<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Investigation of polyhydroxyalkanoate production by an activated sludge microbial consortium treating artificial dairy wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>McCullagh, Mary A.</td>
</tr>
<tr>
<td><strong>Publication date</strong></td>
<td>2013</td>
</tr>
<tr>
<td><strong>Type of publication</strong></td>
<td>Doctoral thesis</td>
</tr>
</tbody>
</table>
| **Rights** | © 2013, Mary A. McCullagh  
http://creativecommons.org/licenses/by-nc-nd/3.0/ |
| **Item downloaded from** | http://hdl.handle.net/10468/1182 |

Downloaded on 2018-12-18T11:37:28Z
Investigation of Polyhydroxyalkanoate Production by an Activated Sludge Microbial Consortium Treating Artificial Dairy Wastewater

Mary Mc Cullagh BSc (Microbiology) & MSc (Biotechnology)

A thesis submitted to the National University of Ireland, Cork in fulfilment of the requirements for the Degree of Doctor of Philosophy.

Department of Microbiology

Head of Department: Professor Gerald F. Fitzgerald

Supervisors:
Dr Niall O’Leary
Professor Alan Dobson

April 2013
ACKNOWLEDGEMENTS

I would firstly like to thank my supervisors Dr Niall O’Leary and Prof. Alan Dobson for their constant support and guidance throughout the entirety of my PhD.

To all who helped me in the ERI especially during all my early mornings and late evenings for your patience, support and understanding, I would like to express my sincere gratitude especially to Dr. Jonathan Kennedy, Mark O’Mahony, Christina Forbes, Sharon Lawton, Bill Ryan, Stephen Jackson, Lekha Menon and Burkhardt Flemer.

To the Microbiology Department of UCC, especially Paddy O’Reilly and Dan Walsh, technicians of the UCC Microbiology Department, who helped and guided me with all my enquiries, thank you for your assistance.

I would also like to thank Dr. Wenger, Krisrtina Kuprovskyte and Rob Healy in the Department Chemistry and Dr. Karen Galvin in Department of Nutrition in UCC for all their help with the Gas Chromatography analysis.

I would like to thank my Aunt Charlotte Holland for all her help throughout my PhD

Finally, I would like to thank my family, my Mother and Father, Frances and Liam, my brothers and Sisters, Kate, Robert Sheila, and William, brother in Law, Neil and friends for their encouragement, without your full support this PhD would not have been possible.
ABSTRACT

Petrochemical plastics/polymers are a common feature of day to day living as they occur in packaging, furniture, mobile phones, computers, construction equipment etc. However, these materials are produced from non-renewable materials and are resistant to microbial degradation in the environment. Considerable research has therefore been carried out into the production of sustainable, biodegradable polymers, amenable to microbial catabolism to CO₂ and H₂O. A key group of microbial polyesters, widely considered as optimal replacement polymers, are the Polyhydroxyalkaonates (PHAs). Primary research in this area has focused on using recombinant pure cultures to optimise PHA yields, however, despite considerable success, the high costs of pure culture fermentation have thus far hindered the commercial viability of PHAs thus produced. In more recent years work has begun to focus on mixed cultures for the optimisation of PHA production, with waste incorporations offering optimal production cost reductions. The scale of dairy processing in Ireland, and the high organic load wastewaters generated, represent an excellent potential substrate for bioconversion to PHAs in a mixed culture system. The current study sought to investigate the potential for such bioconversion in a laboratory scale biological system and to establish key operational and microbial characteristics of same.

Two sequencing batch reactors were set up and operated along the lines of an enhanced biological phosphate removal (EBPR) system, which has PHA accumulation as a key step within repeated rounds of anaerobic/aerobic cycling. Influent to the reactors varied only in the carbon sources provided. Reactor 1 received artificial wastewater with acetate alone, which is known to be readily converted to PHA in the anaerobic step of EBPR. Reactor 2 wastewater influent contained acetate and skim milk to imitate a dairy processing effluent. Chemical monitoring of nutrient remediation within the reactors as continuously applied and EBPR consistent performances observed. Qualitative analysis of the sludge was
carried out using fluorescence microscopy with Nile Blue A lipophillic stain and PHA production was confirmed in both reactors. Quantitative analysis via HPLC detection of crotonic acid derivatives revealed the fluorescence to be short chain length Polyhydroxybutyrate, with biomass dry weight accumulations of 11% and 13% being observed in reactors 1 and 2, respectively. Gas Chromatography-Mass Spectrometry for medium chain length methyl ester derivatives revealed the presence of hydroxyoctanoic, -decanoic and -dodecanoic acids in reactor 1. Similar analyses in reactor 2 revealed monomers of 3-hydroxydodecenoic and 3-hydroxytetradecanoic acids.

Investigation of the microbial ecology of both reactors as conducted in an attempt to identify key species potentially contributing to reactor performance. Culture dependent investigations indicated that quite different communities were present in both reactors. Reactor 1 isolates demonstrated the following species distributions *Pseudomonas* (82%), *Delftia acidovorans* (3%), *Acinetobacter sp.* (5%) *Aminobacter sp.*, (3%) *Bacillus sp.* (3%), *Thauera sp.*, (3%) and *Cytophaga sp.* (3%). Relative species distributions among reactor 2 profiled isolates were more evenly distributed between *Pseudoxanthomonas* (32%), *Thauera sp* (24%), *Acinetobacter* (24%), *Citrobacter sp* (8%), *Lactococcus lactis* (5%), *Lysinibacillus* (5%) and *Elizabethkingia* (2%). In both reactors Gammaproteobacteria dominated the cultured isolates. Culture independent 16S rRNA gene analyses revealed differing profiles for both reactors. Reactor 1 clone distribution was as follows; *Zooglea resiniphila* (83%), *Zooglea oryzae* (2%), *Pedobacter composti* (5%), *Neissericeae sp.* (2%) *Rhodobacter sp.* (2%), *Runella defluvii* (3%) and *Streptococcus sp.* (3%). RFLP based species distribution among the reactor 2 clones was as follows; *Runella defluvii* (50%), *Zoogloea oryzae* (20%), *Flavobacterium sp.* (9%), *Simplicispira sp.* (6%), Uncultured *Sphingobacteria sp.* (6%), *Arcicella* (6%) and *Leadbetterella bysophila* (3%). Betaproteobacteria dominated the 16S rRNA gene clones identified in both reactors. FISH analysis with Nile Blue dual staining
resolved these divergent findings, identifying the Betaproteobacteria as dominant PHA accumulators within the reactor sludges, although species/strain specific allocations could not be made.

GC analysis of the sludge had indicated the presence of both medium chain length as well short chain length PHAs accumulating in both reactors. In addition the cultured isolates from the reactors had been identified previously as mcl and scl PHA producers, respectively. Characterisations of the PHA monomer profiles of the individual isolates were therefore performed to screen for potential novel scl-mcl PHAs. Nitrogen limitation driven PHA accumulation in E2 minimal media revealed a greater propensity among isoates for mcl-pHA production. HPLC analysis indicated that PHB production was not a major feature of the reactor isolates and this was supported by the low presence of scl phaC1 genes among PCR screened isolates. A high percentage distribution of phaC2 mcl-PHA synthase genes was recorded, with the majority sharing high percentage homology with class II synthases from Pseudomonas sp. The common presence of a phaC2 homologue was not reflected in the production of a common polymer. Considerable variation was noted in both the monomer composition and ratios following GC analysis. While co-polymer production could not be demonstrated, potentially novel synthase substrate specificities were noted which could be exploited further in the future.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
</tbody>
</table>

## Chapter 1. An Overview of Current Petrochemical Plastic use and the Development of Environmentally Sensitive Biopolymer Alternatives from Microbial Sources

1. Introduction 2
1.1 Biodegradable Polyesters 4
1.2 Chemically Modified Natural Products for the Production of Biodegradable Plastics 7
1.3 Microbial Polymers 12
1.4 Polyhydroxyalkanoates (PHAs) 15
1.5 Commercial Production of Polyhydroxyalkanoates 36
1.6 PHA Production using Mixed Culture 40
1.7 Dairy Processing Industry Wastewater – a High Volume, High Nutrient Waste Source in Ireland 48
1.8 Bibliography 51

## Chapter 2. Sequencing Batch Reactor Set Up and Optimisation for EBPR Associated PHA Accumulation

2.1 Introduction 86
2.2 Material and Methods 90
2.2.1 Reactor Set-up and Operation 90
2.2.2 Reactor Trials 93
2.2.3 Media and Analysis 95
2.2.4 Microscopic Analysis 96
2.2.5 Determination of PHB Production via Fluorescent Microscopy 96
2.2.6 Determination of PHB Production via Crotonic Acid
Conversion and Spectrophotometric Analysis 97

2.2.7 Quantification of PHB Production via High Performance Liquid Chromatography (HPLC) 97

2.2.8 Determination of PHA Composition using Gas Chromatography-Mass Spectrometry 98

2.3 Results and Discussion 101

2.3.1 Trial 1 101
2.3.2 Trial 2 108
2.3.3 Trial 3 112
2.3.4 Quantitative Analysis of PHA 120
2.3.5 Gas Chromatography-Mass Spectrometry 122

2.4 Conclusion 125

2.5 Bibliography 129

Chapter 3. Investigation of the Microbial Ecology of Sequencing Batch Reactor sludge operated under conditions for EBPR derived PHA Accumulation 140

3.1 Introduction 141

3.2 Material and Methods 147

3.2.1 Media for Culture Dependent Isolation Procedures 147
3.2.2 Culture Dependent Investigation of PHA Accumulation 149
3.2.2.1 Restriction Fragment Length Profiling (RFLP) of 16S rRNA Genes from Cultured Isolates 150

3.2.3 Culture Independent Investigations of PHA Accumulation 151
3.2.3.1 DNA Isolation Procedures 151
3.2.3.2 PHA Synthase PCR Primer Synthesis and Application to Nucleic Acid Samples 152
3.2.3.3 Investigation of Microbial Diversity within the Sludge; 16S rRNA Amplification and RFLP Analysis 154

3.2.4 RNA Isolation 154
3.2.4.1 Reverse Transcription and PCR Analysis (RT-PCR) of Sludge RNA 157

3.2.5 Denaturing Gradient Gel Electrophoresis (DGGE) 158
3.2.6 Fluorescent In Situ Hybridization (FISH) 160

3.3 Results and Discussion 165

3.3.1 Culture Dependent Investigations of PHA Accumulation 165
3.3.2 Culture Independent Investigations of PHA Accumulation 173
Chapter 3. Characterisation of PHA Accumulation in the Sludge of SBRs operated Under EBPR Conditions.

3.3.2.1 Investigation of Microbial Diversity within the Sludge; 173
   16S rRNA Amplification and RFLP Analysis

3.3.2.2 PHA Synthase PCR Primer Synthesis and Application to Nucleic Acid Samples 182

3.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis 188

3.3.4. Denaturing Gradient Gel Electrophoresis (DGGE) 190

3.3.5 Fluorescent In situ Hybridization (FISH) 191

3.4 Conclusion 195

3.5 Bibliography 200

Chapter 4. Characterisation of PHA Accumulation in Pure Cultures isolated from SBRs operated Under EBPR Conditions. 225

4.1 Introduction 226

4.2 Material and Methods 228
   4.2.1 Bacterial Cultures and Media 228
   4.2.2 Induction of PHA Production in Liquid Culture and Visualisation via Fluorescent Microscopy 230

4.2.3 Determination of PHB Production using High Performance Liquid Chromatography (HPLC) 231

4.2.4 Determination of Medium Chain Length PHA Production via Gas Chromatography 232

4.2.5 Molecular Investigation of Reactor Isolates for Polyhydroxyalkanoate Synthesis Genes 234

4.3. Results and Discussion 235
   4.3.1 Qualitative Analysis of PHB production of Isolates from Both Reactors using Lipophilic Staining 235
   4.3.2 Induction of PHA Accumulation in Liquid Culture and Fluorescence Microscopy Analysis 237
   4.3.3 Molecular Investigation of Reactor Isolates for Polyhydroxyalkanoate Synthase Gene Homologues 240
4.3.4. Sequencing and Database Comparative Analysis of \textit{pha} Gene Homologues 245

4.3.5 Quantitative investigation of PHB Production using High Performance Liquid Chromatography (HPLC) 250

4.3.6 Determination of PHA Production using Gas Chromatography (GC) 252

4.4 Conclusion 255

4.5 Bibliography 257

\textbf{Chapter 5. General Discussion} 264

5.1 Bibliography 275
CHAPTER 1

An Overview of Current Petrochemical Plastic use and the Development of Environmentally Sensitive Biopolymer Alternatives from Microbial Sources
1. Introduction

Since the 1940s, synthetic plastics have been replacing glass, wood, metals and other constructional materials in many industrial, domestic and environmental applications (Poirier et al., 1995). Table 1.1 presents a list of common petroleum derived plastics, and their applications, which reflect the versatility and durability of these materials. Plastics are now used in almost every industry as their chemical structures can be manipulated to provide a wide range of physical properties and they also offer a high degree of chemical resistance (Ojumu et al., 2004; Zinn et al., 2001). With respect to the scale of plastics production, U.S. figures for 2009 indicate the generation of 13 million tonnes of plastic for containers and packaging, ~ 11 million tonnes as durable goods such as appliances, and almost 7 million tonnes as nondurable goods, e.g. plates and cups. However, such widespread use is not without consequence. In the same year 30 million tonnes of plastic waste was generated globally, representing 12.3% of all municipal solid wastes. Only 8% of the total plastic waste generated in 2010 was recovered for recycling (EPA, 2010). Due to their relatively low density, (~0.9g/cm$^3$), discarded plastics occupy a relatively high volume fraction of municipal landfill ranging from 10-20%. The desirable synthetic polymer properties of durability result in the majority of them being resistant to microbial attack due to the presence of multiple aromatic rings and unusual bonds, e.g. halogenations (Madison and Huisman 1999; Alexander, 1981). Such polymers are extremely stable and do not readily enter into degradation cycles of the biosphere (Shimao, 2001). Incineration of plastics has been applied as a partial solution. However, in addition to being expensive, elimination approaches incorporating incineration also have negative environmental and health consequences due to the release of toxic derivatives such as hydrogen chloride and hydrogen cyanide (Johnstone, 1990; Atlas, 1993, Reddy et al., 2003, Keshavarz and Roy, 2010). As a result there is a keen
interest from both environmental and industrial interest groups to examine alternative polymer sources which circumvent these issues.

**Table 1.1.** Common petroleum derived plastics.

<table>
<thead>
<tr>
<th>Type of plastic</th>
<th>Commercial Names</th>
<th>Some Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylic</td>
<td>Acrylan, Orlon</td>
<td>sweaters, carpets</td>
</tr>
<tr>
<td>Nylon</td>
<td>Cantrece, Antron</td>
<td>clothing, carpet</td>
</tr>
<tr>
<td>Polyacrylic acid</td>
<td>acrylic paint</td>
<td>cars, homes, art</td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>Orlon, Acrilan</td>
<td>clothing, fabrics</td>
</tr>
<tr>
<td>Polybutadiene</td>
<td>rubber, Buna S</td>
<td>automobile tyres</td>
</tr>
<tr>
<td>Polycarbonate</td>
<td>Lexan, Merlon</td>
<td>football helmets</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>Alathon</td>
<td>shopping bags, electrical insulation</td>
</tr>
<tr>
<td>Polyethylene terephthalate (polyester)</td>
<td>Mylar, Dacron, Avisco, Jetspun, Zantrel</td>
<td>soft drink bottles, photographic film, audiotapes, clothing, fabrics</td>
</tr>
<tr>
<td>Polymethacrylate</td>
<td>Lucite, Plexiglass</td>
<td>aircraft windshields</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>Herculon, Vectra</td>
<td>luggage, fabrics</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Styrofoam</td>
<td>foam cups, packaging.</td>
</tr>
<tr>
<td>Polytetrafluoroethylene</td>
<td>Teflon</td>
<td>stain-proof coating on upholstery, non-stick coating on cooking utensils</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>foam rubber</td>
<td>sofa cushions</td>
</tr>
<tr>
<td>Polyvinyl acetate</td>
<td>Vinylite</td>
<td>chewing gum, adhesives</td>
</tr>
<tr>
<td>Polyvinyl chloride</td>
<td>Naugahyde, Koroseal</td>
<td>raincoats, drain pipes, phonograph records</td>
</tr>
<tr>
<td>Polyvinylidene chloride</td>
<td>Saran Wrap</td>
<td>food wrapping</td>
</tr>
</tbody>
</table>
A second significant concern surrounding global reliance on petrochemical polymers is the non-renewable nature of the feedstock. Various opinions have been posited in relation to the reality of current petrochemical reserves, however a consensus is now being reached that global oil production capacities are decreasing (Murray and King, 2012). The precise timing of any future oil crisis is open to debate. However, future polymer production technologies must incorporate this scenario. Therefore, in conjunction with environmental consequences for excessive plastics use and improper recycling/disposal, the need for replacement polymers for the future which can be synthesised from renewable sources is also critical (Braunegg, 1998).

### 1.1 Biodegradable Polyesters

The majority of alternative, “biological” polymers studied to date belong to the polyester family, in which component monomers are bonded via ester linkages. Many kinds of esters occur in nature and enzymes that degrade them are ubiquitous in living organisms. As a result polymers incorporating these bonds offer varying degrees of biodegradability upon disposal in suitable environments. Indeed, several biodegradable polyesters are now produced on a commercial scale via chemical synthesis, such as polyvinyl alcohol, polylactic acid, polycaprolactone and polyglycolic acid (Table 1.2). Polyvinyl alcohol (PVA) is a vinyl polymer in which the main chains are joined by direct carbon-carbon linkages. These linkages are also found in traditional plastics such a polyethylene, polypropylene, polystyrene and water-soluble polymers, such as polyacrylamide and polyacrylic acid. However, among the vinyl polymers produced industrially, PVA is the only one known to be mineralized by microorganisms (Shimao, 2001). Due to PVAs water solubility and thermo-plasticity it can be moulded in various shapes, such as containers and films. PVA can therefore be used to make water-soluble and biodegradable carriers, which may be useful in the manufacture of delivery systems for chemicals such as fertilizers, pesticides and herbicides.
**Poly-Lactic Acid (PLA)**

PLA is a highly versatile biodegradable aliphatic polyester derived from 100% renewable resources such as cork and sugar beet, which is also produced on a commercial scale. As PLA is absorbed in animals and humans, the use of this polymer in medicine has been extensively developed (Ikada and Tsuji, 2000). The only major limitation with this material is the lack of monomer diversity, being either L-lactide or D-lactide, which limits diversity in the physico-chemical properties of the respective polymers. In addition, Polycaprolactone (PCL) is a biodegradable aliphatic polyester generated via ring-opening polymerization of ε-caprolactone from crude oil. The polymer, with a molecular weight of up to several thousands, can be used in the form of a waxy solid/viscous liquid as a polyurethane intermediate, a reactive diluent for higher solid coatings or a plasticizer for vinyl resins. At molecular weights > 20,000, it is used as a thermoplastic polymer with mechanical properties similar to polyethylene. Polyglycolic acid (PGA) was one of the first biodegradable synthetic polymers investigated for biomedical applications. PGA is a highly crystalline polymer which does not stretch or deform easily and displays very low solubility in organic solvents. The polymer has been fabricated into a variety of forms and structures. PGA can be used for medical sutures as well as in the provision of scaffolding matrices for tissue regeneration due to its excellent degradability and mechanical properties supporting host cell attachment and viability.
Table 1.2. Chemically synthesised biodegradable polymers. The use of petrochemical starting materials is a limitation in the environmentally sensitive credentials of these degradable polymers.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (lactic acid)</td>
<td>Lactic acid derived from petroleum, coal, natural gas and other sources</td>
<td>Sutures, drug delivery, vascular grafts, artificial skins and orthopaedic implants (Mayer, 1994) Packaging and consumer goods (Lipinsky and Sinclair, 1986)</td>
</tr>
<tr>
<td>Poly(ε-caprolactone)</td>
<td>Chemical synthesis of crude oil or chemical treatment of saccharides</td>
<td>Thermoplastic polyurethanes, resins for surface coatings, adhesives and synthetic leather and fabrics Stiffeners for shoes and orthopaedic splints and fully biodegradable compostable bags, sutures and fibres</td>
</tr>
<tr>
<td>Polyglycolic acid (PGA)</td>
<td>Glycerol and other sources</td>
<td>Geo-textiles and plant pots, and it is suitable for thermoformed blown bottles and injection moulded objects</td>
</tr>
<tr>
<td>Polyvinyl alcohol</td>
<td>Ethanol</td>
<td>Adhesives or paper coatings, ceramics, in reprography and photography, in medicine and biotechnology and in manufacturing of biodegradable polymer films</td>
</tr>
</tbody>
</table>
1.2 Chemically Modified Natural Products for the Production of Biodegradable Plastics

Agricultural materials are also being investigated as progenitors of replacement polymers and include low cost natural material sources such as starch, cellulose based agricultural products, as well chitin from marine sources and proteinaceous fractions from soybean, milk, etc. A summary of their applications are provided in Table 1.3 and discussed in more detail below.

Starch

Starch is a major plant storage form of glucose, consisting of two primary components amylase, (unbranched), and amylopectin, (branched). There are three main ways that starch can be used for biodegradable polymers production; (i) preparation of starch composites with other plastics incorporating a low amount of starch to enhance the biodegradability of traditional oil-based polymer materials (de Graaf and Janssen, 2000; Ke and Sun, 2000), (ii) preparations of starch composites with starch content (>50% w/w) which produces plastified, (heat softened), starch that exhibits mechanical properties similar to conventional plastics but at a lower price (Bastioli 1998) and (iii) Starch biodegradable polymers generated via the extrusion process by mixing granular starch in the presence of soy protein, glycerol, alginate, lignin, humic substances and urea, ammonium chloride, etc as plasticizers (Otaigbe et al., 1999, Glenn and Hsu, 1997; Glenn et al., 2001). Starch can be applied in four different technological modes that result in a diverse group of products (Table 1.3). Commercially, such blends can reduce the price of synthetic plastics generating inherent industrial interest. In addition, the process increases the biodegradability of the final product, tackling environmental concerns highlighted previously (Burgesscassler et al., 1991; Imam et al., 1992; Breslin and Swanson, 1993; Wool et al., 2000).
However, further development of this application could contribute negatively to ongoing concerns regarding the redirection of food resources to industrial raw materials. Political instability in major oil-exporting regions, coupled with rapid growth in the economies of China, India and other developing countries have increased global fuel demands. One consequence has been the rapid expansion of biofuel production from food crops in the USA, Brazil, Europe and several Southeast Asian countries (Cassman and Liska, 2007). Higher prices for biofuel crops will indirectly raise prices of all major food crops as farmers shift from lower-yield rice and wheat, to higher-yielding and more profitable biofuel crops such as maize and sugarcane. Therefore net grain importing countries and regions that experience acute food shortages are likely to face greater food insecurity challenges in the short term due to reduced surpluses for export and humanitarian aid. An additional dependence on starch based crops as a resource to meet polymer demands would therefore present a further challenge to this scenario.

**Cellulose**

Cellulose is one of the most abundant biopolymers on earth with more than \(10^{13}\) tons estimated to be synthesized each year on the planet (Brown, 1996). It is the major constituent of plant cell walls, and more than half of the organic carbon on earth is bound within cellulosic materials. Cellulose was first isolated over 150 years ago and can be distinguished from other polysaccharides because it is produced by plants, the molecular chain is very long and consists of one repeating unit, (cellobiose). In nature, it occurs in a crystalline state and cellulose is extracted from the cells walls in microfibrils by chemical extraction. In all forms, cellulose is a very highly crystalline, high molecular weight polymer, which is infusible and insoluble in all but the most aggressive hydrogen bond-breaking solvents. Herbivores subsist largely on cellulose, not because they can digest it themselves, but because their digestive tracts contain microbes that produce hydrolytic cellulases (Flieger et al., 2003). Natural
cellulose fibres are low cost, biodegradable and have strong mechanical properties (Kolybaba et al., 2003). Table 1.3 identifies a number of plastics made from cellulose. Biocomposites made completely from biologically renewable resources offer additional benefits to convenient removal after end of life use, i.e. biodegradation, composting and/or carbon dioxide neutral combustion (Riedel and Nickel, 2001). However, the ability to make fuels and other value added products from lignocellulose depends on the ease with which biomass is separated or broken down into cellulose, hemicellulose and lignin. Pre-treatment of lignocellulose is required which involves rupturing the cell wall and breaking the bonds linking lignin to hemicellulose, while decreasing the crystallinity of cellulose (Lynd, 1996; Chandra and Rustgi, 1998; Hahn-Hägerdal et al., 2006). Although a number of lignocellulose pretreatment methods have been developed to improve hydrolysis, they typically require severe reaction conditions, large capital investment, high processing costs and significant investment risks (Alvira et al., 2010; Kumar et al., 2009).

Chitin

Chitin is the second most abundant polysaccharide in nature, with approximately $10^{11}$ tons generated per year (Kurita, 2006). This biopolymer was first identified in 1884 and is synthesised by a large number of organisms. Chitin occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. Chitin is also produced by a number of other living organisms in the lower plant and animal kingdom, serving in many functions where reinforcement and strength are required. In industrial processing chitin is extracted from crustaceans by acid treatment to dissolve calcium carbonate followed by an alkaline extraction to solubilize proteins. A decolourization step is often added to remove leftover pigments and obtain a colourless product. These treatments must be adapted to each chitin source, owing to
differences in the ultrastructure of the initial materials. The resulting chitin needs to be graded in terms of purity and colour since residual protein and pigment can cause problems for further utilization, especially for biomedical products. Partial deacetylation under alkaline conditions produces chitosan, which is one of the most important chitin derivatives in terms of applications (Rinaudo, 2006). Chitosan has been used in edible coatings and films to extend the shelf life of foodstuffs e.g. fruit, meat, and seafood (Venkatrajah et al., 2011). Chitin and chitosan can be degraded by bacterial species such as Actinomycetes, especially the Streptomyces sp. (Itoh et al., 2002; Schrempf, 2001) (Table 1.3).
Table 1.3. Chemically modified natural products for the production of biodegradable plastics.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch composites with low amount of starch (10-20%)</td>
<td>Copolymerization with maize starch</td>
<td>Broad set of starch based ingredients for a wide range of food and especially non-food applications such as biodegradable plastic</td>
</tr>
<tr>
<td>Starch composites with medium amount of starch (40-60%) (Plastified starch materials)</td>
<td>Typically Corn, wheat and potato starch.</td>
<td>Tyres, personal hygiene and disposable medical products.</td>
</tr>
<tr>
<td>Starch composites with high amount of starch (90%) (Thermoplastics)</td>
<td>Corn and other crops</td>
<td>Starch based tubes, compost bags, agricultural mulch films.</td>
</tr>
<tr>
<td>Foamed Starch (100% of starch)</td>
<td>Corn and other crops</td>
<td>Loose fill packaging material, starch-based sheets for thin walled products such as trays and disposable dishes</td>
</tr>
<tr>
<td>Cellulosic biocomposites</td>
<td>Plant cell walls</td>
<td>Wood-replacement panels, decorative interior materials, wood adhesive applications</td>
</tr>
<tr>
<td>Cellulose acetate</td>
<td>Introducing acetyl groups into cellulose (as cotton or wood fibres)</td>
<td>Non-flammable “safety-film” and other coatings applications requiring high melting point, toughness, clarity and good resistance to UV light, chemicals, oils, and greases</td>
</tr>
<tr>
<td>Chitin</td>
<td>Crustaceans</td>
<td>Textile Industry, medicine and in food production.</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Produced from deacetylation of chitin</td>
<td></td>
</tr>
</tbody>
</table>
1.3 Microbial Polymers

As discussed above, many types of biodegradable polymers can be produced from petrochemicals and natural polymeric storage materials. However, certain microorganisms are also known to generate intracellular polymers such as neutral polysaccharides and polyesters such as polyhydroxyalkanoates (PHAs). Furthermore, these materials are often synthesised from unrelated carbon sources via diverse metabolic processes, allowing a number of starting materials to be incorporated.

Neutral Polysaccharides

The use of neutral polysaccharides in food, pharmaceutical and chemical industries has increased steadily during the past decade. They are natural gelling polysaccharides suitable for new biomedical applications (Murano, 2000). Gellan Gum, Pullulan, Laminarin and Curdlan are commercially important for industrial production of biodegradable plastic. Table 1.4 identifies polysaccharide plastics produced by different organisms.

Commercial Applications of Neutral Polysaccharides

The total gelatin market in Western Europe is 60,000 tonnes per year of which 80% is in foods (Jin et al., 2003). Pullulan production at the Hayashibara company in Japan is 300 metric tonnes per year. ABAC GmbH (Switzerland) and Biopolymer Engineering (USA), are the largest suppliers of medical-grade yeast glucan which is used to stimulate the immune system. The preparation of glucan-enriched alimentary fibers, which are now an important product for cholesterol-controlling functional foods was patented by Karinen and Bergelin (1993) (Cho and Dreher 2001). Furthermore a technique for glucan film preparation was patented by Nurture Inc. (USA) (Potter et al., 1999).
The industrial scale production of curdlan commenced in Takeda Chemical Industries Ltd. (Japan) in 1989 and curdlan was approved and commercialized for food usage in Korea, Taiwan, and Japan. In 1996, Pureglucan®, the trade name of curdlan, was launched in the US market as a formulation aid, processing aid, stabilizer, and thickener or texture modifier for food use (Spicer et al., 1999). There has been no reported evidence of any toxicity or carcinogenicity arising from Pureglucan®. At present, curdlan is mostly used in the food industry, although further developments in the field have allowed it to be used in the production of biodegradable plastic for medical applications (Flieger et al., 2003). The range of alternative polymers under development gives a clear indication of the current political, scientific and commercial interest in this field. Despite this, limitations in process economics, range of applications, polymer properties and downstream processing issues have hampered the competitiveness and global uptake of each of these offerings. However, one additional family of bacterial polymers, the polyhydroxyalkanoates, offer a number of environmental, commercial and industrial advantages over the biological/natural alternatives discussed this far.
Table 1.4. Neutral Polysaccharides.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Applications</th>
<th>Companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gellan Gum</td>
<td>Extracellular polysaccharide produced by <em>Sphingomonas sp.</em> (Pollock 1993)</td>
<td>Food industry as a gelling agent in frostings, glazes, icings, jams and media for tissue cultures</td>
<td>Developed and produced by Kelco Biopolymers (Division Merck) under the trade name Kelcogel and Gelrite</td>
</tr>
<tr>
<td>Pullulan</td>
<td>Water soluble, extracellular polysaccharide by certain strains of the polymorphic fungus <em>Aureibasidium pullulans.</em></td>
<td>Film for foodstuffs, food additives or components for cosmetic products</td>
<td>Commercial production of Pullulan began in 1976 by Hayashibara</td>
</tr>
</tbody>
</table>
| Laminarin and curdlan | Laminarin: Mainly, extracted from the cell walls of baker’s yeast (strains of yeast commonly used as a leavening agent in baking bread) which is the by-product in the production of alcoholic beverages (Osumi, 1998)  
Curdlan: Produced by *Alcaligenes sp.* and *Agrobacterium sp.* (Nakata et al., 1998). | Medical-grade yeast glucan-Orally administered to stimulate the immune system. Cholesterol-controlling functional foods, glucan film preparation. Curdlan is used as a formulation aid, processing aid, stabilizer and thickener or texture modifier for food. | ABAC GmbH (Switzerland) Biopolymer Engineering (USA) |
1.4 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHA) are linear polyesters naturally produced by bacteria under specific growth conditions (Byrom, 1987; Katoh et al., 1999; Anderson and Dawes 1990). Lemoigne was the first to report the discovery of PHAs in the form of polyhydroxybutyrate in Bacillus megaterium (Lemoigne 1926). PHAs are polymers of 3-hydroxyalkanoate monomers that accumulate intracellularly as large granules in the cytoplasm of various bacterial species. Accumulation occurs under specific conditions when an essential inorganic nutrient, e.g. nitrogen or phosphorus, is limiting in the presence of excess carbon (Steinbüchel and Hein, 2001). Intracellular PHA granules act as a carbon reserve material and sink for reducing equivalents, without impacting negatively on cellular fitness, e.g. via osmotic stress (Peters and Rehm, 2005). Three hundred different microbial species are currently known to synthesise PHAs (Coats et al., 2007). A biotechnologically significant aspect of PHAs is that they combine excellent biodegradability with physical properties comparable to conventional plastics currently in use. Most synthetic polymers dispersed in the natural environment are resistant to microbial enzymatic attack, although some can be degraded slowly (Steinbüchel, 1992; Pranamuda et al., 1995). In contrast, PHAs can be solubilised by bacteria, algae and fungi present in the environment via extracellular depolymerases, enabling absorption through the cell wall and intracellular catabolism (Holmes, 1985). The rate of biodegradation of PHA depends on environmental conditions like temperature, moisture, pH, nutrient supply and those related to polymer qualities such as crystallinity and surface area (Abe and Doi, 2002). In aquatic environments, PHAs can be readily degraded by various environmental microorganisms, within 3-9 months (Figure 1.1) (Jendrossek, 2001). Furthermore, PHAs can be synthesised from renewable carbon resources based on agricultural and industrial wastes using established fermentation approaches (Serafim et al., 2008). As a result the last three decades have seen considerable research
efforts focused on the development of PHAs for commercial, industrial and medical applications.

