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The Conversion of Waste PET Plastic into a High Value-Added Biodegradable Plastic

STRIVE

Environmental Protection
Agency Programme

2007-2013

SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

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EPA STRIVE Programme 2007–2013

The Conversion of Waste PET Plastic to a High Value-Added Biodegradable Plastic

An Investigation of the Conversion of Waste Polyethylene Terephthalate to the Biodegradable Polymer Polyhydroxyalkanoate

(2005-ET-LS-9-M3)

STRIVE Report

Prepared for the Environmental Protection Agency

by

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The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Table of Contents

Acknowledgements ii

Disclaimer ii

Details of Project Partners iii

Executive Summary vii

1 Introduction 1

- 1.1 Polyethylene Terephthalate 1
- 1.2 Polyethylene Terephthalate and Other Plastic Waste 1
- 1.3 Pyrolysis 2
- 1.4 Biodiesel and Glycerol 2
- 1.5 Polyhydroxyalkanoate 3

2 Studies on the Conversion of Polyethylene Terephthalate to Polyhydroxyalkanoate 5

- 2.1 Experimental Section 5
- 2.2 Results and Discussion 5
- 2.3 Conclusion 7

3 The Conversion of Polyethylene Terephthalate to Polyhydroxyalkanoate through Molecular Manipulation 9

- 3.1 Experimental Section 9
- 3.2 Results and Discussion 9
- 3.3 Conclusions 12

4 Biomass Manipulation to Improve the Polyethylene Terephthalate to Polyhydroxyalkanoate Conversion Process 13

- 4.1 Experimental Section 13
- 4.2 Results and Discussion 13

5 The Conversion of Benzene, Toluene, Ethylbenzene and Xylene Compounds by Single and Defined Mixed Cultures to Medium-Chain-Length Polyhydroxyalkanoate 17

- 5.1 Experimental Section 17
- 5.2 Results 17
- 5.3 Discussion 20
- 5.4 Conclusion 21

6 Conclusions and Recommendations 22

- 6.1 Conclusions 22
- 6.2 Recommendations 24

References 25

Acronyms 29

Executive Summary

This report details the work carried out in the development of a biotechnological process for the conversion of polyethylene terephthalate (PET) plastic bottles into biodegradable plastic (project 2005-ET-LS-9-M3). Plastic waste is and will continue to be a major problem for Irish society as a whole. Our need to reduce the amounts of waste produced is of utmost importance; however, due to the quick pace of modern society, plastic waste will be a burning issue for the foreseeable future. In tackling the problem of waste plastic, there is an opportunity to develop innovative ways to deal with excess waste that is not currently reused or recycled but incinerated or dumped. The goal of this project was to try to develop a technology that could take plastic waste, namely PET (which is most commonly associated with plastic drink bottles), and convert it into a new biodegradable polymer.

The first task of this project was to develop a biological process that could convert terephthalic acid (TA) into the biodegradable polymer polyhydroxyalkanoate (PHA). The thermal treatment of PET in the absence of air (pyrolysis) generates TA as the major product. The TA generated is potentially a feedstock for the microbial synthesis of the value-added biodegradable polymer PHA. Despite the knowledge that bacteria have been reported to degrade TA and that other bacterial strains can accumulate PHA, the production of PHA from TA has never been reported and, thus, the conversion of PET to a biodegradable plastic has not been achieved. Known TA degraders were tested for PHA accumulation but all strains failed to accumulate it. Consequently, new strains were isolated from soil exposed to PET granules and screened for PHA accumulation. Thirty-two strains were isolated – three of these strains, which were capable of the accumulation of medium-chain-length (mcl) PHA (mcl-PHA) from TA as a sole source of carbon and energy, were selected for further study. These isolates were identified using 16S rDNA techniques as *Pseudomonas putida* (GO16), *P. putida* (GO19), and *P. frederiksbergensis* (GO23). *Pseudomonas putida* GO16 and GO19 accumulate PHA composed predominantly of a 3-hydroxydecanoic acid monomer, while *P. frederiksbergensis* GO23 also accumulates 3-hydroxydecanoic acid as the predominant monomer but with increased amounts of 3-hydroxydodecanoic acid and 3-hydroxydodecenoic acid compared with the other two strains. Polyhydroxyalkanoate was detected in all three strains when nitrogen depleted below detectable levels in the growth medium.

Having investigated the conversion of PET to PHA using soil isolates, the next part of the project aimed to use molecular techniques in order to convert TA to polyhydroxybutyrate (PHB) and to enhance TA conversion to PHA. The first strategy targeted PHB accumulation and involved the cloning of an operon containing the genes necessary for the production of PHB into a selection of organisms known to utilise TA as the sole source of carbon and energy, along with a range of bacteria that were isolated during this project from PET-contaminated soil for their ability to grow on TA but which failed to accumulate PHB or mcl-PHA. The genes responsible for PHB accumulation in the gram-negative soil bacterium *Ralstonia eutropha* are organised on a gene cluster, *phaCAB*. Here, a broad-host range plasmid containing these genes has been successfully created and expressed in a target bacterium producing 4% of cell dry weight (CDW) as PHB. The second strategy was targeted at mcl-PHA and involved two different approaches, one aimed at cloning the genes responsible for TA degradation from *Comamonas testosteroni* and expressing these genes in two organisms that have been extensively studied and shown to produce mcl-PHA, *P. putida* CA-3 and *P. putida* KT2440, but that do not possess the ability to utilise TA as the sole source of carbon and energy. Unfortunately, no TA degradation was achieved with these recombinant organisms during this study. The other approach involved cloning the *phaG* gene from *P. putida* CA-3, which encodes a (*R*)-3-hydroxyacyl-ACP-CoA transferase into *P. putida* GO10, a gram-negative soil organism isolated on TA as a sole source of carbon and energy during the course of this study. The wild-type strain, GO10, was able to accumulate

PHA from alkanoic acids (e.g. octanoic acid) but could not accumulate PHA from TA. The recombinant GO10_ *pJB861-phaG* was recorded to produce up to 12% PHA of CDW from TA when the *phaG* gene was expressed in this strain.

Since the wild-type strains isolated in this study were better than the recombinant strains, it was decided to proceed with the wild-type strains for further development of mcl-PHA production from PET. The improvement of this process by enhancing the fermentation production was then undertaken. Bioprocess manipulation has been previously successfully employed in the production of PHA from a variety of both PHA-related and -unrelated substrates. However, this is the first study to employ TA as the source of carbon and energy in a fermentor to produce mcl-PHA. Bioprocess manipulation allows for increased control over growth as well as PHA production, due to the enhanced ability to closely monitor and control some of the various physical parameters involved in microbial growth. Specific feeding strategies were employed in order to increase the biomass and PHA volumetric productivity (g/l/h) over a 48-h period, and to increase the amount of TA that is utilised during this period. In addition to manipulating the feeding of TA, a co-substrate – glycerol (a waste from the biodiesel industry) – was used to further enhance the process. The co-feeding of TA and waste glycerol in this study's recycling process enabled the provision of a higher biomass and PHA volumetric productivity (g/l/h), a route for another waste material, and allows the production and study of mcl-PHAs with varying monomer compositions. The ability to alter the monomer composition of PHA allows alteration of the material properties, thus offering greater diversity and flexibility for PHA production.

In the final portion of the project, the technology developed for PET conversion to PHA was applied to a major portion of the products from the pyrolysis of mixed plastic waste, namely benzene, toluene, ethylbenzene, *p*-xylene and styrene (BTEXS). Known *P. putida* species that are able to utilise BTEXS as a sole carbon and energy source were assessed for their ability to produce PHA from the single substrates. *Pseudomonas putida* F1 is able to accumulate mcl-PHA when supplied with toluene, benzene, or ethylbenzene. *Pseudomonas putida* mt-2 accumulates mcl-PHA when supplied with toluene or *p*-xylene. The highest level of PHA accumulated by cultures in a shake flask was 26% of CDW for *P. putida* mt-2 supplied with *p*-xylene. A synthetic mixture of BTEXS, which mimics the aromatic fraction of mixed plastic pyrolysis oil, was supplied to a defined mixed culture of *P. putida* F1, mt-2, and CA-3 in the shake flasks and fermentation experiments. Polyhydroxyalkanoate accumulated to 24% and 36% of the CDW of the shake-flask-grown and fermentation-grown cultures, respectively. In addition, a threefold higher cell density was achieved with the mixed culture grown in the bioreactor compared with shake-flask experiments. A run in the 5-l fermentor resulted in the utilisation of 59.6 g (67.5 ml) of the BTEXS mixture and the production of 6 g of mcl-PHA. The monomer composition of PHA accumulated by the mixed culture was the same as that accumulated by single strains supplied with single substrates with 3-hydroxydecanoic acid occurring as the predominant monomer. The purified polymer was partially crystalline with an average molecular weight of 86.9 kDa. It has a thermal degradation temperature of 350°C and a glass transition temperature of –48.5°C.

This project has been successful in its goal to convert waste PET to PHA. In addition to this, another form of plastic waste reuse has been identified and investigated in the conversion of mixed plastic waste to PHA. During this research, two patents, which protect this technology, have been secured, and, while both of these processes require further investigation to achieve commercial viability, the results of this project have laid the foundations for an innovative green technology that may prove valuable to an Ireland striving towards a greener future.

Success in this project represents an important step towards developing a technology with a broad significance to the global community. In an ever-changing world, which is under severe threat from climate change, the value of this indigenous novel technology which can help deal with one of the world's waste problems will be of great advantage to Ireland both environmentally and economically. A large amount of development is still required to make this goal a reality but this research has shown that not only is this process possible for PET but also for other plastic wastes. This expands the horizons of opportunity for a green environmentally friendly technology designed, researched and firmly rooted in Ireland.

A spin-out company, Bioplastech Ltd, was created in 2009 to commercialise technologies developed by Dr O'Connor and his team arising from this Environmental Protection Agency-funded project. Converting waste materials to a value-added, environmentally friendly (i.e. biodegradable) plastic answers the current United States (Bio-preferred initiative) and European Union (Lead Market Initiative) efforts to promote bio-based eco-friendly products arising from waste and sustainable resources, but it is also of great interest to the global polymer market seeking new polymers and applications in areas such as biodegradable packaging, personal-care products, adhesives, biomedical and health-care products, consumer electronics, etc.

1 Introduction

In the last half-century, petrochemical plastics have become indispensable in almost all parts of everyday life. Their durable nature and relatively low production cost have seen them become invaluable in modern society. In 2008, despite a global financial crisis causing a decrease in demand for plastic, 245 million tonnes were produced worldwide, with Europe accounting for 60 million tonnes, contributing about 25% of the global total (PlasticsEurope, 2009). The extremely recalcitrant nature of plastic coupled with the fast-pace disposable culture of today has led to a variety of different plastics becoming a major waste problem worldwide. In an attempt to address this waste problem, various recycling technologies have been developed. While many of these recycling technologies have been successful in diverting plastics away from landfill, the maximum amount of plastic waste being mechanically recycled in Europe is around 30% in Germany, where 95% of all plastic waste is collected and available for recycling (PlasticsEurope, 2007). The main reason for such low recycling rates is the relatively low value of the recycled product. The primary aim of this project was to establish a technology for the conversion of polyethylene terephthalate (PET) (Fig. 1.1) to a higher-value biodegradable plastic polyhydroxyalkanoate (PHA). The application of this technology to other waste recycling and PHA production was also investigated.

