Biodegradation of poly(3-methoxy-4-hydroxy styrene)

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Poly(3-methoxy-4-hydroxy styrene) (MHS) was prepared as a biodegradable polymer; it offers a simplified model of naturally biodegradable polymers related to softwood lignin, having pendant guaiacyl groups. This polymer was decomposed by microorganisms in soil, and four intermediates were identified in the degradation pathway. Judging from the identified intermediates, vanillic acid seems to be the first biodegradation product of polyMHS in the pathway. The ring cleavage of vanillic acid gives rise to monomethyl ester of β-carboxymuconic acid, discovered as an intermediate in the degradation pathway. Monomethyl ester of β-carboxymuconic acid, which was isolated and characterized, is decomposed to maleic and oxalic acids by microorganisms which are essential for utilization of guaiacyl group. Elucidation of the degradation pathway of polyMHS revealed that the step reactions responsible for the conversion of this compound to maleic and oxalic acids are somewhat similar to the step reactions responsible for the degradation of lignin by microorganisms.

INTRODUCTION

In recent years, various kinds of synthetic polymer have been produced and used widely. In particular, the properties of synthetic polymers which are not degradable by microorganisms have been very advantageous in their use as packaging and construction materials. However, the public problem of disposal of these polymers has arisen because of the difficulties of their natural decomposition.

The decomposition of water-soluble polymers have been thought to be easier than that of water-insoluble ones. Poly(ethylene glycol)[1,2] and poly(vinyl alcohol)[3], for example, are known to be attacked easily by microorganisms, although oligomers of water-insoluble polymers such as polyethylene[4], polyurethanes[5], aliphatic polyesters[6], and nylon-6[7] are also known to be decomposed by microorganisms although they are not really suitable for biodegradation.

We have tried to find a good model for a biodegradable synthetic polymer among natural polymers, almost all of which are known to be biodegradable: we paid attention to lignin which exists abundantly next to cellulose in higher plants. Poly(3-methoxy-4-hydroxy styrene) was prepared as a model polymer whose structure is related to softwood lignin. This styrene derivative has a simpler structure than lignin and its biodegradation was expected because of its pendant guaiacyl groups. As is well known, the guaiacyl group is recognized as the main constituent of softwood lignin.

The present investigation was undertaken to see if poly(3-methoxy-4-hydroxy styrene) could be degraded by microorganisms isolated from fresh garden soil. The degradation process of the above polymer was also estimated from the degradation products.

EXPERIMENTAL

Materials

3-Methoxy-4-hydroxy styrene (MHS) was prepared from ferulic acid by a procedure similar to Sovish's method[8]. Ferulic acid (10 g) and hydroquinone (0.3 g) were dissolved in quinoline (10 ml). Copper powder (0.2 g) was added to the above solution. Then the mixture was heated at 190°C under a nitrogen atmosphere until the evolution of carbon dioxide subsided. The resulted mixture was distilled under reduced pressure (0.1 mmHg). The fraction boiling at 85°–90°C was collected. The obtained crude MHS was added to cold 1 N hydrogen chloride solution, extracted by ethyl ether (free from peroxide), and then dried with anhydrous sodium sulphate. After the removal of sodium sulphate and ethyl ether, MHS was purified by vacuum distillation. The yield was 4.8 g, 62%

PolyMHS was obtained by bulk polymerization using 1% of 2,2'-azobisisobutyronitrile (AIBN) as an initiator. MHS was sealed in a glass tube under 10^{-4} mmHg, being polymerized at 90°C for 4 h. The polymerization mixture was dissolved in methanol, then added to water. The white precipitate was collected by filtration (M_n = 2000)[9].

Microbiological materials, media and conditions of cultivation

Cultures were prepared in the following way. Fresh garden soil (50 mg) was added to 1 litre of distilled water. The mixture was stirred for 1 h and filtered through Toyo Filter Paper No. 4. Then the following mineral bases were added to the filtrate: NH_4NO_3 (20 g/l); $K_2HPO_4 \cdot 12H_2O$ (1.5 g/l); $MgSO_4 \cdot 7H_2O$ (0.5 g/l). The pH was adjusted to 7.0 with 4% H_3PO_4 and 1% KOH solutions. The medium (100 ml) was dispensed into sterile shaking flasks. The carbon source polyMHS sample (0.05 g) was aseptically added. The flasks