**Figure 1.1.** Degradation of P(3HB-3HV) in aerobic sewage sludge. Bottles made of P(3HB-3HV) were incubated at ~20°C in aerobic sewage sludge for 0, 2, 4, 6, 8, and 10 weeks, (from left to right), respectively (Madison and Huismann, 1999).
**PHA Granule Characteristics**

PHAs consist of 3-hydroxy fatty acid monomers, which form linear, head to tail polyesters, (Figure 1.2a). PHA is typically composed of $10^3$ to $10^4$ monomers, which accumulate as intracellular inclusions of 0.2-0.5µm in diameter (Figure 1.2b). These inclusions/granules, are synthesised and stored by both Gram positive and Gram negative bacteria without hazardous effects to hosts (Luengo *et al.*, 2003).

![Figure 1.2. PHA polymer structure. (a) General chemical structure of PHAs. R represents the variable side chain; (b) Transmission electron micrograph of ultrathin section of *Azotobacter chroococcum* cell grown on phenylacetic acid (Nuti *et al.*, 1972).](image)

Each PHA granule is coated with a layer of phospholipids and proteins termed phasins, the predominant biomolecules at the cytoplasm-granule interface. Phasins comprise up to 5% of the total cell protein of PHB accumulating cells and have been shown to influence the size and number of PHA granules (Jossek *et al.*, 1998; Pieper-Fürst *et al.*, 1995; Wieczorek *et al.*, 1996). *In vivo*, the hydrophobic polyester core is largely amorphous, with the water component preventing crystallization (de Koning and Lemstra, 1992, Horowitz and Sanders, 1994). However, PHA is often crystalline when purified using mechanical or enzymatic cell lysis.
Degradation of PHAs

The biodegradability of these polymers is dependent on their chemical and physical properties with melting point being an important factor. As the melting point increases, the biodegradability or the enzymatic degradability decreases. In biological systems PHAs can be degraded under both aerobic and anaerobic conditions by enzymatic hydrolysis and in animal tissues, via a combination of non-enzymatic and enzymatic hydrolysis (Gogolewski et al., 1990). Biodegradation of PHA polymers is also influenced by properties associated with the chemical structure, (e.g. crystallinity, orientation, morphology and hydrophilicity-hydrophobicity balances), and the presence of functional groups (Mochizuki and Hirami, 1997). It has been reported that highly ordered structures, i.e. high crystalline materials, demonstrate lower biodegradability (Nishida and Tokiwa, 1992). In addition to inherent polymer properties, the microbial population in a given environment and the temperature also contribute to biodegradability in situ (Tokiwa and Calabia, 2004). In the environment, PHB is mainly degraded by micro-organisms from the families Pseudonocardiaceae, Micromonosporaceae, Thermomonosporaceae, Streptosporangiaceae and Streptomycetaceae (Tokiwa and Jarerat 2003) with most PHA-producing bacteria inherently possessing the ability to degrade polymers intracellularly. Biomer (Germany) owns the technology to produce P(3HB) from Alcaligenes latus on a large scale. The melted polymers have low viscosity, permitting the injection moulding of objects with thin walls. The end product is very hard and can be used at temperatures from -30°C to 120°C. The product degrades within two months in the environment (Chen, 2005).

With respect to biomedical applications, the biodegradability of PHAs in vivo is of great importance as the rate of degradation of the material should equal the regenerative rate of tissue, in order to be used effectively as scaffolds in tissue engineering applications. There are varying degrees of biodegradability in this regard. Miller and Williams suggest that
P(3HB) and P(3HB-co-3HV) monofilaments implanted in animals did not lose their mass and retained their physical and mechanical properties over a period of 6-12 months (Miller and Williams, 1987). However, it has been reported that P(3HB) films lose their mass by 30-80% within a year (Duvernoy et al., 1995).

*In vitro* studies have investigated the stability of the polymers in model systems using varying values of pH, temperature and salinity. The polymers used in these tests had different compositions, molecular mass and crystallinity and revealed that PHA degradation is multiphasic. PHA involves the biodegradation of amorphous regions eroded by random scission in the first few weeks with polymer chains being disrupted and crystallinity of the polymer increasing. Erosion processes are subsequently initiated, leading to a gradual reduction in polymer mass. The process can occur over 2-3 years and is dependent on environmental circumstances and the physiochemical properties of the PHA (Volova, 2004).

*In vivo* PHAs are also degraded by the enzymes present in blood and animal tissues. PHA-depolymerising activity was studied in calf serum, pancreatin and synthetic gastric juice on P(3HB-co-3HV) microspheres and caprolactone with respective weight loss observed in the following progressive order: bovine serum > pancreatin juice > synthetic gastric juice (Atkins and Peacock, 1996).
Classification of PHAs

PHAs are classified as short chain length (scl), medium chain length (mcl) and long chain length (lcl) based on the respective lengths of the hydroxyalkanoic acid monomers (Steinbüchel et al., 1992). Scl monomer lengths range from C$_3$ to C$_5$ carbons, mcl monomers range from C$_6$ to C$_{14}$ carbons and, lcl monomers consist of hydroxyalkanoates $>$C$_{14}$ (Zinn et al., 2001). Scl and mcl-PHAs arise from different biosynthetic routes and therefore are typically produced by different microorganisms.

Scl-PHAs display a high degree of crystallinity, yielding plastics that are rigid and brittle with a limited range of uses. Polyhydroxybutyrate (PHB) is a well characterised scl-PHA, which has a relatively high crystallinity, melting temperature (T$_m$), stiffness and brittleness (Braunegg et al., 1998). PHB exhibits material properties similar to those of polypropylene, however processing can be difficult given that its melting point, (T$_m$=175°C), is slightly lower than the temperature at which it starts to degrade, (185°C). Scl-PHAs are also produced from diverse monomers of 3-hydroxybutyrate, 4-hydroxybutyrate and 3-hydroxyvalerate. The introduction of other monomer units, such as 3-hydroxyvalerate, enhances the material and physical properties of the resulting plastic (Philip et al., 2007). Therefore, scl-PHAs can be quite mechanically diverse and can be used in different applications such as in bulk packaging, hygiene, agriculture and biomedical devices (Table 1.5). It should be noted that 3-hydroxyalkanoates in general are also biocompatible with mammalian tissue, thus PHAs can be hydrolysed within the body to naturally occurring mammalian metabolites and reabsorbed at a slow rate. Therefore, medical applications of scl-PHA are also being investigated (Lütke-Eversloh and Steinbüchel, 2004). Biomedical grade blends of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) has been processed into vascular grafts and artificial heart valves, and the performance of this material to date is very promising (Philip et al., 2007). Additional uses include surgical sutures, wound dressings,
and slow release drug delivery systems. Finally, PHB is reported to have piezo-electric properties similar to those of natural bone. Therefore, it has potential to be used as biodegradable fixative plates that could stimulate bone formation to support and promote healing of injuries or osteodegenerative conditions (van der Walle et al., 2001).
Table 1.5. Scl-PHA applications.

<table>
<thead>
<tr>
<th>Application</th>
<th>Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Packaging</td>
<td>Single use bottles for shampoo, cosmetics. Containers and cups for food and bags</td>
<td>Doi, 1990; Lauzier et al., 1993</td>
</tr>
<tr>
<td>Agriculture</td>
<td>Mulching films, biodegradable flower pots, one-season irrigation tubes. Biodegradable matrices for the controlled release of plant growth factors (nutrients and fertilizers) or pesticides and herbicides.</td>
<td>White BG, 1983</td>
</tr>
<tr>
<td>Hygienic</td>
<td>Sanitary napkins, diapers, disposable razors and cutlery</td>
<td>Doi, 1990; Lauzier et al., 1993</td>
</tr>
<tr>
<td>Hot Melt Adhesives</td>
<td>Bookbinding, bag ending and carton sealing.</td>
<td>Kauffman et al., 1992</td>
</tr>
<tr>
<td></td>
<td>(based on the copolymer P(3HB-co-HV))</td>
<td></td>
</tr>
<tr>
<td>Coatings in the form of aqueous latex for fibrous materials such as paper or cardboard.</td>
<td>Paper cups and trays for holding food and drinks</td>
<td>Lauzier et al., 1993; Marchessault et al., 1995</td>
</tr>
</tbody>
</table>
**Scl-PHA Biosynthesis**

Poly (3)-hydroxybutyrate (PHB) was the first PHA to be discovered and is the best characterised polyester in the scl-PHA class to date. Various microorganisms such as *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), *Alcaligenes latus* and *Pseudomonas sp.* have been reported to produce PHA by the condensation or modification of acetyl-CoAs from sugars (Figure 1.3). PHB synthesis requires 3 enzymatic steps (Figure 1.3). Two acetyl-CoA molecules are condensed by a β-ketothiolase, (PhaA), into acetoacetyl-CoA, which is subsequently reduced to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetyl-coA reductase (PhaB) (Steinbüchel and Schlegel, 1991). The PHB synthase enzyme, (PhaC), polymerises the (R)-3-hydroxybutyryl-CoA monomers to PHB (Rehm *et al.*, 2003; Stubbe and Tian, 2003). All three enzymes for PHB synthesis are located in the cytosol of the cell where PHB accumulation takes place, (Anderson and Dawes, 1990).

![Figure 1.3](image.png)

*Figure 1.3. Microbial production of PHB polymers using Acetate as the substrate. (Taken from Taroncher-Oldenburg, 2000).*
**Mcl-PHA Biosynthesis**

Medium chain length polyhydroxyalkanoates (mcl-PHAs) were first reported in 1983 (de Smet *et al*., 1983). They consist of 3-hydroxyalkanoic acids with 6 or more carbon atoms (Witholt and Kessler, 1999). Typical members of mcl-PHAs are poly (3-hydroxyoctanoate), and poly(3-hydroxynonanoate), which are often formed as copolymers with 3-hydroxyhexanoate, 3-hydroxyheptanoate and/or 3-hydroxydecanoate (Gross *et al*., 1989). Mcl-PHAs tend to be sticky and elastic materials displaying low crystallinity. The melting points can vary from 40°C to 60°C, with glass transition temperatures in the range of -50 to +25°C (Ren *et al*., 2010, Chung *et al*., 2011).

Three different metabolic routes generate mcl-PHA precursor molecules (Figure 4):

(i) **β-oxidation of fatty acids**

(ii) **De novo** fatty acid biosynthesis, which generates (R)-3-hydroxyacyl-CoA monomers from non-related carbon sources such as glucose and gluconate.

(iii) Chain elongation in which acyl-CoA is extended with acetyl CoA (Witholt and Kessler, 1999).

In the β-oxidation dependent route, fatty acid starting materials are first CoA activated before conversion to trans-enoyl-CoA intermediates. Subsequent enoyl-CoA reductase activity generates the (S)-3-hydroxyacyl-CoA, which is converted by an epimerase to the required (R)-3-hydroxyacyl-CoA monomer. In addition, an (R)-specific enoyl-CoA hydratase, PhaJ, can also act on 2-trans-enoyl-CoA to generate (R)-3-hydroxyacyl-CoA (Fiedler *et al*., 2002; Tsuge *et al*., 2003). The latter enzyme functions as a key supplier of (R)-3-hydroxyacyl-CoA monomers in recombinant bacteria when fatty acids or plant oils are used as carbon sources (Fukui *et al*., 1998; Fukui *et al*., 1999). In the case of non-fatty acid
substrates such as sugars etc, which are oxidized to acetyl-CoA, fatty acid \textit{de novo} biosynthesis produces PHA intermediates, \((R)-3\)-hydroxyacyl-acyl carrier protein, (ACP). A \textit{phaG} encoded \((R)-3\)-hydroxyacyl-ACP-CoA transferase produces \((R)-3\)-hydroxyacyl-CoA monomers and has been established as a key link between \textit{de novo} fatty acid biosynthesis and mcl-PHA accumulation (Hoffmann \textit{et al}., 2000; Hoffmann \textit{et al}., 2002, Rehm \textit{et al}., 1998). In the final step of mcl-PHA biosynthesis, a class II PhaC synthase catalyses the conversion of \((R)-3\)-hydroxyacyl-CoA molecules into mcl-PHAs, while simultaneously releasing CoA (Figure 1.4).
A valuable feature of mcl-PHAs is that polymers with different side chain functional groups can be synthesized by some organisms, including *Pseudomonas oleovorans* and *Pseudomonas putida*. *Pseudomonas* species are capable of producing mcl-PHAs with various side chains such as halogens (Doi and Abe 1990; Kim *et al.*, 1992; Kim *et al.*, 1996), branched alkyls (Hazer *et al.*, 1994), phenyl (Kim *et al.*, 1991; Hazer *et al.*, 1996) olefins (Fritzsche *et al.*, 1990) and esters (Scholz *et al.*, 1994) when grown on substrates containing the corresponding chemical structures (Table 1.6). However, the ability to utilize these carbons sources may differ considerably between strains (Kim *et al.*, 2000). Certain functional groups can also be chemically modified to obtain more useful polymers and extend the potential application of mcl-PHAs as environmentally biodegradable polymers and functional biomaterials for biomedical uses. Gamma irradiation was shown to enhance the tensile properties of mcl-PHA film. The greater the cross linking achieved, the higher the tensile properties although the films also endure chain scission which depolymerises the
polymer films to some extent. Similarly the higher the side-chain olefin concentration the higher the cross linking, therefore the greater the tensile properties (Ashby et al., 1998). Physical modification of mcl-PHA by electron beam-induced cross-linking has also been described in the past, with the rubber-like PHA generated remaining fully biodegradable (de Koning et al., 1994). Mcl-PHAs have the potential to be used in coatings, and in medical temporary implants such as scaffolding for the regeneration of arteries and nerve axons. They have recently been used in latex paints, developed by AZO in the Netherlands and also in vein scaffolding for vascular deficient patients, developed by Metabolix in Boston (Ren et al., 2010).
Table 1.6. Mcl-PHAs with various side chains (Table modified from de Roo et al., 2002).

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Hydroxyalkanoic acid monomer</th>
<th>Monomer name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olefin/Alkene</td>
<td><img src="image1.png" alt="Image" /></td>
<td>3-hydroxy-5,7-tetradecadienoic acid</td>
</tr>
<tr>
<td>Branched alkyls</td>
<td><img src="image2.png" alt="Image" /></td>
<td>3-hydroxy-5-methyloctanoic acid</td>
</tr>
<tr>
<td>Epoxy</td>
<td><img src="image3.png" alt="Image" /></td>
<td>3-hydroxy-7-epoxyoctanoic acid</td>
</tr>
<tr>
<td>Halogen</td>
<td><img src="image4.png" alt="Image" /></td>
<td>3-hydroxy-9-fluorononanoic acid</td>
</tr>
<tr>
<td>Phenyl</td>
<td><img src="image5.png" alt="Image" /></td>
<td>3-hydroxysebasic acid benzylester</td>
</tr>
<tr>
<td>Ester</td>
<td><img src="image6.png" alt="Image" /></td>
<td>3-hydroxysebacic acid methylester</td>
</tr>
</tbody>
</table>

Recently a PHA copolymer has been produced consisting of 3-hydroxybutyrate (3HB) and a small amount of medium chain length monomers with side groups of at least 3 carbon units or more (Noda et al., 2005). The mcl-PHAs monomers incorporated consisted of 3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydecanoate. There are several different grades of copolymers possible depending on the average molecular weight, the average mcl-
3-hydroxyalkanoate (3HA) content within the copolymer and the side chain length of the chosen mcl-3HA unit. Commercial copolymers are currently available, e.g. Nodax from Proctor and Gamble is employed in foams, fibers or nonwovens, films and latex and can be used in bulk packaging, flushable hygiene products, agricultural films and medical applications (Noda et al., 2005). There are several different grades of copolymers available within the Nodax family, depending on the average molecular weight, the average mcl-3HA content within the copolymer and the side group chain length of the chosen mcl-3HA unit. The simplest form of this class of copolymer is the Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHx) copolymer comprising of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) units. Other 3HA units such as 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) are also available to form various copolymers comprising 3HB and one or more mcl-3HAs. The unique molecular structure of these co-polymers gives useful properties that traditional PHA polymers like PHB or Polyhydroxybutyrate-co-hydroxyvalerate (PHB-co-HV) do not offer. These include high durability and ductility, coupled with thermal properties similar to those of polyethylene (Noda et al., 2005). The flexibility of the copolymer is determined by the length of the side group chain of the mcl-PHA. Procter and Gamble are further developing the polymer to incorporate anaerobic and aerobic degradability, hydrolytic stability and elastic and mechanical properties to suit specific application needs (Noda et al., 2005).
Polyhydroxyalkanoate Synthases

While multiple metabolic routes may contribute to the provision of monomers for PHA synthesis, polymer generation is dependent on the activity of specific synthases. PHA synthases catalyse the condensation of (R)-3-hydroxyacyl-coenzyme A monomers to PHA and free CoA. The PHA synthase operon was first cloned from Cupriavidus necator (Schubert et al., 1988; Slater et al., 1988; Peoples and Sinsky 1989 a, b). However, nucleotide sequences of 58 PHA synthase genes from 45 different bacteria have since been generated (Rehm and Steinbüchel, 1999). The predicted primary structures generated from these sequences, together with varying substrate specificities among encoded enzymes resulted in four major classes (I-IV) of PHA synthases being established. In summary, class I and class II PHA synthases comprise enzymes consisting of only one type of subunit, PhaC (Qi and Rehm 2001). Class I PHA synthases preferentially utilize CoA thioesters of various (R)-3-hydroxy-fatty acids comprising 3 to 5 carbon atoms, thus giving rise to commercially relevant, scl-PHA homo- and copolymers, such as Poly-3-hydroxybutyrate and poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) produced by Cupriavidus necator (Nomura and Taguchi, 2006). Class II PHA synthases preferentially utilize (R)-3-hydroxy CoA substrates comprising 6 to 14 carbon atoms (Slater et al., 1988 and Schubert et al., 1988). Mcl-PHAs are commonly produced by various Pseudomonads. Pseudomonas putida have the ability to produce both homo- and co-polymer of hydroxy -hexanoic, -octanoic, -decanoic, dodecanoic and tetradecanoic acid (Steinbüchel and Gorenflo, 1997). Pseudomonas oleovorans can produce copolymers of hydroxy -hexanoic, -octanoic and -decanoic acid (Kim et al., 1997). Class III and IV PHA synthase enzymes consist of two different types of subunits. PhaC proteins are involved in both strains, which exhibit amino acid sequence similarity of only 21-28% to class I synthases. In contrast, Class III synthases have a PhaE subunit which does not display any apparent similarity to class I or II PHA synthases (Liebergessell et al., 1992;
Yuan et al., 2001). In Class IV PHA synthases PhaE is replaced by PhaR (McCool and Cannon, 2001). Class III and IV synthases typically utilise CoA thioesters of (R)-3-hydroxy fatty acids with 3 to 5 carbon atoms. However there are a few exceptions to this rule. The synthase from Thiocapsa pfennigii demonstrates approximately 85% amino acid sequence identity to Class III PHA synthase PhaC subunits. However it also exhibits, broad substrate specificity, polymerising CoA thioesters of both short chain length and medium chain length 3-hydroxy fatty acids (Rehm and Steinbüchel, 1999). Similarly, the synthase from Aeromonas punctata hosts a phaC subunit with 45% sequence similarity to class I counterparts. The A. punctata synthase catalyses co-polyester formation between 3-hydroxybutyrate and 3-hydroxyhexanoate. The generation of hybrid class III PHA synthases by interchanging the phaE and phaC genes from Aeromonas vinosum and Thiocapsa pfennigii indicated that the PhaC protein subunit mediates the substrate specificity (Lieberggesell et al., 2000).

**Organisation of PHA Biosynthesis Genes**

The PHA synthase genes and genes for other proteins related to the metabolism are often clustered in the bacterial genomes (Figure 1.5) (Rehm and Steinbüchel 1999; Rehm and Steinbüchel, 2001). In Cupriavidis necator the genes for class I PHA synthase (phaC), β-ketothiolase (phaA) and NADP-dependent acetoacetyl-CoA reductase (phaB) comprise the phaCAB operon (Steinbüchel and Schlegel; 1991, Slater et al., 1988; Schubert et al., 1988; Peoples and Sinskey, 1989 a, b). Although the operon organisation within Cupriavidis necator is conserved among other PHB-accumulating bacteria, some bacteria show a different gene order but the synthase genes are still co-localized with other PHB biosynthesis genes (Rehm and Steinbüchel, 2001).
All *Pseudomonads* which accumulate mcl-PHAs possess two different *phaC* genes encoding class II synthases, which are separated by the structural *phaZ* gene encoding an intracellular PHA depolymerase. In addition, downstream of the synthase gene arrangement, the *phaD* gene (encoding a structural protein with unknown function) is located, followed by the genes *phaI* and *phaF* which are transcribed in the opposite direction. The latter genes encode structural and regulatory proteins (Rehm, 2003). In all bacteria possessing a two component synthase, *phaC* and *phaE* are directly linked in their genomes and most probably constitute a single operon.

**Role of Metabolic Flux**

It has been shown that metabolic networks and carbon flux play a central role in PHA production. It is well known that PHB synthesis is regulated at the enzymatic level (Senior and Dawes, 1971) and that the intracellular concentration of acetyl-CoA and free coenzyme A play a central role in the regulation of polymer synthesis (Haywood et al., 1988). PHB synthesis is initiated by both high intracellular concentrations of NAD(P)H and high ratios of NAD(P)H:NAD(P) (Lee et al., 1995). NADH and NADPH prevents citrate synthase activity. This suggests that PHB accumulation is enhanced by facilitating the metabolic flux of acetyl-CoA to the PHB synthetic pathway and that citrate synthase is a potentially important control point in the whole PHB synthesis process based on its ability to control the availability of CoA (Henderson and Jones, 1997), which regulates the activity of 3-ketothiolase. Studies with recombinant strains showed that the biosynthesis rate of PHB is controlled by 3-ketothiolase and acetoacetyl-CoA reductase, whereas the content of PHB is controlled by PHB synthase (Jung et al., 2000). Metabolic modelling approaches to PHB biosynthesis in *R. eutropha* have been performed in which those factors and regulatory aspects have been investigated (Leaf and Srienc, 1998; Yoo and Kim, 1994). PHB synthase, posttranslationally
activated by acetyl phosphate have been suggested (Miyake et al., 1997). Furthermore, a second enzyme involved, called phosphotransacetylase, which converts acetyl CoA to acetyl phosphate is regulated by the acetyl CoA concentration and carbon to nitrogen ratio (C:N) in the cell. Therefore, acetyl phosphate could act as a signal of C:N balance affecting PHB metabolism (Miyake et al., 1997).

A number of cellular metabolic enzymes have been manipulated in attempts to improve PHA yields in various bacterial species. It has been shown that overexpression of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase redirected catabolic flux through the pentose phosphate pathway resulting in elevated NADPH/NADP⁺ ratio, which favoured PHA accumulation to levels approximately 41% higher than recombinant E. coli harbouring only the PHB synthesis operon from Ralstonia eutropha (Lim et al., 2002). Research by Hong et al (2003) also investigated the role played by the Entner-Doudoroff pathway in PHB synthesis and demonstrated reduced PHB accumulation in an eda deficient mutant lacking 2-keto-3-deoxy-6-phosphogluconate aldolase. In addition to specific metabolic enzymes, signal transduction systems have also been shown to play a role in the detection of extracellular conditions which induce PHA accumulation and the co-ordination of central metabolic pathways involved. The arc system in E. coli, which regulates the expression of several operons, relative to the redox state of the environment, has previously been manipulated to improve E. coli as a host strain for PHB biosynthesis (Nikel et al., 2006). In addition, arc gene mutants demonstrate the expression of TCA cycle components under micro-aerobic conditions, elevating levels of reducing equivalents, which can be channelled into PHB production
**PHA Depolymerases**

Many bacteria and fungi have the capability of producing PHA depolymerases, which act on native PHA granules or denatured PHA (Jendrossek and Handrick, 2002; Jendrossek, 2007). Two different PHA depolymerases exist, extracellular and intracellular. Intracellular PHA depolymerases are produced when the required nutrients are supplied to the medium and actively degrade the endogenously stored native (amorphous) PHA. Such PHA depolymerases degrade PHB in the cell to give 3-hydroxybutyric acid. A dehydrogenase acts on 3-hydroxybutyric acid by oxidising it to acetylacetate followed by a β-ketothiolase acting on acetylacetate to break it down to acetyl-CoA. The β-ketothiolase enzyme plays an important role in both the biosynthetic and the biodegradation pathways. Under aerobic conditions, the acetyl-CoA enters the citric acid cycle and is oxidised to CO₂ (Jendrossek, 2001). Very little is known about the intracellular depolymerases since they are always found to be intimately connected to the PHB granules and the overall process is very complex (Tokiwa and Calabia, 2004; Jendrossek, 2001; Merrick and Doudoroff, 1964). Extracellular PHA depolymerases, (i.e. carboxyesterases), have the capability to hydrolyze the water insoluble PHA to water soluble monomers (Holmes, 1985, Jendrossek and Handrick, 2002, Tokiwa and Calabia, 2004). The PHA depolymerase enzymes act on the polymer mainly by hydrophobic interactions and degradation by these depolymerases initially produces oligomers. The resulting soluble products are then absorbed through the cell walls and utilised (Doi, 1990). Some microbes produce an additional dimer hydrolase which further breaks the oligomers into the corresponding monomer (Tanaka et al., 1981). Various bacterial and fungal species from diverse ecosystems have been reported to degrade PHAs extracellularly, and include isolates from soil, compost, sewage sludge fresh and marine water including deep sea and estuarine sediments. Recent reports have added *Gracilibacillus* and *Enterobacter* species as contributors to PHA degradation (Volova et al., 2011).
Figure 1.5. Molecular Organization of Class I PHA synthase genes (Rehm, 2003).
1.5 Commercial Production of Polyhydroxyalkanoates

Development of Pure Culture Based Processes

PHB was the only recognised PHA for almost five decades until in 1974 a number of additional 3-hydroxy fatty acids were identified in activated sludge (Wallen and Rohwedder, 1974). In the 1970s, Imperial Chemical Industries (ICI) sought to commercialise PHAs, and several bacteria were investigated as potential production organisms. Originally, methylotrophic species were chosen as methanol was a low cost substrate and ICI had considerable experience of fermentations with methanol utilisers. However, the quantity of polymer produced per cell was inadequate and its molecular weight was too low for the intended applications. Azotobacter was subsequently assessed as it was microbiologically well understood as an industrial production species. However, the studied strain was unstable and secreted polysaccharides, an undesirable by-product directly impacting on the yield of PHA. Finally, Ralstonia eutropha was chosen to produce high molecular weight PHB on fructose under nitrogen and phosphate limiting conditions and was shown to be able to accumulate PHB up to 80% of cell dry weight (Byrom, 1987).

The use of recombinant strains for cost-effective PHA production has also been investigated. Key objectives of the approach have focused on achieving rapid growth, high cell density, expanded substrate use, and low cost polymer purification (Vandamme and Coenye, 2004). In this manner, PHA production levels of up to 80-90% cell dry weight have been achieved in recombinant Escherichia coli cells harbouring R. eutropha PHA synthesis genes (Kim et al., 1992). Table 1.7 presents a list of strains studied together with the respective carbon sources and polymers produced. Examining table 1.7 it is clear that Alcaligenes eutrophus, (now Cupriavidus necator), produces the highest amounts of scl-PHA (up to 88% w/v) using various carbon sources.
Table 1.7. Bacterial strains involved in PHA production using different carbon sources.

(Table adapted from Reddy et al., 2003).

<table>
<thead>
<tr>
<th>Bacterial strain(s)</th>
<th>Carbon source(s)</th>
<th>Polymer(s) produced</th>
<th>PHA content (%w/v)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>Gluconate, Propionate, Octonaote</td>
<td>PHB, PHB, PHB</td>
<td>46-85, 26-36, 38-45</td>
<td>Liebergesell et al., 1994</td>
</tr>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>Sucrose</td>
<td>PHB</td>
<td>88</td>
<td>Wang and Lee 1997</td>
</tr>
<tr>
<td><em>Bacillus megaterium QMB1551</em></td>
<td>Glucose</td>
<td>PHB</td>
<td>20</td>
<td>Mirtha et al., 1995</td>
</tr>
<tr>
<td><em>Klebsiella aerogenes recombinants</em></td>
<td>Molasses</td>
<td>PHB</td>
<td>65</td>
<td>Zhang et al., 1994</td>
</tr>
<tr>
<td><em>Methylobacterium rhodesianum MB 1267</em></td>
<td>Fructose/Methanol</td>
<td>PHB</td>
<td>30</td>
<td>Ackermann and Babel, 1997</td>
</tr>
<tr>
<td>M. extorquens (ATCC55366)</td>
<td>Methanol</td>
<td>PHB</td>
<td>40-46</td>
<td>Borque et al., 1995</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Euphorbia/caster oil</td>
<td>PHA</td>
<td>20-30</td>
<td>Eggink et al., 1995</td>
</tr>
<tr>
<td><em>P. denitrificans</em></td>
<td>Methanol, Pentanol</td>
<td>P(3HV), P(3HV)</td>
<td>0.02, 55</td>
<td>Yamane et al., 1996</td>
</tr>
<tr>
<td><em>P. oleovorans</em></td>
<td>Gluconate, Octonoate</td>
<td>PHB, PHB</td>
<td>1.1-5, 50-68</td>
<td>Liebergesell et al., 1994</td>
</tr>
<tr>
<td><em>Pseudomonas putida GPp104</em></td>
<td>Octonoate</td>
<td>PHB</td>
<td>14-22</td>
<td>Liebergesell et al., 1994</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>Palm Kernel Oil, Lauric acid, Myristic acid, Oleic acid</td>
<td>PHA, PHA, PHA, PHA</td>
<td>37, 25, 28, 19</td>
<td>Tan et al., 1997</td>
</tr>
<tr>
<td><em>P. putida BM01</em></td>
<td>11-Phenoxyun-decanoic acid</td>
<td>5POHV</td>
<td>15-35</td>
<td>Song and Yoon, 1996</td>
</tr>
<tr>
<td><em>Sphaerotilus natans</em></td>
<td>Glucose</td>
<td>PHB</td>
<td>40</td>
<td>Takeda et al., 1995</td>
</tr>
</tbody>
</table>
Economic Considerations

The high costs related to high cost of feedstocks and aseptic reactor operation limit the potential replacement of petroleum-based thermoplastics with PHAs (Lee, 1996). In addition to high substrate costs for pure culture fermentations, negative economic impacts are also imparted by; elevated oxygen demands due to high cell concentrations, monitoring and process control requirements and maintenance of sterility in reactor vessels (Shilpi and Srivastava, 2005). There have been few publications on the cost of production of mcl-PHAs. The technology used is very similar to scl-PHAs whose economics have been well studied (Choi and Lee, 1999 a, b). The price of PHA has been reduced to about $4.90 kg$^{-1}$ due to process optimization, growing competition on the PHA market and larger scale production facilities (Lunt, 2008). Despite this, the costs are still unsustainably higher than that of petroleum based plastics such as polypropylene and polyethylene, costing less than $1 Kg^{-1}$ (Philip et al., 2007). The majority of PHA production has involved pure culture fermentation processes. *Cupriavidus necator* is the most studied and frequently used bacteria for PHA production. However, these are expensive processes and there is a major challenge to reduce their production costs (Nath et al., 2008). One of the main production costs is the carbon source price and the use of wastes and by-products as raw material for the fermentation process represents a key goal to improve process economics (Castilho et al., 2009). Fermentations based on pure glucose generate costs of up to US$1.30/kg PHB, whereas crude/waste carbons sources such as whey, the substrate contribution lowers production costs to US$0.22/kg PHB. Therefore, even the most efficient processes for PHA production will not allow PHA to compete with petroleum plastics if expensive substrates such as glucose are employed. However, PHA content and production are usually lower for bacteria grown in crude, inexpensive substrates and the development of efficient processes based on cheaper carbon sources such as agro-industrial by-products and wastes, remains an ongoing challenge.
(Salehizadeh and Van Loosdrecht, 2004). Inexpensive carbon sources which have been used to date include molasses, sucrose, starch-based materials, cellulosic materials, hemicellulosic sugars, whey-based culture media, culture media based on oils, fatty acids, glycerol, gaseous substrates, organic matter from wastes and wastewaters (Castilho et al., 2009). PHA production from organic matter from waste and wastewaters can be achieved using either pure (wild-type or recombinant) or mixed cultures. A PHA content of 72.6% has been reported in Cupriavidus necator fed with fatty acids from food scrap digestion. The PHA produced was a copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV) (Du et al., 2004). An alternative production approach currently being investigated by a number of groups is the use of mixed cultures for PHA synthesis.