1.1 Polyethylene Terephthalate

Polyethylene terephthalate has uses in a wide variety of areas, such as beverage and other liquid containers, food packaging, thermoforming applications and the fibre industry (Petcore, 2010). In the United States (US) alone, 5.4 billion pounds (2.4 billion kg) of PET bottles were on shelves in 2008 (NAPCOR, 2008). Polyethylene terephthalate exists both as a semi-crystalline (opaque and white) and an amorphous (transparent) thermoplastic, and can be made into a resin, film or fibre. The largest outlet for PET is for the production of synthetic fibres, with bottle resin production the next largest application. In textile applications, PET is often referred to as ‘polyester’ while ‘PET’ is used often to refer to the packaging resins. Polyethylene terephthalate packaging resin markets have seen very strong growth over the last 20 years. Polyethylene terephthalate first penetrated the carbonated soft drinks market because it is lightweight and strong – PET bottles are virtually unbreakable, while a typical 1.5-l bottle weighs about 40–45 g, about one-tenth the weight of glass. Polyethylene terephthalate has taken the market share in the bottled drinks market due to its good clarity and not leaving any taste in the beverage. The crystalline form of PET is used for trays for pre-cooked meals that are reheated in either microwave or conventional ovens. It is also used for thick-walled containers for cosmetic and personal-care products as well as for pharmaceuticals (Petcore, 2010).

1.2 Polyethylene Terephthalate and Other Plastic Waste

Petrochemical plastics are extremely recalcitrant and, while some fungi have been shown to degrade

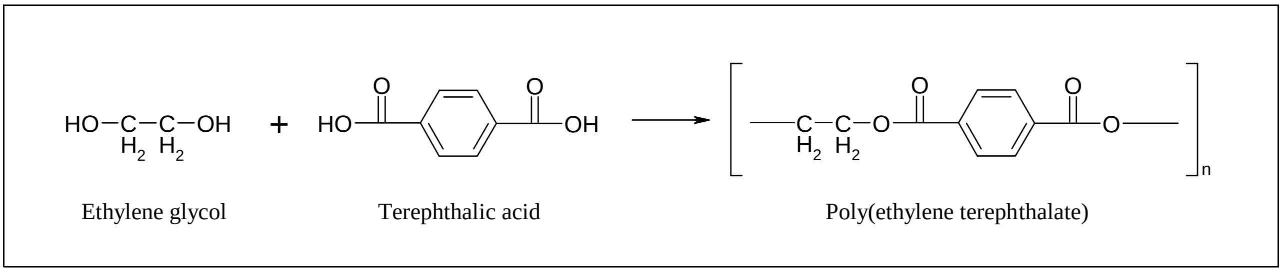

Figure 1.1. Pathway for the synthesis of polyethylene terephthalate (PET).

polystyrene (Kaplan et al., 1979), wide-scale biodegradation of conventional petrochemical polymer has never been shown. The multi-million tonne levels of plastic usage, coupled with our throwaway convenience culture, has led to waste management issues. The figures from the United States Environmental Protection Agency (USEPA, 2009) show clearly the gravity of the plastics waste issue. In 2008, plastics comprised 12% (30 million tons) of total municipal solid waste in the US. Only 7.1% of that plastic waste was recovered for recycling, leaving 27.87 million tons of plastic for landfill or incineration. In Europe, the statistics according to PlasticsEurope show that in countries such as Switzerland, Sweden and Denmark there is virtually no plastic waste being disposed of in landfill. These countries obtain these high ‘recycling’ levels predominantly through energy recovery (achieved through municipal incinerators) (IdentiPlast, 2007) and while energy recovery is a viable option in retrieving energy from highly calorific plastic waste, there is no value-added product or material obtained. In fact a European Union (EU) directive, voted on in 2008, distinguishes between recycling and energy recovery and will lead to it being reported separately from official recycling figures. Around 80% of plastic waste is disposed of in this way in these countries, with the remaining 20% of discarded plastics being materially recycled (IdentiPlast, 2007). In common with other petrochemical plastics, the success of PET as a convenience bulk commodity polymer has led to post-consumer PET products becoming a major waste management problem. To take PET plastic bottles as an example, 2.7 million tons of PET bottles were available on shelves in the USA in 2008, and only 27% of these were being mechanically recycled (USEPA, 2009). In Europe, a combination of incineration and physical recycling only resulted in a total of 40% of all plastic bottles being recycled; thus, the majority of PET bottles end up in landfill (Petcore, 2009). One reason for this is that the current technologies for PET recycling generally lead to low-value products. Recycled PET materials do not compete well on price or performance with virgin PET; consequently, recycled PET is blended with virgin PET to improve the properties of the recycled PET. New PET recycling technologies are coming on-stream, such as layered bottle-to-bottle recycling which involves sandwiching recycled PET between layers of higher-grade virgin polymer. While this technology increases the market for recycled PET resin, the longer-term problem associated with this technology will be to distinguish a blended PET bottle from a full-virgin polymer bottle. As the inner layer of PET’s quality will diminish during successive rounds of recycling, it will lead to supply-stream quality-control issues, which will present major issues for polymer processors.

1.3 Pyrolysis

Pyrolysis is best described as the thermal decomposition of a substance in the absence of air to produce pyrolysis oils, solids or gases (Albright et al., 1983). The energy required for the process is provided for by the process itself which means that there are little or no running costs. Polyethylene terephthalate pyrolysis carried out by Prof. Walter Kaminsky’s group was achieved in a fluidised bed reactor at a conversion rate of 51% back to the monomer form, terephthalic acid (TA), along with 20% comprising dimers and short oligomers that can be easily treated with sodium hydroxide to obtain monomeric TA to achieve a total yield of 71% (Yoshioka et al., 2004). This pyrolysis solid could be used without any further processing or clean-up step in this study’s laboratory. Mixed waste stream pyrolysis was also performed in Prof. Kaminsky’s laboratory from which the monomer constituents were determined. A typical domestic mixed plastic waste stream was pyrolysed by Prof. Kaminsky for this study based on previously described methods (Kaminsky and Kim, 1999).

1.4 Biodiesel and Glycerol

Biodiesel (BD) refers to a diesel fuel that has been produced from either a vegetable oil or animal fat. The process of BD production is generally achieved through the transesterification of oils and fats. The most common method for BD production involves reacting vegetable oils or animal fats catalytically with a short-chain alcohol, typically methanol. Animal and plant fats and oils are made of triglycerides, which are esters of free fatty acids with the alcohol, glycerol. Almost all BD is produced from virgin vegetable oils using the base-catalysed technique, as it has been shown to achieve up to a 98% conversion yield (Ma and Hanna, 1999; Zhang et al., 2003a). However, waste oils are becoming increasingly popular as starting materials due to the price and increased rates of waste oil collection. The prevalence of BD in the marketplace has led to an increase in the by-product glycerol. Approximately 100 kg of glycerol are produced for every 1 t of BD that is manufactured. This glycerol is contaminated with excess alcohol from the reaction, as well as with the base catalyst and water. The dramatic rise in glycerol production combined with its limited uses has resulted in a sharp decrease in its market value. Currently, most by-product glycerol worldwide is sent to water treatment for digestion but this process is slow and expensive with little economic value (Nopharatana et al., 2007). Glycerol is purified by distillation and used in both food and pharmaceuticals. However, distillation is a costly process and the low price of virgin glycerol makes it undesirable economically (Zhang et al., 2003b). By-product glycerol also contains impurities such as BD and methanol, which is a major issue for processing. Many studies have been performed to investigate the utilisation of pure and waste glycerol (WG) as a feedstock for bacterial growth and for the production of PHA (Ashby et al., 2004, 2005; Cavalheiro et al., 2009; da Silva et al., 2009; Ibrahim and Steinbüchel, 2009).

1.5 Polyhydroxyalkanoate

Polyhydroxyalkanoates (PHAs) are a class of optically active biodegradable polyesters accumulated by numerous bacteria as intracellular granules (Anderson and Dawes, 1990). The first reported PHA, a homopolymer of polyhydroxybutyrate (PHB), was discovered by Lemoigne in 1926. It was nearly 60 years later before the first medium-chain-length (mcl) PHA (mcl-PHA) was characterised in *Pseudomonas oleovorans* (De Smet et al., 1983). Polyhydroxyalkanoate accumulation is generally stimulated in response to inorganic nutrient limitation, such as nitrogen or phosphorus, in the presence of excess carbon. The biopolymer becomes mobilised when conditions for normal growth return (Lageveen et al., 1988; Anderson and Dawes, 1990; Madison and Huisman, 1999). Polyhydroxyalkanoate is generally classified into three groupings according to the size of its comprising monomers:

1. PHA made up of short-chain monomers (3 to 5 carbons) is referred to as short-chain-length (scl) PHA;

2. PHA made up of longer-carbon-chain monomers (6 to 14 carbons) is referred to as mcl-PHA; and

3. Hybrid polymers, with scl and mcl hydroxyalkanoate monomers incorporated into the same polymer.

The polymer properties, both chemical and physical, are determined by the monomer composition of the biopolymer. Properties such as stiffness, flexibility, resistance to organic solvents and melting point can be changed by altering the monomer components. Thus, the basis for biopolymer diversity lies in the monomer composition (Steinbüchel and Hein, 2001; Van der Walle et al., 2001). One of the commercially appealing features of PHA is its biodegradability (Lee, 1996). It is this property of PHA that has gained interest for the biopolymer as a potential alternative to traditional environmentally unfriendly plastics. In addition, PHA can be intracellularly and extracellularly degraded. Numerous aerobic and anaerobic PHA-degrading micro-organisms have been isolated from various environments, including *Aspergillus fumigatus* and *P. lemoignei* from soil, and *Alcaligenes faecalis* and *P. fluorescens* from activated sludge. Further features of PHAs are that they are non-toxic and can be highly crystalline or elastic depending on the monomer composition. They are biodegradable, biocompatible and can be synthesised from waste products (Du and Yu, 2002; Koller et al., 2005). Initially, PHAs were used for packaging purposes as a substitute for conventional non-degradable plastics in the manufacture of shampoo and cosmetic bottles as well as bags and containers for cups and food products (Van der Walle et al., 2001). In the body, PHAs are hydrolysed to naturally occurring mammalian metabolites. Potential medical applications in wound management, implants for tissue engineering and drug delivery exist (Williams et al., 1999; Zinn et al., 2001). The biocompatibility of PHB has resulted in its potential as a degradable implant. Suggested implants include pericardial substitutes after cardiac surgery (Duvernoy et al., 1995), patch materials for repair of defects in the gastrointestinal tract (Freier et al., 2002), scaffolds for neuronal rescue and regeneration after spinal cord injury (Novikov et al., 2002), or suture materials (Volova et al., 2003; Kunze et al., 2006). Altering PHA composition to allow controlled biodegradation rates (Chen and Wu, 2005) lends potential to its use in slow-release drug-delivery systems. The major limitation in the commercialisation of PHAs is their much higher price relative to conventional petroleum-based plastics. Bacterially synthesised PHA is five to ten times more expensive than petrochemical plastics such as polyethylene or polypropylene which cost between US $0.25 and US $0.5/kg to manufacture. However, by lowering the cost of substrates for PHA production, this issue may be addressed. By investigating waste materials as PHA substrates, this project aimed to develop a novel method of PET recycling to divert waste from landfill and incineration and to attempt to address a major cost of PHA production.

