Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions

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Abstract

Production of poly(3-hydroxyalkanoates) (PHA) by *Pseudomonas aeruginosa* 42A2 from agro-industrial oily wastes was studied. PHA accumulation, throughout the cell cycle, was observed as intracellular accumulation associated to polyphosphate granules. A 54.6% PHA accumulation was obtained when technical oleic acid (TOA) was used as carbon source. Molecular weight of the polymer was 54.7 Da. The polymer was amorphous, with glass transition at \(-47.5^\circ C\) and thermal degradation at 293°C. PHA production and monomer composition were affected by \(K_{La}\) and temperature. The most relevant characteristic of the polymer produced at low aeration rates (\(K_{La}, 0.06 \, s^{-1}\)) were the unusual C\(_{14}:2\) (13%) and the increase of C\(_{12}:1\) (42.2%). The highest amount of unsaturated monomers was found in the polymer produced at 18°C (64.4%).

PHA accumulation ranged between 66.1% when waste-free fatty acids from soybean oil (WFFA) were used as carbon substrate, 29.4% when waste frying oil (WFO) was used and 16.8% when glucose was used. Depending on the substrate supplied a wide range of components was observed. Major saturated or unsaturated components of the polymer found were C\(_{10}:0\), C\(_{12}:0\) and C\(_{8}:0\) or C\(_{12}:1\) and C\(_{14}:1\), respectively. When glucose was used as carbon substrate C\(_{9}:0\), C\(_{11}:0\) and C\(_{16}:0\) were found.

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Keywords: PHA; Waste-free fatty acids; Low aeration rates

1. Introduction

Since polyhydroxy butyrate polymers (PHB) were first described in *Bacillus subtilis*, almost 90 bacterial strains that accumulate these polyesters have been found. PHAs are seen by electron microscopy as inclusion bodies with a protein boundary envelope [1]. PHAs were first identified as carbon and reduction power reserve material in prokaryotes, but it has become clear they are ubiquitous in nature. Low molecular PHB (100–200 monomer units) was found in bacterial plasma membranes in *Azotobacter vinelandii* and *B. subtilis* and also occurs in diverse plant and animal tissues. Although there is no in vivo evidence of their function, it is assumed that these low molecular PHB forms have a function as ion-selective channels [2].

PHAs are biodegradable thermoplastics consisting of monomers of \(d(-)\) configuration. Their molecular weight is in the range of \(10^3–10^6\) Da. More than 80 different hydroxyalkanoic acids constituent of PHA have been described [3,4]. Depending on PHA accumulation kinetics, bacteria can be divided in two groups. The first group is formed by bacteria that require the limitation of some nutrients. To this group belong *Ralstonia eutropha* or *Pseudomonas oleovorans*. Bacteria of the second group do not depend on nutritional limitation as they accumulate PHA during cell growth. Some examples are *Alcaligenes latus*, *A. vinelandii*, *Pseudomonas putida*, *Pseudomonas aeruginosa* 47T2 and *E. coli* [5–7].

Most of the carbon substrates supplied for PHA production are pure alkanes, fatty acids or carbohydrates [8,9]. Complex...
substrates, such as castor or euphorbia oil, gave PHA<sub>cell</sub> of (C<sub>6</sub>–C<sub>14</sub>) with <i>P. aeruginosa</i> [10]. However, the use of sub-products or wastes has hardly been explored at all, which may well be due to the complexity of their composition. <i>Pseudomonas resinovorans</i> accumulated 15% of the cellular dry weight of PHA<sub>cell</sub> from tallow [11]. Short chain length PHA was produced by <i>R. eutropha</i> or <i>Pseudomonas</i> when incubated with the oil remaining from a rhamnose-producing process [12]. Non-related substrates, such as molasses or brewery multi-processing wastes, were converted into PHB by various <i>Azoarcus</i> strains or <i>A. latus</i>, respectively [13–15]. PHA production with waste frying oils or with sub-products from the vegetable oil refining process has not been reported.

