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Production of Medium-Chain-Length Polyhydroxyalkanoates by *Pseudomonas aeruginosa* With Fatty Acids and Alternative Carbon Sources

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Abstract

In this study, medium-chain-length polyhydroxyalkanoates (mcl-PHAs) were produced by *Pseudomonas aeruginosa* using different carbon sources. Decanoic acid induced the highest (9.71% [±0.7]) mcl-PHAs accumulation in bacterial cells at 47 h. The cells preferred to accumulate and degrade the polyhydroxyoctanoate than polyhydroxydecanoate (PHD) during early stage and final stage of the growth, respectively. The production cost of mcl-PHAs can be reduced by using edible oils as the carbon source. The bacteria accumulated 6% (±0.7) of mcl-PHAs in the presence of olive oil. Besides, reused oil was another potential carbon source for the reduction of the production cost of mcl-PHAs. Overall, PHD was the major constituent in the accumulated mcl-PHAs.

Index Entries: Edible oils; fatty acids; medium-chain-length polyhydroxyalkanoates; *Pseudomonas aeruginosa*.

Introduction

Polyhydroxyalkanoates (PHAs) are polymers that are accumulated as energy storage materials in various microorganisms when cells have adequate supplies of carbon but are limited for another nutrient, such as nitrogen, phosphate, or oxygen *(1–3)*. In general, PHAs can be divided into two groups according to the number of carbon atoms in the monomer units. Short-chain-length PHAs (scl-PHAs) contain 3–5 carbon atoms and are commonly produced by *Ralstonia eutropha* and *Alcaligenes latus*. Medium-chain-length PHAs (mcl-PHAs) contain 6–14 carbon atoms and are typically produced by fluorescent pseudomonads *(4)*. The properties of PHAs follow in part from their bacterial origin. scl-PHAs are highly crystalline materials and are brittle in nature. Owing to their rigid property, they are not suitable

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for making films for medical and special devices (5). Mcl-PHAs are elastomeric thermoplastics. They are rubbery and flexible materials with low crystallinity and can be used in a wide range of industrial and medical applications which cannot be fulfilled by scl-PHAs (6). The mode of biosynthesis of polyhydroxybutyrate (PHB) is well understood. However, the synthesis of mcl-PHAs has been studied in recent years (7). Like all PHAs, accumulation of mcl-PHAs is in the form of granules (8). It is believed that mcl-PHAs synthesis from fatty acids is via the fatty acid β-oxidation to yield acyl-CoAs intermediates such as enoyl-CoA, 3-Ketoacyl-CoA, and/or S-3-hydroxyalcyl-CoA, which are substrates for mcl-PHAs synthase when fatty acids are used as the sole carbon source (7). *Pseudomonas aeruginosa* is a Gram-negative, aerobic rod, belonging to the bacterial family *Pseudomonadaceae* and is reported to produce mcl-PHAs (9). Highly homologous genes have been isolated from *P. aeruginosa* in order to enhance the production of mcl-PHAs (10–12).

Owing to the promising future of mcl-PHAs in industrial and medical areas, the production of mcl-PHAs is highly desired. In this study, we studied mcl-PHAs synthesis by *P. aeruginosa* using different fatty acids. Moreover, edible oils from the local market were used as substitute carbon sources in order to reduce the production cost.

Materials and Methods

Bacterial Cultures and Growth Conditions

A strain of *P. aeruginosa* PAO1 was donated by Institute of Microbiology of Shandong University (11). The strain was stored at –20°C in the presence of 25% (v/v) glycerol and was maintained on a nutrient agar (Oxoid, Hampshire, England) slant at 4°C by monthly subculture. In the shake flask study, the strain was inoculated in 1-L conical flasks containing 200 mL of cultivation media. The cultivation medium is shown in Table 1. Glucose and fatty acids are obtained from International Laboratory USA, whereas edible oils are obtained from the local market. The pH was adjusted to 7.0, and the medium was autoclaved. The cells were cultivated in the above media for 48 h under aerobic conditions in a temperature-controlled shaker set at 250 rpm and at 30°C, harvested by centrifugation, washed and then lyophilized by freeze drier. Triplicates were done for each manipulation and the values (presented later) after the ± symbol in the parentheses indicated the standard deviation.

