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High Cell Density Conversion of Hydrolysed Waste Cooking Oil Fatty Acids Into Medium Chain Length Polyhydroxyalkanoate Using *Pseudomonas putida* KT2440

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Abstract: Waste cooking oil (WCO) is a major pollutant, primarily managed through incineration. The high cell density bioprocess developed here allows for better use of this valuable resource since it allows the conversion of WCO into biodegradable polymer polyhydroxyalkanoate (PHA). WCO was chemically hydrolysed to give rise to a mixture of fatty acids identical to the fatty acid composition of waste cooking oil. A feed strategy was developed to delay the stationary phase, and therefore achieve higher final biomass and biopolymer (PHA) productivity. In fed batch (pulse feeding) experiments *Pseudomonas putida* KT2440 achieved a PHA titre of 58 g/l (36.4% of CDW as PHA), a PHA volumetric productivity of 1.93 g/l/h, a cell density of 159.4 g/l, and a biomass yield of 0.76 g/g with hydrolysed waste cooking oil fatty acids (HWCOFA) as the sole substrate. This is up to 33-fold higher PHA productivity compared to previous reports using saponified palm oil. The polymer (PHA) was sticky and amorphous, most likely due to the long chain monomers acting as internal plasticisers. High cell density cultivation is essential for the majority of industrial processes, and this bioprocess represents an excellent basis for the industrial conversion of WCO into PHA.

Keywords: biopolymers; medium chain length polyhydroxyalkanoates (PHA); hydrolysed waste cooking oil; *Pseudomonas putida* KT2440; biocatalysis; bioprocess

1. Introduction

Petrochemical based plastics have been used in a variety of applications for more than seventy years and have replaced materials like glass and metal due to their high performance, low price, versatility, and durability [1,2]. The worldwide annual production of plastics was 335 million tonnes in 2016, and its production is expected to triple by 2050 [2]. Many of the uses of plastics are short term, and consequently these materials become waste within a short period of time after manufacture and use. The vast majority of petrochemical based plastics are not biologically degraded [3]. Plastic recovery and recycling rates are low [4], and thus millions of tonnes of plastics end up in landfills and in the environment [5–8]. Given the environmental damage caused by non-degradable plastics, there is an urgent need for solutions. Biodegradable plastics can be part of the plethora of solutions to address

a complex global challenge. A critical challenge to providing biodegradable plastic solutions is the development of robust processes for biodegradable plastic production.

It is not just the end-of-life of plastics that is a concern for society, but also the origin of the starting materials. Materials of bio-based origin are being sought, as the origins of the current non degradable polymers are finite (fossil based) and depleting [9,10]. Biobased polymers can be produced from renewable resources such as corn dextrose, and many studies are investigating so called second generation (lignocellulose) sources [11–13]. Wastes, such as waste cooking oil, are also potentially interesting starting materials, but no studies have investigated this substrate for high cell density and high PHA productivity.

Polyhydroxyalkanoates (PHAs) are water-insoluble energy storage microbial polyester synthesized by many Gram-positive and Gram-negative bacteria when exposed to a surplus of carbon and generally a limitation of a vital inorganic nutrient (N, P, S, or Mg) [1]. PHAs vary in the composition of the monomer side chain and hydroxyl position, which affects their material properties [14]. The monomer composition, and thus physical properties of PHA, can be tailored by co-feeding different carbon sources in fermentation cultivation systems [15–17]. For example, incorporation of monomers with unsaturated side chains in PHA will increase the melting temperature and decrease the glass transition temperature of the polymer [18]. Furthermore, these unsaturated bonds could be exploited for chemical or enzymatic modifications [19,20] that can render PHAs water soluble or allow their coupling with functional molecules, and therefore broaden their application potential.

High production costs, compared to the traditional petrol-based plastics, remain the major challenge for polyhydroxyalkanoates entry into the plastics market. The use of inexpensive carbon sources, such as waste products, and a highly productive fermentation process could help to overcome the production costs [21–24].

Waste cooking oil (WCO) is a major waste from human food processing and preparation with over 29 million tonnes produced annually around the globe [25]. As WCO contains high levels of fatty acids, it might be suitable substrates for PHA production. It has been widely reported that fatty acids are excellent substrates for PHA accumulation by *Pseudomonas* strains with a PHA composition related to the chain length of the fatty acid supplied [26–30].

