

Developing Next Generation Algae Bioplastic Technology

by Shawn Price

Thesis submitted in fulfilment of the requirements for
the degree of

Doctor of Philosophy

under the supervision of Peter Ralph, Mathieu Pernice &
Unnikrishnan Kuzhiumparambil

University of Technology Sydney
Faculty of Science

December 2022

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Shawn Price, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctorate of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Signature:

Production Note:
Signature removed prior to publication.

Date: 7/12/22

Preface

Thesis format

This thesis is written in the format of a thesis by compilation— a combination of published chapters and those unpublished but with the intention of publication in a peer reviewed scientific journal in the near future. Given that this thesis is presented as a series of ready to submit manuscripts, there is an element of repetition in the introduction of some chapters since they are each submitted as stand-alone manuscripts.

The first chapter is a literature review which introduces the field of cyanobacteria as a production platform for the bioplastic poly-hydroxy-butyrate (PHB). The second chapter explores random mutagenesis for the creation of novel cyanobacterial mutant strains with enhanced PHB productivities. The third chapter investigates the use of chemical modulators to elicit and inhibit PHB productivity in cyanobacteria. The fourth chapter explores the use of wastewater as a medium for cyanobacterial PHB production. The fifth chapter is a techno-economic assessment of the economic viability of industrial production of cyanobacterial PHB. The final chapter is a synthesis of the thesis with final perspectives on this exciting research area.

Publications

At the time of thesis submission, Chapter 1 and 5 have been published in the peer reviewed *Journal of Environmental Chemical Engineering* (IF 5.909). Chapters 2, 3 and 4 are currently in review in different peer reviewed journals and are expected to be published in 2023.

Table of Contents

Preface.....	3
Thesis format	3
Publications.....	3
List of Figures	6
List of Tables	10
Abstract	11
Chapter 1 - Cyanobacterial Polyhydroxybutyrate for Sustainable Bioplastic Production: Critical Review and Perspectives	14
Authors	14
Affiliations.....	14
Abstract	14
Introduction	14
PHA/PHB in Cyanobacteria and Algae.....	18
PHB Material Properties.....	31
Industrial Cyanobacterial PHB Production	33
PHB Economic Assessment.....	42
Improving Viability of PHB Production from Cyanobacteria	46
Conclusion.....	52
Chapter 2 - Random Chemical Mutagenesis Followed By FACS to Enhance Cyanobacterial PHB Bioplastic Production	54
Abstract	54
Introduction	54
Materials and Methods	56
Results and Discussion	60
Conclusion.....	67
Chapter 3 - Chemical Elicitors and Inhibitors of Cyanobacterial PHB and Biomass	69
Abstract	69
Introduction	69
Materials & Methods	75
Results & Discussion.....	78
Conclusion.....	88
Chapter 4 - Assessing the suitability of domestic wastewater as a medium for cyanobacterial PHB Bioplastic Production	89
Abstract	89

Introduction	89
Materials and Methods	91
Results & Discussion.....	93
Conclusion.....	100
Chapter 5 - Techno-Economic Analysis of Cyanobacterial PHB Bioplastic Production	101
Abstract	101
Introduction	101
Approach, Rationale and Key Assumptions.....	104
Results and Discussion	112
Conclusion.....	121
Synthesis.....	123
Summary of cyanobacterial PHB.....	123
Thesis outcomes, reflections and next steps.....	123
Final perspectives on the future commercial viability of cyanobacterial PHB.....	127
Appendix.....	129
Chapter 5 Appendix.....	129
References	132

