Polyhydroxybutyrate Production in a Photobioreactor Using Spirulina Platensis

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science

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by

Adrianna Mika

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London, Ontario, Canada

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Abstract

Biopolymers and other naturally derived chemicals continue to gain attention over petrochemical based products due to the price volatility of crude oil, increasing need to reduce greenhouse gas emissions and public awareness of environmental issues. Cyanobacterium *Spirulina (Arthrospira) platensis*, a commercially feasible blue-green alga, is able to synthesize poly(3-hydroxybutyrate) (PHB), a biodegradable and biocompatible polymer, as an energy storage product of photosynthesis directly from CO₂. A flat plate photobioreactor (PBR) with LED illumination was designed for the cultivation of *S. platensis*. Computational fluid dynamics (CFD) software was used to improve airflow uniformity and mixing in the photobioreactor. PHB extracted from *S. platensis* biomass was characterized and compared to commercially available PHB produced by microbial fermentation. PHB of high purity was obtained with crystallinity lower than that of commercial PHB. Improved polymer properties, such as reduced brittleness due to a lower crystallinity, could further increase the range of applications of PHB.

Keywords: *Spirulina (Arthrospira) platensis*, polyhydroxybutyrate, photobioreactor design, biomass, biopolymer, cyanobacteria, microalgae
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# Table of Contents

Abstract .......................................................................................................................... ii  
Acknowledgments ........................................................................................................... iii  
Table of Contents .......................................................................................................... iv  
List of Tables .................................................................................................................. vi  
List of Figures ................................................................................................................ vii  
List of Abbreviations ..................................................................................................... ix  
**Chapter 1: Introduction** ............................................................................................... 1  
1.1 Thesis objectives ....................................................................................................... 3  
**Chapter 2: Background and Literature Review** ......................................................... 4  
2.1 Microalgae ................................................................................................................. 4  
2.1.1 Historical background of commercial interest ..................................................... 5  
2.2 Microalgae production ............................................................................................... 7  
2.2.1 Open systems ....................................................................................................... 7  
2.2.2 Closed systems ..................................................................................................... 8  
2.3 Flat plate photobioreactor (PBR) design considerations ........................................... 11  
2.3.1 Light intensity ..................................................................................................... 11  
2.3.2 Light source ......................................................................................................... 12  
2.3.3 Light-emitting diodes (LEDs) as a light source .................................................. 14  
2.3.4 Geometry ............................................................................................................. 15  
2.3.5 Gas sparging ........................................................................................................ 15  
2.3.6 Operating conditions ......................................................................................... 16  
2.4 Poly(3-hydroxybutyrate) (PHB) .............................................................................. 17  
2.4.1 Properties and applications .............................................................................. 17  
2.4.2 Production .......................................................................................................... 18  
2.4.3 Photoautotrophic production using cyanobacteria ........................................... 19  
2.6 *Spirulina (Arthrospira) platensis* .......................................................................... 22  
2.6.1 Production and applications .............................................................................. 22  
2.6.2 Growth considerations ...................................................................................... 23  
2.6.3 *Spirulina* and flat plate PBRs ............................................................................ 24  
2.6.4 *Spirulina* and LEDs ......................................................................................... 25  
2.6.5 *Spirulina* and PHB ......................................................................................... 26  
2.7 Relationship of the literature reviewed with the research plan ................................. 28  
**Chapter 3: Materials and Methods** ......................................................................... 30  
3.1 Chemicals .................................................................................................................. 30  
3.2 Design of a flat plate PBR with LED illumination ..................................................... 30  
3.2.1 Multiphase flow simulation model ..................................................................... 30  
3.2.2 Construction of flat plate PBR .......................................................................... 30  
3.2.3 Simulation validation ......................................................................................... 31  
3.2.4 LED illumination system design ........................................................................ 31  
3.3 Cultivation of *S. platensis* in flat plate PBR under LED illumination ...................... 32
Chapter 4: Results and Discussion

4.1 Design of a flat plate PBR with LED illumination ........................................... 40
   4.1.1 Multiphase flow simulation model ................................................................. 40
   4.1.2 Gas sparger design ......................................................................................... 40
   4.1.3 Geometry modifications .................................................................................. 43
   4.1.4 Simulation validation ...................................................................................... 44
   4.1.5 Implementation of LED illumination ............................................................... 46
   4.1.6 Final design ..................................................................................................... 48

4.2 Selection of cultivation conditions for *S. platensis* biomass production .................. 49
   4.2.1 Growth medium ............................................................................................ 49
   4.2.2 PBR hydrodynamics ...................................................................................... 52
   4.2.3 Effect of PBR lighting ..................................................................................... 56
   4.2.4 Final conditions ............................................................................................. 59

4.3 Cultivation of *S. platensis* in a flat plate PBR ....................................................... 60
   4.3.1 Comparison of growth curves ........................................................................ 60
   4.3.2 Viability of *S. platensis* ................................................................................ 64
   4.3.3 Comparison of PHB content in *S. platensis* .................................................. 65
   4.3.4 Comparison of pigment contents .................................................................... 68

4.3 Characterization of polyhydroxybutyrate (PHB) by *S. platensis* ......................... 71
   4.3.1 Visualization of Intracellular PHB ................................................................. 71
   4.3.2 Characterization of PHB ............................................................................... 72
   4.3.3 Crystallinity of *S. platensis* PHB ................................................................. 78

Chapter 5: Conclusions

5.1 Summary of conclusions ...................................................................................... 80
5.2 Future work ......................................................................................................... 81

References ............................................................................................................... 83
Appendix A: Supplementary Simulation Results ....................................................... 93
Appendix B: Confocal Images .................................................................................. 98
Appendix C: XRD Data ............................................................................................ 100
Curriculum Vitae .................................................................................................... 102
List of Tables

Table 1: Comparison of PHB thermal and mechanical properties to polypropylene .......... 18
Table 2: PHB contents in Spirulina reported in the literature........................................... 27
Table 3: Compositions of modified Zarrouk and Zarrouk media..................................... 33
Table 4: Summary of cultivation conditions investigated.................................................. 34
Table 5: Cultivation conditions investigated in the flat plate PBR........................................ 52
Table 6: Cultivation conditions investigated in PBR with modified sparger ..................... 56
Table 7: Selected cultivation conditions for S. platensis ...................................................... 59
Table 8: Comparison of growth kinetics between runs 9 and 10 with S. platensis ............. 64
Table 9: Absorption by pigments in S. platensis ................................................................. 69
Table 10: Crystallinity parameters of S. platensis PHB and Commercial PHB characterized by FTIR................................................................. 75
Table 11: Crystallinity of S. platensis PHB and Commercial PHB characterized by XRD .. 76
List of Figures

Figure 1: Commercial applications of microalgae................................................................. 1
Figure 2: Schematic diagram of a raceway pond................................................................. 8
Figure 3: Schematic diagram of a horizontal tubular PBR................................................... 9
Figure 4: Schematic diagram of a flat plate PBR. S/V: surface area to volume ratio, and L: light path length (reactor thickness) ........................................................................... 10
Figure 5: Spectral distribution of solar irradiance at the Earth’s surface and after passage through 1 m of pure water .......................................................... 13
Figure 6: Absorbance spectra of major cyanobacteria pigments ......................................... 13
Figure 7: Chemical structure of PHB, \([\text{OCH(CH}_3\text{)CH}_2\text{C}=\text{O}]_n\) ........................................................................... 18
Figure 8: PHB biosynthetic pathway for cyanobacteria (adapted from [53]) ................... 21
Figure 9: Morphology of helical S. platensis filaments under optical microscopy .......... 22
Figure 10: Simulation materials, models and boundary conditions..................................... 40
Figure 11: Gas sparger designs tested in ANSYS CFX ..................................................... 41
Figure 12: Algal slurry velocity contour in PBR with sparger design #4. A: Side view, and B: zoomed-in side view around the sparger ......................................................... 41
Figure 13: Simulations within sparger tube (1 vvm) .......................................................... 42
Figure 14: Pressure (P) vs. position (x) directly above sparger tube across length of sparger ....................................................................................................................... 42
Figure 15: SolidWorks model of the final sparger design showing hole length \(L_h\) and hole diameter \(\phi\) ...................................................................................................................... 43
Figure 16: Front view of algal slurry velocity contour in reactor with sparger design #4 .... 44
Figure 17: Algal slurry velocity contours in reactor with added fillets ................................. 44
Figure 18: Time lapse of dye injection streaklines in the PBR (left) and simulation results of streamlines (right) at 1 vvm ................................................................................. 45
Figure 19: Airflow pattern in A: PBR (left) and in simulation (right) at 1 vvm ................. 45
Figure 20: PBR with LED panels implemented ....................................................................... 46
Figure 21: Photon flux density vs. panel position ................................................................... 47
Figure 22: Final PBR models. A: Final simulation model of fluid within PBR (quartered), and B: final CAD model of PBR internal frame with sparger ................................. 48
Figure 23: PBR post-construction with one panel removed to show microalgal culture within PBR (left) and with LED panels on (right) .................................................... 48
Figure 24: Biomass density versus time for S. platensis using different media ................. 49
Figure 25: PHB content for *S. platensis* using different media ........................................ 50
Figure 26: Phycobiliprotein content for *S. platensis* using different media .................... 51
Figure 27: Biomass density versus time for five *S. platensis* growth experiments in the flat plate PBR ......................................................................................................................... 52
Figure 28: Comparison of healthy and damaged *S. platensis* filaments ......................... 53
Figure 29: Biomass density versus time for three *S. platensis* growth experiments in the modified flat plate PBR ......................................................................................................................... 56
Figure 30: Biomass density versus time for *S. platensis* for run 9 .................................. 61
Figure 31: Biomass density versus time for *S. platensis* for run 10 ............................... 61
Figure 32: Experimental setups. A: run 9 with HPS illumination, and B: run 10 with red LED illumination (one panel removed to display internals of modified PBR) ............... 62
Figure 33: Average light intensity versus time for *S. platensis* for runs 9 and 10 ............. 63
Figure 34: Damaged *S. platensis* filaments observed during run 9 ............................... 65
Figure 35: Comparison of PHB content in *S. platensis* for runs 9 and 10 ..................... 66
Figure 36: Comparison of microbial and cyanobacterial PHB production with respect to solar energy utilization ..................................................................................................................... 67
Figure 37: Freeze-dried *S. platensis* biomass from run 9 (left) and run 10 (right) ............ 68
Figure 38: Comparison of chlorophyll *a*, carotenoid and phycobiliprotein content in *S. platensis* biomass for runs 9 and 10. Statistically significant differences are denoted with an asterick (P<0.05, n=2). ...................................................................................................................... 69
Figure 39: Spectral output of light sources in runs 9 and 10 .............................................. 69
Figure 40: Confocal micrographs of Nile-red stained *S. platensis* filaments during stationary growth phase. A: auto-fluorescence of *S. platensis*, B: true-colour image of Nile red-PHB fluorescence, C: overlaid image ...................................................................................................................... 71
Figure 41: Confocal micrographs of Nile-red stained *S. platensis* filaments during A: exponential phase, and B: stationary phase. Blue: *S. platensis* autofluorescence, red: Nile red-PHB fluorescence ...................................................................................................................... 72
Figure 42: PHB cast from chloroform using *S. platensis* PHB and commercial PHB. A: 50 mg *S. platensis* PHB, B: 10 mg *S. platensis* PHB. ................................................................................. 73
Figure 43: FTIR spectra of *S. platensis* PHB and commercial PHB .................................. 74
Figure 44: FTIR spectra (in the range of 1100–1500 cm⁻¹) of *S. platensis* PHB and commercial PHB .......................................................................................................................... 74
Figure 45: XRD spectra (in the range of 2θ = 10° – 40°) of *S. platensis* PHB and commercial PHB .......................................................................................................................... 76
Figure 46: DSC heating curves for *S. platensis* PHB and commercial PHB ....................... 77
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>cdw</td>
<td>cell dry weight</td>
</tr>
<tr>
<td>CFD</td>
<td>computational fluid dynamics</td>
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<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared imaging</td>
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<tr>
<td>HPS</td>
<td>high-pressure sodium</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>PAR</td>
<td>photosynthetically active radiation</td>
</tr>
<tr>
<td>PBR</td>
<td>photobioreactor</td>
</tr>
<tr>
<td>PFD</td>
<td>photon flux density</td>
</tr>
<tr>
<td>PHB</td>
<td>poly(3-hydroxybutyrate)</td>
</tr>
<tr>
<td>vvm</td>
<td>volume of gas per volume of culture per minute</td>
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<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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Chapter 1: Introduction

Biopolymers, biofuels and other naturally derived chemicals continue to gain attention over petrochemical based synthetic products due to the price volatility of crude oil and public awareness of environmental issues. The growing global demand for energy and petrochemical based products such as plastics, along with the increasing need to reduce pollutant emissions and greenhouse emissions, is driving extensive research efforts towards the development of sustainable production processes. Of special interest are the efforts to capture carbon and harness the energy of the sun using a biological approach through microalgae biotechnology. As a result of their high photosynthetic efficiency, minimal nutrient requirements, ability to sequester CO₂, ability to grow in marine, brackish or wastewaters and on non-arable land, and unique biochemical composition, microalgae are being studied for a wide array of commercial applications, as illustrated in Figure 1 [1].

![Figure 1: Commercial applications of microalgae](image)

The cost of microalgal biomass production, however, is still on the order of $5/kg or more [2]. Many methods to increase microalgal growth rates are being researched, such as strain-
specific optimization of growth conditions (e.g. response surface methodology), genetic engineering, and the development of closed photobioreactors (PBRs). It has been well established through numerous economic evaluations of microalgae to biofuels processes (e.g. biodiesel, <$1/kg value) that selling high value co-products is necessary if any of the technologies are to be profitable [2, 3].

The purpose of this investigation was to first improve mixing, light supply and growth kinetics of microalgal production through PBR engineering. Secondly, we proposed blue-green alga *Spirulina platensis* as a potential host microorganism for the production of poly(3-hydroxybutyrate) (PHB), a biopolymer with medical applications.

Enclosed PBRs are used to produce high-density cultures of microalgae on a large scale in a closed and controlled system. In this work, a flat plate PBR was selected in order to maximize the illumination surface area and light penetration into the culture. Biomass productivities of photoautotrophic microalgal cultures obtained in PBRs are 5-20 times above what is obtained in current commercial open ponds; however, they may cost 10 times more than open systems [4, 5]. Many ways to reduce costs are being researched. For example, demonstration scale studies of algae production have found that mixing can account for almost 25% in energy demands [6].

PHB is a thermoplastic polyester synthesized by a wide variety of bacteria and is receiving notable attention as an alternative to synthetic polymers due to its biodegradability, biocompatibility and similar properties to polypropylene [7]. Blue-green algae (cyanobacteria) are being researched as hosts for low-cost PHB production due to their minimal nutrient requirements and ability to accumulate PHB as an energy storage product of photosynthesis directly from CO\(_2\) – unlike current commercial PHB production via microbial fermentation, which requires costly carbon sources and high oxygen demand [8]. In this work, *S. platensis* was selected as the host for PHB production because large-scale production of *S. platensis* has been shown to be feasible [9]. *S. platensis* has been reported to accumulate up to 10% cell dry weight of PHB [9].

Moreover, if the production of *S. platensis* biomass can be improved in an enclosed PBR, sustainable polymer production directly from CO\(_2\) becomes more feasible. Polymers like polypropylene are produced and consumed in large quantities and are based on depleting
petrochemical feedstocks. Although polypropylene is relatively inexpensive, the other unique properties of PHB are attractive and allow for novel applications. In particular, its biodegradability and biocompatibility make PHB increasingly attractive for biomedical applications such as medical implant materials and drug delivery vehicles [10]. Unlike bacteria currently used for commercial production, blue-green algae do not contain inflammatory lipopolysaccharides, suggesting that algal PHB might be less pyrogenic and so more suitable for biomedical uses [11]. And unlike bacteria that require complex carbon to produce PHB, which is expensive, inefficient and competes with food sources, *S. platensis* produces PHB directly from CO\(_2\) and more efficiently in terms of solar energy utilization.

### 1.1 Thesis objectives

The specific objectives of this work were to: (1) use computational fluid dynamics (CFD) software to further optimize the design of a flat plate PBR by improving mixing and airflow uniformity out of a sparger; (2) implement an LED illumination system to improve the supply of light energy; (3) use the PBR to grow *S. platensis* and produce PHB; and (4) extract and characterize the PHB produced by *S. platensis* to compare the polymer properties with commercial PHB.
Chapter 2: Background and Literature Review

Biotechnology, technology based on biological systems, is now one of the major growing areas in science and engineering. A historical example of biotechnology is fermentation for the production of wine and vinegar. Within the broad disciple of modern biotechnology, the use of microalgae has great potential to manufacture products that improve our lives such as food, animal feed, materials, chemicals and fuels. Additionally, microalgal processes allow us to potentially reduce our global environmental footprint, resulting in their increasing importance in green process engineering.

2.1 Microalgae

Algae are a very large, diverse and polyphyletic group of organisms that have been shown to date back up to 3.5 billion years ago from fossil records. Algae can range from large, filamentous seaweeds (macroalgae) to microscopic, single-celled organisms (microalgae). While there is no universal definition of algae, they are chlorophyll-bearing organisms, which have no true roots, stems and leaves or leaf-like organs that are found in higher plants [12]. All algae have the common characteristic of oxygenic photosynthesis (equation 2.1). For this reason, both prokaryotic cyanobacteria (referred to as blue-green algae) and eukaryotic microalgae can be classified as algae. Moreover, prokaryotic cyanobacteria are also considered to be “microalgae” alongside eukaryotic microalgae with respect to microalgae technology as they are both commercially promising producers of specialty chemicals and biofuels.

\[
6\text{CO}_2 + 6\text{H}_2\text{O} \rightarrow \text{hv} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 \quad (2.1)
\]

The capacity for photosynthesis allows algae to use light as a source of energy to convert carbon dioxide to sugar using water as an electron donor. It is a redox process in which \(\text{H}_2\text{O}\) is oxidized (a very energy demanding reaction) and \(\text{CO}_2\) is reduced. A thermodynamic limitation exists due to the fact that photosynthesis is endothermic; the reaction requires energy (\(\Delta\text{H} \approx 2,800 \text{ kJ/mol glucose}\)) from light to proceed. Furthermore, an efficiency variable exists when considering the conversion of solar energy to biomass due to the energy losses associated with many steps of the photosynthesis process. For example, solar energy
that is reflected and transmitted if photons do not reach light-harvesting complexes, or solar energy with wavelengths that cannot be utilized by chlorophyll and accessory pigments. The region of solar energy that can be utilized for photosynthesis is between 400 to 700 nm.

