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Environmental cleaning mission

Bioconversion of oxidatively fragmented polyethylene plastic waste to value-added copolyesters

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

ABSTRACT

The innovative recycling method, we are proposing, relies upon the controlled oxidative fragmentation of waste LDPE plastic to the inexpensive substrates for future sustainable production of PHAs with the aid of Cupriavidus necator. LDPE oxidized fragments (PE-F) were obtained from the re-engineering LDPE film by means of pro-oxidant/ pro-degradant additives, followed by treatment under natural UV light. Cupriavidus necator was grown in either tryptone soya broth (TSB) or basal salt medium (BSM) supplemented with PE-F for 48 h. PHA production was higher in TSB supplemented with PE-F (29%) than in TSB alone (only 0.6%). No PHA was detected in either BSM alone or BSM supplemented with PE-F. The recovered PHA was characterized using GPC, NMR, and electrospray ionization tandem mass spectrometry (ESI-MS/MS). These analytical tools applied confirmed that the resulting PHA was a terpolymer having an average molar mass of 624 kg/mol and consisting of 3-hydroxybutyrate (HB), 3-hydroxyvalerates (HV) and 3-hydroxyhexanoate (HH) co-monomer 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 daily activities (1). However, their inappropriate usage along with the abuse of these materials in an increased range of applications comes with severe environmental consequences due to their recalcitrance to biodegradation that leads to their accumulation in different environmental compartments (terrestrial and aquatic) in high quantities and difficulties in managing them (1, 2). One severe environmental consequence owing to their littering in marine and freshwater compartments is represented by their detrimental effect on life cycles of aquatic plants and living organisms (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 (1,3). For these reasons, PHAs are perceived to be a better replacement of the fossil fuel-based synthetic polymeric materials used in the production of plastics for various applications including packaging and commodity items. PHAs can be then viewed as a useful means for mitigating the environmental burden associated with petroleum-based plastics (4).

With the high 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 especially waste materials for sustainable and cheap production of PHAs (5). Among the waste products currently being exploited are post-consumer plastic wastes, food waste, bio-waste, and dairy wastes among others (6-9). Researchers have also reported the utilization of post-consumer plastic wastes such as polystyrene (6,10), polyethylene terephthalate (11), pyrolyzed polyethylene (12), oxidized polyethylene wax (13) and low density polyethylene powder (14) for the production of various monomeric units of PHAs. The different kinds of hydrocarbons present in these plastic products could be a source of carbon for bacterial growth and efficient accumulation of PHAs (12). Notable bacteria strains that have been reported to successfully accumulate PHAs intracellularly upon utilization of plastic products include Cupriavidus necator H16 (a genetically stable and well-known strain for the intracellular accumulation of PHAs), Acinetobacter calcoaceticus, Acinetobacter pittii, Pseudomonas putida, Pseudomonas frederiksbergensis and other Pseudomonas spp. (6,10-13).

Low density polyethylene (LDPE) is one of the most consumed petrochemical plastics. It is commonly used in multilayer film for packaging applications, shopping bags, mulching films for agricultural applications and toys (15). 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 (15). Thus, an innovative recycling method proposed in this study is the controlled oxidative degradation of the collected LDPE plastic waste, to the inexpensive substrate for PHAs production. This approach employs an oxidative step that converts LDPE films to oxidized fragments and investigates the ability of Cupriavidus necator to utilize these LDPE fragments for the production of biodegradable PHAs.

MATERIALS AND METHODOLOGY

Carbon Source

LDPE film (thickness 40 µm) engineered with pro-oxidant/ pro-degradant additives consisting of a blend of Manganese stearate(1%-wt) and Iron stearate(0.2%-wt) 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) (16). 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} (17), in agreement with the results observed by the relevant mechanical properties. The weight average molar mass (Mw) of the oxidatively fragmented LDPE sample was 15,1 kg/mol with a dispersity index (Mw/Mn) 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 the 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 K2HPO4, 1 g/L KH2PO4, 1 g/L KNO3, 1 g/L (NH4)2SO4, 0.1 g/L MgSO4.7H2O, 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 oxidative 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 an 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. The chloroform/biopolymer mixture was collected afterward 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):

$$\text{The percentage yield of PHA (PHA \%)} = \frac{\text{Weight of extracted polymer (WPHA)}}{\text{Cell dry weight (CDW)}} \times 100$$

(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 °C. 1,2,4-Trichlorobenzene containing an anti-oxidant 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 (^{1}H-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 II 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 (18)) resulted in lower mass PHA that was dissolved in a chloroform/methanol solution (1:1 v/v).

