PRODUCTION OF POLYHYDROXYALKANOATES FROM MIXED MICROBIAL CONSORTIA DERIVED FROM SOIL: EFFECT OF NITROGEN LIMITATION ON MICROBIAL SHIFT AND PROPERTIES OF RECOVERED POLYESTERS

KOUMELIS I.1,2, NTAIKOU I.1, IATRIDI Z.2, KAMILARI M.3, TSITSILIANIS C.2 and LYBERATOS G.1,4

1Institute of Chemical Engineering and Sciences (ICEHT/FORTH), 10 Stadiou st., Platani, GR 26504, Patras, Greece.
2Department of Chemical Engineering, University of Patras, 1 Karathedori st., GR 26500, Patras, Greece.
3Department of Biology, University of Patras, GR 26500, Patras, Greece.
4School of Chemical Engineering, National Technical University of Athens, Zografou Campus, GR 15780, Athens, Greece

ntaikou@iceht.forth.gr

ABSTRACT

The purpose of the present study was to investigate the effect of nitrogen limitation on PHAs production from a mixed microbial consortium derived from soil, the properties of the final products and the changes of the microbial populations. A synthetic medium with mixed volatile fatty acids was used as carbon source, and the fermentation process was achieved by operating a sequential batch reactor in cycles of carbon efficiency nitrogen limitation (PHAs accumulation phase) and carbon limitation (growth phase). Ammonium sulphate was added during carbon limitation conditions, in order to ensure nitrogen sufficiency for microbial growth. The limitation of nitrogen during the accumulation phase, was achieved by altering the N:C ratio used.

It was shown that PHAs yields measured either as g PHAs/g VSS or g PHAs/g t-COD consumed were higher when the reactor was operated in nitrogen limitation mode. The analysis of the recovered polyesters via 1H-NMR revealed that co-polymers were produced in both cases, whereas the ratio of N:C seemed to affect the composition of the final recovered product. In all cases 3HB was the dominant monomer, ranging from 62% to 91%, whereas 3HV was also detected in all cases and 4HB in one case. Further analysis of the thermal characteristics confirmed the copolymeric nature of the products. Molecular weights ranged from 77.10^4 Da to 180.10^4 Da, while the polydispersities were similar in all cases. The dominant microorganisms were isolated using Nile blue live staining and were further characterized via various biochemical and phenotypical tests. Subsequently, the isolated strains were identified using 16S r-RNA analysis.

Keywords: PHB, PHV, nitrogen limitation, soil bacteria

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters that have similar properties to petrochemical plastics. PHAs production has generally been achieved using either wild or recombinant microorganisms via fermentation processes. A key factor for enhanced production yields is nutrient limitation, that seems to stimulate the polymerization and intracellular accumulation of PHAs by microorganisms. The use of pure microbial cultures has mainly been so far suggested by the literature (Lee et al., 1999). However, mixed consortia for PHAs production could be advantageous especially when complex wastes are used as substrates.
In general, mixed cultures are microbial populations of unknown composition, which are able to perform specific intracellular and extracellular reactions, and are selected by the operational conditions imposed on the biological system. Mixed cultures selected for PHA production can have a high intracellular storage capacity due to operational conditions that limit their primary metabolism. There are three main methodologies for the enrichment and preservation of PHAs producers in mixed culture i.e. the PAO/GAO methodology, which uses alternating anaerobic-aerobic conditions, the microaerophilic-aerobic methodology and the feast and famine methodology, during which the consortium is subjected to consecutive periods of external substrate availability and unavailability (Dias et al., 2006).

The purpose of the present study was to investigate the effect of nitrogen limitation during PHAs production from a mixed consortium initially derived from soil, on the changes of the microbial populations and the on the properties of the final products. A synthetic medium with mixed volatile fatty acids was used as carbon source, and the fermentation process was achieved by operating a sequential batch reactor in cycles of alternating nitrogen (PHAs accumulation phase) and carbon limitation (growth phase). Ammonium sulphate was added during carbon limitation, in order to ensure nitrogen sufficiency for microbial growth. The sufficiency versus limitation of nitrogen was achieved by altering the ratio of N:C used.

