Plant Oils and Products of Their Hydrolysis as Substrates for Polyhydroxyalkanoate Synthesis

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doi: 10.15255/CABEQ.2014.2252
Original scientific paper
Received: September 22, 2014
Accepted: June 4, 2015

Plant oils could provide a sustainable source of carbon for polyhydroxyalkanoate production as they are both renewable and inexpensive. No study to our knowledge has undertaken a comparative study of the use of major European and global commodity plants oils and products of their hydrolysis as substrates for medium chain length polyhydroxyalkanoate (mcl-PHA) production. There have been several studies which have investigated the use of plant oils and their hydrolysis products for short chain length PHA (scl-PHA) production, therefore, in this study, we have focused specifically on mcl-PHA-producing organisms. A comparison between direct growth on oils and the products of their hydrolysis is described here for several mcl-PHA-producing *Pseudomonas* strains. *Pseudomonas putida* KT2440, CA-3, GO16, *Pseudomonas chlororaphis* 555 were screened for their ability to utilize a range of common plant oils (olive, sunflower, rapeseed, and palm) and their hydrolysis products as sole sources of carbon and energy for growth and PHA accumulation. When the oils were supplied in shaken flask experiments, *P. putida* CA-3 and *P. putida* KT2440 showed little or no growth, while *P. putida* GO16 reached a cell dry weight of between 0.33 and 0.56 g L^{-1}, and accumulated mcl-PHA to between 12 and 25 % of CDW, *P. chlororaphis* 555 reached a cell dry weight of between 0.67 and 0.86 g L^{-1}, and accumulated mcl-PHA to between 27 and 34 % CDW in 48 h. In contrast, when the hydrolyzed fatty acid mixtures were supplied, all 4 strains tested grew and accumulated mcl-PHA. *P. putida* CA-3 and GO16 achieved the highest biomass (1.02 – 1.06 g L^{-1}) with the majority of the hydrolyzed plant oil fatty acids, however *P. chlororaphis* 555 accumulated similar levels of PHA as these two strains. Despite being the strain of choice for mcl-PHA accumulation, for the majority of studies, *P. putida* KT2440 achieved less biomass and accumulated less PHA than other strains tested with the majority of oil-derived fatty acids. It is important to note that both biomass and PHA levels varied significantly across strain and hydrolyzed oil type. Due to the fact that *P. chlororaphis 555* was able to grow and accumulate PHA from both plant oils and hydrolyzed oil fatty acids, it was selected for bioreactor trials to try to achieve high cell density and high PHA productivity using rapeseed oil and hydrolyzed rapeseed oil fatty acids. Rapeseed oil (RO) and its hydrolysis product (HROFA) were chosen for these experiments because *P. chlororaphis* 555 accumulated approximately 30 % mcl-PHA from both substrates, and as this oil can be produced globally, it would offer less barriers to scale-up than Palm oil. The mcl-PHA volumetric productivity with RO as the substrate was 0.53 g L^{-1} h^{-1} after 25 h with a yield of 0.22 g PHA g^{-1} oil, while the volumetric productivity with HROFA as the substrate was 0.54 g L^{-1} h^{-1} after 25 h with again a lower yield of 0.15 g PHA g^{-1} HROFA. Thus, under the fermentation conditions tested, HROFA was an inferior substrate for PHA production when compared to RO.

Key words:
polyhydroxyalkanoates, biopolymers, plant oils, fatty acids

Introduction

Plastics are a ubiquitous part of modern life. They can be modified to meet the demands of almost any conceivable application[1]. While plastic is extremely useful and has been very important in the development of society since the beginning of the 20th century, it also presents a problem. It is estimated that 25 Mt of plastic waste was generated in the European Union in 2008. As much as 49 % of this waste ended up in landfill sites[2]. Plastic persists in the environment[3] and plastics buried in landfills can leach harmful chemicals into groundwater[4]. Almost all plastics currently in use are produced using petrochemicals derived from fossil oil and gas. It is

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estimated that 8 % of annual petroleum production is used in the production of plastic[5]. There is a need for new methods of producing plastics that are derived from renewable resources and are biodegradable so the environmental impact of industrially produced materials can be reduced.

Polyhydroxyalkanoates (PHA) are polymers naturally synthesized as carbon storage molecules by a range of bacteria including *Pseudomonas*. Certain bacteria accumulate PHA as a carbon and energy store under conditions where there is an excess of carbon and a limitation in another essential nutrient such as nitrogen or phosphorous[6]. PHA is biodegradable in all biologically active environments and can be produced from renewable resources[7]. Fatty acids have traditionally been the preferred substrates for studies investigating the production of medium chain length (mcl) PHA[8,9], but they are generally expensive as a purified single fatty acid.

Plant oils as substrates for PHA production may be interesting due to their relative low cost and the minimal processing required compared to fatty acids prior to fermentation. In 2014, the oils tested in this study ranged in price from an average of approximately $700/mt for palm oil to an average of approximately $4000/mt for olive oil[10]. While many studies have investigated the accumulation of scl-PHA from oils, we have focused solely on mcl-PHA production. Furthermore, a comparative study of oils versus fatty acid mixtures is not currently available. Mcl-PHA has been accumulated by *Pseudomonas aeruginosa* using palm oil[11], *Brassica carinta* oil[12] and waste frying oil[13], however, this species is an opportunistic pathogen and not suitable for PHA production at an industrial level. *Pseudomonas chlororaphis* HS21 was previously used to produce mcl-PHA from palm oil[14]. *Comamonas testosteroni* produced mcl-PHA from a range of oils, such as castor seed oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, and sesame oil[15]. *Pseudomonas resinovorans* B2649 was shown to utilize lard, butter oil, olive oil, sunflower oil, coconut oil and soybean oil to produce mcl-PHA[16], and *Pseudomonas saccharophilia* B628 was cultured using soybean oil and sunflower oil to produce mcl-PHA[17]. Genetic engineering has also been used to express lipase genes in well-known PHA-producing strains, such as *P. putida*, *P. oleovorans* and *P. corrugata* to allow them to grow directly on oils[18,19]. Surprisingly, none of these studies has examined the ability of the strains to grow to high cell density when cultivated on plant oils, growth has been carried out in shake flasks or to low cell density in batch fermentation. Some of the studies compared the growth on oils to growth on individual pure fatty acids, mainly oleic acid[11,12,13,14,16], but there has been no comprehensive comparison between growth and PHA production using plant oils and crude fatty acid mixtures (generated from the hydrolysis of oils).

