Degradation by micro-organisms of polymers and model compounds containing ester, amide or urethane groups: kinetic and mechanistics aspects

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SUMMARY: This work has been performed using two pure strains of gram positive bacteria isolated from an industrial compost for household refuse and garden soil. These micro-organisms have a stable phenotype, are easy to grow and were shown to be efficient degraders of various organic structures. It has been possible to get an insight both into fundamental mechanistic aspects of the degradation and into the fate of the studied derivatives in composts. Among others, measurement of oxygen consumption as a function of incubation time together with isolation and identification of degradation intermediates and residues has shown that short diester-amides derived from phenylalanine are assimilated without prior hydrolysis. Also the mineralization of typical polyesteramides are limited by the assimilation of the fragments resulting from the ester groups hydrolysis.

Introduction

A given type of micro-organism presents three main types of specificity towards substrates: a chemical specificity which depends on the substrate functional groups, a morphological specificity which depends on its crystalline-amorphous organization and an interaction specificity which depends on the internal flow rate in the degradation vessel. The present work concerns mainly the chemical specificity towards a large variety of substrates of a pure strain of gram positive non sporulating bacteria (strain 2.2) isolated on polycaprolactone (PCL) from an industrial compost for household refuse. It degrades PCL very efficiently by chain-end step-wise erosion and assimilates all aliphatic polyesters which have been tried. The same method, the measurement of oxygen consumption as a function of incubation time, has been used in all cases to allow easy comparison.

Terephthalic acid, naphthalene-2,6-dicarboxylic acid and their methyl or ethylene glycol diesters have been first considered as model compounds of polyethylene terephthalate and polyethylene naphthalene-2,6-dicarboxylate (PET and PEN). Model compounds of

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polyesteramides and one polyesteramide containing phenylalanine units have then been tested. The last part is related to a low molecular weight polycaprolactone (PCL) terminated by a diurethane methanol end group and to the corresponding model diurethane. Amides, aromatic esters and diurethanes have been choosen for this study because they have often been proposed as co-units or chain-extenders to improve the physical properties of aliphatic polyesters which are efficiently mineralized by micro-organisms [1-8].

Special attention has been paid to the quantitative comparison of the degradation rate of the various substrates, to the identification of the degraded intermediates in order to anticipate the most favourable structure for a degradable polymer and to the identification and quantification of the degradation residues in relation with the new legislations [9].

Experimental

The aromatic esters and polyesters PET and PEN were kindly supplied by Agfa-Gevaert. The synthesis of phenylalanine derivatives (VI to IX) was published recently [10]. The preparation of the other amides will be published elsewhere [11]. The polyesteramide was prepared according to the method described by Huang [12]. The diurethane terminated PCL was obtained by reaction of OH-terminated PCL 3000 supplied by Solvay-Interox with a stoechiometric amount of toluene-2,4-diisocyanate (TDI) followed by deactivation of the terminal -N=C=O groups with methanol. The corresponding model compound was prepared by reaction of TDI with an excess methanol. The structure of the low molecular weight compounds and polymers used in the present work was confirmed by ^{1}H NMR.

The respirometric method was outlined previously together with the method of isolation and characterization of the pure strain of gram positive non sporulating bacteria (strain 2.2) used in this work [13, 14]. The substrates were incubated (~ 50 mg/100ml) as sole carbon source in a phosphate buffer at pH 6.8. The products which are not soluble in the culture medium were degraded as fine powders (aromatic esters, polyesteramide and diurethane derivatives). The final concentration of micro-organisms is of 10^7 micro-organisms /ml culture medium. The percentage of oxygen consumed is expressed as the ratio of the oxygen consumed to the quantity of oxygen that would be consumed assuming complete mineralization which means quantitative transformation in CO_2 and H_2O. A value ranging from 40 to 70% is usually obtained for complete degradation since part of the carbon is used to produce biomass. The curves given in figures 1 to 5 are the mean value of duplicate experiments differing by less than 5%. A negative control (no carbon source) hes been substracted from the experimental curves. The results were compared to a positive control containing glucose. The incubation temperature is 37°C in all cases. At the end of the incubation, the soluble residue was

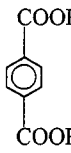
separated from the insoluble fraction (biomass and insoluble substrate) by filtration. Biomass was separated from residual substrate by solvent extraction. The soluble and insoluble residues were then qualitatively and quantitatively characterized by measuring the total soluble carbon [15] and by FTIR and NMR.

