Carbon waste to carbon product:

Converting Oxalate to PHA

Craig Stephen Blake

Supervisors:

Dr Leonie J. Hughes

Dr Damian W. Laird

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Abstract

Waste carbon streams are increasingly viable options for the reduction in production costs for PHA synthesis. Oxalate is a major by-product in the alumina industry and using that carbon provides an opportunity to reduce Bayer liquor processing costs while producing a marketable product. Standard waste water treatment inoculum was adapted to oxalate in a mixed microbial culture (MMC) and co-fed oxalate and propionate (50:50 carbon ratio) under accumulating conditions (C/N ~120). The MMC accumulated the co-polymer polyhydroxybutyrate-co-hydroxyvalerate (PHBV) ~18.6% by weight (~55% HV) after cycling between accumulation and growth conditions. This represents a 4-fold increase in PHA yields from MMC using oxalate as a carbon source reported in the literature. These findings present evidence via GCMS, NMR and thermal analysis preformed on an isolated polymer that more useful co-polymers can be synthesised from oxalate providing further evidence that the valorization of highly oxidised waste carbon streams is possible.

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Craig Stephen Blake (BSc)

HONOURS THESIS

Introduction

The strength, lightness and durability of petroleum derived thermoplastics has seen them become an essential part of everyday life. A promising candidate to replace conventional plastics are the polyhydroxyalkanoates (PHA).[1] PHAs possess similar properties to the various synthetic thermoplastics such as polypropylene and can therefore be used in their place. However, unlike crude oil derived plastics, PHAs are 100% biodegradable.[7]

PHA is predominantly used by micro-organisms as a carbon and energy storage system when an essential nutrient such as N, P, S, Mg or O_2 is limited in the presence of excess carbon.[8] Micro-organisms synthesise PHA in order to convert the soluble carbon into an insoluble compound that applies no osmotic pressure to the cell, preventing the loss of valuable carbon and reduction equivalents.[9-11] PHA exists as intracellular inclusions localised in the cell cytoplasm that bud off as granules approximately 0.2 ± 0.5 µm in diameter from the cells cytoplasmic membrane.[7, 12]

The most common PHA is made up of repeating (R)-3-hydroxybutyrate (HB) monomers forming the homopolymer poly-3-hydroxybutyrate (PHB).[13] PHB is so commonly found because all that is required

intracellularly is acetyl CoA, a common intermediate product of carbon assimilation (Figure 2).[2] Acetyl CoA reacts with the enzymes β-ketothiolase (PhaA) and then acetoacetyl-CoA-reductase (PhaB) to synthesise R-HB monomers (Figure 1).[14-15] PHB synthase (PhaC) then reacts the carboxyl group of one R-HB monomer to form an ester bond in the hydroxyl group of another R-HB to extend the polymer chain (Figure 1). [9, 15] The PHA enzymes are non-specific and therefore any CoA thioester can be synthetised into R-HA monomers and incorporated into PHAs.[14] This is evident when looking at the 150 plus HA monomers that have currently been described in the literature.[1, 14, 16-18]

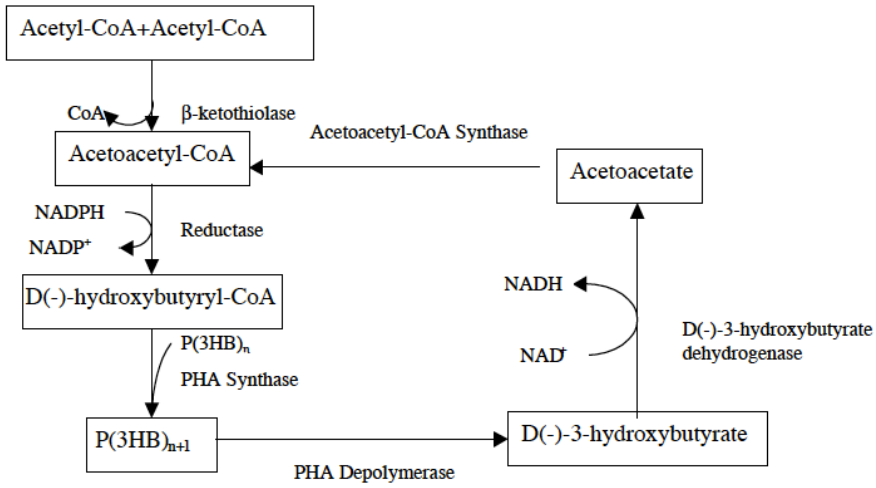

Figure 1: Representative metabolic pathway of PHB synthesis and degradation from *R. eutropha* reproduced from Peoples & Sinskey, 1989.[1]

The degradation of PHAs is performed by micro-organisms and some fungi in soil, seas, lake water and sewage via the secretion of PHA depolymerases (Figure 1).[7, 19] Under aerobic conditions PHA is completely degraded to carbon dioxide and water, and under anaerobic conditions is converted to methane.[7, 12]

Currently there are various products using PHA on the market such as biodegradable packaging, bottles and other disposable products.[20-21] PHAs also have the added advantage of being inherently biocompatible

because they are made up of naturally non-toxic constituents.[22] There is also increasing evidence that PHA is not just a storage polymer but a universal constituent of biological cells in bacteria, fungi, plant and animal tissue.[23-25] This allows PHAs to be used in biomedical applications such as drug delivery systems and tissue engineering by providing a scaffolding for bone, skin, heart and even nerve tissue.[23, 26] The enzymes that synthesis PHAs produce 100% (R)-configured monomers that have easily modifiable —OH and —COOH functional groups. This makes HA monomers perfect to use as starting chemicals for the synthesis of antibiotics, vitamins, aromatics and pheromones.[21, 27-28]

PHAs are an extremely useful renewable product that could potentially replace the traditional plastics that we use today. However, the production of PHAs are approximately 5-10 times more expensive than traditional plastics and are therefore are currently only being explored in the medical field.[7, 10, 13]

Microorganisms require a carbon source for growth and energy, this means that only around 50% of the carbon source supplied actually ends up as the desired product. [29] When the feeds used to produce PHA can account for 30—50% of the production costs, finding cheaper alternatives is necessary to drastically lower production costs.[8, 29] Carbon waste streams provide a potentially cheap feedstock compared to traditional carbon sources like glucose which is 76% more expensive per tonne than whey solids produced from the cheese industry. Other waste carbon sources that have been investigated include sugarcane[30], soy bean oil[31], and paper mill wastewater[32].

The use of sterile pure cultures that require pure sterile substrates and axenic reactors contributes to high production cost.[27, 33] An alternative

approach is the use of mixed microbial cultures (MMC). MMC are generally more robust than pure cultures and require less expensive equipment making them more applicable for use on an industrial scale.[33-34] They also do not require sterilisation of substrates or culture, allowing for cheaper carbon waste steams to be used.[35] Optimisation of an MMC for PHA accumulation can be achieved by using selective pressure, typically feast-famine cycling, to select for the desired metabolic activity.[33, 35]

The aluminium industry in Western Australia produces a large amount of organic waste, specifically oxalate, during the Bayer processing of bauxite ore.[36] This process uses hot caustic soda to extract the aluminium from the crude ore. In the process, any organic matter in the crude ore is also extracted. The liquor is continually recycled within the plant and the organic carbon reaches saturating levels. Therefore, Acetate can be found at concentrations of 3-11 g L^{-1} in the liquor while oxalate, formate, malonate and succinate can all found at concentrations of ~1 g L^{-1}. [34, 37] Oxalate is of particular interest as it co-precipitates as sodium oxalate with aliminium hydroxide ($Al(OH)_3$).[38] The contamination of sodium is detrimental to the downstream processing causing a reduction in the production efficiency and quality of the final product.[36] Therefore, oxalate and the other organic carboxylates must be removed. This presents a problem for oxalate which cannot be disposed of untreated in the environment due to its toxicity in high concentrations to plants and animals. [39-41] Therefore, a number of chemical, biological and bio-electrical oxalate treatments have been developed.[39-41] At the processing pH of the liquor the CO_2 causes sodium carbonate formation, effectively lowering the pH of the process liquor and resulting in further economic cost to re-causticize the liquor. Bacteria could be used to convert the organic material to PHA rather than just oxidising it to CO_2, removing

the need for further processing of the liquor while producing a valuable by-product. Nitrogen makes up about 0.68% of the total organic matter of Bayer liquors as it is mostly present as quinolone and pyridine derivatives that are not bioavailable.[42] The high carbon to nitrogen ratio appears to be favourable for the conversion of carbon to PHA. Previous studies at Murdoch have demonstrated that oxalatrophic bacteria sourced from a waste water treatment mixed microbial culture (MMC), and supplied with oxalate under the appropriate conditions are able to yield ~4.5% w/w of PHB.[43] This is a relatively small yield when compared to other carbon sources. The small yield is likely because oxalate is a highly oxidised substrate and the use of oxalate as a carbon source for biosynthesis must involve reductive reactions.[44] Biosynthesis requires lots of energy in the form of ATP which, is difficult as the catabolism of 1 mol of oxalate theoretically only produces enough energy to synthesise 2.83 mol of ATP.[44] The biochemical pathway for the synthesis of PHA from oxalatrophic bacteria is not yet fully understood, but the proposed metabolism pathway is shown in figure 2. The metabolism pathway indicates where the organic metabolites found in the Bayer liquors may theoretically enter the pathway and allow the synthesis of PHA.[2]