Industrially, fatty acids are predominantly produced through the hydrolysis of plant oils, mainly soybean oil, palm oil, rapeseed oil, sunflower-seed oil, cotton oil, and olive oil), which represent about 85 percent of the fats production worldwide, with the remaining 15 percent coming from animal-origin fats, such as tallow, lard, butter, poultry fat, and fish oil. Industrially, fats and oils are hydrolysed in the splitting process to yield free fatty acids and glycerin. Typical splitting plants operate as continuous or batch processes at temperatures of 210 – 250 °C and vapor pressures of 20 – 40 bar without catalysts. However, in this study, saponification was used to represent fatty acid production, as this is more appropriate for a laboratory setting due to technical and mechanical constraints[20].

Oleic acid prices in 2014 were significantly higher than most raw oil prices at approximately $1800/mt, however, it is true that crude fatty acid mixtures would certainly be cheaper to produce than purified single fatty acids, as no distillation is required, and may also produce PHA with different properties to that produced on single fatty acids[10,21]. There has been a limited number of reports of the use of saponified fatty acids for mcl-PHA production. Saponified palm kernel oil has been used to produce mcl-PHA (3 g L^{-1} CDW 37 % PHA) in *Pseudomonas putida* PGA1[21,22]. Mcl-PHA has been accumulated by *Pseudomonas aeruginosa* (3.5–5.5 g L^{-1} CDW, 66.1 % PHA) from waste free fatty acids from soybean oil[13] and from saponified waste palm oil by *Pseudomonas* sp G101 (5.2 g L^{-1} CDW, 42 % PHA)[23].

In the current study, we have tested both plant oils and fatty acid mixtures generated from the saponification of olive oil, sunflower oil, rapeseed oil and palm oil as substrates for mcl-PHA production by *P. putida* KT2440, CA3, GO16, and *P. chlororaphis* 555. We examined the effect of oil type and bacterial strain on PHA accumulation levels and monomer composition, as well as attempted to grow strains to high cell density and high PHA productivity.

Materials and methods

Bacterial growth medium & strain maintenance

Minimal Salt Medium (MSM), prepared as previously described[24] was used as the growth medium for all strains in shaken flask experiments. *Pseudomonas putida* KT2440, CA-3 and *Pseudomonas chlororaphis* 555 were grown on *Pseudomonas* isolation agar (PIA) (Fluka analytical). *Pseudomonas*

putida GO16 was grown on MSM medium supplemented with sodium terephthalate (TA) (1.95 g carbon L^{-1}) and solidified with 15 g L^{-1} agar as it grew poorly on PIA. TA was used as a selective carbon source, to lower the risk or contamination by other bacterial strains, as *P. putida* GO16 was originally isolated using this carbon source[54]

Growth conditions for PHA accumulation in shaken flask cultures

Growth experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL of nitrogen-limited MSM medium supplemented with the appropriate carbon source at a concentration of 3 g L^{-1}. Ammonium chloride in this medium was limited to 0.25 g L^{-1}. An overnight culture was prepared by inoculating 2 mL of MSM supplemented with the appropriate carbon source with a single colony from a plate and incubating overnight at 30 °C and shaking at 250 rpm. 100 μL of the 2 mL MSM overnight culture was used as an inoculum for the 50 mL flasks, which were then incubated under the same conditions for 48 h. All of the oils used in this study were of technical grade and obtained from Sigma Aldrich. All four strains were tested in this manner on all of the oils and all of their hydrolysis products.

Fermentation of *Pseudomonas chlororaphis* 555 on rapeseed oil and a fatty acid mixture derived from rapeseed oil

Inoculation culture conditions. Four 50 mL cultures of *P. chlororaphis* were prepared in MSM as described above, with either rapeseed oil (RO) or hydrolysed rapeseed oil fatty acids (HROFA) as the carbon source. The cultures were grown for between 16 and 18 h before transfer to the bioreactor.

Fermentation conditions. Fermentations were performed in a Biostat B+ bioreactor with a 5 L working volume (Sartorius). MSM was used as the base media for all fermentations. The fermentations had a final volume of 5 L, with initial agitation at 500 rpm. The pH was controlled at 6.9 +/– 0.1 by addition of 20 % NH_4OH solution or 15 % (v/v) H_2SO_4. The NH_4OH also provided a nitrogen source. Foaming was controlled by addition of antifoam solution (polypropylene glycol P2000, Sigma). Dissolved oxygen (DO) was controlled at 20 % of maximum by increasing agitation. O_2 was supplied as air at a constant flow rate of 5 litres per minute. An oil concentration of 60 g L^{-1} was used for the fermentation on pure rapeseed oil. An initial substrate concentration of 1 g L^{-1} (HROFA) was used for the fed-batch fermentation on fatty acids derived from rapeseed oil. 90 g L^{-1} of HROFA was fed over 25 hours by peristaltic pump, the feed rate was designed to ensure that the concentration of fatty acids in the bioreactor never exceeded 20 mmol L^{-1}. To achieve this, the culture was grown at an average set μ of 0.2 h^{-1} by limiting the rate at which the HROFA were supplied to the fermentation. This rate of feeding was increased exponentially every hour in order to maintain the average set μ.

Oil hydrolysis

An amount of 100 g of oil was added to 100 mL of 6 mol L^{-1} NaOH. The mixture was heated to 60 °C for 1.5 h. The hydrolysed oil was then mixed with 200 mL of 6 mol L^{-1} HCl. The mixture was decanted into a separating funnel and the fatty acid phase separated from the aqueous phase containing salt and glycerol. The fatty acid phase was washed with approx. 10 volumes (1000 mL) of distilled water.

Analytical techniques

Analysis of fatty acids in hydrolysed oils

The mixtures of fatty acids produced from oil hydrolysis were derivatised with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (TMS). 2 mL of chloroform were placed in a gas chromatography vial. 1 μL of the substrate and 20 μL of TMS were added. The vial was sealed and placed at 70 °C for 30 minutes. The derivatised fatty acids were then analyzed using an Agilent 6890N gas chromatograph (GC) 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 minutes, increasing by 10 °C min^{-1} to 250 °C and holding for 1 minute. A 10:1 split was used with helium as the carrier gas and an inlet temperature of 250 °C.

