Guillermo A. D'Alessio Cagnone & Alejandro Luque Gil

**Master's Thesis**

# Optimization of a batch process for production of biopolymers using low-cost feedstocks.

**Faculty of Engineering/Faculty of Agricultural & Food Sciences**

**Department of Biosystems Engineering**

Supervisor: Dr. David Levin

University of Manitoba, Winnipeg, Manitoba

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## ABSTRACT

During the last century, the preoccupation concerning the environment has increased. This fact is due to the high consumption of products from non-renewables sources which processing, seriously damages the ecosystem. One of the most important non-renewables sources nowadays remains petroleum. Petroleum is used, mostly, as a raw material for obtaining energy. According to the International Energy Agency, in 2013 the world consumption of energy was 1,894.28 kg of oil equivalent per capita and more than 80% came from fossil fuel sources. For this reason, one of the main aims of the technology is trying to reduce its consumption in, inter alia, the production of petroleum derivatives.

One important application of petroleum derivates are polymers. Since its invention at the ending of the 19$^{th}$ century, plastics have become very important in our lives. This material has become a focal component in most of the day-to-day activities. From phones to prosthesis, plastic is in our lives due to its properties and easy manufacturing. However, its production can be carried out through other more environmentally sustainable processes.

In 1925, the microbiologist Maurice Lemoigne discovered the first polyhydroxyalkanoate (PHA) which years after will be used for the manufacturing of biodegradable plastics. The discovery of the so-called bioplastics was a hope to reduce the consumption of plastics that come from petroleum. The most important property of bioplastics is its capability for being 100% degradable and recyclable, not only by chemicals but also by microorganisms as bacteria.

These plastics can be produced by different species of bacteria. One of the most employed is *Pseudomonas putida* which will be characterized later in this thesis. To obtain a high performance in the production of bioplastics there are different factors to consider: from the nutrient-limitation during the growth of bacteria to the extraction process.

In spite of the advantages of bioplastics compared to conventional plastics, there is still a limiting factor that slows its manufacturing: the high cost of production. Although increasingly closer to its value, bioplastics should be economized even more to be able to compete with the other plastics.

Due to all these facts, this thesis tries to find an optimal way to produce PHA from *Pseudomonas putida* using a low-cost feedstock, acidified Canola fatty acids. PHA production in bioreactors obtained the maximum amount of 12.75% in 2.00 g/L of CDW. The production in flasks was significantly higher arriving to values as high as 30.97%.

## ACKNOWLEDGMENTS

First of all, we want to thank Dr. David Levin for giving us the opportunity of working on a project in his laboratory. He was always there during our stay to make us feel comfortable. We also want to thank Warren Blunt and Christopher Dartiailh for letting us collaborate in their study about *Pseudomonas putida* LS46 and give us support in our thesis. Finally, we want to thank Riffat I. Munir and Parveen Sharma that both of them helped us in the laboratory every time need was required.

Our experience at University of Manitoba was really gratifying. We had the opportunity of working in a different environment that we were used to. It was a notable chance to learn how to share knowledge with people from different cultures and learn from all of them.

*Guillermo*

Ending another stage, I want to thank my parents and siblings, as they have been a source of inspiration for me and the fact that they always encouraged me to set my thresholds as far as I wanted.

On one hand, I would to thank all the amazing people that I have met during my exchange in both semesters, knowing for sure that we will keep a life-time friendship. On the other hand, I would like to thanks all the friends back home that encourage me and remained me that distance is just a word.

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## NOMENCLATURE

$ACAS$: Acidified canola fatty acids

$CAGR$: Compound annual growth rate

$DOT$: Dissolved oxygen tension

$CDW$: Cell dry weight

$GC$: Gas Chromatography

$h\ pi$: Hours post-inoculation

$MIMS$: Membrane inlet mass spectrometer

$PBS$: Phosphate-buffered saline

$P$HA: Polyhydroxyalkanoate

$PHB$: Polyhydroxybutyrate

$PHBV$: Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

$P.putida$: *Pseudomonas putida*

$RMM$: Ramsay's minimum media

# INTRODUCTION

## Objectives

Professor David Levin, in the Department of Biosystems Engineering, at the University of Manitoba, studies the production of biopolymers called Polyhydroxyalkanoates, (PHAs) which are both biodegradable and biocompatible. The overall objective of the research described in this thesis was to improve the production of PHAs. The specific objectives of this thesis were:

Objective 1: to find the optimal rate of C/N/P for the Ramsay's medium with the aim of obtaining the maximum production of PHA, as well as the maximum growth of the bacteria with a new low-cost substrate (free fatty acids from canola oil).

Objective 2: to study the different production rates of PHA using a bioreactor with different oxygen-limited conditions.

## Organization of the thesis

The thesis is organized in order to accomplish a better understanding of the objectives. Therefore, it is separated in Phase I and Phase II.

While performing Phase I and Phase II simultaneously, new information emerged and additional experiments were performed and have been incorporated into the thesis as "Other experiments"

# LITERATURE REVIEW

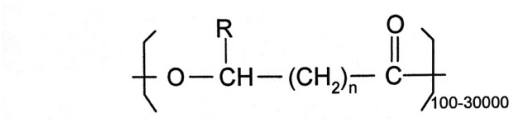
## Polyhydroxyalkanoates

Synthetic polymers derived from petroleum, collectively known as Plastics, have become significant since the 1940s, and since then have replaced many traditional constructional materials, such as glass, wood, and even metals, in many industrial, domestic, and environmental applications (Poirier et al. 1995; Cain 1992; Lee 1996; Lee et al. 1991). These widespread applications are not only due to their favourable mechanical and thermal properties, but mainly due to their stability, durability, and very low cost (Rivard et al. 1995). However, because of these properties petroleum-based plastics have been used in many short-live, disposable products, such as packaging materials, which have become a major environmental problem. The rate of biodegradation of petroleum-based plastics to 100s to 1000s of years, and hence they are considered as "non-biodegradable. Due to their persistence, they accumulate in the environment, and increasing numbers of communities are concerned about the impact of plastic waste (Rivard et al. 1995; Poirier et al. 1995; Witt et al. 1997; Muller et al. 2001).

Polyhydroxyalkanoates (PHAs) are gaining increasing attention in the biodegradable polymer market due to their promising properties, such as high biodegradability. Likewise, there has been considerable interest in the development and production of biodegradable polymer to solve the current problem of pollution caused by the continuous use of petroleum-based polymers.

Most commercially available PHAs are obtained with pure microbial cultures grown on renewable feedstocks (i.e. glucose) under sterile conditions. Recent research has focused on the use of agri-industrial waste streams as low cost carbons sources for bacterial growth media. PHA production by bacterial can utilize carbohydrates such as glucose and sucrose, or fatty acids from used fryer oil or glycerol from biodiesel production. Researchers in industry are also working on methods with which transgenic crops will be developed that express PHA synthesis routes from bacteria to produce PHA as energy storage in their tissues (Bugnicourt et al. 2014).

Polyhydroxyalkanoates are accumulated as intracellular inclusion bodies, or granules, in some bacteria under conditions of physiological stress. The PHA polymers serve as carbon and energy storage molecules for the bacteria when soluble carbon sources ar not available in their environment. PHAs can be extracted from the bacteria cells and then formulated and processed by extrusion to produce rigid or flexible plastic materials suitable for a variety of medical and industrial applications, including biomedical materials, packaging materials, resins in biocomposite materials, moulded goods, non-woven fabrics, adhesives, coatings, and films.




| | | | |
|---|---|---|---|
| n =1 | R = | hydrogen | poly (-3-hydroxypropionate) |
| | | methyl | poly (-3-hydroxybutyrate) |
| | | ethyl | poly(3-horoxyvalerate) |
| | | propyl | poly (-3-hydroxyhexanoate) |
| | | pentyl | poly (-3-hydroxyoctanoate) |
| | | nonyl | poly (-3-hydroxydodecanoate) |
| n = 2 | R = | hydrogen | poly (-4-hydroxybutyrate) |
| n = 3 | R = | hydrogen | poly (-5-hydroxyvalerate) |

**Figure 1.** The general structure of polyhydroxyalkanoates (Ojumu et al. 2004).

PHAs are generally divided into two groups: short-chain-length (SCL-) PHAs and medium-chain-length (mcl-) PHAs. SCL-PHAs consist of (R)-hydroxyalkanoates of $C_3$-$C_5$, while mcl-PHAs are comprised of aliphatic and/or aromatic (R)-hydroxyalkanoates of $C_6$ – $C_{14}$. (Kim 2007).

General properties of polyhydroxyalkanoates

PHAs polymers are thermoplastic and they differ in their properties depending on their chemical composition (homo- or co-polyesters, containing 3-hydroxyl fatty acids). Some SCL-PHA, such as poly(3-hydroxybutyrate), abbreviated PHB, are similar in their material properties to polypropylene (PP), and offer good resistance to moisture and aroma barrier properties. PHB is a fragile material due to re-crystallization with ageing at room temperature. Thus, mechanical properties change with time and samples stored at room temperature for 60 days have lower values for elongation-at-break than samples stored for 30 days. For these reasons efforts in compounding PHA are mainly focused on the search of plasticizer and nucleating agents capable of reducing the crystallization process and improving flexibility and elongation in the final product (Bugnicourt et al. 2014).

**Table 1.** Range of typical properties of PHAs (Bugnicourt et al. 2014).

| Property* [units] | Values |
|---|---|
| $T_g$ [ºC] | 2 |
| $T_m$ [ºC] | 160-175 |
| $X_{cr}$ [%] | 40-60 |
| $E$ [GPa] | 1-2 |
| $\sigma$ [MPa] | 15-40 |
| $\varepsilon$ [%] | 1-15 |
| $WVTR$ [g·mm/m$^2$·day] | 2.36 |
| $OTR$ [cc·mm/m$^2$·day] | 55.12 |

*$T_g$: glass transition temperature; $T_m$: melting temperature; $X_{cr}$: crystallinity degree; $E$: Young's modulus; $\sigma$: tensile strength; $\varepsilon$: elongation at break; $WVTR$: water vapour transmission rate; $OTR$: oxygen transmission rate.

Thermal and mechanical properties of polyhydroxyalkanoates

Isostatic PHB displays a number of properties comparable to petroleum-based polymers (e.g.PP), such as high melting temperature (175 ºC) and relatively high tensile strength (30-35 MPa). However, pure PHB has had only limited commercial use because of its intrinsic brittleness (presenting low strain at break) and the narrow processing window of this plastic. Indeed, the elongation at break is very different between PHB (5%) and PP (400%) (El-Hadi et al. 2002). There are two principal reasons for the brittleness of PHAs: the secondary crystallization of the amorphous phase takes place during storage at room temperature (the glass transition temperature of PHB is close to room temperature), and PHAs in general have a low nucleation density, therefore large spherulites exhibit inter-spherulitic cracks (Bugnicourt et al. 2014).

Moreover, PHB thermally decomposes at temperatures just above its melting point. A short exposure of PHB to temperature near 180 ºC could induce a severe degradation accompanied by production of the degraded products of olefinic and carboxylic acid compounds, e.g., crotonic acid and various oligomers.

The extremely low resistance to thermal degradation seems to be the most serious problem related to the processing of PHB. The main reaction involves chain scission, which results in a rapid decreased in molecular weight (Mohanty et al. 2002). Nevertheless, during the processing, the degradation of the chains may be reduced by the addition of a lubricant that

prevents the degradation of the chains in processing, so that the material can be processed at 170-180 ºC. This leads to a decrease in the molecular weight, as well as a reduction in the melt viscosity. The crystallization temperature shifts to lower values, and crystallization takes longer (Bugnicourt et al. 2014).

The addition of plasticizers is considered as a relatively simple route to modify the thermal and mechanical properties of polymers. Blending polymers with plasticizers may modify the physical properties of polymers and a decrease in processing temperature can be achieved. Thus, PHB is commonly blended with plasticizers and nucleation agents that lead to a lower glass temperature and lower crystallinity due to the formation of numerous, small and imperfect crystallites (Bugnicourt et al. 2014).

Global PHA market situation

In the industrial production of PHA, the polyester is extracted and purified from bacteria cultured with glucose as the carbon source. In the 1980s, Imperial Chemical Industries developed poly(3-hydroxybutyrate-co-3-hydroxyvalerate), commonly known as PHBV (a SCL-PHA-type polymer), obtained via fermentation of glucose, sold under the name 'Biopol', and distributed in the US initially by Monsanto, and later Metabolix (Bugnicourt et al. 2014).
PHA has been used in orthopaedic applications, tissue engineering, and for production of bioplastic materials used by, food services, in packaging materials, as well as in the pharmaceutical industry and agriculture. Innovative properties of PHA can bring additional value to the applications in which they are used. It is expected that advancement in the industrialization process of PHA would drive down the cost of PHA and make it an alternative for conventional plastic (Market research store 2016). The market for Polyhydroxyalkanoate (PHA) was valued at USD $70.0 Million in 2015. It is projected to grow at a CAGR of 4.88% from 2016 to 2021 (Markets and Markets 2017).

Global PHA market is driven by the increasing demand for renewable, eco-friendly, and bio-based materials. Another driving force are potential bio-medical applications such as bone-marrow scaffolds, cardiovascular patches, nerve repair devices, and wound dressing materials High demand for packaging, followed by food services is also contributing to the growth of the market. However, R&D facilities and high cost of raw materials, which is 20% to 80% higher than the cost of raw materials of conventional plastics, are few of the challenges faced by this market. The opportunities that would help in the growth of this market are the rapid technological changes and increased investment in R&D by the developed countries (Market Research Store 2016).

