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Medium chain length polyhydroxyalkanoate produced from ethanol by *Pseudomonas putida* grown in liquid obtained from acidogenic digestion of organic municipal solid waste

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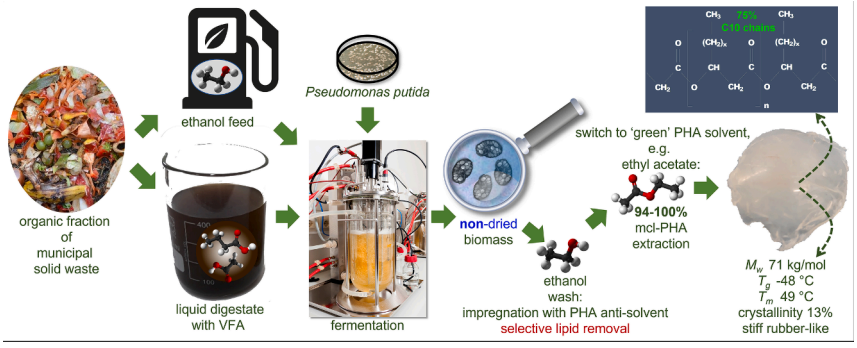
HIGHLIGHTS

- 100% Medium chain length polyhydroxyalkanoate was produced from ethanol.
- Ethanol washing of wet cells obviates the need of drying before PHA extraction.
- Ethanol washing removes solvent-extractable contaminants before PHA extraction.
- PHA is efficiently extracted with green solvents from ethanol-washed *Pseudomonas*.
- *Pseudomonas putida* can grow on liquid digestate of organic municipal solid waste.

GRAPHICAL ABSTRACT



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ABSTRACT

Production of medium chain length polyhydroxyalkanoate (mcl-PHA) up to about 6 $g.L^{-1}$ was obtained by feeding ethanol to *Pseudomonas putida* growing in liquid obtained from acidogenic digestion of organic municipal solid waste. Washing the wet, heat-inactivated *Pseudomonas* cells at the end of the fermentation with ethanol obviated the need of drying the biomass and enabled the removal of contaminating lipids before solvent-mediated extraction of PHA. Using 'green' solvents, 90 to near 100% of the mcl-PHA was extracted and purities of 71–78% mcl-PHA were reached already by centrifugation and decantation without further filtration for biomass removal. The mcl-PHA produced in this way consists of 10–18% C8, 72–78% C10 and 8–12% C12 chains (entirely medium chain length), has a crystallinity and melting temperature of ~13% and ~49 °C, respectively, and is a stiff rubberlike, colourless material at room temperature.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable aliphatic polyesters produced, among else, by a wide variety of bacteria and archaea from a variety of carbon sources (Sudesh et al., 2000; Możejko-Ciesielska and Kiewisz, 2016; Anjum et al. 2016; Reddy et al., 2022;

Abbreviations: OFMSW, organic fraction of municipal solid waste; mcl-PHA, medium chain length polyhydroxyalkanoate; VFA, volatile fatty acids.

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Obulisamy and Mehariya, 2021; Koller and Rittmann, 2022). PHA is accumulated in bacterial cells when nitrogen or other nutrient concentrations are limiting for growth, and carbon sources are present in excess. Monomeric hydroxyalkanoate, polymeric PHA and PHA granules can have several biological roles for microbial cells besides the well-known carbon/energy storage function of PHA, for example provision of heat and osmotic stress tolerance, antioxidant activity, and protection against protein denaturation (Obruca et al., 2020). The polyester is made up of saturated or partially unsaturated 3-hydroxy or 4-hydroxy fatty acids (FA) consisting of 4 to 16 carbon atoms (van der Walle et al., 2001, Singh and Mallick, 2009). The monomer composition of the PHA depends on the bacterial species, the culturing conditions and the carbon sources used (Huijberts et al., 1992; Sudesh et al., 2000; Możejko-Ciesielska and Kiewisz, 2016; Anjum et al. 2016). The mechanical and physical properties of PHA (van der Walle et al., 2001; Noda et al., 2005) are determined (i) by its monomer composition, which can be further tuned by post-synthesis modifications, (ii) by its processing history, and (iii) by the presence of any non-PHA components that were not removed by purification or have been added on purpose. The hydrophobicity (size) of the PHA-constituting monomer units as well as any post-synthesis modifications will affect the biodegradation speed of the polymer. For example, crosslinking of unsaturated PHA to produce rubbers will reduce the accessibility of the PHA chains inside the crosslinked network and generally tend to reduce the biodegradation speed (van der Walle et al., 2001). Two main classes of PHA are distinguished, namely short chain length PHA (scl-PHA), which is composed of hydroxy butyrate (C4) and/or valerate (C5) monomers, and medium chain length-PHA (mcl-PHA), which is composed of 3-hydroxy fatty acid monomers consisting of 6 to 14 carbon atoms (Singh and Mallick, 2009; Li et al., 2016; Reddy et al., 2022). Lcl-PHA containing long chain length hydroxy fatty acids are relatively rare (Singh and Mallick, 2009; Reddy et al., 2022). The variety of available PHA structures and properties implies the potential suitability of PHA for a wide variety of applications varying from thermoplastic materials suitable for replacement of polythene to coatings, rubbers, and polymer additives (van der Walle et al. 2001; Li et al., 2016, Reddy et al., 2022). Especially mcl-PHA can be used as property-modifying ingredient in blends with other polymers (Li et al., 2016).