Microalgae are particularly attractive for their high photosynthetic efficiency (and thus higher CO$_2$ fixation) compared to terrestrial plants that need to spend energy on growing roots and other organelles (4-6%; 1% reported for conventional energy crops) – with cyanobacteria (up to 10%) having an even greater photosynthesis capability compared to eukaryotic algae (5%) [13, 14]. According to endosymbiosis theory, chloroplasts in eukaryotic cells originated by engulfing (but not digesting) cyanobacteria. Having faster growth than other multicellular plants, microalgae can double their biomass in times ranging from 2 to 5 days [15].

In addition to carbon dioxide, water and light, inorganic salts are required. The molecular formula of microalgal biomass can be approximated as C$_{100}$H$_{183}$O$_{48}$N$_{11}$P$_1$ and contains approximately 50% carbon by dry weight [16]. This can be used to estimate some of the nutrient requirements for growth, however phosphorus is typically supplied in excess as phosphates complex with metal ions and thus not all bioavailable, and other essential elements like iron are also required albeit in smaller amounts [16].

2.1.1 Historical background of commercial interest

The much higher growth rates and productivities of microalgae compared to conventional terrestrial crops, along with their minimal nutrient requirements and ability to adapt to live in a wide range of environments have driven the interest in the production of microalgae for industrial applications. In addition to requiring less land than conventional crops, they do not require arable land and thus do not compete for arable soil with crops that are used as a food source for human consumption.

The large-scale culture of microalgae first began in Japan by Nihon Chlorella to produce green alga _Chlorella_ in the early 1960s, followed by cyanobacterium _Spirulina_ in Lake Texcoco, Mexico by Sosa Texcoco and the French Institute of Petroleum during the 1970s [5, 17]. In the 1970s, the first oil crisis led to the launch of an R&D program by the U.S. National Renewable Energy Laboratory, which included biodiesel from microalgae.
However, the program lasted until 1996 as the Department of Energy reduced funding allocated to the program and experiments were discontinued [5].

Since then, a new interest in the production of biodiesel from microalgae has re-emerged due to once again crude oil price instability and environmental motives (e.g. climate change, rising CO₂ levels and depleting resources). There are at least 40 companies currently exploring algae as a fuel source [5]. Species of particular interest for the production of biofuels due to their high lipid contents include *Botryococcus braunii*, *Chlorella*, *Dunaliella*, and *Nannochloropsis*.

Aside from the usage of microalgae for biodiesel, large-scale algae production facilities were already established in Asia, India, the USA, Israel and Australia by the 1980s for human nutrition [18]. Species of commercial interest include *Chlorella* and *Spirulina* as health foods with high nutritional value, *Dunaliella salina* for β-carotene (vitamin A precursor) production, *Haematococcus pluvialis* for astaxanthin production, and *Cryptothecodinium cohnii*, *Shizochytrium* and *Nannochloropsis* for polyunsaturated fatty acid (PUFA) production such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [19]. More than 70 companies are known to produce *Chlorella* [19]. Molecules such as β-carotene and astaxanthin are powerful antioxidants and natural colourants with applications in the health, nutraceutical, cosmetics, food and feed industries. Most notably, these molecules have high market values. For example, naturally derived astaxanthin has a value of $10,000/kg, which is much higher than synthetic sources of astaxanthin valued at $2000/kg [20]. Even PUFAs such as EPA used for human nutrition have values of $200/kg [20].

In the last 30 years, the potential applications of microalgae in modern biotechnology being developed have continued to grow, particularly in the field of genetic engineering due to advancements in DNA sequencing and methods to introduce, delete, or change metabolic pathways [18]. Most notably is applying metabolic engineering and genetic methods to develop algal strains optimized for high productivity (e.g. reducing chlorophyll antenna size) and increased contents of higher value compounds [21]. Researchers are also looking to genomes of algae that have been sequenced to develop new applications. For example, the FDA recently approved human clinical trials for RetroSense Therapeutics/RST-001 (Michigan, USA) to transplant the gene for a light-sensitive protein channelrhodopsin-2 from
unicellular green alga *Chlamydomonas reinhardtii* into the eyes of blind humans and investigate the extent of vision restoration [22].

### 2.2 Microalgae production

While the genetic modification of microalgae is mainly studied on a small scale in laboratories, for any industrial production to be possible, there is a need for the scale up of processes to produce grow microalgae (whether transgenic or not). Genetic engineering can be applied as a means to increase growth kinetics of algae and thus reduce costs, or to increase yields of high value metabolites and thus improve profitability. However, the production costs behind photobioreactors (PBRs) are arguably just as important. Especially considering the fact that if transgenic algae are ever to be used industrially, closed PBR systems will be necessary in order to ensure the transgenic species do not enter the ecosystem [5]. Both open and closed microalgae cultivation systems were evaluated for their suitability in this work.

#### 2.2.1 Open systems

Microalgae are commercially produced in open systems such as raceway ponds, shown in Figure 2, as they are very cost efficient on a large scale. A raceway pond configuration consists of narrow channels with minimal depth (15-50 cm) to allow for appropriate light penetration, arranged into a large closed loop circulation system. Mixing is provided by paddle wheels for gas/liquid mixing, the dispersion of nutrients and preventing the sedimentation of algae [23]. Baffles are incorporated at bends to guide the flow. The supply is CO$_2$ is typically from the atmosphere, but submerged aerators can be incorporated for additional carbonation. Overall, material, energy and maintenance costs are fairly inexpensive.
Although growing microalgae in open ponds is more economical than enclosed PBRs, they suffer many disadvantages [24]. Drawbacks of such systems include losses due to evaporation, difficulty in maintaining a monoculture due to the risk of contamination, poor light penetration and low productivities. Open pond systems typically require the use of strains that thrive in highly selective conditions, such as *Dunaliella salina*, which requires a highly saline media. While economical in tropical and subtropical locations around the Pacific Ocean, it is unlikely that the traditional open pond systems will ever be feasible in Canada, at least during cold winters and lesser levels of solar exposure. Most importantly, cultures grown for pharmaceutical applications (such as for astaxanthin) must be maintained entirely free of any microbial contamination likely to occur in open ponds.

2.2.2 Closed systems

Closed systems may cost ten times more than open systems [5]. Biomass productivities of photoautotrophic microalgae cultures obtained in enclosed PBRs, however, are 5-20 times above what is obtained in open raceways [4]. It is reported that high cost/high yield closed systems and low cost/low yield reactors open systems can theoretically be equally profitable [24].

High-density cultures of microalgae achieved in enclosed PBRs have several advantages, such as the elimination of a concentration step, less chance of contamination, and being less susceptible to seasonal changes.

All PBRs are closed vessels whereby an algal broth is allowed to collect light energy, and all contain a supply of CO$_2$, degassing for the removal of O$_2$ and temperature regulation. High
O₂ concentrations in the algal broth can result in the inhibition of photosynthesis [16]. In open ponds, O₂ can diffuse into the surrounding air. In PBRs, however, the build-up of O₂ within the closed system is an issue. For mixing and the prevention of sedimentation, as opposed to a paddle wheel, PBRs typically require a highly turbulent flow created by means of an airlift pump or a mechanical pump. The use of a mechanical pump, however, is largely avoided to minimize stresses on the organisms and potential cell damage [16]. The major variable that is changed across different PBR configuration is the vessel geometry. The major closed PBR configurations are tubular (horizontal or vertical) or flat plate. Depending on the availability of sunlight, an artificial light supply may be implemented, either externally or internally.

Horizontal tubular PBRs (shown in Figure 3) consist of small diameter tubes (up to 80 m in length) that run horizontally and an external degassing chamber to both supply CO₂ and remove O₂. Vertical tubular configurations (or column PBRs) are larger in diameter and aerated from the bottom of the vessel to allow for the introduction of CO₂ into the system. There is an issue of dark zones near the middle of the column that can be alleviated with heavy mixing, but it is important to keep shear stresses on the surface of cells to a minimum to avoid cell death [25]. As a result, an airlift or bubble column PBR design with internal illumination is typically preferred for this configuration.

![Figure 3: Schematic diagram of a horizontal tubular PBR](image-url)
Flat plate PBRs (shown in Figure 4) consist of two high light-transmitting plates separated by a small distance (1-30 cm) with an air-bubbling tube placed along the bottom [26]. Flat plate PBRs are being studied as a more cost-effective option for mass production over tubular PBRs [27]. Their entire surface area is well illuminated and the thin space between the flat plates allows for light to penetrate with no dark zones in the system; this is an essential advantage for high photoautotrophic productivity. They can also be tilted for direct exposure to sunlight. It is simple to construct once the unique optimal light-path of an algal species is empirically determined [28].

Figure 4: Schematic diagram of a flat plate PBR. S/V: surface area to volume ratio, and L: light path length (reactor thickness)

The major problem in tubular PBRs arises from the O$_2$ and CO$_2$ gradient that develops in the tube [25]. Flat plate PBRs are useful for the mass production of biomass, as it does not allow for a large concentration of dissolved oxygen since the dissolved O$_2$ path (i.e. the height of the reactor) is very small [27]. Since sedimentation is also still a problem in tubular PBRs, highly turbulent flow must be used [16]. The necessary driving force for maintaining a turbulent flow is inversely proportional to the diameter of tubing. A high velocity of the algal culture is thus required to achieve turbulent flow since density and viscosity remain constant and the tubing diameter is small in order to achieve a high surface area-to-volume ratio. The high velocity required can create shear stresses on microalgal cells. Flat plate PBRs, however, are stirred very effectively by the airflow out of the sparger. The airflow rate may also be accurately controlled to set the optimal rate of mixing.
2.3 Flat plate photobioreactor (PBR) design considerations

In general, the light capture efficiency is arguably the most important parameter for PBR engineering. The biochemical reaction of interest, photosynthesis, is dependent on the supply of light energy to proceed. However, any waste of incident light will result in its conversion into heat. The heat energy will affect the temperature of the system, which in turn can affect the microalgal growth rate, in addition to the solubility of the gases within the system.

Secondly, the hydrodynamic parameters within the PBR can be just as important. In flat plate PBRs, mixing is provided by the supply of gas. It is important to achieve a balance between the minimization of hydrodynamic stresses as to not damage cells, while achieving sufficient mixing for gas/liquid mass transfer and exposure to light. Mass transfer rates are affected not only by the fluid properties and velocities, but also the geometry of the vessel. It is important to consider all parameters and their relationships with each other.

2.3.1 Light intensity

The irradiance (flux of energy per unit area) of incident light, $I_o$, depends on the type of light source, intensity, and relative position of the light source from the culture, all of which can be controlled. As mentioned previously in section 2.1, the spectral range that algae can use in photosynthesis, referred to as photosynthetically active radiation (PAR), is between 400 and 700 nm. The intensity of a light source is directly associated with the number of photons that are emitted per unit area. Thus, the unit of measure of incident irradiance in the PAR region is typically reported in the unit of $\mu$mol photons/m$^2$ s (which can approximated to W/m$^2$ s if the light output spectra are known).

Photosynthesis and the resulting biomass production rate is a function of light intensity, referred to as the photosynthesis-irradiance (PI) curve. While the specific irradiance values will vary for every species, it has been well established that there are three regions on the PI curve: (1) a light-limited region, (2) a light-saturation region, and (3) a photo-inhibition region. The light-limited region begins at a critical light intensity at which microalgal cells can begin growing; photosynthetic rate increases linearly with irradiance until a level of light saturation is reached. At light-saturation, the photosynthetic rate has reached its capacity and remains constant at this maximum value; as irradiance increases in this region, excess energy
is dissipated as heat or fluorescence thus reducing the light utilization efficiency of the process. Once a critical irradiance level is reached, the amount of excess energy results in photo-inhibition whereby growth rate decreases due to photo-damage and can lead to cell death. These rates differ between species and even between strains of the same species, and also depend on temperature [17]. Moreover, microalgae acclimated to lower or higher light levels will respond differently to a new light environment they are exposed to [21].

Designing a system whereby the light intensity can be controlled, either by control of the distance between the light source and algal culture, or by means of electronic instrumentation, is beneficial.

2.3.2 Light source

In addition to designing a PBR to effectively provide the correct quantity of light, it is beneficial to provide the correct quality of light as well. The energy of a photon is inversely proportional to its wavelength \((E \propto \lambda^{-1})\), thus shorter wavelengths have higher energy contents and vice-versa. The energy content of wavelengths longer than 750 nm is too low to mediate photo-chemical changes and will only result in a thermal change, while energy below 380 nm results in ionizing effects [21].

Moreover, it is beneficial to consider the relative absorbance in the range of PAR by pigment molecules in microalgae, because photons can be reflected, transmitted, or absorbed by the molecules. Figure 5 shows the spectral distribution of solar irradiation at the Earth’s surface and Figure 6 shows the absorbance spectra of a few major pigment groups in cyanobacteria [29, 30]. As shown in Figure 5, blue light (approximately 425 – 490 nm) penetrates farther into water compared to red light (approximately 620 – 740 nm) [29].
Figure 5: Spectral distribution of solar irradiance at the Earth’s surface and after passage through 1 m of pure water (adapted from [29])

Figure 6: Absorbance spectra of major cyanobacteria pigments

Chlorophyll $a$, the major photosynthetic pigment present in all species of microalgae, most effectively transfers energy from an absorbed photon when excited at 680–700 nm. The energy of photons in this range is similar to the energy input required to raise the energy level of an electron in chlorophyll $a$ from a stable ground state ($S_0$) to a higher energy level excited state ($S_1$) [21]. The excited electron is transferred from chlorophyll $a$ to other molecules along a chain of electron carrier molecules, in order to produce NADPH from NAD$^+$ and ATP from ADP, which are used during the Calvin cycle and carbon fixation, and other cellular processes. Below the range of 680–700 nm, a portion of the absorbed energy
may be lost as fluorescence or heat. Although chlorophyll $a$ has an absorbance peak at 435 nm as well, a portion of the energy that is absorbed is not used for photosynthesis, thus light utilization efficiency is reduced [21]. Accessory pigments such as chlorophyll $b$ (absorbance peaks at 640 and 450 nm, not present in cyanobacteria), carotenoids (absorbances between 400-550 nm), and phycobiliproteins (absorbance peaks at 615 nm for phycocyanin and 650 nm for allophycocyanin), all differ in their absorption spectra to broaden the spectral range the cells can utilize for photosynthesis.

2.3.3 Light-emitting diodes (LEDs) as a light source

Different types of light sources each have different light output spectra. Fluorescent lights are most commonly used with algal growth experiments as they emit light that resembles the spectrum of daylight [21]. However, photosynthetic efficiency can be improved if a light source with a spectral output that resembles the photosynthetic absorption spectrum of the species being cultivated.

Factors that should be considered for light include: energy emission in the region of chlorophyll $a$ absorption, energy emission in the region of major accessory pigment absorption, conversion of energy into heat, lifetime and cost. It is suggested that light-emitting diodes (LEDs) can best meet these requirements. While LEDs are 2-10 times more expensive than fluorescent lamps, their efficiency is high with very low heat generation (less than 10%), and their lifetime can be up to 5 times longer than other types of light sources [21].

What is of most interest is that they can be designed to produce very narrow spectral output (20 nm full width at half maximum) [31]. The emission spectrum of a red LED (e.g. peak wavelength of 630 nm) can overlap with the photosynthetic absorption spectrum of chlorophyll $a$ very closely. It has been reported that LEDs with a peak wavelength of 643 nm have been considered as the most cost-efficient light source to operate PBRs, for over 1 year under continuous mode [21].
2.3.4 Geometry

Because light capture efficiency is one of the most important parameters in PBR design, high surface area-to-volume ratios (S/V) and optimum light path lengths (L) are needed. As discussed previously, a high S/V ensures maximum culture exposure to light and increases volumetric productivity. The light path length will influence this ratio, as it is one the dimensions of the reactor (thickness, L).

Optimization of the light path, L, should be designed to compromise between the growth inhibition in deep layers due to light limitation, and the inhibition of superficial culture layers by excess illumination [32]. The light intensity decreases exponentially with distance from a reactor wall as the biomass density increases. The Lambert-Beer’s law gives the irradiance profile:

\[ I(z,X) = I_o e^{-kzX} \] (2.2)

where \( k \) is the light attenuation coefficient of biomass, \( I_o \) is the measured light intensity at the reactor surface and \( I(z,X) \) is the light intensity at a given depth of optical path, \( z \), and biomass density, \( X \) [33]. In dense cultures, light intensity tends to decrease drastically due to the shading effects of cells, which block other cells from the available light. A reduced light path increases the probability of cell exposure to the optimal light regime, but at some point a further reduction would not result in increased productivity [34]. It is commonly reported that a light path of 1-2 cm may be the practical optimum for designing large-scale enclosed PBRs [34].

An advantage to flat plate configurations is that light can be supplied from either panel to minimize the light-limited region between the panels, without increasing the intensity of light exposed to the superficial layer of cells per one panel.

2.3.5 Gas sparging

Gas sparging is used for the introduction of CO\(_2\) into the system and the removal of O\(_2\) out of the system. The importance of gas sparging, however, is to provide adequate mixing. Turbulence from mixing breaks down diffusion barriers around the cells, resulting in
improved CO₂ transfer from the gas phase to the cell phase [35]. Secondly, turbulence induced by the rising air bubbles will induce a degree of mixing tangential to the flow direction [36]. Cells are carried by the turbulent eddies which results in the random motion of algae across the optical path length of the reactor in a few tens of milliseconds [37]. The random exposure of algal cells between light and dark regimes enhances photosynthetic efficiency by overcoming cell shading and/or potential photosaturation of cells at the superficial layer of the reactor [35]. Finally, heat transfer within the culture is improved with efficient mixing.

At low gas flow rates, dead zones and settling of biomass may occur toward the bottom of the reactor. An accumulation of O₂ in dead zones can lead to cell deterioration and may compromise the viability of the whole culture [38]. Given adequate mixing, freely suspended microalgae and media are typically treated as one liquid phase [38, 39]. Inadequate mixing, however, also allows for the formation of biomass aggregates which leads to a three-phase system within the PBR (gas, liquid, solid) and may decrease mass transfer rates [38]. Additionally, shear sensitive algal cells carried by the rising bubbles may be damaged when the bubbles collapse at the bubble break-up at the liquid-air interface at the top of the reactor [38]. Microalgal species react differently to hydrodynamic forces (i.e. pressure gradients and shear stress) and will have a specific optimal rate of mixing [40]. It has been reported that aerations as low as 0.05vvm can be optimal in flat plate PBR without inducing damage by stress [39].