The industrial ecosystem of the PHA market comprises manufacturers of PHA such as, Metabolix Inc. (US), Kaneka Corporation (Japan), Meredian Holdings Group, Inc. (US), Shenzhen Ecomann Biotechnology Co., Ltd (China), BioMatera (Canada), Biomer (Germany), Bio-On Srl (Italy), Newlight Technologies, LLC (US), PHB Industrial S.A. (Brazil), PolyFerm Canada, Inc. (Canada), TianAn Biological Materials Co. Ltd (China), and Tianjin GreenBio Materials Co., Ltd. (China), and converting industries using PHA in various applications such as, BASF SE (Germany), and The Dow Chemical Company (US) (Markets and markets 2017).

### *Pseudomonas putida*

More than 60,000 organic chemicals are sold worldwide. When one of them enters the environment, whether by accident or design, often they are degraded by microbes. Over the past century, microbes have been isolated from the environment to study their degradative

metabolism in the laboratory. *Pseudomona putida* among others turned to be studied more than any other bacterial specie (Wackett 2003).

*Pseudomonas putida* is comprised of a heterogeneous group of Gram-negative, road-shape bacterium occurring in several environmental niches, due to its metabolic versatility and low nutritional requirements (Timmis 2002; Sharma et al. 2012; Poblete-Castro et al. 2012).
Throughout the past century, *Pseudomonas* species have figured prominently in efforts to unravel how microbes recycle dispersed organic molecules in the environment. In 1926, der Dooren de Jong reported that *Pseudomonas* would grow on 80 different organic compounds (Wackett 2003).

Initiated by the pioneering discovery of its high capability to degrade recalcitrants and inhibiting xenobiotics, extensive biochemical analysis of this bacterium has been carried out in the recent years. In addition, *P. putida* shows a very high robustness against extreme environmental conditions such as high temperature, extreme pH, or the presence of toxins or inhibiting solvents. Moreover, it is genetically accessible and grows fast with simple nutrient demand (Dos Santos et al. 2004).

Different species of *P. putida* vary in their genome content and organization and phenotypic behaviour, creating a wide range of industrial applications to be studied (Poblete-Castro et al. 2012; Sharma et al. 2014). The central carbon routes of carbon metabolism that *P. putida* has is of particular interest, receiving carbon from the various converging pathways of substrate utilization and supplying products, by-products and intermediate products with higher added-value. Its fast growth, high biomass yield, and low maintenance demands are additional features that are important for industrial application (Poblete-Castro et al. 2012).

*Substrate uptake*

In contrast to various other industrial microorganisms, glucose is not the preferred carbon substrate for pseudomonads. Concerning the use of industrial substrates, *P. putida* is capable of using raw glycerol, a technical by-product from the biodiesel industry (Ciesielski et al. 2010). It is reported that some species of *Pseudomonas putida* are able to metabolize other substrates as fatty acids, amino acids, aromatic compounds and waste fryer oils as well as glucose, glycerol and biodiesel glycerol (Sharma et al. 2012; Ebert et al. 2011).

*Metabolism*

For the catabolism of sugars, Entner-Doudoroff pathway is employed instead of the Embden-Meyerhof-Parnas pathway (Fuhrer et al. 2005). Some *P. putida* strains are able to metabolize glycerol and fatty acids by the tricarboxylic acid cycle (TCA), the pentose pathway and the β-oxydation pathway (Sharma et al. 2014) (See Figure 2).

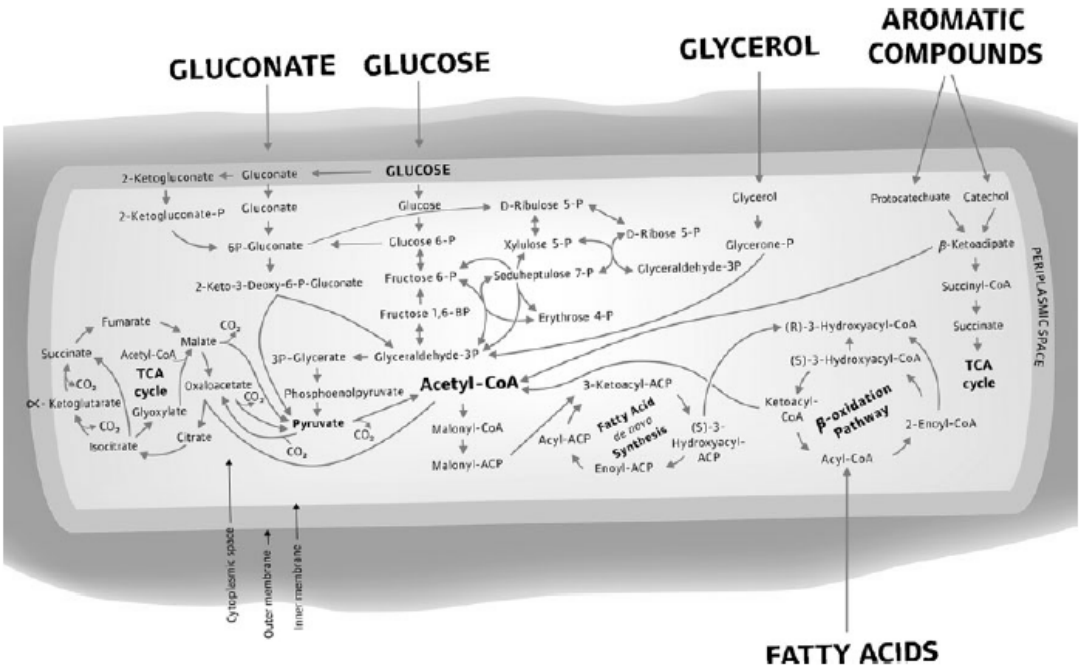



**Figure 2.** Metabolic pathways in the carbon core metabolism of *Pseudomona putida* (Poblete-Castro et al. 2012).

With regard to the environmental applications, huge interest focused on the degradation pathways and the underlying mechanisms that show that some *P. putida* strains have a naturally capacity to tolerate and modify aliphatic, aromatic and heterocyclic compounds (Schmid et al. 2001; Sharma et al. 2014).

Industrial applications of *P. putida* strains

Prompted by their excellent production properties and the advent of genetic engineering, *P. putida* strains have been applied and optimized to a broad portfolio of industrial products, involving bio-based materials, as well as de novo synthesis and biotransformation of high value chemicals and pharmaceuticals.

Beyond its more traditional application for xenobiotic degradation, *P. putida* is gaining more and more importance as host for whole cell bio-catalysis and *de novo* synthesis of chemicals. This is due to significant benefits from its well-known capacity to tolerate and modify aliphatic, aromatic, and heterocyclic compounds.

Furthermore, depending on the cultivation conditions, *P. putida* strains can storage PHAs as carbon and energy source (Hoffmann & Rehm 2004). Their material properties depend on the monomeric composition which can be precisely controlled by fermentation strategies or by metabolic engineering. This illustrates the pronounced potential of *P. putida* species for an economically attractive production of PHA with diverse composition (Poblete-Castro et al. 2012).

The application range of *P. putida* in industrial biotechnology has a great potential to further grow and expand in the future considering the interesting portfolio of novel products becoming available via efficient *P. putida* cell factories (Figure 3).

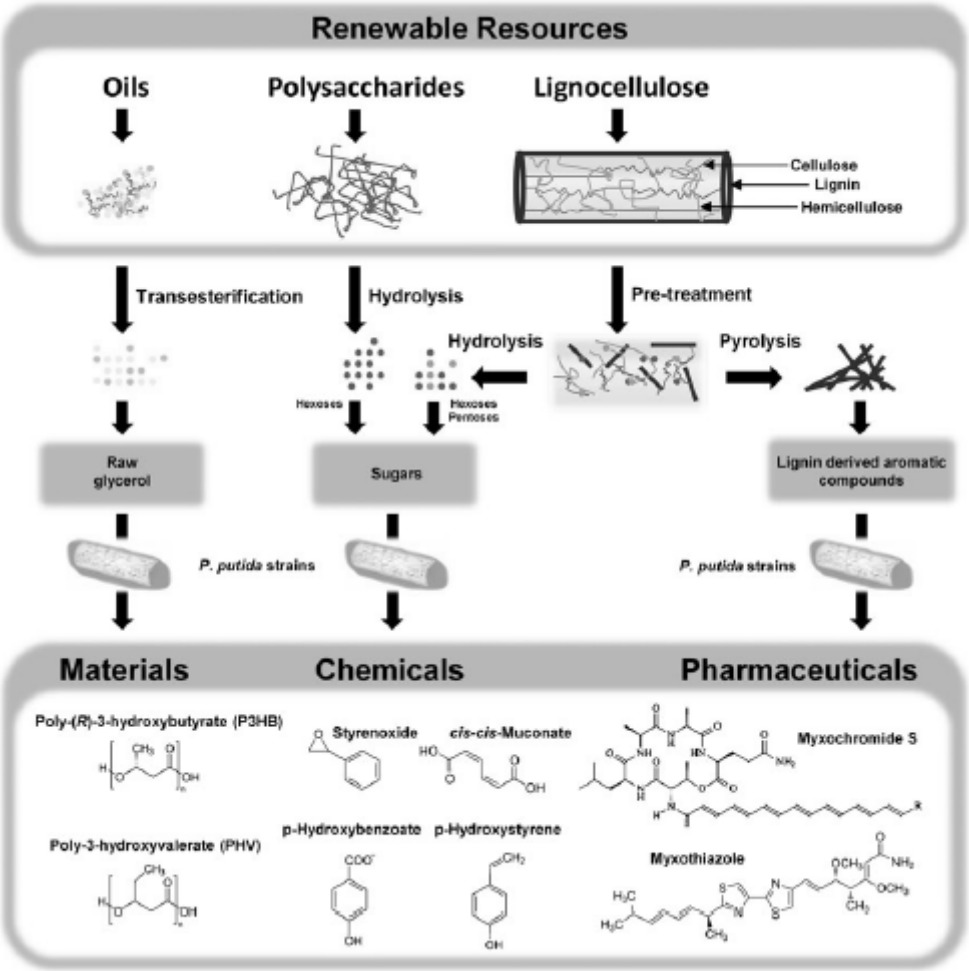



**Figure 3.** Integration of Pseudomonas putida as cell factory in the bio-based production pipelines from renewable resources (Poblete-Castro et al. 2012).

De Smet et al. (1983) detected the inclusion bodies in *Pseudomonas olevorans* for the first time when grown on octane. *P. putida* strains were confirmed to be potential producers of PHAs among *P. fluorescens* and *P. aeruginosa* in a study by Huisman et al. (1989).

*P. putida*, alike other microorganisms, accumulate PHA polymers as carbon and energy storage molecules under conditions of carbon-excess conditions when another major nutrient is limited (Rehm 2010). The accumulation of excess carbon is a general mechanism used by many *Pseudomonas* species, and all *P. putida* strains, that is essential for resource balancing (Escapa et al. 2012). So far both *P. putida* KT2440 and *P. putida* GPo1 are widely used for large-scale production of mcl-PHAs in fed-batch and continuous cultures (Kim 2002; Sun et al. 2007; Elbahloul & Steinbüchel 2009).

<u>*Pseudomonas putida* LS46</u>

Recently, *Pseudomonas putida* LS46, a novel PHAs producer was isolated from a municipal wastewater treatment plant in Winnipeg, Manitoba, Canada on its ability to synthesize medium-chain-length PHAs (Sharma et al. 2012). The *P. putida* LS46 genome has been sequenced (Sharma et al. 2013) and compared with nine other sequenced *P. putida* strains (Sharma et al. 2014).

PHA production by *P. putida* LS46

*P. putida* LS46, like other *P. putida* stains, metabolize glucose, glycerol, and fatty acids by glycolysis, the TCA cycle, the pentose pathway and β-oxidation pathways (Fu et al. 2014, 2015). At least three different metabolic pathways provide the precursors for the synthesis of PHAs (Figure 4).

i. Fatty acid de novo biosynthesis is the main route during growth on carbon sources that are metabolized to acetyl-CoA, like glucose, gluconate, glycerol etc.;

ii. β-oxidation is the main pathway for PHAs production when fatty acids are used as carbon source;

iii. Chain elongation reaction in which acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA is involved is involved in the PHA synthesis when small chain length fatty acids like C6 and C7 are used.