**Pollutant Conversion to PHA**

Styrene is a major toxic environmental pollutant with millions of kilograms of styrene released into the environment each year as industrial effluents. It can also affect people by respiratory tract irritation, central nervous system depression, muscle weakness, and narcosis in humans and other mammals used a substrate for PHA production. Styrene can be converted to (PHA) by Pseudomonas putida CA-3. Therefore the biodegradation of styrene have been extensively researched (O’Leary et al., 2005; Ward et al., 2005; O’Leary et al., 2001) P. putida CA-3 is capable of the complete mineralization of styrene. It does so by epoxidation of styrene and isomerization of the epoxide to phenylacetaldehyde, which is further oxidized to phenylacetic acid (O’Connor et al., 1995). Phenylacetic acid is converted to phenylacetyl-coenzyme A (CoA), which is further oxidized to acetyl-CoA (O’Leary et al., 2002, 2001). The link between de novo fatty acid synthesis and PHA monomer accumulation was investigated, and a functionally expressed 3-hydroxyacyl-acyl carrier protein-CoA transacylase (phaG) gene in P. putida CA-3 was identified. The deduced PhaG amino acid
sequence shared >99% identity with a transacylase from *P. putida* KT2440, involved in 3-hydroxyacyl-CoA MCL-PHA monomer sequestration from de novo fatty acid synthesis under inorganic nutrient-limited conditions. Similarly, with *P. putida* CA-3, maximal *phaG* expression was observed only under nitrogen limitation, with concomitant PHA accumulation. Thus, β-oxidation and fatty acid de novo synthesis appear to converge in the generation of MCL-PHA monomers from styrene in *P. putida* CA-3. (O’Leary *et al*., 2005).

### 1.6 PHA Production using Mixed Culture

The use of inexpensive substrates (i.e. crude carbon substrates from industrial food or agricultural wastes) and mixed cultures, (i.e. activated sludge), have previously been proposed (Braunegg *et al*., 2004; Reis *et al*., 2003; Salehizadeh and Van Loosdrecht, 2004). In general, mixed cultures are microbial populations of unknown composition, such as activated sludge from wastewater treatment plants. In such environments, microbial species are selected for based on the operational conditions of the biological system and the composition of the influent to the system. (Salehizadeh and Van Loosdrecht, 2004; Chua *et al*., 2003; Reis *et al*., 2003; Dionisi *et al*., 2004; Dionisi *et al*., 2006). The application of mixed culture, waste treatment systems for PHA accumulation could potentially mitigate 50% of the costs associated with pure carbon substrate based synthesis and 30 - 40% of the costs associated with aseptic operational demands of current approaches to microbial production of PHAs (Akiyama *et al*., 2003; Braunegg *et al*., 2004). The use of mixed cultures for PHA synthesis brings a number of advantages relative to pure culture applications; (i) the process can be carried out without the need for high cost, sterile reactor systems, (ii) continuous cultures can be maintained without the economic risks of culture contamination and (iii) mixed populations provide for greater diversity of carbon source utilisation (Dionisi
et al., 2005). As PHA accumulation in mixed cultures is not solely dependent on inorganic nutrient deficiencies, the PHA production process can be performed with nutrient-rich, undefined media. The production of PHAs by mixed cultures has recently been reviewed (Dias et al., 2006; Reis et al., 2003, and Salehizadeh and Vans Loosdrecht 2004, Kleerebezem and van Loosdrecht, 2007).

Routes to PHA Production by Mixed Cultures

Aerobic Dynamic Feeding (ADF)

PHA storage by activated sludge can occur under fully aerobic conditions when the population is submitted to consecutive periods of substrate excess (feast), alternated with substrate limitation (famine) (Majone et al., 1996). The process is referred to as “aerobic dynamic feeding” (ADF), or the “feast and famine” approach. The need for physiological adaptation due to the lack of sufficient amount of enzymes or RNA required for growth is considered the most probable mechanism for storage of PHA by mixed cultures subjected to aerobic feast and famine conditions (Daiger and Grady 1982; Anderson and Dawes 1990; Krishna and van Loosdrecht, 1999). Thus, in the presence of excess external carbon substrate, metabolism is predominantly directed toward PHA storage, while biomass production is supported to a lesser extent. Mixed culture PHA production from paper mill white water has been reported previously via a three stage process involving; acidogenic fermentation, culture enrichment for PHA accumulators and fed-batch experiments delivering feast-famine conditions (Bengtsson, et al., 2008). Acidogenic fermentation is necessary as it has been reported that carbohydrate excess can inhibit PHA accumulation in ADF systems. Feast-famine exposure has been carried out under aerobic conditions, at medium-high org: (0.0015), yielding PHA to 48% dry cell weight, comprising of PHB and polyhydroxyvalerate
Serafim and co-workers reported pulse-feeding of acetate to an enriched sludge to achieve a PHB content of up to 68.5% cell dry weight (Serafim et al., 2004). At such values, mixed culture systems begin to approach commercial PHB production by pure producer strains, although careful comparisons of culture densities is required before generalised approximations can be made and economical impacts inferred. Under dynamic substrate feeding (feast-and-famine process), P(3HB) contents of 67% (Beccari et al., 1998) and P(3HB-co-3HV) contents of up to 62% (Dionisi et al., 2001) have been obtained from sludge. Furthermore, aerobic dynamic substrate feeding with pulse acetate addition obtained a high polymer content of 78.5% (Serafim et al., 2004).

**Microaerophilic-Aerobic Process**

A microaerophilic-aerobic process has also been suggested for mixed culture PHA synthesis (Satoh et al., 1998, and Takabatake et al., 2000). Oxygen limiting conditions, (microaerophilic exposure), have also been shown to diminish the growth capacity of microorganisms, resulting in carbon uptake going to PHA production (Satoh et al., 1998). The Microaerophilic–aerobic process involves supplying a limited amount of oxygen to the anaerobic zone of anaerobic–aerobic operation, where sodium acetate is provided as the primary organic substrate. Under such conditions, microorganisms can take up organic substrates and derive energy through oxidative degradation of the organic fraction. If the supply of oxygen is excessive, assimilative activities such as the production of protein and other cellular components will develop. However, if the supply of oxygen is adequately controlled, the assimilative activity will be suppressed and substrates directed to intracellular PHA accumulation. By using these conditions, PHA accumulators are selected regardless of the ability of the microorganisms to accumulate poly-P or glycogen, and the selected PHA accumulators will have a lower tendency to accumulate glycogen (Satoh et al., 1998).
maximum PHA content of 62% was achieved using this process, with acetate as the carbon
source and a 30 hour incubation period.

**Enhanced Biological Phosphorus Removal (EBPR)**

Nitrogen and phosphorus discharges are the primary cause of surface water
eutrophication events, having the capacity to promote excessive growth of algae (Danalewich
*et al.*, 1998). Chemical precipitation has been the main method used for the removal of
phosphate from wastewater streams (Stratful *et al.*, 1999). Chemical removal techniques,
using metal salts are reliable and well established processes. The three main metal salts
available for phosphorus removal are calcium, aluminium and iron. Calcium and aluminium
are less commonly applied. Although iron salts are not the most efficient precipitant, they are
relatively inexpensive. Iron (II) and iron (III) are the most commonly used although iron (II)
is the most attractive because of its cost benefits. One of the problems is the additional
quantities of chemical sludge as it needs to be handled, treated and disposed of to sustain the
Biological Phosphorus Removal (EBPR) has been successfully employed as an alternative to
chemical precipitation for the removal of phosphorus (Brdjanovic *et al.*, 1998; Mino *et al.,*
1998). In 1974, Wallen and Rohwedder reported that PHA production was a feature of
biological phosphorus removal by mixed cultures in wastewater treatment plants (Wallen and
Rohwedder, 1974). EBPR is achieved when an activated sludge plant mixes influent
wastewater with returned sludge (biomass), in a process consisting of alternating anaerobic
and aerobic cycles. In the anaerobic phase the bacteria take up carbon sources, mainly
volatile fatty acids (VFAs) and store them in the form of PHAs and in the aerobic phase,
stored PHA is used as an energy source to enable orthophosphate uptake (Figure 1.6). PHA is
known to play an important role in mixed cultures especially anaerobic processing where
electron donor and acceptor availability are separated (Satoh *et al.*, 1998). The sludge P
content in full scale EBPR processes can reach levels of 4-5% of the sludge dry weight, while some lab-scale EBPR enrichment processes have reported up to 15% P (Crocetti et al., 2000).

In an EBPR process the key microbial species required are known as Polyphosphate Accumulating Organisms (PAOs). Under anaerobic conditions, PAOs take up organic substrates (preferably volatile fatty acids-VFAs) and store them as PHA. The reducing equivalents needed are provided by glycolysis of internally stored glycogen (Mino et al., 1987; Arun et al., 1988; Satoh et al., 1992, Smolders et al., 1994 a, b). The energy for PHA storage is obtained partly from glycogen utilization but mostly from the hydrolysis of intracellularly stored polyphosphate (polyP), resulting in orthophosphate release into solution. In the aerobic phase, PAOs take up excessive amounts of orthophosphate to recover the intracellular polyphosphate levels, using the oxidation of stored PHA as an energy source. Meanwhile, they also grow and replenish the glycogen pool using PHA as both carbon and energy sources (Arun et al., 1988; Pereira et al., 1996). Net phosphorus removal is achieved by wasting sludge after the aerobic period when the biomass contains a high level of polyphosphate. It should be noted that glycogen accumulating organisms, GAOs, can also be present, which compete with the sludge for substrate (Seviour et al., 2003). However, recent work has suggested that well known PAOs such as Candidatus accumilibacter, may also produce glycogen at the expense of PHAs under varying conditions (Acevedo et al., 2012). Although, many studies have been carried out to examine the function and composition of EBPR microbial communities, (Lemos et al., 1998; Pijuan et al., 2009). limited studies have been carried out on the recovery of PHA using this system. PHB accumulations up to 57% mixed liquor suspended solids (MLSS) have been reported, although unstable production conditions were indicated (Takabatake et al., 2000). Rodgers and Wu (2010) and Kasemsap and Wantawin (2007) also reported PHB contents of 50% and 51% PHB and PHV, respectively in laboratory based EBPR systems. Although, the PHA yields were lower than
those of pure cultures, they demonstrate the ability to harvest PHA from mixed culture systems.

However, problems can be encountered with biological phosphorus removal as the BPR process is sensitive to disturbances such as dilution of the wastewater, e.g. in times of heavy rainfall, with prolonged disturbances leading to recovery times of over 4 weeks. After periods of low organic carbon loads, the effluent phosphate is significantly increased on the following 1–2 days. This results in the average phosphate load in the effluent increasing by about 60% due to this effect. Changes in the influent organic composition from VFAs to sugars, such as glucose, may favour the increased presence of glycogen accumulating organisms (GAOs). Furthermore, VFAs are essential for effective BPR process optimisation. VFAs can also be produced on-site with low operational costs and no storage or handling problems, making them an attractive choice as a nutrient removal carbon source.
<table>
<thead>
<tr>
<th>Requirements of EBPR</th>
<th>Effects EBPR System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Availability of potassium, magnesium and calcium</td>
<td>Both potassium and magnesium are simultaneously required and neither was adequate by itself for EBPR</td>
</tr>
<tr>
<td>Temperatures</td>
<td>The growth rates double with approximately every 10 °C increase in temperature until the optimum temperature is reached.</td>
</tr>
<tr>
<td>DO levels greater than 2 mg/l.</td>
<td>The anaerobic zone must be kept with a lack of oxygen (0.0–0.2 mg/l oxygen) as the presence of oxidising substances such as oxygen and nitrate will interfere with the BPR process while maintaining an oxygen concentration of between 3.0 and 4.0 mg/l in the oxic zone has been recommended.</td>
</tr>
<tr>
<td>The ratio of phosphorus to total organic carbon (P/TOC)</td>
<td>Limited phosphate loadings may lead to less PAOs proliferating leading to the establishment of GAO with PAO in an SBR under P-rich loading conditions and GAO dominating under limited P loading conditions.</td>
</tr>
<tr>
<td>The pH of a combined BNR system</td>
<td>Optimal nitrification occurs between pH 7.5 and 9.0. If alkalinity is insufficient, the pH value may fall below pH 7 with which the rate of nitrification decreases as pH values fall, becoming zero at approximately pH 6.0. The pH optimum for denitrification appears to be between pH 7.0 and 8.0. Fluctuation in pH is one of the many characteristics, which lead to sludge “bulking” conditions in BNR systems highlighting that the rate of P-release under anaerobic conditions was increased as the pH was increased</td>
</tr>
<tr>
<td>Filamentous Bulking</td>
<td>Filamentous organisms multiply to such an extent as to interfere with the proper compaction of settling sludge. In general, the sludge settleability index declines with the introduction of nutrient removal, but among nutrient removal plants the best settling characteristics are found in plants with biological P-removal and the poorest in plants that perform simultaneous denitrification.</td>
</tr>
</tbody>
</table>
Figure 1.6. Mechanism of EBPR for PHA accumulation. (a) Anaerobic phase in an EBPR system; (b) Aerobic phase in an EBPR system.
1.7 Dairy Processing Industry Wastewater – a High Volume, High Nutrient Waste Source in Ireland

A number of industrial processing wastewaters have been examined to date as alternative substrates for PHA production with mixed cultures, including swine waste liquor, palm oil mill effluents, vegetable and fruit wastes, olive oil effluents and sugar cane molasses (Hassan et al., 1997a, b; Meesters 1998; Reis et al., 2003; Salehizadeh and van Loosdrecht, 2004; Dionisi et al., 2005; Albuquerque et al., 2007). Significant work has also been carried out to date with dairy wastewater using recombinant and non-recombinant microorganisms (Janes et al., 1990; Lee et al., 1997; Wong and Lee 1998; Ahn et al., 2000; Ahn et al., 2001; Park et al., 2002; Yellore and Desai 1998; Povolo and Casella, 2003). Maximum PHB accumulations achieved using recombinant E. coli has been over 90% of cellular dry weight using shake-flask cultures in whey based media (Janes, 1990). In a separate study, 85% cellular dry weight of PHB was obtained in recombinant E. coli strains in whey-based medium without additional complex nitrogen sources (Lee et al., 1997). A stable high-copy-number plasmid, pSYL105, containing the A. eutrophus PHA biosynthesis operon and plasmid pSYL107, which is pSYL105 containing the E. coli ftsZ gene, have been described previously was used in the above study (Lee et al., 1994). In studies with non-recombinant microorganisms, a Methylobacterium sp. was shown to accumulate PHA when grown up on pure lactose of up to 59% of its dry weight (Yellore and Desai, 1998). The above studies seemed to indicate that lactose was a better carbon substrate than whey. Furthermore, recombinant E. coli was shown to produce greater amounts of PHA than non-recombinant microorganisms.

Dairy industry processing is a major wastewater generator in the agri-food sectors of many countries. The most common sources in dairy industry are pasteurized milk, milk and whey powders, cheese, butter, evaporated milk, ice cream, yoghurt and wastewater from
associated processes such as cleaning and washing of floors, bottles, crates, vehicles, and the cleaning-in-place (CIP) of factory equipment, and tanks, as well as the inside of tankers (Steffen et al., 1989). Dairy wastewaters are more concentrated than domestic sewage effluents, containing high concentrations of organic material such as proteins, carbohydrates, and lipids. These wastewaters are also characterised by suspended solids (SS), high biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogenous compound contents, and large variations in pH. Treatment demands in this industry are rigorous as improperly remediated plant effluents can drastically alter the nutrient profiles of receiving water bodies and cause devastating pollution events. The three main treatment options for dairy industry are (1) levied discharge of wastewater to municipal treatment facilities; (2) removal of semisolid and special wastes from the site by waste disposal contractors; (3) construction of onsite treatment facilities (Robinson, 1994; Gough and McGrew, 1993). The principle concern of most dairy waste treatment plants is cost, comprising either high treatment levies charged by local authorities or the inherent capital requirement for on-site treatment facilities. These problems can be compounded by increasing stringencies in SS, BOD and COD allowable discharge limits, exceeding treatment capacities of municipal systems (Robinson, 1994). Therefore, an increasing amount of dairy industries, certainly the majority of those in Ireland, are forced into onsite wastewater treatment. When choosing the treatment option, it should meet the required demands and reduce the costs associated with long-term industrial wastewater discharge.

In the Irish dairy industry, nitrogen and phosphorus nutrient remediation by biological processes have been investigated in the past (Mulkerrins et al., 2004 a, b). An EBPR based approach was successfully employed. An assumed aspect of this process was the accumulation of PHA by PAO species; however no investigation of the capacity for PHA accumulation was investigated using the EBPR process. Given the sheer scale of dairy
effluent production globally, the outlets for a biotechnological approach to waste organic carbon load conversion to commercially relevant biodegradable polymers are considerable. Indeed, the raw material for dairy processing, milk, is composed of ~5% lactose, ~4% fats, ~3% protein and less than 1% ash. The fat content is largely made up of saturated C₄ - C₁₄ fatty acids, whose inherent availability gives rise to the potential for novel, medium chain length PHA synthesis, not traditionally achieved in ADF reactor systems operated on short chain VFAs (Serafim et al., 2008). The study presented herein presents the author’s research efforts to investigate the feasibility of such a mixed culture, for the microbial conversion of the organic fraction of the dairy wastewater to PHA, facilitated via the EBPR reactor operational model.

Hence, the primary objective of this project was to optimise PHA accumulation under EBPR conditions using dairy wastewater in lab-scale sequencing batch reactor (SBR). This was carried out with activated sludge from a dairy processing plant using varying operational parameters and differing COD:N:P ratios. Once PHA accumulation was observed in the SBRs the microbial ecology was analysed in the SBRs using culture dependent and independent molecular methods. This analysis sought to correlate operational parameters and COD:N:P ratios with alterations in the microbial community and concomitant PHA production profiles. Finally, qualitative and quantitative analysis was carried out on pure cultures isolated from the SBRs that showed PHA production. The molecular and chemical analyses performed in this chapter sought to determine whether any of the microorganisms present in the SBR had the ability to produce high yields of PHA and also whether the reactor media selected for cultures capable of producing novel PHAs.
1.8 Bibliography


http://sepuplhs.org/pdfs/Earths_Resources_Transparency_16_1.pdf


Volova, T.G., A.N. Boyandin, A.D. Vasiliev, V.A. Karpov, and I.V. Kozhevnikov. 2011 Biodegradation of polyhydroxyalkanoates (PHAs) in the South China Sea and identification of PHA-degrading bacteria. Microbiology. 80, 252-260


CHAPTER 2

Sequencing Batch Reactor Set Up and Optimisation for EBPR Associated PHA Accumulation
2.1 Introduction

PHA production using mixed culture systems has received considerable attention recently, both academically and industrially. The use of renewable waste materials and activated sludge for polymer synthesis can reduce process costs by up to 50% when compared with pure culture aseptic reactor operation and optimised substrate costs (Serafim et al., 2004). In the past decade, there has been considerable research in this field (Lemos et al., 2003; Dias et al., 2005, 2006; Dionisi et al., 2006; Serafim et al., 2006, 2007). Key areas of focus have included process configuration, reactor optimisation, process modelling, microbial ecology characterisation and novel polymer synthesis. A number of different strategies have been explored for PHA production using mixed cultures, such as aerobic dynamic feeding (ADF), microaerophilic-aerobic cycling and enhanced biological phosphorus removal (EBPR), discussed previously in the introduction (Section 1.6). Typically, investigations into the application of these strategies have focused on waste streams generated by indigenous industry, e.g. olive mill wastewaters in Greece and Portugal (Serafim et al., 2004). In the current study a similar perspective was employed in selecting a major industrial sector waste stream within the Irish economy upon which to model PHA production potential.

The dairy sector forms the largest component of Ireland’s food and drink industry, contributing 27% of all food and drink exports, generating revenues of €2.36 billion annually. Approximately 5.5 billion litres of milk are produced in Ireland each year, which undergo downstream processing into 4 primary product streams; butter, cheese, milk powders (including casein) or liquid milk for consumption (http://www.ndc.ie/docs/ndc-strategy-report.pdf). With respect to the nutrient composition of milk protein, carbohydrate and fats comprise ~12% (w/v) in a near 1:1:1 ratio, respectively. The inherent 4% fatty acid content
is one feature of particular significance from a PHA production perspective, given the varied routes for assimilation of these molecules into biodegradable polyesters. Table 2.1 below provides a summary of the major nutrients present in milk.

**Table 2.1.** Nutritional Composition of Milk per 100g.

<table>
<thead>
<tr>
<th></th>
<th>Whole milk, pasteurised, average</th>
<th>Skimmed milk, pasteurised, average</th>
<th>Semi-skimmed milk, pasteurised, average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kcal</td>
<td>66</td>
<td>34</td>
<td>46</td>
</tr>
<tr>
<td>kJ</td>
<td>274</td>
<td>144</td>
<td>195</td>
</tr>
<tr>
<td><strong>Protein (g)</strong></td>
<td>3.3</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Carbohydrates (g)</strong></td>
<td>4.6</td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Fat (g)</strong></td>
<td>3.9</td>
<td>0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Saturated fatty acids (g)</td>
<td>2.5</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (g)</td>
<td>1.0</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (g)</td>
<td>0.1</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Trans fatty acids (g)</td>
<td>0.1</td>
<td>Tr</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Adapted from Food Standards Agency (2002) McCance and Widdowson (Roe *et al.*, 2002)

In relation to the waste streams generated by dairy processing, Table 2.2 outlines the high nutrient load and inherent complexity of various production process effluents. Among these whey has the highest COD:N:P levels; (61250mg/l: 2500 mg/l: 533 mg/l, respectively), while waste from yoghurt plant generated effluent with COD:N:P levels of 1500mg/l: 67mg/l: 7.2mg/l, respectively. Therefore, there are huge variations within effluents and optimised strategies for treatment are likely to be site-specific, tailored to the particular waste being generated.
Table 2.2. Composition of wastewater/effluent obtained in dairy plants, as well as the wastewaters generated during cheese and casein production (composition in mg/l, except for pH) (Modified from Omil et al., 2003).

<table>
<thead>
<tr>
<th>Origin</th>
<th>COD</th>
<th>Fats</th>
<th>N&lt;sub&gt;t&lt;/sub&gt;</th>
<th>P&lt;sub&gt;t&lt;/sub&gt;</th>
<th>pH</th>
<th>TS</th>
<th>VS</th>
<th>TSS</th>
<th>VSS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>4000</td>
<td>400</td>
<td>55</td>
<td>45</td>
<td>8–11</td>
<td>675</td>
<td>635</td>
<td></td>
<td></td>
<td>Kasapgil et al., 1994</td>
</tr>
<tr>
<td>DP</td>
<td>4000</td>
<td></td>
<td>200</td>
<td>60</td>
<td>5–9</td>
<td>5100</td>
<td>4300</td>
<td>500</td>
<td></td>
<td>van den Berg and Kennedy, 1983</td>
</tr>
<tr>
<td>DP</td>
<td>2926</td>
<td>294</td>
<td>36</td>
<td>21</td>
<td>6.7</td>
<td>2750</td>
<td>1880</td>
<td></td>
<td></td>
<td>Rico et al., 1991</td>
</tr>
<tr>
<td>DP</td>
<td>633</td>
<td>106</td>
<td></td>
<td></td>
<td>8.9</td>
<td>710</td>
<td>447</td>
<td>240</td>
<td></td>
<td>Lo et al., 1985</td>
</tr>
<tr>
<td>DP</td>
<td>2209</td>
<td>60</td>
<td></td>
<td></td>
<td>7.2</td>
<td></td>
<td>278</td>
<td></td>
<td></td>
<td>Timofeyeva, 1992</td>
</tr>
<tr>
<td>DP</td>
<td>4500</td>
<td>56</td>
<td>33</td>
<td>7.2</td>
<td>2540</td>
<td>1093</td>
<td>816</td>
<td></td>
<td></td>
<td>Harper, 1974</td>
</tr>
<tr>
<td>DP</td>
<td>3190</td>
<td>690</td>
<td>43</td>
<td>7</td>
<td>5-10</td>
<td></td>
<td>820</td>
<td></td>
<td></td>
<td>Rusten et al., 1992</td>
</tr>
<tr>
<td>Whey</td>
<td>61250</td>
<td>2500</td>
<td>533</td>
<td>4.6</td>
<td></td>
<td>5 077</td>
<td>4900</td>
<td></td>
<td></td>
<td>Méndez, 1989</td>
</tr>
<tr>
<td>Whey</td>
<td>6600</td>
<td>650</td>
<td>650</td>
<td>4-6</td>
<td>63000</td>
<td>57000</td>
<td>2000</td>
<td></td>
<td></td>
<td>van den Berg and Kennedy, 1983</td>
</tr>
<tr>
<td>DP</td>
<td>296</td>
<td></td>
<td></td>
<td></td>
<td>8.1</td>
<td></td>
<td>943</td>
<td></td>
<td></td>
<td>Shamir et al., 2001</td>
</tr>
<tr>
<td>DP</td>
<td>2125</td>
<td>70</td>
<td>100</td>
<td>9.8</td>
<td>1500</td>
<td>280</td>
<td>250</td>
<td></td>
<td></td>
<td>Monroy et al., 1995</td>
</tr>
<tr>
<td>DP</td>
<td></td>
<td>191</td>
<td>50.9</td>
<td>8.5</td>
<td></td>
<td>804</td>
<td>804</td>
<td></td>
<td></td>
<td>Monroy et al., 1995</td>
</tr>
<tr>
<td>DP</td>
<td>4500</td>
<td>350</td>
<td>60</td>
<td>50</td>
<td></td>
<td>800</td>
<td>800</td>
<td></td>
<td></td>
<td>Craggs et al., 2000</td>
</tr>
<tr>
<td>DP</td>
<td>4000</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td>800</td>
<td></td>
<td></td>
<td></td>
<td>Koyuncu et al., 2000</td>
</tr>
<tr>
<td>DP</td>
<td>1750</td>
<td>75</td>
<td>9.1</td>
<td></td>
<td></td>
<td>400</td>
<td>355</td>
<td></td>
<td></td>
<td>Koyuncu et al., 2000</td>
</tr>
<tr>
<td>CP</td>
<td>4430</td>
<td>754</td>
<td>18</td>
<td>14</td>
<td>7.32</td>
<td>1100</td>
<td></td>
<td></td>
<td></td>
<td>Koyuncu et al., 2000</td>
</tr>
<tr>
<td>YB</td>
<td>1500</td>
<td>63</td>
<td>7.2</td>
<td></td>
<td></td>
<td>191</td>
<td></td>
<td></td>
<td></td>
<td>Koyuncu et al., 2000</td>
</tr>
</tbody>
</table>

DP - dairy Plant; CP - cheese Plant; YB - yoghurt and buttermilk
Despite these challenges, dairy processing wastewater has previously been shown to be amenable to biological remediation, with an EBPR approach being successfully applied in an Irish pilot scale plant trial (Mulkerrins et al., 2004). However, the primary objective of that study was to determine the phosphate accumulation capacity of the sludge rather than PHA accumulation. EBPR, as discussed earlier, is reliant on an anaerobic phase in which bacteria take up carbon sources, primarily volatile fatty acids (VFAs), and store them in the form of PHAs. The energy required is primarily gained from the degradation of their intracellular polyphosphate (poly-P) which is released into the sludge as orthophosphate. In the aerobic phase, the stored PHA is used as an energy source to enable orthophosphate uptake in the cell to recover the poly-P level (Mino et al., 1998). Therefore, while the polyphosphate accumulation capacity has been successfully demonstrated, the inherent PHA accumulation was not explored to any significant degree. The EBPR method was therefore selected as the operational model for this project, to assess PHA production from artificial dairy industry waste waters.
2.2 Material and Methods

2.2.1 Reactor Set-up and Operation

An extensive review of the existing literature on mixed culture applications in PHA accumulation was performed in an effort to determine common reactor conditions facilitating polymer formation (Table 2.3). It was subsequently decided that our analyses would be conducted using single sequencing batch reactor systems (SBRs) in which all steps would be carried out in a sequenced order. Triplicate reactors were set up in order to allow independent changes in operational conditions to be assessed for optimisation of PHA production. Reactor operational cycles involved an initial anaerobic/anoxic phase designed to induce PHA accumulation, followed by an aerobic phase intended to facilitate COD removal, nitrification of ammonia compounds and polyphosphate accumulation. All reactors had a 2L working volume (Figure 2.1), and were operated on 2-3hr cycles as discussed below. Influent feeding and effluent withdrawal involved 350ml volumes per cycle, resulting in hydraulic retention times of 11.43hr and 17.14hr, respectively, depending on the cycle length (2-3hr). Activated sludge from an aerated mixed waste reactor at the Kerry Ingredients dairy processing facility in Listowel Co. Kerry was used to inoculate the SBRs. Influent and effluent pumping was achieved using Watson Marlow 504S and 520S pumps, respectively. Aeration was provided by a TetraTec APS 400 air pump (DO ≥1-2 mg/l), and sludge mixing was achieved using a VWR VS C4 magnetic stirrer set to 250 rpm. Coordination of reactor equipment was facilitated by Termina TR610 top2 programmable switch timers (Theben, Germany). The reactors had a solids retention time (SRT) of 10 days, maintained via removal of defined biomass volumes at the end of one aerobic cycle each day. MLSS and sludge volume index (SVI) were analysed according to Standard Methods (APHA, 2001). Efforts
were made to sustain the sludge MLSS at minimum of 2 g/L during operation. The reactor was operated without pH control; the temperature was kept between 20-25°C.

The COD:N:P ratios in both reactors were similar. The COD:N:P ratios established in the acetate based feed was 1700:25:25. While the COD:N:P ratios established in the final skim milk based feed was 1800:25:25 with the composition of skim milk (Sigma) consisting of 5.3% total nitrogen and 55% lactose. The COD:N:P ratios used in these reactors were low especially in relation to nitrogen. The reason for this was to try and promote PHA production by maintaining one of the known inducing conditions, low availability of inorganic nutrients, in the presence of relatively high carbon availability.
Figure 2.1. Sequencing Batch Reactors used in trial 1, 2 and 3.
2.2.2 Reactor Trials

3 distinct reactor trials were conducted over the course of the project, with varied operational characteristics, in an effort to establish conditions which would contribute to stable reactor performance and PHA accumulation. Table 2.3 provides a summary of the key variables that differed between them. A key focus was the timing of the anaerobic/anoxic and aerobic stages. Trial 1 investigated the use of a 90 minute anaerobic/anoxic stage coupled with a 60 minute aerobic stage. Analysis of the literature indicated that such SBR cycling conditions had successfully facilitated PHA accumulation in other mixed culture systems. Trial 1 was operated for a period of 92 days, with regular sampling and analysis of influents, effluents and mixed liquors.

Table 2.3. Reactor variables in Trials 1 – 3.

<table>
<thead>
<tr>
<th>Trial</th>
<th>1</th>
<th>2</th>
<th>3 (a)</th>
<th>3(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic (Minutes)</td>
<td>90</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Aerobic (Minutes)</td>
<td>60</td>
<td>90</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Organic Substrate</td>
<td>Skim milk</td>
<td>Skim milk</td>
<td>Sodium Acetate</td>
<td>Sodium Acetate &amp; Skim Milk</td>
</tr>
<tr>
<td>HRT (Hours)</td>
<td>17.1</td>
<td>17.1</td>
<td>11.4</td>
<td>11.4</td>
</tr>
<tr>
<td>SRT (Days)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
In Trial 2, the reactors were reseeded with fresh activated sludge from the dairy plant and set up in essentially the same manner as per Trial 1. The primary modification in operation involved adjustment of the anaerobic/anoxic stage to 60 minutes and that of the aerobic stage to 90 minutes, (Table 2.3). Under these cycling conditions the SBRs were operated for 44 days with regular influent, effluent and mixed liquor sampling to enable investigations for improved PHA accumulation and system stability. The lines were disinfected regularly with 3% sodium hypochlorite to prevent contamination. Trial 3 sought to deliver further system optimisation based on the outcomes of Trials 1 and 2. In Trial 3(a) the organic fraction was sodium acetate while in Trial 3(b) skim milk and sodium acetate was the organic fraction. In Trial 3 the organic fraction was prepared independently by dissolving 3g skim milk powder per litre of distilled H2O and autoclaving at 115°C for 5 minutes. The organic and inorganic influent fractions were subsequently mixed during simultaneous feeding of respective volumes into the reactor. This called for additional pump installation and recalibration of the influent loads to maintain desired COD:N:P ratios. Operationally, the reactor was first maintained on a continuous 90 minute aerobic cycle to encourage the development of a diverse microbial consortium capable of utilising available carbon substrate(s). Subsequently, after 4 SRT periods had elapsed and stable maintenance of MLSS levels had been observed, the skim milk reactor was switched to an anaerobic cycle of 30 minutes and an aerobic cycle of 60 minutes (Table 2.3) to initiate PHA accumulation. Furthermore, the carbon substrate was changed from skim milk to sodium acetate which was also sterilised and separated from the mineral salts component of the media to prevent influent contamination. The carbon substrate change during this initial period was also performed to allow for a comparative assessment of PHA accumulation by the reactor biomass with a readily utilisable substrate. The COD:N:P ratio in this reactor was
A second SBR was also established and fed synthetic media in which the organic fraction was made up of both sodium acetate and skim milk (COD:N:P 1800:25:25). The goal of this reactor was to investigate the contribution of the skim milk component of the wastewater to PHA accumulation in the biomass. Identical cycling conditions were used in both reactors, as per above. Sampling of influent, effluent and mixed liquors for analysis were conducted as before to enable an assessment of both PHA accumulation and overall nutrient remediation/depletion by the respective reactor systems.

