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ENVIRONMENTAL CLEANING MISSION: Bioconversion of Oxidatively Fragmented Polyethylene Plastic Waste to Value-Added Copolyesters

KEYWORDS: *Biodegradable biopolymers; polyhydroxyalkanoates (PHAs); oxidative fragmentation, low density polyethylene (LDPE); bioplastics; bio-based polymers; Cupriavidus necator, marine litter*

ABSTRACT

Biodegradable polyhydroxyalkanoates (PHAs) are evidenced as clean alternative to conventional fossil fuel based mass polymeric materials used for commodity plastic productions. The PHAs high production costs can be made dropped down by using cheap plastic waste substrates that otherwise might negatively affect the environmental soundness. Low density polyethylene (LDPE), commonly used for production of commodity plastic items (bins and shopping bags) becomes the source of a problematic plastic waste impact due to their widespread utilization. A convenient innovative recycling method, we are proposing, is bound to the controlled oxidative fragmentation of the LDPE plastic items, at the end of service life, as inexpensive substrates for future sustainable production of PHAs by Cupriavidus necator, a genetically stable and well known microbial strain for the intracellular accumulation of PHAs. The use of LDPE oxidized fragments as microbial substrate yielded 29% of PHAs (cell dry weight). The PHA, as retrieved upon microbial conversion, was characterized using GPC, NMR and electrospray ionisation tandem mass spectrometry (ESI-MS/MS). The quoted analytical tools confirmed that the attained PHA sample was a terpolymer consisting of 3-hydroxybutyrate (HB), 3-hydroxyvalerates (HV) and 3-hydroxyhexanoate comonomer units randomly distributed along the chain backbone.

INTRODUCTION

Petrochemical plastics have gradually become an integral part of our daily life and it is almost impossible to do without them due to their increased applications in a wide range of the daily activities [1]. However, their inappropriate usage along with the abuse of those materials in an increased range of applications comes with severe environmental consequences due to the littering in marine and fresh water compartments affecting the life cycles of the aquatic plants and living organisms. This is due to their recalcitrance to biodegradation that leads to their accumulation in high quantities and difficulties in managing for its sound impact [1,2]. Alternatively, there is a group of bio-based polymers named Polyhydroxyalkanoates (PHAs) that are synthesized by selected bacterial

strains under unbalanced nutrient conditions. They are biodegradable and have unique properties similar to traditional plastics [1]. For these reasons, PHAs are perceived to be a better replacement of the synthetic polymeric materials used in the production of plastics for various applications including packaging and commodity items, PHAs can be taken as a means for mitigating the environmental burden bound to petroleum-based plastics [3]. With the high wide production cost of PHAs mostly resulting from the high cost of carbon sources (40-50% of total production cost), it has become essential to exploit inexpensive carbon sources for a sustainable and cheap PHAs production [4]. Low density polyethylene (LDPE) is one of the most consumed petrochemical plastics (around 9 million tons in 2017 of demand in EU according to the statistics of Plastic Europe), it is commonly used in multilayer film for packaging applications, shopping bags, mulching film for agricultural applications and toys. LDPE, usually discarded in combination with other polymeric materials, makes it difficult to have it recycled as a mono-material and thus constitutes a problematic waste to be properly managed [5]. The recycling method proposed in this study is the controlled oxidative fragmentation of the collected LDPE plastic items, at the end of their service life, as inexpensive substrate for PHAs production in different culture medium by *Cupriavidus necator*; a genetically stable and well known strain for the intracellular accumulation of PHAs [6].

MATERIALS AND METHODOLOGY

Carbon Source

LDPE film (thickness 40 μm) engineered with pro-oxidant/pro-degradant additives was treated under natural UV light (open-air, sunlight irradiation and weather conditions) by exposition in south direction (coord. 43° 52'42" N 10° 35' 3" E) [7]. The oxidative process was monitored by ATR-FTIR (chemical variations) and by stress-strain test (mechanical variations). The aging process was stopped after 55 days of exposition when the samples had registered an elongation at break of 5% and showed the presence of an increasing band in the carbonyl region with a relative maximum peak at 1715 cm^{-1} [8], in agreement with the results observed by the relevant mechanical properties. The weight average molar mass (M_w) of the oxidatively fragmented LDPE sample was 15,1 kg/mol with a dispersity index (M_w/M_n) of 4.7.

Microorganism

The microorganism used in this study for PHAs production from oxidatively fragmented LDPE (PE-F) was *Cupriavidus necator* H16 (NCIMB 10442, ATCC 17699). This bacterial strain was obtained from the stock culture available at University of Wolverhampton, (UK).