However, next to some remaining technical challenges and regulatory hurdles, the production costs of PHA are still impeding widespread bulk application because they are still significantly higher than at least those of bulk chemical polymers. Downstream processing, fermentation, and feedstock all contribute significantly to the cost of PHA production, the feedstock contributing up to approximately 30% (Atkinson and Mavituna, 1991, de Koning et al., 1997). Therefore, inexpensive feedstocks or waste-derived feedstocks with negative value can reduce the cost of PHA production (Anjum et al. 2016), provided they do not significantly affect the production efficiency and the performance and purity of the PHA. An additional advantage of using waste streams as a feedstock is the valorization of waste and the environmental benefit of increased circularity. Feedstocks such as molasses, whey, agricultural residues, waste fats and oils, waste glycerol and wastewater-derived usable carbon sources have been employed to produce various types of PHA (Singh and Mallick, 2009; Du et al., 2012; Anjum et al. 2016; Favaro et al., 2018).

An alternative and abundant stream is urban waste. The organic fraction of urban or municipal solid waste (OFMSW) contains carbohydrates, lipids and proteins which are suitable for conversion to valuable products by fermentation. Biogas production by anaerobic digestion of organic waste is a way to recover energy from this stream. In the acidogenic phase preceding the methanogenic phase of this process, volatile fatty acids (VFA) are produced. The liquid, VFA-containing fraction of the anaerobically digested OFMSW can serve as feedstock for PHA production, for example production of scl-PHA by mixed microbial cultures (Morgan-Sagastume et al., 2014; Korkakaki et al., 2016; Valentino et al., 2019; Lorini et al., 2021). The balance of usable carbon compounds and other nutrients in the digestate should then be compatible with PHA accumulation, which requires nutrient limitation, and the VFA concentration in the broth should be below the toxic level. The limiting usable VFA concentration implies that in a simple batch process, only a very limited amount of PHA can be produced, such that repeated batch or sequencing batch processes are required to ensure recurrent replenishment of VFA and thus, increased PHA production. For such sequential processes, fast sedimenting (granular) bacterial cultures should be used, for example activated sludge from wastewater treatment plants. Moreover, activated sludge is a mixed microbiome which, in contrast to pure strains, can be adapted for increased (scl)-PHA accumulating capacity. This can be done by alternating carbon-rich and carbon-depleted growth phases ('feast-famine' approach), selecting for species capable of temporary carbon storage by accumulating PHA during the feast phase and using PHA as a carbon source during the famine phase. In contrast to granular microbiomes, efficient centrifugation/sedimentation- or filtration-mediated separation of the free-floating (less well sedimenting) cells of pure microbial strains from the liquid, yet fibre- and particle-rich OFMSW digestate is hardly possible. While this precludes the above-mentioned repeated batch or sequencing batch processes, also a continuous feed of liquid digestate with relatively low VFA concentration is not possible, because it would result in an excessive volume increase, culture dilution, a need for uneconomical over-dimensioning of bioreactors, and a significant increase of volume-related processing costs. In contrast, mcl-PHA production by pure *Pseudomonas* culture is well possible in a fed-batch process with gradual administration of concentrated waste-derived feedstock, such as VFA isolated from digested organic waste (Cerrone et al., 2014), or ethanol obtained by fermentation of (pre-treated and enzymatically hydrolysed) OFMSW (Ballesteros et al., 2010; Moreno et al., 2021), as for example in the URBIOFIN biorefinery (https://www.urbiofin.eu).

In the present study, the liquid fraction of acidogenically digested, VFA-containing OFMSW was used as a growth medium for *Pseudomonas putida* in two-stage, batch/fed-batch fermentations, in which VFA was consumed in an initial batch stage mainly for cell growth, and an ethanol feed was applied in a subsequent stage for mcl-PHA accumulation. To our knowledge, this is the first report describing mcl-PHA production from ethanol, as well as the first report describing solvent-based PHA extraction from biomass that had not been dried.

2. Methods and materials

2.1. Materials

2.1.1. Chemicals

Dibasic ester was purchased from Sigma Aldrich, and is a mixture of dimethyl-succinate, -glutarate and -adipate. TamiSolve® NxG, (1-butylpyrrolidin-2-one) was purchased from Taminco bvba (Gent, BE; subsidiary of Eastman Chemical Co.). Ethyl acetate (99.5%) and ethanol (96%) were purchased from Merck. Antifoam 204 was purchased from Sigma-Aldrich. Ethanol was used as a model compound for concentrated bioethanol produced from OFMSW (Moreno et al., 2021).

