TECHNISCHE UNIVERSITÄT WIEN
Vienna | Austria

Master Thesis

Developing a sustainable PHB extraction method from *Synechocystis* sp. grown on whey

Conducted to obtain the academic degree: **Diplom-Ingenieur (Dipl.-Ing.)**

Under the supervision of:

Associate Prof. Dipl.-Ing. Dr.nat.techn. Oliver Spadiut

Univ.-Ass. Dipl.-Ing. Ricarda Kriechbaum

Institute of Chemical, Environmental and Bioscience Engineering

Submitted at:

TU WIEN
Faculty of Mechanical and Industrial Engineering

By:

Lukas Martetschläger, BSc (01612389)

The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error- Bertolt Brecht

Acknowledgements

First, I want to thank my supervisors, Associate Prof. Dipl.-Ing. Dr.nat.techn. Oliver Spadiut and Univ.-Ass. Dipl.-Ing. Ricarda Kriechbaum for giving me the opportunity to work in their research group and for their support, help and many inspiring discussions throughout the development phase of my Master Thesis.

I would also like to thank my parents, whose support and understanding made my studies possible in the first place and who always had an open ear for me during my time in school and university.

Finally, I would like to thank my grandparents, with whom I was always welcome throughout the years and who have provided for my culinary well-being.

Table of Contents

Abstract

Most plastic products in circulation worldwide are based on fossil petroleum and are not biodegradable, which is known to be one of the biggest burdens and threats to the environment. One possible solution to this problem could be the use of bio-based and biodegradable polyhydroxybutyrate (PHB). Under nitrogen and phosphate depletion, *Synechocystis* sp. PCC 6714 produces PHB. As a positive side-effect, this method also binds the greenhouse gas CO_2 through photosynthesis.

This thesis analyses the results of a series of experiments carried out as part of basic research to better understand a possible PHB production process. The main goal was to investigate the effects of different lactose concentrations between 1 and 10 g/L from concentrated whey on the microorganism, particularly on the PHB production. The following three main results were obtained:

Firstly, the cyanobacterium strain used could not metabolise lactose and even showed lower growth and PHB content of 2.2% at 1 g/L lactose compared to 4.2% without the addition of whey in the shake flask experiment. When upscaling in a photobioreactor, the same ratios were observed between cultivations with and without whey, with the PHB content doubling to 6.5 and 10.5% (volumetric productivity 7.7 and 21.1 mg/L/day).

Secondly, the standard hydrolysation method for PHB quantification with concentrated sulfuric acid was compared with the alkaline sodium hydroxide method using a multivariate data analysis. Subsequently, the more promising acidic method was further optimised to reduce viscosity of sulfuric acid, gaining an optimum at 160 min, 14 M H_2SO_4, 100°C.

Thirdly, as an alternative to the state of the art methods for recovering PHB, which use, e.g. the harmful chloroform, three ionic liquids based on the cation 1-Ethyl-3-methylimidazolium with three different anions Dimethylphosphate, Acetate or Chloride were tested to dissolve the biomass but not the biopolymer. 1-Ethyl-3-methylimidazolium Dimethylphosphate completely dissolved the biomass at 75°C after 1 h and did not decompose or dissolve the PHB so that it could be implemented in a complete recovery process.

Kurzfassung

Die meisten der weltweit im Umlauf befindlichen Kunststoffprodukte basieren auf fossilem Erdöl, sind zumeist biologisch nicht abbaubar. Dies bedeutet zumindest eine große Belastung, wenn nicht sogar eine beträchtliche Gefahr für Umwelt und Lebewesen. An der Lösung dieses Problems wird mannigfaltig geforscht. Eine mögliche Alternative bietet Polyhydroxybutyrat (PHB), ein biobasiertes und biologisch abbaubares Polymer. Dieses kann unter Stickstoff- und Phosphatmangel von *Synechocystis* sp. PCC 6714 produziert werden, parallel dazu wird das Treibhausgas CO_2 durch Photosynthese verstoffwechselt.

Hauptziel dieser Masterarbeit war es, im Rahmen verschiedener Experimente ein detaillierteres Verständnis eines PHB-Produktionsprozesses zu erhalten. Vor allem sollten die Auswirkungen verschiedener Laktosekonzentrationen im Bereich von 1 bis 10 g/L aus konzentrierter Molke auf den Mikroorganismus *Synechocystis* sp. PCC 6714 und in der Folge auf die PHB-Produktion untersucht werden. Folgende drei Haupterkenntnisse wurden daraus gewonnen:

Erstens: Das verwendete Cyanobakterium konnte keine Laktose verstoffwechseln und zeigte sogar ein geringeres Wachstum und einen kleineren PHB-Gehalt von 2,2% bei 1 g/L Laktose im Vergleich zu 4,2% ohne den Zusatz von Molke in Schüttelkulturen. Beim Upscaling in einem Photobioreaktor wurden die gleichen Verhältnisse zwischen der Kultivierungsmethoden mit und ohne Molke beobachtet. Dabei verdoppelte sich der PHB-Gehalt absolut sogar auf 6,5 und 10,5% (volumetrische Produktbildungsrate 7,7 bzw. 21,1 mg/L/Tag).

Zweitens: Die Standard-Hydrolyse-Methode zur PHB-Quantifizierung mit konzentrierter Schwefelsäure zeigte im direkten Vergleich mit der basischen Natriumhydroxid-Methode mittels multivariabler Datenanalyse vielversprechendere Ergebnisse. Daher wurde diese saure Methode weiter optimiert, um die Viskosität der Schwefelsäure zu verringern. Das dabei erreichte Optimum liegt bei 160 min, 14 M H_2SO_4, 100°C.

Drittens: Als Alternative zu den herkömmlichen Methoden der PHB-Gewinnung, bei denen z.B. das schädliche Chloroform verwendet wird, wurden drei ionische Flüssigkeiten auf Basis des Kations 1-Ethyl-3-methylimidazolium mit drei verschiedenen Anionen (Dimethylphosphat, Acetat und Chlorid), mit dem Ziel getestet, nur die Biomasse, nicht aber das Biopolymer aufzulösen. Das Ergebnis zeigte, dass 1-Ethyl-3-methylimidazoliumdimethylphosphat die Biomasse bei 75°C nach einer Stunde vollständig auflöste, das PHB aber nicht zersetzte bzw. löste. Demnach könnte dieses Lösungsmittel in einem umweltfreundliches Rückgewinnungsverfahren von PHB aus der Biomasse eingesetzt werden.

Abbreviations

ATP	Adenosine triphosphate
BG-11	Cyanobacteria medium
BM	Biomass
BMIM	Ionic liquid component (cation):1-Butyl-3-methylimidazolium
BOD	Biochemical Oxygen Demand
CAGR	Compound annual growth rate (%)
Cl	Ionic liquid component (anion): Chloride
COD	Chemical Oxygen Demand
DOE	Design of experiment
DEP	Ionic liquid component (anion): Dimethylphosphate
EDTA	Ethylenediaminetetraacetic acid
EMIM	Ionic liquid component (cation): 1-Ethyl-3-methylimidazolium
FTIR (ATR)	Fourier transform infrared spectroscopy (attenuated total reflection)
GC-MS	Gas chromatography-mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IC	Ion chromatography or ion exchange chromatography
IL	Ionic liquid
LIRFP CD QIT-MS	laser-induced radio frequency plasma charge detection quadrupole ion mass spectrometry
MP	Ionic liquid component (anion): Methylphosphonate
Mt	Mutant strain MT_a24 generated by Kamravamanesh et al.[1]
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (reduced →NADPH)
NMR	Nuclear magnetic resonance
OAc	Ionic liquid component (anion): Acetate
OD_{750}	Optical density (=absorbance) at λ=750 nm
PBR	Photobioreactor
PHA	Polyhydroxyalkanoate
PHB	Poly-(R)-3-hydroxybutyrate
PP	Polypropylene
PE	Polyethylene
RT	Room temperature
U	Enzyme activity $\left(\frac{\mu mol}{min}\right)$
WT	Wilde-type strain *Synechocystis* sp. PCC 6714 Pasteur Culture Collection of Cyanobacteria at the Pasteur Institute (Paris, France)
XRD	X-Ray Diffraction
μ	Growth rate (day^{-1} or h^{-1})

A. Introduction

A.1. Cyanobacteria

Presumably 2.45 billion years ago, the first procaryotic microorganisms capable of photosynthesis emerged. These so-called “cyanobacteria” are gram-negative and represent a diverse group that occurs in various forms, from single-cell organisms to loose cell clusters and filamentous cell assemblies to large colonies. Many of these cyanobacteria are very similar in size to the eukaryotic microalgae. "Kyanós", which means blue in ancient Greek, is eponymous for the blue colour of the blue-green microorganisms due to the phycobiliproteins. “Algae” belong to a broad group of organisms capable of photosynthesis.[2] There is controversy among experts as to whether prokaryotic cyanobacteria should be included in the definition of microalgae. Proponents of inclusion argue that they share lifestyles and habitats.[3] In order to achieve a clear differentiation, the term microalga is used in this thesis only for eukaryotic microorganisms capable of photosynthesis. The first microalgae arose from an endosymbiosis between a heterotrophic eukaryotic cell and a cyanobacterium, which evolved into today’s eukaryotic microalgae, which comprise at least 30 taxonomic classes.[3-5]

Cyanobacteria are adaptable, due to their low nutrient requirements and tolerance to extreme environmental conditions. They are found all over the world, e.g. in freshwater, seawater and salt lakes, but also on land in moist to dry soils or on rocks between the Arctic to the Antarctic. Some cyanobacteria can even fix nitrogen from the atmosphere, which is very important for some ecosystems, e.g. in the sea or swamp rice fields. Compared to other algae, they have higher photosynthesis capacity and growth rates and develop easily under normal nutrient conditions. They grow in a barren environment with sufficient air, water and mineral supply and use light as their only energy source. Therefore, the cultivation of cyanobacteria is considered relatively easy and inexpensive.[3, 4] Cyanobacteria can convert 3 to 9% of solar energy into biomass and have a higher growth rate than plants.[6] Unlike microalgae, cyanobacteria generally produce a relatively low amount of lipids, and advanced genetic engineering tools are broadly available.[7] As a result, cyanobacteria are receiving much attention in research and offer a wide range of potential applications, especially in combination with genetic engineering.[8]

A.1.1. Cultivation methods

For large-scale biomass production, mainly phototrophic cultivation is used, i.e. the microorganisms exclusively perform photosynthesis without an organic carbon source. On an industrial scale, mainly open ponds or closed photobioreactors (PBR) are used, in which only CO_2 and light are applied (see Figure 1). In this case, the energy of light is used to fix CO_2 and synthesise the carbon skeleton via the Calvin-Benson cycle. The disadvantage of the phototrophic cultivation method is the limited and slow biomass production.[9]

The more commonly used open systems, such as natural or artificial ponds, raceways or cascades, have large a space demands. The process parameters can only be controlled to a limited extent. These cultivations highly depend on different variables, such as climatic conditions, local requirements and

available construction materials. The influence of climatic conditions must already be taken into account in the development process when selecting the microorganisms, which must be optimised for the relevant conditions.[10] The ponds, which are usually between 5 and 1,000 cm deep, are photo-limited, not optimised for maximum growth rates and more susceptible to contamination than closed systems. For example, in Western Australia, ß-carotene is produced in a 50 ha artificial shallow pond that struggles with low productivity. In contrast, closed PBRs achieve higher concentration, higher productivity per unit area and higher photosynthetic efficiency, avoid water loss through evaporation and allow better control of process parameters. Tubular, flat-panel, airlift column and plastic bag systems are available for industrial scale, but each of which has its limitations, as discussed by Hossain and Mahlia.[11] For optimised cultivations, influencing factors as pH, light, mixing, mass transfer, temperature and sufficient nutrient supply must be carefully considered. Therefore, closed PBRs are well suited for the production of high-value bioactive substances with high sterility requirements. However, the construction costs for a closed system are about ten times higher than for an open system. In addition, the operating costs for closed PBRs are also higher, e.g. for temperature control, artificial lighting or transport of broth or air. Current research is trying to minimise these disadvantages and make the production of photosynthetic microorganisms more economical, which is being done empirically and with the help of simulations.[11-13]

Heterotrophic growth occurs when no photosynthesis takes place, but the cell obtains energy from organic carbon sources, such as sugar. With this metabolism, higher growth rates can be achieved than with phototrophic growth. The heterotrophic growth of cyanobacteria and microalgae can be used to remove pollutants, as they are used as biological agents in wastewater treatment plants.[14]

The simultaneous assimilation of inorganic carbon (e.g. CO_2) and organic carbon sources is called mixotrophic growth, a combination of phototrophic and heterotrophic growth. Due to the lower light irradiation, less energy is required. According to literature, the increased maximum growth rate μ_{max} corresponds approximately to the sum of the maximum growth rates of phototrophic and heterotrophic cultivations ($\mu_{mixo}=\mu_{photo}+\mu_{hetero}$), e.g. for *Arthrospira platensis* or *Chlorella vulgaris*. In some cases, the measured μ_{mixo} even exceeds this sum.[15, 16] In addition, mixotrophic cultivations are known to yield the highest lipid contents.[17] Despite these promising results of mostly axenic cultures, mixotrophic cultivation has not yet been implemented in large-scale processes. The heterotrophic wastewater treatment mentioned above could also be operated mixotrophically, although the effects would still have to be analysed.[14]

In both heterotrophic and mixotrophic growth, it should be noted that not all cyanobacteria or microalgae can metabolise organic carbon sources. In addition, contamination by other microorganisms could occur and an excess of organic substrate could also limit growth. The additional costs of the organic carbon source and competition with food production must also be considered. With a suitable carbon source, the potential of mixotrophic cultivation is shown in lower production costs and higher growth rates.[18, 19] As shown for *Synechocystis* sp. PCC 6803, the optimal amount of sugar in mixotrophic growth depends on light intensity. Furthermore, glucose is not degraded in the absence of light.[20] Further effects of different organic carbon sources, especially on PHB production, are discussed in chapters A.2.4 and A.3.

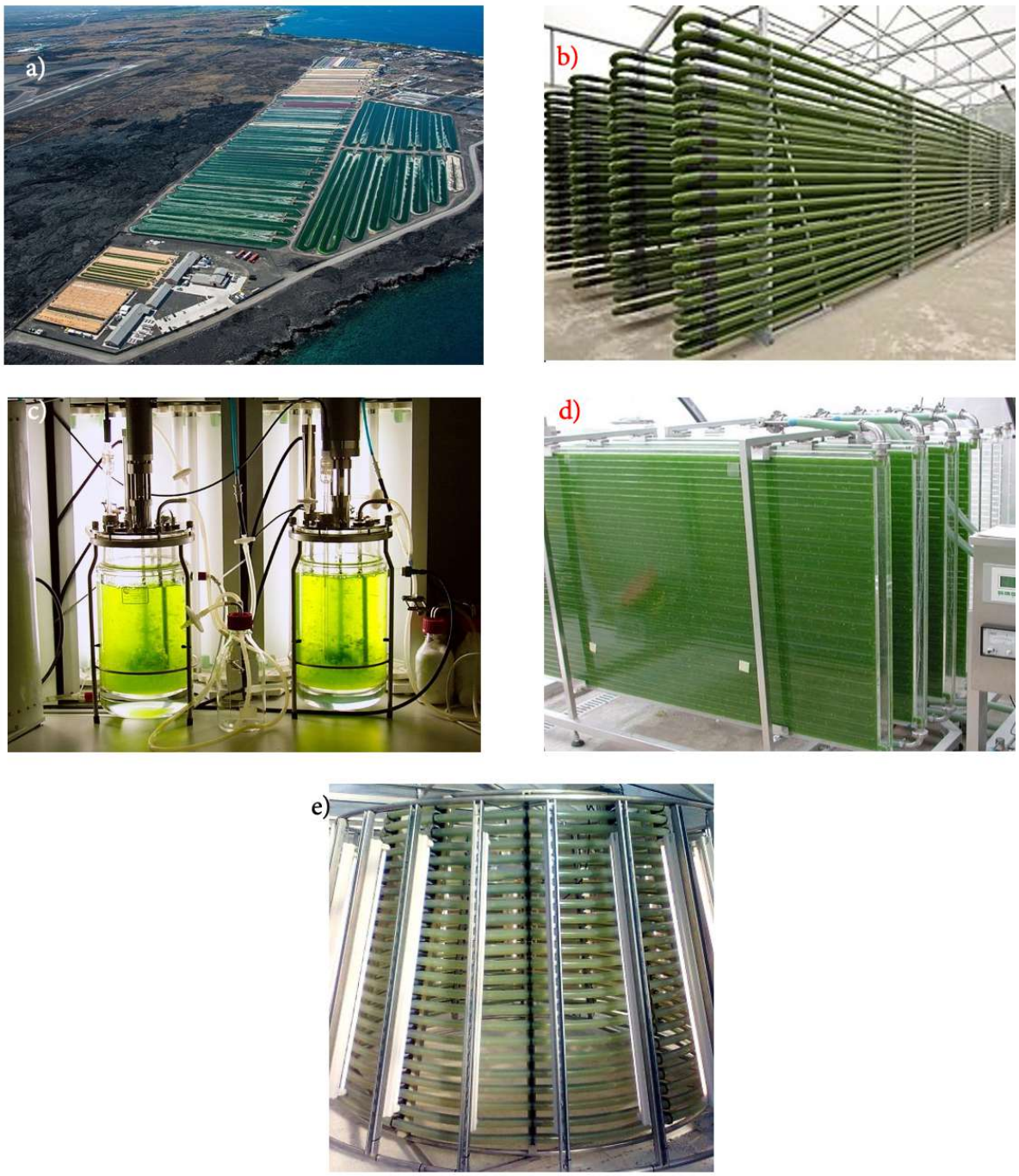


Figure 1: Microalgae and cyanobacteria photobioreactor systems: a) open raceway ponds [21], b) tubular PBR [22], c) laboratory stirred tank PBR [23], d) flat plate PBR [24] and e) Christmas tree reactor (tubular PBR) [25]

A.1.2. Applications

Since cyanobacteria and microalgae can be used for the sustainable production of biochemicals or biofuels, they are considered "green cell factories" and have gained attention in recent years.[26] They are also considered "third generation biomass" as they do not compete with humans for nutrient sources, at least in phototrophic production. Furthermore, commercial plants do not compete with agricultural land and have a smaller footprint. Today, modern industry uses about 220 macroalgae compared to only 15 microalgae and cyanobacteria. Mainly *Arthrospira platensis* (Spirulina) is used as

a procaryotic microorganism, and *Chlorella vulgaris*, *Dunaliella salina* and *Haematococcus pluvialis* as eucaryotic. These microorganisms are primarily used in cosmetics or in food and feed production.[19, 27] In recent years, these microorganisms are also increasingly used for CO_2 mitigation, in wastewater treatment plants or as feedstock for biofuel.[18] In addition, the cyanobacteria *Nostoc* Vaucher ex Bornet & Flahault and the filamentous *Aphanizomenon flos-aquae* are used in China as "hair vegetables" or food additives. Despite the "uncomplicated" cultivation, unicellular cyanobacteria have so far hardly been used for biotechnological applications.[3] Crucial for the economic viability of cyanobacteria are high cell densities and a sufficient light supply.[28]

A.1.3. *Synechocystis* sp.

The unicellular *Synechocystis* sp. PCC 6803 is one of the best-studied cyanobacteria. The strain *Synechocystis* sp. PCC 6714 used in this thesis (see Figure 2) is genetically very similar to the model system *Synechocystis* sp. PCC 6803.[29] It can be cultivated photographically, heterotrophically and mixotrophically, but cannot metabolise lactose. The most commonly used medium is BG-11, as for many cyanobacteria, with a pH between 8 and 9. The microorganisms can even tolerate alkaline conditions with a pH of up to 11.[30] The optimal cultivation temperature is between 25 and 30°C, and with a measured doubling time of less than seven hours, it grows relatively fast for cyanobacteria.[31] The cyanobacterium stores carbon in the form of glycogen, but in the case of nitrogen and phosphorus deficiency, polyhydroxybutyrate (PHB) is produced, which is explained in more detail in chapter A.3. Unlike other cyanobacteria, *Synechocystis* sp. cannot fix nitrogen.[32-34]

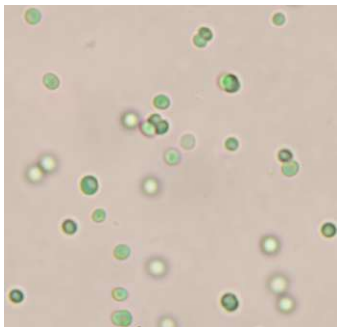


Figure 2: Microscope image of *Synechocystis* sp. PCC 6714

A.2. Whey

A.2.1. Properties and market

Cow milk has been part of the human diet for more than 11,000 years. Whey is a by-product of cheese production, which began about 3,000 years ago.[35] In this process, milk is treated with chemical or biochemical substances at a specific temperature, which causes the casein contained in the milk to coagulate. Depending on whether lactic acid bacteria (acid coagulation) or rennet enzyme (sweet coagulation) are used to coagulate the milk during cheese production, acidic or sweet whey is produced. When the coagulation product is cut, yellow/green-coloured whey is released, which, after heat treatment, is separated from the curd in different ways depending on the type of cheese.[36] Approximately 9 kg (~9 L) of whey is produced while manufacturing 1 kg of cheese.[37] For this reason,

and due to a lack of current data, this paper estimates the amount of annual global whey production (2021) based on the global cheese production published by the United States Department of Agriculture. According to this data, 22 Gt [38] of cheese were produced worldwide in 2021, equivalent to about 198 Gt of whey in the same year. This is less than the estimated 232 to 245 Gt (2009: 180 to 190 Gt) extrapolated by Baldasso et al. [39] when assuming analogous growth of cheese production during the same period (2009: 17 Gt [40], 2021: 22 Gt [38]). In addition, further growth in global whey production is assumed as a result of the expected increase in milk production in the coming years.[41] After separating the cheese, the remaining whey still contains about 80 to 90% of the volume, about 50% of the nutritional value and 20% of the proteins originally contained in the total milk. Therefore, whey is an excellent source of functional proteins and provides humans with a high amount of lactose, minerals (Ca, P, Na, K, Fe, Cu, Zn and Mg) and vitamin B. The average composition of whey compared to milk is shown in Table 1.[35] The vitamin riboflavin (B2) is responsible for the yellow colour of whey.[41]

Table 1: Composition of milk and whey [35]

Components	Concentration (wt/v %)	
	Milk	Whey
Casein protein	2.8	<0.1
Whey protein[a)]	0.7	0.7
Fat	3.7	0.1
Ash	0.7	0.5
Lactose	4.9	4.9
Total solids	12.8	6.3

[a)] *Whey protein composition (approximate): 50% β-lactoglobulin, 20% α-lactalbumin, 15% glycomacropeptide (in sweet whey only) and 15% peptide components/minor protein (e.g., immunoglobulins, serum albumin, lactoferrin, lysozyme, lactoperoxidase and growth factors)*

A.2.2. Applications and environmental impact

In the past, whey was considered a waste product of the cheese industry, for which there were three common recycling methods: (i) disposal into waters, (ii) spreading on agricultural land or (iii) use as animal feed. However, each type of these uses has its disadvantages. The enormous Biochemical Oxygen Demand (BOD) of 40 to 60 g/L and the huge Chemical Oxygen Demand (COD) of 50 to 88 g/L of whey are about 100 to 175 times higher than the same volume of domestic wastewater. Consequently, the disposal of whey into water bodies is prohibited in most major milk-producing countries, making waste disposal very costly. With 70 to 72% of the dry matter, lactose is the main component and the substance mainly responsible for the high COD and BOD values. The spreading of whey over fields increases the risk of soil salination and reduces redox potential. In addition, acidic whey can damage the soil due to its low pH value. Moreover, the valuable resources mentioned above are lost in these disposal methods. Feeding whey in liquid or powder form to animals can be a suitable alternative to utilise the product, just bringing a small financial profit.[42] These options treat whey as a waste stream and assume it has no potential for economically superior applications. 10% of the total amount of whey is not further processed and 40% is discarded, which means a significant loss of the described nutrients.[43] In recent

years, however, it has become evident that new applications for whey have been developed due to regulatory pressure and scientific and technological progress.[35, 41]

Whey is industrially used in the food and beverage industry only to a minor extent, e.g. for butter, alcoholic and non-alcoholic mixed drinks. At the same time, whey protein has recently attracted much attention and is considered a high-quality protein source. After separating lactose and fat by ultrafiltration or diafiltration, the proteins are sold as food supplements. The successful use of whey must include the utilisation of lactose.[35, 43] As summarised by Ryan et al., in recent years, biotechnology discovered the use of whey for conversion in bioprocesses for single-cell proteins/yeast, ethanol, bacteriocins, enzymes, organic chemicals, biohydrogen and for the production of bioplastics. The monosaccharide glucose is probably the best-studied carbohydrate source for microorganisms. Unfortunately, the industrial exploitation of this sugar stands in direct competition with food production. Lactose from whey avoids the fuel vs food dilemma, and the raw material cost of this waste stream (0.071 USD/kg) is significantly cheaper than glucose (0.493 USD/kg).[41, 44, 45]

Bioplastics, like PHA or polylactic acid, derived from whey can be produced by microorganisms using lactose or its metabolites as a C-source or by protein contained in whey. Three strategies for utilising lactose for PHB production can be found in the literature. Microorganisms such as *Hydrogenophaga pseudoflava* or recombinant *Escherichia coli* can metabolise lactose directly using their β-galactosidase activity (see A.2.3), which is the most straightforward strategy. For strains without β-galactosidase activity, there are two other alternatives. First, lactose can be hydrolysed into its monosaccharides glucose or galactose so that it can be metabolised by, for example, *Haloferax mediterranei* or *Pseudomonas hydrogenovora*. Secondly, lactobacilli can convert the disaccharide into lactic acid, which many common PHA-producing organisms can metabolise.[44]

In summary, the potential of whey is far from exhausted and its integration into high-value processes could benefit the environment. With regard to the 17 Sustainable Development Goals (SDGs) adopted by the United Nations in 2015, this would include, in particular “Clean Water and Sanitation” (6th), “Industry, Innovation and Infrastructure” (9th) and “Responsible Consumption and Production” (12th).[41, 44]