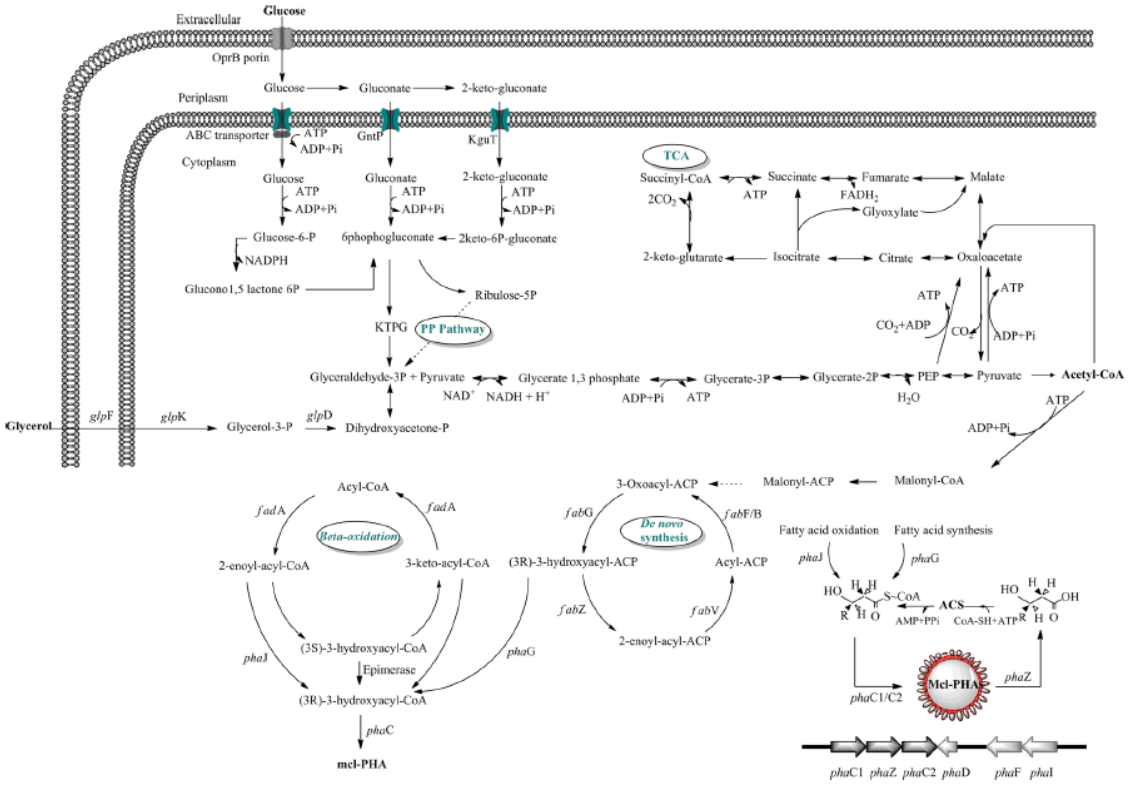



**Figure 4.** Proposed PHA synthesis and degradation pathways for *P. putida* LS46 (Fu et al. 2015).

Major components of PHAs produced by *P. putida* LS46 are either 3-hydroxyoctanoate or 3-hydroxydecanoate depending on the carbon substrate used for PHAs production. There are six genes in the PHA synthesis operon (*pha*) in *P. putida*. These are *phaC1, phaZ, phaC2, phaD, phaF* and *phaI*. The *phaC1* and *phaC2* are PHA synthases that incorporate (R)-3-hydroxyacyl-CoA monomers into the PHA polymer. The *phaZ* encodes a PHA depolymerase, which hydrolyses the PHA monomers, which can be fed into central metabolism for growth. The other three genes (*phaD, phaF and phaI*) are regulatory genes.

Physical and thermal properties of PHAs, such as tensile strength, elongation-to-break ratio, and melting temperature are determined by the polymer subunit composition. *P. putida* LS46 can synthesize polymers with very different subunit composition, depending on the substrate

type used to grow the bacterium. Thus, PHA polymers with different subunit compositions, and thus different physical and thermal properties may be tailored for specific applications.

*P. putida* LS46 shows good PHA production (22% of cell dry weight = CDW) in glucose medium. PHA production was detected as early as 12 hours (h) in both nitrogen-limited and nitrogen-excess conditions. The increase in PHA production after 48 h was higher in nitrogen-limited cultures than in nitrogen-excess cultures. *P. putida* LS46 produced mcl-PHAs when cultured with glucose, glycerol, or $C_6$-$C_{14}$ saturated fatty acids as carbon sources, and mcl-PHAs accounted for 56% of the CDW when cells were batch cultured in medium containing 20mM octanoate.

The following table shows the results of PHA from previous studies performed using different carbon sources on *P. putida* LS46 and the resulting monomer compositions (Table 2).

**Table 2.** Polyhydroxyalkanoate (PHAs) production by *Pseudomonas putida* LS46 on different fatty acids and their monomer composition.

| | | | Monomer composition (mol%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Carbon source | Cell dry mass (g/L) | % PHAs of cell dry mass | $C_6$ | $C_7$ | $C_8$ | $C_9$ | $C_{10}$ | $C_{12}$ |
| Glucose (1%) | 2.57±0.02 | 10.00±0.01 | 2.20 | nd | 15.70 | nd | 76.10 | 5.60 |
| Fatty acid (20 mmol/L) | | | | | | | | |
| $C_6$ | 1.32±0.10 | 13.12±1.05 | 82.34±0.51 | nd | 16.02±0.18 | nd | 1.64±0.39 | nd |
| $C_8$ | 2.36±0.09 | 56.17±1.08 | 6.52±0.44 | nd | 88.50±0.58 | nd | 3.81±1.23 | 1.07±0.31 |
| $C_9$ | 2.29±0.06 | 31.02±2.78 | nd | 18.71±0.98 | nd | 81.29±0.98 | nd | nd |
| $C_{10}$ | 1.47±0.13 | 33.68±3.86 | 4.56±0.14 | nd | 47.50±1.18 | nd | 46.61±1.16 | 1.32±0.50 |
| $C_{12}$ | 3.83±0.11 | 36.90±0.81 | 4.39±0.26 | nd | 55.68±0.95 | nd | 37.80±1.13 | 2.78±0.28 |
| $C_{14}$ | 3.95±0.07 | 33.06±1.57 | 1.49±1.03 | nd | 46.12±3.16 | nd | 51.16±2.43 | 1.21±0.09 |

**Note:** nd, not detected.

# PHASE I: OPTIMIZATION OF C/N/P RATIOS FOR PHA PRODUCTION BY *P. PUTIDA* LS46 IN FLASK CULTURES

## Literature review

Many bacteria store carbon and energy molecules under conditions of nutrient-limitation. *Pseudomonas putida* accumulates mcl-PHAs as carbon and energy storage molecules when the bacterial experience conditions nitrogen-, phosphorous-, or oxygen-limitation in the presence of abundant carbon (Elbahloul & Steinbüchel 2009). Therefore, it is important to find the optimal combination of C/N/P for each specific carbon source. Aldén *et al*. [2001] at Lund University, for example, studied how different ratios of C/N/P affected the bacterial growth in agricultural soil. However, this study was not conducted to evaluate production of a high-value product by a specific bacterium, and also used a different carbon source.

Factorial experiments to optimize C/N/P ratios have been conducted with some bacteria, but no reports on optimization of C/N/P ratios for PHA production by *P. putida* LS46 cultured with canola free fatty acids are available in the literature. Furthermore, the presence of unsaturated fatty acids in the substrate may modifications in the optimum nutrient parameters, as it is known that both the rate of growth and the cell mass accumulation (final cell density) of *P. putida* LS46 is greatly influenced by the carbon source, and this bacterium accumulated PHA polymers with different subunit compositions when cultured with vegetable oils with different amounts of saturated versus unsaturated fatty acids.

## Materials and Methods

Experiment P101

The factorial experiment was carried out to elucidate the optimum ratio between 3 different components, with means 3 factors. The combinatorial possibilities between a high and a low value for each of them makes a total of $2^3$ combinations, in other words 8 different conditions. All tests were conducted with three independently replicated cultures (i.e., three biological replicates).

Twenty-four (24) non-baffled, 250 mL Erlenmeyer flasks were used for all experiments. Luria-Bertani (LB) medium was prepared and autoclaved at 121ºC for 30 min. A glycerol-stock of *P. putida* LS46, stored at -40 ºC, was thawed and used to inoculate 50 mL cultures of LB medium in the 250 mL flasks. The inoculum cultures were grown at 30 ºC on a rotary shaker (150 rpm) for 24 h.

Ramsay's Minimal Medium (RMM) was prepared as previously described (Sharma et al., 2012; Table 3). Eight (8) different conditions. with different concentrations of carbon (C), nitrogen (N), and phosphorus (P) were tested. The Ramsay's medium was modified in each flask for obtaining the quantities of C, N or P required (Table 4). The non-contributing components to the C, N or P concentrations were not modified. Table 4 below displays the conditions for the factorial experiment. Note that the name of each group (triplicate) of samples is composed by the millimolar concentration of each key component (mM) being C/N/P and the rate of C/N/P (last 3 columns) are referenced to carbon, being always a value of 1.0. The carbon source used in all experiments is free fatty acids from canola oils (ACAS).

In the first part of Phase 1, the highest level of nitrogen was the concentration normally used in standard RMM and the low value was the half this concentration. For the carbon and phosphorous amounts, the normal levels in standard RMM were used as the low level and the double this amount was used for the high level (Table 5).

**Table 3**. Composition of Ramsay's Minimal Medium (RMM).

| Component | Concentration (in distilled water) |
|---|---|
| $Na_2HPO_4$ | 6.7 g/L |
| $KH_2PO_4$ | 1.5 g/L |
| $(NH_4)_2SO_4$ | 1.5 g/L |
| $MgSO_4$ | 50 mg/L |
| $FeNH_4$ | 60 mg/L |
| $CaCl_2$ | 10 mg/L |
| Trace elements | 1 mL/L |
| ACAS | 20 mM |

**Table 4.** Eight (8) conditions tested in Experiment P101.

| Sample | C [mol/L] | N [mol/L] | P [mol/L] | C [%] | N [%] | P [%] | C | N | P |
|---|---|---|---|---|---|---|---|---|---|
| 20.0/23.5/58.2 | 0.02 | 0.02 | 0.06 | 19.7 | 23.1 | 57.2 | 1.0 | 1.2 | 2.9 |
| 40.0/23.5/58.2 | 0.04 | 0.02 | 0.06 | 32.9 | 19.3 | 47.8 | 1.0 | 0.6 | 1.5 |
| 20.0/11.8/58.2 | 0.02 | 0.01 | 0.06 | 22.2 | 13.1 | 64.7 | 1.0 | 0.6 | 2.9 |
| 40.0/11.8/58.2 | 0.04 | 0.01 | 0.06 | 36.4 | 10.7 | 52.9 | 1.0 | 0.3 | 1.5 |
| 20.0/23.5/116.4 | 0.02 | 0.02 | 0.12 | 12.5 | 14.7 | 72.8 | 1.0 | 1.2 | 5.8 |
| 40.0/23.5/116.4 | 0.04 | 0.02 | 0.12 | 22.2 | 13.1 | 64.7 | 1.0 | 0.6 | 2.9 |
| 20.0/11.8/116.4 | 0.02 | 0.01 | 0.12 | 13.5 | 7.9 | 78.6 | 1.0 | 0.6 | 5.8 |
| 40.0/11.8/116.4 | 0.04 | 0.01 | 0.12 | 23.8 | 7.0 | 69.2 | 1.0 | 0.3 | 2.9 |

**Table 5.** Low and high levels for Ramsay's medium (Experiment P101).

| Component | Low level | High level |
|---|---|---|
| $Na_2HPO_4$ | 6.70 g/L | 13.40 g/L |
| $KH_2PO_4$ | 1.50 g/L | 3.00 g/L |
| $(NH_4)_2SO_4$ | 0.75 g/L | 1.50 g/L |
| $MgSO_4$ | 50 mg/L | 50 mg/L |
| $FeNH_4$ | 30 mg/L | 60 mg/L |
| $CaCl_2$ | 10 mg/L | 10 mg/L |
| Trace elements | 1 mL/L | 1 mL/L |
| ACAS | 20 mM | 40 mM |

The test cultures were incubated at 30ºC on a rotary shaker (150rpm).

At 48 and 72 hours post-inoculation (h pi), the 30 mL of each culture was harvested, transferred to a fresh, sterile 50 mL Falcon tube, and centrifuged at 4700 x $g$ for 20 min. The supernatant from each tube was transferred to a second fresh sterile 50 mL tube and later used to measure the culture pH. The cell pellets were then resuspended in Phosphate Buffered Saline (PBS), and centrifuged again at 4700 x $g$ for 20 min. This wash step was repeated two additional times. The samples were then placed in a drying oven at 60ºC for 48 h. Once dried, the CDW of each sample was determined.

The PHA content per cell and polymer subunit composition were analysed as follows. Between 5 and 10 mg of CDW of each sample was weighted and introduced into a screw cap vial. Methanolization was carried out by adding 1.0 mL of 15% $H_2SO_4$ in methanol, and 1.0 mL of 1 mg/mL solution of Benzoic Acid in Chloroform. The vials were place in a boiling water bath for 5 to 6 h. The PHA composition was determined by gas chromatography – mass spectrometry (GS-MS) analysis described by Braunegg et al. (1978).

Experiment P102

After the results obtained from P101 and their discussion in the following sections, a decision was made to delete the phosphorus as a study factor. Similarly, as in P101, a total of $2^2$ with their respective biological replicates were performed. In other words, 4 different conditions and a total of 12 test cultures.

The inoculum cultures were prepared in the same manner as the preparation for P101. The test cultures were set in the same way as P101, with three major differences:

i. Due to the results on P101, baffled Erlenmeyer flasks (500 mL) were used in order to avoid mass transfer issues;

ii. The carbon source in this case was autoclaved separately and was added to the flasks containing RMM after they were autoclaved;

iii. The concentration of each component in the test cultures was as describe in Table 6. Samples were taken after 12, 24, 36, and 48 h pi. The conditions for the factorial experiment P102, with identical structure as the one presented for experiment P101 (Table 7).