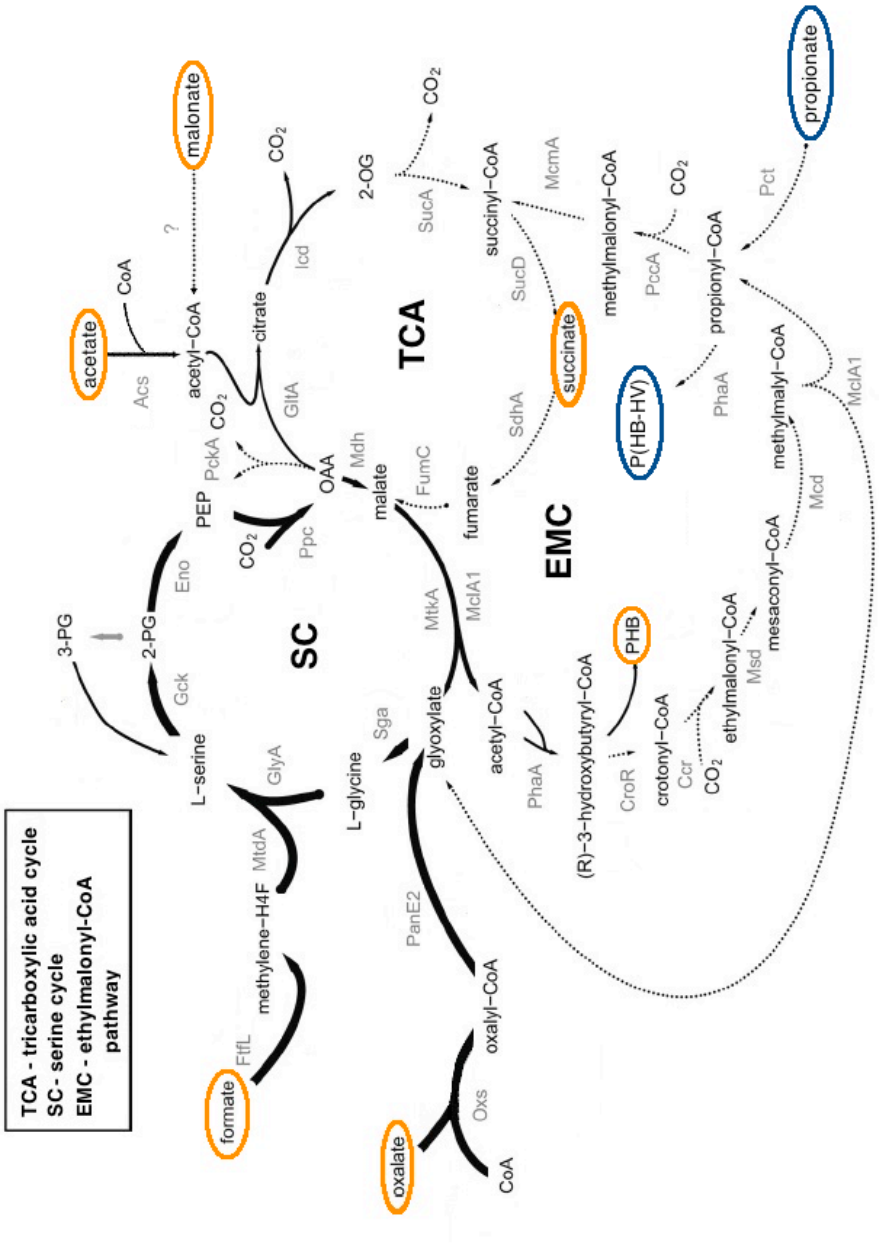

Figure 2: The carbon assimilation pathways of the oxalotrophic *Methylobacterium extorquens* AM1 adapted from Schneider et. al. 2012[2] indicating where the organic metabolites found in the Bayer liquor and potential co-metabolite propionate would theoretically enter the pathway and allow for PHA synthesis.

Co-feeding has been used in the literature as a way to both increase the yield of PHA production and to synthesise more useful copolymers from difficult to use metabolites.[45-46] Octanoate and nonanoic acid are used for the production of specific medium chain length PHAs, these substrates are both expensive and not optimal for cell growth.[45, 47] In

order to improve both growth and PHA yields a cheaper substrate, such as glucose, is introduced as a co-metabolite. The glucose is used for growth and energy production by the cell allowing the octanoate or nonanoic acid to be shuttled into PHA synthesis pathways increasing yield of the desired PHA.[46]

PHB is the least useful of the PHAs because it is a highly stiff, crystalline and relatively brittle thermoplastic.[7] Processing PHB is very difficult as it's melting point is 175°C, and the polymer starts degrading to crotonic acid at ~180°C.[7] However, the addition of 3-hydroxyvalerate monomers produces the much more useful copolymer poly-3-hydroxybutyrate-co-3-valeratemore (PHBV). The EMC pathway allows for various short chain fatty acids such as propionate to be converted into hydroxyalkanoates, in this particular case propionate is converted first into propionyl-CoA and then to PHBHV via the PHA enzymes (Figure 2).[9, 24, 48] Co-polymers are formed when there are two or more different HA's present, the percentage makeup of the polymer depends on monomer availability. The metabolic pathway adapted from Schneider *et. al.*[2] indicates that co-feeding propionate and oxalate could theoretically produce PHBV (Figure 2). Additionally, the oxalotrophic bacteria *Bacillus subtilis* was described as a potential producer of PHBV.[49]

Therefore, this project aims to co-feed a MMC optimised to use oxalate for PHB production under accumulation conditions, to produce and characterise a co-polymer.

Methods

Reactor setup

Parallel 1L sequencing batch reactors, each with a 600mL sludge blanket, were established: one reactor was fed acetate (control) and the other the experimental feed. Air was supplied from an aquarium pump with an air stone to provide O_2 saturating conditions and mixing was performed by an overhead stirrer (IKA RW 20 digital) at 280 rpm with impeller. Calibrated peristaltic Cole-Parmer pumps were used for addition of feed and decant of the reactors. Each cycle ran for a total of 4 or 6 hours split into four phases: feed, aerobic, settle and decant (Table 1). The 5mm plastic tubing connecting feed reservoir, pumps, reactor and decant lines was cleaned weekly to minimise bacterial build up, ensuring consistent feed volume delivery.

Table 1: Breakdown of the phases that make up the 6 and 4-hour batch cycle including phase times and the active equipment during growth and accumulation.

	Feed	Aerobic	Settle	Decant
6h cycle (min)	4.65	320.35	30	5
4h cycle (min)	4.65	215.35	15	5
Air	active	active		
Stirrer	active	active		
Feed pumps	active			
Decant pump				active

The feed pumps were calibrated by measuring the volume of water by weight (assuming that the density of water used for calibration and feed are 1 g mL^{-1}) delivered in 1 minute at various speed settings, each in triplicate, to create a correlation between pumping time and mass of feed delivered. Each cycle the pumps were set to deliver in total 400 mL of feed, 70 ± 3.25 mL of the concentrated feed and 330mL of the diluent mixed together in a mixing funnel before delivered to the reactors.

Return activated sludge (biological floc composed of bacteria and protozoa) from a local Perth wastewater treatment plant was collected, washed with tap water and 600 mL of the resulting sludge was placed into each reactor.

Feed solutions

All feed solutions used during the experiment delivered the following concentrations each cycle: 167.25 mM of sodium hydrogen carbonate, 10.10 mM of potassium dihydrogen orthophosphate and 1.55 g L^{-1} of yeast extract. Trace elements were made up in 20 L buckets and each 20 L bucket contained the following: 25 mg L^{-1} of EDTA, 0.54 mg L^{-1} $ZnSO_4 \bullet 7H_2O$, 0.30 mg L^{-1} $CoCl_2 \bullet 6H_2O$, 1.24 mg L^{-1} $MnCl_2 \bullet 4H_2O$, 0.31 mg L^{-1} $CuSO_4 \bullet 5H_2O$, 0.36 mg L^{-1} $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, 0.24 mg L^{-1} $NiCl_2 \bullet 6H_2O$, 0.26 mg L^{-1} $Na_2SeO_4 \bullet 10H_2O$, 0.018 mg L^{-1} H_3BO_3, 0.06 mg L^{-1} $Na_2WO_4 \bullet 2H_2O$, 18 mg L^{-1} $CaCl_2 \bullet 2H_2O$, 406.25 mg L^{-1} $MgCl_2 \bullet 6H_2O$ and 6.075 mg L^{-1} $FeCl_2 \bullet 6H_2O$.

The stock feeds used for growth conditions contained 74.71 mM sodium nitrate. This was reduced to 2.94 mM under accumulation conditions. The stock acetate feed contained 202.96 mM of sodium acetate. The stock oxalate feed contained 201.59 mM oxalic acid and 403.15 mM sodium hydroxide. The stock 50:50 oxalate propionate feed used during the co-feeding experiment contained 68.63 mM sodium propionate, 100.8 mM oxalic acid and 201.27 mM sodium hydroxide.