Extraction of Polymers

The extraction method (13) was modified. In brief, lyophilized cells were suspended in chloroform (30 mL/g) and extracted overnight at 60°C. The cell debris was then separated by filtration. The chloroform solution was concentrated by rotary evaporator under 60°C and the polymer was precipitated overnight on cold methanol (for better precipitation)

Table 1
The Cultivation Medium for the Production of mcl-PHAs

Component (/L)	Constituents	
E medium	3 g/L $(NH_4)_2HPO_4$	
	5.8 g/L K_2HPO_4	
	3.7 g/L KH_2PO_4	
100 m*M* $MgSO_4 \cdot 7H_2O$		
1 mL microelement solution (/L of 1 *N* HCl)	2.78 g/L $FeSO_4 \cdot 7H_2O$	
	1.98 g/L $MnCl \cdot {}_4H_2O$	
	2.81 g/L $CoSO_4 \cdot 7H_2O$	
	1.67 g/L $CaCl_2 \cdot 2H_2O$	
	0.17 g/L $CuCl_2 \cdot 2H_2O$	
	0.29 g/L $ZnSO_4 \cdot 7H_2O$	
Carbon source	Glucose (20 g/L)	
	Fatty acids (8 g/L)	Citric acid
		Octanoic acid
		Nonanoic acid
		Decanoic acid
		Lauric acid
	Edible oils (8 g/L)	Corn oil
		Soybean oil
		Peanut oil
		Canola oil
		Olive oil
		Reused oil

(1:9 v/v chloroform: methanol). The precipitated polymer was collected by centrifugation (Beckman J2-21) at 9000*g* (Beckman, CA).

Preparation and Analysis of PHAs

Polysters in dried cells (20 mg) or in purified forms (10 mg) were methyl-esterfied in a (1:1; v/v) mixture of chloroform and methanol-sulfuric acid *(14)*. The lower chloroform solution was analyzed by gas chromatography (GC). A Hewlett Packard 5890 Series II Gas Chromatograph system (Hewlett Packard) was used, equipped with an AT-50 Alltech® capillary column (Alltech) and with a flame ionization detector. The operating program was set according to Ballistreri et al. *(13)*. Cell concentration was defined as cell dry weight (g)/L of culture broth. In brief, the cell was centrifuged in order to remove the supernatant and oven dried. The cell weight was measured as the cell concentration. The PHA content (wt%) was defined as the percentage of the ratio of PHA concentration to cell concentration (i.e., g [%] mcl-PHAs/g cell mass).

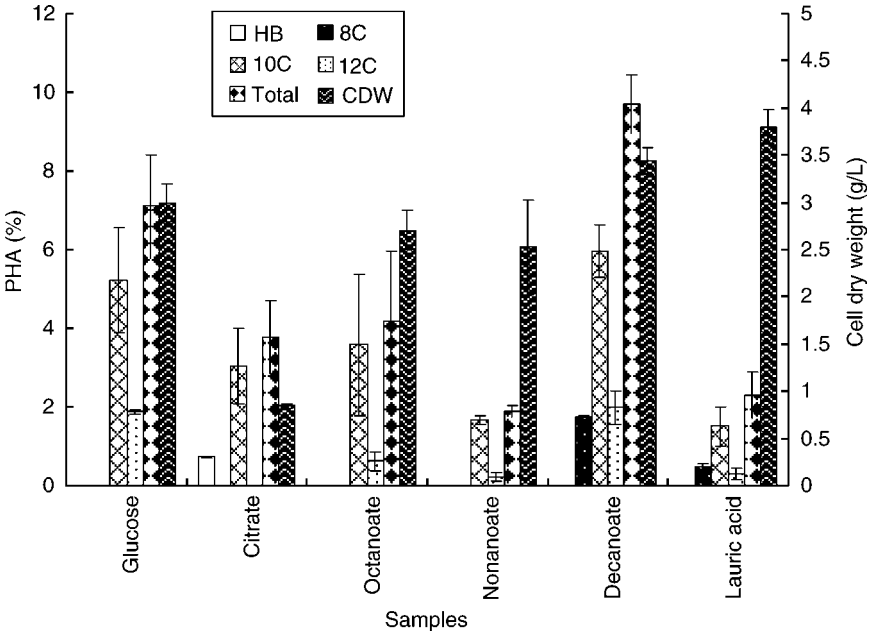

Fig. 1. The percentage of different mcl-PHAs accumulated by *P. aeruginosa* in the presence of glucose and other fatty acids and their relative cell dry weight. HB, hydroxybutyrate; 8C, hydroxyoctanoate (HO); 10C, hydroxydecanoate (HD); 12C, hydroxydodecanoate (HDD); total, total polyhydroxyalkanoates accumulated in the cell; CDW, cell dry weight.