**Table 6.** Low and high levels components in RMM (Experiment P102).

| Component | Low level | High level |
|---|---|---|
| $Na_2HPO_4$ | 6.70 g/L | 6.70 g/L |
| $KH_2PO_4$ | 1.50 g/L | 1.50 g/L |
| $(NH_4)_2SO_4$ | 0.75 g/L | 1.50 g/L |
| $MgSO_4$ | 50 mg/L | 50 mg/L |
| $FeNH_4$ | 30 mg/L | 60 mg/L |
| $CaCl_2$ | 10 mg/L | 10 mg/L |
| Trace elements | 1 mL/L | 1 mL/L |
| ACAS | 20 mM | 80 mM |

**Table 7.** C, N, P concentrations in Experiment P102 flasks.

| Sample | C [mol/L] | N [mol/L] | C [%] | N [%] | C | N |
|---|---|---|---|---|---|---|
| 20.0/23.5 | 0.02 | 0.02 | 46.0 | 54.0 | 1.0 | 1.2 |
| 80.0/23.5 | 0.08 | 0.02 | 77.3 | 22.7 | 1.0 | 0.3 |
| 20.0/11.8 | 0.02 | 0.01 | 63.0 | 37.0 | 1.0 | 0.6 |
| 80.0/11.8 | 0.08 | 0.01 | 87.2 | 12.8 | 1.0 | 0.1 |

**Results**

Experiment P101

Tables 8 and 9 show the CDW [g/L], PHA accumulation [wt% of CDW], PHA production [g/L], and pH of samples taken at 48 and 72 h pi from the P101 test cultures, with their related standard deviations. To achieve a better understanding of the results, the data of Tables 8 and 9 are plotted in Figures 5 and 6, respectively.

**Table 8.** Cell Dry Weight (CDW) [g/L], wt% PHA, and PHA production [g/L] in Experiment P101.

| Sample | CDW [g/L] 48h | CDW [g/L] 72h | PHA [wt%] 48h | PHA [wt%] 72h | PHA [g/L] 48h | PHA [g/L] 72h |
|---|---|---|---|---|---|---|
| 20.0/23.5/58.2 | 1.86 ± 0.13 | 1.71 ± 0.09 | 4.02 ± 0.65 | 3.14 ± 0.26 | 0.07 ± 0.01 | 0.05 ± 0.01 |
| 40.0/23.5/58.2 | 2.82 ± 0.14 | 2.65 ± 0.21 | 8.00 ±0.45 | 4.90 ± 0.39 | 0.23 ± 0.01 | 0.13 ± 0.02 |
| 20.0/11.8/58.2 | 1.72 ± 0.19 | 1.76 ± 0.22 | 4.33 ± 0.50 | 4.54 ± 0.35 | 0.07 ± 0.00 | 0.08 ± 0.02 |
| 40.0/11.8/58.2 | 3.28 ± 0.02 | 2.95 ± 0.31 | 7.65 ± 0.46 | 8.35 ± 0.52 | 0.25 ± 0.02 | 0.25 ± 0.04 |
| 20.0/23.5/116.4 | 1.36 ± 0.14 | 1.66 ± 0.04 | 2.85 ± 0.43 | 2.51 ± 0.37 | 0.04 ± 0.01 | 0.04 ± 0.01 |
| 40.0/23.5/116.4 | 1.71 ± 0.20 | 3.43 ± 0.27 | 2.95 ± 0.45 | 3.94 ± 0.16 | 0.05 ± 0.00 | 0.14 ± 0.02 |
| 20.0/11.8/116.4 | 0.83 ± 0.11 | 1.31 ± 0.28 | 2.74 ± 0.43 | 2.09 ± 0.28 | 0.02 ± 0.01 | 0.03 ± 0.01 |
| 40.0/11.8/116.4 | 1.60 ± 0.21 | 1.82 ± 0.18 | 2.14 ± 0.33 | 4.14 ± 0.46 | 0.03 ± 0.01 | 0.08 ± 0.01 |

**Table 9.** pH values in culture flasks at 48 and 72 h pi in Experiment P101.

| Sample | pH 48h | pH 72h |
|---|---|---|
| 20.0/23.5/58.2 | 6.92 ± 0.02 | 6.94 ± 0.03 |
| 40.0/23.5/58.2 | 6.73 ± 0.10 | 6.75 ± 0.02 |
| 20.0/11.8/58.2 | 6.93 ± 0.03 | 6.97 ± 0.02 |
| 40.0/11.8/58.2 | 6.82 ± 0.07 | 6.93 ± 0.02 |
| 20.0/23.5/116.4 | 7.11 ± 0.01 | 7.09 ± 0.02 |
| 40.0/23.5/116.4 | 7.07 ± 0.04 | 7.00 ± 0.03 |
| 20.0/11.8/116.4 | 7.19 ± 0.02 | 7.15 ± 0.01 |
| 40.0/11.8/116.4 | 7.12 ± 0.01 | 7.13 ± 0.02 |

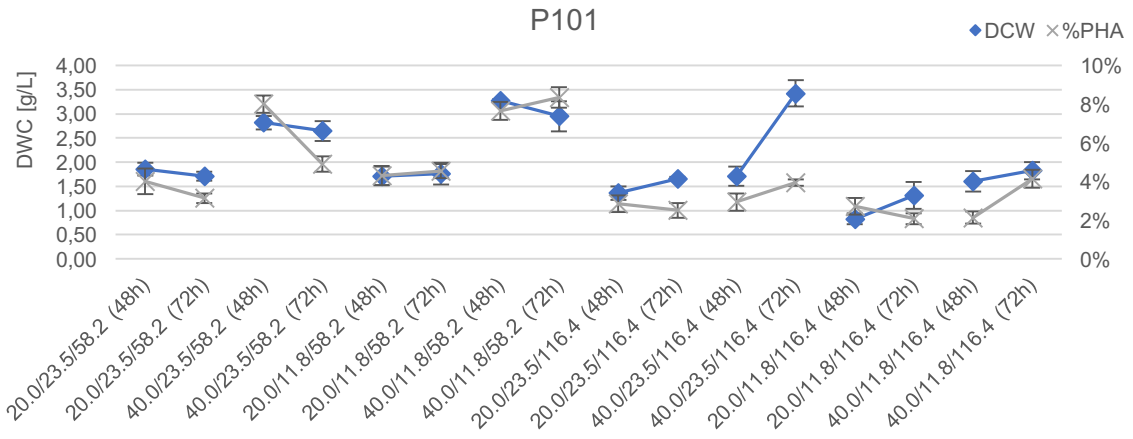



**Figure 5.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in Experiment P101.

The maximum CDW production was 3.43 ± 0.27 [g/L], at 72 h pi, in the test culture with a high concentration of carbon, low concentration of nitrogen, and high concentration of phosphorous (Figure 5). However, the PHA production in these cells was only 3.94 ± 0.16 [%wt CDW] which clearly was not the highest value. The maximum PHA production [%wt of CDW] was detected in the test culture with a high carbon, low nitrogen, and low phosphorous at 72 h pi, with 8.35 ± 0.52 [%wt CDW]. Figure 6 reveals that the pH values remained between a pH of 6.73 and 7.19 over the course of the experiment.

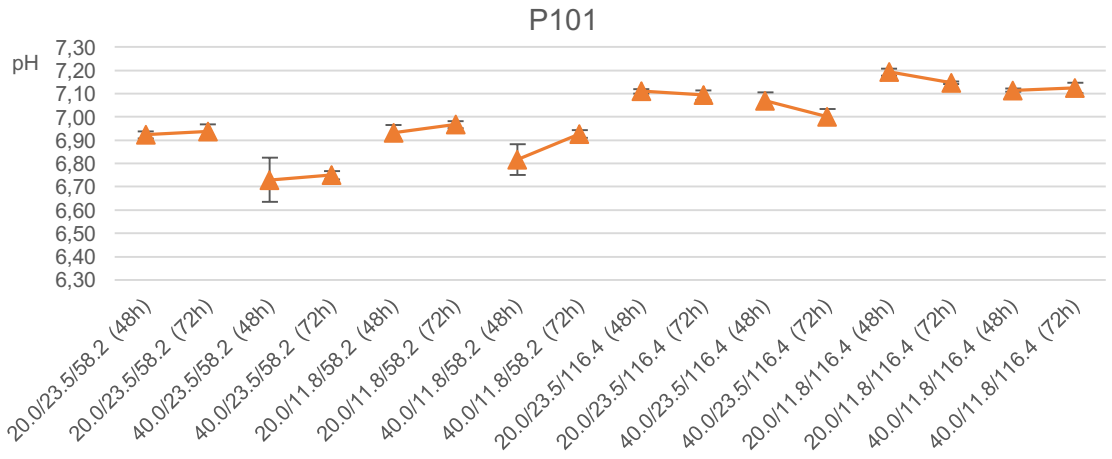



**Figure 6.** pH values in P101 cultures at 48 and 72 h pi in Experiment P101.

Experiment P102

Tables 10 shows the results, of CDW in [g/L], PHA in [wt% of CDW], and pH of the cultures at 12, 36, 24, and 48 h pi from the P102 test cultures, with their associated standard deviations. To accomplish a better understanding of the results, the data of Table 10 are represented in Figures 7 and 8.

**Table 10.** Cell Dry Weight (CDW) production, PHA accumulation (wt% CDW), and pH values at 48 and 72 h pi in Experiment P102.

| Sample | CDW [g/L] 12h | CDW [g/L] 24h | CDW [g/L] 36h | CDW [g/L] 48h |
|---|---|---|---|---|
| 20.0/23.5 | 1.14 ± 0.13 | 1.74 ± 0.10 | 1.94 ± 0.14 | 1.94 ± 0.18 |
| 80.0/23.5 | 1,39 ± 0.11 | 2.66 ± 0.20 | 3.54 ± 0.20 | 4.67 ± 0.16 |
| 20.0/11.8 | 1.05 ± 0.10 | 1.83 ± 0.10 | 2.02 ± 0.11 | 2.01 ± 0.04 |
| 80.0/11.8 | 1.61 ± 0.13 | 2.93 ± 0.14 | 3.87 ± 0.20 | 4.58 ± 0.13 |
| **Sample** | **PHA [wt%] 12h** | **PHA [wt%] 24h** | **PHA [wt%] 36h** | **PHA [wt%] 48h** |
| 20.0/23.5 | 21.53 ± 0.38 | 19.28 ± 0.55 | 16.24 ± 0.82 | 15.97 ± 0.89 |
| 80.0/23.5 | 11.13 ± 0.73 | 22.58 ± 0.64 | 24.68 ± 0.89 | 27.45 ± 0.71 |
| 20.0/11.8 | 16.90 ± 0.49 | 20.77 ± 0.56 | 18.27 ± 0.49 | 18.27 ± 0.54 |
| 80.0/11.8 | 13.04 ± 0.40 | 25.45 ± 0.85 | 27.05 ± 0.88 | 30.97 ± 0.88 |
| **Sample** | **pH 12h** | **pH 24h** | **pH 36h** | **pH 48h** |
| 20.0/23.5 | 6.77 ± 0.05 | 6.91 ± 0.03 | 6.94 ± 0.02 | 6.97 ± 0.01 |
| 80.0/23.5 | 6.69 ± 0.04 | 6.54 ± 0.04 | 6.49 ± 0.02 | 6.54 ± 0.02 |
| 20.0/11.8 | 6.81 ± 0.03 | 6.84 ± 0.02 | 6.96 ± 0.02 | 7.00 ± 0.02 |
| 80.0/11.8 | 6.73 ± 0.05 | 6.61 ± 0.01 | 6.64 ± 0.02 | 6.59 ± 0.04 |

Two well differentiated trends can be identified regarding cell mass production in Experiment P102 (Figure 7). Cultures with low C concentrations entered stationary phase by 36 h pi, and PHA concentrations in the cells plateaued or even decreased (to near 20 wt% CDW). In contrast, cultures with high C concentrations continued to grow and entered stationary phase after 48 h pi, and the PHA concentrations in the cells continued to increase.

