Effect of The Sequential Utilization of Glucose and its Membrane Oxidation Products on The Polyhydroxyalkanoate Synthesis in *Pseudomonas putida*

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**ABSTRACT**

Batch culture of *Pseudomonas putida* mt-2 started on glucose progressed in three successive phases according to the substrates available, respectively: glucose, gluconate and 2-ketogluconate. These two latter compounds derived from the membrane oxidation of glucose. Growth was faster on glucose and it decreased significantly on gluconate then 2-ketogluconate without diauxic phases and showed fast transitions of the respiratory quotient values. FT-IR spectra of cells sampled from each phase were statistically well discriminated and showed a structural modifications. In particular, absorbance at characteristic bands of poly(3-hydroxyalkanoate) increased during the course of the culture. The polymer was identified by GC-MS as medium-chain-length type (mcl-PHA) composed mainly of 3-hydroxydecanoate (~ 52 %). PHA cell contents were 0.8 to 1.3 % on glucose, 2.0 % on gluconate and 5.2 % on 2-ketogluconate. Without nutrient limitation, *P. putida* mt-2 was able to accumulate mcl-PHA. The decreases of the oxygen uptake and of the growth rate concomitant with the sequential utilization of the substrates seemed to enhance the polyester synthesis. Based on the kinetic data and on the involved pathways, the effect of energy production on the PHA synthesis was discussed.

**Key Words:** *Pseudomonas putida* mt-2, glucose metabolism, PHA synthesis, energy production, regulation.

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**INTRODUCTION**

*Pseudomonas putida*, among other pseudomonads, expresses membrane dehydrogenases. Figure (1) enabling the extracellular conversion of glucose to gluconate (Gn) and thereafter to 2-ketogluconate (KGn) (Leslie and Phibbs 1984). As in *P. fluorescens* (Fuhrer et al. 2005), cultures of *P. putida* mt-2 on glucose do not show any diauxic although seemingly it utilizes sequentially the sugar then its oxidation products, i.e. Gn and KGn (Latrach Tlemçani et al. 2008). The transitions from one substrate to the other coincide with variations of the respiratory quotient (RQ), indicating metabolic changes.

Metabolic adaptations can lead to partial deviations of the carbon flux toward the synthesis of new molecules like poly-hydroxyalkanoate (PHA). Many Pseudomonas are able to store PHA, especially under unbalanced nutrient conditions such as a nitrogen limitation which is generally combined to a carbon excess (Diniz et al., 2004 and Huisman et al. 1989). *P. putida* KT2440 and KT2442, strains TOL-plasmid-cured derivatives of *P. putida* mt-2 (Bagdasarian et al. 1981), were reported to accumulate medium-chain-length PHA (mcl-PHA) (Huijberts et al. 1992 and Sun et al. 2007). That must be the case for the parent strain mt-2. The more current strategies to enhance PHA accumulation are often based on nutritive limitations which however disadvantage cell growth (Sun et al. 2007). Recently Sun et al. (2009) obtained unsaturated mcl-PHA without nitrogen or phosphorus limitations using PHA-related substrates, but the imposed specific growth rates were often low and did not exceed 0.26 h−1. According to Prieto et al. (2007), there is very little knowledge about the regulation of PHA synthesis in pseudomonads.

Polyhydroxyalkanoates (PHAs) are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by bacteria to store carbon and energy.
More than 150 different monomers can be combined within this family to give materials with extremely different properties (Chen and Wu 2005). These plastics are biodegradable and are used in the production of bioplastics. PHAs -have potential applications, particularly within the medical and pharmaceutical industries (Doi and Steinbuchel 2002).

This work aims to highlight some metabolic changes consequent to the sequential utilization of glucose, Gn and KGn during growth of P. putida mt-2. We especially attempted to know if there is a relation between the culture progress and the synthesis of PHA. The investigation was based on FT-IR data of cells and the online measurements of oxygen uptake and CO₂ production. Characterization of the stored PHA, i.e. the cell content and the monomer composition, were performed by Gas Chromatography with MS and FID detections.

