PHAs accumulation in *Pseudomonas putida* P5 (wild type and mutants) in lipid containing media

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates synthesized by various bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. In this work, we describe the isolation and characterization of *Pseudomonas putida* P5 strain, as well as some of its derivatives (mutants and recombinants) regarding the accumulation of PHAs. All the strains were subjected to fermentation and showed PHA accumulation with glucose, gluconate or octanoate as carbon source. In order to obtain PHA produced by these strains, a special procedure was developed, which allowed 80% PHA recovered. Differences in msc-PHA accumulation were observed in chemically induced mutants. RAPD analysis of these mutants proved that some rearrangements are produced as result of mutagenesis. The best results concerning msc-PHA accumulation were obtained when 0.4% octanoate was used. Different other lipid substrates (vegetal oils) were tested in order to establish the best substrate that allowed the largest accumulation of PHA. The results showed that the selected strains of *P.putida* (wild type or mutant) could utilize oil waste in the medium as a carbon source better than that of glucose and thus could substantially lower the cost of production of PHA.

Keywords: Polyhydroxyalkanoates, *Pseudomonas putida*, carbon source, mutants, recombinant strains

Introduction

Plastic materials are taking an important place in our every day life. Their physical properties make them very convenient in utilization. But these non-degradable plastics are accumulating in the environment at the rate of 25 million tones per year [6]. That is why replacement solutions like the using of biopolymers (biodegradable polymers) have been envisaged. On that purpose different kinds of polyesters (polyhydroxyalkanoates (PHAs), poly lactides, aliphatic polyesters and polysaccharides) have been studied during the last 80 years. PHAs have very interesting physical properties and biodegradable abilities [14, 15]. They could be as well used in packaging as in medical applications due to their biocompatibility and to their slow hydrolytic degradation [10].

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by various bacteria [4, 11]. They represent products with biotechnological importance due to their special properties [1]. They are accumulated intracellular as carbon and energy reserves under certain conditions and can be detected through various methods based on PHA granule specific staining. To date, approximately 120 different constituents of PHAs have been identified. Various detection methods such as Sudan black B, Nile red or Nile blue staining were described and widely used to isolate bacterial PHA producers.
The biological polyesters known as polyhydroxyalkanoates (PHAs) are mainly produced by microbial fermentation processes, and there is a major challenge to reduce their production costs [10]. The isolation and the purification of bacterial polyhydroxyalkanoates are the key step of the process profitability in the fermentation system. Through the bacterial fermentation approach, PHAs are produced and accumulated in the cytoplasm of the cell [6]. Moreover, due to the large impact of the carbon source price on production costs, one of the most important approaches to reduce costs is to use wastes and by-products as raw material for the fermentation process.

The present work was carried out to evaluate the PHA production and accumulation in three strains of *Pseudomonas putida* (wild strain and mutants derived from it) and to establish the fermentation conditions for biopolymer biosynthesis.

**Material and methods**

**Bacterial strains.** As PHA producing microorganisms a wild type *Pseudomonas putida* strain was used, designated as P5, which was selected following microbiological screening and two mutant strains, M1 and M3 (table 1).

**Table 1.** *Pseudomonas* strains used to study the PHA biosynthesis

<table>
<thead>
<tr>
<th>Code</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 5</td>
<td><em>Pseudomonas putida</em> (wild strain)</td>
<td>Genetic Engineering Laboratory, Faculty of Biotechnology Bucharest</td>
</tr>
<tr>
<td>M1</td>
<td>Mutant P5-derived strain obtained through NTG treatment</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>Mutant P5-derived strain obtained through NTG treatment</td>
<td></td>
</tr>
</tbody>
</table>

The strains M1 and M3 were selected during a mutagenesis process with nitrosoguanidine applied to the P5 wild type strain of *Pseudomonas putida*. The selection of the mutants was based on the intensity of the fluorescence of the colonies grown on selective medium supplemented with Nile blue or Nile red [13].