A.2.3. β-galactosidase properties

The β-D-galactosidase (BGAL) belongs to the subclass of glycosidases. This large group of β-galactosidases hydrolyses the β-D-glycosidic bond of a terminal galactose. The hydrolysed compounds can be disaccharides, oligosaccharides, or synthetic substrates with an aglycone in the full acetal. The enzyme can be obtained from many organisms, such as bacteria, fungi, yeasts, plants and animal cells, both naturally and recombinantly. BGAL is mainly used for the hydrolysis of lactose in the milk and dairy industry to galactose and glucose (as shown in Figure 3).[46, 47] The pH optimum of the enzyme depends on the natural source and therefore lies within a wide range.[48]

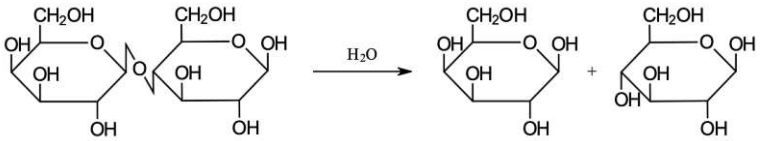


Figure 3: Schematic hydrolyse of lactose to galactose and glucose

A.2.4. Lactose metabolism in cyanobacteria and microalgae

Data on the mixotrophic growth of microalgae and especially cyanobacteria under lactose or whey are very limited. Davies et al. examined cyanobacteria and microalgae for their BGAL activity. They detected BGAL activity in many of the microalgae. However, only the first of the two cyanobacteria examined, *Microcystis* sp. and *Anabaena cylindrica*, showed low activity.[49] In the microalga *Tetradesmus obliquus* (homotypic synonym: *Scenedesmus obliquus*) [50, 51], mixotrophic growth was observed with a whey permeate. This showed larger cell growth than under heterotrophic conditions and even greater growth than under phototrophic conditions. The highest growth rate was achieved with a whey permeate of 40% of the total medium, corresponding to an initial concentration of about 25 g/L lactose. Here, the μ_{max} between mixotrophic (1.08 day^{-1}) and phototrophic (0.27 day^{-1}) was about four times higher (heterotrophic μ_{max}= 0.70 day^{-1}). In another study, *T. obliquus* was used to produce BGAL, with an activity on the seventh day of about 250 U/g at a cell dry weight of about 0.35 g/L (20 g/L lactose and 180 mg/L nitrate). The phototrophic culture had the highest selectivity for the enzyme. However, the mixotrophic conditions showed faster growth than in previous work.[16] When using galactose or glucose individually, higher activities and dry cell weights were achieved.[52] Girard et al. cultured *C. vulgaris* in a medium with whey containing 5 g/L lactose and found no significant difference in cell growth. In contrast, Abreu et al. showed increased growth on non-hydrolysed whey with a lactose concentration of 10 g/L compared to the phototrophic culture. In this case, the microalgae grew faster on whey hydrolysate than on a mixture of glucose and galactose with the same concentrations. It was found that more glucose than galactose was taken up by the cell. The two publications (Girard et al. [16] and Abreu et al. [18]) used different *C. vulgaris* strains, media including lactose concentration, temperatures (22 and 30°C) and pH (7 and 4.5), which could explain the different results. The microalga *Phaeodactylum tricornutum* does not metabolise lactose and shows growth inhibition.[53]

Two strains of the cyanobacterium *Nostoc* sp. showed a mixotrophic metabolism of lactose comparable to that of glucose and maltose.[54] Zanette et al. carried out mixotrophic cultivations of six different microalgae and two cyanobacteria, *Arthrospira platensis* (homotypic synonym: *Spirulina platensis*)[51] and *Synechococcus subsalsus* with lactose (5 g/L). The microalgae *Chlorella minutissima*, *Dunaliella tertiolecta*, and *Nannochloropsis oculate* grew statistically significantly more under lactose than under phototropic conditions, in contrast to the two cyanobacteria. The highest activity was measured in *D. tertiolecta* with about 10 to 75 U/g biomass, which is, however, low compared to other microorganisms. The wide range of variation results from the conversion of activity 30 U/L and a cell density of $1x10^{7}$-$7x10^{7}$ cells/mL, assuming an average mass of a microalga/cyanobacterium of $4x10^{-11}$ g/cell [55]. *S. subsalsus* grew on lactose and showed a lower growth rate than under phototrophic conditions, indicating that no assimilation of disaccharides takes place. After three days, cell death had occurred in 70% of *A. platensis*.[19] In another study, ultra or nano-filtered whey (removal of proteins) was added to the nutrient solution, and even a further positive effect on growth was observed. The lactose concentration in the fed culture was increased to 2 g/L after reaching 0.15 g/L.[56] This result contradicts the first publication by Zanette et al. with the same unmodified strain of *A. platensis*[1]. The

[1] According to the curator of *A. platensis* at the Oceanographic Institute of the University of São Paulo, the A. *platensis* LEB 52 (Vieira-Salla et al.[56]) used in the publications is the old name of *A. platensis* code 159 (Zanette et al.[19]) contained in their collection.[57]

reasons for this could be the different media, the different cultivation conditions (e.g. pH or temperature) or the use of whey permeate instead of pure lactose.[19, 56]

Synechocystis sp. has no BGAL activity. Therefore, the lacZ, which encodes BGAL of the lac operon of *E. coli,* was introduced in some publications. The influence of different promoters or their parts has been studied, sometimes in combination with environmental influences on BGAL activity. This method is not only used for *Synechocystis* sp. [58-61], but also for other cyanobacteria.[26, 62] One publication claims that BGAL accounts for at least 10% of the soluble proteins in *Synechocystis* sp. when lacZ is used.[63] Although the latter publication does not specify where and from what activity the BGAL originates, it should be mentioned that extracellular proteins account for about 80 mg/g DCW in another study.[57]

A.3. Polyhydroxybutyrate

A.3.1. Properties

Polyhydroxybutyrate (poly-(R)-3-hydroxybutyrate, PHB) belongs to the class of polyhydroxyalkanoates (PHA), which are polyesters produced by a variety of microorganisms as an energy reserve. One of the most common representatives and the first PHA to be isolated and characterised was PHB in 1926 (in 1888, Martinus W. Beijerinck was the first to discover PHAs granules in the cytoplasm of microorganisms). So far, researchers have found more than 150 PHAs.[64] PHB is derived from the monomer (R)-3-hydroxybutanoic acid, and the polymer repeating unit is shown in Figure 4.[65, 66]

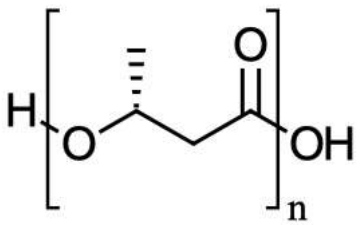


Figure 4: Structure of poly-(R)-3-hydroxybutyrate (PHB) ($M_{Repeating\ unit}$=86.1 g/mol)

PHB is a highly crystalline and biodegradable polymer with thermoplastic properties. The properties of PHB match well with those of polypropylene (PP), as shown by Markl et al., although the former is more brittle in contrast to the latter. The mechanical properties of PHB, other common polymers or other PHA copolymers are compared in the literature.[45, 65] The high stiffness and low elasticity are undesirable properties that can be corrected by blends or copolymers (e.g. 3-hydroxyvaleric acid).[6, 67] PHB is considered a potential green PP substitute because of these similarities.[68] Depending on the molar mass, the melting temperature ranges from 160 to 180°C, with a glass transition temperature of around 5-6°C. PHB contains an average of 60 (5 kDa) and a maximum of 25,000 units (2,150 kDa) of hydroxybutyric acid. For industrial processing, the average length should be at least 46 units or 4 kDa.[69] Other publications consider a molecular weight of more than 500 kDa (5,750 units) acceptable.[70, 71]

The polymer can be rolled, drawn, or pressed into moulds at temperatures above 140 °C. Thermal processing at about 170°C is critical, as melting and decomposition temperatures are close. If the PHB content in the dry cell weight is more than 50%, it can be pressed directly into light to dark-brown bodies above the polymer's melting temperature. Bacterially produced PHB is optically active, UV-resistant, widely resistant to fats and oils, waterproof and relatively gas-impermeable to oxygen, carbon dioxide and water vapour. However, PHB shows poor durability in basic or acidic solutions. Possible solvents for PHB will be discussed in more detail in the later chapter (A.5) on possible recovery methods.[72]

PHB is entirely biodegradable into CO_2, water and energy and does not form toxic degradation products.[73] Numerous bacteria and fungi can meet their carbon and energy needs with PHB, even within a few weeks, depending on the environmental conditions.[15] Unlike PP, PHA sinks in water and degrades anaerobically. According to Policastro et al., various Life Cycle Assessments (LCAs) indicate that PHAs are preferable to conventional plastics and even other bioplastics from a sustainability point of view.[64] However, Dilkes et al. showed, based on an LCA for a material made of biodegradable PHA and thermoplastic starch, that greenhouse gas emission could be higher than for a comparable product made of PP. Most of the emissions come from methane, which is produced during biodegradation and released into the environment in landfills. Capturing this greenhouse gas, which is even more potent than CO_2, with suitable systems is capable to improve the LCA. However, PHB shows that it is ecologically superior to PP or polyethylene (PE).[74] LCA provides a fascinating insight, but more and broader comparisons (e.g. accumulation in the oceans) should be made to introduce possible improvements in the processes or waste management.[72, 75]

A.3.2. Application and market

In the 1970s, Imperial Chemical Industries BioProducts introduced the first commercial PHA product named BIOPOL®. This co-polymer of PHB and 3-hydroxyvalerate is also known as PHBV or under the trade name Biomer L from Biomer. This degradable plastic is mainly used for packagings such as disposable bottles for cosmetics or detergents.[8, 73] Compared to PHB, PHBV is more elastic, harder and has a lower melting point, making PHBV even more similar to PP. PHBV is produced instead of pure PHB by the cell without or only after specific precursors (such as methane, glucose and fructose) and has the same economic disadvantages as PHB.[64]

Although PHAs have been known for a long time, many potential applications are still in development. These include hygiene articles, protein purification, products in the textile industry, printing and biofuels/fuel additives.[8] One main application of PHB lies in medicine: in surgery (nails, fabrics and plates, surgical sutures) and long-term drug applications, e.g. as a retarding matrix for drug embedding. The (R)-3-hydroxybutanoic acid formed during biodegradation is a typical fatty acid metabolite in human blood.[72] Furthermore, this bioplastic could be used in the food packaging industry. The optically pure (R)-3-hydroxybutanoic acid obtained after the hydrolysis of PHB can be used as a reagent for numerous syntheses.[72] Depending on the application area, PHAs can be classified as high-value-added polymers (e.g. biomedicine) or low-value-added (e.g. packaging).[76] Industrial production of PHBs (for an overview of industrial production, see [8, 76]) is currently carried out with heterotrophic bacteria (*Ralstonia eutropha*, or recombinant *E. coli*).[33, 77, 78] These microorganisms grow on a synthetical, well-defined, nutrient-poor medium that utilises only a single substrate.[79]

The main limitations of PHB production are low yields, high production costs, the complexity of the production technology and difficulties in downstream processing.[45] With an annual production of more than 367 Gt of plastic in 2021, bioplastics accounted for 2.41 Gt, less than 1%, which is expected to grow to 7.6 Gt by 2026. According to European Bioplastics, PHAs are expected to account for 1.8% (45 kt) of bioplastics in 2021 and 6.4% (49 kt) in 2026,[80] which is less than 0.1% compared to the PP production capacity (103 Gt, 2021).[68, 81, 82] Lhamo et al. estimated PHAs total production capacity (2021) at around 36kt. The market value of PHA is estimated to be around USD 215.2 million in 2020 and is expected to increase at a compound annual growth rate (CAGR) of 6.1% to USD 327.3 million by the end of 2026.[76] Compared to the price per kilo of less than 1€ for the fossil fuel-based PP, 1.18 to 6.12 €/kg for PHA is above or far above the market price, making large-scale production unattractive.[70]

A.3.3. PHB biosynthesis

Some experts estimate that more than 75 genera, most of which are procaryotic, can synthesise PHB (more than 300 species), including cyanobacteria such as *Chlorogloea fritschii*, *Arthrospira*, *Aphanothece* sp., *Gloeothece* sp. *Synechococcus* sp. and *Synechocystis* sp.[2, 8, 9, 45] In addition, recombinant organisms can produce PHAs, which has been reported for microalgae, bacteria and even plants.[78] Furthermore, mixed consortia with phototrophic microorganisms have also been investigated in the literature for PHB production.[83] In addition to glycogen, *Synechocystis* sp. and its mutants used in this work have been shown to produce PHB as a storage substance. *Synechocystis* sp., like most other PHB-producing microorganisms, synthesises this storage substance only under unfavourable conditions such as nitrogen or phosphorus deficiency (or an unequal carbon: nitrogen or NADPH: ATP ratio).[2, 33, 84] PHB is synthesised in three biosynthetic steps, as shown in Figure 5.[2] In addition, some microorganisms produce PHAs in balanced nutrient ratios.[64] It should also be noted that, besides the mentioned variant, PHB can be obtained by ring-opening polymerisation of β-butyrolactone.[45]

In the cell, PHAs are stored as amorphous granules (0.2±0.5 µm).[74] A layer of proteins and phospholipids is formed on the surface, which has metabolic, structural, biosynthetic and even regulatory functions. To reflect the complexity of these subcellular pseudo-organelles, they are also called carbonosomes.[67] Through (bio)chemical or physical reactions, the PHAs denature and form crystalline sites. In the amorphous stage, the PHB is easier to dissolve than after transformation, which is also reflected in the reduction of possible solvents.[71]

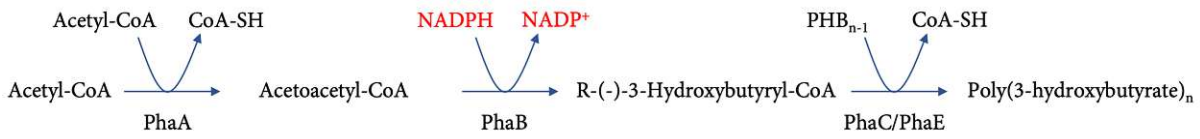


Figure 5: PHB biosynthetic pathway in *Synechocystis* sp. Biochemical steps consuming a reducing equivalent are marked in red. Abbreviations: CoA… coenzyme A, PhaA… 3-ketoacyl-CoA thiolase, PhaB… acetoacetyl-CoA reductase, PhaC/PhaE… two-component poly(3-hydroxyalkanoate) synthase.[86, 87]

[2] A schematic overview of the intermediate stages of the photoautotrophic pathways of *Synechocystis* sp. PCC 6803, is given in the publications of Wijffels et al.[85] or Kamravamanesh et al.[1]

In recent years, genetic engineering techniques have been used to understand metabolic pathways better. Unlike other cyanobacteria, *Synechocystis* sp. strives to synthesise PHB as a carbon store in addition to the primary storage material glycogen when nutrients are scarce.[83] They have different functions for the cell so that only the combination of the two storage substances can ensure survival and the synthesis of proteins and pigments.[88] However, the advantage of PHB production for cyanobacteria has not been entirely understood. In general, PHB production can, for example, increase stress tolerance or decrease redox stress. Chlorosis describes the process in a cell during nutrient limitations, such as the well-studied nitrogen deprivation. Here, glycogen synthesis starts immediately and rapidly, whereas PHB is built up more slowly.[67] Under prolonged limiting conditions, glycogen is degraded, and PHB is built up in *Synechocystis* sp. PCC 6803 under nitrogen limitation.[89] Furthermore, for both *Synechocystis* sp. PCC 6803 and *Synechocystis* sp. PCC 6714, a combination of phosphorus and nitrogen limitation leads to increased PHB production.[34, 90] For economic reasons, the glycogen content should be kept as low as possible while maximising the PHB content.[91]

A.3.4. PHB cultivation conditions

The ratio of the mass (g) of PHB (or possibly PHA) per mass (g) of dried biomass in percent is defined as PHB content and is stated as g PHB / g BM, wt% or in short %.

Generally, most cyanobacteria accumulate less than 10wt% (g PHB /g DCW) under phototrophic conditions.[30] Under optimal conditions, 50% of PHB in cyanobacteria can be achieved under phototrophic growth.[92] However, PHB contents of around 80% are common in heterotrophic organisms[9] and comparable values have been achieved with mixed cultivations.[72] Some publications provide an overview and outlook on the use of cyanobacteria for PHB production.[6, 8, 9, 31, 74, 83, 93]

The main goal of genetic engineering is to create recombinant microorganisms that produce more PHB, grow faster, and are easier to handle in simpler media due to new or improved pathways. In addition to the targeted genetic modification, randomly mutated microorganisms can show an increased PHB content, such as the cyanobacterium used in this thesis (UV mutagenesis).[87, 94, 95] The resulting disadvantages of genetic modification are, on the one hand, the potential environmental threat and, on the other hand, the intercellular limitation of PHB content that can be achieved by modification.[74] Genetic engineering has been used for years in *Synechocystis* sp. PCC 6803 to increase the PHB content, but according to Sirohi et al. has not yet reached an economically reasonable level.[83, 96, 97] In a more recent publication from Koch et al., a recombinant *Synechocystis* sp. PCC 6803 increased the PHB content from 15 to 63% under phototrophic conditions and 32 to 81% with 10 mM acetate under phosphorus and nitrogen limitation compared to the wild type. These measurement results are comparable to those of heterotrophic microorganisms, which could indicate significant progress towards economic viability.[90]

The studies of Kamravamanesh et al.[34] were used to determine the optimal cultivation conditions of PHB in *Synechocystis* sp. PCC 6714. Therefore, a pH of 8.5, a temperature of 28°C and BG-11 as the nutrient medium containing nitrate and not ammonium were chosen as fermentation conditions. In addition, the PHB content was higher when carbonate was used as acetate, in contrast to publications in which *Synechocystis* sp. PCC 6803 was used.[34, 90, 93] There are conflicting results in the literature

on the effects of restricted gas exchange in *Synechocystis* sp., which are not further discussed in this paper.[6, 90]

For high PHB yields, a more operationally complex two-stage cultivation is often described in the literature, where nitrogen is added to a low nitrogen medium after the first growth step. On the other hand, nitrogen limitation can also be done simply after consumption from the N-source.[9] Comparing these two methods for *Synechocystis* sp. PCC 6714, Kamravamanesh et al. found that the single-stage cultivation yielded higher PHB levels. The WT showed a maximum PHB content of 20.4±2 compared to 16.4±2%, and the Mt 35±4 compared to 30±4% under nitrogen and phosphorus limitation in one step.[91] It should be noted that the Mt reached up to 37±4% in previous studies with two-stage cultivation.[1]

In heterotrophic PHB production, the carbon source alone has been reported to account for 30 to 40% of production costs, with the total cost of the medium exceeding 50%.[83] The use of waste streams reduces the cost of feedstocks such as paper mill wastewater, activated sludge, food waste, olive mill wastewater, sugar cane molasses and whey.[65, 76] However, pretreatments, such as degradation processes or inhibition prevention, may be required, increasing the process costs.[64]

Mixotrophic cultivation combines the advantages of phototrophic and heterotrophic growth, resulting in high productivity, rapid growth and faster attainment of nitrogen and phosphorus limitation. However, they are more susceptible to contamination. Acetates, citrates, glucose, fructose, propionates and others are used as substrates for the cultivation of cyanobacteria. Under mixotrophic conditions, cyanobacteria can even reach 85% (wild type *Alusira fertilisima* CCC444).[6] The recombinant *Synechocystis* sp. PCC 6803 produced 32.4% PHB content on a substrate of shrimp wastewater.[83] In the literature, whey has been investigated as a substrate for PHB production.[65, 98-100] A PHB content of up to 80% could be produced by *E. coli* and 60% by *Methylobacterium* sp. The microalga *Chlorella pyrenoidosa* reached almost 80%, with whey as a carbon source.[65, 98]

A.3.5. PHB analysis

The literature shows that the physical properties of PHB depend on the strain, the medium used, the type of cultivation (hetero-, mixo- or phototrophic) and the carbon source. Therefore, the PHB produced, and the influence of different extraction methods should be studied in detail. The main focus should be on the most crucial quality characteristics such as molecular weight, polydispersity, crystallinity and chemical composition.[45] The latter two can be measured by Nuclear Magnetic Resonance (NMR) spectroscopy, Fourier-Transform Infrared Spectroscopy (FTIR), Gas Chromatography-Mass Spectrometry (GC-MS) and X-ray diffraction (XRD).[101] In addition to molecular weight, polydispersity is considered a critical measurement that indicates the heterogeneity of the polymer lengths within the sample. These measurements should be used to investigate potential applications or possible effects of processes, especially downstream processes.[45, 102] For industrial processing, polydispersity should not be bigger than 3.[69] Molecular weight can be determined by viscometry or gel permeation chromatography, the latter being used to determine polydispersity.[8, 65] Furthermore, the mechanical properties of the bioplastic, such as Young's modulus of elasticity, elongation at break and tensile strength, can be measured by differential scanning calorimetry.[8, 65] The methods for quantifying PHB are discussed separately in the following chapter.

A.3.6. PHB quantification

Quantifying PHB in microorganisms has been of interest since the early days of PHB research. Originally it was done gravimetrically after extraction, although this is considered complicated and inaccurate and is still rarely used.[95, 102] Two measurements by HPLC or GC are commonly used after a chemical conversion of PHB. The GC measurement is based on the formation of methyl or propyl ester of 3-hydroxybutyrate in a mixture of methanol or propanol, sulfuric acid and chloroform and subsequent quantification.[3] In the HPLC method, concentrated and highly viscous sulfuric acid is heated to (100°C) with a BM containing PHB, resulting in hydrolysis and the formation of crotonic acid ((2E)-But-2-enoic acid, see Figure 6).[70] Crotonic acid has a 2-alkenoic acid subunit and is UV active (λ_{abs}~210 nm) due to the unsaturated bond in combination with the acid group, which is exploited by the use of a UV detector.[103, 104] Critical issues with both methods are the duration of the sample preparation (drying and reactivity) and the use of harmful organic solvents or highly concentrated acids.[105]

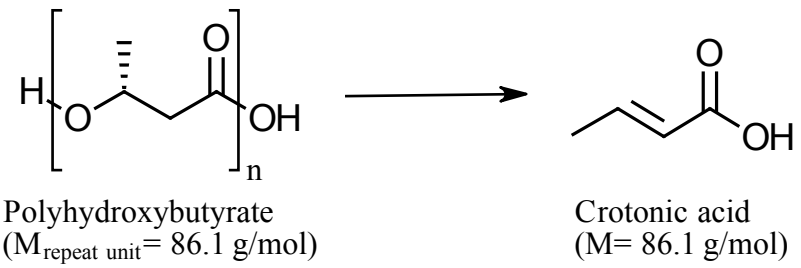


Figure 6: Hydrolysis PHB to crotonic acid (detailed reaction mechanism see [106] and [107])

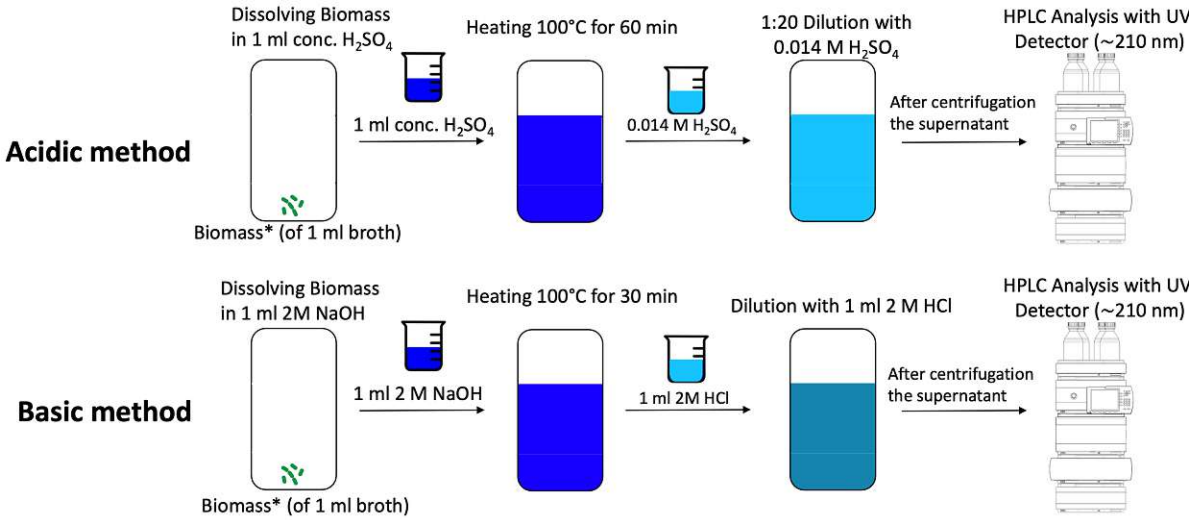


Figure 7: Acidic and Alkaline methods of PHB quantification via HPLC (Acidic [104], Alkaline [108, 109])

In addition to the more frequently used acid hydrolysis, Del Don et al. presented an alkaline hydrolysis with NaOH (2 M). After neutralisation with HCl, the resulting crotonic acid is quantified by HPLC in

analogy to the sulfuric acid method.[108, 109] The schematic sequences for the alkaline and acidic methods are shown in Figure 7. Based on this NaOH method via an HPLC, a composition analysis was presented that would not work with the acidic method.[103] The alkaline method has been shown to hydrolyse amorphous PHB up to 30 times faster than crystalline PHB.[107]

In addition, there are other PHB quantification methods, such as thermal instability of PHB above 180°C (e.g. thermogravimetric analysis, pyrolysis or low-temperature thermolysis).[70] Iijima et al. use an assay kit (Sigma-Aldrich) after ester hydrolysis to β-hydroxybutyrate for quantification. Sudan black B or Nile blue are the two most widely used staining agents for quantifying PHB, among others.[76] A recently introduced and rapid method uses a "laser-induced radio frequency plasma charge detection quadrupole ion trap mass spectrometer" (LIRFP CD QIT-MS).[105]

A.4. Ionic Liquids

The purification of PHB is discussed in the following chapters, preceded by an excursus in Ionic Liquids.