MATERIALS AND METHODS

Bacterium, culture conditions and assays: Pseudomonas putida mt-2 DSM-3931 (from DSMZ, Germany) was used throughout this study. Cultures were performed in a 3-L AppliKon fermentor containing 2 L of a mineral medium supplemented with 20 g L⁻¹ of glucose. Culture conditions, medium composition, determination of substrate concentrations and inlet/outlet gases analysis were described in a previous work (Latrach-Tlemçani et al. 2008). Cell sampling for GC and FT-IR analyses were carried out at well-defined ages of the cultures according to the available substrate: 2 h (growth starting on glucose), 4.5 h (glucose log-phase), 7 h (gluconate log-phase) and 10 h (2-ketogluconate log-phase).

FT-IR analyses: Whole cells were harvested from 10 mL of culture medium by centrifugation (5000 × g, 10 min) then washed twice with 9% (W/V) NaCl solution. Pellet was suspended in an appropriate volume of the saline to obtain 0.7 units of absorbance at 660 nm. Five µL of this suspension was spread out in triplicate on ZnSe spectral windows and then dried for 20 min within a vacuum desiccator. Spectra were recorded in the transmission mode using a Tensor 27 HTS-XT (Bruker, France) between 4000 cm⁻¹ and 700 cm⁻¹ at a resolution of 4 cm⁻¹. Each final bacterial spectrum results from the ratio of sample spectrum to the background one, then registered in absorbance units. Final spectra were submitted to a “quality test” adapted from Helm et al. (1991) then normalized by setting to 1 unit the relative absorbance of the amide-I band located around 1650 cm⁻¹ and 1654 cm⁻¹. Spectral data were compared by using hierarchical cluster analysis based on Ward’s algorithm (Opus 6.0 software, Bruker Optics). The detailed procedure was described in a previous work (Guibet et al. 2003).

Gas Chromatography analyses: Hydrolysis of PHA and derivatization in volatile fatty acid methyl esters:

The method was widely adapted from the work of Huijberts et al. (1992) and Lageveen et al. (1988). After extraction, hydrolysis and methanolysis, the organic phase was recovered by centrifugation (10000 × g, 5 min) and dried over anhydrous MgSO₄ then filtered on deactivated glass wool. The extracts were stored at -20 °C prior to analysis. 0.5 g L⁻¹ of methyl hexanoate ester in chloroform was used as internal standard. 3-Hydroxydecanoic acid (from Sigma) was methanolized then used for calibration in the range of 0 to 56 mg L⁻¹.

GC-MS apparatus and conditions: Analysis of the methyl esters was performed with a Varian 3800 chromatograph equipped with a BP-20 column (50 m × 0.22 mm i.d., SGE Europe Ltd, France). The same temperature program as for GC-MS was used. The injector operated in split mode (1:10), its temperature as that of FID were set at 230 °C. Temperature program was 32 °C to 240 °C with a rate of 10 °C min⁻¹. The mass spectrometer was a Saturn 2000 equipped with an ion trap analyzer, operating at 1 scan s⁻¹ either in electron impact mode (70 eV) or in chemical ionization mode (with acetonitrile as ionization reagent). The detection of methyl 3-hydroxyalkanoates was based on the presence of a specific MS fragment m/z=103 corresponding to a McLafferty rearrangement. For each peak detected, a linear retention index was calculated using GC-Retention Index external standards (hydrocarbons from C7 to C31) (van den Dool and Kratz 1963).

GC-FID apparatus and conditions: The solution of methanolyzed polymer was analyzed with a Varian 3900 chromatograph equipped with a BP-20 column (30 m × 0.25 mm i.d., SGE Europe Ltd, France). The same temperature program as for GC-MS was used. The injector operated in split mode (1:5), its temperature as that of FID were set at 230 °C. For each peak, a linear retention index was calculated using GC-Retention Index external standards (hydrocarbons from C7 to C31) in order to attribute peaks corresponding to methyl 3-hydroxyesters by comparison with GC-MS data.