2.2.3 Media and Analysis

Initially in Trial 1 and 2 dairy industry wastewater was simulated with a synthetic media containing; 0.45g MgSO$_4$.7H$_2$O, 0.33g NaCl, 0.25g Yeast extract, 0.47g (NH$_4$)$_2$SO$_4$, 0.22g KH$_2$PO$_4$ and 0.09g CaCl$_2$ per litre, respectively. The corresponding media in trial 3 (a) and (b), contained 0.09g MgSO$_4$.7H$_2$O, 0.07g NaCl, 0.025g Yeast extract, 0.093g (NH$_4$)$_2$SO$_4$, 0.044g KH$_2$PO$_4$ and 0.017g CaCl$_2$ per litre, respectively. The organic fraction was provided in the form of skim milk powder alone (Trial 1 and 2) or through a combination of sodium acetate and skim milk powder (Sigma Aldrich) to achieve COD:N:P ratios ranging from 1000:40:60 mg/l to 1800:25:25 mg/l (Trial 3 (a) and (b)), respectively. Reactor influent and effluent PO$_4$-P, NH$_4$+-N, NO$_3$-N and COD concentrations were determined via colorimetric assays, quantified on a HACH Odyssey DR/2500 Spectrophotometer (HACH, Loveland, USA). PO$_4$-P and NH$_4$+-N were assayed with ammonium molybdo-vanadate and Nesslers reagent (Reagecon, Ireland), respectively, in accordance with the HACH DR/2500 standard protocols manual ($\lambda = 430$nm). NO$_3$-N was determined via the Nitratetest method (Palintest®, UK) of nitrate reduction to nitrite and subsequent, colour forming diazonium reaction ($\lambda = 570$nm). COD was determined via potassium dichromate/sulphuric acid oxidation.
150mg/L vials, Reagecon), incubated for 2 hours at 150°C in a HACH COD reactor before spectrophotometric quantification. pH values were determined using a WTW pH340 meter, while an Oxical probe and WTW Multiline P4 universal meter were used to measure DO, (WTW, Weilheim, Germany).

2.2.4 Microscopic Analysis

Microscopic analyses of the sludge were conducted on a daily basis, and combined phase contrast and fixed culture staining to monitor the sludge microflora for undesirable proliferations of organisms capable of destabilising the system (e.g. filamentous species, Zoogela and tetrads/cyanobacteria). Analyses of the sludge samples were performed using a Leica DM3000 epifluorescence microscope system, with an EL6000 metal halide external light source and a DFC490 8mp, CCD digital camera, (Leica Microsystems, Germany).

2.2.5 Determination of PHB Production via Fluorescent Microscopy

Visualisations of intracellular polyphosphate granules were achieved via 3 minute staining of heat fixed samples with the fluorescent dye 4', 6-diamidino-2-phenylindole (1 ug/ml). Visualisation of PHAs involved incubation of biomass samples with 1% aqueous solution of Nile blue A at 55°C for 10 minutes before transfer to a microscope slide. In the case of both polymers, analyses were performed using a Leica DM3000 epifluorescence microscope system, with an EL6000 metal halide external light source and a DFC490 8 mp, CCD digital camera, (Leica Microsystems, Germany). A UV/violet range filter D was employed (Leica), offering 335 nm and 455 nm excitation and emission wavelengths, respectively. Image capture and processing was performed with LAS software V3.1.0.
2.2.6 Determination of PHB Production via Crotonic Acid Conversion and Spectrophotometric Analysis

Initially, determination of PHB production in the reactors was carried out using a spectrophotometric approach previously reported by Law and Slepecky, (1961). Samples of sludge were collected from each reactor at the anaerobic stage and centrifuged at 6000 rpm for 30 minutes. The pellets were weighed and a standardised 1 g of biomass suspended in 5 ml of sterile water and homogenised using ultrasonic treatment for 5 minutes. 2 ml of the cell suspension was added to 2 ml of 2 M HCl and heated to boiling for 2 hours in a water bath. The samples were centrifuged at 6000 rpm for twenty minutes. 5 ml of chloroform was added to the resulting precipitate. The samples were left overnight at 28°C on a shaker at 150 rpm, followed by centrifugation at 6000 rpm x 20 minutes, extraction with 0.1 ml chloroform and drying at 40°C. 5 ml of concentrated sulphuric acid were added and the tubes heated in a 100°C water bath for 20 minutes. After cooling to 25°C, the amount of PHB was determined on a U.V. spectrophotometer at wavelength 235 nm.

2.2.7 Quantification of PHB Production via High Performance Liquid Chromatography (HPLC)

HPLC was performed to accurately quantify the amount of PHB present in the reactors using the method of Karr et al. (1983). Samples ranging from 0.01 to 500 mg of PHB containing material can be used. Therefore, samples of 5 mg (sludge cells) were digested in 1 ml concentrated sulphuric acid at 90°C for 30 minutes. The tubes were cooled on ice, after which a 4 ml volume of 0.014N H₂SO₄ was added with rapid mixing. The samples were filtered through a 0.45 µm filtropur syringe filter (Sarstedt) to remove particulate material. The HPLC was carried out using an Agilent 1200 HPLC system with a refractive index detector. A REZEX 8 µm 8% H, Organic Acid Column 300x 7.8mM (Phenomenex, USA) was used with 0.01N H₂SO₄ as the elution fluid, at a flow rate of 0.6 ml/min. The
temperature of the column was maintained at 65°C. Substrate and end-product peaks were identified by comparison of retention peak profiles of samples to those of pure compounds of known concentrations. The standard used was crotonic acid at 2.5 mM, 5 mM, 10 mM and 20 mM. The HPLC solvent was 0.01N Sulphuric acid: 227 µl concentrated H₂SO₄ in a litre of H₂O filtered through a 0.45 µM filter (Sarstedt). Absorbance of the crotonic acid was measured at 210 nm.

2.2.8 Determination of PHA Composition using Gas Chromatography-Mass Spectrometry

Chemical characterisation of sludge PHA was based on methanolysis of the polymer (Lee and Choi, 1995). For each analysis, 5 mg of sludge was transferred to a microfuge tube and spun down at 14,000 rpm for 10 minutes. The pellets were washed with water and re-centrifuged before re-suspending in 100µl dH₂O, (storage at -20°C was possible at this point). The samples were lyophilized overnight and weighed (±0.05mg). The contents were transferred to a glass vial and the tubes were weighed again to determine the transferred mass. 2 ml 15% H₂SO₄ methanol and 2 ml chloroform containing methyl benzoate were added to the samples and allowed to boil at 100°C for 2.5 hours with continuous mixing. The samples were then cooled on ice for 5 minutes and 1ml of dH₂O was added and vortexed for 1 minute. The samples were centrifuged at 4000 rpm for 5 minutes. The complete water phase was discarded, including droplets hanging on the tube wall and the top layer of the chloroform phase. 1 ml of dH₂O was added, vortexed, centrifuged and the water phase was discarded as described previously. Na₂SO₄ powder was added to dry the chloroform phase, as the powder binds any free water and settles quickly with centrifugation at 4000 rpm x5a minutes. 200 µl of the chloroform phase was pipetted to a GC vial without transferring any
of the Na₂SO₄. Appropriate derivatizations are required to determine the chain length of 3-hydroxyalkanoic acid methyl esters via favourable mass fragmentations in a mass spectrometer. The mass spectra of trimethylsilyl (TMSi) derivatives of 3-hydroxyalkanoic acid methyl esters exhibit characteristics fragments of m/e 175, m/e 73/ m/e 89 and molecular ion-related fragments of m/e (M-73) and of m/e (M-15). Fragmentation patterns of unsaturated monomers show a characteristic peak of m/e 138. The derivatization procedure involves the addition of 50 µl bis(trimethyl-silyl)acetamide to 1 ml of methanolised sample. The samples were vortexed and incubated for 10 minutes at 80°C. The 3-hydroxyalkanoic acids methyl esters and TMSi derivatives were analysed with a CP 3800 Gas chromatograph (Varian) and a Saturn 2000 GC/MS/MS (Varian) and a BPX5 Column- 30 m x 0.25 mm, (I.D. x 0.25 µm). The solvent used was Methanol (Sigma). The injection volume was 1 µl sample from the organic phase, with helium as the carrier gas and the oven temperature programmed for peak separation (35°C for 2 minutes and then increments of 15°C/min up to 280°C). The structures of the monomers were identified by analysis of the characteristic peaks and comparison with standard mass spectra if available. The standard used to achieve qualitative analysis was Pseudomonas putida CA3 (Lee and Choi, 1995). The Pseudomonas putida CA3 was inoculated in 5 ml LB agar and left to grow overnight at 28°C and 150 rpms. Cultures were subsequently centrifuged at 5000rpm for 7 minutes. The resulting supernatant discarded and the pellet washed in 5mls of sterile Ringers solution. The culture was centrifuged again, the supernatant discarded and the pellet was resuspended in 10ml of relevant E2 test media. Each culture was grown in 4 different iterations of the E2 minimal media, (i) E2 with nitrogen (8mM) and 10mM acetate (ii) E2 limiting nitrogen (1.5mM) and 10mM acetate, (iii) E2 with nitrogen (8mM) and 10mM glucose and, (iv) E2 limiting nitrogen (1.5mM) and 10mM glucose, respectively. E2 N-Lim + Glucose/Acetate and incubated at 28°C for 4 hours shaking. 1ml was transferred into an appendorf and its
absorbance was read at a wavelength of 600nm. Once the absorbance/optical density (OD) was in the range of 0.5 to 1.0, 1ml was taken from the samples, centrifuged and the pellet stored at -20°C. For analysis each pellet was washed and subjected to Nile Red lipophilic staining using 1ml of 0.5 mg/ml solution of Nile red in acetone. 0.5ul of sample was then added to a labeled slide and observed via a fluorescent microscope (Leica DM3000) with an I3 filter, with an excitation wavelength of 450-490 and an emission filter of 510. Images were captured via a DFC490 8mp, CCD digital camera, (Leica).
2.3 Results and Discussion

The primary objective of our initial investigations was to establish if PHA accumulation from a waste stream modeled on outputs from dairy processing could be achieved. However, despite the apparent simplicity of the experimental query, several concurrent considerations had to be addressed such as; maintaining biomass stability in the system, assessing the impacts of the influent composition, assessing the effluent characteristics for overall nutrient remediation and monitoring non substrate related operational parameters/occurrences negatively affecting the above system. As a consequence, several challenges were encountered which required us to progressively modify our operational strategies.

2.3.1 Trial 1

Prior to commencing the trial an extensive literature survey was carried out to determine cycling conditions to support PHA accumulation in the system (Table 2.4). On the basis of this comparative assessment, the key cycling conditions under investigation in Trial 1 involved a 90 minute anaerobic exposure followed by a 60 minute aerobic period. Initially, the reactors performed stably, (Figure 2.2), with good PO₄-P removal, averaging below 2 mg/l in the effluent. With respect to nitrogen manipulations effluent NO₃-N levels on average were below 3 mg/l while residual NH₄-N nitrogen was below 10 mg/l for all three reactors. In addition, DO levels remained low indicating strong metabolic activity among microorganisms in the reactor. Overall, reactor 2 had the greatest COD removal with effluent levels averaging 58 mg/l, while effluent COD average loads were 149 mg/l and 98 mg/l for reactors 1 and 3, respectively, (Figure 2.2). The stable phosphate and COD removal observations provided preliminary indications that an EBPR process was potentially
becoming established in the reactors with the possibility of PHA accumulation being supported. Indeed, these observations correlated with the nutrient removal profile reported by Rodgers and Wu who used the EBPR system in reactors seeded with sludge from the Tuam wastewater treatment plant, Co. Galway, (Rodgers and Wu, 2010). The authors reported NO₃-N effluent concentrations below 4 mg/L, and PO₄³⁻-P levels circa 1 mg/L for the project, with the influent PO₄³⁻-P concentration of 36.3 mg/l. In EBPR systems as described previously, phosphate is taken up in the aerobic cycle and in the anaerobic cycle it is released out of the cell so that carbon can be taken up at stored in the form of PHA. However, in the anaerobic cycle of this trial PHA was not being accumulated (Figure 2.3)
<table>
<thead>
<tr>
<th>Reference</th>
<th>Cycling conditions</th>
<th>Wastewater</th>
<th>PHA Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dionisi et al., 2005</td>
<td>10 min feed, 1 h 48 min reaction aerobic and final 2 minute’s withdrawal. There was no settling phase and all excess biomass was removed with the liquor</td>
<td>Pilot scale activated sludge process with anaerobic—aerobic-anoxic configuration This plant also received influent wastewater from a municipal wastewater treatment plant. (COD:N:P ratios if available) 1X 2L SBR</td>
<td>Maximum value of about 0.25 g PHA/L/H using Run IIIa</td>
</tr>
<tr>
<td>Chua et al., 2003</td>
<td>Anaerobic 1 h, aerobic 2h, and settling period 40 min. HRT: 6 hour, SRT 3 days</td>
<td>Municipal (Tokyo) (COD:N:P ratios if available) 6 x 20 L SBRs</td>
<td>Maximum amount of PHB, 31% sludge dry weight</td>
</tr>
<tr>
<td>Dai et al., 2007</td>
<td>2h anaerobic, 2.5h aerobic followed by 60 min settling HRT: 24 h, SRT:7 days</td>
<td>Full-scale domestic wastewater treatment plant 1x 8L SBR</td>
<td>PHA concentration was 14% of dry cell weight (DCW).</td>
</tr>
<tr>
<td>Liu et al., 2000</td>
<td>Eight 3-h cycles per day, anaerobic phase (60 min), an aerobic phase (60 min), and a settling phase (60 min). HRT: 6 hours, SRT: 7.5 days</td>
<td>A 1.5 L SBR was used to enrich the activated sludge</td>
<td>The Lpha5 strain; PHA accumulation being approx. 20% of cell dry weight Similar results for Lpha7 grown in the same growth medium.</td>
</tr>
<tr>
<td>Perez-Feito and Noguera, 2006</td>
<td>6h cycle; Anaerobic (2 hrs), aerobic (3 hrs), settling (55 minutes) and idle (5 minutes). At the end of the settling phase one third (0.6 L) was withdrawn resulting in; HRT: 18 hours, SRT: 7.5 days The waste activate sludge was withdrawn at the end of the aerobic stage</td>
<td>Seeded with sludge from the end of the aerobic stage of a wastewater treatment plant that constantly achieves effluent phosphorus concentrations below 1.0 mg phosphorus/L 1 x 1.8L</td>
<td>Maximum PHB production, 25%</td>
</tr>
</tbody>
</table>
Figure 2.2. Effluent Inorganic nutrient analyses and associated dissolved oxygen concentrations in mixed liquor of SBRs (a-c). Inorganic nutrient level fluctuations in reactor 1(a), 2 (b) and 3 (c) were considerable under the conditions tested in the triplicate SBRs and coincided with poor sludge stability.
However from day 40, reactor stability began to degrade with sludge washout being observed and the effluent nutrient profiles becoming erratic. Figure 2.4 demonstrates considerable increases in effluent COD levels in both reactors 1 and 3 between day 63 and 89 of reactor operation. These events coincided with observed sludge washout to varying degrees within the reactors. It was noted that sludge washout events were a feature of reactor 2 also, however COD effluent levels did not appear to reflect this under analyses. The author questions whether feed line impedance or contamination of influent may have reduced carbon levels entering reactors. In relation to disruptive bulking, nitrogen and phosphorus deficiency can result in the overproduction of extracellular polysaccharide in wastewater which builds up in the sludge and leads to poor settling (Jenkins et al., 2004). However, this was unlikely to have been a dominant condition within the reactors given the detectable inorganic nutrient levels in the reactor effluents. The acceptable limits for biological activity for residual ammonia is 1-3.0 mg/L and ortho-phosphate residue 0.5-2.0 mg/L. Alternatively, limiting
oxygen availability can contribute to the proliferation of filamentous species such as *Sphaerotilus* and *Thiotrix*, which can result in reactor sludge wash out. Microscopic analysis of the sludge in these periods confirmed the presence of filamentous proliferations, sharing a number of phenotypic traits with *Thiotrix* species (Figure 2.4 (b)). Concerns arose therefore with respect to the extended anaerobic period within the SBR cycles, as a possible source for the disruption. In addition, considerable gas bubble formation was observed in all reactors at day 40, which further contributed to poor sludge settling, and washout. Denitrification in the anaerobic/anoxic phase was suspected, whereby the dissolved oxygen supply in the sludge is exhausted and the microbes begin to utilise oxygen from the nitrates, ultimately yielding nitrogen gas. Nitrate reductase assays were not carried out but should be considered in any further studies to confirm this hypothesis. This test determines whether the microbe produces the enzymes nitrate reductase and nitrite reductase. The two enzymes catalyse two reactions involved in converting starting compound nitrate into end product nitrogen gas. If a sludge containing microorganisms producing nitrate reductase is grown in a medium containing nitrate, the enzyme converts the nitrate to nitrite. Nitrite reacts with certain chemicals to yield a red-coloured product. If the bacterium also produces nitrite reductase, nitrogen gas will be liberated. Bubbles collecting in an inverted Durham tube indicate that nitrogen has been produced. Irrespective of the underlying cause however, the persistent nature of these performance deficiencies indicated that continued operation of the reactors under these conditions was not a viable prospect and Trial 1 was concluded on day 94.
Figure 2.4. Trial 1 Effluent COD levels from SBRs and microscopic analysis (a) Effluent COD levels in the 3 reactors; (b) Microscopic analysis of sludge from Reactor 2 on day 37, black circle highlights presence of filamentous organisms.
2.3.2 Trial 2

In Trial 2 the cycling conditions were altered in order to address the negative impacts observed in Trial 1. In response to excess gas formation and the outgrowth of filamentous species, the anaerobic\anoxic stage was reduced from 90 to 60 minutes, while aerobic exposure was increased from 60 to 90 minutes. The latter adjustment also sought to enhance catabolism of the skim milk organic fraction of the media. However, upon reinstatement of the SBRs, it was observed that COD effluent readings were significantly elevated relative to those observed in Trial 1 (Figure 2.5). In trial 2, the average effluent COD values for reactors 1, 2 and 3 were 259 mg/L, 215 mg/L and 174 mg/L. A brief period of improved COD removal was observed between days 15 to 25, when effluent averages decreased by ~ 40% across all reactors. Analyses of effluent inorganic nutrients did not reveal any parallel enhanced remediation performance during the same period, (Figure 2.6). However, effluent COD levels increased again to between 390 - 430 mg/L after this period and did not decrease to any sustained degree for the remainder of the trial. It was noted that, while a general common trend emerged vis a vis sustained COD loads in the effluent across all three reactors (Figure 2.5), varying degrees of poor individual reactor performance were identified in the early stages.
Analysis of the inorganic nutrient fluctuations further highlighted instability within the systems (Figures 2.6, (A-C)). Compared to trial 1, phosphate and nitrogen ammonia effluent levels were considerably higher throughout Trial 2, with no apparent phosphate or nitrogen removal during certain periods. Phosphate removal remained low throughout the trial with the highest phosphate removal being 67% compared with PO$_4^{3-}$-P removal of 92% in Trial 1. Furthermore, NH$_4^+$-N ammonium levels also remained high with 50% removal in the trials peak performance compared with a peak NH$_4^+$-N nitrogen removal of 60% percent in Trial 1. The poor remediation of COD and PO$_4^{3-}$-P in Trial 2 were not consistent with potential EBPR operation and questions arose with respect to the integrity of the process set up. Upon testing of all meters, feed and aeration devices, a discrepancy in the OXICAL sensor was revealed, despite daily calibration. Replacement of the probe membrane and
recalibration revealed that DO in the aerobic phase had been overestimated, and that an inadvertent oxygen limitation was imposed on the system. The poor nutrient remediation and sludge stability observed may well have been attributable to this unforeseen sensor malfunction. However, while clear negative impacts were attributable to low DO levels, Trial 2 also saw recurrent contamination of the influent, associated with the presence of an unidentified rod-shaped, Gram negative bacterium. The preparation of non-sterile media in 25 L volumes to sustain reactor feeding for ~ 50 hour periods was found to allow growth of the contaminant up to $1 \times 10^8$ cfu/ml. Testing of the media indicated that a 10-20% nutrient depletion effect was associated with this proliferation. Application of standard disinfection practices, (3% sodium hypochlorite), in the media and frequent flushing of all lines to the reactors could not resolve this contamination issue. These combined challenges to the system resulted in significant filamentous sludge bulking and washout once more. Trial 2 was terminated on day 44 to enable resolution of the system issues. Again, like Trial 2 PHA was not being accumulated in the anaerobic cycle. Firstly, phosphate levels removal remained low in Trial 2 which seemed that EBPR removal was not occurring which resulting in PHA accumulation not occurring in the anaerobic cycle (Figure 2.7).
Figure 2.6. Trial 2 monitoring of effluent inorganic nutrient loading and mixed liquor dissolved oxygen concentrations. (A-C) Effluent Inorganic nutrient levels in the triplicate SBR reactors. Black arrow indicates repair of OXICAL sensor and accurate DO readings.
2.3.3 Trial 3

Having committed significant resources to the performance of Trials 1 and 2, without any significant return in relation to PHA production it was determined that a considerable modification of the experimental strategy be considered. While a number of technical issues had hindered progress toward the objective of PHA accumulation, several questions had begun to emerge also in relation to the complexity of the skim milk as a precursor for PHAs. The use of wastewater for the accumulation of PHA using the Enhanced Biological Phosphorus Removal (EBPR) process is well documented (Wallen and Rohwedder, 1974; Barnard, 1974; Fuchs and Chen, 1975; Satoh et al., 1996; Crocetti et al., 2001). However, many of these studies have employed short chain fatty acids as carbon sources (e.g. acetate and propionate). As a result it was decided, (notwithstanding the resolution of technical issues), to investigate the PHA production capacity of the system using dual carbon sources, acetic acid and skim milk. It was found that pre-sterilisation of the organic sources, and
discrete feeding into the reactors, eliminated the contamination issues associated with Trial 2. Furthermore, the use of triplicate reactors was discontinued as all had suffered similar fates in the earlier trials. Dedicated reactors for acetate alone, (Reactor 1), and acetate + skim milk (Reactor 2) were devised. The sludge inoculum was also allowed a period of 4 SRTs under aerobic conditions to acclimatise to the skim milk media and demonstrate stable COD removal, before the reactors were converted to anaerobic and aerobic cycling with their respective carbon sources. There was little disruption in COD removal during the transition from skim milk media to sodium acetate media. Reactor 1 was subsequently operated for the remainder of the trial with COD:N:P influent ratios of approximately 1700:25:25, during which time the residual effluent COD values ranged from levels below the limits of detection up to 465 mg/L, (Figure 2.8 (a)). Reactor 2, receiving the dual carbon source (sodium acetate/Skim milk) media (COD:N:P = 1800:25:25) demonstrated similar organic nutrient removal capabilities, with a minimum effluent COD below the level of detection and a maximum of 450 mg/l, (Figure 2.8 (b)). The average COD effluent values over the course of approximately 11 months were 105 mg/L and 135 mg/L of reactor 1 and 2 respectively. The combined, nutrient removal profile emerging suggested that the EBPR process was potentially occurring, with concomitant PHA accumulation in the anoxic/anaerobic period.
Figure 2.8. Effluent COD monitoring in (a) Reactor 1 fed Sodium Acetate and (b) Sodium acetate + Skim Milk. Influent (blue) and effluent (red). Black Arrows show an increase COD/Carbon1 on day 322 in both reactors.
Mixed liquor samples were collected at the end of both the anaerobic and aerobic cycles and monitored for inorganic nutrient load patterns indicative of EBPR emergence. Figure 2.9 (a) and (b) presents the phosphate levels within reactors 1 and 2 at the end of each cycle. Day 144-322 showed the greatest phosphate removal at an average of 32% for reactor 1 and 24% for reactor 2. Phosphate levels were monitored as an indicator of any potential shift in metabolism/ecology within the sludge population toward EBPR processes. Given the inverse relationship between polyphosphate and PHA intracellular levels during EBPR, we sought to identify periods of elevated aerobic P removal to undergo further investigation for PHA accumulation during the corresponding anaerobic/anoxic periods within the reactor cycle. Reactor 1 phosphorus removal was optimal between days 149-203 and 295-368, averaging 45% and 71%, respectively. In reactor 2, optimal phosphorus removal occurred between days 146-168, averaging 36%. However, compared to trial 1, PO₄³⁻-P removal of 92%, Trial 3 demonstrated lower overall phosphate removal. However, when compared to trial 2, with the highest phosphate removal being 67%, reactor 1 in trial 3 showed higher removal. After ~11 months, (circa day 322 of continuous operation), both reactors began to show a trend toward increased residual COD levels (Figure 2.8 (a) and (b)) up to the end of the trial, with reactor 1 reaching 620 mg/L and reactor 2 reaching 450 mg/L. Average effluent residual COD during this latter period, was 292 mg/L and 310 mg/L for reactor 1 and 2, respectively. Thus 92-94% COD removal was observed in reactors 1 and 2 up to day 322, which decreased to ~82% in both after this period. Furthermore, effluent NH₄⁺-N ammonium levels remained low with removal of 80% and 64% for reactor 1 and 2 respectively. NH₄⁺-N ammonium levels were lower compared with a peak NH₄⁺-N removal of 60% in Trial 1. Trial 3 showed phosphate removal, although lower than Trial 1 resulted in PHA accumulation occurring in the anaerobic cycle (Figure 2.10).
These changes in the operating performance of both reactors reflect an inherent instability in these reactor systems over prolonged operation, although the causes are unclear and may be due to either a shift in the physiology of key microorganisms, or changes in the microbial ecology of the system (Dionisi et al., 2005). The decrease in poly-p increased the use of the glycolytic pathway in order to compensate for the lack of ATP formed from poly-p hydrolysis. In our system, the P level in the influent was 25mg/l. The P levels might have been too low for our system resulting in our system shifting to glycogen accumulating metabolism resulting in PAO competing with GAOs in the system and making PHA accumulation unstable (Acevedo et al., 2012). Indeed, in a prior study Mulkerrins, et al. proposed that low phosphate levels could suppress the development of PAOs leading to the establishment of GAOs (Mulkerrins, et al., 2004). In contrast, in the aerobic cycle phosphate removal was observed in both reactors with reactor 1 showing the greatest amount of removal going as low as 0 mg/L and from 10-15 mg/L of phosphate removal. Coupled with a stable COD utilization, these periods of reactor operation were subjected to further investigation for PHA accumulation. Furthermore, the sludge age (SRT) may have caused the COD removal to decrease. Kargi and Uyger, 2002 found that a sludge age (SRT) of 10 days was optimal resulting in maximum nutrient removal efficiencies and minimum SVI.
Figure 2.9. Mixed liquor Phosphate levels during anaerobic and aerobic cycling in SBRs 1 and 2 (a) Elevated anaerobic mixed liquor phosphate levels consistently exceeding influent loads (b) Aerobic phosphate levels, consistently lower than influent loadings.
Figure 2.10. Levels of phosphate and PHA in the aerobic and anaerobic stage.

The sludge from both reactors was subjected to the Nile Blue A stain (Ostle and Holt, 1982). Nile Blue A is used in conjunction with fluorescence microscopy to stain for the presence of polyhydroxybutyrate (PHB) granules in prokaryotic and eukaryotic cells. The samples were collected during the anaerobic cycle when PHA accumulation is thought to occur (Marais et al., 1983). The areas of the activated sludge which showed PHA accumulation, after Nile Blue A staining, showed bright orange fluorescence under U.V. exposure, when PHA granules were present in the bacterial cells, Figure 2.11.
Figure 2.11. (a) Fluorescent microscopy of PHA accumulation in the sludge. Area of the activated sludge which demonstrates PHA accumulation, after Nile Blue A staining, showed bright orange fluorescence with u.v light. Sample was taken at the end of the anaerobic stage when maximal PHA accumulation should occur. (b) Fluorescent microscopy of PHA accumulation in the sludge. Area of the activated sludge which does not demonstrated PHA accumulation, after Nile Blue A staining, showed bright orange fluorescence with u.v light. Sample was taken at the end of the aerobic stage when PHA accumulation should not occur. (c) Fluorescent microscopy of PHA accumulation in *Pseudomonas putida* CA3 when grown on E2 minimal media with limited nitrogen (1.5mm) and excess carbon.
2.3.4 Quantitative Analysis of PHA

Nile Blue A staining and fluorescence microscopy provided qualitative detection of PHA accumulation in both reactors. Subsequent quantification of the actual yields involved a multistage process of sludge homogenisation, HCl acid digestion, chloroform extraction, sulphuric acid conversion to crotonic acid and UV spectrophotometric analysis ($\lambda = 265$ nm). The maximum PHA Reactor 1 accumulated was $\sim 10\%$ (w/v) PHA while Reactor 2 accumulated a maximum of $20\%$ (w/v). HPLC analysis, specifically targeting short chain length PHAs (Figure 2.12 a and b), confirmed a maximal $11\%$ accumulation in reactor 1 and $13\%$ for reactor 2. These represent peak accumulation periods and while, in general, scl-PHA accumulation in both reactors were quite low (Figure 2.13). Average PHA accumulations in both Reactor 1 and 2 were between 2-5%, with several extended periods where no PHA could be detected. Although PHA accumulation was occurring it was generally quite low in both reactors. Aerobic sludge where PHA accumulation would not be observed was used as the negative control and *Pseudomonas putida* CA3 was used as the positive control.
**Figure 2.12.** (a) HPLC curve showing crotonic acid standard (20mM) (b) HPLC Curve showing sample analysis of crotonic acid peak
2.3.5 Gas Chromatography-Mass Spectroscopy

Our analyses also focused on the potential for mcl PHA production within the reactor systems and whether skim milk incorporation into the media altered the monomer composition of any such mcl PHAs. Gas chromatography is commonly used for the extraction and quantification of mcl PHA (Braunegg et al., 1978, Findlay and White, 1987). This method involves cells undergoing direct, mild acid or alkaline methanolysis, followed by GC of the 3 hydroxymethyl esters (Comeau et al. 1988). *Pseudomonas putida* CA-3 was used as a positive control as it is a well known producer of mcl PHAs (Ward et al., 2005). Furthermore, de Roo et al., 2002 previously reported methanolytic production of 3HA methyl esters from *P. putida* CA-3. GC MS of acid methanolysed sludge in this study revealed PHA monomers of hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid.
were present in samples from reactor 1. In reactor 2 the presence of methyl esters of 3-
hydroxyhexanoic acid, 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid were
identified (Table 2.5) (Figure 2.14 a and b). In the current study, it became clear that
differences in the media composition/carbon source in both reactors was resulting in distinct
PHAs being accumulated, indicating that skim milk components exert considerable influence
in this process. It has been observed previously that the selection of appropriate production
strains, as well as suitable cultivation conditions, and carbon sources, can yield tailor made
PHA compositions (Suriyamongkol et al., 2007). However, recognition must be given also
to the fact that the nitrogen concentrations in the influent media were low which could have
induced the accumulation of mcl PHAs in any *Pseudomonads* present in the sludge. Such
organisms would not be under the influence of EBPR metabolic processes.

