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Polyhydroxyalkanoates (PHAs) Production Through Conversion of Glycerol by Selected Strains of Pseudomonas Mediterranea and Pseudomonas Corrugata

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Polyhydroxyalkanoates (PHAs) are polyesters synthesized by numerous bacteria as an intracellular carbon and energy storage compounds in the cytoplasm of cells. To cut down the cost of production and optimize the PHA/dried cell weight different concentrations (1, 2 and 5 %) of refined glycerol and a crude glycerol from biodiesel process, were used as carbon source in a batch fermentation process. *Pseudomonas mediterranea* 9.1 as well as *Pseudomonas corrugata* 388 and A1 reference strains, grew and synthesized a medium-chain-length poly(3-hy-droxyalkanoates) elastomer both on crude and refined glycerol carbon sources. The mcl-PHA produced by *P. mediterranea* 9.1 grown on refined glycerol was apparently different as a result of a different metabolic pathway. Gas chromatography PHA's Different monomeric units of side chains, which ranged from C12 to C19 in length in mcl-$PHA_{(CG)}$ and from C5 to C16 in mcl-$PHA_{(RG)}$ were obtained.

1. Introduction

Polyhydroxyalkanoates (PHAs), a family of biopolyesters with diverse structures, are bioplastics completely synthesized by over 30% of soil-inhabiting bacteria (Wu et al., 2000) from many carbon substrates including simple sugars (Huijberts et al., 1992), free fatty acids (Eggink et al., 1993), simple alkanes and alkanols (De Smet et al., 1983), and triacylglycerols (Ashby and Foglia, 1998; Solaiman et al., 2002).
They are generally categorized into two groups based on the length of their monomeric side-chains. Short-chain-length PHA (scl-PHA) polymers consist of 3-hydroxyalkanoic acids with monomeric repeat units of 4–5 carbons, while medium-chain-length PHA (mcl-PHA) polymers are composed of monomeric repeat units that are C6-C14 carbons in length. In an attempt to reduce production costs in the recent years focus has shifted to the use of less valuable, renewable materials as substrates for PHA production (Ashby et al., 2011), as soy molasses (Solaiman et al., 2006), wheat-based co-products (Koutinas et al., 2007; Xu et al., 2010), refined and crude glycerol (Ashby et al., 2004; Cavalheiro et al,, 2009; Shrivastav et al., 2010; Zhu et al., 2010; Mothes et al., 2007; Kawata et al., 2010; Ashby et al., 2011).
Medium-chain-lenght PHAs were first detected in *Pseudomonas oleovorans* (De Smet et al., 1983) and later in a variety of Pseudomonads (Solaiman et al, 2006). In a previous work our group studied waste cooking oils as renewable source for polyhydroxyalkanoate polymer production by a strain of *P. corru-*

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gata A1 producing lipase (Alicata et al., 2005; Conte et al., 2005; Solaiman et al., 2005) and patented a fed batch on stat fermentation process.
In order to cut down the production cost, a new approach has been developed based the conversion of both refined (RG) and crude glycerol (CG) in a batch fermentation process by *P. mediterranea* 9.1 using *P. corrugata* A1 and 388 as reference strains, being already tested good converters of triacylglycerols (Ashby and Foglia,1998; Solaiman et al., 2002). The three strains are able to produce mcl-PHA either from refined (RG) and crude glycerol (CG). *P. mediterranea* 9.1 revealed a different metabolic pathway, as shown by the conversion efficiency and some chemical and technological properties. This paper reports the investigation carried out to optimize the fermentation and preliminary GC characterization of mcl-$PHA_{(RG)}$ and mcl-$PHA_{(CG)}$ extracted from biomass of *P. corrugata* strains.

