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Studies on poly-3-hydroxyoctanoate biosynthesis by a consortium of microorganisms

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Abstract. Polyhydroxyalcanoates (PHAs) are specifically produced by a wide variety of bacteria, as an intracellular energy reserve in the form of homo- and copolymers of [R]-*β*-hydroxyalkanoic acids, depending on the C source used for microorganism growth, when the cells are grown under stressing conditions. In this paper we present microbiological accumulation of poly-3-hydroxyoctanoate (PHO) by using a consortium of bacterial strains, *Pseudomonas putida* and *Bacillus subtilis*, in a rate of 3:1, grown on a fermentation medium based on sodium octanoate as the sole carbon source. The experiments performed in the above mentioned conditions led to the following results: from 18.70 g sodium octanoate (7.72 g/L in the fermentation medium) used up during the bioprocess, 3.93-3.96 g/L dry bacterial biomass and 1.834 - 1.884 g/L PHA, containing 85.83 - 86.8% PHO, were obtained.

Keywords: polyhydroxyalcanoates, *Pseudomonas putida*, *Bacillus subtilis*, sodium octanoate, poly-3-hydroxyoctanoate.

1. Introduction

Poly(*β*-hydroxyalkanoic acid)s (PHAs), also known as polyhydroxyalkanoates or microbial polyester, are a class of natural thermoplastic polymers [1]. Due to their properties, similar to those of conventional plastics and to their biodegradability, have attracted much interest as alternatives to synthetic polymers, the more so as they can be produced from renewable resources and processed with the aid of equipments used for polyolefins or other synthetic materials. The mechanical properties of PHAs depend on monomer structure and on molecular weight of polymers, varying between rubber like elasticity and brittleness of crystalline textolite.

Microbial polyhydroxyalkanoates comprise a wide variety of different polyesters, with more than 150 (R)-hydroxyalkanoates identified as known constituents, who are synthesized intracellularly as carbon and energy storage compounds under unbalanced growth conditions [2].

PHAs are biodegradable, biocompatible and naturally accumulated by several bacteria such as *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cupriavidus* and some species of photosynthetic bacteria under conditions of nutrient stress [3-9].

Numerous researchers have attempted to isolate PHA producing-microorganisms from various sources since the potential to discover and identify novel species with vastly superior production capacity remains untapped [10-13].

In this paper we present microbiological accumulation of poly-3-hydroxyoctanoate (PHO) by using a consortium of bacterial strains, *Pseudomonas putida* and *Bacillus subtilis*, in a rate of 3:1, grown on a fermentation medium based on sodium octanoate as the sole carbon source. The isolation process needs a pre-treatment and degreasing, followed by solvent extraction and purification. The resulted polymer was suspended in chloroform and evaporated until a thin pellicle was obtained.

2. Experimental

2.1. Materials

Components of nutritive media were purchased from Merck and Sigma-Aldrich, organic solvents, analytical reagents and mineral salts, from Merck, except for methyl esters of 3-hydroxy acids (C8, C9, C10), purity 98% purchased from Larodan, Sweden.

The PHA producing microorganisms were three laboratory strains (wild type), *Pseudomonas putida* ICCF 391, *Bacillus subtilis* BSP ICCF 84 and *Bacillus subtilis* BSV ICCF 349, and their

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performances are presented as experiments 1, 2 and 3. Stock cultures were grown at 28ºC and maintained by periodic transfer on M44 (cDSMZ424) agar slants.

2.2. Procedures and methods

Inoculum growth medium and cultivation conditions

A microbial suspension in sterile distilled water was prepared from the stock culture. 10 mL of a medium containing (% g/v): glucose 1%, corn extract 1.5%, KH_2PO_4 1%, NaCl 1%, $MgSO_4$ 0.05% previously sterilized at 115ºC, for 20 minutes was inoculated with 1 mL of the above-mentioned microbial suspension, containing 10^7 cfu/mL. The inoculum (preculture) was prepared in 500 mL Erlenmeyer flasks, by incubation at 30ºC, for 20 – 24 hours, on a laboratory rotary shaker (Heidolph – Germany) at 220 rpm.

Fermentation 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 (medium E): $NaNH_4HPO_4 \cdot 4H_2O$ 0.35 g%, K_2HPO_4 0.75 g%, KH_2PO_4 0.37 g%, trace element solution I 0.1 mL%, trace element solution II 0.1 mL%. Trace element solution I contains 120 g/L $MgSO \cdot 7H_2O$. Trace element solution II contains per liter, 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 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 $ZnSO_4 \cdot 7H_2O$ in 1 M HCl. The sterilized fermentation medium (as mentioned above) was inoculated with 10 % (v/v) preculture and incubated at 30ºC, for 48 hours, on a rotary shaker, at 220 rpm. At 0 and 24 hours of cultivation, the medium was supplemented with octanoic acid, to a final concentration of 0.425% g/v**,** according to the HPLC assay of octanoic acid content, and other several measurements were carried out: pH, dry cells and optical density (OD) to measure the bacterial growth, and mcl-PHA content, measured by GC-FID

Biomass separation and preparation post-biosynthesis

At the end of the fermentation process particles of bacterial cells were separated by centrifugation at 4000 rpm, in a centrifuge (Hettich – Germany), washed with water and methanol and the obtained suspension was centrifuged again, in the same conditions.

