

# Radiation crosslinking of a bacterial medium-chain-length poly(hydroxyalkanoate) elastomer from tallow<sup>1</sup>

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## Abstract

*Pseudomonas resinovorans* produces a medium-chain-length poly(hydroxyalkanoate) (MCL-PHA) copolymer when grown on tallow (PHA-tal). This polymer had a repeat unit composition ranging from C4 to C14 with some mono-unsaturation in the C12 and C14 alkyl side chains. Thermal analysis indicated that the polymer was semi-crystalline with a melting temperature ( $T_m$ ) of  $43.5 \pm 0.2^\circ\text{C}$  and a glass transition temperature ( $T_g$ ) of  $-43.4 \pm 2.0^\circ\text{C}$ . The presence of unsaturated side chains allowed crosslinking by  $\gamma$ -irradiation. Irradiated polymer films had decreased solubility in organic solvents that indicated an increase in the crosslink density within the film matrix. The addition of linseed oil to the  $\gamma$ -irradiated film matrix enhanced polymer recovery while minimizing chain scission. Linseed oil also caused a decrease in the enthalpy of fusion ( $\Delta H_m$ ) of the films (by an average of 60%) as well as enhanced mineralization. The effects of crosslinking on the mechanical properties and biodegradability of the polymer were determined. Radiation had no effect on the storage modulus ( $E'$ ) of the polymer. However, radiation doses of 25 and 50 kGy did increase the Young modulus of the polymer by 129 and 114%, and the tensile strength of the polymer by 76 and 35%, respectively. Finally, the formation of a higher crosslink density within the polymer matrix decreased the biodegradability of the PHA films. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Medium-chain-length poly(hydroxyalkanoate); *Pseudomonas resinovorans*; Radiation crosslinking

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## 1. Introduction

Poly(hydroxyalkanoates) (PHAs) are optically active polyesters biosynthesized by numerous bacteria [1], as intracellular inclusion bodies that serve as carbon and energy reserves. Recently, PHAs have attracted considerable industrial attention because they are biodegradable and, in many cases, their physico-mechanical properties compare fa-

vorably with synthetic polymers [2,3]. Moreover, because they are biocompatible and biodegradable these polymers provide a more 'environmentally friendly' alternative to their synthetic counterparts.

The composition of PHA is dependent on two factors: (1) the enzymatic specificity of the organism, and (2) the carbon substrate provided to the bacteria. Polymerization imparts the [*R*]-stereochemical configuration to the repeat units, resulting in fully isotactic polyesters [4–7]. This stereoregularity enhances crystallization so that PHAs with short alkyl side chains (poly-3-hydroxybutyrate, PHB) are highly crystalline whereas PHAs with longer side chains (poly-3-hydroxyoctanoate, PHO) tend to be more amorphous and

elastomeric [8]. Because of the large number of PHA producers and the broad substrate specificity of the PHA synthases, a wide variety of homo- and co-polyesters have been produced. A number of reviews on PHAs have been published that describe the structural variability of the polymers, the biochemical aspects of polymer formation, genetic control of the process, and potential applications [1,9–12].

Several strains of *Pseudomonas* belonging to the rRNA homology group I accumulate MCL-PHAs [13,14]. These polymers consist of 3-hydroxy fatty acid repeat units ranging in chain length from C6 to C14 when grown on alkanes [15–17], alkanolic acids [13,18,19], or alcohols [20]. Longer alkyl side chains on the  $\beta$ -hydroxy ester polymer backbone have substantial effects on the properties of the polymers, including a decrease in crystallinity and concomitant increase in elastic properties with increased chain length. Also, MCL-PHAs exhibit melting temperatures ( $T_m$ ) that vary between 40 and 60°C and glass transition temperature ( $T_g$ ) values well below 0°C [16,19,21]. As a result MCL-PHA polymers tend to soften at ambient temperatures, an effect that seriously limits potential application.

Fats and oils have only recently been studied as substrates for PHA production. While numerous bacteria can produce MCL-PHA from free fatty acids, the ability to convert triglycerides to PHA has only been demonstrated by *Aeromonas caviae*, which produced poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from olive oil [22], and *Pseudomonas aeruginosa*, which produced a complex copolymer when grown on euphorbia or castor oil [23]. Recently, the formation of another complex MCL-PHA by *Pseudomonas resinovorans* was reported when grown on tallow (PHA-tal) [24]. Because of its high oleic acid content (48%), some of the PHA-tal side chains retain the unsaturation present in the carbon substrate, resulting in a tacky elastomer.

Crosslinking is a well known method used to control polymer properties. Crosslink formation leads to a more uniform polymer matrix, which generally improves the overall physical or chemical properties of the polymer. The presence of double bonds generally enhances crosslinking. It was thought that by inducing the formation of crosslinks the properties of PHA-tal could be enhanced. To date, both chemical (peroxides, sulfur vulcanization) [25,26], and physical (electron

beam irradiation) [27] means have been used to form crosslinks within MCL-PHAs containing unsaturated alkyl side chains. The drawback to chemical crosslinking agents is the production of residual contaminants. Radiation provides an acceptable alternative for crosslinking polymers. Gamma-irradiation (from cesium-137), as opposed to ultraviolet (UV) or electron-beam sources, was chosen as the radiation source for this study because it has high penetration power based on its low linear energy transfer coefficients and undergoes exponential attenuation in the irradiated material. In this study, PHA-tal was isolated, purified, and solution-cast into films. The polymer was characterized before and after crosslinking by tensile testing, dynamic mechanical analysis (DMA), and differential scanning calorimetry (DSC) to compare the effect of  $\gamma$ -irradiation on the physical and mechanical properties of the polymer.