Growth Media and Chemicals

The growth media tryptone soya broth (TSB) and tryptone soya agar (TSA) were purchased from Lab M Ltd, UK. Following the manufacturer's instructions, both media were prepared under aseptic conditions. Basal salts Medium (BSM) (distilled water, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 1 g/L KNO_3 , 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L NaCl and 10 ml/L (Trace elements) and BSM salts were purchased from BDH Chemicals Ltd.,UK.

Shake Flask Fermentation

To get rid of impurities, 0.50g of PE-F was placed in a 100 mL beaker and rinsed with ethanol. The sterile oxidatively fragmented LDPE sample was then sonicated in sterile 50 mL TSB or BSM for 8 min at 0.5 active and passive intervals, with a power of 70% using a Bandelin Electronic sonicator (Berlin, Germany). This was added to 200 mL of sterile TSB or BSM in a 500 mL flask, after which 1000 µl of starter culture was added to give a total fermentation volume of 250 mL. For the experimental control, 250 mL of TSB or BSM was inoculated with 1000 µl of starter culture without the addition of oxidative fragmented LDPE sample. All flasks were incubated in a rotary incubator for 48 h at 30°C and 150 rpm.

PHAs Extraction

After 48 h fermentation, PHAs extraction was performed. The cultures were centrifuged in a Sigma 6-16KS centrifuge for 10 min at 4500 rpm. The biomass obtained was frozen overnight at -20°C, followed by lyophilization using an Edward freeze-drier (Modulyo, Crawley, UK) for 48 h at a temperature of -40°C and at a pressure of 5 mbar. The dried biomass was transferred into extraction thimbles and PHAs were extracted by Soxhlet apparatus with HPLC grade chloroform (Sigma Aldrich) running for 48 h. Chloroform/biopolymer mixture was collected afterwards and concentrated at 50°C using a rotary evaporator to remove the chloroform. The yield of the polymer was recorded using the equation [Eq. 1]:

$$\text{Percentage yield of PHA (PHA \%)} = \frac{\text{Weight of extracted polymer (WPHA)} \times 100}{\text{Cell dry weight (CDW)}} \quad [\text{Eq. 1}]$$

POLYMER IDENTIFICATION

Gel Permeation Chromatography (GPC)

The molar mass and molar mass distribution of the oxidatively fragmented PE-F sample was determined by gel permeation chromatography (GPC, Agilent Technologies PL GPC 220) apparatus equipped with two columns: Agilent PLgelOlexis guard plus 3x Olexis, 30 cm, 13µm, at 160 °CC. 1,2,4-Trichlorobenzene containing an antioxidant was employed as a solvent at a flow rate of 1.0 mL/min. The obtained data were analyzed by using polystyrene calibration.

The number-average molar mass (Mn) and the molar mass distribution index (Mw / Mn) of the obtained PHA samples were determined by GPC experiments conducted in a chloroform solution at 35°C and at a flow rate of 1 mL/min using a Viscotek VE 1122 (Malvern, Worcestershire, UK) pump with two Mixed C PLgelstyragel columns (Agilent, Santa Clara, CA, USA) in series and a Shodex SE 61 RI detector (Showa Denko, Munich, Germany).

Nuclear Magnetic Resonance (NMR)

Proton Nuclear Magnetic Resonance (¹H-NMR) spectra were recorded with a Bruker Avance II (Bruker, Rheinstetten, Germany) operating at 600 MHz, with 64 scans, 2.65 s acquisition times and an 11 µs pulse width.

Electrospray Mass Spectrometry (ESI-MS/MS)

Electrospray Mass Spectrometry analysis was performed by using a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan LCQ Fleet, San Jose, CA, USA). The partly degraded PHA samples (as described in [9]) resulted in lower mass PHA that was dissolved in a chloroform/methanol solution (1:1 v/v).

RESULTS AND DISCUSSION

Bacterial Growth and Yield

The growth curves of *Cupriavidus necator* H16 in TSB only, BSM only and TSB/BSM supplemented with PE-F are shown in Figure 1.

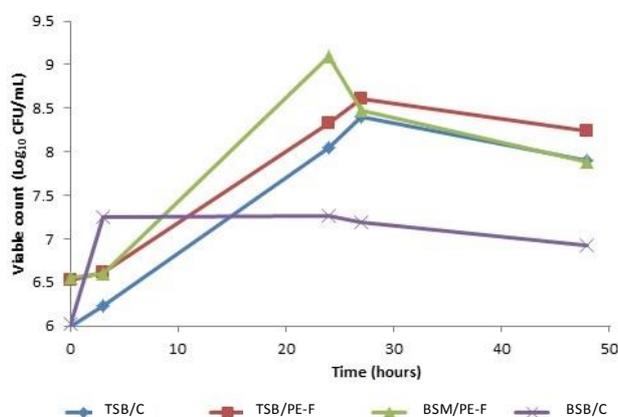


Figure 1. Growth recording of *Cupriavidus necator* H16 with 0.5g/L of oxidatively fragmented (PE-F) in either TSB or BSM incubated at 30°C for 48 h in a shaker incubator. Viable count (Log₁₀ CFU/mL) data points at time 0,3,24, 27 and 48 h are mean values of triplicates experiments (n=3)

