Development of sustainable microbial fermentation strategies for the production of medium-chain length polyhydroxyalkanoates (mcl-PHAs) from biodiesel derived glycerol

by

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Author's Declaration

- ✓ I hereby declare that I, Umesh Sharma, am the sole author of this thesis.
- ✓ This is the true copy of my thesis, including any required final revisions, as accepted by my examiners.
- ✓ I understand that my thesis may be made available to the public electronically.

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Dedication

I dedicate this thesis to my dearest and sweetest mother, my guardian angel, Uma Sharma, and all too loving sister, Mala Sharma. Words cannot express how extremely grateful I am for you and all the good wishes, prayers, and sacrifices you constantly make for me.

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Abstract

Bio-plastics have emerged as a promising alternative to conventional petrochemical derived plastics particularly over the past few decades. Numerous production methods for bio-plastics have been researched; however, work remains towards developing a commercially viable and economical process. The purpose of this research was to develop a sustainable fermentation strategy for production and scale-up of medium-chain length polyhydroxyalkanoates (mcl-PHAs), or bio-plastics, using a novel strain of the gram negative bacterium *Pseudomonas putida*, LS46, with biodiesel derived waste glycerol (WG) as feedstock. Experiments were conducted to gain a basic understanding of the general growth patterns exhibited by LS46. Thereafter, flask-batch experiments were conducted to study effects of variation in media conditions upon cell biomass production and mcl-PHA accumulation. Subsequently, optimal medium conditions observed within flasks were scaled-up and employed in the operation of a pilot-scale fermenter to increase production capacity for mcl-PHAs. It was concluded that mcl-PHA production at commercial levels could be viable with advanced process optimization.

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List of Abbreviations

CDW	Cell Dry Weight
DO	Dissolved Oxygen
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LB	Luria-Bertani
LCL-PHAs	Long-chain length Polyhydroxyalkanoates
MCL-PHAs	Medium-chain length Polyhydroxyalkanoates
N	Nitrogen in the form of ammonia sulfate
OD	Optical Density
PHAs	Polyhydroxyalkanoates
REG-80	Renewable Energy Group 80 % glycerol content of the biodiesel derived waste glycerol as per the manufacturer's Certificate of Analysis
RM	Ramsay medium
RPMs	Revolutions per minute
SCL-PHAs	Short-chain length Polyhydroxyalkanoates
WG	Waste Glycerol

Chapter 1 Literature Review

1.1 Introduction

Over the past decade significant attention has been placed on issues related to the environment and its sustainability. Academic institutes, businesses, and governmental organizations have all been instrumental in emphasizing the impact our current actions and practices have upon the environment and earth as a whole, both short-term and long-term. This new shift in thinking has given rise to various terms and concepts that have now become well known around the world, and a part of our everyday vocabulary such as, biodegradability, carbon footprint, greenhouse gas emissions, just to mention a few. Moreover, it has also lead to the development of many new environmentally friendly products and services for consumers conscientious of their impact on the earth and its environment.

Billions of dollars are invested annually by government and private firms globally for research and development of new products and services that coincide with the "green" or "eco-friendly" concept valued in today's society. At first, such investments may appear costly and quite exorbitant. However, the rewards and savings gathered over time in terms of efficiency and sustainability are highly valuable. These products and services are required to meet specific codes and standards in an effort to limit or mitigate any negative impact it has on the environment during its production or usage.

A product utilized world-wide in large quantities is plastic. Plastics contain a wide range of moldable synthetic or semi-synthetic organic solids of high molecular mass. They are incorporated in a range of products within residential, commercial, and industrial applications and are found in almost every facet of our lifestyle such as, disposable kitchen cutlery, furniture, machinery housings, packaging and wrapping, specialized medical equipment, pharmaceuticals in addition to countless others.

Since the last decade approximately 150 million tonnes of plastic materials have been manufactured and utilized annually. An increase in this trend is expected until the year 2020 and beyond (Castilho et al., 2009). According to some estimates, an annual 4 % increase in the production of plastics is projected until 2050. Such immense reliance on plastic has placed a huge

burden on this single entity leading to many concerns and issues related to the environment and health. Primarily, the means by which plastic is manufactured is a major cause of concern.

Current methods involve intensive processing of fossil fuel for manufacturing of plastics, or polyolefins, that consumers utilize on a daily basis such as, polypropylene, polyethylene, polyvinyl chloride, polystyrene, etc. Reliance upon fossil fuel, a non-renewable resource, for the production of plastics has raised concerns revolving around the long-term sustainability of this practice.

Furthermore, the target end product, plastic itself, is a cause of major concern due to its increasing accumulation in waste landfills. Majority of plastic is found in temporary form factors such as, kitchen utensils or packaging and wrapping materials; therefore, after their short-term use they are tossed away as refuse which ultimately finds its way into waste sites that now have begun to accumulate huge quantities of plastic (Castilho et al., 2009). Plastics hold a very low degree of degradable properties and in some instances hold no degradable properties. In fact, the degradation process for plastics can take hundreds of years to begin. This in itself can pose a threat to all wildlife and ecosystems as plastic continues to accumulate within landfills (Castilho et al., 2009).

Moreover, recycling of plastics for possible reuse is a costly investment as implementation of new laws and regulations are required in addition to the development of effective recycling programs. Incineration of plastics to reduce its accumulation in landfills poses a threat to health and the environment as toxic and carcinogenic chemicals are released into the atmosphere (Castilho et al., 2009). One such toxic chemical heavily discussed in the news and media is Bisphenol A also known as BPA. It is a chemical agent used to harden polycarbonate plastics. Such plastic is used in the lining of food and beverage containers. Researchers at Bayer and General Electric revealed BPA as a toxic chemical leading to adverse effects for women during pregnancy and child development. Furthermore, it is an endocrine disruptor which mimics estrogen leading to negative effects. Based upon these findings the Canadian Department of Environment declared BPA as a toxic substance officially banning its application in the processing and production of plastics in 2008 (Vogel, 2009).

Therefore, as a solution to some of these major dilemmas, biodegradable and sustainable alternatives are now being developed to eventually phase out conventional petrochemical derived plastics. Presently, there are three types of biodegradable plastics that have been ventured into

market by established manufacturers of conventional plastic packaging and wrapping materials such as, BASF, Monsanto, Metabolix to name a few.

The three types of biodegradable plastics currently available today comprise of photodegradable, semi-biodegradable, and completely degradable (Reddy et al., 2003). Photodegradable plastics contain a light sensitive component within the polymer backbone which requires exposure to a constant source of ultraviolet (UV) light over the course of several weeks to months in order to initiate the degradation process. However, in many instances landfills lack sunlight, and thus such plastic remains non-degraded (Reddy et al., 2003).

Semi-biodegradable plastics on the other hand comprise of starch-linked polymers. In other words, starch is incorporated within the polymer backbone to hold together short fragments of polyethylene (Reddy et al., 2003). This allows for bacteria found within the soil to attack the starch, while releasing the polymer fragments to be degraded by other bacteria. The starch is attacked by bacteria, but the polymer fragments prevent further degradation and thus remain non-degraded as well (Reddy et al., 2003).

On the contrary completely degradable plastic is a new form of plastic and holds great promise. However, it largely remains in the phase of research and development for eventual commercial-scale mass production. This form of plastic is interesting as it involves production via sustainable means such as, microbial conversion processes. It includes polyhydroxyalkanoates (PHAs), polylactides (PLAs), aliphatic polyesters, polysaccharides, co-polymers and/or blends of such components (Reddy et al., 2003). Since bio-plastics is a broad topic for discussion, the focus of this thesis will be on PHAs strictly; more specifically their production, applications as well as its relevancy in the market. Furthermore, the research project involving development of a sustainable fermentation strategy incorporating industrial waste streams, in this case, biodiesel-derived waste glycerol will be discussed in depth.

1.2 What are Polyhydroxyalkanoates (PHAs)?

Polyhydroxyalkanoates, or PHAs were first discovered in 1888 by Martinus Beijerinck as inclusion bodies in bacteria when a sample was observed under a microscope. Initially, PHAs were extracted by Maurice Lemoigne in 1926 from the bacterium *Bacillus megaterium* (Sudesh et al., 2000). The functional role of PHAs and the significance of the carbon to nitrogen ratio in the production of PHAs was determined by R.M Macrae and J.F. Wilkinson in 1958 (Rai et al., 2011).

PHAs are an intracellular carbon and energy storage component synthesized by a myriad of gram negative or gram positive bacteria (Rai et al., 2011). This source of carbon and energy storage is triggered due to a nutrient imbalance during microbial growth. Earlier research accounts from the 1950's acknowledge a high carbon to low nitrogen ratio as the key contributor for the accumulation of PHAs within a cell (Sudesh et al., 2000). In other words, during conditions of excess carbon source availability and simultaneous limitation of nitrogen, an essential component for cell development, certain types of bacteria are capable of synthesizing and accumulating PHAs (Sudesh et al., 2000).

In simple terms, the cells utilize the supply of nitrogen available within media for the development and production of new cells. However, once nitrogen is completely exhausted, a famine response is triggered within the cell's metabolism and the excess carbon available within media is incorporated into the production of PHAs, which serves an energy storage reserve for the cells.

By the same token current research literature has mentioned other essential nutritional components such as, magnesium, oxygen, phosphorous, and sulphur to be triggering factors for the accumulation of PHAs within a bacterial cell when found in limited quantities (Rai et al., 2011). PHAs are accumulated as cytoplasmic inclusion bodies for which the number per cell and granule size varies among different species (Ojumu et al., 2004). Usually bacteria produce PHA granules during the stationary phase of their growth period when subjected to the aforementioned nutrient stress conditions (Rai et al., 2011).

There are mainly two major groups of PHAs and are defined based upon their carbon chain-lengths. They include short-chain length (scl) and medium-chain (mcl) length groups. The short-chain length PHAs are comprised of three to five carbons, while the medium-chain length groups are composed of six to fourteen carbons.

At the present time, PHAs are seen as potential plastic replacements by various industries, most particularly the packaging industry. They hold chemical and physical properties similar to conventional plastics, and are a fully biodegradable polymer; thus, making it an attractive eco-friendly alternative to conventional plastics (Rai et. al., 2011).

1.3 Production Processes

Various methods for production of PHAs have been proposed and tested. The majority of these methods are either in laboratory or pilot-scale testing phase. A small proportion of industrial manufacturing has been reported to occur as well, but further research and development is imperative for eventual wide-scale commercial production. Presently, the most common method for PHA production is via microbial means, which will be the focus of this thesis project. But, alternative options have been considered as well such as, genetically modified and transgenic plants (Reddy et al., 2003).

1.3.1 Microbial Fermentation

Microbial PHA accumulation was first reported in 1926 and observed within the bacterium *Bacillus megaterium* (Zhu et al., 2013). Presently, microbial fermentation is a process profoundly researched for PHA production. However, the eventual initiative of scaling-up any such potential process proves to be quite costly and economically unviable due to various constraints related to optimal growth conditions, feedstock selection, media sterilization, and downstream processing.

A major cost contributor to microbial PHA production involves feedstock; accounting for 50 % to 60 % of the total expenditures (Zhu et al., 2013). To address this concern, industrial waste streams such as, fryer oils, waste free fatty acids, and glycerol are currently being investigated as carbon source feedstock for microbial conversion into the target PHA (Castilho et al., 2009). This has the potential to substantially improve the cost-effectiveness, since pure forms of feedstock, for instance carbohydrates and chemical-grade fatty acids can contribute to high costs (Zhu et al., 2013). Furthermore, turning waste into a value added end-product is a highly sustainable and attractive concept given today's firm stance on diverting wastes into value added processing rather than disposal and accumulation within landfills.

For example, in the characterization of a novel *Pseudomonas putida* strain, LS46, has exhibited the capacity to metabolize biodiesel derived waste glycerol into mcl-PHAs (Fu et al, 2014). A comparative analysis was conducted by employing two different substrates, which included pure chemical grade glycerol versus waste glycerol obtained from a biodiesel manufacturing plant. Cell biomass production and PHA accumulation on both substrates was quite comparable. However, minor differences were observed. For instance, the rate of growth was faster

on waste glycerol in comparison to pure glycerol, which was due to the presence of other constituents within the waste glycerol such as, waste free fatty acids. This was to be expected as LS46 has demonstrated a significant level of growth on waste free fatty acids as the sole carbon source (Fu et al., 2014). Thus, the presence of such components within the waste glycerol stream even when present in small quantities promotes a faster growth rate by LS46 by improving waste glycerol uptake. As a matter of fact, incorporation of a co-substrate as feedstock can lead to the development of a highly sustainable fermentation strategy. Furthermore, the diversion of two waste streams into the bio-processing of value-added products, while preventing their accumulation into landfills is highly favourable.

Recently published studies on PHA production have examined various other feeding strategies and evaluated the incorporation of inexpensive industrial waste streams as feedstock such as, waste glycerol (Posada et al., 2012). For instance, addition of waste materials in submerged and solid-state fermentation has reportedly been investigated for their efficacy in maximal yields of PHAs (Castilho et al., 2009). Submerged fermentation was introduced in the 1930's and has now become the preferred industrial method. This process requires a liquid medium and is ideal for unicellular organisms such as, bacteria and yeast. Solid-state fermentation, on the other hand has existed for centuries, and is still widely used in industry for manufacturing of food and food products, cosmetics, pharmaceuticals, etc. The diverse waste sources incoporated by various studies included, molasses, sucrose, starch-based materials, cellulosic materials, hemicelluslosic sugars, whey-based materials, as well fatty acids, glycerol, and oils (Castilho et al., 2009).

In contrast to submerged fermenation, studies on solid-state fermentation have not been discussed extensively in literature (Castilho et al., 2009). Although, recently, some reports have gradually emerged. Studies have incorporated soy based and biodiesel derived wastes for microbial conversion into PHAs by *Cupriavidus nector* (*C. necator*).

Furthermore, integration of a second waste stream in the form of molasses was also examined as a co-substrate. Additionally, agro-industrial wastes such as, sunflower and soy slabs (or cakes), soy bran, and solid residues from biodiesel processing have been examined as well (Castilho et al., 2009). Overall it was concluded that production of PHAs using industrial waste streams as feedstock in either submerged or solid-state fermentation can be viable, but further research into development of such strategies for full-scale production is necessary (Castilho et al.,

2009). Moreover, full feedstock composition analyses would also be required for optimization of PHA production. In fact, a similar conclusion was also reached by a study that investigated production of PHAs using open and mixed cultures from a waste sludge stream that contained varying levels of organics, nitrogen and phosphorous (Morgan-Sagastume et al., 2010). Pretreatment of the waste feedstock was determined necessary to allow for optimized production of the target PHAs.

For instance, milder pretreatment techniques on the waste sludge were suggested in order to yield lower levels of non-volatile fatty acids while maintaining readily available essential nutrients for improved performance in PHA production (Morgan-Sagastume et al., 2010). Furthermore, evaluation of different feeding strategies with industrially available wastes was recommended to promote maximal microbial production of PHAs.

In another independent study, sequential feeding strategies with xylose and octanoic acid for production of mcl-PHAs in bioengineered *Pseudomonas putida* KT2440 was investigated (Le Meur et al., 2012). *P. putida* KT2440 was introduced with xylose specific enzymes to allow processing of the xylose fed media into mcl-PHAs. It was observed that the cells did not produce any mcl-PHAs; instead, in an alternate feeding strategy mcl-PHAs were only produced when provided with a fatty acid co-substrate. In this case, the co-substrate provided was octanoic acid. Overall, it was concluded that xylose stimulated high cell growth rate at 2.5 g/L, while octanoic acid promoted 20 % w/w of mcl-PHA. Thus, a sequential feeding strategy was developd with xylose as the growth substrate and octanoic acid as a precursor for mcl-PHA production.

Current research largely includes PHA producing bacteria of the genera *Alcaligenes*, *Pseudomonas*, *Enterobacter*, *Necator*, *Rhodobacter*, *Ralstonia*, and *Cupriavidus*. Each bacterium holds its own capacities with respect to the type and amount of PHA produced which is correlated to the nutrient conditions provided. Information on certain cyanobacteria and specific halophiles accumulating PHA has been described as well. Of the above aforementioned bacteria, *Cupriavidus* has been observed with the highest polymer content accumulation at 77 % of the total cell dry weight when provided with a carbon to nitrogen (C:N) ratio of 200 g/L:1 g/L (Ienczak et al., 2013).

With the availability of an assortment of bacteria and their diverse strains, this has led into the venture of mixed culture fermentation strategies (Moita & Lemos, 2012). It has enabled the production of variable PHA biopolymers which are composed of different monomer compositions.

Through this method a form of control is maintained by employing a staggered feeding or inoculation technique (Ashby et al., 2005).

All in all, microbial fermentation is highly versatile for PHA production given the range of products that can be generated from a variety of feedstock based upon the numerous studies carried out. In fact, it generally remains the preferred route for biopolymer production. The type of polymer formed, i.e. homopolymers or copolymers has been shown to be controlled based upon feedstock composition as well as feeding strategy approach; namely fed-batch or continuous.

1.3.2 Metabolic engineering

Synthesis of PHAs via metabolic engineering has not gained much interest when compared to the previously discussed method of microbial fermentation with non-engineered (wild-type) bacteria. Nevertheless, with better understanding and research, gradually interest into this field has broadened. Metabolic engineering involves the development of a novel cellular metabolic pathway or the manipulation of an existing one for the production of a target end product. Thus, in the case of biopolymer synthesis, the cellular pathways involved in the biosynthesis of PHAs have been recently studied for increased yields and production (Gumel et al., 2013).

Normally, synthesis of PHA in bacteria begins with the uptake of a specific carbon source and involves a particular cellular metabolic pathway. For instance, uptake of sugars is involved in the process of glycolysis to begin its breakdown into pyruvate, which is then converted into acetyl-CoA via the pyruvate dehydrogenase (PDH) oxidation pathway (Gumel et al., 2013). Thereon, the acetyl-CoA produced moves into the process of PHA production. Likewise, fatty acids are involved in a distinct metabolic process known as the Krebs' cycle or β-oxidation cycle.

Metabolic engineering has allowed for a targeted approach in the production of PHAs. With this method, synthesis of specific monomers has been possible along with improved yields. For example, numerous strains of the bacterium *Escherichia coli* (E. coli) have been metabolically engineered to regulate production of scl-PHAs (Tappel et al., 2012). This has been accomplished by enhancing expression of the short-chain fatty acid catabolic operon to increase expression of the target scl-PHAs. According to a report by Theodorou et al., a 1.7-fold increase in the cPHB operon of the mutant E. coli strain had been achieved when compared to its wild-type strain, and thus, higher production of scl-PHAs (Theodorou et al., 2011).

In another report by Jian et al. (2010), reduction in the expression of product inhibiting intermediates had been accomplished for improved polymer biosynthesis. They described improvements in carbon channeling by reducing production of acetic acid. Acetic acid has been observed to inhibit production of biomass and ultimately PHAs. A 90 % decrease in the excretion of acetate was achieved when compared to its parent strain, and thereby a 2-fold increase in biomass and 3.5-fold increase in PHA accumulation (Jian et al., 2010).

Comparable observations of increases in cell biomass and PHA production have been reported by Liu et al. in relation to inhibition of the β-oxidation pathway within a mutant strain of *Pseudomonas putida* (KTQQ20). Six genes involved in the relevant pathway were knocked out to significantly decrease fatty acid oxidation activity and consequently increase PHA accumulation. The mutant strain resulted in mcl-PHA accumulation at 10 % w/w of total cell biomass. A homo-copolymer was produced with 16 % by mol 3-hydroxydecanoate and 84 % by mol 3-hydroxydodecanoate (Liu et al., 2011). A change in carbon source provided some interesting results. For instance, with decanoic acid as feedstock, total PHA accumulation resulted in 5 % w/w of biomass, while tetradecanoic acid provided more than 78 % w/w PHA of the total cell biomass obtained (Liu et al., 2011).

Similarly, in a separate study, a mutant strain of *Pseudomonas entomophila* accumulated more than 90 % w/w of PHA with inhibition of the β-oxidation cycle. This was accomplished by knocking out genes relevant to the pathway (Chung et al., 2011). Furthermore, the PHA product consisted 99 % by mol of 3-hydroxydodecanoates (3-HDD). Other metabolically engineered mutant strains have also been reported such as, *Aeromonas hydrophila*, *Sacchromyces pombe*, *Ralstonia eutropha* (Chung et al., 2011). It is apparent that through metabolic engineering higher yields of the target PHA can be achieved in addition to monomer specific products.

1.3.3 Genetically Modified and Transgenic Plants

Production of PHAs using genetically modified plants is a novel concept that is highly cost-effective since conventional methods of biopolymer synthesis can be expensive. Extensive research in this area has recently been reported, while promoting the use of readily available atmospheric carbon-dioxide and sunlight to produce PHAs.

A commonly discussed aspect of plant synthesized PHA in literature involves the low costs related to separation and recovery techniques (Gumel et al., 2013). This is due to the low input

required for harvesting of the plants. Moreover, low water content within plant derived PHA was observed when compared to microbial methods of PHA production. Thus, extraction methods can avoid the use of harsh and invasive chemicals to dissolve cellular mass for release of the target PHA. Therefore, genetically modified and transgenic plants can play a major role in sustainable production of PHA for the near future (Gumel et al., 2013).

A frequent approach in the development of genetically modified plants for synthesis of PHAs involved introduction of the necessary machinery to equip non-PHA producing plants into PHA producing ones. This transformation was carried out by constructing a plasmid comprising of PHA synthesis genes from bacteria capable of PHA biosynthesis such as, *Acinetobacter* and *Bacillus megaterium* (Gumel et al., 2013). Recently, a tobacco plant was modified for PHA production and accumulated approximately 20 % w/w PHA within leaf tissue, nearly 9 % of the plant's total biomass (Bohmert-Tatarev et al., 2011).

In another approach, a method known as codon-optimization was used to improve expression of PHA within the tobacco plant (Matsumoto et al., 2011). This was accomplished by inserting PHA synthesis genes of the bacterium *Ralstonia eutropha* into the plant. By optimization of the *pha*B gene, a two-fold increase in PHA content of the plant tissue occurred compared to the non-optimized gene (Matsumoto et al., 2011). In contrast, optimization of the *pha*C gene had no significant effect on PHB accumulation, thereby, concluding the *pha*B gene to have a rate-determining effect on the accumulation of PHB within leaves of the plant (Matsumoto et al., 2011).

Despite encouraging results with increased PHA production, it was found that high levels of PHB led to chlorosis; a condition characterized by low chlorophyll production and consequently decreased production of carbohydrates, reducing plant growth (Reemmer, 2009). Therefore, to overcome such concerns numerous alternatives were suggested; one strategy included delaying synthesis of PHA until photosynthetic tissues become well-developed. This was achieved by a chemically induced gene-switch that was developed within a plant called *Arabidopsis thaliana*. A PHB level of approximately 14 % w/w was obtained in newer leaves while 7 % in mature ones (Reemmer, 2009).

An alternative approach led to the production of PHA within plant peroxisomes (Tilbrook et al., 2011). To study this approach *Arabidopsis thaliana* and *Saccarum* (species of the sugarcane plant) were subjected with PHA genes from *Ralstonia eutropha* yielding 1.8 % and 1.6 % of PHB based on dry biomass, respectively (Tilbrook et al., 2011).

Therefore, with advanced research into PHA production within plants, the potential for a cost-effective and low-carbon emitting system is promising. Furthermore, effective use of renewable resources such as, light energy from the sun and atmospheric carbon-dioxide can ensure the development of a sustainable system for PHA production eliminating costs involved due to feedstock source.

1.4 Categories and Polymer Characteristics of PHAs

The basic structural unit of PHAs consists of an ether and a ketone group forming an ester bond. As mentioned earlier in section 1.2, PHAs can be divided into two major structural groups based upon carbon chain length and the repeating unit size. These include short-chain length (scl) PHAs that are comprised of a three carbon (C_3) to five carbon (C_5) chain and medium-chain length (mcl) PHAs that are comprised of a six carbon (C_6) to fourteen (C_{14}) carbon chain (Gao et al., 2011). Moreover, a third group of PHAs also exists and is known as the long-chain length (lcl) PHAs. Based upon literature, they were first discovered in 1983 and are comprised of carbon chains that are C_{16} and higher in length. However, they have not been studied as widely as scl- and mcl-PHAs due to their low production.

Studies describing physical properties of scl- and mcl-PHAs, characterize scl-PHAs as highly crystalline and brittle, while mcl-PHAs as elastomeric and flexible in nature. Such properties have been further tested and exploited in varying measures to produce different constituents for their potential in commercial and household applications (Lee & Na, 2012). In fact, several different grades of copolymers have been developed with their properties varying based upon average molecular weight, chain-length, and side group branching as well as branch size (Noda et al., 2005; . Moreover, scl- and mcl-PHA copolymers have demonstrated a variety of properties, which is dependent upon the monomer composition. An increase in mcl-PHA content of the scl- and mcl-PHA copolymer has demonstrated lower melting temperature and reduced crystallinity providing improved ductility and toughness making it much easier to process the end-product polymer for a wide array of applications.

1.4.1 Homopolymers vs. Copolymers

Two major categories of PHAs have been described in literature, which include homopolymers and heteropolymers (or copolymers) (Lee & Na, 2012). In homopolymers, a single type of PHA polymer is repeated, while a heteropolymer is comprised of two or more PHA polymers (Lee & Na, 2012). Both categories of PHA demonstrate an array of properties favourable for specific applications and conditions. Homopolymers currently investigated include, poly-3-hydroxybutyrate (P3HB), poly-3-hydroxyvalerate (P3HV), poly-4-hydroxybutyrate (P4HB), poly-3-hydroxyhexanoate (P3Hx), poly-3-hydroxyoctanoate (P3HO), poly-3-hydroxydecanoate (P3HD), and poly-3-hydroxydodecanoate (P3HDD) (Chen & Wu, 2005). Each type of homopolymer has demonstrated different physical properties ranging from brittle and rigid to flexible and tough (Chen & Wu, 2005).

Copolymers presently developed include, P3HB-co-3HV, P3HB-co-mcl-PHAs, P3HB-co-3HHx as well as an elaborate blend of P3HB-co-3HV-co-4HB (Zhu et al., 2013). Such polymers are developed using varying ratios of the individual monomer units. Compared to their homopolymer counterparts, copolymers have exhibited improved and favourable mechanical properties such as, tensile strength, and elongation to break (Zhu et al., 2013). Numerous applications can be served by exploiting the most favourable properties of various PHA copolymers to replace petrochemical derived plastics (Zhu et al., 2013). Furthermore, thermal and mechanical properties of PHAs can be manipulated based upon the different sizes of the side chains in the repeating polymer unit (Zhu et al, 2013).

1.5 Extraction and Recovery Processes

With on-going research in the field of PHA production, numerous developments in the methods of downstream processing for fermentation systems have ensued. Isolation and purification of PHAs are a key step of the overall process profitability specifically in fermentation systems (Jacquel at al., 2008). For instance, new techniques involving intracellular extraction and isolation of the target PHA polymer from non-PHA cell biomass have been studied, albeit, possessing their own advantages and disadvantages (Gumel et al., 2013).

A major concern often involves the cost-effectiveness and environmental impact of a specific procedure. In some extraction methods, the use of harsh and expensive chemicals has led

to the search for alternate procedures (Gumel et al., 2013). Economics of PHA extraction are dependent upon equipment, energy, and chemicals, which contribute to the overall product recovery yields and potential to reutilize the applied chemicals.

1.5.1 "Classical" Chloroform Extraction

This method is often carried out in lab settings and involves continuous stirring with chloroform under ambient conditions. Dried cell biomass from microbially produced PHA is subjected to this procedure for extraction of the target PHA. Furthermore, this is a time demanding process in order to allow for complete extraction of the PHA. Despite excellent isolation of PHAs, concerns related to toxicity of the chloroform solvent constitutes the main drawback of this method (Kunasundari & Sudesh, 2011).

After extraction of the polyester, solubility of the extracted PHA from the chloroform solvent is reduced by adding a PHA anti-solvent resulting in precipitation of highly pure PHA. Research studies on this extraction method have found low to non-existent solubility of scl-PHAs in most anti-solvent or non-halogenated solvents such as, acetone under conditions of industrial scale extraction (Kunasundari & Sudesh, 2011; Riedel et al., 2012). Since this is a time-consuming process, it can take up to more than 12 hours in some cases. Moreover, extraction solvents at a 20-fold quantity of the cell biomass being treated are required, as well as an additional 10 L of the PHA anti-solvent per liter of PHA solution (Kunasundari & Sudesh, 2011).

Variations to this methodology of "classical" chloroform extraction have been mentioned in literature. For instance, lyophilization, also known as freeze-drying, of the cell biomass targeted for extraction has been reported (Kunasundari & Sudesh, 2011). The freeze-dried cell biomass is treated with a chloroform and methylene chloride solvent. This treatment allows for dissolution of cell lipids such as, the phospholipids making up the cell membrane. By adding chloroform to an excess of methanol, the PHAs are precipitated out from the solution while other cell lipids are dissolved in the chloroform/methanol solution and left to evaporate. The dried and purified PHA is re-dissolved in chloroform and placed on a flat thin glass dish with a loose cover to allow the chloroform to evaporate gradually. This method is best suited for producing thin PHA films (Kunasundari & Sudesh, 2011).

