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STUDIES ON BIOSYNTHESIS OF POLYHYDOXYALKANOATES (PHA) FOR BIOMEDICAL ENGINEERING

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ABSTRACT. Performed researches during recent years have encouraged PHA experiments on their use in development of biological materials (biodegradable) in medical applications. The purpose of our research was to study the capacity of some *Pseudomonas* strains to synthesize medium chain length PHA (mcl-PHA) containing monomers C6 – C14 from different substrates and to characterize the obtained biopolymers with respect to their monomer compositions. From three strains cultivated on media containing structural related, non-related or both C sources, a strain of *Pseudomonas putida (*ICCF 391) showed the highest bio productive capacity when grown on glucose and octanoate as C sources and a strain of *Pseudomonas fluorescens* (ICCF 392) had the best bio productivity when cultivated on citrate and octanoate as C sources. The GC-FID characterization of the biopolymers, performed after a mild methanolysis, disclosed the prevalence of the monomer induced by the structural related compound used as an additional carbon sources.

Keywords: polyhydroxyalkanoates, biodegradable polymers, biopolymers, mcl-PHA, *Pseudomonas*

INTRODUCTION

The use of polyhydroxyalkanoates (PHA) for biomedical applications is well known in literature as they are biodegradable and biocompatible polymers with lots of possible applications (Liu X *et al.*, 2004; Pompe T *et al.*, 2007). Our attention turned to the development of some PHA suitable for new composite materials with biomedical applications like: bone tissue repairs and blood vessel engineering. It is well known that mcl-PHA is a family of bio-polyesters which differ from each other depending on their monomer composition. Up to now, more than 100 monomers have been revealed in these polymers. Among these monomers, there are 3-hydroxy acids with 6-16 carbon atoms, with a wide variety of chains (saturated, unsaturated, linear or branched), containing aliphatic and aromatic side chains, with different functional groups (Steinbüchel , 2001).

Physical and mechanical properties of mcl-PHA depend on their structure, namely the structure of the monomers from which they are composed. It follows that these properties can be designed at the stage of fermentation, by using different substrates. Conversion of the substrates is specific, depending on the involved structure and metabolic pathways.

Substrates for mcl-PHA production can be divided into three categories, depending on the function that they can perform during the fermentation process (Kessler *et al.*, 2001).

- substrates that support both cell growth and production of mcl-PHA;

- substrates that support cell growth but not the production of mcl-PHA;

- substrates that support the production of mcl-PHA, but does not support cell growth .

In general, the literature mentions two strategies used in fermentation processes for the production of mcl-PHA:

- batch culture system, in the presence of both carbon source, a substrate for cell growth and another as the precursor for the structural incorporation of a specific monomer (Kim *et al.*, 1996).

- culture system in two stages: in the first stage, cell mass is produced, in the second stage the substrate that induces the synthesis and the incorporation of a specific monomer is added (Kim *et al.*, 1997; Kim, 1997).

For example, it was found that batch cultivation systems can use simultaneously a mixture of carbon sources such as citrate/octanoate (Durner *et al*., 2000) or glucose/octanoate (Kim *et al.*, 1996; Kim *et al.*, 1997) supporting cell growth as well as the production of mcl-PHA. Fatty acid acts as a precursor for the synthesis of mcl-PHA with an appropriate monomer composition and structurally unrelated carbon source is used to provide the energy support of the bacterial cells.

The purpose of the experiments presented in this work was to study the capacity of some *Pseudomonas* strains to synthesize PHA from different substrates and to characterize the obtained biopolymers with respect to their monomer compositions.

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MATERIALS AND METHODS

Bacterial strains, media and growth conditions.