2 Studies on the Conversion of Polyethylene Terephthalate to Polyhydroxyalkanoate

The first task of this project was to prove that PET could be converted into the biodegradable polymer PHA. The thermal treatment of PET in the absence of air (pyrolysis) generates TA as the major product. The TA generated is potentially a feedstock for the microbial synthesis of the value-added biodegradable polymer PHA. Consequently, the conversion of PET to a desirable high-value material was investigated. The conversion of PET to a biodegradable plastic had never been demonstrated and few bacteria are known to degrade TA. Of those that do, none have been demonstrated to accumulate PHA and, thus, a contaminated soil was screened to isolate new TA-degrading bacteria capable of accumulating PHA.

2.1 Experimental Section

Polyethylene terephthalate manufactured by Krupp-Formoplast was supplied to a laboratory-scale pyrolysis plant in order to obtain TA for conversion to PHA. The minimal mineral salts medium (MSM), E2, was prepared as previously described (Vogel and Bonner, 1955) and then used, supplemented with TA, as the sole source of carbon and energy for all culture techniques discussed in this chapter. One kilogram of soil sample was collected from PET-exposed soil at an industrial site used to mould PET granules to PET products. It was reasoned that leaching of TA from PET might occur and that this soil would be a good source of TA-degrading bacteria. Dilutions of the soil were spread-plated on solid E2 medium containing 1.1 g/l of TA as the sole source of carbon and energy. Plates were incubated at 30°C for 48 h. Various isolates were selected by visual differentiation of contrasting colony morphology. These isolates were then assessed for their ability to accumulate PHA. Both soil isolates and known TA degraders were grown in shake-flask experiments. To screen for organisms capable of PHA accumulation, the inorganic nitrogen source, sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$), was limited to 1 g/l (67 mg nitrogen). Thirty-two soil isolates and three commercially obtained strains were grown in shake flasks for 48 h and tested for PHA accumulation as previously described (Braunegg and Sonnleitner, 1978). The concentration of nitrogen in the growth medium was monitored as ammonium over time using the previously described method of Scheiner (Scheiner, 1976). The concentration of TA in the medium was monitored by taking 1-ml samples from the culture flask at various time points and analysing by high-performance liquid chromatography (HPLC). Five soil isolates capable of achieving high optical densities, with TA as the sole carbon and energy source, were selected and identified by sequence analysis of 16S ribosomal ribonucleic acid (rRNA) genes. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) and the sequences were determined by GATC-Biotech, Germany. The resulting sequences were searched on the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database. Solution nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), dynamic mechanical analysis (DMA), gel permeation chromatography (GPC) and X-ray diffraction (XRD) were performed on the resulting polymers produced during this section of the project to determine polymer properties.

2.2 Results and Discussion

The pyrolysis of PET resulted in the generation of solid, liquid and gaseous fractions. Seventy-two per cent weight to volume (w/v) of the TA present in PET was recovered as monomeric TA (solid fraction). Oligomers of TA made up almost 26% of the solid fraction. The addition of the solid fraction of PET pyrolysis to a solution of sodium hydroxide resulted in the hydrolysis of the oligomers and increased the proportion of TA making up the solid fraction to 97% (weight to weight) w/w, which was used as a feedstock for biodegradable plastic synthesis by bacteria. Three bacterial strains known to utilise TA as the sole source of carbon and energy for PHA accumulation were tested for their ability to accumulate PHA. However, these strains were incapable of PHA accumulation with TA derived from PET or commercially available TA (control). Since these strains were unable to synthesise PHA, screening for TA-degrading strains from soil was performed.

Thirty-two strains isolated from PET-contaminated soil, with different morphologies growing on TA as the sole source of carbon and energy, were selected for further study. All of these isolates were screened for PHA after growth in shake flasks with limited nitrogen and with TA as the sole source of carbon and energy. Of the 32 isolates screened, only three accumulated detectable levels of PHA. These three organisms were identified using 16S ribosomal deoxyribonucleic acid (rDNA) techniques as *P. putida* GO16, *P. putida* GO19 and *P. frederiksbergensis* GO23. All three strains accumulated PHA to between 23% and 27% of the total cell dry weight (CDW) when supplied with TA either from a commercial source or from the pyrolysis of PET. The PHA levels and composition were identical from both sources. All three organisms were grown in shake flasks under nitrogen-limited conditions with 4.2 g/l of TA generated by PET pyrolysis (Fig. 2.1).

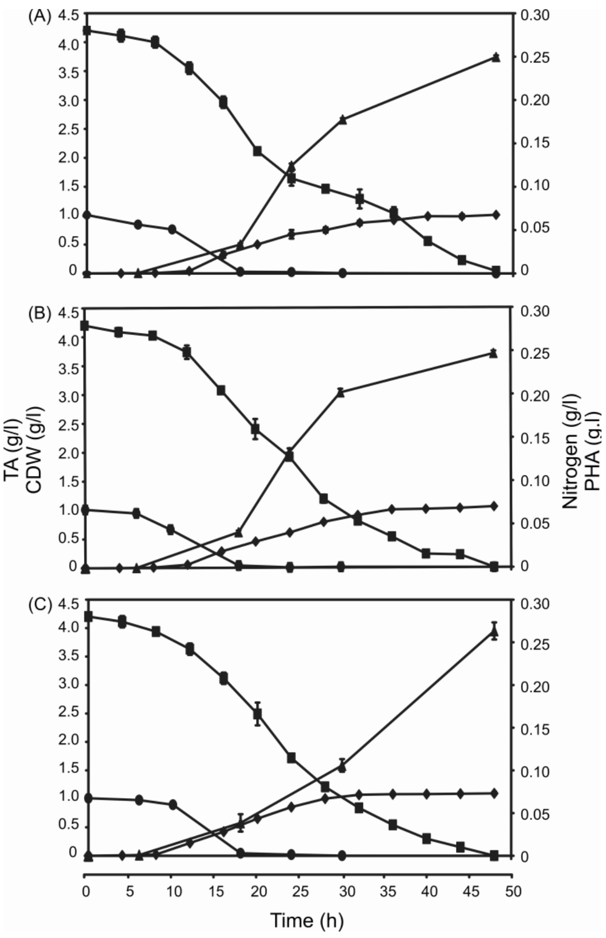

Figure 2.1. Polyhydroxyalkanoate (PHA) accumulation by (A) *Pseudomonas putida* GO16, (B) *P. putida* GO19, and (C) *P. frederiksbergensis* GO23 in shake flasks containing growth medium consisting of 4.202 g/l of terephthalic acid (TA) and 67 mg/l of nitrogen at 30°C. Cell dry weight (CDW) g/l (closed squares), PHA accumulation g/l (closed triangles), TA concentration (closed diamonds) and nitrogen concentration g/l (closed circles) supplied as sodium ammonium phosphate were all monitored over a 48-h period. All data shown are the average of at least three independent determinations.

The ^{1}H-NMR spectra for each PHA derived for GO16, GO19 and GO23 were established. Peak assignments are typical of mcl-PHA derivatives. The ^{13}C spectrum showed chemical shift assignments that are prominently associated with mcl monomer structural units of 3-hydroxydodecenoic acid (3HDDE), 3-hydroxydecanoic acid (3HD) and 3-hydroxyoctanoic acid (3HO). Gas chromatography–mass spectrometry (GC-MS) analysis of the PHA samples confirmed the presence of 3-hydroxyhexanoic acid (3HHX), 3HO, 3HD, 3-hydroxydodecanoic acid (3HDD) and 3HDDE, with 3HD as the predominant monomer. While the sequence homology of 16S rDNA indicated a strong similarity between these bacteria, often closely related species have differing PHA accumulation abilities (Tobin and O'Connor, 2005). Indeed, the PHA composition of all three strains appears extremely similar (Table 2.1). The PHA from *P. putida* GO23 contains a higher proportion of 3HDD (22%) and 3HDDE (21%) compared with PHA from the other two strains. It has been documented that the monomer composition of the PHA dictates the polymer properties (Yoshie and Inoue, 2002) and, while the difference between the three polymers presented in this study may appear small, the PHA polymer isolated from *P. frederiksbergensis* GO23 was physically different compared with plastic from GO16 and GO19. The DSC, TGA, DMA, GPC and XRD results are shown in Table 2.2.

2.3 Conclusion

The goal of this portion of the project was to prove that PET could be converted into the biodegradable polymer PHA. The pyrolysis of PET provided a relatively pure feedstock of TA which when converted to the sodium salt was extremely soluble and thus capable of being supplied as a sole source of carbon and energy. Strains previously reported to grow on TA were tested but none showed PHA accumulation. Bacteria were therefore isolated from soil exposed to PET granules at a PET bottle processing plant. Of the 32 strains isolated, three were capable of accumulation of mcl-PHA from TA as a sole source of carbon and energy and were selected for further study. These isolates were identified using 16S rDNA techniques as *P. putida* (GO16), *P. putida* (GO19) and *P. frederiksbergensis* (GO23). Strains GO16 and GO19 accumulate PHA composed predominantly of a 3HD monomer, while strain GO23 accumulates an even ratio of 3HD and 3HDD. Strains GO16 and GO19 accumulate PHA when nitrogen drops below 15 mg/l after approximately 13 h of growth, while GO23

Table 2.1. Composition of polyhydroxyalkanoate (PHA) accumulated from terephthalic acid.

Bacterial strain	PHA (% CDW)	3HHX	3HO	3HD	3HDD	3HDDE
***Pseudomonas putida* GO16**	27	1	21	48	14	16
***Pseudomonas putida* GO19**	23	1	23	45	14	17
***Pseudomonas frederiksbergensis* GO23**	24	1	14	42	22	21

CDW, cell dry weight; 3HHX, 3-hydroxyhexanoic acid; 3HO, 3-hydroxyoctanoic acid; 3HD, 3-hydroxydecanoic acid; 3HDD, 3-hydroxydodecanoic acid; 3HDDE, 3-hydroxydodecenoic acid.

Table 2.2. Properties of polyhydroxyalkanoate polymer extracted from *Pseudomonas putida* (GO16), *P. putida* (GO19), and *P. frederiksbergensis* (GO23).

Strain	ΔH_m	T_m (°C)	T_G (°C)	MW	MN	Q (MW/MN)	Crystallinity (%)
***Pseudomonas putida* GO16**	12.75	35.36	–53.13	7.43×10^4	3.76×10^4	1.97	26.8
***Pseudomonas putida* GO19**	10.75	34.19	–53.14	12.32×10^4	5.19×10^4	2.37	18.71
***Pseudomonas frederiksbergensis* GO23**	11.78	35.75	–53.38	9.38×10^4	4.4×10^4	2.10	31.09

ΔH_m, melting enthalpy; T_m, melting temperature; T_G, glass transition temperature; MW, molecular weight; MN, molecular number; Q (MW/MN), polydispersity.

accumulates PHA earlier when nitrogen concentration drops below 45 mg/l. Strain GO23 accumulates PHA at a maximal rate for longer than strains GO16 or GO19. The PHA polymers produced by each of these strains were analysed using spectroscopic and thermal techniques. Each of these polymers is thermoplastic, with the onset of thermal degradation occurring at about 235°C. The molecular weight ranged from 74.3 to 123 kDa. X-ray diffraction indicated crystallinity of the order of 18–31%. Thermal analysis showed a low glass transition (–53°C) with a broad melting endotherm between 0° and 45°C.

