Extraction of Lipid-Grown Bacterial Cells by Supercritical Fluid and Organic Solvent to Obtain Pure Medium Chain-Length Polyhydroxyalkanoates

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ABSTRACT: A simple two-step process was developed to extract and purify medium chain-length polyhydroxyalkanoates (MCL-PHA) from bacterial cells (*Pseudomonas resinovorans*) grown on lard and tallow. The process consists of supercritical fluid extraction (SFE) of the lyophilized cells with carbon dioxide to remove lipid impurities, followed by chloroform extraction of the cells to recover the MCL-PHA. SFE conditions were varied as to temperature (40–100°C), pressure (2000–9000 psi), and carbon dioxide flow rate (0.5–1.5 L/min, expanded gas). Lipid material, usually 2–4%, but in some cases as high as 11%, was extracted from the dried cells by SFE. A pressure range (5000–9000 psi, increased stepwise), a temperature of 60°C, and a carbon dioxide flow of 1.5 L/min were routinely used to extract the bacterial cells (4–5 g) after 3 h. Higher flow rates could shorten the extraction time even more. SFE did not extract MCL-PHA from the cells. Yield of MCL-PHA after chloroform extraction at room temperature was a maximum of 42.4% based on dry cell weight. The results show that the two-step process saves time, uses much less organic solvent, and produces a purer MCL-PHA biopolymer than previous extraction and purification methods. A more environmentally friendly clean-up procedure based on SFE and organic solvent recovery was developed to remove contaminating lipid materials from the fermentation biomass, allowing for the recovery of higher purity MCL-PHA that are suitable for more demanding applications.

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Polyhydroxyalkanoates (PHA) are poly-β-esters produced by numerous bacteria as intracellular carbon and energy storage materials that can be accumulated to a high percentage during fermentation (1). PHA are thermoplastic materials that have various mechanical properties ranging from hard crystalline polymers to elastic rubbers, depending on the monomer units. PHA are used where biodegradable polymers are desired. Recently the use of PHA has been expanding and the market is expected to grow. However, the present cost of production must be reduced. Major expenses in producing PHA are cost of the fermentation substrate, extraction of the biopolymer, and treatment of the fermentation and extraction wastes (2). Fortunately for the United States, there are large supplies of inexpensive fats and oils (tallow, lard, soybean oil, corn oil) that can be used as fermentation substrates to produce PHA.

PHA are classified as either short or medium chain-length polyesters, depending on the number of carbon atoms in the monomer. Among PHA, medium chain-length (MCL-PHA) with monomers containing 6–14 carbon atoms are our primary interest. In previous reports from this laboratory (3–5), we found that one bacterial species of *Pseudomonas* (*P. resinovorans*) possessed an extracellular lipase that enabled it to use triglycerides directly as feedstocks for MCL-PHA biosynthesis. In many cases, fatty acid unsaturation was conserved in the PHA synthesis, resulting in side chains containing double bonds. This is important because a double bond is a useful functional group for derivatizing and tailoring the physical properties of the bacterial polyester for targeted applications.

Previous extraction and purification of MCL-PHA from lipid-grown bacterial cells was time-consuming and difficult because the PHA biopolymer and lipid substrates had similar solubility characteristics in chlorinated solvents (3,4,6). Consequently, a new purification process that saved time, used less organic solvent, and produced a purer product was needed to replace the former extraction methods.

Supercritical fluid extraction (SFE) is now frequently used to extract lipids from many materials (7–9). However, there are only a few reports using SFE on bacterial cell biomass. One such report used SFE to extract carotenoids from bacterial cells (10). Another report described the SFE of bacterial lipids for use in the classification of bacteria by fatty acid profiles (11). Previously, we have used SFE to extract fungal lipids (12). The specific objective of this research was to determine whether SFE could be used to remove lipid contaminants from bacterial-cell biomass obtained from lard and tallow fermentation in hopes of obtaining a more efficient purification procedure for the fermentation product, PHA.

EXPERIMENTAL PROCEDURES

Materials. Tallow was obtained from Miniat Inc. (Chicago, IL). Lard was obtained from Holsum Foods (Waukesha, WI).

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Carbon dioxide, SFE grade, was purchased from BOC Gases (Lebanon, NJ). Chloroform, high-performance liquid chromatography grade, was purchased from Burdick and Jackson (Muskegan, MI). Polypropylene wool was purchased from Aldrich Chemical (Milwaukee, WI). Hydromatrix, a celite, was purchased from Varian Inc. (Harbor City, CA).