**Table 2.5.** Types of hydroxyalkanoic acids produced in reactor 1, 2 a *Pseudomonas putida*
CA3.

<table>
<thead>
<tr>
<th>Methanolysed Sample</th>
<th>Medium Chain length Monomers Identified.</th>
<th>Retention times</th>
</tr>
</thead>
</table>
| Reactor 1 Sludge    | 3- hydroxyoctanoic acid, 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid | 9.47 min  
12.05 min  
13.59 min |
| Reactor 2 Sludge    | 3- hydroxyhexanoic acid, 3-hydroxydodecenoic acid, 3-hydroxytetradecanoic acid | 7.19 min  
13.59 min  
15.56 min |
| *Pseudomonas putida* CA-3 | 3-hydroxyhexanoic, 3-hydroxyoctanoic, 3-hydroxydecanoic, 3-hydroxydodecanoic acid | 5.01 min  
8.08 min  
10.54 min  
13.08 min |
Figure 2.14. (a) 1 Mass spectra of acid methyl esters, (a) 2 Gas chromatographic analysis of the TMSi derivatives of 3-hydroxyalkanoic acid methyl esters of PHA synthesized by *Pseudomonas putida* CA3 (Peaks indicate the retention time in which a TMSi derivatives was detected). (b)1 Mass spectra of the acid methyl esters, (b)2 Gas chromatographic analysis of the TMSi derivatives of 3-hydroxyalkanoic acid methyl esters of PHA synthesized in the reactor (Peaks indicate the retention time in which TMSi derivatives were detected).
2.4 Conclusion

Dairy wastewater is one of the many wastewaters that have been studied for PHA production with a lot of studies carried out using recombinant and non-recombinant microorganisms (Janes et al., 1990; Lee et al., 1997; Wong and Lee, 1998; Ahn et al., 2001; Ahn et al., 2000; Park et al., 2002; Yellore and Desai 1998; Povolo and Casella, 2003). A lot of the work has been done using recombinant E. coli strains with a plasmid containing the Ralstonia eutropha PHA biosynthesis genes (Choi et al., 1998). PHB of 80 to 90% cell dry weight (Lee et al., 1999) has been achieved using whey, in laboratory settings. Studies with non-recombinant microorganisms such as Methylobacterium sp. grown on pure lactose produced 59% PHB when the concentration of lactose in the medium was 0.9 g/L (Yellore and Desai, 1998). However, the problem with using pure cultures for PHA production is the high costs due to sterilization, a risk of contamination and pure substrate costs. The use of pure substrates can contribute up to 50% of the overall production cost (Choi and Lee, 1997). These problems are not encountered in mixed cultures and there are a variety of carbon substrates in the wastewater allowing for greater diversity of PHA producers within the diverse populations supported by same (Dionisi et al., 2005). It should be noted that a common feature of the studies noted above involved a sole focus on scl-PHA production.

Several applications of mixed culture systems for industrial wastewater conversions to polyhydroxyalkanoates have been described in recent years, by which to benchmark the observations in this project. Liu and co-workers reported PHA production using tomato cannery waste coupled with a mixed microbial culture during wastewater treatment (Liu et al., 2008). The two-stage PHA production process comprised an SBR, operating under a periodic feast/famine regime, to accomplish simultaneous wastewater treatment and selection for PHA accumulating microbes. This was followed by a batch reactor for the production of PHA-rich biomass. PHA content on a cell-weight basis was within the range 7 to 11% in non-
filtered wastewater and 2 to 8% in filtered wastewater. Subsequently, batch studies were implemented with varying loading rates, ranging from 0.4 to 3.2 food-to-microorganisms ratios. A maximum 20% PHA content on a cell-dry weight basis was obtained. In a separate study, a three stage process for production of PHA from a paper mill wastewater was examined (Bengtsson et al., 2008). The system involved using an acidogenic fermentation to convert organic matter to volatile fatty acids (VFAs), an activated sludge system operating under feast/famine conditions to enrich for PHA producing organisms and PHA accumulation in batch reactors. The maximum PHA content achieved was 48% of the sludge dry weight. PHA production was also observed from organic wastes by mixed bacterial cultures using anaerobic-aerobic fermentation systems from Palm Oil Mill Effluent (POME). POME was used as an organic source, which was cultivated in a two-step-process of acidogenesis and VFA being utilised by PHA producers for PHA production. Even though the maximum PHA content was observed at only 40% of the cell dry weight (CDW), their production and performance are significant in mixed microbial culture (Salmiati et al., 2007). Two recent studies used an EBPR system approach for PHA accumulation, however the outputs reported were quite variable (Rodgers and Wu, 2010, Kasemsap and Wantawin, 2007). Rodgers and Wu, 2010, found that with 12.8% phosphorus in the biomass under anaerobic conditions, the system achieved PHA accumulation of 28.8% dry biomass weight. Kasemsap and Wantawin, (2007) produced 51% PHA when there was a polyphosphate content of 8% in the biomass.

The present study used dairy wastewater to try and assess the feasibility of PHA production using a similar EBPR model. However, there were challenges throughout the project in establishing a stable, continuous process. Optimisation of the reactors proved difficult and it was not until trial 3 that phosphate removal and COD removal in both reactors indicated that EBPR system had become established. Furthermore, qualitative analysis of the sludge in
both reactors showed that PHA accumulation was occurring. In terms of directly comparing PHA production levels the outputs of the EBPR approach adopted in this study were relatively low. While the preliminary yields of ~10-13% PHB are far below any economically relevant standard, the potential for further development of the system would seem to exist. Furthermore, it was interesting to note that medium chain length PHAs were also synthesised in the system, suggesting the possibility of co-polymer producers within the sludge. This aspect of the system was investigated separately (Chapter 4). In summary, the SBRs operated in this project provided a baseline output in relation to the potential for PHA production for dairy based wastewater. The author recognises that several avenues for optimisation of the system remain to be carried out. Indeed, in the original design of this project it was intended that real time dairy wastewater would be fed into a stably maintained reactor to offer real-time insights into the bioconversion potential. Furthermore, it was also envisaged that pre-fermentation of the dairy waste would be conducted to increase the VFA content and enhance the PHA accumulation capacity of the system. In reality however, the EBPR model proved challenging from the outset to establish in a stable format and considerable time was dedicated to this objective at the cost of other, planned system optimisations. Acknowledging this fact, consideration should be given to the nature of dairy processing and the seasonal variations associated with milk production, particularly in Ireland. The dairy industry is one of the most important sectors of Irish agriculture and accounts for 27% of agricultural output (Department of Agriculture and Food, 2006) with the production of 5.35 million tonnes of milk per annum. However, the problem with producing PHA using dairy wastewater is that there are seasonal fluctuations in the dairy industry in Ireland, with peak deliveries during the peak supply month (May), a multiple of six times the volume of the lowest month’s production (January) (Dillon et al., 2008). This seasonal fluctuation has resulted in varying nutrient levels in effluents generated in Irish diary
processing plants at different times of year. In addition, the volume and the composition of the effluent from a dairy plant is dependent on the type of product being processed, the production program, operating methods, design of the processing plant, the degree of water management being applied and subsequently, the amount of water being conserved (Britz et al., 2006). On a lab scale SBR it is possible to control different parameters and the carbon source. However, in a real dairy industry setting one faces an array of additional challenges which may influence the quantity and composition of PHAs generated from plant effluents. Thus, given the performance difficulties experienced in this study, one might suggest that an EBPR based system for PHA production may struggle to cope with fluctuating influent nutrient compositions, as reflected in varying seasonal loadings within the processing industry. Although the current project did not offer the scope to examine an application of the aerobic dynamic feeding approach, such a system may offer a more robust opportunity in future considerations of dairy wastewater bioconversion to PHAs.
2.5 Bibliography


CHAPTER 3

Investigation of the Microbial Ecology of Sequencing Batch Reactor Sludge Operated under Conditions for EBPR Derived PHA Accumulation
3.1 Introduction

Activated sludge systems for the treatment of wastewaters represent a rather unique tool in relation to process design. Essentially, process engineers must examine potential plant wastewater volumes and nutrient loads to determine appropriate biological treatment steps to achieve licensed effluent discharge limits. Stable operation of the process subsequently falls to control systems design engineers, who determine the operational models that control flow rates, sludge recycling, effluent return etc to each of the treatment steps (Andrews, 1992). Interestingly, within their design strategies, both engineering sets do not concern themselves with understanding the complexity of the key working components, i.e. the mixed microbial consortia. Activated sludge is simply regarded as a “dynamic” input/output module within the process design, whose performance can be controlled by responsive monitoring of key operational variables. As a result, critical bioreactor parameters have been identified, which meet the dual functional demands of process application and control. Table 3.1 presents a number of these variables, viewed from a mechanistic perspective, where grossly defined changes in the microbial community act as indicators of deteriorating reactor fitness.
Table 3.1. Key, mechanistic operational considerations for Biological nutrient removal (BNR).

<table>
<thead>
<tr>
<th>Controlling factor</th>
<th>Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid Retention Time (SRT)</strong></td>
<td>Longer SRTs are required for nitrification and is a direct function of the wastewater temperature.</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>Biological reaction rates are temperature dependent increasing with temperature until a maximum rate is reached.</td>
</tr>
<tr>
<td><strong>Dissolved Oxygen</strong></td>
<td>When DO levels are maintained between 0.5-1.0 mg/L carbonaceous and nitrogenous demands may be avoided. Control of DO is essential for systems operating simultaneous nitrification/denitrification within sludge flocs.</td>
</tr>
<tr>
<td><strong>Filamentous Growth</strong></td>
<td>Elimination or control of key operating conditions that cause filamentous growth, (low DO, low F:M ratio, SRT, complete mixing , etc.)</td>
</tr>
<tr>
<td></td>
<td>Chlorination of the return activated sludge can also kill filaments, but must ensure excess chlorine is not entering the system and disrupting the BNR process.</td>
</tr>
<tr>
<td><strong>Scum and Foam</strong></td>
<td>Remove as quickly and completely as possible. Clarifiers should be designed with good scum removal facilities and foam may be removed directly from the bioreactor by selective wasting from the surface.</td>
</tr>
<tr>
<td><strong>Recycle Loads/recycle streams from sludge processing operations</strong></td>
<td>Leads to nutrient overloading in the system. The magnitude of the problem is dependent on the type of sludge processing and handling operations.</td>
</tr>
</tbody>
</table>
While such considerations are indeed critical for standard operation of biological reactors, specialist applications of activated sludge systems for specific nutrient removal, (e.g. phosphate), requires greater ecological investigation and performance correlations. Biochemical activities and microbial interactions in nutrient removal systems are fairly complex and process design, optimisation, control and troubleshooting depend upon their elucidation (Jeyanayagam, 2005). An example of this is provided in Table 3.2, where key steps, and the required microbial communities, associated with enhanced biological phosphate removal, EBPR, are identified. In summary, key microbial species required in this process are referred to as Polyphosphate accumulating organisms (PAOs). These bacteria are responsible for high P content of EBPR sludge. Under anaerobic conditions, PAOs take up organic substrates, (volatile fatty acids-VFAs), and store them as PHA. (Mino et al., 1987; Arun et al., 1988; Satoh et al., 1992; Smolders et al., 1994; Smolders et al., 1995). The energy for PHA storage is obtained partly from glycogen utilization but mostly from the hydrolysis of the intracellular stored polyphosphate resulting in orthophosphate release into solution. In the aerobic phase, PAO take up excessive amounts of orthophosphate to recover the intracellular polyphosphate levels using the oxidation of stored PHA as energy source. Meanwhile, they grow and replenish the glycogen pool using PHA as both carbon and energy sources (Arun et al., 1988; Pereira et al., 1996). Glycogen accumulating organisms (GAOs) are known to compete within the sludge for the same molar substrates as PAOs but do not perform EBPR (Seviour et al., 2003).
Table 3.2. Processes involved in different cycles in Biological Phosphorus Removal. (Modified from Jeyanayagam, 2005).

<table>
<thead>
<tr>
<th>Zone</th>
<th>Process</th>
<th>Mediating Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>Phosphorus release/PHB uptake</td>
<td>Heteroraphs (PAOs)</td>
</tr>
<tr>
<td></td>
<td>Fermentation: Complex organisms converted to VFAs</td>
<td>Heterotrophs (non-PAOs)</td>
</tr>
<tr>
<td>Pre-Anoxic</td>
<td>Denitrification: Nitrate to nitrogen gas via the:</td>
<td>Heterotrophs (non-PAOs)</td>
</tr>
<tr>
<td></td>
<td>• Use of influent substrate BOD removal and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Use of stored substrate</td>
<td></td>
</tr>
<tr>
<td>Post-Anoxic (if provided)</td>
<td>Denitrification: Nitrate to nitrogen gas via the:</td>
<td>Heterotrophs (Non-PAOs)</td>
</tr>
<tr>
<td></td>
<td>• Use of cellular substrate (endogenous reaction)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Use of methanol</td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>BOD Removal</td>
<td>Heterotrophs (non-PAOs)</td>
</tr>
<tr>
<td></td>
<td>Ammonification: Organic Nitrogen to ammonia nitrogen</td>
<td>Heterotrophs (non-PAOs)</td>
</tr>
<tr>
<td></td>
<td>Nitrification: Ammonia Nitrogen to nitrate nitrogen</td>
<td>Autotrophs (Nitrosococcus)</td>
</tr>
<tr>
<td></td>
<td>PHB Degradation and excess phosphorous uptake</td>
<td>Heterotrophs (PAOs)</td>
</tr>
</tbody>
</table>

PAO- Phosphorous accumulating organism, PHB: Polyhydroxybutyrate, DePAO- Denitrifying PAO, VFAs: Volatile fatty acids.
There are a number of important considerations to be borne in mind when assessing literature on the microbial ecology of such systems. The key organisms in the EBPR process are polyphosphate-accumulating organisms (PAOs). These PAOs remove most of the P from the wastewater. Original growth dependent studies indicated that *Acinetobacter* spp. from the γ-Proteobacteria were of major importance in EBPR activated sludge systems. However, the emergence of accurate, molecular investigative approaches has debunked this theory (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999; Liu *et al.*, 2001). Indeed, members of the β-proteobacteria genus *Rhodococcus*, are now thought to be key (Nielsen *et al.*, 2011). Uncultured Rhodococcus-related ‘*Candidatus Accumulibacter*’ (*Accumulibacter*) is an important PAO in full-scale plants (He and McMahon, 2011). Some strains of *Accumulibacter* can become highly enriched in laboratory-scale reactors allowing for detailed analysis of the processes involved (Oehmen *et al.*, 2007). In EBPR systems they have the capacity for substrate uptake and formation of polyhydroxyalkanoates (PHA) as storage products without growth under anaerobic conditions while most other fast-growing bacteria are inactive. This is followed by growth and uptake/storage of phosphorus in the subsequent aerobic conditions where nitrate and oxygen are present and PHAs are degraded to provide energy for same. This is the first bacterium involved in wastewater treatment for which a genome sequence was generated (Martin *et al.*, 2006), which has allowed subsequent investigations using transcriptomics (He and McMahon, 2011), and proteomics (Wilmes *et al.*, 2008) to describe the unique PAO metabolism. A high level of diversity is observed among *Accumulibacter* in lab-scale reactors and full-scale plants (He and McMahon 2007; Slater *et al.*, 2010), but the physiological differences and functional importance for such plants is still poorly resolved. 16S rRNA gene cloning and sequencing has identified other phosphate accumulators also present in EBPR systems; including various α- and γ-Proteobacteria and members of the high G+C, gram positive bacteria (Bond *et al.*, 1995;
Kawaharasaki et al., 1999; Liu et al., 2001; Wagner et al., 1994). However, only a few studies have attempted to substantiate the presence/roles of these emerging species in full-scale EBPR processes and further work is required in this area (Crocetti et al., 2000; Zilles et al., 2002; Lee et al., 2003; Wong et al., 2004; Wong et al., 2005; Nielsen et al., 2011). Although several other bacteria are claimed to be PAOs (Oehmen et al., 2007), only actinobacterial Tetrasphaera are found in significant abundance in full-scale EBPR plants (Nguyen et al., 2011). The two groups of PAOs have been studied extensively in situ in full-scale systems and Tetrasphaera seem to have a more versatile physiology (e.g. fermentation) than Accumulibacter (Nguyen et al., 2011; Kong et al., 2008). However, other PAOs are likely still present in full-scale plants and remain to be described to understand more in detail the ecological niches the various PAOs occupy and their relative importance for plant function and stability.

What is clear from the above studies is that the microbial diversity identified in laboratory and full scale EBPR reactor systems is heavily dependent on the system design, the influent wastewater and also on the analytical methods applied. In this study, it was observed that PHA accumulation differed between the two EBPR modelled SBRs. Reactor 1 received acetate as a sole carbon source and accumulated PHAs dominated by hydroxyoctanoic, 3-hydroxydecanoic and 3-hydroxydodecanoic acid monomers. Reactor 2 received a combined substrate of skim milk + acetate and accumulated PHAs composed largely of 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid monomers. To determine whether these differing outputs reflected divergent microbial communities, culture dependent and independent ecological investigations were performed in an effort to profile both systems.
3.2 Material and Methods

3.2.1 Media for Culture Dependent Isolation Procedures

Minimal salts agar contained 7g K$_2$HPO$_4$, 3g KH$_2$PO$_4$, 1g (NH$_4$)$_2$SO$_4$ and 15g agar per litre demineralized water. Furthermore, post autoclaving 2ml 10% (w/v) MgSO$_4$ (sterile) and 10mls of a 20% w/v solution of the carbon source (Glucose, acetate or skim milk) was added.

E2 minimal media with a full nitrogen complement (8mM) or with limited nitrogen (1.5 mM) contained 1.1g or 0.2g (NH$_4$)$_2$SO$_4$ respectively, 5.72g K$_2$HPO$_4$, 3.7g KH$_2$PO$_4$ and 15g agar per litre demineralized water. The pH was adjusted to 7.1 using 10N NaOH. 1ml 1M MgSO$_4$, 1ml E2 vitamin solution (Table 3.3), 1ml MT Stock (Table 3.4) was added post-autoclaving. 10mM carbon source (Glucose, acetate or skim milk) was added to the medium (wt/vol). LB agar contained 10g tryptone, (peptone from casein), 5g yeast extract and 10g NaCl added to 800ml of demineralized water. The pH was adjusted to 7.5 with NaOH and the volume adjusted to 1 litre with demineralized water. Nutrient agar contained 5g peptone, 3g yeast extract, 15g agar added to 800ml of demineralized water. It was adjusted to pH 7.0 using NaOH and the final volume adjusted to 1 litre with demineralized water before autoclaving. Skim milk agar contained 5g tryptone (peptone from casein), 2.5g yeast extract, 1g skim milk powder (no inhibitors) 1g glucose, 15g of agar per litre of demineralized water. The skim milk agar was transferred into a waterbath and heated gently, with frequent shaking until completely dissolved. The medium was autoclaved at 110°C for 5 mins.
**Table 3.3.** Composition of MT Stock Vitamin solution.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin H (B7, Biotin)</td>
<td>Sigma</td>
<td>20</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>BDH</td>
<td>20</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride (B6)</td>
<td>Sigma</td>
<td>100</td>
</tr>
<tr>
<td>Riboflavin (B2)</td>
<td>Sigma</td>
<td>50</td>
</tr>
<tr>
<td>Thiamine hydrochloride (Aneurine vitamin B1)</td>
<td>Sigma</td>
<td>50</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>Sigma</td>
<td>50</td>
</tr>
<tr>
<td>Pantothenic acid (Vit B5)</td>
<td>Sigma</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Sigma</td>
<td>1</td>
</tr>
<tr>
<td>Aminobenzoic acid (p-)</td>
<td>GPR</td>
<td>50</td>
</tr>
<tr>
<td>Thiocitic Acid (DL-6,8-)</td>
<td>Sigma</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 3.4. MT Microelements stock in 1N HCl.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>2.78</td>
</tr>
<tr>
<td>MnCl$_2$.4H$_2$O</td>
<td>1.98</td>
</tr>
<tr>
<td>CoSO$_4$.7H$_2$O</td>
<td>2.81</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>1.47</td>
</tr>
<tr>
<td>CuCl$_2$.2H$_2$O</td>
<td>0.17</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.29</td>
</tr>
</tbody>
</table>

(Lageveen *et al.*, 1988)

3.2.2 Culture Dependent Investigations of PHA Accumulation

As the PHA accumulation was observed in both reactors by fluorescent microscopy using the Nile blue stain, 1ml of sludge was collected from both reactors and centrifuged and the supernatant taken off. The pellet was washed with distilled water again, centrifuged for a further five minutes to obtain a pellet. The pellet was washed using 1.5ml sterile phosphate buffered saline solution, (PBS), pelleted by centrifugation and resuspended in 1ml sterile PBS. Tenfold PBS serial dilutions were carried out on respective sludge samples down to $10^{-6}$. 0.1 ml aliquots of dilutions $10^{-3}$ to $10^{-6}$ were spread plated onto; skim milk, nutrient or LB agar. Minimal salts agar with skim milk or acetate as carbon source, respectively, was also employed. PHA production was also incorporated into the culture-dependent screen via, E2 minimal media with a full nitrogen complement (8mM) or with limited nitrogen (1.5 mM) and one of the following carbon sources glucose, acetate or skim milk, respectively. In the case of each media, plates were incubated in a 28°C incubator for 2 to 7 days to facilitate
slow growing bacteria. The above procedure was also carried out on Luria Bertani (LB) agar plates.

3.2.2.1 Restriction Fragment Length Profiling (RFLP) of 16S rRNA Genes from Cultured Isolates

In order to perform RFLP profiling of pure culture isolates, 16S rRNA gene sequences were amplified by performing colony PCRs with universal forward primer 27F (5’- AGAGTTTGATCCTGGCTCAG-3’) and reverse primer 1492R (5’-GGTTACCTTGTTACGACTT-3’) (Sigma-Aldrich) (Brodie et al., 2006). Reaction mixtures were 50 µl and consisted of 2µl a colony suspension, (prepared by resuspending a picked colony sample in 50 µl of sdH2O and heating to 95°C for 5 minutes), 1µl each from 10pmol stocks of primers (Sigma), 8µl dNTPs (50µM), 3µl of MgCl₂ (1.5mM), 5 µl Buffer (1x) and 0.5 µl Taq (2.5 units). All PCR reagents and Taq polymerase were obtained from Fermentas, (UK). The following thermocycling conditions were applied, 95°C for 3 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minute. This was followed by a final 5 minute extension step at 72°C. Sterile water was used in place of cultures for negative control PCR reactions to eliminate false positive results. DNA from a previously cloned and sequenced 16S rRNA gene from Pseudomonas putida CA-3 was employed as the positive control. PCR reactions were subjected to electrophoresis on 1 % wt/vol agarose gels stained with Ethidium Bromide and visualised under UV light using a GeneWizard Gel Doc system, and the image captured using GeneSnap software. The PCR products had to be purified and then subjected to restriction digest with 0.25 µl BSuR1 and 0.25 µl ALuI enzymes (Fermentas), 1 µl of Tango buffer (Fermentas) and 0.5 µl of sterile water and this 2 µl was out in 20 µl reaction and placed in a 37°C incubator overnight to maximise the reaction Profiles were then examined to identify discrete restriction patterns to
enable grouping of isolates into discrete, related clusters, from which representative 16S amplicons were sequenced and the data comparatively analysed against the GENBANK DNA database.

3.2.3 Culture Independent Investigations of PHA Accumulation

3.2.3.1 DNA Isolation Procedures

Genomic DNA from sludge samples taken from both reactors was extracted using a method adapted from Zhou and co-workers, (Zhou et al., 1996). 300 µl of mixed liquor was transferred to 2ml eppendorf tubes with 500 µl of 1% CTAB and 500 µl lysis buffer. 40 µl lysozyme (10mg/ml) was subsequently added, the sample vortexed briefly and then incubated at 37°C for 30 minutes. 200 µl 10% SDS and 6 µl proteinase K (10mg/ml) were subsequently added and the samples incubated at 50°C for a further 30 minutes with mixing at 5 minute intervals. Acid washed beads (0.5g glass beads) were added and the samples subjected to ballistic mixing in a “Bead-beater” (Biospec, USA) at a low speed for 1 minute. Samples were centrifuged at 12,000 rpm for 15 minutes, the supernatants removed to a fresh tube and an equal volume of phenol:chloroform:isoamyl added and mixed for 1 minute. Upon separation into aqueous and organic phases, the supernatant was again removed to a new tube and equal volume of ice cold isopropanol added. The samples were incubated at -20°C for 30 minutes and centrifuged at 12,000 rpm for 15 minutes to pellet any nucleic acid, and the supernatants discarded. The pellets were washed in ice cold 70% ethanol and centrifuged as before. The supernatant was removed and the pellet air dried at 37°C, before being resuspended in 150 µl of sterile water. The samples were run on a 1% gel to observe if DNA was present and spectrophotometric analysis of O.D. 260/280nm values used to assess purity and concentration.
3.2.3.2 PHA Synthase PCR Primer Synthesis and Application to Nucleic Acid Samples

Amplification of *phaC* gene homologues was attempted in order to determine as full a profile as possible of synthase diversity present in the reactors during PHA accumulation periods. Romo *et al.*, 2007 have previously reported the use of degenerate primer pairs for *phaC* gene cloning with mixed success. However, due to the increased sequence availability of current nucleotide databases we designed novel, specific primers for this study. Class I *pha* synthase gene sequences from a range of organisms from α, β and γ proteobacteria were gathered from NCBI. The sequences were translated to amino acid sequence and aligned using the DNASTAR MegAlign, Clustal W Algorithm method. Based on these alignments, the most conserved protein regions were identified. The corresponding regions on the gene sequences were subsequently examined for suitable sites from which to design PCR primer pairs. A focus on strong base-pairing between the 3’ end of the primer and the template was maintained, in addition to regions encoding aromatic and/or low degeneracy amino acids, e.g phenylalanine, tryptophan, tyrosine and methionine, respectively. Forward and reverse primers were chosen from regions reflecting these characteristics and tested for any undesirable secondary structures/primer pairings using DNASTAR’s Primer Select program. None were identified. The following Class I PHA synthase primer pair were subsequently ordered (Sigma); *Forward*: 5’- CCGCCGTGGATAACAAGTTCTAC-3’ with a melting Temperature (Tm): 71.1 °C and *Reverse*: 5’-GCGTGGTGTAGTCGGCCGTTTCCAAATA-3’ (Sigma Aldrich) with a melting Temperature (Tm): 59.6 °C. The following thermocycling conditions were applied: Step 1: 95°C for 5 minutes, Step 2: 95°C x 1 minute, Step 3: 55°C x 45 seconds, Step 4: 72°C x 1 minute followed by 35 cycles of steps 2 to 4. Gels were analysed under UV light using a The primers were validated by using as a positive control a *Rhodococcus* isolate from this study. *R. opacus* strain MR22, *R. erythropolis* and *R. fascians* accumulate small amounts of poly(3HB), whereas *R. ruber* and *Nocardia coralline*
accumulate significant amounts of a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in addition from glucose (Haywood et al., 1991; Alvarez et al., 1997). The resulting PCR product was sequenced to establish accurate amplification. Successful amplicons from the sludge were separated into a clone library using a TOPO-TA Cloning Kit vector pCR2.1-TOPO (Invitrogen). The plasmid vector pCR2.1-TOPO is linearized with single 3’-thymidine (T) overhangs for TA cloning. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3’ ends of PCR products. This allows PCR inserts to ligate efficiently with the vector. The selection markers provided by the vector include ampicillin and Kanamycin resistance, the latter of which was used in our screen of transformants. Traditional blue white screening is also facilitated by disruption of the \textit{lacZ} alpha fragment following successful clone insertion events. Therefore, screening media also contained IPTG inducer and \(\beta\)-galactosidase to identify appropriate transformants. Kanamycin resistant, white colonies were therefore selected and transferred to 96 well plates containing LB-Kanamycin for replica plating onto solid media for storage at 4°C. These colonies were then subjected to colony PCR using M13 forward and reverse primers; (M13 (-20)f: 5’-GTAAAACGACGGCCAGT-3’; M13R: 5’-CAGGAAACAGCTATGAC-3’). RFLP profiling of the \textit{phaC1} homologues was performed as above, before representative clones from dominant RFLP groups were sent for sequencing and BLAST analysis.
3.2.3.3 Investigation of Microbial Diversity within the Sludge; 16S rRNA Amplification and RFLP Analysis

16S rRNA gene amplification was carried out using sludge DNA as the template to obtain an overview of the microbial diversity of both reactors. PCR primers and reaction conditions previously described for the pure culture isolates were employed in this task. Upon successful amplification of the appropriately sized target, a clone library was constructed using the pCR2.1 TOPO vector, and transformed into an *E. coli* host, as per the generation of the *phaC1* gene clone library above. Successful clones were identified via blue white screening on LB-Kan-IPTG-β-Gal solid media, and M13 primer directed amplification of cloned inserts was performed via colony PCR on relevant isolates. RFLP was carried out on all amplicons using the procedure described previously, and representatives from dominant RFLP groups were sequenced and comparatively analysed against DNA databases using the NCBI BLAST algorithm.

3.2.4 RNA Isolation

In addition to assessing the overall diversity of 16S rRNA and *phaC1* genes within sludge DNA, investigation of the dominant, metabolically active species during periods of PHA accumulation was also conducted via RNA analyses. Extraction of total RNA from sludge samples was performed using Ambion’s Totally RNA Kit. 200 mg sludge samples from both reactors were centrifuged at 12,000 g for 5 min, the supernatants discarded and the pellets resuspended in 100 µl TE containing 1 mg/ml lysozyme. The samples were allowed to sit at room temperature for 5 minutes. Following the addition of denaturation solution (300 µl), some modifications were made to the manufacturer’s protocol, as this kit does not have a protocol for mixed microbial sludge. The entire mixture was placed in a 2 ml screw-cap micro centrifuge tube containing 1 g 0.1 mm zirconia/silica beads (Biospec Products,
The mixture was subjected to three x 1 min bead beats at 5000 rpm with 10 min cooling on ice between each. The resulting lysed mixture was centrifuged at 13,000 g for 3 min. The supernatant was measured (this measurement was referred to as one starting volume) and transferred to a sterile micro centrifuge tube. One starting volume of Phenol:Chloroform:Isoamyl alcohol was added to the lysate and vortexed vigorously for one minute. The tubes were left on ice for 5 minutes and then centrifuged at 12,000 x g for 5 minutes at 4°C. The upper, aqueous phase was transferred to a new eppendorf tube, taking care to avoid the material at the aqueous/organic interface. The volume of the aqueous phase was measured and a 10% volume of sodium acetate solution (0.3 M, pH 5.2) added and mixed by inversion for ten seconds. One starting volume of Acid-Phenol:Chloroform was added, vortexed vigorously for one minute, placed on ice for 5 minutes and then centrifuged at 12,000 x g for 5 minutes. The upper, aqueous phase was transferred to a new Raze-free vessel with a capacity of at least twice the volume of the lysate at this point of the procedure. An equal volume of ice-cold isopropanol was added to the prepared RNA prep and mixed well. The preparation was placed at -20°C for at least 30 minutes, followed by centrifugation at 12,000 x g for 15 minutes. The supernatant solution was removed carefully so as not to disturb RNA any pellets adhered to the tube. The residual salts were removed by washing the pellet with 70% ethanol as follows: 70% ethanol (300 µl) was added to the RNA pellet and vortexed for 30 seconds. The sample was then centrifuged for 5-10 minutes at 7,500 rpm at 4°C and the ethanol supernatant carefully aspirated using a fine tipped pipette and low pressure vacuum. The pellets were allowed to dry in a laminar flow hood and resuspended in a desired volume of diethyl-pyrocarbonate treated sdH2O. The purity/integrity of the RNA samples was confirmed visually by running samples on Ethidium Bromide (EtBr) stained 1% Tris-Borate (TB)E gels for 20 minutes at 120mV, and identifying key ribosomal bands upon UV exposure. In addition, spectrophotometric determination of 260/280nm absorbance
levels was conducted to determine the concentration of nucleic acid in the samples and the possibility of protein contamination.

While all methods claim to generate DNA-free RNA, it is prudent to treat all samples as being contaminated with DNA. Hence RNA was DNase treated using Ambion’s TURBO DNA-free™. However, treated RNA occasionally required an additional DNase treatment, (1U DNase enzyme), and sample incubation for 30 min. In order to verify that the treated RNA was DNA free, a PCR of the 16S rRNA gene was carried out on the DNase-treated sample. The PCR reaction and cycling conditions were as follows: in a 25 µl reaction, 1.5 mM MgCl₂, 0.2 mM each dNTP, 5 pmol of each primer (27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG ACT T-3’) (Lane et al., 1991)), 1 X buffer (20 mM Tris pH 8.4, 50 mM KCl) and 1 U Taq polymerase (Invitrogen, UK). The cycles for the reaction were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 92°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final elongation step of 72°C for 5 min. All PCR amplifications were carried out using an MJ Research PTC-200P Thermal cycler.
3.2.4.1 Reverse Transcription and PCR Analysis (RT-PCR) of Sludge RNA

For each sample, 2 µg of total RNA was reverse transcribed with 5 µmol of random decamers and mixed and heated for 3 minutes at 85°C. The tubes were placed on ice and the remaining RT components; 2 µl 10x RT buffer, 4 µl of dNTP Mix (2.5 mM), 1 µl RNase inhibitor (10 units/µl) and 1 µl MMLV-RT (100 units/µl) were added. Samples were mixed and incubated at 42-44°C for one hour. Inactivation of the reverse transcriptase was carried out by incubation at 92°C for 10 minutes. The resulting cDNA was used as a template for the amplification of 16S and \textit{phaC1} gene transcript from metabolically active fractions within the sludge. Where poor amplification results were deemed to be potentially related to low cDNA levels, a GenomiPhi HY DNA amplification kit (GE Healthcare) was applied to increase cDNA levels. The process consisted of heat denaturation of the template in sample buffer by mixing 2.5 µl of template (at least 10 ng/µl) with 22.5 µl of sample buffer and heating to 95°C for 3 minutes, followed by cooling on ice to 4°C. Each amplification reaction was combined with 22.5 µl of reaction buffer with 2.5 µl of enzyme mix on ice and added to the cool sample. The sample was incubated at 30°C for 4 hours and the sample was heated to 65°C for 10 minutes and cooled to 4°C. The amplified material was purified using a microcentrifuge filter column extraction kit (Qiagen) as per the manufacturer’s instructions.
3.2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Efforts were also made to assess changes in the microbial biomass over time and to potentially highlight any intrinsic bias in the gene cloning strategies, via the application of DGGE analyses. The bacterial 16S rDNA targeted primer pair consisting of; 341F (5’-CCTACGGGAGGCAGCAG-3’) with a GC clamp (5’-CGCCCCGCCCGCAGCCGAGCTCCGCAGCCCCGCCG-3’) and 907R (5’-CCGTCAATTCTTTRAGTTT-3’) has been widely used for DGGE analysis of bacterial communities (Muyzer et al., 1993). The primer pair were employed here with 10ng/μl and 50 ng/μl of DNA, using Touch W (Table 3.5) and the EUB16S PCR programmes. The latter consisted of the following thermocycling conditions; 94°C for 5min, followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minute, and a final 10 minute extension. Initially, reactions contained 1 μl of 341- GC F (10pmol/μl), 1 μl 907 R (10 pmol/μl), 8μl dNTPs (1.25mM), 6 μl of 25mM MgCl₂ (3mM), 5 μl 10x buffer, 0.5 μl of Taq polymerase (2.5 units) (Fermentas), 26.5 μl sterile water and 2 μl of DNA sample. Also Dimethyl sulfoxide (DMSO) (2.5 μl) was added to each reaction to allow for strand separation in potential GC rich regions. Numerous attempts to optimise the DGGE approach were undertaken via modification of thermocycling conditions, reagent concentrations and re-isolation of DNA from samples to ensure purity.
Table 3.5. Touch W programme used on the DNA extracted from reactor 1 and 2.