3 The Conversion of Polyethylene Terephthalate to Polyhydroxyalkanoate through Molecular Manipulation

Having investigated the conversion of PET to PHA using soil isolates the next part of the project aimed to use molecular techniques in order to enhance TA conversion to PHA and to convert TA to PHB. Two distinct strategies were devised to investigate the production of PHB and mcl-PHA from TA. The first strategy was targeted at PHB and involved the cloning of an operon containing the genes necessary for the production of PHB into a selection of organisms known to utilise TA as the sole source of carbon and energy but which screened negative for PHA accumulation. The genes responsible for PHB accumulation in the Gram-negative soil bacterium *Ralstonia eutropha* have been extensively studied. The current study attempted to create a broad-host range plasmid containing these genes and expressing them in the target bacteria. The second strategy was targeted at mcl-PHA and involved two different approaches. The first approach aimed at cloning the genes responsible for TA degradation from *Comamonas testosteroni* (Wang et al., 1995) and expressing these genes in two organisms that have been extensively studied and shown to produce mcl-PHA – *P. putida* CA-3 and *P. putida* KT2440 – but that do not possess the ability to utilise TA as the sole source of carbon and energy. The other approach involved cloning the *phaG* gene from *P. putida* CA-3 which encodes an (*R*)-3-hydroxyacyl-ACP-CoA transferase into *P. putida* GO10, a Gram-negative soil organism isolated on TA as a sole source of carbon and energy during the course of this study. *Pseudomonas putida* GO10 was assayed positive for PHA production from PHA-related substrates but negative from TA. The expression of *phaG* has been shown to either increase PHA accumulation levels in *Pseudomonas* strains or to allow PHA synthesis in strains that were previously incapable of PHA accumulation from unrelated substrates such as gluconate.

3.1 Experimental Section

The strains, plasmids and primers used in this study are listed in Tables 3.1–3.3. Strains were grown in 50 ml of MSM broth, supplemented with 4.2 g/l TA at 30°C, and shaken at 200 rev./min for PHA screening. The growth medium for the recombinants contained the appropriate antibiotic (50 µg/ml). For the purpose of PHA accumulation experiments, the MSM used contained NH_4Cl supplied at a concentration of 0.25 g/l (65 mg N/l). The genomic DNA of three bacteria was extracted as previously described (Nikodinovic et al., 2003). The 16S rRNA genes were amplified by PCR and the sequences were determined by GATC-Biotech, Germany. The resulting sequences were searched on the NCBI BLAST database (Lane, 1991). All general molecular biology techniques were performed following the procedures described by Sambrook et al. (1989). Isolation of plasmid DNA from all strains was carried out using plasmid isolation procedures as described in the QIAprep plasmid preparation kit The primers to amplify the gene clusters from each source are listed in Table 3.3. Polymerase chain reaction was performed using a DNA Engine® Thermal Cycler (Bio-Rad, Hercules, USA).

3.2 Results and Discussion

3.2.1 PHB gene expression

Two strains capable of achieving high optical densities with TA as the sole carbon and energy source but that were unable to accumulate PHA from any carbon source tested were selected and identified by sequence analysis of 16S rRNA genes. Strain GO14 shared 99% homology with a known *Burkholderia glathei* species and strain GO13 shared 99% homology with a known *Pseudomonas* species. Primers PhaC1_HinDIII (F) and PhaB1_BamHI (R) (Table 3.3) were used to amplify *phaCAB* from genomic DNA isolated from *R. eutropha* H16. The cleaned PCR product *phaCAB* fragment was ligated into pJB864 and pJB866 using T4 DNA ligase to

Table 3.1. *Pseudomonas* strains used in this study.

Strain	Relevant characteristics	Source or reference
***Escherichia coli* DH5α**	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *gal*- *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen, USA
***Ralstonia eutropha* H16**	Wild-type strain, source of *phaCAB* genes	Steinbüchel and Schlegel (1991)
***Pseudomonas putida* CA-3**	Wild-type strain	O'Connor et al. (1995)
***Pseudomonas putida* KT2440**	Wild-type strain	Hume et al. (2009)
***Burkholderia glathei* GO14**	Wild-type strain	This study
***Pseudomonas putida* GO13**	Wild-type strain	This study
***Comamonas testosteroni* YZW-D**	Wild-type strain, source of *tph* operon	Wang et al. (1995)
***Pseudomonas putida* GO10**	Wild-type strain	This study
***Pseudomonas jessenii* C8/pPhaG**	Recombinant organism, source of pPhaG vector	Tobin et al. (2007)
***Burkholderia glathei* GO14/pJB866-phb**	Recombinant organism, expressing *phaCAB* from pJB866-phb vector	This study
***Pseudomonas putida* CA-3/pJB861-tph**	Recombinant organism, expressing *tph* from pJB861-tph vector	This study
***Pseudomonas putida* KT2440/pJB861-tph**	Recombinant organism, expressing *tph* from pJB861-tph vector	This study
***Pseudomonas putida* CA-3/pUCP30NT-tph**	Recombinant organism, expressing *tph* from pUCP30NT-tph	This study
***Pseudomonas putida* KT2440/pUCP30NT-tph**	Recombinant organism, expressing *tph* from pUCP30NT-tph	This study
***Pseudomonas putida* GO10/pPhaG**	Recombinant organism, expressing *phaG* from pPhaG	This study
***Pseudomonas putida* GO10/pJB861-phaG**	Recombinant organism, expressing *phaG* from pJB861-phaG	This study

Table 3.2. Plasmids used in this study.

Plasmid	Relevant characteristics	Source or reference
pJB864	Broad-host range, expression under Pm promoter, amp^R	Blatny et al. (1997)
pJB866	Broad-host range, expression under Pm promoter, tet^R	Blatny et al. (1997)
pJB861	Broad-host range, expression under Pm promoter, kan^R	Blatny et al. (1997),
pUCP30NT	Broad-host range, expression under T7 promoter, gm^R	Kang et al. (2007)
pAR1219	Broad-host range co-expression vector under UV5 promoter, amp^R + tet^R	Davanloo et al. (1984)
pPhaG	Broad-host range cloning vector containing 888 bp *phaG* fragment from CA-3, gm^R	Tobin et al. (2007)
pGEMT-Easy	For cloning of PCR products	Promega
pJB864-phb	4.4 kb *phaCAB* fragment from H16 in pJB864, amp^R	This study
pJB866-phb	4.4 kb *phaCAB* fragment from H16 in pJB866, tet^R	This study
pJB861-tph	5.5 kb *tph* fragment from YZW-D in pJB861, kan^R	This study
pUCP30NT-tph	5.5 kb *tph* fragment from YZW-D in pUCP30NT, gm^R	This study
pJB861-phaG	888 bp *phaG* fragment from CA-3 in pJB861, kan^R	This study

Table 3.3. Primers used in this study.

Primer	Relevant characteristics	Source or reference
PhaC1_HinDIII (F)	ATCAACAAGCTTGGCGACCGGCAAAGGCGCGGCAG	This study
PhaB1_BamHI (R)	ACATATGGATCCTCAGCCCATATGCAGGCCGCCGT	This study
tphC_NotI_JB (F)	ATAGCTGCGGCCGCACGCAACGCATCCATTCGCAGACG	This study
tphA1_EcoRI_JB (R)	ATCCACGAATTCCTAATGGTTGCCAGTCGGAT	This study
pUC30NT_NotI ins (F)	ATGCTAGTTATTGCGGCCGCGTGGCAGCAGCC	This study
pUC30NT_ NotI ins (R)	GGCTGCTGCCACGCGGCCGCAATAACTAGCAT	This study
pUC30NT_EcoRI ins (F)	GCCATATCGACGACGAATTCAAGCATATGAACGT	This study
PUC30NT_ECORI INS (R)	ACGTTCATATGCTTGAATTCGTCGTCGATATGGC	This study
phaG_NotI (F)	TTATAGCGGCCGCAGGCCAGAAATCGCTGT	This study
phaG_EcoR1 (R)	TTATTATTAGGATCCTCAGATGGCCAA GC	This study
27F	AGAGTTTGATCMTGGCTCAG	GATC BIotech
1392R	ACGGGCGGTGTGTGTRC	GATC BIotech

generate pJB864-phb and pJB866-phb (Table 3.2). Exhaustive attempts were made to transform GO13, GO14 and YZW-D with pJB864-phb and to transform pJB866-phb into YZW-D. After multiple attempts under different transformation conditions, strain GO14 was successfully transformed. Therefore, only *B. glathei* GO14/pJB866-phb was used for expression studies and PHB screening. After successfully engineering *B. glathei* GO14/pJB866-phb, PHB assays were performed. The PHB assays revealed that the recombinant strain accumulated low levels (4% of CDW) of PHB, while no PHB was detected in the wild type. This represents a very low level of PHB accumulation when compared with other studies expressing *phaCAB* in *Escherichia coli.*

3.2.2 tph gene expression

In order to prepare pUCP30NT for the insertion of the *tph* gene cluster, restriction sites that did not cut the *tph* insert or the vector were successfully engineered using site-directed mutagenesis. Primers tphC_NotI_JB (F) and tphA1_EcoRI_JB (R) (Table 3.3) were successfully used to amplify the *tph* genes from genomic DNA isolated from *C. testosteroni* YZW-D. The cleaned PCR product *tph* was ligated into two vectors using T4 DNA ligase to generate pJB861-tph and pUCP30NT-tph (Table 3.2). These two vectors were transformed into *P. putida* CA-3 and *P. putida* KT2440 to create the mutants *P. putida* CA-3/pJB861-tph, *P. putida* KT2440/pJB861-tph, *P. putida* CA-3/pUCP30NT-tph and *P. putida* KT2440/pUCP30NT-tph (Table 3.1). An attempt was made to transform *P. putida* CA-3/pUCP30NT-tph and *P. putida* KT2440/pUCP30NT-tph with the co-expression vector pAR1219 (Table 3.2) to create the recombinant strains *P. putida* CA-3/pUCP30NT-tph/pAR1219 and *P. putida KT2440/*pUCP30NT-tph/pAR1219 in order to express the T7 polymerase in conjunction with the pUCP30NT vector; however, the pAR1219 plasmid was never successfully re-isolated from the above mutants due to antibiotic resistance issues. After successfully engineering the mutants *P. putida* CA-3/pJB861-tph, *P. putida* KT2440/pJB861-tph, *P. putida* CA-3/pUCP30NT-tph and *P. putida* KT2440/pUCP30NT-tph, all carrying the *tph* gene cluster, growth experiments were attempted using limited nitrogen MSM supplemented with TA and glucose, with induction with *m*-toluic acid or isopropyl-β-D-thiogalactoside (IPTG) to promote the expression of the genes responsible for the TA degradation pathway. All four mutants grew under these conditions and reached biomass levels of between 0.4 and 0.6 g/l; however, no TA utilisation was monitored in any of the experiments. These cells were also assayed for PHA, all of which were negative.