This paper describes: (1) the taxonomic classification of the strain <i>Pseudomonas</i> sp. 42A2 (NCIMB 40405); (2) the chemical characterization of the PHA accumulated by this strain when cultivated on complex substrates, such as technical oleic acid (TOA), waste frying oil (WFO) and waste-free fatty acids from soybean oil (WFFA), or with glucose; (3) the influence of culture parameters.

2. Material and methods

2.1. Media and growth conditions

<i>Pseudomonas</i> 42A2 NCIMB 40405 was grown in a mineral medium (MM) containing (g/l): 0.1 g KCl, 1 g KH<sub>2</sub>PO<sub>4</sub>, 2 g K<sub>2</sub>HPO<sub>4</sub>, 0.01 g CaCl<sub>2</sub>, 0.5 g MgSO<sub>4</sub>, 0.012 g FeSO<sub>4</sub>, 3.5 g NaNO<sub>3</sub>, supplemented with 0.05 ml trace element solution per liter (100 ml of trace element solution contains 0.05 g Ca, 0.035 g B, 0.05 g Mn, 0.07 g Zn and 0.06 g Mo). The final pH was adjusted to 6.8 with NaOH 0.1 M if necessary. Cultures were incubated on an orbital shaker (100 rpm) in Erlenmeyer baffled flasks at 30 °C.

Technical oleic acid (kindly supplied by LASEM) (20 g/l), waste frying oil (20 g/l), waste-free fatty acids from soybean oil (kindly supplied by ERASOL, Santiago, Cuba) (60 g/l) and glucose (Panreac, Barcelona, Spain) (20 g/l) were used as carbon sources for growth and PHA accumulation. A 2% (v/v) cell suspension on saline serum of an overnight culture (72 h) was added (1 ml) to the medium (100 ml) and adjusted to pH 9.0. Cultures were incubated at 30 °C. The final pH was adjusted to 6.8 with NaOH 0.1 M if necessary. Cultures were harvested on an orbital shaker (100 rpm) in Erlenmeyer baffled flasks at 30 °C.

2.2. 16S rRNA sequences

DNA was isolated from late-exponential phase cells by lysis with sodium dodecyl sulphate (SDS)-proteinase k, and treatment with cetyltrimethylammonium bromide (CTAB) as described elsewhere [16]. The 16S rRNA gene was amplified from genomic DNA using standard PCR protocols [17]. Universal oligonucleotide primers described by Lane [18] to amplify nearly the entire gene encoding the 16S rRNA were used. Universal eubacterial primers were 16F27 (5′-AGAGTTTGA TCMTGGCTCAG) and 16R518 (5′-CGTTTACCTGTGACTTCACC) hybridizing at positions 8–27 and 1488–1511 relative to <i>E. coli</i> numbering. PCR was performed in 50 µl reactions containing 2.5 U of Taq DNA polymerase (Amersham Pharmacia Biotech), 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub> each of the deoxyxynucleoside triphosphate (Amersham Pharmacia Biotech) at a concentration of 200 µM, 0.5 µM of each primer and 1 µl of DNA solution. DNA was amplified with a Gene Amp PCR System 2400 (Perkin-Elmer). Thermal cycling conditions were: 5 min at 94 °C, followed by 30 cycles consisting of denaturation (1 min at 94 °C), annealing (1 min at 58 °C), extension (90 s at 72 °C) and a final extension at 72 °C for 10 min. Amplified DNA was confirmed by gel electrophoresis through a 1.5% (w/v) agarose gel in TBE buffer followed by staining with ethidium bromide [19].

The nucleotide sequences of the PCR product were determined in both with a DNA Sequencing Kit (ABI PRISM dRhodamine Terminator Ready Reaction Kit, Perkin-Elmer) following the manufacturer’s instructions. Universal forward and reverse primers (16F27, 16F357, 16F945, 16R518, 16R1087, 16R1488) were used. The products of the sequencing reactions were analyzed with a model 3700 DNA sequencer (Perkin-Elmer). The 16S rRNA gene sequence was aligned with published sequences from the GenBank database using NCBI BLAST [20] and RDP [21] comparison software.