Currently, there are a limited number of studies on the use of hydrolysed and saponified cooking oils, or other waste oils, for PHA production [31–34]. Surprisingly, none of these studies have examined the ability of the strains to grow to high cell density on the hydrolysed substrates, nor have they achieved high PHA productivity.

The aim of the current study was to develop a bioprocess to achieve high cell density and high medium chain length PHA (mclPHA) productivity using the fatty acid fraction of hydrolysed waste cooking oil as the sole carbon source and *P. putida* KT2440, a generally recognised as safe (GRAS) and robust organism used in many biotechnological applications [35]. We also examined the properties of the mclPHA polymer accumulated during this bioprocess.

2. Results

2.1. Fatty Acid Composition of Hydrolysed Waste Cooking Oil (HWCO)

Hydrolysed waste cooking oil (HWCO) was separated into the glycerol and fatty acid fractions using a separation funnel. The fatty acid fraction of hydrolysed waste cooking oil (HWCOFA) contained predominantly oleic and linoleic acid (Figure 1). The high content of C18:2 and C18:1 is in keeping with previously reported contents of fatty acids in waste cooking oils [36–38]. The HWCOFA also had a relatively high content of saturated C16 fatty acid, which makes the hydrolysed oil solid at room temperature. This poses challenges for feeding to a bioreactor and bioavailability in an aqueous growth medium. To address the feeding challenge, the HWCOFA mixture was maintained at a temperature of 40 °C so that it could be poured into the bioreactor.

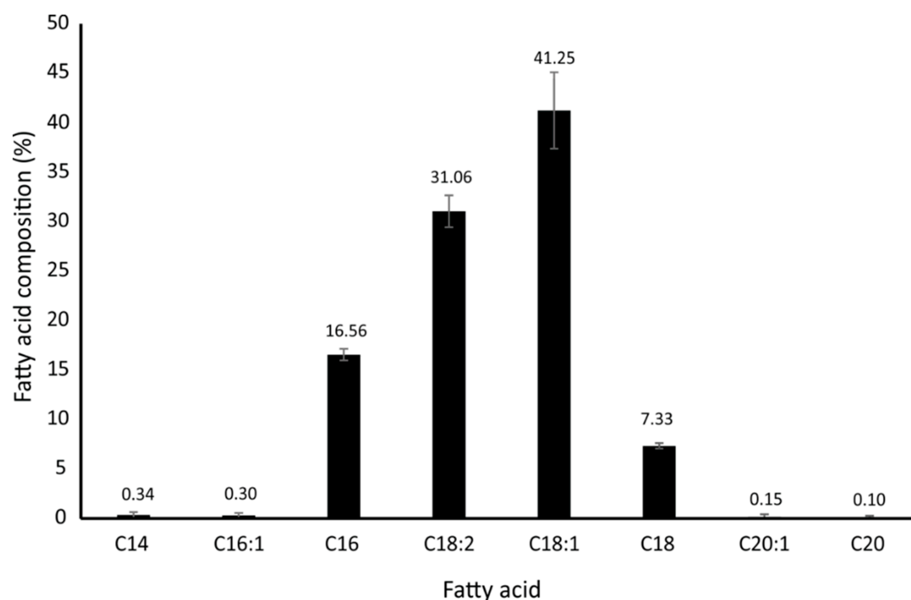

Figure 1. Fatty acid composition (expressed as a % of total fatty acids present) of hydrolysed waste cooking oil (HWCO) analysed by GC/MS. C14—myristic acid; C16:1—palmitoleic acid; C16—palmitic acid; C18:2—linolelaidic acid; C18:1—oleic acid; C18—stearic acid; C20:1—gondoic acid; and C20—arachidic acid. The error bar represents standard deviation among three separate hydrolysis experiments.