The viability of *Cupriavidus necator* H16 was reduced, between 28h and 48 h of incubation, from 8.4 log₁₀ CFU mL⁻¹ to 7.8 log₁₀ CFU mL⁻¹ for TSB only and from 8.6 log₁₀ CFU mL⁻¹ to 8.2 log₁₀ CFU mL⁻¹ in TSB with PE-F. A significant difference in growth was also found between BSM only and TSB/BSM supplemented with PE-F (P<0.05).

The average cell dry weight (CDW) and the average PHA yield after 48 h of bacterial growth are reported in Table 1.

Table 1. PHA yield by *Cupriavidus necator* in TSB/BSM only and TSB/BSM supplemented with PE-F after 48 hours of incubation at 30°C.

Incubator Medium	Average CDW (g/L)	Average PHA (g/L)	PHA (% w/w)
TSB only	0.48	0.003	0.6%
TSB with PE-F	0.52	0.15	29%
BSM only	0.13	ND ^(a)	ND ^(a)
BSM with PE-F	0.19	ND ^(a)	ND ^(a)

^(a)Not detectable

It was observed that PHA was produced in both incubation media (TSB only and TSB supplemented with PE-F). However, a greater yield and percentage PHA per CDW was obtained in TSB supplemented with PE-F than with TSB only. This clearly shows that the addition of PE-F into TSB may have stimulated further bacterial growth resulting in an increased nutrient utilization with subsequently better production of PHA. Although *Cupriavidus necator* was able to grow in BSM only and BSM supplemented with PE-F, no PHA production was detected under those growth conditions after 48 h of culture period. This suggests that while PE-F alone can maintain bacterial growth in BSM culture, it is not sufficient enough for PHA production. This also suggests that perhaps a

synergistic effect does occur between the TSB culture medium and the presence of oxidized PE fragments. Two hypothesis can be envisaged: 1) the *Cupriavidus necator* H16 was able to synthesize PHA by a complex bio-transformation process that is taking into account a mechanism of biofilm formation in which the PE-F fragments behaved as a good substrate since at possess a wettable surface or 2) the *Cupriavidus necator* H16 was able to synthesize PHA by using the glucose monohydrate and organic carbon present in the culture media. To better understand the bioconversion process and to better assign the role of the carbon source, experiments by using ^{13}C labeled oxidized PE fragments are in progress [10].

PHA Identification and Characterization

The GPC analysis revealed the weight average molar mass (M_w) of the PHA obtained with PE-F to be 624 kg/mol, the number-average molar mass (M_n) to be 212 kg/mol and hence the molar mass dispersity index (M_w/M_n) resulted to be 2.6.

The $^1\text{H-NMR}$ was used to further investigate and determine the overall molecular structure of the polyester produced by using TSB supplemented with PE-F. The presence of three of signals in which sets are corresponding to the protons of HB repeating units: a doublet at 1.26 ppm attributed to CH_3 group, a doublet at 2.57 ppm attributed to CH_2 group and a multiplet at 5.24 ppm characteristic of a CH group was observed. The $^1\text{H-NMR}$ spectrum also showed signals characteristic of HV repeating units: a triplet at 0.9 ppm, a methylene resonance at 1.60 and 2.57 ppm and a methyne resonance at 5.17 ppm. This analysis shows the presence of both 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerates (3-HV) monomers units in the produced polyester [11]. However, due to the overlapping of proton signals of the other eventual PHA structural units the ESI-MS/MS analysis was been performed, but we did not obtained any valuable information on the PHA monomeric unit composition.

The ESI-MS was also used for the structural characterization of the obtained PHA. After controlled thermal degradation of the polyester as obtained *via* E1cB mechanism induced by sodium bicarbonate (NaHCO_3) [12], the ESI-MS/MS spectrum of the selected parent ion of resulting oligomer was acquired (Figure 2).