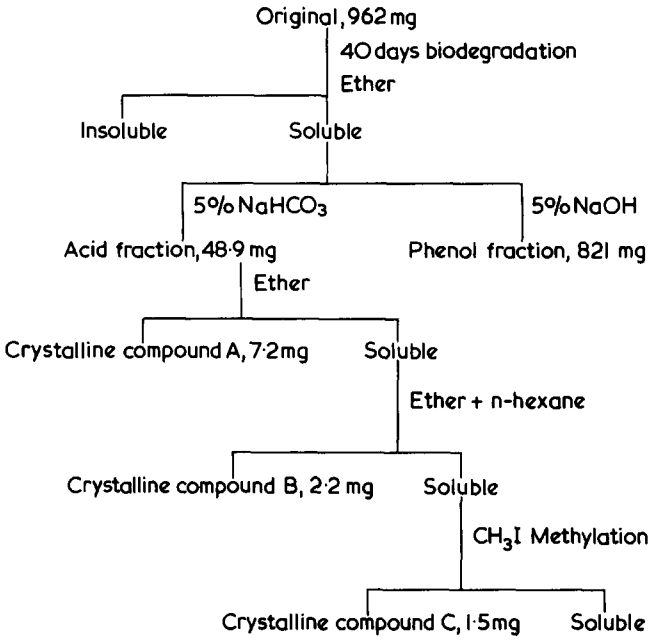

Figure 1 Fractionation of biodegraded polyMHS

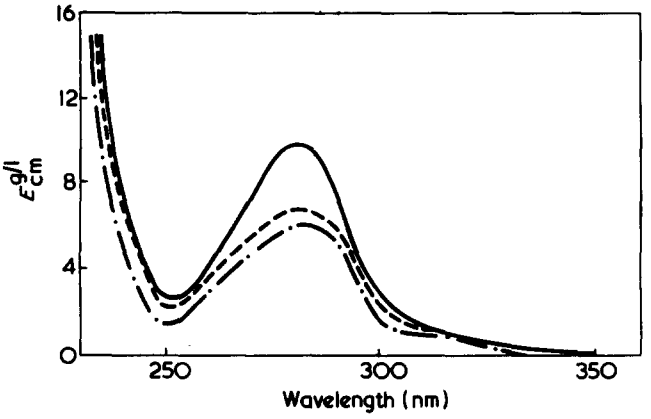

Figure 2 Change of ultraviolet spectra of polyMHS with incubation time: ——, original; — — — —, 10 days; — . —, 20 days

were shaken reciprocally at 30°C for a definite length of time.

When large quantities of degradation products were required for chemical and physical determination, they were grown on a large scale. In order to check the autoxidation of the carbon source, blank tests were carried out with the same media containing no microorganisms.

Chemical and physical determination

Ultra-violet absorption spectra were determined on a Hitachi Model 139 recording spectrophotometer. Infra-red absorption spectra were determined on a Perkin–Elmer model 180 grating spectrophotometer. Mass spectrograms were obtained by using JEOL model JMS 07. The molecular weight of the samples was measured with a Hitachi Model 115 molecular weight apparatus.

Biodegraded products were fractionated by the following method (*Figure 1*). After incubation in a thermostat at 30°C for 40 days, the cells were removed by centrifugation. The remaining reaction mixture was washed and extracted with ethyl ether. Then the ether-soluble part was separated into acid and phenol fractions by washing with 5% $NaHCO_3$ and 5% NaOH solutions. From the acid fractions crystalline compounds A and B were obtained. The crystalline compound C was obtained after CH_3I methylation of the solution. The methyl esters of the acid products were analysed using a Hitachi gas-chromatograph model 163-FID under the following conditions: isothermally at 80°C and 150°C; 1m x 5mm column of SE-30, 80 mesh; carrier gas highly pure N_2 at 35 ml/min; detector, flame ionization detector.

RESULTS AND DISCUSSION

The cultures isolated from soil by the enrichment technique[10] gave at least two organisms which were effective for the degradation of polyMHS. The isolates were identified as *Moraxella* and *Penicilium* species as reported elsewhere[10].

The ultra-violet spectrum of polyMHS which was obtained from the polyMHS cultures, stopped at different stages of substrate utilization, was measured after dissolving in EtOH. The absorption peak of phenyl groups appeared at 280 nm decreased with incubation time as shown in *Figure 2*. The spectral change in the ultra-violet spectrum shows that polyMHS was degraded by the microorganisms. In particular, the decrease of intensity of the absorption peak at 280 nm is considered to be the result of the cleavage of the phenyl groups of polyMHS.

