

PRODUCTION AND CHARACTERIZATION OF MICROBIAL POLY(HYDROXYALKANOATES) FROM AGRICULTURAL LIPIDS

Introduction

Fats and oils have been the primary sources of aliphatic carbon compounds used by industry. The inexpensive petroleum feedstocks, however, have gradually reduced their importance as industrial commodities. In the food-use arena, health conscious consumers have decreased the demands for highly saturated oils/fats. These surplus agricultural lipids thus constitute potentially inexpensive feedstocks for the production of biopolymers from microorganisms.

Poly(β -hydroxyalkanoates) (PHA) are carbon- and energy- reserve materials produced by numerous microorganisms¹. These biomaterials are potentially useful in various applications that could benefit from their biodegradability². The medium-chain-length PHA (mcl-PHA) have side-chains with 6-14 carbon atoms and are produced by the pseudomonads³. Our laboratory has shown that several species of the pseudomonads produce mcl-PHA by using agricultural triacylglycerols and their saponified products⁴. In this communication, we report the synthesis of PHA from triacylglycerols by *Pseudomonas saccharophila*, and the properties of the mcl-PHA produced by *Ps. resinovorans* grown on agricultural lipids.

Experimental

Bacteria, Media, and Growth. *Pseudomonas resinovorans* NRRL B-2649 and *Ps. saccharophila* NRRL B-628 were obtained from National Center for Agriculture Utilization Research (ARS/USDA, Peoria, IL). Cells were routinely grown in Nutrient Broth (Difco Laboratories, Detroit, MI) or LB medium (1%, w/v tryptone; 0.5%, w/v yeast extracts; 0.5% NaCl) in an incubator shaker operated at 30°C and 250 rpm orbital shaking. PHA production was performed by a shake-flask fermentation protocol in minimal medium E* as described⁵.

Cell Growth Determination. The growth of *Ps. saccharophila* in minimal media containing agricultural triacylglycerols was followed by a serial dilution cell-counting protocol. Culture samples were withdrawn at time intervals and serially diluted in LB medium. Diluted samples were spread on LB solid medium (1%, w/v agar) and incubated at 30°C for 1-2 days. The number of colonies that appeared on the solid medium was converted cell density expressed as colony-forming-unit (cfu)/ml culture.

PHA Isolation. PHA was isolated from the bacteria by Soxhlet extraction in chloroform as described by Cromwick *et al.*⁴. The polymers were purified by solid-phase extraction with Sep-Pak Plus cartridges containing silica gel according to the method of Ashby and Foglia⁵. The purity of the final product was verified by TLC analysis on silica gel plates with hexane/ether/formic acid (80:20:2, v/v/v) as the solvent system, visualized by 50% sulfuric acid spraying and heating.

Polymer Characterization. The compositions of the polymer repeating units were determined by gas chromatography (GC) and GC/mass spectrometry (GC/MS) analyses of the β -hydroxymethyl ester derivatives. Samples were prepared according to the procedure of Brandl *et al.*⁶ and analyzed under the conditions described by Cromwick *et al.*⁴. The molar mass averages of the polymers were estimated by gel permeation chromatography (GPC)⁴. The calibration standards were the low polydispersity polystyrene samples with molar masses ranging from 1.28×10^3 g/mol to 3.15×10^6 g/mol (Polyscience Corp., Warrington, PA). The glass transition temperature (T_g) and melting temperature (T_m) were determined using a Perkin-Elmer DSC-7 differential scanning calorimeter⁷.

Electron Microscopy. Bacteria were fixed in a 1% glutaraldehyde/0.1M imidazole-HCl solution for 1 hr at room temperature. Post-fixation treatment was performed in a 2% osmium tetroxide/0.1M imidazole solution.

*Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Cells were embedded in epoxy resin and baked at 55°C for 2 days. Thin sections of 50-60 nm were prepared with a diamond knife. The sections were stained with lead citrate and uranyl acetate solution. Observations were made with a Philips Model CM12 electron microscope operated in the bright field imaging mode.

Results and Discussion

Cell growth in triacylglycerols. Cromwick *et al.*⁴ first showed that *Ps. resinovorans* NRRL B-2649 could grow and synthesize PHA using tallow as the sole carbon source. We have subsequently demonstrated that this bacterium also grows and produces mcl-PHA in a minimal medium containing other sources of triacylglycerols⁵. Under shake-flask fermentation conditions, we generally obtained 3-4 gr cell dry weight/liter culture by using soybean-, sunflower- (high oleic) or coconut-oil as the carbon source.