PHA accumulation

Single carbon source

Cultures were supplied growth feed until the biomass concentrations stabilised indicating the carbon supplied equalled carbon required to

maintain the maximum biomass possible. Once this had occurred the first accumulation experiment saw the two vessels supplied oxalate and acetate respectively under accumulation conditions for 16 days. Accumulation was maintained for as long as possible by monitoring the biomass concentration of the culture and the experiment stopped when the concentration dipped below 1 g L^{-1}. Once this occurred the cultures were supplied with growth feed until the biomass concentration had once again stabilised. This experiment was performed twice due to time constraints.

Cofeeding

Feed with 50:50 carbon mole ratio of oxalate and propionate was supplied to the reactor under growth conditions until the culture adapted to the new feed as indicated by the stabilisation of the biomass concentration. The culture was then placed under accumulation conditions for 16 days. The culture was then switched back to growth conditions for 12 days in order to allow the biomass concentration to recover. Then the culture was placed back under accumulation conditions for a further 14 days. Finally, the culture was placed under growth conditions until the biomass concentration stabilised.

PHA isolation

The culture was placed under accumulation conditions and the biomass concentration closely monitored. After 7 days the biomass concentration dipped below 4 g L^{-1} and half the culture was sacrificed, with the other half placed back under growth conditions. Excess liquor was removed by spinning down the biomass at 1500 rpm and stored at –15 °C.

Monitoring of culture state

Biomass concentration (g L^{-1} dry cell weight (DCW)) was determined by taking a 5 mL sample of reactor liquor containing biomass while mixing was taking place, filtering through a pre-weighed glass

microfiber filter paper pore size 1.2 µm, followed by drying at 80 °C to constant weight.

The average pH over the course of the experiments was measured to be 9.4 ± 0.4 for oxalate and 9.2 ± 0.6 for acetate.

Analysis of carbon source utilisation

Clear liquor samples were collected as needed by pelleting a 2.0 mL mixed liquor sample by centrifugation at 5000 rpm for 5 minutes. 1.8 mL of clear liquor was recovered and treated with 80 µL of concentrated HCl to halt residual biological activity and stored at –15 °C for carbon source analysis. Soluble carbon (acetate, oxalate, proprionate) from the clear liquor samples collected as above was esterified in a one-step closed reflux.[43] 1 mL of acidified sample was treated with 1 mL of AR grade 1-butanol and 20 µL of benzoic acid internal standard (prepared accurately at approximately 20 mg/mL in 1-propanol), sonicated for 5 minutes and refluxed in Pyrex 9826 culture tubes for 1 hour at 120 °C.[43] After allowing the reaction mixture to cool to room temperature, sufficient $NaHCO_3$ (in most cases, 0.5 mL of 0.87 M) was added to neutralise solution pH. Samples were spun for 2 min at 1,500 rpm to separate layers and the top organic layer collected. The resulting butyl esters were analysed via GCMS (Table 2). Soluble carbon standards, containing 0 to 25 mM were prepared from AR grade acetate, oxalate and propionate sodium salts. 1mL of each of the standards was treated as above with the addition of 44.4 µL conc HCl.

Identification and quantification of PHA

Biomass

Slide samples were prepared to visualise the presence of PHA in the cells. This was achieved by air drying liquor samples on glass microscope slides. Staining was prepared by exposing the dried slide to Sudan Black for 10 seconds before rinsing, then exposing the slide to safranin which was immediately washed off. The slides were then viewed under oil emersion at x100 on an Olympus BX41 with SPUT INSIGHT color 3.2.0 for image capture.

GCMS analysis

For PHA analysis, solid samples were prepared by pelleting a 10.0 mL mixed liquor sample by centrifugation at 1,500 rpm for 2 minutes. The clear liquor was removed, and the pellet freeze dried. PHB content of lyophilised biomass was extracted, hydrolysed and esterified in a one-step closed reflux.[43] Up to 20 mg of biomass was placed in a sample tube to which 1.45 mL of a 1:4 mixture of concentrated (32%) hydrochloric acid:1-propanol was added. 50 μL of 20 mg/mL benzoic acid was used as an internal standard. An additional 1.5 mL of AR grade dichloromethane (DCM) was added before sealing and refluxing at 150°C for 2 hours with regular agitation. The organic phase was washed with deionised water and sufficient $NaHCO_3$ to neutralise the solution pH and remove free acids, dried over sodium sulphate and analysed by GCMS (Table 2).[50] Hydroxybutyric acid standards, containing 0 to 3.44 mg of hydroxybutyrate, were prepared from the sodium salt of L-(+)-β-hydroxybutyric acid and treated as above.

PHBV copolymer

After attempting to maximise PHA production, half the culture was removed from the reactor and the cells concentrated via centrifugation (10 min at 1500 rpm) and stored in a -15°C freezer. The

pellets were freeze dried and the PHA extracted from the lyophilised biomass by submerging in DCM and sonicating for 10 min. Biomass was then removed via filtration and the solution concentrated using a rotary evaporator until a viscous residue remained. The PHA was precipitated by the addition of ice cold methanol and polymer was purified by recrystallization before further analysis.[51]

GCMS analysis

0.0016g of the isolated biopolymer was hydrolysed and esterified in a one-step closed reflux as described previously. Poly-hydroxybutyric-co-hydroxyvaleric acid standards, containing 0 to 0.01109 g of HB 88% and HV 12%, was prepared from the poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (natural origin) obtained from Sigma-Aldrich and treated as above. Due to the small sample size available the GCMS protocol used a 5μL injection volume.

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance

Solution ^{1}H and ^{13}C NMR spectra were recorded using a Varian MR400 shielded NMR (400MHz) in 5mm ID NMR tubes at 25 ^{o}C and standard Varian pulse sequences. The 10.7mg sample of isolated PHA was dissolved in a minimal amount of deuterated chloroform ($CDCl_3$). ^{13}C NMR data was collected over 1000 scans and H using 8 scans. SpinWorks 4 was used to process the data and the chemical shifts were calibrated using the TMS internal standard in the $CDCl_3$ solvent.

Differential scanning calorimetry (DSC) and Thermal analysis (TGA)

TGA and DSC were performed simultaneously on a Perkin Elmer Simultaneous Thermal Analyzer (STA). A sample of 8 mg was heated at 10 ^{o}C min^{-1} from room temperature to 500 ^{o}C in a dynamic nitrogen atmosphere (flow rate. 10 mL min^{-1}).[52]

Table 2: GCMS parameters for soluble carbon and PHA analysis (Column name: DB-5ms, Thickness: 0.25 μm, Length: 30.0 m, Diameter: 0.25 mm).

	Soluble carbon source analysis	PHA analysis
Solvent	Hexane	Dichloromethane
Injection volume	1 μL	1 μL
	Sampler	
Number of rinses with solvent pre-run	3	3
Number of rinses with solvent post-run	3	3
Number of rinses with sample	3	3
Plunger speed (suction)	High	High
Viscosity comp. time	0.2 sec	0.2 sec
Plunger speed (injection)	High	High
Syringe insertion speed	High	High
Injection mode	Normal	Normal
	GC	
Column oven temp program	50 °C hold 2 min; 150 °C at 10 °C min^{-1} hold 5 min	60 °C hold 2 min; 150 °C at 15 °C min^{-1}; 165 °C at 4 °C min^{-1}; 220 °C at 15 °C min^{-1} hold 1 min
Injection temp	300.0 °C	200.0 °C

Injection mode	Split	Split
Carrier gas	He – Prim. Press. : 72.5-130.5	He – Prim. Press. : 72.5-130.5
Flow control more	Linear velocity	Linear velocity
Pressure	5.4 psi	8.1 psi
Total flow	11.8 mL min–1	77.6 mL min–1
Column flow	0.80 mL min–1	0.98 mL min–1
Linear velocity	32.4 cm sec–1	36.1 cm sec–1
Purge flow	3.0 mL min–1	3.0 mL min–1
Split ratio	10.0	75.1
	MS	
Ion source temp.	200 °C	200 °C
Interface temp.	250 °C	250 °C
Solvent cut time	3.5 min	2.5 min
Detector voltage	Relative to the tuning result	Relative to the tuning result
Micro scan width	0 u	0 Kv
Use MS program	No	No
Threshold	1000	1000
Start time	4.00 min	2.5 min
End time	17.00 min	16.00 min
Acq. Mode	Scan	Scan
Event time	0.50 sec	0.50 sec
Scan speed	666	909
Start m/z	45.00	41.00
End m/z	350	450.00

Single carbon source

In order to obtain a baseline of oxalate conversion to PHB produced, the oxalate culture was placed under accumulation conditions for 16 days. The biomass concentration of the culture was monitored over the accumulation period to ensure that the culture stayed healthy. The oxalate culture experienced an increase in biomass concentration over the first 7 days of accumulation (Figure 3) from 2.63 g L^{-1} to 3.83 g L^{-1}. This is likely due to the accumulation of PHB in the biomass rather than increase in cell growth as PHB accumulation is observed over this same period (Figure 5). However, after this the concentration decreased slightly for the remaining 10 days of the experiment.