The infra-red spectrum at 1500 to 2000 cm^{-1} in polyMHS incubated with microorganisms is shown in *Figure 3*. The absorption of the C=O stretching vibrations appeared[11] at about 1675 to 1725 cm^{-1}. The spectral change in the infra-red spectrum of polyMHS obtained from the cultures, stopped at different incubation times, shows that carbonyl

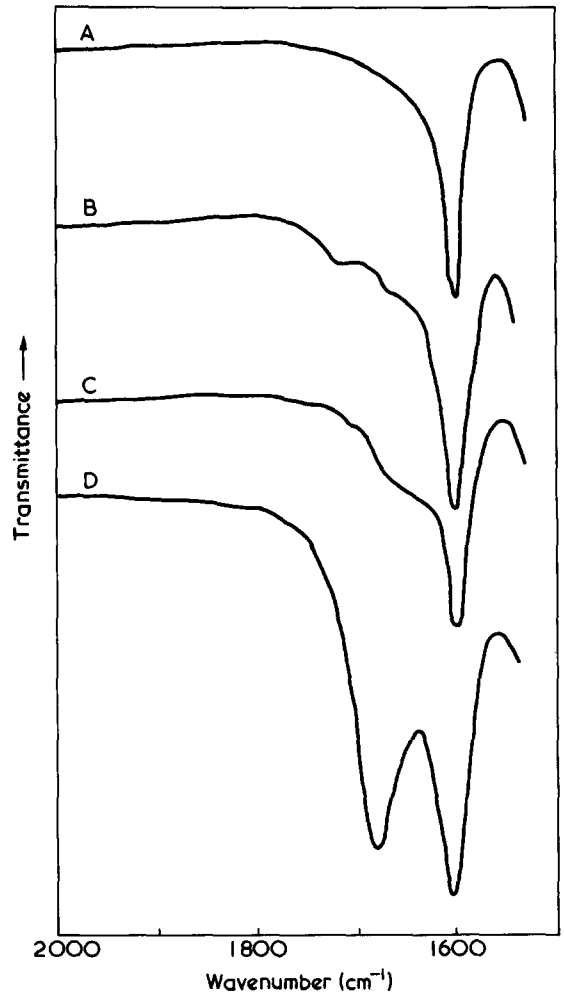

Figure 3 Change of infra-red spectra of polyMHS with incubation time: A, original; B, 5 days; C, 10 days; D, 20 days

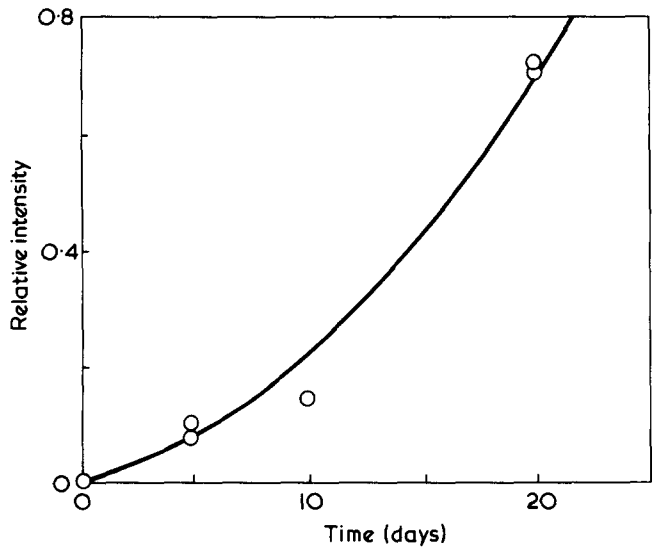

Figure 4 Change of relative intensity of C=O stretching band at about 1700 cm^{-1} in infra-red spectrometry of polyMHS