2. MATERIALS AND METHODS

2.1. Seed, medium and culture conditions

For the startup of the bioreactor an enriched mixed culture derived from soil was used. A 10% w/v soil suspension was prepared with distilled water and was kept at ambient temperature under constant agitation (250-300rpm) for two hours. Subsequently, it was heated to 70°C for 10 minutes, cooled, and the suspended solids were left to settle. The supernatant was filtered via 0.5mm sieve and was supplemented with acetic, butyric and propionic acids, 1g/L each, 1g/L (NH₄)₂SO₄, 2.5 g/L K₂HPO₄ and 1.5 g/L KH₂PO₄. The enhancement of the initial culture with PHAs producers was performed by subjecting it to sequential nitrogen/carbon limitation. A 0.5 L draw-and-fill reactor was used, operated in cycles of 23h of continuous aeration and agitation and 1 h of settling, for 15 days. For the initial enrichment procedure described above, as well as for the operation of the main bioreactor, a basal synthetic medium (BSM) of the following composition was used: 0.2 g/L MgSO₄·7H₂O, 0.05 g/L CaCl₂·2H₂O, 2.5 g/L K₂HPO₄ and 1.5 g/L KH₂PO₄ and 1 ml/L of a trace elements solution. The trace elements solution was prepared separately as follows: 10 mg/L CuSO₄·2H₂O, 2 g/L FeSO₄·7H₂O, 30 mg/L NaMoO₄·2H₂O, 20 mg/L NiCl₂·6H₂O, 1 g/L ZnSO₄·7H₂O, 0.2 g/L CoCl₂·6H₂O, 0.3 g/L KI, 0.3 g/L H₃BO₃, 30 mg/L MnCl₂·4H₂O and 2.5 g/L EDTA. A mixture of acetic, butyric and propionic acids in ratio 1:1:1 was used as carbon source, whereas (NH₄)₂SO₄ was used as nitrogen source.

The bioreactor was of 1.5 L working volume and was operated in sequential batch mode. Carbon and nitrogen sources were provided to the reactor separately as follows: carbon efficiency phase (accumulation), settling/decanting phase, nitrogen sufficiency (growth) phase, settling/decanting phase. The reactor was kept at ambient temperature, with magnetic agitation of 200 rpm and constant aeration, with moisturized air via sterilized filter, with volumetric rate of 0.83-1.2 L/min.

2.2. Analytical techniques

Total and volatile suspended solids (TSS, VSS), chemical oxygen demand (COD) and ammonium nitrogen (phenate method) were determined according to the Standard
Methods (APHA, AWWA & WEF, 1995). Volatile fatty acids (acetate, propionate, butyrate) were quantified by gas chromatography (Varian CP-3800), with flame ionization detector and capillary column. Prior to analysis, samples were acidified with H$_2$SO$_4$ (0.6% v/v). For the recovery of PHAs at the end of each operational period, 1 L of mixed fermentation broth was removed from the reactor under agitation. The broth was centrifuged at 4500 rpm for 15 min and the microbial pellet was re-suspended in 95% ethanol, washed twice with distilled water and subsequently lyophilized at -50°C (CRYODOS-50, TelStar). PHAs were extracted from the dry biomass with chloroform (50ml/g biomass) at 70°C, for 1h in a SER 148 (VELP) extraction apparatus. Recovery of the solvent was carried out until its initial volume was reduced by half, and then methanol was added at a ratio 1:6 (v/v). The precipitated PHAs were then recovered by filtration and the yield of produced PHAs was estimated gravimetrically. For further chemical and thermodynamic analysis, chloroform casting of PHAs pellets was conducted (20ml/g PHAs) at 70°C for 15min. The solvent was slowly evaporated in proper vials in order to receive PHAs films with thickness 0.15-0.25mm.

The composition of the recovered PHAs was determined by $^1$H NMR spectroscopy using a 400 MHz Bruker Advance DPX spectrometer. The solvent that was used was deuterated chloroform, CDCl$_3$. The molecular weight of the polymers was measured by using size exclusion chromatography (SEC) at 25°C with a Polymer Lab chromatograph comprising two PI gel (5 μm) columns, a refractive index detector (RI) and a UV/VIS (254 nm) detector. Chloroform was used as an eluent at a flow rate of 1mL/min. Polystyrene standards with low polydispersity were used to generate calibration curves. Solutions in CHCl$_3$ of the products at a concentration of 4 mg/mL were prepared. In the abovementioned solutions, a small quantity of toluene was added, so as to know when the experiment was ended. The eluent (CHCl$_3$) was filtered with a 5μm filter, while all the prepared solutions were filtered using 0.45μm filters. Differential scanning calorimetry (DSC) was performed by using DSC 2920 CE, TA Instruments. Samples of 9-10 mg were weighed in aluminum pan and an empty pan was used as reference. Measurements were performed in a nitrogen atmosphere (50mL/min), in two heating steps from −30 to 200°C and at a heating rate of 10°C/min.