Methods. SFE extraction. A schematic of the SFE apparatus and extraction cell has been published in previous reports (13,14). Lyophilized cells (4–5 g) from the fermentation of *P. resinovorans* NRRL B 2649 were ground in a mortar with pestle to a uniform particle size. The cells were then packed tightly into a 24-mL stainless steel high-pressure cylinder (rated at 10,000 psi) from Keystone Scientific (Bellefonte, PA). In some experiments, Hydromatrix (4–5 g) was mixed in with the cells to create greater surface area. Polypropylene wool was placed in front and back of the cells to protect the 2-µm frits in the extraction cylinder. The extraction cylinder was then placed in the oven of the SFE apparatus model Speed, obtained from Applied Separations Corp. (Allentown, PA) and connected to the lines supplying the pressurized carbon dioxide. A thermocouple was attached directly to the wall of the extraction vessel to monitor the temperature. After equilibrium at the preset temperature, the cylinder was pressurized to the initial starting pressure, and thereafter the pressure was adjusted stepwise to higher pressures, usually on an hourly basis. Flow of CO_2 gas was recorded with a "Floline" mass flowmeter obtained from Horriba, Inc. (Sunnyvale, CA) at various flow rates at atmospheric pressure as the CO_2 exited the traps. The traps were 25-mL empty glass bottles obtained from Pierce Chemical Co. (Rockford, IL) that were fitted with lids containing septa. A 14-gauge needle punctured the septa and served as the exhaust. Traps were changed hourly and weighed to record the amount of material extracted from the cell biomass until there was nothing being collected.

Solvent extraction. Lyophilized cells (4–5 g) from the SFE were suspended in 50 mL of chloroform, stirred for 10 min, and then vacuum filtered through Whatman #1 filter paper with the aid of a Büchner funnel. The cells were removed from the filter paper and resuspended in another 50 mL of chloroform and again filtered. The accumulated solvent (100 mL) was evaporated in a beaker on a steam bath to constant weight to recover the MCL-PHA.

RESULTS AND DISCUSSION

The results of exhaustive extraction of tallow- or lard-grown *P. resinovorans* lyophilized cells first by SFE and second by organic solvent are listed in Table 1. The lipid extracted and the PHA recovered are based on one sample of lyophilized cells from each of several individual fermentations. Cells grown on a lard substrate usually had more lipid contaminants due to the difficulty of removing the cell biomass from the fermentation broth. It was easier to separate the biomass from the tallow fermentation because the tallow fermentation broth had more lipid solids that were not as difficult to separate from the biomass. At the fermentation temperature (30°C) lard had more oil and less fat solids present than did tallow. Extracts from cells grown on lard also showed a greater variability in the results compared to tallow. The results from multiple samplings from a single batch (50 g) of cells grown on tallow are also included in the table and show the same results as the individual batches from tallow. The PHA recovered from the cells grown on lard or tallow indicate that there is no difference in the PHA from the two substrates.

TABLE 1
Exhaustive Extraction of Lipid-Grown *Pseudomonas resinovorans* Cells by Supercritical CO_2 Followed by Chloroform Extraction[a]

	Lyophilized cell weight (mean%)	
Substrate	Lipid	PHA
Tallow	3.87 ± 0.62 (6)	41.7 ± 3.60 (4)
Lard	5.75 ± 3.74 (4)	42.4 ± 4.30 (3)
Tallow	3.70 ± 0.77 [11]	n.d.

[a]PHA, polyhydroxyalkanoate; n.d., not determined; values in parentheses are individual fermentations; values in brackets are simple fermentations with 50-g batches of lyophilized cells extracted 4–5 g at a time.

