Biotechnology Letters (2006) 28: 157–162
DOI 10.1007/s10529-005-5329-2

Biosynthesis of medium-chain-length poly(hydroxyalkanoates) from soy molasses

Daniel K.Y. Solaiman[1,*], Richard D. Ashby[1], Arland T. Hotchkiss Jr[2] & Thomas A. Foglia[1]

[1]*Fats, Oils and Animal Coproducts Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania, 19038, USA*
[2]*Crop Conversion Science and Engineering Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania, 19038, USA*
**Author for correspondence (Fax: +1-215-233-6795; E-mail: dsolaiman@errc.ars.usda.gov)*

Received 8 November 2005; Accepted 10 November 2005

Key words: biopolymer, PHA, *Pseudomonas*, *Pseudomonas corrugata*, raffinose, short-chain galactooligosaccharides, stachyose

Abstract

Pseudomonas corrugata was selected from a screening process for the bioconversion of inexpensive soy molasses into medium-chain-length poly(hydroxyalkanoates) (mcl-PHA). We obtained yields of 1.5 g cell dry weight (CDW)/l culture with growth medium supplemented with 2% (w/v) soy molasses, and of an average of 3.4 g CDW/l with 5% (w/v) soy molasses. Crude PHAs were obtained at 5–17% of CDW. The most prominent repeat-unit monomers in the PHAs were 3-hydroxydodecanoate, 3-hydroxyoctanoate, 3-hydroxydodecanoate, and 3-hydroxytetradecenoate. This work represents the first description of fermentative mcl-PHA production from the soy molasses.

Introduction

Poly(hydroxyalkanoates) (PHAs) are bacterial polyesters produced and sequestered as intracellular granules by many microorganisms. PHAs are commonly grouped into two major categories: short-chain-length (scl-) PHA in which the repeat units of the polymer are the hydroxy fatty acids (HFAs) of 4–6 carbon chain length; and medium-chain-length (mcl-) PHA where the repeat units are HFAs are >6 carbon chain length. The scl-PHAs are thermoplastic-like because of their relatively high crystallinity. On the other hand, mcl-PHAs have minimal crystallinity and hence are amorphous and exhibit elastomeric and adhesive properties. Because PHAs are biodegradable and biocompatible, and can be produced by fermentation of renewable feedstocks, they are thus considered as attractive ''green'' substitutes for petroleum-derived polymers in such applications as in medicine, drug-delivery agents, agriculture and horticulture, fibers, and other consumer products (Zinn *et al.* 2001, Lenz & Marchessault 2005).

To help lower the production cost, an active area of PHA research is the use of inexpensive renewable agricultural and industrial coproducts as feedstocks for PHA production. Various molasses streams with high carbohydrate content are generated in many industrial processes and several studies have shown that these streams can support PHA biosynthesis (Gouda *et al.* 2001,

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Wu *et al*. 2001). Soy molasses is a coproduct stream of soybean processing. It is high in potentially fermentable carbohydrates (up to 30% w/v) and is less expensive ($\sim$ one-fifth) than the commonly used glucose substrate. Since the cost of the carbon substrate has been suggested to contribute to >50% of the production cost of bioproducts (Braunegg *et al*. 2004), the inexpensive soy molasses is thus an attractive feedstock to use to reduce fermentative production costs. Examples of the use of soy molasses to produce industrial chemicals or bioproducts include lactic acid synthesis with *Lactobacillus salivarius* (Montelongo *et al*. 1993), butanol production with *Clostridium beijerinckii* (Qureshi *et al*. 2001), and sophorolipid synthesis with *Candida bombicola* (Solaiman *et al*. 2004). In this paper, we describe the use of soy molasses as a potential low-cost substrate to produce mcl-PHA from *Pseudomonas corrugata*.

Materials and methods

Bacteria and culture media

Pseudomonas corrugata 388, originally isolated from alfalfa roots by F.L. Lukezic (Pennsylvania State University, University Park, PA), and *P. chlororaphis* NRRL B-2075 were obtained from Dr. W.F. Fett (Eastern Regional Research Center/ARS/USDA, Wyndmoor, PA). *P. oleovorans* strains NRRL B-778, NRRL B-14682, and NRRL B-14683; and *P. resinovorans* NRRL B-2649 were obtained from the ARS Culture Collection (NCAUR, Peoria, IL). *P. putida* KT2442 was a gift of Prof. R.A. Gross (Polytechnic University, Brooklyn, NY). Bacteria were grown in E* medium (for medium composition see Brandl *et al*. 1988) with soy molasses as the sole carbon source. Incubation of shake-flask cultures was carried out at 30 °C at 200–250 rpm rotary shaking. Soy molasses in the form of soy solubles (50% moisture and 30% carbohydrates) was supplied by Central Soya (Gibson City, IL).