The *Pseudomonas* strains used for the degradation of naphthalene derivatives were provided by M. Mergeay (VITO, Mol, Belgium) [16]. The characteristics of the S_{TA} strain isolated from garden soil are the following: long rods, gram+, spore, catalase -, oxydase - and capable to use glucose. It is possibly a *Bacillus sp.*

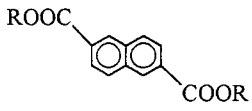
Results and discussion

Aromatic diacids, their esters and polymers

Polyethylene terephthalate (PET) is an example of aromatic polyester well-known to be non biodegradable. Replacing terephthalic acid (I with R=H) by naphthalene-2,6-dicarboxylic acid (II with R=H) gives polyethylene naphthalene-2,6-dicarboxylate (PEN), the new support for photographic emulsions. To our knowledge, its biodegradability has never been reported. Since the probability for degrading PET and PEN was low, we started with model compounds I and II.



(I)



(II)

where R = -H, $-CH_3$ or $-CH_2-CH_2-OH$

As no growth was observed neither with the strain isolated from compost (strain 2.2) nor with 14 strains of naphthalene degrading *Pseudomonas*, eight strains have been then isolated from a suspension of industrial compost or of garden soil (six strains on I and two strains on II). Among the six strains obtained on terephthalic derivatives, one (strain S_{TA}) was tested on solid agar medium. It showed important growth (+++) on plate containing terephthalic acid, medium growth (++) on naphthalene 2,6-dicarboxylic acid and low growth (+) on its dimethyl esters. The measurement of the oxygen uptake (figure 1) shows that the rate of mineralization of

terephthalic acid is quite the same as that of glucose and reach a plateau after 100 hours. On the contrary, the mineralization of the other derivatives is very low. Indeed, the residual concentration determined by UV-spectrometry is 1 to 2 % for terephthalic acid, showing a complete degradation. Similar results were obtained with the other strains. As seen, in spite of some growth on solid agar medium, reproducible significative results could not be obtained with naphthalene derivatives. We could not detect any sign of PET or PEN biodegradation. The same conclusion could probably be drawn for most of the new products having no equivalent in nature.

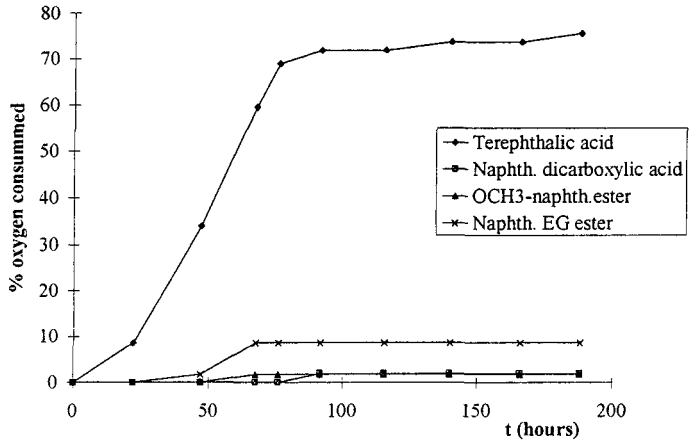


Figure 1. Oxygen consumption on terephthalic acid, naphthalene dicarboxylic acid, its dimethyl ester and its ethylene glycol ester

Amides, ester-amides and acid-amides

Model compounds

The eight model compounds are presented hereafter in order to better visualize and compare their structure. Compounds VI, VIII and X were synthetized using L-phenylalanine; compounds VII and IX were prepared from D-phenylalanine.