2.1.1.1. OFMSW digestate. A liquid fraction of a hydrolytic digestate was supplied by Dr. A. Serna Maza, Technological Innovation Centre (CIAM), Urbaser, Zaragoza, Spain. The digestate (batch of November 2019) was produced in an acidogenic digester fed with an organic fraction of municipal solid waste from Zaragoza, Spain. OFMSW was treated in a wet digester and for this the OFMSW was diluted with water to a maximum total solid (TS) content of 10%. The liquid fraction rich in VFA was obtained after polyelectrolyte-induced flocculation and solid–liquid separation. It contained 7.55 $g.L^{-1}$ VFA consisting of 2.77 $g.L^{-1}$ acetic, 1.56 $g.L^{-1}$ propionic (propanoic), 1.89 $g.L^{-1}$ butanoic, 1.11 $g.L^{-1}$ pentanoic and 0.22 $g.L^{-1}$ hexanoic acid, and furthermore 350 $mg.L^{-1}$ nitrogen (NH_4^+), 1.13 $mg.L^{-1}$ phosphorus (of which 0.142 mg L^{-1} P-

PO_4^{3-}), 139 mg.L^{-1} sulphur, 1576 mg.L^{-1} Ca, 0.012 mg.L^{-1} Co, 0.082 mg.L^{-1} Cu, 1.46 mg.L^{-1} Fe, 322 mg.L^{-1} K, 97.7 mg.L^{-1} Mg, 1.37 mg.L^{-1} Mn, 558 mg.L^{-1}Na, and 0.029 mg.L^{-1} Zn. The VFA content was within the range of values reported for similar processes of anaerobic digestion of OFMSW with maximum values up to 23 g.L^{-1} (Cavinato et al., 2017). The ammonium or nitrogen content was low and the digestate hardly contained any phosphorus. On the other hand, it was rich in the macro-nutrients calcium and to a lesser extent magnesium.

The content of some of the micro-nutrients in the liquid digestate, in particular the elements Co, Cu and Zn, is rather low and might be insufficient for good growth of *P. putida* (Preusting et al., 1991; Huijberts and Eggink, 1996). Because of the limitations of some of the components for good growth, the digestate was supplemented with additional minerals and nutrients.

2.2. Microorganism and medium

Precultures of *Pseudomonas putida* KT2442 (Bagdasarian et al., 1981) were prepared by inoculation of 100 mL of LB medium (tryptone 10 g.L^{-1}, yeast extract 5 g.L^{-1}, NaCl 5 g.L^{-1}) in Erlenmeyer flasks. Cultivation was for 6 h at 30 °C and 150 rpm. The precultures were used for inoculation of 1 L of digestate medium. Digestate medium was prepared by adding the following chemicals to 1 L of digestate: yeast extract 2.5 g (run 4) or 5.0 g (run 1–3), glucose.H_2O 2 g, KCl 1 g, $(NH_4)_2SO_4$ 2 g (run 1), 3 g (run 4) or 4 g (run 2, 3) and antifoam 204 1 mL. After sterilisation by autoclaving (121 °C, 15 min) $MgSO_4{\cdot}7H_2O$ (2 g) and a trace element solution (2 mL) were added. The trace element solution contained: 2.78 g.L^{-1} $FeSO_4{\cdot}7H_2O$, 1.98 g.L^{-1} $MnCl_2.4H_2O$, 2.81 g.L^{-1} $CoSO_4{\cdot}7H_2O$, 1.47 g.L^{-1} $CaCl_2.2H_2O$, 0.17 g.L^{-1} $CuCL_2.2H_2O$, and 0.29 g.L^{-1} $ZnSO_4{\cdot}7H_2O$.

Prior to inoculation, the pH of the medium was adjusted to 6.3 by addition of a H_3PO_4 solution (8.5%, v/v). Next the pH was adjusted to 6.8 with NaOH (10%, w/v). The rest of the H_3PO_4 solution (20 mL in total per L digestate) was added manually during the first 24 h of the fed-batch phase. The addition of the H_3PO_4 solution in two parts prevented as much as possible precipitation of phosphate salts, especially calcium salts, because the digestate contained a lot of calcium.

2.3. Fed-batch fermentation

The 1.1 L batch fermentations were performed in a Labfors 5 bioreactor (volume 3.6 L, Infors HT, CH). The conditions at the start of the fermentation were as follows: temperature 30 °C, stirrer speed 300 rpm, air flow of 0.3 L.min^{-1}. The pH was controlled at a minimum value of 6.8 (NaOH, 10% w/v). During growth of *Pseudomonas* stirrer speed and air flow were gradually increased to 850 rpm and 0.7 L.min^{-1} respectively, to maintain the pO_2 level at approximately 35%.

At the end of the batch phase, when the pO_2 level increased and CO_2 production ceased, the fed-batch phase was started by feeding the medium with ethanol. Ethanol feeding was controlled by the dissolved oxygen level. Addition was in small doses (resulting in an initial ethanol concentration in the reactor of ~ 0.29 g.L^{-1}) with an overall rate of approximately 2 g.L^{-1}.h^{-1} (~7 pulses per hour). During the fed-batch phase, pH was controlled at 6.8 with NaOH (10%, w/v). The stirring and aeration were kept at high levels, viz. 850 rpm and 0.7 L.min^{-1}, respectively. A Hamilton Visiferm DO Arc 120 probe was used to measure the dissolved oxygen level in the medium. CO_2 production was determined by measuring the CO_2 content of the off gas by a Servomex 4100 Gas Purity Analyser.

Bacterial growth was monitored by measuring the optical density of the cultures at 600 nm (OD600). The dry biomass content was determined at the end of the fermentation.