A.4.1. Properties

Paul Walden, who synthesised ethylammonium nitrate (melting point 12.5°C) in 1914, is considered the father of room-temperature Ionic Liquids (ILs). Organic chemists probably developed ILs before Walden but called these products "intractable oils" and discarded them. Despite their early discovery, ILs have only become the focus of scientific interest since the 1990s, with increased attention in the last 20 years. The most practical definition of an ILs, according to MacFarlane et al. in their book "Fundamentals of Ionic Liquids", is "a liquid comprised entirely of ions". Unlike many other definitions, the authors do not refer to the melting point below 100°C, as this would be unnecessarily restrictive. For comparison: table salt (NaCl) has a melting point of 801°C. For the tests considered in this thesis, only ILs with a melting point below 100°C are used, which means they also meet the more tight definitions.[110, 111] It should be noted that several different ions can be present in the liquid, not only one, as in the case of melted NaCl. Moreover, the same example illustrated the difference to the term "molten salt", which contains only one type of anion and cation.[112]

The following properties are in principle typical for ILs, but they may vary due to the wide range of ILs. Compared to volatile organic solvents, the handling and safety of ILs are better due to almost negligible vapour pressures and non-flammability. The properties ILs vary widely, e.g. polarity, mixing behaviour, density and melting point. Kamlet-Taft solvent parameters have been established for describing the solubility of ILs, where three independent values are measured. α the hydrogen bond donor, β hydrogen bond acceptor and π^* dipolarity/polarizability describe the solubility behaviour of the solvent.[113] Some experts estimate the possible total number of ILs to be as high as 10^{18}. This variability, combined with experience and quantitative parameters such as those of Kamlet-Taft, provides opportunities for easy and precise selection of ILs. For example, a suitable IL can be selected as a substitute based on the Kamlet-Taft parameters of a volatile organic solvent.[110-112]

One of the most critical properties of ILs, is their viscosity, which is at least ten times higher than water or even more. To enable better handling in a lab or on an industrial scale, researchers are trying to minimise these parameters, which is considered one of the "holy grails" of ILs research. It should also

be noted that viscosity also influences ion transport, which is especially important for electrochemical applications. Viscosity can be reduced by adding solvents, but this has drawbacks and limitations. Such as solvents can influence the viscosity, dissolving substances can also change the viscosity during the process. Unfortunately, an increase in viscosity is observed during the biomass-dissolving process. Furthermore, it has been shown that the viscosity decreases sharply with increasing temperature. The electrically conductive liquids exhibit good electrochemical stability (towards oxidising/reducing agents) and have high heat capacity, thermal conductivity, and good thermal stability. Applications of ILs include solution processes, catalysis, synthesis, sensor technology, heating, cooling, analytics, battery technology and biotechnology. Because ILs can replace volatile organic solvents, they are often referred to as "green" chemicals. When ILs are used as solvents or catalysts, they usually have to be recycled due to their price. This critical process step depends on the type of ILs used and can be very complex.[110-112]

In general, the toxicity and the environmental effects, such as biodegradability, have been little studied. In the case of the toxicologically studied ILs, the comparison shows that the effects depend very much on the composition, with even minor changes, such as the length of the alkyl chain, having a noticeable influence. These toxic compounds could even be used in the pharmaceutical industry, e.g. as disinfectants.[111, 112] Scientists who have examined various publications warn of the potential danger to health and the environment posed by ILs. From today's perspective, however, the numerous advantages of using ILs outweigh their risks. Nonetheless, the processes should be constantly improved with a particular focus on removing ILs from the production stream.[114]

A.4.2. Application and market

In the "life cycle cost analysis" of an ILs process, they usually cause higher acquisition costs than established processes. However, that can be compensated by lower operating and disposal costs. That is achieved in particular through more efficient dissolution behaviour and recycling of ILs. The price of ILs is between USD 10 to 100 per kg, thus in the range of processes to fine chemicals. In addition to the economic perspective, the potential environmental benefits that can be achieved by recycling ILs or replacing fossil or other harmful resources should also be considered. Likely, taxes and stricter regulations expected in the context of climate change will bring financial benefits to those adopting greener production practices, where the sustainable use of ILs can play an important role.[115, 116]

In the last ten years, ILs have become established in many commercial processes and applications, e.g. stationary phase in capillary GC columns or scrubbing of mercury vapour from natural gas.[112, 116] Interesting for biotechnology are the good dissolving properties of biopolymers, which are otherwise difficult to dissolve in conventional solvents. It is possible to dissolve all or only certain parts of biomass. Examples are cellulose and lignin, which can be dissolved individually or together. Selected ILs can also stabilise proteins, DNA and RNA and are a suitable solvent for biocatalysis.[112, 117]

In 2020, the global ionic liquids market revenue was estimated at USD 43 million, despite the pandemic and was expected to reach USD 55.8 million by 2026, based on an expected CAGR of 5.4%. The biotechnology market was valued at USD 6 million in 2021 and is expected to reach USD 17.8 million by 2026, based on a CAGR of 7.2%. In upcoming years, the versatile and adaptable ILs will find further applications through extensive research and will be used in various commercial processes, often increasing sustainability compared to an alternative process.[118]

A.5. PHB Recovery

A.5.1. State of the art

In addition to the crucial measurements of molecular weight and polydispersity mentioned in the previous chapters, the crucial factors of yield and purity provide essential information about the recovery.[70] When selecting the method and process conditions, including pretreatment, special attention should be paid to economic efficiency and environmental compatibility and ensuring that the PHB does not decompose (even partially) or degrade in quality, which could otherwise jeopardise the overall economic success.[69, 71, 102, 119] There are two different standard methods for the recovery of PHB, (i) solvent extraction, where only PHB is dissolved out from the cell, or (ii) cellular lysis, where the cell is dissolved down to PHA, although both can be mixed in one process.[120] For a list of potential extraction and cellular lysis processes, see Pagliano et al. [70] or Koller et al. [121]

The general process steps for solvent extraction consist of: Mixing the BM with the solvent, heating the mixture, separating the extraction residues and the solvent (including the dissolved PHA), separating of PHA and the solvent (evaporation or precipitation) in combination with the recovery of the solvent (for a schematic procedure, see Pagliano et al.[70]). Solvent evaporation or water removal is the main contributor to the high energy costs. It should be noted that the solvent is added at a very high biomass-solvent ratio, up to 1:20,[121] which can lead to the following problems: e.g. that organic solvents cannot be 100% recovered or wholly removed from the PHA product. In addition, many organic solvents are highly volatile that can create flammable atmospheres or endanger health and the environment.[70] 30% of the total production costs are caused by the extraction process, of which solvents alone account for two-thirds (about 20%).[69, 122] Despite all these impracticalities, this is nevertheless the most commonly used production process.[69]

Halogenated solvents are often used as a benchmark for PHA extractions because of their high recovery, purity and molecular weight, but they are all derived from fossil fuels. The most commonly used solvent is chloroform, which is non-flammable but harmful to the environment and health (among other things, it is irritant and potentially carcinogenic).[76] Other solvents and mixtures of alcohols, alkanes, amides, carbonates, ethers, ketones, organosulphur compounds and linear and cyclic esters are also described in the literature. In addition, supercritical fluids, aprotic solvents (e.g. anisole DMSO or DMF) or ionic liquids can be used, although the latter will be discussed in the following chapter.[123] Pretreatment by chemical (e.g. oxidants, bases or salts), mechanical methods (e.g. heat treatment) or combinations thereof can make the PHA granules more accessible to the solvents and thus improve the yield, purity or amount of solvent required.[70]

For the cellular lysis method, the biomass is mixed with the agent and heated, PHAs are separated from the solution (possibly purified), and the solution is treated, with the dissolved biomass considered a waste stream. A wide range of substances is used for this purpose, e.g. oxidising agents such as the commonly used NaClO, surfactants, phages, enzymes alkaline and acid compounds.[122] In addition, mechanical methods such as bread mill, high-pressure homogenizer or ultrasound can be used for cellular lysis.[124] The biomass can never be dissolved entirely and therefore contaminates the PHA. Less energy is usually consumed than during a solvent extraction, but the material input is comparable, if not more expensive.[70] In addition, living organisms such as mealworms digest PHA-rich biomass

and extract the undigested PHAs.[76] Cellular lysis usually consumes less energy, but the material input is ideally comparable or even more expensive.

In general, it should be noted that the results of the studies vary significantly due to the different PHA producing microorganisms, the methods used to determine PHA content and the experimental setup. This should also be taken into account when selecting the method used.[121] Comparing the costs of, for example, a chloroform solvent extraction with a sodium hypochlorite cellular lysis shows that the latter is generally cheaper. Solvent extraction becomes economically competitive when large quantities are involved, or harsh cellular lysis processes cannot be used due to quality conditions. Higher purity, higher yields and lower PHA degradation can be achieved with solvent extraction. In addition, cellular lysis is generally less harmful to the environment, but optimisation could further reduce this gap.[70]

A.5.2. Recovery using ILs

Analogous to solvent extraction, i.e. extraction of the bioplastic and separation of the cell debris, the patent by Hecht et al. [49] describes a process for the extraction of PHA from biomass using ILs. In this patent, the following relevant examples are given, such as the extraction of PHA (copolymer hydroxybutyrate/hydroxyhexanoate) with Terrasail[3] or this PHA in biomass using 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]).[126] For the ILs diethanolammonium acetate, a higher solubility of 3% (wt/v) was found with a lower recovery of PHB compared to the standard solvent chloroform. In addition, 2-hydroxyethylammonium acetate (2.5g/L), diethanolammonium formate, 2-hydroxyethylammonium formate, ethylammonium acetate and ethylammonium formate dissolve PHB but do not degrade it.[127] This thesis focuses on the reverse approach, i.e. biomass dissolution, and is based on the studies discussed below.

Previous studies showed that ILs could completely dissolve *Synechocystis* sp. PCC 6803.[128] Building on this, the same research group attempted to dissolve 1 mg BM with 5wt% PHB in 1 g 1-ethyl-3-methylimidazolium methylphosphonate [EMIM][MP] without decomposing the PHB. Due to the sharp increase in viscosity and the associated handling problems, the mass ratio of biomass to IL was not increased. The undissolved PHB was separated by membrane filtration (3 µm,) and the methanol-washed solid was analysed for PHB. The IL was successfully removed from the biomass with some antisolvent (preferably water), and the IL could be reused after drying. More than 98% PHB was recovered from the microorganism, with about 70% impurities measured.[129]

Two other papers deal with the recovery of PHA from microorganisms. First, Filippi et al. mixed biomass (*Rhodovulum sulfidophilum* DSM-1374, 14.3wt% PHB) at a ratio of 1:10 and 1:30 (w/w) at 60°C for 24h with ILs (1-ethyl-3-methylimidazolium dimethylphosphate…[EMIM][DMP], [EMIM][DEP] and [EMIM][MP][4]). Only [EMIM][DMP] could be separated by centrifugation without adding methanol as an antisolvent. The filtration described by Kobayashi et al. was difficult due to the high viscosity. The high amount of impurities of 70% of residue could be decreased to 50% by increasing

[3] According to "chempedia.info" Terrasail is an IL from Sachem (United States, Austin, Texas) based on the anion docusate (dioctylsulfosuccinate).[125]

[4] For reasons of clarity and in accordance with the preferred IUPAC designation, the anion declared as methylphosphite in the publication by Filippi et al., will be referred hereinafter as methylphosphonate [MP].[130, 131]

the biomass:IL ratio from 1:10 to 1:30 (w/w).[132] The second publication by Dubey et al. was relatively similar. A biomass of *Halomonas hydrothermalis* (74wt% PHB) and one of the just mentioned ILs [EMIM][DEP] was used at a mixing ratio of 1:10 (w/w) at 60°C for 24 h. As in the previously mentioned work, methanol was added for better separation of the PHB, resulting in a yield of 60% and a purity of 86%. After adding methanol and treating the IL with charcoal, the biomass impurities were separated, and then IL was distilled to remove excess menthol and any water it contained.[133]

Table 2: PHB recovery dissolving BM using ILs in the literature

IL	Microorganism	PHB content (%)	Ratio BM:IL	T (°C)	t (h)	Purity (%)	Recovery (%)	Ref.
[EMIM][MP]	*Synechocystis* sp. PCC 6803	5	1:1,000	RT	0.5	30	98	[129]
[EMIM][DEP]	*Halomonas hydrothermalis* MTCC 5445	74	1:10	60	24	86	60	[133]
[EMIM][MP] [EMIM][DMP] [EMIM][DEP]	*Rhodovulum sulfidophilum* DSM-1374	14.2	1:10/ 1:30	60	24	~30/ ~50	-	[132]

-No data available

B. Objective

There are two major problem areas when dealing with plastic: the raw material and the disposal. First, almost all plastics are based on petroleum, a non-renewable resource with a finite capacity.[134] Secondly, non-biodegradable plastics are incinerated, deposited in landfills or disposed of improperly. In the latter two cases, the polymers remain in ecosystems for decades, including in the form of microplastics, and can have adverse effects on health and the environment.[135] As a bio-based and biodegradable polymer, PHB offers solutions to both problems. Global plastic production is estimated to double in the next 20 years and reach 2.5 times that amount by 2050.[136] Plastic production consumes up to 12% of the world's fossil fuel demand as feedstock.[45]

In addition, phototrophic microorganisms metabolise CO_2, which reduces this potent greenhouse gas.[82] One of these organisms is the cyanobacterium *Synechocystis* sp., which produces PHB under nitrogen starvation conditions. The slow growth under phototrophic conditions and low productivity, a problem with phototrophic microorganisms, can be accelerated by adding a carbon source. This so-called mixotrophic cultivation leads to faster biomass growth and higher product output but is associated with challenges. On the one hand, carbon sources should be sustainable and not directly compete with human nutrition. On the other hand, the process's economic viability should be considered, as the carbon source accounts for about 80% of the fermentation medium and up to 30 to 40% of the total cost.[18, 64] Lactose from whey offers itself as a renewable, sustainable and cost-effective carbon source as whey is considered a waste product of the dairy industry.[35, 44] Unfortunately, *Synechocystis* sp. cannot metabolise lactose.[58-61]

The primary HPLC method to quantify PHB is based on using concentrated sulfuric acid (96wt%, 18 M) to hydrolyse the polyester into the UV-active crotonic acid.[70] This corrosive chemical is very viscous and hence difficult to pipet accurately. A full factorial multivariate data evaluation (DOE) assesses the influence of the concentration and time on the decomposition of PHB using the standard acid H_2SO_4 and a protocol with the base sodium hydroxide.[108, 109] The more promising method is further optimised by a composite design to verify the standard operating procedure.

Solvent extraction with a halogenated solvent derived from fossil sources, which is harmful to health and the environment, is mainly used to obtain PHB. ILs offer safe handling due to their low vapour pressure and well-adjustable properties due to their extensive combination possibilities.[112] In contrast to the solvent extraction procedure, based on recent publications, ILs should dissolve the biomass, which is otherwise very difficult to dissolve with conventional solvents, and the undissolved PHB can then be separated. Decomposition of the bioplastic or other quality loss should be avoided.[129, 132, 133] Through proper recycling and optimized processes, the comparatively expensive ILs should become economical and reduce the process costs of recovery, which account for 30% of the total costs.[70]

This results in the following tasks, which are examined as an objective in this thesis:

- Observation of differences in cell growth and PHB content as a function of lactose concentration and comparing that to phototrophic cultivation
  - Screening experiments in shake flasks (different lactose concentrations)
  - Upscaling into a stirred tank photobioreactor
- Investigation and optimisation of alkaline and acidic hydrolysis for quantification of PHB
- Investigation of the solubility of biomass and PHB in IL

C. Materials & Methods

C.1. Chemicals and microorganisms

The chemicals used in this thesis are purchased from Carl Roth (Karlsruhe, Germany), except otherwise stated, like the PHB and standards for the ion chromatography (IC) from Sigma Aldrich (Darmstadt, Germany). Unless otherwise specified, ultrapure water was used to prepare solutions, provided by an Arium Mini system from Sartorius (Goettingen, Germany) or Synergy system by Merck Millipore (Billerica, Massachusetts, USA). The wild-type strain of *Synechocystis* sp. PCC 6714 (WT or wild type) was taken from the Pasteur Culture Collection of Cyanobacteria of the Pasteur Institute (Paris, France). Based on this microorganism, the mutant strain MT_a24 generated by Kamravamanesh et al.[1] was also used as a comparison (Mt or mutant) for shake flask experiments.

C.1.1. BG-11 medium

BG-11 medium at pH 8.5 was used in this work for fermentation experiments and precultures in this thesis. The required stock solutions are listed in Table 3. According to Table 4, the components were added except for the last two. Using 1 M NaOH, the pH value was adjusted to 8.5 using a WTW SenTix 41 or 81 pH electrode from Thermo Fisher Scientific (Waltham, MA, USA). After autoclaving at 120°C for 20 min, Fe-Ammonium-Citrate and Na_2CO_3 were added via a sterile filtration (0.22µm) and stored at 4°C if not used immediately. For agar plates, 0.75wt% agar was added before autoclaving.

Table 3: BG-11 medium stock solutions

Stock Solution	Volume (L)	Chemical	Mass (g)
100x BG-11	0.25	$NaNO_3$	37.4
		$MgSO_4 \times 7H_2O$	1.875
		$CaCl_2 \times 2H_2O$	0.9
		Citric acid	0.15
HEPES (pH 8)	0.25	HEPES	59.6
K_2HPO_4	0.050	K_2HPO_4	2.0
Trace-Metal-Mix	0.5	H_3BO_3	1.43
		$MnCl_2 \times 4H_2O$	0.9
		$ZnSO_4 \times 7H_2O$	0.11
		$Na_2MoO_4 \times 2H_2O$	0.195
		$CuSO_4 \times 5H_2O$	0.0395
		$Co(NO_3)_2 \times 6H_2O$	0.0247
EDTA	0.05	Na_2EDTA x $2H_2O$	43.6
Na_2CO_3	0.05	Na_2CO_3 (anhydrous)	5.3
Fe-Ammonium-Citrate	0.05	Fe-Ammonium-Citrate	0.3

Table 4: Recipe for 1 L of BG-11 medium

Stock Solution	Volume (mL)
100x BG-11	10
HEPES (pH 8)	10
K_2HPO_4	1
Trace-Metal-Mix	1
EDTA	1
Na_2CO_3*)	1
Fe-Ammonium-Citrate*	1

*) *Sterile filtrated compounds added after autoclaving*

C.1.2. Whey

NÖM AG (Baden, Austria) supplied a concentrated acidic whey (see Table 5), which was further purified via tangential flow filtration (0.2µm). Screening experiments revealed a precipitate from a whey component at alkaline pH values (see D.1.1). Therefore, the pH of the milk product was adjusted to pH 9, and the liquid phase was used for further experiments, as indicated by *whey pH 9*.

Table 5: Composition of concentrated acidic whey

Compound	Concentration (g/L)
Lactose	155
Glucose	1.4
Galactose	17.6
Lactate	3.1

C.2. Screening experiments in shake flask

The shake flasks experiments were performed in 100 mL Erlenmeyer flasks with a total liquid volume of 50 mL. The screening experiments were carried out in a Minitron Shaker from Infors AG (Bottmingen, Switzerland), and sampling and preparation were done in a laminar hood. For all screening experiments, the parameters in the shaker were set to a 3 V% CO_2 atmosphere, 28°C, a shaking speed of 150 rpm and a 14/10 h light/dark cycle. These experiments were performed in triplicates (see Figure 8).

C.2.1. Preculture

The precultures of *Synechocystis* sp. PCC 6714 and its mutant strain MT_a24 by Kamravamanesh et al. [1] in BG-11 pH 8.5 were stored at 28°C, 14/10 light-dark period and atmospheric air, corresponding to a CO_2 content of about 0.04 V%[137]. These phototrophic precultures were used for further screening experiments and batch fermentation.

Figure 8: Shake flask experiment with lactose (FLTR 10, 5, 2 and 1 g/L lactose)

C.2.2. Phototrophic screening

A specific volume of the precultures was inoculated in a BG-11 pH 8.5 medium to obtain an initial OD_{750} of 0.1.

C.2.3. Mixotrophic screening

For the mixotrophic screening, whey was added to achieve specific lactose concentrations in BG-11 pH 8.5 medium. For the initial mixotrophic screening, 1, 2, 5 and 10 g/L lactose were selected, with 160 g/L lactose in the whey, which was sterile filtered (0.22 µm pore size) and added into the autoclaved medium. Wild-type and mutant strains were added, reaching a starting OD_{750} of 0.1. Finally, this experiment was repeated for WT with the whey pH 9, with the 2 g/L lactose bottles removed.

C.2.4. Sampling and Harvest

A sample of 1 mL was transferred to a 1.5 mL Eppendorf tube, and the OD_{750} was measured. After centrifugation of the broth for 10 min, 14,000 g and 4°C, the pellet was dried at 75°C for PHB analysis (see C.4.3). As described in C.4, the supernatant was analysed with IC and HPLC, and the last procedure is performed only for mixotrophic regimes to analyse the sugar contents. The sampling interval was between four to seven days, where the exact intervals can be seen in D.1.

Finally, the harvested fermentation broth was centrifuged at 6,000 g for 10 min at 4°C in 15 mL Falcon, and the supernatant was discarded. The pellet stored at -20°C or frozen with liquid nitrogen was lyophilised at -40°C and at 0.02 mbar using a Freezone 2.5 L Benchtop Freeze Dry System from Labconco (Kansas City, Missouri, USA). This dried biomass was then stored at -20°C.

C.3. Photobioreactor cultivations

For batch fermentation, 1.5 L and 5 L R'ALF PLUS laboratory fermenters from Bioengineering (Wald, Switzerland, see Figure 9) were used with the Supervisory Control and Data Acquisition (SCADA)-software InTouch developed by AVEVA (Cambridge, United Kingdom). Temperature, pH, four

peristaltic pumps, agitator speed and dO_2 were monitored, and everything except the final value were controlled via the SCADA software, although the pH was manually adjusted with 1 M Na_2CO_3 when necessary. This EasyFerm Plus pH probe (Hamilton, Bonaduz, Switzerland) was calibrated with a two point-calibration (standards pH 7 and 4). This probe, the dO_2 sensor, the temperature sensor, a septum, a flask containing 1 M Na_2CO_3 (with a 0.22 µm gas filter), a gas inlet and outlet, including a condenser with a gas filter in front of each port (0.22 µm pore size) were connected to the reactor and filled with autoclavable BG-11 pH 8.5 chemicals (1.5 or 5 L) and autoclaved for 20 min at 120°C.[34]

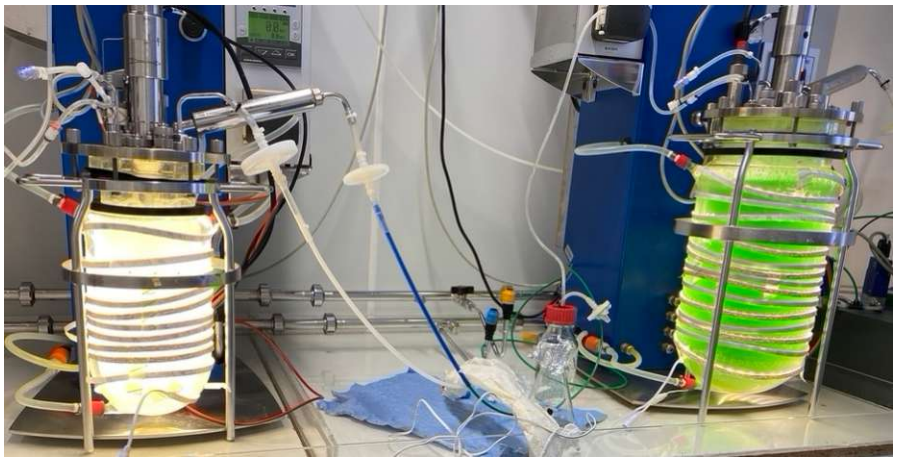


Figure 9: Photobioreactors R'ALF Plus (left 1.5 L and right 5 L)

After autoclaving, the water jacket and cooling water for the condenser were attached, and the sensors were connected. 1.5 mL or 5 mL (1.5 or 5 L reactor) of 1 M Na_2CO_3 and the same volume of Fe-ammonium citrate were added via the septum and a sterile filter (0.22 µm pore size). At a stable medium temperature of 28°C, the dO_2 sensor (Hamilton, Bonaduz, Switzerland) was calibrated by defining 50% of dO_2 as 300 mL/min air at 1500 rpm and 0% as no signal (disconnected cable). In the "Model 0254" from Brooks Instruments (Hatfield, Pennsylvania, USA), the inlet gas flow was set to 300 mL/min air and 10 mL/min CO_2. This device controlled the two 4850 mass flow controllers, one for each gas flow. These flow rates, including the approximately 0.04 V% CO_2 in air, for a gas flow of 10.12 mL/min CO_2, or a total of 3.3 V% CO_2. Based on the results of the screening, a lactose concentration of 1 g/L was chosen for the PBR cultivations. Thus, the sterile filtrated whey pH 9 was added via the septum for mixotrophic cultivations (9.4 mL whey pH 9 for 1.5 L medium or 31.3 mL for 5 L). One or two (1.5 or 5 L rector) LED strips were wrapped around the glass reactors and operated 24 h/per day without dark phase. The agitator speed was set to 300 rpm (pitched-blade impellers). The CO_2 concentration in the exhaust gas was determined by a DASGIP GA 4[5] from Eppendorf (Hamburg, Germany).

Only *Synechocystis* sp. wild type with an initial OD_{750} of 0.1 was used for batch fermentation, with preculture handling described in C.2.1. Every 4 to 6 days, 5 mL samples were taken via the sample port after disposing of the first 5 mL. The OD_{750} of this sample was measured, and 1 mL was transferred three times each into a 1.5 mL Eppendorf tube. The centrifuged (14,000 g) pellets were dried at 75°C for PHB

[5] Note that the device indicates that maintenance is required and that different channels measure different concentrations.

analysis, and the supernatant was analysed once with the IC and HPLC (for mixotrophic fermentation only) as described in C.4.

After completing the experiment, the fermentation broth was centrifuged in 50 mL Falcons at 6,000 g for 10 min at 4°C for the 1.5 L reactor and 10,000 g for 25 min at 4°C for the 5 L reactor. Finally, the supernatant was discarded, and the pellet lyophilised as described in C.2.4 and stored at -20°C until further usage.

