

# Biodegradation of Polyaromatics Synthesized by Peroxidase-Catalyzed Free-Radical Polymerization

Richard Farrell,<sup>1</sup> Madhu Ayyagari,<sup>2,3</sup> Joseph Akkara,<sup>2</sup> and David Kaplan<sup>3</sup>

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Polymers formed from peroxidase-based free-radical polymerization reactions were characterized for rates of mineralization against lignin and humic acid controls. Degradation studies were carried out in soil systems over 202 days and cumulative net CO<sub>2</sub> was determined. Whereas mineralization of the humic acid and alkali lignin controls totaled ca. 20% at the end of the test exposure, there was essentially no net mineralization of the hydrolytic lignin control. Mineralization of the test samples totaled 5% for poly(*p*-ethylphenol) and 11% for poly(*m*-cresol). At the same time, mineralization of the poly(*p*-phenyl phenol) totaled 64%. Conversely, the readily biodegradable polymers cellulose and PHB reached values of 91 to 97% in less than 60 days. Our data suggest that the mineralization kinetics of the enzymatically derived polyaromatics mimic those of the naturally occurring heteropolymers.

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**KEY WORDS:** Polyaromatics; free-radical polymerization; biodegradation; peroxidase.

## INTRODUCTION

A variety of polyaromatic compounds is present in the biosphere. These compounds include heteropolymers and heterooligomers such as lignins, tannins, lignans, and humic acid, among others. Lignins and humic acids contain a high content of aromatic residues linked by aliphatic bridges in complex configurations. Lignins are derived from enzyme-based free-radical polymerizations, usually catalyzed by the enzyme peroxidase, and, together with microbially synthesized aromatic and other reactive compounds, are considered an important source of structural units for the formation of humic acid in soils [9, 14, 19]. The biodegradation or mineralization of lignins and humic acid is significantly slower than that of other organic biopolymers present in the biosphere. The resistance of these polymers to biodegradation under

natural conditions is a result of the complex heteropolymeric structure of the polymers, the high percentage of aromaticity and ether linkages in the polymer core, and the fact that these polymers are water insoluble and difficult to wet. Nevertheless, the bio-/environmental degradation of these polymers is attested to by the fact that there is no long-term accumulation of these polymers in the soil; i.e., there is an equilibrium concentration characteristic of each soil ecosystem [19]. Moreover, the slow turnover of these polymers serves an important function by maintaining a high content of organic matter in the soil to modulate water holding capacity, metal chelation, and related critical ecological functions.

Though it was long thought that only the white-rot fungi and related litter-degrading basidiomycetes were capable of extensively degrading lignin, biodegradation studies with <sup>14</sup>C-labeled lignins have shown that several species of brown-rot fungi and a large number of bacteria also are capable of degrading these materials [2, 8, 12]. Likewise, there exists a wide variety of soil microorganisms capable of degrading the lower molecular weight polyaromatics produced during the breakdown of lignin [7] as well as catabolizing humic acid [19]. Thus, it is to

<sup>1</sup>NSF—Biodegradable Polymer Research Center, University of Massachusetts Lowell, Department of Biology, Lowell, Massachusetts 01854.

<sup>2</sup>Biotechnology Division, Natick Labs, Natick, Massachusetts 01760.

<sup>3</sup>Tufts University, Biotechnology Center, Department of Chemical Engineering, Medford, Massachusetts 02155.

be expected that a number of microorganisms capable of degrading synthetic polyaromatics of commercial interest exist in nature. Indeed, Haraguchi and Hatakeyama [10] reported that a mixed population of soil microorganisms was capable of biodegrading a series of lignin-related model polystyrenes.

We have focused our studies on controlling enzyme-based free-radical polymerization reactions *in vitro* to explore the synthesis of new polyaromatic materials [1, 3–6, 11, 13, 17]. These reactions can be carried out with a wide range of aromatic monomers including phenols, modified phenols, aniline, and modified anilines. Complex polymer products are generated that have been characterized in detail for elemental composition; structure by Fourier transform infrared (FTIR) spectroscopy, gel permeation chromatography (GPC), and nuclear magnetic resonance (NMR); and thermal properties by thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC). The structures of the polymers can be modulated based on the reaction conditions, as well as the monomers and solvent mixtures used in the reactions. The conjugated polymeric backbone generated by the enzymatic synthesis suggests applications for these polymers as resins, electromagnetic shielding, photoresists, and battery electrodes.

