight loss [Fig. 2(F)] when a biofilm is formed. This suggests that the amorphous phase is degraded preferentially when cells are growing in suspension in the medium, because spherulites were already observed in the early degradation steps. In contrast, we suggest that amorphous and crystalline phases were degraded at about the same rate when a biofilm was formed. In agreement with this, holes, visible with the naked eye (not shown), appeared near 52% weight loss. After a long incubation time under low-speed stirring (67 days), bacterial cells were clearly visible in the cavities of the polymer film (not shown). Such holes never appeared in the absence of bacterial colonization, even at high weight loss.

FTIR Observations

The composition of the biofilm and biomass was analyzed by FTIR (spectra not shown). The most important bands common to both spectra are situated near 2950 cm^{-1} (CH_2 and CH_3 stretching), 1660 cm^{-1} (C═O stretching in proteins), 1545 cm^{-1} (N—H bending in proteins), and 1040 cm^{-1}

(polysaccharides). These spectra are in agreement with those published.[20,21] In the biofilm spectrum, a band appears in addition to the previously cited ones. It is located between 1725 and 1740 cm^{-1} (C═O absorption band of saturated aliphatic esters) and can be attributed to residual polycaprolactone in the biofilm. This band was not observed in the spectrum of biomass because this sample was obtained after elimination of the polycaprolactone films. Although the spectra of biomass and biofilm are qualitatively similar, they are quantitatively different. Indeed, the ratio of proteins to polysaccharides absorbance (A_{1660}/A_{1040}) is much lower for the biofilm (1.6) than for the biomass (2.4). This observation is in agreement with the fact that polysaccharides play a role in adhesion phenomenon.[22] According to the literature, various cell-surface structures were observed and are thought to be associated with the attachment process.[23,24] Direct observation by scanning and transmission electron microscopy has revealed that the attached microbial population exists within a matrix of extracellular polymeric substances produced by the microorganisms themselves.[10,21,25] Exopolysaccharide production is required for the formation of the complex three-dimensional structure of biofilms.[26]

Mechanism of Degradation

The experimental data suggest that polycaprone biodegradation could occur by at least two different mechanisms. Efficient stirring, preventing the formation of a biofilm on the surface of the polymer, leads to the preferential degradation of the amorphous phase, releasing the spherulites. In conditions favoring the colonization of the polymer film and the subsequent formation of an orange biofilm, nonpreferential spherical degradation of the amorphous and crystalline phases occurs with the final formation of holes through the film. Different enzymes could be involved in the two cases. Genetic studies have shown that many genes are differently expressed in the bacteria of the biofilm and in their counterpart growing in the medium. Changes in multiple environmental physicochemical conditions in the biofilm account for the reprogramming of gene expression.[27] It is possible that the two extreme mechanisms coexist in various proportions according to the growth conditions. In laboratory conditions, formation of the biofilm prevented total degradation, probably by limiting oxygen diffusion at the interface of the polymer. The coexistence of two different degradation mechanisms was proposed recently by Nishida et al.[28] in a study of the effect of crystallinity on the microbial degradation of polyhydroxybutyrate. As in our case, one degradation mechanism implicated preferential spherical degradation of the amorphous regions, leaving the lamellae intact, whereas the other degradation mechanism was a nonpreferential spherical degradation due to the colonization by the bacteria, resulting in the formation of holes.

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