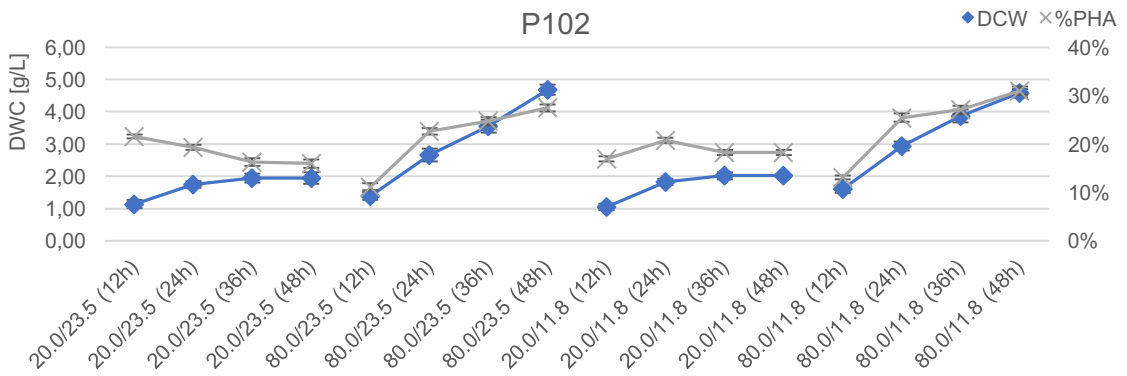



**Figure 7.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in Experiment P102.

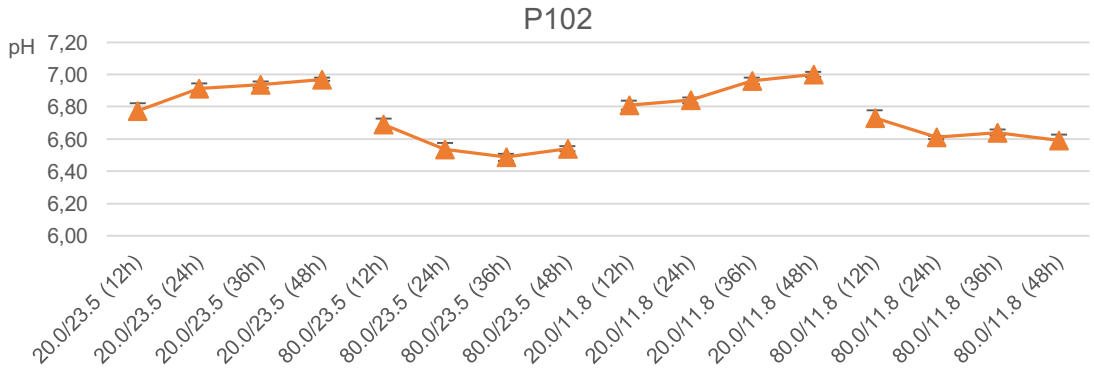



**Figure 8.** pH values in P102 cultures over the course of Experiment P102.

## Discussion

Experiment P101

The cell dry weight production data showed some clear trends. First, in cultures with low versus high C/N/P in the same ratios (20.0/11.8/58.2 versus 40.0/23.5/116.4) it was apparent that it is the molar concentration of each nutrient that matters, not necessarily the ratios. Cultures with higher molar concentrations of each nutrient displayed greater growth. Cultures with high P concentration continued to grow after 48 h pi, meanwhile cultures with low P concentrations had entered stationary phase and were relatively steady after 48 h pi. However, cultures with high C and P concentrations accumulated less PHA, while cultures with high C and low P concentrations displayed greater PHA production. These results are consistent with previous observation that P. putida accumulates PHAs under conditions of carbon excess when some other nutrient is limiting.

Experiment P102

In Experiment P101, ACAS was added to the RMM, and then autoclaved. This can result in solidification of the ACAS in the medium and introduce an artefact in to the experimental results. Therefore, an alternative approach to making the media was used in which the ACAS solution and RMM were autoclaved separately, and then the substrate was added to the autoclaved RMM after both solutions had cooled to room temperature. This alternative method of preparing the media was used for Experiment P102. The results showed that the

maximum PHA production in Experiment P101 was 8.35 ± 0.52 wt% CDW, while the minimum PHA production in Experiment P102 was 11.13 %w CDW. These results will be discussed in Phase III, Experiment P301, titled *Other experiments*.

# PHASE II: EFFECTS OF OXYGEN-LIMITATION ON *P. PUTIDA* LS46 GROWTH AND PHA SYNTHESIS WITH A LOW COST FEDSTOCK

## Literature review

PHAs have been classified as short-chain-length scl-PHAs, with 3-hydroxyalkanoate subunits of 3 to 5 carbon chains, and as medium-chain-length mcl-PHAs, with 3-hydroxyalkanoate subunits containing 6- to 14-carbon chains (Fu et al. 2014). The mcl-PHAs are semi-crystalline elastomers with a low melting point, low tensile strength, and high elongation-to-break ratios, and they have wider applications in the health science due to their biocompatibility and biodegradability (Sharma et al. 2012).

PHAs differ from petroleum plastics in their speed and degree of biodegradability, although, the end products are mostly $CO_2$ and water. A huge advantage in this process is that the carbon released is already part of the current carbon cycle; thus, no net gain or loss is produced (Bernard 2014).

Inside the living cell, PHA acts as an intracellular, granular energy storage reserve. It's synthesis and accumulation is triggered by unfavourable growth conditions (Bernard, 2014). For this reason, it is considered important to know the effects of oxygen-limitation, not only on cellular growth, but also on PHA production. A previous study has revealed that *Pseudomonas putida* LS46 is capable of growth at high rate at dissolved oxygen tensions (DOT) $\geq$ 10%, but that this condition supported little PHA accumulation. When the DOT was lowered to 1%, the growth rate was reduced, but the accumulation of PHA increased more than 3-fold (and more than five-times when the DOT was 0%) (Blunt et al. 2016). In that study, it was also demonstrated that a DOT of approximately 5%, cell growth and PHA accumulation were somewhat variable, indicating that this could be close to a possible switch point where oxygen becomes limiting.

Also, it has been demonstrated that the carbon chain length of the substrate determines the range of monomer units incorporated into PHA, with the 3-hydroxy acids possessing the same chain length as the substrate being at least a major component of the polymer in all cases (Anderson & Dawes 1990). Thus, the substrate used to grow the bacteria plays a significant role in the composition of the polymers synthesized. The study of low cost feedstocks has become very important. Therefore, results from this study will be compared to those of a previous study that evaluated the role of dissolved oxygen on cell growth and PHA accumulation by *P. putida* LS46 cultured on octanoic acid (Blunt et al. 2016). Like Phase 1 of this thesis, Phase II is separated in two parts: Experiment P201 and Experiment P202.

## Materials and Methods

Experiment P201

The study of the oxygen limitation with a bioreactor was executed in parallel with the first phase of the thesis. So that, the fact of adding the substrate before autoclave it or after is an inflection point that separates the phase in these two experiments.

In the first part of Phase II, three different oxygen conditions were investigated: DOTs = 0%, 5% and 10%. This parameter was the only one differing among bioreactors, and all other parameters or conditions, were the same in all cases. It is important to remark that a DOT = 0% does not mean that there is no oxygen flow. A 0% DOT condition means that all the oxygen is consumed by the bacteria at the same rate that it is introduced into the bioreactor.

All reactor experiments were conducted in a 7L, glass, round-bottom Applikon bioreactor, with a 3L working volume. For its preparation, 3 L of RMM (Table 11) were prepared. Before

autoclaving, 20 mM of ACAS were added in the reactor medium. The conditions of the autoclave were the same explained in Phase I.

The bioreactor setup and preparation followed the conditions described by Blunt and Dartiailh, (2016). Once it was autoclaved, the bioreactor was cooled down by means of the cooling circuit. At the same time, the controllers were activated for adjusting the pH the temperature to proper levels and the stirrer was set up at 250 rpm to homogenize the medium. The pH was adjusted with 2M HCl and 4M NaOH dilutions.

**Table 11.** Bioreactor operating conditions for Experiment P201.

| Parameter | Value |
|---|---|
| Temperature | 30ºC |
| pH | 6.5 |
| DOT | 0 / 5 / 10% |
| Air flow | 6 LPM |
| Stirrer | 250 – 1200 rpm |
| Substrate | 20 mM |
| Working volume | 3 L |

When the bioreactor was at the appropriate temperature, 30 ºC, the air-flow was connected at 6 litres per minute (LPM) and bubbled throughout the bioreactor via spargers. Simultaneously, a Membrane Inlet Mass Spectrometer (MIMS) was connected and let the bioreactor overnight for the stabilization of the electrodes.

The bioreactors were inoculated with 1% of the final volume (30 mL) from test cultures (RMM), prepared as described in Experiment P101. Samples were taken every two hours post-inoculation, and processed as described in Phase I. In this case, the supernatant was used to analyse the residual carbon (amount of ACAS) and residual ammonium in the media. For substrate or residual carbon and PHA analyses, 1 mL of supernatant was dried in the oven, and processed as previously described in Phase I. Nitrogen analysis was performed using 0.5 mL of supernatant, which was filtered to remove the substrate and diluted in 4.5 mL of distilled water. Ammonia concentrations were determined via the Lachat QuikChem® Method 10-107-06-1-I, and the measured concentrations were rounded-off and reported to 2 significant digits.

After acquisition of first results and discussion in the upcoming sections, a new condition was set. This condition implied keeping all parameter as they were, but substituting the carbon source concentration for 80 mM instead of 20mM. The DOT conditions in which the experiment was carried out was 0%.

Experiment P202

Second part of Phase II was designed because there was suspicion that there was a problem related to mass transfer when the substrate was autoclaved all together with the Ramsay's Minimal Media

Three new conditions were proposed. First, the same conditions for the previous bioreactor experiment were replicated (DOT = 0%; 20 mM ACAS), but the substrate and the media were autoclaved separately, and the substrate was added both autoclaved solutions had cooled to room temperature. In the second, the rate of oxygen sparged was reduced to 1 LPM and in the third, nitrogen was limited by preparing the RMM with 0.5 g/L of $(NH_4)_2SO_4$ instead of 1.5 g/L.

**Table 12.** Bioreactor operating conditions for Experiment P202.

| Parameter | P202-1 | P202-2 | P202-3 |
|---|---|---|---|
| Temperature | 30ºC | 30ºC | 30ºC |
| pH | 6.5 | 6.5 | 6.5 |
| DO | 0% | 0% | 0% |
| Air flow | 6 LPM | 1 LPM | 6 LPM |
| Stirrer | 250 rpm | 250 rpm | 250 rpm |
| Substrate | 20 mM | 20 mM | 20 mM |
| $[(NH_4)_2SO_4]$ | 1.5 g/L | 1.5 g/L | 0.5 g/L |
| Working volume | 3 L | 3 L | 3 L |

**Results**

Experiment P201

Tables 13 and 14 show the cell mass production [CDW, g/L], PHA accumulation [wt% CDW], the PHA production [g/L], and pH of samples taken every 2 hours for a total of 24 h pi, under 4 different conditions, and their related standard deviations. To better visualize the results, the data in Tables 13 and 14 are plotted in Figures 9, 10, 11, and 12, respectively.

**Table 13.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in cultures under different conditions in Experiment P201.

| | DWC [g/L] | | | | PHA content in DWC [%wt] | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 mM | | | 80 mM | 20 mM | | | 80 mM |
| t [h] | 0% | 5% | 10% | 0%-Sx4 | 0% | 5% | 10% | 0%-Sx4 |
| 00 | 0.06 ± 0.05 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.00 | - | - | - | - |
| 02 | 0.21 ± 0.07 | 0.04 ± 0.06 | 0.22 ± 0.31 | 0.00 | - | - | - | - |
| 04 | 0.21 ± 0.09 | 0.24 ± 0.08 | 0.23 ± 0.24 | 0.61 | - | - | 2.70 ± 0.00 | - |
| 06 | 0.51 ± 0.28 | 0.21 ± 0.21 | 0.51 ± 0.27 | 0.72 | 2.18 ± 0.33 | - | 1.48 ± 0.00 | 3.73 |
| 08 | 0.91 ± 0.24 | 0.62 ± 0.01 | 1.01 ± 0.05 | 1.20 | 2.90 ± 1.06 | 4.27 ± 3.88 | 2.50 ± 0.19 | 5.95 |
| 10 | 1.18 ± 0.20 | 1.19 ± 0.21 | 1.33 ± 0.36 | 1.79 | 2.00 ± 0.42 | 3.38 ± 2.23 | 2.21 ± 0.00 | - |
| 12 | 1.46 ± 0.21 | 1.36 ± 0.03 | 1.62 ± 0.21 | 2.56 | 2.82 ± 0.46 | 7.72 ± 1.01 | 1.86 ± 0.40 | 19.96 |
| 14 | 1.50 ± 0.20 | 1.38 ± 0.35 | 1.60 ± 0.24 | 3.09 | 2.83 ± 0.81 | 6.28 ± 1.57 | 2.22 ± 0.98 | 15.70 |
| 16 | 1.36 ± 0.05 | 1.24 ± 0.22 | 1.66 ± 0.16 | 3.35 | 2.87 ± 0.66 | 5.44 ± 0.00 | 2.23 ± 0.45 | - |
| 18 | 1.14 ± 0.11 | 1.24 ± 0.21 | 1.51 ± 0.06 | 3.21 | 3.19 ± 0.58 | 2.50 ± 1.22 | 1.66 ± 0.40 | 10.42 |
| 20 | 0.96 ± 0.09 | 1.19 ± 0.27 | 1.44 ± 0.19 | 3.65 | 2.88 ± 0.71 | 7.59 ± 6.70 | 2.13 ± 0.28 | 9.10 |
| 22 | 0.68 ± 0.11 | 1.19 ± 0.18 | 1.40 ± 0.42 | 4.12 | 2.37 ± 0.95 | 4.25 ± 1.78 | 2.12 ± 0.52 | - |
| 24 | 0.46 ± 0.09 | 1.24 ± 0.27 | 1.26 ± 0.47 | 3.83 | 2.12 ± 0.80 | 3.28 ± 2.35 | 1.47 ± 0.24 | 9.48 |

**Table 14.** Residual carbon (ACAS) concentrations [g/L] and ammonium nitrogen concentrations [mg/L] in cultures under different conditions in Experiment P201.