Results

The results of the different mcl-PHAs accumulated by *P. aeruginosa* in the presence of glucose and other fatty acids and their relative cell dry weight are shown in Fig. 1 and Table 2. The results showed that the 3-hydroxydecanoate (HD) and 3-hydroxyoctanoate (HO) were the major types of mcl-PHAs accumulated in the bacterial cell. Polyhydroxydecanoate-co-Polyhydroxydodecanoate (PHD-co-PHDD) was accumulated in the cells in medium containing glucose, octanoic acid, and nonanoic acid. Polyhydroxybutyrate-co-Polyhydroxydecanoate (PHB-co-PHD) was accumulated in the cells cultured in citric acid medium. For the decanoic acid and lauric acid medium, the bacteria produced Polyhydroxyoctanoate-co-Polyhydroxydecanoate-co-Polyhydroxdodecanoate (PHO-co-PHD-co-PHDD), with Polyhydroxyoctanoate (PHO) and Polyhydroxydro-decanoate (PHD) being accumulated in greater proportion.

Figure 2 showed the growth curve of *P. aeruginosa* and time points for different mcl-PHAs accumulation using the decanoic acid as the carbon source. The results showed that *P. aeruginosa* in medium containing decaonic acid grew exponentially from 16–33 h. The cell produced more PHO than PHD between 22 and 60 h. However, the accumulation of PHD was higher after 60 h of fermentation. However, only a low percentage

Table 2
The PHA Accumulation in the Bacterial Cell and Their Cell Dry Weight After 48 h Cultivation in Medium Containing Different Carbon Sources

Carbon sources	mcl-PHAs accumulated (%)	Cell dry weight (g/L)
Glucose	7.1 (±1.3)[a]	3.0 (±0.2)
Citric acid	3.78 (±0.9)	0.84 (±0.02)
Octanoic acid	4.19 (±1.8)	2.7 (±0.21)
Nonanoic acid	1.89 (±0.1)	2.5 (±0.5)
Decanoic acid	9.71 (±0.7)	3.4 (±0.14)
Lauric acid	2.28 (±0.6)	3.8 (±0.18)

[a]The values in the parentheses were standard deviations.

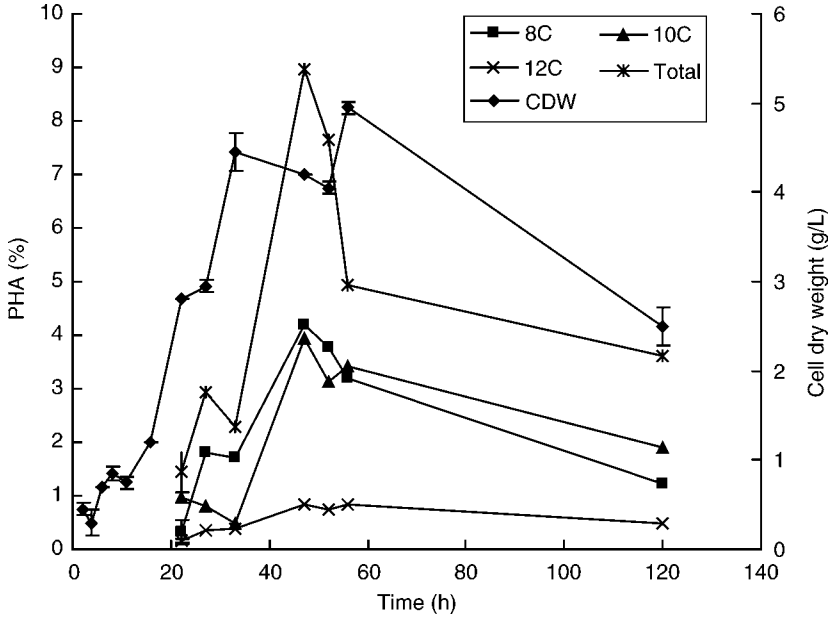

Fig. 2. The growth curve and the percentage of different mcl-PHAs accumulated by *P. aeruginosa* in 120 h shake flask fermentation by using decanoic acid as the sole carbon source. 8C, hydroxyoctanoate (HO); 10C, hydroxydecanoate (HD); 12C, hydroxydodecanoate (HDD); total, total polyhydroxyalkanoates accumulated in the cell; CDW, cell dry weight.

of Polyhydroxydodecanoate (PHDD) was accumulated in cell during the fermentation. The highest percentage of mcl-PHAs accumulation was up to 8.96 (±0.5)% at 47 h.