Because these polymers are generated by an enzymatic process, and based on analogies with the biological heteropolymers that they mimic most closely (i.e., lignins and humic acid), it has been presumed they should be biodegradable. To determine the validity of this assumption, the mineralization of a series of peroxidase-catalyzed polyaromatic products was studied in a controlled soil environment.

## MATERIALS AND METHODS

### Polymer Synthesis

The methods for synthesis of the polymers have been described elsewhere [1, 4]. Briefly, two types of reaction were used. In monophasic organic solvent reactions, peroxidase (Type II, Sigma Chemical Co., St. Louis, MO) was reacted with the monomer (*p*-phenyl phenol or *m*-cresol) in dioxane with HEPES buffer, pH 7.5, at room temperature overnight. The polymers that formed precipitates were subsequently washed exhaustively to remove enzyme and any unreacted monomer. In the second type of reaction, reversed micellar systems were used to carry out the synthesis [4]. The peroxidase was dissolved in buffer, as above, and this mixture was

added to isooctane containing a surfactant (AOT) with stirring to form reversed micelles. The monomer, ethyl phenol, was added to the system followed by hydrogen peroxide to initiate polymerization.

### Polymer Characterization

The three polymers were characterized by FTIR, GPC, NMR, TGA, and DSC as described previously. In addition, the polymers were assessed for solubility in dimethylformamide (DMF), chloroform, acetone, alcohol, and water. Details of the chemical characterizations have been reported elsewhere [4].

### Biodegradation

Polymer mineralization (i.e., conversion of polymer-C to CO<sub>2</sub>) was measured using a respirometric method based on that described by Bartha and Pramer [21]. Powdered samples totaling ca. 250 mg polymer-C (i.e., 295- to 323-mg total weight) were buried in test reactors (250-ml biometer flasks; Belco Glass Inc., Vineland, NJ) containing 50 g (oven-dry weight) of a standard soil mix (1 : 1 : 0.1, w/w/w, mix of potting soil, sand, and composted manure, pH 7.1) maintained at 55–60% water-holding capacity (WHC) and incubated in a controlled-environment chamber at 30°C. Carbon dioxide produced during the biodegradation process was trapped in 20 ml of 0.50 M KOH added to the side-arm portion of the biometer flasks. The CO<sub>2</sub> traps were changed at 0.5- to 30-day intervals for a total of 202 days; at each sampling period, a 4-ml aliquot of the KOH from each trap was titrated with standardized 0.10 M HCl. Daily and cumulative CO<sub>2</sub> production (total and net) and percentage mineralization were calculated relative to a control flask (soil without added polymer). Initially the polymers were air-dried to a powder and used directly in the biodegradation assays. Subsequently, polymers were prepared by first removing residual entrapped solvent by drying in a vacuum oven at 40°C for 48 h. The samples were then ground to pass a 100-mesh sieve but be retained on a 270-mesh sieve (i.e., particle size, 53 to 150 μm) before being incorporated into the soil.

### Controls

Two lignins, a hydrolytic lignin and an alkali lignin, and one humic acid sample (Sigma Chemical Co.) were used as control materials in the biodegradation studies. The hydrolytic lignin was from a commercial hydroly-

sis pilot plant with sugar cane bagasse as the main input; it had a carbon content of 63%, a methoxyl content of 9–11%, an  $M_n$  of 1700 and an  $M_w$  of 19,300. The alkali lignin was a kraft lignin from a commercial pulp mill with spruce as the main wood input; it had a carbon content of 49%, a thiol content of 2%, an  $M_n$  of 1750, and an  $M_w$  of 14,200. The humic acid sample had a carbon content of ca. 35% but was otherwise uncharacterized. In addition to the polyaromatics, microcrystalline cellulose (Sigma Chemical Co.) and the bacterial polyester, poly-3-hydroxybutyric acid (P3HB) (Monsanto—formerly Zeneca BioProducts; Billingham, UK; grade G044, batch 5100P,  $M_n \approx 130,000$ ) were included in the biodegradation study as “positive controls,” i.e., materials known to be readily biodegradable in soil.