2.2. Bioprocess Development Using HWCOFA Mixture as the Carbon and Energy Source

In order to develop a robust growth of *P. putida* KT2440 in a bioreactor using hWCO as carbon substrate, three fed batch strategies were undertaken. All fermentations had the same concentration of 12 g/l HWCOFA at the time of inoculation. Cells were incubated for three hours before any additional HWCOFA was supplied to the bioreactor. The first feed strategy (FS1) is based on the supply of substrate through a pulse feed starting with 12 g of HWCOFA at T3, followed by pulses of the same amount of HWCOFA at T5, T6, and T7 hours (Figure 2). Over the next two hours the pulse feeds were 18 g and 24 g, followed by a 30 g pulse at T11, a 34 g pulse at T12, two 35 g pulses at T13 and T14, and two 40 g pulses at T15 and T16 (Figure 2). Between T17 and T25, 10 pulses of 45 g of HWCOFA were fed. This was followed by 48 g pulses at time 26 and 27 hours, and finally 50 g and 55 g at time 28 and 29 hours, respectively (Figure 2). This feed strategy was based on a feed strategy for nonanoic acid and designed to delay oxygen limitation typically occurring at high cell density growth [39]. The dissolved oxygen in the liquid medium was used as a tool to determine when substrate feeding should occur, and in this strategy, the dissolved oxygen was kept below 5%. A total of 967 g of HWCOFA were supplied over a 30 h incubation, with *P. putida* KT2440 achieving a final CDW of 115 g/l (Figure 3).

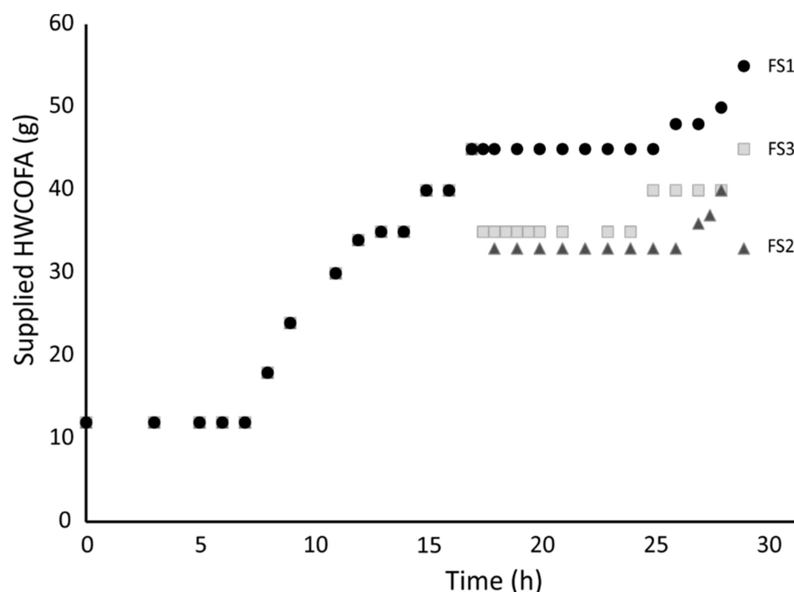

Figure 2. Hydrolysed waste cooking oil fatty acids (HWCOFA) pulse feeding strategies. Feed strategies FS2 and FS3 have the same feed profile as FS1 up to T17 hours after, at which time the number of pulses and the amount of HWCOFA supplied was different to FS1. FS1 supplied a total of 967 g of HWCOFA, FS2 804 g of HWCOFA, and FS3 844 g of HWCO, in 30 h fermentation. The starting volume of the culture was 3 l, which increased to 4.2 l for FS1 and FS3 and to 4.1 l for FS2.

We have observed that the growth rate started to slow at 19 hours (Figure 3). Therefore, we designed a second feed strategy (FS2), building on the FS1. The substrate was supplied to the same amount until T17 hours, followed by 9 pulses of 33 g of HWCOFA until T26 hours, and pulses of 36 g, 37 g, 40 g, and 33 g at T27, T27.5, T28, and T39 (Figure 2). FS2 supplied a total of 804 g of HWCOFA, achieving a final biomass of 145 g/l with a delayed onset of the stationary phase observed (Figure 3). The final strategy, FS3, was also an adaptation of FS1 with the same amount of HWCOFA supplied to the bioreactor up to 17 hours, followed by nine 35 g pulses between T17.5 and T23, four pulses of 40 g between T24 and T28, and a final 45 g pulse at T29 (Figure 2). The total HWCOFA supplied to the fermentation medium was 844 g, which resulted in a final CDW of 159 g/l (Figure 3). PHA accumulation was detected after approximately 10 hours of incubation and linearly increased for longer in FS2 and FS3 (Figure 4). The highest substrate to biomass yield of was achieved with FS3 resulting in 1.5-fold improvement compared to FS1, while substrate to PHA yield increased 1.9-fold, with FS3 compared to FS1 (Table 1). Interestingly, the PHA content of cells decreased towards the end of the bioprocess with FS2 and FS3 (Figure 4), but without the corresponding drop in biomass (Figure 2).