2.4. Mcl-PHA quantification and analysis

Lyophilized biomass (approximately 10–20 mg) and purified PHA (approximately 10–15 mg, used for reference) were dissolved, each in 2 mL of 15% (v/v) H_2SO_4 in methanol. Two mL of 0.5 mg/mL of methyl benzoate in chloroform was added as an internal standard to each of the two sulphuric acid–methanol mixtures. The mixtures were incubated while stirring for 4–5 h at 110 °C to hydrolyse PHA and obtain the methyl esters of the released 3-hydroxy fatty acid monomers. The samples were subsequently cooled on ice and 1 mL of demineralized water was added to each. After thorough mixing and phase separation by centrifugation (10 min, 1500 rpm in Eppendorf 5804R centrifuge with S-4–72 rotor) the organic phase was collected and dried over Na_2SO_4. Samples were analysed by gas chromatography–mass spectrometry (GC–MS). Analysis and quantification of the methyl esters were performed on a Trace 1300 GC + ISQ7000 (Thermo Scientific) equipped with a Rxi-5 ms column (30 m × 0.25 mm; df 0.25 μm; Restek, PA, US). Helium was used as the carrier gas with a flow rate of 1.2 mL per min. Injection of 1 μL sample was at 300 °C with a split ratio of 1.8. The column temperature increased from 50 °C (1 min) to 275 °C at a rate of 7.5 °C per min. Detection was by MS (mass range 35–800 amu).

2.5. Mcl-PHA extraction

Fresh (wet) *P. putida* biomass (dry weight content 25–30 %) from fermentation run 2 was pelleted by centrifugation (~10 min at 13500 × g in a rotor with a K-factor of 4300 at this RCF), heated to 80 °C for 15 min, subsequently cooled, mixed with ethanol (96%, v/v) in the proportion of 1 (biomass) to 3 (ethanol, w/w), incubated for 30 min at room temperature while stirring with a magnetic bar, and finally filtered over a Büchner funnel with Whatman 595 filter paper. The ethanol was discarded, and the biomass was mixed with fresh ethanol in the same proportion on top of the Büchner funnel, using a spoon to mix. After 30 min incubation and regular mixing with a spoon, the ethanol was removed from the funnel by applying vacuum. This latter procedure was repeated 3 times (total 5 extractions, of which 4 in the Büchner funnel) to replace the water in the biomass with ethanol and to remove lipids and other solvent-soluble non-PHA material that would otherwise be extracted together with the PHA. PHA does not dissolve in ethanol at room temperature (as previously verified by us). Subsequently mcl-PHA was extracted from the ethanol-soaked biomass using a suitable solvent. The following solvents were tested in view of their potential suitability for PHA extraction and their relatively low toxicity, carcinogenicity and/or environmental impact: methyl-ethyl ketone (2-butanone), ethyl acetate, cyclohexanone, dibasic ester, TamiSolve. Chloroform, as the most-used and best-performing PHA solvent for analytical and laboratory purposes (although toxic and carcinogenic), was used for comparison. The extraction was carried out by mixing ethanol-soaked biomass (approx. 45 % dry weight) and solvent in the proportion of 1 to 10 w/w, respectively, and incubating for 16 h at 60 °C or room temperature, stirring at 600 rpm with a magnetic bar. The solvent fraction, collected by careful decantation after centrifugation, was dried under a N_2 flow and the biomass fraction was used for two subsequent extraction cycles with a tenfold excess of solvent (total 3 successive extractions). The residual biomass was dried. Analysis of mcl-PHA in the solvent fractions and residual biomass was as described above.

2.6. Mcl-PHA characterisation

2.6.1. Gel permeation chromatography (GPC)

The absolute molecular weight of mcl-PHA was determined by GPC. Mcl-PHA extracted with chloroform from dried *P. putida* cell biomass, produced in fed-batch fermentation run 1, was dissolved in 3 mL of 1,1,1,3,3,3-hexafluoro-2-propanol to a concentration of 3–4 mg/mL and filtered through 0.45 μm PTE filters. Samples were analysed on a Viscotek HP-SEC system as described by Thiyagarajan et al. (2019), using 1,1,1,3,3,3-hexafluoro-2-propanol as a mobile phase. Calibration of the system was performed with a narrow standard PolyCAL PMMA 50 kD (from Viscotek, Malvern Panalytical, UK).

2.6.2. Differential scanning calorimetry (DSC)

Phase transition temperatures of mcl-PHA were determined by DSC. DSC curves were recorded on a power-compensating Perkin Elmer Diamond 8000 calorimeter provided with liquid nitrogen cooling. The temperature range used was −90 °C to +150 °C and the heating and cooling rates were 10 °C/min. Analyses were done with mcl-PHA extracted with chloroform from *P. putida* cell biomass produced in fed-batch fermentation run 1.

2.7. Analysis of medium components

VFA, ethanol and glucose were analysed by HPLC. Measurements were done using a Waters HPLC system equipped with a refractive index detector (Waters model 2414) and a Shodex KC-811 300 × 8 mm column at 65 °C with 3 mM H_2SO_4 as mobile phase and a flow rate of 1.0 mL per min. Samples were mixed with an equal volume of 1 M H_2SO_4 containing 100 mM valeric acid or 250 mM propionic acid as the internal standard.