(III) CH_3-NH-CO-CH_2-CH_3

(IV) CH_3-$(CH_2)_9$-NH-CO-$(CHOH)_3$-CH_2OH

(V) $HOOC$-$(CH_2)_5$-NH-CO-$(CH_2)_2$-$COOH$ (aliphatic diacid-amide)

(VI and VII) HOOC-C*HR-NH-CO-$(CH_2)_2$-COOH (L-AA and D-AA)

(VIII and IX) CH_3-OOC-C*HR-NH-CO-$(CH_2)_2$-COO-CH_3 (L-EE and D-EE)

(X) HOOC-C*HR-NH-CO-$(CH_2)_2$-CO-NH-C*HR-COOH (LL-AA)

with R = -CH_2-Ph

The three aliphatic model compounds (III to V) containing one amide function have been tested with the strain 2.2 without success. The compounds (VI to X) derived from an aromatic amino-acid, L or D-phenylalanine, have shown better results in agreement with the data concerning amides derived from amino-acids reported in the litterature [17, 18].

The oxygen consumption on these L- and D-(diacid-amide) (VI and VII) and L- and D-(diester-amide) (VIII and IX) (figure 2) shows that the D-derivatives are assimilated more slowly than the L-derivatives.

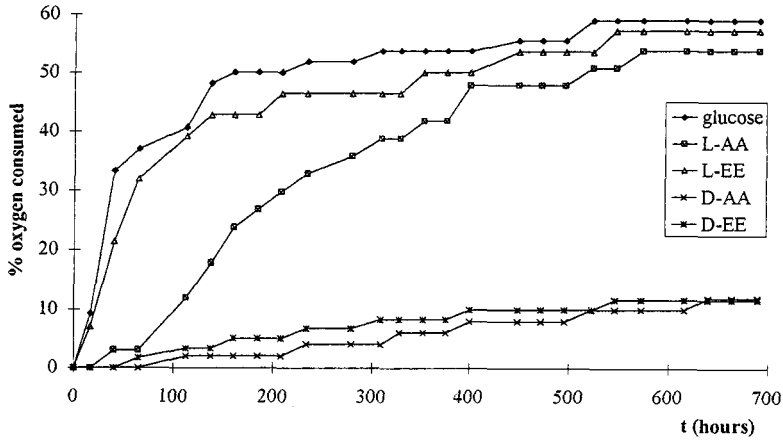


Figure 2. Oxygen consumption on diester-amides (VIII and IX) and diacid-amides (VI and VII) derived from L- and D-phenylalanine

The L-(diester-amide) (VIII) is degraded faster than the L-(diacid-amide) (VI) but the percentage of oxygen consumed at the plateau is similar. This proves that assimilation of the L-diester-amide) (VIII) is not preceded by hydrolysis of the ester functions. As bio- and chemical

hydrolysis of the amide groups are usually slower than that of ester group, the L-(diester-amide) (VIII) probably penetrates in the micro-organism without any prior degradation into smaller fragments. On the contrary to the L derivatives, there is no significative difference between the D-(diacid-amide) (VII) and the D-(diester-amide) (IX).

The residual concentration of the L-(diacid-amide) (VI) and L-(diester-amide) (VIII) found by UV-spectrometry is of the order of 10%. This value can be compared with that found for glucose. In this case, an organic residue corresponding to about 10% of the initial glucose is usually found by soluble carbon method [15] at the plateau of oxygen consumption. Specific glucose analysis by the glucose-oxidase method has demonstrated the absence of glucose, the 10% residue being thus due to low molecular weight fragments from glucose degradation but also to molecules excreted by the micro-organisms. The residual concentration is of 65 to 70% for the D-(diacid-amide) (VII) and D-(diester-amide) (IX) during the same incubation time, in accordance with their low oxygen consumption.

Chemical hydrolysis of the ester bonds (0.016% per day) is very low when compared with the assimilation (12% per day). However, chemical hydrolysis of the amide group of the D-(diacid-amide) (VII) which is 0.9% per day could be responsible of the degradation of this compound (0.8% per day).