2.3. Electron microscopy

Cells, harvested after 72 h of incubation in TOA, were washed with 0.3M Störelsen buffer pH 7.5 for 1.5 h at 4 °C, fixed with 2.5% glutaraldehyde and washed in 0.1 M Störelsen buffer pH 7.5 for 90 min and then post-fixed with 1% osmium for 2 h at 22 °C. The fixed cells were embedded in 1% agar and then dehydrated in a graded solution of ethanol. Ultra-thin sections 40–90 nm thick were viewed in a Philips EM 301 electron microscope with accelerating voltages of 80 kV. The lipid inclusions and polyphosphate granules were microanalysed by X-ray and monitored in a Hitachi 80 MT electron microscope with accelerating voltages of 200 kV, coupled to a EDS KEVEX-800 detector S-S-Li working at 104 eV.

2.4. Substrate composition

Gas chromatography (GC): The free fatty acid composition of the substrates used was determined by GC after methanolysis with NaOH 0.5N solution of sodium methoxid
by standard techniques [22]. Samples were methylated with diazomethane [23], diluted with ether and injected on a Shimadzu GC-14A. The monomers were analyzed on a Hewlett Packard 5890 series II Chromatograph (Hewlett Packard, Palo Alto, CA) equipped with splitless injector (Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA) in electron impact (EI) and chemical ionization (CI) mode. CI used methane as ionizing gas. Derivatized compounds, such as TMS derivatives, were separated on a methyl silicone fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The temperature program was injection at 30 °C, increase at 8 °C/min up to 310 °C and hold at this temperature for 10 min more. Helium was used as carrier gas at a flow rate of 1 ml/min.

Nuclear magnetic resonance (NMR): Purified PHA was dissolved in deuterated chloroform, and 1H and 13C NMR spectra were recorded on a Varian GEMINI 300 (Varian Assoc., Palo Alto, USA) operating at 300 MHz for 1H and 75.4 MHz for 13C. The values are expressed in δ scale relative to the chloroform signal (δ, 7.26 ppm) of the non-deuterated material.

2.7. $K_{La}$ determination

Oxygen solution transfer coefficients were calculated using fresh medium with the static gassing-out method [27], under the operating conditions of the experiments.

2.8. Molecular weight distributions

Molecular weight distributions (MWD) were determined by gel permeation chromatography (GPC) coupled with a light-scattering (LS) detector, miniDAWN (Wyatt Technology, USA), which provides precise molecular weights, $M$. This instrument uses a 20-mW semiconductor laser at a wavelength of 690 nm with three photodiode detectors placed at 45°, 90° and 135°. The solvent delivery system was a model 510 Waters pump. The separation system was a Styragel 5E Column (Waters, Milford, Mass., USA) working at 40 °C. Tetrahydrofuran (THF) was used as eluent at a flow rate of 0.5 ml/min. 50 μL of a 5 mg/ml solution was injected for each sample.

The curve, $y = 0.54227x + 12.48221; \quad R^2 = 0.99961$, was created with polystyrene standards of low polydispersity ($M_M/M_S < 1.1$), whose $M_S$ were: 9.100, 18.100, 37.900, 96.400, 190.000 and 355.000 Da.

2.9. Thermal properties: differential scanning calorimetry (DSC) and thermogravimetry (TGA)

The thermal properties of the microbial polyesters were examined by a DSC–TGA Mettler-Toledo 50 (New York, USA), using STARe System acquisition and processing software (Mettler-Toledo). For DSC analysis, samples were cooled to −150°C and then heated at a rate of 10–340°C/min under a nitrogen atmosphere (80 ml/min). Thermogravimetric analysis (TGA) was carried out at air atmosphere (100 ml/min). The heating rate was 10 °C/min.
3. Results and discussion