Analysis of free fatty acid concentration in oils

2 g of oil were dissolved in 40 mL of a 50:50 mixture of diethylether and ethanol. Two drops of 1 % Phenolphthalein indicator (in ethanol) were added and the mixture titrated against 0.01 mol L^{-1} NaOH. The following equation was used to determine the free fatty acid content of the oil. OA = oleic acid[25].

$$\mathrm{A} = \frac{C_{\mathrm{NaOH}} V_{\mathrm{NaOH}} MW_{\mathrm{OA}}}{m_{sample} \cdot 1000} \cdot 100\ (\mathrm{wt\ \%})$$

Nutrient and biomass analysis

Samples were taken at 0.5-, 1-, or 2-hour intervals during the fermentations. Two 2-mL samples were taken and centrifuged at 17960 g for 3 minutes. The supernatant was decanted into a separate

Table 1 – *Mcl-PHA production from oils*

Bacterium	Substrate(s)	Cultivation method	CDW (g L^{-1})	PHA (% CDW)	Reference
Pseudomonas aeruginosa	palm oil	batch fermentation	2.7	39	11
Pseudomonas aeruginosa	*Brassica carinata* oil	shake flasks	1	5	12
Pseudomonas aeruginosa	waste frying oil	shake flasks	3.5 – 5.5	29	13
Pseudomonas chlororaphis HS21	Palm kernel oil	batch fermentation	3.3	45	14
Comamonas testosteroni	castor seed oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, sesame oil	shake flasks	ND	79 – 88	15
Pseudomonas resinovorans	lard, butter oil, olive oil, high oleic sunflower oil, coconut oil, soybean oil	shake flasks	2.9 – 3.8	40 – 51	16
Pseudomonas saccharophilia	soybean oil, sunflower oil	shake flasks	ND	ND	17

tube, and the cell pellets and supernatant frozen at –80 °C. The cell pellets were then lyophilysed. The lyophilysed pellets were weighed to give an estimation of the cell biomass during the fermentation. 50 mL flask cultures were centrifuged at 3220 g for 10 minutes, 2 mL of supernatant were retained and the remainder discarded. Pellets were resuspended in 1 mL of DI water and transferred into Eppendorf tubes. These were then centrifuged at 17960 g for 3 minutes, the supernatant discarded, and the pellets treated as the fermenter samples. The concentration of nitrogen in the supernatant was determined using the method described by Scheiner[26]. The soluble inorganic phosphate concentration was determined using the USA EPA colorimetric method[27].

Determination of PHA content and monomer composition

PHA content was determined by subjecting lyophilised cells to acidic methanolysis[28,29]. From 5 to 10 mg of dried cells were resuspended in 2 mL of acidified methanol (15 % H_2SO_4, v/v), and 2 mL of chloroform containing 6 mg L^{-1} 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 hours. The tubes were then placed on ice for 1 minute. 1 mL of water was added to each tube and the solution mixed by vigorous vortexing. 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 analyzed by gas chromatography (GC) using an Agilent 6890N chromatograph equipped with a HP Innowax column (30 m x 0.25 mm x 0.5 μm), and a flame ionization detector (FID). An oven ramp cycle was employed as follows, 120 °C for 5 minutes, increasing by 3 °C min^{-1} to 180 °C, 180 °C for 10 minutes. 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 samples, and used as standards to identify PHA monomers.

Polymer isolation

Cells were harvested from cultivations in a Thermo Sorvall Contifuge Stratos continuous flow centrifuge (Fisher Scientific, Dublin, Ireland) at 25040 × 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 hours. The mixture was allowed to decant, 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[30]. The supernatant was then decanted, and the precipitated PHA was allowed to dry before further analysis.

PHA characterisation

Gel permeation chromatography (GPC). The average molecular weight (M_w), 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) with PELV 290 UV-vis detector set at 254 nm. Spectroscopic grade chloroform was used as the eluent at flow rate of 1.0 mL min^{-1}. 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[31].

Differential scanning calorimetry (DSC). The polymer was analyzed by DSC with a Perkin-Elmer Pyris Diamond calorimeter calibrated to Indium standards to determine the glass transition temperature (T_g), melting temperature (T_m), and degradation temperature (T_d). The samples were encapsulated in hermetically sealed aluminum pans and heated from –70 to 100 °C at a rate of 10 °C min^{-1}. To determine the glass transition temperature (T_g), the samples were held at 100 °C for 1 minute, and rapidly quenched to –70 °C. The samples were then reheated from –70 to 100 °C at 10 °C min^{-1} to determine the melting temperature (T_m) and T_g. Finally, the samples were heated to 350 °C at a rate of 10 °C min^{-1} to determine the T_d[31].

Results and discussion

Hydrolysed oil fatty acid (HOFA) composition

Each of the HOFA were analyzed for their fatty acid content by GCMS (Table 2). HROFA and HOOFA were predominantly composed of oleic acid, while the HSOFA was predominantly composed of linoleic acid. HPOFA was the only oil tested to have a saturated fatty acid predominating with palmitic acid, the predominant acid detected.

P. putida KT2440, the most commonly studied bacterial strain for mcl-PHA production, cannot grow on pure oils, therefore the oils were hydrolysed to produce fatty acid mixtures and glycerol. Glycerol is not a focus of the current study, but numerous studies have shown its usefulness for the production of polyhydroxyalkanoate[40,41,42].

The fatty acid mixtures produced by hydrolysing oils were analysed by GC-MS to determine their fatty acid composition (Table 2). The fatty acid compositions of the fatty acid mixtures derived from oils in this study agree well with published fatty acid compositions of these oils[43,44]. The common European plant oils; olive, sunflower and rapeseed, consist mainly of unsaturated C_{18} fatty acids. Due to this fatty acid profile, these mixtures are liquid at room temperature (~20 °C). Palm oil consists of predominantly C_{16} and $C_{18:1}$ fatty acids. Palm oil-derived fatty acids are solids at temperatures up to 35 °C. This may pose problems for supply of these substrates to laboratory-scale bioreactors in a fed-batch scenario, as they would need to be heated.

Growth and PHA production in shaken flasks with plant oils

The oils used in this study were olive oil (OO), sunflower oil (SO), rapeseed oil (RO) and palm oil (PO). These oils are among the nine major vegetable oils produced globally[31]. The EU is the world's leading producer and consumer of olive oil and rapeseed oil, and a major producer of sunflower oil. Palm oil is not produced in Europe, but is a major import, and is a globally important oil[33]. The pure oils were tested as substrates for growth and PHA production in a range of PHA-producing strains.