RESULTS

Figure (2) represents the successive phases characterizing growth of P. putida mt-2 on glucose. Glucose phase (I), during which Gn and KGn are produced, takes place until complete depletion of the sugar. Then Gn phase (II) starts whereas KGn continues to accumulate. At Gn exhaustion, cells utilize KGn: The KGn phase (III).

RQ values (CPR/OUR) increased with the progress of the culture and were characteristic of each growth phase.
Concomitantly specific growth and oxygen uptake rates decreased while CO₂ production remained rather constant at 12 ± 2 mmole g⁻¹ h⁻¹ (Table 1).

By the hierarchical cluster analysis, FT-IR spectra of cells harvested from the different growth phases could gather in four well-discriminated groups (Figure 3).

The subtraction of Sample-1 spectrum, sampled at 2 h and taken as reference, with the three others spectra Figure (4) showed several structural differences, in particular at a characteristic wavenumbers of PHA: 2927 cm⁻¹, 1745 cm⁻¹ and 1158 cm⁻¹ (Hong et al. 1999 and Randriamahefa et al. 2003).

Detection and analysis of the polyester were performed using GC-MS. Five different monomers could be characterized using both electron impact and chemical ionization as detection modes. Calculation of retention index was carried out to confirm the identification. Four methyl 3-hydroxyalkanoates were saturated monomers (labelled C₈:₀, C₁₀:₀, C₁₂:₀ and C₁₄:₀) while only one was mono-unsaturated (labelled C₁₂:₁). The biggest peak was recorded for methyl 3-hydroxydecanoate (C₁₀:₁). The relative response factors for C₈:₀, C₁₀:₀, C₁₂:₀, C₁₂:₁ and C₁₄:₀ were, respectively: 3.12 ± 0.77, 6.24 ± 0.24, 5.85 ± 0.24, 1.54 ± 0.11 and 5.92* (*SD could not be calculated). PHA represents about 0.8 % of the dry biomass harvested at the beginning of the culture (sample-1). This content increased during the culture to reach 5.2 % in the KGn phase (Table 1). However, PHA composition did not much vary; it was always made of about 52 % of the C₁₀:₀ monomer. The fraction of C₁₂:₀ decreased while the amount of C₈:₀ and C₁₂:₁ monomers increased. The level of C₁₄:₀ remained very low, less than 2 % of the total composition of PHA.

Table 1: Growth kinetics, PHA content and PHA composition in P. putida mt-2 grown on glucose (nd: non-determined).

<table>
<thead>
<tr>
<th>Growth substrate (Sample n°, age)</th>
<th>µ (h⁻¹)</th>
<th>q₉O₂ (mmol g⁻¹ h⁻¹)</th>
<th>q₉CO₂ (mmol g⁻¹ h⁻¹)</th>
<th>PHA content (% w/w)</th>
<th>PHA composition (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (S1, 2h)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.76</td>
<td>C₈:₀ 3.8 C₁₀:₀ 54.0 C₁₂:₀ 35.9 C₁₂:₁ 6.2 C₁₄:₀ 0.0</td>
</tr>
<tr>
<td>Glucose (S2, 4.5h)</td>
<td>0.60</td>
<td>34.0</td>
<td>13.6</td>
<td>1.32</td>
<td>C₈:₀ 3.0 C₁₀:₀ 48.7 C₁₂:₀ 36.4 C₁₂:₁ 10.0 C₁₄:₀ 1.9</td>
</tr>
<tr>
<td>Gluconate (S3, 7h)</td>
<td>0.29</td>
<td>11.8</td>
<td>10.3</td>
<td>1.96</td>
<td>C₈:₀ 4.3 C₁₀:₀ 52.4 C₁₂:₀ 27.9 C₁₂:₁ 13.1 C₁₄:₀ 2.3</td>
</tr>
<tr>
<td>2-ketogluconate (S4, 10h)</td>
<td>0.18</td>
<td>10.6</td>
<td>14.0</td>
<td>5.25</td>
<td>C₈:₀ 11.7 C₁₀:₀ 53.5 C₁₂:₀ 16.3 C₁₂:₁ 16.6 C₁₄:₀ 1.9</td>
</tr>
</tbody>
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Figure 2: Growth profile of P. putida mt-2 on glucose.