2.3.6 Operating conditions

A PBR should be designed to allow for accurate control of culture parameters such as pH, temperature and nutrient concentrations. If the supply of CO₂ cannot be balanced with the consumption of CO₂ by the algae, the pH of the culture will vary and may affect growth. Temperature should be controlled to be at or near the optimum for the cultivated organism. The optimum temperature for growth varies between species, between strains of the same species, and also depends on the previous history of growth [17]. If a low cultivation temperature is required and/or heat generation from the light source is high, outer-temperature-regulation chamber can be added outside the vessel for cooling with water [34].
If the PBR is intended for operation under sterile conditions to ensure cultivation of a monoculture, the construction material should be chosen to allow for sterilization before operation.

2.4 Poly(3-hydroxybutyrate) (PHB)

2.4.1 Properties and applications

Poly(hydroxyalkanoates) (PHAs) are a versatile family of linear polyesters of different D-(−)-hydroxyalkanoic acids and are considered to be biopolymers as they are naturally derived. PHAs are biologically synthesized by a wide variety of bacteria and some cyanobacteria under stress conditions as carbon and energy reserves in the form of intracellular, water-insoluble granules. Although PHAs are good candidates for biodegradable plastics with many potential applications, so far only several PHAs have been produced in large scale (via microbial fermentation) to be available for application studies, including poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(4-hydroxybutyrate) (P4HB), and poly(3-hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) [41]. Moreover, their high price compared with conventional plastics has limited their use in a wide range of applications. As a result, many researchers over the past 20 years have been directed to develop medical applications using PHAs as high value added applications due to their biocompatibility [10]. With successful approval of P4HB as a long-term absorbable surgical suture, more PHA-based materials are expected to go into clinical trials soon [42]. Biomedical applications of PHAs include bio-implant materials, drug delivery vehicles, tissue engineering materials and smart materials [42].

PHB is the first, most common, and most well characterized biopolymer belonging to PHAs [43]. It was first characterized in the mid-1920s by French microbiologist Maurice Lemoigne from his work with the bacterium *Bacillus megaterium* [44]. The chemical structure of PHB is shown in Figure 7; it is isotactic, with 100% of the asymmetric carbons in the (R)-stereochemical configuration [45]. As a result, PHB is highly crystalline and brittle. Its molecular weight can vary from 50,000 – 5,000,000 Da [43]. It is particularly attractive for its mostly similar mechanical properties and thermoplastic processability to polypropylene (shown in Table 1) [46].
**Figure 7:** Chemical structure of PHB, \([OCH(CH_3)CH_2C=O]_n\)

**Table 1:** Comparison of PHB thermal and mechanical properties to polypropylene

<table>
<thead>
<tr>
<th>Property</th>
<th>Commercial PHB [8]</th>
<th>Polypropylene [47]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T_m) (°C)</td>
<td>171-182</td>
<td>176</td>
</tr>
<tr>
<td>(T_g) (°C)</td>
<td>0-5</td>
<td>-10</td>
</tr>
<tr>
<td>(X_c) (%)</td>
<td>60-80</td>
<td>50-70</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Young’s modulus (GPa)</td>
<td>3.5</td>
<td>1.5-2</td>
</tr>
<tr>
<td>Elongation-to-break (%)</td>
<td>5</td>
<td>400</td>
</tr>
</tbody>
</table>

Because PHB is brittle and stiff with a relatively high mechanical strength, it is inherently suitable for hard tissue engineering applications, such as bone tissue, where a high mechanical strength is required [48]. PHB has been proposed for use in bone tissue engineering owing to their favourable (i.e. slow) degradation rates and non-toxic degradation products [49]. *In vivo* degradation of PHB yields D-3-hydroxybutyrate, which is a normal constituent of blood. PHAs also exhibit piezoelectricity, which stimulates bone growth and aids in wound healing [48]. Additionally, the chiral monomer units of PHB can be used as basic molecules for the chemical production of complex chiral pharmaceutical agents [46].

### 2.4.2 Production

Bacteria synthesize PHB as an energy storage reserve under stress conditions. High quantities of PHB are not produced until depletion of an essential growth component, such as nitrogen, while an excess amount of carbon is present.

PHB is currently only available from microbial fermentation technology. W.R. Grace (USA) began producing pound quantities of PHB from microbes for commercial evaluation in the 1960s but the project was abandoned because yields were relatively low and the solvent extraction process was expensive. The petroleum crisis of the mid-1970s renewed interest in alternatives to synthetic plastics and ICI (UK) began researching PHB commercialization. Conditions that resulted in high yields of PHB were found, but mechanical properties showed no advantage over polypropylene due to excessive brittleness [44]. In the late 1980s, ICI
began commercializing a co-polymer of PHB and PHBV under the trade name Biopol. Although the biopolymer’s properties were improved, oil prices stabilized and production costs remained higher than those from petroleum products [44]. High production costs due jointly to expensive carbon sources and continuous oxygen demand during the fermentation process continue to limit the use of PHAs today [8].

2.4.3 Photoautotrophic production using cyanobacteria

The capability of cyanobacteria to synthesize PHB was first reported in 1966 [50]. PHB was extracted from cultures of the unicellular cyanobacterium Chlorogloea fritschii and characterized by infrared (IR) spectroscopy. Since then, over 90 cyanobacterial strains belonging to over 25 different genera have been tested for the presence of PHAs. It was found that 60% contain PHAs, and all authors report that PHB is the dominant (if not only) PHA synthesized by cyanobacteria [45]. It is worth noting there is a non-occurrence of PHB in some species and inconsistency of PHB content in species belonging to the same genus, indicating that accumulation of PHB in cyanobacteria appears to be species specific. For example, under the same photoautotrophic growth conditions, the occurrence of PHB in Nostoc commune cannot be detected, while PHB contents of 3.6% and 8.5% cell dry weight (cdw) were detected in N. linckia and N. muscorum, respectively [8]. Most known cyanobacteria capable of synthesizing PHB are reported to accumulate <10% cdw PHB under photoautotrophic growth [8].

Cyanobacteria are being studied as alternative host systems for low-cost PHB production because of their minimal nutrient requirement, short generation time and photoautotrophic nature [8]. Cyanobacteria synthesize their own organic carbon from inorganic carbon using solar energy. In bacteria, carbon substrates such as sugars or lipids are required. The cost of the carbon source is approximately 40% of the total operating cost [51]. Currently efforts are being made to grow the bacteria on inexpensive carbon sources such as different renewable vegetable oils and various waste products [47].

Of high significance is that unlike bacteria currently used for commercial PHB production, cyanobacteria do not contain inflammatory lipopolysaccharides, suggesting that cyanobacterial PHB might be less pyrogenic and so more suitable for biomedical uses [11].
Lipopolysaccharides are found in the outer membrane of gram-negative bacteria and act as endotoxins eliciting strong immune responses, which must be removed.

Still, most known cyanobacteria accumulate <10% cell dry weight (cdw) PHB under photoautotrophic growth [8] whereas bacterial strains are shown to accumulate as much as 50-80% cdw PHB [42]. While sun utilization may be more efficient and potentially less extensive purification required using cyanobacteria to produce PHB, their PHB yields must still be improved in order to further reduce the cost of materials and downstream processing.

Using organic compounds such as acetate, pyruvate, propionate, valerate, glucose or citrate has stimulatory effects on PHB production [8, 45]. Most strains, both bacterial and cyanobacterial, show highest accumulation of PHB in the presence of acetate [45]. Thus, many researchers are using mixotrophic growth conditions with cyanobacteria for enhanced PHB yield [7, 9]. A PHB content as high as 45.6% cdw was achieved in *N. muscorum* when supplemented with acetate and glucose [52].

The presence of acetate in growth medium is known to increase the intracellular concentration of acetyl-CoA, a universal intermediate compound that can easily be converted into PHB by many microorganisms [7]. The PHB biosynthetic pathway described for cyanobacteria is shown in Figure 8 [53, 54]. Studies have shown that only three enzyme groups are involved in the biosynthesis of PHB from acetyl-CoA in both bacteria and cyanobacteria: 3-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase [53-55]. In general, the biosynthetic pathway of PHB starts with two acetyl-CoA molecules reacted via condensation into acetoacetyl-CoA through 3-ketothiolase. Acetoacetyl-CoA is then reduced by acetoacetyl-CoA reductase to form the monomer (R)-3-hydroxybutyryl-CoA. Finally, the key enzyme group PHA synthase carries out the polymerization reaction to produce PHB [55, 56].

It is known that PHB biosynthesis is regulated at the enzymatic level [56]. In addition to high intracellular concentrations of acetyl-CoA, it has been shown that a high intracellular concentration of NADPH (or high ratios of NADPH/NADP) stimulates PHB production [56]. It is reported that the role of PHB in cyanobacteria is to provide cells with a mechanism of removing excess reducing equivalents due to a disruption of the balanced formation of ATP and NADPH from photosynthesis [7]. Furthermore, in the cyanobacterium *Synechococcus*
sp. MA19, it has been shown that PHB synthase activity is activated due to nitrogen deprivation under light conditions [56].

**Figure 8:** PHB biosynthetic pathway for cyanobacteria (adapted from [53])

Thermal and mechanical properties of PHB extracted from cyanobacterium *Nostoc muscorum* showed similar properties when compared to the range of values reported in the literature for commercial PHB [8].

In general, PHB production is typically a two-step cultivation process whereby cells are grown in nutrient-rich medium for biomass production, then transferred into a deficient medium to initiate high PHB production. Supplementation with an additional carbon source is typically applied during the nutrient-limitation step. During the first step, cultivation, PHB content reaches a maximum during the start of stationary phase, and then begins to decrease as the microorganisms begin to mobilize the energy storage molecules [8, 9, 50].
2.6 *Spirulina (Arthrospira) platensis*

*S. platensis* (shown in Figure 9) is a multicellular, filamentous cyanobacterium (blue-green alga) of significant commercial importance. The filaments can range in size in the hundreds of microns. *Spirulina* is the most cultivated microalgae due to its high protein content (~60% cdw), ease to cultivate as a monoculture, tolerance of alkaline conditions, and larger size than single celled algae, thus being easier to harvest [4, 57]. The molecular formula of *S. platensis* biomass can be approximated as $C_{100}H_{173}O_{38}N_{18}S_{1}$ [58].

![Figure 9: Morphology of helical *S. platensis* filaments under optical microscopy](image)

The term “Spirulina” is commonly used as the name for two most important edible species belonging to the genus *Arthrospira*: *A. platensis* and *A. maxima*. These species were originally believed to belong to the genus *Spirulina*, but were reclassified in the early 1990s [59]. The continuing designation of *Spirulina* for these species holds a more traditional and technological meaning than a taxonomic one, due to the considerable amount of time that manufacturers have spent commercially marketing their products under the name “Spirulina” [17, 59]. Both classifications are used in the literature to date. Both *Arthrospira* and *Spirulina* share a helical morphology (Figure 9), but differ in morphological characteristics such as cell wall structure and pore pattern, helicity and filament size, and thylakoid pattern [17].

### 2.6.1 Production and applications

Annual biomass production of *S. platensis* is over 3000 t dry weight, surpassing that of *Chlorella* (2000 t dry weight) and *Dunaliella salina* (1200 t dry weight), making it the most mass cultivated microalga worldwide [19]. Major producer DIC LIFETEC (Japan) owns two companies, Earthrise Nutritionals (California, USA) and Hainan DIC Microalgae (China),

![100 μm]
which produce more than 700 t annually in about 1.85 km² [26]. Cyanotech (Hawaii, USA) produces about 300 t in about 0.36 km² [26]. Currently, all the major producers of Spirulina in the world use raceway ponds for its mass cultivation and the applications are mainly for human and animal nutrition, and cosmetics.

*S. platensis* is currently the organism used for commercial production of the blue phycobiliprotein (pigment-protein) phycocyanin [4]. Phycocyanin has been used in the food and beverage industry and in the cosmetic industry as a natural dye, replacing synthetic pigments [19]. More recently, phycocyanin has been garnering attention for the medicinal properties that it possesses. For example, phycocyanin is used in medical diagnostics as fluorescent antibody tags in cancer, and in biomedical research as a potential pharmaceutical product owing to its antioxidant, anti-inflammatory and immune-enhancing properties [60]. The overall phycocyanin market value has been estimated at $10-50 million/year, making it a high value extract from *S. platensis* (~15% phycocyanin cdw) [57, 61].

### 2.6.2 Growth considerations

The optimal temperature for laboratory cultivation of *S. platensis* has been reported to be 35-38 °C. It has been shown that even at extreme minimum and maximum temperatures of 10 °C and 50 °C, growth can be 28% and 23% of the optimum, respectively [17]. The optimum growth temperature will differ between strains. Cultivation at 24-25 °C is commonly reported in laboratory experimentation [9, 62-65].

*S. platensis* occurs naturally in very alkaline waters and grows favourably within a pH range of 9-10, thus carbon in the form of sodium carbonate or sodium bicarbonate is essential [17]. As discussed previously, CO₂ is the carbon source used by photosynthesis. In aqueous environments, CO₂ exists in equilibrium with the forms H₂CO₃, HCO₃⁻ and CO₃²⁻, and the relative concentrations are a function of pH [17, 38]. In the alkaline region, HCO₃⁻ and CO₃²⁻ species predominate. In some species of microalgae and many species of cyanobacteria such as *S. platensis*, inorganic carbon in the HCO₃⁻ form can be taken up as well due to carbon dioxide concentrating mechanisms [36, 66, 67].
A detailed study on the nutrient requirements of *Spirulina* was performed by Zarrouk as part of a Ph.D. thesis in 1966 [68]. It was never published in a scientific journal, but to date it remains as the source for the medium most commonly used to cultivate Spirulina [17].

It is known that environmental factors such as light and temperature can affect the filament morphology, including a spontaneous change to a straight morphology [17]. This is commonly observed in laboratory settings [69]. Once thought to be an irreversible process, it has been shown that *S. platensis* can randomly convert back into its original helical morphology [70].

It is commonly stated in the literature that *S. platensis* is sensitive to shear stress [71, 72] likely due to its filamentous morphology and the fragility of the sheath (similar to Gram negative bacteria cell wall, mainly composed of peptidoglycans and lipids) surrounding *S. platensis* filaments [35, 73]. As a result, the shear sensitivity can impose constraints in PBR design.

### 2.6.3 *Spirulina* and flat plate PBRs

Currently, all the major producers of *Spirulina* in the world use raceway ponds for its mass cultivation [26]. However, there are several reports in the literature of *Spirulina* cultivation in flat plate PBRs.

Samson and Leduy reported volumetric productivities of 1.17 g/L g/L·d for *S. maxima* in 64 L reactor under continuous cultivation in one of the first descriptions of flat plate PBRs [74, 75]. A production rate of 1.31g/L day has been shown under natural illumination in 20 cm thick flat panel reactors [76]. Tredici et al. proposed a reactor with 1.25 cm thickness and 80 m⁻¹ S/V ratio for the outdoor cultivation of *S. platensis*. It was reported that a biomass density of 5-8 g/L could be maintained when tilted to the sun in the summer [76]. It was shown that a low airflow per unit culture volume per minute (0.36 vvm) could provide a similar biomass yield compared to a high rate of 1.21 vvm. Hu et al. were able to maintain optimal cell densities of 8 g/L *S. platensis* in an outdoor 0.7 m high x 0.9 m long x 2.6 cm thick reactor (12 L) operated in semi-continuous mode and tilted towards the sun throughout the day [74]. They found that an aeration rate of 2.1 vvm resulted in a maximum biomass production, which began to decrease as aeration was increased further [74]. When the optical light path
was reduced to 1.3 cm, a biomass production rate of 4.3 g/L·d and biomass density of 15.8 g/L were achieved (in the summer).

Since then, more experimentation with *S. platensis* in laboratory settings has been reported to utilize smaller scaled flat plate PBR configurations. For example, a 10 mm wide reactor (0.06 L) was used to study the effects of *S. platensis* under intermittent illumination with white LEDs to enhance specific growth rate and light efficiency [77]. A 2 L flat plate PBR using an aeration rate of 0.1 vvm was used to study the reduction of *S. platensis* growth media costs and reported to reach a biomass density of about 2.5 g/L (0.2 g/L·d productivity in batch operation) [65].

It is worth noting that the large scale experimentations, which reported higher biomass yields and productivities, was performed at the optimum temperature for *S. platensis* growth with higher light intensities compared to experimentations done under artificial illumination in laboratory settings, and were optimized for the continuous cultivation of *S. platensis*.

### 2.6.4 *Spirulina* and LEDs

A few reports have studied the effects of LED illumination of *S. platensis* growth and biomass composition [78-80]. The first study that used LEDs with different wavelengths to grow *S. platensis* flask cultures showed that higher light intensities generated more biomass, the use of red LEDs (620-645 nm) showed the largest specific growth rate, and the use of blue LEDs (460-475 nm) showed the lowest [78]. The use of the red LED resulted in a higher specific growth rate than with a white LED (380-760 nm) at the same intensity, and also consumed the least power, which supports the claim that red LEDs can be considered as the most cost-efficient light source to operate PBRs for over 1 year under continuous mode [21]. It is worth noting that blue LEDs are typically used due to the absorption band of chlorophyll *a* at 430 nm and of chlorophyll *b* at 453 nm. However, cyanobacteria do not contain chlorophyll *b*, so the blue LED used in this work would not have been efficiently used by *S. platensis*.

The same group went on to model chlorophyll *a* and phycocyanin production by *S. platensis* under the illumination conditions tested previously [79]. They reported that both chlorophyll *a* and phycocyanin content were highest under blue LEDs and lowest under red LEDs.
Markou [80] observed a similar trend with respect to biomass production, showing the highest biomass production (0.03 g/L·d) was obtained in a 0.4 L cylindrical PBR under monochromatic red and dichromatic pink (red and blue) LEDs, and lowest under blue LEDs. The same trend was observed for chlorophyll a and phycocyanin content that was reported by Chen et al. [79]. The study went on further to demonstrate the effects of wavelength on other biomass components. It was observed that lipid and carbohydrate contents of S. platensis were highest under blue LEDs and lowest under white and pink (red and blue) LEDs [80].