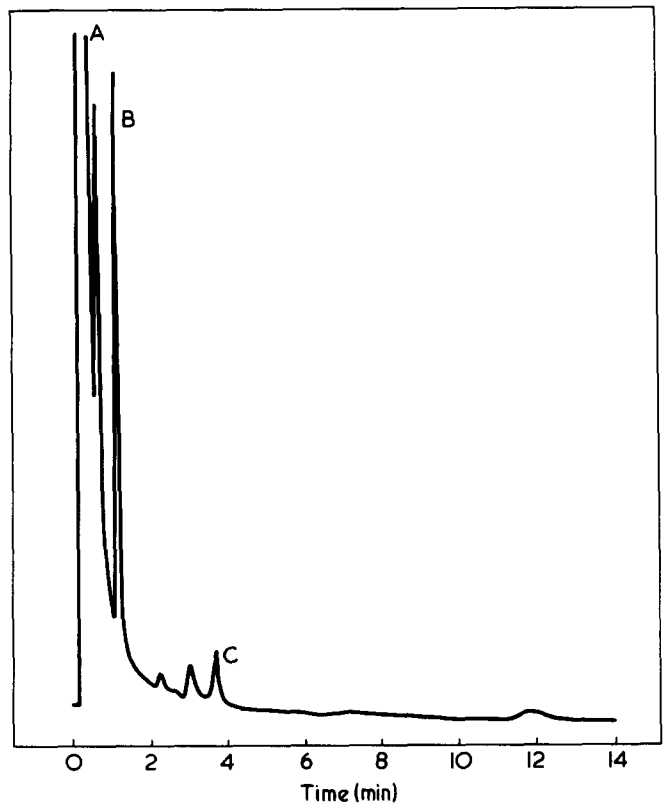

Figure 5 Gas chromatographic separation of the methylated acid fraction of biodegraded polyMHS; isothermal at 80°C: A, solvent; B, dimethyl oxalate; C, dimethyl maleate

groups were formed in the molecules when polyMHS was incubated with microorganisms.

The intensity of the C=O bands evidently increased with incubation time (*Figure 3*). The intensity of each peak due to C=O stretching vibrations is expressed in the form of the relative intensity measured by the base-line method[12]. The relative intensity, which is defined as the ratio of the intensity of the band to that of a suitable band in the same spectrum, is not significantly affected by concentration in the pellet and variation in scattered light. In this experiment, the relative intensity is expressed as a_x/a_{2900}, where x refers to the bands at about 1700 cm^{-1}.

The relative intensity at the C=O stretching band increased markedly with incubation time, as shown in *Figure 4*. This change of the band intensity shows that the degradation of polyMHS increased exponentially with incubation time.

The acid-ether-soluble fraction was prepared as described above from the polyMHS cultures grown on a large scale and stopped at 40 days. Substrate utilization was examined by gas liquid chromatography (g.l.c.) after methylation by CH_3I. The methyl ester fractions gave g.l.c. peaks with retention times of 1.6 min (similar to dimethyl oxalate) and 3.8 min (corresponding to dimethyl maleate) at 80°C as shown in *Figure 5*. The g.l.c. peaks with retention times of 3.8 min (corresponding to trimethyl β-carboxymuconate) and 5.8 min (identical with methyl veratrate) at 150°C are as shown in *Figure 6*.

The acidic and methylated components of the fraction were separated by preparative crystallization and three compounds A, B and C were obtained as crystals as described above. Compound A was obtained as colourless needles. The recrystallized needles melted at 101°–102°C. Physcial and chemical investigation indicated that compound A is a dibasic acid. Its molecular weight is 126 in agreement with the value for empirical formula $C_2H_2O_4 \cdot 2H_2O$. Elemental analysis for the anhydride showed: calculated, C 26.68; H 2.24; found, C 26.70, H 2.20. Mass spectrometry of compound A showed fragments identical with oxalic acid (*m/e*, 44, 45, 46 corresponding to COO, COOH and HCOOH, respectively). The infra-red spectrum of compound A is also identical with oxalic acid as shown in *Figure 7*. The colourless plates crystallized by adding small amounts of n-hexane to the residual ether solution melted at 143°–144°C. Its molecular weight is 200. Elemental analysis showed, for $C_8H_8O_6$: calculated, C 48.01, H 4.03; found, C 48.06, H 4.01. The infra-red spectrum of compound B is identical with monomethyl ester of β-carboxymuconic acid, as shown in *Figure 8*, which was previously synthesized as an authentic sample according to the