## RESULTS AND DISCUSSION

Characteristics of the three synthetic polymers are summarized in Table I. The molecular weights were in the oligomer to low polymer range and all three polymers were insoluble in water.

Data from the initial polymer biodegradation assay revealed no evidence for mineralization and in some cases produced negative net CO<sub>2</sub> curves (Fig. 1), prompting a more detailed look at the polymers and the assay system. The results suggested that the observed inhibition could have been caused by the presence of residual solvent in the powders, which would have to be removed prior to reinitiating the studies. In addition, the original powders exhibited a fairly wide range of particle sizes, which, due to the fact that biodegradation occurs from the surface of the polymer in toward the center, was believed to have contributed to the large standard deviations observed in the initial assays (Fig. 1). Once the residual solvent was removed from the polymers (by drying *in vacuo* at 10  $\mu$ m Hg and 40°C for 48 h) and the powders ground to a more uniform particle size, the biodegradation assays were repeated. The results of these assays are presented in Fig. 2.

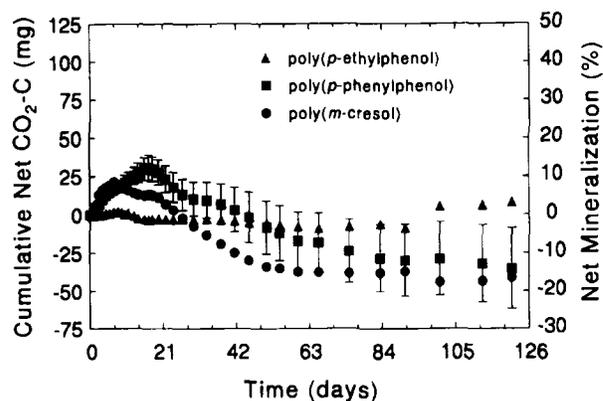


Fig. 1. Inhibition of mineralization of the three enzymatically derived polymers due to residual entrapped solvent.

All three of the enzyme-derived polyaromatics exhibited some degree of biodegradation, and in general, net mineralization of the polyaromatics increased in the order poly(*p*-ethylphenol)  $\approx$  poly(*m*-cresol)  $\ll$  poly(*p*-phenylphenol). However, whereas there was essentially no change in the cumulative net mineralization of the poly(*p*-ethylphenol) and poly(*m*-cresol) after Day 6, mineralization of the poly(*p*-phenylphenol) contin-

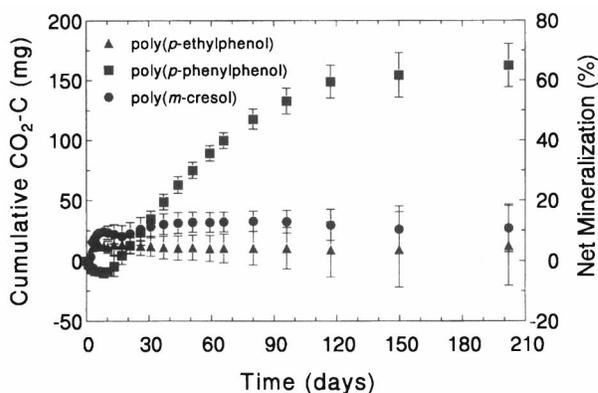


Fig. 2. Mineralization of the three polymers in soil systems during a 202-day test exposure.