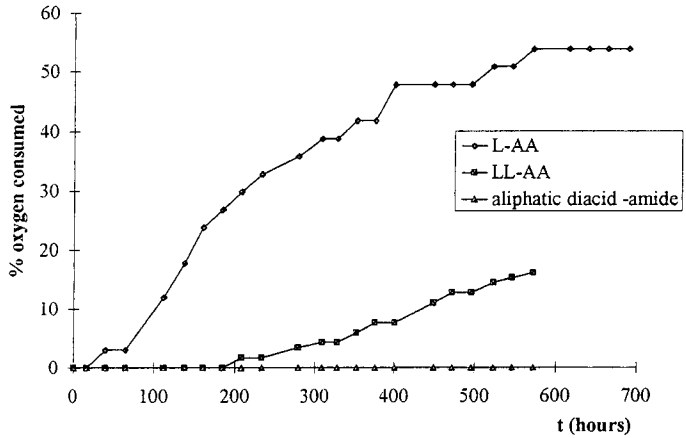


Figure 3. Oxygen consumption on a diacid-amide (VI) containing one L-phenylalanine, a diacid-diamide (X) containing two L-phenylalanine and an aliphatic diacid-amide (V)

Figure 3 compares the mineralization of diacid compounds (V, VI and X) containing one or two amide functions. They are completely soluble in the culture medium at the concentration

used in the present work. Figure 3 shows that the rate of assimilation is very low when the diacid contains two amide groups, even if derived from amino-acid. The LL-(diacid-diamide) (X) is assimilated very slowly, probably because of its larger size. After 600 hours, the percent of recovered residue is less than 10% for (V) and 60% for (X) while the aliphatic diacid-amide (VIII) is not mineralized and quantitatively recovered. Phenylalanine and not terminal acid or ester groups is thus responsible for assimilation of molecules containing amide groups.

Polyesteramide

Figure 4 presents the oxygen consumption of the corresponding L-polyesteramide with structure

$$[\text{-}(CH_3)_2OOC\text{-}C^*HR\text{-}\underline{NH\text{-}CO}\text{-}(CH_2)_2\text{-}\underline{CO\text{-}NH}\text{-}C^*HR\text{-}COO\text{-}CH_2)_3\text{-}]_n \qquad (XI)$$

It is compared with the L-(diester-amide) (VIII) and LL-(diacid-diamide) (X). The rate of assimilation is much lower for the polymer and the LL-(diacid-diamide) (X) than for the L-(diester-amide) (VIII).

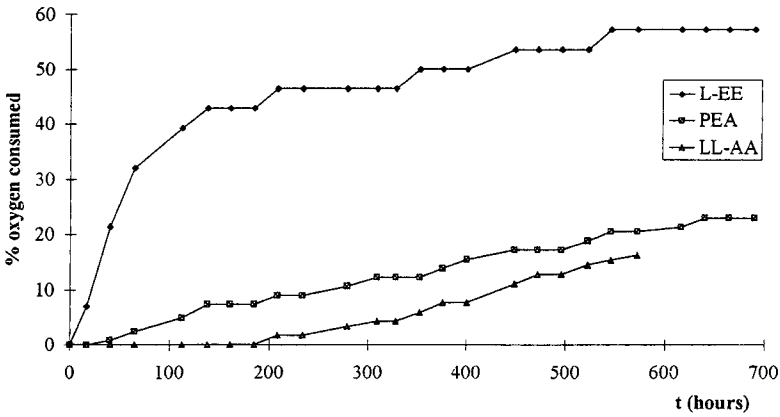


Figure 4. Oxygen consumption on a model diester-amide derived from L-phenylalanine, a model diacid-diamide derived from two L-phenylalanine and its corresponding polyesteramide