The ammonium content of the digestate and fermentation samples was photometrically determined using the Spectroquant® method (Merck, Darmstadt, Germany). The orthophosphate content of the digestate was determined by the LCK 349 assay from Hach Lange. The content of a selection of chemical elements in the digestate was measured by ICP-AES and ICP-MS. The analyses were performed by the Soil Chemistry Laboratory (Wageningen University and Research, CBLB, NL).

3. Results and discussion

3.1. Batch-Fed-batch fermentation for mcl-PHA biosynthesis

Batch fermentations were started by inoculation of 1.0 L liquid digestate medium with a *P. putida* culture (10%, v/v). Glucose and yeast extract were added to the liquid digestate to boost the initial growth of *P. putida*. After a lag phase of approximately 8 h, consumption of the substrates started, as indicated by the sharp increase in CO_2 production at that time (Fig. 1). Approximately 20 h after inoculation, the batch phase appeared to have ended: the CO_2 production ceased, most of the VFA were consumed except for acetic acid, of which 24% was consumed by then, and 73% of the glucose was consumed (Fig. 1). At that moment, the ethanol feeding (fed batch phase) was started, the feeding rate being controlled by the O_2 partial pressure in the fermentation broth. This resulted in CO_2 production giving rise to 2.1–3.8% CO_2 in the off-gas during the next 2 days, at a constant air flow of 0.7 L per min (Fig. 1). The average ethanol feeding rate turned out to be approximately constant, initially 1.6 $g.L^{-1}.h^{-1}$ and gradually declining a little to 1.4 $g.L^{-1}.h^{-1}$ at the end of the fermentation. The administered ethanol was fully consumed, and no acetic acid was accumulated, except for some accumulation near the end of the fermentation, leading to a final acetate concentration of maximally 1.5 $g.L^{-1}$, which is still low and presumably sub-toxic at the pH applied. Accumulation of acetate was shown earlier in fermentations at high ethanol concentrations with *P. putida* strain KT2440 (Yang et al., 2019a; Bator et al., 2020).

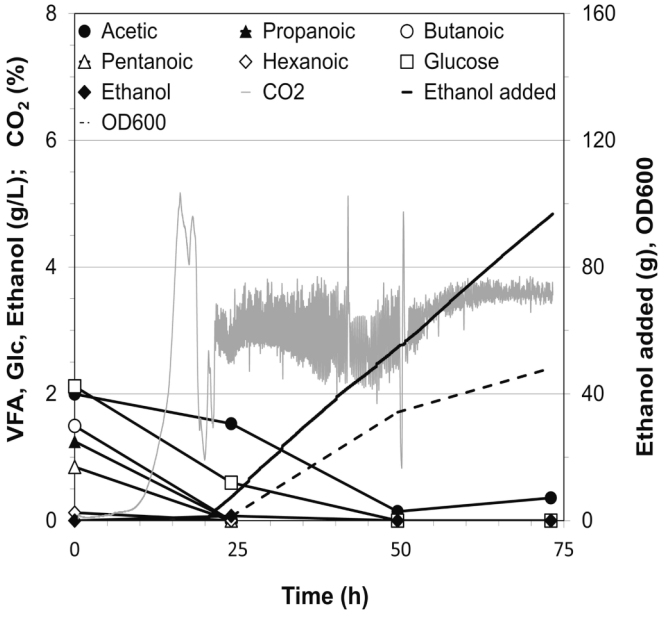

Fig. 1. Fed-batch *P. putida* KT2442 fermentation of liquid OFMSW digestate supplemented with nutrients. The medium composition was as described in Materials and Methods. The data correspond to fermentation run 4 as in Table 1. Ethanol feeding started 20 h after inoculation. The medium volume in the batch phase preceding the ethanol feed was 1.1 L.

After 24 h of fermentation most of the ammonium was used by *P. putida* and nitrogen limitation occurred when the N-NH_4^+ concentration in the medium was less than 10 $mg.L^{-1}$. The fermentation thus continued with an excess of carbon in the broth (relative to nitrogen), originating from the ethanol feed. Since it was previously shown that under these conditions of nitrogen limitation, this strain accumulates mcl-PHA in its cells when grown on one out of a variety of carbon sources (Huijberts and Eggink, 1996; Poblete-Castro et al., 2012), high C:N ratios were applied to induce mcl-PHA production also in the present study. In the four representative *P. putida* KT2442 fermentations addressed in Table 1, C:N ratios varied from 41 to 45 (mol/mol). After the batch phase in which nearly 7 $g.L^{-1}$ of carbon source (mostly VFA) was consumed from the liquid digestate, 96 to 126 g of ethanol was fed to the culture medium. At the end of the fermentations 20 to 28 $g.L^{-1}$ of cell biomass was formed, the biomass yield on total carbon substrate being 0.21 to 0.27 g per g (Table 1). Mcl-PHA was produced by *P. putida* and accumulated to a content of 20 to almost 30% (w/w) of dry biomass after 3 to 4 days of fermentation (Table 1). The mcl-PHA yield on ethanol (or total carbon substrate) was 0.06 to a maximum of 0.08 g per g. The highest amount of mcl-PHA, 7.7 g, was obtained in fermentation run 4 with the higher C:N ratio of 45.7 mol/mol (Table 1). The mcl-PHA volumetric productivity in these fermentations varied from approximately 0.05 $g.L^{-1}.h^{-1}$ in run 1 to approximately 0.08 $g.L^{-1}.h^{-1}$ in run 4, based on total fermentation time. Assuming that PHA accumulation started upon nitrogen limitation, which occurred approximately 24 h after inoculation, the accumulation speed during that phase varied from 0.07 $g.L^{-1}.h^{-1}$ in run 1 to 0.12 $g.L^{-1}.h^{-1}$ in run 4. While the rate of