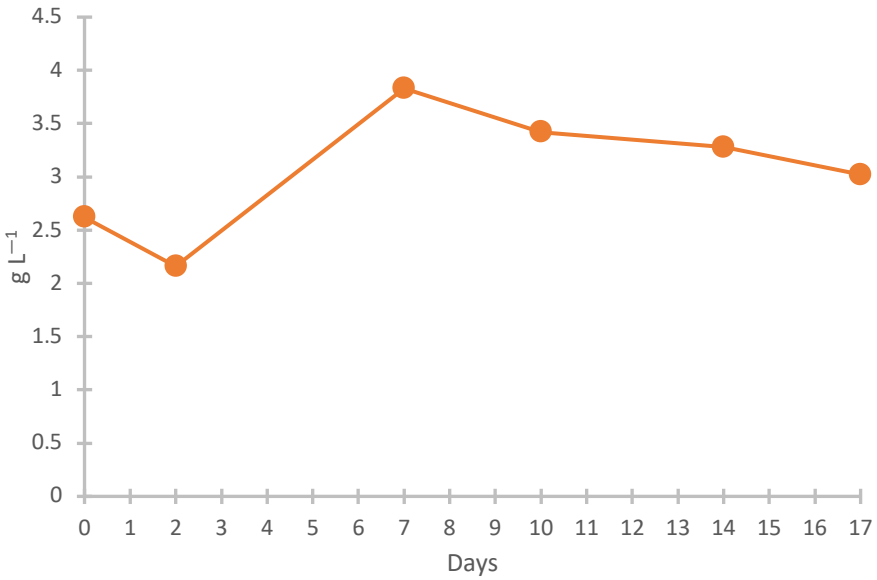

Figure 3: biomass concentration (g L^{-1}) of the oxalate (Ox) fed culture over the duration of the accumulation experiment.

In order to determine if the rate of consumption of oxalate changed between growth and accumulation, soluble carbon in the liquor was monitored over the length of a 6-hour cycle (Figure 4). The uptake of the soluble carbon by the biomass from the liquor under both growth and accumulation conditions appear to follow a similar pattern. The specific consumption rate for the culture was 5.20 mmol (chemical

oxygen demand (COD)) g^{-1} (CDW) h^{-1} under growth conditions, and 4.86 mmol (COD) g^{-1} (CDW) h^{-1} under accumulation conditions. The uptake rate of the wild type strain *Methylobacterium extorquens* AM1 has been reported as 16.9 ± 2 mmol (COD) g^{-1} (CDW) h^{-1} under sterile conditions.[2] The difference in the consumption rate reported in the literature compared to the consumption rate recorded in this experiment is likely due to the population makeup of the culture. This experiment selected for oxalaotrophic bacteria by supplying oxalate as the sole carbon source indicating the population consisted of generalist oxalotrophic species, whereas Schenider *et. al.*[2] studied the major plant colonizer *M. extorquens* AM1. The uptake of oxalate by bacteria is dependent on an oxalate formate antiporter, coded for by the oxlT gene.[53] This antiporter has dual functions; when $oxalate^{2-}$ is exchanged at the cell membrane with $formate^{-}$ this creates an electrochemical gradient that is used to drive ATP synthesis.[2] The uptake of oxalate is also dependant on the conversion of oxalate to formate. This relies on the enzyme formyl-CoA transferase encoded for by the *frc* gene.[12]

Specialist oxalatrophic bacteria like *M. extorquens* AM1 use the methanol and oxalate plants release into the environment as the main source of carbon. Therefore, they would possess a higher copy number of oxlT gene compared to generalist oxalotrophic species resulting in a higher specific consumption rate than seen by the culture in this experiment.

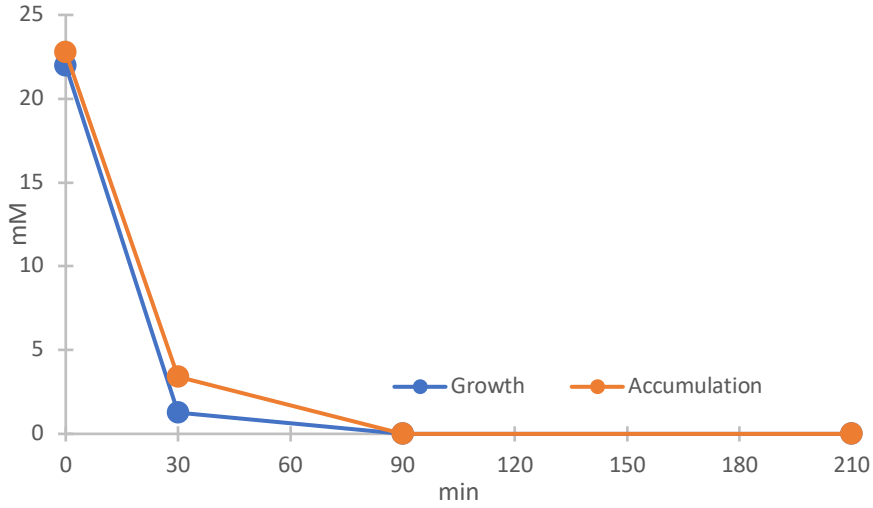

Figure 4: Soluble oxalate detectable in the liquor over the course of a single cycle for growth and accumulation.

The sample collected on day zero indicated there was a small amount of PHB (0.052 % w/w) in the culture (Figure 5). However, the accumulation conditions resulted in a significant increase of PHB accumulation in the cells after only two days. Biomass analysis indicated that up to 0.65 % w/w PHB was stored within the cells (Figure 5). These first two days (8 cycles) of accumulation resulted in the highest conversion of oxalate to PHB with 0.16% of carbon converted to PHB each cycle. PHB stored in the cells reached its highest point on day 8 with PHB making up 0.81 % w/w. The average conversion of oxalate to PHB over the 24 cycles between day 2 and 8 was calculated to be 0.14% each cycle. After this the PHB concentration in the cells remained relatively constant until day 15 where the PHB stored in the cells dropped from 0.72 % w/w down to 0.38 % w/w.

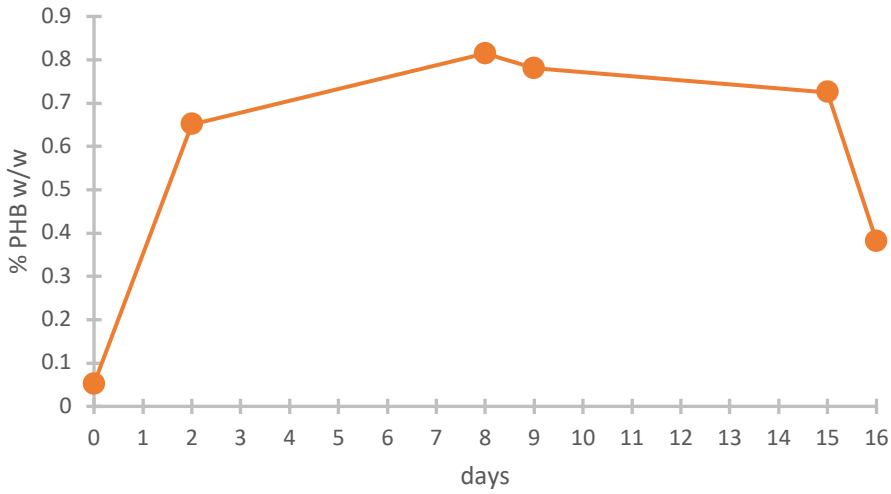

Figure 5: Accumulation of PHB w/w in the oxalate adapted culture under accumulating conditions (C/N ratio ~120).

The reduction of accumulated PHB is likely due to the duration of the experiment and the culture reaching the limits of its maintenance abilities resulting in the cells using the polymer as a carbon source.

Previous studies at Murdoch have been the most successful in the accumulation of PHB from oxalate reporting 4.5% PHB w/w under similar accumulation conditions (120 C/N ratio).[43] This figure is much larger than the 0.81 % w/w PHB yield achieve in this experiment (Figure 3). The wider literature regarding PHB accumulation from oxalate is small. However, PHB concentrations have been reported between 33 mg L^{-1} and 233 mg L^{-1} on specific strains of *Bacillus* tested.[54] Unfortunalty no information on the biomass concentration was provided in the paper and so direct comparisons to the data collected here are difficult.

The low conversion of oxalate to PHB is not surprising when viewing the literature regarding the metabolic pathways of the oxalotrophic bacteria *M. extorquens* AM1. Schenider *et. al.*[2] reported that 92% of the oxalate taken up by oxalotrophic bacteria was catabolised to CO_2, 8% was used for biosynthesis with 0.5% converted to PHB.[2]

Cofeeding

A common technique used to increase both the production yield of PHA and the production of more useful copolymers from difficult substrates is the addition of a co metabolite.[45, 47, 55] Co-feeding with a metabolite that yields a higher ratio of ATP per mole and is more easily incorporated into biosynthesis pathways allows for higher efficiency in the conversion of carbon source to energy (ATP), biomass and PHA. Acetate metabolism has been demonstrated in a number of oxalotrophic species with some even requiring its presence in order for the bacteria to grow on oxalate.[12] However, acetate only allows for the synthesis of PHB.[12] Therefore, in order to determine if the oxalate culture could produce more useful PHAs such as the co-polymer PHBHV propionate was chosen as the co metabolite.