3.1. Taxonomic classification

Pseudomonas 42A2 (NCIMB 40045) was selected due to its capacity to accumulate hydroxy-fatty acids when grown on mineral medium with technical oleic acid as the sole carbon source [28,29]. Strain 42A2 is a motile Gram-negative rod-shaped bacterium. Activities of cytochrome oxidase catalase and arginine dihydrolase were present. Denitrification was observed. The strain could grow on McConkey. Citrate, gelatine, glucose, geraniol, fructose, glycerol, pyruvate, succinate, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid and malic acid were used for growth. Other carbon substrates which supported growth were: oleic acid (C₁₈:1), ricinoleic acid (C₁₈:1), linolenic acid (C₁₈:3), α-eloestearic acid (C₁₈:3) and the n-alkanes: decanoate (C₁₀), undecanoate (C₁₁), dodecanoate (C₁₂), tridecanoate (C₁₃), tetradecanoate (C₁₄) and pentadecanoate (C₁₅). No pyocianine was produced on King A; pyoverdine was observed on King B. No growth was observed on lactose (ONPG), lisine (LDH), ornitine (ODC), urea, indol (TDA), arabinose, mannose, mannotol, maltose, phenylacetica acid, rhamnose, inositol, sorbitol, sacarose, melibiose or amylose. The strain was able to grow at 4 and 42 °C.

The results obtained by the API (20 NE strip test) identified the isolate as P. aeruginosa (99.9%). On this premise, molecular taxonomical characterization of strain 42A2 was carried out by 16S rRNA gene sequence analysis and an almost complete gene was sequenced, consisting of 1476 nucleotides. EMBL database alignments showed 100% similarity with the 16S rRNA gene sequence of P. aeruginosa AL98 (AJ249451) [30], the clinical strain P. aeruginosa PA01 (AE004501) [31] and Pseudomonas sp. strain 61716 (AF227866) [32]. An unidentified gamma proteobacterium 16S rRNA gene, strain JTB12 isolated from environmental samples (AB015249) [33], showed 99.9% similarity with a high RDP match (0.995).

The results showed that strain 42A2 was identical to several previously characterized P. aeruginosa strains. These reference strains, reported in several environments, did not exhibit poly(3-hydroxyalkanoic acid) PHA accumulation. The 16S rRNA gene sequence data were submitted to the EMBL nucleotide sequence database and are listed under the accession number AJ309500.

3.2. Polymer detection and production of poly(3-hydroxyalkanoate) from technical oleic acid

During oleic acid biotransformation studies, two types of intracellular inclusions (transparent and dense) were seen easily in the electron microscopy micrographs (Fig. 1a). The size and number of these electron-transparent inclusions increased with incubation time and seemed to be associated with electron-dense inclusions (black granules). A similar association was described in other Pseudomonas strains [7]. X-ray microanalysis of the thin sections (Fig. 1b) indicated the presence of phosphorus, calcium and magnesium in the electron-transparent inclusion, suggesting that polyphosphate with calcium and magnesium formed the core helix of the PHA macromolecule, similar to the PHB structure already described [2].

The production of PHA from sub-products or residual hydrophobic substrates is desirable, since these are abundant and inexpensive materials. To study polymer production kinetics, strain 42A2 was first investigated at 30 °C in mineral medium with TOA as carbon source (Fig. 2). The medium was inoculated with a cell suspension from an overnight culture on TSA. Samples were taken at time intervals at different stages of cell growth. Cell dry weight and polyester accumulation increased rapidly during the exponential growth (up to 15 h) until nitrogen was depleted from the medium. The...
highest productivity (0.08 g PHA/(l h)) was obtained during this stage. However, accumulation continued until, at 24 h of incubation, 3.2 g/l CDW and 1.6 g/l of PHA had accumulated, which corresponded to a PHA cell content of 49.4%. After 36 h the PHA production rate slowed down, as growth did, and remained steady during the stationary phase. At the end of the culture (72 h), PHA concentration was 2.3 g/l with a PHA content of 54.6%. Similar results were found with P. putida, grown on mineral medium with lard as carbon source, monomer components were C 8:0 (31%), C 10:0 (28%) and C 14:1 (14%) [34]. When tallow was supplied as substrate for PHA production to P. resinovorans cultures, the main monomer components found were: C 8:0 (36%), C 10:0 (33%) and C 14:1 (6%) [11]. Interestingly, Keller-hals et al. reported the PHA composition produced by a strain of P. putida from technical oleic acid with 80% oleic acid content, similar to that used in this study. The main monomers found in the PHA were C 8:0 (49.7%), C 10:0 (29.2%), with only 6.2% of unsaturated monomers [36].