## 2. Materials and methods

### 2.1. Strain information and polymer biosynthesis

*Pseudomonas resinovorans* NRRL B-2649 was obtained from the NCAUR, ARS, United States Department of Agriculture, Peoria, IL. Stock cultures of the organism were produced in shake flasks by growing the organism in medium E\* [18] (100 ml volumes, 250 rpm, 30°C, pH 7.0) with a carbon source consisting of 10–15 mM sodium octanoate. At late log phase (~18 h) the culture was diluted 1:2 with sterile 20% glycerol and the bacteria aseptically transferred in 1.5-ml volumes to sterile cryogenic vials. The vials were frozen in a dry ice-ethanol bath for 15 min and stored at -70°C until use as the inocula for the experiments described below.

Polymer production was done under batch culture conditions in Medium E\* at a volume of 45 l in a 70-l ABEC fermentor (Allentown, PA). The carbon substrate was edible grade tallow (10 g/l) obtained from HRR Enterprises (Chicago, IL). The air flow was less than 15 ml/min, with an agitation rate of 150 rpm. The initial pH of the media was adjusted to 7.0 but the fermentation proceeded with no further pH control. At the conclusion of the fermentation (48 h post-inoculation), the cells were pelleted using a Sharples laboratory super-centrifuge (Pennwalt, Wynnwood,

PA). The cells were lyophilized and stored at 4°C. The crude polymer was extracted from lyophilized cells by Soxhlet extraction with chloroform. The chloroform extract was concentrated on a rotary evaporator and the polymer precipitated by dropwise addition into cold methanol.

## 2.2. Polymer purification

The crude polymer was purified using a flash chromatography technique described by Still et al. [28]. Silica gel (60 Å, 35–75 µm, Analtech, Newark, DE) was used to pack the column (50 mm i.d.). The column was packed dry at a ratio of approximately 45 g silica gel to 1 g crude polymer and washed with 700 ml methylene chloride at a flow rate of approximately 100 ml/min to finalize the packing. Crude polymer (3 g) was loaded onto the column as a 12–15% (w/v) solution in methylene chloride and allowed to enter the column head. The column was then flushed (flow rate approx. 60 ml/min) with 1 l methylene chloride using nitrogen pressure to elute the lipid impurities, followed by 1 l ethyl acetate to elute the PHA polymer. Fractions (10 ml) were periodically monitored by thin layer chromatography (TLC) to determine polymer purity. The ethyl acetate fraction was collected and the solvent removed by rotary evaporation. The polymer residue was dissolved in chloroform and precipitated by dropwise addition into cold methanol. After the polymer precipitate had settled, the supernatant was decanted, and the precipitate was dried under vacuum (25°C, 1 mmHg, 8 h).

## 2.3. Polymer composition

PHA-tal repeat unit compositions were determined by gas chromatography (GC) and GC-mass spectrometry (GC-MS) of the β-hydroxymethyl esters of the hydrolyzed polymers following the procedure of Brandl et al. [18]. The methyl esters were prepared by reacting 10 mg polymer with 6 ml chloroform:methanol:sulfuric acid (6.6:5.6:1, v/v/v), and heating at 100°C for 2 h. After cooling to room temperature, 1.5 ml water was added and the mixture vortexed. The layers were allowed to separate, and the organic layer (bottom) containing the methyl esters was removed and dried over anhydrous sodium sulfate. The solution was then filtered and the methyl esters assayed on a Hewlett Packard (HP, Wilm-

ington, DE) 5890 gas chromatograph equipped with an HP-INNOWax (30 m × 0.53 mm × 1 µm) column and a flame ionization detector. A 2-µl volume was analyzed with split injection (20:1). Helium was used as the carrier gas at 8 ml/min. The temperature of the injector and detector was 250°C. A temperature program of 120–220°C at 3°C/min effectively separated the methyl esters. Quantitation was performed by relative peak areas assuming equal response factors for each methyl ester. The identity of the methyl ester monomers was confirmed by GC-MS using an HP 5890 GC with an HP 5972 Mass Selective Detector. Separations were made with an HP-5 column (30 m × 0.25 mm × 0.25 µm). The injector and detector temperatures for the GC-MS were 230 and 240°C, respectively, with an oven temperature program of 80°C (hold for 2 min) to 230°C at 10°C/min.

## 2.4. Film preparation and irradiation

Films were cast either from solutions of PHA-tal, or PHA-tal with 10% (w/w) linseed oil. Solutions were prepared by dissolving 1.14 g of purified PHA-tal in 15 ml chloroform, and in some cases adding 114 mg linseed oil (cell culture grade, Sigma, St. Louis, MO). Films were cast in glass petri dishes (100 × 15 mm), and the solvent evaporated under a nitrogen atmosphere. The films were subsequently dried under vacuum at 20°C for 7 h. The resulting films were approximately 0.1 mm thick, and were stored desiccated in the dark under a nitrogen purge prior to irradiation. The films in the glass petri dishes were irradiated with 25 or 50 kGy of radiation using a cesium-137 source at 20°C in a nitrogen atmosphere.