2. Materials and methods

2.1 Microrganism and Fermentation Process

Three gram-negative rod shaped ubiquitous bacteria were used throughout this work: *Pseudomonas mediterranea* 9.1 and two strains of *P. corrugata: P. corrugata* 388, originally isolated from alfalfa roots by F.L. Lukezic (Pennsylvania State University, University Park, PA, USA) was supplied by Dr. D.K.Y. Solaiman (Eastern Regional Research Center/ARS/USDA, Wyndmoor, PA, USA) and *P. corrugata* PSTS A1-DSM18227, originally isolated from tomato, obtained through culturing in E* medium added with triolein.
Either commercial refined glycerol (Sicania chimica s.r.l., purity 99.8 %, pH 7.0) or crude glycerol (pH 8.6), a mixture of glycerol and free and methyl esters fatty acids, obtained in our laboratory as by-product of rapeseed (*Brassica napus)* squeezing (MPS80 MT, Mailca) and oil transesterification (TEAG 50 F, Mailca) by sodium metoxide as catalyst, were tested in batch fermentation.
The fermentations were carried out in 2L Erlenmeyer flasks containing E* medium (pH 7.0) as reported by Ashby et al. (2004). The glycerol was added in each test flask at 1, 2 and 5 %. The flasks were inoculated with 25 mL of bacteria from a seed culture and incubated at 30 °C with shaking at 220 rpm. After a preliminary screening at 72 h, a proper timing of fermentation was explored at 48, 72 and 96 h; the process was monitored by Nile Red fluorescent dye to check the PHA production. The cells were harvested by centrifugation at 5000 g, for 20 min, at 4 °C (Beckman Coulter, avanti J-HC), washed with saline solution at 0.9 % and lyophilized overnight to constant weight (Virtis, benchtop 2K).

2.2 PHA extraction

The PHA was extracted by automatic Soxhlet extractor (Buchi extraction system B-811) after dissolving freeze-dried cells in a same volume of acetone, specific solvent for mcl-PHA extraction (Jiang et al, 2006), at boiling point (56 °C) for 2 hs 30 min. After washing for 20 min the PHA was air dried for 15 min at 30 °C. PHA rate in the biomass was routinely evaluated after 48, 72 or 96 h of fermentation, expressed as yield per liter of fermentation substrate.

2.3 Gas Chromatography/Mass Spectrometry PHA Analysis

PHA repeat unit composition was determined by gas chromatography/mass spectrometry (GC/MS) of the 3-hydroxymethyl esters. Prior to analysis, PHA polymers were re-precipitated 3 times into cold methanol in order to remove all residual free glycerol. A mixture of 5.0 mg of PHA, 1.0 mL chloroform, 1.0 mL of methanol and 15 % of sulphuric acid was heated at 100 °C for 140 min, under steady shaking. The reaction mixture was then washed twice with 2.0 mL of water, and the chloroform fraction dehydrated with magnesium sulphate, concentrated and analyzed by a gas chromatography apparatus (Perkin Elmer Clarus 600T) equipped with a column elite 5-MS (5 % diphenyldimethylpolysiloxane, 30 m length, an outside diameter of 0.25 mm and an inside one of 0.25 µm), and with mass detector carrier gas (He) set to 1.30 mL/min. The temperature was maintained at 90 °C for 3 min, then increased up to 190 °C with a rate of 7 °C/min and held for 5 min at this temperature, then increased up to 270 °C with a rate of 8 °C/min and held for 5 min; the injected volume was 1 µL. The identification of peaks was carried out by comparing the mass spectrum peak in the experimentally derived gas chromatogram to the NIST/EPA/NIH library of known mass spectra associated with the instrument and the use of some standard reference.

3. Result and discussion

3.1 Fermentation Process and PHA Extraction

In preliminary tests carried out by conversion of refined or crude glycerol at 1, 2 and 5 %, different yields of biomass were obtained according to the metabolic pathway of the species and to the quality of carbon source (Table 1). In fact, the repeat-unit composition of the biopolymer reflects the fatty acyl composition of substrate used for the synthesis (*P. mediterranea* 9.1 and *P. corrugata* strains A1 and 388 yielded 2.88 to 4.77 g/L of biomass after 72 h batch fermentation, with an average yield of 3.90 g/L when CG was provided as carbon source and 3.49 g/L with RG. *P. corrugata* 388 and *P. corrugata* A1 produced 4.25 g/L and 4.72 g/L of $biomass_{(CG)}$, respectively. *P. mediterranea* 9.1 grew better on 2 % crude glycerol (4.77g/L). The best growth of $biomass_{(RG)}$ for *P. corrugata* A1 was 4.10 g/L at a carbon source of 5 %, for *P. corrugata* 388 was 4.01 g/L at 1 %, and for *P. mediterranea* 9.1 3.31 g/L at 5 %.
Overall PHA recovery ranged from 0.16 g/L to 2.93 g/L and was constantly higher from biomass obtained through CG. *P. mediterranea* grown on 2 % carbon source yielded 2.93 g/L from $biomass_{(CG)}$ and dropped to 0.16 g/L at 5 %. As refers to PHA/dried cell weight, biomass obtained from 2 % crude glycerol fermentation by *P. mediterranea* 9.1 was close to 60 %, whereas declined to 42 % by increasing the carbon source concentration supplied by 5 % (Table 1).
On average *P. corrugata* A.1 $biomass_{(CG)}$ yielded 1.93 g/L (47.3 %), whereas $biomass_{(RG)}$gave 1.03 g/L (27.9 %). The PHA yield from *P. corrugata* 388 $biomass_{(CG)}$ was 1.02 g/L (28.0 %) and 0.59 g/L from $biomass_{(RG)}$ (16 %). *P. corrugata* 388 $biomass_{(CG)}$ gave almost the same values (1.2 g/L) at 1 % and 2 % and dropped to 0.9 g/L at 5 % (Table 1), whereas extraction from *P. corrugata* A1 $biomass_{(CG)}$ gave 1.98,1.80 and 2.0 g/L.

