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Studies on *mcl*-Polyhydroxyalkanoates Using Different Carbon Sources for New Biomedical Materials †

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Abstract: Polyhydroxyalkanoates (PHAs) are microbial homo- and copolymers of [R]-β-hydroxyalkanoic acids, produced by a wide variety of bacteria as an intracellular carbon and energy reserve. To obtain *mcl*-PHAs of microbial origin, we used a Pseudomonas spp. strain (from the National Institute for Chemical-Pharmaceutical Research and Development (ICCF) culture collection of micro-organisms), by varying the carbon sources and the precursors. In this work, assays were performed with fermentation media seeded with inoculum cultures of strain Pseudomonas putida in a proportion of 10%. The influence on *mcl*-PHA production of carbon sources for strain development, hexanoate (C6), heptanoate (C7), octanoate (C8) and nonanoate (C9) acids, as polymers precursors, were analyzed. Due to their properties, similar to those of conventional plastics and their biodegradability, PHAs are suitable for many applications and for biomedical materials useful in surgical sutures, tissue engineering and drugs carriers, which leads us to the deepening of the study of obtaining micro/nanofibers by the electrospinning method.

Keywords: polyhydroxyalkanoates; bioprocess; biomaterials; electrospinning

1. Introduction

Polyhydroxyalkanoates (PHA) are microbial homo- and copolymers of [R]-β-hydroxyalkanoic acids, are produced by a wide variety of bacteria as an intracellular carbon and energy reserve [1,2]. Other environmental factors on which the fermentation medium depends are nature and concentration of the substrate used as carbon and energy source, presence and concentration of other nutrients in the culture medium, temperature, pH, dissolved oxygen concentration, cultivation system. These factors influence the growth rate of the microorganism, cell density at the end of fermentation, substrate grade conversion and intracellular PHA content [3,4]. Depending on the number of carbon atoms, contained by the monomers units, PHAs isolated can be classified as follows: (i) short chain length (*scl*) PHAs—3 to 5 carbon atoms/monomer, (ii) medium chain length (*mcl*) PHAs—6 – 14 carbon atoms/monomer, and *scl*-co-*mcl* with repeat-unit monomers containing 3–14 carbon atoms [2]. Many studies confirmed that *scl*-PHA type is much more flexible and resistant than *scl*-PHAs [5,6].

Due to their properties, similar to those of conventional plastics and their biodegradability, PHAs have attracted much interest as alternatives to synthetic polymers [8]. These are promising materials due to their useful characteristics: thermoplastic and elastomeric properties, biodegradability, biocompatibility and nontoxicity. Consequently, they are good candidates for

various applications in industry (replacements for petroleum-derived plastics, packaging industry, laminate papers and cardboards), fine chemical industry (starting materials for the synthesis of antibiotics and other fine chemicals) or medicine (scaffolds for bone tissue engineering, drug delivery system) [7,9–11].

In this paper, we studied the optimal concentration of fatty acids to obtain new biomaterials used in medical domain.

2. Experimental

2.1. Materials

Components of nutritive media were purchased from Merck (Kenilworth, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA), organic solvents, analytical reagents and mineral salts, from Merck, except for methyl esters of 3-hydroxy acids (C6-C11), purity 98% purchased from Larodan, Sweden.

The PHA producing microorganism was *Pseudomonas putida* ICCF 391. The stock culture was grown at 29 ± 1 °C and maintained by periodic transfer on M44 (cDSMZ424) agar slants.

2.2. Methods

2.2.1. Inoculum Growth Medium and Cultivation Conditions

The pre-inoculum medium had the following composition (% g/vol): yeast extract 1.0%, peptone 1.0%, glycerol 5.0%, agar 2.0%. All the components were dissolved in distilled water, pH adjusted to 6.5, then sterilized at 120 °C, 20 min. Stock cultures were stored at 4 °C. The inoculum containing (% g/v): glucose 1.00%, corn extract 1.50%, KH_2PO_4 1.00%, NaCl 1.00%, $MgSO_4$ 0.05% was sterilized at 115 °C, for 20 min. The inoculum was developed in 500 mL shake flasks (100 mL medium), at 29 ± 1 °C and 220 rpm for 24 h.