## 2.5. Solubility experiments

The solubility of PHA-tal was determined in the following solvents before irradiation: hexane, diethyl ether, chloroform, ethyl acetate, tetrahydrofuran, methylene chloride, acetone, ethanol, methanol, and dimethylformamide. The PHA-tal was added to the above solvents at 1% (w/v) concentrations and each of the solutions magnetically stirred for 24 h at 20°C. The solutions were then visually inspected for clarity and dissolution of solid.

## 2.6. Sol/gel analysis

Sol/gel tests on both non-irradiated and irradiated films were performed with slight modifications according to the method of Gagnon et al. [25]. For each film, a 60–100 mg section was accurately weighed, and placed in a glass vial containing 2 ml chloroform. The film remained in the solvent at room temperature for 24 h, and was occasionally agitated by vortexing. After 24 h, the swollen film or gel was retrieved by filtering off the chloroform solution onto a pre-weighed 0.45  $\mu\text{m}$  nylon filter membrane (Nalge, Rochester, NY) that had been dried overnight in a 60°C oven. The insoluble film was then washed with 3–5 ml chloroform to remove any soluble material. The filter and insoluble film were placed in a 60°C oven overnight and reweighed. The percentage of insoluble film was calculated and the data reported as the average and standard deviation of triplicate determinations.

## 2.7. Molecular weights

Molecular weights were determined by gel permeation chromatography (GPC) using a Waters Model 510 solvent pump, equipped with a Waters refractive index detector Model 410, and 500,  $10^3$ ,  $10^4$  and  $10^5$  Å Ultrastyrigel columns (Waters Chromatography, Milford, MA) in series. Data were collected and tabulated using Millenium software version 2.10 (Waters Chromatography). Polystyrene standards (Polyscience, Warrington, PA) with narrow polydispersities were used to generate a calibration curve. Chloroform was used as the eluent at a flow rate of 1 ml/min. The sample concentration and injection volume were 0.5% (w/v) and 50  $\mu\text{l}$ , respectively.

## 2.8. Mechanical and thermal testing

Tensile testing of rectangular film specimens (7 cm  $\times$  5 mm  $\times$  0.1 mm) was performed using an Instron Model 1122 (Canton, MA). A clamp separation of 25 mm and a crosshead speed of 20 mm/min were used. Dynamic mechanical analyses (DMA) of films were conducted on a Rheometric RSA II Dynamic Analyzer (Piscataway, NJ) using Rhios software version 3.0.1. Films 2 days old and films aged for 12 weeks were cut into rectangular strips (38  $\times$  6.4  $\times$  0.1 mm<sup>3</sup>) and were tested over a temperature range of –125 to 60°C at a ramp rate of 10°C/min and a constant strain frequency of 10.0

rad/s. Differential scanning calorimetry (DSC) was conducted on samples using a Perkin Elmer DSC-7 at a heating rate of 20°C/min over a temperature range of –50 to 100°C.

## 2.9. Biodegradation testing

Two methods were conducted to determine the effects of crosslinking and linseed oil on the biodegradation of the PHA-tal polymer. The first method was performed at the University of Massachusetts-Lowell and involved polymer mineralization (i.e. conversion of polymer carbon to CO<sub>2</sub>). The test was performed using a respirometric method based on that described by Bartha and Pramer [29]. Film samples totaling approximately 115 mg polymer-C (i.e.  $165 \pm 2$  mg total weight; cut into 10  $\times$  10 mm pieces) were buried in test reactors (250-ml biometer flasks; Belco Glass, Vineland, NJ) containing 35 g of a standard soil mix (1:1:0.1, w/w/w mix of potting soil, sand and composted manure; pH  $7.0 \pm 0.5$ ) maintained at 60% water-holding capacity 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 24–168 h intervals and a 4-ml aliquot of the KOH from each trap was titrated with 0.10 M HCl. Daily and cumulative CO<sub>2</sub> production (total and net) and percent mineralization were calculated relative to a control flask (without added polymer films).

The second method involved the fermentation of the polymer-producing bacteria (*P. resinovorans*) using crosslinked and non-crosslinked PHA-tal films as the sole carbon sources. Films were solution cast as described above. One of the films was dried under nitrogen for 6 h and immediately placed into a dry box containing a nitrogen purge to prevent autocatalytic oxidation and crosslinking. Sol/gel analysis (described above) of this film showed that only 1.3% of the film was chloroform-insoluble, suggesting that this polymer was relatively crosslink-free. The second film was dried in a fume hood for 6 h and subsequently stored at 4°C for 10 months. By exposing this film to atmospheric oxygen, it was possible to crosslink the film while minimizing the possibility of chain scission. Sol/gel analysis showed that 81.1% of the film was chloroform-insoluble, indicating a relatively high crosslink density within the film matrix.

Fermentations were conducted at 100-ml volumes in Medium E\* (composition described above). The crosslinked and non-crosslinked polymers were added to the fermentation media at a 0.5% (w/v) concentration. The inoculum was from a frozen cryovial (see above) at a concentration of 0.1%. The cultures were grown at 30°C with shaking at 250 rpm. Aliquots were removed periodically and growth was measured at 660 nm on a Beckman DU 650 spectrophotometer (Beckman, Somerset, NJ) using deionized water as the standard.