The effect of edible oils as carbon sources on the accumulation of mcl-PHAs by *P. aeruginosa* are shown in Table 3 and Fig. 3. The results showed that all edible oils used except corn oil could induce the PHO-co-PHD-co-PHDD accumulation in the bacterial cells. As in the fatty acid fermentation,

Table 3
The PHA Accumulation in the Bacterial Cell and Their Cell Dry Weight Using Various Edible Oils as Carbon Source

Edible oil	Total PHA accumulated in cell (%)	Cell Dry Weight (g/L)
Corn oil	0.72 (±0.1)[a]	4.04 (±0.3)
Soybean oil	2.38 (±0.4)	2.55 (±0.1)
Peanut oil	3.84 (±0.5)	3.99 (±0.3)
Canola oil	3.31 (±0.5)	4.19 (±0.3)
Olive oil	6.0 (±0.7)	4.49 (±0.2)
Reused oil	5.72 (±1.0)	5.41 (±0.05)

[a]The values in the parentheses were standard deviations.

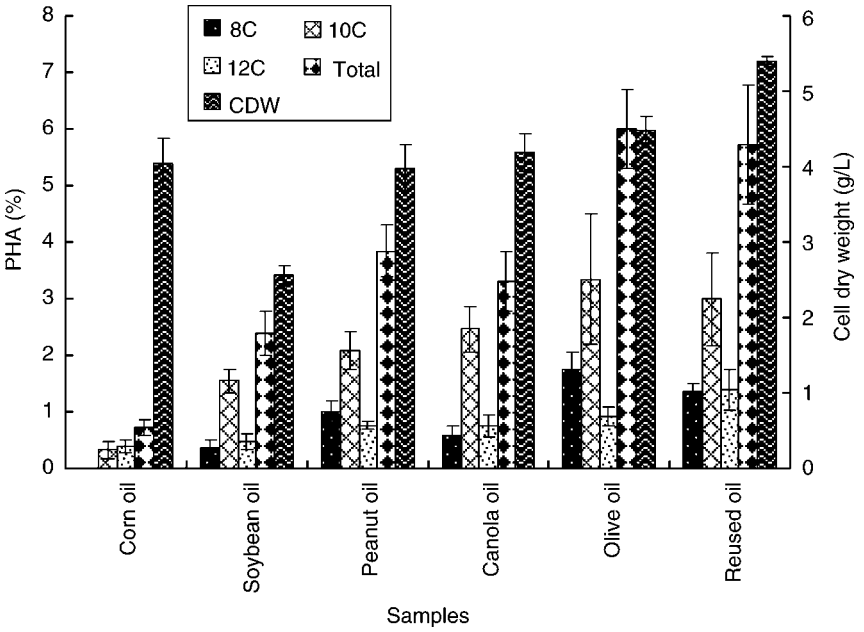

Fig. 3. The percentage of different mcl-PHAs accumulated by *P. aeruginosa* in the presence of different edible oils. HB, hydroxybutyrate; 8C, hydroxyoctanoate (HO); 10C, hydroxydecanoate (HD); 12C, hydroxydodecanoate (HDD); Total, total polyhydroxyalkanoates accumulated in the cell; CDW, cell dry weight.

the bacteria accumulated higher percentage of PHD in medium containing edible oils. The bacteria grew better in the medium containing reused oil. The highest amount of mcl-PHAs accumulated was achieved with the medium containing olive oil. The estimated costs for carbon source for producing mcl-PHAs are set out in Table 4. The costs are around $0.68, $1, $0.16, $0.18, and $0.35/g of mcl-PHA for corn oil, soybean oil, peanut oil, canola oil, and olive oil, respectively.

Table 4
The Estimated Carbon Source Costs
Used for Producing/L g of mcl-PHAs

Sample	Cost (USD)[a]
Decanoic acid	24.1
Corn oil	0.68
Soybean oil	1
Peanut oil	0.16
Canola oil	0.18
Olive oil	0.35

[a]The carbon source costs are calculated according to the prices of the carbon sources and their performance on the mcl-PHAs accumulation. Others costs (e.g., ingredients of medium, manpower, and so on) did not included in these costs.