2.2.2. Biosynthesis Conditions

Batch fermentations were performed in 750 mL Erlenmeyer flasks, containing 250 mL of fermentation medium. Bioprocess medium had the following composition in mineral salts: $NaNH_4HPO_4{\cdot}4H_2O$ 3.5 g/L, K_2HPO_4 7.5 g/L, KH_2PO_4 3.7 g/L, structural correlated carbon source (sodium hexanoate, heptanoate, octanoate and nonanoate) 0.835–2.5 g/L, trace element solution I 1.0 mL/L, trace element solution II 1.0 mL/L [12]. Trace element solution I contains 120.0 g/L $MgSO{\cdot}7H_2O$. Trace element solution II contains per litre: 2.78 g of $FeSO_4{\cdot}7H_2O$, 1.47 g of CaCl2 $\cdot 2H_2O$, 1.98 g of $MnCl_2{\cdot}4H_2O$, 2.81 g of $CoSO_4{\cdot}7H_2O$, 0.17 g of $CuCl_2{\cdot}2H_2O$, and 0.29 g of ZnSO4$\cdot 7H_2O$ in 1 M HCl. The sterilized fermentation medium (as mentioned above) was inoculated with 10% (v/v) preculture and incubated at 300 C, for 48 h, on a rotary shaker, at 220 rpm. At 0 and 24 h of cultivation, the medium was supplemented with fatty acids investigated (C6, C7, C8 and C9), in different combinations. The cultivation was performed in two steps of nutrient addition. Optical density (OD) of cell suspension was measured at λ = 550 nm, 1:25 dilution, with a UV-VIS spectrophotometer Jasco V-Able 630. The quantity of dry biomass was determined with a thermobalance A & D, MF-50.

2.2.3. Post-Biosynthesis Processing

At the end fermentations for biopolymer, isolation was carried out according to following steps: biomass separation through centrifugation; methanol pretreatment and vacuum drying of separated biomass; acetone Soxhlet extraction of *mcl*-PHA from biomass using biomass: acetone ratio of 1:20; concentration of acetone extract at ¼ of the initial volume; *mcl*-PHA precipitation with cold methanol at a ratio of 1:10 concentrated extract: methanol; chloroform dissolution of precipitated *mcl*-PHA; slow evaporation of *mcl*-PHA chloroform solution [2].

2.2.4. *mcl*-PHAs Assay

The gas chromatographic method used for the determination of the polymers obtained by laboratory fermentations with the three bacterial strains above mentioned consisted of a mild acid methanolysis of the polymers followed by gas chromatography of the resulted methyl ester mixture [13]. The polymer composition and purity degree resulted by summing all of the contained monomers, determined by GC-FID. For the monomer composition determination, a capillary column has been used with an HP 5 (5% phenyl-methylpolysiloxane) stationary PHAs. Methyl esters of C6–C11 hydroxy acids have been used as standard substances.

3. Results and Discussion

3.1. PHAs Bacterial Biosynthesis

Several experiments were performed going through all the specific stages of a microbial biosynthesis process. We tested the evolution of the fermentation process for different precursor additions, namely: C8, C9 and combinations of C8-C9, C6-C8, C7-C9 added at different times of the bioprocess. The evolution of fermentation was followed by measurements of pH, optical density and dry cell weight, as shown in Table 1.

Table 1. Fermentations in system fed batch for PHA biosynthesis.

Samples	Precursors Added (g/L)		Fermentation Evolution		
	0 h	24 h	pH	OD [1]	DC [2] (g/L)
P5	8.35 C8	8.35 C8	7.32	0.5198	3.708
P7	8.35 C9	8.35 C9	7.35	0.241	1.619
P13	8.35 C8	8.35 C9	7.16	0.353	1.619
P14	8.35 C9	8.35 C8	7.15	0.422	1.648
P17	8.35 C9	8.35 C7	7.19	0.287	9.795
P18	8.35 C7	8.35 C9	7.25	0.300	8.025
P19	8.35 C8	6.88 C6	7.37	0.527	8.727
P21	6.88 C6	8.35 C8	7.31	0.353	6.147

* [1] Optical Density masured at 550 nm, [2] Dry Cell Weight/L.

Correlating the data from the experiments performed, we noticed that using a mixture of 2 precursors a higher amount of dry biomass (g/L) was obtained, compared to the fermentation in which a single precursor was used (C8 or C9).

