# Biosynthesis of Polyhydroxyalkanoate with a Thiophenoxy Side Group Obtained from *Pseudomonas putida*

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ABSTRACT: Poly(3-hydroxyalkanoate) (PHA) with a thiophenoxy group at the end of the side chain was obtained from *Pseudomonas putida* 27N01, using 11-thiophenoxy undecanoic acid as the sole carbon source. NMR and GC/MS analyses indicated that the polymer consisted of 3-hydroxy-5-thiophenoxypentanoate as the primary monomer unit and 3-hydroxy-7-thiophenoxyheptanoate as a minor unit. The polymer had a white cream color and was elastometric. X-ray analysis suggested that the PHA had an amorphous-like diffraction pattern. The final dry cell concentration was 693 mg/L, and the PHA content of the dry cells was 19.5%. GPC measurement with polystyrene as a standard showed that the number-average molecular weight was 81 000 with a polydispersity of 1.8. The glass transition temperature was approximately 4 °C according to DSC. Thus, we were able to successfully characterize this PHA and to show that a sulfur atom can be incorporated into the bacterial polymer produced from *P. putida* 27N01.

## Introduction

A wide variety of bacteria accumulate poly(3-hydroxyalkanoate) (PHA) from several carbon sources, including sugars, alcohols and fatty acids. The production of novel PHAs has been attempted particularly in *Ralstonia eutropha* and *Pseudomonas oleovorans*, which are known to produce several types of PHAs. *R. eutropha* has the ability to synthesize polyesters consisting of short chain units, which have three to five carbon atoms. Polymers containing 3-hydroxybutyrate, 3-hydroxypentanoate,[1,2] 3-hydroxypropanoate,[3] and 4-hydroxybutyrate units[4,5] have been produced by *R. eutropha*. On the other hand, a polyester consisting of 6−14 carbon atom repeating units has been produced by *P. oleovorans*.[6−8] In addition, various functional groups have been incorporated into PHAs, including halogen,[9] unsaturated,[10] cyano,[11] phenyl,[12] phenoxy,[13] and branched alkyl,[14] as observed in PHAs obtained from the *Pseudomonas* species. PHAs with *n*-alkyl side chains longer than the ethyl group form elastomers with a melting point below 60 °C and glass transition temperatures below −20 °C. Thus, such PHAs demonstrate superior physical properties. We have had particular interest in PHAs with thiophenoxy side chains due to their potentially unique physical properties. Using *P. putida* 27N01, a newly isolated bacterial strain, we investigated the fermentation and production of PHAs bearing thiophenoxy groups from corresponding fatty acids, and report some of the results herein.

## Experimental Section

**Synthesis of Carbon Sources.** The general synthesis procedure used for the preparation of 11-thiophenoxyundecanoic acid (TPUDA), 5-thiophenoxypentanoic acid (TPPA), and 11-(4-methylthiophenoxy)undecanoic acid (4MTPUDA) is as follows. We added 0.11 mol of potassium hydroxide and 0.07 mol of benzenethiol (Aldrich Chemical Co., 97%) or 4-methylbenzenethiol (Aldrich Chemical Co., 98%) to a 500-mL round-bottom flask containing 200 mL of ethanol. To this mixture was added0.06 mol of either 11-bromoundecanoic acid (Aldrich Chemical Co., 99%) or 5-bromopentanoic acid (Aldrich Chemical Co., 97%). The reaction was allowed to continue at the reflux temperature of ethanol for 24 h. The corresponding acids were precipitated by adding the reaction solution to 200 mL of 0.1 M aqueous HCl. The solution was then filtered, dried in vacuo, and purified by recrystallization from hot methanol. The yields of the purified acids ranged from 40 to 80%. The structure was confirmed by NMR spectroscopy, and the purity was checked by TLC.