C.4. Fermentation analytics

C.4.1. Sugar and lactate analysis

The Vanquish Core high-performance liquid chromatography (HPLC) system from Thermo Fisher Scientific (Waltham, Massachusetts, USA) was used to quantify the lactose, galactose, glucose and lactate content. For this purpose, an Aminex HPX-87H carbohydrate column was installed with a guard column from Bio-Rad (Hercules, California, USA) at a pressure of about 65 bar, a column temperature of 60°C and a flow rate of 0.6 mL/min mobile phase. Isocratic elution was performed with 4 mM H_2SO_4 (HPLC grade) for 30 min. A refractive index detector from Ecathech AG (Bern, Switzerland) was used to detect the three sugars, and lactate was measured with an ultraviolet detector at λ=210 nm from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Chromeleon Studio software (7.2.10) from Thermo Fisher Scientific (Waltham, Massachusetts, USA) recorded and analysed the chromatograms. For quantification, standard solutions were prepared and used for all four components.

C.4.2. Nitrate and Phosphate analysis

Nitrate and phosphate were analysed with a Dionex ICS-6000 from Thermo Fisher Scientific (Waltham, Massachusetts, USA) using the Dionex IonPac AS11 from the same company with a guard column (30°C) and conductivity detector combined with an AERS suppressor. Gradient elution with 0.1 M NaOH (IC grade) and ultrapure water at a total flow rate of 2 mL/min was performed as follows: 0-1 min 0.2% NaOH (rest water), 1-6 min increase to 0.2-5% NaOH, 6-13 min increase to 24% NaOH, 13-13.5 increase to 38% NaOH, 13.5-14.5 min hold at 38% NaOH, 14.5-15 min decrease to 0.2% NaOH and 15-20 min equilibrate (end) at 0.2% NaOH. Chromeleon Studio (7.2.8) from Thermo Fisher Scientific (Waltham, Massachusetts, USA) was used to record and analyse the chromatograms. The measured standards were purchased IC-grade chemicals with a defined concentration from Sigma Aldrich (Darmstadt, Germany).

C.4.3. PHB analytics

As described optimised in C.5, 1 mL of 14 M H_2SO_4 was added to the dried biomass and heated for 160 min. After diluting the supernatant 1:20 with 14 mM H_2SO_4 and centrifugation at 14,000 g and 4°C for 10 min, the solution was analysed using the same HPLC-method as in C.4.1 with the UV detector at λ=210 nm. A defined amount of PHB was prepared accordingly and then diluted as a standard.

C.4.4. Dry cell weight

The optical density at 750 nm (OD_{750}) wavelength of the biomass samples was measured using a NanoDrop One spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA) or a spectrophotometer, visible, ONDA V-10 PLUS from Giorgio Bormac S.r.l. (Capri, Italia). For further calculations with only one OD750, the values of the first spectrometer were converted to values of the second spectrometer using a calibration curve (as shown in appendix G.1). A specific volume of the phototrophic fermentation broth and its dilutions were centrifuged (5,000 g, 4°C), the pellet dried at 100°C and weighed. With a triple repetition of this experiment, a calibration curve for the relation between OD_{750} and biomass was calculated (as shown in Figure 10).

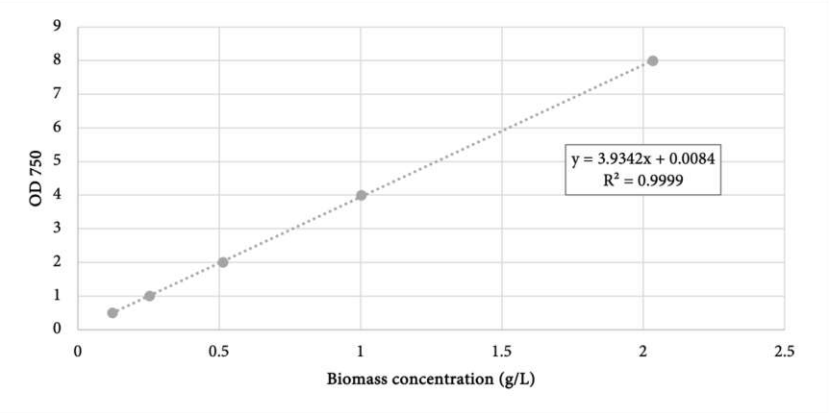


Figure 10: Calibration of dry cell weight

C.5. Optimisation of the PHB quantification method

First, the NaOH and H_2SO_4 quantification methods for PHB were to be examined and compared with multivariate data analysis. The second step was further investigating and refining the most promising experimental setup.

C.5.1. Comparison of NaOH and H_2SO_4 PHB - quantification methods

Experimental design and multivariate data analysis were performed using Sartorius's (Göttingen, Deutschland) MODDE Pro 13 software. The chosen method for the screening experiment was a full factorial experiment design with three centre points, and for higher accuracy, each of those proposed experiments was conducted in triplicate. As controllable factors in this screening experiment were the concentration of the base or acid and the heating time. The boundaries were derived from state of the art. In the case of sulfuric acid, the time interval was chosen between 30 and 240 min and the concentration between 10 and 16 M. In contrast, the standard condition would be 18 M (concentrated) sulfuric acid and a heating time of 60 min. The standard conditions of the alkaline method (30 min heating with a 2 M NaOH) were chosen as the lower limit and 150 min and 6 M as the upper limit. Recovery of crotonic was the chosen response, considering the different dilution steps. The factors were implemented in the MODDE software, which outputted the experiential design according for a full factorial design. In addition, the edge centres were measured, as shown in Figure 11. Thus, about 20 mg (actual mass noted) was poured into an Eppendorf tube, the predefined solution was added and heated

at 100°C with a heating block while shaking at 600 rpm for the indicated duration (each done in triplicates).

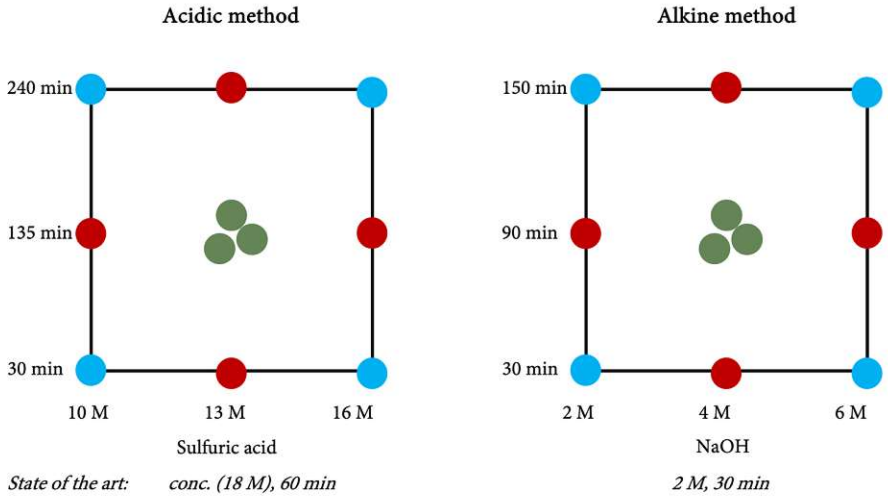


Figure 11: Acidic and alkaline PHB quantification DOE experimental plan

After an ice bath stopped the reaction, the samples were treated according to state of the art. For the acidic method, this means a 1:20 dilution with 14 mM sulfuric acid, while the basic treatment requires a 1:2 dilution with hydrochloric acid of the same concentration. The use of pure PHB eliminated a centrifugation step that would be necessary to make the solution particle-free for the HPLC measurement, e.g. biomass samples. Using the HPLC method described in C.4.1, the supernatants were measured, and the crotonic acid was detected with the UV detector at λ=210 nm. A crotonic acid standard was used for quantification. Based on this results the acidic method was chosen to be optimised in a second DOE.

C.5.2. Optimisation of H_2SO_4 PHB quantification method

The contour plots created with MODDE showed the highest PHB values at higher concentrations and short durations compared to the set limits. Based on these results and to reduce the viscosity and thus the concentration, the limit values for the sulfuric acid and heating duration were set between 12 and 14 M acid and 60 to 160 min. Furthermore, the influence of different PHB concentrations was simulated by choosing a PHB range of 5 to 25 mg, defined as c(PHB) in %, which corresponds to mg. To determine possible quadratic interactions, a composite experimental design was used in which 6 points (red) were added compared to a full factorial analysis, as shown in Figure 12. All the above experiments were performed in triplicate.

Six trials were conducted at the optimised spot conditions of this experiment (14 M, 160 min and 3.2 mg), a triple with 5 mg PHB and with 15 mg. Dilutions of the latter were prepared for a standard. In addition, three additional experiments were carried out under the standard conditions with concentrated H_2SO_4 (60 min, 15 mg).

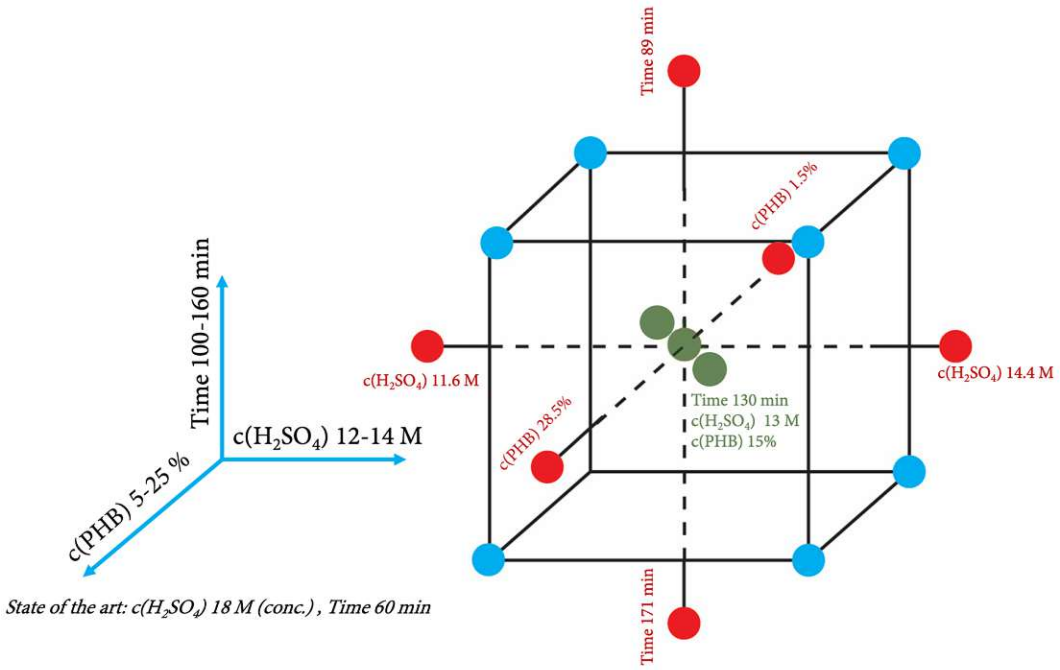


Figure 12: DOE experimental plan (composite design) of the acidic PHB quantification (figure modified from [138])

C.5.3. Comparison of standard and the optimised sulfuric acid method

The optimum found by DOE should be compared with the concentrated sulfuric acid method to investigate possible varieties. Statistically, it was to be checked whether the mean values of the data sets differed statistically significantly. First, it had to be analysed whether the data set was normally distributed, which - like the following investigations - was carried out with Python (3.8) by using the "shapiro" function from the "scipy.stats" package. In the second step, a statistically significant difference between the two variances was tested by checking whether the quotient of the variances was at most 4. The significance level was set to 5% for all tests. For a pair of data that retained the test for normal distribution and a quotient below 4, a two-sided t-test could be performed using the function "stats.ttest_ind" (scipy.stats package). If the variance were significantly different, the t-test would not be applicable, so a Welch's t-test would be performed with the same function "stats.ttest_ind", changing the value "equal_var" (by default on true) to false. In addition, three different *Synechocystis* sp. PCC 6714 with different PHB contents were analysed in triplicate with the 14 and 18 M methods.

C.5.4. The necessity to shake

In prior experiments, the samples were constantly shaken at 600 rpm during heating. Therefore, the influence on the measured value was investigated. For this purpose, three different biomass grown under nitrogen limitation were measured three times with and without shaking according to the 14 M H_2SO_4 method described above. In addition, about 15 mg PHB was analysed three times each with and without shaking by the acidic method.

C.6. PHB solvent extraction with chloroform

The standard recovery method described in the literature uses chloroform to extract the PHB from the biomass.[119] Therefore, three samples of around 1.5 g each of dried biomass from phototrophic and mixotrophic cultivations were transferred to a round-bottom flask and mixed in a 15:1 (V/m) chloroform to biomass and boiled under reflux (bp. $CHCl_3$=61.2°C [139]) for 120 min using a magnetic stirrer according to Mongili et al.[119]. Centrifugation was attempted at 14,000 g for up to 45 min. However, the biomass always floated on top of the chloroform, and both phases were easily mixed. Due to this inadequate and impractical separation, filtration was applied, first separating large particles via a cellulose mash and in the second step, a Whatman glass microfiber filters GF/F (pore size 0.7 µm) from Cytiva (Marlborough, Massachusetts, USA) with a fritted glass and an Erlenmeyer flask connected to a vacuum pump. Before precipitating the filtrate with hexane in a 1:3 ratio ($V_{CHCl_3}:V_{C_6H_{14}}$), the solution was condensed to a volume less than 3 mL using a rotary evaporator to reduce the consumption of required antisolvent. The centrifuged (6,000 g, 15 min) precipitate was separated from the supernatant, dissolved and transferred into a small glass flask (10 mL), where the solution was finally evaporated using a rotary evaporator. Unfortunately, the precipitate was contaminated with something that coloured it yellow/red. Therefore, the steps of dissolution and precipitation were carried out twice to purify the solid material. The mass of the product was measured via a differential weighing of the glass vial with and without the product. The samples were analysed via the 14 M H_2SO_4 method.

C.7. Ionic Liquids

After a short problem statement regarding the solvation of biomass without the PHB dissolving or even decomposing, three different ILs were provided by propionic (Raaba-Grambach, Austria) and stored in closed containers at room temperature. All three ILs share the same cation: 1-Ethyl-3-methylimidazolium [EMIM], an imidazole derivative in which a methyl and an ethyl group have been substituted on two nitrogen atoms. In this publication, the anions used were acetate [OAc], diethylphosphate [DEP] and chloride Cl, the first two of which are liquid at room temperature and the last one is solid with an operating temperature at about 90°C (according to the supplier). They were highly viscose and transparent in liquid form with a slightly yellowish colour. Figure 13 shows the structural formula.

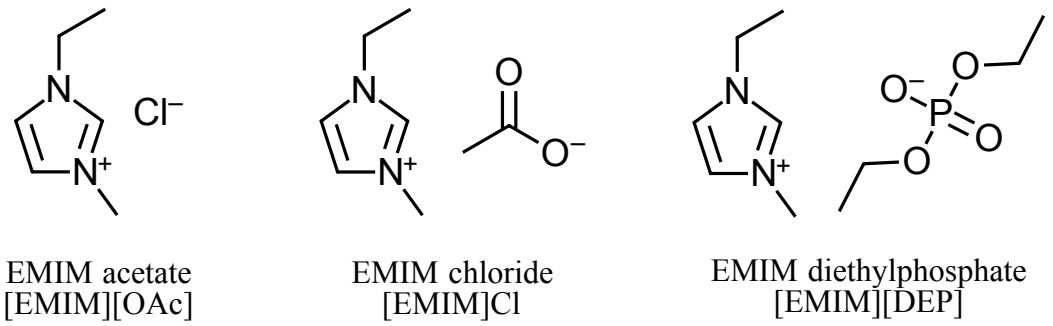


Figure 13: Structural formula of the ionic liquids used in this thesis (EMIM=[EMIM]…1-Ethyl-3-methylimidazolium)

Three preliminary experiments were conducted to investigate the possible use of ILs for PHB extraction, where:

- ILs were mixed with biomass (0wt% PHB),
- ILs were mixed with biomass (0wt% PHB) and adding PHB,
- ILs were mixed with PHB only,

as described below in detail. Furthermore, water was added to ILs mixed with biomass (0wt%) to investigate the viscosity.

C.7.1. IL solubility of biomass

This experiment was conducted at room temperature (RT), 50°C and 75°C, for the two ILs liquid at room temperature and only at 90°C the [EMIM]Cl. This experiment aimed to investigate the solubility of biomass without PHB in ILs and determine solubility kinetics. Biomass was provided by the project partner (Center ALGATECH – Institute of Microbiology of the CAS, Trebon, Czech Republic), which contained no PHB (14 M H_2SO_4 method). According to the IL manufacturer's information, a 10wt% biomass per IL ratio was chosen. 10 g of IL with a magnetic stir bar was added to a 50 mL round-bottom flask using a magnetic stirrer and heated to the target temperature with an oil bath. The 1 g of biomass was added stepwise: 0 min 100 mg, 10 min 200 mg, 20 min 200 mg, 30 min 200 mg and 40 min 300 mg. The liquid was photographed before and after every step. A very viscose sample was taken with a piston-operated pipette adjusted to a volume of 500 µL after specific time intervals into tared Eppendorf tubes and then weighed, as shown in Table 6. After centrifugation at 14,000 g for 10 min, an attempt was made to separate the dark green and highly viscose supernatant in a second tared tube. The pellet was washed twice with water, dried at 100°C and weighed.

Table 6: Biomass sampling times of biomass in IL for kinetics

Sample	Time (h)		
	[EMIM][DEP]	[EMIM][OAc]	[EMIM]Cl
1	0.5	0.5	0.33
2	0.75	0.75	0.5
3	1*)	2*)	0.75
4	2	5	1
5	3	20	2*)
6	5	24	3
7	17	-	-
8	24	-	-

*) *full dissolution of biomass at 75°C*

After the experiment, the IL-biomass mixture was transferred into a 50 mL Falcon and centrifuged for 30 min at 6,000 g. Subsequently, about 0.5 g of this supernatant (done in triplicates) was transferred into a tared 15 mL falcon, and the step of washing with water and centrifugation (6,000 g, for 10 min) was repeated three times before the pellet was dried at 100°C.

C.7.2. IL solubility of PHB

In this experiment, the PHB was mixed with the IL and heated for a defined period to study solubility. As mentioned earlier, 10wt% biomass was added to the IL, assuming a PHB concentration of 20wt% in biomass and 5 g of IL, yielding 100 mg PHB prepared in 50 mL round bottom flasks. These were left for [EMIM][DEP] and [EMIM][OAc] at room temperature, and 50°C for 24 h or at 75°C for 1 h [DEP]/2 h [OAc] and [EMIM]Cl at 90°C for 2 h, based on the experiments discussed in C.7.1. The transferred mixtures were centrifuged at 6,000 g in 15 mL Falcons for 10 min, the pellet was washed twice with water before drying, and the supernatant was mixed with 10 mL of water. The water/IL mixture was measured using the HPLC method described in C.4.3. However, [EMIM]Cl solidified during centrifugation with PHB, hence could not be separated and analysed as described.

C.7.3. PHB mixed with biomass solved in IL

The purpose of this test was to investigate how the IL-biomass fluid behaves when PHB is added. As in the previous chapter C.7.2 the same duration for different temperatures was used for the 0.5 g of IL-biomass mixture prepared in Eppendorf tubes and doped with 20 mg of PHB. The samples were centrifuged at 14,000 g for 30 min and then visually examined for possible PHB pellets.

C.7.4. IL-biomass mixed with water

The IL-biomass mixture prepared in C.7.1. was mixed with water in various ratios between 0-100wt% to improve the operability, as shown in Table 7. However, the results were only evaluated visually as no analytical equipment was available.

Table 7: Transferred mass of IL-biomass mixture

Water (wt%)	Mass (g)		
	[EMIM][OAc][a)]	[EMIM]Cl [b)]	[EMIM][DEP] [c)]
0	0.5027	-	0.5175
1	0.5067	0.5294	0.5214
2	0.5007	-	0.5814
5	0.5322	0.5548	0.5239
10	0.5260	0.5378	0.5132
20	0.5035	-	0.5175
50	0.5072	0.4963	0.4955
100	0.5276	-	0.5341

[a)] All IL-biomass mixtures were used from the 75°C experiment (except the 100wt%, which was taken from 50°C)
[b)] Just enough IL-biomass for four experiments
[c)] 0-5wt% IL-biomass mixtures from 75°C experiment and 10-100wt% IL-biomass from 50°C experiment were used

D. Results

D.1. Screening experiments

Although Synechocystis sp. PCC 6714 does not metabolise lactose, the experiments in which whey is added are referred to as mixotrophic in the following to distinguish them. If no whey is added, the experiment is referred to as phototrophic.

In the following, the results of two mixotrophic screenings with added whey are presented, whereby only the WT was selected for the repetition (second screening). In addition, the WT and the Mt from the two phototrophic screenings are shown.

D.1.1. Whey pretreatment

Firstly, the concentrated whey and its pretreatments are discussed. Microfiltration separates proteins, which leads to further decolourisation of the whey. These proteins, if not separated, could subsequently change the nitrogen content, which, as mentioned earlier, is an essential parameter in PHB production and should therefore be avoided.[140] However, the same step would occur at the latest during sterile filtration with the same pore size.

According to the literature, the precipitate detected in the first mixotrophic lactose screening could be caused due to precipitation of calcium phosphate. This precipitation occurs above a pH of 6, which sometimes causes problems in milk processing.[18, 141] GC-MS analyses showed that only a tiny part of the precipitate consists of lipids. The precipitate brought into the solution by reacidification also has no significant concentration of sugars or proteins. In future pH adjustments, attention should be paid to the choice of base (e.g. NH_4OH, KOH NaOH), as this can influence the PHB content.[100]

Further experiments showed that despite adjusting the whey to pH 9, a precipitate formed with a smaller volume than the first, which a combination of whey, EDTA and ammonium ferric citrate could have produced. In parallel, the lactose concentration decreased while the very low protein content remained stable. However, these results only provide an incomplete picture that should be investigated in more detail.

D.1.2. Biomass concentration

In Figure 14 the biomass concentrations are displayed (see Figure 35 in appendix G.3 for pH). The first mixotrophic screening shows that the higher lactose concentrations have a slightly higher biomass concentration than the lower concentrations. An approximately 0.5 g/L higher cell density was measured for the recombinant microorganisms compared to its non-mutant strain. The second mixotrophic screening with whey pH 9 shows a different picture, with the lowest lactose concentration of 1 g/L producing the highest cell density of 1.4 g/L. In contrast, 5 and 10 g/L lactose quickly reach a stationary phase at 0.4 g/L cell density. The biomass concentration of 1.4 g/L in the second mixotrophy screen is slightly lower than the values measured in the first experiment at the same lactose

concentration (1.8 g/L WT and 2 g/L Mt). Based on the $OD_{750,}$ the cell density was determined, which can be increased by precipitate as it alters the absorbance values, as described at the beginning of this chapter (D.1.1). This then leads to a ostensible higher cell density and could explain at least part of the deviating densities, especially at the higher concentrations. The significantly lower amount of precipitate, which also forms at whey pH 9, could still influence the measurements.