2.3. Isolation of microorganisms

At the end of each operational period bacterial strains were isolated from the mixed culture by repeated streaking on agar plates. The solid medium was prepared by supplementing BSM with 20g/L agar, acetate, butyrate and propionate 0.5g/L each, 0.2g/L (NH$_4$)$_2$SO$_4$ and 2mg/L of the hydrophobic dye Nile Blue, previously dissolved in DMSO (2 mg per ml). Cultures were incubated at 23°C in order to apply the viable colony staining method (Spiekermann et al. 1999). After 48h, 72h and 96h of incubation, agar plates were exposed to UV light (312nm). Several fluorescent colonies were selected based on their morphological differences and were transferred to the same medium to isolate single colonies.

2.4. DNA Extraction and PCR Amplification of Pure and Mixed Cultures

Total DNA extraction using in average 10$^6$ cells, was carried out using the Macherey-Nagel Tissue kit following the manufacturer’s protocol. The genetic diversity and similarity of the bacterial community was analyzed by amplification of the 16S rRNA marker (corresponding to positions 341-534 in E. coli), using eubacteria specific primers, as described by Muyzer et al. (1993). PCRs were carried out in 50 μL volumes (1 unit KAPA Taq DNA Polymerase, 1X KAPA PCR buffer A, 0.2 mM dNTPs, 1 mM MgCl, 0.5 μL DNA template, filled to 50 μL with sterile H$_2$O). The thermocycling program for the touchdown PCR was as follows: initial denaturation was performed at 93°C for 5 min and then at
93°C for 1 min, followed by touchdown primer annealing from 65°C to 53°C (the annealing temperature was decreased 0.5°C every cycle for 25 cycles, to touchdown at 53°C), followed by extension at 68°C for 1 min (for each of the 25 cycles), with a final extension step at 68°C for 10 min. The PCR results were analyzed by horizontal electrophoresis in 1% agarose gel stained with ethidium bromide (1 μg/ml), after which they were inspected under UV light and photographed. Furthermore, in order to detect subtle differences in the amplified fragments, a second electrophoresis was performed in a 3% gel for 4 hours.

3. RESULTS AND DISCUSSION

3.1. Effect of nitrogen limitation on PHAs yields and properties

The sequential batch reactor was operated continually in two distinct periods denoted as cycles A, B and C and cycles D and E, as illustrated in figure 1. For the startup of the reactor in cycle A, an enriched microbial culture derived from soil was used, as described above. For the startup of cycle D, effluent from cycle C was used as inoculum. The main difference between the two operational periods was the sufficiency of nitrogen during the accumulating phases (carbon excess). As shown in table 1, N:C ratios ranged from 5.2 to 17.7 during the accumulation phases of the first operational period whereas during the second operational period nitrogen limitation was complete.

Table 1. N:C ratios during accumulation phases and final yields of recovered PHAs at the end of each operational cycle of the SBR.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>N/C (mgN-NH₄/g COD)</th>
<th>COD cons (mg/L)</th>
<th>pH</th>
<th>Y_PHAS/VSS (g/100g VSS)</th>
<th>Y_PHAS/COD (g/g COD cons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle A</td>
<td>17.7 ± 0.9</td>
<td>2249 ± 20.2</td>
<td>7.2-8.1</td>
<td>24.5 ± 2.12</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Cycle B</td>
<td>6.5 ± 0.3</td>
<td>2331 ± 14.9</td>
<td>7.2-8.5</td>
<td>39.5 ± 2.76</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Cycle C</td>
<td>5.2 ± 0.1</td>
<td>6562 ± 33.3</td>
<td>7.5-8.7</td>
<td>59.6 ± 3.6</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Cycle D</td>
<td>0</td>
<td>3220 ± 7.6</td>
<td>7.7-8.5</td>
<td>38.0 ± 4.5</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>Cycle E</td>
<td>0</td>
<td>3213 ± 16.1</td>
<td>7.7-8.7</td>
<td>39.4 ± 2.8</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