We have recently studied *Ps. saccharophila* in our search for additional microorganisms capable of growing in and synthesizing polymer from agricultural triacylglycerols. A growth study demonstrated that *Ps. saccharophila* could utilize a triacylglycerol such as tallow, soybean oil, sunflower oil, or coconut oil as a carbon substrate for cell growth. Tallow and coconut oil supported cell growth to a high density of 1.8×10^{11} and 1.8×10^{10} cfu/ml, respectively, after a 3-day incubation. *Ps. saccharophila* grown with soybean- or sunflower-oil as carbon source grew to a cell density of 1.2×10^9 and 3.4×10^8 cfu/ml, respectively, in 3 days. These growth characteristics suggest that *Ps. saccharophila* prefers short chain length and/or saturated fatty acids for growth.

PHA Synthesis. *Ps. resinovorans* grown with any one of the four triacylglycerols tested, produced mcl-PHA at high yields. The PHA content reached as high as 51.0 ± 3.2 % of the cell dry weight with coconut oil as substrate. The lowest yield was observed with cells grown in tallow, in which the PHA content was estimated as 39.8 ± 2.0 % of the cell dry weight. These values corresponded to 1.2-1.9 g PHA produced per liter of culture.

Ps. saccharophila grown in any of the four tested triacylglycerols contained refractive inclusion bodies when viewed with a microscope under oil-immersion/ phase-contrast conditions. These intracellular structures were characteristic of PHA granules seen in the typical PHA-producing bacteria. Thin-section transmission electron microscopy study of the tallow-grown *Ps. saccharophila* further revealed that these granules contained biomaterial with a refractive index closely matching the embedding medium plastic (Fig. 1). We have proceeded to isolate the PHA from cells that had been cultured in coconut oil-containing medium at a yield of approximately 1.0 g/l of culture.

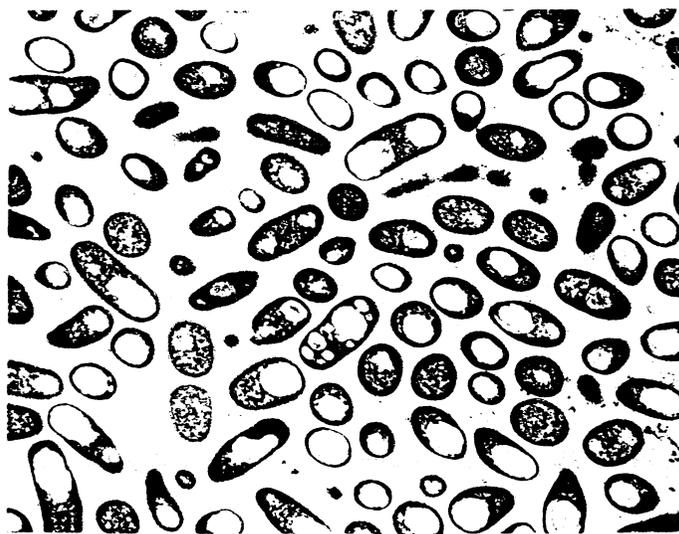


Figure 1. Thin-section electron microscopic images of *Ps. saccharophila* NRRL B-628 grown in a minimal medium with tallow as the sole carbon source.

Characterization of PHA. The repeat-unit compositions of PHA produced by *Ps. resinovorans* in various triacylglycerols were similar. The major monomeric units in all PHA examined are the β -hydroxyoctanoate and β -hydroxydecanoate. In view of the significant difference in the fatty acid compositions of the triacylglycerol substrates used, this observation indicates that the PHA synthases of *Ps. resinovorans* appear to have high specificity for substrates containing acyl chain of 8- to 10-carbon atoms.

Molar mass (m_n) estimation of the PHA yielded values in the range of 6.5×10^4 to 10.1×10^4 g/mol. It is interesting to note that sunflower- and soybean-oil with high olefin contents yielded PHA with low m_n values, while the highly saturated coconut oil and tallow produces high m_n polymers.

The glass transition temperatures (T_g) of the polymers were determined as -46°C to -38°C . The coconut oil-derived PHA with its highly saturated side chains has the highest T_g value. Similarly, the melting temperature of the PHA produced from coconut oil is 48°C . This value is 7°C higher than the sunflower oil-derived polymer which has a high olefin content. The PHA obtained from soybean oil did not exhibit a melting transition, indicating that the polymer exists in an amorphous state. These results suggest that PHA with higher degree of saturation is more structured than those containing larger amounts of unsaturated side chains.

Conclusions

Ps. resinovorans and *Ps. saccharophila* are shown to grow and to produce poly(β -hydroxyalkanoates) by using various agricultural triacylglycerols as the carbon source. The properties of the biopolymers such as the melting temperature and the degree of unsaturation could be manipulated through the choice of the triacylglycerol substrate.

Acknowledgement. The authors thank Dr. Peter Cooke for the electron microscopic study.

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