2.6.5 *Spirulina* and PHB

Because *S. platensis* is non-toxic and already commercially established as a health food product, it was selected as the host cyanobacterium for the production of PHB as opposed to other potentially higher PHB accumulating cyanobacteria.

Reports in the literature for the PHB content in *Spirulina* vary, but it has been shown that nitrogen limitation and supplementation with acetate can increase PHB content; the findings are summarized in Table 2 in the order of their appearance in the literature. One study showed that PHB content in *S. platensis* could reach 3.5% cdw under phosphate limited conditions, but not until after 60 days of cultivation [64]. A control experiment with complete medium was not performed. Until recently, the maximum PHB content that has been reported for *S. platensis* to date is 10% cdw under nitrogen limitation and 0.5% (w/w) acetate supplementation [9]. The study showed that other carbon sources such as 1,4-butaneediol and propionic acid could increase PHB content under nitrogen limitation, but the highest increase was observed with acetate [9].

In 2008, a new *Arthrospira* strain was isolated and characterized from Mangueira Lagoon in Brazil [81]. The new strain, named *Spirulina* LEB 18, has recently been reported to accumulate 44% cdw photoautotrophically under reduced nitrogen conditions [82]. In their investigation, acetate supplementation did not stimulate the synthesis of PHB. However, it is worth noting that PHB content was calculated as the mass of extracted PHB per gram of biomass. In another paper published by the same group investigating PHB extraction methods, it was reported that PHB samples contained up to 34.4% impurities as determined by thermogravimetric analysis (TGA) [83]. As a result, there is a level of inconsistency
between these measurements compared to other reports, which used other PHB quantification methods (e.g. PHB conversion to methyl esters for gas chromatography).

### Table 2: PHB contents in *Spirulina* reported in the literature

<table>
<thead>
<tr>
<th>Year [Reference]</th>
<th>Species</th>
<th>Carbon source</th>
<th>Nutrient limitation</th>
<th>PHB content (% cdw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982 [84]</td>
<td><em>S. platensis</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂/Acetate</td>
<td>n/a</td>
<td>6</td>
</tr>
<tr>
<td>1992 [85]</td>
<td><em>S. jenneri</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td><em>S. laxissima</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>0.30</td>
</tr>
<tr>
<td>1992 [85, 86]</td>
<td><em>S. maxima</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>0.53</td>
</tr>
<tr>
<td>1992 [86]</td>
<td><em>S. maxima</em></td>
<td>CO₂/Acetate</td>
<td>n/a</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>N-limited</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>P-limited</td>
<td>1.2</td>
</tr>
<tr>
<td>1992 [85]</td>
<td><em>S. platensis</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂/Acetate</td>
<td>n/a</td>
<td>2.9</td>
</tr>
<tr>
<td>1996 [45]</td>
<td><em>S. platensis</em></td>
<td>CO₂</td>
<td>n/a</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂/Acetate</td>
<td>n/a</td>
<td>0.8</td>
</tr>
<tr>
<td>2005 [64]</td>
<td><em>S. platensis</em></td>
<td>CO₂</td>
<td>P-limited</td>
<td>3.5</td>
</tr>
<tr>
<td>2005 [9]</td>
<td><em>S. platensis</em></td>
<td>CO₂</td>
<td>N-limited</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂/Acetate</td>
<td>N-limited</td>
<td>10</td>
</tr>
<tr>
<td>2010 [8]</td>
<td><em>S. subsalsa</em></td>
<td>CO₂</td>
<td>N-limited</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>N-limited, increased salinity</td>
<td>7.45</td>
</tr>
<tr>
<td>2015 [82]</td>
<td><em>Spirulina</em> LEB 18</td>
<td>CO₂</td>
<td>n/a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>Spirulina</em> LEB 18</td>
<td>CO₂/Acetate</td>
<td>N-limited</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Spirulina</em> LEB 18</td>
<td>CO₂</td>
<td>N-limited</td>
<td>44</td>
</tr>
</tbody>
</table>

Until recently, only one report from 2010 characterized the thermal and mechanical properties of cyanobacteria derived PHB, using *Nostoc muscorum* [8]. As mentioned in section 2.4.3, it was shown that the properties were similar to what is reported for commercial PHB. However, less than one year ago, thermal and mechanical properties of PHB from *S. platensis* were published by the same group working with *Spirulina* LEB 18 [87]. *S. platensis* derived PHB was electrospun into nanofibers. Compared to electrospun fibers using commercial PHB, *S. platensis* PHB nanofibers had a lower crystallinity and lower melting point. Comparisons for mechanical properties were made with commercial co-polymer PHBV instead of PHB, which is typically reported to have more desirable polymer properties than PHB [88]. It was found that *S. platensis* PHB nanofibers had a higher stiffness, tensile strength, and breaking elongation.
Finally, the group also reported very recently on the biological effects (and potential benefits) of incorporating *Spirulina* LEB 18 biomass or *Spirulina* biomass extracts such as phycocyanin into nanofiber scaffolds due to their anti-inflammatory and antioxidant properties [89]. It was observed that stem cells had greater adhesion to polylactic acid (PLA) scaffolds with *Spirulina* biomass than to PLA alone; *Spirulina* biomass released from the scaffolds during degradation could increase the number of viable cells compared to PLA alone; and the *Spirulina* containing scaffold could successfully be used in an animal model as an artificial skin tissue for a burn injury in an animal model for 7 days without complications, although no differences were observed compared to the control [7]. Although the studies are in their infancy, only dating back to 2010, the principles behind the work are supported by the large amount of research that exists in the literature on the health benefits of *Spirulina* extracts [4]. There is a potential to produce biomaterials directly from CO$_2$ using *Spirulina*, as well as a potential to add *Spirulina* biomass to the PHB system as a bioactive compound to promote cell growth.

2.7 Relationship of the literature reviewed with the research plan

Because the primary focus of this work is the use of microalgae for the production of a biopolymer with implications in medicine, a closed system was selected as the most suitable option for this work. A flat plate PBR configuration was selected for its high surface area to volume ratio, small light path length, low construction cost, and absence of an additional gas transfer unit. The optimization of mixing via gas sparging was investigated in this work.

Designing a system whereby the light intensity can be controlled, either by control of the distance between the light source and algal culture, or by means of electronic instrumentation, is ideal. For this reason, artificial light can be beneficial in order to select a light source that will output light in the wavelength ranges microalgae can utilize without being wasted or negatively impacting photosynthesis. Because this work was aiming to develop a controlled system, artificial lighting was selected as opposed to solar radiation, which cannot be controlled. In this work, we aimed to implement LEDs as a light source that would have a controllable light intensity, as well as a controllable spectral composition, in order to improve the light utilization efficiency of our system and reduce costs.
The PBR design performed within this work was done in two parts. First, hydrodynamic parameters were investigated using computational fluid dynamics software in order to select vessel and sparger geometries that would improve PBR performance while minimizing energy requirements. Second, an LED illumination system was implemented to further improve the performance and minimize energy requirements.

In terms of PBR design scale-up, the important considerations are the economics, energy requirements, environmental impacts and product value. However, scale-up considerations are not included within the scope of this work. Instead, the remainder of the work following the design of the flat plate PBR focuses on its implementation for an alternative production method of a niche product, polyhydroxybutyrate, using a specific strain of algae.

In general, PHB production is typically a two-step cultivation process whereby cells are grown in nutrient-rich medium for biomass production, then transferred into a deficient medium to initiate high PHB production. Supplementation with an additional carbon source is typically applied during the nutrient-limitation step. During the first step, cultivation, PHB content reaches a maximum during the start of stationary phase, and then begins to decrease as the microorganisms begin to mobilize the energy storage molecules [8, 9, 50]. However, a second step of nutrient limitation to enhance intracellular PHB content was not performed in this work due to time constraints. Thus, obtaining a high PHB yield in *S. platensis* was not within the scope of this work. Instead, the polymer properties of PHB that could be extracted from the native PHB content in *S. platensis*, under photoautotroph conditions, were investigated.

The recent reports in the literature of improved properties of *S. platensis* derived PHB compared to commercial PHB, as well as the potential advantages of adding *Spirulina* biomass to the PHB system, support the present efforts being done in this work to contribute to research on PHB from *S. platensis* [87, 89].
Chapter 3: Materials and Methods

3.1 Chemicals

All chemicals used for PBR flow visualization, *Spirulina platensis* growth experiments, pigment extractions, and PHB extraction and characterization were purchased from Sigma-Aldrich (Oakville, ON, Canada) and ACS reagent grade. Ultrapure water (18.2 MΩ cm at 25 °C) from an ultrapure water system (arium® pro, Sartorius) was used for all experiments.

3.2 Design of a flat plate PBR with LED illumination

3.2.1 Multiphase flow simulation model

SolidWorks was first used to design a 3D model of a flat plate PBR. Computational fluid dynamics software ANSYS CFX was then used for its multiphase flow module.

The simulation model was used to: (1) test various sparger geometries and observe effects on the liquid velocity profiles and streamlines of algal slurry particles to select the best sparger geometry, (2) study pressure differentials within the sparger to further refine the sparger design, (3) determine the optimal rate of mixing, and (4) study the liquid velocity profiles to eliminate potential dead zones by implementing baffles and fillets.

Input parameters that were varied in subsequent investigations were bubble size and gas inlet mass flow rate. Output parameters that were later investigated were wall shear and turbulence eddy dissipation.

3.2.2 Construction of flat plate PBR

After consulting with University Machine Services (The University of Western Ontario, London, ON), high-density polyethylene was selected as the frame material as it is easily machined and inexpensive. Transparent ⅜” thick polycarbonate sheets were used for the flat panel material. The use of big washers with the polycarbonate sheets eliminated the need for an additional outer frame for support.
3.2.3 Simulation validation

Once the PBR was constructed, flow visualization was performed to validate the simulation results. 10 mL of 0.27% (w/v) aqueous fluorescein solution was discharged into water using a pipette into the top right corner of the PBR. Results were obtained by a video camera. Streaklines in the PBR were compared to streamlines from simulation results of the PBR operating under the same conditions. Flow visualization was performed using only air and water in the PBR; therefore, simulations using air and water were used for comparison.

The simulation results were further validated by testing the suitability of the PBR for the use of microalgal biomass production using microalga *Nannochloropsis oculata* (UTEX LB 2164) (see Figure A6 in Appendix A).

3.2.4 LED illumination system design

The LED illumination system was designed around previously purchased red/blue/green (RGB) surface mount device (SMD 5050) LED strips (CMY-5050-IP65-60-RGB, Chuangmingyu). Mylar reflective film was used to cover the surface area of two fiberboard panels having the same dimensions as the PBR flat plates. The panels were strategically mounted with one 5 m long LED strip each. The instrumentation for the system included a 12 V power supply (SE-450-12, Meanwell), RGB amplifier (RGB-WA4, Super Bright LEDs), RGB LED controller (LDK-RGB3, Super Bright LEDs) and DC 12 V power programmable time relay switch (THC15A, eBay), enclosed in a 5x17” aluminum enclosure (CHA-5X17, General Devices).

A LI-COR LI-190SA quantum sensor connected to a LI-250A light meter was used to measure photosynthetically active radiation (PAR). Light intensity was measured ½” from the head of the LEDs which allowed for obtaining reproducible results.

The specific light attenuation constant of the PBR wall material ($k_{PC}$) was determined by measuring light intensity exiting a ⅜” thick sheet of polycarbonate ($I_0'$) and solving for the value in the following equation based on the Beer–Lambert law:

$$I_0' = I_0 e^{-k_{PC}L_{PC}} \quad (3.1)$$
where \( I_0 \) is the intensity of the light source and \( L_{PC} \) is the thickness of the polycarbonate sheet [90].

### 3.3 Cultivation of *S. platensis* in flat plate PBR under LED illumination

#### 3.3.1 *S. platensis* seed culture

*S. platensis* (UTEX LB 2340) was obtained from The UTEX Culture Collection of Algae (Austin, TX, USA). *S. platensis* was grown photoautotrophically in autoclaved modified Zarrouk medium [9] supplemented with Vitamin B\(_{12}\) solution as shown in Table 3. The culture was allowed to acclimate to the medium for 5 months prior to any experimentation in the PBR.

The culture was maintained as a flask culture in a 2 L Erlenmeyer flask at a temperature of 23.5 ± 1.5 °C. Filter-sterilized air (~400 ppm CO\(_2\)) was continuously bubbled into the flask and provided agitation. A 1:1 mix of 6500 K and wide spectrum 3100 K fluorescent tubes (GE Lighting) supplied continuous illumination set at a photon flux density (PFD) of 100 \( \mu \text{mol photons/m}^2 \text{s} \).

Prior to any experimentation, the suitability of the modified Zarrouk medium as a growth medium for *S. platensis* was first confirmed by comparing a flask culture of *S. platensis* acclimated to the medium to a flask culture of *S. platensis* acclimated to Zarrouk medium [68] as shown in Table 3. The experiment was performed after 3 months of acclimation to each media. The two flask cultures were grown under the conditions described above for 60 days. The growth kinetics of *S. platensis* were monitored according to section 3.3.3, phycocyanin content was determined according to section 3.3.4 and polyhydroxybutyrate (PHB) content was determined according to section 3.4.2.

Prior to experimentation in the PBR, the culture was tested for the presence of contaminating microorganisms by counting colony-forming units (cfu) after aseptically plating the culture onto Petri dishes with nutrient agar (27 g/L) and incubation in the dark at 28.5 ± 0.5 °C for up to 7 days.
Table 3: Compositions of modified Zarrouk and Zarrouk media

<table>
<thead>
<tr>
<th>Medium component</th>
<th>Modified Zarrouk medium [9]</th>
<th>Zarrouk medium [68]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>9</td>
<td>16.8</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.25</td>
<td>2.5</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>FeSO₄·2H₂O</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Micronutrient solution</td>
<td>0.5 mL</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Vitamin B₁₂ solution</td>
<td>1.0 mL</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

a Composition of micronutrient solution (g/L): H₃BO₃ (2.3), MnCl₂·4H₂O (1.81), ZnSO₄·7H₂O (0.22), CuSO₄·5H₂O (0.08), Na₂MoO₄ (0.014).

b Composition of Vitamin B₁₂ solution (g/L): MOPS buffer (12), thiamine hydrochloride (1.34) cyanocobalamin (0.135), biotin (0.025). pH adjusted to 7.8 and filter-sterilized.

3.3.2 Cultivation conditions in PBR

After construction and validation of the 4 L flat plate PBR, it was used to batch-culture *S. platensis*. The PBR was sterilized with 70% (v/v) aqueous isopropanol and rinsed three times with autoclaved water prior to each experiment. For each experiment, approximately 10% (v/v) inoculum of *S. platensis* in the exponential growth phase was added to modified Zarrouk medium for a working volume of 3 L. The top of the PBR was enclosed with autoclaved glass wool and aluminum foil to prevent contamination but allow for gas exchange.

The flow rate of filter-sterilized air (~400 ppm CO₂) was varied. Values between 0.3 – 6 L/min, corresponding to aeration rates of 0.1 – 2 L_air/L_culture min (vvm), respectively, were tested. For two experiments, the addition of the non-ionic surfactant Pluronic F68 was investigated at concentrations of 0.5 and 1 g/L. A 10% (w/v) Pluronic F-68 solution (sold commercially as Poloxamer 188 solution) was filter-sterilized and added drop-wise to the medium after autoclaving.

*S. platensis* was cultivated under continuous illumination for each experiment, but illumination source and incident light intensity were varied. The LED system described in
section 3.2.4 and section 4.1.5 was used to provide red, blue and green light with a PFD of 68 and 138 µmol photons/m² s before and after modification of the system, respectively. The LED system was also used to provide red light at a PFD of 45 µmol photons/m² s. A high-pressure sodium (HPS) lamp (150 HPS 900490, Sun System) was used to provide a PFD of 100 µmol photons/m² s.

Extensive experimentation varying these parameters was performed to determine suitable conditions that would achieve \textit{S. platensis} growth without any damage or death. Table 4 provides a summary of the cultivation conditions of each experiment. Once suitable conditions for the growth of \textit{S. platensis} were found (runs 9 and 10), the experiments were carried out in duplicate to confirm reproducibility.

**Table 4**: Summary of cultivation conditions investigated

<table>
<thead>
<tr>
<th>Run</th>
<th>Illumination source</th>
<th>PFD (µmol photons/m² s)</th>
<th>Aeration rate (vvm)</th>
<th>Sparger configuration</th>
<th>Addition of Poloxamer 188 solution (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LEDs (red, blue, green)</td>
<td>68</td>
<td>2</td>
<td>Original (tube inside reactor frame)³</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>138³</td>
<td>0.1</td>
<td>Modified (perforated tube inside reactor)⁴</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.35</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8²</td>
<td>LEDs (red)</td>
<td>45</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>HPS</td>
<td>100</td>
<td>0.35</td>
<td>Original</td>
<td></td>
</tr>
<tr>
<td>10³</td>
<td>LEDs (red)</td>
<td>45</td>
<td>0.15</td>
<td>Modified</td>
<td></td>
</tr>
</tbody>
</table>

² Same cultivation conditions but light acclimation of seed culture differed.
³ LED illumination system design modified as described in section 4.1.5.
⁴ Sparger designed in this work as described in section 4.1.2; small hole diameter.
⁵ Sparger used in past work as described in section 4.2.2; large hole diameter.

**3.3.3 Analysis of \textit{S. platensis} growth kinetics**

Biomass density was measured indirectly by turbidity measurements at 750 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (DU-520, Beckman-Coulter) and recorded daily to obtain a growth curve for each experiment [91]. A standard curve to relate the absorbance of light at 750 nm to the biomass density was generated for each run during the middle of the growth cycle by determining biomass dry weight gravimetrically after its drying at 50 ºC in a vacuum oven for 24 h. Temperature and pH (SB20, VWR) were
monitored daily. Antifoam B (10% aqueous emulsion of polydimethylsiloxane) was added at 0.3% (v/v) as necessary to prevent foaming.

The morphology of *S. platensis* filaments was observed daily using an optical microscope (BX60, Olympus). A dye exclusion test with Trypan blue was periodically performed to confirm the viability of *S. platensis*. A suspension of *S. platensis* in Hanks’ balanced salt solution was prepared. 0.2 mL of the suspension was added to 0.5 mL of Trypan blue solution (0.4% for microscopy) and 0.2 mL of Hanks’ balanced salt solution and mixed thoroughly [92]. After 5 minutes, the Trypan blue-cell suspension was transferred to a hemocytometer and observed using an optical microscope.