List of Figures

Figure 1-1: Molecular structure of PHAs with R representing possible aliphatic functional groups.....	17
Figure 1-2: Four species of algae capable of PHB production; (A) <i>Athrospira maxima</i> (B) <i>Oscillatoria jasarvensis</i> (C) <i>Synechocystis PCC6803</i> (D) <i>Nostoc muscorum</i>	18
Figure 1-3: Major cyanobacterial storage polymers (information adapted from (Flores & Herrero 2014)).	19
Figure 1-4: (A) Metabolic pathway of PHB synthesis in <i>Synechocystis PCC 6803</i> (B) Molecular structures of PHB, PHV and PHBV.....	23
Figure 1-5: Molecular structure of PHB and polypropylene (PP).	25
Figure 1-6: Methods of cultivating algae. (A) Bubbled column PBR (B) closed horizontal tubular PBR with pump for mixing.....	35
Figure 2-1: High level overview of experimental procedure.....	57
Figure 2-2: Kill curve showing viability of cultures 21 days after treatment with varying EMS concentrations, with each concentration in triplicate by column.	60
Figure 2-3: (A) PHB Yield % Dry Cell Weight, (B) Volumetric PHB Density and (C) Biomass Density achieved by wild type and 13 mutant strains by day 28 of culturing.	62
Figure 2-4: Conceptual categories of mutants produced.	63
Figure 2-5: Scatter plot of biomass density and PHB yield of characterised 13 EMS mutants.	63
Figure 2-6: Optical density (750nm) of top 5 strains by PHB yield (A), middle 4 strains by PHB yield (B) and bottom 4 strains by PHB yield (C).....	65

Figure 2-7: Maximum quantum yield and maximum electron transport rate of wild type and 13 mutant strains during exponential and stationary growth phases.	66
Figure 2-8: Exponential growth maximum transport rate correlated with (A) final biomass density and (B) final PHB Yield. Stationary growth maximum transport rate correlated with (C) final biomass density and (D) final PHB Yield.....	67
Figure 3-1: Experimental workflow overview including an initial screen of all 10 chemicals at 3 concentrations and a further test with more time points for the top performing chemical triggers.....	76
Figure 3-2: PHB Yield of the cultures grown with 10 different chemicals at 0.1 μ M, 1 μ M and 10 μ M (mean \pm standard error, n=3).	79
Figure 3-3: Biomass density of the cultures grown with 10 different chemicals at 0.1 μ M, 1 μ M and 10 μ M (mean \pm standard error, n=3).	80
Figure 3-4: PHB volumetric density of the cultures grown with 10 different chemicals at 0.1 μ M, 1 μ M and 10 μ M (mean \pm standard error, n=3).	80
Figure 3-5: (A) Biomass density, (B) PHB Yield and (C) PHB volumetric density of IAA 0.1 μ M and methyl jasmonate 1 μ M cultures (mean \pm standard error, n=3).....	83
Figure 3-6: (A) Biomass density, (B) PHB Yield and (C) PHB volumetric density of ethynylestradiol 10 μ M and allopurinol 10 μ M cultures (mean \pm standard error, n=3).	87
Figure 4-1: Overview of the experimental procedure.	91
Figure 4-2: Biomass accumulation measured gravimetrically every 5 days for primary wastewater culture (blue line) and BG11M culture (yellow line). There was no statistically significant difference in the biomass accumulation across the entire 30 days of culturing between the primary wastewater and BG11M culture (p>0.05). All measurements were in triplicate with standard error of the mean shown by error bars.	94

Figure 4-3: PHB % yield normalised to dry biomass determined by HPLC every 5 days for primary wastewater culture and BG11M culture. * denotes a statistically significant difference ($p < 0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.	95
Figure 4-4: Optical density measurements at 675nm corrected for turbidity (by subtracting optical density at 750nm) taken every 2 days of primary wastewater cultures and BG11M culture (mean \pm SEM).....	96
Figure 4-5: Phosphate of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference ($p < 0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.	97
Figure 4-6: Ammonium of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference ($p < 0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.	98
Figure 4-7: Nitrate of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference ($p < 0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.	99
Figure 5-1: Process flow diagram for cyanobacterial PHB production plant.	111
Figure 5-2: Capital cost breakdown (total \$193.5M USD), (A) by individual items and (B) by production section.	113
Figure 5-3: Annual operating cost breakdown (total 147.3M USD per annum), (A) by individual items and (B) by production section.	115
Figure 5-4: Breakeven analysis to determine minimum PHB sell price of \$18,399 USD / tonne for a zero 20-year Net Present Value (NPV).	116
Figure 5-5: Sensitive analysis on factors affecting breakeven PHB sell price.	117
Figure 5-6: Effect of PHB yield on minimum PHB sell price.	118

Figure 5-7: Breakeven PHB sell price of different scenarios explored..... 121

List of Tables

Table 1-1: Summary of different PHB related proteins and their roles.....	23
Table 1-2: Comparison of physical properties between PHB and polypropylene (PP) (data adapted from (Anbukarasu, Sauvageau & Elias 2015; Balaji, Gopi & Muthuvelan 2013; Samper et al. 2018)).	32
Table 1-3: Global manufacturers and production volumes of PHA plastic (data adapted from (Singh et al. 2017)).	42
Table 3-1: List of 10 chemical compounds screened for their effect on cyanobacterial PHB yield and biomass productivity. Chemicals were dosed at 0.1 μ M, 1 μ M and 10 μ M.	76
Table 5-1: Hypothetical scenarios explored for their impact on the base business case.....	107

Abstract

Plastics enable the modern world to function. They have applications in agriculture, medical biotechnology, consumer products, electronics, construction and much more.