Table 1
Characteristics of four representative two-stage batch/fed-batch *P. putida* KT2442 fermentations in OFMSW digestate-based medium with ethanol feed in the second stage.

Fermentation run		1	2	3	4 [a]
Fermentation time	h	97	93	70	73
C/N ratio [b]	$mol.mol^{-1}$	43.9	43.8	41.2	45.7
N-NH_4^+ content [c]	g	0.77	1.20	1.20	0.99
Ethanol feed	g	106	126	118	96
Dry biomass concentration	$g.L^{-1}$	22.1	28.3	20.3	19.8
Dry biomass	g	28.7	36.8	26.4	25.7
Biomass yield	$g.g^{-1}$ substrates [d]	0.25	0.27	0.21	0.24
PHA content	% (w/w dry)	21.7	20.0	26.0	29.9
PHA end concentration	$g.L^{-1}$	4.8	5.7	5.3	5.9
PHA produced	g	6.3	7.4	6.9	7.7
PHA yield	$g.g^{-1}$ ethanol	0.059	0.059	0.058	0.080
Volumetric PHA productivity	$g.L^{-1}.h^{-1}$	0.049	0.061	0.076	0.081

[a] This run is shown in Fig. 1.
[b] Based on total nitrogen as present in NH_4^+, yeast extract, and peptone.
[c] Ammonium from the liquid OFMSW digestate, and from added $(NH_4)_2SO_4$.
[d] Substrates, sum of VFA, glucose and ethanol.

ethanol addition was approximately constant (see Fig. 1), the specific ethanol consumption rate decreased during the two days of feeding because of the increasing cell biomass in this period. This indicates a slowing down of *P. putida* KT2442 activity. Further improvements of the mcl-PHA titre and productivity may be achieved in high cell density cultures by optimisation of medium composition based on nutrient requirements. Yet, the obtained mcl-PHA yield of 0.08 $g.g^{-1}$ and mcl-PHA content of 30% of dry biomass are comparable to those obtained in *P. putida* fermentations with other small molecules as the carbon source.

In the present fermentations in OFMSW medium, most if not all mcl-PHA was produced from ethanol and the OFMSW-derived VFA was mainly used for biomass production, because nitrogen limitation occurred only when the OFMSW-derived VFA were consumed. To our knowledge, this is the first report on mcl-PHA production from ethanol. Even production of the more common scl-PHA from ethanol was reported in only very few studies, typically using a mixed feed and/or genetically modified strains (Gomaa, 2014; Sun et al., 2020), except for one study on *Paracoccus* (Yamane et al., 1996).

Ethanol was previously used as a carbon source in fermentations with another *P. putida* strain, KT2440, to produce mevalonate as a precursor for terpenoid products (Yang et al., 2019a), or to produce rhamnolipids (Bator et al., 2020), or fatty acid ethyl esters (Sarwar et al., 2022). For these applications the *P. putida* strain was adapted by metabolic engineering to optimize product yields. In this engineered strain ethanol was converted to acetyl CoA via acetaldehyde and acetate (Thompson et al., 2020). Acetyl-CoA is the precursor in the formation of the end products in the fermentations (Yang et al., 2019a). The rate limiting step in the ethanol conversion seemed to be the step from acetate to acetyl CoA indicated by the accumulation of acetate in the medium at high ethanol concentrations (Yang et al., 2019a; Bator et al., 2020). The mevalonate/terpenoid (Yang et al., 2019a) and fatty acid ethyl ester production processes of *P. putida* KT2440 were improved by engineering a direct conversion of acetaldehyde to acetyl-CoA thereby preventing accumulation of acetate (Yang et al., 2019a; Sarwar et al., 2022). This could be advantageous as well for mcl-PHA production from ethanol in *P. putida* KT 2442.

Only few reports describe the production of mcl-PHA directly from acetate or VFA by *P. putida* strains. Huijberts showed by applying [1-^{13}C] acetate as the substrate in *P putida* KT2442 fermentations that mcl-PHA formation also occurred via conversion of acetate to acetyl-CoA and *de novo* fatty acid biosynthesis (Huijberts et al., 1994). This pathway is also involved in the formation of mcl-PHA from ethanol since acetic acid is an intermediate in the formation of acetyl-CoA from ethanol. Cerrone et al. (2014) described mcl-PHA production by *P. putida* CA-3 from VFA derived from anaerobic digestion of grass. The isolated VFA consisted of a concentrated mix of C2 to C5 acids with butyric acid as the predominant type. In nitrogen-limited shake flask cultivations with such a VFA mix, mcl-PHA was accumulated to 39% of the dry biomass (Cerrone et al., 2014). In other *P. putida* KT2440 fed-batch cultivations with a cumulative total of approximately 25 $g.L^{-1}$ of acetate as sole carbon source, mcl-PHA was produced to 0.35 $g.L^{-1}$, with a final mcl-PHA content of 22% (w/w) of dry biomass (Yang et al., 2019b).