After a latency phase, a linear increase in cell density can be seen in all four diagrams. This effect, known in the literature, is attributed to the limited light situation, which also occurs at high dilutions.[34, 142] The stationary phase was reached in the second mixotrophic and phototrophic screening. In the phototrophic screening, it is interesting to note that the wild type of *Synechocystis* sp. reaches the stationary phase much faster than the Mt at a density of about 1.8 g/L, slightly lower than the 2.2 g/L reached in about the same period according to the literature.[34] The slower growth of the mutant cannot be explained based on the available data, contradicting the previous publication.[1] Comparing the phototrophic WT with the mixotrophs shows slower growth in the latter, which could indicate the stress caused by the lactose, as also shown for different microalgae and cyanobacteria.[19, 49, 53] However, the phototrophic growth rates, as shown in Table 8 (p. 37), exceed the μ_{max} of 0.26 day^{-1} (WT) from the previous publication.[34]

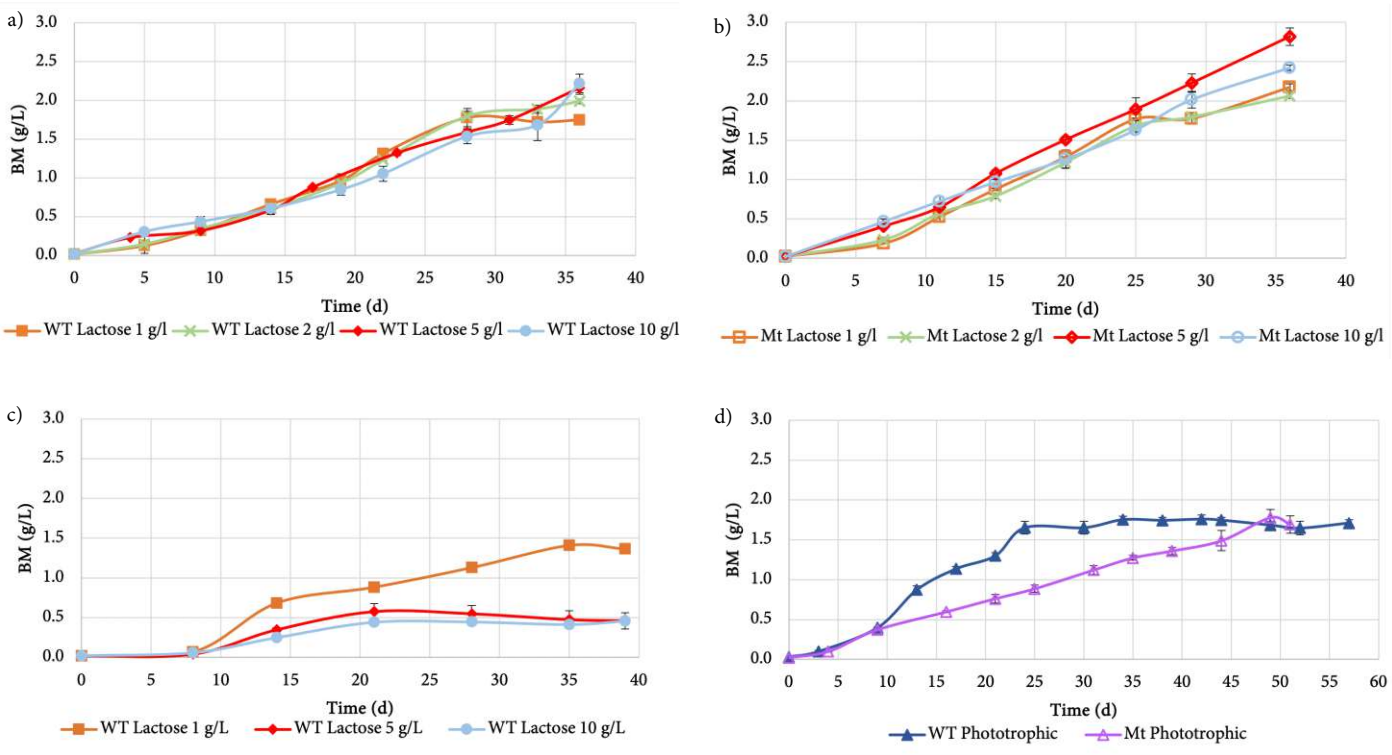


Figure 14: Biomass concentration: a) and b) first mixotrophic screening WT and Mt, c) second mixotrophic screening, d) phototrophic shake flask experiment

D.1.3. Nitrate and Phosphate concentration

Nitrate and phosphate, as mentioned, are closely linked to PHB accumulation in *Synechocystis* sp. (see Figure 15). PHB synthesis can be divided into three phases. In the first phase, the cell grows without limitation, i.e. with low PHB and glycogen production. In the second phase, limitation occurs, and the

microorganisms produce glycogen and some PHB, resulting in a decrease in glycogen and an increase in PHB content in the third phase.[6]

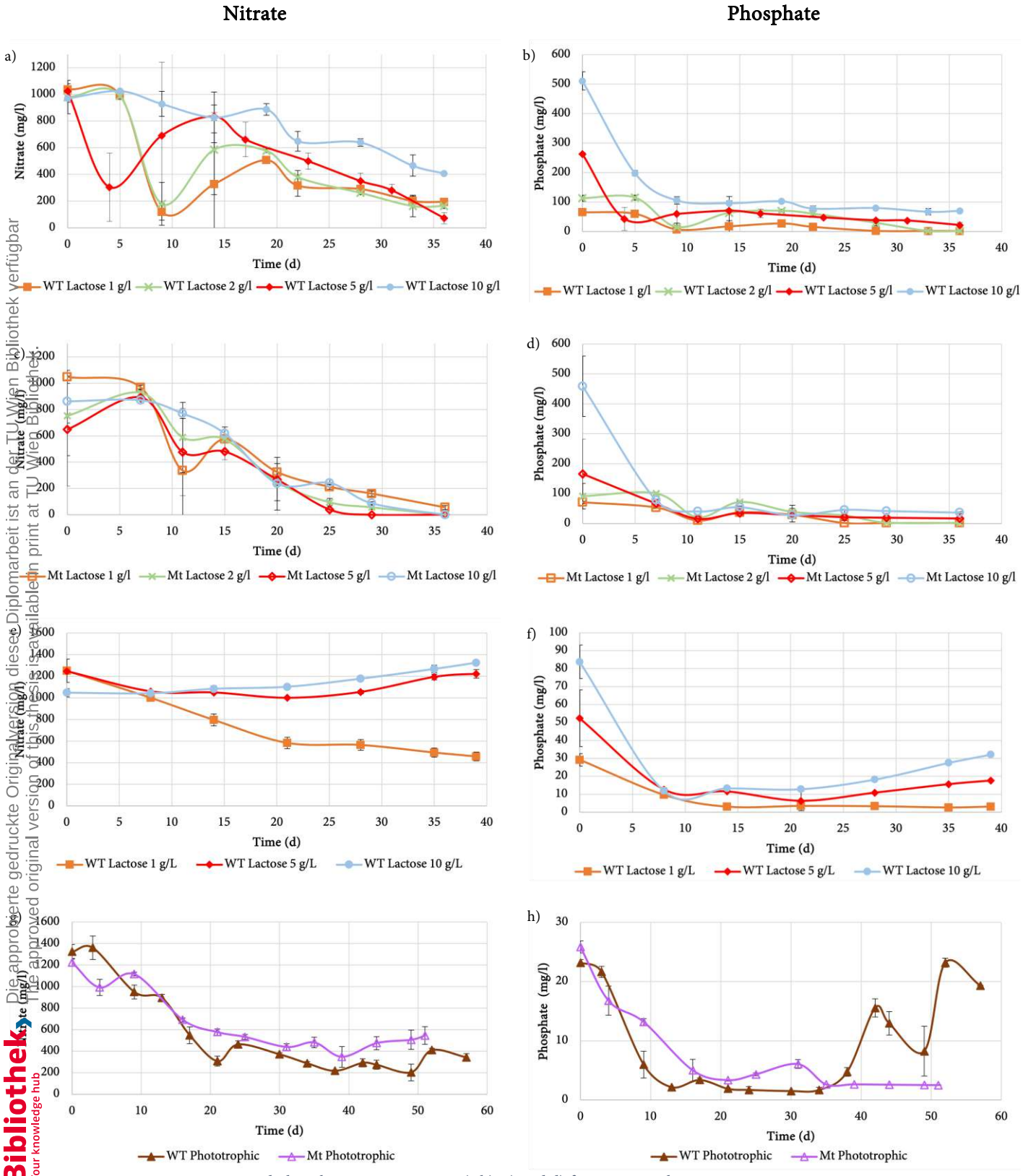


Figure 15: Nitrate and phosphate concentration: a), b), c) and d) first mixotrophic screening, e) and f) second mixotrophic screening, g) and h) phototrophic shake flask experiments

Phosphate concentration decreased rapidly in all experiments, indicating fast limitation. However, studies suggest that phosphate limitation influences PHB content to a lesser extent than nitrogen depletion.[2] In the first mixotrophic screening, the concentration was about five times higher than when whey was used at pH 9 in the second one. The drastic reduction could be caused by the precipitate separated from the whey, which may contain calcium phosphate. In addition to the already discussed distortion of the biomass concentration determination by the precipitate, the increased phosphate content could have increased cell growth and led to more cell growth, as known from the literature.[91]

The nitrate concentration decreased in almost all experiments but more slowly than described in the literature. Unlike earlier publications, this concentration did not drop entirely in some experiments.[1, 34, 91] In photobioreactor cultivations (see D.2), this effect only occurred in the phototrophic, but not in the mixotrophic cultivations. Only at the 5 and 10 g/L lactose measurements of the second mixotrophic screening the N-concentration decreased slightly, which could be related to the low growth already observed at the biomass concentration. During nitrogen starvation, the cells continue fixating CO_2, but the carbon cannot be converted into proteins, and the NADPH concentration increases, so the carbon/energy has to be stored as PHB.[34, 82] When nitrogen is added again, the PHB and glycogen reserves would be almost entirely used up again.[88]

Figure 16: Stress-induced colour change of *Synechocystis* sp. Mt, here caused by different sugar concentrations (FLTR: lactose concentration 10, 5, 2 and 1 g/L)

The colour shift towards a more yellowish colour is an effect also described in the literature, as exemplified in Figure 16 for different lactose concentrations. Stress, especially nitrogen deficiency, triggers the metabolism of phycobiliprotein as a nitrogen storage, which gives the cell's blue colouration.[143] In the case of phosphorus deficiency, the cells cannot regularly synthesise nucleic acids, leading to a blockage of protein and enzyme production, thus severely restricting the cell. Furthermore, as with nitrogen deficiency, degradation of phycobiliprotein is observed.[2]

D.1.4. PHB content

In the longer-lasting phototrophic screening, a reduction of the PHB was observed after the maximum PHB content had been reached, with 4.2% for the WT and approximately 0.8% for the Mt, indicating that the cell had to draw on these energy reserves due to starvation.[101] The maximum PHB content, volumetric productivity and growth rate for the screening experiments are shown in Table 8. The Mt,

which according to the literature, should have higher PHB content, only reaches one-fifth of the WT in this case, which should be rechecked by repetition of the experiment.[1] In contrast, the values in the first mixotrophic screening for the Mt, which is up to 1.1% at 1 g/L and 5 g/L, are higher than the values of the WT, which did not surpass a value of 0.5%. The PHB content is calculated from the biomass concentration, which in the first mixotroph screening was probably artificially increased by precipitation, which would lead to a lower PHB content. In the second mixotroph screening, values up to 3.5% were reached at 5 and 10 g/L, with very low biomass density. At 1 g/L, about 2.2% was achieved, which is still below the phototrophic results but above those of the first mixotroph. PHBs were not detected in any of the inoculated precultures.

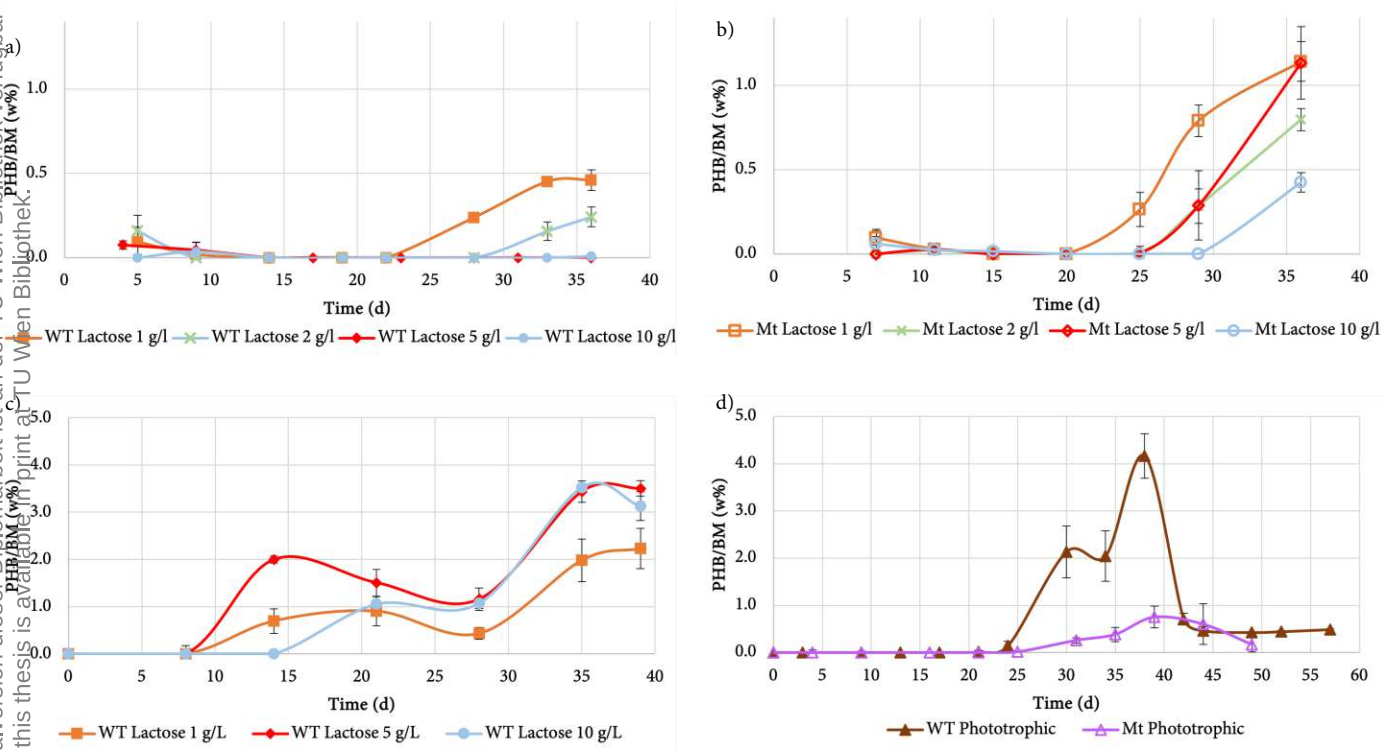


Figure 17: PHB content: a) and b) first mixotrophic screening, c) second mixotrophic screening, d) phototrophic shake flask experiments

The PHB content in the mixotrophic screening did not reach the value of the phototrophic screening. In general, whey, containing sugars and salts, especially lactose, exerts osmotic pressure on the microorganism. The cell needs ATP to deal with the osmotic stress, which disturbs the NADPH/ATP balance, which the cell counteracts by producing PHB from glycogen. However, too much osmotic stress can reduce PHB production.[67, 144] Kanwal et al. showed that a lactose concentration of 1 g/L did not affect the enzyme activity of glutamate decarboxylase in *Synechocystis* sp. PCC 6803. Here, the concentration was only stored for 24 h in the modified medium after the log phase, in contrast to over a month, as done in this thesis. The short duration allows no prediction of possible influence on the growth rate.[145] The inhibitory effect of lactose on cyanobacteria was already discussed in chapter A.2.4. These data indicate that osmotic stress affects growth and PHB production even at the lowest concentration of 1 g/L lactose.

The achieved PHB values of 4.2% for the WT are low compared to the previous 20% in a single-stage cultivation in a photobioreactor, while only 11% were achieved in shake flask experiments.[91] As

described in more detail in the next chapter, the values in a photobioreactor achieved about twice that. Reported PHB content in the literature range from 1% after 21 days (N-limited, *Synechsocystis* sp. PCC 6803)[143], over 5% after two weeks (N-limited, *Synechsocystis* sp. PCC 6803) [129], over reports around 10% [82, 83, 92, 93, 146] to the 20 to 40% already mentioned in chapter A.3.4 partially with genetically modified microorganisms, climaxing in a recent one reaching 80% with a recombinant strain an on acetate (60% phototrophic).[34, 90] Some are also cultivated in photobioreactors, resulting in higher yields. In addition, most cultivations are produced in two-stage cultures, although this could have a negative effect, according to the results investigated by Kamravamanesh et al.[91]

Table 8: Maximum volumetric productivity, μ and PHB content during the screening experiments

Cultivation	Strain	Lactose (g/L)	Volumetric productivity$_{max}$ (mg/L/day)	μ_{max} (day^{-1})	PHB content (%)
1st mixotrophic	WT	1	0.71	0.44	0.5
	WT	2	0.62	0.46	0.2
	WT	5	0.04	0.56	0.1
	WT	10	0.07	0.59	0.0
	Mt	1	2.34	0.31	1.1
	Mt	2	1.62	0.35	0.8
	Mt	5	3.65	0.40	1.1
	Mt	10	1.47	0.43	0.4
2st mixotrophic	WT	1	3.29	0.25	2.2
	WT	5	1.15	0.21	3.5
	WT	10	1.40	0.18	3.5
phototrophic	WT	0	5.49	0.37	0.8
	Mt	0	1.33	0.39	4.2

D.1.5. Lactose

In addition to the lactose concentration, glucose, galactose and lactates were also examined as part of the sugar analysis. However, negligible amounts and no increases in concentration were found for the monosaccharides of lactose. The glucose and galactose concentrations indicate that the lactose was not broken down. Otherwise, an increase in concentrations could be measured unless the sugars would be metabolised immediately. In principle, glucose from whey, like acetate, fructose or valerate, should stimulate PHB production by increasing the acetyl-CoA pool in the cell.[144] However, in mixotrophic glucose metabolism of *Synechocystis* s. PCC 6803 under nitrogen limitation, less PHB but more glycogen was measured compared to phototrophic cultivation. As expected, cell growth was faster in mixotrophic cultivation in literature.[2] This thesis has not covered the effects of galactose on PHB production, whereby *Synechocystis* sp. PCC 6803 possesses a metabolic pathway for galactose.[147] As shown in *E. coli*, lactate can affect PHB production, but only at concentrations higher than those used in this thesis.[140]

The decrease in lactose concentration observed in the first mixotrophic screening (see Figure 18) was not observed in the second screening. The difference between the two cultures also originates from the pretreatment of the whey. In contrast to the first, the whey in the second screening was adjusted to a

pH of 9. In the mixotrophic photobioreactor, to which the whey was also added at a pH of 9, a decrease in lactose concentration was measured. Comparing the WT and Mt samples from the first mixotrophic screening, it is noticeable that the sugar concentrations in the Mt samples dropped more than in the WT samples. In principle, the precipitation discussed should be the same in both, and therefore the same decline should be observed (if the precipitate was the reason for the decrease in concentration). However, the difference could be caused by the slightly higher pH value of the Mt samples.

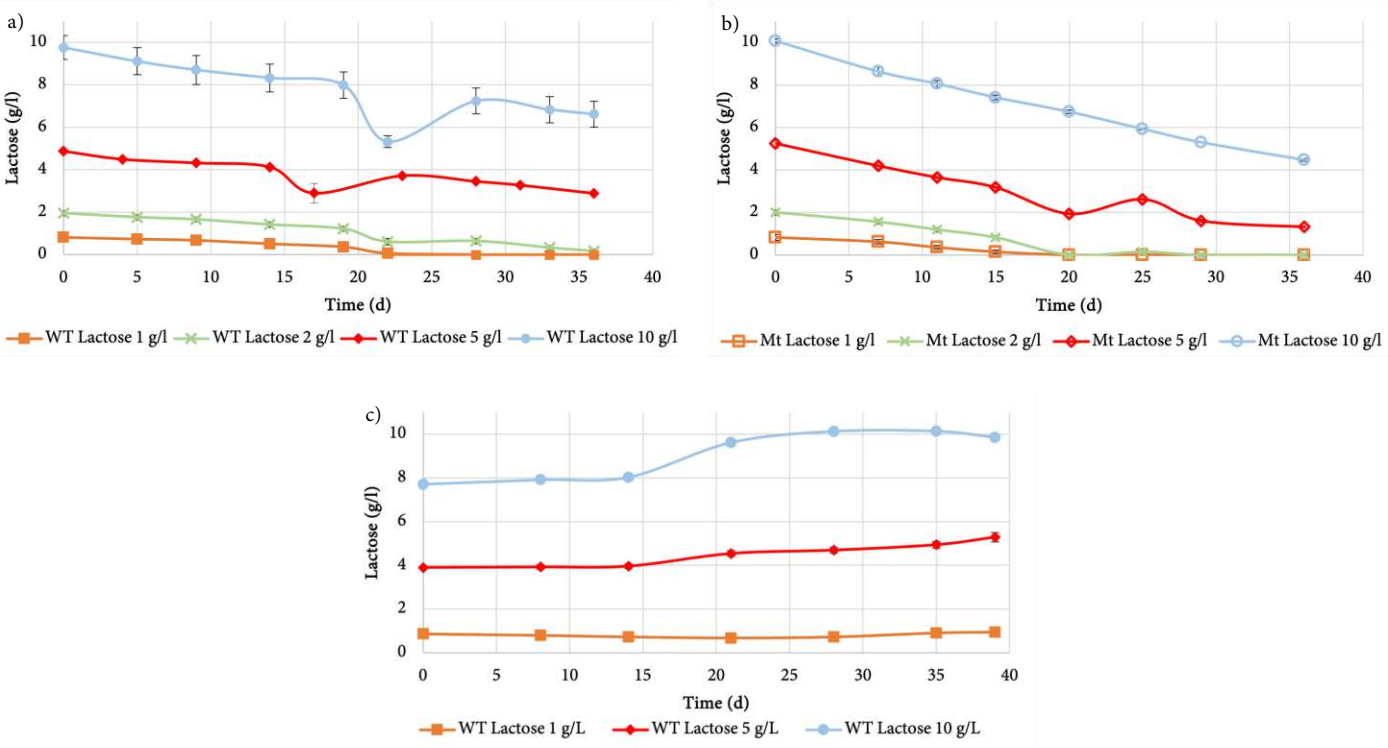


Figure 18: Lactose concentration: a) and b) first mixotrophic screening, c) second mixotrophic screening

D.2. Photobioreactor

Based on the results of the screening experiments, a lactose concentration of 1 g/L was chosen for the mixotrophic experiments, as a comparatively high cell density was achieved in combination with a high PHB content. Furthermore a phototrophic cultivation, is presented as a comparative example in this thesis. The cell density, PHB content and ion concentrations are illustrated in Figure 19 (for CO_2-offgas, temperature and pH see Figure 36 in appendix G.4). The biomass growth of the phototrophic PBR, at just under 2.5 g/L, is about 1 g/L larger than that of the mixotrophic PBR. This tendency could already be observed in the screening experiments. A difference to the shake flask experiments is the maximum cell density, which could be reached earlier in the PBR, as reported in the literature.[34] In the mixotrophic experiments, later reach of maximum was observed compared to the culture without lactose, regardless of the experimental setup.

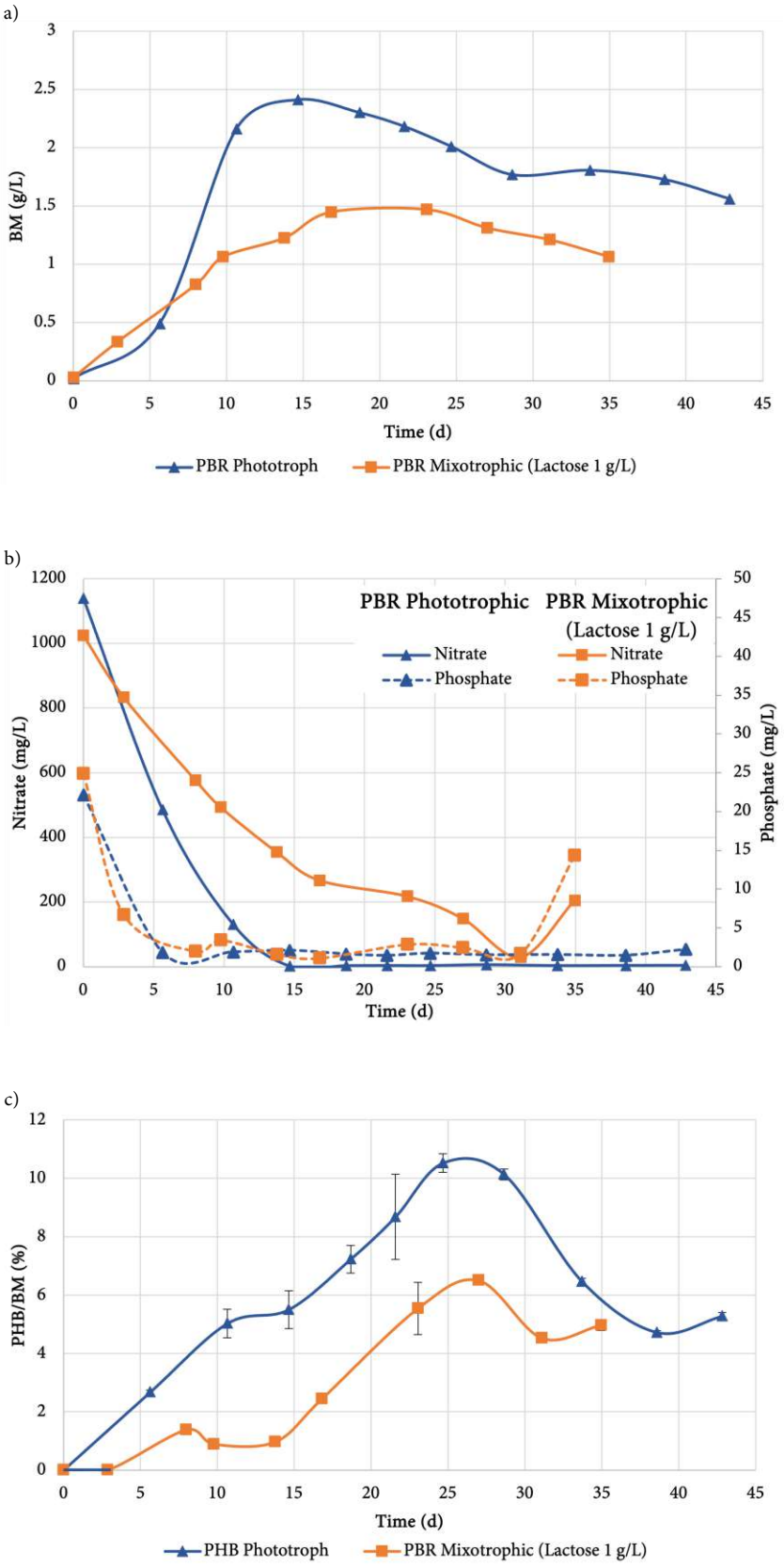


Figure 19: a) Biomass concentration, b) Nitrate and Phosphate concentration and c) PHB-content during the phototrophic and mixotrophic (1 g/L Lactose) PBR cultivation

In the phototrophic cultivation, both phosphate and nitrate are used up after 14 days latest. Cyanobacteria can continue to grow in nutrient-poor media by metabolising intercellular nutrients such as nitrogen or phosphorus.[82, 148] However, as expected, a decrease in cell density was also observed afterwards.[88] In the mixotrophic cultivation, nitrogen was not depleted, indicating the inhibitory effect of lactose, as already observed in biomass growth. The μ_{max} (0.86 day^{-1}) of mixotrophic PBR, despite a lower cell density, is higher than phototrophic (0.57 day^{-1}) because the cells show a stronger growth in the first days. In previous experiments a μ_{max} of about 0.8 day^{-1} was achieved.[34]

The PHB contents in the reactor setups were twice as high as in the screening experiments. With 10.5% in the phototrophic screening and 6.5% in the mixotrophic, the difference found in screening is also evident. Furthermore, similar to biomass growth, the PHB maximum was reached earlier.[34] With the same wild-type strain, PHB contents of 20.4% were measured in a single-stage phototrophic PBR.[91] After reaching the maximal PHB content, the storage substance was metabolised again by the cell, which was reflected in a decrease in the PHB content.[101] According to the literature, most PHB is formed in the static and death phases, which can be observed in the mixotrophic cultivation.[91] As explained in the previous chapter, the lactose concentration drops to zero during the experiment (see Figure 20).

Volumetric productivity reported data is very limited.[83, 93] The values achieved from previous experiments with the WT (59 mg/L/day) or with the Mt (139 mg/L/day) could not be reached (see Table 9).[1, 34] Beyond that, Wang et al. reported a maximum of 263 mg/L/day (recombinant *Synechocystis* sp. PCC 6803).[149] At 21 mg/L/day, the phototrophic reactor reached about one-third of the comparable experiment (59 mg/L/day), which was already indicated by the PHB content. In the mixotrophic PBR (7 mg/L/day) it drops again to one third compared to no lactose addition.