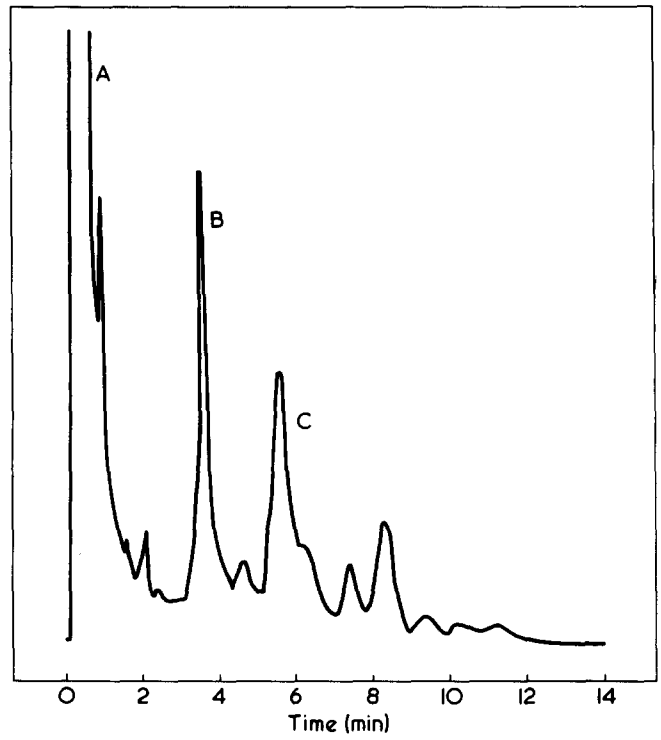

Figure 6 Gas chromatographic separation of the methylated acid fraction of biodegraded polyMHS; isothermal at 150°C: A, solvent; B, trimethyl β-carboxymuconate; C, methyl veratrate

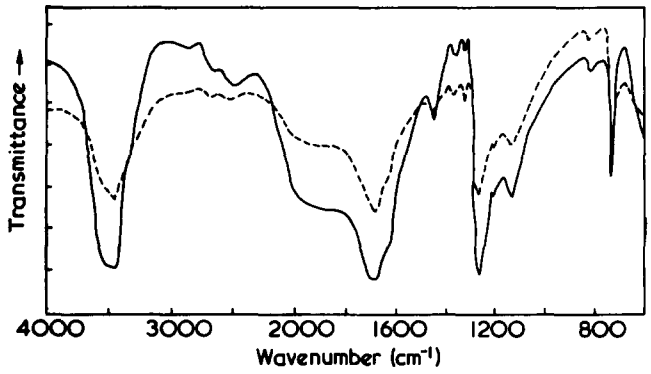

Figure 7 Infra-red spectra of crystalline compound A (——) and oxalic acid (— — — —)

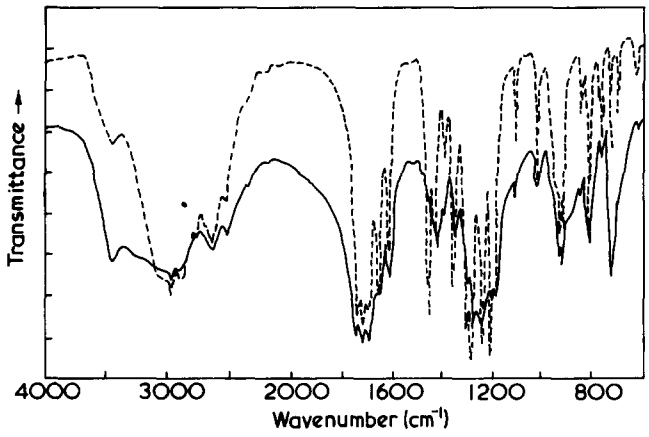

Figure 8 Infra-red spectra of crystalline compound B (——) and monomethyl ester of β-carboxymuconic acid (— — — —)

procedure reported[13] by Husband *et al.* Compound C, crystallized from the ether solution of the acid fraction methylated by CH_3I as colourless needles, melted at 59°–60°C. Its molecular weight is 196. The infra-red spectrum of compound C corresponds to methyl veratrate, as shown in *Figure 9*.

As *Moraxella, Penicilium* and some other species of microorganisms in soil seem to be essential for the degradation of polyMHS, the polyMHS sample used in this experiment is considered to be degraded via a pathway analogous to the degradation of lingnin and its model compounds by those microorganisms. *Figure 10* is tentatively put forward as the most likely representation of the events (identified intermediates are numbered in Roman numerals but the intermediate III is hypothetical). PolyMHS is degraded to β-carboxymuconic acid derivative (IV) presumably through cleavage of the benzene ring between C_3 and C_4 via either or both of the following two pathways. One possible pathway is the degradation of the benzene ring through vanillic or protocatechuic acid (II). The other pathway can be possibly through the intermediate III. Then the β-carboxymuconic acid derivative (IV) is degraded to maleic (V) and oxalic acid (VI) derivatives.