To avoid total loss of the biomass during accumulation the concentration was again measured over the duration of the experiment (Figure 6). Just like in the initial experiments (Figure 3) the biomass concentration increased initially after accumulation began. However, unlike previously the concentration did not level out but continued to increase until it reached the end of the accumulation period. Again, appears to be the result of the accumulation of PHA in the biomass rather than increase in cell growth as PHBV accumulation is observed over this same period (Figure 8). When the culture was switched back to growth the biomass concentration appeared to level out. Once accumulation began again the biomass concentration reached its peak of 5.24 g L^{-1} on day 35. However, after this point the biomass concentration crashed to 1.91 g L^{-1} on day 39 and then appeared to level out. This crash seems to signify the limit at which the culture can handle the conditions and is no longer able to sustain itself.

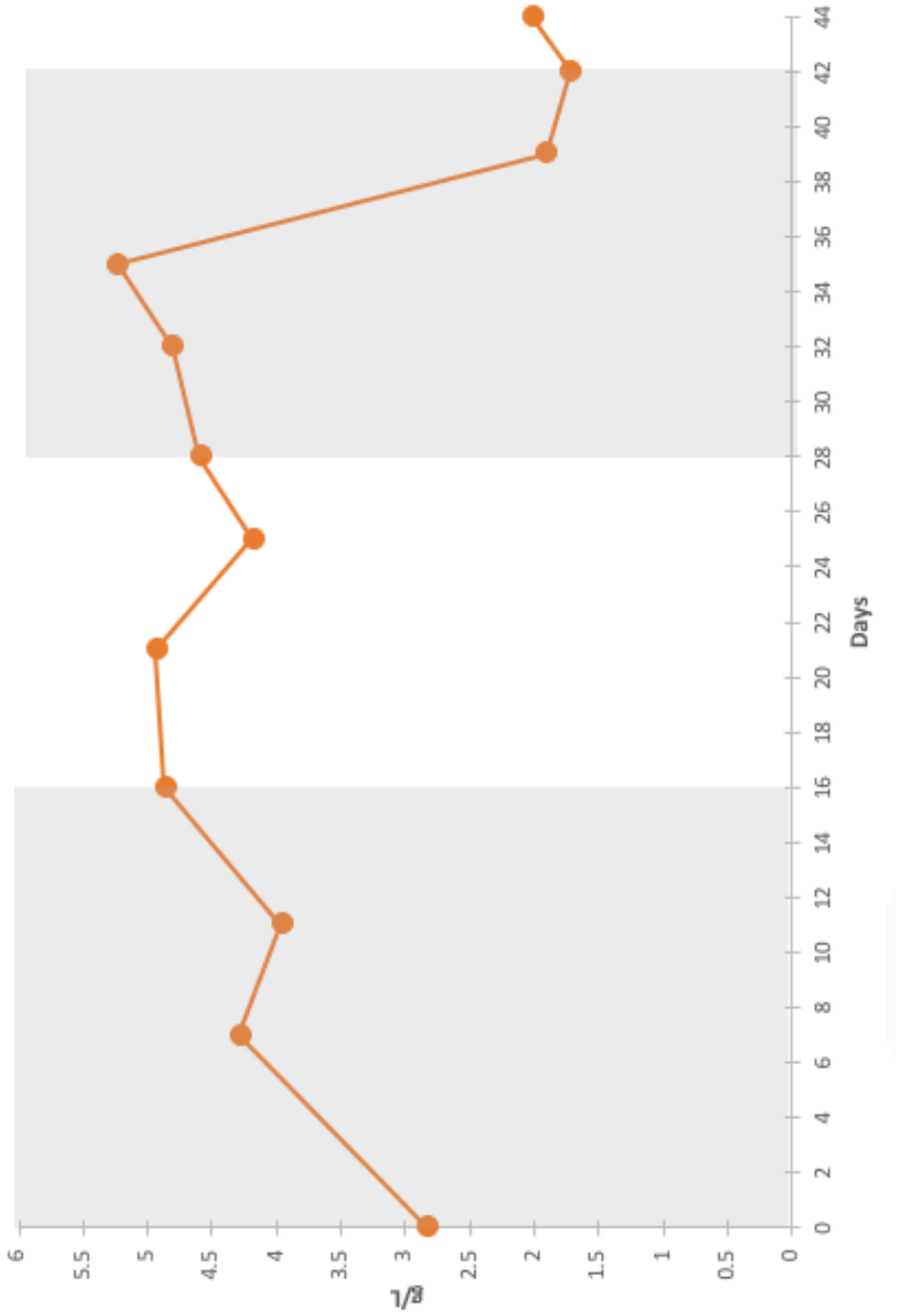

Figure 6: Biomass concentration (g L^{-1}) of the cofed culture during alternating accumulation (grey) and growth (white) conditions.

The uptake of soluble carbon in the cultures was monitored over the course of a single cycle for accumulation and growth to determine the rate of consumption (Figure 7). Oxalate appears to follow the same consumption pattern as seen in Figure 4 and is totally consumed near the start of the cycle. However, the uptake rate decreased drastically

compared to the single carbon source down to 1.42 mmol (COD) g^{-1} (CDW) h^{-1} under growth conditions and 0.865 mmol (COD) g^{-1} (CDW) h^{-1} under accumulation conditions. Further testing is required to determine if this is due to the lower concentration of oxalate in the liquor or if it is due to the presence of propionate.

The uptake rate for propionate was 1.26 mmol (COD) g^{-1} (CDW) h^{-1} under growth conditions and 0.81 mmol (COD) g^{-1} (CDW) h^{-1} under accumulation conditions. One observation to note is that unlike oxalate there is still propionate detectable in the liquor at the end of the cycle. There appears to be a slowing of the uptake rate of the culture for both oxalate and propionate under accumulation conditions compared to growth. This was not observed in the earlier experiment where oxalate was the sole carbon source.

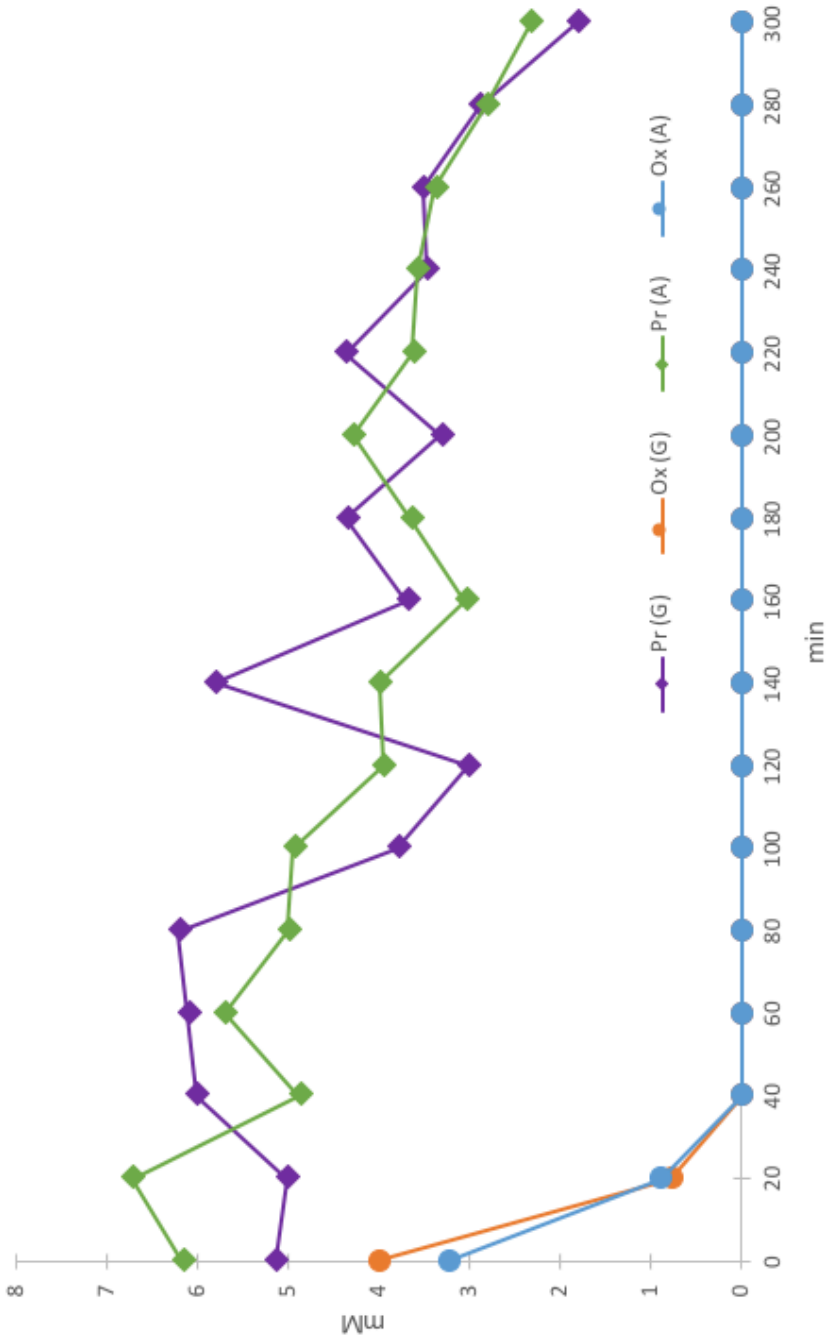

Figure 7: Soluble oxalate (Ox) and propionate (Pr) detectable in the liquor over the course of a single cycle for growth (G) and accumulation (A).