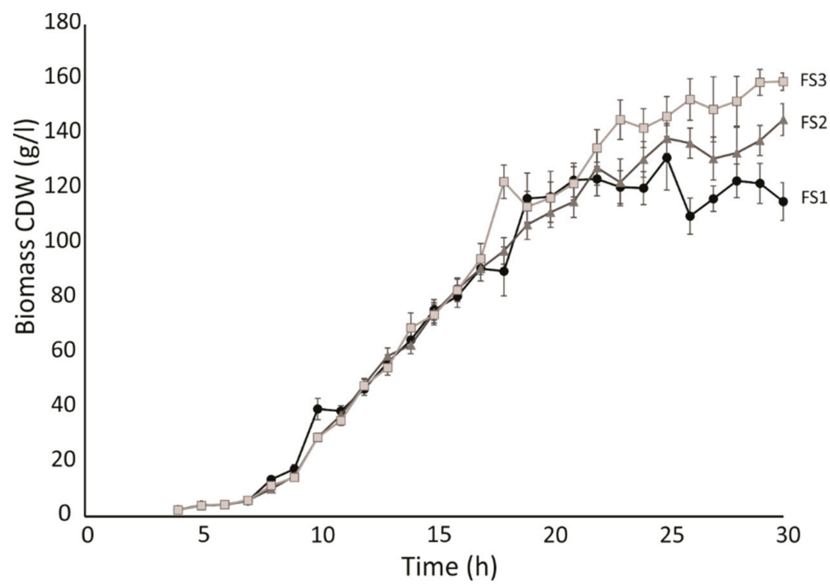

Figure 3. Growth of *P. putida* KT2440 on hydrolysed waste cooking oil fatty acids (HWCOFA) using three different feed strategies: FS1, FS2, and FS3. Biomass is represented as cell dry weight (CDW; g/l). Data are the average of three independent biological replicates and the error bars represent standard deviation among these.

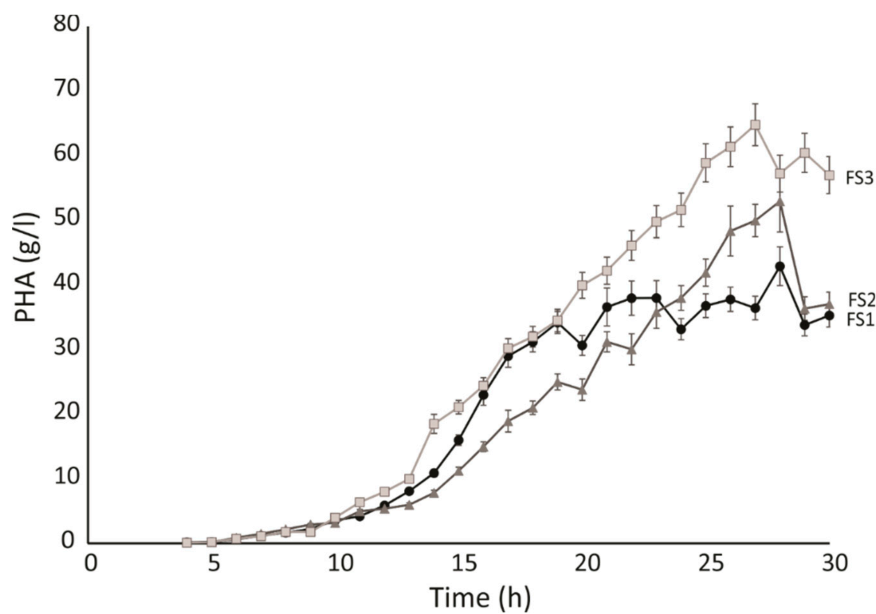

Figure 4. The dynamics of PHA (g/l) accumulation by *P. putida* KT2440 resulting from feed strategies FS1, FS2, and FS3. The error bars represent standard deviation among three independent biological replicates.

Table 1. Growth data for *P. putida* KT2440 using different feeding strategies.