Pseudomonas aeruginosa has been shown to convert pure plant oil to mcl-PHA[11,13]. However, as *P. aeruginosa* is an opportunistic pathogen and is not relevant industrially, it was not used in this study. A number of *Pseudomonas* strains (*P. putida* KT2440, CA-3 and GO16, *P. chlororaphis* 555) were tested for their ability to grow and produce PHA on a range of plant oils. The *Pseudomonas putida* strains have previously been used to produce mcl-PHA from a variety of substrates[34,35,36]. Other *P. chlororaphis* strains have been used to produce mcl-PHA from palm kernel oil[14]. *P. putida* GO16 and *P. chlororaphis* 555 were capable of utilizing all of the plant oils tested as the sole source of carbon for both growth and PHA accumulation (Table 3). The level of free fatty acids present in the oils (0.2–1.1 % (Table 4)) was not high enough to support growth to the biomass levels seen with plant oils, therefore these bacteria must be using the oils as their carbon source. The low levels of growth observed for *P. putida* KT2440 and CA-3 can be attributed to the free fatty acids present in the oils, and not growth on the oil itself. *P. chlororaphis* 555 grew to a higher cell density and accumulated more PHA than *P. putida* GO16 on all of the oils tested. *P. putida* GO16 achieved the highest growth and PHA from olive oil (0.56 g L^{-1} CDW, 25 % PHA), and *P. chlororaphis* 555 achieved the highest levels of growth and PHA from palm oil (0.86 g L^{-1} CDW, 34 % PHA). To the best of our knowledge, there have been no reports of wild type *P. putida* growth or PHA production directly on plant oils; however,

Table 2 – *Fatty acid composition of hydrolysed plant oils (mol %)*

	C8	C10	C12	C14	C16:1	C16	C18:2	C18:1	C18
Olive Oil Fatty Acids	1 %	–	–	–	1 %	18 %	5 %	73 %	4 %
Sunflower Oil Fatty Acids	6 %	–	–	–	–	6 %	50 %	33 %	5 %
Rapeseed Oil Fatty Acids	1 %	–	–	–	–	5 %	16 %	75 %	3 %
Palm Oil Fatty Acids	6 %	–	–	1 %	–	42 %	6 %	39 %	6 %

Table 3 – *Growth and PHA accumulation by a range of bacterial strains supplied with commercially available plant oils (3 g L^{-1}) in shake flask cultures after 48 h incubation at 30 °C. Strain annotations: P. putida KT2440, P. putida CA-3, P. putida GO16, P. chlororaphis 555 (triplicate results). (*Percentage of CDW)*

Oil	KT2440		CA3		GO16		555	
	CDW (g L^{-1})	PHA (%)*	CDW (g L^{-1})	PHA (%)	CDW (g L^{-1})	PHA (%)	CDW (g L^{-1})	PHA (%)
Olive Oil	–	–	0.05 ± 0.00	–	0.56 ± 0.08	25.29 ± 5.1	0.81 ± 0.10	34.14 ± 3.6
Sunflower Oil	0.03 ± 0.00	–	0.05 ± 0.00	–	0.50 ± 0.10	16.35 ± 4.6	0.74 ± 0.12	27.08 ± 6.1
Rapeseed Oil	–	–	0.08 ± 0.02	–	0.41 ± 0.09	11.83 ± 2.4	0.67 ± 0.10	29.28 ± 2.4
Palm Oil	0.09 ± 0.06	–	0.22 ± 0.04	5.66 ± 1.4	0.33 ± 0.05	13.24 ± 1.0	0.86 ± 0.11	34.34 ± 2.2

Table 4 – *Free fatty acid content of plant oils (Weight %)*

Oil	% Free Fatty Acids
Olive Oil	1.1
Sunflower Oil	0.2
Rapeseed Oil	0.2
Palm Oil	0.3

P. chlororaphis has previously been shown to produce mcl-PHA from palm kernel oil[14].

Growth and PHA production in shaken flasks with hydrolysed oil fatty acid (HOFA) mixtures

All strains grew and produced PHA on all of the HOFA substrates tested (Table 5). The other strains in this study varied in their optimal substrate for growth. The highest biomass observed for *P. putida* KT2440 was with fatty acids derived from SO (0.82 g L^{-1}), while the maximum PHA (28.61 %) accumulated by this strain was when fatty acids derived from PO were supplied. *P. putida* CA3 achieved its highest PHA on fatty acids derived from OO and RO (1.02 g L^{-1} and 36–39 % PHA). The highest biomass observed for GO16 was when it was supplied with fatty acids from hydrolysed OO (1.06 g L^{-1}), but it accumulated the highest level of PHA on fatty acids derived from PO (44 %). *P. chlororaphis* 555 grew to the highest biomass and produced the highest amount of PHA on fatty acids from OO (0.94 g L^{-1}, 39 %). In another study, *Pseudomonas putida* PGA1, was grown on saponified palm kernel oil achieving similar biomass yields and PHA content to that reported here[49]. However, to the best of our knowledge, there have been no reports of *Pseudomonas putida* strains grown directly on any of the other saponified oil fatty acid mixtures used in this study.

Batch and fed batch fermentation using plant oils and *P. chlororaphis 555*

P. putida CA-3 and *P.* putida KT2440 showed no significant growth on any of the oils, and since *P. chlororaphis* 555 produced significantly higher biomass and PHA than *P. putida* GO16 when grown on plant oils, it was chosen as the most appropriate strain for fermentation studies (Table 2). While the other oils tested allowed for higher levels of growth than Rapeseed oil, economic and geographical factors were taken into account. Rapeseed was used as the substrate because the production of rapeseed oil in Europe, particularly for industrial applications, has been increasing rapidly since 2003[50].

As oil is not toxic to cells at high concentrations, a batch fermentation can be carried out with a high initial oil concentration. This process is easier to scale up than fed-batch fermentation. Therefore, batch fermentations of *P. chlororaphis* on 60 g L^{-1}

Table 5 – *Growth and PHA production of a range of bacterial strains on fatty acids produced from plant oils in shake flask culture after 48 h incubation at 30 °C. Strain annotations: P. putida KT2440, P. putida CA-3, P. putida GO16, P. chlororaphis 555 (triplicate results). (*Percentage of CDW)*

HOFA$^{\tau}$	KT2440		CA3		GO16		555	
	CDW (g L^{-1})	PHA (%)*	CDW (g L^{-1})	PHA (%)	CDW (g L^{-1})	PHA (%)	CDW (g L^{-1})	PHA (%)
HOOFA	0.70 ± 0.05	23.57 ± 2.1	1.02 ± 0.06	39.32 ± 2.4	1.06 ± 0.13	34.76 ± 2.9	0.94 ± 0.10	38.65 ± 1.9
HSOFA	0.82 ± 0.02	13.91 ± 1.3	0.99 ± 0.09	32.77 ± 1.6	0.91 ± 0.08	26.78 ± 0.8	0.78 ± 0.01	21.33 ± 0.8
HROFA	0.70 ± 0.07	26.01 ± 5.0	1.02 ± 0.07	35.95 ± 1.4	0.96 ± 0.07	32.29 ± 8.1	0.81 ± 0.08	32.92 ± 2.3
HPOFA	0.71 ± 0.09	28.61 ± 3.7	0.63 ± 0.01	29.88 ± 0.1	0.74 ± 0.01	44.23 ± 0.3	0.62 ± 0.07	29.92 ± 2.4

$^{\tau}$HOFA = hydrolysed oil fatty acids, HOOFA = hydrolysed olive oil fatty acids, HSOFA = hydrolysed sunflower oil fatty acids, HROFA = hydrolysed rapeseed oil fatty acids, HPOFA = hydrolysed palm oil fatty acids.