3.4. Molecular weight and thermal properties of PHA

Polystyrene standards with low polydispersity were used to generate a calibration curve from which product molecular weights of PHA TOA were determined. The complete molecular weight distribution for the PHA produced from TOA showed that the distribution was normal-logarithmic. Average molecular weights were $M_n$ of 39.148 Da and $M_w$ of 54.783 Da with a low polydispersity index ($I = M_w/M_n$) of 1.4. The molecular weight is quite a bit lower than the $M_n$ 135 kDa and $M_w$ 49 kDa found for the PHA produced from technical oleic acid by P. putida [36], whereas the polydispersity index is higher, 2.53. The DSC analysis of PHA obtained from TOA indicated that the PHA was amorphous. (1) No enthalpy changes due to cold crystallization or melting of crystalline fractions were found. (2) Glass transition was at $-47.5^\circ C$ (midpoint). (3) Thermal degradation was at about 293$^\circ C$. TGA analysis corroborated the thermal degradation temperature. No ashes were found in the thermal degradation of these polymers. The residual carbonaceous materials (7.3%) produced in thermal degradation completely burnt out at about 281$^\circ C$.

3.5. Effect of the substrate on composition of poly(3-hydroxyalkanoates)

Pseudomonads belonging to the rRNA-homology-group I synthesize 3PHA mcl [37]. The actual composition and hence the properties of PHA's produced depend on the bacterial strain, carbon source and culture conditions. P. aeruginosa 42A2 showed reasonable growth (between 3.5 and 5.5 g/l) with all the substrates studied. PHA accumulation differs with the substrate supplied (Table 1). The highest PHA content was
Table 1
Composition of PHA synthesised by *P. aeruginosa* 42A2 during growth on different carbon sources at 30 °C and a *K* <sub>La</sub> of 0.1 s<sup>-1</sup>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PHA (%)</th>
<th>Relative monomer composition of purified PHA (% w/w)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.6</td>
<td>14.5 ND 40.2 ND 4.2 32.8 ND 8.2 ND ND</td>
</tr>
<tr>
<td>WFO&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4</td>
<td>19.4 0.47 47.1 ND 1.5 18.2 3.0 7.9 2.2 ND</td>
</tr>
<tr>
<td>WFFA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.1</td>
<td>ND 39.2 ND ND 20.1 31.8 8.8 ND ND</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.8</td>
<td>9.8 0.27 48.7 0.6 11.5 11.3 ND 6.5 7.2 4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> C<sub>8:0</sub>, 3-hydroxyoctanoate; C<sub>9:0</sub>, 3-hydroxynonanoate; C<sub>10:0</sub>, 3-hydroxydecanoate; C<sub>11:0</sub>, 3-hydroxyundecanoate; C<sub>12:1</sub>, 3-hydroxydodecenoate; C<sub>12:0</sub>, 3-hydroxydodecanoate; C<sub>14:1</sub>, 3-hydroxytetradecenoate; C<sub>14:0</sub>, 3-hydroxytetradecanoate; C<sub>16:0</sub>, 3-hydroxyhexadecanoate.

<sup>b</sup> COA comprises the following major fatty acids: 80.7% oleic acid (C<sub>18:1</sub>), 5.3% linoleic acid (C<sub>18:2</sub>) and 5.1% palmitic acid (C<sub>16:0</sub>).

<sup>c</sup> WFO comprises the following major fatty acids: 41.65% oleic acid (C<sub>18:1</sub>), 27.9% linoleic acid (C<sub>18:2</sub>) and 8.2% palmitic acid (C<sub>16:0</sub>) and 4.74% stearic acid (C<sub>18:0</sub>).

<sup>d</sup> WFFA comprises the following major fatty acids: 50.8% linoleic acid (C<sub>18:2</sub>), 22.8% oleic acid and 6.8% linolenic acid (C<sub>18:3</sub>).

<sup>e</sup> ND, not detectable.

Achieved with the related-carbon substrates: WFFA (66.1%) followed by TOA (52.7%) and WFO (29.8%). With glucose, the non-related carbon source, only 16.8% PHA content was obtained.