The selected strains were maintained on solid medium, with following composition: 0.3% meat extract, 1% peptone, 0.5% NaCl and 2% agar. The medium was prepared by dissolving all the components in hot distillate water (pH 7.0), then was sterilized through autoclave at 121°C, 20 minutes. The bacterial cells were cultivated in static conditions, for 48 h, at 28°C.

**Fermentation.** Fermentation experiments were achieved by cultivation of the bacterial strains (table 1) on nutritive media with different concentration of Na-octanoate (0.1 – 0.6%), mineral salts, at 30 – 40°C, for 48 – 72 h, with 220 rpm stirring. The influence of octanoate concentration and fermentation parameters on polyester biosynthesis and accumulation and the correlation between biomass and PHA concentration were determined by measuring optical density, octanoate content (GC method), as well as dry cell weight (gravimetric method) as characteristics of fermentation media [8, 9]. The characterization of PHA was performed using a FT-IR method (IR spectrometer Perkin Elmer FTIR 1600) as well as a GC-MS method (Hewlett-Packard model 5859/Hewlett-Packard model 5972). The recovery of intracellular PHA was performed using NaClO digestion and/or solvent extraction [5].
Results and discussion

1. msc-PHA biosynthesis using *Pseudomonas putida*

PHAs are biodegradable thermoplastics consisting of monomers having a D(-) configuration. Depending on PHA accumulation kinetics, bacteria can be divided in two groups. The first group is formed by bacteria (like *Ralstonia eutropha* or *Pseudomonas oleovorans*) that require the limitation of some nutrients. The second group does not depend on nutritional limitation as they accumulate PHA during cell growth (like *Alcaligenes latus*, *P. putida*, *P. aeruginosa* and recombinant *E.coli*) [2]. Most of the carbon substrates supplied for PHA production are pure alkanes, fatty acids or carbohydrates but various wastes products as well new bacterial isolates were tested in order to reduce production costs [2, 7].

Two approaches were followed in our experiments. First: characterization of some bacterial strains for their ability of PHA production and accumulation during the fermentation using octanoate as carbon source and second: optimization of fermentation conditions and of biopolymer recovery procedures in order to obtain increased quantities of PHA.

Three bacterial strains were examined in our experiments for PHA production: the wild type *P. putida* P5 and two mutants, designated as M1 and M3. Two parameters were surveyed during bacterial cultivation in similar conditions, on medium containing sodium octanoate as carbon source: biomass production and PHA biosynthesis and accumulation.

Increased level of bacterial biomass, during fermentation, for both mutant strains comparing with the wild type strain was observed. The best results were obtained with M1 mutant strain (fig. 1).

![Figure 1. Growth rate of the selected bacterial strains](image-url)

The biomass evolution for these three strains is different, due to the following aspects: for the wild type strain, the stationary phase (characterized by the maximum biomass concentration) is reached after 42 h and lasts for 18 h; for the M1 recombined strain, the maximum is reached at 42 h and the stationary phase lasts for 12 h; for the M3 recombined strain, the stationary phase is reached after 48 h and last only 6 h.

Regarding the quantities of dry biomass obtained by the cultivation of these three strains for 48 h, the mutant M1 strain presented the most favorable evolution. It has to be mentioned that the biomass development has to be correlated with both PHA biosynthesis and PHA accumulation levels (fig. 2).
Fig. 2. The maximum dry biomass accumulation obtained by the cultivation of the selected strains.

From bio productivity related to dry biomass point of view, it was observed that, although both mutant strains have a superior capability to produce and accumulate PHA against P5 wild type strain, the M3 mutant strain was able to accumulate higher quantities of PHA, comparing to M1 strain (fig. 3).

Figure 3. The variation of msc-PHA bioprocessivity related to dry biomass for the selected strains.

Because the bioproductivity related to dry biomass for P5/M3 strain is higher than that of P5/M1 strain, it could be considered that the P5/M3 strain is the recommended one to post-biosynthesis processing and this fact would involve reduced costs for materials in the following processing steps.

The same quantities of PHA (1.5g/L) were determined for both mutant strains, after cultivation in 1L fermentation medium, being increased with 67% compared with P5 wild strain (fig. 4).