The remainder of the work was performed on the two successful growth experiments: runs 9 and 10. The growth curve data was used to calculate specific growth rate ($\mu$), daily biomass production rate ($P$) and average biomass production rate ($P_{avg}$) according to the following equations:

\[
\mu = \frac{\ln(X_2 - X_1)}{t_2 - t_1} \quad (3.2)
\]

where $X$ is biomass density, $t$ is time, and subscripts 1 and 2 denote consecutive data points during the exponential growth phase;

\[
P = \mu(t) \times X_{avg} \quad (3.3)
\]

where $\mu(t)$ is the growth rate between two consecutive days, and $C_{avg}$ is the average biomass density; and

\[
P_{avg} = \frac{X_t - X_o}{\Delta t} \quad (3.4)
\]

where $X_t$ and $X_o$ are the final and initial biomass densities, respectively, and $\Delta t$ is the number of days of batch cultivation.

Once biomass density appeared constant and indicated the cells have reached the stationary phase of growth, *S. platensis* biomass was harvested from the medium by centrifugation at 3500 rpm for 10 min (Sorvall ST 40R, Thermo Scientific) and washed three times with
water. The final biomass yield was measured gravimetrically after freeze-drying for 24 hours. Biomass was grinded to a fine powder using a mortar and pestle for all subsequent analyses.

The results for specific growth rate, daily biomass production, average biomass production and final biomass yield represent the mean value of two independent runs.

3.3.4 Analysis of *S. platensis* pigments

1 mg/mL solutions of freeze-dried *S. platensis* biomass in hot 90% (v/v) aqueous methanol were sonicated at 90 W for 3 min (Q125, QSonica) for the extraction of chlorophyll *a* and carotenoids. The solutions were kept in the dark overnight at 4 °C before analysis. Pigment content was measured by spectrophotometry of the supernatant and quantified according to the following equations:

\[
[\text{Chlorophyll } a] \left( \frac{\text{mg}}{\text{L}} \right) = 16.80D_{665} - 9.28D_{652} \quad (3.5)
\]

\[
[\text{Total carotenoids}] \left( \frac{\text{mg}}{\text{L}} \right) = \\
\frac{10000D_{470} - 1.91[\text{Chlorophyll } a] - 95.15(36.92D_{652} - 16.54D_{665})}{225} \quad (3.6)
\]

where *OD*<sub>665</sub>, *OD*<sub>650</sub> and *OD*<sub>470</sub> are the absorbance values at the wavelengths of 665, 650 and 470 nm, respectively [93].

2 mg/mL solutions of freeze-dried *S. platensis* biomass in 50 mM phosphate buffer (pH 6.8) were subjected to five successive freezing and thawing cycles for the extraction of water-soluble phycobiliproteins [80]. Solutions were centrifuged at 3500 rpm and 4 °C for 10 min. Phycobiliprotein content was measured by spectrophotometry of the supernatant and quantified according to the following equations:

\[
[\text{Phycocyanin}] \left( \frac{\text{mg}}{\text{mL}} \right) = \frac{OD_{615} - 0.474OD_{652}}{5.34} \quad (3.7)
\]

\[
[\text{Allophycocyanin}] \left( \frac{\text{mg}}{\text{mL}} \right) = \frac{OD_{652} - 0.208OD_{615}}{5.09} \quad (3.8)
\]
where OD$_{615}$ and OD$_{652}$ are the absorbance values at the wavelengths of 615 and 652 nm, respectively [94].

The results represent the mean value of two independent runs.

### 3.4 Production of PHB by *S. platensis*

#### 3.4.1 Visualization of intracellular PHB

To confirm the presence of intracellular PHB in *S. platensis* cells, PHB granules were stained using Nile red and imaged using a laser scanning confocal microscope (LSM 5 Duo Vario, Zeiss) with a 63x/1.3 ImmKor DIC LCI Plan Neofluar objective at the Biotron Experimental Climate Change Research Centre (The University of Western Ontario, London, ON). 1 mL samples of *S. platensis* were preserved with 20 µL of Lugol’s iodine solution and stored at 4 °C overnight. The pellet was re-suspended in 10% (v/v) aqueous dimethyl sulfoxide and stained with 10 µL of 0.0025% (w/v) Nile red in acetone [95-97]. A 405 nm 50 Diode laser was used to collect images of *S. platensis* autofluorescence (emission wavelengths > 450 nm). Spectral imaging of Nile red stained PHB granules was performed using a LSM 510 META detector (Zeiss). A 543 nm HeNe laser was used to collect images of Nile red-PHB fluorescence at emission wavelengths between 570–613 nm (lambda mode) and confirm the location of PHB granules within the cells.

#### 3.4.2 Quantification of PHB content in *S. platensis*

First, intracellular PHB was reacted into its constituent 3-hydroxybutanoic acid methyl esters by acidic methanolysis to allow for analysis using gas chromatography (GC). 50 mg samples of freeze-dried *S. platensis* biomass was added to a mixture of 2 mL chloroform and 2 mL methanol (15% v/v sulfuric acid) inside thick-walled 20 mL test tubes and capped tightly with stoppers and parafilm [9, 98]. The solutions were heated at 100 °C for 140 minutes using a thermo-stat equipped oil bath. After the reaction, 1 mL of distilled water was added to the cooled mixtures and shaken vigorously for 1 min to permit the separation of an aqueous phase (top layer) and organic phase (bottom layer) with *S. platensis* biomass lying at the interphase. The organic phase was transferred into a glass vial and stored at 4 °C until analysis. The aforementioned procedure was also performed on varying amounts of pure
PHB to prepare pure 3-hydroxybutanoic acid methyl ester solutions for the purpose of a calibration curve.

The methyl ester samples were analyzed using a gas chromatograph (7890B GC, Agilent Technologies) equipped with a flame iodization (FID) detector, mass spectrophotometer (5977A MSD, Agilent Technologies), Agilent J&W DB-35MS column (30 m by 0.250 mm) and helium as the mobile phase (2.6 mL/min). The temperature of the injector was 280 °C and splitless injection mode was used to inject 1 µL of sample. The column was used under the following temperature conditions adapted from Brandl et al. [98]: initial temperature of 80 °C held for 4 min, temperature ramp of 8 °C, and final temperature of 120 °C held for 1 min. The temperature of the FID detector was 300 °C. The PHB content of samples was measured indirectly by relating the areas of the methyl ester peaks (3.3 s retention time as confirmed by mass spectrophotometry) using a calibration curve.

### 3.4.3 PHB Extraction

1% (w/v) solutions of freeze-dried *S. platensis* biomass in methanol were prepared and left overnight at 4 °C for the removal of pigments [99]. After centrifugation (3500 rpm for 10 min at 4 °C), the pellet was dried at 60 °C. PHB was extracted in 1% (w/v) solutions of biomass in chloroform at 60 °C for 36 h under reflux. The hot solution was then passed through a hot glass funnel with a glass wool plug to remove insoluble biomass and the filtrate was allowed to cool to room temperature. PHB was precipitated by drop-wise addition of the solution into two volumes of rapidly stirring cold diethyl ether [100]. The precipitate was centrifuged (3500 rpm for 20 min at 4 °C), washed with diethyl ether, washed with acetone, and then dissolved again into hot chloroform for solution casting into a thin film using a glass Petri dish.

### 3.5 PHB characterization

Commercially available PHB was dissolved in hot chloroform and solution casted using a glass Petri dish to obtain a film with the same mass as the *S. platensis* PHB film obtained in section 3.4.2. Characterization was performed on both films to compare the structural and thermal properties between the PHB extracted from *S. platensis* and commercially available PHB derived from bacteria.
3.5.1 Fourier transform infrared spectroscopy (FTIR)

A Bruker Vector 22 spectrometer equipped with an attenuated total reflectance module (MIRacle, PIKE Technologies) and diamond crystal was used to obtain FTIR spectra of PHB at room temperature and confirm the functional groups of PHB were present in the polymer film extracted from *S. platensis*. The scanned wavenumber range was 4000 – 500 cm\(^{-1}\).

3.5.2 X-ray diffraction (XRD)

XRD was used to study the crystal structure and determine the crystallinity of PHB. PHB films were cut into numerous pieces and spread in random orientation onto glass slides. A Rigaku-Rotaflex RTP 300RC diffractometer with a Co-K\(\alpha\) radiation source (\(\lambda = 1.79\ \text{Å}\)) at the Laboratory for Stable Isotope Science (The University of Western Ontario, London, ON) was used to collect XRD spectra from 2\(\theta\) diffraction angles of 2 – 82° and a step size of 0.02°. The diffraction patterns were analyzed by fitting peaks to the Pearson VII function:

\[
f(x) = y_o \left[1 + \frac{4(2m - 1)}{w^2} (x - x_c)^2\right]^{-m} \tag{3.9}
\]

where \(y_o\) is the peak maximum from the collected data, \(w\) is the full width at half maximum, \(x_c\) is the center of the peak and \(m\) is the shape factor (set at 1) [101]. The degree of crystallinity \((X_c)\) was calculated using the equation:

\[
X_c = \frac{A_c}{(A_c + A_a)} \tag{3.10}
\]

where \(A_c\) and \(A_a\) are the area of the crystalline and amorphous regions, respectively [101].

3.5.3 Differential scanning calorimetry (DSC)

PHB films were condensed tightly into 2.5 mg samples and placed into Tzero pans (TA Instruments). A differential scanning calorimeter (Q20, TA Instruments) was used to subject PHB to two heating and one cooling cycle between temperatures of 0 °C and 185 °C at a rate of 10 °C/min. Data from the second heating cycle was used to measure the melting temperature.
Chapter 4: Results and Discussion

4.1 Design of a flat plate PBR with LED illumination

4.1.1 Multiphase flow simulation model

The initial SolidWorks geometry model began as a 34 cm high x 40 cm long x 2.5 cm thick box to model the fluid domain within the PBR for simulation modeling in CFD software ANSYS CFX. A two-phase model of a gas phase dispersed as bubbles in a continuous liquid phase was used to simulate the fluid flow within the reactor. Air was selected to be the dispersed fluid. The continuous fluid was selected to be water and viscosity was varied from 1 – 5 times the viscosity of water to represent the algal slurry. The settings used in ANSYS CFX to develop the simulation model are shown in Figure 10 and are provided in full detail in Appendix A.

![Simulation materials, models and boundary conditions](image)

**Figure 10:** Simulation materials, models and boundary conditions

4.1.2 Gas sparger design

The design of the PBR began with the selection of the gas sparger. The 5 different designs tested are shown in Figure 11 with the air outlets highlighted in green. A simulation with
each sparger was run using a mass flow inlet rate of 1 vvm (optimal rate of mixing) to observe their effects on the liquid velocity profiles. Streamlines of algal slurry fluid particles from the top of the reactor were also studied to observe their movement and whether or not the sparger design would lead to their entrapment at the bottom of the PBR, which may promote algal settling.

![Figure 11: Gas sparger designs tested in ANSYS CFX](image)

It was observed that design #4 shown in 8 had the most uniform flow around the sparger (shown in Figure 12) and was the only design in which no algal slurry fluid particles were observed to get stuck at the bottom of the reactor. The other designs suffered the drawbacks of large low-velocity regions and non-uniform flow around the sparger. The liquid velocity contour and streamline simulation results the spargers tested are shown in Figures A3 and A4 in Appendix A.

![Figure 12: Algal slurry velocity contour in PBR with sparger design #4. A: Side view, and B: zoomed-in side view around the sparger](image)

After sparger design #4 was selected, a model of half of the sparger (with a symmetry boundary condition) was modeled on its own to observe the fluid domain within the sparger. Pressure differentials within the sparger were studied to further refine the design. It was observed that pressure was uniform for all the hole exits and uniform across the length of the sparger tube, L (Figure 13). Also, the flow rate through the holes was uniform across the length of the sparger (Figure A5 in Appendix A). It was assumed that this meant the sparger
would allow for a uniform flow across the total length of the sparger. However, further
simulations demonstrated otherwise. Going back to the original fluid domain within the
reactor (using sparger design #4), it was observed that the pressure directly above the sparger
showed a large rise and fall in pressure along the length of the sparger, L, at air flows ≤ 2
vvm (Figure 14).

![Figure 13: Simulations within sparger tube (1 vvm)](image13.png)

![Figure 14: Pressure (P) vs. position (x) directly above sparger tube across length of sparger](image14.png)

The pressure gradient above the sparger holes (ΔP = 24 Pa) was also greater than the pressure
gradient in the sparger hole exits (ΔP = 6 and 20 Pa at 1 and 2 vvm, respectively), which
could result in zero flow out of some holes as the gas would travel through the holes where
there is a lower pressure. In order to ensure flow uniformity, it was determined that an
increase in ΔP in the sparger was necessary. To achieve a sufficient ΔP within the sparger for
flow uniformity, the sparger tube was lowered further down to increase the length of the
holes (L_h), and a hole diameter (φ) of 0.2 mm was selected. A SolidWorks model of the
internal of the PBR illustrating the final sparger design is shown in Figure 15.
Figure 15: SolidWorks model of the final sparger design showing hole length ($L_h$) and hole diameter ($\phi$)

An advantage of a reduced sparger hole diameter is a lower risk of clogging from microalgae biomass. Additionally, small air bubble diameters can be advantageous if bubbling with CO$_2$ or CO$_2$-enriched air due to a greater CO$_2$ uptake by microalgae [102]. A disadvantage, however, is that a smaller bubble diameter can result in high turbulence energy dissipation rates, especially at bubble rupture at the air-liquid interface. The small microeddies that form can be particularly damaging to shear-sensitive cells [103]. This will be discussed more in section 4.2.2.

4.1.3 Geometry modifications

Following sparger selection, dead zones in the reactors were considered. The dark blue regions in Figure 16 in the previous section illustrate regions of zero flow in the front and back bottom edges of the reactors, as well as above the sparger. As a result, a fillet with a 6 mm radius for these edges and a 7 mm tall x 5 mm wide triangle above the sparger were added to the initial design. In Figure 16, the dead zones in the left and right corners are approximately 5 mm wide, thus a fillet with a 12 mm radius to these corners was also added.
Simulations using the modified design confirmed that dead zones were eliminated. Figure 17 shows the algal slurry velocity contours of the updated design halfway through the XY and YZ planes of the reactor, as well as zoomed in views around the sparger.

4.1.4 Simulation validation

A model is as good as the assumptions and approximations it is based on. It is up to the design engineer to accurately validate the assumptions and approximations.

Fluid flow visualization using fluorescein solution was performed to visualize the fluid flow patterns within the reactor and compare with the fluid flow patterns obtained in simulations. Figure 18 shows a time-lapse of the fluorescein solution discharged at the top right of the
PBR. As the solution is injected into the fluid at the fixed position, it extends along a streakline showing where the fluid particles have passed. Streamlines from ANSYS CFX showing the direction that water located at the top of the reactor will travel at any point in time resembled what was observed by the streaklines.

![Figure 18: Time lapse of dye injection streaklines in the PBR (left) and simulation results of streamlines (right) at 1 vvm](image)

It was also noted that uniform airflow from the sparger could be achieved. A comparison between the pattern of airflow from the simulation and the pattern of air bubbles in the reactor are shown in Figure 19. Thus, testing of the PBR post-construction validated the accuracy of the assumptions made in the simulations.

![Figure 19: Airflow pattern in A: PBR (left) and in simulation (right) at 1 vvm](image)

Finally, a growth experiment with a test microalga (*Nannochloropsis oculata*) was successfully performed (results shown in Figure A6 in Appendix A). *N. oculata* is a type of marine microalgae of high interest for biofuel production for its high lipid content of up to 60% cdw [104]. *N. oculata* is also very commonly used in flat plate PBRs industrially [26]. It was decided that the PBR designed in this work should be able to support the growth of this species before further experimentation. A maximum biomass production rate of 0.65 g/L·d and biomass density of over 1 g/L was achieved, thus validating the suitability of the reactor for microalgae biomass production.
4.1.5 Implementation of LED illumination

There were several design requirements that were considered for the LED illumination system. The surface mount device (SMD) LED strips must be mounted onto a flat surface, the distance of the light source from the reactor should be controllable, the loss of light should be minimized, ease of cleaning and maintenance of the reactor should not be impeded, and the instrumentation must be connected and contained safely. Instrumentation included a 12 V power supply, a timer to control the light/dark cycle, and a dimmer to control the red/blue/green spectral output.

First, long screws were added to the corners of each side of the reactor to allow for two panels to be placed at varying distances of the reactor, or removed altogether. Mylar sheets were placed on the panels to reflect any incident light on the panels and side panels to reduce loss of light.

The LED strips could not be placed very closely together because they cannot be greatly bent. Additionally, too many strips could not be placed in series without an amplifier to correct for a voltage drop, which limited the amount of LEDs that be mounted onto one panel. The best implementation was to have one LED strip (5 m, 60 LEDs) for each panel. This resulted in 300 LEDs per panel, but only 225 were directly exposed directly to the culture volume due to the reactor frame and slight bends in the LED strips. The final design is shown in Figure 20.

Figure 20: PBR with LED panels implemented
Initially, the 2 LED strips were connected in series and it was noted that panel #2 \((I_o = 20 \, \mu \text{mol photons/m}^2 \text{s})\) was less than half the intensity of panel #1 \((I_o = 48 \, \mu \text{mol photons/m}^2 \text{s})\). The first 4 growth experiments with \textit{S. platensis} were used with this configuration \((I_o = 68 \, \mu \text{mol photons/m}^2 \text{s})\) before the design was modified to implement amplifiers. Implementing amplifiers and connecting the LED strips in parallel doubled the total light supply from when the strips were connected in series.

The distribution of photon flux density (PFD) per panel is shown in Figure 21. The three colours represent the three colours that comprise the spectral output: red (625 nm), green (520 nm) and blue (465 nm). The irradiance level across the surface of the panel varies, due to the voltage drop effect as distance from the power supply increases. However, when the panels are placed facing each other, the light distribution is completely uniform.