Table 1: Yields of biomass and mcl-PHA obtained from Pseudomonas strains grown on RG and CG at different concentrations after 72 h of fermentation

Microbial Strain	Carbon sources % RG or CG	Biomass $_{RG}$ (g/L)	Biomass $_{CG}$ (g/L)	PHA $_{RG}$ (g/L)	PHA $_{CG}$ (g/L)
P. mediterranea 9.1	1	3.07	3.40	0.76	1.71
	2	3.17	4.77	0.81	2.93
	5	3.31	4.19	0.71	0.16
P. corrugata A1	1	3.53	4.72	1.04	1.98
	2	3.44	3.49	1.14	1.80
	5	4.10	4.12	0.91	2.0
P. corrugata 388	1	4.01	4.25	0.75	1.2
	2	3.56	3.79	0.56	1.28
	5	3.20	2.88	0.46	0.90

Table 2: Yields of biomass and mcl-PHA obtained at different fermentation times from Pseudomonas mediterranea 9.1.grown on *1-2 % (RG) and (CG)*

Substrate concentration	Fermentation time	RG		CG	
		Biomass (g/L)	PHA (g/L)	Biomass (g/L)	PHA (g/L)
1 %	48	3.43	0.51	3.48	1.44
	72	3.07	0.76	3.40	1.71
	96	2.95	0.52	3.22	1.30
2 %	48	3.00	0.59	4.75	2.88
	72	3.17	0.81	4.77	2.93
	96	4.12	0.28	4.35	0.54

The production efficiency of P.mediterranea 9.1 on CG and RG was evaluated after 48,72 and 96 h of fermentation (Table 2). Regardless of carbon source and concentration rate in substrate the highest

PHA results were obtained after 72 h, either on CG and RG. Nevertheless, after 48 h PHA(CG) was almost the same. All the PHA's yields were severely affected after 96 h, except PHA(CG).

3.2 Gas Chromatography/Mass Spectrometry

The GC/MS analysis of the 3-hydroxyalkanoate methyl esters performed by methanolysis was used to determine the co-polyesters composition. The chromatographic profile of $PHA_{(CG)}$ obtained by *P. corrugata* A1 and 388 strains showed four most relevant peaks, referred to fatty acids (Figure 1) and a with a diversified presence of minor peaks. On the basis of mass spectral library and reference standard migration three peaks of $PHA_{(CG)}$ were identified as methyl 9-do-decenoate ($C_{12:1}$), methyl hexadecanoate ($C_{16:0}$) and methyl 9-hexadecenoate ($C_{16:1}$). Interestingly, *P.mediterranea* 9.1 chromatogram of $PHA_{(CG)}$ revealed also oleic acid methyl ester (C18:1) and linoleic acid methyl ester ($C_{19:3}$) Chromatogram of $PHA_{(RG)}$ obtained from *P. corrugata* A1 showed four relevant peaks out of eight: pentanoic acid ester ($C_{5:0}$), decanoic acid ester ($C_{10:1}$), dodecenoic acid methyl ester ($C_{12:1}$) and hexadecanoic acid methyl ester ($C_{16:0}$) (Figure 2). Side chains C10 and C12 in length where also detected on $PHA_{(RG)}$ obtained form *P.corrugata* 388. Instead, *P.mediterranea* 9.1 $PHA_{(RG)}$ showed five peaks; among them pentanoic acid methyl ester ($C_{5:0}$), decenoic acid methyl ester ($C_{10:1}$), dodecenoic acid methyl ester ($C_{12:1}$) were identified.