Substrate	Feed Strategy	Initial Volume (l)	Total Substrate Used (g)	Final CDW (g/l)	Final Volume (l)	Total Biomass (g)
HWCOFAs *	1	3	967	115.1	4.2	483.4
	Final PHA (%CDW)	**Final PHA (g/l)**	**PHA productivity (g/l/h)**	**Total PHA produced (g)**	**Biomass Yield (g/g)**	**PHA Yield (g/g)**
	30.6	35.2	1.17	147.9	0.50	0.15
HWCOFAs	**Feed strategy**	**Initial Volume (l)**	**Total substrate used (g)**	**Final CDW (g/l)**	**Final Volume (l)**	**Total Biomass (g)**
	2	3	804	145.2	4.1	595.3
	Final PHA (%CDW)	**Final PHA (g/l)**	**PHA productivity (g/l/h)**	**Total PHA produced (g)**	**Biomass Yield (g/g)**	**PHA Yield (g/g)**
	25.5	37.0	1.23	151.8	0.74	0.19
HWCOFAs	**Feed strategy**	**Initial Volume (l)**	**Total substrate used (g)**	**Final CDW (g/l)**	**Final Volume (l)**	**Total Biomass (g)**
	3	3	881	159.4	4.2	669.5
	Final PHA (%CDW)	**Final PHA (g/l)**	**PHA productivity (g/l/h)**	**Total PHA produced (g)**	**Biomass Yield (g/g)**	**PHA Yield (g/g)**
	36.4	58.0	1.93	243.7	0.76	0.28

* HWCOFAs: hydrolysed waste cooking oil fatty acids. Data are the average of three independent biological replicates (SD < 5%).

2.3. Polymer Analysis

The specific industrial application of PHAs is determined by the mechanical and thermal properties of the biopolymer. These characteristics are affected by the monomer composition, which varies according to the metabolic machinery of the organisms and the substrate supplied [40,41].

The polymer accumulated by *P. putida* KT2440 using HWCOFA is sticky, waxy, and has low molecular weight (Table 2). It contained 42 mol% of (*R*)-3-hydroxydecanoic acid, 39 mol% of (*R*)-3hydroxyoctanoic acid, 8 mol% of (*R*)-3-hydroxydodecanoic acid, 6 mol% of (*R*)-3-hydroxyundodecanoic acid, and 5 mol% of (*R*)-3-hydroxyhexanoic acid. The polydispersity value of just under 2 is typical for mclPHAs (Table 2). Thermal analysis showed a similar low glass transition temperature for both polymers (Table 2). These properties indicate the polymer could be useful for adhesive applications.

Table 2. Properties of PHA polymer extracted from *P. putida* KT2440 grown on hydrolysed waste cooking oil fatty acids (HWCOFAs).

Substrate Conditions	Td (°C)	Tg (°C)	Tm (°C)	Mn	Mw
HWCOFAs	270.61 ± 1.6	-56.1 ± 0.5	20.7 ± 0.5	22954 ± 975	45317 ± 62

Td—thermal degradation temperature; Tg—glass transmission temperature; Tm—melting temperature; Mn—number; Mw—molecular weight.

3. Discussion

We have developed a successful bioprocess for the high cell density conversion of HWCOFA into mclPHA with high PHA volumetric productivity (Table 1). WCO represents a major pollutant due to the poor end-of-life management [42]. The dominant route for the WCO management is incineration, which leads to the loss of the valuable resource [43]. The bioprocess reported here contributes to the end-of-life management through valorisation of this waste material and production of a biodegradable polymer.

While the use of modelling informs the strategy to optimise processes and reduces unnecessary experimentation [44], the building of the model depends upon the accurate measurement of variables, such as substrate concentration. Long-chain length fatty acids such as those found in HWCO are insoluble in water [45]. The hydrophobicity of the fatty acids results in a lack of uniformity in their dispersion in an aqueous medium and the sticking of fatty acids to the wall of the bioreactor. Thus, it was not possible to accurately measure substrate utilisation and a mathematical model of the process could not be constructed. Therefore, an empirical approach was taken in developing a bioprocess for conversion of HWCOFA to mclPHA. Oxygen limitation is a typical reason for termination of aerobic high cell density fermentations, causing the onset of the stationary phase [39]. Oxygen uptake rate is proportional to the cell generation rate, and therefore a gradual decrease in growth rate by decaying the amount of substrate fed should result in high cell density while avoiding the negative physiological effects of growth halt. To initiate the fed batch fermentation, 12 g/l of HWCOFA was used as the starting concentration, as this is known to be below the inhibitory concentration of fatty acid substrates [46]. The fed batch process began with the first feed of HWCOFAs 3 h after inoculation. The rate of substrate feeding then was varied by increasing amounts of the fed substrate, followed by constant feed of the substrate throughout the exponential to early stationary phase (Figure 2). Decaying feeds in FS2 and FS3 caused a delayed onset of stationary phase, and therefore higher final biomass (Figure 3). Decaying feeds also resulted in increased PHA accumulation, likely due to preventing the accumulation of the inhibitory level of fatty acids.