When the PFD of each colour on the LEDs were measured individually, by setting one colour to 100% on the controller and the others to 0%, the values are not equal. The intensities of red, green, and blue (on one LED in the strip) were about 18, 22 and 45 \(\mu \text{mol photons/m}^2 \text{s}\), respectively. However, the PFD of the LED when red, blue and green are all set to 100% on the controller \((66 \, \mu \text{mol photons/m}^2 \text{s})\) is less than the sum of the individual measured intensities of each colour. While the total irradiance level could be measured, it was not possible to determine the compositions of red, blue and green light when all three colours are used. This will be discussed in section 4.2.3.
Considering all the LEDs on the strip on both panels, the output of the LED system, when all three colours are used, is 138 \(\mu\)mol photons/m\(^2\)s.

4.1.6 Final design

The final simulation model (modelling the inside of a quarter of the simulation, with symmetry boundary conditions applied) and the internal frame that was designed in this work are shown in Figure 22. The final 4 L PBR with 2.5 cm thickness and LED illumination is shown in Figure 23.

**Figure 22:** Final PBR models. **A:** Final simulation model of fluid within PBR (quartered), and **B:** final CAD model of PBR internal frame with sparger

**Figure 23:** PBR post-construction with one panel removed to show microalgal culture within PBR (left) and with LED panels on (right)
4.2 Selection of cultivation conditions for *S. platensis* biomass production

4.2.1 Growth medium

As mentioned in section 2.6.2, Zarrouk medium is the standard medium for the cultivation of *S. platensis*. In this work, a modified Zarrouk medium was proposed to follow the work by Jau et al. [9] that achieved a 10% cdw PHB content in *S. platensis*. A comparison was performed in between *S. platensis* grown under the two media in flask cultures to investigate whether the lower nutrient concentration of the modified Zarrouk medium would significantly reduce biomass yield. It was also speculated whether the lower nutrient concentration could promote PHB synthesis, since PHB is primarily produced when there is excess carbon supply but another essential nutrient is limited.

The growth curves of the two flasks are shown in Figure 24. The final biomass densities were 2.0 and 2.2 g/L using modified Zarrouk medium and Zarrouk medium, respectively.

![Biomass density versus time for *S. platensis* using different media](image)

**Figure 24:** Biomass density versus time for *S. platensis* using different media

While it cannot be stated conclusively, it appears that the culture in modified Zarrouk medium may have reached stationary phase based on the last two data points of the curve, while the culture in Zarrouk medium continued to grow. However, the experiment was not continued further as the length of the experiment is far beyond what would be tested in the
PBR. It was concluded that final biomass density is comparable between both media over a reasonable cultivation period.

Although biomass yield was comparable, there was a difference in the composition of *S. platensis* biomass between the two media. Of most significance is that a higher PHB content was obtained using the modified Zarrouk medium (Figure 25).

![PHB Content Graph](image)

**Figure 25: PHB content for *S. platensis* using different media**

It was hypothesized that Zarrouk medium would be able to support a higher biomass content on the consideration of the nitrogen content in the media. The modified medium contains 1.25 g/L NaNO₃ (0.21 g/L nitrogen). Assuming no nitrogen is wasted, all nitrogen is converted to protein, and a nitrogen-to-protein ratio of 1-to-6.25, then there would be 1.29 g/L protein. *S. platensis* typically contain 60% protein, which means it could be possible to obtain 1.29 g/L / 0.6 = 2.15 g/L biomass [57, 105]. If the same calculations are used for Zarrouk medium, which contains 2.5 g/L NaNO₃, it could be possible to obtain 4.39 g/L biomass. The results for phycobiliprotein content (Figure 26) seem to correspond with these calculations, as a higher phycobiliprotein (pigment-protein) content was observed in the higher nitrogen-containing flask with Zarrouk medium (under the same temperature and PFD).
These results support the observation that the flask culture in the modified medium seems to have reached stationary phase (i.e. limitation of an essential nutrient), as reduced phycocyanin content and increased PHB content are both associated with nitrogen depletion [9]. *S. platensis* can degrade phycobiliproteins into amino acids for other cellular processes when nitrogen supply is depleting [9]. It is possible that acclimation to the modified medium (with lower nutrient concentrations) may have positive results on PHB production, as was observed with Jau et al. [9], although a different strain of *S. platensis* was used. However, flask cultures in *S. platensis* in this work were able to grow for up to 60 days and reach a higher biomass density, whereas cells reached stationary phase after 10 days (< 1.5 g/L biomass density) in the work by Jau et al. The difference is perhaps due to less aeration/mixing or less light, or due to supplementation with vitamin B$_{12}$ in this work, which has been observed to enhance the growth of *S. platensis* [106].

Because the purpose of the experiments in this work was to produce PHB from *S. platensis*, a modified Zarrouk medium was selected as the better alternative. However, it is worth noting that if the cultivation of *S. platensis* is for the purpose of producing biomass or phycocyanin, using Zarrouk medium could possibly result in higher biomass densities owing to the larger amount of nutrients available. Because the experiment was only conducted once, a test for statistical significance in differences could not be determined. However, the results were considered sufficient in showing the modified medium was suitable for use with *S. platensis* for the production of PHB.

**Figure 26:** Phycobiliprotein content for *S. platensis* using different media
4.2.2 PBR hydrodynamics

Initial conditions used in the PBR (run 1 in Table 5) resulted in extensive cell death of *S. platensis* and very low biomass production, indicating that even though the reactor had demonstrated its value in *N. oculata* cultivation, it may not be the most suitable for the growth of *S. platensis*. Since *S. platensis* growth is known to be sensitive to shear forces it is exposed to, extensive experimentation varying the aeration rate was performed to determine suitable conditions that would achieve *S. platensis* growth without any damage or death. Table 5 provides a summary of the cultivation conditions of each experiment. The results for runs 1 through 5 are shown in Figure 27.

Table 5: Cultivation conditions investigated in the flat plate PBR

<table>
<thead>
<tr>
<th>Run</th>
<th>Illumination source</th>
<th>PFD (µmol photons/m² s)</th>
<th>Aeration rate (vvm)</th>
<th>Addition of Pluronic F68 (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LEDs (red, blue, green)</td>
<td>68</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.35</td>
<td></td>
<td>0.35</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.35</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>138&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> LED illumination system design modified as described in section 4.1.5.

Figure 27: Biomass density versus time for five *S. platensis* growth experiments in the flat plate PBR
A high aeration rate of 2 vvm (run 1) was first tested for high mixing, but rapid death of the cell culture was observed. Between runs 1, 2 and 3, there was a progressive improvement in the survival of *S. platensis* when aeration was reduced from 2 vvm to 0.35 vvm. In run 4, the addition of 0.05% (w/v) surfactant at low aeration rate (0.35 vvm) significantly improved biomass production and *S. platensis* survival. In run 5, however, an increase in light intensity became detrimental to the growth and survival of *S. platensis*.

Images of the damaged *S. platensis* filaments in this work are shown in Figure 28.

![Image](image.png)

**Figure 28**: Comparison of healthy and damaged *S. platensis* filaments

It is important to consider the shear sensitivity of *S. platensis*. In addition to the microorganism’s filamentous morphology, the biochemical composition of the cell wall has been associated with the cell’s susceptibility to hydrodynamic stresses [107]. Individual cells are encapsulated in a mucilaginous sheath (similar to Gram negative bacteria cell wall, mainly composed of peptidoglycans and lipids) and are dependent on adhesion to each other for survival [35, 73].

It was found that *S. platensis* has been grown successfully at aeration rates as high as 10 vvm [108]. However, the hydrodynamics at a given aeration rate will depend on the geometry of the reactor among other parameters.

A report in the literature on the shear sensitivity of dilute suspensions of *S. platensis* was considered [72]. The study showed that shear stress could cause mechanical destruction of *S.*
*S. platensis* and impair their photosynthetic ability, and that the critical shear stress was dependent on temperature and light intensity. For the conditions used in this study, a critical shear stress of 0.4 – 0.5 Pa was reported [72]. Simulations of the wall shear in the reactor under the conditions in runs 1 to 3 were performed (Figure A7 in Appendix A). The maximum wall shear for runs 1, 2 and 3 were 0.81, 0.85, and 0.46 Pa, respectively. These values are either above or very close to the critical shear stress reported for *S. platensis*.

It has been proposed that for high aeration rates, the mechanism of cell damage for shear-sensitive cells is primarily due to shear [109]. However, even at low aeration rates, there cell damage can be due to the presence of small microeddies (e.g. in the wake of a rising bubble) [38, 103]. The length of a microeddy ($\lambda_L$) is given by equation 4.1:

$$\lambda_L = \left( \frac{\mu_L}{\rho_L} \right)^{3/4} \varepsilon^{-1/4} \quad (4.1)$$

where $\mu_L$ is the viscosity of the medium, $\rho_L$ is the density of the medium, and $\varepsilon$ is the energy dissipation rate. If the calculated length of the microeddies is larger than the size of shear sensitive cells, the cells are simply carried by the fluid eddy and do not experience disruptive forces. But, if they are smaller than the size of shear-sensitive cells, the cells experience pressure differentials on their surface that they cannot withstand, leading to cell damage [110].

Simulations of the turbulence energy dissipation in the reactor were performed in order to calculate the length scale of microeddies using equation 4.1 (Figure A8 in Appendix A). The range of microeddy lengths for runs 1, 2 and 3 were 53–200, 81–250, and 113–665 µm, respectively. These values are either smaller than or similar to the length of *S. platensis* filaments in this work, which range from 100 – 600 µm.

It was thought that the above hypothesis could explain the observations so far. First, simulation results showed undesirable hydrodynamic conditions in the reactor with respect to shear and microeddy lengths. Second, *N. oculata* could successfully be grown in the reactor at a high aeration because it is very resistant to shear [111]. Finally, *S. platensis* could be grown in the PBR prototype because the hole diameters of the sparger were large ($\phi = 1$ mm). Energy dissipation rate is a function of bubble diameter [103]. As shown in equation 4.1, an
increase in $\varepsilon$ will lead to a decrease in $\lambda_L$. It is worth noting that the simulations could report energy dissipitation rates within the bulk of the fluid, but the specific energy dissipitation rates in the wake of a rising bubble or bursting bubble can be much higher [103].

Because there is still the requirement for mixing, a careful balance must be achieved between minimizing shear and achieving good mixing. For example, going below the optimal rate of mixing (1 vvm) results in the formation of potential dead zones in flow. To keep the aeration rate at 0.35 vvm, the addition of a non-ionic surfactant was tested in attempt to improve the hydrodynamic conditions within the reactor. Pluronic F68 has been shown to protect against turbulence-associated damage, such as by reducing the adherence of cells to bubbles, which would reduce the effects of bubble rupture [103]. Moreover, 0.05-1% (w/v) has shown enhanced growth in aerated cultures of shear-sensitive microalgae (red alga *Porphyridium cruentum* and dinoflagellates *Protoceratium reticulatum* and *Phaeodactylum tricornutum*) [107, 112].

Growth with *S. platensis* was observed in run 4 which contained 0.05% (w/v) Pluronic F68. A biomass density of 0.87 g/L was achieved after 9 days of cultivation. Ultimately, the culture began to decline similar to the previous runs. As shown in Figure 27, the initial biomass density for run 4 was greater in the other runs. This is because the same inoculum volume was used for each experiment. For run 4, the seed culture used was dense. Thus, it could not concluded whether the favourable results were due to the protective effects of the surfactant, or protective effects of a more dense culture.

Finally, in an attempt to improve upon these results, two variables were changed at once for run 5 after deciding the use of a lower aeration rate and 0.1% (w/v) Pluronic F68 were both suitable options [39, 103, 107]. However, the results from run 5 were not an improvement from run 4 and were similar to the runs prior.

Pluronic F68 has never been reported for use with *Spirulina* in the literature. It was decided to focus to address the hydrodynamics of the PBR directly by using larger (i.e. less energy dissipating) bubbles. A modification was done by simply implementing the sparger from a prototype PBR used in past work into one of the ports near the bottom of the reactor. The sparger is an aluminum tube perforated with 16 holes spaced apart by 2 cm, with a hole diameter of 1 mm.
It was hypothesized that if cell damage was due to hydrodynamic stresses attributed to the small air bubbles, implementing the previous sparger which sustained the growth of *S. platensis* in past work would allow for shear-sensitive *S. platensis* to be grown in the current flat plate PBR.

### 4.2.3 Effect of PBR lighting

The cultivation conditions tested using the modified PBR are shown in Table 6. The results for runs 6 through 8 are shown in Figure 29.

**Table 6:** Cultivation conditions investigated in PBR with modified sparger

<table>
<thead>
<tr>
<th>Run</th>
<th>Illumination source</th>
<th>PFD (µmol photons/m² s)</th>
<th>Aeration rate (vvm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>LEDs (red, blue, green)</td>
<td>138</td>
<td>0.35</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>LEDs (red)</td>
<td>45</td>
<td>0.15</td>
</tr>
</tbody>
</table>

![Graph: Biomass density versus time for three *S. platensis* growth experiments in the modified flat plate PBR](image)

**Figure 29:** Biomass density versus time for three *S. platensis* growth experiments in the modified flat plate PBR

When the modified PBR was first tested with an aeration rate of 0.35 vvm (which gave the “best” results previously, in runs 3 and 4), rapid cell death was observed once again. The
results of run 6 implied that the size of air bubbles was not the cause of cell damage. The aeration rate was reduced even further to a low but reasonable aeration rate of 0.1 vvm (run 7) and the same result was observed (run 7), thus confirming that cell damage was still occurring even with large bubbles.

The findings led to the investigation of the light supply. As stated previously, this was not investigated initially because of the great amount of support found in the literature to the hypothesis that damage of shear-sensitive *S. platensis* was likely due to hydrodynamic stress. All studies which reported using LEDs to grow *S. platensis* generated positive results [78-80].

While run 7 was being performed in the modified PBR, a simultaneous run was tested in the original PBR designed in this work. An aeration rate of 0.35 vvm (which gave the “best” results previously, in runs 3 and 4) was used, but instead of LEDs (which were being used with the modified PBR), a high pressure sodium (HPS) lamp was used instead. Within the first three days, it was evident that *S. platensis* was thriving without any damage, and continued to do so afterward (results shown later in section 4.3.1).

At this point, it was evident that the light supplied by the LEDs was negatively affecting *S. platensis*. It was considered unexpected because the irradiance level of the LEDs (138 µmol photons/m²s) is below any irradiance level of photoinhibition that has been reported for *S. platensis* [17, 77]. A study that used white LEDs under continuous illumination in a thin layer (10 mm) flat panel PBR reported photosaturation and photoinhibition of *S. platensis* began around 435 and 1308 µmol photons/m² s, respectively [77]. The LEDs were stated to be comparable to that of direct sunlight at summer noon; the spectral data provided showed a high amount of blue (~460 nm) compared to the other wavelengths. The cells were acclimated to each light intensity tested prior to the experiments which measured specific growth rate, but only for one or two days. Still, the irradiance level is far below the level for photo-inhibition.

Next, the spectral output of the LED system was considered. In this work, three monochromatic light sources (red, green and blue) were being used to provide a “white” light. The potential effects of these photons were considered, however, it was difficult to diagnose a specific cause. For example, LEDs of all three colours have been used
individually to cultivate *S. platensis* without negative effects (as well as yellow LEDs) [78, 79, 80]. Moreover, monochromatic light from LEDs using each of these colours individually has been used at intensities as high as 3000 µmol photons/m²s [78, 79].

These studies claimed that biomass produced by *S. platensis* while grown when the light intensity was below 300 µmol photons/m²s were minimal and similar to when *S. platensis* was grown under no illumination, and that the highest productivities were achieved when the light intensity was 3000 µmol photons/m²s [78]. Although photoinhibition of *S. platensis* generally occurs above 500 µmol photons/m²s, there are reports that expose *Spirulina* to high PFD up to 3500 µmol photons/m²s [17]. It is worth noting that light intensity of PAR from the sun ranges from 500 – 2000 µmol photons/m²s during the day, depending on the geographical location [74]. Still, for *S. platensis* to display no growth inhibition at such a high intensity for a very narrow wavelength distribution of which it does not have a high utilization in (e.g. blue light at 460-475 nm, absorbed mainly by carotenoids and phycoerythrin which are found in small amounts in *Spirulina* [80, 113]) is a questionable observation, but it is not known at what irradiance level the cultures were acclimated to. Cultures grown at high light intensity exhibit a higher resistance to photoinhibition [114]. It is possible that a heightened light tolerance was developed in this strain.

In this case, the seed culture flask of *S. platensis* is maintained under fluorescent lights at a light intensity of 100 µmol photons/m²s, which is comparable to the irradiance level of the LEDs. Thus, the negative results in this work are likely not due to light acclimation.

Another study showed no negative effects using red, blue or green monochromatic LEDs on *S. platensis* [80]. The irradiance levels were given to be 9000 lux (lumens/m²), which is a unit of measurement weighted towards green light. For reference, the light intensity of the LEDs used in this work ranged from 500-1500 lux individually and was about 2500 lux with all three colours on.

White LEDs that were used in these studies were polychromatic, providing a range of photons (cool white). What was not investigated in these studies, however, were the combinations of monochromatic LEDs used in the present study (i.e. red, green and blue), although Markou [80] showed high growth with *S. platensis* using pink light by combining red (620 nm) and blue LEDs (440 nm). It was implied that the light intensities were set to be
equal for each colour. As mentioned in section 4.1.5, the exact composition of the spectral output when all three lights are used could not be determined. It is not known whether the outputs of red, green and blue are equal, or which colour dominates. It is possible that the combination used in this work provokes a stress response in *S. platensis*.

The growth of *S. platensis* under red LED illumination has been repeatedly shown to result in a higher growth rate compared to other colours of LEDs [78-80] In run 8, growth of *S. platensis* without damage was finally observed when the LEDs in this work were used to output red light without blue and green. The main photosynthetic pigments in *S. platensis*, chlorophyll *a* and phycocyanin, have peak absorbances at 668 and 615 nm, respectively, were able to effectively utilize the light energy supplied by the red LEDs (peak wavelength of 625 nm). However, the red LEDs used in this work could only output a maximum light intensity of 45 µmol photons/m²·s, which could not support the growth of *S. platensis* to a higher biomass density. It is possible that the improved results that were observed previously with reductions in aeration rates may have been due to a less frequent exposure of the culture to a damaging incident light, or due to a combination of both parameters (reduced turbulence-associated damage and less frequent exposure to damaging incident light).

### 4.2.4 Final conditions

The two sets of conditions that were found suitable for the cultivation of *S. platensis* and used in subsequent experiments are shown in Table 7.