As shown in table 1, the lowest PHA yields, measured either as g PHAs /g COD consumed (Y_PHAS/COD) or g PHAs/g VSS (Y_PHAS/VSS) were observed in cycle A, during which the N:C ratio was the highest. It is reported that PHAs accumulating mixed consortia, when subjected to aerobic dynamic feeding, metabolize acids towards acetyl-CoA, which is partially channeled to the tricarboxylic acid cycle (TCA) for growth and NAD(P)H production, and partially used for PHA production (Dias et al., 2006). It seems that in excess of nitrogen, a higher ratio of carbon source is forwarded to growth rather than PHAs production. Lower ratios of N:C and absence of N during accumulation phases, did not seem to affect significantly either Y_PHAS/COD or Y_PHAS/VSS. On the contrary, Y_PHAS/VSS seems to be affected by the initially offered carbon concentration. Indeed in all cases, with the exception of cycle A, the COD consumed was up to ~3g/L Y_PHAS/VSS reached almost the same (~40%), whereas when COD consumption was doubled Y_PHAS/VSS reached ~60%.
Figure 1. N-NH₄ and COD removal throughout the operation of the SBR with partial (left, cycles A, B, C) and complete (right, cycles D, E) nitrogen limitation.

Table 2. Composition, according to ¹H-NMR analysis, and molecular weights of the recovered PHAs from the end of each operational cycle of the SBR.

<table>
<thead>
<tr>
<th></th>
<th>3HB %</th>
<th>3HV %mol</th>
<th>4HB %mol</th>
<th>Mw (10⁴ Da)</th>
<th>Mn (10⁴ Da)</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle A</td>
<td>90.9</td>
<td>9.1</td>
<td>0</td>
<td>87.48</td>
<td>54.36</td>
<td>1.61</td>
</tr>
<tr>
<td>Cycle B</td>
<td>62.2</td>
<td>17.1</td>
<td>20.7</td>
<td>76.74</td>
<td>46.22</td>
<td>1.66</td>
</tr>
<tr>
<td>Cycle C</td>
<td>72.4</td>
<td>27.6</td>
<td>0</td>
<td>154.43</td>
<td>112.49</td>
<td>1.37</td>
</tr>
<tr>
<td>Cycle D</td>
<td>78.0</td>
<td>22.0</td>
<td>0</td>
<td>171.54</td>
<td>94.87</td>
<td>1.81</td>
</tr>
<tr>
<td>Cycle E</td>
<td>85.0</td>
<td>15.0</td>
<td>0</td>
<td>181.59</td>
<td>113.58</td>
<td>1.59</td>
</tr>
</tbody>
</table>

In table 2, the composition of the produced PHAs is shown. 3HB is the main monomer detected in all cases, implying that carbon is mainly forwarded towards its homopolymer, P3HB or its co-polymers with other monomers. It must be noticed that during the first operational period of the reactor, the composition of the produced polymers seems to be rather random, with the 3HB molecular ratio ranging significantly. Moreover in cycle B, 4HB units are also dominant, without however being detected in any other case. Mean molecular weights and numbers also vary significantly during the first operational period. It is reported that differences in PHAs composition are mainly affected by the types of the carbon source used (Gumel et al., 2012). Since however, the carbon feed composition was the same in all cases (acetate:propionate:butyrate = 1:1:1), the observed differences cannot be attributed to this parameter. One possible explanation is that different acids are consumed selectively for growth and others for accumulation due to dynamic changes on the microbial populations. On the contrary, PHAs from cycles D and E (second operation
period) seem to have more common characteristics, thus implying that microbial consortia of cycles D and E have more similarities, too.

Figure 2. VSS and TSS concentrations throughout the operation of the SBR with partial (left, cycles A, B, C) and complete (right, cycles D, E) nitrogen limitation.

As further revealed by DSC analysis, the polymer produced by cycle A exhibited the most diverse characteristics. Results from all cycles, are presented in table 3. The samples presented melting temperatures (T_m) from ~124 to 158 °C. These values are intermediate between the P3HB T_m value (168–180°C) and the P3HV T_m value (105–110°C) (Pereira et al. 2008), suggesting the co-polymeric nature of the produced polymer. The polymer with highest 3HB contents also exhibited the highest melting temperature (sample A with 91% 3HB), whereas the lowest T_m value was observed for sample B, which has the lowest 3HB contents (62%) and contains also P3HB and P4HB. Generally, it is clear that the reduction in melting point compared to a pure P3HB sample is due to the introduction of the 3HV to form a copolymer, which bears longer side chains. The presence of two T_m values, however, proves that in most cases the produced PHAs are a mixture of different co-polymers and/or pure polymers.

The higher ΔH_m values indicate the existence of polymer chains of high crystallinity in the sample. These chains are probably linear and symmetrically distributed. Moreover, it is apparent that (with the exception of sample A), a higher content of 3HB leads to higher ΔH_m values, an observation that is in agreement with the literature (Bengtsson et al. 2010). Another thermal result is that glass transition is observed at -3°C or at ~12°C. The transition for pure P3HB is around +3°C (Pereira et al., 2008). The T_g at -3°C indicates that an amorphous phase seems to be present. The presence of two T_g's indicates that the sample under study is either a random copolymer or a mixture of a copolymer with some oligomers or a homopolymer. The fact that in some samples only one of the two T_g's is observed may be due to a low content of the second comonomer or to the incapability of the instrument to detect the second T_g.