By genetic engineering, the *acs* gene (acetyl-CoA synthetase) was overexpressed to enhance acetate assimilation and thereby, *de-novo* fatty acyl synthesis/elongation and mcl-PHA accumulation (Yang et al., 2019b). Mcl-PHA production in the *acs* strain was indeed substantially increased to 0.67 $g.L^{-1}$, with a final mcl-PHA content of 43% w/w. The mcl-PHA yield on acetate was 0.02 and 0.03 $g.g^{-1}$ in the wild type and mutant strain, respectively. Such an approach could also be advantageous to produce mcl-PHA in *P. putida* KT 2442 from ethanol, or from acetate or VFA isolated and concentrated from acidogenic digestates of OFMSW and other organic waste streams.

3.2. Preparative extraction of mcl-PHA from biomass

Solvent extraction was presently used for recovery of mcl-PHA from *P. putida* biomass, because the buoyant density of approximately 1 as well as the stickiness of mcl-PHA particles, in contrast to scl-PHA particles, imply severe problems for downstream processing by centrifugation or membrane filtration in an aqueous system. In general, good results have been obtained by solvent extraction of various PHA types with respect to yield, purity, and polymer quality (reviewed by Pérez-Rivero et al., 2019; Pagliano et al., 2021).

In this study, several relatively environment-friendly, low toxicity solvents were tested with the aim to obtain high extraction yields of mcl-PHA, viz. ethyl acetate, dibasic ester, 2-butanone (methyl-ethyl ketone/MEK), TamiSolve NxG (biodegradable multi-purpose solvent), and cyclohexanone (even though this is ranked as problematic because of non-sustainable chemical synthesis). Chloroform, as commonly used laboratory benchmark solvent for PHA extraction, was taken along for comparison, even though it is not suitable for large-scale processes because of its environmental burden and carcinogenicity.

Wet *P. putida* biomass from an ethanol-fed fermentation in liquid OFMSW digestate was pre-treated by washing with ethanol to extract ethanol-soluble components such as membrane lipids, which would otherwise be extracted as contaminants together with the mcl-PHA and compromise its purity. The ethanol also served to remove water from the biomass and increase the accessibility of the PHA polymer to the PHA-extracting solvent. The PHA itself is not soluble in ethanol at room temperature. By removing hydrophobic contaminants, the ethanol pre-treatment decreased the dry biomass by 33%. Thereby, it increased the mcl-PHA content from 20 to 30% w/w, relative to dry biomass. In addition, it increased the PHA purity from maximally 38% to maximally 100%, because the ethanol-extracted contaminant mass (0.33 g per g biomass) was 1.65 times the mass of accumulated PHA (originally 0.2 g per g biomass). The ethanol washing step also avoided the need to dry the biomass before solvent extraction, thereby avoiding the cost and energy required for drying.

The ethanol-treated, ethanol-soaked biomass was mixed with a tenfold excess (w/w) of mcl-PHA-solvent and the PHA was extracted at 60 °C. The extraction was repeated twice with fresh solvent. As shown in Table 2, more than 90% of the mcl-PHA was recovered after three rounds of extraction, except in the case of dibasic ester, where only 24% was extracted. The highest mcl-PHA extraction yields were obtained with methyl-ethyl ketone and cyclohexanone. Yields of 98% were already achieved after 2 extraction steps. The extracted mcl-PHA was slightly transparent, colourless, and elastic (rubber-like).

For comparison, extractions with methyl-ethyl ketone and ethyl acetate were also performed at room temperature but resulted in an extraction yield of only 29.5 and 38.5%, respectively, in the first extraction step (not shown). This poor yield shows that an increased temperature improved the extraction efficiency. Dimethyl carbonate, a green, non-toxic, VOC-exempt solvent for scl-PHA (Samorì et al., 2015; de Souza Reis et al., 2020) was also tested at room temperature for extraction of mcl-PHA. However, the extraction yield after one or three extraction cycles, 14.3% and 19.1%, respectively, was much lower than that of the two other solvents under the same conditions and therefore,

Table 2
Solvent extraction of mcl-PHA from wet *P. putida* KT2442 biomass.

Solvent	Yield of extracted mcl-PHA (% w/w)				Purity
Extraction [a]	1st	2nd	3rd	Sum	(% w/w)
Chloroform	76.3	14.5	2.7	93.6	48
Methyl-ethyl ketone (2-butanone)	87.0	11.9	0.7	99.6	78
Ethyl acetate	78.8	14.1	1.3	94.3	71
Cyclohexanone	82.1	15.6	2.2	99.9	67
Dibasic ester	16.8	5.8	1.9	24.6	50
TamiSolve	75.1	13.3	1.7	90.1	62

[a] Solvent/biomass ratio was 10 (w/w); extraction temperature was 60 °C; wet biomass (from fermentation run 2, as detailed in Tables 1 and 3) was first extracted with ethanol.

dimethyl carbonate was not further tested.