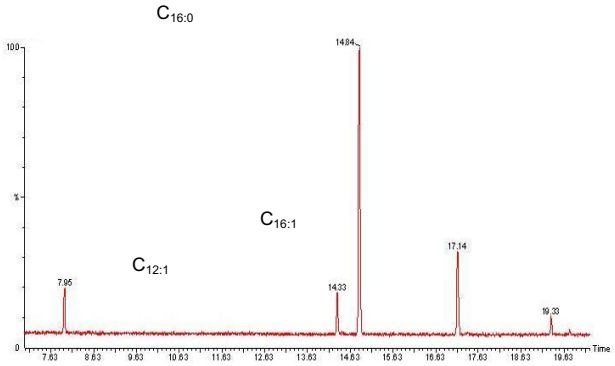

Figure 1: GC analysis of PHA obtained from P. corrugata A1 grown on 1 % of CG

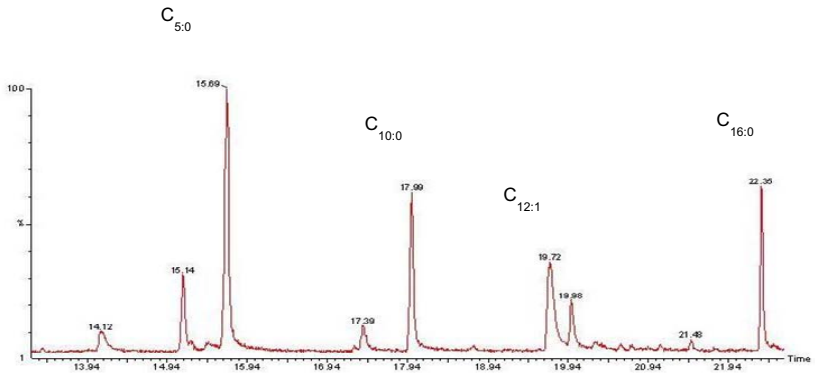

Figure 2: GC analysis of PHA obtained from P. corrugata A1 grown on 1% of RG

4. Conclusions

In order to find out a cheaper microbial production of biomasses and PHAs we have investigated the capability of *P. mediterranea* 9.1 to convert a substrate containing (1, 2 and 5 %) refined or crude glycerol. *P. corrugata* A1 and 388 strains, already known for their attitude to produce PHAs were used as reference strains. The suitability of refined and crude glycerol as carbon source for microbial conversion to PHA was already demonstrated by Ashby et al. (2004) in *Pseudomonas* spp., including *P. corrugata* 388 strain, and in other microbial species (Ashby et al., 2011), but the strains we used - *P. corrugata* A1 and *P. mediterranea* 9.1 - have not been tested before on this carbon sources.

In our conditions *P. mediterranea* 9.1 has shown to be able to produce a mcl-PHA from RG (0.81 g/L) and CG (2.93 g/L). The values are better than the results obtained with *P. corrugata* strains and comparable with the production rate reported by Ashby et al. (2005, 2004). The two polymers showed to have different properties, which are still investigated. The best PHA/dried cells ratios have been obtained when CG was supplied at 2 % of glycerol, giving more than 60 % PHA (Table 2). Diversely, both strains of *P. corrugata* produce an elastomer mcl-PHA very similar regardless of carbon source.

As expected, according to preliminary GC/MS profiles, the composition of the mcl-PHAs are quite different from the one previously obtained in our laboratory by *P. corrugata* A1 conversion of waste fried cooking oils as carbon source (Alicata et al. 2005) and show different monomeric units of side chains, which range from C12 to C19 in length in mcl-$PHA_{(CG),}$ and from C5 to C16 in mcl-$PHA_{(RG).}$ The enhanced properties of mcl-$PHA_{(RG)}$ produced by *P. mediterranea* 9.1 look promising for an improvement of the technological properties required for application as pa-per and tissue coatings. Since also the conversion efficiency of *P.mediterranea* 9.1 was different from *P. corrugata* strains the results confirm that the two species have a different metabolic pathway as suggested by Solaiman et al. (2005, 2007), according to granules accumulation inside the cells growing on oleic acid.

Batch fermentation of *P. mediterranea* 9.1 has shown effective as concern PHA yield, is simple and cheap. Moreover, since a 2 % rate of CG added to substrate allows to obtain high yields of $PHA_{(CG)}$ after 48 h instead of 72 h and the Soxhlet extraction with acetone is highly efficient, it is reasonable that the production cost of the polymer will be affordable after the scale up. Therefore, the results encourage a further investigation aimed to functional applications of the microbial conversion products.

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