| | Residual carbon, ACAS [g/L] | | | | Residual ammonia, $NH_4^+$ [mg/L] | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 mM | | | 80 mM | 20 mM | | | 80 mM |
| t [h] | 0% | 5% | 10% | 0%-Sx4 | 0% | 5% | 10% | 0%-Sx4 |
| 00 | - | - | - | - | 233.3 ± 32.0 | 272.0 ± 5.7 | 220.5 ±24.7 | 235.0 |
| 02 | 0.63 ± 0.22 | 0.75 ± 0.51 | 0.53 ± 0.03 | 2.22 | 268.0 ± 13.1 | 265.0 ± 12.7 | 251.0 ± 35.4 | 248.0 |
| 04 | 0.60 ± 0.13 | 0.79 ± 0.25 | 0.55 ± 0.07 | 2.21 | 265.3 ± 20.0 | 258.5 ± 17.7 | 235.5 ± 41.7 | 242.0 |
| 06 | 0.56 ± 0.17 | 0.77 ± 0.23 | 0.60 ± 0.32 | 2.00 | 235.0 ± 14.4 | 248.5 ± 10.6 | 219.0 ± 8.5 | 169.0 |
| 08 | 0.41 ± 0.19 | 0.77 ± 0.03 | 0.53 ± 0.33 | 1.88 | 204.0 ± 13.7 | 208.0 ± 18.4 | 154.5 ± 55.9 | 69.1 |
| 10 | 0.38 ± 0.27 | 0.61 ± 0.08 | 0.16 ± 0.00 | 1.87 | 173.7 ± 1.2 | 134.5 ± 29.0 | 98.8 ± 28.6 | 36.4 |
| 12 | 0.18 ± 0.05 | 0.17 ± 0.03 | 0.21 ± 0.06 | 1.84 | 147.0 ± 6.2 | 95.4 ± 2.8 | 57.1 ± 52.2 | 5.0 |
| 14 | 0.18 ± 0.04 | 0.16 ± 0.04 | 0.18 ± 0.06 | 1.81 | 125.3 ± 6.4 | 82.0 ± 11.6 | 29.8 ± 15.8 | 1.5 |
| 16 | 0.17 ± 0.04 | 0.13 ± 0.01 | 0.18 ± 0.09 | 1.75 | 119.0 ± 4.6 | 76.8 ± 14.2 | 24.8 ± 17.5 | 0.0 |
| 18 | 0.15 ± 0.03 | 0.14 ± 0.01 | 0.18 ± 0.07 | 1.41 | 116.0 ± 8.9 | 75.3 ± 17.5 | 21.2 ± 15.8 | 0.0 |
| 20 | 0.15 ± 0.03 | 0.14 ± 0.00 | 0.20 ± 0.06 | 1.24 | 116.0 ± 6.2 | 76.3 ± 15.3 | 24.8 ± 20.9 | 0.0 |
| 22 | 0.15 ± 0.03 | 0.13 ± 0.00 | 0.18 ± 0.06 | 1.01 | 108.6 ± 10.1 | 75.9 ± 14.4 | 19.8 ± 13.4 | 0.0 |
| 24 | 0.15 ± 0.03 | 0.15 ± 0.01 | 0.15 ± 0.02 | 0.96 | 111.0 ± 5.0 | 75.7 ± 17.2 | 19.6 ± 13.2 | 0.0 |

As observed in Figure 9, the cell mass production (CDW) under conditions of 20 mM ACAS with DOTs = 0, 5, and 10% were very similar. Cultures with DOT = 5% and 10% reached cell mass concentrations of approximately 1.5 g/L at 12 h pi, and then remained constant, while the culture with DOT = 0% achieved a maximum cell mass concentration at 14 h pi, and then decreased to 0.5 g/L by 24 h pi. The maximum CDW concentration of the cultures with DOTs = 0, 5, and 10% were 1.50 ± 0.20, 1.38 ± 0.35, and 1.66±0.16 g/L, respectively. The culture with four times the substrate concentration (80mM), achieved a cell mass concentration that was significantly greater than the cultures with 20 mM ACAS substrate. The maximum CDW concentration in these cultures was 4.12 g/L at 22 h pi.

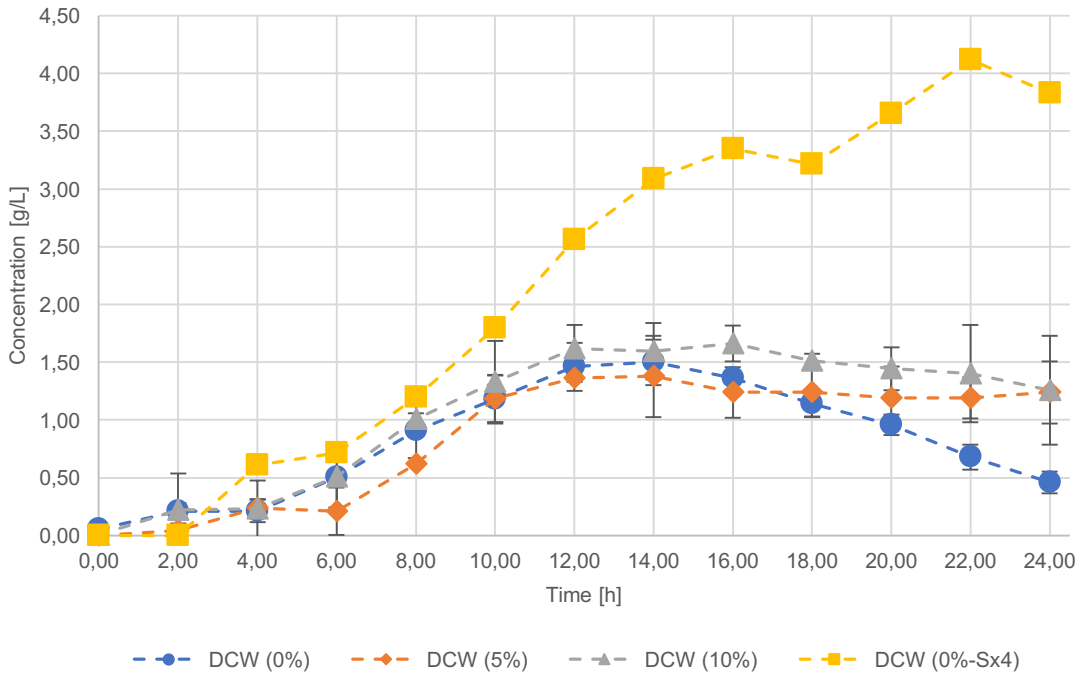



**Figure 9.** Cell Dry Weight (CDW) production [g/L] in cultures under different conditions in Experiment P201.

All values of PHA production were between 2.0% and 7.8% of CDW, and the PHA accumulated to 19.96% CDW at 12 h pi, and decreased thereafter (Figure 10).

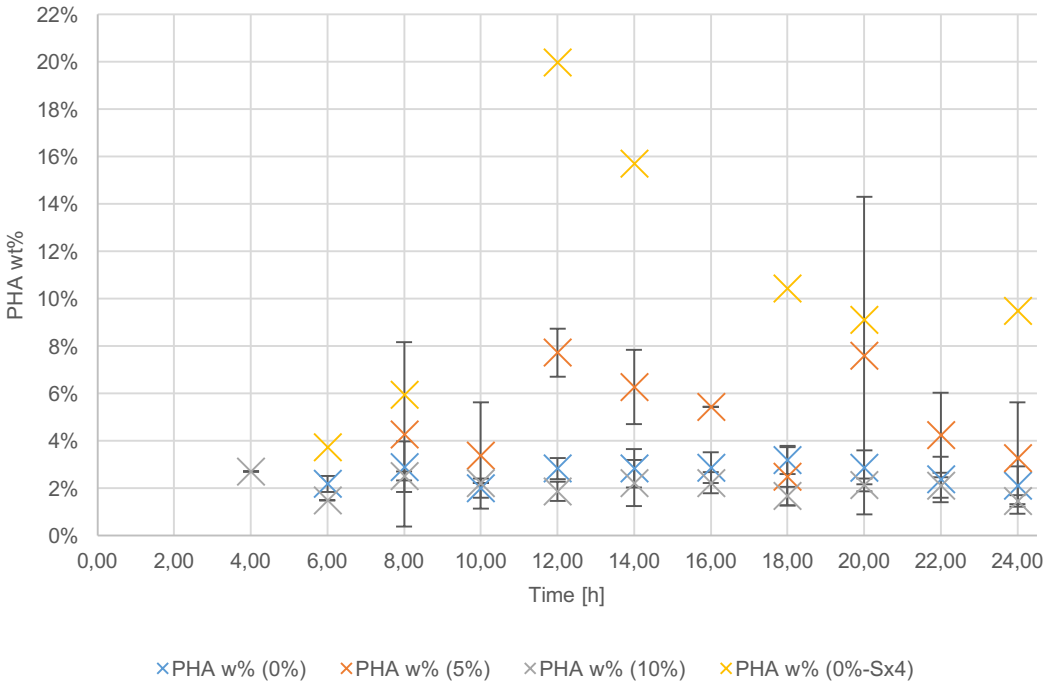



**Figure 10.** PHA accumulation [wt% CDW) in cultures under different conditions in Experiment P201.

Ammonium nitrogen concentrations rapidly after 4 h pi and plateau after 12 h pi. In cultures containing 20mM ACAS substrate, nitrogen concentrations plateaued at different values, depending on the DOT %, corresponding to approximately 150, 76, and 22 mg/L for DOTs = 0, 5 and 10%, respectively. In contrast, nitrogen concentrations in the cultures containing 80 mM ACAS substrate were depleted by 12 h pi (Figure 11). In cultures containing 20 mM ACAS substrate, most of the carbon source was consumed by 12 h pi, and the cultures were substrate-limited therafter (Figure 12). In contrast, the cultures with 80 mM ACAS substrate were always in carbon-excess condition.

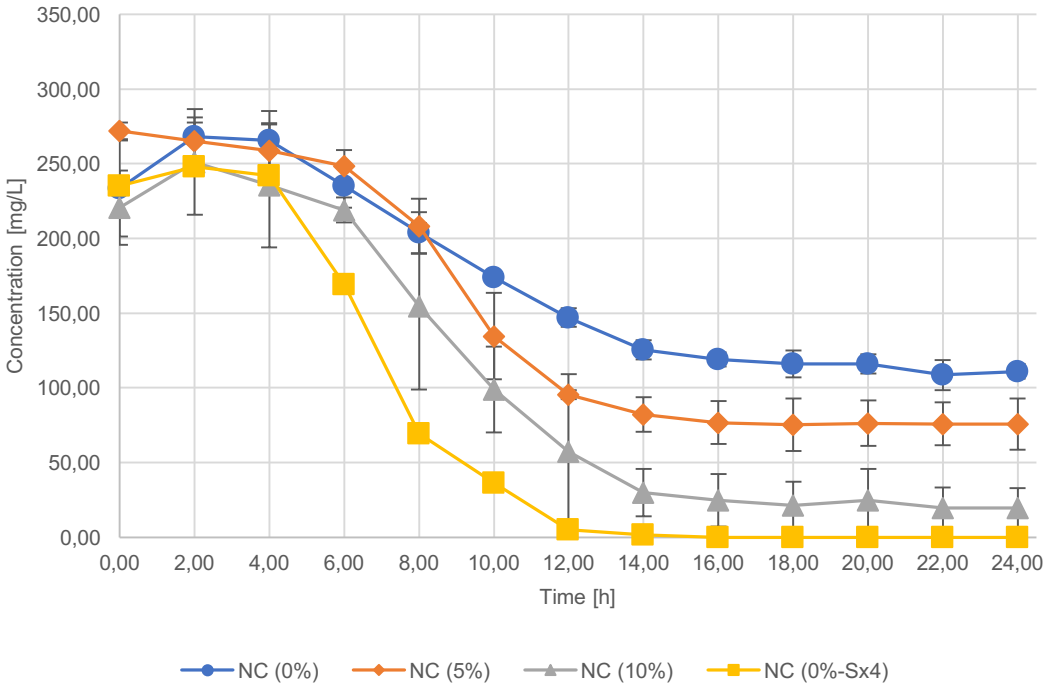



**Figure 11.** Nitrogen concentrations [mg/L] in cultures containing 20 mM or 80 mM ACAS with DOTs = 0, 5, and 10% in Experiment P201.