As expected, the composition of the polymer varied with the carbon substrate added. In all the related substrates (TOA, WFO, WFFA) supplied, a wide variety of saturated or unsaturated monomers, ranging between C<sub>8</sub> and C<sub>14</sub> chain length in the PHA, were detected (Table 1). The main PHA components were C<sub>10:0</sub>, C<sub>12:0</sub> and C<sub>8:0</sub> and minor quantities of unsaturated monomers (C<sub>12:1</sub> and C<sub>14:1</sub>) were found. Special emphasis should be placed on the polymer produced from waste frying oils. 3-Hydroxynonanoic acid (C<sub>9:0</sub>, 0.47%) was detected in it (Fig. 3a), though in small amounts. This suggested the presence of a low concentration of uneven fatty acids in the waste frying oil. Few reports have been published on the presence of uneven carbon atom fatty acid in PHAs from related-carbon sources. C<sub>7</sub>, C<sub>9</sub> and C<sub>11</sub> monomers were detected in the PHA produced by *P. mendocina* [38], when uneven chain-length fatty acids were supplied; C<sub>5</sub> and C<sub>9</sub> were found in the polymer produced by *P. fluorescens* or *P. mendocina* [39,40]. The presence of C<sub>14:2</sub> (3%) also reflects the composition of the substrate, which contained nearly 29% of linoleic acid.

Similarly, the presence of linoleic (50.8%), oleic (22.8%) and linolenic (6.8%) acids in WFFA is reflected in the proportion of unsaturated components in polymer composition (Table 1). Most pseudomonads belonging to the rRNA-homology-group I derive the 3-hydroxyacyl-CoA substrates of C<sub>6</sub>-C<sub>14</sub> for PHAMCL synthase from the intermediates of the fatty acid β-oxidation pathway [41]. A small amount of C<sub>14:1</sub> (8.8%) was found, though few examples were given in the literature [42]. The high amount of the unusual di-unsaturated unit, C<sub>14:2</sub> (31.8%) should be noted. Only low quantities of this monomer (7%) were reported from *P. putida* when grown on lard [34].

Table 2
Composition of PHA synthesised by *P. aeruginosa* 42A2 during growth on crude oleic acid at different oxygenation rates

<table>
<thead>
<tr>
<th><em>K</em> &lt;sub&gt;La&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>PHA (%)</th>
<th>Relative monomer composition of PHA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>54.6</td>
<td>14.5 ND 40.2 ND 4.2 32.8 ND 8.2 ND ND</td>
</tr>
<tr>
<td>0.06</td>
<td>46.2</td>
<td>24.3 30.8 3.8 24.2 3.8 13.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> C<sub>8:0</sub>, 3-hydroxyoctanoate; C<sub>10:0</sub>, 3-hydroxydecanoate; C<sub>12:1</sub>, 3-hydroxydodecenoate; C<sub>12:0</sub>, 3-hydroxydodecanoate; C<sub>14:2</sub>, 3-hydroxytetradecenoate with two double bonds; C<sub>14:1</sub>, 3-hydroxytetradecenoate.

<sup>b</sup> ND, not detectable.

Table 3
Composition of PHA synthesised by *P. aeruginosa* 42A2 incubated at *K* <sub>La</sub> 0.003 s<sup>-1</sup> in submerged culture with TOA as carbon source at different temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>PHA (%)</th>
<th>Relative monomer composition of PHA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>31.9</td>
<td>ND 32.4 36.2 7.1 16.8 7.4</td>
</tr>
<tr>
<td>30</td>
<td>46.2</td>
<td>24.3 30.8 24.2 3.8 13.1 3.7</td>
</tr>
<tr>
<td>37</td>
<td>46</td>
<td>24.3 30.8 24.2 3.8 13.1 3.7</td>
</tr>
<tr>
<td>42</td>
<td>15.4</td>
<td>5.38 37.39 21.4 8.5 27.3 tr&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> C<sub>8:0</sub>, 3-hydroxyoctanoate; C<sub>10:0</sub>, 3-hydroxydecanoate; C<sub>12:1</sub>, 3-hydroxydodecenoate; C<sub>12:0</sub>, 3-hydroxydodecanoate; C<sub>14:2</sub>, 3-hydroxytetradecenoate with two double bonds; C<sub>14:1</sub>, 3-hydroxytetradecenoate.