<table>
<thead>
<tr>
<th>Run</th>
<th>Illumination source</th>
<th>PFD (µmol photons/m²·s)</th>
<th>Aeration rate (vvm)</th>
<th>Sparger configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>HPS</td>
<td>100</td>
<td>0.35</td>
<td>Tube inside reactor frame (original)³</td>
</tr>
<tr>
<td>10</td>
<td>LEDs (red)</td>
<td>45</td>
<td>0.15</td>
<td>Perforated tube inside reactor (modification)²</td>
</tr>
</tbody>
</table>

³ Sparger designed in this work as described in section 4.1.2; small hole diameter.
² Sparger used in past work as described in section 4.2.2; large hole diameter.

Ideally, conditions would be the same except for one parameter, instead of three differing parameters, to allow for better comparisons to be made. However, a different sparger (i.e. different hydrodynamics) between the reactors could not be avoided because an additional reactor would not have been constructed otherwise. The additional (albeit slightly different)
reactor provided the opportunity for more experiments to be performed using both HPS and LED illumination.

When the illumination source was switched to HPS, the aeration rate was selected to be the same as run 4 (the “best” results observed) in order to compare what could be achieved. Since it was first thought that hydrodynamic stress was the problem, we simply decided to select the average between the PFD used in previous runs.

When *S. platensis* was finally observed to thrive in the flat plate PBR, even with the small air bubbles, it became evident that the original issue must be mainly due to the LEDs. To test this hypothesis, the last run performed (run 7) was repeated, except using only red, instead of red/blue/green, since it has been well established that microalgae can thrive under red LEDs [21].

Because of the length of time required to grow *S. platensis*, the first conditions that produced growth curves of *S. platensis* in the flat plate PBR were selected in order to move on to the next area of work.

### 4.3 Cultivation of *S. platensis* in a flat plate PBR

Culturing of *S. platensis* onto nutrient agar plates before and after experimentation in the PBR for runs 9 and 10 showed no growth even after 7 days (colony forming units = 0), indicating the purity of the culture for the following experiments.

#### 4.3.1 Comparison of growth curves

The growth curve for *S. platensis* in the flat plate PBR for run 9 is shown in Figure 30. The maximum specific growth rate was $0.43 \pm 0.04$ per day, observed after 3 days of growth. The final biomass yield was $1.81 \pm 0.04$ g/L. The temperature was $25.2 \pm 2.2$ °C.
The growth curve for *S. platensis* for run 10 is shown in Figure 31. The maximum specific growth rate was $0.52 \pm 0.03$ per day and was observed after 2 days of growth. The final biomass yield was $0.64 \pm 0.08$ g/L. The temperature was $28.6 \pm 1.5 ^\circ C$.

The growth curve for *S. platensis* for run 10 differs from the growth curve shown for run 8 in section 4.2.3 although both runs had the same conditions. During run 8, the light intensity of the seed culture were reduced from 100 to 43 $\mu$mol photons/m$^2$ s. When run 8 was repeated, it was observed that *S. platensis* did not experience a lag phase and grew at a higher rate. This is likely due to the acclimation to low light levels that occurred in the seed culture, which more closely resembles the low light levels in the PBR under red LED illumination. It is known
that the response to changes in light quality or intensity can be in the time scale of days [35]. Thus, the duplicate runs shown in Figure 31 differ from the results shown for run 8, as the inoculum used for these runs was pre-adapted to a lower irradiance level. In the case of the HPS growth curves shown in Figure 30, the seed culture was adapted to the same irradiance level that they were inoculated into (100 µmol photons/m² s).

The differences in temperature are due to the distance of the reactors from the light sources. While LEDs generate less heat compared to the HPS lamp, they were placed a few cm away from both sides of the reactor. The HPS lamp, which generates a substantial amount of heat, was placed 1 m away from the reactor, with only one side exposed. As a result, the HPS culture was more prone to variations in the ambient temperature (19.7 ± 2.0 °C). The experimental setups are shown in Figure 32. The different sparger tube is visible in the modified reactor in Figure 32B.

**Figure 32:** Experimental setups. **A:** run 9 with HPS illumination, and **B:** run 10 with red LED illumination (one panel removed to display internals of modified PBR)

Under both conditions, pH continuously increased from 9.2 to 10.3 over the course of growth. During growth, bicarbonates (HCO₃⁻) are transported through the cell membrane and converted into CO₂ and carbonate [115]. The CO₂ produced is used for photosynthesis while the carbonate ions are released into the culture medium, which will increase the pH of the medium. An increased pH can lead to growth inhibition, but it is frequently reported that pH from 9.5 to 10.5 are considered ideal for the cultivation of *S. platensis* [116]. The tropical and subtropical bodies of water in which *S. platensis* grows naturally have pH values up to 11 [116]. *S. platensis* is able to uptake both HCO₃⁻ and CO₂ due to carbon dioxide concentrating
mechanisms [36, 66, 67]. These results indicate the CO$_2$ concentration was low, because CO$_2$ concentration mechanisms (e.g. bicarbonate transporters) in cyanobacteria are active under low CO$_2$ concentrations and are not functional at high CO$_2$ concentrations [66]. In this work, the CO$_2$ fraction in the gas phase was about 0.04% (v/v), whereas air enriched with 1-20% (v/v) CO$_2$ is commonly used in the literature for *S. platensis* to obtain higher growth rates [102, 117]. Furthermore, it is worth noting that PHB content in cyanobacterium *Synechococcus* MA19 has been shown to be higher by bubbling air enriched with CO$_2$ than with air alone [54].

Cells within a suspension culture experience an average light intensity that sets the average specific growth rate of the culture [90, 102]. Considering one-dimension light transfer across the width of the reactor, the average light intensity ($I_{\text{avg}}$) was determined by the equation:

$$I_{\text{avg}} = \frac{\alpha (I_o e^{-k_{PC}L_{PC}})}{\gamma L} (1 - e^{-\gamma L}) \quad (4.2)$$

where $\alpha$ is the view factor for one-dimension light transfer (1 and 2 for one-sided and two-sided illumination, respectively), $I_o$ is the incident light intensity, $\gamma$ is turbidity and $L$ is the optical light path (width of the reactor), $k_{PC}$ is the light attenuation coefficient of polycarbonate (the material used for the PBR) and $L_{PC}$ is the thickness of the polycarbonate sheet [90, 118]. The value of $k_{PC}$ was determined experimentally. The average light intensity for the growth curves is shown in Figure 33.

![Figure 33: Average light intensity versus time for *S. platensis* for runs 9 and 10](image)
The average light intensity when *S. platensis* cultures reached stationary phase (specific growth rate $\approx 0$) was 19 $\mu$mol photons/m²s under both conditions, suggesting that light became the limiting nutrient for both.

A comparison between the maximum specific growth rate, biomass yield, and biomass production rates calculated from the growth data is shown in Table 8. The average biomass production rate was calculated over the total number of days in batch cultivation, while the maximum biomass production rate shows the highest value obtained for daily biomass production rates. The highest values were obtained at the end of the exponential growth phase (day 16 and 12 for HPS and red, respectively).

**Table 8**: Comparison of growth kinetics between runs 9 and 10 with *S. platensis*

<table>
<thead>
<tr>
<th></th>
<th>Run 9</th>
<th>Run 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum specific growth rate, $\mu_{\text{max}}$ (day⁻¹)</td>
<td>0.43 ± 0.04</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>Final biomass density, $X_f$ (g/L)*</td>
<td>1.81 ± 0.04</td>
<td>0.64 ± 0.08</td>
</tr>
<tr>
<td>Average biomass production rate, $P_{\text{avg}}$ (g/L·d)*</td>
<td>0.09 ± 0.00(2)</td>
<td>0.04 ± 0.00(4)</td>
</tr>
<tr>
<td>Maximum biomass production rate, $P$ (g/L·d)*</td>
<td>0.31 ± 0.03</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

* denotes variables with statistically significant differences (P<0.05, n=2)

In a batch process, cultures are harvested entirely once the stationary phase of growth is reached and a maximum biomass density is achieved. It was necessary to batch-culture *S. platensis* in the flat plate PBR in this work due to the fact that the product of interest, PHB, reaches a maximum during stationary phase.

Because the conditions differ in several parameters, it is hard to make a direct comparison. However, it is clear that “HPS” conditions supported the growth of *S. platensis* to a much higher biomass density, likely due to a higher amount of light energy and better mixing from a higher aeration rate (i.e. better gas transfer and more frequent exposure to light).

### 4.3.2 Viability of *S. platensis*

The viability of *S. platensis* filaments was monitored under optical microscopy to confirm the selected cultivation conditions did not result in the damage observed in section 4.2.2. It is interesting to note, however, that the reactor with the small hole size sparger occasionally showed cells that would stain with Trypan blue which indicates they are not alive (Figure
This was never observed for *S. platensis* in the modified reactor at a lower aeration rate and larger air bubbles.

![Damaged filaments of S. platensis](image)

**Figure 34:** Damaged *S. platensis* filaments observed during run 9

It was concluded that some hydrodynamic stress due to aeration at 0.35 vvm does negatively affect *S. platensis*, but the effect is negligible. It is possible that the effect may become more pronounced above 0.35 vvm (e.g. 2 vvm where wall shear is greater than the critical shear stress reported for *S. platensis*).

4.3.3 Comparison of PHB content in *S. platensis*

PHB contents as a percentage of *S. platensis* dry weight for run 9 and 10 were 0.13 ± 0.02% and 0.75 ± 0.19%, respectively (Figure 35). The values are low, but consistent with what is reported for PHB in *S. platensis* without nitrogen limitation or acetate supplementation as *S. platensis* under photoautotrophic growth has been reported to range in PHB contents from 0.6 – 0.75% cdw [45, 85]. The first study in which PHB from *S. platensis* was quantified reported a PHB content of 6% cdw, but all subsequent studies contradict these findings [84].
It is interesting to note that the difference between PHB contents between the two growth experiments was statistically significant (P<0.05, n=2). It is possible that PHB content is affected by the quality and/or quantity of illumination. PHB granules are localized near thylakoid membranes and it has been suggested that PHB formation may serve as an electron sink in conditions where excess an excess of reducing equivalents are generated (e.g. when CO₂ fixation is hindered in photosynthesis) [119]. In this case, PHB accumulation could mean that growth conditions are far from optimal, which is consistent with the substandard growth kinetics of *S. platensis* under “red” conditions. The results are also consistent in the case of “HPS” conditions, where it is believed that the growth conditions were better due to a higher photon flux density and better mixing that was able to support the grown of *S. platensis* to a higher biomass density. In this case, a lower PHB content was observed.

It is well known that the spectral composition of light can affect the growth and pigment composition of microalgae. However, It is interesting to consider recent reports in the literature that showed the content of other storage molecules in *S. platensis* (e.g. lipids) are increased when using certain wavelengths of LEDs [80]. It has been shown that lipid and carbohydrate content in *S. platensis* is highest using blue LEDs [80]. There is an opportunity to further study the effects of LEDs and investigate the possible effects of wavelength on PHB content.

**Figure 35:** Comparison of PHB content in *S. platensis* for runs 9 and 10
In general, PHB production is typically a two-step cultivation process whereby cells are grown in nutrient-rich medium for biomass production, then transferred into a deficient medium to initiate high PHB production. Supplementation with an additional carbon source is typically applied during the nutrient-limitation step. During the first step, cultivation, PHB content reaches a maximum during the start of stationary phase, and then begins to decrease as the microorganisms begin to mobilize the energy storage molecules [8, 9, 50]. However, a second step of nutrient limitation to enhance intracellular PHB content was not performed in this work due to time constraints. Thus, obtaining a high PHB content in *S. platensis* was not within the scope of this work. Instead, the polymer properties of PHB that could be extracted from the native PHB content in *S. platensis*, under photoautotrophic conditions, were investigated.

Although a low PHB content in *S. platensis* was obtained in this work, considering the efficiency of solar energy utilization can emphasize the advantage of using *S. platensis* for PHB production. This concept is illustrated in Figure 36.

![Figure 36: Comparison of microbial and cyanobacterial PHB production with respect to solar energy utilization](image)

Conventional terrestrial plants typically convert 0.5-2% of solar energy into plant biomass, whereas cyanobacteria have light energy utilization of 5% on average, and can potentially exceed 10% [15, 120]. Considering an average of values in the literature for biochemical composition of plants, the fraction that can be used as a suitable carbon source for PHB production in bacteria, an average conversion efficiency for carbon to biomass for bacteria, and a typical PHB content of 50% (w/w) in bacteria, the overall efficiency of solar energy utilization is 0.045% [42, 121-123]. Although cyanobacteria have lower PHB contents than bacteria, a solar conversion efficiency of 5% to produce biomass that contains 2% (w/w) PHB (low estimate for photoautotrophic growth) results in an overall solar conversion
efficiency of 0.1% to produce PHB [9, 64, 85]. Thus, even at a low PHB productivity that is more than 10 fold lower than in a bacterium, the overall solar energy conversion efficiency doubled. If considering that even 0.5% (w/w) supplementation with acetate can increase PHB contents in \(S. platensis\) to as high as 10% (w/w), the efficiency can become almost 0.5% overall [9]. Furthermore, it must be noted that the use of LEDs as artificial illumination can further increase the efficiency of energy utilization for \(S. platensis\).

\(S. platensis\) only require CO\(_2\), whereas bacteria require carbon sources from plants, which can compete with food sources. Furthermore, while CO\(_2\) is consumed during photosynthesis in both processes, carbon sources used for fermentation using bacteria are converted back into CO\(_2\). Overall, we can see that the carbon footprint is lower using \(Spirulina\). While carbon tax is not in place everywhere, there are many places where there is a cost for CO\(_2\) emissions (e.g. British Columbia). In the case of cyanobacterial production, however, there is further profit from the other co-products in the biomass, such as protein for animal feed. Thus, even with a low PHB content compared to PHB-accumulating bacteria, there are many advantages to the cyanobacterial production route.

### 4.3.4 Comparison of pigment contents

The appearance of freeze-dried \(S. platensis\) biomass obtained from the reactor under each condition and biomass after chlorophyll/carotenoid extraction is shown in Figure 37. It is evident that the differences in both quantity and quality of light affected the photosynthetic pigment contents in \(S. platensis\).

![Figure 37: Freeze-dried \(S. platensis\) biomass from run 9 (left) and run 10 (right)](image)

The amount of chlorophyll \(a\), carotenoids, and phycobiliproteins as a percentage of dry weight for \(S. platensis\) grown under both growth conditions is shown in Figure 38.
**Figure 38**: Comparison of chlorophyll $a$, carotenoid and phycobiliprotein content in *S. platensis* biomass for runs 9 and 10. Statistically significant differences are denoted with an asterick ($P<0.05$, n=2).

The characteristic spectral output of a HPS lamp and the spectral output red LEDs with a peak wavelength of 625 nm (used in this work) are shown in Figure 39. The main pigments of *S. platensis* and their peak light absorption are provided in Table 9 [80].

**Figure 39**: Spectral output of light sources in runs 9 and 10

**Table 9**: Absorption by pigments in *S. platensis*

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Peak absorption (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll $a$ (green)</td>
<td>665 and 435</td>
</tr>
<tr>
<td>Carotenoids (yellow-orange)</td>
<td>400-550</td>
</tr>
<tr>
<td>Phycocyanin (blue)</td>
<td>615</td>
</tr>
<tr>
<td>Allophycocyanin (aqua blue)</td>
<td>652</td>
</tr>
</tbody>
</table>
The major function of carotenoids is to absorb and dissipate excess light to protect the integrity of chlorophyll, though they are present in small amounts [21]. It has been reported that carotenoid content is not affected by the colours of various LEDs, which suggests that there is no adaptation of carotenoids in *S. platensis* to qualitative light changes [80]. The results in this work are consistent with the results in the literature.

The yellow colour of *S. platensis* biomass under HPS illumination appears to suggest a high amount of carotenoids, however, pigment analysis shows that the appearance is actually due to a small amount of chlorophyll *a*, which is the main pigment in *S. platensis*. Similar to fall foliage, once chlorophyll is broken down, the remaining pigments will determine the appearance of cells. It is possible that the amount of chlorophyll *a* was reduced to allow for the protection from carotenoids to be sufficient, as a very high-energy input in the yellow-orange range of light from HPS lamp was present.

Under red illumination, there is a higher amount of chlorophyll *a*, likely to capture as much light energy as possible. It is known that the content of photosynthetic pigments increases as light intensity decreases [80]. In addition to the supply of the red light being low (45 µmol photons/m² s), the peak wavelength of the red LEDs in this work is 625 nm whereas the peak absorption of chlorophyll *a* is 665 nm. Red LEDs with a peak wavelength closer to 655 nm would allow for much better growth of *S. platensis*.

The role of phycobiliproteins is to pass energy absorbed from other wavelengths onto chlorophyll *a* [80]. Both light sources output photons that could be absorbed by phycocyanin. Because the intensity of HPS illumination was high (100 µmol photons/m² s), phycocyanin content was reduced. Because the intensity of the red LEDs was low, phycocyanin content increased. The peak absorbance of allophycocyanin is similar to chlorophyll *a*, thus the same reasoning (low light level) can be applied to suggest why the content was higher under red LED illumination.
4.3 Characterization of polyhydroxybutyrate (PHB) by *S. platensis*

4.3.1 Visualization of Intracellular PHB

Nile red staining is commonly used to screen microorganisms for the presence of PHB [95]. Dimethylsulfoxide (DMSO) pre-treatment prior to staining has been shown to improve the efficacy of Nile red permeability into cells [96]. Confocal micrographs of Nile red stained *S. platensis* filaments are presented in Figure 40, illustrating both the presence and location of PHB granules within the cells. A comparison between confocal micrographs of Nile red stained *S. platensis* filaments taken from the exponential phase and stationary phases of growth is shown in Figure 41. Additional confocal micrographs are provided in Appendix B.

**Figure 40:** Confocal micrographs of Nile-red stained *S. platensis* filaments during stationary growth phase. A: auto-fluorescence of *S. platensis*, B: true-colour image of Nile red-PHB fluorescence, C: overlaid image.
Figure 41: Confocal micrographs of Nile-red stained *S. platensis* filaments during A: exponential phase, and B: stationary phase. Blue: *S. platensis* autofluorescence, red: Nile red-PHB fluorescence.

The results resemble what has been shown in the literature for Nile blue stained PHB granules in *S. platensis* using fluorescence microscopy [9], which is another common method used to detect PHB in microorganisms.