3.2.3 phaG gene expression

One strain capable of achieving high optical densities with TA as the sole carbon and energy source but able to accumulate PHA from related carbon sources only (data not shown) was selected and identified by sequence analysis of 16S rRNA genes. Strain GO10 shared 99% homology with a known *P. putida* species. Primers phaG_NotI (F) and phaG_EcoR1 (R) (Table 3.3) were successfully used to amplify the *phaG* gene from plasmid DNA isolated from *P. jessenii* C8. The cleaned PCR product *phaG* was ligated into pJB861 using T4 DNA ligase to generate pJB861-phaG (Table 3.2). This vector was transformed into *P. putida* GO10 to create the mutant *P. putida* GO10/pJB861-phaG (Table 3.1). The original cloning vector pPhaG was also transformed into *P. putida* GO10 to create the recombinant *P. putida* GO10/pPhaG as a previous study had utilised this vector to enhance PHA production (Tobin et al., 2007). After successfully engineering the mutants *P. putida* GO10/pJB861-phaG and *P. putida* GO10/pPhaG, both carrying the *phaG* gene from *P. putida* CA-3, growth experiments were attempted using limited nitrogen MSM supplemented with TA as the sole source of carbon and energy. Both mutants grew under these conditions and reached biomass levels of 1.4 and 1.05 g/l, respectively, after 48 h. Both recombinants were assayed for PHA production – *P. putida* GO10/pPhaG accumulated mcl-PHA to 3% of CDW, while *P. putida* GO10/pJB861-phaG accumulated PHA to 12% of CDW.

3.3 Conclusions

This portion of the project focused on the conversion of TA to PHA using molecular techniques. In the pursuit of this goal, some of the genes responsible for PHB production in *R. eutropha* H16 were cloned and expressed in a native TA degrader. While some PHB was accumulated by this mutant, the overall levels were low. The genes responsible for TA degradation in *C. testosteroni* YZW-D were cloned and expressed in native PHA-producing organisms. None of these mutants tested positive for TA degradation. The gene which codes for PhaG (an (R)-3-hydroxyacyl-ACP-CoA transferase) in *P. putida* was cloned and expressed in a native TA degrader that had been shown to produce PHA from related substrates (e.g. octanoic acid). Similar to observations made with the PHB accumulating recombinant, these recombinants tested positive for PHA accumulation, again at relatively low levels when compared with wild-type organisms or *phaG* mutants in other studies. While further study on all three of these approaches would be warranted to try to overcome some of the issues and improve the levels of PHA accumulation, it is better to focus on bioprocess improvements for wild-type organisms.

4 Biomass Manipulation to Improve the Polyethylene Terephthalate to Polyhydroxyalkanoate Conversion Process

This study established that PET can be converted into the biodegradable polymer PHA (Chapter 2). The improvement of this process by enhancing the fermentation production was undertaken. Bioprocess manipulation has been successfully employed previously in the production of PHA from a variety of both PHA-related and -unrelated substrates. However, this is the first study to employ TA as the source of carbon and energy in a bioreactor to produce mcl-PHA. Bioprocess manipulation allows for increased control over growth as well as PHA production. Using the bacteria isolated previously in this project, specific feeding strategies were employed in order to increase the biomass and PHA volumetric productivity (g/l/h) over a 48-h period, and to increase the amount of TA that is utilised during this period. In addition to manipulating the feeding of TA, a co-substrate – glycerol – was used to further enhance the process. The objective in these experiments was to increase biomass and PHA productivity and to increase the utilisation of TA

4.1 Experimental Section

Pseudomonas putida GO16 was grown on solid MSM medium supplemented with TA. A culture of *P. putida* GO16 was prepared by inoculating a single colony from MSM/TA plates into 3 ml of Luria Bertani (LB) broth; 1 ml of this culture was then inoculated into 200 ml of MSM broth supplemented with 4.2 g/l TA and 1.8 g/l WG and incubated at 200 rev./min and 30°C for between 18 and 24 h. Two hundred millilitres of preculture were used as the inoculum for the 19.5 l (5 l initial working volume) stirred tank reactor containing 5 l of MSM broth. Fermentations were performed for 48 h at 30°C. The initial concentration of the carbon source in each fermentation depended upon the feeding strategy to be employed. In total six different fermentation strategies with different carbon source combinations were employed (Table 4.1). All fermentation strategies were designed with two distinct phases: In the first phase (24 h), ammonium chloride (NH_4Cl) was in excess to promote growth and, in Phase 2 (24 h), NH_4Cl was limited to promote PHA accumulation. Samples were taken during each fermentation in order to analyse the concentration of TA, glycerol, NH_4Cl, biomass and PHA.

4.2 Results and Discussion

4.2.1 *Fermentation Condition 1 (growth and PHA substrate: TA)*

The first fermentation condition described here involved supplying *P. putida* GO16 with TA alone. As shown in the shake-flask experiments, GO16 accumulates mcl-PHA when supplied with TA as the sole source of carbon and energy when nitrogen availability is limited. Based on these data, a feeding strategy of TA and NH_4Cl was employed to obtain biomass over a 24-h period. For the following 24 h, the NH_4Cl feed was reduced in order to limit the concentration of nitrogen available. Strain GO16 accumulated PHA to 30% of total CDW when grown under these conditions – an increase of 7% from the shake-flask culture. During the biomass accumulation phase, TA was supplied to the medium at a rate of 2.92 g/h, while NH_4Cl was supplied at 0.49 g/h. During the NH_4Cl-limited growth phase (24–48 h), the rates at which TA and NH_4Cl were supplied to the culture were reduced to 1.7 g/h and 0.0075 g/h, respectively. Feeding of both TA and NH_4Cl was designed to prevent accumulation in the medium during the nitrogen-limited growth phase. Both of these feeds were stopped at 46 h to allow complete utilisation of TA in the fermentation. The total amounts of biomass and PHA accumulated from this feeding strategy were 52.2 g and 15.6 g, respectively, which equate to a biomass productivity of 0.18 g/l/h and a PHA productivity of 0.05 g/l/h (Table 4.1).

4.2.2 Fermentation Condition 2 (growth and PHA substrate: glycerol)

The second fermentation condition involved supplying *P. putida* GO16 with WG alone. Initial shake-flask experiments based on those described in Chapter 2 were performed to investigate if GO16 could utilise WG as a sole source of carbon and energy. These experiments were positive for both growth and PHA accumulation (data not shown). Based on these flask results and Fermentation Condition 1, a feeding strategy of WG and NH_4Cl was employed to obtain biomass over a 24-h period, followed by a reduced nitrogen feed for 24 h. Strain GO16 accumulated PHA to 33% of total CDW when grown under these conditions, an increase of 3% from Fermentation Condition 1. During the biomass accumulation phase of growth (0–24 h), WG was supplied to the media at a rate of 6 g/h, while NH_4Cl was supplied at 0.98 g/h. During the first 24 h of the fermentation, GO16 consumed WG at 0.98 g/l/h; during the nitrogen-limited phase of growth (24–48 h), the rates at which WG and NH_4Cl were supplied to the culture were reduced to 4.5 g/h and 0.015 g/h, respectively. Both of these feeds were stopped at 46 h to allow complete utilisation. The total amounts of biomass and PHA accumulated from this feeding strategy were 108 g and 35.64 g, respectively, which equate to a biomass productivity of 0.40 g/l/h and a PHA productivity of 0.13 g/l/h (Table 4.1).

4.2.3 Fermentation Condition 3 (growth substrate: glycerol; PHA substrate: TA)

The subsequent fermentation conditions described here were designed to combine the utilisation of TA and glycerol. In pursuing the co-utilisation of these substrates, it was attempted to increase the amount of TA being utilised and to investigate the effect on monomer composition of the PHAs accumulated when different levels of each substrate were supplied to *P. putida* GO16. Fermentation Condition 3 involved feeding WG and NH_4Cl to obtain biomass over a 24-h period, followed by switching the carbon source to TA and reducing the nitrogen feed for the subsequent 24 h for PHA accumulation. Strain GO16 accumulated PHA to 36.5% of total CDW when grown under these conditions, an increase of 6.5% from Fermentation Condition 1. The growth conditions, including WG and nitrogen feeding, were the same for this fermentation as for Fermentation Condition 2 for the first 24 h. During the nitrogen-limited phase of growth (24–48 h), TA and NH_4Cl were supplied to the culture at rates of 4.5 g/h and 0.015 g/h, respectively. Both TA and NH_4Cl feeds were stopped at 46 h to allow complete utilisation of these nutrients prior to cell harvesting. The total amounts of biomass and PHA accumulated from this feeding strategy were 92.6 g and 33.80 g, respectively, which equate to a biomass productivity of 0.30 g/l/h and a PHA productivity of 0.11 g/l/h (Table 4.1). These totals represent a 1.6-fold increase in biomass productivity and an increase of 15 g of TA utilised from Fermentation Condition 1 but a 1.3-fold drop in productivity from Fermentation Condition 2.

4.2.4 Fermentation Condition 4 (growth substrate: glycerol; PHA substrates: glycerol and TA)

Fermentation Condition 4 involved feeding WG and NH_4Cl to obtain biomass over a 24-h period, as in Fermentation Conditions 2 and 3, followed by co-feeding WG and TA and reducing the nitrogen feed for the subsequent 24 h for PHA accumulation. Strain GO16 accumulated PHA to 35.2% of total CDW when grown under these conditions, an increase of 5.2% from Fermentation Condition 1. The growth conditions, including WG and nitrogen feeding, were the same for this fermentation as for Fermentation Conditions 2 and 3 for the first 24 h. During the nitrogen-limited phase of growth (24–48 h), WG, TA and NH_4Cl were supplied to the culture at rates of 2.25 g/h, 2.25 g/h and 0.015 g/h, respectively. Both TA and NH_4Cl feeds were stopped at 46 h to allow complete utilisation of these nutrients. The total amounts of biomass and PHA accumulated from this feeding strategy were 94.13 g and 33.13 g, respectively, which equate to a biomass productivity of 0.31 g/l/h and a PHA productivity of 0.11 g/l/h (Table 4.1). As expected, due to predetermined carbon availability, the productivity of this fermentation was similar to that of Fermentation Condition 3.

4.2.5 Fermentation Condition 5 (growth substrates: glycerol and TA; PHA substrates: glycerol and TA)

Fermentation Condition 5 involved feeding TA, WG and NH_4Cl to obtain biomass over a 24-h period, followed by co-feeding WG and TA and reducing the nitrogen feed for the subsequent 24 h for PHA accumulation. Strain GO16 accumulated PHA to 35.3% of total CDW when grown under these conditions, an increase of 5.3% from Fermentation Condition 1. The feed rates for this fermentation condition were based on maintaining the same quantity of carbon available as in Fermentation Conditions 2, 3 and 4, while splitting the feed of TA and glycerol over both growth phases. Terephthalic acid, WG and NH_4Cl were supplied at 3 g/h, 3 g/h and 0.98 g/h, respectively, for the first 24 h. During the nitrogen-limited phase of growth (24–48 h), TA, WG and NH_4Cl were supplied to the culture at rates of 2.25 g/h, 2.25 g/h and 0.015 g/h, respectively, in order to deliver the approximate amount of carbon available for PHA accumulation in Fermentation Conditions 2, 3 and 4. All feeds were stopped at 46 h to allow complete utilisation of these nutrients. The total amounts of biomass and PHA accumulated from this feeding strategy were 101.6 g and 35.86 g, respectively, which equate to a biomass productivity of 0.29 g/l/h and a PHA productivity of 0.11 g/l/h (Table 4.1). As with the previous experiments, predetermined carbon availability influenced the productivity of this fermentation, providing similar results to those of Fermentation Conditions 3 and 4.