**Cell Growth.** We used a new isolate, *P. putida* 27N01, throughout the experiment. This strain was isolated from soil and identified by the National Collections of Industrial and Marine Bacteria Limited (NCIMB) in Scotland. As a preculture, *P. putida* was cultivated under aerobic conditions at 30 °C and pH 7.0 for 48 h on a reciprocating shaker in 10 mL of medium containing defined mineral salts and 20 mM octanoic acid as a carbon substrate. The composition of the mineral salt medium was as follows (per liter): 1.5 g of $(NH_4)_2SO_4$, 1.4 g of $KH_2PO_4$, 1.4 g of $Na_2HPO_4$, 0.5 g of $NaHCO_3$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of yeast extract (Difco), and 0.05 g of ammonium iron(III) citrate. In addition, 0.1 mL of trace element solution was added to the medium. The trace element solution contained the following salts (per liter): 3.0 g of $H_3BO_4$ 2.0 g of $CoCl_2 \cdot 6H_2O$, 1.0 g of $ZnSO_4 \cdot 7H_2O$, 0.3 g of $MnCl_2 \cdot 4H_2O$, 0.2 g of $NaMoO_4 \cdot 2H_2O$, 0.28 g of $NiSO_4 \cdot 7H_2O$, and 0.1 g of $CuSO_4 \cdot 5H_2O$. After preculture, 1 mL of the cells was inoculated with modified medium containing TPUDA, TPPA, or 4MTPUDA as the unusual carbon sources. The first cultivation was carried out in a 500-mL flask containing 100 mL of medium under the same conditions as the preculture for up to 2 weeks. Thereafter, the cells were inoculated and grown for a week in a 5-L jar-fermenter (model KMJ-5C; Mitsuwa Bio Systems Co., Osaka, Japan) in a culture volume of 3 L, containing the same medium composition. In the fermenter, agitation speed, aeration rate, and temperature were routinely set at 500 rpm, 0.7 $vol \cdot vol^{-1} \cdot min^{-1}$, and 30 °C, respectively. The pH of the culture was adjusted by addition of 3 N KOH or 2 N HCl using a pH

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controller. Antifoam (Adecanol LG-109; Asahi Denka Kogyo Co., Tokyo, Japan) was added to the medium at a concentration of 0.03% (vol/vol). Direct monitoring of cell growth by measuring optical density was not possible due to emulsified liquid particles of undissolved carbon sources. Therefore, the mixture was filtered through a cellulose filter paper (Toyo Roshi Kaisya, Ltd., porosity no. 2) to remove the particles and was measured daily at 600 nm to obtain growth curves.

**Polymer Isolation.** The cells were harvested by centrifugation, washed with 30% methanol solution in water, dried, and weighed. Dried cells were suspended in 100 mL of chloroform at room temperature for 24 h. The mixture was filtered through a cellulose filter paper, and the filtrate was concentrated by evaporation. The polymer was reprecipitated in methanol and dried to determine the PHA yield.

**Analytical Procedure.** To identify the composition of the polymer by GC−MS, samples were prepared as previously described.[15]Methyl esters of PHA monomers were analyzed by a Hewlett-Packard 5890 system equipped with a J&W Scientific capillary column (30 mm × 0.25 mm) and a total ion detector.

The $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra of PHA samples were recorded on a JEOL JNM-EX 400 FT-NMR spectrometer. The 400 MHz $^1$H NMR and the 100 MHz $^{13}$C NMR spectra were recorded at 25 °C in a $CDCl_3$ solution of the polymer.

The average molecular mass and molecular mass distributions of the polymer were obtained at 30 °C by gel permeation chromatography (Shodex GPC system 11; Showa Denko K. K.; Tokyo, Japan) using AC-800P, AC-80M, and AC803 sample columns. Chloroform was used as an eluent at a flow rate of 1.0 mL·min$^{-1}$. Polystyrene standards with a low polydispersity were used to construct the calibration curve.

Thermal characterization was carried out using a Seiko DSC-20 differential scanning calorimeter (DSC) equipped with an SSC580 data station. The sample was sealed in an aluminum pan, and analysis was carried out while maintaining a dry nitrogen purge. The polymer sample was analyzed at a heating rate of 10 °C/min from −30 to +30 °C (lower temperature heating scan) and from 25 to 140 °C (higher temperature heating scan). The sample was cooled rapidly by quenching in liquid nitrogen, and then analyzed again during a second heating scan from −30 to +30 °C and from 25 to 140 °C.