Another challenge we encountered when developing this bioprocess was foaming. The same issue was described when crude fatty acids were supplied as a carbon substrate in bioreactors at concentrations greater than 10 g/l [31]. Thus the implementation of this technology at scale will require good foam breakage and dispersion.

High PHA productivity when fatty acids are used as a substrate was reported earlier. Lee and co-workers observed *Aeromonas hydrophila* cells with 45.2% of the CDW as PHA accumulation, and a PHA productivity of 1.01 g/l/h with lauric acid and oleic acid [47]. *P. putida* KT2442 grown on oleic acid and *P. putida* KT2440 grown on nonanoic acid have achieved productivities at 1.9 g/l/h and 2.3 g/l/h, respectively [39,47]. In this work, when HWCOFA was used as a substrate, the PHA volumetric productivity was 1.9 g/l/h (Table 1). Thus, low cost HWCOFA show as much potential for PHA production as more expensive virgin fatty acids.

While a number of studies investigated the production of mclPHAs from vegetable oils, waste cooking oils, and saponified waste palm oil [48–51], this is the first study to use HWCOFA for mclPHA production, with high cell density and high PHA productivity in a bioreactor. For example, a mixture of glucose and 2% free fatty acids was used for the growth and PHA accumulation by *P. aeruginosa* 47T2 in shaken flasks [52]. While achieved PHA levels were comparable between this and our study, the biomass (7.9 g/l CDW) reported by Haba et al. [52] is 18.7-fold lower than the biomass achieved in the present study. Similarly, the ability of *P. oleovorans*, *P. resivorans,* and *P. putida* to produce mclPHA from tallow was demonstrated only at low cell density [53]. Conversion of saponified waste palm oil as the sole source of carbon and energy into PHA in a bioreactor by *Pseudomonas sp.* Gl01 was reported by Możejko and Ciesielski [51]. However, our bioprocess based on FS3 achieves 33-fold higher PHA productivity compared to productivity reported by Możejko and Ciesielski [51].

The vast majority of studies report on the need for inorganic nutrient limitation in order to observe PHA accumulation in *Pseudomonas* and other strains [24,54,55]. In the current study, *P. putida* KT2440 accumulated PHA early in the growth cycle when no inorganic nutrient limitation occurred. The fatty acids present in HWCO feed directly into beta-oxidation. The resultant intermediates are PHA precursors. Given the high energy state of cells as a result of growth on long chain fatty acids and the presence of precursors in the cell it is not surprising that we observed PHA accumulation, as the conditions for accumulation of PHA were ideal.

PHA accumulated by bacteria supplied with vegetable oil derived free fatty acids and animal fat derived free fatty acids display liquid properties at room temperature similar to the polymers arising from the supply of HWCOFAs to bacteria in the current study [21,56]. These polymer properties can be explained by the high degree of disorder and by the long chain monomers acting as internal plasticisers [30]. However, the molecular weight (Mw) of PHA polymers accumulated by *Pseudomonas species* supplied with other fatty acid substrates, such as oleic acid, lauric acid [57], and mystiric acid [34], were between 2 and 4.2-fold higher than those seen in our study.

In conclusion, we have developed a high cell density cultivation with the highest known PHA productivity bioprocess using WCO as a source of waste fatty acids. The PHA productivity is similar to that achieved with pure single source fatty acids [28,34,47] and shows great promise for process scale up and polymer product development.