1.5.2 Chloroform Extraction under Reflux

Similar to the previously discussed method of "classical" chloroform extraction, chloroform extraction under reflux has been another technique carried out in lab settings. The main advantage of this process is the shorter period of time required for extraction. Literature reports have indicated this method to provide improved yields when compared to the simple "classical" solvent extraction methods particularly for mcl-PHAs (Jiang, Ramsay, & Ramsay, 2006). Low-boiling point chlorinated partial solvents such as, dichloromethane and chloroform have been shown to isolate high quality PHA in addition to improved yields with decomposition over long periods of extraction (Jiang et al., 2006).

To carry forth this extraction method, a pre-treatment procedure is required. Oven-dried cell biomass is treated with short-chain alcohols to disrupt the cell walls and dissolve impurities without affecting the PHAs. Thus, acetone and other short chain alcohols such as, methanol, ethanol, and n-propanol, are used since they hold favorable cell lytic properties. Furthermore, bursts of agitation or a round of centrifugation is performed which accelerates the pre-treatment step and significantly reduces any possible chance of polymer degradation, thereby increasing isolation yields for extraction (Jiang et al., 2006).

Precipitate from pre-treatment is then transferred to a cellulose extraction thimble and inserted in the Soxhlet extractor containing chloroform as the extracting solvent. After approximately 5 hours, the solvent is evaporated under a vacuum leaving behind a residue. This residue is then subjected to 2 mL of cold methanol for re-precipitation to obtain a dull white solid, which is then filtered and dried into a thin film (Jiang et al., 2006).

1.5.3 Anti-solvent Extraction under High Pressure and Temperature

This method has been recently developed and is currently in the test phase. It is based on, and in fact enhances, the well-known and established anti-solvent extraction method for PHAs. This method is best applied for scl-PHA extraction. Anti-solvents are described as a broad range of volatile liquids, mainly low molecular weight ketones and alcohols. It involves extraction of scl-PHAs using acetone under elevated temperature and pressure.

A recently published study has compared this novel method to an existing and established approach, which utilizes chloroform at ambient pressure within a closed system combining

components for extraction, filtration, and product work-up (Koller et al., 2013). This study reported similar product purity levels following performance of both methods; 98.4 % purity was reported by employing anti-solvent extraction at high temperature and pressure and 97.7 % following the original chloroform method at ambient temperature. Additionally, the novel method posed to be considerably faster than the established approach; while the original chloroform method spent 12 hours, the new strategy completed the task in 20 minutes (Koller et al., 2013).

The separation in the aforementioned new method was achieved by cooling the acetone solution of scl-PHA. This allowed for a nearly quantitative recovery of the solvent, which was conveniently reused for carrying out additional extractions (Koller et al., 2013). Moreover, the study used 21 g of lyophilized and powdered biomass. A three unit setup was developed for the experimental setup, which included a unit for extraction, filtration, and precipitation (Koller et al., 2013). No significant differences in terms of molar mass and thermo analytical parameters were reported when the extracted scl-PHA was characterized.

However, the main drawback of this method involved ensuring complete removal of oxygen from the system as acetone and oxygen can combine to become an explosive mixture (Koller et al., 2013). Otherwise, overall productivity achieved with this method of extraction was reported as favourable as previous anti-solvent extraction methods.

1.5.4 Ultrasonication and Enzymatic Digestion

The use of mechanical disruption in combination with solvent extraction has been reported in various studies as a means of PHA extraction (Penloglou et al., 2011). Yield purities of approximately 95 % has reported to been achieved (Gumel et al., 2013). After disruption of the cell biomass by ultrasonication, the varying efficacy of chemicals and detergents was evaluated for its influence on PHA recovery (Yang, Brigham, Willis, Rha & Sinskey, 2011). The effectiveness of a particular detergent is most presumably dependent upon the microbial species, but further research into this aspect is vital for any decisive conclusions. Detergents such as, sodium dodecylsulfate have been used to rupture cell membranes for recovering granules of the crude PHA polymers, which has allowed for 95 % recovery of the intracellular PHA (Yang et al., 2011). Improvements in purity of the PHA were observed to occur with increased detergent treatment.

On the other hand, a more specific and targeted approach to extracting PHA within cell biomass was investigated which included enzymatic digestion of cells (Yasotha et al., 2006; Kathiraser et al., 2007). This approach led to a purity of nearly 93 %. By combining enzymatic digestion with chemical treatment, approximately 90 % of the PHA was extracted with a purity level around 93 % (Kathiraser et al., 2007). This method requires further investigation for any conclusive results. This method may not be considered as attractive when compared to solvent extraction which provides approximately 96 % purity. Furthermore, enzymes may prove not to be cost-effective for use in large-scale extraction processes. In fact, the possibility of digesting the PHA polymer itself has deemed the method ineffective. Thus, evaluating the type of enzyme required for the specific microorganism and the optimal conditions for the reaction would be necessary to ensure effective results (Kathiraser et al., 2007).

In an alternate case, enzyme and subsequent mechanical cell disruption treatments for recovering PHAs has been studied. Since bacteria contain a significant proportion of their biomass as DNA, a jelly-like polymer, disruption of the cells can release the DNA into the cell homogenate leading to a viscous mass making it difficult to process (Yasotha et. al., 2006). Thus, the use of enzymes in this procedure can ensure proper removal of the non-PHA biomass while leaving behind the target PHA.

1.6 Applications

As previously described in Sections 1.2 and 1.4, PHAs hold thermoplastic properties quite comparable to petroleum derived plastics (or polyolefins) and are becoming an attractive solution to the global issue concerning the use of petrochemical plastics. Current and progressive research into the chemical and physical properties of PHAs has confirmed their use in various other applications and industries, which can range from very basic to highly specialized such as, paper and packaging to a variety of medical devices. Table 1.1 provides a comparative look between some of the important mechanical and thermal properties of both biopolymers and conventional polymers (Sudesh et al., 2000; Bax & Mussig, 2008; Luo et al., 2009; Bengtsson et al., 2010).

Table 1.1. Mechanical and thermal properties of various biopolymers and conventional polymers

Polymer	Characteristic	Melting Temperature (ºC)	Tensile Strength (MPa)	Elongation to Break (%)
Scl-PHAs	Brittle and stiff	40 - 180	43	5
Mcl-PHAs	Elastomeric and flexible	45 - 55	1 - 4	445
Scl-co-mcl-PHA	Highly variable based upon monomer composition of scl- and mcl-PHAs	80 - 100	20 - 104	250 - 450
Low-Density Polypropylene	Elastomeric and flexible	110	30	500
High-Density Polypropylene	Flexible and tough	125	35	150
Polystyrene	Brittle and hard	240	10	3 - 4

Based upon Table 1.1, it is apparent that biopolymers are highly comparable to petrochemical polymers and can be employed in an array of applications. Furthermore, biopolymers are fully biocompatible and biodegradable which makes them highly favourable over conventional polymers. However, further research into the production and processing for biopolymers is necessary for manufacturing at a commercial scale level.

1.6.1 Textiles, Resins, and Polymers

The main sector primarily associated with applications of PHAs involves the packaging industry. PHAs have reportedly been used in articles such as, shampoo bottles, shopping bags, containers, and paper coatings in addition to various disposable items including razors, utensils, and diapers, just to name a few (Chanprateep, 2010). In fact, corporations such as, Proctor and Gamble have integrated PHAs in packaging of their products to limit dependence on conventional plastics, and in the process set a trend for other leading manufacturers to adopt (Chanprateep, 2010). As industrial interest in biopolymers grows, many advanced PHA polymers have been developed. These include homopolymers, copolymers, and block copolymers (Chanprateep, 2010).

According to literature, PHBs (or scl-PHAs) were the first exclusive homopolymers discovered. However, with progressive research into biopolymers, non-PHB (or mcl-PHA) homopolymers have recently emerged. Despite growing interest in this area, findings are limited, particularly related to their physical and polymer characteristics (Chanprateep, 2010).

PHA homopolymers ranging from four to ten carbon atoms in length have been produced (Chanprateep, 2010). Recently, laboratories have succeeded in exclusively producing poly[(*R*)-3-hydroxyundecanoate] and poly[(*R*)-3-hydroxydecanoate] mcl-PHA homopolymers with reports on development of additional mcl-PHA homopolymers for the near future. Among the aforementioned mcl-PHA homopolymers poly[(*R*)-3-hydroxyundecanoate] has exhibited the ability to form a unique crystal and lamellar structure based upon crystallography studies, proving a suitable component for packaging materials (Chanprateep, 2010).

Previously, PHB fibers with high tensile strength were the preferable form of PHAs in packaging applications. To obtain such strength, an increased time for isothermal crystallization of PHB fibers was essential. This resulted in a shift of the molecular orientation to obtain the targeted favourable properties (Garcia et al., 2013). However, with possibilities of producing

various copolymers comprised of scl- and mcl-PHAs, this appears to be the most viable method for wider commercial scale applications of PHAs. For instance, PHBV (poly-3-hydroxybutyrate-co-3-hydroxyvalerate) has now been commonly used in an assortment of packaging materials and holds enhanced physical properties when compared to conventional plastics (Garcia et al., 2013).

Scl-PHA copolymers consisting of C_3 to C_5 chain lengths have been synthesized some of which include, poly[(*R*)-3-hydroxypropionate-*co*-(*R*)-3-hydroxybutyrate], poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate], poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate-*co*-4-hydroxybutyrate]. However, with the discovery of *Pseudomonas* spp.'s ability to accumulate mcl-PHAs, copolymers comprising of C_6 to C_{12} monomers have also been developed (Poblete-Castro et al., 2013). Typical mcl-PHA copolymers include; poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxydecanoate] and poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyl-octanoate-*co*-(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate] as well as a new copolymer poly[(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate)].

Furthermore mixed copolymers of scl- and mcl-PHAs have been reported in literature as well and possess flexible mechanical properties (Shah et al., 2008; Castilho et al., 2009). A successful model includes PHBHHx as previously discussed in Section 1.4.1. It has been produced on an industrial scale by US based Procter & Gamble under the trademarked name of Nodax (Castilho et al., 2009). It consists of scl- and mcl-PHA copolymers comprised of a C_4 carbon chain and a C_6 to C_{12} carbon chain, respectively.

According to literature, new developments in PHA polymers have produced block copolymers. For instance, the capacity to synthesize PHA-containing block copolymers within *Cupriavidus necator* (also known as *Ralstonia eutropha*) with periodic substrate addition has been developed (Ienczak et al., 2013). PHB segments were formed during fructose utilization, but with concomitant pulse feeds of pentanoic acid, the synthesis of (*R*)-3-hydroxyvalerate (3HV) monomers occurred. This resulted in the formation of random PHBV copolymers.

Polymer tests performed on block copolymers demonstrated retention of elasticity over time with respect to films of similar random copolymers with a comparable composition (Gumel et al., 2013). Two PHBV films that contained either 8 % or 29 % 3HV exhibited a quick transition to brittle behaviour, while decreasing to less than 20 % elongation at fracture within a few days after annealing. Conversely, similar block copolymer samples had greater than 100 % elongation

at fracture a full 3 months after annealing. This was attributed to the fact that block copolymers covalently linked polymers that would otherwise form thermodynamically separate phases. The rate and degree of crystallization of the block copolymers was reported to be less than those of random copolymer samples. These differences translated into materials that extended the physical properties of biologically synthesized scl-PHA.

Other recent developments with PHA copolymers include combinations of bio-based plastics such as, starch-based plastics, protein (soy) based plastics, and cellulose-blended plastics (Lee & Na, 2013). Presently, PHAs have been blended with petroleum-derived plastics such as, polyethylene, polypropylene, and poly-vinyl alcohol. Since such polymers are only semi-biodegradable, their production has been limited due to the added costs of their production (Lee & Na, 2013). Thus, additional research into a fully biocompatible and biodegradable alternative is necessary.

1.6.2 Renewable Energy

With growing concerns over the depletion of current fossil fuel reserves, considerations of other alternative and economical fuel sources has become inevitable. As a result, PHAs have been examined for potential applications as biofuels (Zhang et al., 2009). Some of the potential chemical schemes that could possibly be carried out in the production of biofuels from PHAs have been discussed. For instance, both scl- and mcl-PHAs could undergo esterification with methanol to become hydroxyalkanoate methyl esters (3HBME and 3HAME), which demonstrated heat of combustion comparable to ethanol (Gao et al., 2011).

3HBME and 3HAME had heat of combustions valuing at 20 kJ/g and 30 kJ/g, respectively, while ethanol had a heat of combustion at 27 kJ/g (Zhong et al., 2009). With such comparable combustion properties, it is apparent that PHAs are suitable candidates for use as fuel additives at 10 % to 30 % ratios in gasoline and diesel. Furthermore, it was reported that 3HBME held similar, if not improved, properties as a fuel additive when compared to ethanol based upon oxygen content, dynamic viscosity, flash point, and boiling point (Zhang et al., 2009).

Similarly, another biofuel source, butanol, was reportedly produced from glucose in combination with an adapted PHB synthesis pathway. Thus, the application of PHAs as a source for biofuel production is quite promising especially since purified forms of PHA are not required. Furthermore, future increase in gasoline costs could eventually propel competition from PHA-

based biofuels for the future. Currently, the rate of gasoline is approximately $800 per ton; however, as existing oil reserves deplete, costs are projected to increase (Zhong et al., 2009). Thus, despite the higher production costs of PHA-based biofuels, which is estimated to be $1200 per ton, PHA-based biofuels may provide a more sustainable and viable prospect.

1.6.3 Specialized Medical Applications

Studies published in the last decade have reported PHAs to be valuable material aiding in highly specialized areas such as, tissue engineering as well as other biomedical devices (Kose at al., 2003, Chen & Wu, 2005; Wang et al., 2005). Scl-PHAs such as, PHB, PHBV, and P4HB have exhibited promising results (Williams & Martin, 2003; Sevastianov et al., 2003). Observations reported successful development of PHA incorporated devices, some of which include sutures, repair devices and patches, slings, orthopedic pins, nerve guides, and vein valves. Additionally, scl-PHAs had also been integrated in the development of matrices for *in vitro* cell proliferation. PHB and PHBV components in contact with blood did not activate the hemostasis system (or platelet adhesion/formation) at the cellular response level, which would normally be triggered by any form of unrecognized cellular factor (Cheng et al., 2003; Sevastianov et al., 2003). This was attributed to the inert properties of PHAs as well as the blending of polyethylene glycol (PEG) with the PHA.

Similarly, *in vitro* and *in vivo* studies carried out with P4HB and PHO demonstrated results comparable to conventional materials used in cardiovascular surgery, wound healing, and drug delivery (Martin & Williams, 2003; Shishatskaya & Volva, 2004). Although, PHA produced from gram negative bacteria are known to contain pyrogens in the form of lipopolysaccharides, which is an endotoxin formed during the process of PHA production, treatment with an oxidizing agent such as hydrogen peroxide or benzoyl peroxide could eliminate such effects; thus, ensuring prevention of any unforeseen reactions that may occur within the body (Williams et al., 2003).

For purposes of drug delivery, scl-PHAs were designed with controlled degradation rates by chemically modifying their backbones (Martin et al., 2003). The capability for alterations in the PHA compositions allowed favourable mechanical properties, biocompatibility as well as degradation times under physiological conditions, thereby promoting application of PHAs for tissue engineering and implants. Some implant structures that had been developed with scl-PHA included bone marrow scaffolds, skin substitutes, and outer ears (or pinnae). Experiments carried

out on dogs for the implantation of an artificial esophagus using PHBHHx as the material had successfully stimulated the regeneration of the removed esophagus holding good elasticity and strength (Chen & Wang, 2013).

Bone tissue engineering experiments performed on animals proved scl-PHA (PHB) materials to produce a consistent and favourable bone tissue adaptation response with no evidence of any undesirable chronic inflammatory response after implantation periods of up to 12 months. In fact, bone formation had been reported to occur rapidly within the vicinity of the PHA implant as well as subsequent establishment and organization of a new bone structure. Also, incorporation of particulate hydroxyapatite, an essential component found in bones, initiated a bioactive and biodegradable composite effect for hard tissue replacement and regeneration (Doyle et al., 1991; Chen & Wang, 2002; Ni & Wang, 2002).

Recently, another study reported applications of PHB copolymers in tendon repairs performed on animal models consisting of rats (Webb et al., 2013). Results obtained in the study exhibited comparable mechanical properties particularly in load bearing tests. PHB constructed rat tendon scaffolds demonstrated higher maximal load bearing when compared to rats with undamaged tendons at 23.73 ± 1.08 N versus 17.35 ± 1.76 N, respectively. Furthermore, a shorter recovery period was observed post-surgery in rats implanted with PHBHHx scaffolds at 20 days with no observable secondary immune response observed over a 40 day period.

Another review compiled in 2011 and published in the Journal of Materials Science discussed promising new PHA based biomedical materials for the future. Majority of the applications discussed in this review were similar as previously described by Chen and Wu; however, applications of mcl-PHAs was specifically the major focus in this review. Mcl-PHAs were described to exhibit a wider range of physical and mechanical properties when compared to their scl-PHA counterparts. This was based upon the diversity of their chemical composition and structures (Rai et al., 2011).

Two major forms of mcl-PHAs were discussed comprehensively in the review, which included the homopolymer poly (3-hydroxyoctanoate) (P(3HO)) and copolymer poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HHx)). They were produced from various strains of fluorescent *Pseudomonads* under unbalanced growth conditions and held full biocompatible, biodegradable, and thermoprocessable properties (Rai et al., 2011). However, other primary mcl-PHA producing microorganisms were mentioned as well. They included *Alcaligenes*

latus, *Cuprivadus necator*, and *Aeromonas hydrophila*. An interesting fact regarding *Alcaligenes latus* was reported. It involved no nutritional constraints for accumulation of PHAs as required in other PHA producing bacteria (Rai et al., 2011).

Rai et al. described some of the major polymer properties tests performed on mcl-PHA as presented throughout literature. Reports of low crystallinity, low glass transition temperature, low tensile strength, and high elongation before breakage were discussed. Thus, supporting mcl-PHAs as a suitable candidate for a wide array of applications, particularly biomedical, especially where flexible material is most essential (Rai et al., 2011).

Furthermore, mcl-PHAs demonstrated comparable piezoelectric properties similar to current biomedical materials to aid in the stimulation of bone growth and wound healing. Other biomedical applications of mcl-PHAs were described as well, such as, artificial heart valves and other cardiovascular applications in addition to matrices for controlled drug delivery (Murueva et al., 2013). For instance, investigation into *in vitro* drug release behaviour from microparticles composed of degradable PHAs with varying chemical compositions was conducted. A high degree of biocompatibility was reported given the absence of cell cytotoxicity. Furthermore, cell attachment and proliferation was similar to those achieved with polystyrene based drug devices. Cytotoxic drugs encapsulated within copolymer P3HB/3HV microparticles proved to be effective against HeLa tumor cells (Murueva et al., 2013).

To study artificial heart valves, fabricated composites were developed by combining single walled carbon nanotubes with copolymers of mcl-PHA, such as, P(3HO) (Rai et al., 2011). An mcl-PHA copolymer, P(3HB-co-3HHx), was combined with hydroxyapatite for the development of osteosynthetic components and matrices for the purposes of drug delivery (Rai et al., 2011). In another study, carbon nanotube based biopolymer composites demonstrated enhanced mechanical and electrical conductivity for the improved function of osteoblast cell proliferation offering novel approaches for stimulation of bone reformation and repair (Misra et al, 2010).

However, additional research for the continued progress and development of PHAs in biomedical applications is crucial to enhance the abovementioned promising outlook for the future.

1.7 Economics and Market Relevance

Despite the need for additional research required to more broadly scale-up production of PHAs, a small number of global companies have developed their own strategies and technologies

to produce PHAs. The PHA manufactured, although quite limited in quantity, is produced based upon demand from other industries. This is due to a significantly high unit price for overall production and manufacturing of PHAs. In some instances, it is more than ten times higher when compared to their petrochemical counterparts (Castilho et al., 2009).

The industrial production of PHA is originally dated to the 1970s, which predominantly began with scl-PHAs (Zhu et al., 2013). In 1982, UK based Imperial Chemical Industries (ICI), now known as AstroZeneca, produced PHAs in the form of the scl-homopolymer P3HB, but due to high production costs and unfavourable mechanical properties related to high crystallinity, it resulted in limited production given the restricted range of applications (Reddy et al., 2003). But, in the late 1980s an enhanced and improved product, in contrast to the previous scl homopolymer P3HB, was manufactured by AstroZeneca. A copolymer P(3HB-co-3HV) by the name Biopol was mass produced on a commercial level. It possessed enhanced and vastly improved properties related to toughness and flexibility. However, in 2001 Biopol was acquired by Metabolix, a U.S. company based in Cambridge, Massachusetts that currently produces a range of PHA copolymers that are now sold under the name Mirel (Castilho et al., 2009).

Other companies currently producing PHAs include U.S. based Proctor and Gamble, which are known for the introduction of novel PHA copolymers comprised of scl- and mcl-PHAs; for instance, the scl-PHA 3HB in combination with 3HHx (C_6), 3HO (C_8), or 3HD (C_{10}) as the mcl-PHA component (Castilho et al., 2009). Such products are sold under the trademark name Nodax. Presently, the Nodax family of polymers is being produced by another American manufacturer, Meridian Inc., located in Bainbridge, Georgia (Castilho et al., 2009).

Interestingly, since the early 1980s BASF, a company based out of Germany, produced scl-PHAs in the form of P(3HB) and a copolymer of scl- and mcl-PHAs, which included P(3HB-co-3HV) similar to the recent Nodax, but also blended these PHAs with its biodegradable polymer Ecoflex (Shah et al., 2008).

Since expiration of the original patents for PHA copolymers held by Zeneca, other companies have pursued their efforts into commercial level scale-up of PHA production (Zhu et al., 2013). One such company is based in China, TianAn Biologic Materials Co., that has developed the technology and resources for high-scale production of scl- and mcl-PHA copolymers to approximately 2000 metric tons per year (Zhu et al., 2013).

With only a few global companies capable of PHA production at commercial levels, further progress into making the technology and resources readily available and cost-effective still remains elusive, but consistent progress seems highly possible, given the great strides currently made.

1.8 Advantages

As described throughout Chapter 1, it is apparent that PHAs hold both chemical and physical properties that are similar to their petrochemical derived counterparts. This establishes PHAs as a suitable alternative to conventional plastics. However, a significant and highly favourable difference between the two polymers exists, which is that PHAs are fully biodegradable once discarded and disposed in landfills.

Furthermore, numerous polymers of PHAs can be produced and utilized in various aspects ranging from residential, commercial to industrial applications similar to conventional plastics. Applications in highly specialized aspects such as, medical devices have been deemed possible as well. PHAs have been produced using environmentally friendly means which relieve a huge strain currently placed upon depleting fossil fuel reserves, and in the process, decrease the carbon foot-print of producing conventional plastics using petrochemical processing.

1.9 Challenges

Despite great advantages and promise exhibited by PHAs over conventional plastics, a major challenge still remains, which is the commercial scale production of PHAs. As previously described in section 1.7, PHA production is not widely commercialized at the moment. Only a few global companies are capable of producing PHAs; however, production has been driven based upon product demand and need, given the high costs involved in the production of PHAs.

Currently, the most highly preferred method for PHA production has been microbial fermentation. However, concerns related to high processing costs remain. More specifically, feedstock itself can account for 50 % to 60 % of the total operational costs for PHA production. Thus, to mitigate such high expenditures alternatives have been examined.

1.10 Sustainable Fermentation Strategies for Scale-up

Presently, microbial production of PHAs with the incorporation of industrial waste streams as feedstock has been evaluated. A variety of bacteria have been employed in fermentation strategies as described previously in Section 1.3.1. However, since the past few decades *Pseudomonas putida* has become popular and grabbed the attention of scientists and researchers given its ability to convert an assortment of substrates into the highly desired mcl-PHAs for bioplastics.

If literature were any indicator, a variety of strains grown on diverse substrates, pure and waste by-products, under different fermentation strategies have now burst on to the scene (Huijberts et al., 1992; Sanchez et al., 2003; Sun et al., 2007). The bacterium used in this thesis project is also a strain of *Pseudomonas putida* which will be described in the section ahead.

Since pure forms of feedstock can prove costly, the integration of industrial waste streams is considered enticing. Firstly, it aims to significantly reduce costs related to feedstock in the overall PHA production processing, and in some instances perhaps eliminate such costs entirely since an unusable waste stream can be recycled and fed into an alternate value-added bioprocess. Thus, industrial plants producing significant amounts of waste streams can invest in the development of a secondary bioprocess that can become an additional source of revenue.

Secondly, accumulation of industrial waste streams into landfills or costly treatment can be significantly off-set with recycling of the industrial waste stream into a value-added product preventing detrimental effects upon the environment.

Therefore, research efforts must be focused into the understanding of microbial production of PHAs as well as the conditions most optimal for maximal PHA production with incorporation of industrial waste streams as feedstock.

1.11 *Pseudomonas putida* and LS46

Pseudomonas putida is an aerobic, rod-shaped, flagellated, gram-negative bacterium prevalent in most soil and water habitats (Anzai et al., 2000; Ballerstedt et al., 2007)). It grows optimally at 25 ^{o}C to 30 ^{o}C. There are several strains of *P. putida* that have been discovered, however, the KT2440 strain remains the most thoroughly studied throughout literature, and has been fully sequenced, serving as a standard reference by which other strains of *P. putida* are

compared (Espinosa-Urgel et al., 2000; Nelson et al., 2002). *P. putida* KT2440 is a derivative of the soil isolate *P. putida* mt-2 and possesses the ability to degrade aromatic compounds (Nakazawa & Yokota, 1973; Williams & Murray, 1974). Furthermore, it is an ideal host for expanding the range of substrates that it can degrade and transform into value-added products by the recruitment of genes from other microorganisms (Jimenez et al., 2002).

P. putida is a highly robust bacterium given the harsh environmental conditions in which it is found with a diverse aerobic metabolism (Gomes et al., 2005; Ward et al., 2006; Poblete-Castro et al., 2012). It holds the ability to metabolize and breakdown aromatic or aliphatic hydrocarbons and other organic solvents such as, toluene and styrene oil, and thus engage in a variety of metabolic activities within the environment for carbon and nutrient cycling as well as degradation of biogenic and xenobiotic pollutants (Timmis, 2002). This has enabled researchers and scientists to employ *P. putida* in various capacities of bioremediation and environmental restoration efforts such as, oil spills, plant development, and soil regeneration (Molina et al., 2000).

Furthermore, *P. putida* has demonstrated applications in biotechnology processes given its ability to metabolize and convert a variety of industrial waste by-products into polyhydroxyalkanoates (PHAs), or bio-plastics (Puchalka et al., 2008; Verlinden et al., 2007: Davis et al., 2013). These industrial waste by-products include, waste free fatty acids, oils, sugars, just to name a few. Moreover, the conversion of such waste substrates into the more desirable mcl-PHAs has made *P. putida* the bacterium of choice for the development of a practical fermentation strategy at a commercial-scale.

A new strain of *Pseudomonas putida*, LS46, was isolated in the labs of University of Manitobafrom a sample of waste sewage sludge and hog barn wash obtained from a local wastewater treatment plant (Sharma et al., 2012). Overall, LS46 was characterized on a glucose based medium accumulating about 22 % cdw as PHA. It is closely related to other strains of *P. putida*, however, it is genetically distinct based upon nucleotide sequencing analysis. Overall, LS46 demonstrates the flexibility to metabolize a variety of waste sources into mcl-PHAs with 3-hydroxyoctanoates and 3-hydroxydecanoate as the predominant monomers.

A comparative analysis study on LS46 grown on pure chemical grade glycerol and biodiesel derived waste glycerol has exhibited comparable cell biomass production and mcl-PHA accumulation (Fu et al., 2014). In fact, the major difference between the two substrates was the growth rate of LS46. A faster growth rate was observed on waste glycerol mostly attributed to the

availability of waste free fatty acids within the waste stream albeit in a small proportion to the waste glycerol as described earlier in Section 1.3.1.

This research project also employed biodiesel derived waste glycerol as the choice of substrate to study mcl-PHA production by LS46. Similarly, other studies have reported the use of glycerol although with different strains of *Pseudomonas putida* such as, KT2440, in high nitrogen conditions, which exhibited significantly low amounts of PHA accumulation, below 2 % of cdw, when compared to other carbon substrates (Wang & Nomura, 2010). However, in another study, a comparative analysis within flask-batch for mcl-PHA accumulation by different strains of *P. putida* was conducted in minimal medium containing 30 g/L of raw (biodiesel) glycerol (Poblete-Castro et al., 2014).

These strains included, KT2440, KT2442, F1, and S12. Overall, KT2440 demonstrated the highest cell biomass production at 4.23 g/L and mcl-PHA accumulation near 35 % cdw, while S12 exhibited the lowest productivity for both cell biomass production and mcl-PHA accumulation at 3.20 g/L and 12.6 % cdw, respectively. All in all, a similar approach was implemented for the purposes of studying mcl-PHA synthesis by LS46 in this research project, which will be described in greater depth within the sections ahead.

1.12 Thesis Objectives

As discussed earlier in this chapter, production of bioplastics can be achieved via various methods. Some of these methods can be described as conventional, while others as novel. With the concept of sustainability in mind, this project employed the conventional process of microbial fermentation for production of the target mcl-PHAs.