For this experimental recovery procedure, a preliminary methanol treatment of dried bacterial biomass was performed, in order to dissolve certain non PHAs components, susceptible to be further extracted in acetone. Methanol was added to the dried biomass at a ratio of 20:1 (v/g); the resulted suspension was stirred 30 minutes at room temperature, and then centrifuged, and the sediment washed with distilled water and vacuum dried. Soxhlet extraction with acetone was conducted at 55-57ºC, during 5 hours. The PHA containing acetone solution was filtered (for removing bacterial cell components carried by acetone vapors) and concentrated at 2/3 from the initial volume. Methanol was added to the concentrated acetone and PHAs precipitated as a clay, which was dissolved in chloroform. PHA chloroform solution was evaporated slowly at room temperature to form a polymer film.

PHA 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 [14]. The polymer composition and purity degree resulted by summing all of the contained monomers, determined by GC-FID, and expressed in g/100 g product or in % moles. For the monomer composition determination, a capillary column has been used with a HP 5 (5% phenyl - methylpolysiloxan) stationary PHAs. Methyl esters of C6 – C 10 hydroxy acids have been used as standard substances.

3. Results and Discussions

3.1. PHAs bacterial biosynthesis

Several experiments were performed going through all specific stages of a microbial biosynthesis process. We tested two consortia of microorganisms, *P. putida* and *B. subtilis* / *B. subtilis* BSV in a ratio of 3:1, compared to PHA biosynthesis with *P. putida.*

Evolution of the fermentation process was followed by measurements of pH, optical density, dry cell weight and consumption of C8 during fermentation (HPLC), as shown in Table 1.

Table 1. Fermentations in system fed batch for PHA biosynthesis

No.	Strains	C8 (g/L)	Fermentation evolution		
			pH	OD[1]	DC[2] (g/L)
1	*P.putida*	8.51	7.74	0.559	1.86
2	*P.putida* : *B.subtilis* BSP (3:1)	8.51	7.55	0.599	3.96
3	*P.putida : B.subtilis* BSV (3:1)	8.51	7.60	0.562	3.93

[1] Optical Density measured at 550 nm
[2] Dry Cell Weight /L

Correlating data from the experiments carried out, we observed that using a sole microorganism (*P. Putida*), a smaller amount of dry biomass (g/L) was obtained, comparing with the fermentation of the microorganism consortium.

From all the precursor amount (octanoate) used up by the consortium during the fermentation, 18.72 g (8.51 g/L in the fermentation medium), 3.93 - 3.96 g/L dry bacterial biomass and 1.834 - 1.884 g/L PHA, containing 85.83 - 86.8% PHO were obtained (Fig. 1).

3.2. PHA extraction

For Soxhlet acetone extraction of PHA, dry biomass was used after a methanol treatment for removing impurities susceptible to be extracted in acetone. The isolation-purification yields varied between 44 and 50% depending on the polymer content of dry cells.

The composition and purity of polymers isolated by Soxhlet extraction with acetone were determined by GC-FID and expressed in g/100 g product analyzed or mole% (Table 2).

Table 2. Analytical characteristics of polymers biosynthesized

No.* Experiment	PHA %	Hydroxy acids			
		C6 (%)	C8 (%)	C10 (%)	Molar ratio C6:C8:C10
1	47.6	5.38	85.83	6.45	3.75:50:3.2
2	44.0	5.52	86.80	5.65	3.8:50:5.7
3	47.5	5.55	86.56	5.68	3.8:50:2.9

*The polymers presented are obtained from the fermentation above mentioned in experiments No. 1, 2 and 3.

The analytical results show that in the conditions experimentally established for isolation and purification of PHAs obtained using octanoate as a precursor fed every six hours, polymers with content in C8 ranging between 85.83 to 86.8%, C6 between 5.38 and 5.55 % and C10 between 5.65 and 6.45% are obtained.

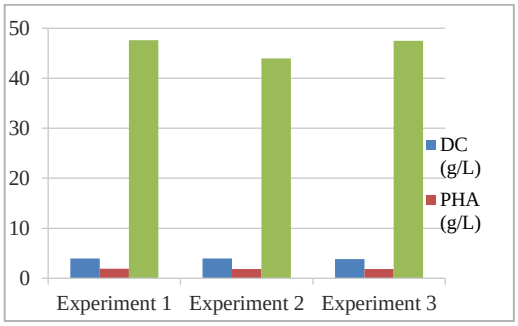

Figure 1. Biomass and mcl-PHA production with consortium of microorganisms

A PHO film obtained in conditions above mentioned is showed in Fig. 2.

Figure 2. Examples of polymers biosynthesized from *Ps. Putida* and *B. Subtilis* containing the PHO / PHA between 85.83-86.8%

4. Conclusions

The experiments carried out in this work were focused on the use of the consortium formed of one strain of *Ps. putida* and two strains of *B. subtilis* selected as PHA producers, due to their ability to use octanoic acid as unique sources of C and energy for cell growth.

The results revealed the consortium performances to produce mcl-PHA by conversion of C8 at a maximum limit of 18.87 g octanoic acid.

Thus, polymers containing (in percentages) 5.38-5.55 C 6, from 85.83 to 86.8 C8, and 5.65 to 6.45 C10 were obtained.

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