4. Materials and Methods

4.1. Bacterial Growth Medium & Strain Maintenance

Minimal Salt Medium (MSM) was used as the growth medium for all strains in shaken flask experiments. This medium contains (per litre): 9 g $Na_2HPO_4x12_XH_20$, 1.5 g KH_2PO_4, 1 g NH_4Cl, 200 μg $MgSO_4$, and 1000 μL trace elements solution. The trace elements solution contained (per litre in 1M HCl): 4 g $ZnSO_{4X}7H_20$, 10 g $FeSO_{4.\ X}\ 7H_20$, 1 g $CuCl_{2X}2H_20$, 1 g $MnCl_{2X}4H_20$, 1 g $Na_2B_4O7_X10H_20$, 0.2 g $NiCl_{2X}6H_20$, and 0.3g $Na_2MoO_{4X}2H_20$. *Pseudomonas putida* KT2440 [ATCC® 47054™] was grown on *Pseudomonas* isolation agar (PIA) (Fluka analytical).

4.2. Waste Cooking Oil (WCO) Hydrolysis

The WCO was supplied by Frylite® (Dublin, Ireland). A measure of 100 g of WCO was hydrolysed using 100 mL of 6 M NaOH. The mixture was heated at 60 °C for 90 min. The resultant saponified fatty

acid mixture was precipitated through the addition of 400 mL of 6 M HCl. The mixture was decanted into a separating funnel and the fatty acid phase separated from the aqueous phase, containing salts and glycerol. The fatty acid phase was washed with approximately 1000 mL of distilled water, filtered using a vacuum system (Fisher FB59037 Range QL100, Dublin, Ireland), and supplied as the sole carbon and energy substrate in fermentations.

4.3. Fermentation Conditions

A single colony of *P. putida* KT2440 from a PIA plate was inoculated into 50 mL Minimal Salt Media (MSM) in flask experiments, as previously described [58]. Flasks were supplemented with 3 g/l of technical oleic acid (Sigma Aldrich, Dublin, Ireland). Flasks were incubated at 30 °C, shaking at 200 rpm for 16–18 h. Four 50 mL cultures of *P. putida* KT2440 overnights were prepared for inoculation into a fermenter.

Fermentations were performed in a Biostat B+ bioreactor with a 5 litre working volume (Sartorius). MSM was used as the base media for all fermentations. The fermentations had an initial volume of 3 litres, with an initial agitation of 500 rpm. The temperature was maintained at 30 ºC and pH was controlled at 6.9 +/− 0.1 by the addition of 20% NH_4OH solution or 15% (v/v) H_2SO_4. The NH_4OH also acted as a nitrogen source. Foaming was controlled by the addition of antifoam solution (polypropylene glycol P2000, Sigma). Dissolved oxygen (DO) was set at 20% of saturation and was increased by increasing agitation. O_2 was supplied as air at a constant flow rate of 5 litres per minute. To start, the fermentation substrate was supplied at a concentration of 12 g/l. Three pulse feeding strategies for HWCOFA were undertaken and are described in the results section.

4.4. Analysis of Fatty Acids in Hydrolysed Waste Cooking Oil

Fatty acids generated by hydrolysis of WCO and virgin plant oil standards (Sigma Aldrich) were derivatised with *N*-Methyl-N-(trimethylsilyl)trifluoroacetamide (TMS). A measure of 2 mL of chloroform was placed in a gas chromatography vial. Then, 1 µL of the substrate and 20 µL of TMS were added. The vial was incubated at 70 °C for 30 min. The fatty acids were then analysed using an Agilent 6890N gas chromatograph (GC) (Cork, Ireland) fitted with a 5973 series inert mass spectrophotometer (MS). A HP-5 column (12 m × 0.2 mm × 0.33 µm; Hewlett Packard) was used with an oven method of 50 °C for 3 min, increasing by 10 °C /min to 250 °C and holding for 1 min. A 10:12 split was used with helium as the carrier gas and an inlet temperature of 250 °C.