Thus, the overall objective of this research was to develop a sustainable fermentation strategy for the microbial production of medium-chain length polyhydroxyalkanoates (Figure 1.1) using a novel strain of the gram-negative bacterium *Pseudomonas putida*, known as LS46 (Figure 1.2), by incorporating waste glycerol derived from a biodiesel production plant.

Figure 1.1. Solvent (chloroform) extracted mcl-PHA polymer from *Pseudomonas putida* LS46

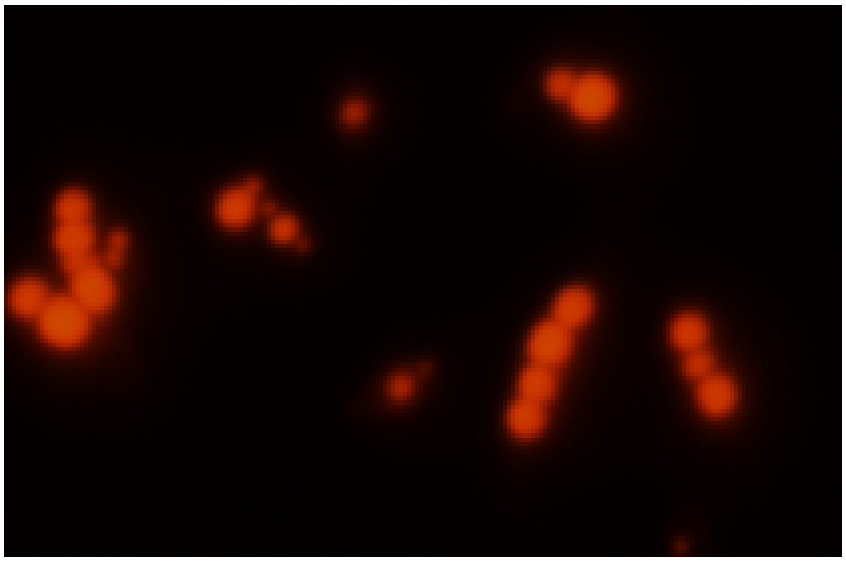

Figure 1.2. Fluorescent microscope view of Nile Red dye stained *Pseudomonas putida* LS46 containing intracellular accumulated mcl-PHAs

The waste glycerol used for this research project is a by-product in the manufacturing process of biodiesel production via transesterification of fatty acids (Figure 1.3).

Figure 1.3. Biodiesel derived waste glycerol (REG-80)

Approximately 100 kg of waste glycerol is produced per tonne of biodiesel that is manufactured (Zhang et al., 2003a). Presently, excess waste glycerol is digested within wastewater treatment plants, but since this process is slow and expensive with minimal economic value, alternate methods for the utilization of this waste by-product have been considered (Nopharatana et al., 2007), for instance, purification of waste glycerol for use in a range of chemical and synthetic compounds; however purification can become costly and the low price of pure glycerol makes the process undesirable (Zhang et al., 2003b).

Up until recently, European directives permitted blending of waste glycerol with fuel oil to utilize as fuel (Slinn et al., 2008). Though, this activity was terminated given the fear of pollution from unburnt combustion products.

In general, to reach the overall objective of scale-up this thesis project was involved in investigating;

- ✓ maximal yields for cell biomass production and mcl-PHA accumulation by LS46 within flask based conditions
- ✓ effects of media sterilization, i.e. autoclaved versus unautoclaved media on LS46 activity
- ✓ effects of carbon loading on LS46 activity
- ✓ effects of nitrogen enrichment on LS46 activity
- ✓ scale-up of the most optimal conditions observed within flask in a pilot-scale fermenter (bioreactor) to obtain higher yields of mcl-PHAs

Chapter 2 General Materials and Methods

2.1 Chemicals and Substrates

Chemicals used throughout the course of this thesis project were of reagent grade and obtained from Sigma-Aldrich and Thermo Fisher Scientific. These chemicals were used as components in the preparation of media for growing *Pseudomonas putida* LS46.

2.2 Medium Components

The source of medium used in this study was a minimal medium known as Ramsay (Ramsay et al., 1990). The following constituents comprised of Ramsay medium (RM) and were added based upon the ratio for a 1 L volume (1X) of medium preparation;

Table 2.1. Chemical components for 1 L volume (1X) Ramsay medium

Component	Amount
Sodium Phosphate (Na_2HPO_4)	6.70 g
Potassium Phosphate (KH_2PO_4)	1.50 g
Ammonium Sulfate $(NH_4)_2SO_4$	1.00 g
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.20g
Ammonium Ferric Citrate ($FeNH_4$)	0.060 g
Calcium Chloride ($CaCl_2$)	0.020 g
Trace Elements	1 mL

Each liter of trace elements solution contained 0.3 g H_3BO_3, 0.2 g $CoCl_2.6H_2O$, 0.1 g $ZnSO_4.7H_2O$, 30 mg $MnCl_2.4H_2O$, 30 mg $NaMoO_4.2H_2O$, 20 mg $NiCl_2.6H_2O$, and 10 mg $CuSO_4.5H_2O$. Prior to beginning any experimentation pH was measured using a pH probe and adjusted to 7.0 with 1 M HCl and 1 M NaOH. The carbon substrate added to the Ramsay medium consisted of waste glycerol, which was obtained from a commercial-scale biodiesel production plant (Renewable Energy Group (REG) in Danville, IL, USA). Based upon the product identification and certificate of analysis information provided by REG, the waste glycerol (REG-80) comprised of approximately 80 % glycerol with the remaining 20 % as various other components such as, ash, methanol, salts, trace metals, water as well as some free fatty acids.

For purposes of confirmation a sample of the REG-80 was analyzed in our lab using the WATERS HPLC system (Milford, MA, USA). Free fatty acids present in the REG-80 sample were analyzed using Agilent 7890A GC coupled with a split-splitless injector and a FID detector with a split ratio at 1:10. Separation of target chemicals was achieved using a DB-23 capillary column (30 m x 320 µm x 0.25 µm; Agilent, CA, USA). Helium was used as carrier gas at a flow rate of 1.78 mL/min. The oven ramping program was set with the initial temperature at 50 ^oC for 4 minutes and thereon from 50 ^oC to 250 ^oC with incremental increases of 15 ^oC per minute with a final hold at 250 ^oC for 10 minutes. Supelco 37 Component FAME Mix purchased from Sigma-Aldrich was used as an external standard for compositional confirmation and identification of fatty acid peaks observed in the REG waste glycerol sample.

2.3 Microorganism, Growth Conditions, and Inoculation Technique

A novel strain of the gram-negative bacterium *Pseudomonas putida* known as LS46 was used over the course of this thesis project. This strain was isolated in the labs of University of Manitoba from waste sewage sludge using a glucose based medium. Complete details on the LS46 strain isolation and characterization study can be found in the publication by Sharma, et al. (2012). Master cell banks of the LS46 strain were prepared and kept as glycerol stocks and stored in a -80 °C freezer. Working cell banks of the LS46 strain were maintained, which limited use of the master cell banks and prevented them from being potentially contaminated. LS46 was streaked on Luria-Bertani (LB) agar plates from a master cell bank and placed for incubation at 30 °C.

Thereafter, these plates were maintained periodically as the working cell bank. A single colony was obtained off of a LB plate and inoculated into a 500 mL shake-flask containing 100

mL of liquid Rich medium, or Luria-Bertani (LB) broth. The LB (Lennox) utilized throughout this project was pre-made as a powdered microbial growth medium and obtained from Sigma-Aldrich. It was prepared as a broth by mixing it in distilled water based upon a 20 g/L ratio. To prepare LB agar plates, a solidifying agent (Bacteriological agar for molecular biology) was added to the broth, which was also obtained from Sigma-Aldrich.

The LB broth flask was placed in a shaking incubator set at 30 °C and 150 rpm to grow overnight for approximately 12 hours. After overnight incubation, 1 mL of the Rich medium inoculum was pipette into another 500 mL shake-flask containing Ramsay medium and 10 g/L waste glycerol (WG) as a means of sub-culturing. A 1 % v/v, or 10 mL/L ratio by volume of inoculum was introduced within the experimental medium. This step was necessary to limit carry-over of Rich medium into the treatment medium used throughout the purposes of this research project. Furthermore, it ensured acclimation of the inoculum to the treatment medium prior to commencing experimentation. Similar to the Rich medium flask, the sub-culture flask was placed in a shaking incubator at 30 °C and 150 rpm.

2.4 Experimental Design

The experiments carried out as well as the methodologies involved have been described in the chapters ahead. Chapters 3, 4, and 5 are dedicated towards describing all major experiments carried out in their complete details. The protocols executed were experiment specific. However, treatment of samples for analysis and processing of data was similar throughout this thesis project, which has been described in this chapter in section 2.5.

In order to best carry out this project, a three-phase approach was established to allow for a gradual transition into the eventual third phase of pilot-scale production of mcl-PHAs. Small-scale tube and flask experiments were initially conducted, which were followed by larger lab-scale pilot runs involving the operation of a bioreactor (Figure 2.1).

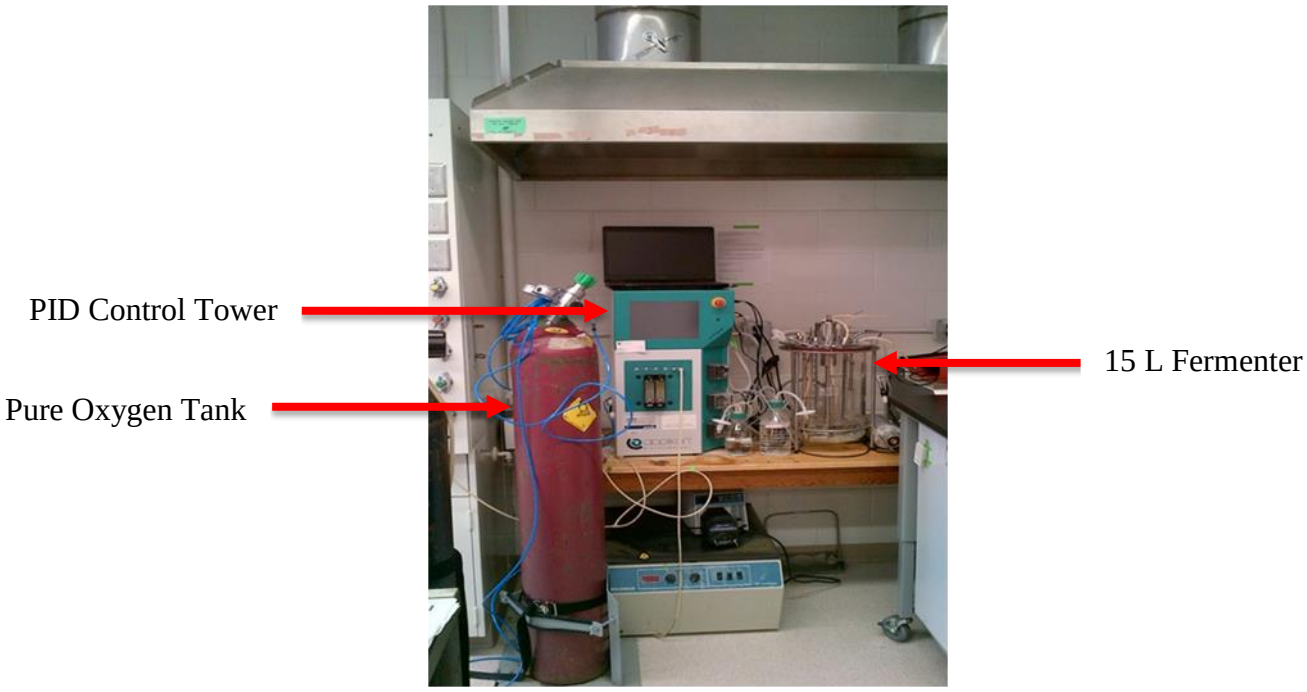

Figure 2.1. 15 L Applikon fermenter connected to a PID controller tower

This approach allowed for obtaining a basic understanding of the underlying behaviour involved in the microbial production of mcl-PHAs by LS46 with incorporation of industrial waste glycerol as a carbon source feedstock. Thus, the basic strategies observed from small-scale were implemented in the operation of a larger pilot-scale lab fermenter for increased production of the target mcl-PHAs.

2.5 Analysis

In each of the experiments conducted throughout this project, various information and data was monitored and collected for analysis. This comprised of cell growth, total cell dry-weight (or biomass), percent mcl-PHA accumulation, mcl-PHA monomer composition as well as measuring levels of residual glycerol and residual nitrogen within the media.

2.5.1 Cell Growth Monitoring

Cell growth of *P. putida* LS46 was monitored and measured based upon optical density (OD), or turbidity, at 600 nm using the Biomate3 Thermo Scientific Spectrophotometer (Madison WI, US).

2.5.2 Total Biomass, PHA Accumulation, and Monomer Composition

The methods employed for quantification and characterization of mcl-PHAs have been previously established (Sharma et al., 2012; Fu et al., 2014). Overall, culture samples were collected, centrifuged, and washed at fixed time points. A small amount of the supernatant was collected to measure residual levels of WG and nitrogen within the medium. The resulting wet pellet was placed to dry overnight and then treated via methylation for analysis on GC for total PHA accumulated as well as PHA monomer composition.

PHA analysis was conducted by samples (1 µL) being injected into the Agilent 7890A GC coupled with a split-splitless injector and a FID detector with a split ratio at 1:10. Separation of target chemicals was achieved using a DB-23 capillary column (30 m x 320 µm x 0.25 µm; Agilent, CA, USA). Helium was used as carrier gas at a flow rate of 1.78 mL/min. The oven ramping program was set with the initial temperature at 75 ^{o}C for four minutes, subsequently, incremental increases of 20 ^{o}C per minute from 75 ^{o}C to 250 ^{o}C, and a final hold at 250 ^{o}C for four minutes. A

fixed mcl-PHA standard containing known monomer compositions was used as an external standard for identification of the peaks detected within the samples by the GC.

2.5.3 Residual Glycerol and Residual Nitrogen Levels

Residual glycerol in media was measured using the WATERS Breeze^{TM} 2 HPLC system (Milford MA, US). Pure glycerol was used an external standard for quantification purposes. Residual Ammonia concentrations in the medium were measured using the Quikchem method 10-107-06-1-I, by flow injection analysis (Lachat Instrument, Colorado, USA).

Chapter 3 *Pseudomonas putida* LS46 Growth Curve

3.1 Background

Prior to conducting any major experiments, it was necessary to begin with a growth curve of the *P. putida* LS46 bacterium in the treatment medium based upon optical density (turbidity). This provided rough estimates of the four phases for cell growth, i.e. lag, growth, stationary, and decline.

Moreover, by observing rough approximations for the length of each phase, it allowed for obtaining a healthy and potent LS46 inoculum prior to beginning any future experimentation. This was vital in ensuring a live and robust culture medium.

3.2 Materials and Methods

Two varieties of media were prepared. The first was Rich medium (LB broth), while the other was Ramsay medium with 10 g/L WG. A single colony of LS46 was obtained from a LB agar plate and inoculated into 100 mL of Rich medium. This flask was then placed for incubation overnight for approximately 12 hours at 30 ^{o}C and 150 rpms.

After this initial period of incubation, 1 mL of the Rich medium inoculum was pipette into a flask containing 100 mL of the treatment medium as a means of sub-culture. Thereafter, another flask containing 100 mL of Ramsay medium with 10 g/L WG was inoculated with 1mL of sub-cultured inoculum. Optical density readings were obtained at 0, 6, 12, 24, 36, 48, and 72 hours. Optical density was measured on an Ultraspec 500 Spectrophotometer set at 600 nm.

3.3 Experimental Design

Overall, majority of the experimental design has been outlined in the previous section, Section 3.2. Readings were measured at the set time points mentioned previously beginning with time zero and plotted on a logarithmic graph. The treatment flask was placed back for incubation after each reading was obtained.

3.4 Results and Discussion

Optical density measurements obtained for LS46 as a means of monitoring cell growth within the treatment medium were plotted on a logarithmic graph as illustrated in Figure 3.1.

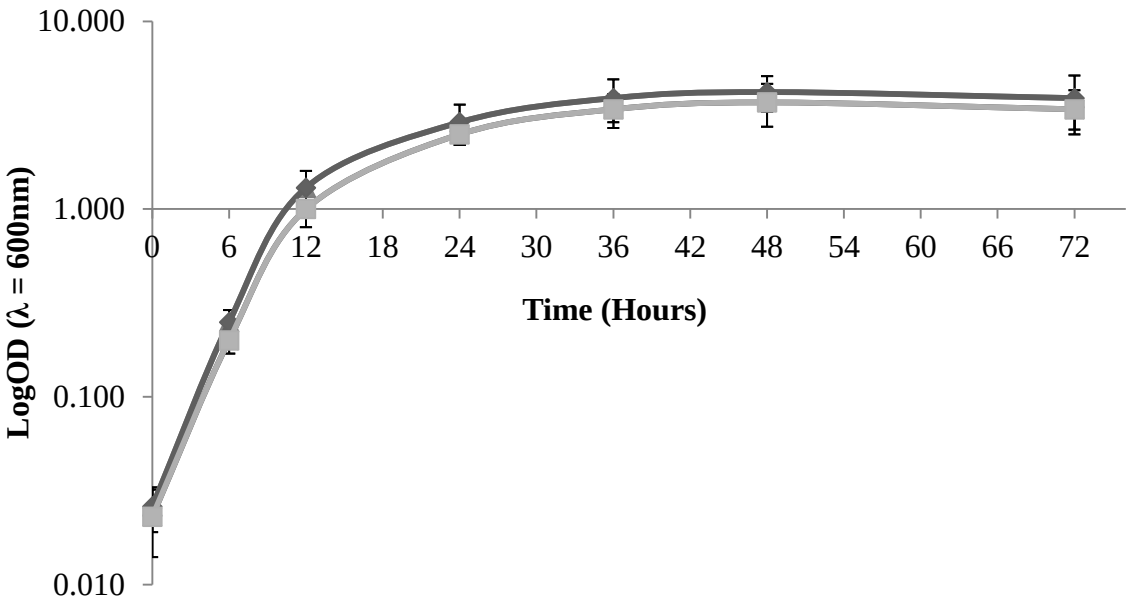

Figure 3.1. Optical density monitored cell growth curve for LS46 grown in 10g/L waste glycerol + 1X Ramsay medium

Doubling time was obtained based upon the following calculations for the exponential phase (Widdel, 2007);

$$\mu = \frac{2.303\ (log\ OD_2 - log\ OD_1)}{(t_2 - t_1)}$$

$$= \frac{2.303\ (log\ 1.300 - log\ 0.250)}{(12\text{hr} - 6\text{hr})}$$

$$= 0.275\text{hr}^{-1}$$

where; μ is the specific growth rate, OD is the measured optical density, and *t* is the specific time point (in minutes or hours) at which OD is measured.

$$t_d = {\ln 2}/{\mu}$$

$$= {\ln 2}/{0.2166\text{hr}^{-1}}$$

$$= 2.52\text{hr}$$

where; t_d is the doubling time and μ is the specific growth rate calculated based upon the previous formula.

Time points of 6 and 12 hours were chosen for calculating doubling time as they were within the desired exponential phase and since the cells are found actively dividing at that stage of cell growth.

To compare the above calculated growth rate and doubling time to estimates reported in literature, calculations for the KT2440 strain grown on pure glycerol have been discussed, which resulted in a lower growth rate and doubling time of 0.22 h^{-1} (Poblete-Castro et al., 2014) and 1.65 h (Wang & Nomura, 2010), respectively. However, such variances in rate can be attributed to the contents of the glycerol substrates, i.e. pure versus waste.

Chapter 4 Effects of Different Medium Conditions on *Pseudomonas putida* LS46

4.1 Background

As outlined earlier in Section 2.4, the overall investigation for maximal mcl-PHA yield by LS46 was a three-pronged approach. These next series of experiments conducted in flasks was the second phase of this project prior to transitioning into the third phase of scale-up. This approach allowed a close study and analysis of variables that affected both cell biomass production as well as intracellular mcl-PHA accumulation by LS46 via successive batch runs.

Medium parameters optimal for maximal LS46 biomass production and intracellular mcl-PHA accumulation were investigated by varying specific growth conditions. Therefore, parameters such as, media sterilization, carbon-loading, and nitrogen enrichment were studied in this phase of the research.

Since media sterilization can account for approximately 10 % of the overhead costs involved in microbial production of PHA, the potential for eliminating such a step was evaluated (Reddy & Mohan, 2012). Thus, media sterilization was tested in a comparative analysis study, i.e. sterilized (autoclaved) versus unsterilized (unautoclaved) media to investigate the possibility of LS46 overcoming contamination within unsterilized media.

Effects of carbon-loading were tested by growing LS46 in varying concentrations of WG. Through this investigation, the potential for imposing a selective pressure on the system was considered (Reddy & Mohan, 2012). Lastly, nitrogen enrichment effects were studied as means to increase overall cell biomass production and as a result higher PHA accumulation with an increased uptake of the available WG substrate. All in all, observations for the best suitable medium conditions promoting high yields of mcl-PHA accumulation by LS46 in flask were considered in the development of the third phase of this project.

4.2 Materials and Methods

A large batch of Ramsay media was prepared. 100 mL of the medium was poured into 500 mL shake flasks with waste glycerol added as the carbon source. The amount of the waste glycerol

used in each flask was experiment specific and has been outlined in the sections ahead. Furthermore, triplicates of each respective condition were setup as well as control flasks.

A 1:5 ratio of media volume to flask volume was employed to ensure optimal head space above the liquid media to allow for sufficient aeration within each of the flasks, which was achieved via shaking. A separate 500 mL shake flask containing 100 mL of Rich medium was prepared. The Rich medium flask was used to inoculate a single colony of LS46, which was obtained from a LB agar plate stored and maintained in a 4 ^{o}C freezer.

Additionally, another 500 mL shake flask was prepared and autoclaved, which contained medium being used in the treatment conditions. Specifically, this flask contained 100 mL of Ramsay medium with 10 g/L (1 % v/v) of waste glycerol as a sub-culture flask to passage the Rich medium grown LS46.

4.3 Results

These series of experiments have been sub-divided into separate sections to organize the overall investigation in terms of the aspect and condition investigated. Details and specifics regarding each experiment as well as all pertinent observations and results have been outlined in their respective section.

4.3.1 Cell Biomass Production and mcl-PHA Accumulation by *Pseudomonas putida* LS46

This preliminary experiment was conducted to observe the fundamental growth patterns and behaviour exhibited by LS46 within flask conditions. Moreover, conditions most suitable for maximum cell biomass production as well as peak mcl-PHA accumulation were investigated. Therefore, a basic shake flask setup was employed in this experimentation. All conditions within flask comprised of the same medium formulation; however, the only variable was the WG added.

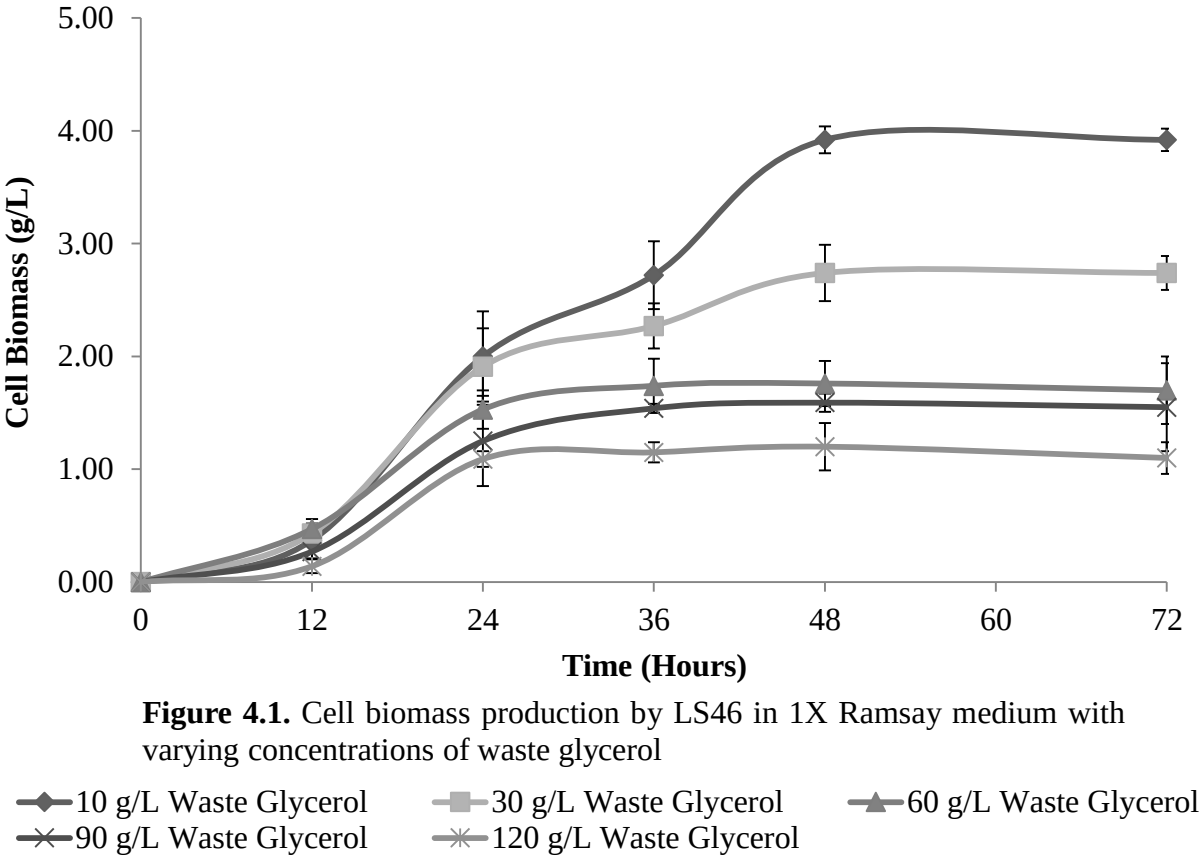

Figure 4.1. Cell biomass production by LS46 in 1X Ramsay medium with varying concentrations of waste glycerol

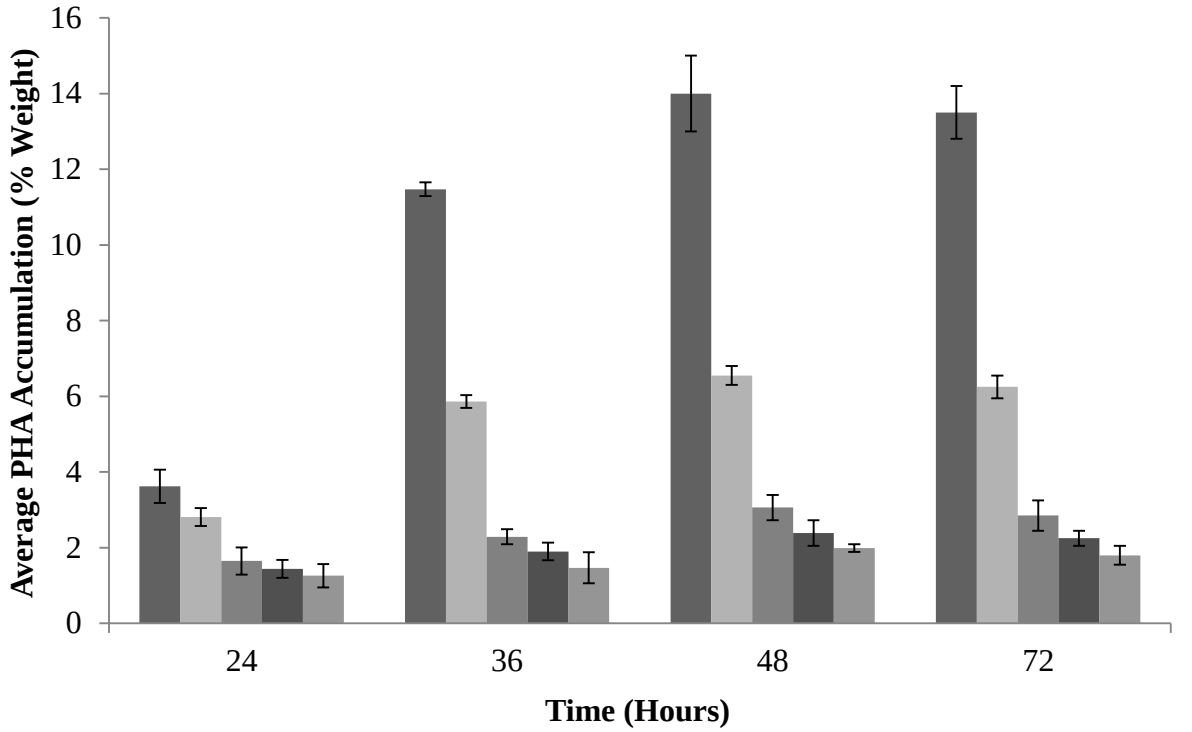

Figure 4.2. PHA accumulation by LS46 in 1X Ramsay medium with varying concentrations of waste glycerol

Based upon Figures 4.1 and 4.2, the 10 g/L WG conditions exhibited the highest cell biomass production as well as mcl-PHA accumulation at approximately 4.0 g/L and 15 % cdw, respectively. As concentration of WG increased there was a noticeable decrease in both cell biomass and mcl-PHA.