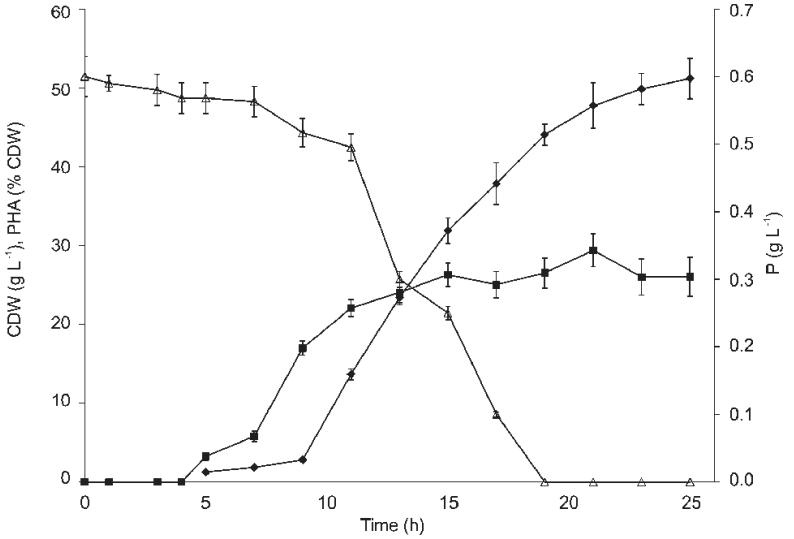

Fig. 1 – *CDW and PHA production in batch fermentation of P. chlororaphis 555 on 60 g L^{-1} rapeseed oil (duplicate results) PHA (% CDW) [■] CDW g L^{-1} [♦] Phosphorus g L^{-1} [Δ])*

rapeseed oil were carried out. The fermentations produced 51 g/l biomass containing only 26 % PHA in 25 hours (Figure 1). This represents a high biomass yield (85 %), but the PHA content is still as low as in the shaken flask experiments. While pure oils show potential as low-cost renewable substrates for PHA production, further bioprocess development is required.

Pure fatty acids have been shown to give very high biomass and PHA yields when used as single substrates for PHA production. 141 g L^{-1} biomass containing 51.4 % PHA was achieved using *P. putida* KT2440 grown on oleic acid under inorganic phosphorous limitation for 38 hours with oxygen enriched air[47]. Therefore, mixtures of fatty acids produced by hydrolysing oils may give higher yields of biomass and PHA than the pure oils themselves. To investigate this, we carried out a fermentation of *P. chlororaphis* 555 using HROFA as the substrate. As fatty acids are reported to be toxic to cells at high concentrations[51] a fed-batch strategy was employed to grow 555 with HROFA in a 5L fermenter. This fermentation strategy yielded 76 g L^{-1} biomass containing 17 % PHA (Figure 2) in 25 hours. Inorganic phosphorous was limited in this fermentation after 18 h as was the case for the RO fermentation. The level of PHA was lower than expected as 34 % of the CDW was PHA in shaken flasks (Table 5). In general, PHA accumulation levels should be higher in bioreactor fermentations compared to shaken flasks where process conditions can be controlled to a higher degree. This PHA yield could potentially be improved by imposing inorganic phosphorous limitation earlier or using nitrogen limitation which may act as a better stimulant for PHA production.

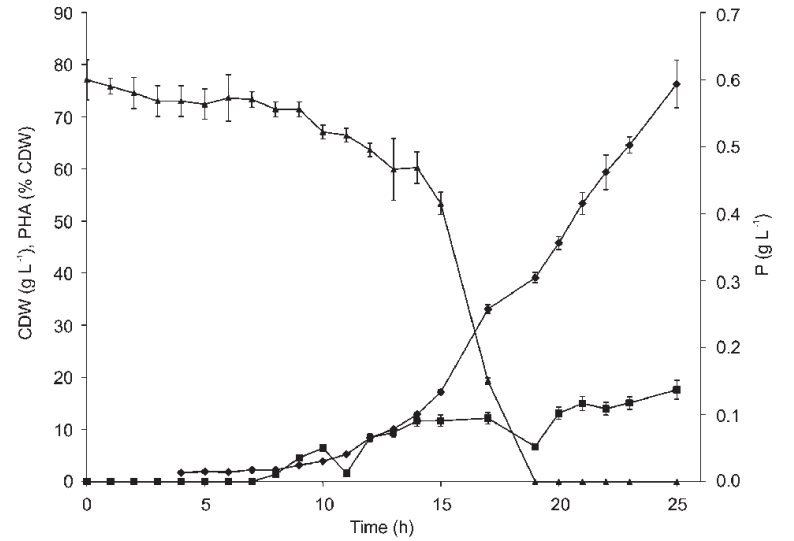

Fig. 2 – *CDW and PHA production in fed batch fermentation of P. chlororaphis 555 on HROFA (duplicate results) PHA (% CDW) [■] CDW g L^{-1} [♦] Phosphorus g L^{-1} [Δ]).*

Polymer properties

The monomer composition of a polymer is important in determining its physical and material properties[52]. The monomer composition of the PHA produced by *P. putida* GO16 and *P. chlororaphis* 555 on pure oils and all strains on the HOFA were analysed by GC.

The monomer composition produced on pure oils varied according to the strain and the oil used (Figure 3). The monomer composition of the PHA produced by the fatty acid mixtures was quite similar in *P. putida* KT2440 (Figure 4A), GO16 (Figure 4B) and CA-3 (Figure 4C), except for the PHA accumulated from fatty acids derived from palm oil. The monomer composition of the PHA produced by *P. chlororaphis* 555 varied considerably from the PHA produced by the other strains, and also varied depending on the substrate used (Figure 4D). The monomer composition of PHA produced by *P. chlororaphis* 555 on pure oil and on fatty acids derived from the same oil is similar. However, the monomer composition of the PHA produced by *P. putida* GO16 varies considerably between pure oil and fatty acids derived from the same oil. While this is not in keeping with the other strains tested, potentially *P. putida* GO16 may produce some monomers from the glycerol portion of the oil, and thus the substrate specificity of its phaG may play a role in monomer composition[53]. Interestingly, the