It has to be noted that sample A was the only one that exhibited double T_m's, T_g's and ΔH_m's, indicating that the recovered bioplastic was more probably a mixture of different PHAs rather than a co-polymer. It seems that during this initial cycle of bioreactor operation, during which nitrogen was abundant, the microbial consortium was more heterogeneous since stress conditions were not that strict, and large populations of different strains coexisted in the culture.
Table 3. Thermodynamic properties of the recovered PHAs from the end of each operational cycle of the SBR.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>$T_{g1}$ ($^\circ$C)</th>
<th>$T_{g2}$ ($^\circ$C)</th>
<th>$\Delta H_{cc}$ (J/g)</th>
<th>$T_{cc}$ ($^\circ$C)</th>
<th>$T_{m1}$ ($^\circ$C)</th>
<th>$\Delta H_{m1}$ (J/g)</th>
<th>$T_{m2}$ ($^\circ$C)</th>
<th>$\Delta H_{m2}$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle A</td>
<td>-0.37</td>
<td>15.07</td>
<td>50.3</td>
<td>60.79</td>
<td>135.14</td>
<td>6.85</td>
<td>158.36</td>
<td>31.5</td>
</tr>
<tr>
<td>Cycle B</td>
<td>-</td>
<td>12.13</td>
<td>n.i.</td>
<td>n.i.</td>
<td>123.63</td>
<td>6.24</td>
<td>146.59</td>
<td>-</td>
</tr>
<tr>
<td>Cycle C</td>
<td>-3.23</td>
<td>12.97</td>
<td>16.25</td>
<td>72.86</td>
<td>145.67</td>
<td>29.51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycle D</td>
<td>-</td>
<td>13.13</td>
<td>22.21</td>
<td>84.35</td>
<td>149.05</td>
<td>24.82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycle E</td>
<td>-3.06</td>
<td>-</td>
<td>36.89</td>
<td>60.75</td>
<td>154.78</td>
<td>43.48</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.i. : not identified

3.2. Effect of nitrogen limitation on microbial populations

The touchdown PCR technique was used to amplify the 16S rDNA V3 region of the microbial communities. This touchdown PCR technique was performed to minimize the nonspecific annealing primers to the non-target DNA, and the PCR product was approximately 233 bp (Fig. 3). No specific differentiation was observed, since in all samples i.e from pure and mixed cultures (noted as $C_A$, $C_B$, $C_C$, $C_D$ and $E$) a single, seemingly, band appeared.

![Figure 3: Image of PCR products of extracted DNA by a 16S rDNA primer, with a 100bp DNA ladder, after electrophoresis in a 1% agarose gel for 1 hour.](image1)

Further investigation by a second electrophoresis of the PCR products, using a 3% agarose gel and run for 4 hours, revealed additional bands (Fig. 4). It can be noted that in many cases (such as in samples D2 and D5), what was supposed to be a single colony product revealed more than one bank, thus suggesting that the initial bacterial cells were
actually taken from a mixed culture. In terms of mixed culture PCR products (Fig. 4, right), an apparent alteration in the microbial profiles can be noted. As an early observation, we could say that the initial microbial population of Cycle A consists of more strains with no specific dominant species, whereas in the following cycles, domination of some species starts to occur. In the final cycle (E), it seems that a least diverse population prevails, since only one clear band is observed. Each band was carefully excised from the gel, purified and sent for direct sequencing in order to delineate whether they are nonspecific PCR products and/or the result of additional strains/species in the culture samples. In any case all the above hypotheses need to be further clarified by additional electrophoresis methodologies as well as by the results of sequencing.

4. CONCLUSIONS

It was shown that alterations in N:C ratios during the accumulation phase in the SBR reactor, affect significantly the composition and the properties of the produced PHAs. A high initial N:C ratio favours the diversity of microbial populations leading thus to a more heterogenous final product, as revealed by DSC analysis. The findings implied that during initial running of the SBR with high N:C ratio, mixtures of homopolymers and co-polymers were formed. The strict limitation of nitrogen (followed in Cycles D and E) resulted to a more homogenous product, and was attributed to the elimination of some microbial strains and prevalence of certain ones. These observations seem to be supported by PCR product analysis and need to be further clarified.

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