In the above scheme, the presence of the intermediate II was estimated from methyl veratrate obtained as crystals. Accordingly, it is difficult to elucidate that either or both of vanillic (R=CH_3 in II) and protocatechuic (R=H in II) acids are essential in the pathway of the degradation from compound I to compound IV. It is well known that protocatechuic acid is degraded to β-carboxy-*cis, cis*-muconic acid[14]. Kawakami[15] reported that vanillic acid is degraded to protocatechuic acid by *Pseudomonas ovalis* and *Pseudomonas fluorescens*. This report seems to suggest that demethylation of vanillic acid to protocatechuic acid takes place before cleavage of the aromatic ring. On the contrary, Zabinski *et al.*[16] showed that methyl ester of α-hydroxy-γ-carboxy-*cis, cis*-muconic acid formed when PCA-4,5-oxygenase cleaves 5-methoxy gallic acids. As described previously, monomethyl ester of β-carboxymuconic acid was obtained and identified as crystal plates in our experiment. These results seem to show that the ring fission could take place without prior demethylation. Therefore, it may be estimated that the degradation of polyMHS via vanillic acid followed by aromatic ring cleavage to monomethyl ester of β-carboxymuconic acid is the most likely process. Judging from the observations that maleate was detected by gas chromatography and that oxalic acid was obtained and identified as crystals, β-carboxymuconate (IV) may be degraded to maleate (V) and oxalate (VI) via some similar pathway of the degradation reported[17] by Ornston *et al.*

It is difficult at present to gauge how the catabolic pathway of polyMHS operates in nature, although the hypothetical process of dissimilation can possibly be assumed by comparing it with the better known process of lignin and its model compounds. Considering the soil environment, this overall reaction probably requires the cooperation of several microorganic species.

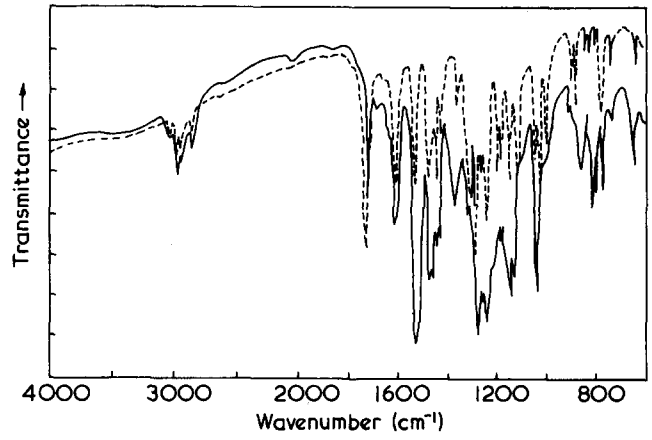

Figure 9 Infra-red spectra of crystalline compound C (——) and methyl veratrate (— — — —)

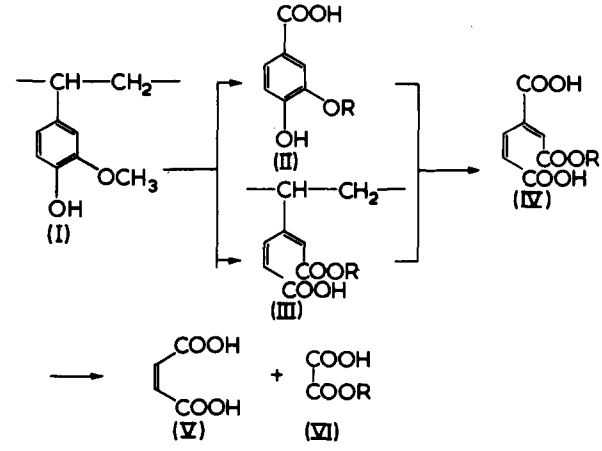

Figure 10 Tentative degradation scheme of polyMHS by microorganisms in soil. The intermediates numbered in Roman numerals have been chemically identified in the forms shown, where R is CH_3 in II and IV, and H in VI; III is hypothetical

ACKNOWLEDGEMENTS

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