4.3.2 Media Sterilization Effects on Cell Biomass Production and mcl-PHA Accumulation

Considering the overall time and costs involved in the preparation of media for microbial PHA production, sterilization is a step that is necessary to ensure a contamination free environment within the medium for the bacterium of choice to predominate over others.

Sterilization is a significant contributor to the overall costs involved with PHA production, which can increase the cost of PHAs by four to nine times higher when compared to conventional plastics (Reddy & Mohan, 2012). Thus, these next series of experiments were conducted as a means to investigate the possibility of eliminating such a step, and thereby considerably decreasing the time and costs invested in the process.

To accomplish this task, a comparative flask experiment was carried out. Two groups comprised of triplicates were setup, one contained sterilized (autoclaved) media while the other comprised of unsterilized (unautoclaved) media. Five concentrations of waste glycerol were employed, i.e. 10 g/L (1 % v/v), 30 g/L (3 % v/v), 60 g/L (6 % v/v), 90 g/L (9 % v/v), and 120 g/L (12 % v/v) of waste glycerol.

It was hypothesized that the use of higher waste glycerol concentrations could aid as a passive method of sterilization within unautoclaved medium. Thus, serving as a means of preferential treatment for LS46 and allowing it to predominate within the medium versus other bacteria. In this investigation, a 1 % v/v of sub-cultured inoculum in the exponential phase was utilized. Furthermore, to avoid any experimental variations all experiments conducted in this project employed the same starting inoculum volume. However, it can be anticipated that a larger inoculum such as, 5 % v/v or 10 % v/v would exhibit different results as a higher number of cells (increased cell density) would be available at the start to initiate microbial growth when compared to a smaller inoculum (Zhang et al., 2002; Koutsomanis & Sofos et al., 2005).

All necessary materials and steps outlined in Chapter 2 were employed to prepare flasks for this experiment. However, in the case of unsterilized media, the step for autoclaving was not employed. Figures 4.1 and 4.3 exhibit the differences in cell biomass production between sterilized and unsterilized media, respectively.

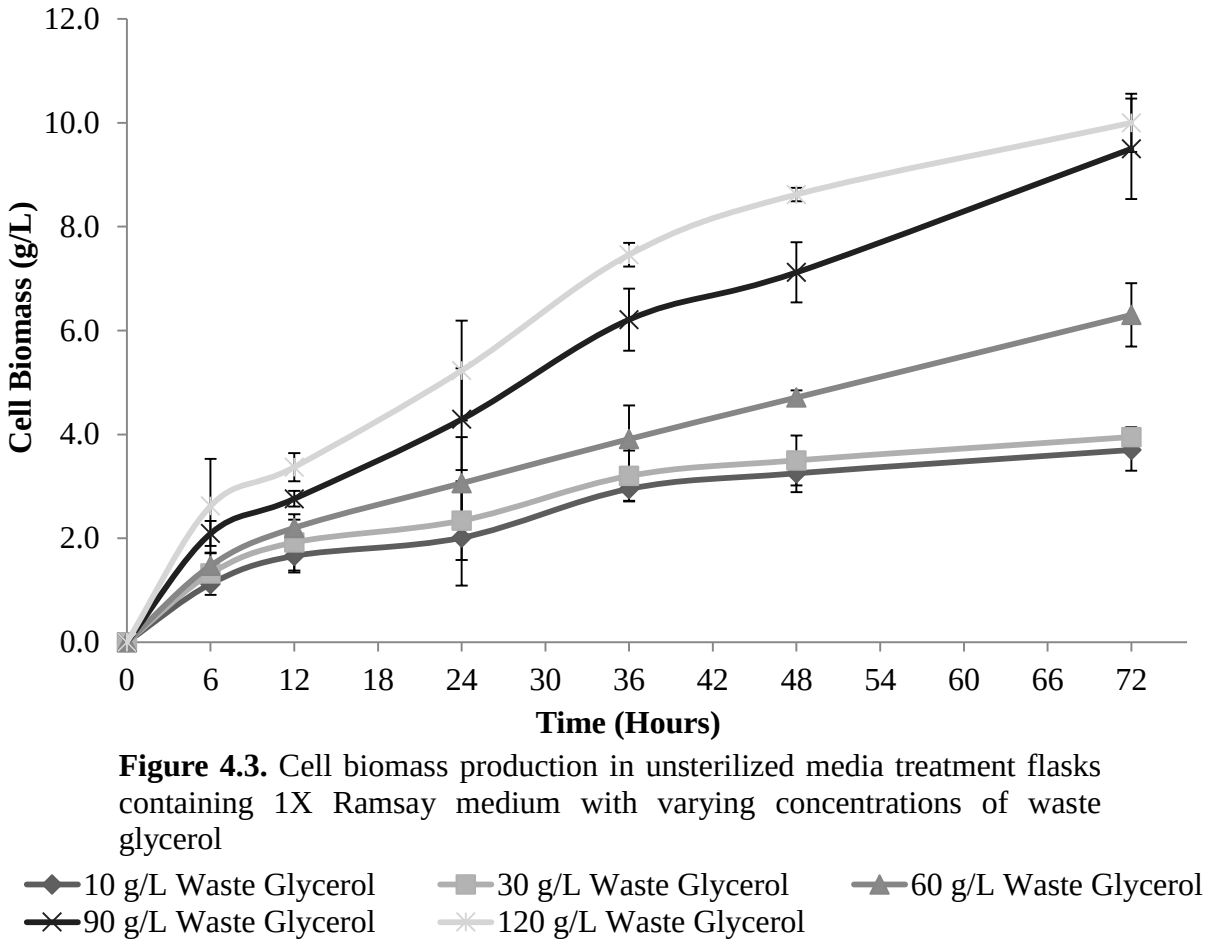

Figure 4.3. Cell biomass production in unsterilized media treatment flasks containing 1X Ramsay medium with varying concentrations of waste glycerol

Based upon this comparative analysis, it would appear that unsterilized media treatment conditions produced higher yields of cell biomass (Figure 4.3) compared to sterilized media treatment conditions (Figure 4.1). However, the presence of LS46 within both media was necessary to be confirmed.

Thus, to confirm that both media treatments contained LS46 as the sole microbe within the cell culture medium, PHA production was evaluated using GC analysis. Figures 4.5 and 4.6 exhibit the overall average PHA accumulated at different time points in sterilized and unsterilized media treatments, respectively.

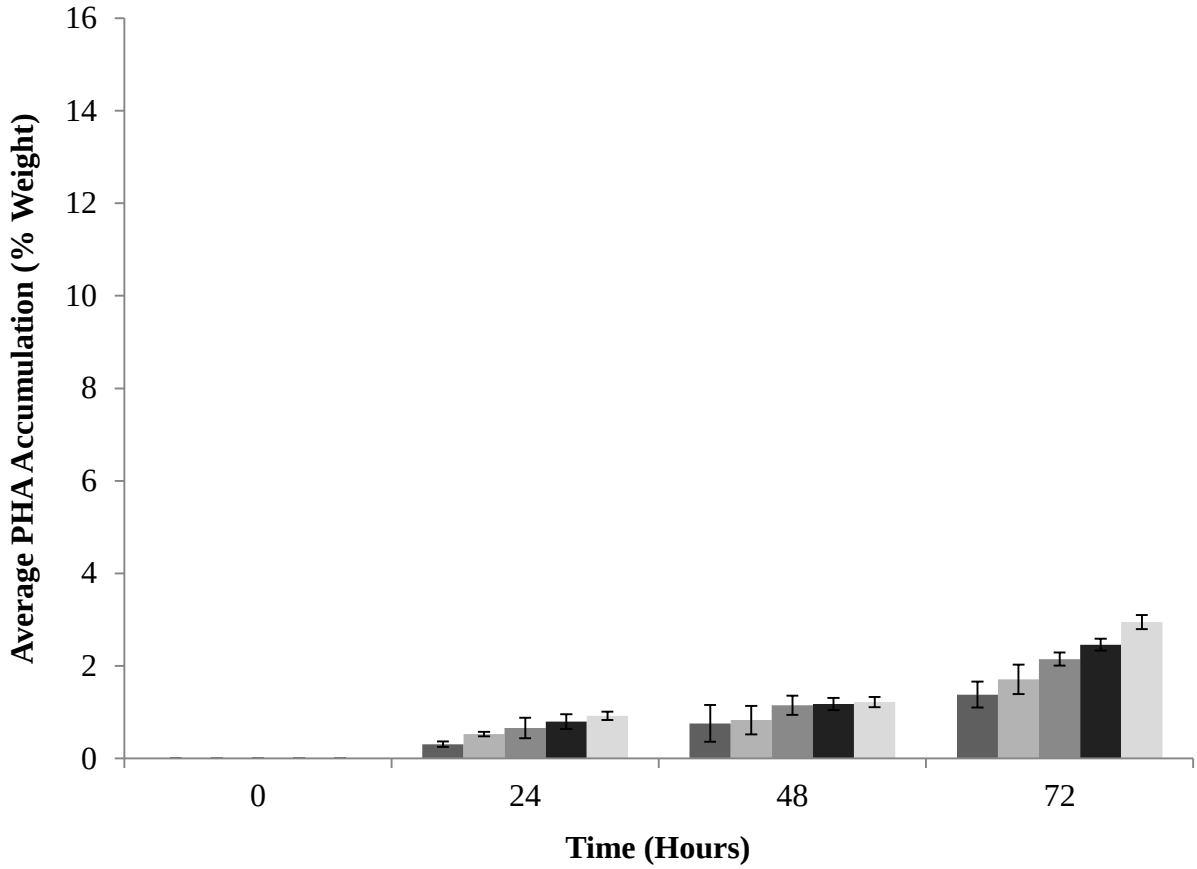

Figure 4.4. PHA accumulation in unsterilized medium treatment flasks containing 1X Ramsay medium with varying concentrations of waste glycerol

The differences in PHA accumulated were significant between sterilized and unsterilized media conditions. In Figure 4.2, the sterilized media conditions exhibited higher levels of PHA accumulation when compared to the unsterilized media conditions. This verified that the culture medium had LS46 predominate within it. Furthermore, the highest PHA levels were found in the 10 g/L of WG conditions within a range of 10 % to 15 % cdw.

The low PHA levels detected in the unsterilized conditions were consistent with the hypothesis stated earlier, and thus LS46 did not predominate within the media culture. In fact, LS46 was accompanied by unwanted contaminants, which appeared to outgrow and outcompete LS46 for resources, while in the process limiting its metabolic activity. This resulted in significantly higher levels of overall cellular biomass production; however, PHA accumulation was drastically low. This confirmed the need for media sterilization when growing LS46 on WG as the substrate.

4.3.3 Variable Carbon-Loading Effects on Cell Biomass Production and mcl-PHA Accumulation

Based upon Figures 4.1 and 4.2, it was evident that a maximum level of cell biomass and intracellular PHA accumulation was attained based upon the medium and growth conditions provided in flask-batch, i.e. sterilized medium containing 10 g/L WG + 1 g/L N. Thus, it was necessary to further test factors and variables to increase these yields beyond the observations achieved, which would inform eventual strategies for commercial level scale-up.

Therefore, studying the effects of increased concentrations of the carbon source feedstock, i.e. waste glycerol, the potential for LS46 to accumulate higher levels of PHA was evaluated in flask. Fifteen shake flasks were divided into five groups of three. Each group contained a specific concentration of WG mixed in Ramsay medium, i.e. 10 g/L (1 % v/v), 30 g/L (3 % v/v), 60 g/L (6 % v/v), 90 g/L (9 % v/v), and 120 g/L (12 % v/v). All Ramsay medium components were made available based upon the 1 L ratio as outlined in Section 2.2.

To commence experimentation, steps outlined in Chapter 2 were employed. Regular time point samples were collected and centrifuged over the 72 hour study. A small sample of the supernatant formed was also collected for eventual analysis of residual glycerol and residual nitrogen concentrations. Figure 4.1 exhibits the effects of higher carbon-loading on cell biomass

production, while Figure 4.2 illustrates the effects of higher carbon-loading on mcl-PHA accumulation.

Based upon Figure 4.1 an inverse relationship between the concentration of WG and amount of cell biomass produced was observed. In other words, lower WG concentrations exhibited highest cell biomass growth of LS46 when compared to the higher WG concentrations, which was counterintuitive to the hypothesis stated earlier. Thus, 10 g/L of waste glycerol exhibited approximately 4.0 g/L of cell biomass production, whereas 120 g/L of waste glycerol provided about 1.0 g/L of cell biomass.

Furthermore, PHA accumulation demonstrated a similar relationship with the increasing WG concentrations. According to Figure 4.2, PHA accumulation was highest in the 10 g/L WG conditions at approximately 15 % of cell dry weight, while less than 2 % of cell dry weight was accumulated as PHA in 120 g/L WG. Thus, a significant decline in intracellular PHA accumulation was observed as the concentration of WG was increased above 10 g/L. Cell biomass produced in each WG condition was treated via methylation and analyzed on an Agilent 7890A GC to confirm accumulation of mcl-PHAs. The monomer composition of the mcl-PHA accumulated has been provided in Table 4.1.

Table 4.1. Percent monomer composition of mcl-PHA accumulated by LS46 in varying concentrations of waste glycerol

Time	Substrate	Monomer Composition (%)					
		C_6	C_8	C_{10}	C_{12}	$C_{12:1}$	C_{14}
24	10 g/L WG	2.69 ± 0.07	24.45 ± 0.53	65.50 ± 0.41	2.42 ± 0.37	4.55 ± 0.32	0.39 ± 0.02
	30 g/L WG	2.98 ± 0.05	25.67 ± 0.63	63.90 ± 0.42	2.62 ± 0.07	4.32 ± 0.29	0.51 ± 0.03
	60 g/L WG	2.74 ± 0.03	23.04 ± 0.18	67.04 ± 1.01	2.57 ± 0.18	4.17 ± 0.22	0.44 ± 0.01
	90 g/L WG	2.26 ± 0.04	25.49 ± 1.83	64.86 ± 0.63	2.36 ± 0.25	4.28 ± 0.12	0.75 ± 0.03
	120 g/L WG	2.31 ± 0.09	28.77 ± 1.97	61.47 ± 1.14	2.45 ± 0.17	4.67 ± 0.42	0.33 ± 0.10
36	10 g/L WG	2.58 ± 0.06	26.85 ± 1.78	63.36 ± 1.27	2.22 ± 0.23	4.41 ± 0.30	0.58 ± 0.07
	30 g/L WG	2.30 ± 0.02	24.26 ± 1.96	66.11 ± 1.02	2.52 ± 0.12	4.24 ± 0.32	0.57 ± 0.13
	60 g/L WG	2.70 ± 0.03	28.50 ± 0.92	61.59 ± 0.68	2.62 ± 0.18	4.12 ± 0.24	0.47 ± 0.01
	90 g/L WG	2.91 ± 0.07	24.97 ± 1.39	64.75 ± 0.89	2.31 ± 0.28	4.25 ± 0.26	0.81 ± 0.09
	120 g/L WG	2.89 ± 0.09	25.89 ± 1.71	63.75 ± 1.71	2.55 ± 0.22	4.21 ± 0.31	0.71 ± 0.16
48	10 g/L WG	2.56 ± 0.06	27.01 ± 0.50	63.16 ± 0.49	2.21 ± 0.20	4.50 ± 0.27	0.56 ± 0.09
	30 g/L WG	2.37 ± 0.01	23.55 ± 0.20	66.70 ± 0.31	2.52 ± 0.11	4.36 ± 0.20	0.50 ± 0.13
	60 g/L WG	2.38 ± 0.08	26.79 ± 0.78	63.37 ± 1.59	2.61 ± 0.19	4.45 ± 0.26	0.40 ± 0.12
	90 g/L WG	2.71 ± 0.10	27.08 ± 1.63	62.20 ± 1.29	2.60 ± 0.28	4.49 ± 0.32	0.89 ± 0.09
	120 g/L WG	2.79 ± 0.12	24.74 ± 1.68	62.00 ± 1.38	2.40 ± 0.22	4.46 ± 0.28	0.61 ± 0.16
72	10 g/L WG	2.59 ± 0.04	25.06 ± 1.16	64.72 ± 1.05	2.50 ± 0.29	4.47 ± 0.25	0.66 ± 0.15
	30 g/L WG	2.29 ± 0.02	28.47 ± 0.48	62.14 ± 1.69	2.24 ± 0.14	4.37 ± 0.30	0.49 ± 0.10
	60 g/L WG	2.53 ± 0.09	24.90 ± 0.05	65.19 ± 0.34	2.32 ± 0.12	4.39 ± 0.23	0.67 ± 0.17
	90 g/L WG	2.46 ± 0.02	25.06 ± 1.16	64.79 ± 1.05	2.31 ± 0.13	4.40 ± 0.40	0.98 ± 0.16
	120 g/L WG	2.33 ± 0.03	26.05 ± 1.38	64.08 ± 1.10	2.40 ± 0.28	4.34 ± 0.38	0.80 ± 0.09

Based upon Table 4.1, specific ranges for the monomer composition of mcl-PHA accumulated by LS46 were observed within the varying WG conditions. Overall, a total of five different monomers were detected with both C_8 and C_{10} as the predominant monomers detected. Furthermore, an unsaturated C_{12} monomer was also detected by GC as well as some trace amounts of the C_{14} monomer. This pattern was exhibited throughout each of the waste glycerol conditions. As an in-depth analysis of the substrate consumption involved in the production of cell biomass and mcl-PHA accumulation, concentrations of residual nitrogen and glycerol levels in the supernatant of the centrifuged time point samples collected were measured, as illustrated in Figures 4.5 and 4.6, respectively.

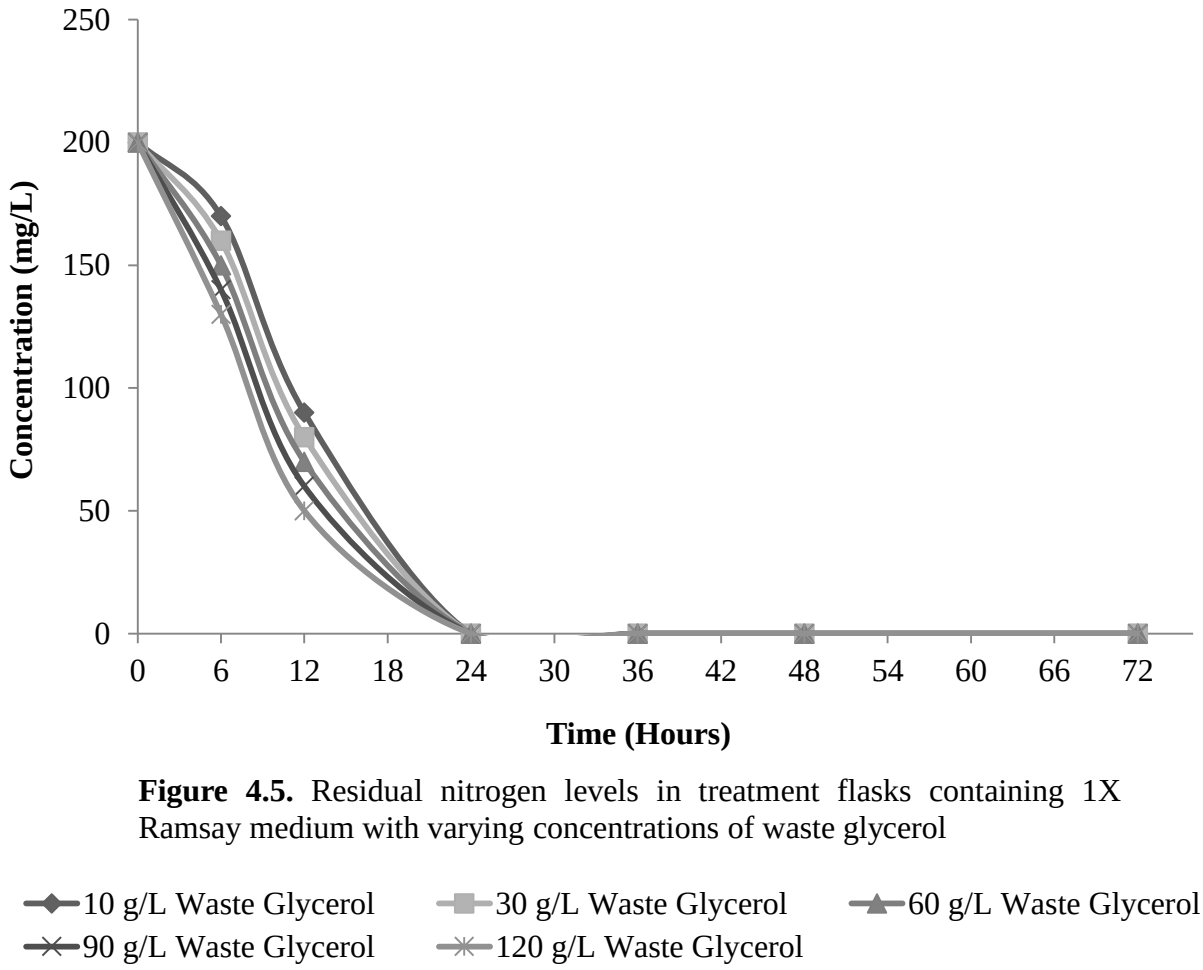

Figure 4.5. Residual nitrogen levels in treatment flasks containing 1X Ramsay medium with varying concentrations of waste glycerol

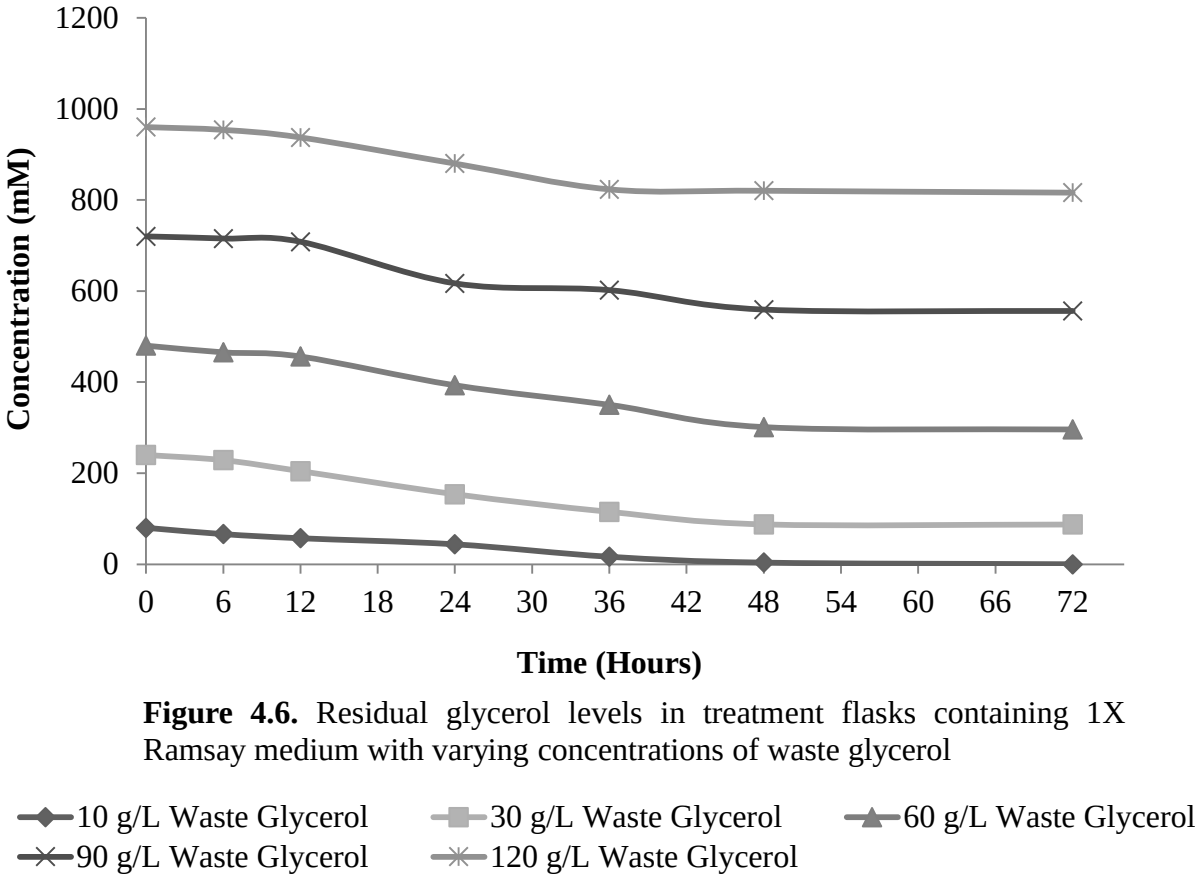

Figure 4.6. Residual glycerol levels in treatment flasks containing 1X Ramsay medium with varying concentrations of waste glycerol

Obtaining these measures was necessary as the amount and rate of nitrogen invested into cell biomass production served as a gauge for developing a system to allow for maximal cell biomass yield in the long term. Similarly, measuring the amount of glycerol uptake by LS46 provided an indicator for the efficiency and the most optimal concentration levels of waste glycerol for highest intracellular accumulation of PHA.

It was apparent that the starting 1 g/L concentration of nitrogen depleted completely between the 18 and 24 hour time point in each of the flask conditions, at which point biomass growth was expected to stop and the accumulation of WG as PHAs were to initiate. There were obvious differences in the rates of nitrogen uptake by LS46. More specifically, in the first 12 hours, the rate of nitrogen uptake was faster as WG concentrations increased (Figure 4.5).

Presently, not enough information or evidence is available in literature to explain this observation at this time. However, it can be inferred that components other than glycerol found within WG may play a role in the increased rate of nitrogen uptake within the higher WG concentrations. For instance, waste free fatty acids may play a role in the increased uptake of nitrogen. Reports on PHA production by other strains of *Pseudomonas* such as, *Pseudomonas* sp. strain DR2 grown on waste oils and waste free fatty acids in flask-batch exhibited improved yields of cell biomass as well as PHA content in comparison to the results achieved on glycerol as a substrate (Song et al., 2008). Thus, a possibility exists that LS46 may prefer waste free fatty acids over glycerol based upon the observations in Figure 4.7.

All in all, WG concentrations in conditions higher than 10 g/L were not utilized completely, but rather high residual amounts of WG were detected within the culture medium (Figure 4.6). Yet, the total amount of WG consumed was consistent amongst each of the WG concentrations with approximately 80 mM to 100 mM of WG being utilized. Despite this consistency in WG uptake the biomass production in each of the conditions varied. However, the potential of osmotic stress on LS46 biomass due to the presence of high contaminants and salts found within WG remain a potential factor in the decreased biomass production in high WG concentrations (Ashby et al., 2005). Furthermore, two studies reported a decrease in the growth rate of the respective bacteria used in their investigation.

In the first study, WG obtained from different manufacturers were tested on two different bacteria, *Cupriavdus necator* JMP 134 and *Paracoccus denitrificans* (Mothes et al., 2007). The

presence of Na^+ ions in the WG was found to have a negative impact on both growth rate and polymer yields as substrate loading was increased.

In the second study, similar observations were reported which also tested PHA production with *C. necator*, however, a different strain named DSM 545 (Cavalheiro et al., 2009). As the concentration of WG increased above 30 g/L, a high accumulation of Na^+ ions in the culture medium was detected, and as a result drastically decreased cell growth.

This led to the inference that an increase in LS46 cell biomass could possibly improve WG uptake for storage as mcl-PHAs, and thereby decrease residual WG within the culture medium. Therefore, increasing nitrogen availability for LS46 could be deemed favourable for additional cell biomass production. Thus, the next section will discuss effects of nitrogen variability on both cell biomass production as well as mcl-PHA accumulation by LS46.

4.3.4 Nitrogen Enrichment Effects on Cell Biomass Production and mcl-PHA Accumulation

Observations gathered from the previous section led to the inference that increasing nitrogen levels from the baseline 1 g/L could improve overall cell biomass yields and PHA accumulation by LS46 within flask. Therefore, to increase both cell biomass and intracellular PHA accumulation, an increase in levels of nitrogen (N) within the culture medium was considered given the key role of nitrogen in cell protein synthesis.

Three different WG conditions in Ramsay media were prepared; 10 g/L WG, 30 g/L WG, and 60 g/L WG. For each condition only N was increased proportionally, i.e. 1 g/L in 10 g/L WG, 3 g/L in 30 g/L WG, and 6 g/L in 60 g/L WG, while the other Ramsay medium components remained consistent. Figure 4.7 illustrates the cell biomass produced within each of the treatment conditions, while Figure 4.8 shows the PHA accumulated.