PHA production from P. corrugata

E* medium (0.5 and 1.0 L) was prepared and sterilized (by autoclaving) in 1- and 2-L beveled-bottom (3 prongs) Erlenmeyer flasks, respectively. Soy molasses was added to the medium to a final concentration of 2 or 5% (w/v) before sterilization. Inoculation was done by the addition of a 1/100-volume aliquot of an overnight (16–20 h) culture of *P. corrugata* 388 to the medium. Unless otherwise specified, incubation was performed at 30 °C and 250 rpm shaking for 3 days. Cells were collected by centrifugation and washed once with cold distilled water. Cells were then lyophilized and weighed to obtain cell dry weight (CDW). To obtain the crude PHA, the lyophilized cells were extracted with chloroform; cell debris was removed by filtration through Whatman No. 1 paper, and the solvent evaporated on a rotary-evaporator. The solvent-free residue was weighed to obtain the crude PHA yields.

Repeat-unit analysis of PHA

Crude PHA samples were subjected to acid-catalyzed methanolysis, the liberated hydroxy fatty acid (HFA) monomers were silylated, and the compositions of these HFA monomers were analyzed as detailed in Ashby *et al*. (2004).

HPLC analysis of sugars in soy molasses

Sucrose, raffinose, and stachyose were determined using a Dionex DX-500 HPLC system consisting of a GP50 gradient pump, a CarboPac PA1 column and guard column, an ED40 electrochemical detector (gold working electrode, pH reference electrode and the quadruple potential waveform), an LC25 chromatography oven (30 °C), a PC10 pneumatic controller (post-column addition of 500 mM NaOH), and an AS3500 autosampler. The isocratic mobile phase was 100 mM NaOH for 30 min. Individual sugars were identified by comparison to standards. As shown in Figure 1, the HPLC protocol used in this study satisfactorily resolved the three major sugars in soy molasses.

Results and discussion

Several strains of PHA-producing *Pseudomonas* were surveyed for their ability to metabolize the

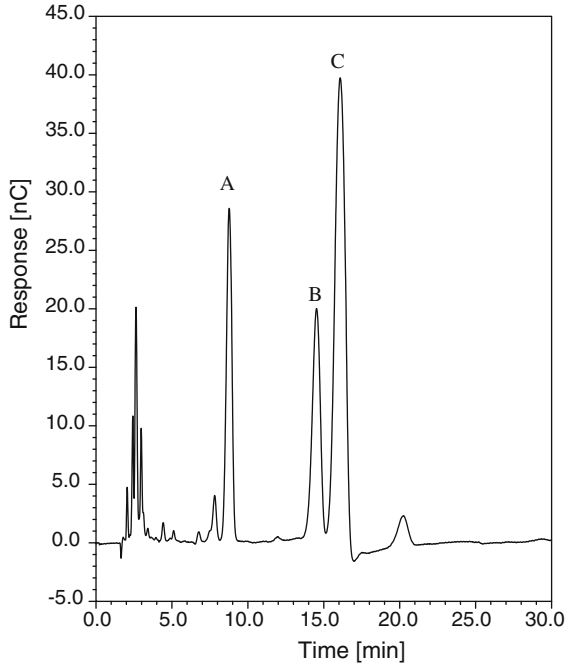

Fig. 1. High performance anion exchange chromatography – pulsed amperometric detection analysis of soy molasses. Sucrose (A), raffinose (B) and stachyose (C) were identified by comparing retention times with standards injected separately.

Table 1. Consumption of sucrose, raffinose, and stachyose in soy molasses by *Pseudomonas* strains.

Bacterial strain[a]	Sugar Consumption[b]		
	Sucrose	Raffinose	Stachyose
P. chlororaphis NRRL B-2075	±	–	–
P. corrugata 388	+	–	–
P. oleovorans NRRL B-778	–	–	–
P. oleovorans NRRL B-14682	–	–	–
P. oleovorans NRRL B-14683[c]	–	–	–
P. putida KT2442	–	–	–
P. resinovorans NRRL B-2649	–	–	–

[a]Cells were grown in culture tubes containing 3 ml E* medium supplemented with 1 and 2% soy molasses. Incubation was performed at 30 °C and 250 rpm rotary shaking for 3 days.