According to literature the production of MCL-poly(3HA) is restricted to fluorescent Pseudomonas belonging to rRNA homology group 1 (Fiedler *et al.*, 2000; Klinke *et al.*, 2000) including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas oleovorans*, *Pseudomonas lemonnieri* and *Pseudomonas putida*. In laboratory fermentations we used three strains: *Pseudomonas fluorescens* ICCF 392, *Pseudomonas putida* ICCF 391 and *Pseudomonas aeruginosa* ICCF 90. *Pseudomonas fluorescens* was isolated from rotten beech wood in the Pharmaceutical Biotechnology Department of ICCF. *Pseudomonas aeruginosa* was purchased from American Type Culture Collection. *Pseudomonas putida* strain comes from USAMV Bucharest collection. Selected strains were maintained in a vegetative stage on a solid medium, having the following composition: yeast extract 1%, peptone 1%, glycerol 1% and 2% agar. The composition of the inoculum medium was: glucose or glycerol 1%, corn extract 1.5%, KH_2PO_4 1%, NaCl 1%, $MgSO_4$ 0.05%. Inoculum culture was developed for 24 hours at 30°C in Erlenmeyer flasks of 500 ml capacity, containing 100 ml medium, under continuous agitation on a rotating shaker, at 220 rpm. Bioprocess medium had the following composition in mineral salts (medium E): $MgSO_4 \cdot 7H_2O$ 0.2 g %, $NaNH_4HPO_4 \cdot 4H_2O$ 0.35 g%, K_2HPO_4 0.75 g%, KH_2PO_4 0.37 g%, trace element solution I 0.1mL%, trace element solution II 0.1mL% and, as C source, citric acid$\cdot H_2O$ (or glucose or glycerol) 2 g % and/or octanoate or decanoate 0.25 – 0.5 g %,. Trace element solution I contains 120g/L $MgSO.7H_2O$. 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. Fermentation medium, with an initial pH of 7 -7.2, was supplemented at different times with a solution containing the additional carbon source. This solution was added under sterile conditions.Two stock solutions have been made, sodium octanoate and sodium decanoate, both of 8.33% concentration. From these, 3mL or 6mL (double portion) were added in the fermentation media, to a final concentration of octanoate and decanoate of 0.25% or 0.5%.

Polymer biosynthesis was performed in 500 ml shaking flasks, containing 100 ml nutrient medium. This was inoculated with 10% inoculum culture. Fermentations were conducted for a period of 48 hours at 30°C on a rotary shaker. Regular measurements of bioprocess parameters were made: optical density (measured at 550 nm), pH, dried biomass and PHA content.

PHA recovery

The biomass resulted after centrifugation of the final culture was treated with a mixture of 1:1 NaOCl : $CHCl_3$ for 2 hours, under stirring, at 40 - 45°C; then it was filtered and the inferior layer of the filtrate was separated. $CHCL_3$ evaporated at room temperature and PHA were recovered as transparent films. This chemical processing for polymer isolation leads to the recovery of mcl-PHA as 35 – 47% of the dry cells.

Analytical characterization of synthesized biopolymers.

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 (Koller *et al.*, 2007). 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 PHAe. Methyl esters of C4 – C18 hydroxy acids have been used as standard substances.

FTIR spectral analysis of PHA samples was performed using a Perkin Elmer FTIR 1600 IR spectrometer. Sample insertion was done in the form of film. Spectra were recorded in the 4000 - 667 cm-1.

RESULTS AND DISCUSSION

Cultivation of *Pseudomonas* strains on media containing different carbon sources and in different conditions was performed in order to compare the fermentation processes with respect to cell growth, PHA biosynthesis and accumulation, PHA composition.

The cell growth of the three *Pseudomonas* strains was compared based on their cultivation on medium E.