4.2.6 Fermentation Condition 6 (growth substrates: glycerol and TA; PHA substrate: TA)

Fermentation Condition 6 involved feeding TA, WG and NH_4Cl to obtain biomass over a 24-h period, followed by feeding TA as a carbon source and reducing the nitrogen feed for the subsequent 24 h for PHA accumulation. Strain GO16 accumulated PHA to 36% of total CDW when grown under these conditions, an increase of 6% from Fermentation Condition 1. The feed rates for this fermentation condition were based on maintaining the same quantity of carbon available as in Fermentation Conditions 2, 3, 4 and 5, while removing the glycerol feed for the PHA accumulation phase. Terephthalic acid, WG and NH_4Cl were supplied at 3 g/h, 3 g/h and 0.98 g/h, respectively, for the first 24 h. During the nitrogen-limited phase of growth (24–48 h), TA and NH_4Cl were supplied to the culture at rates of 4.5 g/h and 0.015 g/h, respectively; however, due to a lower level of biomass at 24 h and the absence of WG during the nitrogen-limited phase, a lower rate of TA utilisation was observed. In order to avoid a build-up of TA in the medium and to enable complete degradation within 48 h, the feed rate of TA during the nitrogen-limited phase of growth was reduced to 4 g/h. All feeds were stopped at 46 h to allow complete utilisation of nutrients prior to harvesting of the culture. The total amounts of biomass and PHA accumulated from this feeding strategy were 81.62 g and 30.96 g, respectively, which equate to a biomass productivity of 0.24 g/l/h and a PHA productivity of 0.09 g/l/h (Table 4.1).

4.2.7 PHA monomer analysis

Using nutrient feeding strategies, the amount of TA being utilised was increased in a 48-h fermentation period (Table 4.1). The second rationale was to investigate whether feeding both TA and WG to *P. putida* GO16 over different phases of growth would lead to a variation in the monomer composition of the PHA accumulated. Gas chromatography and GC-MS analysis of the PHA samples confirmed the presence of 3HHX, 3HO, 3HD, 3HDD and 3HDDE in all fermentation conditions, while 3-hydroxytetradecanoic acid (3HTD) and 3-hydroxytetradecenoic acid (3HTDE) were only present in fermentations when WG was supplied (Table 4.2). The PHA from Fermentation Condition 1, where only TA was supplied to GO16, had a similar monomer composition to that found in the shake-flask experiments; however, a 1.3-fold lower level of 3HDDE was detected. In all fermentation conditions in which WG was supplied, higher proportions of 12- and 24-carbon monomers were detected in the PHA compared with PHA from Fermentation Condition 1 when TA was the sole substrate. The difference between these polymers presented in this study require further study; however, isolated polymer samples show noticeable physical differences.

Table 4.1. Growth data from *Pseudomonas putida* GO16 utilising six different carbon feeding strategies.

Growth (biomass) substrate	PHA substrate	Final volume (l)	Final biomass (g)	Total biomass CDW (g/l)	Total PHA (g/l)	Biomass productivity (g/l/h)	PHA productivity (g/l/h)	TA utilised (g)	Glycerol utilised (g)
TA	**TA**	6.00	52.2	8.7	2.61	0.18	0.05	90	0
Glycerol	**Glycerol**	5.70	108	19.06	6.25	0.40	0.13	0	222
Glycerol	**TA**	6.47	92.6	14.3	5.22	0.30	0.11	105	116
Glycerol	**TA and glycerol**	6.25	94.13	15.06	5.30	0.31	0.11	55	165
TA and glycerol	**TA and glycerol**	7.20	101.6	14.11	4.98	0.29	0.10	109	111
TA and glycerol	**TA**	7.00	81.62	11.66	4.42	0.24	0.09	147	61

TA, terephthalic acid; PHA, polyhydroxyalkanoate; CDW, cell dry weight.

Table 4.2. Monomer composition of polyhydroxyalkanoate (PHA) accumulated from six different carbon feeding strategies.

Growth (biomass) substrate	PHA substrate	Final PHA (% CDW)	3HHX	3HO	3HD	3HDD	3HDDE	3HTD	3HTDE
TA	**TA**	30	1	21	48	20	10	ND	ND
Glycerol	**Glycerol**	33	3	18	35	13	15	7	9
Glycerol	**TA**	36.5	2	21	45	20	8	2	2
Glycerol	**TA and glycerol**	35.2	2	17	36	19	14	5	7
TA and glycerol	**TA and glycerol**	35.3	2	20	40	17	14	2	5
TA and glycerol	**TA**	36	2	22	46	22	6	1	1

CDW, cell dry weight; 3HHX, 3-hydroxyhexanoic acid; 3HO, 3-hydroxyoctanoic acid; 3HD, 3-hydroxydecanoic acid; 3HDD, 3-hydroxydodecanoic acid; 3HDDE, 3-hydroxydodecenoic acid; 3HTD, 3-hydroxytetradecanoic acid; 3HTDE, 3-hydroxytetradecenoic acid. TA, terephthalic acid; ND, not determined.

5 The Conversion of Benzene, Toluene, Ethylbenzene and Xylene Compounds by Single and Defined Mixed Cultures to Medium-Chain-Length Polyhydroxyalkanoate

As a result of the pyrolysis of mixed plastics, a mixture of monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *p*-xylene and styrene (BTEXS)) is produced as the main component of the oil fraction generated (Kaminsky and Kim, 1999; Angyal et al., 2007). In conjunction with this, benzene, toluene, ethylbenzene and xylene (BTEX) are widely used in industry as solvents and as starting materials for the production of pharmaceuticals, polymers and paints. Indeed BTEX compounds are among the top 50 chemicals produced and used worldwide. As a consequence of their wide usage, they are common waste materials from the industry. The metabolism of BTEX compounds by micro-organisms is well known, with physiological, biochemical and molecular investigations of their degradation reported (Smith, 1990; Pieper et al., 2004). However, the focus over the last three to four decades has been towards biodegradation of BTEX compounds, and not their conversion to valuable end products. This study viewed BTEX compounds as a potentially valuable feedstock for the biosynthesis of PHA in the same manner as TA from PET pyrolysis. This section of the report describes the accumulation of PHA from single aromatic substrates by single cultures, as well as mixed aromatic substrates by defined mixed cultures.

5.1 Experimental Section

A synthetic mixture composed of BTEXS in a ratio of 16:11:1.5:1:15 was used to mimic the aromatic components of a mixed plastic pyrolysis sample. Reference *Pseudomonas* strains were obtained for their ability to degrade aromatic hydrocarbons. *Pseudomonas putida* F1 (DSM 6899) is a benzene, toluene and ethylbenzene degrader, *P. putida* mt-2 (NCIMB10432) is a toluene and *m*- and *p*-xylene degrader, and *P. putida* CA-3 (NCIMB41162) is a styrene degrader. The utilisation by the isolates of the single BTEX compounds as the sole carbon and energy source was determined by growth on MSM agar plates and supplemented via the vapour phase. Single colonies of *P. putida* strains grown on solid media were transferred to 3 ml of MSM containing 20 mM benzoic acid as the carbon source. Conical flasks containing 50 ml of nitrogen-limited MSM were then inoculated with this culture. The corresponding BTEX compound (350 µl) or a mixture (350 µl) was placed into a central column and flasks were tightly closed. One hundred millilitres of a precultured mixed cell culture were used as the inoculum for the 7.5-l (5-l working volume) stirred tank reactor containing 5 l of MSM. For mcl-PHA accumulation, nitrogen was limited, with a starting concentration of 65 mg/l, and a feeding rate of 1.5 mg/l/h. The BTEXS mix was supplied using the external single channel peristaltic pump at a substrate flow rate of 12.5 µl/min for the first 6 h of the growth. This was increased to 25 µl/min for the remainder of the fermentation. The colony-forming units of various *Pseudomonas* strains when supplied with the synthetic mixed substrate (BTEXS) was determined. The polymer content of lyophilised cells from all experiments was determined as previously described (Brandl et al., 1988). Solution NMR, DSC, TGA, DMA, GPC and Fourier transform infrared spectroscopy (FTIR) were performed on the resulting polymers produced during this section of the project to determine polymer properties.

5.2 Results

Pseudomonas putida strains F1 and mt-2 were examined for their ability to grow and accumulate PHA from a variety of aromatic hydrocarbons (Table 5.1). *Pseudomonas putida* CA-3 is a well-known styrene degrader capable of accumulating PHA from styrene. Given the individual abilities of these strains to accumulate PHA from the various aromatic substrates tested, it is possible that a mixed culture will grow and accumulate PHA when supplied with a mixture of the aromatic compounds. The ability of a defined mixed culture of *P. putida* F1, mt-2 and CA-3 to metabolise a

Table 5.1. Polyhydroxyalkanoate (PHA) accumulation from benzene, toluene, ethylbenzene, xylene, and styrene (BTEXS) by *Pseudomonas putida* strains.

***P. putida* strain**	**Substrate**	**Benzene**	**Toluene**	**Ethylbenzene**	***p*-Xylene**	**Styrene**	**BTEXS mix**
F1	**CDW (g/l)**	0.34 ± 0.03	0.72 ± 0.11	0.67 ± 0.04	NG	NG	
	PHA (g/l)	0.048 ± 0.002	0.16 ± 0.02	0.098 ± 0.001			
	C_8:C_{10}:C_{12}:$C_{12:1}$[1]	17:65:14:4	18:62:16:4	17:64:15:4			
mt-2	**CDW (g/l)**	NG	0.37 ± 0.03	NG	0.53 ± 0.01	NG	
	PHA (g/l)		0.081 ± 0.003		0.14 ± 0.02		
	C_8:C_{10}:C_{12}:$C_{12:1}$		17:63:15:5		16:66:14:4		
CA-3	**CDW (g/l)**	NG	NG	NG	NG	0.79 ± 0.02	
	PHA (g/l)					0.26 ± 0.03	
	C_8:C_{10}:C_{12}:$C_{12:1}$					18:65:11:6	
F1 + mt-2 + CA-3	**CDW (g/l)**						1.03 ± 0.04
	PHA (g/l)						0.25 ± 0.04
	C_8:C_{10}:C_{12}:$C_{12:1}$						16:64:15:4

[1] Traces of C_6 monomer were also detected, but these were not accounted for in the calculation of the total yield of the polymer.

Cell yield is given as cell dry weight (CDW); the PHA yield is calculated by multiplying the cell yield (g/l) by the PHA content (% CDW) of the cells. Monomeric unit composition of PHA is given as percentage ratio of C_8:C_{10}:C_{12}:$C_{12:1}$.