Carbon dioxide pressure was raised in a stepwise manner during SFE as a precaution to avoid plugging the restrictor by having too much lipid coming off in a short time at the very beginning of the extraction. The restrictor was maintained at only 100°C to prevent breakdown of lipids at low CO_2 flows. Usually the pressure was increased at 1-h intervals. In Figure 1, the pressure was increased from 2,000 to 7,000 psi, at 1-h intervals. This stepwise pressure increase at a CO_2 flow rate of 0.5 L/min illustrates the effect of pressure and temperature on the extraction. For example, lyophilized cells (4 g) from a lard fermentation were subjected to SFE at two temperatures (40 and 60°C). At 2,000 psi, very few contaminants were ex-

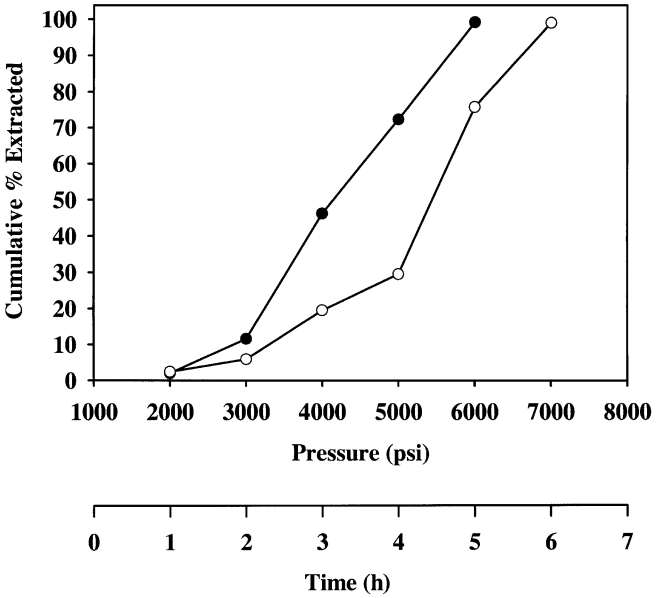

FIG. 1. Supercritical fluid extraction (SFE) of lipids from lyophilized *Pseudomonas resinovorans* cells. Efficiency at two temperatures, 40°C (●) and 60°C (○).

tracted in the first hour. This was also true in the second hour at 3,000 psi. The amount extracted, however, increased at 4,000 psi and continued thereafter. The extraction was more efficient at the lower temperature because the solubility of lipid contaminants is dependent primarily on the density of CO_2 due to the low volatility of lipids at these temperatures. Two advantages of using the lower extraction temperature are that the extraction can be completed in 5 h as opposed to 6 h at the higher temperature, and that less CO_2 is required since the flow rate (0.5 L/min) is the same.

The effect of pressure on lipid extracted from cell biomass in the first hour at 60°C and 1 L/min CO_2 flow was that CO_2 density rather than pressure was making the difference. As the density of CO_2 increased, the amount of total lipid extracted also increased. At a CO_2 density of 0.5545 g/mL (2,000 psi), only 4.8% lipid was extracted; at 0.7350 g/mL (3,000 psi), 24.0% was extracted; and at 0.8604 g/mL (5,000 psi), 84.3% lipid was extracted in the first hour.

The effect of a higher temperature on the lipid extracted from cell biomass in the first hour at 5,000 psi and 1 L/min CO_2 flow was that at 100°C, only 28.4% lipid was extracted, whereas at 60°C, 48.1% lipid was extracted. Again, the extraction is density dependent rather than temperature dependent. As is already known, at constant pressure, the gas density is less at a higher temperature, i.e., 0.8604 g/mL at 60°C vs. 0.7108 g/mL at 100°C (15). Because lipids are not very volatile at the temperatures used here, their solubility in supercritical CO_2 depends primarily on the gas density. Extraction of volatile materials, however, would be expected to increase at the higher temperature. No MCL-PHA were extracted from the biomass at this higher temperature (100°C).

The progress of a typical SFE at constant pressure (5,000 psi) and flow (1 L/min) is shown in Figure 2. This was the extraction of a 50-g batch of lyophilized cells from a tallow fermentation. The cells were extracted 4–5 g at a time; 11 extractions total. Almost 80% of the lipid was extracted in the first hour under these conditions. In the second hour, only 9% was extracted. In the third and fourth hour, only about 3% was extracted in each hour. Hexane (5 mL) was used to clean out the lines in the extractor to get complete recovery of the lipid contaminants. Subsequent gel permeation chromatography of the recovered PHA confirmed the thoroughness of lipid removal by SFE (4).

The effect of CO_2 flow rate on a typical SFE is shown in Figure 3. The amount of lipid extracted is actually independent of the flow rate, but dependent on the total amount of CO_2 passing through the extractor. There is an advantage to using higher flow rates, however, because with the higher flow, the extraction was completed in three hours instead of four hours at the lower flow rate. However, it has been reported that too high a flow may cause a decrease in SFE efficiency (16). Higher flow rates will be tested to find the limit that can be used in our study.