[b]Sugar consumption was monitored by HPLC peak areas. For *P. chlororaphis*, an ~50% decrease of HPLC peak areas attributed to sucrose was observed regardless of the initial soy-molasses concentration.

[c]Variously known as strain ATCC 29347, GPo1, or TF4-1L.

sucrose and major short-chain galactooligosaccharides (i.e. raffinose and stachyose) of soy molasses. The strains selected for the survey were *P. resinovorans*, *P. corrugata*, *P. oleovorans* (3 strains), *P. chlororaphis*, and *P. putida*. We previously had identified these organisms as having certain unique characteristic in regard to PHA production such as metabolizing intact triacylglycerols (Ashby & Foglia 1998), growing and synthesizing mcl-PHA at moderately elevated temperatures (Solaiman *et al.* 2002), synthesizing scl-PHA only or a scl/mcl-PHA blend (Solaiman & Ashby 2005), or producing rhamnolipid biosurfactants (Gunther *et al.* 2005). In this study, their ability to metabolize sucrose (Su), raffinose (Ra) and stachyose (St), which are the major sugars in soy molasses, was investigated in a chemically defined medium E* originally formulated for an improved mcl-PHA synthesis in *Pseudomonas*. Accordingly, cells were incubated in E* medium supplemented with 1 and 2% (w/v) soy molasses for 2 days at 30 °C and 250 rpm. At the end of incubation, cells were pelleted by centrifugation and the resultant cell-free spent medium was collected and analyzed for sugar content by HPLC.

The results of the survey showed that none of the strains metabolized Ra and St (Table 1). This is not surprising in view of the absence of α-galactosidase enzyme in *Pseudomonas*. To date, only one instance of the identification of an α-galactosidase 27A in *P. fluorescens* subsp. *cellulosa* has been described (Halstead *et al.* 2000). The fate of Su in the culture medium, however, varied with each organism (Table 1). Our results showed that *P. corrugata* completely consumed the Su in the medium, and *P. chlororaphis* reduced the Su content of the medium (~50%) with a concomitant production of an unidentified metabolite (data not shown). The other *Pseudomonas* strains, however, did not metabolize sucrose under the experimental conditions used in this work. Although the ability of *P. corrugata* to metabolize sucrose is well documented (Catara *et al.* 1997), our results established for the first time that the organism is still capable of utilizing this sugar in a complex medium environment. Sucrose metabolism by *P. chlororaphis* also has been described (Bergey's

Table 2. Cell-mass productivity and PHA yield of *P. corrugata* grown on soy molasses[a].

Culture volume	Soy-soluble content (w/v)	Cell mass productivity (g CDW/l)[b]	Crude PHA yield (% CDW)[c]
500 ml	2%	1.5	17
	5%	3.2	7
1 l	2%	1.5	6
	5%	3.6	5

[a]The shake-flask cultures were grown in 1- (for 500 ml culture) or 2-l (for 1 l culture) capacity Erlenmeyer flasks having 3-pronged beveled bottom. Incubation was performed at 30 °C and 250 rpm for 3 days.
[b]Lyophilized cell mass was expressed in g cell dry weight (CDW).
[c]Crude PHA was extracted from lyophilized cell with chloroform and subsequently dried to an adhesive-like substance by removal of the solvent in a rotory evaporator.

Manual of Systematic Bacteriology, 1984). Our results show that as a component of the E* + soy molasses medium, the sucrose is only partially metabolized by *P. chlororaphis* NRRL B-2075 under the present experimental conditions. The literature lacks information on sucrose utilization by *P. oleovorans*. The results of this study showed that three strains from this species did not metabolize sucrose in soy molasses. We did not observe consumption of sucrose in soy molasses by *P. resinovorans* and *P. putida* as these organisms are not known to metabolize this dissaccharide (Bergey's Manual of Systematic Bacteriology 1984, Hasanuzzaman *et al.* 2004).