Table 9: Maximum volumetric productivity, μ and PHB content during PBR

Cultivation	Volumetric $productivity_{max}$ (mg/L/day)	μ_{max} (day^{-1})	PHB content (%)
Mixotrophic (1 g/L)	7.7	0.86	6.5
Phototrophic	21.1	0.57	10.5

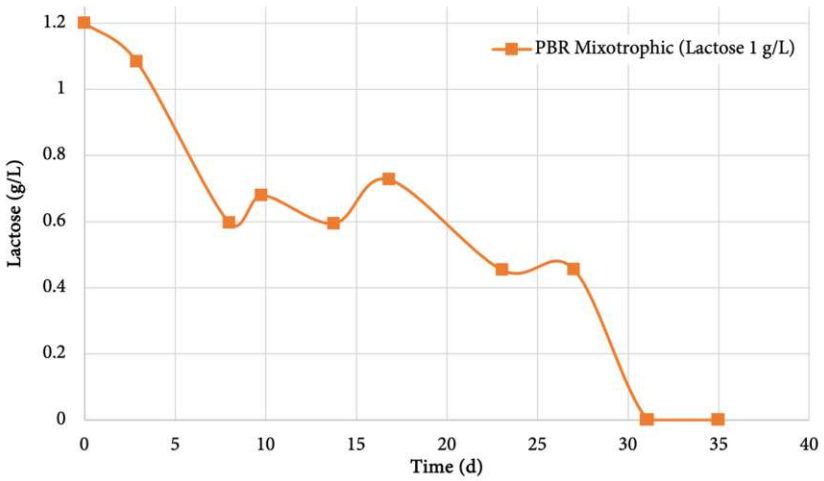


Figure 20: Lactose concentration of the mixotrophic (1 g/L Lactose) PBR cultivation

In contrast to the continuous 24 h illumination in the PBR, a light/dark cycle (14/10) was performed in screening experiments. In *Synechocystis* sp. PCC 6714, no effect of light-dark cycles (16/8) was found on PHB content compared to 24 h illumination.[34] However, for *Synechocystis* sp. PCC 6803, light/dark cycles (12/12) showed a positive effect on PHB production and a slightly negative effect on cell growth.[150, 151] This should be considered in future studies.

D.3. PHB quantification

D.3.1. Comparison of acidic and alkaline PHB-quantification

PHB, like other polyesters, can be degraded by chemical, mechanical, enzymatic or thermal treatment, which can occur individually or in combination.[152] Figure 21 shows typical by-products of the alkaline and acidic hydrolysis from PHB to crotonic acid. For example, in the NaOH method 3% of UV-active isocrotonic acid were measured,[109] which should elute just before the crotonic acid.[108] In the HPLC measurement of the alkaline and acidic samples, a substance eluted with about 3% of the area of the crotonic acid and just before the crotonic acid, which was confirmed by the standard. Therefore, it can be assumed that the peak is the by-product of the crotonic acid, isocrotonic acid.

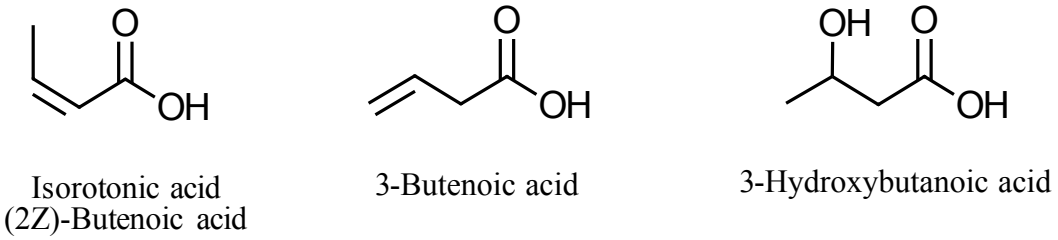


Figure 21: Suggested side-products PHB hydrolysis [107, 153]

The full factorial analysis of the alkaline and acidic method was designed and evaluated using MODDE. A negative logarithmic relationship was found for acidic data sets, whereas no transformation was required for the NaOH method. A summary of the fit plot, the effect plot and the contour plot are shown in Figure 22. The Summary of fit for both studies fulfil the conditions for a good model, such as a difference between R^2 and Q^2 of less than 0.3, $Q^2 > 0.5$, model validity > 0.25 and reproducibility > 0.5. The model for H_2SO_4 describes the system better than the model for NaOH, as higher values are displayed for the acid method.

Both the time and the NaOH concentration negatively influenced the hydrolysis of PHB. This resulted in a contour plot with its maximum in the lower left corner at just below 50% conversion. In the literature, more crotonic acid was measured with increasing experiment duration (0.5-5 h) and NaOH concentration (0.1-4 M), with up to about 20%, whereby the test was carried out at 70°C.[107] At 105°C, concentrations between 0.4 to 1 M NaOH show an almost constant crotonic acid concentration after one hour, and with a constant concentration of 0.67 M between 0.75-2 h. This publication's recovery (38%) with the optimal method (0.67 M NaOH for 1 h at 105°C) is slightly lower than shown in this

thesis (42%).[154] No statistically significant difference between GC and alkaline HPLC methods has been shown in the literature.[154, 155]

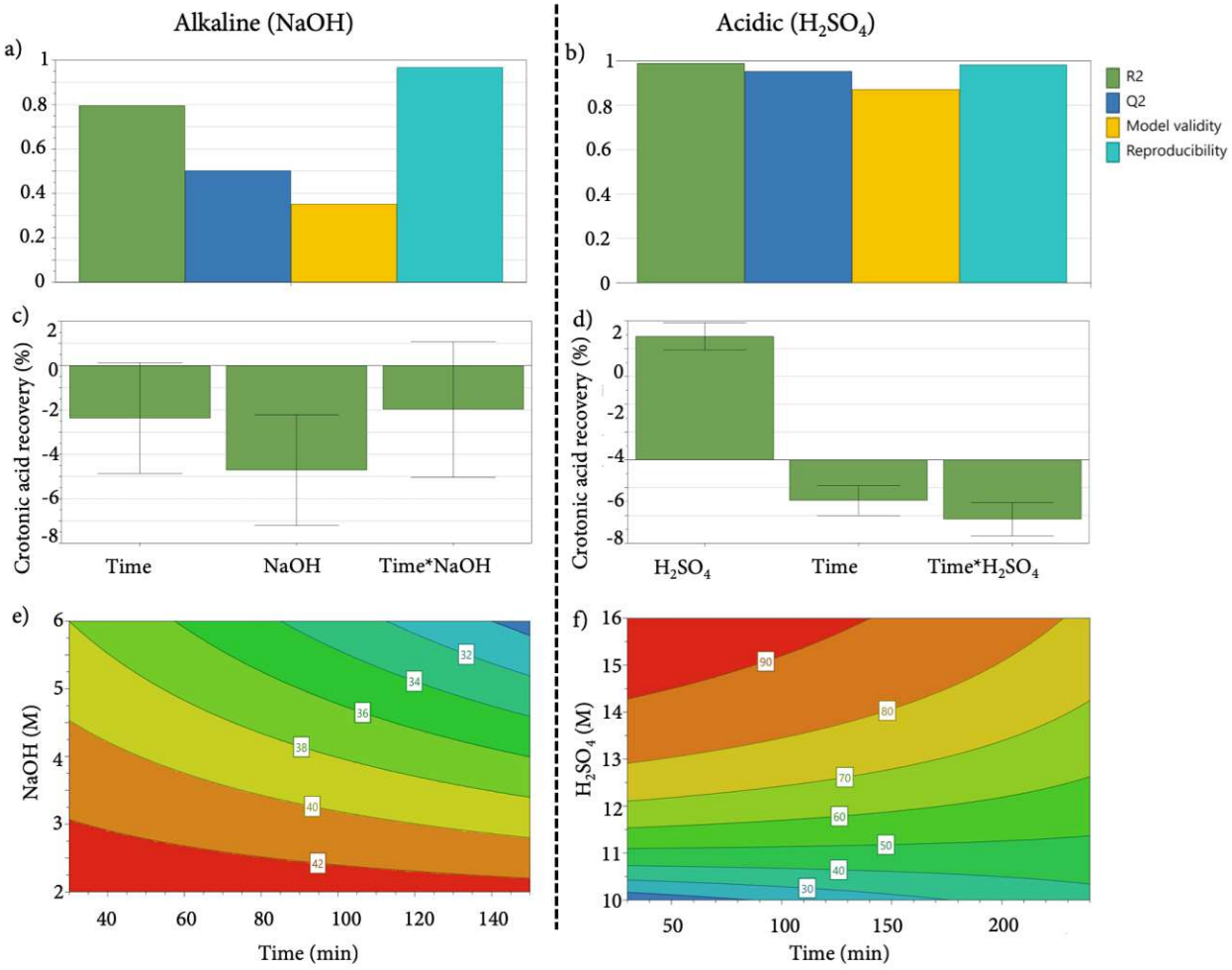


Figure 22: Full factorial analysis of alkaline and acidic method: a) and b) summary of fit for alkaline and acidic, c) and d) coefficients (scaled and centred) of the alkaline and acidic method, e) and f) contour plot time vs concentration of base and acid

Sulfuric acid strongly influenced the concentration, whereas time and the combination of time and sulfuric acid had a negative effect on crotonic acid production. The contour plot illustrates that the highest yields were obtained with short periods and high concentrations. For 0.1 to 4 M sulfuric acid, no decomposition of PHB was measured in the literature, confirming the trend. The up to 90% recovery of crotonic acid is consistent with measurements with concentrated sulfuric acid. [107] The instability of the PHB in a slightly alkaline solution but the relatively good resistance in a slightly acidic solution illustrates the importance of the neutralisation step in the alkaline method for accurate measurement results. Furthermore, the experiments indicate slightly lower sulfuric acid concentrations for this measurement were also possible for further trials.

In contrast to sulfuric acid, hydroxyl anions attack the PHB backbone even at low concentrations. Alkaline ester cleavage is irreversible, whereas acidic catalysed one stands in chemical equilibrium, which explains the high conversion in the NaOH method. Acidic esterification between carboxylic acid and alcohols can only be shifted to the side of the cleavage product by a high acid concentration (80-98%).[107] In both methods, the highest crotonic acid recoveries occur at short heating periods, possibly due to further degradation of crotonic acid under these conditions.

Since the PHB conversion with sulfuric acid was about twice as high as with NaOH, the sulfuric acid method was chosen for further optimisation. Based on these measurements, a time between 100 and 160 min and a concentration between 14 and 12 M was chosen to increase the manageability by lowering the viscosity, as described in the next chapter.

D.3.2. Optimisation of H_2SO_4 PHB- quantification method

A composite design was chosen for the optimisation, and the results are shown in Figure 23. The values of validity and reproducibility of the model, R^2 and Q^2 lie above 0.75, and the difference between the last two is about 0.15, indicating a robust model. As in the previous DOE, the conversion to crotonic acid was most affected by the sulfuric acid concentration. In contrast to the previous experiment, the small positive influence of time on the yield indicates a slower conversion in the low-concentration systems used for the optimisation. The influence of the different PHB concentrations (CPH), their square and the sulfuric acid concentration were proven significant for the model but show a minor influence compared to the sulfuric acid. As can be seen in the contour plot of time vs H_2SO_4 concentration, a yield of over 86% was obtained at the highest concentration (14 M), slightly increasing with prolonged duration. Analogously the literature shows a minor impact of PHB concentrations on the chemical reaction.[154]

With the help of the “optimizer” tool in MODDE, the parameters for the highest recoveries could be determined, namely 160 min and 14 M H_2SO_4 at the boundary of the DOE, as indicated in the contour plot. However, the question remains to what extent the recoveries differ between the 14 M and the 18 M standard methods, which will be addressed in the next chapter.

D.3.3. Comparison of standard and the optimised sulfuric acid method

When comparing the recovery rate of crotonic acid with the 14 M and the 18 M H_2SO_4 standard (data in appendix G.2), the Shapiro-Wilk test was first used to check for normal distribution as described in C.5.3. Since the calculated p-values of 0.97 and 0.97 (18 and 14 M) are above the required significant level (0.05) in both cases, the null hypothesis cannot be rejected. The standard deviations are both the same size and are approximately 4.7%, which also fulfils the variance condition, which is why a two-sided t-test could be performed. With a p-value of 0.16, the null hypothesis is not rejected. Thus, no statistically significant difference was found between the means of the two methods, which shows that they archive the conversion to crotonic acid. Table 10 shows for the 14 and 18 M methods the crotonic acid/biomass ratio and PHB content, based on the corresponding standard analysis, for three different biomasses. For the 18 M method, there was a minimal increase in crotonic acid recovery, while the calculated PHB content remained the same, indicating convergence between the two methods.

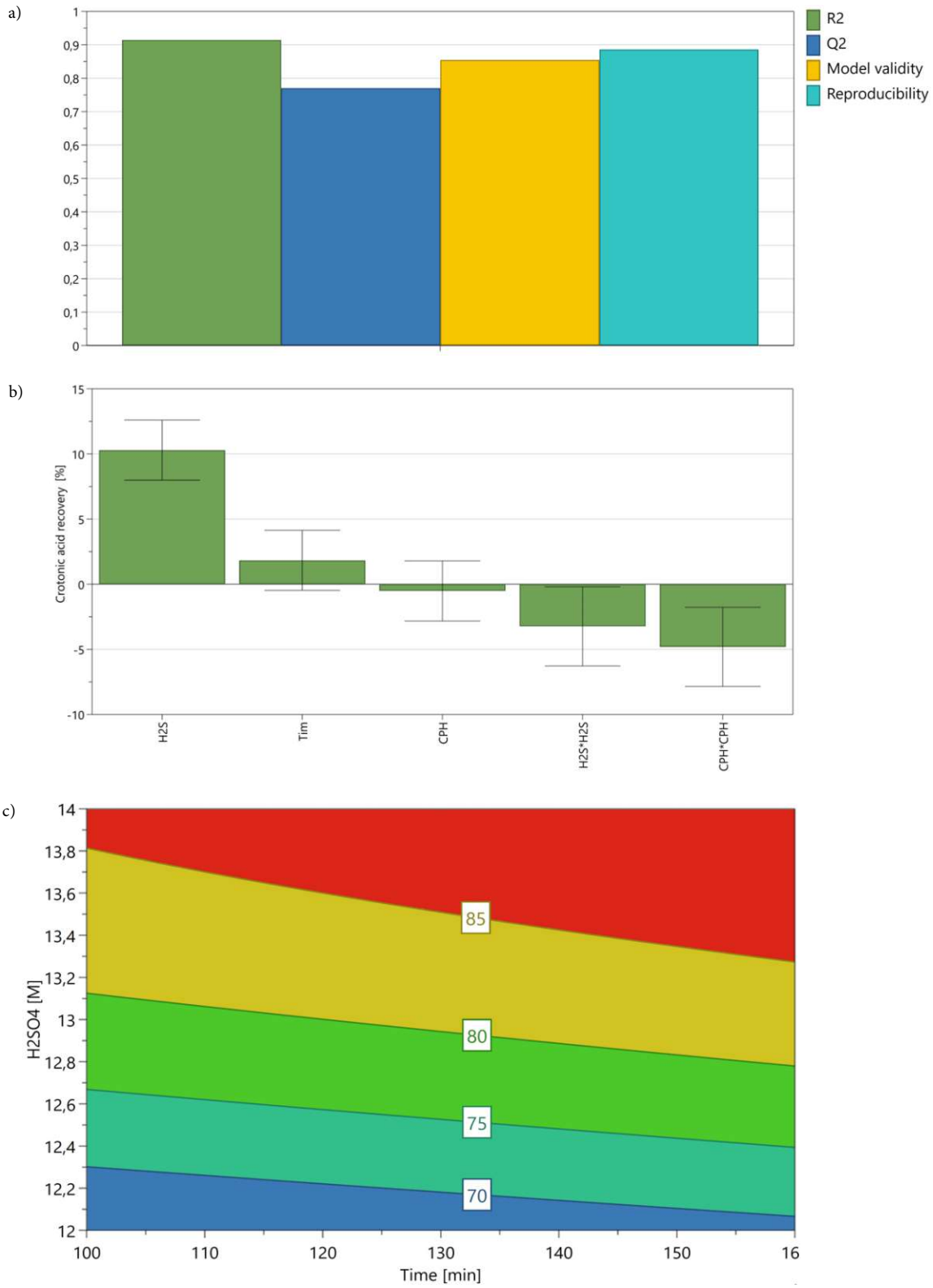


Figure 23: Composite analysis of acidic method: a) summary of fit, b) coefficients (scaled and centred) and c) contour plot time vs concentration. (CPH… PHB Concentration)

This DOE aimed to improve the manageability of sulfuric acid by reducing the concentration. As a result, the dynamic viscosity of sulfuric acid (see Figure 24) decreases from 18 M (concentrated) to 14 M (80wt%). In summary, the 14 M method proved to be a reliable quantification tool, hence was used in future measurements.

Table 10: Three biomass analysed with 14 and 18 M methods

BM	c(H_2SO_4) (M)	Crotonic acid/BM AVG (wt%)	Crotonic acid/BM SD (wt%)	PHB content (based on standard calibration) AVG (%)	PHB content (based on standard calibration) SD (%)
1	14	3.86	0.46	5.34	0.64
	18	4.09	0.05	5.23	0.07
2	14	5.44	0.19	7.53	0.26
	18	5.83	0.13	7.45	0.16
3	14	2.42	0.04	3.33	0.06
	18	2.64	0.03	3.36	0.04

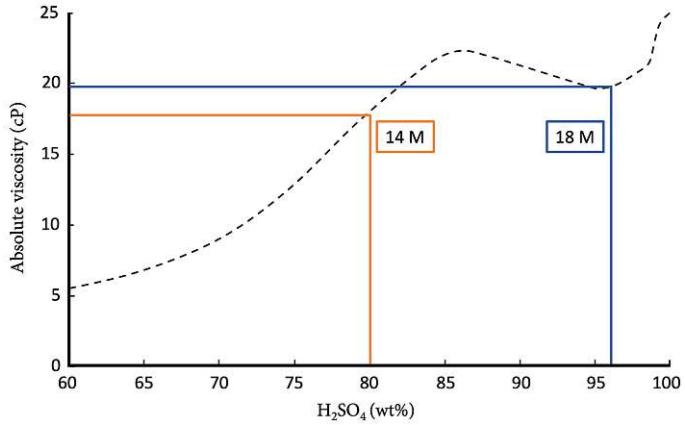


Figure 24: Dynamic viscosity (25°C) depending on the mass fraction of sulfuric acid (1 cP=·0.001 kg m^{-1} s^{-1}) [156]

D.3.1. The necessity to shake

In this experiment, the extent to which shaking influences the PHB content was investigated, which is graphically summarised in Figure 25. The results for the acidic BM hydrolysation are very similar, and no trend can be derived from them. Furthermore, when using pure PHB, no deviation was observed between the recovery with (100.44 ± 1.14wt%) and without shaking (100.31 ± 1.91wt%). Therefore, it can be concluded that shaking is not significant in the described method.

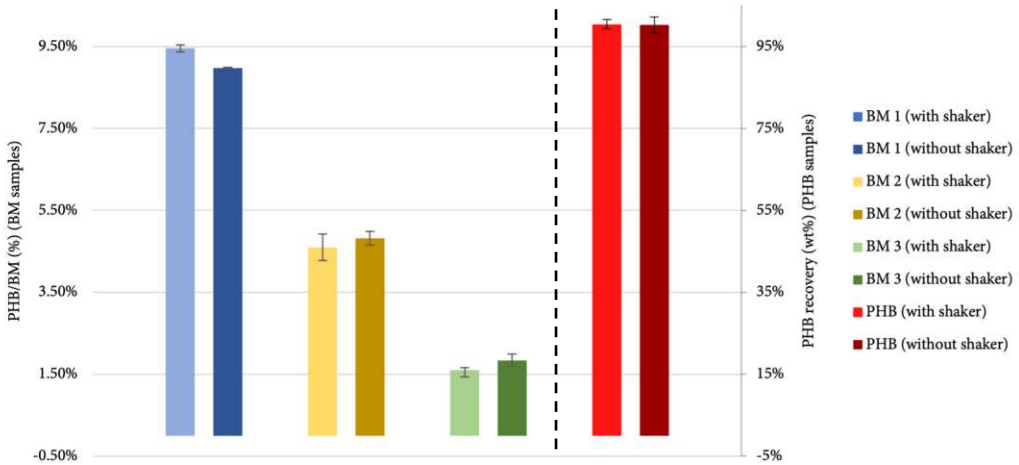


Figure 25: PHB content/recovery using the 14 M sulfuric acid analysis of biomass and PHB without and with shaking (600 rpm). The experiments with biomass samples are shown on the left axis and PHB samples on the right axis.

D.4. Chloroform Extraction

The solvent extraction with chloroform was carried out as described in the literature.[71, 119] The fact that the biomass floats above the chloroform after centrifugation is also a phenomenon known in the literature.[123, 128] Due to the very small amount of precipitate, the sample should have been used to analyse the PHB content with the 14 M H_2SO_4 method. Unfortunately, after the analysis it turned out that the acid was probably highly diluted for unknown reasons, which did not allow a quantitative analysis. This was reflected in the fact that the product did not dissolve or dissolved incompletely due to the low acid concentration. Apart from the fact that the previous treatment affected the analyse, the centrifuged pellet (14,000 g, 15 min) of the undissolved material was treated again with freshly prepared 14 M H_2SO_4, and the now completely dissolved solution was analysed by HPLC after appropriate dilution. From this it can at least be concluded that part of the precipitation contains PHB.

The yield is shown in Table 11 In the literature, large variations in the extraction with chloroform may be due to the biomass used, the pretreatment, the temperature, or the duration, among other factors. The maximum yield of 21wt% achieved in this work, combined with the wide range variation, indicates that the extraction was not satisfactory, as at least yields above 50wt% would be expected.[70, 157] In the literature used, the dried biomass was mechanically crushed before extraction, which possibly makes the PHB easily accessible.[71, 119] Manangana and Shawaphuna have shown that pretreatment can increase the yield from about 39 to 84wt%.[157]. A repetition of the experiments with pretreatment on which this work is based is recommended to clarify the effects.

Table 11: Recovery of chloroform extraction of PHB

Cultivation	PHB content in BM (%)	Extracted PHB (mg)	Recovery (wt%)	Recovery AVG±SD (wt%)
Phototrophic	9.51	6	4.18	3.15 ± 1.04
	9.51	3	2.11	
	9.51	5	3.15	
Mixotrophic	1.64	2	11.41	14.09 ± 6.18
	1.64	2	9.70	
	1.64	5	21.16	

D.5. Ionic Liquid

D.5.1. IL solubility of biomass

During the stepwise addition of the biomass, a green discolouration and an increase in viscosity quickly occurred. Due to the intense colour, no assessment could be made about the degree of dissolution of the biomass in the flasks. Therefore, the total dissolution was determined optically over the sample taken after centrifugation, as exemplified in Figure 26. Unfortunately, the values of the dissolution kinetics do not allow a consistent statement. This is probably due to the high viscose samples and the small quantities taken. Therefore, conclusions were drawn about the amount of dissolved biomass with water-precipitated biomass taken from the supernatant at the end of the experiments. The results are

shown in Figure 27. For [EMIM][DEP], about 80wt% of the biomass could be recovered at 75 and 50°C and less (40wt%) at room temperature. [EMIM][OAc] shows a slightly decreasing solubility trend with temperature, a statement that can be made considering the standard deviations influenced by the high viscosity. The [EMIM]Cl, with about 80wt% recovery of biomass, shows a similarly good result as the previous ones in the experiments. The [EMIM]Cl, which is solid in its pure form at room temperature, remains liquid with the dissolved biomass even at room temperature.

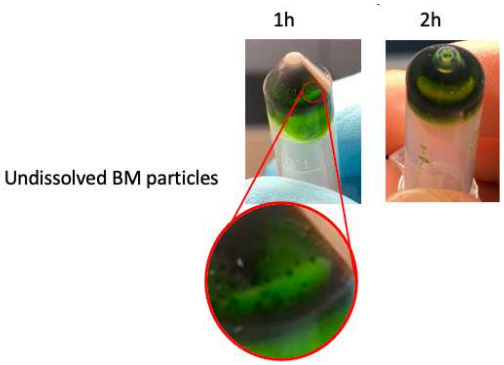


Figure 26: Exemplary illustration of finding the complete biomass dissolution. Sample [EMIM]Cl with BM after 1 h (l) and 2 h (r). After 1 h (see magnification), particles are still visible that were dissolved after 2 h.