### 3. Results and discussion

#### 3.1. Polymer production and properties

It was previously reported that cultures of *P. resinovorans* NRRL B-2649 grown on tallow in shake flask culture produced a MCL-PHA polymer equal to 15% of the overall cell dry weight at PHA volumetric yields of 120–150 mg/l [24]. Larger quantities of PHA-tal, needed for characterization of the mechanical and physical properties of the polymers, were produced in a 45-l batch fermentation. From the fermentation, approximately 40 g crude polymer was obtained; an equivalent of approximately 31% of the cell dry weight. Although fermentation conditions were not optimized, the increase in yield indicated that PHA production by *P. resinovorans* grown on tallow can be improved.

The composition of the PHA-tal polymer was determined by GC and GC-MS analysis of the  $\beta$ -hydroxymethyl esters obtained by acid hydrolysis and methylation of the polymer. The normalized compositional data for the repeat units are listed in Table 1. The monomer chain lengths varied from C4 to C14, with 11% mono-unsaturation present in the C12 and C14 subunits. The

Table 1  
The repeat unit composition of PHA-tal

$\beta$ -Hydroxymethyl ester <sup>a</sup>							
C <sub>4:0</sub>	C <sub>6:0</sub>	C <sub>8:0</sub>	C <sub>10:0</sub>	C <sub>12:0</sub>	C <sub>12:1</sub>	C <sub>14:0</sub>	C <sub>14:1</sub>
Tr <sup>b</sup>	5	20	49	16	5	3	6

<sup>a</sup> Average relative percent ( $n = 3$ , standard deviation  $\leq 0.2$ ) as determined by GC of the  $\beta$ -hydroxymethyl esters obtained by acid hydrolysis of the PHA polymer.

<sup>b</sup> Trace, less than 1%.

weight-average molecular weight ( $M_w$ ) of the polymer was 415000 g/mol with a polydispersity of 2.8. GPC analysis of the crude polymer revealed the presence of an impurity with an elution volume of 35.7 ml (data not shown). A solution of tallow injected into the GPC columns also resulted in a peak at 35.7 ml. This suggested that the extraneous GPC peak observed in the crude polymer was due to triglyceride impurities derived from residual tallow or cellular lipids. GC analysis of the methyl esters from the crude polymer revealed the presence of methyloleate, stearate and palmitate, which are the major components of tallow. This result supported the idea that the extraneous peak in the GPC chromatogram was a tallow impurity and not a lower molecular weight polymer. The tallow contaminant accounted for  $2.3 \pm 0.3\%$  of the total area of the crude polymer sample.

To purify the polymer, the solubility of the PHA-tal in a range of solvents was determined. The PHA-tal was soluble in hexane, diethyl ether, chloroform, ethyl acetate, tetrahydrofuran, methylene chloride, and acetone (partially soluble), while insoluble in ethanol, methanol, and dimethylformamide. Interestingly, the polymer solubilities were similar to the solubility of the tallow substrate. Hence, purification of the PHA-tal polymer based solely on solubility differences was not possible. Polymer purification was achieved by flash chromatography using silica gel as the column packing material (see Section 2). Elution of the column with methylene chloride gave approximately 2% of the starting material. GC analysis of this material showed the presence of methyloleate, stearate and palmitate (data not shown), and no  $\beta$ -hydroxymethyl esters. This indicated that the PHA-tal remained on the column while the impurities eluted in the methylene chloride. The polymer was eluted from the column with ethyl acetate and recovery was 86.4%. The remaining 11.6% of the crude polymer sample was not recovered. GPC analysis of the purified polymer showed an absence of the impurity peak at 35.7 ml. In addition, GC analysis of the purified polymer showed that methyloleate, stearate and palmitate were absent. The molecular weight and monomer composition of the purified PHA was similar to its crude counterpart, indicating that the polymer was not fractionated by the purification method. Furthermore, the crude polymer was yellow in color, whereas the purified polymer was

Table 4

The effects of radiation on the thermal properties of PHA-tal and PHA-tal (linseed oil)<sup>a</sup> films

Film sample	Radiation dose (kGy)	$T_m$ (°C) <sup>b</sup>	$T_g$ (°C) <sup>c</sup>	$\Delta H_m$ (J/g) <sup>b</sup>
PHA-tal	Control	43.5	-43.4	9.6
	25	43.3	-42.3	8.9
	50	43.1	-41.7	9.0
PHA-tal (linseed oil)	Control	43.3	-43.2	3.7
	25	43.1	-43.2	3.4
	50	43.3	-43.3	3.9

<sup>a</sup> Linseed oil was added to the PHA-tal sample at a concentration of 10% (w/w) prior to film formation and irradiation.

<sup>b</sup> Measured by DSC.

<sup>c</sup> Measured by DMA (the maximum value exhibited by the loss modulus,  $E''$ ). The accuracy of each measurement was  $\pm 2.0^\circ\text{C}$ .

that were absent in the control film. In contrast, GC analysis of the sol fraction from the naturally crosslinked (air-exposed) film had a composition identical to the control film. These results indicated that, besides crosslinking, radiation induced some chain scission in the polymer matrix and also that there was a small polymer fraction whose composition did not foster crosslinking. As a result, the formation of a continuously crosslinked film was not possible.