a: Arrows indicate the dates of sampling for the FT-IR and GC-MS analyses (S1: 2h, S2: 4.5h, S3: 7h and S4: 10h). CPR (– –), OUR (—) and RQ (– –).

b: Cell dry weight (O), glucose (○), gluconate (▲), 2-ketogluconate (■), glucose phase (I), gluconate phase (II) and 2-ketogluconate (III).

Figure 3: Hierarchical cluster analyses of FT-IR spectra of P. putida mt-2 cells sampled at different culture ages.

Figure 4: FT-IR spectral subtraction curves showing, in particular, the increase of absorbance at the characteristic wavenumbers of PHA.
DISCUSSION

The cultures of *P. putida* mt-2 were carried out without nutrient limitation what, as expected, favoured growth. PHA content was overall low; the highest value was reached in the KGn growth phase. The polyester was a mcl-PHA of composition similar to those reported in other strains of *P. putida* grown on nonrelated substrates (Huijberts et al. 1992 and Sanchez et al. 2003). Growth kinetics, FT-IR spectra and PHA contents evolve with the substrate available, sign of metabolic changes.

During the culture, RQ increased in consequence of the drastic fall of the specific oxygen uptake rate while the specific CO$_2$ production rate was relatively constant. The changes of RQ values coincide well with the changes of the carbon substrate. In the presence of glucose, growth and oxygen uptake rates were the highest. Utilization of the sugar seems to be more advantageous for growth than that of Gn or KGn, the PHA content was then the lowest. During glucose utilization, a significant part of energy is provided by the membrane oxidations of glucose to Gn, then to KGn, in addition to the energetic contribution of the TCA cycle. The contribution of the last can be supposed proportional to the specific CO$_2$ production which itself was rather constant. The exhaustion of glucose has several consequences. Gn and KGn would only be utilized via the KGn pathway by assuming direct assimilation of Gn is minor contribution (Fuhrer et al. 2005 and del Castillo et al. 2007). Carbon uptake via this pathway involves early consumption of ATP at KGuK and especially of NADPH at KGuD. Hence, the metabolism will have to adjust itself to maintain a suitable NADPH level, paramount for KGn utilization. Deprived of the membrane dehydrogenases contribution (drop of the oxygen uptake), the energy production decreases and becomes dependent on the TCA cycle activity. This cycle would be saturated considering that specific rate of CO$_2$ production was constant, from where the decrease of growth. Consequently, NADPH level would raise. Surpluses of reduced cofactors and carbon are then deviated towards PHA synthesis. From this point of view, reduced cofactors could have a regulatory role in the PHA synthesis by comparison to Ralstonia eutropha in which PHB synthesis is stimulated by both high concentration of NAD(P) H and high ratios of NAD(P)H/NAD(P) (Haywood et al. 1988 and Kessler et al. 2001).

On another side, deviation of carbon and reduced cofactors towards the synthesis of PHA is similar to a fermentation pathway ensuring partial regeneration of cofactors. Cell anabolism, in priority, would adjust according to the ATP production flux. Decrease of the latter would act like a bottleneck reducing the carbon flux towards biosynthesis. The surplus of assimilated carbon would increase the levels of reduced cofactors and acetyl-CoA resulting in the PHA synthesis Figure (5).

This mechanism can be extended to the nutrients indispensable for growth, like nitrogen, phosphorus. These nutrients and the energy production would act through a gauge system which modulates the anabolic bottleneck to adjust carbon flux to be converted into cell material. It is enough that a factor becomes limiting to shift the metabolism towards the synthesis of PHA.

REFERENCES