The PHA accumulated in the biomass (% PHA w/w) was monitored over the course of the accumulation and growth cycles (Figure 8). The most notable observation is that when the culture was fed oxalate as the sole carbon source only PHB was synthesised. However, when the

same culture was supplied propionate as a co-metabolite both HB and HV monomers were detected. Previous studies indicate that the oxalotrophic bacteria *Bacillus subtilis* could be a potential producer of PHBV.[49] This study is the first recorded instance of PHBV synthesis using oxalate as a carbon source. The MMC is clearly metabolising oxalate as shown in Figures 4 and 7, however analysis of the population dynamics of the MMC is required to determine if these PHBV synthesising bacteria are specialist or generalist oxalotrophs. The synthesis of HV monomers requires the precursor propionyl-CoA and acetyl-CoA both of which are products of propionate metabolism.[56] Therefore, the HV detected in the culture can be solely attributed to the bioconversion of source propionate. Attributing the carbon source responsible for the synthesis of HB is not as easy. This is because propionate will also result in the synthesis of HB monomers and cultures fed propionate under accumulating conditions produce a PHBV (28.1 % HB and 71.9 % HV) co-polymer.[57]

After the first 7 days (28 cycles) of accumulation 10.89 % w/w PHBV was detected in the cells, with HV making up 23.7 % of the polymer and the other 76.3 % detectable as HB (Figure 8). The data shows a significant increase in the accumulation of PHB in the co-fed culture compared to the PHB accumulated over 32 cycles in the oxalate experiment (Figure 5). Therefore, the addition of propionate as a co-metabolite appears to have increased the PHA yield in the culture by ~10-fold over a similar number of cycles. The presence of propionate also seems to have increased the conversion of carbon to PHA, with 3.42% of the total oxalate and propionate consumed being converted to PHA per cycle compared to the 0.16% per cycle observed in the initial experiment.

The PHBV yield continued to increase and 13.28 %w/w of the polymer was detected in the biomass on day 11. The conversion of carbon to

the polymer over those 4 days was calculated to be 1.91% per cycle. The conversion of carbon to PHA increased to 2.58% per cycle between day 11 and 16. There was a minimal increase in the PHBV yield with 14.16% w/w detected in the biomass, however, the percentage of HV that made up the co polymer rose to 37.5% (Figure 8).

Switching to growth conditions resulted in a reduction in the PHBV accumulated in the biomass, dropping to as low as 6.23% w/w on day 25. The biomass concentration remained stable between day 16 and 28 and combining this with the data showing the reduction in PHBV in cells indicates that the polymer was being used for cell growth. This is expected as intracellular PHA is accumulated in bacterial cells when essential nutrients to growth like nitrogen are limited and carbon sources are available in excess.[10, 13, 16] Therefore when nitrogen becomes available again the intracellular PHA is used by the bacterial cell as a readily available carbon source for energy and biosynthesis.[9-11]

After the culture was switched back to accumulation conditions there was an increase in PHBV yield from 7.61 % w/w on day 28 to 11.34 % w/w on day 32 (Figure 8). However, when looking at the data for the accumulation of HV in the culture it shows that it jumped from 51.75 mg g^{-1} to 103.36 mg g^{-1} biomass. This is much larger than the increase in the accumulation of HB between day 28 and 32 from 61.66 mg g^{-1} to 82.85 mg g^{-1} of biomass respectively. The carbon conversion rate of propionate to HV from day 28 to 32 was 12.63% per cycle and on day 32 HV made up 45.63% of the PHBV polymer (Figure 8).

Day 35 of the experiment indicated a PHBV yield of 18.62% w/w with HV making up 55.5% of the polymer. The conversion of carbon to PHBV per cycle reached its highest point between day 32 and 35 at 9.10%

with the specific conversion of propionate to HV at 33.95% per cycle (Figure 8).

After day 35 the PHBV yield detected in the culture increased to 20.45% w/w, however this also coincided with the crash of the biomass concertation (Figure 6). There appears to be no reduction in the % PHBV w/w of the culture until it is returned to growth conditions (Figure 8). However, the dramatic drop in biomass indicates limits of accumulation of the culture has been reached and the optimal harvest point would be on day 35.

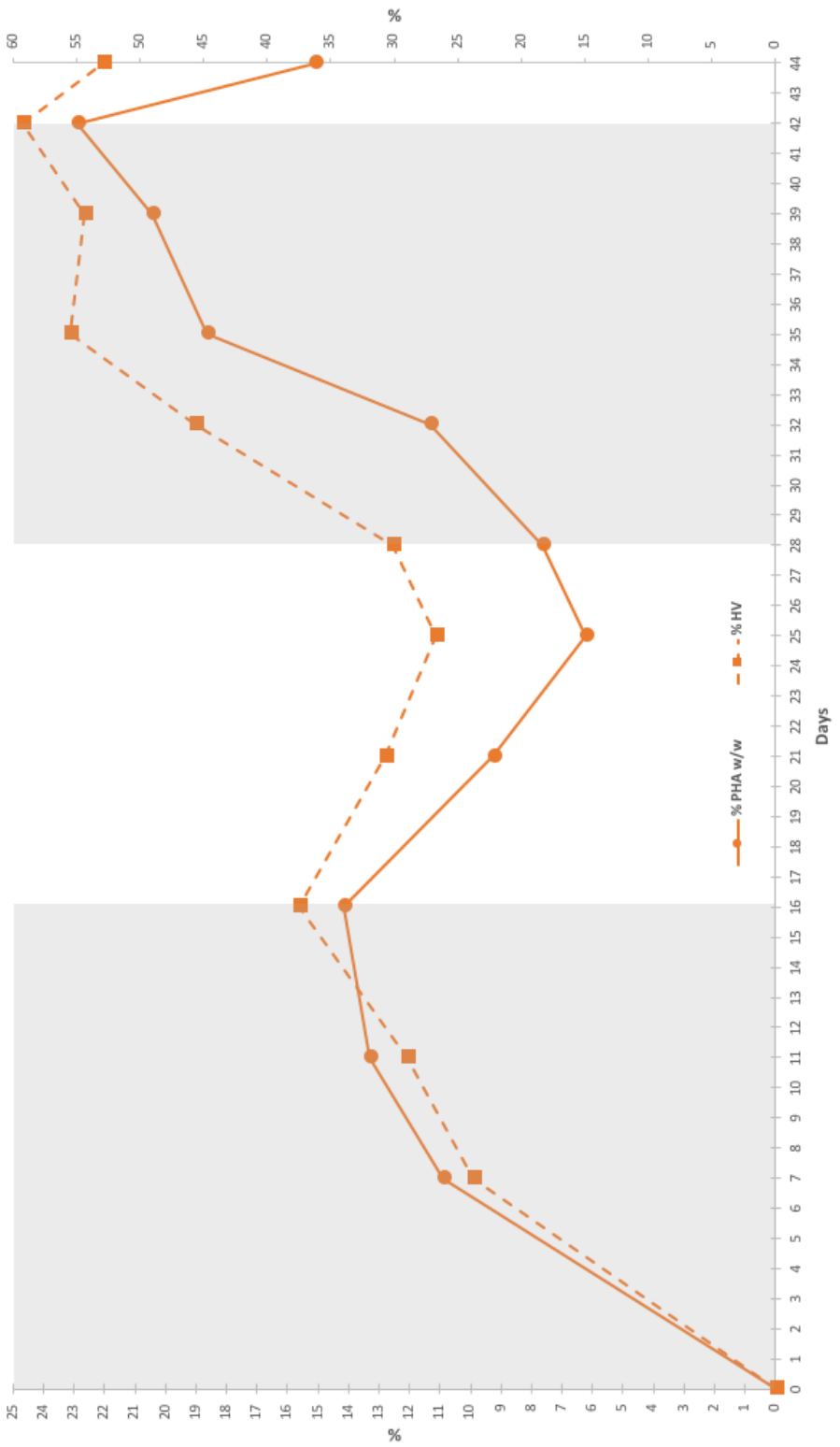

Figure 8: Solid line represents the % PHA w/w (HB+HV) accumulated in the biomass over the course of the accumulation (grey) and growth (white) cycles (primary axis), the dotted line represents the percentage of hydroxyvalerate (HV) that makes up the copolymer (secondary axis).