It was thought that an increase in the concentration of olefinic groups in the polymer matrix prior to irradiation might increase the crosslink density of the polymer film. To increase the number of olefinic groups in the polymer matrix, 10% (w/w) linseed oil was added to the polymer mixture prior to film formation and irradiation. Linseed oil, because of its high degree of unsaturation (> 50% linolenic acid), polymerizes under ambient conditions. For this reason, all polymer films (with and without linseed oil) were stored in the dark under nitrogen before irradiation to minimize autocatalytic oxidation and crosslinking reactions. Table 2 shows that the addition of linseed oil increased (~15%) the insoluble content (gel) of the PHA film. Specifically, 75% of the irradiated samples containing linseed oil were recovered as a gel after radiation doses of 25 or 50 kGy. While this was an increase over the irradiated PHA-tal films, these results showed that the formation of a continuously crosslinked film was not possible even with an increased concentration of olefinic groups to the polymer matrix. The addition of linseed oil also resulted in a decrease in the amount of chain scission caused by radiation. While linseed oil did not eliminate chain scission reactions, the molecular weights of the PHA-tal (linseed oil) from the sol fractions showed a more gradual decrease in

comparison to the PHA-tal samples (Table 3). This was further evident by relatively constant polydispersities for the PHA-tal (linseed oil) samples even up to a radiation dose of 50 kGy.

The major objectives of polymer crosslinking are two-fold; first, to reduce or eliminate the crystal structure of the polymer so that the elasticity of the matrix is based primarily on chemical crosslinks, and second, to improve the elastic response of the polymer. DSC and DMA analyses were performed on each film to determine the effects of radiation on the thermal properties of the polymer films. The thermal properties of each film are shown in Table 4. The irradiation of the PHA-tal film showed little effect on the  $T_m$ ,  $T_g$ , or  $\Delta H_m$  of the films. This implied that, under the test methods used, irradiation only slightly affected the crosslink density and crystallinity of the polymer films. The addition of linseed oil to the polymer matrix did not effect either the  $T_m$  or the  $T_g$  of the polymer films. It did, however, decrease the  $\Delta H_m$  by an average factor of 2.5 in both the irradiated and non-irradiated films when compared to the PHA-tal films. It has been reported that the  $\Delta H_m$  of poly(ethylene terephthalate) is directly related to the crystallinity of the polymer [32]. It was concluded, based on the absolute values for  $\Delta H_m$ , that the addition of linseed oil decreased the crystallinity of the polymer but had little effect on the crosslink densities of the films. This was probably due to the high concentration of double bonds within the fatty acid side chains of the triglyceride.

By measuring the tensile strength of the samples, it was possible to show the effect of radiation dose on the strength and flexibility of the polymer. Fig. 2a shows the stress-strain curves of both the non-irradiated and irradiated PHA-tal samples. The results indicated that the non-irradiated film