Processing time is costly and therefore the shortest amount of time required to recover the product is desirable. In previous reports from this laboratory (3–5), a Soxhlet extraction

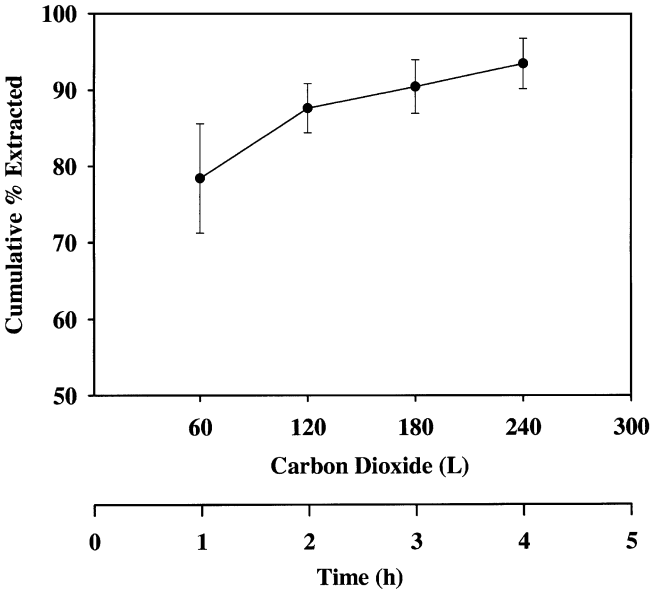

FIG. 2. SFE of lipids from lyophilized *P. resinovorans* cells grown on tallow (5000 psi, 60°C, 1 L/min). See Figure 1 for abbreviations.

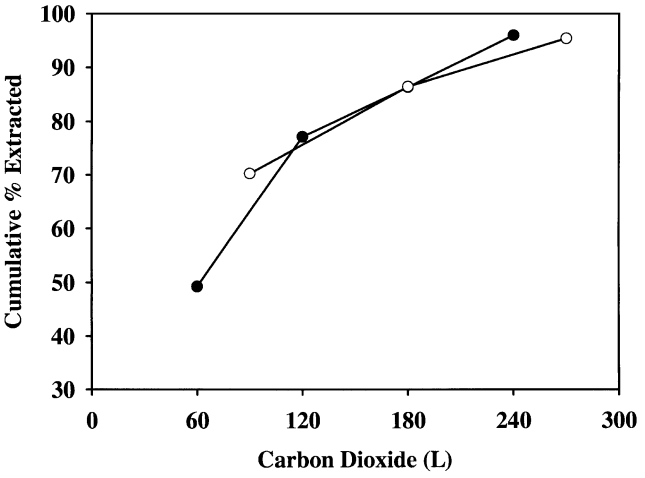

FIG. 3. SFE of lipids from lyophilized *P. resinovorans* cells. Efficiency at two flow rates: 1 L/min = ●, 1.5 L/min = ○. See Figure 1 for abbreviations.

was necessary just to extract the MCL-PHA from the lyophilized cells. However, the greatest amount of time was spent and the most solvent used during the purification of the MCL-PHA by flash chromatography (17). Thus, after a series of experiments, we have been able to shorten the time for SFE and chloroform extraction and product recovery to about 6 h without affecting yield, while at the same time recovering a product that is purer than can be obtained by solvent extraction without SFE. The purity of these MCL-PHA has been established by gel permeation and gas chromatography (4,5). It is expected in the future that the time for the SFE can be further reduced to 1–2 h by a combination of stepwise pressure increases and higher flow rates.

Although this report emphaizes SFE , it should be pointed out that the previous amount of organic solvent used to recover and purify product is dramatically reduced. Here, there is a 20 to1 ratio of organic solvent to dried bacterial cells for the extraction of MCL-PHA from the cells. Another report (6) used a ratio of 200 to 1. In addition, the large quantities of solvents (dichloromethane and ethyl acetate) formerly used by our laboratory for purification of the MCL-PHA by flash chromatography are no longer required. Also, the soxhlet extraction was eliminated. In most experiments (data not presented), the chloroform from the extraction was recovered by using a rotary evaporator with a condenser.

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