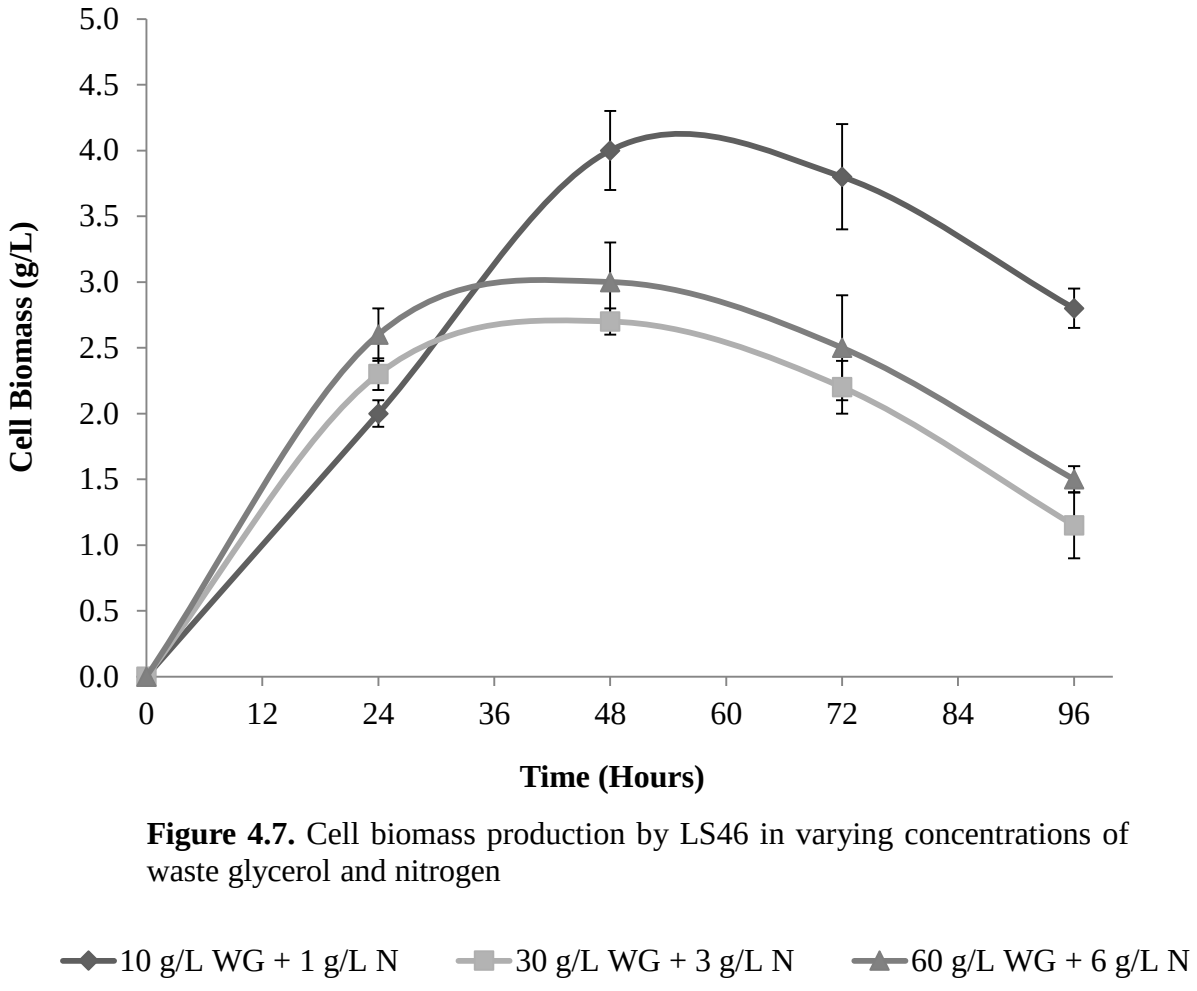

Figure 4.7. Cell biomass production by LS46 in varying concentrations of waste glycerol and nitrogen

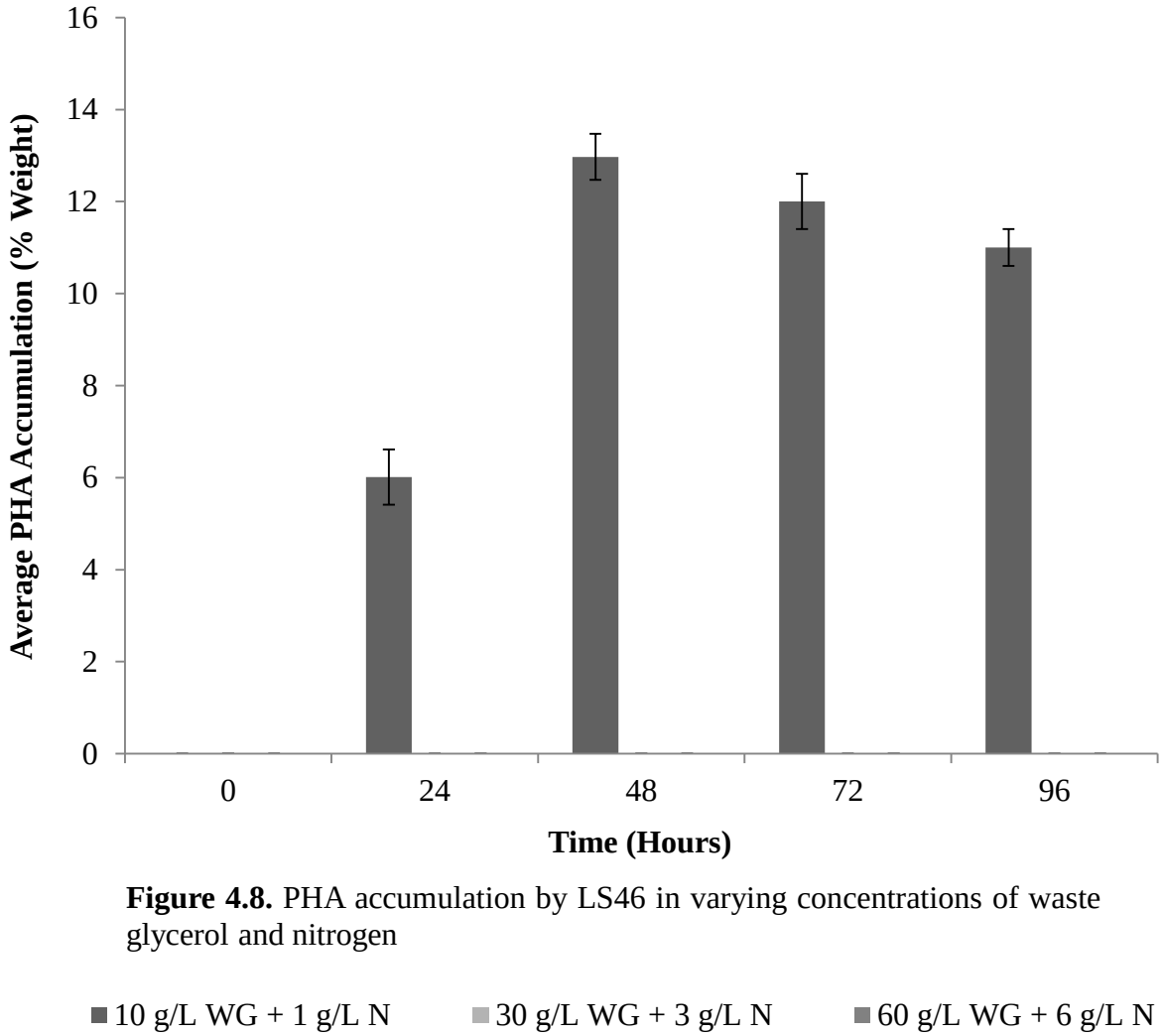

Figure 4.8. PHA accumulation by LS46 in varying concentrations of waste glycerol and nitrogen

The conditions of 10 g/L WG + 1 g/L N correlated quite closely with the results achieved in Figure 4.1 and Figure 4.2, which tested the same media condition. As a matter of fact, the 10 g/L WG + 1 g/L N treatment condition served as a baseline reference measure to validate the activity and growth pattern exhibited by LS46 within WG as the carbon source.

In spite of higher concentrations of nitrogen available within the 30 g/L and 60 g/L WG conditions, LS46 did not exhibit increased cell biomass production as was hypothesized. Furthermore, PHA accumulation by LS46 was absent. Since low nitrogen conditions are vital in triggering PHA accumulation within LS46, high residual nitrogen concentrations were detected within the 30 g/L and 60 g/L WG conditions when compared to the 10 g/L WG conditions. As a result the required nutrient stress was not present to initiate carbon storage. Instead, the baseline conditions demonstrated higher productivity when compared to the other two media conditions.

Substrate consumption analysis of the time point samples collected provided a closer look at the kinetics involving cell biomass production and PHA accumulation as well as confirmation of the observations described earlier. Residual levels of nitrogen and glycerol within the media were analyzed as shown in Figures 4.9 and 4.10, respectively.

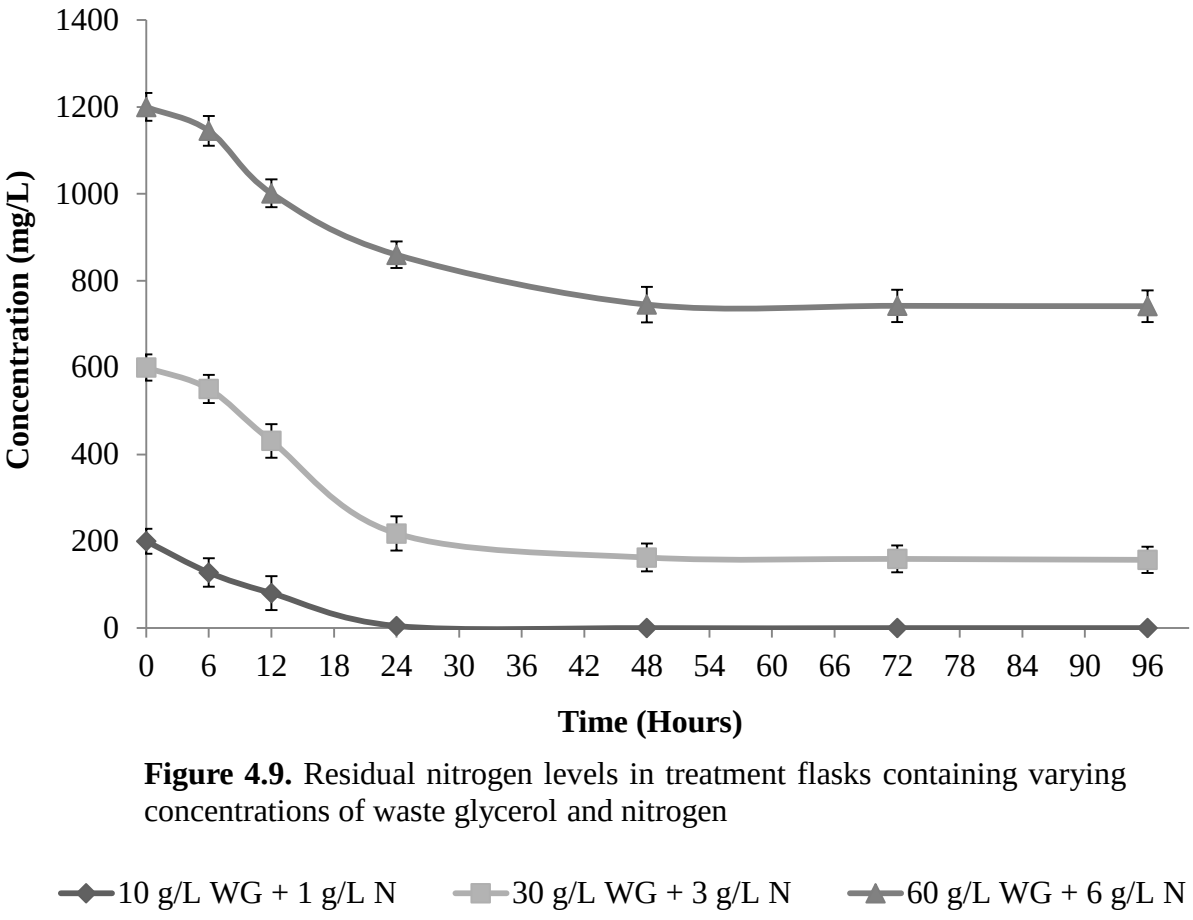

Figure 4.9. Residual nitrogen levels in treatment flasks containing varying concentrations of waste glycerol and nitrogen

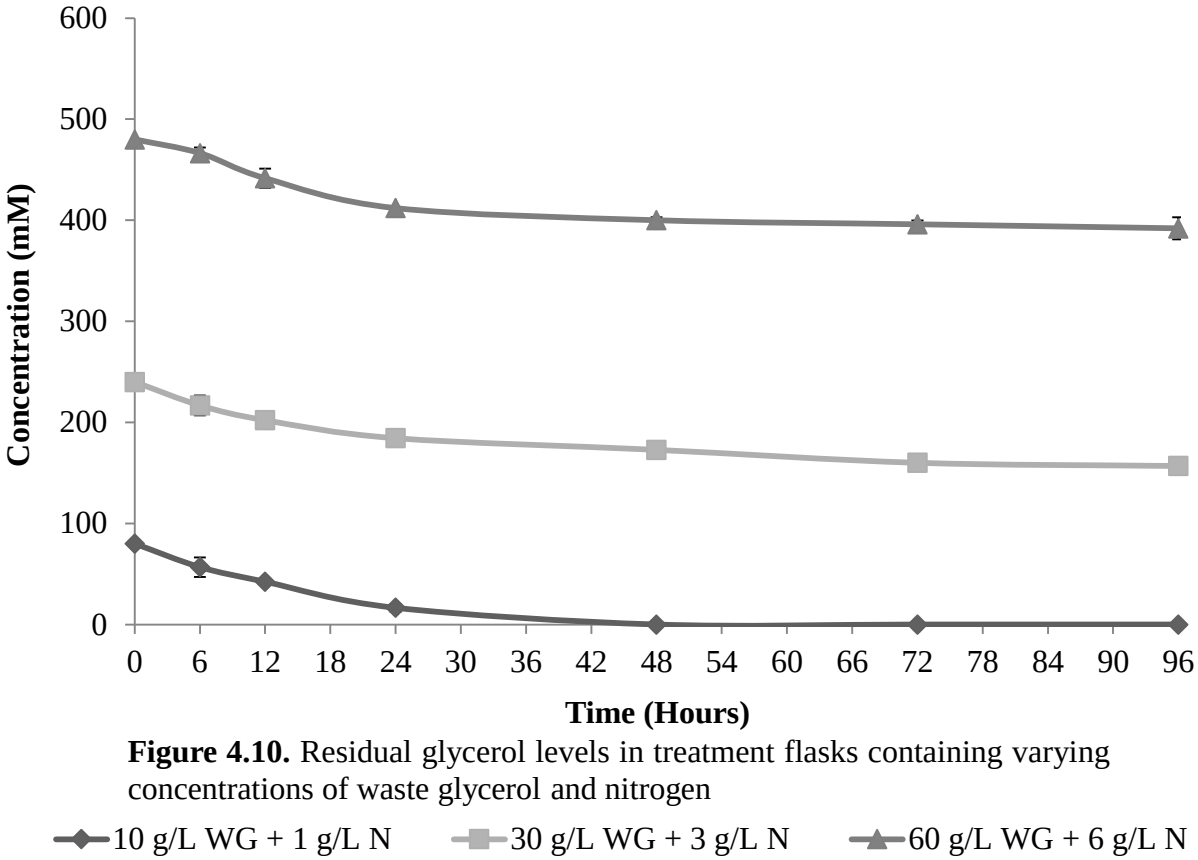

Figure 4.10. Residual glycerol levels in treatment flasks containing varying concentrations of waste glycerol and nitrogen

It was apparent that both nitrogen and waste glycerol were exhausted within the 10 g/L WG + 1 g/L N treatment conditions, while a considerable amount of N and WG remained within the other two treatment conditions. After further investigation into the results gathered, observation led to the conclusion that a limitation within the composition of the Ramsay medium resulted in high residual levels of N and WG. The media composition prepared involved variation in concentrations of WG and N only, thus it was important to proportionally increase other constituents within Ramsay medium as well. Therefore, another experiment was conducted, and all Ramsay medium constituents were proportionally increased with nitrogen.

However, for a focused approach, and an improved comparative analysis study, while limiting experimental variability and error, the culture medium prepared comprised of the 30 g/L WG conditions only. A 144 hour comparative analysis study examining effects of N variation was carried out through this approach. One triplicate condition of flasks contained 30 g/L WG with 1 g/L N, while a second triplicate condition comprised of 30 g/L WG with 3 g/L N. To observe any potential deviation from the basic growth patterns due to contamination within the two different media conditions, an optical density growth curve was obtained as illustrated in Figure 4.11.

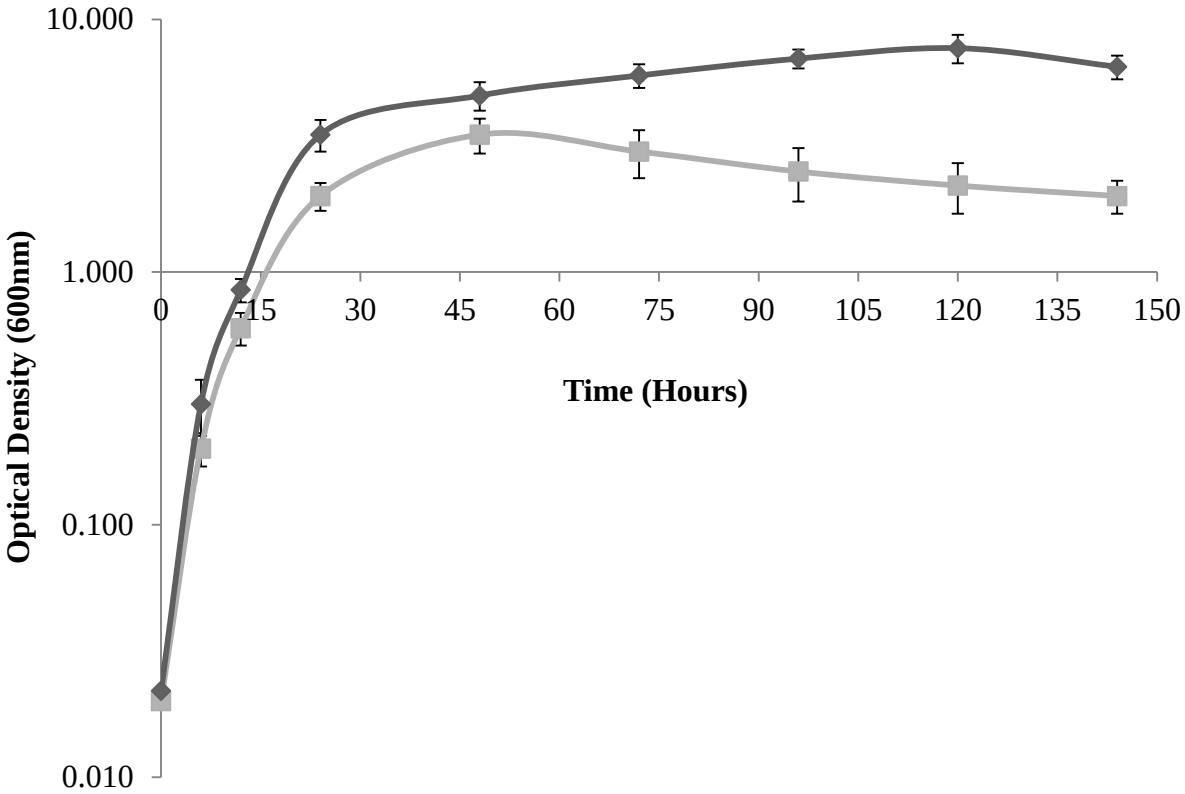

Figure 4.11. Optical density monitored growth for LS46 in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

Cell biomass production was anticipated to be higher in the 3 g/L N in 3X concentration Ramsay medium condition versus the 1 g/L N condition in normal strength Ramsay medium, which has been exhibited in Figure 4.12.

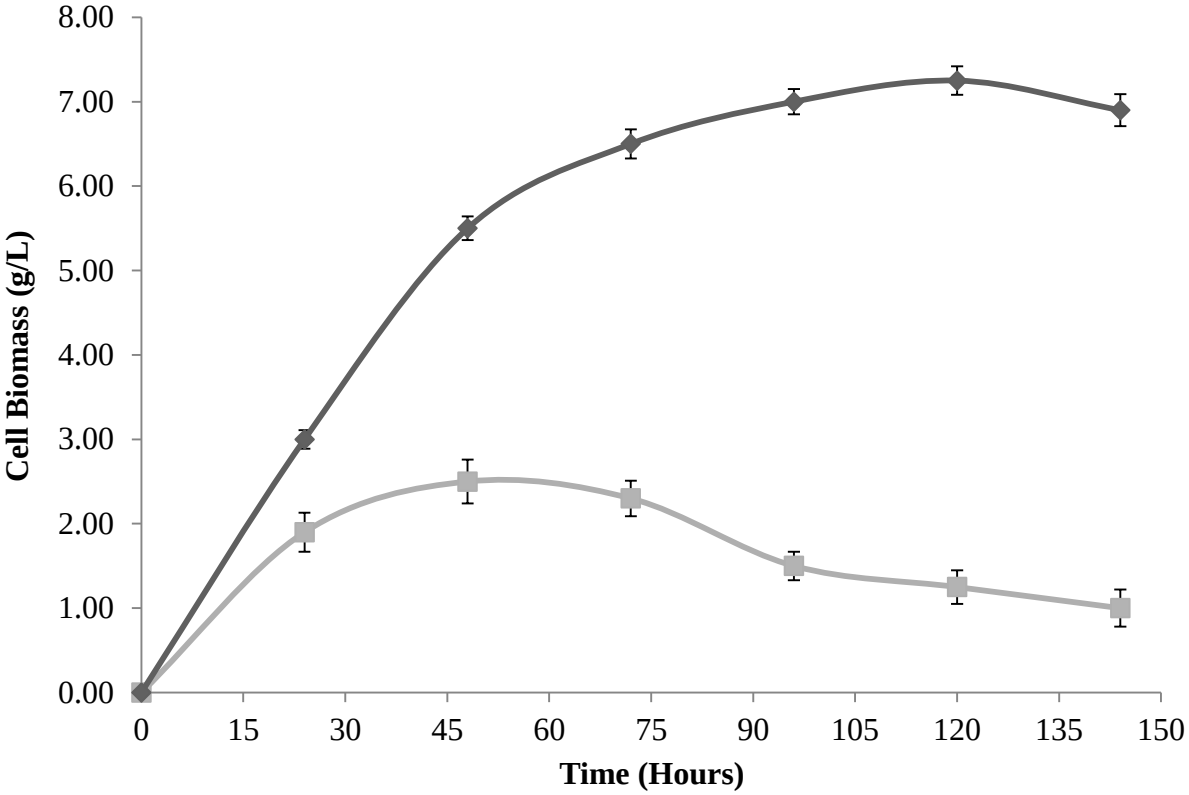

Figure 4.12. Cell biomass production by LS46 in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

With increased cell biomass productivity in the 3X strength Ramsay medium (N corresponding to 3 g/L) conditions, the overall accumulation of PHA was also anticipated to increase. Figure 4.13 illustrates the PHA accumulated within LS46, while Table 4.2 exhibits the monomer composition of the PHA accumulated.

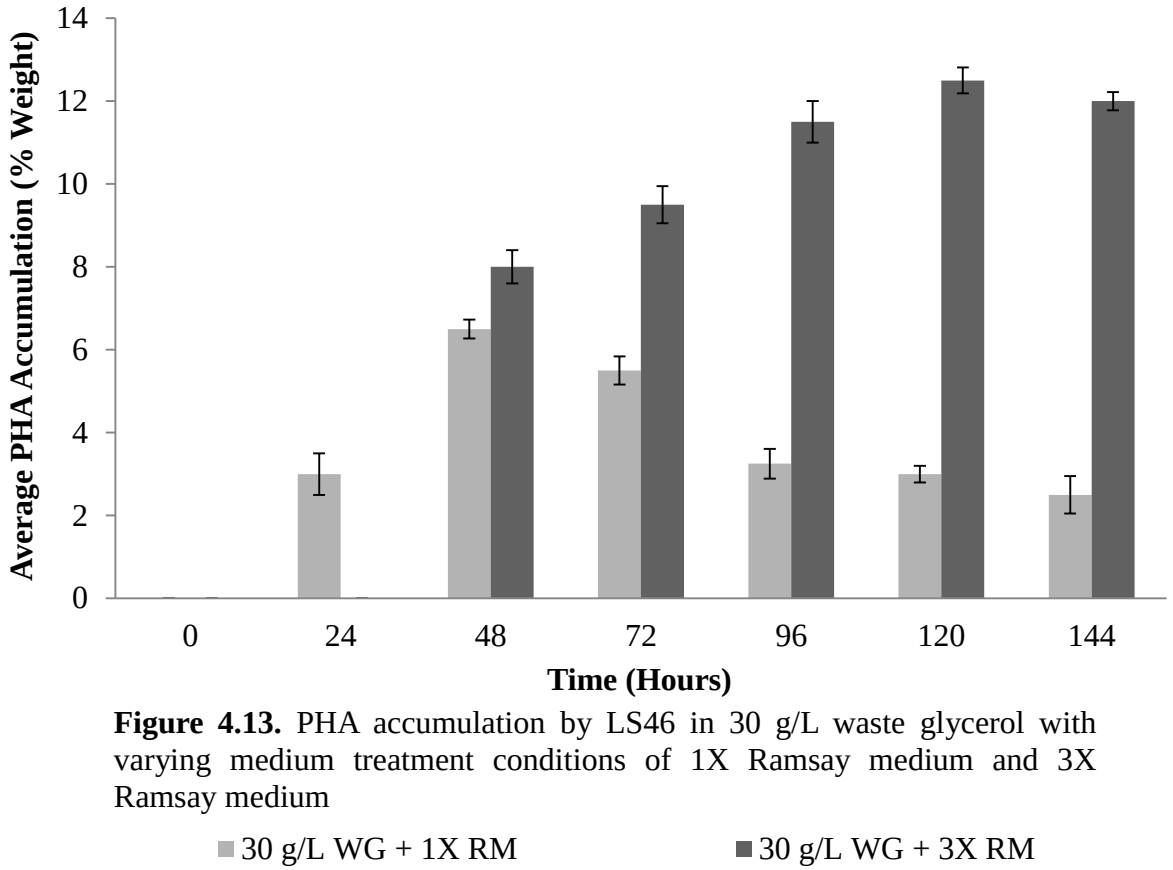

Figure 4.13. PHA accumulation by LS46 in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

Table 4.2. Percent monomer composition of mcl-PHA accumulated by LS46 in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

Time	Substrate	Monomer Composition (%)					
		C_6	C_8	C_{10}	C_{12}	$C_{12:1}$	C_{14}
24	30 g/L WG + 1X RM	2.34 ± 0.06	26.42 ± 0.49	64.12 ± 1.69	2.26 ± 0.15	4.36 ± 0.29	0.50 ± 0.10
	30 g/L WG + 3X RM	--	--	--	--	--	--
48	30 g/L WG + 1X RM	2.71 ± 0.03	25.49 ± 0.92	64.59 ± 0.68	2.64 ± 0.18	4.10 ± 0.24	0.47 ± 0.01
	30 g/L WG + 3X RM	2.43 ± 0.02	24.09 ± 1.16	65.75 ± 1.05	2.34 ± 0.13	4.40 ± 0.40	0.99 ± 0.16
72	30 g/L WG + 1X RM	2.59 ± 0.04	26.11 ± 1.13	63.67 ± 1.15	2.50 ± 0.26	4.47 ± 0.23	0.66 ± 0.12
	30 g/L WG + 3X RM	2.30 ± 0.06	24.46 ± 0.49	66.14 ± 1.69	2.24 ± 0.15	4.37 ± 0.29	0.49 ± 0.10
96	30 g/L WG + 1X RM	2.35 ± 0.01	25.57 ± 0.20	64.71 ± 0.31	2.50 ± 0.10	4.38 ± 0.21	0.50 ± 0.13
	30 g/L WG + 3X RM	2.38 ± 0.08	26.79 ± 0.78	63.37 ± 1.59	2.61 ± 0.19	4.45 ± 0.26	0.40 ± 0.12
120	30 g/L WG + 1X RM	2.45 ± 0.02	24.07 ± 1.16	65.79 ± 1.05	2.30 ± 0.13	4.41 ± 0.40	0.98 ± 0.16
	30 g/L WG + 3X RM	2.31 ± 0.03	25.07 ± 1.38	65.10 ± 1.10	2.40 ± 0.25	4.32 ± 0.36	0.80 ± 0.09
144	30 g/L WG + 1X RM	2.23 ± 0.07	25.52 ± 1.83	64.86 ± 0.63	2.37 ± 0.25	4.27 ± 0.12	0.75 ± 0.03
	30 g/L WG + 3X RM	2.32 ± 0.09	26.76 ± 1.97	63.47 ± 1.14	2.43 ± 0.17	4.69 ± 0.42	0.33 ± 0.10

*-- (Biomass production nil)

The PHA levels accumulated within the 1 g/L N were approximately 6 % of cdw similar to the levels observed in the previous section, Section 4.3.3, which examined the effects of carbon variation. By the same token, the 3X Ramsay medium nitrogen conditions demonstrated a noticeable increase near 12 %.

As done in earlier flask experiments, analysis of residual nitrogen levels (Figure 4.14) as well as residual glycerol concentrations (Figure 4.15) was carried out to obtain a closer look at the possible factors affecting both cell biomass production and PHA accumulation, respectively.