The data clearly shows that the presence of propionate as a co-substrate not only causes a dramatic increase in the accumulation of PHA with up to 18.62% w/w being accumulated within the cells while maintaining a healthy biomass concentration. Co-feeding also allows for the synthesis of the more useful copolymer PHBV. Further analysis is required to determine the percentage contribution of propionate and oxalate to the HB monomers copolymer produced in this experiment. The literature shows that propionate results in the synthesis of a PHBV copolymer.[57] Therefore, it is undetermined if the increase in HB yield observed in the co-feeding experiment is due to the synthesis of HB monomers from propionate, or if the presence of propionate allows the biomass to shuttle oxalate directly into the PHA pathways. It is also not clear why the % HV content of the co-polymer increased over the duration of the experiment before stabilising at ~50%. Further investigation is needed to determine if the %HV would increase further if cycling between growth and accumulation continued or if 55-59% HV content is the limit.

Characterisation of the co-polymer

Biomass concentration was monitored to determine the optimal time to harvest the culture to ensure maximum polymer accumulation while at the same time having a large biomass sample to extract the polymer (Figure 9).

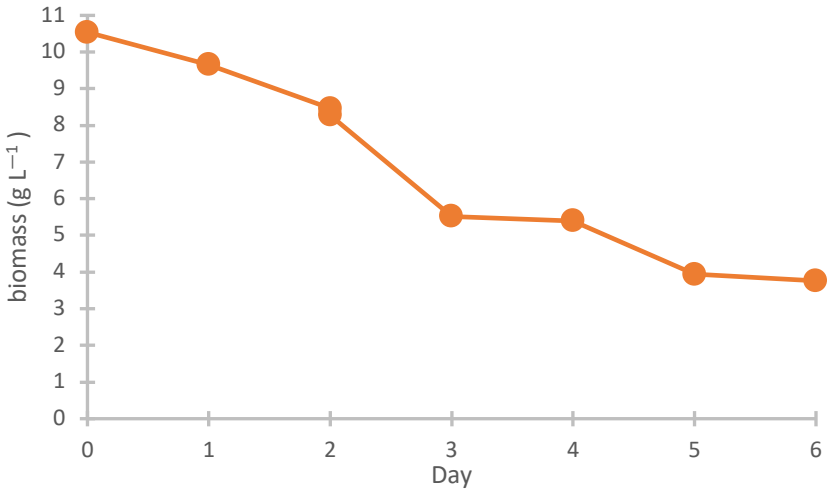

Figure 9: Biomass (g L^{-1}) of the co-fed culture placed under accumulation on a reduced 4-hour cycle.

GCMS results

Isolated polymer (1.6 mg) was digested and analysed via GCMS to determine the make up the polymer and to quantify it. The analysis produced two peaks of interest one at a retention time of 6.13 minutes and a second at RT 7.20 (Figure 10).

Figure 10: GC spectrum of the polymer isolated from the biomass, HB = hydroxybutyrate; HV = Hydroxyvalerate, BA = Benzoic acid internal standard.

The mass spectrum of the first peaks can be viewed in Figure 11 (a) which was similarity search matched at 87% with 3-hydroxybutyric acid propyl ester along with a mass spectrum from the literature of a HB propyl ester standard (Figure 11, b). The ions of interest are the peaks at the signals at 60, 61, 87, 89, 113, 117 and 147 m/z. Compare this with the mass spectrum (Figure 11, c) for the second peak in figure 8 at RT 7.20 which was similarity search matched at 81% to 3-hydroxypentanoic acid ethyl ester and a propyl ester of HV standard form the literature (Figure 11, d). Points of interest in the mass spectrum are the peaks at 89, 101, 131 and 142 m/z.

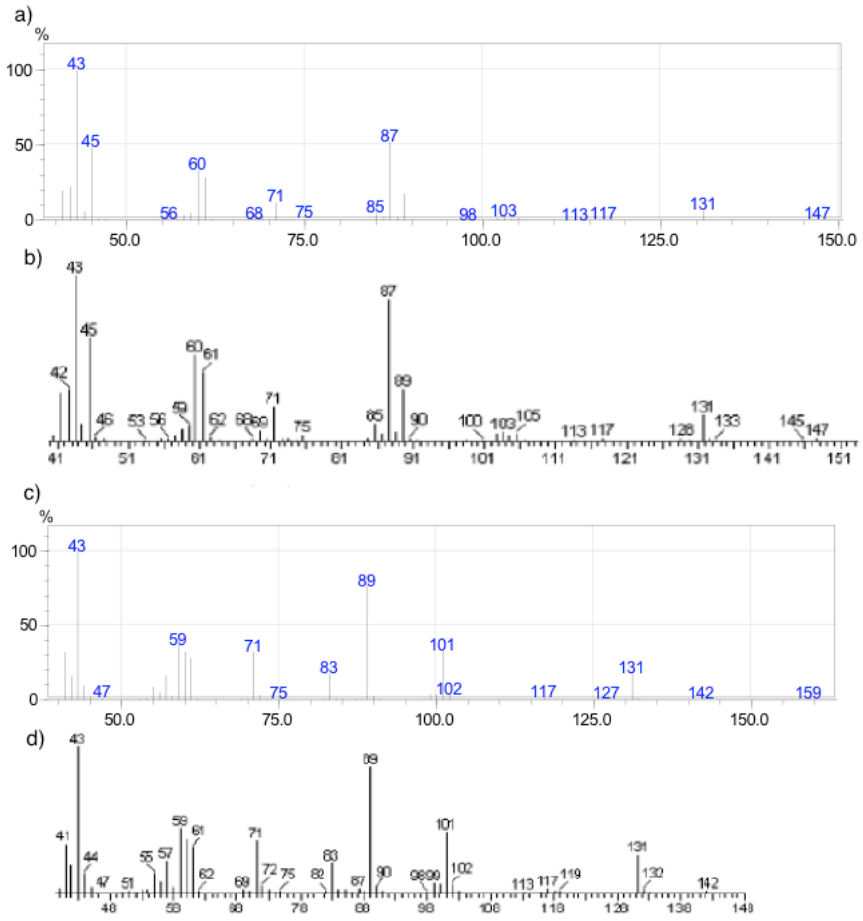

Figure 11: Mass spectrum of (a) the peak at RT 6.136 compared with (b) HB propyl ester literature mass spectrum[3] in the literature and (c) the peak at RT 7.20 compared with (d) HV propyl ester literature mass spectrum.[3]

Quantitative GCMS analysis of the polymer determined that the HB consisted of 1.28 mg and the HV content made up 0.34 mg of the

polymer. The HV content in the polymer was calculated to make up 21%, this is consistent with the data in the earlier samples of the biomass analysed in the initial cofeeding experiment where after 7 days the HV content of the PHBHV accumulated in the biomass made up 24% (Figure 6).

NMR results

The extracted polymer (10.7 mg) of the extracted PHA polymer was analysed via NMR to determine its structure (Figure 12, Table 3). Both the ^{13}C NMR and the ^{1}H NMR spectra produced was consistent with that reported in the literature for a PHBHV copolymer.[4-5]

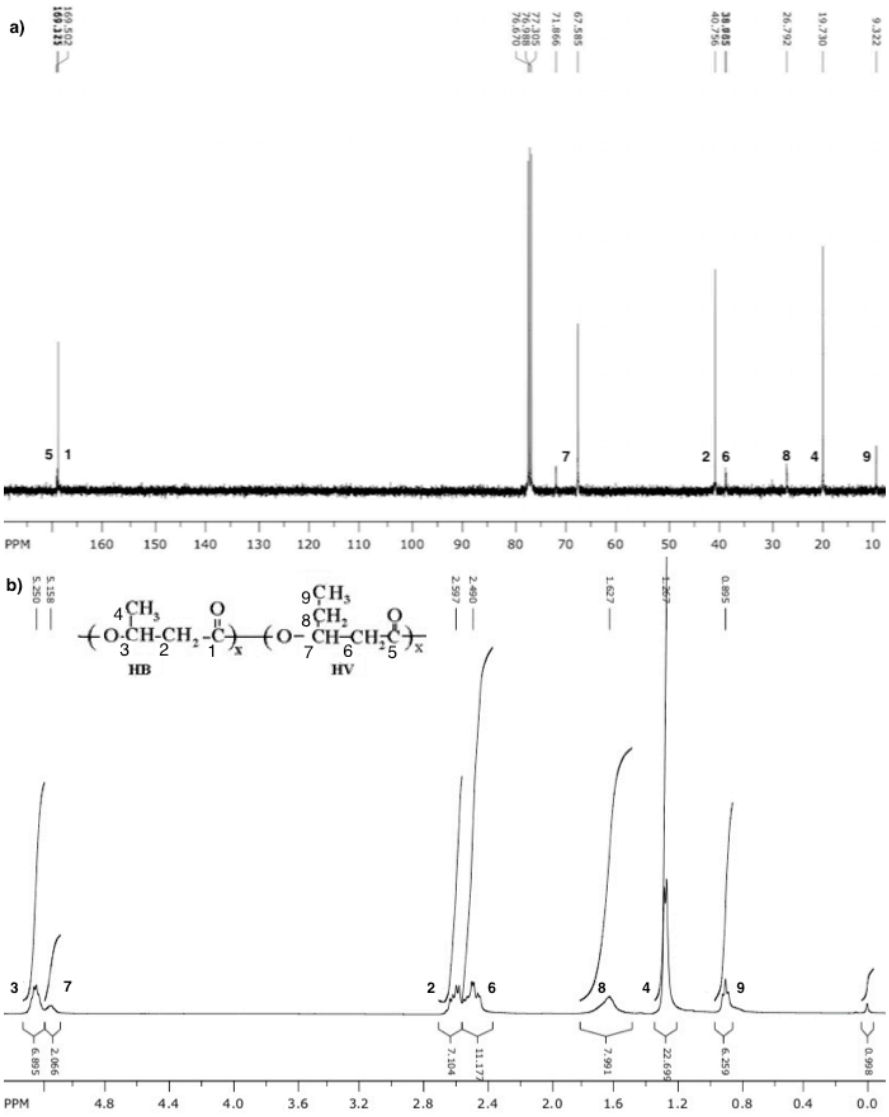

Figure 12: ^{13}C NMR spectra (a) and ^{1}H NMR spectra (b) of isolated polymer with insert of the chemical structure of PHBHV to indicate the corresponding carbon atom and protons with their peaks.