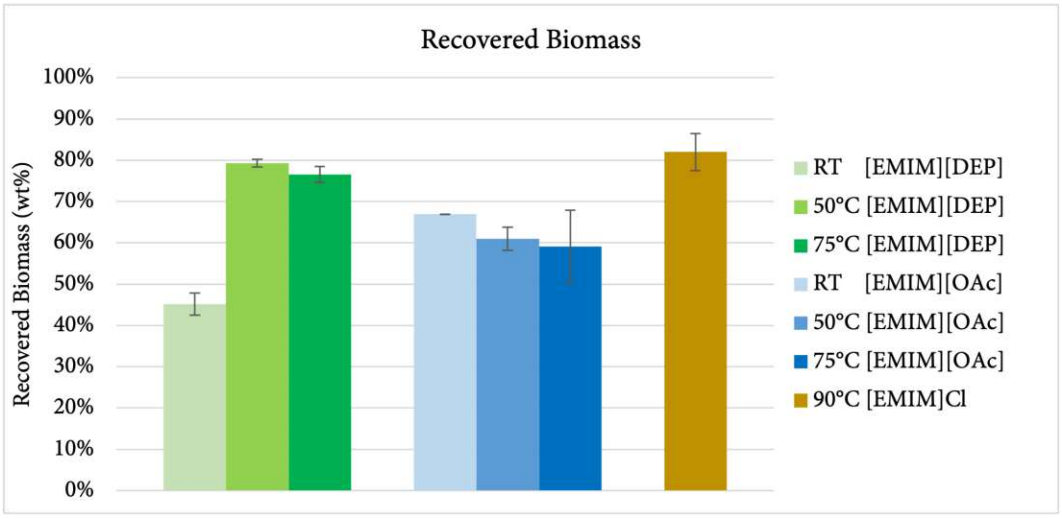
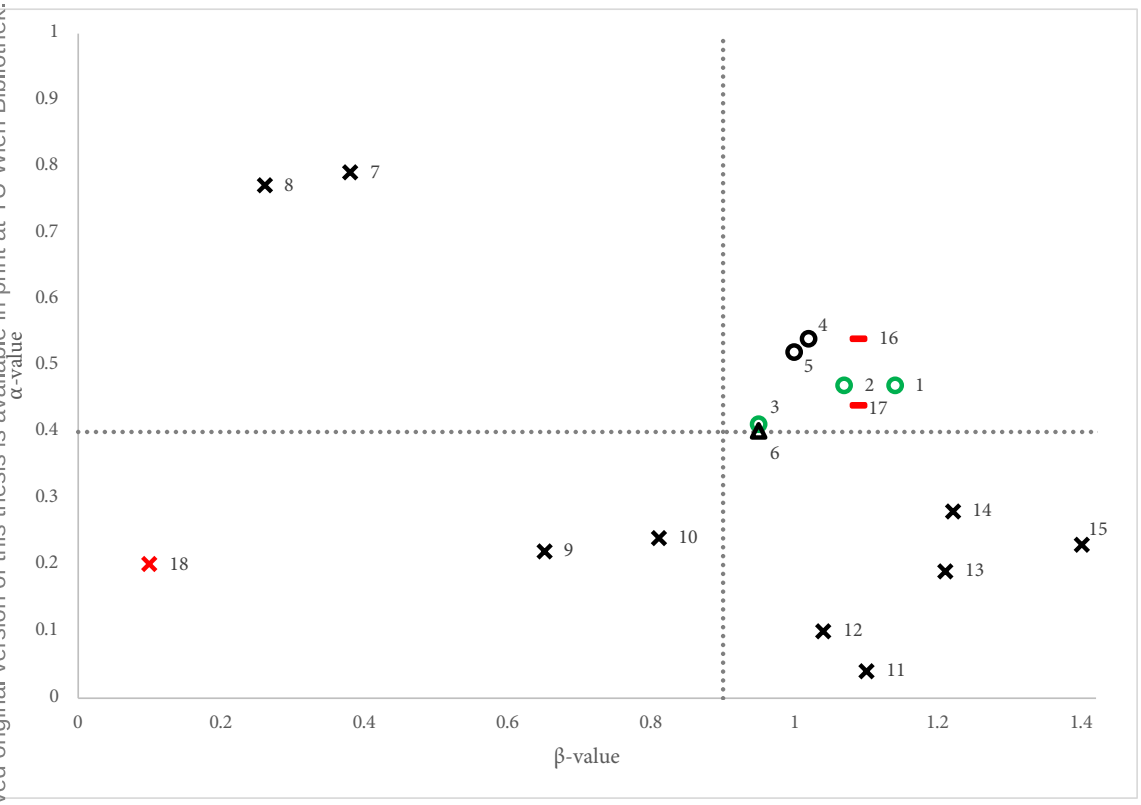


Figure 27: Recovered biomass after precipitation with water

[EMIM] dissolves the biomass by breaking the hydrogen bonds or electrostatic interactions, e.g. with the negatively charged phospholipids or peptidoglycans of the membrane.[70, 158] The total dissolution at the highest measured temperature of [EMIM][OAc] and [EMIM]Cl is 1 h, that of [EMIM][DEP] is 2 h. In comparison, the duration of [EMIM][MP], even at room temperature, is 0.5 h.[129] Measurements in experiments with [EMIM][DEP] and [EMIM][DMP] are carried out at 60°C for 24 h, a more detailed description of the duration until complete dissolution is not explicitly discussed.[128, 132] Fujita et al. showed that [EMIM][OAc] dissolved a part of the biomass after 24 h at RT with stirring. In this experiment, wet (95wt% water) saliferous microalgae were used, and the

temperature was not increased, which could hinder complete dissolution. For [EMIM]Cl, dissolution was observed after less than 1 h at 80°C (also Kobayashi et al.).[129]. After 30 min at RT, [EMIM][MP] dissolved and showed similar properties [EMIM][DEP] [128] when comparing the Kamlet-Taft parameters. The products formed during the dissolution of biomass could be water soluble and undetectable by precipitation with the solvent. This effect could partly explain the apparent limit of about 80wt% biomass recovery. Other precipitants, such as toluene, or the analysis of the antisolvent could shed light on this in the future.

Kobayashi et al. concluded from their measurements that the dissolution behaviour of cyanobacteria strongly depends on two of the three Kamlet Taft parameters, namely hydrogen-bond donating ability (α-value) and hydrogen-bond acceptor ability (β-value). For complete biomass dissolution, the α-value should be above 0.4 and the β-value above 0.9.[129] The α- and β-values for ILs from the publication just mentioned, this thesis and other sources are shown in the graph in Figure 28. The ILs used in this work lie within the solubility window just described.



		Ref.
1:	[EMIM][OAc]	[159]
2:	[EMIM][DEP]	[159]
3:	[EMIM]Cl [a]	[128]
4:	[BMIM][EP]	[129]
5:	[EMIM][MP]	[129]
6:	[EMIM][EP]	[129]
7:	[EMIM]BF_4	[129]
8:	[EMIM]$[Tf_2N]$	[129]
9:	[TBP][Bz-o-SO_2]	[129]
10:	[TBP]$[C_2H_2(COO)_2]$	[129]
11:	[TBP]Ser	[129]
12:	[TBP]Asp	[129]
13:	[TBP]$[H_2PO_2]$	[129]
14:	[TBP][BzCOO]	[129]
15:	[TBP][MP]	[129]
16:	[EMIM][OPr]	[159]
17:	[BMIM][OAc]	[159]
18:	Chloroform	[160]

Figure 28: Structural formula of the ionic liquids used in this thesis (Diagram based on [129])([TBP]… Tetra-n-butylphosphonium, [EP]… Ethylphosphonate, BF_4… Tetrafluoroborate, $[Tf_2N]$… Bis(trifluoromethanesulfonyl)imide, [Bz-o-SO_2]… Orthosulfuric benzolate, $[C_2H_2(COO)_2]$… Maleate, Ser… Serinate, Asp… Asparaginate, $[H_2PO_2]$… Hypophosphorate and [BzCOO]… Benzolate)

[a] *[EMIM]Cl was approximated by means of the value of 1-ethyl-3-butylimidazolium chloride [128, 129]*

D.5.2. IL solubility of PHB

After mixing and heating (RT, 50 and 75°C) the ILs with the PHB (see Figure 29 for the experimental setup), the liquid was centrifuged. A residue was observed for [EMIM][DEP] and [EMIM][OAc], except in the case of [EMIM][OAc] at the highest temperature (75°C). The [EMIM]Cl PHB mixture became solid during centrifugation, in contrast to the sample solution with the biomass (see Figure 30). A white solid appeared on the surface, indicating that the PHB did not dissolve completely. Due to the more complex purification, the quantification of PHB in [EMIM]Cl was not performed.

The recovery rates of PHB in the ILs are shown in Figure 31. For [EMIM][DEP], a recovery rate of 73 to 83wt% is achieved for all temperatures. No precipitation of PHB was observed after the addition of water, and no crotonic acid was detected in the aqueous phase by HPLC. This observation suggests that the losses were caused by handling. This is also supported by the study of Kobayashi et al. according to which the relatively similar IL [EMIM][MP] does not dissolve PHB.[129]

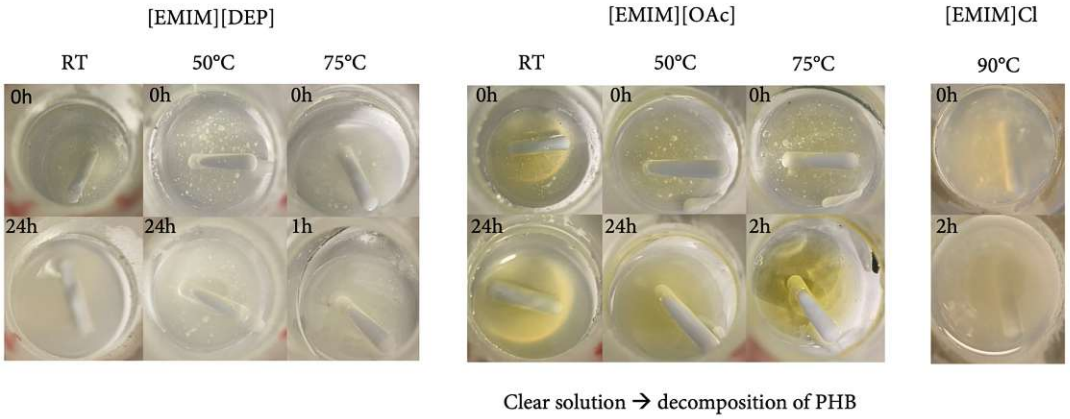


Figure 29: Mixing of PHB with IL: Upper pictures show IL with PHB at the beginning of the experiment and the lower pictures IL with PHB at the end of the experiment

Figure 30: [EMIM]Cl with 2wt% PHB after centrifugation. In contrast to dissolving BM, the IL with PHB solidifies. PHB has probably settled on the surface (white spots).

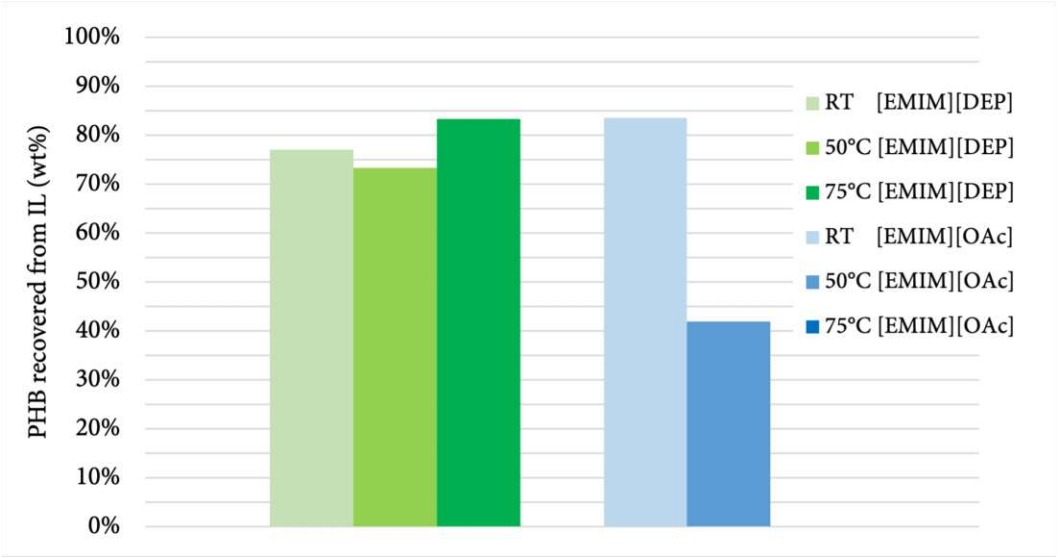


Figure 31: Recovered PHB after IL treatment: For [EMIM][OAc] 75°C no PHB was detected; [EMIM]Cl solidified during centrifugation → no data available

While the recovery rates of PHB for [EMIM][OAc] at RT are comparable to those of [EMIM][DEP], they drop to half at 50°C and to zero at 75°C. In the HPLC analysis of the latter two, crotonic acid was found in the supernatant mixed with water. To produce crotonic acid from PHB, [EMIM][OAc] converted 97% PHB to crotonic acid after 3 h at 140°C. A directly proportional temperature dependence of the conversion was found, as only 15% were converted after 3 h at the lowest temperature tested at 80°C. In this study, 20wt% (PHB/IL) was used compared to the 2wt% used in this thesis. The catalytic effect described in this work, including its temperature dependence, coincides with the trends of the measurements. The acetate probably catalysed this hydrolysis due to its basicity. If the slightly alkaline to neutral chloride is used instead of the acetate, neither crotonic acid formation nor bioplastic depolymerisation was observed.[158] The example from the patent by Hecht et al. in which [BMIM][OAc] (85°C) is used to "dissolve" PHB should also be questioned, as this would probably lead to decomposition.[161] When the strongly acidic anion $FeCl_4$ is used in combination with 1-(3-sulfonic acid)-propyl-3-methylimidazole or [BMIM], PHB can also decompose into crotonic acid [126], but with [BMIM]Cl, which is very similar to the [EMIM]Cl used, no decomposition of PHB was observed. These two publications also support the assumption that [EMIM]Cl did not decompose PHB.

D.5.3. PHB mixed with IL-biomass solution

The ILs mixed with biomass and PHB were heated and afterwards centrifuged. Due to the dark green colour of the IL-biomass mixture, it is difficult to recognise the contrast with the biomass, as attempted in Figure 32. This correlates with the result of the previous chapter. Pellets are visible for [EMIM][DEP] and [EMIM][OAc] at room temperature and appear to be smaller for [EMIM][OAc] at 50°C and [EMIM]Cl. However, no pellet is visible for [EMIM][OAc] at 75°C. Regarding separation in a centrifuge, reference is made to the results of Filippi et al., who achieved sedimentation without methanol addition at a similar biomass to IL ratio only with [EMIM][DEP], but not with [EMIM][DMP] or [EMIM][MP]. Filtration at a mixture of 1:10 (biomass:IL) proved to be very

difficult.[132], therefore methanol is used in the literature for separation at a ratio of 1:10 (biomass:IL), but filtration was only possible at a ratio 1:1,000 biomass:IL.[129, 132, 133]

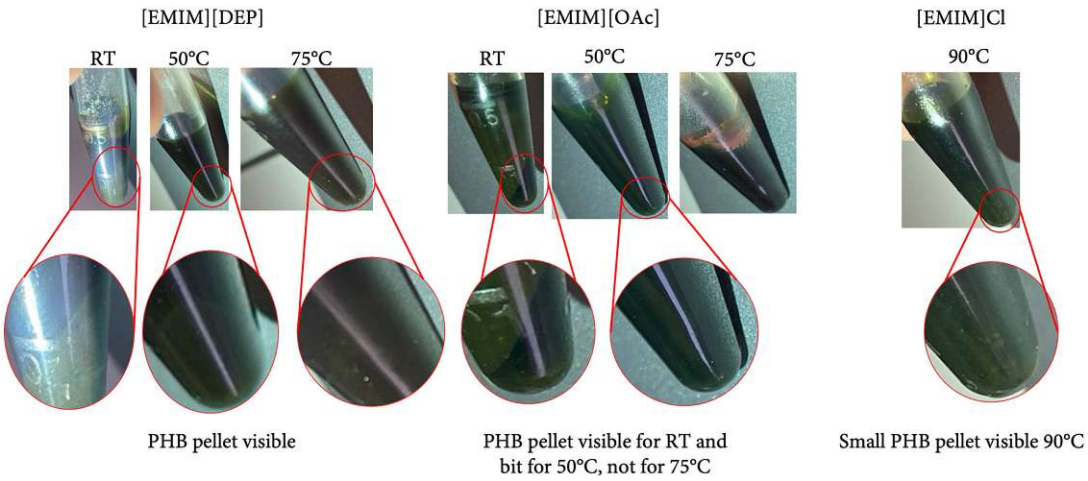


Figure 32: IL-biomass solutions doped with PHB contain a pellet after centrifugation in all but the 75°C [EMIM][OAc] sample

D.5.4. IL-biomass mixed with water

Water can be added to reduce the viscosity and thus improve manageability, which according to manufacturer proionic (Graz, Austria) is sufficient up to 10wt% (V/w). Phase separation was observed at 20, 50 and 100wt%. For the smaller ratios, no statement is possible without further measurements (see Figure 33). Similar to antisolvent methanol used in the literature, water could also influence the precipitation and thus the purity of PHB.[132, 133]

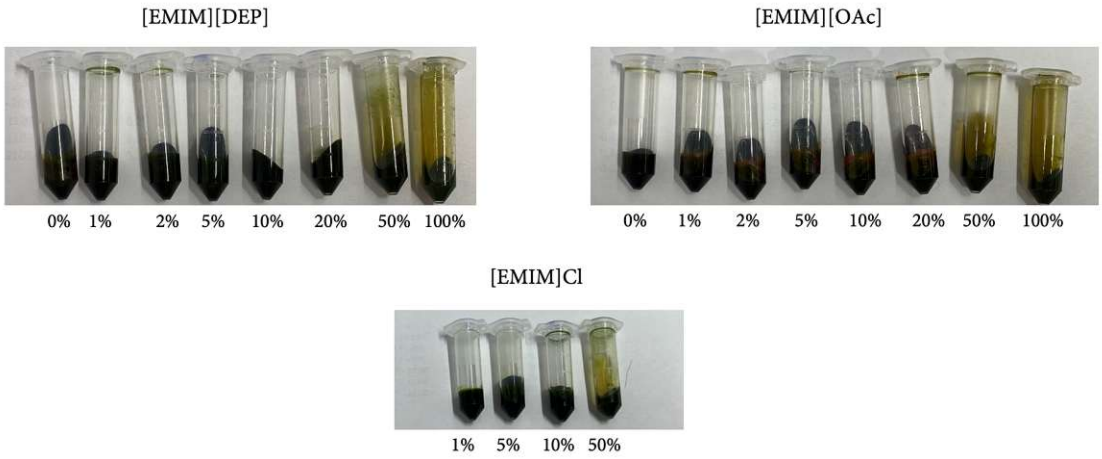


Figure 33: IL-biomass solution mixed with water for three ILs: Visually, no noticeable difference for 0 to 20wt% is evident

E. Conclusion

In screening experiments, the PHB production in *Synechocystis* sp. PCC 6714 was examined under different lactose concentrations. In the first mixotrophic screening, the precipitation of the whey probably alters the OD_{750} measurements, leading to a higher biomass concentration and a lower PHB content. Adjusting the pH to 9 reduces the problem, but precipitation was still observed. *Synechocystis* sp. PCC 6714 cultured on whey showed a lower growth than the phototrophic one, especially at higher concentrations (in the second mixotrophic screening). In all experiments, the phosphate concentration reached the limitation relatively quickly. For the extensively investigated nitrogen concentration, slower degradation was observed in the shake flask experiments, which was never completely limited in some cases. The PHB content of the mixotrophically grown microorganisms is with a maximum of 3.5% at the highest lactose concentrations (5 and 10 g/L) and 2.2% with 1 g/L below the 4.2% of the phototrophic culture. The comparatively poor performance of phototrophic-grown Mt cannot be explained with the available data. The first mixotrophic screening observed better PHB production of Mt, but the precipitate most likely influenced the data. The microorganism does not metabolise lactose, so there is yet no comprehensible explanation why a decrease in lactose concentration was measured in the experiments. The screening showed that added whey negatively affects PHB and biomass production as long as the *Synechocystis* sp. PCC 6714 (and its mutant) used here cannot metabolise lactose or it is added hydrolysed. The lowest lactose concentration of 1 g/L showed in combination with the biomass concentration the best results, which was also used in the PBR.

The biomass concentration and growth in the PBR were larger than in the shake flask and the nitrate and phosphate limitation were reached earlier. Furthermore, the PHB contents achieved were also about twice as high as in the screening, at 10.5% (21.1 mg/L/day) for the phototrophic and 6.5% (7.7 mg/L/day) for the mixotrophic cultivation. Thus, the PHB content is below 20.4% from the earlier publication by Kamravamanesh et al.[91] As in the screening experiment, the lactose concentration decreased, and the sugar negatively affected the PHB content and growth.

The comparison of the acidic and alkaline PHB hydrolysis showed that sulfuric acid converted almost twice as much PHB into crotonic acid as NaOH. Therefore, the sulfuric acid method was chosen for further optimisation because of the high PHB decomposition to crotonic acid. The results show that 16 M H_2SO_4 at 100°C and 160 min (without shaking) slightly reduces the viscosity compared to the concentrated acid (18 M). However, the recovery rate of crotonic acid does not change statistically significantly. Hence, the method presented here provides a reliable procedure for quantifying PHB.

The low recovery rate in chloroform extraction (max. 21wt%) is probably due to the poor accessibility of PHB in dried biomass. Therefore, various pretreatment steps are available to be used to improve recovery.

The solubility area of α-and β-values proposed by Kobayashi et al. was confirmed, as all three ILs values were in the range and solubilised the biomass. The [EMIM][OAc] catalyses the decomposition of PHB, which is probably due to the alkaline properties of the anion, especially at high temperatures, making it unsuitable for this application. [EMIM][DEP] and [EMIM]Cl do not dissolve the PHB, the latter becoming solid at room temperature. Complete dissolution of the biomass (10wt% biomass) after 1 h

at 75°C with [EMIM][DEP] did not reach the solubility of [EMIM][MP] presented by Kobayashi et al., which dissolved 0.1% biomass:IL after 0.5 h at room temperature.[129] The experiments showed that the PHB could be separated by centrifugation, making [EMIM][DEP] the preferred IL for further PHB recovery approaches.

F. Outlook

The following presents some essential open questions and future tasks for establishing a sustainable and economic PHB production with *Synechocystis* sp. PCC 6714. The composition of whey precipitate should be analysed in detail before and after pH adjustment, especially for calcium phosphate. If a recombinant *Synechocystis* sp. PCC 6714 capable of metabolising lactose was available, the effects of different lactose concentrations on the microorganism should be analysed and compared analogously to this work. In the absence of a recombinant lactose-metabolising microorganism, the addition of a BGAL to the cultivations could simulate this. In addition, the difference between whey and the mere addition of sugar would need to be investigated, which has been shown in *C. vulgaris* for hydrolysed whey compared to glucose and galactose.[18, 140] Furthermore, different pretreatments of the whey, e.g. the difference between ultra or nanofiltration, influence fermentation.[56] As shown in the literature, instead of time-consuming HPLC measurements, sugar concentrations in a biological system can be analysed for future experiments or industrial applications, the using FTIR/ATR (attenuated total reflection) in combination with an external light guiding fiber probe, which can be carried out automatically on-line or at-line.[162] In addition, other physicochemical analyses (Raman spectrometry, SERS), flow cytometry, photon density wave spectroscopy, and FTIR/ATR can quantify PHAs at-line, the latter of which can be performed in-line in combination with an ultrasound particle tool.[142] Furthermore, in-line hyperspectral imaging can indicate whether the cell is growing or producing PHB, thus indicating PHB content, even on larger scales.[163]

The drastic reduction of process costs by exceeding the threshold of 30% PHB content mentioned in the literature could not be achieved in the experiments of this thesis.[144] The recombinant *Synechocystis* sp. 6803 by Koch et al. exceeds this significantly with 80% (employing acetate) and is even comparable to heterotrophic microorganisms, showing great economic potential.[90] Therefore, a detailed cost analysis should be carried out. For a continuous process, a sophisticated two- or multi-step continuous process should be established, which could increase the productivity and quality of PHB and thus simplify downstream processing.[45, 83] When upscaling, which is crucial in phototrophic processes, care should be taken in the selection and design of the reactor to ensure sufficient and uniform illumination.[3, 7, 83] Process costs could also be reduced by switching from an expensive, chemically unstable medium to a complex medium, or at least by switching to cheaper medium components (HEPES), taking into account possible effects on metabolism.[30] Flue gases can be used as an low-cost source of CO_2 [83] and optimising the concentration could bring benefits.[8] Potential aging effects of PHB should be considered in experimental design and sample analysis.[45] PHB production in *Synechocystis* sp. can be further simulated using a kinetic/dynamic model, as described in the literature,[82] but possible mixotrophic growth would need to be included in the future.[31]

Despite the higher conversion of PHB into crotonic acid by the acid method, the alkaline, in addition to its low concentration, offers a methodological advantage that can be used in the future. The biomass does not have to be centrifuged and dried, but the desired base concentration can be adjusted by mixing the broth with NaOH solution. This dramatically reduces the time between sample collection and result to sample collection, mixing with NaOH, heating (e.g. 30 min), neutralisation with HCl, filtration (or centrifugation) and HPCL analysis (approx. 30 min). This could also be used for screening experiments

on a 96-well plate.[103] If the NaOH method is to be further optimised, lower concentrations (down to 0.5 M) and shorter heating times (down to 30 min) as boundaries should be chosen.[107-109]

Repeating the extraction should be done by pretreating the biomass, for which there are various methods. One of the most common methods is the use of NaClO[70, 120, 124] or, following the literature already discussed [71, 119], it would also be possible to grind the biomass to increase the recovery to at least 50wt%.

In a further step, PHB-containing biomass can be carried out with [EMIM][DEP]. Furthermore, additives should reduce the viscosity of IL to increase manageability. Fragmentation of PHB was reported in the literature when treated with [EMIM][DEP], which should be investigated in further work.[133] Unfortunately, there is evidence that imidazole-based ILs could be toxic.[114] Dubey et al. [133] and Filippi et al. [132] found a large amount of impurities in the PHAs, which could be minimised by changes in the process or further process steps. In this context, Pagliano et al. also argue that at a purity of about 70 to 80% one should no longer speak of extracted PHA but of a "refined microbial biomass". Therefore, these authors also state that the achieved purity and the corresponding separation problems in the two studies with realistic IL-to-biomass ratios (1:10 w/w) [132, 133] rather represent a pretreatment step, i.e. without further improvement of the process and, if necessary, adjustment of the ILs, this process does not represent a complete recovery process.[70] A subsequent chloroform extraction could increase purity.[129, 133]

Furthermore, it has to be discussed to what extent this process could be regarded as solvent extraction in the sense of Pagliano et al.[70] or as cell lysis, since the PHB is not dissolved by the ILs used, but methanol was used for precipitation. In addition, residues of the ILs could influence the crystallisation behaviour of the plastic via an ion-dipole interaction between the positively charged imid ring (of 1-ethyl-3-propylimidazolium bis(trifluoromethanesulfonyl) imide) and the ester group of the PHB.[164] For the bioplastic, further processing should be ecologically and economically compatible, which would make the recycling of the ILs mandatory and would further be improve by continuous extraction process.[70] The complete regeneration of the ILs could be analysed by measuring the viscosity, the ion mobility or with an infrared spectrometer (NMR would be possible but not practical for a large-scale process control).[112] The points presented here can lead to reduced production costs, which will expand the application range of PHB and help to replace non-renewable and non-biodegradable plastics.