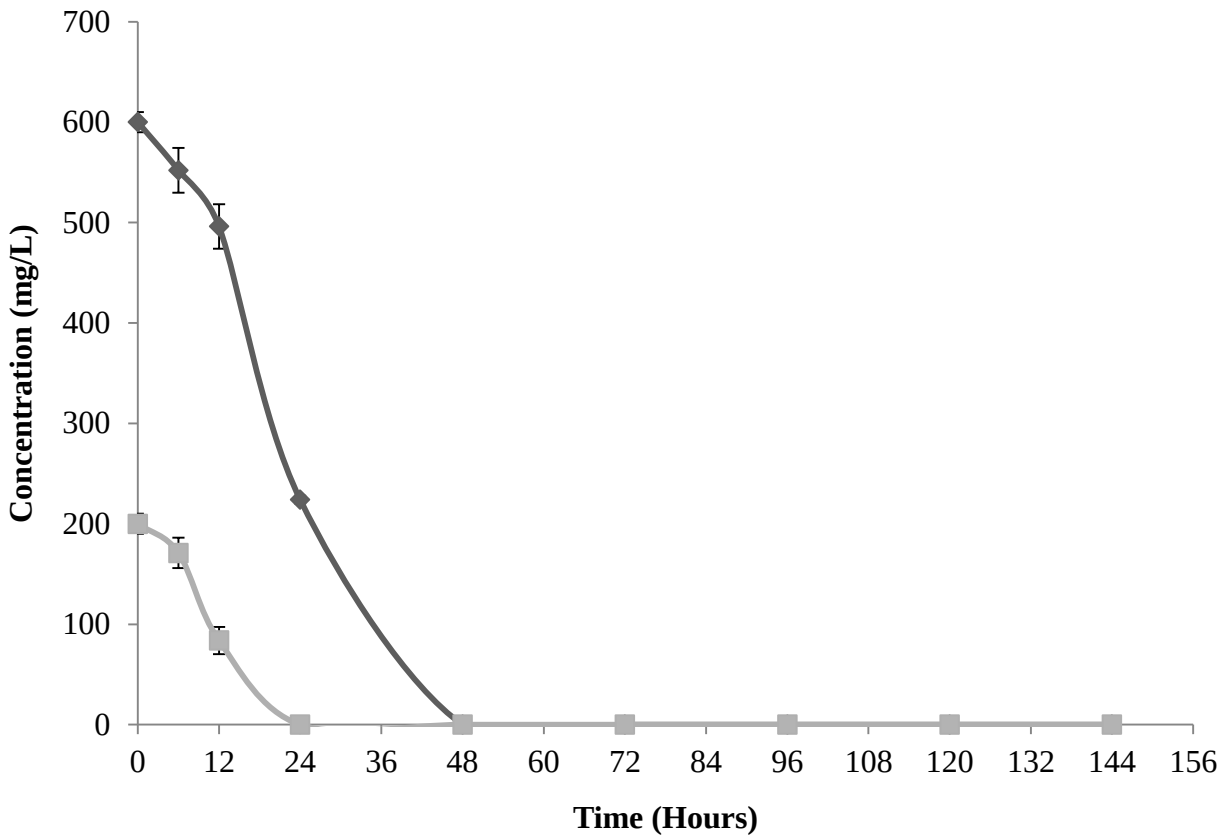

Figure 4.14. Residual nitrogen levels measured in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

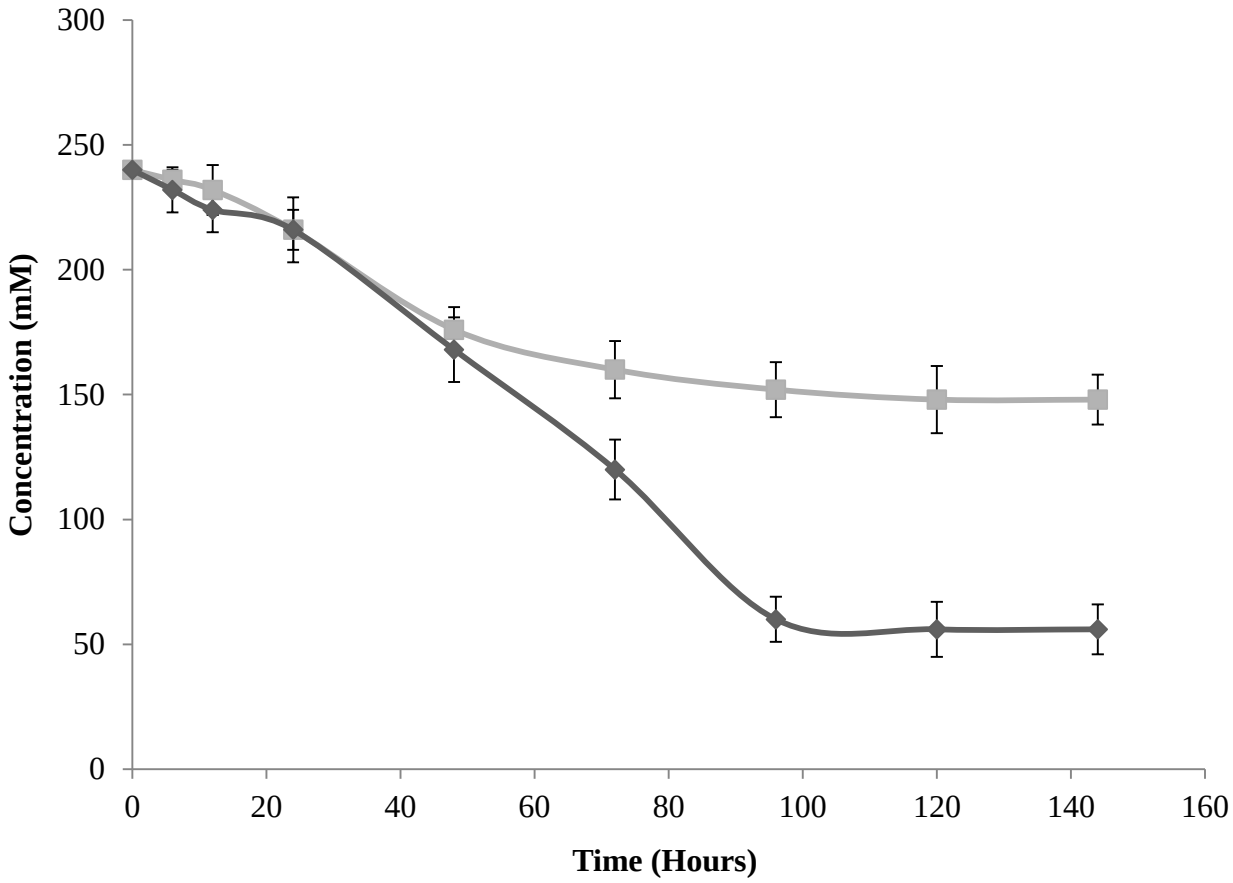

Figure 4.15. Residual glycerol levels measured in 30 g/L waste glycerol with varying medium treatment conditions of 1X Ramsay medium and 3X Ramsay medium

Utilization of nitrogen for cell biomass production by LS46 went to completion in both media conditions. Nitrogen levels were exhausted near the 24 hour time point in the 1X RM concentrations, and near the 48 hour time point in the 3X RM concentrations.

Glycerol utilization did not go to completion as evident by the residual glycerol levels detected in both of the media conditions. However, a significant difference in residual glycerol levels was observed between the two media conditions as anticipated. Since biomass production and PHA accumulation was greater in the 3X RM conditions, glycerol uptake by LS46 was significantly higher. Thus, a lower residual amount of glycerol was detected when compared to the 1X RM conditions. All in all, observations achieved in this experiment provided a general guideline in further developing strategies for scale-up involving increased cell biomass production as well as increased intracellular PHA accumulation.

4.4 Discussion

Each of the previous sections in this chapter provided background as well as imperative data and results pertaining to an aspect or condition examined within flask conditions. In the first section of this chapter, a baseline level of cell biomass and PHA accumulation by LS46 in flask containing varying concentrations of WG was established. Since PHA accumulation is activated in carbon excess and nitrogen limiting conditions (Lee et al., 1999), a carbon to nitrogen ratio most optimal for triggering the highest levels of intracellular PHA accumulation was considered through this strategy. Thus, a 10 g/L WG to 1 g/L N ratio was determined ideal for maximal cell biomass synthesis and PHA accumulation by LS46 in flask.

Thereon, specific media conditions were varied accordingly to analyze effects on overall cell biomass synthesis and PHA accumulation by LS46. Effects of media sterilization, carbon-loading, and nitrogen enrichment were examined.

In evaluating effects of media sterilization, flasks containing unautoclaved (unsterilized) medium exhibited significant levels of cellular growth (Figure 4.3) compared to sterilized media conditions (Figure 4.1). Furthermore, this illustrated that the bacterial contaminants present within the flasks have the capacity to grow on Ramsay medium with WG as the carbon substrate, therefore, the need for proper media sterilization will be mandatory when developing strategies for scale-up in the long-term.

Differences between total PHA accumulation were significant and reinforced this observation as sterilized media conditions (Figure 4.2) exhibited highest PHA accumulation when compared to unsterilized media conditions (Figure 4.4).

Effects of carbon-loading on LS46, especially within unsterilized media conditions, were tested as a means of creating a selective pressure treatment for LS46. Nevertheless, LS46 could not compete against the mixture of bacteria within such an environment, thus, development of a strategy with unsterilized media conditions was deemed unfeasible.

Within sterilized media conditions, an inverse relationship was observed between LS46 activity and the concentration of WG. LS46 biomass production was highest in low WG concentrations compared to high WG concentrations (Figure 4.1). Furthermore, PHA accumulation exhibited similar trends with highest levels of PHA detected in low WG concentrations, while low PHA accumulation were detected in high WG concentrations (Figure 4.2).

This observation implied an inhibitory effect by high WG concentrations on the activity of LS46. Such inhibitory effects have been reported in literature where excess carbon is made available; however, for different PHA producing bacteria. For instance, production of scl-PHAs by *Paracoccus denitrificans* and *Cupriavidus necator* on crude glycerol exhibited decreased accumulation of PHB in high crude glycerol conditions (Mothes et al., 2007).

According to the study, when grown on pure glycerol, 70 % cdw of PHB has been detected, whereas growth on crude glycerol exhibited a 20 % decrease in PHB content. It was found that the presence of contaminants within the crude glycerol such as, ash, methanol, salts, or fatty acids was a major contributor to the decrease in the scl-PHA content of the cells. More specifically, salt contaminants such as, NaCl had a negative effect on osmoregulation particularly during the PHA accumulation phase. Therefore, over the course of fermentation contaminants within the WG can accumulate and effect both cell growth and PHA synthesis especially when WG is used in excess quantities.

Another study also employed biodiesel derived wastes as feedstock, which included crude glycerol as well, for the production of scl-PHA copolymers by *C. necator* (Garcia et al., 2013). A similar decrease in the polymer accumulation was reported due to an increase in the levels of salts such as, NaCl and K_2SO_4.

However, when comparing the results obtained with LS46 grown on WG to other *P. putida* strains grown on glycerol, there have been limited reports shared in literature. Though, discussion of a long lag phase has been reported for the parent strain, KT2440 (Escapa et al., 2013). To eliminate such a delay, co-feeding of another substrate such as, octanoic acid has been suggested. Furthermore, manipulation of regulator genes essential to the metabolism of glycerol in KT2440 has also been investigated. In both scenarios, a drastic two-fold increase in glycerol metabolism and conversion into PHAs has been observed when compared to the fully regulated KT2440 grown on glycerol as the sole carbon source.

Additionally, an alternative strain of *P. putida*, KT2442 grown on glycerol has been reported in literature as well (Huijberts et al., 1992). This strain has exhibited approximately 22 % of cdw as mcl-PHAs. Furthermore, the predominant monomer detected via GC analysis has been C_{10}, similar to what has been observed with LS46. Another strain, KT217, grown on glycerol as well reported a significant accumulation of PHA near 30 % of cdw, although, within a fed-batch operation (Javers et al., 2012). Nevertheless, effects of substrate loading or nitrogen enrichment were not investigated.

Though, reports on other *Pseudomonas* species such as, *oleovorans* NRRL B-14682 and *corrugate* 388 have suggested effects of osmotic stress placed upon cells in high glycerol concentrations leading to lower biomass yields (Ashby et al., 2005). This is the result of a decrease in enzymatic efficiency resulting in a slower growth rate, and thus a decrease in polymer production. To overcome inhibitory effects of high WG concentrations, significantly increasing cell biomass was considered. With such an increase, an improvement in substrate consumption was hypothesized. Thus, the effects of nitrogen enrichment were investigated to improve WG uptake and thereby increase overall PHA accumulation.

Figure 4.12 illustrates a drastic increase in cell biomass between 30 g/L WG + 1X RM and 30 g/L WG + 3X RM. In both media conditions nitrogen utilization went to completion, although the rate of nitrogen utilization was prolonged in the 3X RM conditions given the availability of higher concentrations of N (Figure 4.14). Cell biomass drastically increased from approximately 2.5 g/L in 1 g/L N with 1X Ramsay medium conditions to approximately 8.5 g/L in 3 g/L N with 3X Ramsay medium conditions (Figure 4.12).

Moreover, WG uptake and the overall intracellular accumulation of mcl-PHAs was higher in the 3X RM conditions versus 1X RM given the improved levels of cell biomass production

when the increase in N occurred in the context of increasing the other components of the Ramsay medium 3 fold (Figure 4.15). As a result levels of intracellular PHA accumulation demonstrated a significant improvement from approximately 5 % cdw to approximately 12 % cdw (Figure 4.13).

Overall, these series of flask studies provided a starting point for the development of a feeding strategy most suitable for improving cell biomass yields and intracellular PHA accumulation by LS46. It was established that sterilized media conditions are crucial in order to ensure a LS46 predominant culture and to prevent potential contaminants entering and overrunning the culture medium, which can otherwise become counterproductive to the efforts of producing the target mcl-PHAs.

Furthermore, the baseline flask conditions comprising of 10 g/L WG + 1 g/L N provided the highest level of biomass and PHA accumulation within flask when compared to higher WG concentrations. Therefore, WG to N ratios comparable to the condition of 10 g/L WG + 1 g/L N can be considered as a reference point in the development efforts of scale-up strategies as was evidenced from significant improvements in both cell biomass and PHA yields within the 30 g/L WG + 3 g/L N conditions.

Chapter 5 Bioreactor Experiments

5.1 Background

These next series of experiments were the final steps taken towards the conclusion of the three pronged approach established for the development of a sustainable fermentation strategy to improve and scale up production of mcl-PHAs by LS46. In this phase of the project a large volume bioreactor was employed to analyze and test media conditions and growth parameters evaluated within the flask based studies.

Based upon reports in literature regarding batch fermentations with other *Pseudomonas* sp., it was hypothesized that with improved control of general process parameters such as, aeration, dissolved oxygen, foaming, pH, temperature, and stirring significant improvements in PHA accumulation were expected (Lee et al., 1999; Ashby et al., 2005).

5.2 Materials and Methods

All chemicals and substrates utilized in these next series of bioreactor experiments have been outlined in Chapter 2. Furthermore, protocols and steps for media preparation and inoculation procedures have also been indicated in Chapter 2 as well.

5.3 Experimental Design

Overall two feeding strategies were evaluated, the first strategy comprised of large volume single batch runs, while the second strategy tested the potential of a fed-batch run. Details and specifics regarding the setup and operation of each strategy have been discussed in the sections ahead.

5.3.1 Batch Runs

To scale-up production levels of the targeted intracellular mcl-PHA accumulation, a pilot-scale experiment was conducted. Thus, conditions and parameters studied in flask experiments were tested and scaled-up within a lab grade fermenter, specifically the 15 L ez-Control from Applikon Biotechnology.

5 L of media was directly prepared within the fermenter and then placed for autoclaving at 121 ^{o}C and exposed to a large liquid cycle for 60 minutes. The media composition comprised of 30 g/L WG + 2X Ramsay medium. Despite 30 g/L WG + 3X RM exhibiting significant yield improvements based upon results observed in flask (Section 4.3.4), a 30 g/L WG + 2X RM composition was pursued instead. These starting media conditions for this phase of the project were not based upon a firm scientific reasoning other than the fact that it was simply a starting point to begin investigation of the targeted high-yield production of mcl-PHAs at pilot-scale. Furthermore, this ensured carbon excess and nitrogen limiting conditions were maintained within the fermenter to initiate PHA accumulation over the course of the batch run.

Process parameters including aeration, dissolved oxygen, foaming, pH, stirring, and temperature were controlled with an adaptive PID system for accurate control and measure. This maintained optimal growth conditions and a favourable environment for LS46 proliferation, which of course are not possible to control within flask. Aeration was kept constant at 10 LPM, while dissolved oxygen was set at 50 %. DO was calibrated at 100 % air saturation, which was at ~8 mg/L oxygen. pH was calibrated and set at 7.0. The reactor temperature was set for 30 ^{o}C. Stirring was controlled based upon oxygen demands of the reactor culture. Lower oxygen demands required slow stirring (low rpms), while higher oxygen demands led to fast stirring (high rpms). This corresponded to a lower limit setting of 250 rpms, with an upper limit setting of 750 rpms.

Biological triplicates were conducted to confirm reproducibility of the observations and results achieved within batch. Each run was conducted over a 96 hour period with fixed time point sampling for analysis of cell biomass production, PHA accumulation, PHA monomer composition, residual nitrogen, and residual glycerol.

5.3.2 Fed-Batch Run

An alternate feeding strategy for the scale-up of mcl-PHA accumulation by LS46 was evaluated via fed-batch. However, only a single run of this strategy was conducted. In this process, a specific level of media nutrient was added in the fermenter with the goal of increasing cell biomass yields by prolonging the growth phase of LS46, and thereby enhancing PHA accumulation. The amount of media nutrient for feeding was established based upon rough estimates gathered from the data and results of nutrient and substrate consumption profiles

obtained from flask as well as batch runs. This involved regular feeding of medium nutrients into the fermenter at fixed time points.

5 L of media was prepared within the fermenter. Feeding of the nutrient media was conducted manually using the aseptic technique to prevent contamination of the fermenter. Times for feeding were also established based upon observations of the nutrient and substrate consumption profiles obtained from both flask and batch runs conducted earlier. Thus, all feeding was provided within the first 30 hours of operation of the fermenter at 15, 22, 25, 27, 28, and 28.5 hours.

5.4 Results and Discussion

With the capacity to control process parameters and conditions within a fermenter, an increase in LS46 cell biomass and intracellular PHA accumulation was hypothesized. Figure 5.1 illustrates LS46 cell biomass production based upon the optical density measures. However, as a direct measure, Figure 5.2 exhibits total cell biomass synthesis in terms of cell dry weight collected during the different time point sampling.

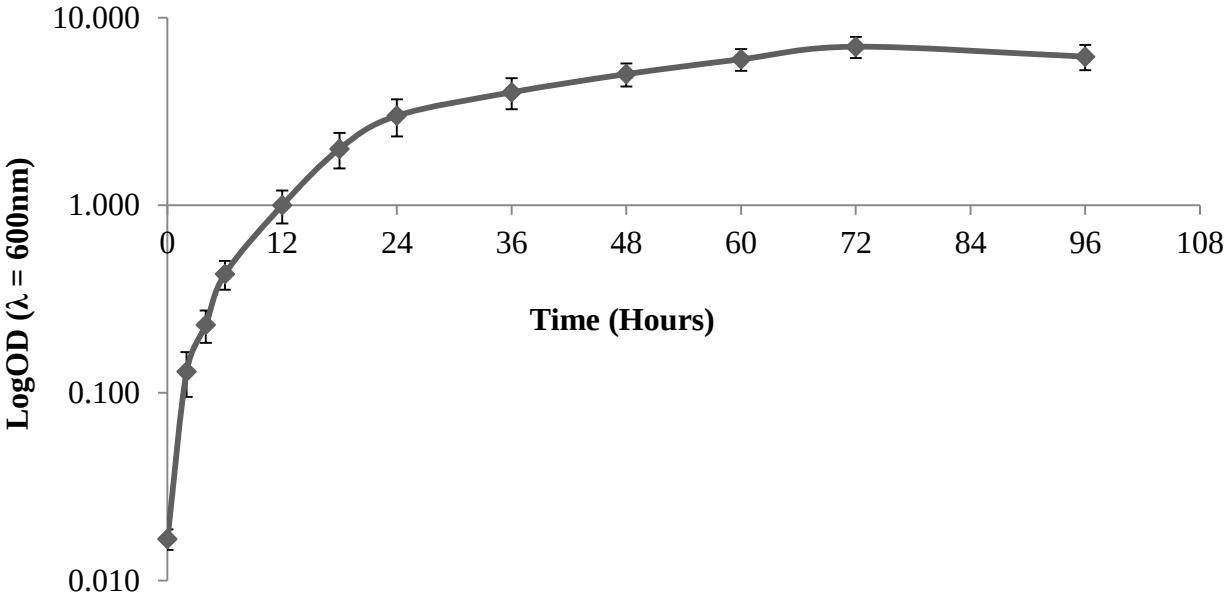

Figure 5.1. Optical density monitored growth of LS46 in batch containing 30 g/L waste glycerol + 2X Ramsay medium

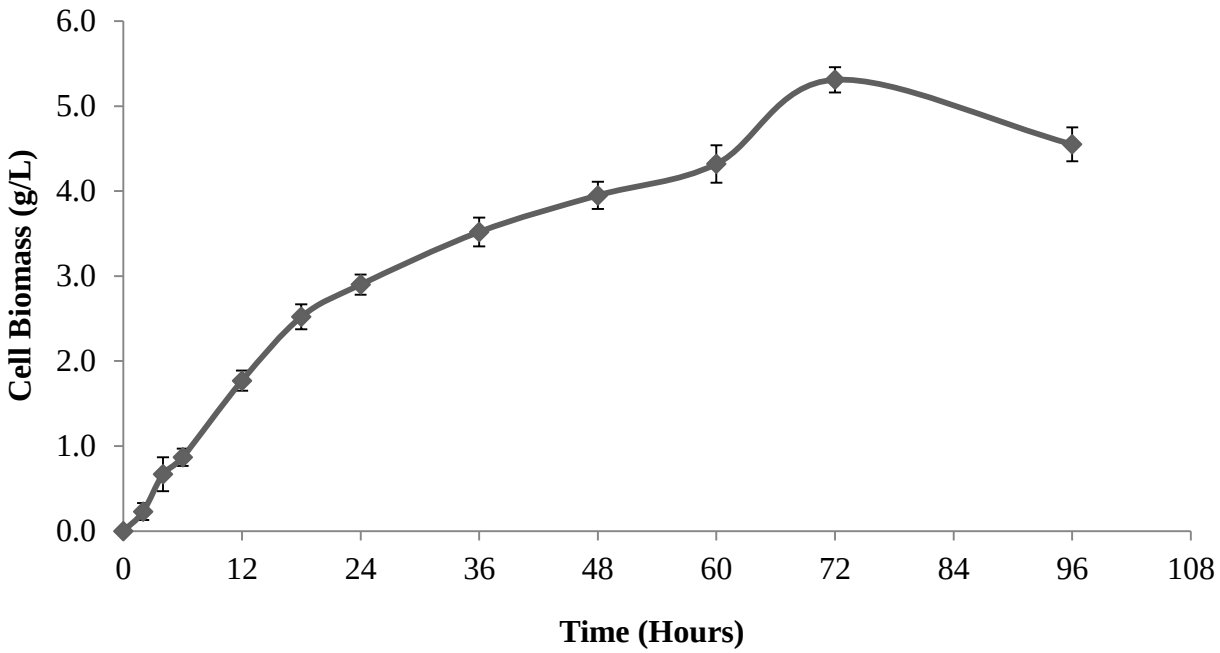

Figure 5.2. Cell biomass production by LS46 in batch containing 30 g/L waste glycerol + 2X Ramsay medium

With the fixed media conditions provided, LS46 appeared to have reached a maximum yield of biomass near 5 g/L. However, it is important to note that this total may not be a true representation of the actual biomass yield achieved. Excessive foaming over the course of fermentation was becoming an issue despite the use of a foaming controller to add anti-foaming agent as required, which led to the loss of some biomass. This was largely due to technical difficulties causing the foaming detector to perform ineffectively.

Analysis of residual nitrogen concentrations within the culture medium demonstrated complete uptake by LS46 near the 24 hour time point (Figure 5.3).

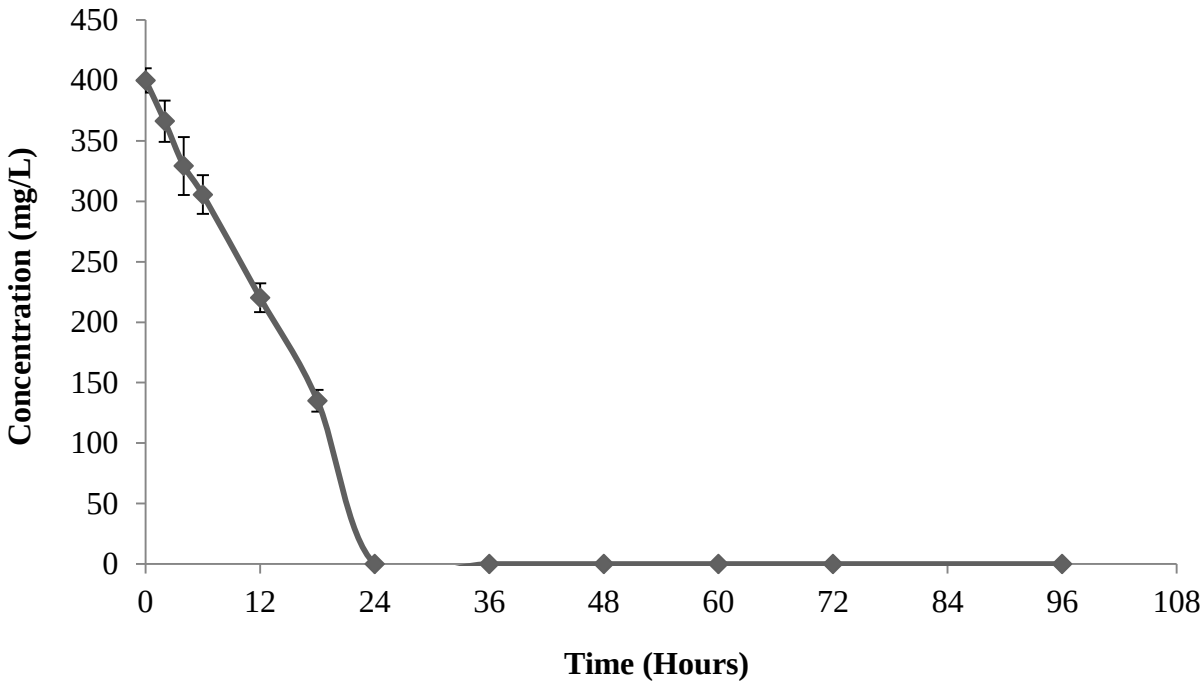

Figure 5.3. Residual levels of nitrogen in batch containing 30g/L waste glycerol + 2X Ramsay medium

With nitrogen fully exhausted within the culture media, PHA accumulation was triggered based upon the noticeable spike in PHA levels detected at 24 hours according to Figure 5.4.

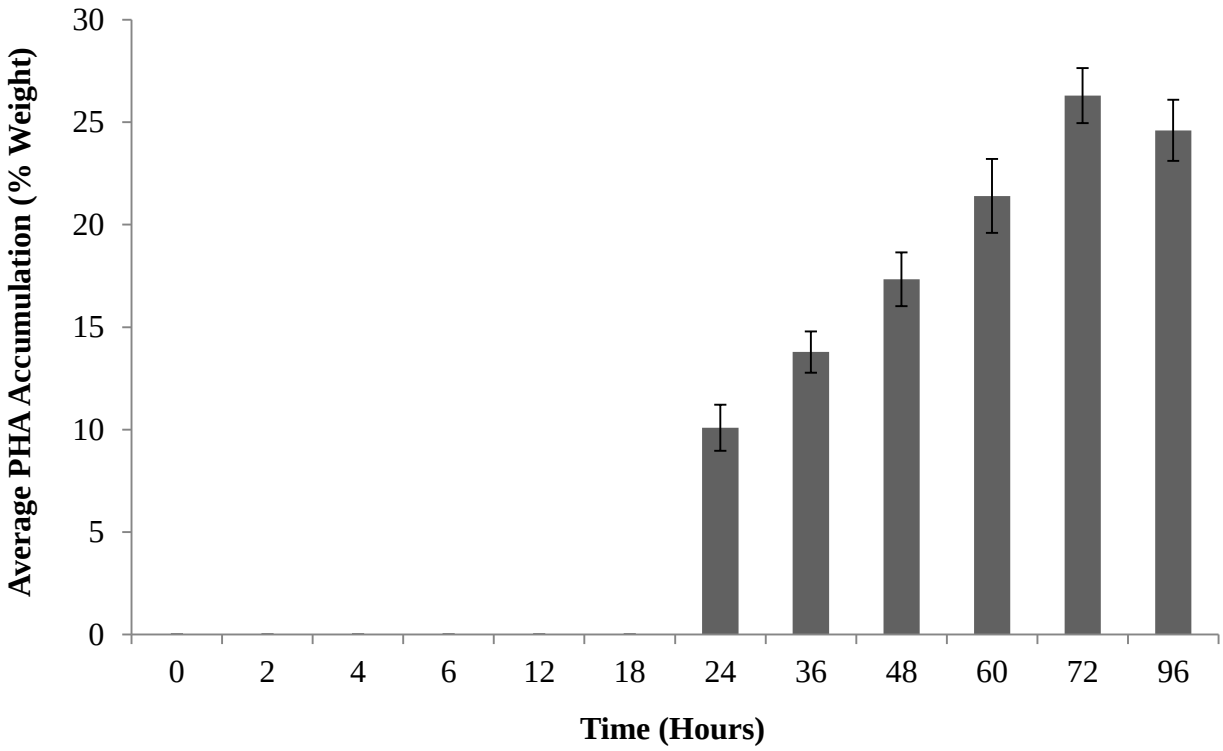

Figure 5.4. PHA accumulation by LS46 in batch containing 30 g/L waste glycerol + 2X Ramsay medium

A monomer composition analysis of the cell biomass collected was conducted using GC detection to confirm accumulation of the targeted mcl-PHAs. Table 5.1 exhibits the results achieved.