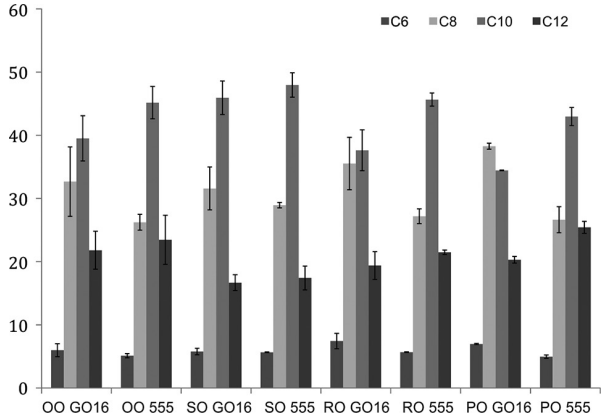

Fig. 3 – *Monomer composition of PHA produced by P. putida GO16 and P. putida 555 on a range of oils. OO – olive oil, SO – sunflower oil, RO – rapeseed oil, PO – palm oil (triplicate results).*

monomer composition of the PHA produced in both fermentations by *P. chlororaphis* 555 were almost identical, and matched very closely the monomer composition observed in shaken flask experiments. These polymers were completely amorphous as evidenced by the lack of a defined T_m (Table 6). While the polymer produced from RO had a slightly lower M_w and M_n as well as a higher T_g compared to the polymer produced from ROFA, these variations were minor and had little effect on the polymers physical characteristics. However, these variations

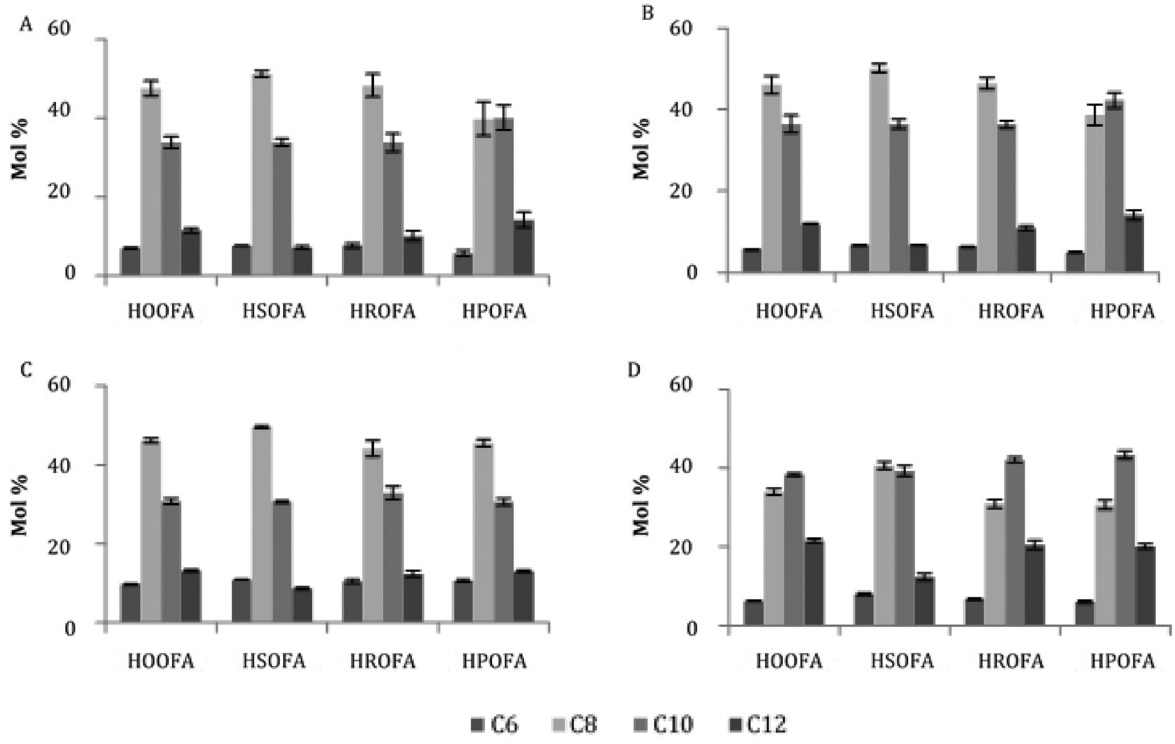

Fig. 4 – *Monomer composition of PHA produced by P. putida KT2440 (A), GO16 (B), CA3 (C) and P. chlororaphis 555 (D) on fatty acids produced by hydrolysing oil. HOOFA – hydrolysed olive oil fatty acids, HSOFA – hydrolysed sunflower oil fatty acids, HROFA – hydrolysed rapeseed oil fatty acids, HPOFA – hydrolysed palm oil fatty acids (triplicate results).*

Table 6 – *Properties of PHA polymer extracted from P. chlororaphis 555 grown on RO and HROFA. Nd = not detected*

Substrate	T_d/° C	T_g/° C	T_m/° C	M_n/Da	M_w/Da	P.D.
RO	290	–64.9	Nd*	52649 ± 644	94796 ± 188	1.7 ± 0
HROFA	289.4	–55.2	Nd*	59746 ± 482	103652 ± 390	1.7 ± 0

may play a role in determining the final application of these types of polymers, and so should not be discounted completely. Considering the variations in monomer composition between strains and on the various oils and fatty acid mixtures, it is important to consider both strain and substrate when designing fermentation strategies, not only in terms on growth and PHA productivity, but also in terms of polymer properties.

Conclusion

Plant oils and fatty acid mixtures from hydrolysed plant oils are good substrates for bacterial growth and mcl-PHA accumulation in shake flask experiments. However, PHA productivity from these substrates in bioreactor experiments needs to be improved in order to progress such processes towards scale-up and application. As shown, untreated oils have potential for mcl-PHA production. As these substrates cost less and are more readily scaled, we would suggest that further study and optimization could provide the further productivity gains required for cost effectiveness.

ACKNOWLEDGEMENT

Meg Walsh is funded by the Irish Research Council Employment Based Postgraduate Programme (Ref no. 39826).