X-ray diffraction analysis was carried out using a Routaflex RU-200 generator operating at 40 kV and 140 mA. Nickel-filtered Cu Kα radiation ($\lambda$ = 1.540 50 Å) was used. An approximately 0.3 mm polymer sample was exposed to the X-rays.

## Results and Discussion

**Cell Growth.** Although the carbon sources were insoluble in water, *P. putida* grew slowly on the emulsified carbon source. Despite altering the monomer concentrations from 3 to 10 mM, cell growth was not improved, probably due to attachment of the floating carbon source on the inside wall of the glass vessel. The growth curves on the carbon sources are shown in Figure 1. When 11-thiophenoxyundecanoic acid (TPUDA) or 11-(4-methylthiophenoxy)undecanoic acid (4MTPUDA) was used as the carbon source and the optical density was measured at 600 nm, a lag time of 2 days was observed. The cells then increased for 3 days followed by a plateau. In contrast 5-thiophenoxypentanoic acid (TPPA) took 6 days to reach a plateau. These data suggest that the distance between the functional group and the carboxyl group is related to cell growth. This result agrees with the findings of another report on *P. oleovorans*.[16] The dry cell weight, polymer weight, and polymer yield from each carbon source are summarized in Table 1. When *P. putida* was cultivated on

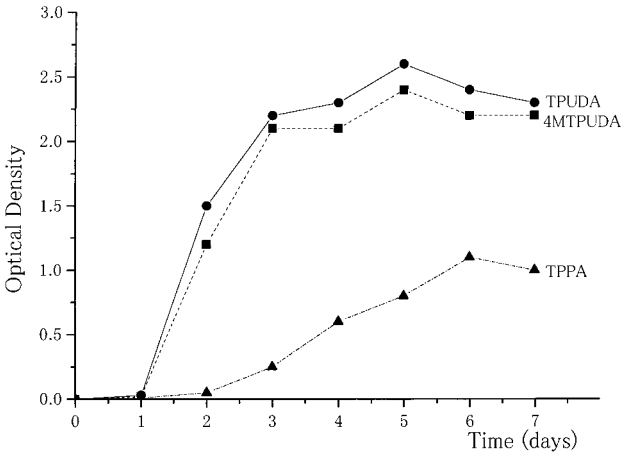


**Figure 1.** Growth of *P. putida* as determined by optical density measurement, on different unusual carbon sources (3 mM/L): (■) 4MTPUDA; (●) TPUDA; (▲) TPPA.

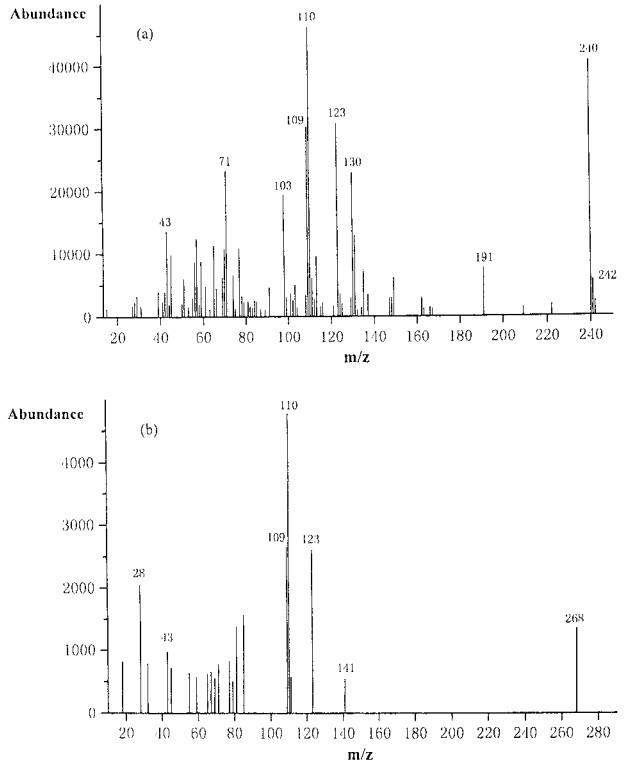


**Figure 2.** (a) Mass spectrum of the peak with retention time of 13.14 min in the gas chromatogram of the methanolyzed sample of the PHA obtained from TPUDA. (b) Mass spectrum of the peak with retention time of 15.49 min in the gas chromatogram of the methanolyzed sample of the PHA obtained from TPUDA.