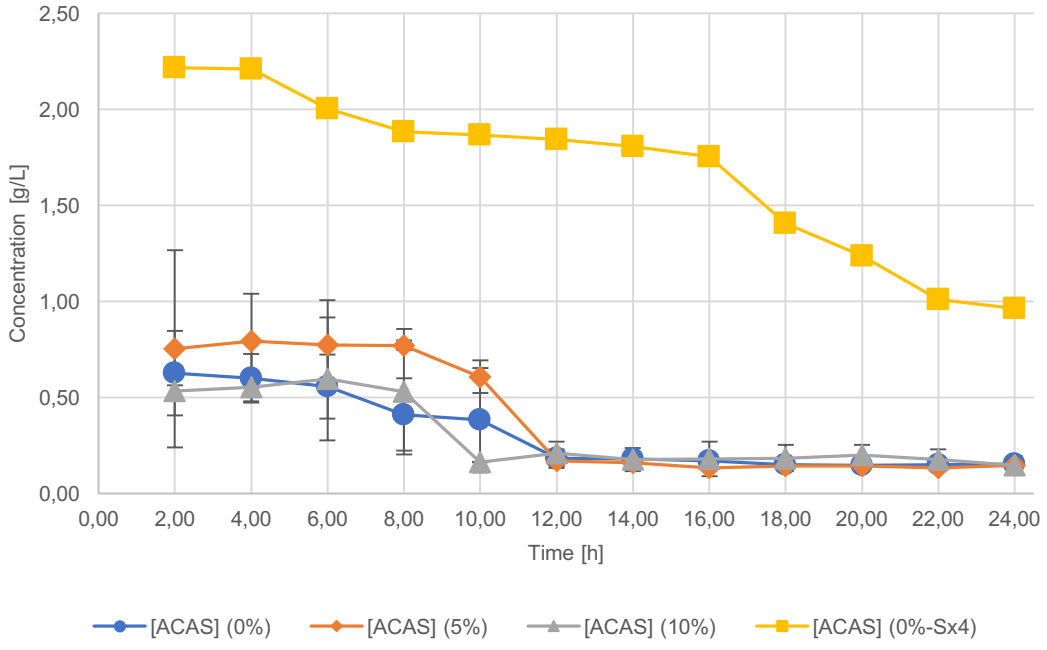



**Figure 12.** Carbon (ACAS) substrate concentrations [mg/L] in cultures containing 20 mM or 80 mM ACAS with DOTs = 0, 5, and 10% in Experiment P201.

Experiment P202

Tables 15 and 16 show the cell mass production [CDW, g/L], PHA accumulation [wt% CDW], the PHA production [g/L], and pH of samples taken every 2 hours for a total of 24 h pi, under 3 different conditions. To better visualize of the results, the data from Tables 15 and 16 are plotted in Figures 13 and 14 respectively.

In experiment P202-1, cell mass production increased up to 16 h pi and then plateaued, achieving a maximum CDW of 2.00 g/L. In experimentP202-2, cell mass production achieved its highest value, 0.81 g/L, at 12 h pi, and then decreased to 0.49 g/L by 24 h pi. In experiment P202-3, cell mass continued to increase up to 22 h pi, and then decreased at 24 h pi, where the CDW was 1.24 g/L (Figure 13).

PHA production was greater under the conditions of experiments P202-1 and P202-2 than under the conditions in experiment P101. PHA production was approximately 12 wt% CDW from 10 to 18 h pi under the conditions in experiment P202-1 shows and reached a maximum of 12.75% at 20 h pi. Overall, PHA production under the conditions of experiment P202-2 was lower than in experiment 202-1. Although PHA production reached a maximum of 13.2% at 10 h pi, it decreased thereafter for the remaining course of the experiment, reaching a CDW of 6.46% at 24 h pi.

**Table 15.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in cultures under different conditions in Experiment P202.

| | CDW [g/L] | | | %wt PHA | | |
|---|---|---|---|---|---|---|
| t [h] | P202-1 | P202-2 | P202-3 | P202-1 | P202-2 | P202-3 |
| 00 | 0.06 | 0.05 | 0.00 | - | - | - |
| 02 | 0.19 | 0.20 | 0.10 | - | - | - |
| 04 | 0.37 | 0.35 | 0.28 | - | - | - |
| 06 | 0.46 | 0.38 | 0.40 | 9.35 | 9.72 | 7.16 |
| 08 | 0.79 | 0.51 | 0.46 | 10.24 | 9.72 | - |
| 10 | 0.94 | 0.76 | 0.55 | 11.43 | 13.20 | 18.92 |
| 12 | 1.32 | 0.81 | 0.76 | 11.95 | 10.44 | 22.50 |
| 14 | 1.77 | 0.72 | 0.86 | 12.15 | 9.52 | 23.77 |
| 16 | 1.89 | 0.66 | 0.92 | 12.03 | 9.57 | 23.06 |
| 18 | 1.89 | 0.59 | 1.07 | 11.71 | 9.98 | 24.21 |
| 20 | 2.00 | 0.56 | 1.15 | 12.75 | 8.68 | 21.29 |
| 22 | 1.94 | 0.55 | 1.24 | - | 7.27 | - |
| 24 | 1.84 | 0.49 | 1.10 | 10.76 | 6.46 | 26.15 |

**Table 16.** Residual carbon (ACAS) concentrations [g/L] and ammonium nitrogen concentrations [mg/L] in cultures under different conditions in Experiment P202.

| | $NH_4^+$ [mg/L] | | |
|---|---|---|---|
| t [h] | P202-1 | P202-2 | P202-3 |
| 00 | 189 | 248 | 105 |
| 02 | 233 | 280 | 78 |
| 04 | 268 | 228 | 49 |
| 06 | 265 | 213 | 42 |
| 08 | 235 | 215 | 28 |
| 10 | 219 | 210 | 4 |
| 12 | 209 | 205 | 3 |
| 14 | 175 | 200 | 3 |
| 16 | 105 | 196 | 2 |
| 18 | 64 | 190 | 2 |
| 20 | 54 | 189 | 2 |
| 22 | 37 | 185 | 2 |
| 24 | 37 | 184 | 2 |

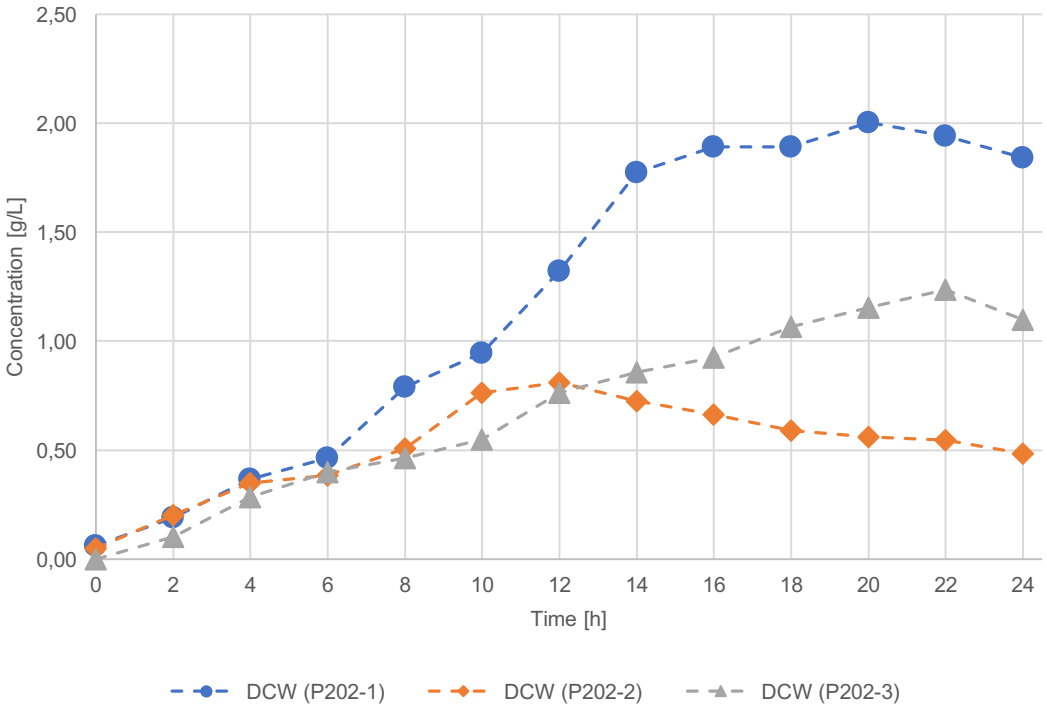



**Figure 13.** Cell Dry Weight (CDW) production [g/L] in cultures under different conditions in Experiment P202.

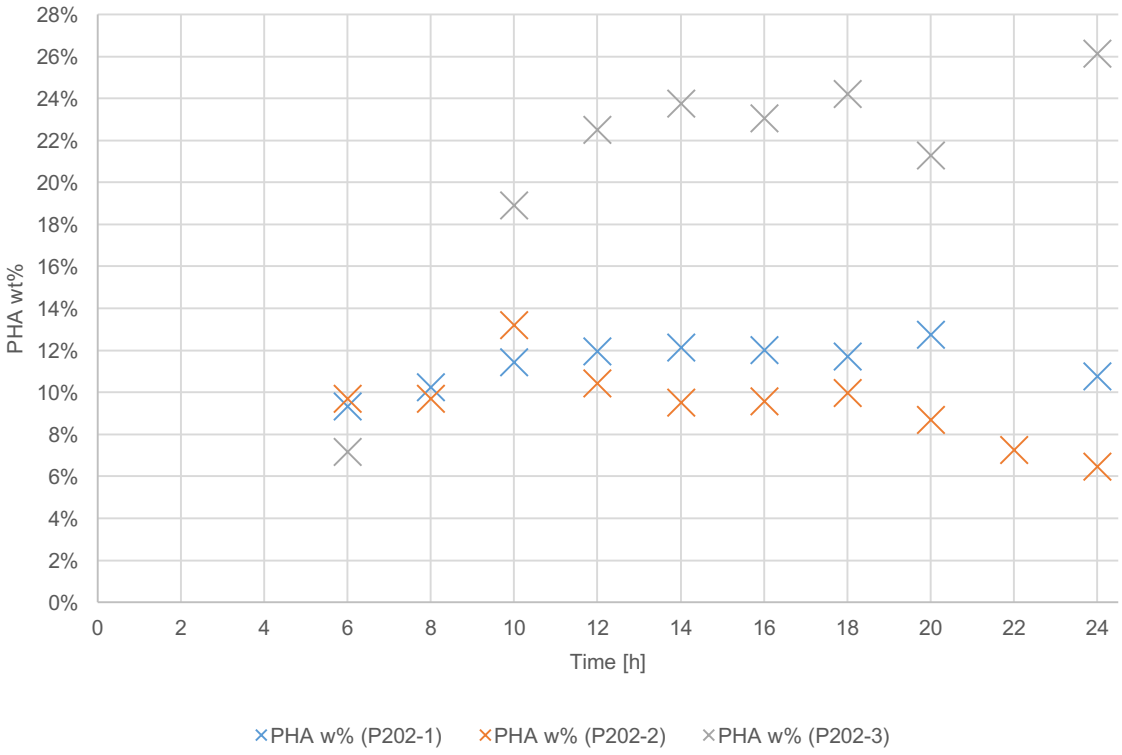



**Figure 14.** PHA accumulation [wt% CDW) in cultures under different conditions in Experiment P202.

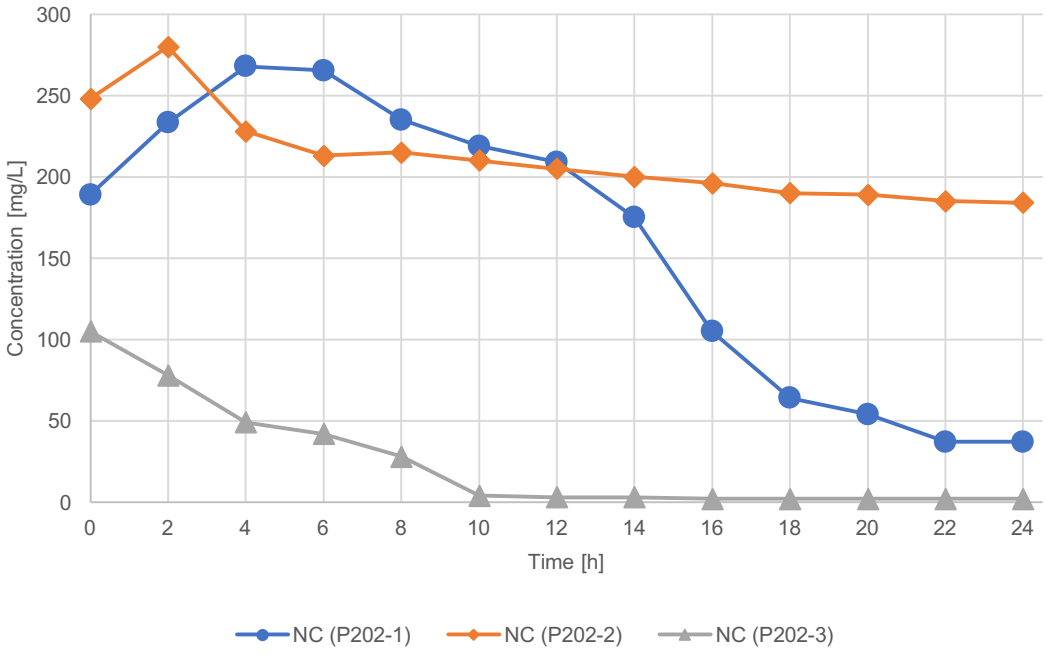



**Figure 15.** Nitrogen concentrations [mg/L] in cultures containing 20 mM ACAS in Experiment P202.

## Discussion

General discussion

Due to a difference in the method by which the carbon source was added to the RMM, Phase II was separated into two parts: P201 and P202. In experiment P201, the experiments were conducted in bioreactors with biological replicates.

Autoclaving the substrate and media solutions separately, and then adding the substrate to the RMM after each solution had cooled to room temperature resulted in greater cell mass and PHA production. Under the same culture conditions, cell mass production was 1.50 ± 0.20 g/L with 2.83 ± 0.81 wt% PHA in experiment P201, while cell mass production reached 2.00 g/L CDW with a maximum of 12.75 wt% PHA in experiment 202.