Figure 4. The variation of the msc-PHA concentration related to bacterial strain type.
2. The biomass separation in presence of Ponilit CS2

Optimization of fermentation conditions for PHA production and recovery is the aim of various experiments [12]. It was observed that the concentration of bacterial cells after fermentation is influenced by flocculation agents [3].

In our experiments, Ponilit CS2 was used as flocculation agent. In order to determinate the optimum concentration of this flocculation agent, 100 ml from final fermentation, performed with all three strains, were used for PHA biosynthesis and accumulation. The results obtained for these experiments revealed the fact that, using 0.1% v/v concentration of Ponilit CS2 or Ponilit GT1 (in previous experiments), the sedimentation time is considerably reduced and the bacterial biomass can be fully recovered, through centrifugation (table 2).

Table 2. The biomass recovering using Ponilit CS2 as flocculation agent

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ponilit CS2 concentration (%v/v)</th>
<th>Sedimentation time (min)</th>
<th>Recovered quantity of dry biomass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>-</td>
<td>30</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>5</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3</td>
<td>0.26</td>
</tr>
<tr>
<td>M1</td>
<td>-</td>
<td>25</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>4</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3</td>
<td>0.33</td>
</tr>
<tr>
<td>M3</td>
<td>-</td>
<td>30</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10</td>
<td>0.30</td>
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<td></td>
<td>0.2</td>
<td>6</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4</td>
<td>0.30</td>
</tr>
</tbody>
</table>

3. The isolation of PHA

Three methods of biopolymer extraction were tested in order to select the best conditions of PHA recovery:

**Method 1**
- NaClO digestion of thawed biomass, in order to transform and render soluble the non-PHA material, followed by centrifugation;
- washing successive operations of PHA containing cellular rests, using distillate water, followed by acetone treatment for desalination and lipids dissolution;
- PHA extraction from water insoluble cellular material, using CHCl₃ treatment and stirring for 24 h at 30°C;
- filtration of the resulted suspension;
- concentration of the chloroformed filtrates at 1/3 from initial volume;
- PHA precipitation (3-5):1 MeOH : concentrate chloroform solution.

**Method 2**
- it was eliminate the NaClO digestion + centrifugation phase, from method 1.

**Method 3**
- it was eliminate the NaClO digestion + centrifugation phase, from method 1;
- MeOH for dissolution of the remained lipids.
The best results were obtained when biopolymer extraction method 3 was used (fig. 5). The lowest recovering yield of mcl-PHA was obtained with the first variant of extraction method, due to the NaClO oxidative degradation effect. When the second variant of extraction method was used, the yield was lower than the variant, due to msc-PHA solubility in acetone used to biomass washing.

![Graph showing PHA isolation efficiency vs extraction method]

**Figure 5.** The variation of the PHA isolation efficiency related to the extraction method.

The biopolymer produced by the selected strains was examined using microscopy, a characteristic structure being observed (fig. 6).

![Microscopical aspect of the PHA biopolymer produced by P.putida 5-3 strain.]

**Figure 6.** Microscopical aspect of the PHA biopolymer produced by *P.putida* 5-3 strain.

The chemical composition of PHA produced by selected strains was determined by GC-MS (data not shown). It was observed that the main content was represented by the PHO as follows: 20–25% 3-hidroxybutirate, 20-30% 3-hidroxyhexanoate and 35–50% 3-hidroxyoctanoate.

**Conclusions**

The performed experiments allow the following conclusions:

- During fermentation, increased biomass level was obtained with both mutant strains, the best results being obtained with M1 strain.
- Mutant strains registered the same PHA quantities recovered from biomass resulted in 1L fermentation medium, but these were higher than for wild strain.
During fermentation, using Ponilit CS2 as flocculation agents allowed the reduction of sedimentation time and fully recovering of the bacterial biomass, through centrifugation.

Using GC-MS to characterize the PHA obtained from each selected strain, the main content was represented by the PHO, as follows: 20–25% 3-hydroxybutirate, 20-30% 3-hydroxyhexanoate, 35–50% 3-hydroxyoctanoate.

Biopolymer extracted from bacterial strains was subjected to microscopic analysis, specific aspects being observed.

Acknowledgments

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References