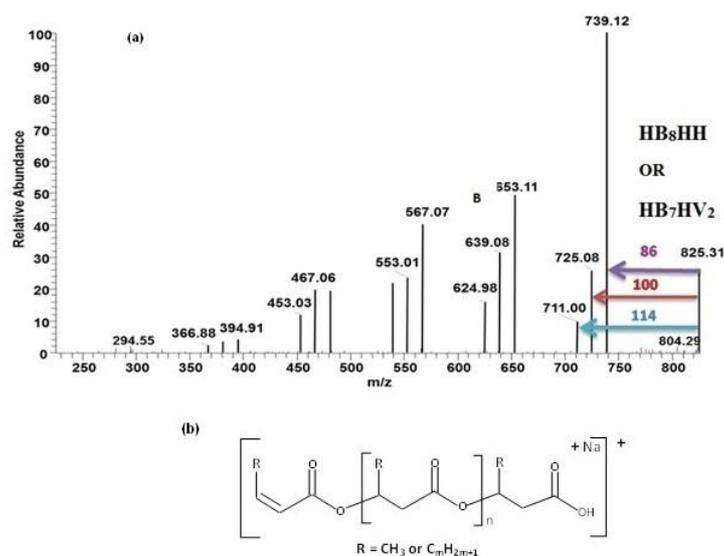


Figure 2. (a) The ESI-MS/MS spectrum (positive-ion mode) spectrum obtained for the selected sodium adduct of oligomer at m/z 825, obtained via partial thermal degradation of the PHA produced by *Cupriavidus necator* H16 in TSB using PE-F as an additional carbon source (b) The general formula of ions observed in the ESI-MS spectra.

As shown in Figure 2, three fragmentation paths were detectable in the spectrum of the parent ion at m/z 825 (this may have corresponded to the isobaric ions contained in two HV units or one HH unit $[\text{HB}_7\text{HV}_2 + \text{Na}]^+$ or $[\text{HB}_8\text{HH} + \text{Na}]^+$ respectively). The formation of the first series of product ions at m/z 739, 653 and 567 (terminated by carboxyl and crotonate end groups) resulted from the displacement of regular molecule of crotonic acid (86 Da). The fragmentation spectrum of this ion further confirms that the most intensive ions in the clusters correspond to sodium adducts of 3-hydroxybutyrate oligomers. The second series of product ions at m/z 725, 639, 553, 467 may have been formed by the loss of valeric acid (100 Da) thus confirming that the obtained oligomer was also containing 3-hydroxyvalerate co-monomer units. However, the third series of product ions at m/z 711, 625, 539, 453 and 367 were created by the expulsion of 2-hexenoic acid (114 Da; thus indicating the presence of HH unit in the oligomer chain). In conclusion, the ESI-MS/MS fragmentation results confirmed that on the use of PE-F as carbon source for *Cupriavidus necator*, the obtained PHA comprises of 3-hydroxybutyrate, 3-hydroxyvalerates and 3-hydroxyhexanoate co-monomeric units, randomly distributed along the backbone chain.

CONCLUSION

This study demonstrated that oxidatively fragmented LDPE is a valuable carbon source for the production by *Cupriavidus necator* in TBS culture medium, supplemented with oxidatively LDPE fragments, of bacterial PHA (Fig. 3). The NMR and ESI-MS/MS analyses revealed, that the controlled oxidative fragmentation of the LDPE plastic items is leading, after bioconversion of the attained fairly low molar fragments, to production of a PHA random terpolymer consisting of the following repeating monomeric units: 3-hydroxybutyrate, 3-hydroxyvalerates and 3-hydroxyhexanoate.

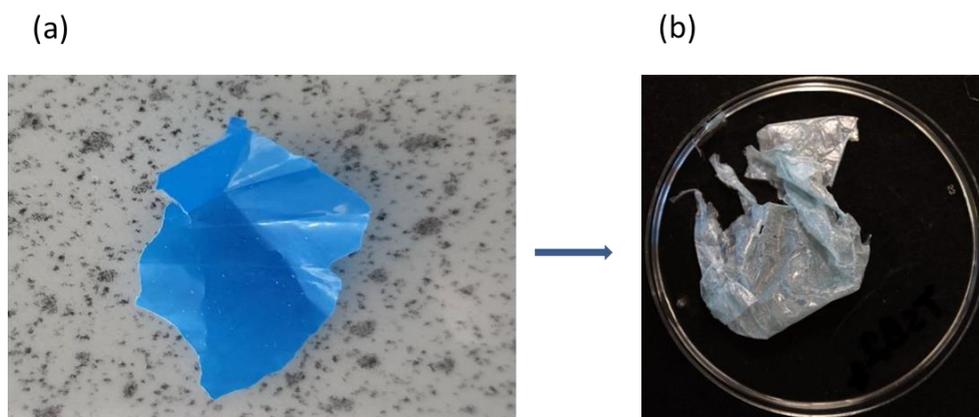


Figure 3. (a) Oxidatively fragmented LDPE (sample PE-F); (b) PHA extracted from *Cupriavidus necator* using TSB supplemented with PE-F.

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