Discussion

Mcl-PHAs produced by *P. aeruginosa* in medium containing different carbon sources were extracted and analyzed. The results in Fig. 1 and Table 2 demonstrated that besides related carbon sources (fatty acids), mcl-PHAs could also be produced from an unrelated carbon source, i.e., glucose. The results showed that the high cell mass will not contribute to the high PHA production. This is because the growth of the cell is not related to the production of PHAs. The growth of the cell only depends on the environmental conditions and richness of the nutrient presented in the medium. Once the environmental conditions are suitable and the nutrient is rich in the medium, the bacteria can grow well. However, the production of PHA is not related to the growth of the cell. The metabolic condition of the cell contributes to the accumulation of PHA as PHA is a secondary metabolite. The bacteria will accumulate the PHA as the carbon storage compound under limited nutrient, such as nitrogen, phosphate, or oxygen but excess carbon source. Also, when the carbon source is limited in the medium, the bacteria will breakdown the PHA as the carbon source. Therefore, the cell mass is not directly proportional to the PHA concentration. The results also demonstrated that HD was the major constituent in PHAs and HO being the second most common monomers in the polymer. The highest mcl-PHAs yield of 9.71 (±0.7)% was obtained with decanoic acid mineral medium.

Because the bacteria accumulated the highest percentage of mcl-PHAs in the presence of decanoic acid, therefore, decanoic acid was chosen for studying cell growth and the mcl-PHA accumulation between time intervals. The results demonstrated that the exponential phase of *P. aeruginosa* was between 16 h and 33 h. The results in Fig. 2 showed that the cells accumulated more 3-HO than 3-HD between 22 and 60 h and the reverse after

60 h. This demonstrated that the bacteria preferred to accumulate PHO than PHD when decanoic acid is still rich in the medium. After 60 h fermentation, the nutrient began to be depleted and the bacteria preferred to hydrolyze the PHO than PHD as the carbon source for growth. Therefore, the higher accumulation of PHD was found at the end of the fermentation. The bacteria accumulated up to 8.96 (±0.5)% mcl-PHAs at 47 h and started to decrease accumulation after 47 h. Therefore, the bacterial cells should be harvested at 47 h after fermentation in order to obtain the optimal percentage of mcl-PHAs.

Wide application of PHAs is mainly limited by the high cost, especially for the carbon sources, of producing PHA by bacterial fermentation. Therefore, synthesis of PHAs using cheaper carbon sources has been regarded as an attractive alternative for the production of PHA at low cost. Different edible oils purchased from local markets were used as alternative carbon sources for the production of mcl-PHAs. Edible oils are cheaper carbon sources compared with fatty acids. The estimated carbon source costs used for producing per gram of mcl-PHA are calculated in Table 4 based on the prices of different edible oils and their performance on inducing production of mcl-PHAs in this study. Compared with decanoic acid ($24.1/g), the estimated carbon source cost used for producing mcl-PHAs can be reduced by around 93.8–99%. Reused oils (after fried for several times) and maltwaste are even cheaper carbon sources that can further reduce the production cost. However, preliminary results (data not shown) demonstrated that maltwaste is not a suitable carbon source for the production of mcl-PHAs. The results in Fig. 3 showed that 3-HD content is still the major constituent in the polymer as the same in the fatty acid containing medium. Olive oil is the most promising edible oil for producing mcl-PHAs as it can induce the highest mcl-PHAs accumulation. Although bacteria only yielded 5.72 (±1)% of mcl-PHAs in reused oil medium compared with 6 (±0.7)% for olive oil medium, it is usually obtained free from restaurant. Therefore, it is also a good choice for reducing the production cost of mcl-PHAs.

Conclusions

The highest yield of up to 9.71 (±0.7)% mcl-PHAs by *P. aeruginosa* was obtained in decaonic acid mineral medium. The highest amount of mcl-PHAs was found to be accumulated at 47 h. The cells preferred to accumulate and degrade PHO at different time intervals during the cell growth. Among the edible oils, olive oil was found to be a good choice for inducing the production of mcl-PHAs in *P. aeruginosa*, with mcl-PHAs yields as high as 6 (±0.7)%. Reused oil from restaurant is another potential carbon sources that can further reduce the production cost of mcl-PHAs. Overall, PHD was the major constituent in the accumulated mcl-PHAs.

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