References

1. *PlasticsEurope*, 2014. URL: http://www.plasticseurope.org/use-o plastics.aspx Accessed 14/4/14
2. *European Commission*, 2013. Green Paper: On a European Strategy on Plastic Waste in the Environment.
3. *Andrady, A.L. Neal, M.A.*, Applications and societal benefits of plastics, Philos. Trans. R. Soc., B. **364** (2009) 1977.
4. *Teuten, E.L., Saquing, J., Knappe, D., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Daisuke, O., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H.*, Transport and release of chemicals from plastics to the environment and to wildlife, Philos. Trans. R. Soc., B. **364** (2009) 2027.
5. *Hopewell, J., Dvorak, R., Kosior, E.*, Plastics recycling: challenges and opportunities, Philos. Trans. R. Soc., B. **364** (2009) 2115.
6. *Witholt, B., Kessler, B.,* Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics, Curr. Opin. Biotechnol. **10** (1999) 279. http://dx.doi.org/10.1016/S0958-1669(99)80049-4
7. *Snell, K.,* PHA bioplastic: A value-added coproduct for biomass biorefineries Biofuels, Bioprod. Biorefin. **3** (2009) 456. http://dx.doi.org/10.1002/bbb.161
8. 8. *Madison, L.L., Huisman, G.W.,* Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic, Microbiol. Mol. Biol. Rev. **63** (1999) 21.
9. *Solaiman, D.K.Y., Ashby, R.D., Foglia, T.A.*, *Marmer, W.N.*, Conversion of agricultural feedstock and coproducts into poly(hydroxyalkanoates), Appl. Microbiol. Biotechnol. **71** (2006) 783. http://dx.doi.org/10.1007/s00253-006-0451-1
10. *Index Mudial*, 2014 URL: http://www.indexmundial.com Accessed 14/4/14
11. *Marsudi, S., Unno, H., Hori, K.,* Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*, Appl. Microbiol. Biotechnol. **78** (2008) 955. http://dx.doi.org/10.1007/s00253-008-1388-3
12. *Impallomeni, G., Ballistreri, A., Carnemolla, G.M., Guglielmino, S.P., Nicolò, M.S., Cambria M.G.,* Synthesis and characterization of poly(3-hydroxyalkanoates) from *Brassica carinata* oil with high content of erucic acid and from very long chain fatty acids, Int. J. Biol. Macromol. **48** (2011) 137. http://dx.doi.org/10.1016/j.ijbiomac.2010.10.013
13. *Fernández, D., Rodríguez, E., Bassas M., Viñas M., Solanas A.M., Llorens J., Marqués, A.M., Manresa, A.,* Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions, Chem. Eng. J. **26** (2005) 159.
14. *Yun, H. S., Kim, D.Y., Chung, C.W., Kim, H.W., Yang, Y.K., Rhee, Y.H.,* Characterization of a tacky poly (3-hydroxyalkanoate) produced by *Pseudomonas chlororaphis* HS21 from palm kernel oil, J. Microbiol. Biotechnol. **13** (2003) 64.
15. *Thakor, N., Trivedi, U., Patel, K.C.,* Biosynthesis of medium chain length poly(3-hydroxyalkanoates) (mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils, Bioresour. Technol. **96** (2005) 1843. http://dx.doi.org/10.1016/j.biortech.2005.01.030
16. *Ashby, R.D., Solaiman, D.K., Foglia, T.A., Liu, C.K.,* Glucose/lipid mixed substrates as a means of controlling the properties of medium chain length poly(hydroxyalkanoates), Biomacromolecules **2** (2001) 211. http://dx.doi.org/10.1021/bm000098+
17. *Solaiman, D.K., Ashby, R.D., Foglia, T.A.,* Medium-chain-length poly(beta-hydroxyalkanoate) synthesis from triacylglycerols by *Pseudomonas saccharophila*, Curr. Microbiol. **38** (1999) 151. http://dx.doi.org/10.1007/PL00006779
18. *Solaiman, D.K.Y., Ashby, R.D., Foglia, T.A.,* Production of polyhydroxyalkanoates from intact triacylglycerols by genetically engineered *Pseudomonas*, Appl. Microbiol. Biotechnol. **56** (2001) 664. http://dx.doi.org/10.1007/s002530100692
19. *Solaiman, D.K.Y., Ashby, R.D., Foglia, T.A.*, Physiological Characterization and Genetic Engineering of *Pseudomonas corrugata* for Medium-Chain-Length Polyhydroxyalkanoates Synthesis from Triacylglycerols, Curr. Microbiol. **44** (2002) 189. http://dx.doi.org/10.1007/s00284-001-0086-5