PHB is known to accumulate in small amounts in *S. platensis* during the exponential phase of growth, and reaches a maximum during the start of stationary phase before the microorganisms begin to mobilize the energy storage molecules [8, 9, 50]. A smaller quantity of PHB granules was observed in *S. platensis* filaments sampled from the exponential phase (Figure 41A) compared to from the stationary phase (Figure 41B).

While these results allow us to confirm and visualize PHB granules within the cells, these observations must be supported by chemical analyses, as it is possible to mistake other cell inclusions for PHB granules.

4.3.2 Characterization of PHB

The proximity between PHB and thylakoid membranes hinders the separation of PHB and chlorophyll *a* and can result in green PHB products, thus pigment extraction using methanol...
prior to PHB extraction is beneficial for obtaining a pure polymer product [83, 99]. PHB films cast from chloroform using PHB extracted from *S. platensis* and commercially available PHB are shown in Figure 42. Provided a sufficient amount of PHB is used, it is possible to cast a coherent film (Figure 42A), but it is more difficult when a smaller quantity of PHB is used (Figure 42B).

![Figure 42: PHB cast from chloroform using *S. platensis* PHB and commercial PHB. A: 50 mg *S. platensis* PHB, B: 10 mg *S. platensis* PHB.](image)

PHB produced from *S. platensis* was characterized and compared to that of a commercial product (Sigma-Aldrich) that was produced by microbial fermentation. FTIR was used to investigate whether the functional groups of PHB were present in the polymer film extracted from *S. platensis*. FTIR spectra of *S. platensis* PHB and commercial PHB are presented in Figure 43 and Figure 44.

A close resemblance of the FTIR spectra between the commercial sample and *S. platensis* product confirms that the PHB produced is of high purity.
**Figure 43:** FTIR spectra of *S. platensis* PHB and commercial PHB

**Figure 44:** FTIR spectra (in the range of 1100–1500 cm\(^{-1}\)) of *S. platensis* PHB and commercial PHB
Among the IR absorption peaks, the sharp peak at 1720 cm\(^{-1}\) is related to the ester C=O stretching group from the crystalline region of PHB while the shoulder at 1740 cm\(^{-1}\) is associated with the C=O stretching group from the amorphous region [124, 125]. PHB has characteristic peaks within the 1100-1300 cm\(^{-1}\) region due to C-O-C groups in the crystalline (1274 and 1227 cm\(^{-1}\)) and amorphous phases (1261 and 1180 cm\(^{-1}\)) [124].

Although peak intensities cannot be used because the sample size is unknown, the ratios between peaks can be used to make comparisons. It is observed that the peak intensity at 1274 cm\(^{-1}\) (crystalline phase) in commercial PHB is higher relative to the peak at 1261 cm\(^{-1}\) (amorphous phase), which is not observed in \textit{S. platensis} PHB. Furthermore, the band ratios of 1720/1740 (C=O index, I\(_{\text{C=O}}\)) and 1227/1454 (C-O index, I\(_{\text{C-O}}\)) can be used to examine the crystallinity of PHB polymers [125, 126]. In the I\(_{\text{C-O}}\) index, the intensity of the crystalline peak at 1227 cm\(^{-1}\) (which has been shown to change with sample crystallinity) is compared to the intensity of a reference peak at 1454 cm\(^{-1}\) (C-H bending of CH\(_2\)), which does not change with sample crystallinity [126]. The results of the IR crystallinity ratios for the PHB films are given in Table 10. The results indicate that there is reduced crystallinity in PHB derived from \textit{S. platensis}.

\begin{table}[h]
\centering
\caption{Crystallinity parameters of \textit{S. platensis} PHB and Commercial PHB characterized by FTIR}
\begin{tabular}{|c|c|c|}
\hline
 & \textit{S. platensis} PHB & Commercial PHB \\
\hline
I\(_{\text{C=O}}\) & 2.24 & 2.30 \\
I\(_{\text{C-O}}\) & 3.22 & 3.59 \\
\hline
\end{tabular}
\end{table}

While FTIR could provide a qualitative indication of sample crystallinity, XRD was used in order to quantify the degree of crystallinity of both samples. XRD powder patterns of \textit{S. platensis} PHB and commercial PHB are shown in Figure 45 which both have a similar diffraction pattern. The XRD pattern shows peaks at \(2\theta\) values of 15.7°, 19.7°, 25.4° and 29.7°, and possibly at 35.4°.
Figure 45: XRD spectra (in the range of $2\theta = 10^\circ - 40^\circ$) of *S. platensis* PHB and commercial PHB

The peaks are consistent with what has been reported in the literature for PHB, including their relative intensities [127-129]. The values of $2\theta$ for the peaks differ due to a difference in the incident X-ray energy used. A Co-K\(\alpha\) radiation source ($\lambda = 1.79$ Å) was used in this work, whereas Mo-K\(\alpha\) radiation ($\lambda = 0.71$ Å) and Cu-K\(\alpha\) radiation ($\lambda = 1.54$ Å) were used in the other studies.

The peak at 35.4\(^\circ\) has been reported in the literature [127, 128, 130], but not identified in other reports [128]. It was difficult to confirm the peak in this work due to the relatively low intensity and small sample size used, which resulted in a relatively high signal to noise ratio. However, it was concluded that the *S. platensis* product was PHB.

The degree of crystallinity ($X_c$) (Table 11) was determined by calculating the ratios of the areas under the crystalline peaks and the total area (i.e. crystalline and amorphous regions). The crystallinity determined for *S. platensis* PHB and commercial PHB was 81.7\% and 86.8\%, respectively. The lower crystallinity of *S. platensis* PHB compared to commercial PHB is consistent with the crystallinity trend determined by FTIR.

Table 11: Crystallinity of *S. platensis* PHB and Commercial PHB characterized by XRD

<table>
<thead>
<tr>
<th></th>
<th><em>S. platensis</em> PHB</th>
<th>Commercial PHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_c$ (%)</td>
<td>81.7</td>
<td>86.6</td>
</tr>
</tbody>
</table>
PHB samples extracted from bacteria usually exhibit crystallinity in the range of 60-80%, thus the calculated values in the present work are considered to be high [85]. Again, this could be due to the sample size limitation which resulted in a high signal to noise ratio. The full XRD spectra and overall crystallinity calculations for both PHB samples are provided in Appendix C.

DSC was used to determine the thermal properties of the PHB samples. PHB samples first underwent an initial heating scan to remove any thermal history. Data from the second heating scan is shown in Figure 46.

![DSC heating curves for S. platensis PHB and commercial PHB](image)

**Figure 46:** DSC heating curves for *S. platensis* PHB and commercial PHB

The measured melting point of *S. platensis* PHB (167 °C) is lower than the measured melting point of commercial PHB (175 °C), which is consistent with the lower crystallinity of the sample. Values of the melting point of commercial PHB range from 171–182 °C in the literature, which could be vary due to different heating rates used during analysis [8]. A similar decrease in melting point was observed recently with electrospun nanofibers using *S. platensis* PHB compared to commercial PHB, which also had a lower crystallinity [87].

A lower melting point may also be due to a lower molecular weight, however, molecular weight determination was not performed in this work, and thus its effect on melting point in this work cannot be concluded. Although a lower melting point could also be an indication of eutectic impurities, FTIR data showed that the *S. platensis* PHB sample was pure.
4.3.3 Crystallinity of *S. platensis* PHB

The FTIR, XRD and DSC results all suggest a reduced crystallinity in PHB derived from *S. platensis*.

The value for $I_{\text{C-O}}$ for commercial PHB in this work (3.59) is higher than in the literature for commercial PHB (2–2.6) [125, 126]. It may be difficult to compare these values due to differences in sample preparation, which can cause differences in crystallinity. For example, the crystallinity of PHB determined by XRD for Wei at al. was 61%, whereas it was 87% in this work [125]. Wei at al. did not discuss sample preparation for PHB [125]. In the present work, films were cast using heat during solvent evaporation. It has been shown that heating films after casting can increase the crystallinity (as well as the intensity of absorbance at 1726 cm$^{-1}$) due to the further crystallization that can occur from improved mobility of the molecules compared to at room temperature (FTIR). However, the comparisons between the results in this work to reports in the literature are consistent the work by Xu et al. that demonstrated an increase in $I_{\text{C-O}}$ corresponds to higher crystallinity [126].

When comparing the crystallinity ($X_c$) between *S. platensis* PHB (81.7%) and commercial PHB (86.6%), the findings agree with a study published very recently that reported electrospun nanofibers with *S. platensis* PHB had a lower crystallinity ($X_c = 50.2\%$) compared to nanofibers produced with commercial PHB ($X_c = 57.2\%$) [87]. However, there is a discrepancy between the crystallinity values. In the work done by Morais et al., electrospinning was carried out at 21 °C [87]. It is known that electrospun fibers can have a lower crystallinity because the samples are prepared under kinetically controlled conditions [101]. Thermal annealing of electrospun PVA fibers has been shown to increase crystallinity with each treatment of increasing temperatures, as the heat will allow the molecules to be mobile and further crystallize [101]. The values obtained by Morais et al. are consistent with another study, which reported a degree of crystallinity of 53.1% for PHB electrospun fiber mats [131].

Although mechanical properties were not tested in this work, the expectation is that the mechanical properties of *S. platensis* PHB would be more favourable with reduced crystallinity (i.e. reduced brittleness).
In addition to the effects of crystallinity on polymer properties, it is worth noting that molecular weight determination was not performed in this work, which can also affect the melting temperature and mechanical properties. Thus, more work is still required to further investigate the improved properties of PHB produced by *S. platensis*. It was, however, recently reported that electrospun nanofibers produced from *S. platensis* PHB, which had a higher molecular weight, exhibits higher stiffness, tensile strength, and breaking elongation than nanofibers produced from commercial PHBV [87]. In addition to the biodegradability and biocompatibility of PHB [7], and the lack of inflammatory lipopolysaccharides in *S. platensis* [11], improved mechanical properties of PHB derived from *S. platensis* could further increase the range of applications in biomedical applications (e.g. tissue engineering scaffolds).
Chapter 5: Conclusions

5.1 Summary of conclusions

Enclosed PBRs can be designed to cultivate microalgae to high biomass densities in closed and controlled systems. There are many commercial applications of microalgae, but many large-scale applications are limited by the need to reduce production costs and improve profitability with co-production of high value added products [2, 3]. In this work, both PBR design and a unique product from microalgae were investigated in one integrated study. The cyanobacterium (blue-green alga) *S. platensis* was used for the production of the biopolymer PHB.

Using computational fluid dynamics software, a flat plate PBR was successfully designed. In particular, potential dead zones in the reactor were minimized and a sparger that would provide uniform airflow was designed in order to improve mixing. Testing of the PBR post-construction validated the accuracy of the assumptions made in the simulations. In order to improve light efficiency, an LED illumination system that could provide a controllable output of red, blue and green light was implemented.

Initial studies aimed at selecting suitable cultivation conditions for *S. platensis* the PBR showed the importance of PBR hydrodynamics. It was determined that an aeration rate below 0.35 vvm be used in the flat plate PBR to minimize the exposure of shear sensitive *S. platensis* to hydrodynamic stresses. Furthermore, it was shown that *S. platensis* were not able to grow under combined red, blue and green illumination with the LEDs that were used in this work, but the exact cause of damage could not be definitively diagnosed.

Growth of *S. platensis* under a high aeration/polychromatic (HPS) light condition was compared to a low aeration/monochromatic (red LED) condition. The first set of conditions could support a higher biomass density in the PBR (1.81 g/L versus 0.64 g/L) due to better mixing and higher light availability, but the second set of conditions resulted in a higher PHB content (0.13% versus 0.75%) likely due to the growth conditions being less optimal. Despite the relatively low PHB contents in *S. platensis* compared to in a PHB accumulating
bacterium, it was showed that the overall solar energy conversion efficiency for PHB production is higher than that by microbial fermentation.

Confocal fluorescence microscopy images qualitatively showed Nile red-stained PHB granules within *S. platensis* were present in higher amounts during the stationary phase of growth. PHB produced by *S. platensis* was determined to be of high purity using FTIR.

A key finding in this work was the lower crystallinity of *S. platensis* PHB produced in this work compared to that of commercial PHB (produced by microbial fermentation) determined using XRD, which was consistent with the crystallinity trend determined by FTIR and a lower melting point determined by DSC. In addition the lack of inflammatory lipopolysaccharides in *S. platensis* produced PHB points to its promising biomedical applications ranging from medical sutures to tissue regeneration [10, 11].

### 5.2 Future work

There was an apparent issue with the LEDs used in this study. *S. platensis* was not able to grow under combined red, blue and green illumination, but could grow under red illumination. In order to diagnose the problem, it is recommended to perform additional experiments under blue illumination, green, illumination, and other combinations between the three colours. However, due to the low power of the LEDs, it may be beneficial to employ LEDs with a higher light intensity to take better advantage of the system. The low power LEDs used in this work did not provide sufficient light energy to support the growth of *S. platensis* to a high biomass density.

An LED illumination system provides a potential for further investigating the effects of the incident light wavelength on the biochemical composition of *S. platensis*. Further studies should be conducted to determine a possible effect of LED wavelength on PHB accumulation in *S. platensis*, which has not yet been reported in the literature.

In this work, a nutrient limitation step to enhance intracellular PHB content in *S. platensis* was not performed due to time constraints. It is recommended the designed system be repeated with nutrient limitation and supplementation with acetate to improve PHB content in *S. platensis*. 
It is recommended that mechanical testing be performed to study the potential of improved mechanical properties owing to the lower crystallinity of PHB produced by *S. platensis*. Additionally, molecular weight determination should be performed to better understand the factors that govern the polymer’s thermal and mechanical properties.
References


Appendix A: Supplementary Simulation Results

Materials, models and boundary conditions used in ANSYS CFX

Mesh size: fine (see Figure A1)

Default Domain Setup

Dispersed fluid: Air 25 °C
Bubble diameter = 5 mm (varied throughout simulations)
Continuous fluid: Algal slurry (or used water, i.e. 1x viscosity of water)
Viscosity = 0.005 Pa·s (5x viscosity of water)
Set gravity
Buoyancy ref \( \rho = 1.2 \text{ kg/m}^3 \)
Buoyancy model: buoyant
Turbulence: fluid dependant homogenous model: no
For air
Turbulence: zero equation model (recommended choice for dispersed fluids)
Buoyancy model: density difference
For slurry
Turbulence: k-e model with scalable wall function (prominent turbulence model, considered
to be an industry standard for general purpose simulations)
Buoyancy model: density difference
Two-phase model
Buoyancy driven mixing
Pair model surface tension= 0.073 N/m
Drag force: grace drag model (good for air-water systems; well suited to gas-liquid flows
with bubbles of different shapes)
Turb trans: Sato enhanced eddy (for particle induced turbulence; large dispersed particles
tend to increase turbulence in continuous phase due to presence of wakes behind particles)

Boundary Conditions
Walls are smooth no slip for both phases
Symmetry
Outlet degassing condition
Inlet subsonic 0.00006 kg/s (varied throughout simulations) = 0.0036 kg/min= 3 L/min
normal to BC medium 5% turbulence pure air

Figure A1: Mesh generated in simulation software
Sparger design selection

**Figure A2:** Sparger geometries (1–5) tested in simulations

**Figure A3:** Liquid velocity around sparger for spargers 1–5
Figure A4: Streamlines from top of reactor for spargers 1–5
Uniform velocity through hole exits across length of sparger

**Figure A5:** Velocity though hole exits in sparger design #4

Validation with test microalga *Nannochloropsis oculata*

*N. oculata* (UTEX LB2164)
Modified Erdshreiber’s medium
pH 8, 30 °C

**CO₂ input:**
Air at 2 vvm

**Light input:**
High pressure sodium lamps
750 µmol photons/m² s

**Figure A6:** Biomass density versus time for *N. oculata* in the flat plate PBR
Investigation of PBR hydrodynamics

**Figure A7:** Wall shear at 1 vvm (left) and 0.35 vvm (right)

**Figure A8:** Turbulence eddy dissipation rate at 1 vvm (left) and 0.35 vvm (right)
Appendix B: Confocal Images

Nile-red stained *S. platensis* filaments during exponential growth phase

Figure B1: Confocal micrographs of *S. platensis* auto-fluorescence (left) and Nile red-PHB fluorescence (right) during exponential growth phase

Nile-red stained *S. platensis* filaments during stationary growth phase
Figure B2: Confocal micrographs of *S. platensis* auto-fluorescence (left) and Nile red-PHB fluorescence (right) during stationary growth phase
Appendix C: XRD Data

Figure C1: Full XRD powder pattern

Sample of peak fitting

Figure C2: Sample of peak fitting to Gaussian model in MATLAB for determination of $y_0$, $x_c$ and $w$.

The coefficients for $y_0$, $x_c$ and $w$ obtained for the Gaussian model were used in the Pearson VII function (equation 3.9 in section 3.5.2) for each peak:

$$f(x) = y_0 \left[ 1 + \frac{4(2\bar{m} - 1)}{w^2} \left( x - x_c \right)^2 \right]^{-m}$$ (3.9)
Figure C3: Full XRD pattern with each crystal structure (4 peaks) fit to the Pearson VII function for *S. platensis* PHB

Figure C4: Full XRD pattern with each crystal structure (4 peaks) fit to the Pearson VII function for commercial PHB

Using equation 3.10 in section 3.5.2, $X_c$ was calculated from $2\theta = 10–70^\circ$ by the sum of $y$ for the 4 Pearson VII curves (excluding the overlaps between peaks) divided by sum of $y$ of raw data. Equation C1 shows the calculation *S. platensis* PHB where 0.02 is the step size of $2\theta$:

$$X_c = \frac{A_c}{(A_c + A_a)} \quad (3.10)$$

$$X_c = \frac{\sum_{2\theta=5}^{17.74} f(2\theta) \times 0.02 + \sum_{2\theta=17.76}^{21.32} f(2\theta) \times 0.02 + \sum_{2\theta=21.34}^{28.78} f(2\theta) \times 0.02 + \sum_{2\theta=28.8}^{70} f(2\theta) \times 0.02}{y_{\text{data}} \times 0.02} \quad (C1)$$
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EDUCATION

Chemical and Biochemical Engineering
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2013  First Place Award for Undergraduate Research Project Presentation
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Chemical Engineering Club  
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2010 – 2011  **Student Program Co-chair**  
61\(^{st}\) Canadian Chemical Engineering Conference (CSChE 2011)  
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