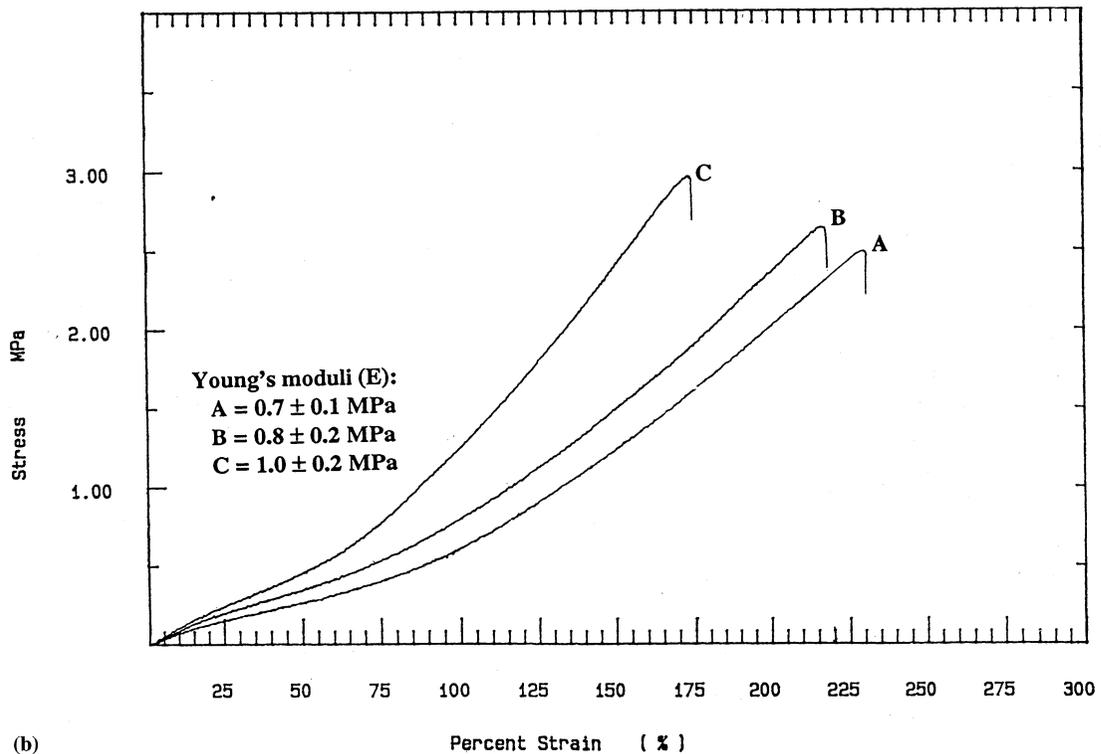
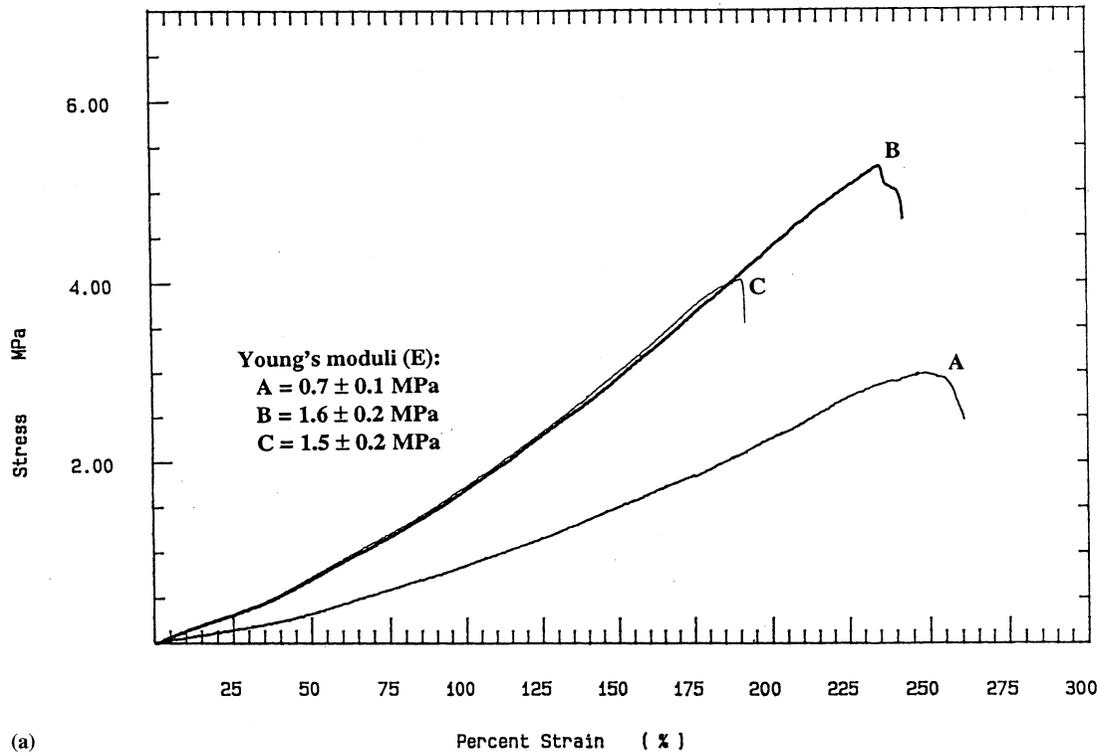


Fig. 2. Stress-strain results for irradiated and non-irradiated PHA-tal films: (a) PHA-tal; (b) PHA-tal + 10% (w/w) linseed oil. The A-curves correspond to non-irradiated control samples for each sample set. The B-curves correspond to samples that were irradiated at 25 kGy. The C-curves correspond to samples that were irradiated at 50 kGy.

was more flexible, but weaker compared to the irradiated films. This was evident by absolute values of 248% strain at peak, 3.0 MPa stress at peak, and

a Young modulus ( $E$ ) of 0.7 MPa in the control film. By applying a radiation dose of 25 kGy, the polymer exhibited a 5% decrease in the percent strain at