<table>
<thead>
<tr>
<th>Touch W Programme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 94°C for 10 minutes</td>
</tr>
<tr>
<td>2. 94°C for 1 minute</td>
</tr>
<tr>
<td>3. 66°C for 1 minute</td>
</tr>
<tr>
<td>4. 72°C for 3 minutes</td>
</tr>
<tr>
<td>5. Go to 2, two times</td>
</tr>
<tr>
<td>6. 94°C for 1 minute</td>
</tr>
<tr>
<td>7. 64°C for 1 minute</td>
</tr>
<tr>
<td>8. 72°C for 3 minutes</td>
</tr>
<tr>
<td>9. Go to 6, two times</td>
</tr>
<tr>
<td>10. 94°C for 1 minute</td>
</tr>
<tr>
<td>11. 62°C for 1 minute</td>
</tr>
<tr>
<td>12. 72°C for 3 minutes</td>
</tr>
<tr>
<td>13. Go to 10, for two times</td>
</tr>
<tr>
<td>14. 94°C for 1 minute</td>
</tr>
<tr>
<td>15. 60°C for 1 minute</td>
</tr>
<tr>
<td>16. 72°C for 3 minutes</td>
</tr>
<tr>
<td>17. Go to 14, two times</td>
</tr>
<tr>
<td>18. 94°C for 1 minute</td>
</tr>
<tr>
<td>19. 58°C for 1 minute</td>
</tr>
<tr>
<td>20. 72°C for 3 minutes</td>
</tr>
</tbody>
</table>
3.2.6 Fluorescent In Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) was employed in an attempt to determine the spatial mapping of specific bacteria within sludge samples. FISH has been carried out on reactor 1 and 2 biomass samples with hybridization of probes for EUB Mix Alpha-, Beta- and Gammaproteobacteria. The FISH probes are single stranded oligos and 5’ labelled with fluorophore. Each probe was ordered with a different fluorophore as it is possible to apply multiple probes which can be detected separately. The probes used were EUB Mix (50 ng/µl of EUB 3381 and 100 ng/µl of EUB 338 II/III), Alf 1b, Bet 42a and Gam 42a (Table 3.6). The test organisms were Pseudovibrio (Alphaproteobacteria), Ralstonia eutropha (Betaproteobacteria) and E. coli DH5α (Gammaproteobacteria). Probes were dissolved in 100 µl sterile TE at -80°C and working concentrations of 50ng/µl made up in sterile water and stored at -20°C.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe Sequence (5’-3’)</th>
<th>rRNA target</th>
<th>Specificity</th>
<th>% Formamide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB Mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUB I</td>
<td>GCTGCCTCCCGTGGACTGAGT</td>
<td>16S, 338±355</td>
<td>Diverse Bacteria</td>
<td>0-70</td>
<td>Amann et al., 1990</td>
</tr>
<tr>
<td>EUB II</td>
<td>GCAGCCACCCGTAGGTGT</td>
<td>16S, 338±355</td>
<td>Planctomycetales</td>
<td>0-50</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>EUB III</td>
<td>GCTGCCACCCGTAGGTGT</td>
<td>16S, 338±355</td>
<td>Verrucomicrobiaceae</td>
<td>0-50</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>Alf 1b</td>
<td>CGTTCG(C/T)TCTGAGCCAG</td>
<td>16S, 19±35</td>
<td>α-Proteobacteria</td>
<td>20</td>
<td>Manz et al., 1992</td>
</tr>
<tr>
<td>Bet 42a</td>
<td>GCCTCCCACCTCGTTT</td>
<td>23S, 1027±1043</td>
<td>β-Proteobacteria</td>
<td>35</td>
<td>Manz et al., 1992</td>
</tr>
<tr>
<td>Gam 42a</td>
<td>GCCTCCCACATCGTTT</td>
<td>23S, 1027±1043</td>
<td>γ-Proteobacteria</td>
<td>35</td>
<td>Manz et al., 1992</td>
</tr>
</tbody>
</table>
8 µl of each hybridization buffer was added to each well in the slide. The hybridization buffer consisted of 360 µl 5M NaCl (0.9M), 40 µl 1M Tris/HCl, pH 7.2 (0.02M), formamide, and sterile water to bring the volume to 2 mls. 2 µl of 10% SDS was added last to avoid precipitation. The percentage formamide added in the hybridization buffer depends on the probe. EUB Mix requires 0-50% (25% used), 20% for Alf 1b, 35% for Bet 42a and 35% for Gam 42a. Probes were added to each well as follows: 50ng probes were employed for pure cultures (1µl of working stock) and 100ng probes used for environmental samples (2 µl of working stock). Slides were placed horizontally in a 50 ml tube (coated in tin foil) containing filter paper/tissue saturated in remaining hybridization buffer at the bottom. The slides were hybridized at 46°C for 1.5-3 hours. Meanwhile, a tube containing washing buffer was preheated using a waterbath at 48°C. For the washing buffer, the amount of 5M NaCl added depended on the Formamide concentration (Table 3.7). In addition to the 5M NaCl, wash buffer contained Tris/HCl, (pH 7.2), 0.5M EDTA (only required if formamide >20%), sterile water and 2 µl of 10% SDS (added last to avoid precipitation). As per pcr cloning experiments, a *Rhodococcus* isolate acted as positive control.
### Table 3.7. Probes and relevant Formamide concentrations.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Formamide in hybridisation buffer (%)</th>
<th>Amount 5M NaCl in was buffer</th>
<th>Control organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB MIX</td>
<td>0-50 (try 25% initially)</td>
<td>1490ul (0.159M)</td>
<td><em>E. coli</em> DH5α</td>
</tr>
<tr>
<td>Alf 1b</td>
<td>20</td>
<td>2150ul (0.225M)</td>
<td><em>Pseudovibrio</em></td>
</tr>
<tr>
<td>Bet 42a</td>
<td>35</td>
<td>700ul (0.080M)</td>
<td><em>Ralstonia eutropha</em></td>
</tr>
<tr>
<td>Gam 42a</td>
<td>35</td>
<td>700ul (0.080M)</td>
<td><em>E. coli</em> DH5α</td>
</tr>
</tbody>
</table>

Slides were removed from the hybridization chambers and 3 mls of preheated washing buffer was used to briefly rinse slides prior to immersing them in the 50 ml tubes containing the wash buffer. Efforts were made to prevent the sample temperature dropping significantly during change of buffers which can result in non-specific binding of probes. The slides were washed for 25 minutes at 48°C in hybridization buffer in a rotary oven at 100rpm. Slides were rinsed with cold, deionized water and left to air dry in the dark. The samples were subsequently stained with Nile Red to allow PHA visualisation. 20 µl of a 1 mg/ml Nile Red solution was placed on the slides for 10 seconds. Slides were then washed briefly with distilled water and dried at room temperature in the dark. Analyses of the samples were performed using a Leica DM3000 epifluorescence microscope system, with an EL6000 metal halide external light source and a DFC490 8mp, CCD digital camera, (Leica Microsystems, Germany) following the appropriate filter for the probe (Table 3.8).
Table 3.8. Probes used in FISH and Nile Red staining.

<table>
<thead>
<tr>
<th>Probe/Stain</th>
<th>Target organism(s)</th>
<th>Abs/Emission</th>
<th>Filtercube</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB MIX</td>
<td>Bacteria</td>
<td>560/580</td>
<td>N2.1</td>
</tr>
<tr>
<td>Alf 1b</td>
<td><em>Alphaproteobacteria</em></td>
<td>495/520</td>
<td>I3/L5</td>
</tr>
<tr>
<td>Bet 42a</td>
<td><em>Betaproteobacteria</em></td>
<td>552/570</td>
<td>Cy3/Cy5</td>
</tr>
<tr>
<td>Gam 42a</td>
<td><em>Gammaproteobacteria</em></td>
<td>649/670</td>
<td>Cy3/Cy5</td>
</tr>
<tr>
<td>Nile Red</td>
<td>PHA</td>
<td>543/598</td>
<td>I3</td>
</tr>
</tbody>
</table>
3.3 Results and Discussion

3.3.1 Culture Dependent Investigations of PHA Accumulation

The importance of obtaining pure cultures from a mixed microbial system such as EBPR sludge is a key step if basic understanding of how these systems work and how they might then be manipulated is to be obtained (Palleroni, 1997; Amann, 2000). Pure cultures allow biochemical pathways or genes in isolation to be studied. However problems with analyzing natural microbial communities using culture-dependent methods sometimes referred to as the ‘plate count anomaly’ have been highlighted previously (Amann et al., 1995; Schramm and Amann; 1999). A key issue is ending up with what Amann and Ludwig (Amann and Ludwig, 2000) refer to as ‘laboratory-weeds’. Therefore, culture-independent techniques provide an essential, complementary approach to isolation based investigations. In this project culture dependent and independent characterizations were performed on both reactors during the periods of peak PHA accumulation.

With respect to pure culture isolations, several differing solid media were employed in the screens in an attempt to cover a range of nutrient conditions which might be influential within the reactors. Interestingly, nutritionally complex LB agar supported the most heterogeneous growth with gross, differing morphologies used in the selection of isolates. In total, 110 pure cultures were isolated from reactor 1 (fed with acetate alone), while reactor 2 (fed with acetate and skim milk) yielded 252 pure cultures. The reason for this considerable disparity cannot be categorically stated. However, it is reasonable to suggest that the complex skim milk substrate present in reactor 2 contributed to an increased diversity in the culturable fraction within the sludge. Efforts were made to amplify the 16S rRNA genes from each of the 362 total isolates, to enable a reductive grouping process and identify key microbial species.
However, the PCR approach proved far from routine and multiple rounds of PCR with considerable modifications to reaction conditions and reagents were necessary to achieve even partial sampling of the full complement of isolates. Relevant amplicons from only 55 of the isolates from reactor 1 and 65 of the isolates from reactor 2 could be generated using this method. It is possible therefore that while the 27f/1429r 16S rRNA gene primers are well publicised in the study of eubacterial diversity, they may not have been sufficiently degenerate to enable amplification from a considerable proportion of the isolates generated. It should be noted redundancy within the actual isolates may have contributed to the overall percentage failures also. RFLP profiling of these gene targets was successful in identifying potential species clusters within the isolates (accepting that some of these may have been repeat isolates). There were seven dominant RFLP groups in reactor 1 and thirteen dominant RFLP groups in reactor 2 (Figure 3.1). RFLP profiling of these gene targets was successful in identifying potential species clusters within the isolates (accepting that some of these may have been repeat isolates). The 16S PCR products were purified and were sent away for sequencing and the resulting data analysed using the NCBI BLAST.
Gel presents sample RFLP profile outputs for reactor 1 (Top row) and reactor 2 (Bottom row). Red boxes highlight similar profiles generated with reactor 1 isolates.

**Figure 3.1.** Sample RFLP analysis of 16S rRNA Clones obtained from both reactors.

BLAST analysis of the sequence data from the representative 16S rRNA amplicons from both reactors was used to identify the respective isolates to at least the species level (>98% sequence identity). Table 3.9 presents the results of these analyses. Interestingly, the 2 reactors showed a very low level of overlap with only *Acinetobacter* and *Thauera* species being commonly identified in both. Thus the cultivable communities appeared to be strongly influenced by the carbon nutrient composition of the respective reactors influents, despite acetate being common to both.
Applying these species identities to the proportionate RFLP profile distributions overall, one can propose the relative species distributions within the cultivable fraction in each reactors accessed by this technique. These samples that showed accumulation of PHA in the previous chapter in HPLC and GC analysis underwent molecular analysis (Section 2.3.4). Reactor 1 isolates demonstrated the following species distributions *Pseudomonas* (77%), *Delftia acidovorans* (3%), *Acinetobacter sp.* (5%), *Aminobacter sp.*, (3%) *Bacillus sp.* (3%), *Thauera sp.*, (3%), *Agromyces* (3%), and *Cytophaga sp.* (3%). With respect to PHA accumulation, many of the species identified have the potential to accumulate polyesters. For example *Delftia acidovorans* is known as a PHA producer (Loo and Sudesh, 2007) and it can efficiently accumulate PHA containing high molar fractions of 4-hydroxybutyrate (Saito and Doi, 1994). While *Pseudomonas sp.*, which were the most dominant bacteria among the isolates profiled, have been heavily investigated on the basis of their capacity for mcl-PHA production (Huisman et al., 1989; Timm and Steinbuchel, 1990; Preusting et al., 1990; Gross et al., 1989; Fritzsche et al., 1990). Relative species distributions among reactor 2 profiled isolates were more evenly distributed between *Pseudoxanthomonas* (32%), *Thauera sp* (24%), *Acinetobacter* (24%), *Citrobacter sp* (8%), *Lactococcus lactis* (5%), *Lysinibacillus* (5%) and *Elizabethkingia* (2%). One of the dominant species in the reactors were *Thauera* which is known to be a PHA producer (Oshiki et al., 2008) and also has been shown to produce PHA in sugar cane molasses (Albuquerque et al., 2010) and *Pseudoxanthomonas*. It was decided to look at the overall phylotypes to give a better sense of the overall perspective on the similarities/differences between them than comparing them to individuals. The results are presented in figure 3.2(A and B) and highlight a dominance of Gamma-proteobacteria within both reactors. Figure 3.3(A
and B) show the evolutionary taxa of the dependent cultures obtained from reactor 1 and 2. It is not possible to rule out a potential bias within the specificity of the primers as the full complement of isolates did not yield amplicons. However, what is clear is that a significant number of potential PHA accumulating bacteria are viably present within the reactor systems.

**Table 3.9.** Identities of RFLP profile representative pure cultures from Reactor 1 and 2.

<table>
<thead>
<tr>
<th><strong>16S rRNA gene identities (&gt; 98%)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reactor 1 (110)</strong></td>
<td><strong>Reactor 2 (252)</strong></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Acinetobacter sp.</td>
</tr>
<tr>
<td>Pseudomonas mendocina</td>
<td>Elizabethkingia sp</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>Lysinibacillus fusiformis</td>
</tr>
<tr>
<td>Delftia acidovorans</td>
<td>Thauera sp.</td>
</tr>
<tr>
<td>Cytophaga sp.</td>
<td>Citrobacter sp.</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>Lactococcus lactis</td>
</tr>
<tr>
<td>Agromyces sp.</td>
<td>Pseudoxanthomonas sp.</td>
</tr>
<tr>
<td>Thauera sp.</td>
<td></td>
</tr>
<tr>
<td>Aminobacter sp.</td>
<td></td>
</tr>
</tbody>
</table>
A. Reactor 1.

B. Reactor 2.

**Figure 3.2.** Chart showing relevant proportion of phylotypes among reactor isolates.
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.71881629 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 888 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
Figure 3.3 B. Evolutionary relationships of taxa for Reactor 2.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.16796435 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 299 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
3.3.2 Culture Independent Investigations of PHA Accumulation

3.3.2.1 Investigation of Microbial Diversity within the Sludge; 16S rRNA Amplification and RFLP Analysis

As stable reactor conditions facilitating PHA accumulation became established, considerable effort was directed toward optimizing DNA extraction methods enabling downstream molecular investigations. 16S rRNA gene amplification and clone library generation was successfully carried out using sludge DNA as template to obtain information on the microbial community composition in both reactors and identify any dominant members. 224 clones were obtained with DNA from reactor 1 and 56 clones from the reactor 2 sample (Figure 3.4).
Figure 3.4. M13 amplicons obtained from both reactors. Lane 1, 50, 51 & 100: 1kb DNA Plus ladder. Lane 2-49 & 52-99: M13 Profiles 16S rRNA clones from the reactors.

The clone library yields, particularly for reactor 2, seemed low and no obvious root cause could be attributed. Several potential contributing factors are possible for the disparate returns from the respective reactor samples including differences in the number of rRNA operons, different efficiencies of cell lyses and DNA extraction or shifts due to PCR amplification bias (Eschenhagen et al., 2003).

Following digestion with appropriate nucleases, 32 RFLP groups were found to be dominant in reactor 1 while 11 RFLPs were found to be dominant in reactor 2 (Figure 3.5). Compared to the culture dependent method there were a greater number of RFLPs found in reactor 2 (considering that reactor 2 only had 56 clones with 11 RFLP groups generated). The greater numbers of RFLP obtained in the culture independent method are typical of such analyses and indicate that certain bacteria
present in the reactor were unculturable on the media employed. Thus the culture
independent method was more successful in accessing species diversity within the
reactors.

**Figure 3.5.** RFLP analysis of 16S rRNA Clones obtained from both reactors. Lane 1,
50, 51 &100: 1kb DNA Plus ladder. Lane 2-49 & 52-99: 16S rRNA clones from the
reactors 1 and 2, respectively.
Sequencing of representative cloned 16S rRNA genes and BLAST analysis allowed identification to species level of the respective RFLP groups (Table 3.10). In a similar fashion to the culture dependent screen only limited species overlap was observed between reactor 1 and 2, confined to Runella defluvii and Zoogloea oryzae.

Overlaying the species identities generated by the RFLP profiling and representative sequencing approach, the relative distribution of species reported in the reactors by this method were determined. Reactor 1 clone distribution was as follows; Zoogloea resiniphila (83%), Zooglea oryzae (2%), Pedobacter composti (5%), Neissericeae sp. (2%) Rhodobacter sp. (2%), Runella defluvii (3%) and Streptococcus sp. (3%). It can be clearly observed that Zooglea dominated the 16S RFLP profiles in reactor 1. Although, Zooglea species such as Zoogloea ramigera have been considered an important organism in wastewater treatment, there is little information on the distribution and concentration of this organism in wastewater treatment processes (Lu et al., 2001). Zooglea are capable of producing intracellular PHB and utilizing organic acids as substrate (Park et al., 1999). However, it has also been observed that they produce exocellular polymeric material which acts as a matrix to enmesh other microorganisms (Horan and Eccles, 1986) and large masses of Z. ramigera may physically interfere in the sludge compaction and settling. Typically the appearance of an excess of Zooglea in EBPR reactors negatively affects process performance because the overabundance of Zooglea results in additional substrate consumption (VFA) and contributes to effluent quality deterioration (Montoya et al., 2008). In contrast, the relatively poorly represented Rhodobacter sp. are strongly associated with high level PHA accumulation under appropriate conditions and polyphosphate kinase genes from these hosts have been reported in established EBPR systems (McMahon et al., 2002). RFLP based species distribution among the reactor
2 clones was as follows; *Runella defluvii* (50%), *Zooglea oryzae* (20%), *Flavobacterium* sp. (9%), *Simplicispira* sp. (6%), Uncultured *Sphingobacteria* sp. (6%), *Arcicella* (6%) and *Leadbetterella bysophila* (3%). The results presented significantly different population composition, as detectable by the approach used. *Runella sp.* dominated reactor 2 and have been previously linked with activated sludge performing EBPR, although a definitive contribution by this strain to the process has not been demonstrated (Ryu *et al.*, 2006). A similar finding has recently been reported for *Simplicispira sp.* also (Lu *et al.*, 2007).

Table 3.10. Sequence identities of 16S clone RFLP representatives from Reactors 1 and 2.

<table>
<thead>
<tr>
<th>16S rDNA</th>
<th>Reactor 1 (224 clones) % Similarity</th>
<th>Reactor 2 (56 clones) % Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoogloea oryzae</td>
<td>97</td>
<td><em>Simplicispira sp.</em> 98</td>
</tr>
<tr>
<td>Zoogloea sp.</td>
<td>99</td>
<td><em>Sphingobacteria sp</em> 93</td>
</tr>
<tr>
<td>Rhodobacter sp.</td>
<td>98</td>
<td><em>Leadbetterella bysophila</em> 78</td>
</tr>
<tr>
<td>Runella defluvii</td>
<td>97</td>
<td><em>Runella defluvii</em> 93</td>
</tr>
<tr>
<td>Pedobacter composti</td>
<td>99</td>
<td><em>Flavobacterium sp.</em> 87</td>
</tr>
<tr>
<td>Neisseriaceae sp.</td>
<td>93</td>
<td><em>Zooglea oryzae</em> 97</td>
</tr>
<tr>
<td>Streptococcus sp.</td>
<td>99</td>
<td><em>Arcicella sp.</em> 77</td>
</tr>
</tbody>
</table>
As per the culture dependent approach, broad phylotype distributions were allocated to the reactors on the basis of the 16S rRNA gene identities to enable crude, but direct comparison of the separate reactors. The results, presented in Figure 3.6(A and B) highlight a common dominance of β-proteobacteria while β-proteobacteria and Bacteroides were the two classes in reactor 2. Figure 3.7(A and B) show the evolutionary taxa of the dependent cultures obtained from reactor 1 and 2.
Figure 3.6. Chart showing relevant proportion of phylotypes among reactor 16S RFLP clones.
**Figure 3.7 A.** Evolutionary relationships of taxa for Reactor 1.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.70222284 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 762 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
Figure 3.7 B. Evolutionary relationships of taxa for Reactor 2.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.77769289 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 885 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
3.3.2.2 PHA Synthase PCR Primer Synthesis and Application to Nucleic Acid Samples

The PhaC1 primers specifically targeting genes for short chain length PHA synthesis were applied to sludge DNA in an attempt to generate as complete a profile as possible of the diversity of potential PHA producers present in the reactors. The *PhaC1* amplicons obtained underwent RFLP profiling and 7 dominant RFLPs emerged from reactor 1 while 14 appeared to be associated with reactor 2 (Figure 3.8).

![Sample RFLP analysis of PhaC1 Clones obtained from both reactors.](image)

Red boxes indicate lanes displaying similar profiles used in establishing groupings.

**Figure 3.8.** Sample RFLP analysis of PhaC1 Clones obtained from both reactors.

BLAST analysis of sequence identities were subsequently performed (Table 3.11). *Rhodobacter sp.*, and *Dechloromonas aromatica pha* synthases were found to be present in both reactors using this method. With respect to *Rhodobacter sp.*, this finding correlated with the earlier identification of this species via the culture
independent 16S rRNA gene screening. *Thauera sp.* phaC1 gene cloning via PhaC1 primers was also further supported by the isolation of this species during the culture dependent method. However no other species identified by the PhaC1 primers were highlighted via the culture dependent or independent methods. Many of the bacteria associated with the identified synthase genes are known to produce PHAs in diverse wastewaters. *Rhodobacter sp.*, has been shown to produce 30% cell dry weight (PHB) in Palm Oil Mill Effluent (POME) treated anaerobically (Hassan et al., 1997). *Burkholderia cepacia* has been shown to produce PHA from different waste also such as palm oil, producing yields of 57.4% cell dry weight (Alias and Tan 2005). Indeed, *Thauera sp.*, *Azoarcus sp.*, and *Dechloromonas sp.*, are also known to produce PHA to varying degrees (Oshiki et al., 2008; Lemos et al., 2008; Ahn et al., 2007; Wolterink et al., 2005).

**Table 3.11.** phaC1 gene clone identities isolated from Reactor 1 and 2.

<table>
<thead>
<tr>
<th>PhaC1</th>
<th>Reactor 1 (11 clones)</th>
<th>% Similarity</th>
<th>Reactor 2 (18 clones)</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodobacter sp.</em></td>
<td>79</td>
<td><em>Rhodobacter sp.</em></td>
<td>81</td>
<td></td>
</tr>
<tr>
<td><em>Dechloromonas aromatica</em></td>
<td>93</td>
<td><em>Dechloromonas aromatica</em></td>
<td>92</td>
<td></td>
</tr>
<tr>
<td><em>Thauera sp.</em></td>
<td>80</td>
<td><em>Burkholderia cepacia</em></td>
<td>87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Azoarcus sp.</em></td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>
In relation to the phylotype distributions within the phaC1 RFLP groups, figure 3.9 demonstrates the $\beta$-proteobacterial genes were dominant in the reactors. *Betaproteobacteria* have been shown to be dominant in EBPR processes as well as producing PHA in these processes (Kämpfer *et al.*, 1996, Wagner *et al.*, 1994 and Bond *et al.*, 1999a). Figure 3.10(A and B) show the evolutionary taxa of the dependent cultures obtained from reactor 1 and 2.
**Figure 3.9.** Chart showing relevant proportion of phylotypes among reactor using the *phaC1* gene clones.
Figure 3.10 A. Evolutionary relationships of taxa for Reactor 1.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.39096490 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 441 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
Figure 3.10 B. Evolutionary relationships of taxa for reactor 2.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.85312636 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 123 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
3.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

In order to investigate the metabolically active fraction within the reactor during periods of high PHA accumulation, RNA was successfully isolated from reactor 1 and 2 during periods of PHA accumulation and subjected to reverse transcription to cDNA. The cDNA was used as a template to carry out PCRs for both 16S rRNA and \textit{phaC1} synthase genes and the products cloned and transformed into \textit{E. coli}, with blue-white screening employed to identify successful cloning events. In the case of 16S rRNA genes, the cDNA procedure appeared to have been successful with 480 clones being obtained from reactor 1 and 336 cDNA derived clones from reactor 2. However, when M13 primer driven colony PCRs were performed, many were found to lack amplifiable inserts, despite numerous attempt at PCR optimisation. RFLP profiling of those that could be amplified reduced the overall potential diversity to 39 distinct clone groups from reactor 1 and 48 from reactor 2, respectively (Figure 3.11).

Strongly dominant profile among Reactor 1 samples is highlighted in red boxes. \textit{Rhodococcus} was employed as the positive control (result not shown).

**Figure 3.11.** Sample RFLP analysis of the clones from cDNA.
Sequencing of clone representatives and BLAST analyses against the GenBank nucleic acid database revealed that many of the sequences contained vector nucleotides or sequence sharing no known identity to previously sequenced genes. Sequencing of alternative clones from representative groups could not resolve this issue. As a result, despite the considerable apparent starting volumes, a very limited number of species were finally identified, which were difficult to reconcile with the earlier findings of the culture dependent and independent methods. The species identified in reactor 1 were *Escherichia coli* and *Bacillus sp*, while in Reactor 2 *Escherichia coli*, *Citrobacter freundii*, *Escherichia fergusonii* and an uncultured *Proteobacteria* were identified. It has been shown that even though 16S rDNA analysis is a very useful technique for culture-independent analysis of complex microbial communities, the problem is that the initial clone library volumes do not reflect the *in situ* quantities of the respective microorganisms. Thus interpreting key issues for troubleshooting can be problematic. Variation between clone numbers and genuine diversity could be a consequence of varying numbers of rRNA operons within species, different efficiencies of cell lyses and DNA extraction or shifts due to PCR amplification bias (Eschenhagen et al., 2003).

In a similar approach *phaC1* gene amplification and cloning from cDNA was carried out, to determine how the synthase diversity identified using cloning from the sludge DNA was reflected in the expression profile. RFLP was carried out to identify the dominant species and representative amplicons sequenced. However, in a similar fashion to the outcome of 16S cDNA investigation, the species diversity was not only highly limited, but also dominated by species which did not correlate with earlier findings. Synthase gene expression from a *Saccharomyces cerevisiae* strain was reported in reactor 1, while the presence of *Escherichia coli* synthase expression was
again noted for reactor 2. It is difficult to envisage any meaningful contribution of *E. coli* to PHA accumulation within the reactors. *E. coli* has typically been used as a recombinant strain to harbour *pha* genes from other bacteria (Liu *et al.*, 2007). Furthermore, while *Saccharomyces* species have been associated with PHA production in the past (Nuti and Lepidi, 1974) the nature of the dairy effluent and the near neutral pH of the system, would be unlikely to favour their growth in the reactor. It must be considered therefore that some contaminating aspect or depreciation in the accuracy of the amplification strategy likely contributed to the findings reported.

### 3.3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

As the culture dependent and independent methods yielded contrasting insights into the potential community composition different profiles, efforts were made to perform corroborating molecular characterisations. The PCR-DGGE method has been widely recognised as a powerful tool for use in microbial technology. DGGE is based on the electrophoresis of PCR-amplified 16S ribosomal DNA (rDNA) fragments in polyacrylamide gels containing a linearly increasing denaturant gradient (Muyzer *et al.*, 1993). The PCR products, which are essentially all the same size, are separated into bands as they electrophorese through a polyacrylamide gel of increasing denaturant. These bands can then be excised and the nucleotide sequences determined without the need for cloning. PCR-DGGE assay allowing the microbial composition and diversity of a given system to be analysed without the need to isolate individual species (Muyzer and Ramsing, 1995; Ishii *et al.*, 2000). It is particularly effective in analyzing the microbial community structure of nonculturable environmental samples such as microorganisms responsible for EBPR. A variety of
conditions were employed for DGGE optimisation in this study. These included different concentrations of DNA, varying PCR programmes and primers combinations along with the addition of DMSO. The GenomiPhi kit (GE Healthcare) was used to increase the amount of potential template. However, despite our efforts it was not possible to produce appropriate gel bands. This may have been caused by a few problems that are associated with DGGE from complex microbial samples. Firstly, a DGGE experiment is limited to DNA fragments typically below 500 bp in size (Myers et al., 1985). Since common complementary technique involves isolating bands from DGGE for sequencing (for taxonomic identification of bands of interest), this size limit restricts the amount of sequence available for identification (Muyzer and Smalla, 1998). Thirdly, DGGE requires large quantities of DNA for effective resolution, often in the order of 500ng of PCR product for complex environmental samples (Nakagawa and Fukui, 2002). Therefore, PCR DGGE of the 16S rRNA gene was not successfully employed in this project.

3.3.5 Fluorescent In Situ Hybridisation (FISH)

The FISH technique with group-specific oligonucleotide probes targeting rRNA has previously demonstrated that EBPR sludge contained alpha-, beta- and gamma-subclasses of proteobacteria, the Cytophaga group, and Gram-positive bacteria with high G+C DNA contents (Wagner et al., 1993, Manz et al., 1994; Kämpfer et al., 1996, Snaidr et al., 1997; Sudiana et al., 1998, and Bond et al., 1999). FISH with oligonucleotides specific for rRNA has been used successfully to analyze the bacterial populations of other sludges (Snaidr et al., 1997). The culture independent method carried out on reactor 1 and 2 had shown that Betaproteobacteria was the dominant class while the culture dependent method suggested that Gammaproteobacteria was dominant in both reactors. Therefore, alpha-, beta- and
gamma-subclasses of proteobacteria oligonucleotide probes were used to detect these groups in both reactors. The probed samples were dual stained with Nile Red to enable co-localisation of FISH probes with PHA accumulation within the sludge. Binding of BET42a probes at sites displaying enhanced fluorescence with Nile Red staining suggested that Betaproteobacteria were the likely dominant PHA accumulators within the sludge samples from both reactors (Figure 3.12 (a-c)). β-proteobacteria have been shown as prominent species in EBPR sludges analysed by FISH (Kämpfer et al., 1996, Wagner et al., 1994 and Bond et al., 1999) and in carbon removal activated sludge (Hiraishi, 1988; Hiraishi et al., 1989) Although β-proteobacteria are commonly found in activated sludge, it should be noted that different types of β-proteobacteria can be present, depending on the operational conditions (Bond et al., 1999).
(a) Brightfield image.