One of the tested solvents with low toxicity and environmental impact, cyclohexanone, has been tested previously for the extraction of poly(3-hydroxybutyrate) (PHB), an scl-PHA type, produced by the bacterial strain *Cupriavidus necator* (Jiang et al., 2018). A yield of 95% was obtained with a PHB purity of 99.5% after extraction of acetone-washed dry biomass, at 120 °C for 30 min and precipitation of the extracted PHB with methanol from the cyclohexanone fraction. Lower extraction temperatures resulted in lower yields of 16 and 90% at 80 and 100 °C, respectively.

3.3. Characterisation of mcl-PHA

The monomer composition of mcl-PHA produced by *P. putida* KT2442 from ethanol is shown in Table 3. The chain length of the 3-hydroxy-fatty acids ranged from C6 to C14, with 3-hydroxydecanoate (C10) as the dominant type accounting for more than 70% (w/w) of the mcl-PHA monomer units. A high content of the C10 monomer in mcl-PHA was reported in other studies using *P. putida* strains cultivated on a variety of low molecular weight substrates, i.e. VFA (mix and separate C2–C5 acids), sugars (glucose, fructose) and glycerol (Huijberts et al., 1992; Cerrone et al., 2014; Yang et al., 2019b; Xu et al., 2021a; Xu et al., 2021b; Dartiailh et al., 2021).

The molecular weight and physical properties were determined for mcl-PHA produced in fermentation run 1, which run is detailed in Tables 1 and 2. A weight-average molecular weight (M_w) of 71.1 kg.mol^{-1} and a number-average molecular weight (M_n) of 42.5 kg.mol^{-1} was obtained, in line with reported data on mcl-PHA produced in *P. putida* from other low molecular weight substrates like glucose and glycerol, or from mcl-fatty acids (Dartiailh et al., 2021; Xu et al., 2021a; Xu et al., 2021b; Ouyang et al., 2007; Huijberts and Eggink, 1996; van der Walle et al., 2001). The corresponding dispersity ($Đ = M_w/M_n$) of 1.67 was also in line with the values of 1.2 – 2.2 reported in those studies. The observed glass transition temperature (T_g) was –48 °C and the observed melting temperature (T_m) 49 °C, which values are comparable to those reported previously by Dartiailh et al. (2021) and Xu et al. (2021a) for mcl-PHA produced from glycerol (T_g –47 °C and T_m 43 °C) and glucose (T_g –40 °C and T_m 54 °C). The presently observed low melting enthalpy ΔH_M of 19.8 J.g^{-1} indicates the crystallinity to be low. Tentatively assuming ΔH_M to be ~ 146–150 J.g^{-1} for 100% crystalline PHA (Barham et al., 1984; De Jesus Assis et al., 2019; Rigouin et al., 2019), the observed ΔH_M would correspond to a crystallinity of ~ 13%. In agreement with the high content of one particular monomer type (3-hydroxy decanoic acid) in the present mcl-PHA, this 13% crystallinity is roughly intermediate between literature data (Barham et al., 1984; De Jesus Assis et al., 2019; Rigouin et al., 2019) for poly(3-hydroxy dodecanoic acid) (~30% crystallinity) and PHA consisting of a more equal mixture of 3-hydroxy octanoic, decanoic, dodecanoic and tetradecanoic acid (~5% crystallinity). In general, mcl-PHA is considered to be an elastomer in view of its T_g below room temperature, its relatively low T_m, and its low degree of crystallinity (van der Walle et al., 2001). This agrees with the firm, rubberlike consistency of the material.

4. Conclusions

Mcl-PHA was produced by *P. putida* KT2442 for the first time in liquid OFMSW digestate with ethanol feeding. The yield, PHA content, and PHA properties were comparable to those obtained with other low-molecular weight carbon sources. Before 'green' solvent extraction of PHA, the wet biomass can be pre-treated at room temperature with ethanol, as anti-solvent of PHA, (i) to replace the water in the biomass with ethanol, (ii) to increase the final PHA purity by removing lipids and other ethanol-soluble non-PHA components, and (iii) to avoid the cost and energy required to dry the biomass before solvent extraction.

Table 3
Composition of 3-hydroxy fatty acid monomer units in mcl-PHA produced in four representative two-stage batch/fed-batch *P. putida* KT2442 fermentations in OFMSW digestate-based medium with ethanol feed in the second stage.

Fermentation run	1	2	3	4
3-OH-C6 [a]	0.7	0.8	1.4	1.4
3-OH-C8 [a]	10	12.9	16.8	18.4
3-OH-C10 [a]	78.2	73.7	73.4	71.9
3-OH-C12 [a]	10.5	11.8	8.3	8.3
3-OH-C14 [a]	0.7	0.8	ND [b]	ND [b]

[a] Unsaturations were not determined.
[b] ND, Not detectable.

CRediT authorship contribution statement

Truus de Vrije: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Ricardo M. Nagtegaal:** Investigation, Formal analysis. **Ruud M. Veloo:** Investigation, Formal analysis. **Frans H.J. Kappen:** Methodology. **Frits A. de Wolf:** Conceptualization, Methodology, Visualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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