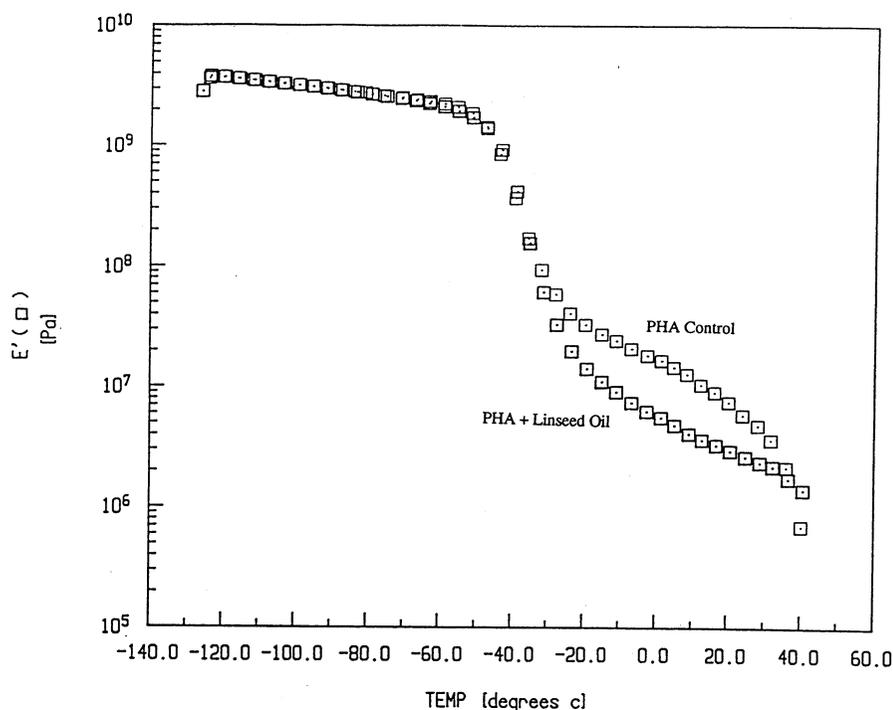


Fig. 3. DMA analysis showing the relative average effect of linseed oil on the storage modulus ( $E'$ ) of the polymer films ( $n = 3$ ). Radiation doses up to 50 kGy showed no effect on either the PHA control or the PHA + 10% (w/w) linseed oil.

peak, a 76% increase in its stress at peak and a 1.3-fold increase in modulus. These results implied that the polymer was made slightly stronger and more rigid by inclusion of a higher crosslink density. Typically, elastomers show a reduction in tear resistance as the crosslink density of the polymer increases. At a radiation dose of 50 kGy, the percent strain at peak further decreased by 19%, while the stress at peak (tear resistance) of the polymer film decreased by 24%. This reduction in tear resistance indicated that a threshold was reached between radiation doses of 25 and 50 kGy where the film became more brittle. At the same time, the modulus remained relatively constant. These results indicated that this polymer was indeed reacting in a manner similar to other crosslinked elastomeric materials with regard to tensile strength.

Based on the stress-strain curve (Fig. 2b), the addition of linseed oil to the film matrix caused a slight decrease in polymer flexibility (from % strain at peak) as radiation dose increased. Without radiation, the flexibility of the PHA-tal (linseed oil) was 7.1% less than its PHA-tal counterpart. At radiation doses of 25 and 50 kGy, the flexibility of the PHA-tal (linseed oil) decreased from 230.1 to 216.2 and 173.4% strain at peak, respectively. This indicated that the addition of linseed oil marginally increased the crosslink density of the films. How-

ever, it also was possible that linseed oil molecules self-polymerized and phase-separated to cause the slight decrease in polymer flexibility. Interestingly, the tensile strength at peak for the linseed oil-containing samples was relatively depressed compared to the PHA-tal films. At 50 kGy of radiation, the tensile strength of PHA-tal (linseed oil) (2.96 MPa) was identical to the non-irradiated PHA-tal control film (2.99 MPa). These results, along with the thermal properties and molecular weight data, suggested that the linseed oil was functioning more like a plasticizer in the polymer matrix rather than providing loci for increased crosslink formation. DMA analysis of the PHA-tal and PHA-tal (linseed oil) (Fig. 3) showed that the polymer film containing linseed oil had a lower modulus regardless of radiation dose, further supporting the idea that linseed oil was functioning as a plasticizer within the film matrix.

### 3.3. Biodegradation

All bacterial PHAs have been classified as biodegradable because, while numerous bacterial species produce PHAs, many bacterial species, including *Pseudomonas fluorescens* and *Pseudomonas lemoignei*, also produce the extracellular depolymerases necessary for polymer breakdown

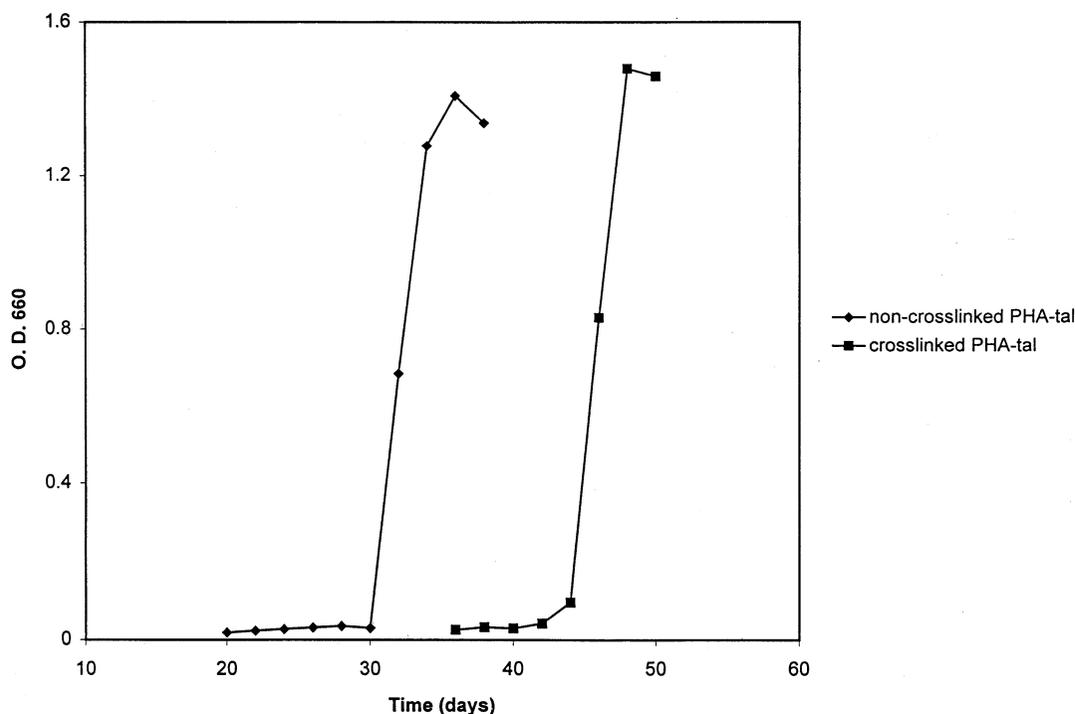


Fig. 4. Growth of *P. resinovorans* on non-crosslinked and crosslinked PHA-tal films.