Unfortunately, their widespread use comes at the cost of the environment in the form of aquatic and terrestrial pollution. However, bioplastics made from renewable resources that can biodegrade in the environment offers a solution to this problem, but they are held back due to high substrate costs of sugar feedstock required for fermentation.

Cyanobacteria are microscopic photosynthetic organisms capable of converting atmospheric CO₂ into a widely used bioplastic, PHB (poly-hydroxy-butyrate). Although this feedstock is significantly cheaper compared to current bioplastic production, the industrial production of cyanobacterial PHB is still not economically viable due to lower PHB productivity rates and high cultivation equipment costs compared to fermentation. In order to progress towards economic viability, four areas were explored as separate data chapters in this thesis:

Chapter 2: Creation of novel mutant strains through random chemical mutagenesis with superior cyanobacterial PHB productivity

Targeted genetic engineering requires advanced technical manipulation and prior knowledge of which genes to target. However, random mutagenesis can create mutants with novel mutations which result in a desired phenotype that can be sequenced to learn about new PHB metabolic mechanisms. In this study, ethyl methane sulfonate (EMS) was used to create a mutant library which was screened using fluorescent activated cell sorting (FACS) to sort single cells with BODIPY 493/503 (a neutral lipid dye) into well plates. These mutants were then screened for growth rate before being tested for PHB productivity. Two mutant strains were created with enhanced PHB yields (29% and 26% higher than wild type), biomass densities (36% and 33% higher than wild type) and PHB volumetric densities (75% and 67% higher than wild type).

Chapter 3: Identifying chemical enhancers and inhibitors of cyanobacterial PHB metabolism

Chemical modulators which affect cyanobacterial metabolism can be used to increase PHB production at industrial scales. The mechanisms of enhancers and inhibitors of PHB production can also be studied to identify genetic and regulatory information on PHB metabolism. Thus, 10 different compounds (including oxidants, antioxidants, phytohormones) were screened at 3 concentrations (0.1 μM , 1 μM and 10 μM) to identify compounds which boosted and reduced PHB production. Two treatments, 0.1 μM IAA and 1 μM methyl jasmonate were found to increase PHB yield (55% and 19% compared to control). Two treatments, 10 μM allopurinol and 10 μM ethynylestradiol, were found to decrease biomass density, PHB yield and PHB density.

Chapter 4: Exploring municipal wastewater as a media for cyanobacterial PHB production

Using wastewater as a substrate not only reduces demand for fresh water, but also reduces production costs through not requiring synthetically made media. However, wastewater as a substrate introduces the possibility of culture contamination, presence of inhibitory pollutants and provided a unique nutrient profile. This study demonstrated the potential for primary domestic wastewater as a nutrient source of cyanobacterial biomass cultivation with no significant difference between biomass densities compared to the control culture. However, PHB yield was significantly inhibited (85% lower than control) which may have been linked to non-cyanobacterial biomass.

Chapter 5: Techno-economic modelling to identify key financial drivers of cyanobacterial PHB profitability

This techno-economic modelling study breaks down the key capital and operating costs and identifies the major financial barriers to profitability. For a base case scenario, a 10,000 tonnes of PHB bioplastic resin per year facility in Australia was used with breakeven and sensitivity analysis to assess economic viability. The financial model was then used to explore potential paths to financial viability such as examining the effect of the scale of production volume, additional revenue from utilising a biorefinery approach to cyanobacterial biomass, use of holding or ripening tanks to reduce cultivation costs and geographic location of the hypothetical production facility. The base case revealed that the

cost of production was \$18.1k USD/tonne which is over four times the current market price of PHB. However, through the combination of several optimistic scenarios, the breakeven price could potentially reach \$7.7k USD/tonne.