(b) Nile Red Stain; Red circles indicate PHA

(c) Betaproteobacteria probe (Green circles indicating co-localisation sites).
(d) Brightfield image of a positive control *Ralstonia eutropha*

(e) Nile Blue A staining of *Ralstonia eutropha*

**Figure 3.12.** FISH and Nile Blue fluorescence microscopy of sludge (a-c) and *Ralstonia eutropha* as the positive control (d) and (e).
3.4 Conclusion

Culture-dependent (Prakasam and Dondero, 1967) and independent (Schuppler et al., 1995; Wagner et al., 1994) methods have been used to analyse and compare the microbial structure of activated sludge. However, culture-dependent methods are biased by selecting of species which may not represent the true dominant microorganisms present (Wagner et al., 1994). However, this has been improved by culture-independent molecular techniques like fluorescent in situ hybridization (FISH), 16S rRNA polymerase chain reaction (PCR) or Denaturing Gradient Gel Electrophoresis (DGGE). These techniques have been used to investigate different kinds of wastewater treatment plants: laboratory-scale sequencing batch reactor (SBR), laboratory plants with continuous operation (Crocetti et al., 2000; Gieseke et al., 2001; Liu et al., 2001; Pijuan et al., 2004, and Lu et al., 2006) and municipal treatment plants (Prakasam and Dondero, 1967; Kämpfer et al., 1996; Snaird et al., 1997; Hesselmann et al., 1999; Martin et al., 2006, and Oehmen et al., 2007).

In this study, several methods were implemented to investigate the microbial diversity from each reactor in an effort to determine whether identified patterns of species correlated across the various methods used. Culture dependent and independent methods revealed (a) that quite different communities were found to be present in both reactors and that (b) a great deal of variation was produced by the distinct approaches. In terms of clear outcomes, one could suggest that the skim milk in reactor 2 was having a considerable influence on the diversity and composition of microbial community. Biological waste treatment is dependent on the populations’ capability to carry out multiple microbial activities and interactions in high C/N (carbon/nitrogen) ratio wastewaters (Daims et al., 1999; Figuerola and Erijman,
The selection of these microbial communities occurs through the physiochemical properties of the wastewater, the design and operation of the system and the ambient environmental conditions (Yu and Mohn, 2001). For a biological treatment system to be effective a stable microbial community is required for consistent and reliable breakdown and transformation of waste constituents (Smith et al., 2003). However, in these systems, the communities are subject to both environmental and process changes such as variations in pH, temperature, seasonal climate changes, organic loading rates and toxicants (Pokhrel and Viraraghavan, 2004). In an EBPR process, proposed key candidates include *Microlunatiss phosphovorus*, *Rhodocyclus* related species, *Tetrasphaera*-related species, and *Malikia spp.* (Nakamura et al., 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Liu et al., 2001; Spring et al., 2005). The culture dependent approach had indicated that the *Gammaproteobacteria* was the dominant class. However, *Betaproteobacteria* was dominant in reactor 1 while *Betaproteobacteria* and *Bacteroidetes* were dominant in reactor 2 in both the culture independent and FISH analysis methods. Furthermore, the dual staining with Nile Red showed that the *Betaproteobacteria* was dominant for PHA accumulation. *Thauera sp.*, *Azoaracus sp.* and *Zoogloea sp* are all related to *Rhodocyclus* being found in the same class (*Betaproteobacteria*) and family *Rhodocyclaceae*. *Dechloromonas aromatica* is also found in this family and has been described previously as a PHA producer (Oshiki et al., 2008; Ahn et al., 2007; Wolterink et al., 2005). Microorganisms such as found in this system have also been found in EBPR systems. For example, *Azoarcus, Thauera, Zooglea, Rhodobacter, Simplicispira*, and *Runella* have all been found in EBPR systems, although definitive roles have yet to be attributed to them (Thomsen et al., 2007). Furthermore, many of the bacteria isolated in this system were also known to accumulate PHA, such as
Thauera sp. Zoogloea sp. (Oshiki et al., 2008), Azoarcus sp., (Lemos et al., 2008), Pseudomonas sp., (Kim et al., 1997) Rhodobacter sp. (Hassan et al., 1997), and Bukrholderia cepacia (Keenan et al., 2004; Nakas et al., 2004; Alias and Tan 2005; Celik et al., 2005). Therefore, taken together, the results of the community analysis appear to be consistent with the emergence of an EBPR system under the operating conditions imposed, which appeared to be occurring in trial 3 (Section 2.3.3).

Microbial diversity in pure cultures and mixed cultures for PHA production has been widely studied (Table 3.12). A variety of microorganisms are able to produce PHA in using a diverse range of carbon sources as well as wastewaters. Furthermore, these bacterial strains have been shown to produce a variety of different PHAs from these carbon sources (Table 3.12)
Table 3.12. Bacterial strains that produce PHA from different carbon sources and wastewater

<table>
<thead>
<tr>
<th>Bacterial strain(s)</th>
<th>Carbon source(s)</th>
<th>Polymer(s) produced</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes latus</em></td>
<td>Malt, soy waste, milk waste, vinegar waste, sesame oil</td>
<td>PHB</td>
<td>(Wong et al., 2004, 2005)</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Glucose, e-caprolactone, Sugarbeet, Molasses</td>
<td>PHB, terpolymer</td>
<td>(Labuzek and Radecka 2001; Yilmaz and Beyatli 2005; Valappil et al., 2007)</td>
</tr>
<tr>
<td><em>Bacillus spp.</em></td>
<td>Nutrient broth, glucose, alkanoates, e-caprolactone, soy molasses</td>
<td>PHB, PHBV, Copolymers</td>
<td>(Katircioğlu et al., 2003; Shamala et al., 2003; Tajima et al.. 2003; Yilmaz et al., 2005; Full et al., 2006)</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses</td>
<td>PHB, PHBV</td>
<td>(Keenan et al., 2004; Nakas et al., 2004; Alias and Tan 2005 ; Celik et al., 2005)</td>
</tr>
<tr>
<td><em>Escherichia coli mutants</em></td>
<td>Glucose, glycerol, palm oil, ethanol, sucrose, molasses</td>
<td>(UHMW)PHB</td>
<td>(Mahishi et al., 2003; Kahar et al., 2005; Park et al., 2005; Nikel et al., 2006; Sujatha and Shenbagarathai 2006)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Glucose, technical oleic acid, waste free fatty acids, waste free frying oil</td>
<td>mcl-PHAs</td>
<td>(Hoffmann and Rehm 2004; Fernández et al., 2005)</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>Glucose, soybean oil, alcohols, Alkanoates</td>
<td>mcl-PHAs</td>
<td>(Xu et al., 2005)</td>
</tr>
<tr>
<td><em>Rhizobium meliloti, R. viciae, Bradyrhizobium japonicum</em></td>
<td>Glucose, sucrose galactose, mannitol, trehalose, xylose, raffinose, maltose, dextrose, lactose, pyruvate, sugar beet molasses, whey</td>
<td>PHB</td>
<td>(Mercan and Beyatli 2005)</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Malt, soy waste, milk waste, vinegar waste, sesame oil</td>
<td>PHB</td>
<td>(Wong et al., 2004, 2005)</td>
</tr>
<tr>
<td><em>Cupriavidus necator</em></td>
<td>Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil</td>
<td>PHB, copolymers</td>
<td>(Kim et al., 1995; Kichise et al., 1999; Taguchi et al., 2003; Kahar et al., 2004; Khanna and Srivastava 2005; Volova and Kalacheva 2005; Volova et al., 2005)</td>
</tr>
</tbody>
</table>

(Modified from Verlinden et al., 2007).
As a result, it was determined that, going forward, an investigation of the pure cultures isolated for novel polymer production capacities could prove to be biotechnologically useful.

Finally, not all of the experimental approaches adopted were equally productive. Efforts were made to optimise DGGE, to obtain profiles of microbial diversity in the sludge to account for any intrinsic bias in the cloning approaches. Although, several approaches were used to optimise DGGE, 16S PCR and GC clamps, profiles were difficult to obtain. Although DGGE did not work in this project it could be used in the future in an effort to account for any intrinsic bias in the cloning approaches. In addition, the findings of the reverse transcription PCR (RT-PCR) approach did not facilitate any readily interpretable data on the diversity of expressed genes from active PHA accumulating populations within the community. Multiple rounds of PCR were employed in conjunction with repeated sequencing of cloned inserts, but it was not possible to resolve the issues influencing this task. The use of *E. coli* as the library host and the subsequent appearance of *E. coli* as a dominant species in the data undermined our ability to support the validity of that experimental approach. However, it would certainly be of value to revisit this aspect of the study in any future iteration of the work.
3.5 Bibliography


CHAPTER 4

Characterisation of PHA Accumulation in Pure cultures Isolated from SBRs Operated under EBPR Conditions
4.1 Introduction

The economic feasibility of PHAs, in competition with petrochemical plastics, has greatly improved as progress continues in the optimisation of PHA production design, coupled with efficient and selective PHA recovery. Significant advances have accrued through a better understanding of the PHA biosynthetic pathway, allowing the use of natural and recombinant microbial organisms and plant cells, to produce high PHA yields (Suriyamongkol et al., 2007, Reddy et al., 2003, Luengo et al., 2003). However, these pure culture systems are still confined by varying degrees to the financial demands of pure substrates, sterile reactor conditions and efficient PHA recovery to high levels of quality and purity (Ogawa et al., 2005). In this study, attempts have been made to circumvent the issues of pure substrate costs and sterile reactor demands through the employment of an activated sludge culture and wastewater feed model. While some degree of feasibility has been demonstrated in this study with respect to the potential dairy wastewater conversion, (albeit at low levels of 10-20% PHA), the issue of recovery represents a key limitation of the system. The complexity of the activated sludge matrix is compounded by the relatively low PHA content within an apparently limited fraction of the overall population. Therefore, while certain financial constraints may be offset by this approach, the energy demands to process high volumes of sludge using temperature or physical disruption, would likely incur costs that would undermine the profitability of the exercise. If one examines the history of industrial exploitation of PHAs there is a faltering process of development followed by technology transfer and subsequent re-development as different challenges are encountered. In 1994 a shift toward genetically altering plants to express the metabolic pathway for PHA accumulation occurred (Gross and Kalra, 2002). This was further developed by Monsanto® who
engineered canola plants producing 14% plastic, in addition to generating Canola oil. However, Monsanto® believes that plants should produce at least 20% PHA in order to be commercially viable and in 1998 abandoned further development. Metabolix (Cambridge, MA) is currently trying to develop PHA production in both plant crops and by fermentation to make them commercially viable (Gross and Kalra, 2002). Interest in co-polymers has also continued to develop due to the increased diversity of properties/applications they offer, relative to the respective monomer homo-polymers. There are several different grades of co-polymers possible depending on the average molecular weight, the average mcl-3HA content within the co-polymer and the side chain length of the chosen mcl-3HA unit. Commercial co-polymers are currently available, e.g. Nodax from Proctor and Gamble is employed in foams, fibers, films and latex and can be used in bulk packaging, flushable hygiene products, agricultural films and several medical applications (Noda et al., 2005). The simplest form of this class of co-polymer is PHBHX, comprised of 3HB and 3-hydroxyhexanoate (3HHx) units. Other 3HA units such as 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) are also available to form various co-polymers comprising 3HB and one or more mcl-3HAs. The unique molecular structure of these co-polymers gives useful properties that traditional PHA polymers like PHB or PHB-co-HV do not offer. These include high durability and ductility, coupled with thermal property ranges similar to those of polyethylene (Noda et al., 2005). The current most economically competitive use of PHAs is in medical applications of medium chain length or blended mcl-scl co-polymers, where product cost prices are not an issue due to a limited range of competing materials (Kim et al., 2007; Noda, 2005). Thus the discovery and development of cultures capable of producing such compounds continues to be of considerable interest (Ren et al., 2010; Philip et al., 2007; Noda et
It was determined therefore that chemical and molecular characterisation for novel or mixed polymer production potentials should be carried out with pure culture isolates in this study.

4.2 Material and Methods

4.2.1 Bacterial Cultures and Media

Stock samples (-80°C) of the seventeen bacterial cultures (Table 4.1) previously isolated from two distinct sequencing batch reactors (SBRs) operated under enhanced biological phosphate removal conditions were plated on standard Luria-Bertani (LB) agar. The LB agar contained 10g tryptone, (peptone from casein), 5g yeast extract, 10g NaCl and 15g agar added to 800ml of distilled water. The volume was made to 1 litre of distilled water and autoclaved. The plated cultures were maintained at 4°C prior to analyses. In order to induce PHA accumulation, cultures were grown in E2 minimal media, as described in Section 3.2.1, (Vogel and Bonner, 1956), with either a full nitrogen complement (8mM) or limited nitrogen (1.5mM) via modification of the ammonium sulphate component. Usually, PHA accumulation occurs in a suitable bacterium in the presence of a relative abundance of utilisable carbon with an inorganic nutrient limitation, (e.g. nitrogen) (O'Leary et al., 2005). With respect to carbon source provision, glucose, acetate and skim milk were respectively incorporated into the media to a final concentration of 10mM. The pH was adjusted to 7.1 using 10N NaOH. 1ml of 1M MgSO₄ and 1ml of MT Stock-Lösung and 1ml E2 vitamin solution was added post-autoclaving (Section 3.2.1.). Isolates were analysed for PHA accumulation using the E2 mineral medium agar (Lageveen et al., 1988) plates. Isolates were spotted on the E2 mineral medium agar and incubated for 48 hours. This was followed by the plates being flooded with Nile
blue A (1% w/v) in ethanol. On decanting the stain and exposing the plate to U.V. light, bright orange colonies were scored as accumulators of PHA.

**Table 4.1.** List of Pure Culture Isolates.

<table>
<thead>
<tr>
<th>Reactor 1 (Acetate fed)</th>
<th>Reactor 2 (Acetate+Sim Milk fed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SM2- <em>Pseudomonas sp.</em></td>
<td>16S2M1- <em>Acinetobacter johnsonii</em></td>
</tr>
<tr>
<td>16SM3- <em>Delftia acidovorans</em></td>
<td>16S2M2- <em>Acinetobacter sp. OM E81</em></td>
</tr>
<tr>
<td>16SM4- <em>Aminobacter sp.</em></td>
<td>16S2M3- <em>Elizabethkingia</em></td>
</tr>
<tr>
<td>16SM5- <em>Bacillus sp.</em></td>
<td>16S2M4- <em>Uncultured gamma Acinetobacter</em></td>
</tr>
<tr>
<td>16SM6- <em>Cytophaga sp.</em></td>
<td>16S2M5- <em>Acinetobacter sp. Anoxii</em></td>
</tr>
<tr>
<td>16SM7- <em>Pseudomonas sp.</em></td>
<td>R2MOS2- <em>Citrobacter sp.</em></td>
</tr>
<tr>
<td>R1MOS1- <em>Pseudomonas sp.</em></td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>R1MOS5- <em>Pseudomonas mendocina</em></td>
<td></td>
</tr>
<tr>
<td>R1MOS7- <em>Thauera sp.</em></td>
<td></td>
</tr>
<tr>
<td><em>Agromyces sp.</em></td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 Induction of PHA Production in Liquid Culture and Visualisation via Fluorescent Microscopy

A colony from each pure culture was inoculated into 5 ml LB broth, respectively, and incubated overnight at 28°C, in a shaking incubator operated at 150 rpm. Cultures were subsequently centrifuged at 5000 rpm for 7 minutes, the resulting supernatant discarded and the pellet washed in 5mls of sterile Ringer’s solution. Each sample was then re-centrifuged, the supernatant discarded and the respective pellets resuspended in 10ml of relevant E2 test media. Each culture was grown in 4 different iterations of the E2 minimal media, (i) E2 with nitrogen (8mM) and 10mM acetate (ii) E2 limiting nitrogen (1.5mM) and 10mM acetate, (iii) E2 with nitrogen (8mM) and 10mM glucose and, (iv) E2 limiting nitrogen (1.5mM) and 10mM glucose, respectively and incubated at 28°C for 4 hours shaking. Furthermore, *Rhodococcus* was used as the positive control were subjected to the same conditions as the pure cultures. *Rhodococcus opacus* and *Rhodococcus ruber* have cultivated for 24 hours 30°C in NB medium harvested and incubated for several days in low-nitrogen MSM medium with gluconate and glucose (Alvarez et al., 2000). 1ml was transferred into an Eppendorf and each sample’s absorbance was read at a wavelength of 600nm. When the absorbance/optical density reached 0.5 to 1.0, 1ml of the culture was taken from the growth media, centrifuged and analysed immediately or the pellet stored at -20°C. To identify PHA accumulation each pellet, (fresh or defrosted), was washed and subjected to Nile Red lipophilic staining using 1ml of 0.5 mg/ml solution of Nile red in acetone. 0.5ul of sample was then added to a labeled slide and observed via a fluorescent microscope (Leica DM3000) with an I3 filter, with an excitation wavelength of 450-490nm and an emission filter of 510nm. Images were captured via
a DFC490 8mp, CCD digital camera, and modified using LAS software V3.1.0 programme (Leica Microsystems, Germany).

4.2.3 Determination of PHB Production using High Performance Liquid Chromatography (HPLC)

Quantitative analysis of short chain length-PHAs/PHBs is dependent on high performance liquid chromatographic detection of crotonic acid formation, derived by chemical depolymerisation of PHB (Campbell et al., 1982, Nickerson, 1982, Ostle and Holt, 1982, Findlay and White 1983). To achieve this samples ranging from 0.01 to 500mg of PHB containing material can be used. The pure cultures were centrifuged and the supernatant removed and weights of the pure cultures were found to be between 25-40mg. These were digested in 1ml concentrated sulphuric acid at 90°C for 30 minutes. The tubes were cooled on ice, after which a 4ml volume of 0.014N H₂SO₄ was added with rapid mixing. The samples were filtered through a 0.45µm filtopur syringe filter (Sarstedt) to remove particulate material. HPLC was carried out using a previously published method (Karr et al., 1983) on an Agilent 1200 HPLC system with a refractive index detector, A REZEX 8µ 8%H, Organic Acid Column 300x 7.8mM (Phenomenex, USA) with 0.01N H₂SO₄ as the elution fluid, at a flow rate of 0.6ml/min. The temperature of the column was maintained at 65°C. Substrate and end-product peaks were identified by comparison of their retention times with those of a commercial preparation of crotonic acid, [Sigma-Aldrich]. Relative concentrations were determined from a plot of peak areas generated with standards of known concentrations. The standard used was crotonic acid at 2.5mM, 5mM, 10mM and 20mM. The HPLC solvent was 0.01N Sulphuric
Acid: 227µl concentrated in a litre of dH₂O filtered through a 0.45µM filter (Sarstedt). Absorbance of the crotonic acid was measured at 210nm.

4.2.4 Determination of Medium Chain Length PHA Production via Gas Chromatography

Gas chromatography has long been used for the extraction and quantification of PHA (Braunegg et al., 1978). This method involves cells undergoing direct, mild acid or alkaline methanolysis, followed by gas chromatographic separation of the 3HA methyl ester(s) (Lee and Choi, 1995). Cell suspensions containing 5mg of cells for sludge and between 25 to 40 mg of the pure culture cells were transferred to an Eppendorf tube and centrifuged at 14,000 rpm for 10 minutes. The cell pellets were washed with water and centrifuged again and supernatants discarded. The cell pellets were resuspended in 100 µl dH₂O and either processed immediately or stored at -20°C. The PHA extraction and detection procedure began with overnight lyophilisation of the samples. The samples were weighed (±0.05mg), the contents transferred to a glass vial, and the tubes weighed again to determine the transferred mass. 2 ml 15% H₂SO₄, methanol and 2 ml chloroform containing methyl benzoate was added to each sample and boiled at 100°C for 2.5 hours while stirring. The samples were cooled on ice for 5 minutes and 1ml of dH₂O was added and vortexed for 1 minute. The samples were subsequently centrifuged at 4000 rpm for 5 minutes, the water phase discarded, including droplets on the tube wall and the top layer of the chloroform phase. 1 ml of dH₂O was added, vortexed, centrifuged and the water phase was discarded as described previously. Na₂SO₄ powder was added to dry the chloroform phase, as long as there is free water present, the Na₂SO₄ powder binds the water and settles fast. The samples were centrifuged at 4000 rpm for 5 minutes to accelerate the settling. 200 µl of the chloroform phase was transferred to a GC vial,
avoiding transferral of any Na$_2$SO$_4$. Appropriate derivatizations are required to determine the chain length of 3-hydroxyalkanoic acid methyl esters via favourable mass fragmentations in a mass spectrometer. The mass spectra of trimethylsilyl (TMSi) derivatives of 3-hydroxyalkanoic acid methyl esters exhibit characteristic fragments of m/e 175, m/e 73/ m/e 89 and molecular ion-related fragments of m/e (M-73) and of m/e (M-15). Fragmentation patterns of unsaturated monomers show a characteristic peak of m/e 138. The derivatization procedure involved the addition of 50µl bis(trimethyl-silyl)acetamide to 1 ml of methanolized sample, which was then vortexed and incubated for 10 minutes at 80°C. The samples were then subjected to GC analysis, with comparative standards (3mM, 6mM and 12mM) of hydroxyhexanoic acid (6C), hydroxyoctanoic acid (8C) and hydroxydecanoic acid (10C) methyl esters, respectively. The GC system used for the analyses of these samples differed to that described in Chapter 1 (Section 2.2.8) due to a mechanical issue at the time of sampling. Instead a Varian 3800 GC with flame ionisation detector and 8200 autosampler with an Rtx-5 column, (60m, 0.25mm i.d., 0.25um film thickness), was employed, (Restek Corporation, Bellefonte, PA, USA). A 1 μl sample from the organic phase was injected, (split ratio 1:5), with helium as the carrier gas. Oven temperature was programmed for peak separation as follows; 35°C for 2 minutes, increase to 280°C at 15°C/min; hold at 280°C for 10 minutes. Injector temperature was 250°C and detector temperature was 300°C. The structures of the monomers were identified by analysis of characteristic peaks in relation to the standards. The samples were quantified by generating a standard curve (Area versus Concentration) using the 3-12mM hydroxyl-hexanoic, -octanoic and -decanoic monomer concentrations.
4.2.5 Molecular Investigation of Reactor Isolates for Polyhydroxyalkanoate Synthesis Genes

As previously described, sequences from organisms (Class I *pha* synthase sequences α, β and γ proteobacteria) were gathered from NCBI. The sequences were translated to amino acid sequence and aligned using Lasergenes MegAlign Clustal W Algorithm method. Based on these alignments, the most conserved regions of the amino acid sequences were identified. Universal primers were then designed with a focus on (a) strong base-pairing between the 3’ end of the primer and the template, (b) conserved aromatic amino acid encoding regions and (c) regions containing low codon usage amino acids. The primers were further checked for undesirable features such as hairpin loops or dimers. In an attempt to clone the PHA synthases the following Class I *Pha* Synthase primers (short chain length primers) were developed; phac1F-5’-CCGCCGTGGATAAACAAGTTCTAC-3’ and phaC1R-5’-GCGTGGTGTAGTCGGCCGTTTCAATAA-3’, respectively. The forward and reverse primers designed for Class II (medium chain length) PHA synthase screening were; phaC2F-5’-CGCAACTGAAGACTGCTGGAAGCTG-3’ and phaC2R-5’TGTTGGGGGCGATCCAGGC-3’, respectively. Colonies of the respective isolates were picked from solid media (LB Agar) and resuspended in distilled water to act as the template for subsequent PCR reactions. Amplification of *phaC1* homologues employed PCR cycling conditions described earlier and a DNA Taq (Bioline) polymerase was utilised. The negative control was sterile water, while a well characterised short chain polymer producing *Rhodococcus* sp provided the positive control. Thermocycler conditions for the amplification of *phaC2* homologues were as follows; 95°C x 5 minutes followed by 35 cycles of 95°C x 1 minute, 66°C x 45 seconds, and 72°C x 45 seconds. *Pseudomonas putida* CA-3, a
well-known medium chain polymer producer, provided a positive control for the reactions. Successful amplicons were extracted from the gel using the GeneJet™ extraction kit, in accordance with the manufacturer’s instructions, and sequenced (GATC, Konstanz Germany). Nucleotide sequence data generated was comparatively analysed against GENBANK using the BLASTn algorithm.

4.3. Results and Discussion

4.3.1 Qualitative Analysis of PHB Production of Isolates from Both reactors using Lipophilic Staining

Our work, and that of previous investigators of EBPR, has shown that the induction of PHA accumulation in activated sludge requires the optimised delivery of appropriate anoxic/oxic cycling conditions combined with an appropriate carbon feedstock (Serafim et al., 2008). In contrast, PHA accumulation in pure cultures typically presents a far more manageable challenge. In its simplest form the bacterial goal of PHA production is to capture a suitable carbon when some growth limiting conditions prevents immediate catabolism of same. The limitations are often critical inorganic nutrients such as phosphorus, nitrogen, oxygen or sulphur (Suriyamongkol et al., 2007). Thus, the incorporation of such limitations into defined media, with an appropriate carbon source, can trigger a metabolic shift in pure cultures toward PHA storage, if they possess appropriate molecular machinery for the production of such polymers. A commonly used trigger is the provision of limited nitrogen (Lageveen et al., 1988). Indeed, the accumulation of PHAs in cultures grown on such solid media can induce an increase in opacity of colonies, an attribute that has been used in the past to identify pha gene disrupted mutants in P. putida CA-3 (Goff et al., 2009). As
a result, our initial attempts at determining the bioplastic production capacities of the reactor isolates focused on qualitative solid media screens using E2 media containing varying amounts of nitrogen. Furthermore, the isolates were screened directly for isolation of the PHA accumulating bacteria by the plate assay method whereby PHA production can be correlated with increasing colony opacity (Section 4.2.1). Some cultures did not grow well using this method although the samples were incubated for 48 hours and involved the use of acetate as a carbon source, which is known to be readily utilised by bacteria for PHA production. Indeed, a number of the isolates that did grow were known PHA accumulators, which would have been expected to produce a positive result. However, an initial problem was encountered whereby growth of pure cultures on the nitrogen limited media proved quite difficult (Figure 4.1). Therefore, it was decided that other methods would be used for qualitative and quantitative analysis of the isolates for PHA.

Figure 4.1. Plate screen of isolates obtained from the reactor. The black circle indicates were colonies were opaque on the plate that may have the ability to produce PHA.
4.3.2 Induction of PHA Accumulation in Liquid Culture and Fluorescence Microscopy Analysis

As the solid media screen failed to provide any significant or reliable indication of the PHA production potential of the reactor isolates a second strategy, again involving nitrogen limitation-based induction, was attempted using a liquid media approach. To overcome difficulties associated with the poor growth of a number of isolates in E2 minimal liquid media, pre-growth on LB broth was performed, followed by cell collection, washing and re-suspending in E2 glucose or acetate (10mM) with both normal and limiting nitrogen concentrations. It was hoped that even if cultures should fail to achieve further growth in the defined media, sufficient biomass would already be available to deliver demonstrable PHA accumulation under the nitrogen limiting conditions. Figure 4.2 (a) and (b) indicate that sufficient biomass for qualitative and quantitative analyses were generated by this approach on the carbon sources acetate and glucose, respectively.
(a) E2-Acetate (N-Lim)

![Graph showing optical density of isolates after four hours on E2 minimal media for E2-Acetate (N-Lim).]

(b) E2-glucose (N-Lim).

![Graph showing optical density of isolates after four hours on E2 minimal media for E2-glucose (N-Lim).]

**Figure 4.2.** Optical density of isolates after four hours on E2 minimal media.
Upon Nile Red staining and fluorescence microscopy a number of cultures, isolated from both reactors, were found to be capable of accumulating PHAs under the conditions provided. Figure 4.3 provides an example of PHA granule visualization in the *Bacillus sp* reactor isolate 16SM5. In the presence of adequate nitrogen no induction of polymer synthesis was detected (Fig 4.3, (a)). However, in the same media with limiting nitrogen, PHA accumulation is clearly discernible after 4 hours, (Fig 4.3, (b)).

**Figure 4.3**. Fluorescent visualisation of Nile Blue stained PHAs. 16MS5-*Bacillus sp* cultured for 4 hours on E2 minimal media containing (a) 8mm or (b) 1.5mM ammonium sulphate.

In total, eight cultures were found to produce PHA under nitrogen limiting conditions with both acetate and glucose; R2MOS2-*Citrobacter sp.*, 16S2M1-*Acinetobacter johnsonii* Agromyces sp., R1MOS7-*Thauera sp*, 16SM7-*Pseudomonas sp.*, 16SM5-*Bacillus sp.*, 16SM4-*Aminobacter sp.* and 16SM2-*Pseudomonas sp.*. The inclusion of acetate as a carbon source in the screen was intended to facilitate the selection of short chain length PHB production. The first step involved acetate conversion directly to acetoacetyl-CoA by a beta-ketothiolase, prior to dehydrogenase directed formation of
the hydroxybutyrate monomer. However, only one isolate, 16SM6-\textit{Cytophaga}, demonstrated PHB accumulation on acetate alone. The finding is consistent with the previously reported production of polyhydroxybutyrate in this species (Höfle, 1984). In contrast, glucose proceeds via glycolysis to acetyl-CoA which can enter fatty acid synthesis, generating the medium chain length hydroxyacyl-acyl carrier protein (ACP) precursors of hydroxyacyl-CoA monomers. Thus, despite the qualitative nature of the screen, early indications seemed to point to a potential dominance of mcl-PHA production metabolism within the isolates, while the possible co-synthesis of scl and mel polymers was not ruled out. Three strains failed to show any detectable PHA granule formation, \textit{Delftia acidovorans}, 16S2M2-\textit{Acinetobacter sp. OM E81} and \textit{Lactococcus lactis}. It should be noted that among these \textit{Delftia acidovorans} is a known PHA producer (Loo and Sudesh, 2007) and would be expected to be induced under the conditions tested. Failure to detect PHA may have been due to the small volume of sample being tested coupled with very low level induction in the particular strain of \textit{Cytophaga} isolated. A summary of the visualisation results are provided in Table 4.2.

\subsection*{4.3.3 Molecular Investigation of Reactor Isolates for Polyhydroxyalkanoate Synthase Gene Homologues}

Following the qualitative identification of PHA accumulation in many of the reactor isolates, efforts were made to investigate whether they possessed genes encoding specific synthases (i.e. short chain length or medium chain length) and to what extent were they similar or different from known synthase genes. \textit{phaC1} synthase gene amplification was attempted with each of the isolates with only a limited number giving a positive amplification (Figure 4.4 (a)). Among these were
16SM6-Cytophaga sp., 16SM5-Bacillus sp., 16SM7-Pseudomonas sp., 16SM2-Pseudomonas sp. 16SM4-Aminobacter sp. and 16S2M3-Elizabethkingia.

**Table 4.2.** Qualitative fluorescent determination of PHA accumulation among reactor isolates.

<table>
<thead>
<tr>
<th>Reactor 1 cultures</th>
<th>Acetate</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SM2-Pseudomonas sp</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16SM3-Delftia acidovorans</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16SM4-Aminobacter sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16SM5-Bacillus sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16SM6-Cytophaga sp.</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16SM7-Pseudomonas sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R1MOS1-Pseudomonas sp.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R1MOS5-Pseudomonas mendocina</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R1MOS7-Thauera sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Agromyces sp.</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reactor 2 cultures</th>
<th>Acetate</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S2M1-Acinetobacter johnsonii</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16S2M2-Acinetobacter sp. OM E81</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16S2M3-Elizabethkingia</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16S2M4-Uncultured gamma Acinetobacter</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16S2M5-Acinetobacter sp. Anoxii</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R2MOS2-Citrobacter sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactococcus lactis sp.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) indicates the detection of PHA, (-) no detection of PHA granules.
The apparent presence of a short chain length synthase in 16SM6-*Cytophaga* corellated well with the isolate’s ability to accumulate PHAs with acetate as the sole carbon source (Table 4.2). It was interesting to note also that the *Delftia sp*, which earlier failed to accumulate PHA from acetate under N-limiting conditions, did not appear to host a scl synthase, amplifiable with the primer pair employed. This might explain the earlier *pha* negative phenotype. Both *Pseudomonas* species which generated a positive *phaC1* amplicon were shown to accumulate PHAs under N-limiting growth on acetate. Group II *Pseudomonads* typically produce medium chain length polymers, and these 2 isolates were also found to have accumulated PHAs from glucose (Table 4.2). These observations suggest a potential for naturally diverse co-polymer synthesis within certain *Pseudomonas* species, which have largely been considered solely for their mcl homopolymer production capacities. In contrast, the other 2 *Pseudomonas* isolates, R1MOS1-*Pseudomonas sp.* and R1MOS5-*Pseudomonas mendocina*, were found to neither accumulate PHA when grown on acetate, nor to host amplifiable scl-synthase homologues. Thus a number of the findings appeared to correlate well at this early stage. However, it was noted that the *Elizabethkingia* isolate, which accumulated PHA from glucose but not from acetate, appeared to host a scl *phaC1* gene homologue. This suggested that scl synthetic apparatus may have been present in the cell, but may not be inherently functional or actively expressed under the conditions tested.
Figure 4.4. Degenerate PCR amplification of poly-hydroxyalkanoate synthase genes.