With respect to the influence of dissolved oxygen during fermentation, we observed that there was no significant effect of different DOT concentrations in cultures containing 20 mM substrate. However, cell mass and PHA production were much greater in cultures containing 80 mM substrate and at DOT = 0. Comparing experiments P202-1, P202-2 and P202-3, it was clear that as the air flow decreased from 6 to 1 LPM, cell mass and PHA production decreased significantly, but is it not clear that the lack of dissolved oxygen acted as a trigger for PHA synthesis, as seen with octanoic acid in previous studies (Blunt et al. 2016). Regarding the PHA production, it is seen when the system is under nitrogen limitation the production increased significantly, suggesting that nitrogen limitation is the trigger of PHA production.

## PHASE III: OTHER EXPERIMENTS

### Experiment P301

As stated in previous sections, concern about the low wt% PHA production was a concern from the data obtained in experiments P101 and P201. Four hypotheses were suggested for the low wt% PHA in the cells:

*Hypothesis 1*: The stock strain of *P. putida* LS46 used for all experiments P101 and P202 (from Christopher Dartiailh) was a contaminant or a mutant because it was not behaving, metabolically, as expected;

*Hypothesis 2*: Differences in cell mass production and PHA accumulation may be due to differences in the effectiveness of the carbon sources. Thus, cell mass production and PHA accumulation were evaluated using octanoic acid (a medium chain length free fatty acid) versus ACAS (long chain free fatty acids derived from canola oil);

*Hypothesis 3*: Aeration in flasks may account for differences in cell mass production and PHA accumulation. Thus, cultures were grown in smooth-sided flasks versus baffled flasks;

Hypothesis 4: The carbon source is free fatty acids from canola oil, which was prepared in different batches for experiments P101 and P201. The first and second batches were different colours (the first batch was lighter in colour and the second batch), it was possible that the different productivities of cell mass and PHA production were a consequence of different ACAS batches;

To test these hypotheses, experiment P301 was performed.

### Materials and Methods

LB media (100 mL) was prepared, transferred into non-baffled 250 mL Erlenmeyer flasks, and inoculated with P. putida LS46. This culture was used to inoculate the experimental flasks. Eight (8) test cultures of 250 mL capacity where prepared with RMM and with different carbon sources: 20 mM octanoic acid, 20 mM light ACAS, and 20 mM dark ACAS (See Table 17) The RMM and substrates were autoclaved separately, and mixed only after each solution had cooled to room temperature. Figure 17 represents the scheme for experiment P301, with different conditions represented by flasks 1 to 8. The test cultures were harvested at 48 h pi and processed as described for experiment P102.

**Table 17.** Conditions for eight test cultures in experiment P301.

| **Flask nº** / **Component** | [1] | [2] | [3] | [4] | [5] | [6] | [7] | [8] |
|---|---|---|---|---|---|---|---|---|
| $Na_2HPO_4$ [g/L] | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 |
| $KH_2PO_4$ [g/L] | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| $(NH_4)_2SO_4$ [g/L] | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| $MgSO_4$ [mg/L] | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| $FeNH_4$ [mg/L] | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| $CaCl_2$ [mg/L] | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Trace elements [mL/L] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Carbon Source 20mM | Octanoic acid | Light ACAS | Dark ACAS | Octanoic acid | Light ACAS | Dark ACAS | Octanoic acid | Light ACAS |
| Baffled flask? | NO | NO | NO | NO | NO | NO | YES | YES |

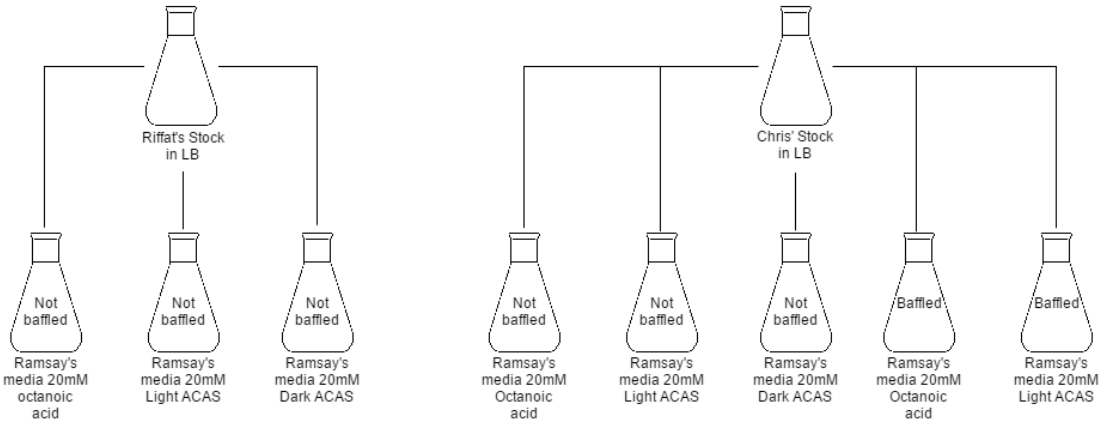



**Figure 17.** Experimental design for eight test cultures in experiment P301.

**Results and Discussion**

Tables 18 show the cell mass production [CDW, g/L], PHA accumulation [wt% CDW], the PHA production [g/L], and pH of samples taken at 48 h pi from experiment P301. The data presented in Table 18 are plotted in Figure 18.

**Table 18.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in cultures under different conditions in Experiment P301.

| Sample | CDW [g/L] 48h | PHA [wt%] 48h | PHA [g/L] 48h |
|---|---|---|---|
| [1] | 2.07 | 57. 66% | 1.19 |
| [2] | 0.71 | 4.38% | 0.03 |
| [3] | 1.54 | 11.80% | 0.18 |
| [4] | 2.26 | 52.79% | 1.19 |
| [5] | 0.82 | 5.76% | 0.05 |
| [6] | 1.95 | 15.88% | 0.31 |
| [7] | 2.37 | 55.80% | 1.32 |
| [8] | 0.88 | 6.46% | 0.06 |

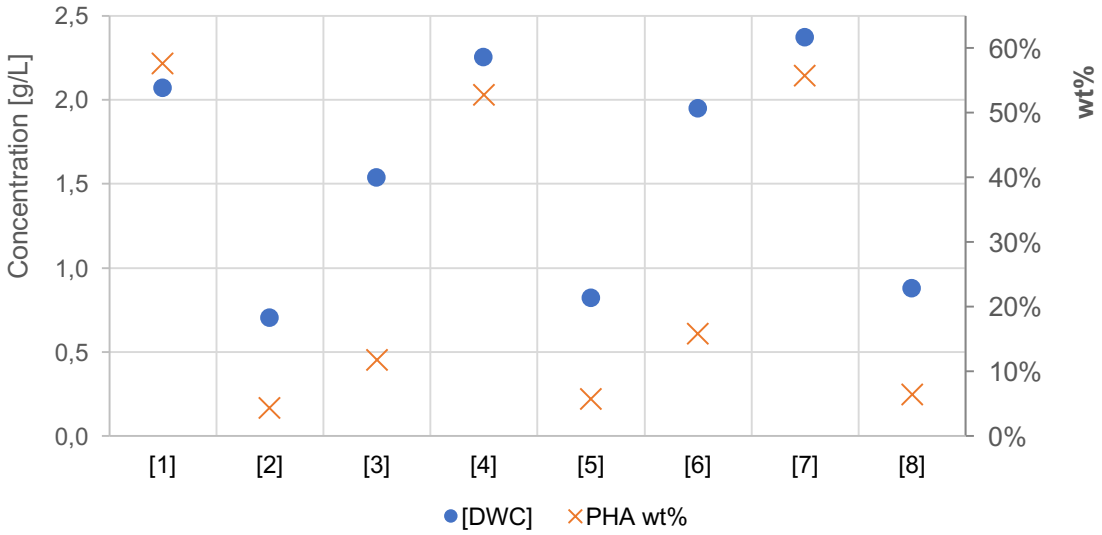



**Figure 18.** Cell Dry Weight (CDW) production and PHA accumulation (wt% CDW) in cultures under different conditions in Experiment P301.

Experiment P301 was designed to test four hypotheses. The first hypothesis tested was that the stock strain of *P. putida* LS46 used for experiments P101 and P202 were different. To test this hypothesis two different stocks of *P. putida* LS46 were tested side-by-side. Flasks 1, 2, and 3 were inoculated with a stock of *P. putida* LS46 from Dr. Riffat Munir ("Riffat's Stock"), while flasks 4, 5, and 6 were inoculated with a stock of *P. putida* LS46 from Mr. Chris Dartiailh ("Chris's Stock"). The results of experiment P301 show that cell mass production (CDW) and PHA accumulation (wt% CDW) were very similar in cultures 1 and 4, 2 and 5, 3 and 6, demonstrating that the source of the bacteria was not the problem, and thus the hypothesis was rejected.

The second hypothesis was that the differences in CDW and PHA production resulted from differences in the carbon sources. The data show that cell mass production (CDW) and PHA accumulation (wt% CDW) was significantly greater in flasks 1, 4, and 7, which contained 20 mM octanoic acid, than in flasks, 2, 3, 5, 6, and 8, which contained 20 mM ACAS (Table 18; Figure 18). Thus, the hypothesis was supported.

The third hypothesis was that aeration may account for differences in cell mass production and PHA accumulation. However, cell mass production (CDW) and PHA accumulation (wt% CDW) in cultures 4 and 7 were very similar, even though culture 4 was in a smooth-sided (non-baffled) flask, while culture 7 was in a baffled flask. Thus, the hypothesis was rejected.

The fourth hypothesis was that differences in cell mass production and PHA accumulation were due to differences in batches of the ACAS substrate. The data generated by experiment P301 clearly support this hypothesis. Cell mass production (CDW) and PHA accumulation (wt% CDW) were significantly lower in flasks 2 and 5, containing "light" ACAS compared with flasks 3 and 6, which contained "dark" ACAS.

## CONCLUSION

A year project long, involving the use of a novel PHA producing strain Pseudomonas putida (strain L46) and long chain free fatty acids derived from canola oil (ACAS) as carbon source was conducted. During the project, experiments were carried out in flasks (Phase I) and batch bioreactors (Phase II). Cell mass production and PHA accumulation were found to be very low in the intial experiments of both Phases, and thus a third experiment (Phase III) titled "Other experiments" was conducted. A summary of each Phase parts involving the thesis are presented as follows.

### Phase I

Experiment P101: The data from experiment P101 shows that cultures containing higher concentrations of carbon (C) substrate concentrations produced greater amounts of cell mass than cultures with low C concentrations. Also, cultures containing high concentrations of phosphorus (P) supported good cell growth (i.e. delayed the on set of stationary phase) at 48 h pi, cultures with higher P concentrations had lower PHA accumulation, while cultures with high C and low P concentrations produced less cell mass, but accumulated greater amounts of PHA.

Experiment P102: Little difference in either cell mass production or PHA accumulation were seen in cultures with high C concentrations, regardless the nitrogen (N) concentration.

### Phase II

Experiment P201: Cell mass production and PHA accumulation were evaluated at three different dissolved oxygen (DOTs = 0%, 5% and 10%) in cultures containing 20 mM or 80 mM ACAS. Cell mass production and PHA accumulation were very similar in all cultures containing 20 mM ACAS, regardless of the dissolved oxygen concentration (DOT – o%, 5%, or 10%). However, cell mass production and PHA accumulation were significantly greater in cultures containing 80 mM ACAS and a dissolved oxygen concentration (DOT) of 0%,

Experiment P202: Cell mass production and PHA accumulation were evaluated under different air-flow rates, 6 LPM versus 1 LPM, and different N concentrations (1.5 g/L versus 0.5 g/L). When the air-flow was decreased from 6 to 1 LPM with the same N concentration (1.5 g/L), the cell mass production was similar up to 6 h ppi, but then decreased steadily in the culture with 1 LPM air-flow. PHA accumulation followed a similar pattern in that PHA production was similar in they both cultures up to 8 h pi, and then began to decrease steadily in the culture with 1 LPM air-flow. In the culture with an air-flow of 6 LPM and an N concentration of 0.5 g/L, the cell mass production was similar to the culture with an air-flow of 1 LPM and 1.5 g/L N up to 12 h pi, and it continued to steadily increase up to 22 h pi, while the culture with 1 LPM air-flow and 1.5 g/L N steadily decreased. The PHA production in P202-3, nitrogen limited, is higher than the other cases, suggesting this condition as a trigger for PHA production.

### Phase III

Experiment P301: Four hypotheses related to culture conditions were tested. The data showed that: 1) the strain of *P. putida* LS46 used for Phase I and Phase II experiments was the correct strain: 2) cell mass production and PHA accumulation were greater with octanoic acid than with ACAS; 3) cell mass production and PHA accumulation were the same regardless if the flasks had smooth, non-baffled sides or baffled-sides; and 4) different batches of substrate ("light" ACAS versus "dark" ACAS) had a significant impact on cell mass production and PHA accumulation.

**Further Studies**

It may be concluded that further studies must be done with ACAS as a low-cost carbon source as other low-cost carbon sources, as crude biodiesel-derived (waste) free fatty acids can produce greater cell mass production and PHA accumulation with the same bacterial strain. More investigation is also required regarding scaling-up procedures, as there is a notable difference between the results generated in bioreactors compared with those obtained in flasks.

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