20. *Dieckelmann, G., Heinz, H.J.,* The basics of industrial oleochemistry: a comprehensive survey of selected technologies based on natural oils and fats, Oleochemical Consulting International, Mulheim (Ruhr)-Saarn, Germanay, 1989, pp 82–102.
21. *Tan, I.K.P., Sudesh Kumar, K., Theanmalar, M.,Gan, S.N., Gordon, B.,* Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1, Appl. Microbiol. Biotechnol. **47** (1997) 207. http://dx.doi.org/10.1007/s002530050914
22. *Annuar, M.S.M., Tan, I.K.P., Ibrahim, S., Ramachandran, K.B.*, Production of Medium-Chain-Length Poly(3-hydroxyalkanoates) from Crude Fatty Acids Mixture by *Pseudomonas putida*, Food Bioprod. Process. **85** (2007) 104. http://dx.doi.org/10.1205/fbp06017
23. *Możejko, J., Ciesielski, S., J.,* Saponified waste palm oil as an attractive renewable resource for mcl-polyhydroxyalkanoate synthesis, Biosci. Bioeng. **116** (2013) 485. http://dx.doi.org/10.1016/j.jbiosc.2013.04.014
24. *Guzik, M.W., Narancic, T., Ilic-Tomic, T., Vojnovic, S., Kenny, S.T., Casey, W.T., Duane, G.F., Casey, E., Woods, T., Babu, R.P., Nikodinovic-Runic, J., O'Connor, K.E.,* Identification and characterization of an acyl-CoA dehydrogenase from Pseudomonas putida KT2440 that shows preference towards medium to long chain length fatty acids, Microbiol. **160** (2014) 1760. http://dx.doi.org/10.1099/mic.0.078758-0
25. *Rao, Y., Xiang, B., Zhou, X., Wang, Z., Shaofei, X., Xu, J.,* Quantitative and qualitatitve determination of acid value of peanut oil using near-infrared spectrometry, Food. Eng. **93** (2009) 249. http://dx.doi.org/10.1016/j.jfoodeng.2009.01.023
26. *Scheiner, D.,* Determination of ammonia and Kjeldahl nitrogen by indophenol method. Water Res. **10** (1976) 31. http://dx.doi.org/10.1016/0043-1354(76)90154-8
27. *USEPA*, 1978. Colorimetric method for phosphorous determination. *Method no. 365.3*.
28. *Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., Pseudomonas oleovorans* as a Source of Poly (B-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters, Appl. Environ. Microbiol. **54** (1988) 1977.
29. *Lageveen, R.G., Huisman, G.W., Preusting, H., Ketelaar, P., Eggink, G., Witholt, B.,* Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalenoates Appl. Environ. Microbiol. **54** (1988).
30. *Elbahloul, Y., Steinbuchel, A.,* Large-scale production of poly(3-hydroxyoctanoic acid) by *Pseudomonas putida* GPo-1 and a simplified downstream process, Appl. Environ. Microbiol. **75** (2009) 643. http://dx.doi.org/10.1128/AEM.01869-08
31. *Kenny, S.T., Nikodinovic, J., Babu, R.P., Woods, T., Blau, W.J., O'Connor, K.E.,* Up-Cycling of PET (Polyethylene Terephthalate) to the Biodegradable Plastic PHA (Polyhydroxyalkanoate), Environ. Sci. Technol. **42** (2008) 7696. http://dx.doi.org/10.1021/es801010e
32. *Murphy, D.*, The status of industrial vegetable oils from genetically modified plants. European Chemicals Agency. 2012.
33. *Spencer, P., Krautgartner, R., Audran, X., Rehder, L.E., Boshnakova, M., Dobrescu, M., Flach, B., Wilson, J., Bettini, O., Guerrero, M., Bendz, K.*, USDA Foreign Agricultural Service Gain Report (2014) AU1407.
34. *Sun, Z., Ramsay, J.A., Guay, M., Ramsay, B.A.,* Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440, Appl. Microbiol. Biotechnol. **74** (2007) 69. http://dx.doi.org/10.1007/s00253-006-0655-4
35. *Ward, P.G., de Roo, G., O'Connor, K.E.,* Accumulation of Polyhydroxyalkanoate from Styrene and Phenylacetic Acid by Pseudomonas putida CA-3, Appl. Environ. Microbiol. **71** (2005) 2046. http://dx.doi.org/10.1128/AEM.71.4.2046-2052.2005
36. *Kenny, S.T., Runic, J.N., Kaminsky, W., Woods, T., Babu, R.P., O'Connor, K.E.,* Development of a bioprocess to convert PET derived terephthalic acid and biodiesel derived glycerol to medium chain length polyhydroxyalkanoate, Appl. Microbiol. Biotechnol. **95** (2012) 623. http://dx.doi.org/10.1007/s00253-012-4058-4
37. *Steinbuchel, A., Schlegel, H.G.,* Physiology and molecular genetics of poly(*β*-hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus,* Mol. Microbiol. **5** (1991) 535. http://dx.doi.org/10.1111/j.1365-2958.1991.tb00725.x
38. *Fukui, T., Doi, Y.,* Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain, Appl. Microbiol. Biotechnol. **49** (1998) 333. http://dx.doi.org/10.1007/s002530051178
39. *Verlinden, R.A., Hill, D.J., Kenward, M.A., Williams, C.D., Radecka, I.,* Bacterial synthesis of biodegradable polyhydroxyalkanoates, J. Appl. Microbiol. **102** (2007) 1437. http://dx.doi.org/10.1111/j.1365-2672.2007.03335.x
40. *Ashby, R., Solaiman, D., Foglia, T.,* Synthesis of short-/medium-chain-length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol, Biomacromolecules **6** (2005) 2106. http://dx.doi.org/10.1021/bm058005h
41. *DaSilva, G.P., Mack, M., Contiero, J.,* Glycerol: a promising and abundant carbon source for industrial microbiology, Biotechnol. Adv. **27** (2009) 30. http://dx.doi.org/10.1016/j.biotechadv.2008.07.006
42. *Mothes, G., Schnorpfeil, C., Ackermann, J.U.,* Production of PHB from Crude Glycerol, Eng. Life Sci. **7** (2007) 475. http://dx.doi.org/10.1002/elsc.200620210
43. *O'Brien, R.D.,* Fats and Oils. Formulating and Processing for Applications. CRC Press, Boca Raton, Florida, 2009.
44. *Stauffer, C.E.,* Fats and Oils. Eagan Press, St. Paul, Minnesota, 1996. http://dx.doi.org/10.1094/9780913250907
45. *Barnebey, H., Brown, A.,* Continuous fat splitting plants using the colgate-emery process, J. Am. Oil Chem. Soc. **25** (1948) 95. http://dx.doi.org/10.1007/BF02579733
46. *Ramachandra Murty, V., Bhat, J., Muniswaran, P.K.A.,* Hydrolysis of oils using immobilized lipase enzyme: A review, Biotechnol. Bioprocess Eng. **7** (2002) 57. http://dx.doi.org/10.1007/BF02935881
47. *Goswami, D., Basu, J.K., De, S.,* Lipase applications in oil hydrolysis with a case study on castor oil: a review, Crit. Rev. Biotechnol. **33** (2013) 81. http://dx.doi.org/10.3109/07388551.2012.672319
48. *Ayorinde, F.O., Saeed, K.A., Price, E., Morrow, A., Collins, W.E., McInnic, F., Pollack, S.K., Eribo, B.E.,* Production of poly-(*β*-hydroxybutyrate) from saponified *Vernonia galamensis* by *Alcaligenes eutrophus*, J. Ind. Microbiol. Biotechnol. **21** (1998) 46. http://dx.doi.org/10.1038/sj.jim.2900555

49. *Tan, I.K.P., Sudesh Kumar, K., Theanmalar, M., Gan, S.N., Gordon, B.,* Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1, Appl. Microbiol. Biotechnol. **47** (1997) 207. http://dx.doi.org/10.1007/s002530050914
50. *Rosillo-Calle, F., Pelkmans, L., Walter, A.,* A global overview of vegetable oils with reference to biodiesel. A report for the IEA Bioenergy Task 40., 2009
51. *Lee, S.Y., Wong, H.H., Choi, J.I., Lee, S.H., Lee, S.C., Han, C.S.,* Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of *Pseudomonas putida* under phosphorus limitation. Biotechnol. Bioeng. **68** (2000) 466. http://dx.doi.org/10.1002/(SICI)1097-0290(20000520)68:4<466::AID-BIT12>3.0.CO;2-T
52. *Van der Walle, G.A., de Koning, G.J., Weusthuis, R.A., Eggink, G.,* Properties, modifications and applications of biopolyesters, Adv. Biochem. Eng. Biotechnol. **71** (2001) 263.
53. *Tobin, K.M., O'Leary, N.D., Dobson, A., O'Connor, K.E.,* Effect of heterologous expression of *phaG* [(*R*)-3-hydroxyacyl-ACP-CoA transferase] on polyhydroxyalkanoate accumulation from the aromatic hydrocarbon phenylacetic acid in *Pseudomonas* species, Fems Micro. Lett. **268** (2007) 9. http://dx.doi.org/10.1111/j.1574-6968.2006.00607.x
54. *Kenny, S.K., Runic, J.N., Kaminsky, W., Woods, T., Babu, R.P., Keely C.M., Blau, W., O'Connor, K.E.,* Up-Cycling of PET (Polyethylene Terephthalate) to the Biodegradable Plastic PHA (Polyhydroxyalkanoate**)**, Environ. Sci. Technol. **42** (2008) 7696. http://dx.doi.org/10.1021/es801010e