Table 5.1. Percent monomer composition of mcl-PHA accumulated by LS46 in 30g/L WG + 2X RM batch conditions

Time (Hours)	**Monomer Composition (%)**					
	C_6	**C_8**	**C_{10}**	**C_{12}**	**$C_{12:1}$**	**C_{14}**
24	2.45 ± 0.02	24.07 ± 1.16	65.79 ± 1.05	2.30 ± 0.13	4.41 ± 0.40	0.98 ± 0.16
36	2.31 ± 0.03	25.07 ± 1.38	65.10 ± 1.10	2.40 ± 0.25	4.32 ± 0.36	0.80 ± 0.09
48	2.38 ± 0.08	26.79 ± 0.78	63.37 ± 1.59	2.61 ± 0.19	4.45 ± 0.26	0.40 ± 0.12
60	2.41 ± 0.07	25.07 ± 1.08	64.83 ± 1.01	2.30 ± 0.10	4.40 ± 0.40	0.99 ± 0.15
72	2.56 ± 0.09	25.09 ± 1.16	64.70 ± 1.05	2.52 ± 0.24	4.45 ± 0.25	0.68 ± 0.13
96	2.26 ± 0.06	26.50 ± 0.46	66.10 ± 1.69	2.28 ± 0.14	4.35 ± 0.30	0.49 ± 0.10

The monomer compositional analysis confirmed accumulation of mcl-PHAs with C_8 and C_{10} as the predominating monomers as well as some unsaturated C_{12} monomers similar to the results achieved in flask-batch. HPLC analysis for the residual levels of WG within the culture media provided a closer look at the efficiency of substrate utilization by LS46 as well as the correlation between carbon uptake and PHA accumulation (Figure 5.5).

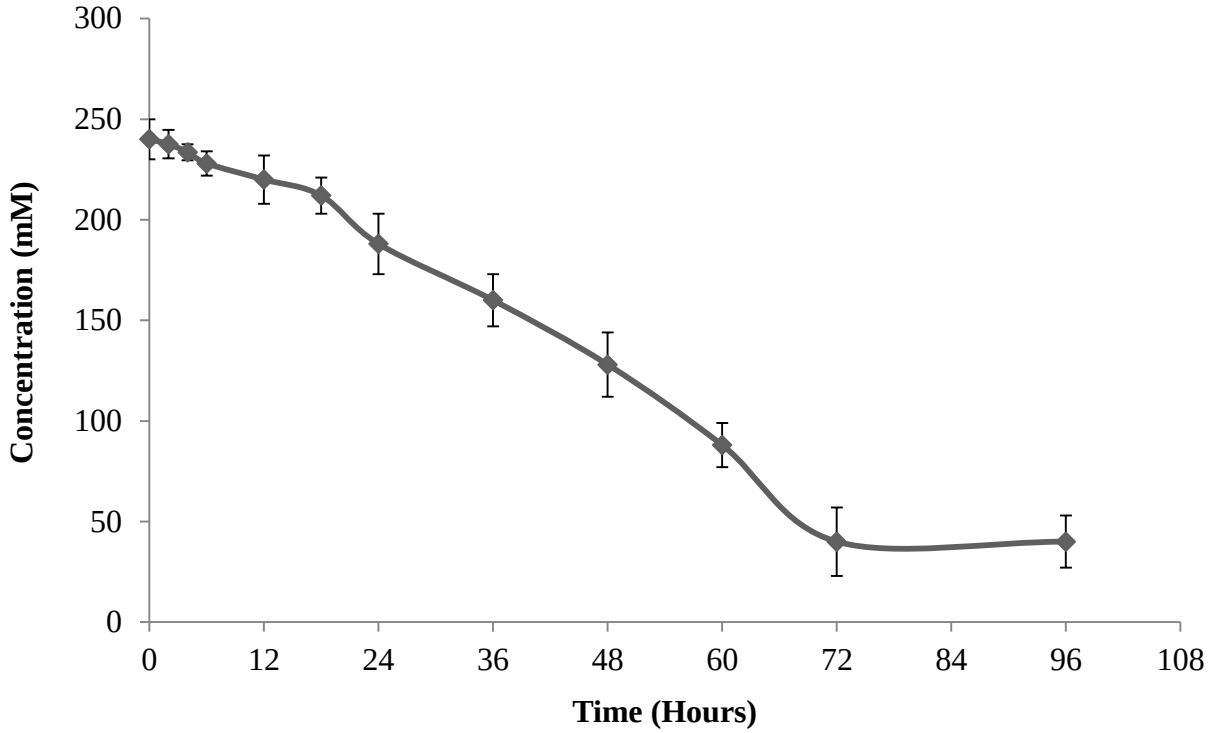

Figure 5.5. Residual levels of glycerol in batch containing 30 g/L waste glycerol + 2X Ramsay medium

Based upon Figure 5.5 it was apparent that less than one-third of the available WG remained within the culture media after 72 hours. In other words WG was not fully exhausted similar to the trends reported within the flask-batch experiments conducted in Chapter 4.

A significant increase in the maximum average of intracellular PHA accumulation was achieved via the batch reactor system when compared to flask-batch conditions. With the capacity to adjust conditions on demand within the fermenter and provide a controlled environment for the culture medium, improvements in PHA synthesis and yields were anticipated. However, a drastic increase in cell biomass was not observed in batch reactor compared, which was most likely the result of excessive foaming as stated earlier.

To examine the potential of further increasing LS46 cell biomass within a fermenter, a fed-batch system was setup as a final test run. The feeding strategy in this process varied from the normal batch run as media nutrients were fed into the fermenter containing media culture at pre-determined fixed time points.

As stated earlier, the time points chosen for the feedings were determined based upon rough estimates from the observations gathered for the growth patterns demonstrated by LS46 within flask conditions as well as batch runs. In this case the feeding strategy was based upon maintaining both carbon and nitrogen levels within the fermenter during the growth phase to ensure a significant increase in cell biomass. 20 g/L WG and 1 g/L nitrogen were provided via the feeds. Addition of the media nutrients have been indicted by arrows in Figures 5.6 and 5.8, respectively. It is important to state that foaming was not a significant concern in fed-batch as it was in batch. This was the result of manual addition of anti-foaming agent as needed especially during feeding, which prevented the fermenter from foaming over.

Figure 5.6 exhibits the level of nitrogen maintained within the fermenter as well as the feeding patterns followed based upon the time points illustrated. As discussed previously, nitrogen, which is essential in cell biomass production, was fed into the culture medium throughout the six feeds consecutively to prolong the cell growth phase of LS46. The six feeds delivered over the course of this experiment provided a total of 8 g/L of nitrogen.

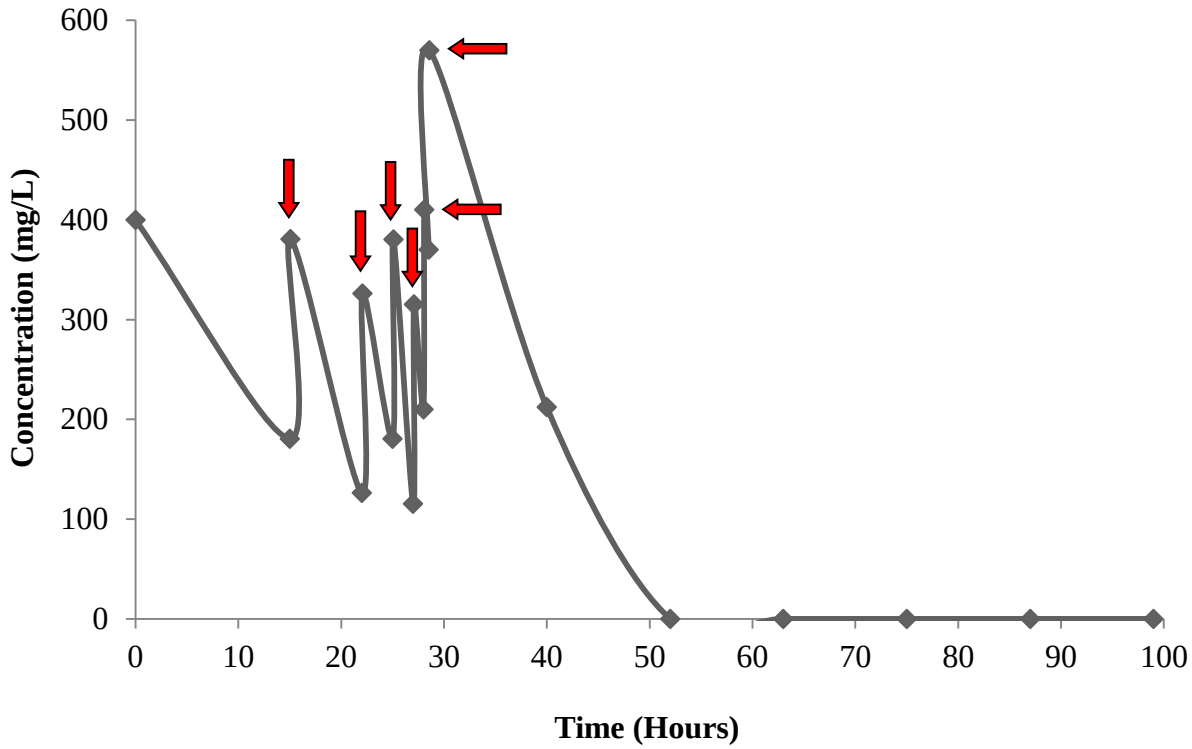

Figure 5.6. Residual levels of nitrogen in fed-batch operated for 99 hours containing a total of 8g/L nitrogen

Addition of 1g/L nitrogen within fermenter

As shown in Figure 5.6, efforts were made to maintain nitrogen levels above half of the starting concentration within the first 30 hours of the fed-batch run. Thus, nitrogen was fed into the system when levels were anticipated to drop near the 200 mg/L levels based upon estimates observed in flask and batch runs conducted previously. This prevented nitrogen limited conditions from occurring too prematurely, while maintaining levels near 400 mg/L of nitrogen. Otherwise, metabolic activity for PHA accumulation within LS46 would be triggered once nitrogen was exhausted. This prolonged the phase for cell biomass synthesis, which was fundamental to meet the goals of increasing cell biomass as illustrated in Figure 5.7.

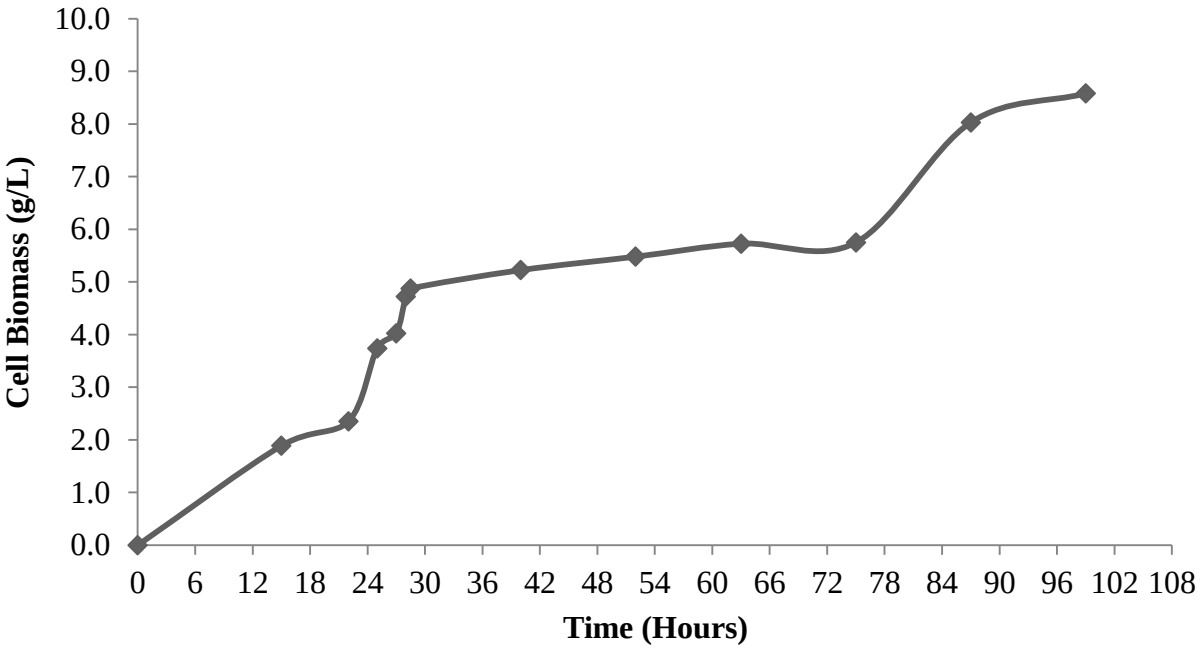

Figure 5.7. Cell biomass production by LS46 in fed-batch operated for 99 hours

Since the final two feedings were provided at closer time intervals, it is apparent that nitrogen did not decrease near the range of 200 mg/L prior to the feeding. The addition of nitrogen to the fermenter during the final two feeds increased levels well beyond the starting concentration of 400 mg/L.

As for WG, it was provided in excess at the start of the run at a concentration of 30 g/L. Thus, only three WG feeds were introduced into the fermenter to prevent excessively high levels of WG within the medium culture which could potentially have inhibitory and toxic effects on the metabolic activity of LS46 due to high salt accumulation as described in Section 4.3.3. Residual levels of glycerol as well as the feedings of WG have been illustrated in Figure 5.8.

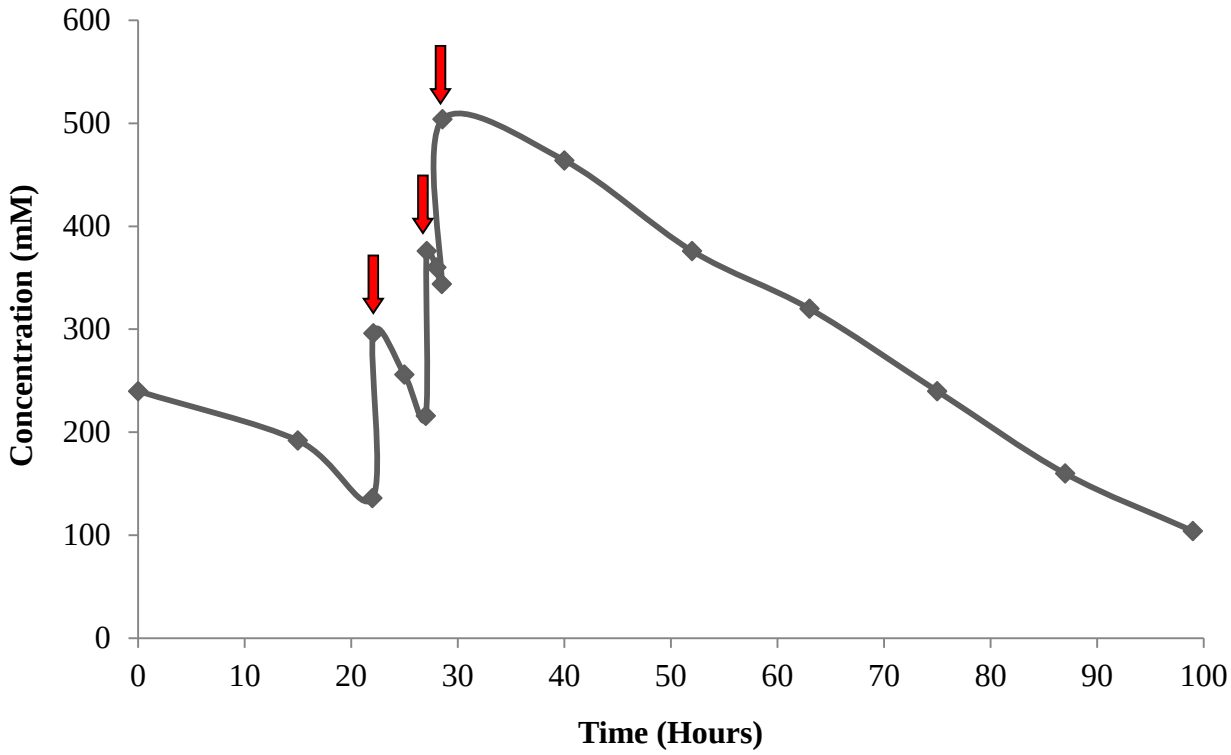

Figure 5.8. Residual levels of glycerol in fed-batch operated for 99 hours containing a total of 90g/L waste glycerol

➡ Addition of 20g/L WG within fermenter

20 g/L of WG was fed into the system at 22, 27, and 28.5 hours as indicated by the arrows in Figure 5.8. A total of 90 g/L of WG had been made available for LS46 by the conclusion of the entire run. This ensured a carbon excess environment throughout the fed-batch run which was vital to promote maximal intracellular PHA accumulation by LS46 once nitrogen levels were exhausted. Moreover, by gradually introducing additional WG into the fermenter the risk of any inhibitory effects with high WG concentrations, as was observed in flask experiments, were minimized. Additionally, substrate consumption improved based upon the lower residual levels of glycerol detected in Figure 5.8.

A steady uptake of WG was observed after the final WG feeding which was provided at the 28.5 hour time point. In combination with nitrogen levels completely exhausted near the 52 hour time point, intracellular PHA accumulation by LS46 was triggered (Figure 5.9).

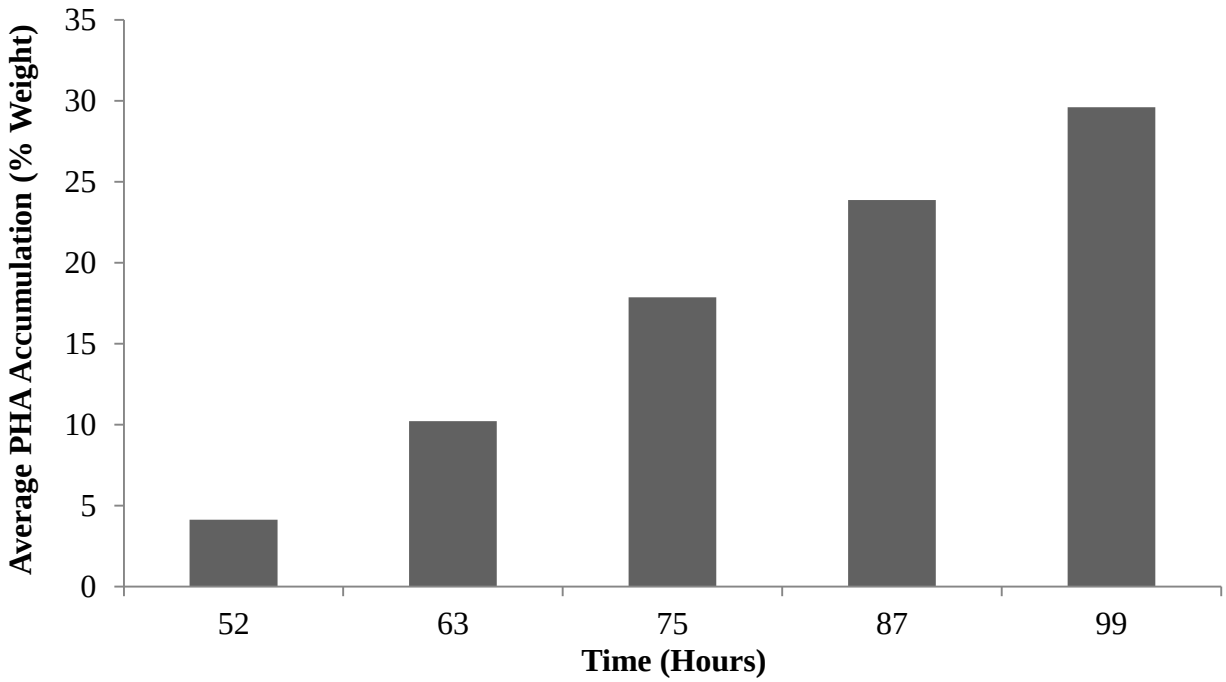

Figure 5.9. PHA accumulation by LS46 in fed-batch operated for 99 hours

Monomer composition of the PHA accumulated was detected using GC and reported in Table 5.2. Similar to previous experiments mcl-PHAs were detected with both C_8 and C_{10} monomers as the predominant monomers produced by LS46.

Table 5.2. Percent monomer composition of mcl-PHA accumulated by LS46 in fed-batch conditions						
Time (Hours)	**Monomer Composition (%)**					
	C_6	**C_8**	**C_{10}**	**C_{12}**	**$C_{12:1}$**	**C_{14}**
52	2.30	26.77	63.46	2.42	4.71	0.34
63	2.58	26.85	63.36	2.22	4.41	0.58
75	2.32	24.28	66.10	2.53	4.24	0.57
87	2.42	25.06	64.80	2.33	4.40	0.99
99	2.52	24.12	65.71	2.49	4.47	0.69

Accumulation of the targeted mcl-PHAs confirmed the absence of contamination within the fermenter over the course of the fed-batch run especially since a manual approach for feeding of the media nutrients was employed. The monomer composition achieved in this fed-batch run followed the pattern of ratios obtained in the flask-batch experiments with C_8 and C_{10} as the predominant monomers detected. Furthermore, an unsaturated C_{12} monomer was detected as well.

Overall, differences in batch versus fed-batch for mcl-PHA accumulation were not drastic. According to average estimates obtained, a maximum of 5.31 g/L cell biomass and 26.30 % cdw of PHA were achieved in batch, while a maximum of 8.58 g/L cell biomass and 29.61 % cdw of PHA were obtained in fed-batch. Nevertheless, the targeted increase in both cell biomass and mcl-PHA accumulation was achieved via fed-batch. Productivity measured in terms of PHA accumulation was higher in fed-batch versus batch at 2.54 g/L to 1.40 g/L, respectively. However, decisive conclusions regarding preference for a specific fermentation approach cannot be established considering a single run for fed-batch was conducted. Additional runs would be necessary to confirm the observations achieved.

An overall cost analysis for the operation of a fed-batch run would be the most appropriate means of determining the effectiveness and productivity of the process, i.e. evaluation for the input of energy, time, and resources must be taken into consideration. A fermentation strategy minimizing cost and input, while maximizing productivity and output is most economically desired especially when considering the realm of commercialization in industry.

Chapter 6 General Discussion and Conclusions

Based upon the experiments conducted in this research project, it is apparent that *Pseudomonas putida* LS46 is a reliable candidate for the bioconversion of biodiesel derived waste glycerol into mcl-PHAs. However, to develop a sustainable and viable fermentation strategy at large-scale commercial levels, improvements in process optimization are necessary.

To examine the potential of scaling-up production of mcl-PHAs with LS46, it was necessary to chart a progressive plan, and thus, a three stage approach was established in this case in order to gain a fundamental understanding of the general growth patterns exhibited by LS46. Synthesis of mcl-PHAs within flask was highest at approximately 15 % of cdw, while these yields were increased to near 30 % in a large volume 15 L fermenter given drastic improvements in control parameters that are not possible in flask.

Referring to reports in literature, it is obvious that additional mcl-PHA producing strains of *Pseudomonas putida* exist as well as other microorganisms that demonstrate the capacity to metabolize glycerol and a wide variety of other carbon substrates. However, for the purposes of this section the discussion will focus on glycerol as the carbon substrate.

For instance, two strains of *Pseudomonas putida*, KT2440 (Huijberts et al., 1992) and KT217 (Javers et al., 2012) have demonstrated the capacity to convert glycerol into mcl-PHAs at a cdw of approximately 22 % and 30 %, respectively. Obvious differences in cdw exist of course due to variations in medium composition and growth conditions. In this case, KT2440 was grown within 1000 mL shake-flasks, while KT217 was studied in a fermenter. Based upon GC analysis, monomers detected ranged between C_6 to C_{14}, which confirmed the accumulation of mcl-PHAs.

In another report, a few different strains of *Pseudomonas putida* were studied in a comparative analysis evaluating the conversion of waste glycerol into mcl-PHAs (Poblete-Castro et al., 2014). These strains included KT2440, KT2442, F1, and S12. Each of the strains demonstrated the capacity to convert glycerol into mcl-PHAs, however, differences in the rate of glycerol uptake and total % cdw accumulation as PHA varied. KT2440 exhibited the highest PHA accumulation at 35 % cdw, while F1 had the lowest PHA accumulation at 10 % cdw.

Compositional analysis of the mcl-PHAs accumulated by the various strains of *Pseudomonas putida* as reported throughout literature detected C_8 and C_{10} as the predominant

monomers (Ashby et al., 2005, Javers et al., 2012, Escapa et al., 2014). This pattern was also observed with LS46 in this project (Fu et al., 2014).

In comparison to other PHA producing microorganisms grown on glycerol, productivity levels exhibit obvious variations. Such variances can be attributed to the type of substrate and bacterium being used. Also, differences in the category of PHAs and their monomer composition become apparent. However, reports of different microorganisms grown on glycerol have exhibited the capacity to convert such a substrate into PHAs.

Reports on *Cupriavidus necator* have shown the conversion of waste glycerol into scl-PHAs to 38 % cdw, while on pure glycerol accumulation has been increased to 62 % cdw (Cavalheiro et al., 2009). Another scl-PHA producer, *Halomonas* sp.KM-1, was also grown on both pure and waste glycerol with PHA production being most effective on pure glycerol (Kawata & Aiba et al., 2010). In fact, results observed in 2 % pure glycerol were comparable to results achieved in 3 % waste glycerol at 40.5 % cdw versus 39 % cdw, respectively.

A characterization study of *Burkholderia* (formerly *Pseudomonas*) *cepacia* exhibited the capacity to convert biodiesel derived glycerol into scl-PHAs similar to the previous two bacteria discussed. Observations achieved showed a high accumulation of PHAs at 81.9 % cdw when using 30 g/L WG. However, increasing WG concentrations above 30 g/L resulted in decreased PHA synthesis as was observed in this research project with LS46.

All in all, it is apparent that many fermentation strategies exist for the production of PHAs given the availability of a wide array of bacteria capable of metabolizing many waste streams such as, biodiesel derived waste glycerol. However, when comparing LS46 to other strains or species, productivity may not appear to be as ideal since many commercial-scale strategies seek a high intracellular accumulation of PHAs (Wang et al., 2014). A 90 % to 95 % cdw of accumulation is considered ideal for sufficient extraction. Furthermore, to compete against current cheaper petrochemical processes, biological methods must take into consideration the overall costs and resources involved in the process. Thus, the input to output ratio must be as low as possible. Nevertheless, with further investigation into optimization of the current conditions and process parameters studied with LS46, potential for scale-up of mcl-PHA synthesis is highly feasible.

Chapter 7 Concluding Remarks

7.1 Current Investigations

Recently a heightened interest in bio-products has ensued as the search for biodegradable and eco-friendly materials such as, bioplastics develops. Heavy reliance upon fossil fuel and fossil fuel derived products has led to this rapid surge given the negative impact it has upon the earth and atmosphere as well as human health and surrounding fragile wildlife. Moreover, as current oil reserves continue to dwindle, the search for alternatives has become inevitable.

In this research project microbial production of PHAs was the main focus of study. More specifically, the production of PHAs using a novel strain of the mcl-PHA producing bacterium *Pseudomonas putida*, LS46, by growing on waste glycerol obtained from a biodiesel production plant was investigated. Crude glycerol is the main by-product of biofuel processing. Glycerol in its pure form is employed in the production of chemicals and paints as well as various food and pharmaceutical agents.

However, treatment and purification of crude glycerol is highly inefficient and costly. Therefore, by incorporating WG into a value-added bioprocess can address numerous concerns. Firstly, diversion of wastes from landfills. Secondly, the development of a cost-effective and sustainable process, and thirdly, an additional revenue stream.

Nonetheless, other studies have also focused upon PHA production albeit in different methods. For instance, genetic engineering of PHA producing microorganisms and plants has been discussed (Poirier, 1999; Suriyamonkol et al., 2007), while newer approaches have considered the introduction of PHA producing genes within non-PHA producing microorganisms and plants to initiate production of PHAs at increased rates (Valentin, 1999). However, since these are novel methods for PHA production, work on the overall application and viability at a commercial scale still remains.

At this time, microbial PHA production remains the most favourable approach given the flexible options available for feedstock and microorganisms. Moreover, it is a highly sustainable and eco-friendly method of PHA production as waste streams are diverted from landfills and directly processed into value added products.

Current investigations have tested different waste streams available from the agriculture and farming industry. For example, non-lignocellulosic agro-wastes such as, sugar cane, cassava, and beet root to mention a few, whey from the dairy industry as well as waste by-products from the production of biofuels and bio-products such as, glycerol and low-quality fatty acid esters have been employed as feedstock for microbial production of PHAs (Castilho et al., 2009; Silva-Queiroz et al., 2009). Variations in PHA polymers have been reported which include copolymers and heteropolymers with short-chain length and medium-chain length variants based upon the type of substrate used as feedstock.