RESULTS AND DISCUSSION

This research investigates the microbial conversion of oxidatively fragmented LDPE plastic to biodegradable PHAs with the aim of providing an economical alternative for producing environmentally friendly biopolymers and an efficient method for recycling LDPE wastes within the framework of the Environmental Cleaning Mission vision. From earlier studies, it was shown that the addition of pro-oxidant additives able to promote the photo- and thermo- degradation of PE films increases the carbonyl index of PE and makes the carbon chains of PE sensitive to biodegradation (14,19,20). This could be responsible for the increased growth observed in the growth analysis of

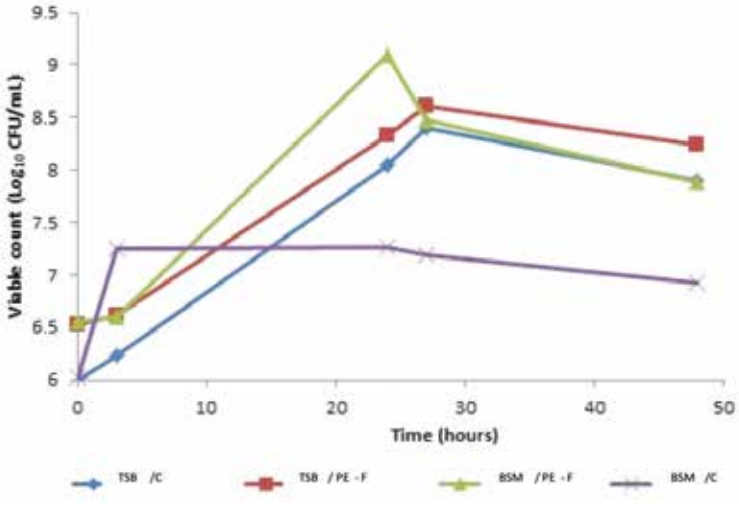

Figure 1. Growth recording of *Cupriavidus necator* H16 with 0.5g/L of oxidatively fragmented (PE-F) in either TSB or BSM incubated at 30°C for 48 h in a shaker incubator. Viable count (Log_{10} CFU/mL) data points at time 0,3,24, 27 and 48 h are mean values of triplicates experiments (n=3)

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

[a]Not detectable

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.

Cupriavidus necator H16 in TSB/BSM supplemented with PE-F (Figure 1) than in controls (TSB/BSM only) without the addition of PE-F; suggesting that the pretreatment of LDPE made it easier for Cupriavidus necator to hydrolyze PE-F.

The increased value observed in the growth curve from time 0 to 28 h also suggests that PE-F had no inhibitory effect on the growth of *Cupriavidus necator*. Besides, Seyfriedsberger *et al*. had earlier noted that linear low density polyethylene (LLDPE) pose little to no antimicrobial activity against Gram-positive and Gram-negative bacteria (21). 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.

It was observed that PHA was produced in both incubation media (TSB only and TSB supplemented with PE-F). However, greater yield and percentage PHA per CDW was obtained in TSB supplemented with PE-F (29%) than with TSB only (0.6%). 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. In addition, the cell dry weight of *Cupriavidus necator* (0.52 g/L CDW) observed in this study was slightly higher than that observed (0.339 g/L CDW) in a previous report where untreated LDPE was used (14), even after 21 days incubation of *Cupriavidus necator*. This further demonstrates the positive influence of PE-F on PHA productions by the indicated microbial strain.

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 the 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 BSM culture medium and the presence of oxidized PE fragments. Two hypotheses can be envisaged: 1) *Cupriavidus necator* H16 was able to synthesize PHA by a complex biotransformation process that is taking into account a mechanism of biofilm formation in which the PE-F fragments behaved as a good substrate since it possesses a wettable surface or 2) *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 (22).

PHAs Identification and Characterization

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

The ^{1}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 signals whose sets correspond to the protons of HB repeating units were observed: 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. The ^{1}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 methine resonance at 5.17 ppm. This analysis shows the presence of both 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV) monomeric units in the produced polyester (23). The content of non-HB units, on the level of 14 mol %, was estimated from 1H NMR spectrum, similarly as in the case of PHA derived from PS (6). However, due to the overlapping proton signals of the other eventual PHA structural units, the ESI-MS/MS analysis was performed, although no valuable information on the PHA monomeric unit composition was obtained.

The ESI-MS was also used for the structural characterization of the obtained PHA. After controlled thermal degradation of the polyester as obtained *via* the E1cB mechanism induced by sodium bicarbonate ($NaHCO_3$) (13), the ESI-MS/MS spectrum of the selected parent ion of resulting oligomer was acquired (Figure 2).