TPUDA as the sole carbon source, a final cell concentration of 693 mg/L and a polymer content of 19.3% calculated from the dry cell weight were attained. No polymeric material was obtained from fermentation with 4MTPUDA, although the cell growth was the same as that of TPUDA. In the case of TPPA, cell growth was slower, and only trace amounts of the polymer were found. The polymer obtained from TPUDA was the only polymer obtained in sufficient quantities for determination of its properties.

**Polymer Characterization.** The polymer obtained from *P. putida* was characterized by both GC/MS and NMR spectroscopy. For GC/MS analysis, both the dry cell sample and the isolated polymer were methanolized

**Table 1. Final Cell Concentration and Polymer Content of the Dry Cells of *P. putida* Grown on Different Carbon Sources**

| carbon source | culture time (days) | biomass (mg/L) | polymer (mg/L) | polymer content (%) |
|---|---|---|---|---|
| thiophenoxyundecanoic acid (TPUDA) | 7 | 693 | 135 | 19.5 |
| (4-methylthiophenoxy)undecanoic acid (4MTPUDA) | 7 | 606 | | |
| thiophenoxypentanoic acid (TPPA) | 10 | 31 | 3 | 9.6 |

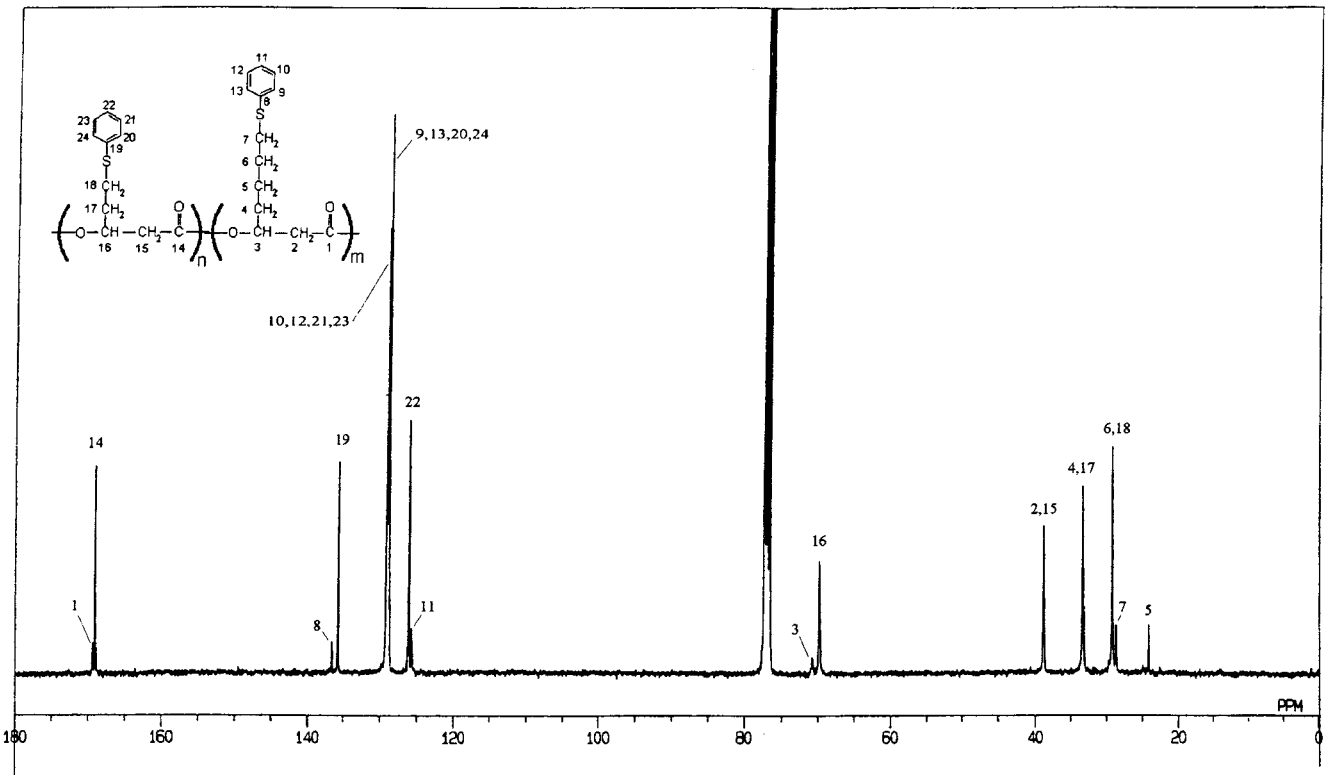


**Figure 3.** $^{13}$C NMR spectrum of the polymer produced by *P. putida* grown with TPUDA.

to convert the polymer into methyl 3-hydroxyalkanoate with the functional group.[17] The chromatograph of the mathanolized polymer produced from TPUDA fermentation had two major peaks that were observed at 13.14 and 15.49 min. To identify these two peaks, a mass spectroscopy measurement was performed. The results are presented in parts a and b of Figure 2. On the basis of the two peak areas, R.T.13.14 min was predominant and R.T.15.49 min was minor, at a ratio of approximately 15:1. Because of the low concentration of the R.T.15.49 compound, Figure 2b has a smaller number of *m*/*z* peaks. In Figure 2a, characteristic *m*/*z* peaks (43, 71, 103) from methyl 3-hydroxyalkanoates were detected. Similar *m*/*z* peaks (71,43) were detected in Figure 2b. Characteristic *m*/*z* peaks derived from thiophenoxy structure $C_6H_5S$ (109, 77, 32) were detected in both R.T. compounds. Judging from the maximum peak (242 and 268) in each mass pattern, R.T.13.14 min and R.T.15.49 min were assigned as methyl 3-hydroxy-5-thiophenoxypentanoate and methyl 3-hydroxy-7-thiophenoxyheptanoate, respectively.