Table I. Characteristics of the Polyaromatics Studied for Biodegradation

Polymer	Poly( <i>p</i> -phenylphenol)	Poly( <i>p</i> -ethylphenol)	Poly( <i>m</i> -cresol)
Molecular weight	3850	2000	2433
Polydispersity	2.8	1.4	2.6
$T_m$	200°C	125°C	150°C
Solubility	DMF, complete; CHCl <sub>3</sub> & acetone, partial; alcohol & water, insoluble	DMF, CHCl <sub>3</sub> , acetone, soluble; alcohol, partial; water, insoluble	DMF, complete; CHCl <sub>3</sub> , acetone, alcohol, partial; water, insoluble

**Table II.** Aerobic Biodegradability of Natural and Synthetic Polyaromatics in Soil at 30°C and a Moisture Content of 60% WHC

Test material <sup>a</sup>	CO <sub>2</sub> -C (mg)			$r_{MAX}^b$ (mg CO <sub>2</sub> -C day <sup>-1</sup> ) <sup>d</sup>	Net mineralization	
	Theoretical	Total	Net		Theoretical (%)	RBI <sup>c,d</sup>
Cellulose	251	466.6	229.2 a	18.2 a	91.4 ± 4.5	1.00 a
Poly(3-hydroxybutyrate)	250	499.9	242.5 a	14.4 a	97.0 ± 4.5	1.06 a
Lignin (alkali)	250	294.8	57.4 c	0.35 b	23.0 ± 10.6	0.25 c
Lignin (hydrolytic)	251	239.3	1.9 d	0.00 b	0.8 ± 7.8	0.01 d
Humic acids	250	289.0	51.7 c	0.36 b	20.6 ± 16.0	0.23 c
Poly( <i>p</i> -ethylphenol)	254	399.7	12.5 <sup>e</sup> cd	2.51 b	4.9 ± 9.0	0.05 cd
Poly( <i>p</i> -phenylphenol)	254	264.4	162.3 b	1.90 b	64.0 ± 5.4	0.70 b
Poly( <i>m</i> -cresol)	256	264.4	27.0 <sup>e</sup> cd	4.69 b	10.6 ± 5.0	0.12 cd

<sup>a</sup>Values for cellulose and poly(3-hydroxybutyrate) were recorded on Day 55; all other values were recorded on Day 202.

<sup>b</sup>Maximum rate of CO<sub>2</sub> production.

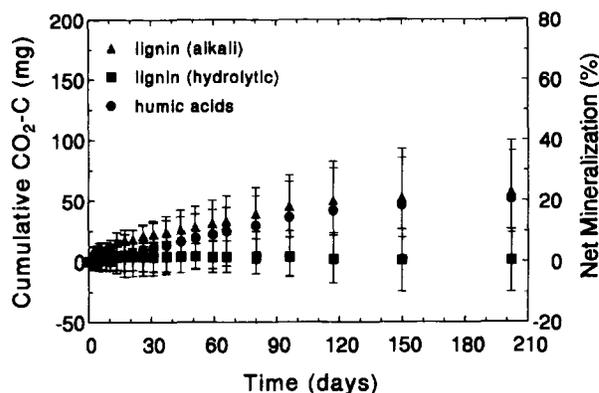
<sup>c</sup>Relative biodegradation index = net mineralization of the test polymer + net mineralization of the positive reference material (i.e., cellulose).

<sup>d</sup>Values followed by the same letter are not significantly different at the  $P \leq 0.05$  level of probability (least-significant difference test).

<sup>e</sup>Net mineralization of the polymer had essentially ceased by Day 6 of the test exposure.

ued throughout the soil test exposure. These results suggest (i) that poly(*p*-ethylphenol) and poly(*m*-cresol) are fairly recalcitrant and (ii) that these samples may have been “contaminated” with small amounts of low molecular weight oligomers, which were more readily degraded. On the other hand, after a lag period of about 10 days, during which total CO<sub>2</sub> production in the polymer amended soils was less than that in the control soils, mineralization of the poly(*p*-phenylphenol) proceeded slowly—with a sustained rate of mineralization of ca. 2 mg CO<sub>2</sub>-C day<sup>-1</sup>—for about 85 days. Though net CO<sub>2</sub> evolution from the polymer-amended soil continued to increase after Day 96, there was no significant increase in percentage net mineralization after Day 120. Upon completion of the test exposure, cumulative net mineralization of the poly(*p*-phenylphenol) totaled 64 ± 5% (Table II). Current FDA test protocols state that an organic compound can be presumed “biodegradable” if more than 50% of its carbon content is converted to CO<sub>2</sub> during the test exposure [20]. Thus, in accordance with this guideline, poly(*p*-phenylphenol) is the only one of the synthetic polyaromatics that can be considered biodegradable.