<sup>b</sup> ND, not detectable.

<sup>c</sup> Tr, trace amounts (<1%, w/w).
In *P. aeruginosa*, when grown on glucose, the main way to produce acetyl-CoA and 3-OH-acyl-CoA is catabolism through the Entner-Duodoroff pathway, which leads to de novo synthesis of the monomer components of PHAs [41,43].

The composition of the polymer produced from glucose by *P. aeruginosa* 42A2 is given in Table 1. A wide range of components was found (C₈–C₁₆), though almost 50% was C₁₀:0. The proportion of C₈:0 (9.8%) in the case of *Pseudomonas* 42A2 was similar to those polymers produced by *P. fluorescens* [39] or *P. putida* [44]. Two uneven monomers from glucose C₉ (0.3%) and C₁₁ (0.6%) (Fig. 3a and b) were detected. The presence of uneven fatty acids may be feasible when propionil-CoA is used as a primer for de novo fatty acid biosynthesis [45]. To our knowledge, 3-hydroxyhexadecanoic acid (C₁₆:0) is reported for the first time (Table 1; Fig. 3c) in a PHA produced from glucose by *Pseudomonas*.

3.6. Effect of *K*ₐ on PHA production

Though it is well known that nutrient limitation of N, P, S, Mg or oxygen affects PHA accumulation in presence of an excess of carbon source [8,42,46–49], little information is available about the effect of other culture parameters. When *P. aeruginosa* 42A2 cultures were incubated at 30°C at two different oxygen transfer rates, polymer accumulation was similar, with 2.3 and 2.4 g/l of PHA at *K*ₐ of 0.1 s⁻¹ and 0.06 s⁻¹, respectively. The relative monomer composition was modified when oxygen transfer was changed (Table 2). Major monomer components of the polymer formed at high *K*ₐ (0.1 s⁻¹) were C₁₀:0 (40.3%) and C₁₂:0 (32.8%) and a low amount of C₁₂:1 (4.2%), whereas, in the polymer produced at a *K*ₐ of 0.06 s⁻¹, the main proportions were C₁₀:0 (24.3%), C₁₁:0 (30.8%) and C₁₂:1 (24.2%). The low amount of C₁₂:0 (3.8%) and the presence of the unusual C₁₄:₂ (13%) were the most relevant characteristics.

3.7. Effect of the incubation temperature on PHA production

We examined whether the incubation temperature affected PHA composition. PHA accumulation (Table 3) at 18°C was 31.9%, with maximum accumulation at 30°C (46.2%). No major differences were found in monomer composition, although different amounts of the components were found: C₁₀:0 (between 30 and 39%), C₁₂:1 and C₁₄:₂ (13–27%) at all incubation temperatures assayed. 24.3% C₁₀:0 was found in the PHA produced at 30°C, but not in the polymer produced at 18°C. The highest total amount of unsaturated monomers was found in the polymer produced at 18°C, with 60.4% of unsaturated components. At 30°C the proportion of unsaturated components was 41%; at 37°C, 54.2%; and at 42°C, 48.7%. These findings suggested that no direct relationship may be established between cultivation temperature and PHA composition. Similar results were found [35] when decanoate was supplied as carbon source, whereas the saturated/unsaturated components ratio increased when cultivation was with glucose.

In this study, we reported that residual waste frying and other oily wastes are suitable substrates for PHA production. The new strain *P. aeruginosa* 42A2 accumulated up to 54.6% of PHA. The monomer composition of the polymer varies with the substrate supplied. This is the first time C₇, C₉, C₁₄:₂ and C₁₆:₀ monomers are described in a PHA produced by *P. aeruginosa*.

Acknowledgements

The financial support of the Comissió Interdepartamental de Recerca i Tecnologia CIRIT project (199956R 00024) and of the Comisión Interministerial de Ciencia y Tecnología...
References