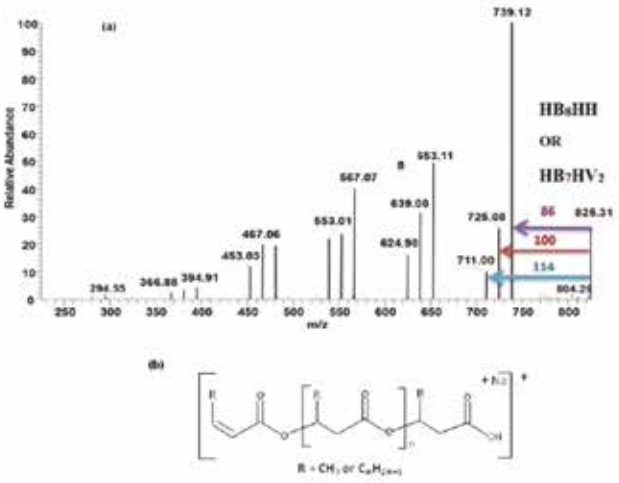

Figure 2. (a) The ESI-MS/MS spectrum (positive-ion mode) obtained for the selected sodium adduct of PHA oligomer at *m/z* 825. The sample for ESI-MS/MS analysis was obtained via controlled moderate-temperature degradation of the PHA produced by *Cupriavidus necator* H16 (b) The general formula of ions 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 $(HB_7HV_2+ Na)^+$ or $(HB_8HH+Na)^+$ 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 a 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 2-pentenoic acid (100 Da) thus confirming that the obtained oligomer also contained 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). Thus, the ESI-MS/MS fragmentation results confirmed that when PE-F is used as a carbon source by *Cupriavidus necator*, the obtained PHA comprises of 3-hydroxybutyrate, 3-hydroxyvalerates, and 3-hydroxyhexanoate co-monomeric units, randomly distributed along the backbone chain.

Cupriavidus necator has previously been shown to convert PE to 3-hydroxybutyrate and 3-hydroxyvalerates units of PHA (6,13,14). In one of this study by Montazer *et al.*, 3-HB and 3-HV comonomers of PHA was produced when *Cupriavidus necator* was made to grow in minimal salt polyethylene medium supplemented with LDPE powder (100mg) as the sole carbon source for 21 days (14). Findings from our study with the use of oxidative LDPE fragments also support that work, although our study further revealed the presence of 3-hydroxyhexanoate units; demonstrating that the growth of *Cupriavidus necator* on PE-F in TSB not only improves PHA production and yield but also influence the compositional changes in the structure of the resulting PHA polymer.

CONCLUSION

This study demonstrated that LDPE oligomers obtained *via* oxidative degradation of LDPE plastic waste are, as promoted by the presence of pro-oxidant/pro-degradant additives, a valuable carbon source that can be used for the production of bacterial PHAs by *Cupriavidus necator* in TSB culture medium (Figure 3). The NMR and ESI-MS/MS analyses revealed that the bioconversion, as guided by *Cupriavidus necator* of LDPE oxidized oligomers obtained (characterized by fairly low molar masses) led to the production of a PHA random terpolymer consisting of the 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate repeating monomeric units.

We think that the reported findings give rise to an interesting breakthrough in the production of valuable biodegradable/compostable polymeric materials such as PHAs and relevant plastic items.
This is occurring indeed by bioconversion of plastic waste based on a mass full carbon backbone polymeric material such as Polyetylene within the challenging vision of the Environmental Cleaning Mission.
That approach has been found to be valid also for the management of plastic waste based on the two other full carbon backbone polymeric materials, such as Polystyrene (6) and Polypropylene (24).

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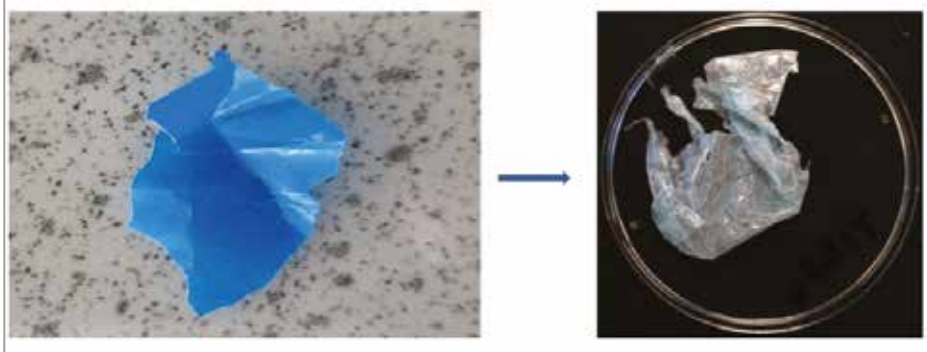

Figure 3. (a) Oxidative fragmented LDPE (sample PE-F); (b) PHA extracted from *Cupriavidus necator* using TSB supplemented with PE-F.