Based on the above results, we next focused on studying PHA production from soy molasses by *P. corrugata*. *P. corrugata* was cultured in 500 ml and 1l E* medium supplemented with 2% (w/v) and 5% (w/v) soy molasses. Consumption of the three major soy sugars (i.e., Su, Ra, and St), cell-mass and PHA yields, and the repeat-unit composition of PHA at the end of the 3-day fermentation runs were determined. The clear spent culture medium was analyzed by HPLC to determine the consumption of soy sugars. The results showed that the sucrose was completed consumed by *P. corrugata* in both the 2% and 5% soy molasses cultures for both the 0.5 and 1l cultures. As found in the survey described above, raffinose and stachyose were not consumed by the organism under the large-scale (1l) shake-flask growth conditions. We observed that cell-mass yields were proportional to the amounts of soy solubles present in the medium. Table 2 shows that the amounts of cellular materials produced in the 5% soy molasses medium were 2.1–2.4 times those obtained with 2% soy molasses. Yields of 3.2–3.6 g CDW/l obtained with the 5% soy-molasses cultures were higher than those obtained with *P. corrugata* grown on E* medium supplemented with glucose (1.52 g CDW/l) or oleic acid (1.62 g CDW/l) (Solaiman *et al.* 2002). However, since the soy molasses-containing medium was not treated (e.g., by filtration or centrifugation) to remove insoluble substances that might be present in the feedstock, the apparently higher cell yields with soy molasses as substrate cannot be definitively ascertained. Table 2 also lists crude PHA yields of *P. corrugata* grown on soy molasses. Because of the low yields (5–17% CDW), we did not attempt to purify the PHAs by solvent precipitation. We previously had obtained crude PHA yields of 31 and 61%-CDW from *P. corrugata* grown on glucose or oleic acid, respectively (Solaiman *et al.* 2002). The low PHA yields obtained with *P. corrugata* on soy molasses reflect the general observation that unrelated fermentation feedstocks support lower mcl-PHA production than fatty-acid substrates. As a high nitrogen content of growth medium might also affect total mcl-PHA accumulation (Solaiman *et al.* 2003), the nitrogenous substances in soy molasses (up to 5% w/v) could contribute to the observed low yields of this study.

The effect of feedstock on PHA repeat-unit composition has been the subject of several investigations. Table 3 shows that 3-hydroxydecanoic acid is the most prominent repeat-unit monomer of the mcl-PHA extracted from *P. corrugata* grown on soy molasses. This was followed by 3-hydroxyoctanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxytetradecenoic acid. This repeat-unit composition is similar to

Table 3. β-Hydroxyalkanoate repeat-units of mcl-PHA from *P. corrugata* grown on various feedstocks.

Feedstock	Composition (mol %)[e]						
	C_6	C_8	C_{10}	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$
Soy molasses (2%)[a]	3	17	40	14	6	3	17
Soy molasses (5%)[a]	Tr[e]	20	49	21	Tr	Tr	12
Glucose (0.5%)[b]	2	10	56	11	n.d.[e]	2	9
Oleic acid (0.5%)[c]	4	42	30	10	Tr	Tr	14
Glycerol (5%)[d]	1	14	46	9	29	1	Tr

[a]Data were averages of values obtained from 500 ml and 1 l experiments where no significant difference was observed for each entrée
[b]From Solaiman *et al.* (2002).
[c]From Solaiman *et al.* (2002, 2005). Values represent the averages of those obtained in the two separate studies described in these references.
[d]From Ashby *et al.* (2005). The mcl-PHA was produced by *P. corrugata* 388 in a mixed-culture fermentation with *P. oleovorans* NRRL B-14682, a scl-PHA-producing organism.
[e]The values typically have standard errors of $< 10\%$ due to the crude nature of the preparations. Tr, trace amounts detected (≤ 0.5 mol %). N.d., not detected. C_6 3-hydroxyhexanoate, C_8 3-hydroxyoctanoate, C_{10} 3-hydroxydecanoate, $C_{12:0}$ 3-hydroxydodecanoate, $C_{12:1}$ 3-hydroxydodecenoate, $C_{14:0}$ 3-hydroxytetradecanoate, $C_{14:1}$ 3-hydroxytetradecenoate.

that of PHA obtained from *P. corrugata* grown on glucose (Table 3). Glycerol also directed the production of mcl-PHA with 3-hydroxydecanoic acid as the predominant repeat-unit monomer (Table 3). These observations suggest that mcl-PHA produced in *P. corrugata* via the *de novo* fatty acid biosynthesis pathway contains the 10-carbon hydroxy fatty acid as its most prominent repeat-unit monomer.

These results conform to the long-held observation that unrelated substrates such as glucose and gluconate yielded mcl-PHA having 3-hydroxydecanoate as the predominant monomer (see Steinbüchel 1991). In contrast, the polymer produced by *P. corrugata* from oleic acid via the β-oxidation pathway contains 3-hydroxyoctanoate as the predominant monomer, followed closely by 3-hydroxydecanoate (Table 3).