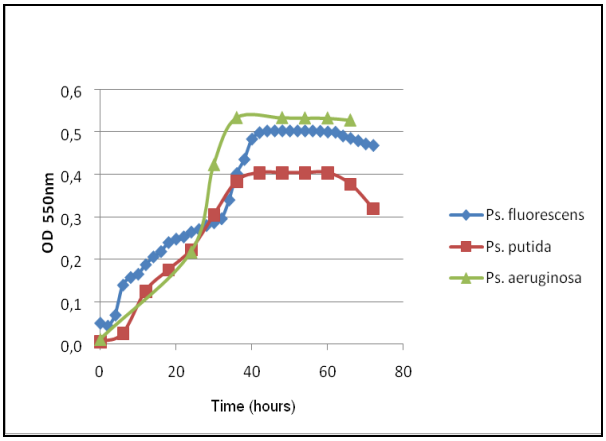

Fig.1 Growth curves of studied *Pseudomonas* strains

The following aspects can be pointed out from these experimental results:

- *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* strains developed up to a higher level of cell density as *Pseudomonas putida* strain.

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- in the case of *Pseudomonas putida* strain, the stationary phase (characterized by maximum cell density) is reached after 42 hours and lasts 18 hours, meanwhile the stationary phase of *Pseudomonas fluorescens* culture is reached at 46 hours and lasts 10 hours, and of *Pseudomonas aeruginosa* at 36 hours and lasts 24 hours.

Biomass quantities and the amounts of mcl-PHA obtained by cultivating the three strains on different media, are presented in figure no. 2.

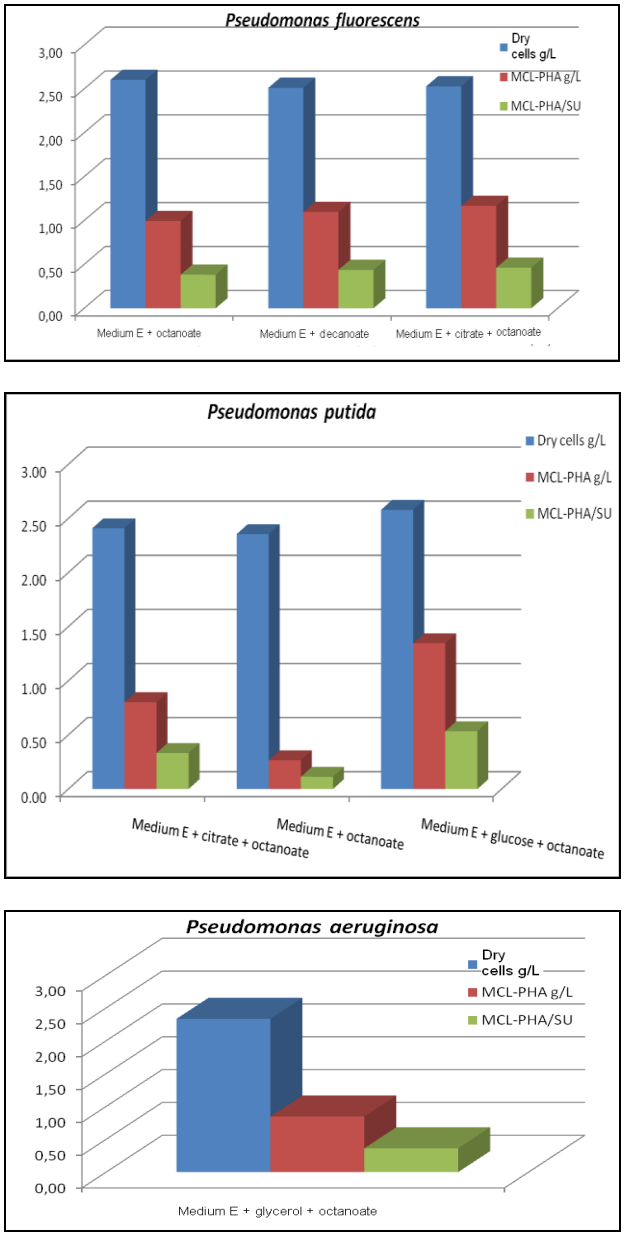

Fig. 2 Biomass and mcl-PHA production with three *Pseudomonas* strains

Correlating the biomass quantity with PHA biosynthesis and accumulation level, the results of the performed experiments showed that the optimal medium for mcl-PHA production varies from a strain to another, according to the figure no.3:

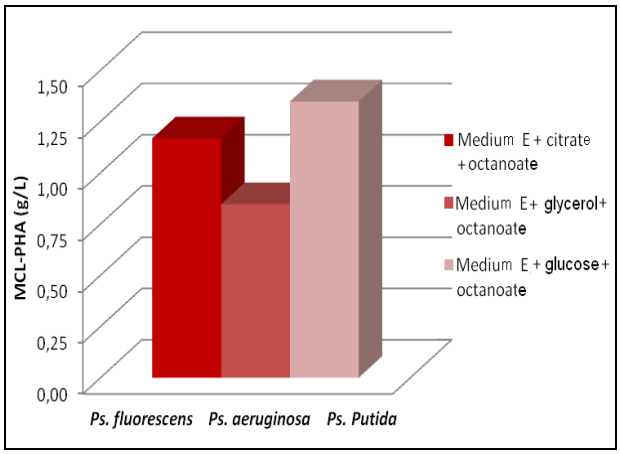

Fig. 3 Mcl-PHA bio-productivity of *Pseudomonas* strains

In terms of bio-productivity, the experiments carried out demonstrated that *Pseudomonas putida* strain grown on medium E modified with glucose and octanoate as C sources presented the highest biosynthesis capacity (1,347 g mcl-PHA/L fermentation medium), in comparison with all the other strains grown on media with different combinations of C sources.

GC-FID analysis of the obtained biopolymers highlighted the following results:

1. *Ps. putida* synthesized mcl-PHA whose content in PHO varies depending on the concentration of octanoate added to the fermentation medium as follows:

– 0,25% C8 in medium led to the following composition of mcl-PHA: C6:**C8**:C10:C11:C14 = 8:**57**:2:4:1, at total content of PHA of 72%;

– 0,5% C8 in medium resulted in a mcl-PHA containing: C6:**C8**:C10:C11:C14 = 13:**77**:2:4:1, at total content of PHA in the analyzed sample of 97%;

2. *Ps. fluorescens* produced a mcl-PHA containing mainly PHO or PHD, depending on the use of correlated C source:

– 0,25% C8 in medium led to mcl-PHA containing: C6:**C8**:C10:C11 = 13:**79**:6:2, at total content of PHA in the analyzed sample of 99,7%;

– 0,25% C10 in medium resulted in mcl-PHA with the following composition: C6:C8:C9: **C10**:C11:C14 = 9,5:46:0,5:**27**:8:1 at total content of PHA in the analyzed sample of 92%.

CONCLUSIONS

The experiments performed with three strains of *Pseudomonas* sp. in order to examine their capacity to produce mcl-PHA by conversion of structural related or non-related C sources or combinations of these, demonstrated that each of the strains has a specific behavior relative to this parameter.

Thus, the *Pseudomonas putida* ICCF 391 strain presented the best bio-productive capacity when grown on medium E with glucose and octanoate as C sources (1,347 g mcl-PHA/L fermentation medium) in

comparison with *Pseudomonas fluorescens* ICCF 392 that produced a maximum of 1,167 mcl-PHA/L when grown on citrate and octanoate and with *Pseudomonas aeruginosa* ICCF 90 *(*ATCC 9027), that produced a maximum of 0,83 mcl-PHA/L fermentation medium, when grown on glycerol and octanoate as C sources.

Regarding the composition of biopolymers, the experiments disclosed the following aspects:

- the strains tested on different media containing additional C sources as octanoate or decanoate produced only mcl-PHA, since C4 si C5 monomers have not been identified.
- the content in certain monomers can be controlled by the nature and concentration of the additional C source used in the fermentation process, whereas the biopolymers obtained during this study contained predominantly the monomer induced by the additional substrate.

According to these results, our study confirmed the fact that mcl-PHA biosynthesis strategy can be designed in a suitable manner as to lead to the prevalence of a certain monomer or ratio of monomers, of which a special, desired polymer property depends.

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