As expected, the data for the lignin and humic acid controls (Fig. 3, Table II) indicate that these materials are highly resistant to biodegradation. Indeed, the hydrolytic lignin exhibited essentially no net mineralization during the soil test exposure; i.e., cumulative net CO<sub>2</sub> evolution from the soil amended with hydrolytic lignin amounted to less than 1.0% of the added lignin-C. The alkali lignin and humic acid controls, on the other hand, were very slowly mineralizable ( $r_{MAX} \approx 0.4$  mg CO<sub>2</sub>-C day<sup>-1</sup>), and upon completion of the soil test exposure,



**Fig. 3.** Mineralization of control aromatic heteropolymers in the soil degradation systems during a 202-day test exposure.

net CO<sub>2</sub>-C accounted for only about 20% of the added polymer-C. The observed differences in mineralization of the alkali and hydrolytic lignins presumably reflect compositional differences in these compounds. Nevertheless, our results are consistent with those of Haider *et al.* [9] and Martin *et al.* [16], who reported that lignins were resistant to complete mineralization and that the greatest conversion of lignin-C to CO<sub>2</sub> occurred during the earliest stages of decomposition. Likewise, given that humic acid is perhaps the most formidable, naturally occurring molecule that soil microbes are called upon to degrade [19], it is not surprising that little mineralization occurred during the 202-day soil test exposure.

Mineralization data for the synthetic and natural polyaromatics can be put into context when considering the data obtained from polymers generally consid-

ered to be readily biodegradable. For example, in the same soil system, both cellulose and P3HB degraded at a much more rapid rate and to a much greater extent than any of the polyaromatics (Table II). Indeed, net mineralization of these positive controls reached the 50% mark—the FDA-defined threshold for concluding that an organic compound is “biodegradable”—in only 10 days. In comparison, poly(*p*-phenylphenol)—the only polyaromatic (synthetic or natural) to achieve 50% net mineralization during the soil test exposure—required about 90 days to reach this benchmark. Results for the positive controls reflect the fact that these biopolymers are widespread throughout the environment, being both produced and degraded by a wide range of soil microorganisms. In addition, the microorganisms that degrade these biopolymers derive sufficient energy and carbon from their decomposition to support the synthesis and maintenance of new cells. It is unlikely, however, that the energy yielded from biodegradation of the polyaromatics is sufficient to support the growth and development of new microbial biomass [19]. Indeed, decomposition of these materials is most likely a result of cometabolism, wherein the microorganisms involved derive neither energy nor carbon from the polymer substrate itself. Such processes are considerably more complex and slower than direct catabolism, and as a consequence, lignin and humic acids generally exhibit decomposition times of the order of years to decades [18]. With this in mind, it is our conclusion that the enzyme-derived, synthetic polyaromatics exhibit mineralization kinetics that mimic those of naturally occurring lignins and humic acids. This finding is to be expected, given the similarities in structure of the enzymatically generated and naturally occurring heteropolymers, and supports our original hypothesis.

Though it was somewhat surprising that the poly(*p*-phenylphenol) degraded to the extent observed during the soil test exposure (i.e., RBI = 0.70), the presence of the phenyl side chain suggests similarities to lignins as a possible explanation. On the other hand, the resistance to biodegradation of poly(*m*-cresol) may reflect the fact that *m*-cresol itself is a common constituent of the core molecule of soil humic acids [15]. However, it is difficult to extend these observations without first obtaining a more systematic structure/mineralization profile for the enzyme-derived polyaromatics. A more systematic study in which the side chains on the conjugated main chain of these polymers are varied would be useful in elucidating trends in mineralization kinetics. In particular,

varying side-chain size and chemistry would be useful in correlating these structural features with similar features found in naturally occurring lignins and humic acid.

## ACKNOWLEDGMENTS

Financial support for R. Farrell was provided by the National Science Foundation—Industry/University Cooperative Research Center for Biodegradable Polymer Research at the University of Massachusetts Lowell. The technical assistance of John Mitchell during the biodegradation phase of this study is gratefully acknowledged.