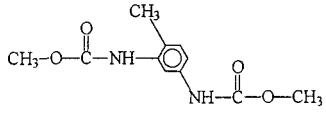
After one month incubation, 25% of the initial solid polymer is recovered. The culture medium contains a soluble fraction which corresponds to 33% of the initial substrate. This soluble fraction has been shown by NMR to be constituted of pure LL-(diacid-diamide) (X). This

demonstrates that the first step of the polyesteramide degradation is the biohydrolysis of all the ester functions giving the LL-(diacid-diamide) (X). Hexanediol which is the second hydrolysis product of the ester bonds cleavage is not detected in the soluble fraction because it is very rapidly assimilated by the micro-organisms. The rate of mineralization of the polymer is thus limited by the rate of assimilation of the intermediate LL-(diacid-diamide) (X). A polyesteramide based on the more rapidly assimilated L-(diester-amide) (VIII) would probably degrade much faster.

Urethane and urethane terminated polycaprolactone

Model compound

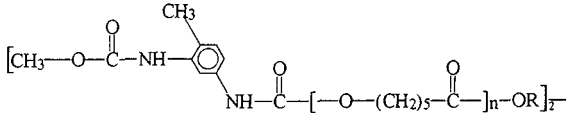
The model compound toluene 2,4-dimethylurethane (XII) was hardly degraded after 380 hours incubation as indicated by the very weak oxygen consumption given in figure 5.



(XII)

Urethane terminated polycaprolactone

Polycaprolactonediol is degraded by chain-end attack as previously demonstrated [19, 20]. After 380 hours incubation, residual polymer has been shown to be absent. The diurethane terminated PCL of structure (XIII) shows a short induction period (figure 5) which probably corresponds to incipient endo-attack. The oxygen consumption curve then grows parallel to that of polycaprolactonediol.



(XIII)

After 380 hours incubation, 25% of the initial polymer XIII is recovered. The soluble fraction has been shown by NMR to be constituted of the diurethane-methanol chain-ends bound to a mean value of 1.5 caprolactone units per diurethane-methanol group. During this incubation

time, degradation of the diurethane chain ends is thus very inefficient when compared with PCL chain.

In other systems involving aliphatic diurethane, other low molecular weights aliphatic polyesters and the same micro-organisms, the terminal diurethane molecules were quantitatively recovered after complete degradation of the polyester [21].

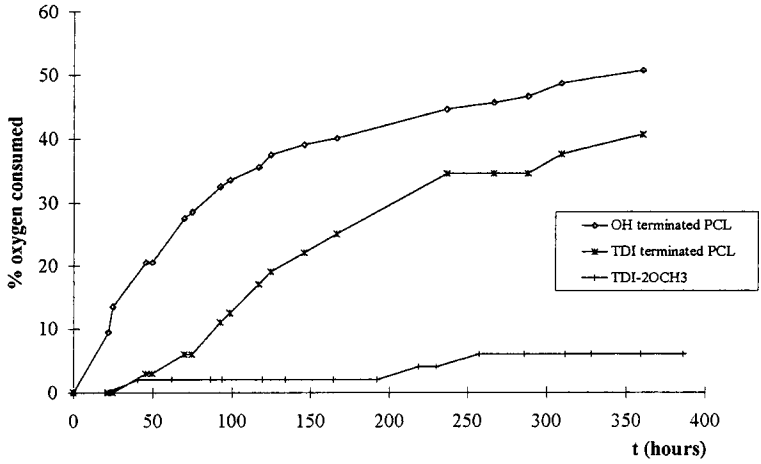


Figure 5. Oxygen consumption on di OH-terminated PCL, TDI terminated PCL and TDI-2OCH$_3$

Conclusion

The introduction of aromatic acid or amide as co-units, or of urethane chain extenders in polyesters to improve their physical properties sets the problem of the mineralization of these groups. This work indicates that phthalic acids are rather efficiently degraded and could be used if they can be separated from the mixed aromatic-aliphatic polyester by hydrolysis. The use of amide co-units derived from amino-acids is, to our opinion, of restricted applicability unless for biomedical applications. Chain extension of aliphatic polyesters using diisocyanate, although apparently promising, probably yields undegradable diurethane residue.

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