(a) Lanes 1 and 22 contained DNA ladder, lanes 20 and 21 contained positive and negative control reactions, respectively. Lanes 2-19 contained isolate amplifications as follows: 1 = DNA Ladder, 2 = 16SM6-Cytophaga sp., 3 = 16SM5-Bacillus sp., 4 = Agromyces sp., 5 = R MOS5-Pseudomonas mendocina, 6 = 16SM7-Pseudomonas sp., 7 = 16SM2-Pseudomonas sp., 8 = 16SM3-Delftia acidovorans, 9 = R MOS7-Thauera sp., 10 = 16SM4-Acinetobacter sp., 11 = 16SM2M4-Uncultured gamma Acinetobacter, 12 = R MOS1-Pseudomonas sp., 13 = 16SM5-Acinetobacter sp. Anoxii, 14 = R MOS2-Citrobacter sp., 15 = 16SM1-Acinetobacter johnsonii, 16 = 16SM3-Elizabethkingia, 17 = Lactococcus lactis sp., 18 = 16SM1-Acinetobacter johnsonii, 19 = Agromyces sp., 20 = Positive control, 21 = Negative control, 22 = DNA Ladder. (b) Lanes 1 and 23 contained DNA ladder, lanes 21 and 22 contained positive and lane 20 contained negative control reactions, respectively. Lanes 2-19 contained isolate amplifications as follows: 1 = DNA Ladder, 2 = 16SM6-Cytophaga sp., 3 = 16SM5-Bacillus sp., 4 = Agromyces sp., 5 = R MOS5-Pseudomonas mendocina, 6 = 16SM7-Pseudomonas sp., 7 = 16SM2-Pseudomonas sp., 8 = 16SM3-Delftia acidovorans, 9 = R MOS7-Thauera sp., 10 = 16SM4-Acinetobacter sp., 11 = 16SM2M4-Uncultured gamma Acinetobacter, 12 = R MOS1-Pseudomonas sp., 13 = 16SM5-Acinetobacter sp. Anoxii, 14 = R MOS2-Citrobacter sp., 15 = 16SM1-Acinetobacter johnsonii, 16 = 16SM3-Elizabethkingia, 17 = Lactococcus lactis sp., 18 = 16SM1-Acinetobacter johnsonii, 19 = Agromyces sp. (a) phaC1 degenerate PCR reactions (b) phaC2 degenerate PCR reactions. Target amplicon band sizes are indicated by black boxes.
PCR screening for \textit{phaC2} gene homologues indicated a greater potential distribution of this synthase among the isolates (Figure 4.4 (b)). A number of the positive results were in keeping with published observations of mcl-PHA producers and also our own observations in the induction experiments. For instance, 3 of the \textit{Pseudomonas} isolates R1MOS5-\textit{Pseudomonas mendocina}, 16SM7-\textit{Pseudomonas sp.}, 16SM2-\textit{Pseudomonas sp.}, which accumulated PHA on glucose, all gave the target size amplicon for \textit{phaC2}, as expected. A correlation was also noted between the apparent presence of a \textit{phaC2} amplicon in the \textit{Elizabethkingia sp.} and its ability to accumulate PHA from glucose, but not acetate (Table 4.2). 16SM5-\textit{Bacillus sp.}, which accumulated PHA on both substrates, also hosted an apparent \textit{phaC2} homologue. Medium chain length PHA accumulation in environmental isolates of \textit{Bacillus} has been previously reported (Khiyami \textit{et al.}, 2011). Indeed PHA accumulation on both acetate and glucose was a common feature of the majority of the \textit{phaC2} positive isolates, which also included \textit{Agromyces sp.} 16SM4-\textit{Aminobacter sp.}, 16S2M1-\textit{Acinetobacter johnsonii} and R2MOS2-\textit{Citrobacter sp.} One anomalous finding was the amplification of a potential mcl synthase homologue in 16SM6-\textit{Cytophaga sp.} This strain was only found to accumulate PHA from acetate. Furthermore, it was not possible to find any precedence in the literature, or available nucleotide/protein databases of a \textit{Cytophaga sp} associate mcl-PHA synthase. The presence of this gene may not therefore be associated with functional expression in this strain. In summary, the screen for \textit{pha} synthase homologues appeared to mirror the induction experiments, with a dominance of medium chain length production correlating well with an apparent dominance of \textit{phaC2} homologue distribution.
4.3.4. Sequencing and Database Comparative Analysis of pha Gene Homologues

While the PCR approach sought to capitalise on the presence of highly conserved sequences within known synthases, homology to previously published synthase sequences were investigated to assess whether any potentially novel synthases could be identified via low overall percentage identities. Amplicons generated via the Class II PHA synthase PCR screens were sent for sequencing by GATC and the nucleotide sequences comparatively analysed against the NCBI GenBank non redundant nucleotide database using the BLASTn algorithm ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). Table 4.3 presents the top 3 BLAST search query results for each synthase amplicon, together with the percentage nucleotide identity and the associated source organisms. It became immediately apparent that the majority of the gene sequences shared high homology with previously sequenced *Pseudomonas* species, even in non-*Pseudomonas* reactor isolates from which the homologue was cloned. However, as table 4.3 shows there are distinct differences in (a) the respective % identities of the homologues and (b) in the identity of the source organisms for previously sequenced genes in GenBank. Thus the incorporation of a common contaminant in the PCR reaction is not a viable explanation.
Table 4.3. Nucleotide sequences subjected to Database comparison using NCBI BLAST database results.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Homolog ID</th>
<th>% identity</th>
<th>Source Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SM2</td>
<td>Pseudomonas sp.</td>
<td>98</td>
<td>P. putida KCTC1639</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>P. putida</td>
<td></td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>P. putida</td>
<td></td>
</tr>
<tr>
<td>R1MOS5</td>
<td>Pseudomonas</td>
<td>88</td>
<td>Pseudomonas sp. g106</td>
</tr>
<tr>
<td>mendocina</td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>P. pseudoalcaligenes</td>
<td></td>
</tr>
<tr>
<td>16SM7</td>
<td>Pseudomonas sp.</td>
<td>90</td>
<td>Streptoalloteichus sp.</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>P. fluorescens SBW25</td>
<td></td>
</tr>
<tr>
<td>16SM5</td>
<td>Bacillus sp.</td>
<td>99</td>
<td>P. putida KCTC1639</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>P. putida S16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>P. putida U</td>
<td></td>
</tr>
<tr>
<td>16SM6</td>
<td>Cyophaga sp.</td>
<td>99</td>
<td>P. putida KCTC1639</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>P. putida S16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>P. putida U</td>
<td></td>
</tr>
<tr>
<td>16S2M1</td>
<td>Acinetobacter</td>
<td>98</td>
<td>P. putida</td>
</tr>
<tr>
<td>johnsonii</td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>P. putida KCTC1639</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>P. putida</td>
<td></td>
</tr>
<tr>
<td>16SM4</td>
<td>Aminobacter sp.</td>
<td>100</td>
<td>P. putida</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>P. putida</td>
<td></td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>P. putida</td>
<td></td>
</tr>
<tr>
<td>Agromyces sp.</td>
<td>Class II synthase</td>
<td>81</td>
<td>Pseudomonas sp. g106</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>P. pseudoalcaligenes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>P. oleovorans</td>
<td></td>
</tr>
<tr>
<td>16S2M3</td>
<td>Elizabethkingia sp.</td>
<td>71</td>
<td>P. putida sp.</td>
</tr>
<tr>
<td></td>
<td>Class II synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>P. putida GB-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>P. putida S16</td>
<td></td>
</tr>
</tbody>
</table>
With regard to the gene similarities to *Pseudomonas*, it should be noted that the vast majority of class II synthases identified to date have been associated with these species (Huisman *et al.*, 1989; Timm and Steinbüchel, 1990; Preusting *et al.*, 1990; Gross *et al.*, 1989; Fritzsche *et al.*, 1990). Therefore, the targeting of Class II PHA synthase gene is likely biased toward the isolation of genes reflecting this. Figure 4.5 presents a phylogenetic analysis of Class II PHA synthase genes found in the source organisms which were similar to the Pha synthase genes found in the organisms isolated from the reactors (Table 4.3). This phylogenic tree shows how all the source organisms found are closely related and all within the genus *Pseudomonas*. Figure 4.6 presents a phylogenetic analysis of 59 pha synthase gene isolates, broadly grouped into class I-IV, which demonstrates this concentration of class II synthases within the *Pseudomonads* (Rehm, 2003). In addition it is possible that the phaC2 amplicons generated from unrelated isolates in this study were xenologs, although the PHA accumulation profiles suggest the function of mcl synthases in the strains. *Agromyces* (Actinobacteria) and *Elizabethkingia* (Flavobacteria) did display some level of divergence from published *Pseudomonas* mcl synthases, but overall the results did not identify the likelihood of the isolates producing novel polymers on the basis of considerable differences at the nucleotide and/or protein level. However, efforts were committed to providing further clarity on the findings via chemical characterisation of polymer compositions from isolates.
**Figure 4.5.** An Evolutionary Taxa of the source organisms containing the *pha* Class II synthase genes.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.20750550 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 396 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).
4.3.5 Quantitative Investigation of PHB Production using High Performance Liquid Chromatography (HPLC)

Despite a number of indicators within the data that the induction and screening strategy appeared to favour the production of mcl-PHAs, the isolates were subjected to HPLC analyses for potential scl (PHB) granules, via sulphuric acid digestion to crotonic acid. It was observed that crotonic acid derivatives could not be detected in all but 1 of the isolates grown under PHA inducing conditions. 16S2M5-Acinetobacter anoxii yielded a minor peak at 25.880 min corresponding to crotonic acid (standard elution 25.790min) (Figure 4.7 (a) and (b)), despite earlier acetate Nitrogen-limiting culture experiments failing to produce detectable granule accumulation. Other isolates for which crotonic acid derivative production from PHB would have been anticipated included Delftia acidovorans, Bacillus sp, and Thauera sp, but none was detected. It is unclear whether the failure to detect PHB in these microorganisms reflected poor sample yields under the conditions tested, or a lack PHB synthetic apparatus. As mentioned previously, poor growth of many isolates were noted on E2 media, which appears better suited to inducing medium chain length PHA production. Medium chain length PHAs are not detected using the HPLC method, due to the volatile nature of the longer chain methyl ester derivates generated during sample preparation. Analysis of the latter required gas chromatography.
(a) HPLC curve showing crotonic acid standard (5mM)

(b) 16S2M5 *Acinteobacter sp. Anoxic* sample showing weak crotonic acid production.

**Figure 4.7.** HPLC analyses for Polyhydroxybutyrate. Black arrows indicates crotonic acid elution.
**4.3.6 Determination of PHA Production using Gas Chromatography (GC)**

GC analysis was carried out to identify the possible types and quantity of medium chain length PHAs present in the reactor biomass and those potentially produced by the pure culture isolates. Concentrations of methyl esters were determined via comparison with standard curves produced with hydroxyhexanoic acid, hydroxyoctanoic acid and hydroxydecanoic acid (3mM, 6mM and 12mM). The retention times of the TMSi derivative were as follows: hydroxyhexanoic acid, 12.49 minutes, hydroxyoctanoic acid, 14.43 minutes and hydroxydecanoic acid, 16.27 minutes. While HPLC analyses in chapter 2 identified PHB production in both reactor sludges, (Section 2.3.4), as per the EBPR model, the GC analysis revealed differing methyl ester profiles relative to the different influent carbon compositions (Table 4.6). Derivatised samples from Reactor 1 were found to contain high levels of hydroxyhexanoate monomers under acetate feeding. However, in reactor 2, where acetate and skim milk were both present in the influent, a more complex PHA profile with hydroxyoctanoic and -decanoic monomers was revealed. It would appear therefore that the skim milk component may play a considerable role in modifying the composition of polymers within the biomass. In relation to the production of mcl-PHAs by the pure culture isolates, many of the samples appeared to lack detectable methyl ester derivatives. However, some notable observations arose. In contrast to *Acinetobacter anoxii* PHB production, *Acinetobacter johnsonii* was shown to produce 3 hydroxy-hexanoic, -octanoic and -decanoic acid derivatives as well as producing the greatest amount of medium chain length PHA among the isolates (Table 4.6). Previous analysis of *Acinetobacter* production of mcl-PHAs indicated a limited capacity only for hydroxyoctanoic acid monomer packaging (*Alvarez et al.,* 1997). Our findings expand this narrow categorisation, demonstrating an alternative enzyme
substrate specificity of the mcl pha synthase(s) in this isolate. Medium chain length-
PHAs are produced by a number of Gram negative bacteria, mainly Pseudomonads
(Huisman et al., 1989; Timm and Steinbüchel, 1990). Pseudomonas mendocina has
two phaC genes separated by a gene for intercellular PHA depolymerase (phaZ).
Pseudomonas mendocina has also been shown to produce mcl-PHA from various
carbon sources. Hydroxyhexanoate, hydroxyoctanoate and hydroxydecanoate being
the major components when grown on different carbon sources such as glucose, citric
acid and carbon sources related to their monomeric structure such as myristic acid,
octanoate or oleic acid (Tian et al., 2000). Despite this only one Pseudomonas isolate,
16S2M7, yielded monomer derivatives detectable by GC. Furthermore, the analysis
revealed the uncharacteristic, singular production of hydroxyoctanoate monomers by
this strain (Table 4.6). Longer chain length monomers in the reactor sludge might be
attributable to direct incorporation of long chain fatty acids derived from the skim
milk element of the influent media. However, the generation of hydroxyoctanoate
monomers from glucose grown cultures suggest an altered synthase specificity or
precursor provision mechanism in this isolate, compared with other Pseudomonas.
Indeed, reference to the BLAST analysis of the 16S2M7 Class II PHA synthase
reveals that it shares greater similarity with non putida strains P. fluorescens and P.
extremiaustralis, which do not routinely accumulate hexanoic, octanoic and decanoic
monomers. The Bacillus sp and Agromyces isolates were also found to produce mcl-
PHA monomer derivates at either end of the scale. The Bacillus sp uniquely
produced hydroxyhexanoates, in a similar fashion to the reactor 1 sludge sample,
(Table 4.4) despite the cloned synthase sharing a high degree of similarity to P. putida
sequences (Table 4.3). In contrast, the Agromyces synthase appeared to favour longer
chain monomers of hydroxyoctanoate and hydroxydecanoate in equal ratios (Table
The latter corresponded with a reduced similarity at the nucleotide level to mcl-synthases from *P. putida* (Table 4.4).

**Table 4.4.** Quantitative analysis of Pure Cultures and sludge obtained for reactor 1 and 2 for mcl-PHA production.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydroxyhexanoic acid (mM)</th>
<th>Hydroxyoctanoic acid (mM)</th>
<th>Hydroxydecanoic acid (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sludge from Reactor 1</td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sludge from Reactor 2</td>
<td></td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>16S2M1 <em>Acinetobacter johnsonii</em></td>
<td>0.7</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>16S2M7 <em>Pseudomonas sp.</em></td>
<td>–</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
<td><em>Agromyces sp.</em></td>
<td>–</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>16SM2 <em>Bacillus sp.</em></td>
<td>0.4</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
4.4 Conclusion

The results of the analyses of pure cultures for polyhydroxyalkanoates production potential were, to an extent, at odds with the central objective of examining an EBPR based PHA production model. EBPR operates largely on the basis of selecting for organisms which are highly proficient in accumulating scl-PHAs (Takabatake et al., 2000; Rodgers and Wu 2010; Kasemsap and Wantawin 2007). As a result, one would consider it likely that a pure culture isolation strategy operated upon such a system would produce a concentration of scl-PHA synthesising microbes. However, our analyses produced the opposite observation, revealing a higher percentage of mcl-PHA synthetic genes and related activities. Our objective was to determine whether novel co-polymers might potentially be synthesised by identifying scl/mcl co-expression capabilities among isolates. Such polymers have considerable commercial potential as they combine the demands of bulk applications with improved flexibility (Noda et al., 2005). The challenge in achieving this objective came, rather surprisingly, from a lack of scl (PHB) synthetic capacities. HPLC analysis indicated that PHB production was not a major feature of the reactor isolates and this appeared to be confirmed by the molecular screens. Despite an awareness of the potential bias within the PCR approach and the E2 media based induction strategy, the initial pure culture isolation strategy did not discriminate in any way for species producing either class of PHA (see Section 3.3.1). It did become clear however that a considerable diversity of mcl-PHAs can be produced within a relatively limited range of bacterial species and that such cultures might be amenable to engineering of specialist plastics were production costs would be offset by greater profitability (e.g via medical applications). Thus in the isolates analysed herein, the common presence of a phaC2 homologue was not reflected in the production of a common polymer.
Considerable variation was noted in both the monomer composition and ratios. The incorporation of, (or stimulation of nascent copies of) scl \textit{pha} synthase genes in these differing isolates could therefore potentially yield novel co-polymer compounds for future exploitation.


CHAPTER 5

General

Discussion
Despite ever optimistic, industry-led projections of global oil reserves, sustained crude oil price increases in recent years provide a more realistic perspective of a decreasing global oil production capacity, unable to keep pace with increasing world demand (Murray and King, 2012). While society at large is keenly aware of the need for sustainable fuels development, awareness of our dependence on petrochemicals for the provision of everyday polymers/plastics is not as strong, despite a near constant interaction with same in developed society. Common uses of plastic include packaging materials, synthetic household items, mobile telecommunications devices, medical equipment, computer housing and materials for the construction industry (Clark and Hardy, 2004). The scale of petrochemical plastics consumption in everyday living is reflected in an estimated global market value of $180 billion in 2011. By way of comparison, the global antibiotic market in 2009 was estimated at $42 billion (Hamad, 2010). As a result polymer applications will have to be facilitated in any future shift toward a “renewables” based global economy. In addition, as petrochemical plastics can persist in the environment for extended periods upon disposal, the incorporation of bio-degradability into novel polymer properties is also a key area of focus. According to the US EPA, $2.5 \times 10^8$ tonnes of municipal solid waste was generated in the US in 2010, of which 12.4% was composed of plastic solid waste, PSW (USEPA, 2010). It is estimated that 18-25% of the total volume of landfill sites can be occupied by accumulated PSW, although compaction due to landfill mass is likely to reduce this value over time (Andrady, 2003). Legislative pressure is already mounting in relation to end of life treatment of petrochemical polymers and the unsustainability of landfill disposal. The amended European Parliament and Council directive 94/62/EC on Packaging and Packaging Waste called for recycling and/or energy recovery targets of 60% for packaging
waste, to be implemented within member states by 2008 - 2011. In response to the issues outlined above, significant research effort has been committed over the last number of decades toward the investigation and commercialisation of sustainable, eco-friendly polymers (Flieger et al., 2003). However, despite varying successes, commercial applications of biodegradable polymers still account for less than 1% of the global plastics market (Nampoothiri et al., 2010). Despite public, political and industrial good will towards these materials, a critical barrier of end product has yet to be effectively bridged (Widdecke et al., 2008).

Polyhydroxyalkanoates have received considerable interest as genuine, environmentally sensitive alternatives to petrochemical plastics. Current synthetic polymers are dependent on non-renewable feedstocks and are recalcitrant to biological degradation, resulting in their persistence in our environment upon disposal (Madison and Huisman 1999; Alexander, 1981). Efforts to produce PHAs have largely focussed on pure cultures of natural and/or recombinant bacteria such as E. coli, achieving yields of up to 80-90% PHA cell dry weight (Byrom, 1987; Liebergessell et al., 1994; Wang and Lee 1997, Kim et al., 1992). However, the scale of industrial production to date indicates the economic limitations in this approach, typically due to costs of feedstock, sterile bioreactor design and operation and downstream processing chemical/energy needs. On this basis, the use of renewable waste materials and non sterile activated sludge systems has received considerable attention for polymer synthesis, offering process cost reductions by up to 50% (Serafim et al., 2004). In the past decade, there has been considerable research into the use of mixed cultures for PHA production focusing on configuration, reactor optimisation, process modelling, microbial ecology characterisation and novel polymer synthesis (Lemos et al., 2003; Dias et al., 2005, 2006; Dionisi et al., 2006;
Serafim et al., 2006, 2007). A number of different strategies have been explored for PHA production using mixed cultures such as; aerobic dynamic feeding (ADF), microaerophilic-aerobic cycling and Enhanced Biological Phosphorus removal (EBPR).

Dairy processing is a major industry in Ireland, with volumes set to increase in the future. In 2015 EU milk quotas are to be abolished and, with increasing global market demand, Ireland is one of the few countries poised to benefit from a dramatic increase in dairy production and related exports. The Department of Agriculture’s Food Harvest 2020 strategy estimates a 50 percent increase in output from the dairy sector as a realistic outcome over the next decade. In quantitative terms, this amounts to a 2.75 billion litre increase in milk volumes. While this has prompted the dairy industry to initiate programmes for increased plant processing capacities, consideration must also be given to sustainable management of the inevitable increase in wastewater production such an expansion will bring. Indeed, a central element of the Harvest 2020 strategy is the promotion of sustainable/green technology for an Irish agri-food sector that is “innovative, efficient, and a global leader in environmentally sustainable production”. In the current study we conducted preliminary investigations into the potential for dairy ingredient processing wastewater to facilitate PHA production using the EBPR process which has already been implemented in Irish dairy wastewater (Mulkerrins et al., 2004).

Initially in trial 1, three reactors were set up using skim milk as its carbon source and cycling conditions, Anaerobic 90 minutes and aerobic 60 minutes with a COD:N:P ratio, 1000:40:60. This trial showed good PO₄-P removal, averaging below 2 mg/l in the effluent, NO₃⁻-N levels on average were below 3 mg/l while residual NH₄⁺-N nitrogen was below 10 mg/l for all three reactors. In addition, DO levels
remained low indicating strong metabolic activity among microorganisms in the reactor with reactor 2 giving the best performance. Reactor 2 had the greatest COD removal with effluent levels averaging 58 mg/l. The COD levels were also good for the other two reactors, with average loads of 149 mg/l and 98 mg/l 1 and 3, respectively. The stable phosphate and COD removal observations seemed to show preliminary indications that an EBPR process was potentially becoming established in the reactors with the possibility of PHA accumulation. However, stability of the reactor began to degrade resulting in sludge washout. This was caused by filamentous growth (*Thiotrix*) occurring due to the extended anaerobic period within the SBR cycle. The filamentous growth resulted in denitrification due to the low oxygen levels encountered and the microorganisms utilising oxygen from the nitrates ultimately leading to nitrogen gas (gas bubbles). The filamentous growth and the gas bubbles resulted in poor settling of the sludge and therefore sludge washout, and it was decided to terminate the first trial. Trial two was set up in the same manner as Trial 1 and the cycling conditions amended to 60 minute anaerobic and 90 minute aerobic phase, while the influent maintained a COD:N:P ratio, 1000:40:60. However sub-critical oxygenation in the reactors, due to faulty sensor probe, resulted in poor remediation and washout. Furthermore, as with the previous trial, contamination was also a problem in large volume influent causing a 10-20% depletion of nutrients before entry to the reactors. The reactors became unstable, with filamentous proliferations causing sludge washout and Trial 2 was terminated. Following the experiences of Trial 1 and 2, it was decided in Trial 3 that organic fraction be sterilised and fed independently from the inorganic nutrients to prevent contamination. Furthermore, cycling times were decreased to two hours and carbon substrate levels were increased to try and optimise PHA production. Sodium acetate
was also implemented in the reactors, one reactor receiving sodium acetate alone as the carbon source and reactor 2 being fed influent containing both sodium acetate and skim milk. High phosphorus removal, indicative of EBPR operation, became established in the system. Spectrophotometric analyses showed that PHA was being produced in both reactors reactor 1 and 2 producing 10% and 20% respectively. Quantification of potential PHB production by HPLC revealed 11% and 13% sludge dry wt accumulations in reactors 1 and 2, respectively. Although PHA accumulation was relatively low, compared with other mixed culture systems it did reveal that PHA production was greater in reactor 2 with skim milk and sodium acetate than in reactor 1 although sodium acetate is known to be easily utilisable for PHA production. It should be noted that as time progressed, the reactor performance in relation to P removal deteriorated. Recent studies have reported similar occurrences in other EBPR based systems, with PHA accumulation of 28.8% of dry biomass weight with residual phosphorous of 12.8% (Rodgers and Wu, 2010) and 52% PHA accumulation when residual effluent phosphate was 8% (Kasemsap and Wantawin, 2007) used the EBPR system. The challenges encountered in this study in relation to successful, continuous operation of the EBPR system consumed a considerable amount of time and further work, such as pre-fermentation of dairy wastewater to increase VFA contents and feeding to reactors was unable to be carried out. As a result there is considerable scope for the optimisation of the system and the reported PHA yields achieved at this stage represent a preliminary demonstration of the potential of the system. In this regard it was noted also that as well as producing 3-hydroxybutyrate, PHA monomers of hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid were also identified in reactor 1, and 3-hydroxydodecenoic acid and 3-hydroxytetradecanoic acid identified in reactor 2 (Chapter 2, Figure 12 a
and b). The skim milk component of the media, mimicking dairy wastewater, was therefore seen to have a significant impact on the composition of the polymers being produced, due to longer chain fats likely being present. In terms of industrial relevance, it is possible that dairy wastewater may offer a source of novel polymers, influenced by the inherent composition of the feedstock. In conclusion, one would have to query, (based on the instability we encountered), how an EBPR based approach would react to seasonal fluctuations associated with dairy processing outputs annually in Ireland (i.e. spring/summer peak production). The inherent fluctuation in influent nutrient compositions and related oxygen consumption demands would require careful control systems design in order to maintain EBPR based polyhydroxyalkanoate synthesis.

In chapter 3, culture dependent and independent approaches were applied in an effort to establish key PHA producers within reactors 1 and 2, and to establish whether the microbial profiles differed considerably, based on the influent compositions. Our analyses suggested that; (a) diverse communities were found to be present in both reactors, (b) little overlap occurred in the profiles obtained for both reactors, and (c) data correlation between the differing approaches was observed, but to a limited extent. The physiochemical properties of wastewater, the design and operation of the system and the ambient environmental conditions typically influence the microbial communities within activated sludge (Yu and Mohn, 2001). Investigation of our system reflects this, as the skim milk in reactor 2 appeared to have a considerable impact on the microbial ecology profile. It was noted that none of the analytical approaches specifically identified currently accepted, key EBPR candidate species such as Candidatus accumilibacter, Tetrasphaera-related species, or Malikia spp. (Nakamura et al, 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Liu et al., 2001;
Spring et al., 2005). However, Thauera sp., Azoaracus sp. and Zooglea sp. which were identified are related to Rhodocyclus sp, with other strains previously being reported to be important in EBPR. In addition, Rhodobacter, Simplicispira and Runella have all been found in association with published EBPR systems, although definitive roles have yet to be attributed to them (Thomsen et al., 2007). Many of the bacterial genera identified in this system were also known PHA accumulators such as Pseudomonas sp., Rhodobacter sp., Burkholderia cepacia, Azoarcus sp. and Thauera sp. Furthermore, phylogenetic trees were generated for the culture dependent and independent method as well as the bacteria found to contain the Class I pha synthase genes. As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the topology at that branch is considered "correct" (Nei and Kumar, 2000). All the interior branches of each phylogenetic tree had a topology of 95% and higher that indicated the branching pattern for each tree was similar. Overall, the findings of the culture dependent and independent analyses suggested a broad ecological network existed in both reactors, capable of facilitating the observed accumulations of PHAs. Comparatively however, the approaches suggested differing phylotypes were dominant in the reactors. The culture independent method carried out on reactor 1 and 2 suggested that Betaproteobacteria was the dominant class, while the culture isolations and profiling experiments indicated a Gammaproteobacterial dominance in both. To resolve this issue, FISH probes for alpha-, beta- and gamma-subclasses of proteobacteria were applied to sludge samples during periods of maximal PHA accumulation. Dual staining with Nile Red was performed to facilitate co-localisation with PHA accumulation within the sample. It was determined from this approach that Betaproteobacteria appear to contribute significantly to PHA accumulation in both reactors. Analysis of past literature has revealed that β-
proteobacteria have previously been shown as prominent species in EBPR sludges analysed by FISH (Kämpfer et al., 1996, Wagner et al., 1994 and Bond et al., 1999). While the FISH analyses may correlate with the 16S rRNA gene analyses, in highlighting the importance of β-proteobacteria within the system, it does not facilitate identification of the particular species. As a result, the characterisations performed to date on the sludge communities carry an element of subjectivity, due to the potential for inherent bias present in the methodologies. DGGE profiling and RT-PCR analyses of total RNA from the reactors was also attempted in an effort to further enhance our understanding of key microbial contributions within the system. However these were unsuccessful, despite repeated attempts at optimisation. As a result, the PHA accumulation observed in both reactors could not be definitively associated with any of the species identified by the various approaches.

As discussed earlier, a strong focus has emerged in recent years in the production of PHA co-polymers of scl and mcl monomer compositions. Both reactors in this study were found to yield both scl derived crotonic acid and mcl esters ranging from hydroxy hexanoate (6C) - hydroxytetradecanoate (14C), suggesting the possibility for co-polymer producers within the respective sludges. Furthermore, the 16S rRNA derived identities of the pure cultures isolated from the reactors revealed species which are commonly associated with scl and mcl PHA synthesis. On this basis we investigated the potential for such polymer production among the isolates. Following both qualitative and quantitative analyses, it was determined that the majority of the microorganisms isolated demonstrated mcl PHA. Ironically, the presence of scl-PHAs was noticeably absent, despite the reactors operating on an EBPR model, which requires scl PHA accumulation from acetate or other VFAs as a fundamental characteristic. These findings were further reflected in our efforts to
clone scl and mcl genes from the isolates, to account for a potential failure of the induction media to meet the signalling requirements of PHA pathways in the differing species. Once again, mcl-PHA sequences dominated the findings, with a common relatedness to *Pseudomonas* species genes available on GenBank being clearly noted. Chemical characterisations of the PHAs produced were carried out in an attempt to determine if the species therefore produced a generic polymer, however considerable diversity was observed in this regard. *Pseudomonas* species produced the greatest amounts of hydroxyhexanoic acid, however this was not a typical observation within this species. Numerous studies have shown mcl PHA accumulation in *Pseudomonas* species, with polymer compositions containing varying ratios of hydroxy-hexanoic, -octanoic and -decanoic acids (Huisman et al., 1989; Timm and Steinbüchel, 1990; Preusting et al., 1990; Gross et al., 1989; Fritzsche et al., 1990). The singular production of hydroxyhexanoic acid by our isolate suggests some novel synthase properties therefore. Sequence analysis of the gene suggested a divergence from common class II synthases, with only 80% sequence identity to other *Pseudomonas* species synthases being noted. The *Bacillus sp* and *Agromyces* isolates were also found to produce divergent mcl PHA monomer derivates. The *Bacillus sp* uniquely produced hydroxy-hexanoates, in a similar fashion to the *Pseudomonas* isolate above. In contrast, the *Agromyces* synthase appeared to demonstrate a preference for hydroxyoctonate and hydroxydecanoate monomers, in equal ratios. The latter corresponded with a reduced similarity at the nucleotide level to mcl-synthases from *P. putida* (~75% identity). The observations of varying polymer compositions within the isolates were reflected in the overall GC profiles of reactor 1 and 2 sludge samples also, which demonstrated differing mcl monomer profiles in conjunction with the varying influent compositions. Reactor 1 sludge GC profiling revealed a mcl
monomer composition limited to high concentrations of hydroxyhexanoate, while the presence of skim milk in the influent of reactor 2 appeared to facilitate longer chain length complexity, with hydroxy-octanoate and hydroxyl-decanoate monomers being detected. Therefore, while co-polymer production could not be demonstrated among the isolates, it became clear that the reactor sludges represent an excellent resource for the cloning of atypical synthases which could be further exploited in designer PHA approaches.

In conclusion this study has demonstrated the feasibility of achieving PHA accumulation in an EBPR model system, receiving artificial influents designed in part to mimic wastewater components and nutrient concentrations encountered in dairy processing sectors. Considerable challenges remain to be overcome before any real time application of the process might be envisaged, but the findings of this study suggest the merits of continued exploration in this area. A clear impact of the skim milk/ dairy component of the influent was demonstrated throughout each stage of the study in terms of the remediation performance of the reactor, the microbial ecology determinations, the PHA accumulation profile and the diversity of isolates accumulated. It is likely therefore that any industrial scale design one might envisage for the system would have to be cognisant of the fact that a site specific microbial community is likely to emerge, rather than a text book reference system becoming established. From a biotechnological perspective this is particularly exciting however, as it suggests that variations in influent feeds can result in completely altered community dynamics, which are reflected in the diversity or PHA synthase genes presented within same. Thus a vast scope for the cloning of novel synthases exists via the sampling of such varied reactor systems.
5.1 Bibliography


Biosynthesis and characterization of poly(β hydroxyalkanoates) produced by 

Hesselmann, R.P.X., C. Werlen, D. Hahn, J.R. van der Meer, and A.J.B. 
Zehnder. 1999. Enrichment, phylogenetic analysis and detection of a bacterium that 


3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. 

Kämpfer, P., R. Erhart, C. Beimfohr, J. Böhringer, M Wagner, and R. Amann. 
1996. Characterization of bacterial communities from activated sludge plant: culture-
dependent numerical identification versus in situ identification using group and 