## REFERENCES

1. J. A. Akkara, K. J. Senecal, and D. L. Kaplan (1991) *J. Polym. Sci.* **29**, 1561.
2. G. I. Amer and S. W. Drew (1980) *Annu. Rep. Ferment. Process.* **4**, 67–103.
3. M. S. Ayyagari, J. A. Akkara, and D. L. Kaplan (1996) *Acta Polym.* **47**, 193–203.
4. M. S. Ayyagari, K. A. Marx, S. K. Tripathy, J. A. Akkara, and D. L. Kaplan (1995) *Macromolecules* **28**, 5192.
5. F. F. Bruno, J. A. Akkara, L. A. Samuleson, D. L. Kaplan, B. K. Mandal, K. A. Marx, J. Kumar, and S. K. Tripathy (1995) *Langmuir* **11**, 889.
6. F. F. Bruno, J. A. Akkara, D. L. Kaplan, P. Sekher, K. A. Marx, and S. K. Tripathy (1995) *Ind. Eng. Chem. Res.* **34**, 4009–4015.
7. R. B. Cain (1980) in T. K. Kirk, T. Higuchi, and H.-M. Chang (Eds.), *Lignin Biodegradation* CRC Press, Boca Raton, FL, Vol. 1, pp. 21–60.
8. D. L. Crawford and R. L. Crawford (1980) *Enzyme Microb. Technol.* **2**, 11–22.
9. K. Haider, J. P. Martin, and E. Rietz (1977) *Soil Sci. Soc. Am. J.* **41**, 556–562.
10. T. Haraguchi and H. Hatakeyama (1980) in T. K. Kirk, T. Higuchi, and H.-M. Chang (Eds.), *Lignin Biodegradation*, CRC Press, Boca Raton, FL, Vol. 2, pp. 147–159.
11. C. F. Karayigitoglu, N. Kommareddi, R. D. Gonzalez, V. T. John, G. L. McPherson, J. A. Akkara, and D. L. Kaplan (1995). The morphology of phenolic polymers enzymatically synthesized in surfactant microstructures. *Mater. Sci. Eng.* **C2**, 165–171.
12. T. K. Kirk, T. Higuchi, and H.-M. Chang (1980) in T. K. Kirk, T. Higuchi, and H.-M. Chang (Eds.), *Lignin Biodegradation*, CRC Press, Boca Raton, FL, Vol. 2, pp. 235–243.
13. N. S. Kommareddi, M. Tata, C. Karayigitoglu, V. T. John, G. L. McPherson, M. F. Herman, C. J. O'Connor, Y.-S. Lee, J. A. Akkara, and D. L. Kaplan (1995) Enzymatic polymerizations using surfactant microstructures and the preparation of polymer-ferrite composites. *Appl. Biochem. Biotechnol.* **51**, 241.
14. J. P. Martin and K. Haider (1986) in P. M. Huang and M. Schnitzer (Eds.), *Interactions of Soil Minerals with Natural Organics and Microbes*, SSSA Spec. Pub. No. 17, Soil Science Society of America, Madison, WI, pp. 283–304.
15. J. P. Martin, K. Haider, and C. Saiz-Jimenez (1974) *Soil Sci. Soc. Am. J.* **38**, 760–765.

16. J. P. Martin, K. Haider, and G. Kassim (1980) *Soil Sci. Soc. Am. J.* **44**, 1250–1255.
17. R. S. Premachandran, S. Banerjee, X.-K. Wu, V. T. John, G. L. McPherson, J. Akkara, and D. L. Kaplan (1996) *Macromolecules* **29**, 6452–6460.
18. J. D. Stout, K. M. Goh, and T. A. Rafter (1981) *Soil Biochem.* **5**, 1–73.
19. R. L. Tate (1987) *Soil Organic Matter: Biological and Ecological Effects*, John Wiley & Sons, New York.
20. U.S. Food and Drug Administration (1987) Environmental assessment technical assistance handbook PB87-175345, National Technical Information Service, Washington, DC.
21. R. Bartha and D. Pramer (1965) *Soil Sci.* **100**, 68–70.