The chemical shifts match very closely with those reported in the literature,[4-5] the one difference is the chemical shift for carbon number

7. The chemical shift for C7 from the extracted polymer matches the chemical shift recorded in Kemavongse *et. al.*[4] however, it does not match the chemical shift reported in Mumtaz *et. al.*[5] which has the chemical shift of C7 at 76.68 ppm.[4-5]

The significantly lower signals in the ^{13}C and ^{1}H NMR spectrum correspond to carbons present in the HV monomer, indicating that the HV is the minor component in the extracted co-polymer. Using the integration preformed on the ^{1}H NMR analysis of the CH atom signals produced from the HB and HV monomers (C3 and C7 respectively) the HV content of the polymer was calculated to be 23% which is consistent with the other data.

Table 3: Chemical shift signals obtained from ^{13}C and ^{1}H NMR spectroscopy preformed on the isolated polymer with chemical shifts reported in the literature for comparison.[4-6]

Carbon atom	Chemical shift (ppm)				
	^{13}C	^{1}H	Mumtaz et. al.	Kemavongse et. al.	Shamala et. al.
1	169.50	–	169.29	169	–
2	40.756	2.597	40.75	40	~2.5
3	67.585	5.250	67.4	68	5.28
4	19.730	1.26, d	19.74	19.66	1.29, d
5	169.12	–	169.12	169	–
6	38.665	2.490	38.75	38	~2.5
7	71.866	5.158	76.68	72	5.15
8	26.792	1.627	26.8	27	1.59
9	9.322	0.89, t	9.31	9	0.9, t

Thermal properties

The results of the thermal analysis (Figure 13) showed that the isolated copolymer possessed a higher thermal-degradation temperature 270.45 °C than compared to commercial PHB (260.39 °C) and PHBHV (261.47 °C), but lower than other bacterially synthesised PHBHV polymers (281.93 °C) reported in the literature.[5] This event resulted in a 92.93 % reduction in mass of the sample suggesting complete thermal degradation.

Due to time constraints, only preliminary testing of the thermal properties was performed to give an indication of the crystallization (T_c) and melting temperature (T_m) of the isolated polymer. These temperatures were recorded as ~130 °C for T_c and 170.7 °C for T_m both of which were slightly higher than those recorded in the literature of 121 and 166 °C respectively.[4] This data indicates that the crystallinity of the copolymers is above 55 %. This is consistent with the literature which reports crystallinity at ~56 % for PHBV polymers.[1]

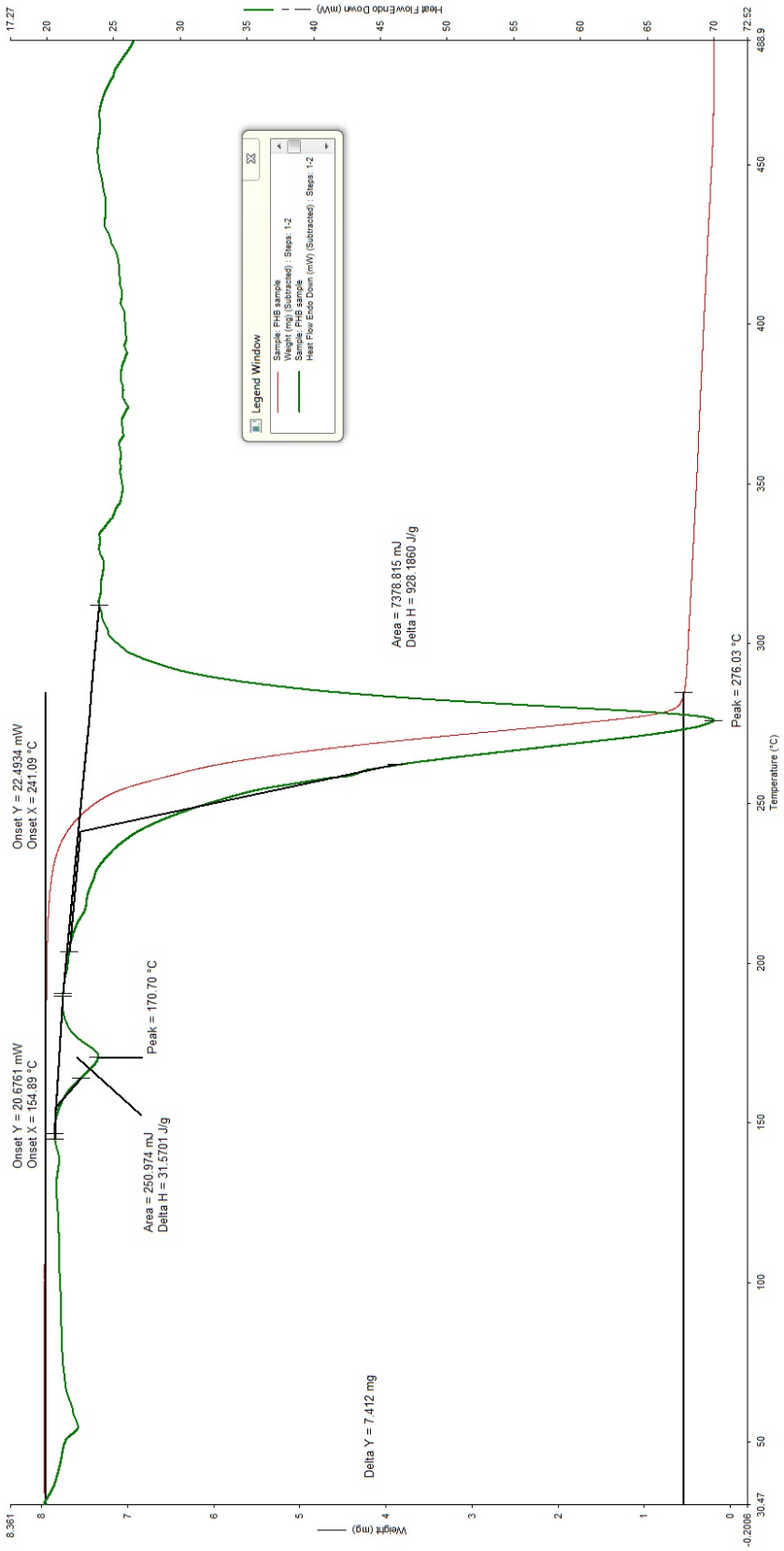

Figure 13: Thermal analysis of 7.95 mg of the extracted polymer, the green line showing the heat flow (exothermic down) and the red line showing the sample weight under a nitrogen atmosphere heated at 10 °C per min.

Conclusion

Industrial waste streams are a largely untapped resource that provide a cheap alternative to expensive traditional carbon sources such as glucose. The waste carbon found in the Bayer liquors used to process bauxite ore in the aluminium industry represents a unique opportunity. Current best practice involves the oxidation of the organic material to CO_2, however, as discussed this results in further economic cost due to the need to re-causticise the liquor. The literature and previous studies at Murdoch indicate that oxalate is a difficult substrate for bacteria use for growth and to synthesise PHB from. However, it is possible to produce modest yields of ~4.5 % PHB w/w when fed oxalate as the sole carbon source. This experiment has shown that the PHA yield can be dramatically increased to 18.62 % PHA w/w at a biomass concentration of approximately 5 g L^{-1} via co-feeding. In addition to the increase in the PHA yield, the co-feeding resulted in the more useful copolymer PHBV (HV ~55%). Analysis via GCMS, NMR and thermal analysis of a polymer sample isolated from biomass, confirmed the co-fed MMC with propionate was able to synthesise a PHBV co-polymer. The data showed that oxalate was totally consumed by the culture even when propionate was available. However, further investigation is required to determine if the presence of propionate allows the bacteria to shuttle oxalate into PHA synthesis pathways or if the oxalate is used by the cell in some other way. The data also showed that the percentage of HV increased with the increasing time under accumulation, why this occurred is not yet understood and requires further research. Lastly, further investigation is recommended into the optimal oxalate to propionate ratio that would further maximise the PHA yield achieved from oxalate.

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