The $^{13}$C NMR spectrum of the polymer is shown in Figure 3. The obtained chemical shift supports the GC/MS result. Judging from the $^{13}$C NMR peaks appearing from 110 to 160 ppm, the signal pattern of the polymer resembles that of TPUDA, indicating that the thiophenoxy structure is well preserved in the polymer side chain.

The molecular weights of the polyester were determined by GPC in comparison with the polystyrene standard. The weight-average molecular weight, and the number-average molecular weight was 146 000 and 81 000, respectively. The polydispersity had a narrow value of 1.8.

The DSC experiment revealed a glass transition point around 4 °C, with no clear melting point. The polymer had a white cream color and was elastomeric. The X-ray diffraction pattern supported the finding that the polymer obtained from solvent casting was amorphous-like.

The present study investigated a polymer with a thiophenoxy group in the structure produced by *P. putida*. To the best of our knowledge, this is the first report of PHA containing a sulfur atom in the structure. A previous study has shown that PHAs with phenoxy groups at the side chain can be produced by *P. oleovorans*.[18] Although the thiophenoxy group is bulkier than the halogen atom or phenyl or other functional groups, the cell yield and polymer content were almost the same or higher. However, 4MTPUA, which has a methyl group substituted on the aromatic site, did not induce polymer production. This result agrees with a previous study on *P. oleovorans* grown on 4-methylphenoxyundecanoic acid.[18] The present results suggest that the bulky size of the side group of alkanoyl−CoA limits its incorporation into the polymer, whereas 4MTPUDA, which has a longer methylene group between the sulfur atom and carbonyl group, is assimilated to support cell growth. On the other hand, TPPA, which has shorter methylene groups, does not promote cell growth. We investigated the PHA with a thiophenoxy group at the end of the side chain and showed that the bacterial PHA

can be biosynthesized from isolated *P. putida*. We believe that the PHA with a sulfur atom will usher in a new era of bacterial polymers.

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