3.2. PHAs Extraction

Following the processing of biomass and the isolation of PHAs by the mentioned methods, yields between 53 and 56% were achieved depending on the polymer content of the dry cells. The composition and purity of polymers isolated were determined by GC-FID and expressed in g/100 g of analyzed product (Table 2).

Table 2. Experimental values obtained for PHAs biosynthesized.

Samples	PHAs (%)	Hydroxyacids					
		C6 (%)	C7 (%)	C8 (%)	C9 (%)	C10 (%)	C11 (%)
P5	51.16	7.32	-	**88.00**	3.29	1.29	-
P7	40.40	1.25	21.58	13.23	**59.63**	0.75	0.47
P13	47.66	2.91	13.57	33.79	**45.72**	0.93	0.86
P14	35.81	1.33	19.55	14.9	**59.59**	1.44	0.77
P17	43.22	0.11	14.23	0.36	**79.32**	1.44	2.54
P18	48.79	-	**66.18**	0.52	26.77	2.20	0.57

P19	56.29	9.66	-	**79.46**	0.13	7.59	0.96
P21	52.64	8.28	-	**82.65**	-	5.53	1.08

The analytical results show that by biosynthesis, isolation and purification of PHAs, using different precursors co-polymers with other content of monomers were obtained ranging between 66.18 for C7, 79.46–88% for C8, 45.72–79.32% for C9.

From all the precursor amount used up by microorganism during the fermentation, 16.70 g (8.35 g/L in the fermentation medium), 1.619–9.795 g/L dry bacterial biomass and 0.54–1.95 g/L PHA, containing mostly PHO were obtained (Figure 1).

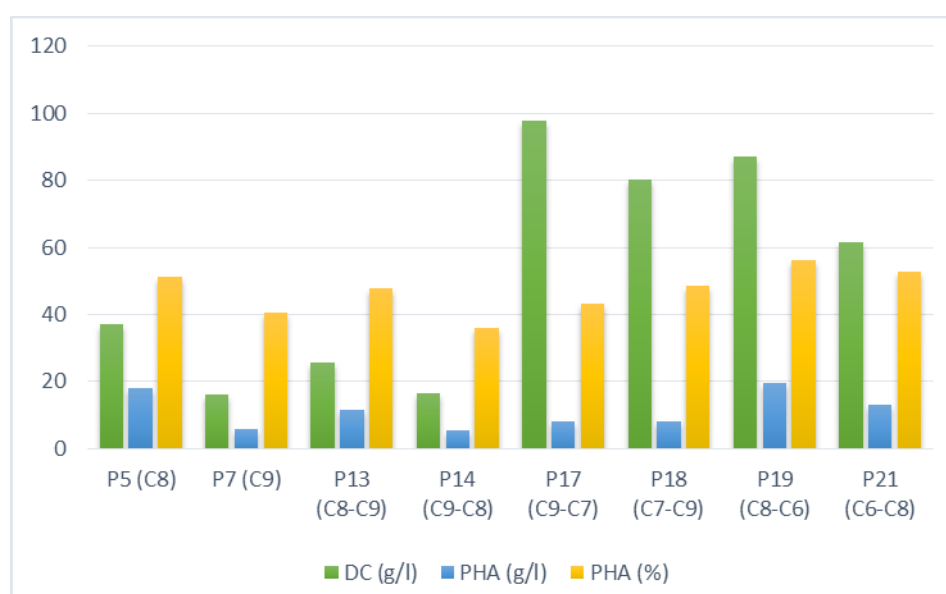

Figure 1. Biomass and *mcl*-PHAs production by *Ps. putida.*

4. Conclusions

Following the studies performed to obtain *mcl*-PHAs, several sets of experiments were performed that followed the effect of each of the C6–C9 fatty acids and their combinations. The results showed the optimal conditions and the maximum limit of metabolism with four fatty acids and the ability of the microorganism to metabolize octanoic acid easier and more productive than the other three fatty acids, these results are further verified in the experimental model of biosynthesis and processing to obtain PHAs. The results revealed the performance of the microorganism to produce *mcl*-PHA by converting the monomers tested as precursors to a maximum limit of 16.70 g/L. Thus, polymers containing (in percentage) 66.18 C7, from 79.46 to 88 C8, from 45.72 to 79.32 C9.

And following the obtained results, an in-depth study of these biopolymers can be continued for their use as a material in the electrospinning method to obtain fibres and scaffolds for tissue engineering applications.

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