NG, no growth; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; C_{12}, 3-hydroxydodecanoate; $C_{12:1}$, 3-hydroxydodecenoate. All data are an average of three independent measurements.

mixture of BTEXS in a ratio of 16:11:1.5:1:15 was examined with a view to assessing PHA accumulation. The ratio of each substrate to the other was based on the composition of the mixed plastic pyrolysis oil reported by Kaminsky and Kim (1999). The final CDW achieved after 48 h of growth was 1.03 g/l. Twenty-four per cent of the CDW was composed of PHA, representing a PHA productivity of 0.25 g/l (Table 5.1). To determine the fate of each of the individual strains in this mixed culture, samples were taken periodically over the 48-h growth period and analysed for total colony-forming units (TCFU) on LB as well as CFU for each individual strain on selected aromatic carbon substrates (see Experimental Section) (Fig. 5.1A). In order to improve the PHA yield from the BTEXS substrate, the mixed culture was grown in the bioreactor. The BTEXS mixture was supplied as a liquid and a 1.5-fold increase in the level of PHA accumulation and a 3-fold increase in CDW were observed relative to that obtained in the shake-flask experiments (Fig. 5.1B). The PHA levels increased 8.5-fold from time 6 h to time 24 h, with a further 1.8- and 2.1-fold increase over the subsequent 6 and 18 h of incubation, respectively (Fig. 5.1B).

Five grams of mcl-PHA were obtained from the 15-g of lyophilised cells grown in the bioreactor. The ^{1}H-NMR spectrum of the PHA sample was determined to establish the structure of the monomers unambiguously. Peak assignments are typical of mcl-PHA derivatives. Chemical shift assignments for the ^{13}C spectrum of PHA are prominently associated with mcl monomer structural units of 3-hydroxydodecanoate (C_{12}), 3-hydroxydecanoate (C_{10})

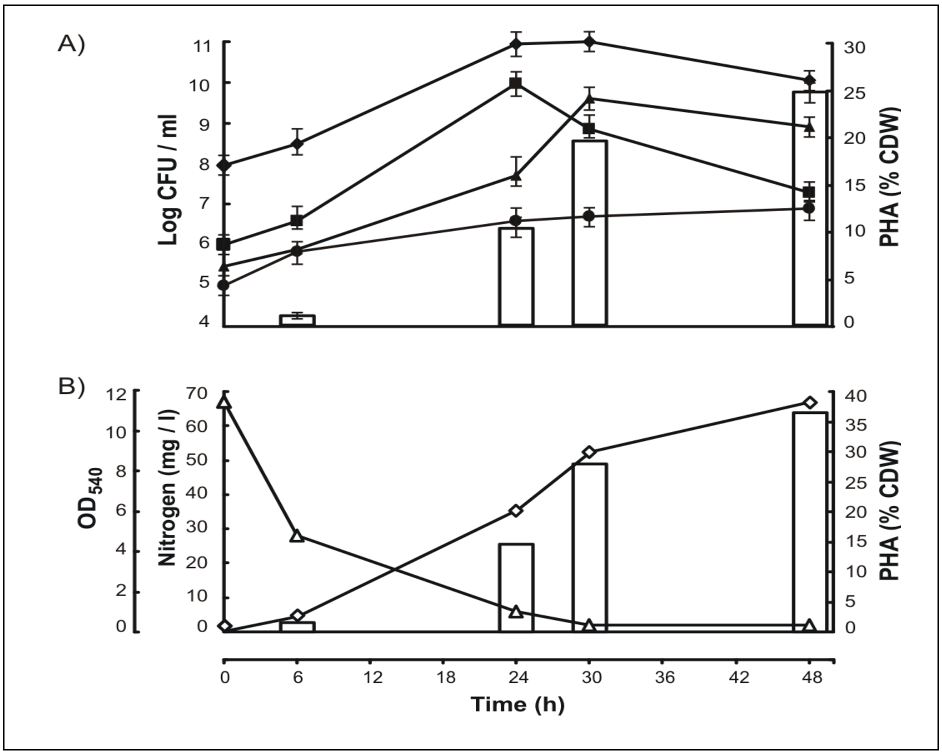

Figure 5.1. Polyhydroxyalkanoate (PHA) accumulation (bars) and mixed culture population analysis when supplied with a mixture of aromatic hydrocarbons in (A) shake flasks and (B) stirred tank bioreactor (fermentor). Total colony-forming units (CFU)/ml (closed diamonds), CFU/ml for *Pseudomonas putida* CA-3 (closed triangles), *P. putida* F1 (closed squares) and *P. putida* mt-2 (closed circles). Mixed cultures were grown for 48 h in minimal mineral salt medium under nitrogen-limiting conditions (65 mg N/l). Nitrogen levels (open triangles) and optical density (OD_{540}, open diamonds) were monitored during fermentation. All shake-flask experiments are an average of at least three independent determinations, while the values in the bioreactor study are an average of two independent determinations. CDW, cell dry weight.

and 3-hydroxyoctanoate (C_8). The FTIR spectrum of the PHA sample is indicative of the typical structure of a highly saturated polyester.

5.3 Discussion

From the research described here, it is clear that the possibility exists to convert a mixed petrochemical waste into an environmentally friendly product, as well as to add value to the recycling (pyrolysis) of petrochemical plastics. *Pseudomonas putida* CA-3A is a well-studied and successful PHA-accumulating bacterium from an aromatic hydrocarbon (styrene) and can accumulate mcl-PHA at up to 27% of the CDW in shake flasks and 43% of the CDW in a bioreactor with a specific nitrogen feeding strategy. Thus, the level of PHA accumulated by *P. putida* F1 (toluene 22%) and mt-2 (*p*-xylene 26%) is very promising as higher PHA and biomass can be achieved in a bioreactor. However, low levels of the PHA accumulation from benzene and ethylbenzene by *P. putida* F1 and from toluene by mt-2 will require further improvement. Having successfully established that *P. putida* F1 and mt-2 were capable of accumulating PHA when supplied with single aromatic hydrocarbon substrates, the ability of these strains to grow and accumulate PHA when supplied with a mixture of aromatic hydrocarbons was assessed. A 350-µl quantity of a BTEXS mixture (ratio 16:11:1.5:1:15) was put in the central glass column of a shake flask containing 50 ml of liquid medium (309.5 mg of substrate was present in the 350-µl BTEXS mixture). A total of 51.5 mg of CDW (1.03 mg/ml) was achieved by the mixed culture supplied with BTEXS. This equates to a growth yield of approximately 0.17 g CDW/g substrate supplied. The supply of volatile substrates in a bioreactor generates much higher cell growth yields due to much more efficient feeding of the substrate to the liquid medium (Ward et al., 2005; Goff et al., 2007) and, thus, higher cell growth yields are predicted when these strains are grown in a bioreactor. In the bioreactor study, when the mixed culture of *P. putida* was supplied with the BTEXS mixture as a liquid (fed-batch), a 1.5-fold improvement in the PHA yield (% CDW) was achieved, which was comparable with a previous study of PHA production from the polystyrene pyrolysis oil (Ward et al., 2006). Furthermore, a 3-fold increase in the biomass yield was also achieved in this study, which resulted in a 4.5-fold overall PHA productivity increase (from 0.25 g/l to 1.16 g/l). The growth yield of 0.27 g CDW/g substrate supplied represents a 1.6-fold improvement in comparison with the shake-flask experiments. The monomer composition of the PHA did not change when the strains were switched from growth on a single substrate to the aromatic mixture. Polyhydroxyalkanoate accumulation from aromatic hydrocarbons proceeds through the fatty acid biosynthetic pathway where 3-hydroxyacyl-CoA intermediates serve as the precursors for the PHA synthesis. It has been shown by Rehm and co-workers (Rehm et al., 1998) that the enzyme responsible for transferring (*R*)-3-hydroxyacyl-ACPs to their corresponding CoA equivalents has a preference for 3-hydroxydecanoyl units. The mcl-PHA polymer obtained from the cells grown in the bioreactor is a partially crystalline bioplastic, as evidenced by the presence of a melting peak (Table 5.2). This was not present in PHA accumulated by *P. putida* CA-3 grown on styrene alone (Ward and O'Connor, 2005; Ward et al., 2005). The low glass transition temperature of the polymer may be attributed to an internal plasticiser effect of randomly placed side chains. However, the molecular weight of the PHA accumulated from BTEXS, although comparable with that obtained from styrene, is relatively low, and the polydispersity is relatively high (Table 5.2) when compared with other mcl-PHAs or synthetic polyesters (Preusting et al.,

Table 5.2. Polymer properties of the medium-chain-length polyhydroxyalkanoate (mcl-PHA) accumulated from benzene, toluene, ethylbenzene, xylene and styrene (BTEXS) by *Pseudomonas putida* strains.

MW (Da)	MN	Q (MW/MN)	T_g (°C)	T_m (°C)	T_d (°C)
86,990	23,110	3.76	–48.46	43.95	350

MW, molecular weight; MN, molecular number; Q (MW/MN), polydispersity; T_g, glass transition temperature; T_m, melting temperature; T_d, decomposition temperature. All values are the means of two independent determinations.

1990; Van der Walle et al., 2001). The authors shall attempt in the future to improve the polydispersity value through optimisation of the PHA extraction method as well as by examining fermentation parameters.

5.4 Conclusion

The objective of this portion of the project was to convert petrochemical aromatic hydrocarbons such as BTEX compounds into biodegradable PHA by bacterial fermentation. In conclusion, the conversion of petrochemical aromatic hydrocarbons (BTEXS) to mcl-PHA by *Pseudomonas* species either as single strains or defined mixed cultures has been demonstrated. Furthermore, it has been demonstrated that the supply of the BTEXS through liquid feeding in a bioreactor improved the growth yield and PHA accumulation rates comparable with shake-flask experiments. As BTEXS compounds are the major components of the oil generated from pyrolysis of mixed plastic, the conversion of mixed plastic waste to value-added mcl-PHA is now possible; however, further bioprocess manipulation is needed in order to improve both biomass and PHA productivity.

6 Conclusions and Recommendations

6.1 Conclusions

In this study, the main achievement was the two-step chemo–biotechnological conversion of the waste plastic PET to the biodegradable polymer, mcl-PHA. This involved isolation of soil bacteria, screening, testing and subsequent bioprocess optimisation to improve the efficiency of the process. A molecular manipulation of TA degradation, as well as PHA and PHB accumulation, showed that the wild-type organisms were better suited to process optimisation than were micro-organism-expressing genes on a plasmid. The application of the TA to PHA technology was also used to convert the petrochemical aromatic hydrocarbons (BTEXS) to PHA. This is the first study to demonstrate the conversion of PET to the biopolymer PHA. The combinatorial approach involved the initial conversion of PET to its predominant monomer form (TA) by means of pyrolysis, and the subsequent utilisation of the TA pyrolysis product to isolate bacteria capable of PHA accumulation under nitrogen limitation. The pyrolysis of PET was carried out for this study by Prof. Walter Kaminsky in Hamburg, Germany (Grause et al., 2004) and provided a relatively pure feedstock of TA – 72% of the TA present in PET is recovered as monomeric TA (solid fraction). Oligomers of TA make up almost 26% of the solid fraction. The addition of the solid fraction of PET pyrolysis to a solution of sodium hydroxide resulted in the hydrolysis of the oligomers and increased the proportion of TA making up the solid fraction to 97% w/w. The resulting TA was supplied as a sole source of carbon and energy. Strains previously reported to grow on TA failed to accumulate PHA. As bacteria capable of growth on aromatic carbon sources have been isolated from soil contaminated with those substrates (Fries et al., 1994; Beller et al., 1996; Andersen et al., 2000; Kim and Jeon, 2009), samples were obtained from soil exposed to PET granules at a PET bottle processing plant. It was reasoned that some TA may have leached into the soil from PET granules visualised in the soil or from the processing operation in the plant adjacent to the site. Bacteria were then isolated from the PET-exposed soil. From the 32 strains isolated, three strains capable of accumulation of mcl-PHA from TA as the sole source of carbon and energy were selected for further study.