4.5. PHA Content and Monomer Composition Determination

Determination of the PHA content of cells and monomer composition of PHA was determined by subjecting lyophilised cells to acidic methanolysis [59,60]. Dried cells were weighed (5 to 10 mg) and suspended in 2 mL of acidified methanol (15% H_2SO_4, v/v) and 2 mL of chloroform, containing 6 mg/l benzoate methyl ester as an internal standard. The solution was placed in 15 ml Pyrex test tubes, sealed, and incubated at 100 °C for 3 h. The tubes were then placed on ice for 1 min. A meausre of 1 mL of water was added to each tube, and the solution was mixed by vigorous vortexing for 1 min. The phases were allowed to separate, and the organic phase was removed and passed through a filter before further analysis. The 3-hydroxyalkanoic acid methyl esters were analysed by gas chromatography (GC) using an Agilent 6890N chromatograph equipped with a HP Innowax column (30 m × 0.25 mm × 0.5 µm) and a flame ionisation detector (FID). An oven ramp cycle was employed as follows: 120 °C for 5 min, increasing by 3 °C/min to 180 °C, and at 180 °C for 10 min. A 20:1 split was used with helium as the carrier gas and an inlet temperature of 250 °C. Commercially available 3-hydroxyalkanoic acids (Bioplastech Ltd Dublin Ireland) were methylated as described above for PHA isolated from *P. putida* KT2440, and they were used as standards to identify individual PHA monomers. The unsaturated monomers were detected using the programme described for the analysis of fatty acids.

4.6. Nutrient and Biomass Analysis

Samples were taken at 1 or 2 h intervals during the fermentations. Two 2 mL samples were taken and centrifuged at 17.960× g for 3 min. The supernatant was decanted into a separate tube, and the cell pellets and supernatant were frozen at –80 °C. Cell dry weight (CDW) was determined by first freezing the cell pellet and then lyophilising (freeze-drying) the cell pellet at –80 °C overnight and subsequently weighing the cells. For cultures grown in a 50 mL flask, cell suspensions were centrifuged at 3,220× g for 10 min, and 2 mL of supernatant was retained and the remainder discarded. Pellets were resuspended in 1 mL of DI water and then transferred into Eppendorf tubes. These were then centrifuged at 17.960× g for 3 min; the supernatant was discarded and the pellets treated as the samples from the bioreactor. The concentration of nitrogen in the supernatant was determined using the method described by Scheiner [61]. The soluble inorganic phosphate concentration was determined using the USA EPA colorimetric method (USEPA, 1978).

4.7. Polymer Isolation

Cells were harvested from the bioreactor in a Sorvall centrifuge (Fisher Scientific, Dublin, Ireland) at 25,040 g. Harvested cells were frozen at −80 °C for 24 h and then lyophilised (Labconco, Fisher Scientific). PHA was isolated from freeze-dried cells using room temperature acetone. This involved the stirring of 10 g of cells suspended in 100 mL acetone for 24 h. The mixture was allowed to settle, and the supernatant was filtered using a 0.2 µm PTFE filter. Acetone containing PHA was then subjected to rotary evaporation under vacuum until approximately 90 mL of acetone had been recovered. The polymer was precipitated using 2 vol of a wash solution consisting of 35% methanol, 35% ethanol, and 30% distilled water [62]. The supernatant was then decanted, and the precipitated PHA was allowed to dry before further analysis.

4.8. PHA Characterisation

4.8.1. Gel Permeation Chromatography (GPC)

The average molecular weight (Mw), the molecular number (Mn), and the polydispersity index (PD) of the polymer were measured by GPC using PL gel 5 mm mixed-C +PL gel column (Perkin-Elmer, Dublin, Ireland)) with the PELV 290 UV-vis detector set at 254 nm. Spectroscopic grade chloroform was used as the eluent flow rate of 1.0 mL/min. A sample concentration of 1% (w/v) and injection volumes of 500 µL were used. A molecular weight calibration curve was generated with polystyrene standards with low polydispersity [63].

4.8.2. Differential Scanning Calorimetry (DSC)

The polymer was analysed by DSC (Perkin-Elmer, Dublin,) with a Perkin–Elmer Pyris Diamond calorimeter calibrated to Indium standards to determine the glass transition temperature (Tg), melting temperature (Tm), and degradation temperature (Td). The samples were encapsulated in hermetically sealed aluminium pans and heated from −70 °C to 100 °C at a rate of 10 °C/min. To determine the glass transition temperature (Tg) the samples were held at 100 °C for 1 min and rapidly quenched to −70 °C. The samples were then reheated from −70 to 100 °C at 10 °C/min to determine the melting temperature (Tm) and glass transition temperature (Tg). Finally, the samples were heated to 350 °C at a rate of 10 °C/min to determine the thermal destruction temperature (Td) [63].

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Conflicts of Interest: The authors declare no conflict of interest.

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