Reports similar to the fermentation strategy employed in this project have been discussed in literature, however, with different strains of *Pseudomonas putida*. Two strains of *P. putida* classified as *P. oleovorans* NRRL B-14682 and *P. corrugata* 388 exhibited the production of mcl-PHAs when grown on WG, similar to LS46 (Asbhy et al., 2005). Fermentation in different WG concentrations was conducted, which demonstrated a decrease in cell biomass and mcl-PHA accumulation due osmotic stress. More specifically, higher WG concentrations had inhibitory effects on the enzymatic efficiency of both strains due to high accumulation of salts present within the WG.

In another study with *P. oleovorans* NRRL B-14682 grown on crude glycerol in batch fermentation, molecular sizes of PHA polymers was shown to be dependent upon the impurities found within the crude glycerol (Ashby et al., 2011). Furthermore, concentrations of certain contaminants such as, MeOH within the crude glycerol directly correlated with the increased rate of end-capping causing premature chain termination on the PHAs accumulated, which led to a decrease in the molecular weight of the polymers.

Thus, potential solutions to overcoming such inhibitory effects included improved purification techniques to completely remove methanol within the crude glycerol, or implementation of a staggered inoculation and feeding technique during fermentation by which polymer lengths can be controlled.

PHA producing bacteria other than *Pseudomonas putida* have also demonstrated the ability to metabolize WG such as, *Cupriavidus necator* (Cavalheiro et al., 2009), *Halmonas* sp. KM-1 (Kawata and Aiba, 2010), or recombinant strains of *Escherichia coli* (Koller et al., 2005) which provides even more potential fermentation options in lieu of current strategies.

All in all, based upon the countless choices available for microbial production of PHAs, fermentation remains a flexible option and strategy that is currently expanding as both knowledge and understanding increases.

7.2 Future Research

Based upon the data and results achieved in this research project, it would be worthwhile to consider conducting follow-up experiments, particularly in the case for fed-batch, as it would be a valuable strategy to consider for the future. Since results achieved within fed-batch went in a promising direction, it would be interesting to delve deeper with this feeding strategy and observe reproducibility of the results achieved.

Furthermore, testing different aspects within fed-batch could be considered such as, changes in the rate of feeding or the media contents of the feed itself. Moreover, some studies have tested the effects of pressure within fed-batch to enhance production of mcl-PHAs, which has demonstrated improved oxygen transfer to the cells (Follonier et al., 2012). Such a strategy can also be employed to test LS46.

Testing effects of different concentrations for dissolved oxygen within the fermenter system on overall cell biomass production and PHA synthesis by LS46 is another aspect that can be investigated as well. Lastly, evaluating different methods and procedures for the extraction of the mcl-PHAs produced by LS46 for eventual properties analysis and testing would be important to consider. Since the overall target of this research project is to produce high-yields of mcl-PHAs, it would be crucial to perform chemical and properties testing on the mcl-PHA produced to confirm the overall viability of the product for use as bio-plastic. This can also affirm the overall viability of this project for applications at the commercial scale.

7.3 Engineering Significance

It is obvious that production of alternatives to petrochemical derived plastics is highly regarded within the industry. Bio-plastics hold a significant degree of interest worldwide, however, due to the major costs and investments involved in the conventional processes of bio-plastic manufacturing further investigation into sustainable means of production are being considered.

In this research project, the development of a sustainable fermentation strategy for microbial production of mcl-PHAs with a novel strain of the gram-negative bacterium *Pseudomonas putida*, LS46, has been considered. Experiments conducted in this study have demonstrated LS46 to successfully grow and synthesize the targeted mcl-PHAs with waste glycerol as feedstock in Ramsay medium. The waste glycerol used in this study was obtained from a biodiesel production plant.

Therefore, since the glycerol produced is a waste stream in the process of biodiesel production, it would be worthwhile for the biodiesel industry to consider integration of a downstream microbial bioprocess for the production of a value added product in the form of bio-plastic. Moreover, this can help in limiting their carbon footprint upon the environment and in the process provides an added stream of profitable revenue. All in all, this research project presents a potentially viable industrial process as bio-plastics are a highly-regarded alternative to conventional petrochemical derived plastics. Moreover, the capacity to expand to commercial scales of production in a sustainable manner appears feasible.

References

Agnew, D.E., Stevermer, A.K., Youngquist, J.T., & Pfleger, B.F. (2012). Engineering *Escherichia coli* for production of C(1)(2)-C(1)(4) polyhydroxyalkanoate from glucose. *Metabolic Engineering*, 14(6), 705-713.

Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H., & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology*, 50(4), 1563-1589.

Ashby, R.D., Solaiman, D.K., & Foglia, T.A. (2005). Synthesis of short-/medium-chain-length poly (hydroxyalkanoate) blends by mixed culture fermentation of glycerol. *Biomacromolecules*, 6(4), 2106–2112.

Ashby, R.D., Solaiman, D., & Strahan, G.D. (2011). Efficient utilization of crude glycerol as fermentation substrate in the synthesis of poly (3-hydroxybutyrate) biopolymers. *Journal of the American Oil*, 88, 949-959.

Ballerstedt, H., Volkers, R.J., Mars, A.E., Hallsworth, J.E., Dos Santos, V.A.M., Puchalka, J., van Duuren, J., Eggnik, G., Timmis, K.N., de Bont, J.A.M., & Wery, J. (2007). Genomotyping of Pseudomonas putida strains using P. putida KT2440-based high-density DNA microarrays: implications for transcriptomics studies. *Applied Microbiology and Biotechnology*, 75(5), 1133-1142.

Bax, B., & Müssig, J. (2008). Impact and tensile properties of PLA/Cordenka and PLA/flax composites. *Composites Science and Technology*, 68 (7), 1601-1607.

Bengtsson, S., Pisco, A. R., Johansson, P., Lemos, P. C., & Reis, M. A. (2010). Molecular weight and thermal properties of polyhydroxyalkanoates produced from fermented sugar molasses by open mixed cultures. *Journal of Biotechnology*, 147 (3), 172-179.

Bohmert-Tatarev, K., McAvoy, S., Daughtry, S., Peoples, O., & Snell, K. (2011). High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. *Plant physiology*, 155(4), 1690-1708.

Castilho, L.R., Mitchell, D.A., & Freire, D.M. (2009). Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology*, 100(23), 5996-6009.

Cavalheiro, J.M., de Almeida, M.C.M., Grandfils, C., & Da Fonseca, M.M.R. (2009). Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry*, 44(5), 509-515.

Chanprateep, S. (2010). Current trends in biodegradable polyhydroxyalkanoates. *Journal of Bioscience and Bioengineering*, 110(6), 621-632.

Chee, J.Y., Tan, Y., Samian, M.R., & Sudesh, K. (2010). Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment*, 18(4), 584-592.

Chen, L.J., & Wang, M. (2002). Production and evaluation of biodegradable composites based on PHB–PHV copolymer. *Biomaterials*, 23(13), 2631-2639.

Chen, G.Q., & Wu, Q. (2005). The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials*, 26(33), 6565-6578.

Chen, G.Q., & Wang, Y. (2013). Medical applications of biopolyesters polyhydroxyalkanoates. *Chinese Journal of Polymer Science*, *31*, 719-736.

Cheng, G., Cai, Z., & Wang, L. (2003). Biocompatibility and biodegradation of poly (hydroxybutyrate)/poly (ethylene glycol) blend films. *Journal of Materials Science: Materials in Medicine*, 14(12), 1073-1078.

Chung, A.-L., Jin, H.-L., Huang, L.-J., Ye, H.-M., Chen, J.-C., Wu, Q., & Chen, G.-Q. (2011). Biosynthesis and characterization of poly(3-hydroxydodecanoate) by β-oxidation inhibited mutant of *Pseudomonas entomophila* L48. *Biomacromolecules*, 12(10), 3559-3566.

Davis, R., Kataria, R., Cerrone, F., Woods, T., Kenny, S., O'Donovan, A., Guzik, M., Shaikh, H., Gearoid, D., Gupta, V.K., Tuohy, M.G., Padamatti, R.B., Casey, E., & O'Connor, K.E. (2013). Conversion of grass biomass into fermentable sugars and its utilization for medium chain length polyhydroxyalkanoate (mcl-PHA) production by *Pseudomonas strains*. *Bioresource Technology*, 150, 202-209.

Doyle, C., Tanner, E. T., & Bonfield, W. (1991). In vitro and in vivo evaluation of polyhydroxybutyrate and of polyhydroxybutyrate reinforced with hydroxyapatite. *Biomaterials*, 12(9), 841-847.

Escapa, I.F., Del Cerro, C., García, J. L., & Prieto, M.A. (2013). The role of GlpR repressor in *Pseudomonas putida* KT2440 growth and PHA production from glycerol. *Environmental Microbiology*, 15(1), 93-110.

Espinosa-Urgel, M., Salido, A., & Ramos, J.L. (2000). Genetic analysis of functions involved in adhesion of Pseudomonas putida to seeds. *Journal of bacteriology*, 182(9), 2363-2369.

Follonier, S., Henes, B., Panke, S., & Zinn, M. (2012). Putting cells under pressure: a simple and efficient way to enhance the productivity of medium-chain-length polyhydroxyalkanoate in processes with *Pseudomonas putida* KT2440. *Biotechnology Bioengineering*, 109(2), 451-461.

Follonier, S., Panke, S., & Zinn, M. (2011). A reduction in growth rate of *Pseudomonas putida* KT2442 counteracts productivity advances in medium-chain-length polyhydroxyalkanoate production from gluconate. *Microbial Cell Factories*, 10(1), 1-11.

Fu, J., Sharma, U., Sparling, R., Cicek, N. Levin, D.B.. (2014). Evaluation of medium-chain length polyhydroxyalkanoates production by *Pseudomonas putida* LS46 using biodiesel by-products stream. *Canadian Journal of Microbiology*, 60(7), 461-468.

Gao, X., Chen, J.C., Wu, Q., & Chen, G.Q. (2011). Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels. *Current Opinions in Biotechnology*, 22(6), 768-774.

Garcia, I.L., López, J.A., Dorado, M.P., Kopsahelis, N., Alexandri, M., Papanikolaou, S., & Koutinas, A.A. (2013). Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly (3-hydroxybutyrate-co-3-hydroxyvalerate) production by *Cupriavidus necator*. *Bioresource Technology*, 130, 16-22.

Gomes, N.C., Kosheleva, I.A., Abraham, W.R., & Smalla, K. (2005). Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. *FEMS Microbiology Ecology*, 54(1), 21-33.

Gumel, A.M., Annuar, M., & Chisti, Y. (2013). Recent advances in the production, recovery and applications of polyhydroxyalkanoates. *Journal of Polymers and the Environment*, 21(2), 580-605.

Gumel, A.M., Annuar, M.S.M., & Heidelberg, T. (2013). Current application of controlled degradation processes in polymer modification and functionalization. *Journal of Applied Polymer Science*, 129(6), 3079-3088.

Huijberts, G.N., Eggink, G., De Waard, P., Huisman, G.W., & Witholt, B. (1992). Pseudomonas putida KT2442 cultivated on glucose accumulates poly (3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. *Applied and Environmental Microbiology*, 58(2), 536-544.

Ienczak, J., Schmidell, W., & Aragão, G. (2013). High-cell-density culture strategies for polyhydroxyalkanoate production: a review. *Journal of Industrial Microbiology & Biotechnology*, 40(3), 275-286.

Jacquel, N., Lo, C.-W., Wei, Y.-H., Wu, H.-S., & Wang, S. S. (2008). Isolation and purification of bacterial poly(3-hydroxyalkanoates). *Biochemical Engineering Journal,* 39(1), 15-27.

Javers, J., & Karunanithy, C. (2012). Polyhydroxyalkanoate Production by *Pseudomonas putida* KT217 on a Condensed Corn Solubles Based Medium Fed with Glycerol Water or Sunflower Soapstock. *Advances in Microbiology*, 2(3), 241-251.

Jian, J., Zhang, S.-Q., Shi, Z.-Y., Wang, W., Chen, G.-Q., & Wu, Q. (2010). Production of polyhydroxyalkanoates by *Escherichia coli* mutants with defected mixed acid fermentation pathways. *Applied Microbiology and Biotechnology*, 87(6), 2247-2256.

Jiang, X., Ramsay, J., & Ramsay, B. (2006). Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440. *Journal of Microbiological Methods*, 67(2), 212-219.

Jiménez, J.I., Miñambres, B., Garcia, J.L., & Díaz, E. (2002). Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environmental Microbiology*, 4(12), 824-841.

Kathiraser, Y., Aroua, M.K., Ramachandran, K.B., & Tan, I.K.P. (2007). Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. *Journal of Chemical Technology & Biotechnology*, 82(9), 847-855.

Kawata, Y., & Aiba, S. (2010). Poly (3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol. *Bioscience,* 74(1), 175-177.

Kenny, S.T., Runic, J.N., Kaminsky, W., Woods, T., Babu, R.P., & O'Connor, K.E. (2012). Development of a bioprocess to convert PET derived terephthalic acid and biodiesel derived glycerol to medium chain length polyhydroxyalkanoate. *Applied Microbiology & Biotechnology*, 95(3), 623-633.

Kim, G.K., Lee, I.Y., Yoon, S.C., Shin, Y.C., & Park, Y.H. (1997). Enhanced yield and a high production of medium-chain-length poly (3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. *Enzyme and Microbial Technology*, 20(7), 500-505.

Koller, M., Bona, R., Braunegg, G., & Hermann, C. (2005). Production of polyhydroxyalkanoates from agricultural waste and surplus materials. *Biomacromolecules*, 6, 561-565.

Koller, M., Bona, R., Chiellini, E., & Braunegg, G. (2013). Extraction of short-chain-lengthpoly-[(R)-hydroxyalkanoates] (scl-PHA) by the "anti-solvent" acetone under elevated temperature and pressure. *Biotechnology Letters*, 35(7), 1023-1028.

Köse, G.T., Ber, S., Korkusuz, F., & Hasirci, V. (2003). Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) based tissue engineering matrices. *Journal of Materials Science: Materials in Medicine*, 14(2), 121-126.

Koutsoumanis, K.P., & Sofos, J.N. (2005). Effect of inoculum size on the combined temperature, pH and a w limits for growth of *Listeria* monocytogenes. *International Journal of Food Microbiology*, 104(1), 83-91.

Kunasundari, B., & Sudesh, K. (2011). Isolation and recovery of microbial polyhydroxyalkanoates. *Express Polymer Letters*, 5(7), 620-634.

Laycock, B., Halley, P., Pratt, S., Werker, A., & Lant, P. (2014). The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science*, 39(2), 397-442.

Lee, G., & Na, J. (2012). Future of microbial polyesters. *Microbial Cell Factories*, 12(1), 54-57.

Lee, S., Choi, J., & Wong, H. (1999). Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. *International Journal of Biological Macromolecules*, 25(1), 31-36.

Le Meur, S., Zinn, M., Egli, T., Thony-Meyer, L., & Ren, Q. (2012). Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered *Pseudomonas putida* KT2440. *BioMed Central*, 12(1), 53-64.

Liu, Q., Luo, G., Zhou, X.R., & Chen, G.Q. (2011). Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by beta-oxidation pathway inhibited *Pseudomonas putida*. *Metabolic Engineering*, 13(1), 11-17.

Luo, L., Wei, X., & Chen, G. Q. (2009). Physical properties and biocompatibility of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) blended with poly (3-hydroxybutyrate-co-4-hydroxybutyrate). *Journal of Biomaterials Science, Polymer Edition*, 20 (11), 1537-1553.

Martin, D.P., & Williams, S.F. (2003). Medical applications of poly-4-hydroxybutyrate: a strong flexible absorbable biomaterial. *Biochemical Engineering Journal*, 16(2), 97-105.

Matsumoto, K., Morimoto, K., Gohda, A., Shimada, H., & Taguchi, S. (2011). Improved polyhydroxybutyrate (PHB) production in transgenic tobacco by enhancing translation efficiency of bacterial PHB biosynthetic genes. *Journal of Bioscience and Bioengineering* 111(4), 485-488.

Misra, S.K., Ohashic, F., Valappil, S.P., Knowles, J.C., Roy I., Silva, S.R.P., Salih, V., Boccaccini, A.R. (2010). Characterization of carbon nanotube (MWCNT) containing P(3HB)/bioactive glass composites for tissue engineering applications. *Acta Biomaterialia*, 6(3), 735–742.

Moita, R., & Lemos, P.C. (2012). Biopolymers production from mixed cultures and pyrolysis by-products. *Journal of Biotechnology*, 157(4), 578-583.

Molina, L., Ramos, C., Duque, E., Ronchel, M.C., García, J.M., Wyke, L., & Ramos, J.L. (2000). Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. *Soil Biology and Biochemistry*, 32(3), 315-321.

Morgan-Sagastume, F., Karlsson, A., Johansson, P., Pratt, S., Boon, N., Lant, P., & Werker, A. (2010). Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus. *Water Resource*, 44(18), 5196-5211.

Mothes, G., Schnorpfeil, C., & Ackermann, J.U. (2007). Production of PHB from crude glycerol. *Engineering in Life Sciences*, 7(5), 475–479.

Murueva, A.V., Shishatskaya, E.I., Kuzmina, A.M., Volova, T.G., & Sinskey, A.J. (2013). Microparticles prepared from biodegradable polyhydroxyalkanoates as matrix for encapsulation of cytostatic drug. *Journal of Materials Science: Materials in Medicine*, 24(8), 1905-1915.

Nakazawa, T., & Yokota, T. (1973). Benzoate metabolism in *Pseudomonas putida* (arvilla) mt-2: demonstration of two benzoate pathways. *Journal of bacteriology*, 115(1), 262-267.

Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A. P., ... & Fraser, C. M. (2002). Complete genome sequence and comparative analysis of the

metabolically versatile Pseudomonas putida KT2440. *Environmental microbiology*, 4(12), 799-808.

Ni, J., & Wang, M. (2002). In vitro evaluation of hydroxyapatite reinforced polyhydroxybutyrate composite. *Materials Science and Engineering*, 20(1), 101-109.

Noda, I., Green, P. R., Satkowski, M. M., & Schechtman, L. A. (2005). Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. *Biomacromolecules*, 6(2), 580-586.

Nopharatana, A., Pullammanappallil, P.C., & Clarke, W.P. (2007). Kinetics and dynamic modelling of batch anaerobic digestion of municipal solid waste in a stirred reactor. *Waste Management*, 27, 595-603.

Ojumu, T.V., Yu, J., & Solomon, B.O. (2004). Production of polyhydroxyalkanoates, a bacterial biodegradable polymers. *African Journal of Biotechnology*, 3(1), 18-24.

Penloglou, G., Chatzidoukas, C., & Kiparissides, C. (2012). Microbial production of polyhydroxybutyrate with tailor-made properties: an integrated modeling approach and experimental validation. *Biotechnology Advances*, 30(1), 329-337.

Poblete-Castro, I., Escapa, I.F., Jäger, C., Puchalka, J., Lam, C.M.C., Schomburg, D., & dos Santos, V.A.M. (2012). The metabolic response of *P. putida* KT2442 producing high levels of polyhydroxyalkanoate under single-and multiple-nutrient-limited growth: Highlights from a multi-level omics approach. *Microbial Cell Factories*, 11(1), 113-123.

Poblete-Castro, I., Becker, J., Dohnt, K., dos Santos, V.M., & Wittmann, C. (2012). Industrial biotechnology of *Pseudomonas putida* and related species. *Applied Microbiology and Biotechnology*, 93(6), 2279-2290.

Poblete-Castro, I., Binger, D., Rodrigues, A., Becker, J., dos Santos, V.A.M., & Wittmann, C. (2013). In-silico-driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates. *Metabolic Engineering*, 15, 113-123.

Poblete-Castro, I., Binger, D., Oehlert, R., & Rohde, M. (2014). Comparison of mcl-Poly (3-hydroxyalkanoates) synthesis by different *Pseudomonas putida* strains from crude glycerol: citrate accumulates at high titer under PHA-producing conditions. *BMC Biotechnology*, 14(1), 962-973.

Poirier, Y. (1999). Production of new polymeric compounds in plants. *Current Opinion in Biotechnology*, 10, 181-185.

Posada, J.A., Naranjo, J.M., López, J.A., Higuita, J.C., & Cardona, C.A. (2011). Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol. *Process Biochemistry*, 46(1), 310-317.

Puchałka, J., Oberhardt, M.A., Godinho, M., Bielecka, A., Regenhardt, D., Timmis, K.N., Papin, J.A., Vitor, A.P., & dos Santos, V.A.M. (2008). Genome-scale reconstruction and analysis of the *Pseudomonas putida* KT2440 metabolic network facilitates applications in biotechnology. *PLoS computational biology*, 4(10), e1000210.

Rai, R., Keshavarz, T., Roether, J.A., Boccaccini, A.R., & Roy, I. (2011). Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Materials Science and Engineering*, 72(3), 29-47.

Ramsay, B., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., & Ramsay, J.A. (1990). Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. *Applied and Environmental Microbiology*, 56(7), 2093–2098.

Reddy, C.S.K., Rashmi, G., & Kalia, V.C. (2003). Polyhydroxyalkanoates: an overview. *Bioresource technology*, 87(2), 137–146.

Reddy, V.M., & Mohan, V.S. (2012). Effect of substrate load and nutrients concentration on the polyhydroxyalkanoates (PHA) production using mixed consortia through wastewater treatment. *Bioresource Technology*, 114, 573–582

Reemmer J. (2009). Advances in the synthesis and extraction of biodegradable polyhydroxyalkanoates in plant systems: a review. Basic Biotechnology, 5(1), 44–49.

Ren, Q., de Roo, G., Witholt, B., Zinn, M., & Thony-Meyer, L. (2010). Influence of growth stage on activities of polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida* U. *BMC Microbiology*, 10, 254.

Riedel, S.L., Brigham, C.J., Budde, C.F., Bader, J., Rha, C., Stahl, U., & Sinskey, A.J. (2012). Recovery of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from *Ralstonia eutropha* cultures with non-halogenated solvents. *Biotechnology Bioengineering*, 110 (2), 10.

Sánchez, R.J., Schripsema, J., da Silva, L.F., Taciro, M.K., Pradella, J.G., & Gomez, J.G.C. (2003). Medium-chain-length polyhydroxyalkanoic acids (PHA mcl) produced by *Pseudomonas putida* IPT 046 from renewable sources. *European Polymer Journal*, 39(7), 1385-1394.

Sevastianov, V.I., Perova, N.V., Shishatskaya, E.I., Kalacheva, G.S., & Volova, T.G. (2003). Production of purified polyhydroxyalkanoates (PHAs) for applications in contact with blood. *Journal of Biomaterials Science: Polymer Edition*, 14(10), 1029-1042.

Shah, A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: a comprehensive review. *Biotechnology Advances*, 26(3), 246–265.

Shishatskaya, E.I., & Volova, T.G. (2004). A comparative investigation of biodegradable polyhydroxyalkanoate films as matrices for in vitro cell cultures. *Journal of Materials Science: Materials in Medicine*, 15(8), 915-923.

Sharma P.K., Fu J., Cicek N., Sparling R., & Levin D.B. (2012). Kinetics of medium-chain-length polyhydroxyalkanoate production by a novel isolate of *Pseudomonas putida* LS46. *Canadian Journal of Microbiology*, 58(8), 982-989.

Silva-Queiroz, S.R., Silva, L.F., & Pradella, J. (2009). PHA MCL biosynthesis systems in Pseudomonas aeruginosa and Pseudomonas putida strains show differences on monomer specificities. *Journal of Biotechnology*, 143, 111-118.

Slinn, M., Kendall, K., Mallon, C., & Andrews, J. (2008). Steam reforming of biodiesel by-product to make renewable hydrogen. *Bioresource Technology*, 99(13), 5851-5858.

Song, J.H., Jeon, C.O., Choi, M.H., & Yoon, S.C. (2008). Polyhydroxyalkanoate (PHA) production using waste vegetable oil by Pseudomonas sp. strain DR2. *Journal of Microbiology*, 18(8), 1408-1415.

Sudesh, K., Abe, H., & Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science*, 25(10), 1503-1555.

Sun, Z., Ramsay, J.A., Guay, M., & Ramsay, B.A. (2007). Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by Pseudomonas putida KT2440. *Applied Microbiology and Biotechnology*, 74(1), 69-77.

Suriyamongkol, P., Weselake, R., & Narine, S. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants - a review. *Biotechnology Advances*, 25(2), 148-175.

Tappel, R.C., Wang, Q., & Nomura, C.T. (2012). Precise control of repeating unit composition in biodegradable poly(3-hydroxyalkanoate) polymers synthesized by *Escherichia coli*. *Journal of Bioscience & Bioengineering*, 113(4), 480-486.

Tilbrook, K., Gebbie, L., Schenk, P.M., Poirier, Y., & Brumbley, S. M. (2011). Peroxisomal polyhydroxyalkanoate biosynthesis is a promising strategy for bioplastic production in high biomass crops. *Plant Biotechnology Journal*, 9(9), 958-969.

Timmis, K.N. (2002). *Pseudomonas putida*: a cosmopolitan opportunist par excellence. Environmental Microbiology, 4, 779 – 781.

Valentin, H.E., Broyles, D.L., & Casagrande, L.A. (1999). PHA production, from bacteria to plants. *International journal of Biological Macrocmolecules*, 25, 303-306.

Vogel, S.A. (2009). The Politics of Plastics: The Making and Unmaking of Bisphenol A "Safety." *American Journal of Public Health*, 99(3), 559-566.

Verlinden, R.A., Hill, D.J., Kenward, M.A., Williams, C.D., & Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology*, 102(6), 1437-1449.

Wang, F., & Lee, S.Y. (1997). Poly (3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of *Alcaligenes latus* under Nitrogen Limitation. *Applied and Environmental Microbiology*, 63(9), 3703-3706.

Wang, Q., & Nomura, C.T. (2010). Monitoring differences in gene expression levels and polyhydroxyalkanoate (PHA) production in *Pseudomonas putida* KT2440 grown on different carbon sources. *Journal of bioscience and bioengineering*, 110(6), 653-659.

Wang, Y., Yin, J., & Chen, G. Q. (2014). Polyhydroxyalkanoates, challenges and opportunities. *Current Opinion in Biotechnology*, 30, 59-65.

Wang, Y.W., Wu, Q., Chen, J., & Chen, G.Q. (2005). Evaluation of three-dimensional scaffolds made of blends of hydroxyapatite and poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) for bone reconstruction. *Biomaterials*, 26(8), 899-904.

Ward, P.G., Goff, M., Donner, M., Kaminsky, W., & O'Connor, K.E. (2006). A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. *Environmental Science & Technology*, 40(7), 2433-2437.

Webb, W.R., Dale, T.P., Lomas, A.J., Zeng, G., Wimpenny, I., El Haj, A.J., & Chen, G.Q. (2013). The application of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) scaffolds for tendon repair in the rat model. *Biomaterials*, 34(28), 6683-6694.

Widdel, F. (2007). Theory and measurement of bacterial growth. *Di dalam Grundpraktikum Mikrobiologie*, 4, 11.

Williams, P.A., & Murray, K. (1974). Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. *Journal of Bacteriology*, 120(1), 416-423.

Williams, S.F., & Martin, D.P. (2003). *U.S. Patent No. 6,623,730*. Washington, DC: U.S. Patent and Trademark Office.

Williams, S.F., Martin, D.P., Gerngross, T., & Horowitz, D.M. (2003). *U.S. Patent No. 6,623,749*. Washington, DC: U.S. Patent and Trademark Office.

Yang, Y.H., Brigham, C., Willis, L., Rha, C., & Sinskey, A. (2011). Improved detergent-based recovery of polyhydroxyalkanoates (PHAs). *Biotechnology Letters*, 33(5), 937-942.

Yasotha, K., Aroua, M.K., Ramachandran, K.B., & Tan, I.K.P. (2006). Recovery of medium-chain-length polyhydroxyalkanoates (PHAs) through enzymatic digestion treatments and ultrafiltration. *Biochemical Engineering Journal*, 30(3), 260-268.

Zhang, C.H., Wu, J.Y., & He, G.Y. (2002). Effects of inoculum size and age on biomass growth and paclitaxel production of elicitor-treated *Taxus yunnanensis* cell cultures. *Applied Microbiology and Biotechnology*, 60(4), 396-402.

Zhang, Y., Dube, M.A., McLean, D.D., & Kates, M. (2003a). Biodiesel production from waste cooking oil: 1. Process design and technological assessment. *Bioresource Technology*, 89(1), 1-16.

Zhang, Y., Dube, M.A., McLean, D.D., & Kates, M. (2003b). Biodiesel production from waste cooking oil: 2. Economic assessment and sensitivity analysis. *Bioresource Technology*, 90(3), 229-240.

Zhang, X., Luo, R., Wang, Z., Deng, Y., & Chen, G.Q. (2009). Application of (R)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. *Biomacromolecules*, 10(4), 707-711.

Zhu, C., Chiu, S., Nakas, J.P., & Nomura, C.T. (2013). Bioplastics from waste glycerol derived from biodiesel industry. *Journal of Applied Polymer Science*, 130(1), 1-13.