Much research of the molecular aspect of PHA accumulation has led to a greater understanding of the bacterial genetics involved in PHA accumulation and has given rise to the notion that genetic manipulation may lead to increased yields of PHA. Some of these techniques were successfully employed in order to produce both PHB and mcl-PHA in soil organisms isolated in this study that can utilise TA but which screened negative for either polymer prior to molecular manipulation. An attempt was also made to express the genes for TA degradation in bacteria known to accumulate PHA.

The well-studied PHB-accumulating bacterium *R. eutropha* H16 has been shown to have 37 PhaA isologs, 15 PhaB isologs, PhaC2 and at least four phasins involved in PHB accumulation (Pohlmann et al., 2006). Despite this, the *phaCAB* gene cluster containing one copy of *phaA*, *phaB* and *phaC* has been shown to be sufficient for transferring PHB accumulation to non-native host strains (Peoples and Sinskey, 1989; Kidwell et al., 1995; Langenbach et al., 1997). In this study, this gene cluster was cloned to a broad-host range expression vector and expressed in *B. glathei* GO14 – a TA-degrading soil organism isolated during this study. The levels of PHB accumulated by this recombinant were low in comparison with previous studies (Schubert et al., 1988; Slater et al., 1988); however, this result represents the first time PHB has been produced using this gene cluster in this species and the first time PHB has been produced from TA. One reason for the low levels of PHB accumulation may be low levels of plasmid expression; in the future, this could be investigated by checking the levels of mRNA produced after induction. Another possible reason for these low levels of PHB accumulation could be strain related; this could be overcome by further attempts to transform the pJB-phb plasmids into other TA degraders.

A number of genes have been implicated in mcl-PHA biosynthesis in *Pseudomonas* species. The mcl-PHA locus, comprising PHA polymerase (*phaC*1 and *phaC*2) as well as PHA depolymerase (*phaZ*), is a well-accepted model for the organisation of these genes in *P. oleovorans* and *P. putida* (Huisman et al., 1991). Regulatory and structural genes further downstream from the PHA locus genes have also been identified (Prieto et al., 1999). Accumulation of PHA from carbon sources structurally unrelated to that of PHA such as glucose proceeds through acetyl-CoA and de novo fatty acid biosynthesis (Eggink et al., 1992; Huijberts et al., 1994; Rehm et al., 1998). An important step in this process is catalysed by 3-hydroxyacyl ACP:CoA transacylase (PhaG) converting (*R*)-3-hydroxyacyl-ACP to its CoA derivative, the substrate for PHA polymerase (PhaC). Expression studies of *phaG* have shown either an increase in PHA accumulation levels from unrelated substrates in *Pseudomonas* strains already producing PHA or novel PHA synthesis in strains that were previously incapable of PHA accumulation from unrelated substrates such as gluconate (Fiedler et al., 2000; Hoffmann et al., 2000; Matsumoto et al., 2000; Tobin et al., 2007). During this study, the *phaG* gene from *P. putida* CA-3 was cloned and expressed in a native TA degrader *P. putida* GO10. This is the first report of the use of a recombinant organism producing PHA from TA; however, as with the work on PHB synthesis in *B. glathei* GO14, the levels of PHA accumulated were low when compared with previous studies where PhaG was employed to alter the PHA-accumulating abilities of an organism (Fiedler et al., 2000; Hoffmann et al., 2000; Matsumoto et al., 2000; Tobin et al., 2007). Further studies could be attempted to improve the *P. putida* GO10/pJB861-phaG mutant by altering the induction conditions; however, large increases in PHA accumulation would be difficult.

The genes responsible for TA degradation in several organisms have been studied (Wang et al., 1995; Junker et al., 1996; Shigematsu et al., 2003a,b; Choi et al., 2005). In these studies, TA was shown to be degraded to the protocatechuate. In this study, the genes described to metabolise the conversion of TA to protocatechuate in *C. testosteroni* YZW-D were cloned into two expression vectors. These vectors were transformed into two protocatechuate-degrading organisms – *P. putida* CA-3 and *P. putida* KT2440 – both known PHA-producing organisms (Hume et al., 2009). No expression of either vector resulted in TA degradation in these strains and, thus, PHA was not produced via this strategy during this study. Many possibilities for the failure of this method may exist and study of the level of expression of any of the *tph* genes would allow for further understanding. Also, an investigation into the process of TA uptake in different organisms may provide a solution. However, further study was not possible within the time constraints of this project.

Given that the wild-type organisms proved to be more robust at PHA production, the bioprocess manipulation focused on these strains. Bioprocess manipulation has previously been successfully employed in the production of PHA from a variety of both PHA-related and -unrelated substrates (Lee et al., 1999; Goff et al., 2007; Sun et al., 2007; Elbahloul and Steinbüchel, 2009). This portion of the project involved the use of fermentation techniques to improve the biological portion of the conversion of PET to the biodegradable polymer PHA. The co-feeding of WG with TA was also attempted to improve biomass and PHA productivity. A number of studies have reported the utilisation of pure and waste glycerol as a feedstock for bacterial growth and for the production of PHA (Ashby et al., 2004, 2005; Cavalheiro et al., 2009; da Silva et al., 2009; Ibrahim and Steinbüchel, 2009). When TA was supplied as the sole carbon and energy source (Fermentation Condition 1), 2.61 g/l of PHA were produced in 48 h compared with 0.25 g/l in flask culture, an increase of 10.5-fold. When WG was supplied in conjunction with TA, further improvements in the amount of PHA were observed, ranging from 4.32 g/l from Fermentation Condition 6 to 5.28 g/l from Fermentation Conditions 3, 4 and 5. This represents up to a 21.1-fold increase in PHA production from initial flask experiments. As a consequence of these improvements, a 5-fold increase in the amount of TA utilised in the 48-h period was also achieved. In Fermentation Condition 1, a total of 15 g/l TA were utilised, 3.5-fold more than was utilised in flasks. In Fermentation Condition 6, the increase was higher, with a 5-fold increase in TA utilisation relative to flask experiments. In conjunction with the improvement in TA utilisation, the addition of a second feed stream to the process provides the opportunity to integrate the recycling of two different wastes. As shown in Table 4.2, the variation in the feeding strategy (ratio of the wastes) affects the monomer composition of the polymer produced, allowing for the possibility to tailor the polymer for a required process. Further fermentation processing with closely controlled exponential feeding strategies could further improve the levels of biomass and PHA accumulated from WG and, thus, increase the amount of both waste streams utilised and converted to PHA.

An added portion of this project involved the conversion of the petrochemical aromatic hydrocarbons (BTEXS) into biodegradable PHA by bacterial fermentation. This process is again based on this study's chemo–biotechnological approach to recycling. As a result of the pyrolysis of mixed plastics, a mixture of the BTEXS compounds is produced as the main component of the oil fraction generated (Kaminsky and Kim, 1999; Angyal et al., 2007). In conjunction with this fact, BTEX compounds are widely used in the industry as solvents and as starting materials for the production of pharmaceuticals, polymers and paints (APA, 2001). In fact, BTEX compounds are among the top 50 chemicals produced and used worldwide (Energetics, 2000). As a consequence of their wide usage, they are common waste materials from the industry (Caprino and Togna, 1998; ATSDR, 2004). The metabolism of BTEX compounds by micro-organisms is well known with physiological, biochemical and molecular investigations of their degradation reported (Smith, 1990; Pieper et al., 2004). Using bioprocess manipulation, this study has demonstrated the conversion of petrochemical aromatic hydrocarbons (BTEXS) to mcl-PHA by *Pseudomonas* species, either as single strains or as defined mixed cultures. Furthermore, it has demonstrated that supply of the BTEXS through liquid feeding in a bioreactor improved growth yield and PHA accumulation rates compared with shake-flask experiments. As mentioned, BTEXS compounds are the major components of the oil generated from pyrolysis of mixed plastic. The conversion of mixed plastic waste to value-added mcl-PHA was demonstrated in this research for the first time; however, due to relatively low yields, further bioprocess manipulation is needed in order to improve both biomass and PHA productivity.

6.2 Recommendations

The continued demand for plastic in multiple aspects of daily life continues to put pressure on waste management infrastructure, which in turn increases the demand for innovative recycling technologies that will remove society's reliance on landfill for waste management.

The conversion of PET plastic to high-value products such as biodegradable plastic is technically feasible. The scaling of the PET to PHA process is the next step in its development. The co-utilisation of glycerol and TA is essential to improve biomass and PHA productivity. This co-feeding strategy also allows the modification of PHA composition which affects the physical properties of the polymer. The integration of multiple technologies and waste streams (waste refinery) needs to occur to improve the opportunities for emerging technologies such as the conversion of PET to PHA. The integration of recycling technologies will improve the economics and offer recyclers multiple market opportunities, e.g. the integration of PET recycling and biodiesel manufacture from waste plant oils in a single site will increase the number of value-added green outputs from the site. The expansion of this approach to include other wastes will lead to an integrated solution dealing with a variety of wastes while reducing the costs of the overall waste refining process. A major limiting factor to the mechanical recycling of petrochemical polymers is the low value of the product. A switch to recycling technologies that produce a high-value product is needed to create a market pull that will increase waste plastic recycling rates. The production of higher-value products will promote recycling within Ireland and reduce the current practice of waste plastic exportation. The latter is part of a 'diversion from landfill' practice which is not recycling and is unsustainable.

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Acronyms

3HD	3-Hydroxydecanoic acid
3HDD	3-Hydroxydodecanoic acid
3HDDE	3-Hydroxydodecenoic acid
3HHX	3-Hydroxyhexanoic acid
3HO	3-Hydroxyoctanoic acid
3HTD	3-Hydroxytetradecanoic acid
3HTDE	3-Hydroxytetradecenoic acid
BD	Biodiesel
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BTEX	Benzene, toluene, ethylbenzene and xylene
BTEXS	Benzene, toluene, ethylbenzene, *p*-xylene and styrene
CDW	Cell dry weight
CoA	Coenzyme A
DMA	Dynamic mechanical analysis
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EU	European Union
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectroscopy
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria Bertani
mcl	Medium chain length
MSM	Mineral salts medium
N	Nitrogen
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium chloride
NMR	Nuclear magnetic resonance

OD	Optical density
PACoA	Phenylacetyl-CoA
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
pH	Minus log of hydrogen ion concentration
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PTFE	Polytetrafluorethylene
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
TA	Terephthalic acid
TCFU	Total colony-forming units
T_d	Decomposition temperature
TEM	Transmission electron micrograph
T_g	Glass transition temperature
TGA	Thermogravimetric analysis
T_m	Melting temperature
US	United States
w/v	Weight to volume
w/w	Weight to weight
WG	Waste glycerol
XRD	X-Ray diffraction

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomhnithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil, Pobal agus Rialtais Áitiúil.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail;
- scardadh dramhuisce.

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

National Development Plan 2007 - 2013

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

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SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Pobal agus Rialtas Áitiúil
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