[27,33]. The introduction of crosslinks into the polymer matrix was evaluated for its effect on polymer film degradation. This was tested by growing *P. resinovorans* (the polymer-producing organism) in cultures containing non-crosslinked and crosslinked PHA-tal as the sole carbon source. The increased crosslink density in the polymeric carbon source resulted in an increased lag time (from 32 to 46 days) in the culture (Fig. 4). Optical density (660 nm) measurements revealed that the non-crosslinked PHA-tal supported noticeable bacterial growth 14 days prior to the culture containing the crosslinked polymer. Based on these results, it was evident that the presence of covalent crosslinks within the matrix had a detrimental effect on bacterial growth and consequently biodegradation. However, the fact that the organism could grow on the polymer at all indicated that, with or without a high crosslink density, the PHA-tal was at least partly biodegradable.

The effect of linseed oil on the biodegradability of PHA-tal was also studied using a standard soil test [29]. It is shown in Fig. 5 that, although neither PHA sample was appreciably biodegradable under the conditions employed, PHA-tal (linseed oil) was approximately 26% (on average) more biodegradable after 66 days than was PHA-tal. Many species of soil-associated pseudomonads

produce extracellular lipases in the presence of triglyceride substrates [34]. Because of this, the difference in biodegradability between PHA-tal and PHA-tal (linseed oil) may be due to the leaching of linseed oil from the polymer matrix over time and the subsequent action of extracellular bacterial lipases on the linseed oil. In addition, it seems that irradiation had little effect on the biodegradation of the polymers in the soil test.

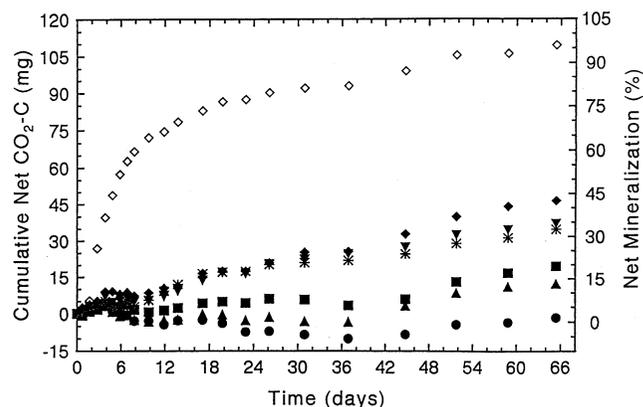


Fig. 5. Biodegradability of irradiated and non-irradiated PHA-tal and PHA-tal + 10% linseed oil based on a standard soil test. ( $\diamond$ ) Cellulose (S.D. = 9.3%); ( $\bullet$ ) PHA-tal, 0 kGy (S.D. = 6.2%); ( $\blacktriangle$ ) PHA-tal, 25 kGy (S.D. = 7.3%); ( $\blacksquare$ ) PHA-tal, 50 kGy (S.D. = 7.7%); ( $\blacklozenge$ ) PHA-tal + 10% linseed oil, 0 kGy (S.D. = 7.9%); ( $*$ ) PHA-tal + 10% linseed oil, 25 kGy (S.D. = 8.7%); ( $\blacktriangledown$ ) PHA-tal + 10% linseed oil, 50 kGy (S.D. = 8.3%).

This could be due to air-induced crosslink formation within the non-irradiated films over time.

In conclusion, it has been demonstrated that *Pseudomonas resinovorans* produces an MCL-PHA copolymer containing unsaturated aliphatic side chains from tallow. While MCL-PHAs have previously been classified as thermoplastic elastomers, their low  $T_g$  and crystallization rates have placed limits on their potential applications. This paper shows that these shortcomings can be overcome to some extent by inducing the formation of physical crosslinks within the polymer matrix by  $\gamma$ -irradiation. Hence, the crosslink density and the elasticity modulus of the polymer could be controlled by varying the radiation dose to which the polymer was exposed. Although not shown, results imply that it is also possible to regulate these parameters by utilizing polymers with varying mole fractions of unsaturated monomer subunits. In this way, the polymer matrix can be made more rigid and consequently more useful. One drawback to radiation-induced crosslinking is its effect on the tear resistance of the polymer. It was thought that the addition of linseed oil into the matrix might act as a filler to overcome or lessen the effects of radiation on the tear resistance of the polymer. It was found, however, that linseed oil did not lead to increased crosslink density, but acted more like a plasticizer within the polymer matrix and did not enhance the physical properties of the films. Results indicated that the presence of a higher crosslink density within the polymer slowed biodegradation but did not completely eliminate it.

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