Summary

We have demonstrated in this study that soy molasses can be used as a feedstock for the fermentative production of mcl-PHA using *P. corrugata* as the producing strain. The repeat-unit composition of the PHA polymer was determined and compared to those obtained from other feedstocks. Further study to manipulate the fermentation conditions and to genetically engineer the producing strain could result in a high-yield production system for converting the inexpensive soy molasses to the value-added mcl-PHA.

Acknowledgement

The authors thank Nicole Crocker, Marshall Reed and Bun-Hong Lai for their technical assistance.

References

Ashby RD, Foglia TA (1998) Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates. *Appl. Microbiol. Biotechnol.* **49**: 431–437.

Ashby RD, Solaiman DKY, Foglia TA (2004) Bacterial poly(hydroxyalkanoate) polymer production from the biodiesel co-product stream. *J. Polymers Environ.* **12**: 105–112.

Bergey's Manual of Systematic Bacteriology (1984) Vol. 1, Krieg HR, Holt JG, eds. Baltimore: Williams & Wilkins, p. 166.

Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl. Environ. Microbiol.* **54**: 1977–1982.

Braunegg G, Bona R, Koller M (2004) Sustainable polymer production. *Polym.-Plast. Technol.* **43**: 1779–1793.

Catara V, Gardan L, Lopez MM (1997) Phenotypic heterogeneity of *Pseudomonas corrugata* strains from southern Italy. *J. Appl. Microbiol.* **83**: 576–586.

Gouda MK, Swellam AE, Omar SH (2001) Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiol. Res.* **156**: 201–207.

Gunther IV NW, Nuñez A, Fett W, Solaiman DKY (2005) Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. *Appl. Environ. Microbiol.* **71**: 2288–2293.

Halstead JR, Fransen MP, Eberhart RY, Park AJ, Gilbert HJ, Hazlewood GP (2000) α-Galactosidase A from *Pseudomonas fluorescens* subsp. cellulosa: cloning, high level expression and its role in galactomannan hydrolysis. *FEMS Microbiol. Lett*. **192**: 197–203.

Hasanuzzaman M, Umadhay-Briones KM, Zsiros SM, Morita N, Nodasaka Y, Yumoto I, Okuyama H (2004) Isolation, identification, and characterization of a novel, oil-degrading bacterium, *Pseudomonas aeruginosa* T1. *Curr. Microbiol.* **49**: 108–114.

Lenz RW, Marchessault RH (2005) Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *Biomacromol.* **6**: 1–8.

Montelongo J-L, Chassy BM, McCord JD (1993) *Lactobacillus salivarius* for conversion of soy molasses into lactic acid. *J. Food Sci.* **58**: 863–866.

Qureshi N, Lolas A, Blaschek HP (2001) Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101. *J. Ind. Microbiol. Biotechnol.* **26**: 290–295.

Solaiman DKY, Ashby RD (2005) Genetic characterization of the poly(hydroxyalkanoate) synthases of various *Pseudomonas oleovorans* strains. *Curr. Microbiol.* **50**: 329–333.

Solaiman DKY, Ashby RD, Foglia TA (2002) Physiological characterization and genetic engineering of *Pseudomonas corrugata* for medium-chain-length polyhydroxyalkanoates synthesis from triacylglycerols. *Curr. Microbiol.* **44**: 189–195.

Solaiman DKY, Ashby RD, Foglia TA (2003) Effect of inactivation of poly(hydroxyalkanoates) depolymerase gene on the properties of poly(hydroxyalkanoates) in *Pseudomonas resinovorans*. *Appl. Microbiol. Biotechnol.* **62**: 536–543.

Solaiman DKY, Ashby RD, Nunez A, Foglia TA (2004) Production of sophorolipids by *Candida bombicola* grown on soy molasses as substrate. *Biotechnol. Lett.* **26**: 1241–1245.

Solaiman DKY, Catara V, Greco S (2005) Poly(hydroxyalkanoate) synthase genotype and PHA production of *Pseudomonas corrugata* and *P. mediterranea*. *J. Ind. Microbiol. Biotechnol.* **32**: 75–82.

Steinbüchel A (1991) Polyhydroxyalkanoic acids. In: Byrom D, ed. *Biomaterials - Novel Materials from Biological Sources*, New York: Stockton Press, pp. 125–213.

Wu Q, Huang HH, Hu GH, Chen JC, Ho KP, Chen GQ (2001) Constitutive production of poly-3-hydroxybutyrate by strain of *Bacillus aureus* JMa5 cultivated in molasses media. *Ant. van Leeuwenhoek* **80**: 111–118.

Zinn M, Witholt B, Egli T (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Adv. Drug Delivery Rev.* **53**: 5–21.