G. Appendix

G.1. Calibration curve of two used spectrometers for OD_{750}

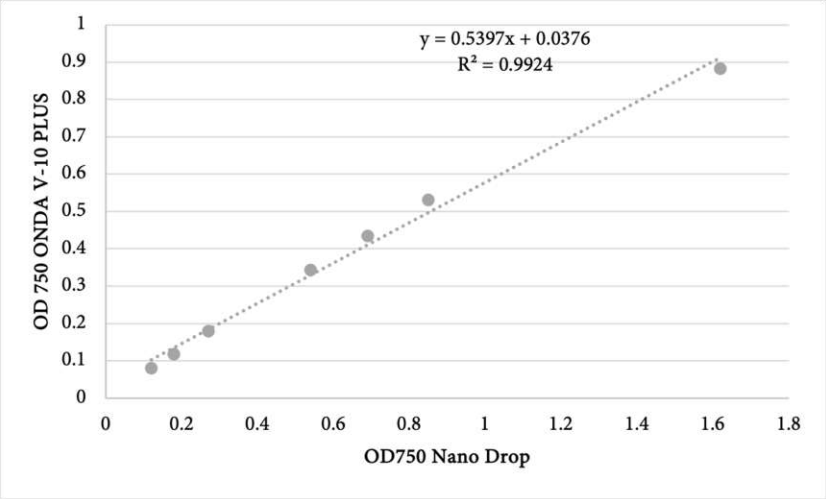


Figure 34: Calibration curve for conversion of OD750 of NanoDrop and ONDA V-10 Plus

G.2. Data of 14 and 18 M PHB quantification

Table 12: Data for statistical analysis of the quantification of 14 and 18 M sulfuric acid PHB

	Recovery crotonic acid	
H_2SO_4	18 M	14 M
	0.885	0.774
	0.789	0.765
	0.832	0.707
	0.787	0.735
	0.806	0.811
	0.779	0.778
		0.731
		0.839
		0.855
		0.802
		0.740
		0.790
		0.721
		0.773
		0.799
AVG	0.813	0.775
SD	0.040	0.043

G.3. pH– value in shake flask experiments

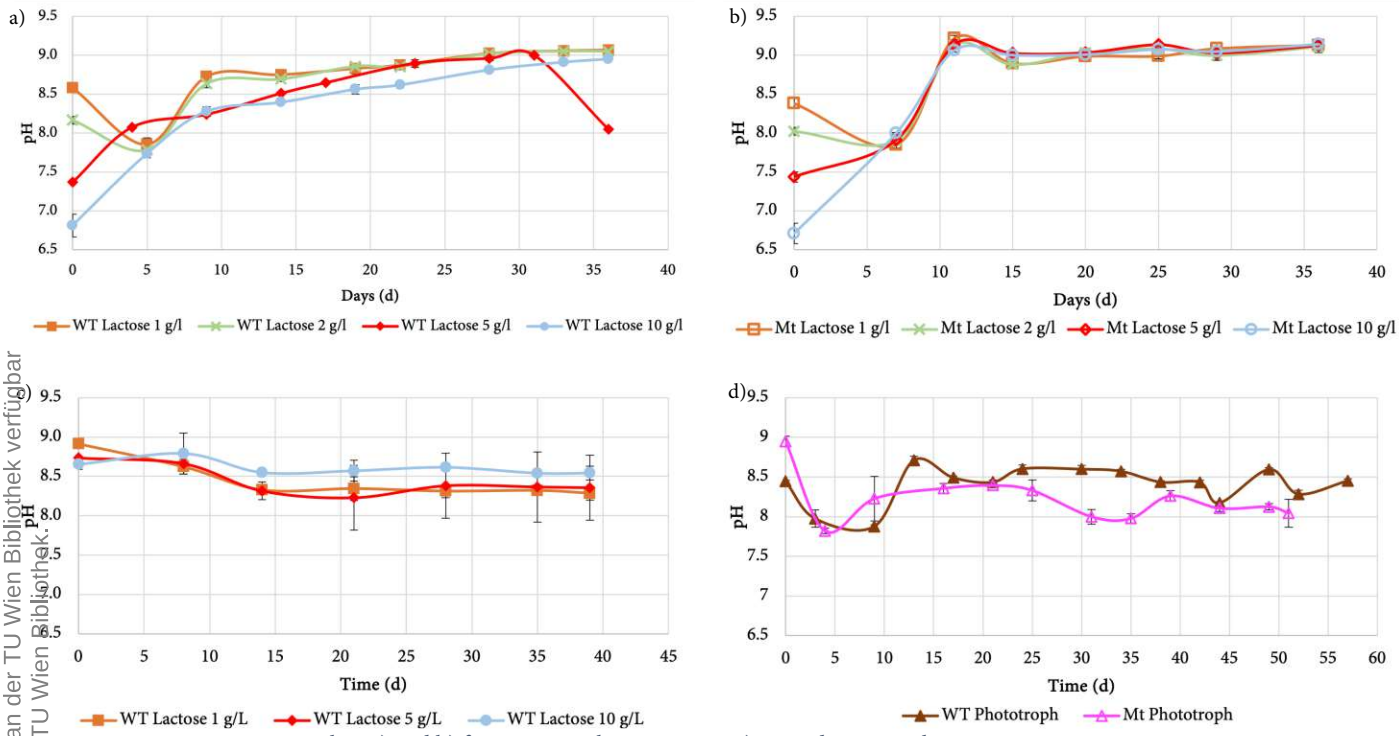


Figure 35: pH value: a) and b) first mixotrophic screening, c) second mixotrophic screening, d) phototrophic shake flask experiments

G.4. pH, CO_2-offgas and temperature in PBR

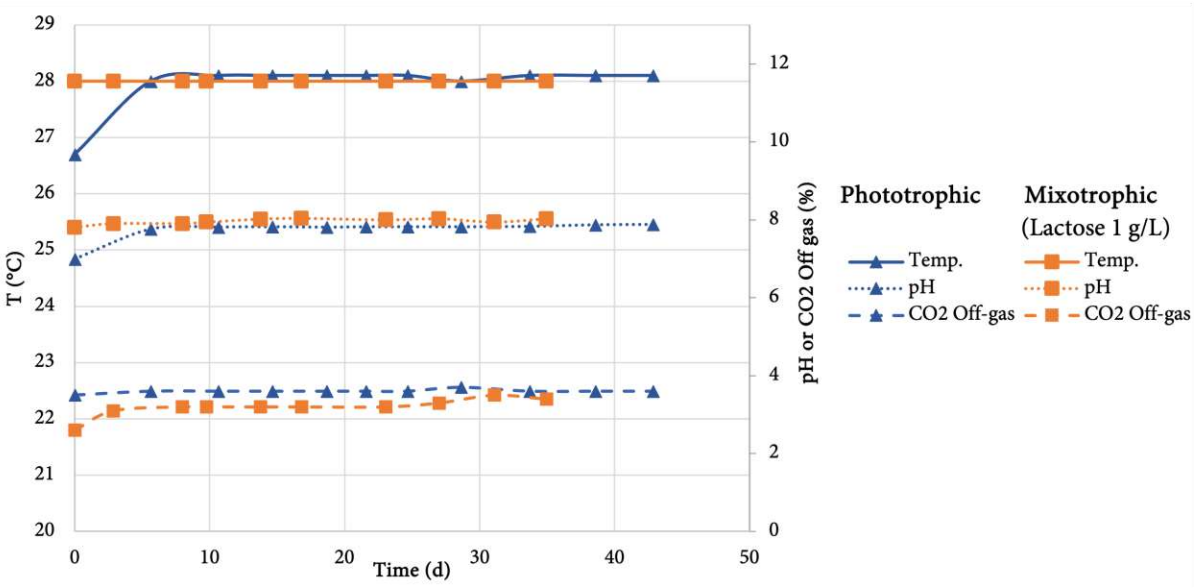


Figure 36: pH, CO_2 Off-gas and temperature during phototrophic and mixotrophic (1 g/L Lactose) fermentation in PBR

H. List of figures

I. List of tables

J. References

1. Kamravamanesh, D., et al., *Increased poly-beta-hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain Synechocystis sp. PCC 6714: Mutant generation and characterization.* Bioresour Technol, 2018. **266**: p. 34-44.
2. Wu, G.F., Q.Y. Wu, and Z.Y. Shen, *Accumulation of poly-β-hydroxybutyrate in cyanobacterium Synechocystis sp. PCC6803.* Bioresource Technology, 2001. **76**(2): p. 85-90.
3. Posten, C. and C. Walter, *Microalgal Biotechnology: Potential and Production*. 1. Aufl. ed. 2012, Berlin/Boston: Berlin/Boston: Walter de Gruyter GmbH Co.KG.
4. Wingender, J., *Cyanobakterien*. 2012, Thieme Gruppe.
5. Ruffing, A.M., *Engineered cyanobacteria: Teaching an old bug new tricks.* Bioengineered Bugs, 2011. **2**(3): p. 136-149.
6. Kamravamanesh, D., M. Lackner, and C. Herwig, *Bioprocess Engineering Aspects of Sustainable Polyhydroxyalkanoate Production in Cyanobacteria.* Bioengineering (Basel), 2018. **5**(4).
7. Johnson, T.J., et al., *Photobioreactor cultivation strategies for microalgae and cyanobacteria.* Biotechnology Progress, 2018. **34**(4): p. 811-827.
8. Samantaray, S., R. Bhati, and N. Mallick, *Cyanobacterial polyhydroxyalkanoates: an alternative source for plastics*, in *Cyanobacteria*. 2014. p. 227-244.
9. Drosg, B., et al., *Photo-autotrophic Production of Poly(hydroxyalkanoates) in Cyanobacteria.* Chemical and Biochemical Engineering Quarterly, 2015. **29**.
10. Cheregi, O., et al., *Microalgae biotechnology in Nordic countries - the potential of local strains.* Physiol Plant, 2019. **166**(1): p. 438-450.
11. Hossain, N., T. Meurah, and T.M.I. Mahlia, *Progress in physicochemical parameters of microalgae cultivation for biofuel production.* Critical Reviews in Biotechnology, 2019: p. 1-26.
12. Huang, Q., et al., *Design of Photobioreactors for Mass Cultivation of Photosynthetic Organisms.* Engineering, 2017. **3**(3): p. 318-329.
13. Masojídek, J. and G. Torzillo, *Mass Cultivation of Freshwater Microalgae*, in *Encyclopedia of Ecology*, S.E. Jørgensen and B.D. Fath, Editors. 2008, Academic Press: Oxford. p. 2226-2235.
14. Subashchandrabose, S.R., et al., *Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation.* Environment International, 2013. **51**: p. 59-72.
15. Ogbonna, J., E. Ichige, and H. Tanaka, *Interactions between photoautotrophic and heterotrophic metabolism in photoheterotrophic cultures of Euglena gracilis.* Applied Microbiology and Biotechnology, 2002. **58**(4): p. 532-538.
16. Girard, J.-M., et al., *Mixotrophic cultivation of green microalgae Scenedesmus obliquus on cheese whey permeate for biodiesel production.* Algal Research, 2014. **5**: p. 241-248.
17. Yadav, K., et al., *Chapter 4 - Algal physiology and cultivation*, in *Handbook of Algal Biofuels*, M. El-Sheekh and A.E.-F. Abomohra, Editors. 2022, Elsevier. p. 79-96.
18. Abreu, A.P., et al., *Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source.* Bioresource Technology, 2012. **118**: p. 61-66.
19. Zanette, C.M., et al., *Microalgae mixotrophic cultivation for β-galactosidase production.* Journal of Applied Phycology, 2019. **31**(3): p. 1597-1606.
20. Wang, Y., et al., *Characteristics of mixotrophic growth of Synechocystis sp. in an enclosed photobioreactor.* Biotechnology Letters, 2002. **24**(19): p. 1593-1597.
21. Commons, W. *File:Microalgae cultivation facility along the Kona Coast of the Big Island of Hawai'i.jpg --- Wikimedia Commons{,} the free media repository*. 2022; Available from: https://commons.wikimedia.org/w/index.php?title=File:Microalgae_cultivation_facility_along_the_Kona_Coast_of_the_Big_Island_of_Hawai%E2%80%99i.jpg&oldid=711282906.
22. Commons, W. *File:Photobioreactor PBR 4000 G IGV Biotech.jpg --- Wikimedia Commons{,} the free media repository*. 2021; Available from: https://commons.wikimedia.org/w/index.php?title=File:Photobioreactor_PBR_4000_G_IGV_Biotech.jpg&oldid=528518295.
23. Commons, W. *File:Bioreaktor quer2.jpg --- Wikimedia Commons{,} the free media repository*. 2022; Available from: https://commons.wikimedia.org/w/index.php?title=File:Bioreaktor_quer2.jpg&oldid=660826121.
24. Commons, W. *File:Photobioreactor PBR 500 P IGV Biotech.jpg --- Wikimedia Commons{,} the free media repository*. 2020; Available from: https://commons.wikimedia.org/w/index.php?title=File:Photobioreactor_PBR_500_P_IGV_Biotech.jpg&oldid=487891588.
25. Commons, W. *File:20120927 Tannenbaumreaktor.jpg --- Wikimedia Commons{,} the free media repository*. 2020; Available from: https://commons.wikimedia.org/w/index.php?title=File:20120927_Tannenbaumreaktor.jpg&oldid=460754133.
26. Sun, X., et al., *Development of a N-Acetylneuraminic Acid-Based Sensing and Responding Switch for Orthogonal Gene Regulation in Cyanobacterial Synechococcus Strains.* ACS Synthetic Biology, 2021. **10**(8): p. 1920-1930.
27. Borowitzka, M.A., *Commercial production of microalgae: ponds, tanks, tubes and fermenters.* Journal of Biotechnology, 1999. **70**(1): p. 313-321.

28. Socher, M.L., et al., *The challenge of scaling up photobioreactors: Modeling and approaches in small scale.* Engineering in Life Sciences, 2016. **16**(7): p. 598-609.
29. Kopf, M., et al., *Finished Genome Sequence of the Unicellular Cyanobacterium Synechocystis sp. Strain PCC 6714.* Genome Announc, 2014. **2**(4).
30. Iijima, H., et al., *Simultaneous increases in the levels of compatible solutes by cost-effective cultivation of Synechocystis sp. PCC 6803.* Biotechnology and Bioengineering, 2020. **117**(6): p. 1649-1660.
31. Carpine, R., et al., *Industrial Production of Poly-β-hydroxybutyrate from CO2: Can Cyanobacteria Meet this Challenge?* Processes, 2020. **8**(3): p. 323.
32. Bandyopadhyay, A., et al., *Novel Metabolic Attributes of the Genus Cyanothece, Comprising a Group of Unicellular Nitrogen-Fixing Cyanobacteria.* mBio, 2011. **2**(5): p. e00214-11.
33. Kamravamanesh, D.Q.K.D., *A quantitative, time-resolved approach to bioprocess understanding for the economic production of polyhydroxyalkanoate.* 2019, Wien: Wien. VII, 142 Blätter, Illustrationen, Diagramme.
34. Kamravamanesh, D., et al., *Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium Synechocystis sp. PCC 6714.* AMB Express, 2017. **7**(1): p. 143.
35. Smithers, G.W., *Whey and whey proteins—From 'gutter-to-gold'.* International Dairy Journal, 2008. **18**(7): p. 695-704.
36. Fox, P.F. and P.L.H. McSweeney, *Chapter 1 - Cheese: An Overview*, in *Cheese (Fourth Edition)*, P.L.H. McSweeney, et al., Editors. 2017, Academic Press: San Diego. p. 5-21.
37. Gigli, I., *Whey.* 2019: IntechOpen.
38. United States Department of Agriculture, F.A.S., *Dairy Production and Trade Developments.* 2021.
39. Baldasso, C., T.C. Barros, and I.C. Tessaro, *Concentration and purification of whey proteins by ultrafiltration.* Desalination, 2011. **278**(1): p. 381-386.
40. Choi, S.H. and S.J. Oh, *World production and consumption of cheese.* 2011. p. 301-308.
41. Zandona, E., M. Blažić, and A. Režek Jambrak, *Whey Utilization: Sustainable Uses and Environmental Approach.* Food technology and biotechnology, 2021. **59**(2): p. 147-161.
42. Schingoethe, D.J., *Whey Utilization in Animal Feeding: A Summary and Evaluation1, 2.* Journal of Dairy Science, 1976. **59**(3): p. 556-570.
43. Panghal, A., et al., *Whey valorization: current options and future scenario – a critical review.* Nutrition & Food Science, 2018. **48**.
44. Ryan, M.P. and G. Walsh, *The biotechnological potential of whey.* Reviews in Environmental Science and Bio/Technology, 2016. **15**(3): p. 479-498.
45. McAdam, B., et al., *Production of Polyhydroxybutyrate (PHB) and Factors Impacting Its Chemical and Mechanical Characteristics.* Polymers, 2020. **12**: p. 2908.
46. Lang, C. and J. Hartmann-Schreier, *β-Galactosidase.* 2009, Thieme Gruppe.
47. Oliveira, C., P.M.R. Guimarães, and L. Domingues, *Recombinant microbial systems for improved β-galactosidase production and biotechnological applications.* Biotechnology Advances, 2011. **29**(6): p. 600-609.
48. Husain, Q., *β Galactosidases and their potential applications: a review.* Critical Reviews in Biotechnology, 2010. **30**(1): p. 41-62.
49. Davies, C.M., et al., *Plant and algal interference in bacterial beta-D-galactosidase and beta-D-glucuronidase assays.* Appl Environ Microbiol, 1994. **60**(11): p. 3959-64.
50. Wynne, M.J. and J.K. Hallan, *Reinstatement of Tetradesmus G. M. Smith (Sphaeropleales, Chlorophyta).* Feddes repertorium, 2016. **126**(ISSN: 0014-8962): p. 86.
51. Schoch, C.L., et al., *NCBI Taxonomy: a comprehensive update on curation, resources and tools* Database (Oxford), 2020. **2020**.
52. Bentahar, J., et al., *Investigation of β-galactosidase production by microalga Tetradesmus obliquus in determined growth conditions.* Journal of Applied Phycology, 2019. **31**(1): p. 301-308.
53. Cerón-García, M.C., et al., *Mixotrophic production of marine microalga Phaeodactylum tricornutum on various carbon sources.* Journal of microbiology and biotechnology, 2006. **16**: p. 689.
54. Ghorbani, E., et al., *Metal removal capability of two cyanobacterial species in autotrophic and mixotrophic mode of nutrition.* BMC Microbiology, 2022. **22**(1): p. 58.
55. Hu, w., *Dry Weight and Cell Density of Individual Algal and Cyanobacterial Cells for Algae*, in *Faculty of the Graduate School.* 2014, University of Missouri-Columbia: Missouri-Columbia.
56. Vieira Salla, A.C., et al., *Increase in the carbohydrate content of the microalgae Spirulina in culture by nutrient starvation and the addition of residues of whey protein concentrate.* Bioresource Technology, 2016. **209**: p. 133-141.
57. Shen, L., et al., *Characterization of extracellular polysaccharide/protein contents during the adsorption of Cd(II) by Synechocystis sp. PCC6803.* Environmental Science and Pollution Research, 2018. **25**(21): p. 20713-20722.
58. Qi, F., et al., *Construction, characterization and application of molecular tools for metabolic engineering of Synechocystis sp.* Biotechnology Letters, 2013. **35**(10): p. 1655-1661.
59. Thelwell, C., N.J. Robinson, and J.S. Turner-Cavet, *An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10728-33.
60. Sun, T., et al., *Re-direction of carbon flux to key precursor malonyl-CoA via artificial small RNAs in photosynthetic Synechocystis sp. PCC 6803.* Biotechnology for Biofuels, 2018. **11**(1): p. 26.
61. Fang, F. and S.R. Barnum, *Expression of the Heat Shock Gene hsp16.6 and Promoter Analysis in the Cyanobacterium, Synechocystis sp. PCC 6803.* Current Microbiology, 2004. **49**(3): p. 192-198.

62. Li, S., et al., *Development and optimization of genetic toolboxes for a fast-growing cyanobacterium Synechococcus elongatus UTEX 2973.* Metabolic Engineering, 2018. **48**: p. 163-174.
63. Mermet-Bouvier, P. and F. Chauvat, *A conditional expression vector for the cyanobacteria Synechocystis sp. strains PCC6803 and PCC6714 or Synechococcus sp. strains PCC7942 and PCC6301.* Curr Microbiol, 1994. **28**(3): p. 145-8.
64. Policastro, G., A. Panico, and M. Fabbricino, *Improving biological production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) co-polymer: a critical review.* Reviews in Environmental Science and Bio/Technology, 2021. **20**(2): p. 479-513.
65. Kumar, A., et al., *Commercialization of Bacterial Cell Factories for the Sustainable Production of Polyhydroxyalkanoate Thermoplastics: Progress and Prospects.* Recent Patents on Biotechnology, 2015. **9**: p. 4-21.
66. Steinbüchel, A. and H.E. Valentin, *Diversity of bacterial polyhydroxyalkanoic acids.* FEMS Microbiology Letters, 1995. **128**(3): p. 219-228.
67. Koch, M. and K. Forchhammer, *Polyhydroxybutyrate: A Useful Product of Chlorotic Cyanobacteria.* Microbial Physiology, 2021. **31**(2): p. 67-77.
68. Markl, E., *PHB - Bio Based and Biodegradable Replacement for PP: A Review.* Novel Techniques in Nutrition & Food Science, 2018. **2**(5).
69. Aramvash, A., F. Moazzeni Zavareh, and N. Gholami Banadkuki, *Comparison of different solvents for extraction of polyhydroxybutyrate from Cupriavidus necator.* Engineering in Life Sciences, 2018. **18**(1): p. 20-28.
70. Pagliano, G., et al., *Recovery of Polyhydroxyalkanoates From Single and Mixed Microbial Cultures: A Review.* Frontiers in Bioengineering and Biotechnology, 2021. **9**.
71. Fei, T., et al., *Effective recovery of poly-β-hydroxybutyrate (PHB) biopolymer from Cupriavidus necator using a novel and environmentally friendly solvent system.* Biotechnology Progress, 2016. **32**(3): p. 678-685.
72. Kripp, T.C. and W. Caseri, *Poly(β-hydroxybuttersäure).* 2012, Thieme Gruppe.
73. Matavulj, M. and H.-P. Molitoris, *Biodegradation of polyhydroxyalkanoate-based plastic (BIOPOL) under different environmental conditions I. Weightloss of substrate.* Hoppea, 2000(61): p. 735-749.
74. Nanda, N. and N. Bharadvaja, *Algal bioplastics: current market trends and technical aspects.* Clean technologies and environmental policy, 2022. **24**(ISSN: 1618-954X): p. 2679.
75. Dilkes-Hoffman, L.S., et al., *Environmental impact of biodegradable food packaging when considering food waste.* Journal of Cleaner Production, 2018. **180**: p. 325-334.
76. Lhamo, P., S.K. Behera, and B. Mahanty, *Process optimization, metabolic engineering interventions and commercialization of microbial polyhydroxyalkanoates production – A state-of-the art review.* Biotechnology Journal, 2021. **16**(9): p. 2100136.
77. Jiang, Y., et al., *Polyhydroxybutyrate production from lactate using a mixed microbial culture.* Biotechnol Bioeng, 2011. **108**(9): p. 2022-35.
78. Hempel, F., et al., *Microalgae as bioreactors for bioplastic production.* Microbial Cell Factories, 2011. **10**(1): p. 81.
79. R, V., *Overview on Polyhydroxyalkanoates: A Promising Biopol.* Journal of Microbial & Biochemical Technology, 2011. **03**.
80. Bioplastics, E. *BIOPLASTICS MARKET DEVELOPMENT UPDATE 2021.* 2022; Available from: https://www.european-bioplastics.org/market/.
81. echemi. *There Was A Period of Loss Last Year, And PP Has Increased Its Production Capacity by 8.83 Million Tons This Year!* 2022 1.11.2022]; Available from: https://www.echemi.com/cms/603815.html#:~:text=In%202021%2C%20the%20total%20global,year%20increase%20of%20about%208.59%25.
82. Carpine, R., et al., *Poly-β-hydroxybutyrate (PHB) production by Synechocystis PCC6803 from CO2: Model development.* Algal Research, 2018. **29**: p. 49-60.
83. Sirohi, R., et al., *Sustainable production of polyhydroxybutyrate from autotrophs using CO2 as feedstock: Challenges and opportunities.* Bioresource Technology, 2021. **341**: p. 125751.
84. Anfelt, J., et al., *Genetic and nutrient modulation of acetyl-CoA levels in Synechocystis for n-butanol production.* Microbial Cell Factories, 2015. **14**(1): p. 167.
85. Wijffels, R.H., O. Kruse, and K.J. Hellingwerf, *Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae.* Current Opinion in Biotechnology, 2013. **24**(3): p. 405-413.
86. Ruiz, J.A., et al., *ESCHERICHIA COLI REDOX MUTANTS AS MICROBIAL CELL FACTORIES FOR THE SYNTHESIS OF REDUCED BIOCHEMICALS.* Computational and Structural Biotechnology Journal, 2012. **3**(4): p. e201210019.
87. Katayama, N., H. Iijima, and T. Osanai, *Production of Bioplastic Compounds by Genetically Manipulated and Metabolic Engineered Cyanobacteria.* Adv Exp Med Biol, 2018. **1080**: p. 155-169.
88. Kaewbai-ngam, J., et al., *Production of glycogen, PHB, biohydrogen, NAD(P)H, and proteins in Synechocystis sp. PCC 6803 disrupted in metabolically linked biosynthetic pathway(s).* Journal of Applied Phycology, 2022. **34**(4): p. 1983-1995.
89. Koch, M., et al., *PHB is Produced from Glycogen Turn-over during Nitrogen Starvation in Synechocystis sp. PCC 6803.* Int J Mol Sci, 2019. **20**(8).
90. Koch, M., et al., *Maximizing PHB content in Synechocystis sp. PCC 6803: a new metabolic engineering strategy based on the regulator PirC.* Microb Cell Fact, 2020. **19**(1): p. 231.
91. Kamravamanesh, D., et al., *Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain Synechocystis sp. PCC 6714: Bioprocess understanding and evaluation of productivities.* Bioresour Technol, 2019. **273**: p. 277-287.

92. Panda, B. and N. Mallick, *Enhanced poly-β-hydroxybutyrate accumulation in a unicellular cyanobacterium, Synechocystis sp. PCC 6803.* Letters in Applied Microbiology, 2007. **44**(2): p. 194-198.
93. Lee, J., et al., *Recent progress and challenges in microbial polyhydroxybutyrate (PHB) production from CO2 as a sustainable feedstock: A state-of-the-art review.* Bioresource Technology, 2021. **339**: p. 125616.
94. Lackner, M., et al., *Characterization of photosynthetically synthesized poly(3-hydroxybutyrate) using a randomly mutated strain of Synechocystis sp. PCC 6714.* International Journal of Biobased Plastics, 2019. **1**(1): p. 48-59.
95. Wang, B., et al., *Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO2.* Metabolic Engineering, 2013. **16**: p. 68-77.
96. Sudesh, K., K. Taguchi, and Y. Doi, *Effect of increased PHA synthase activity on polyhydroxyalkanoates biosynthesis in Synechocystis sp. PCC6803.* International Journal of Biological Macromolecules, 2002. **30**(2): p. 97-104.
97. Tyo, K.E., et al., *Identification of gene disruptions for increased poly-3-hydroxybutyrate accumulation in Synechocystis PCC 6803.* Biotechnol Prog, 2009. **25**(5): p. 1236-43.
98. Amadu, A.A., et al., *A review of biopolymer (Poly-β-hydroxybutyrate) synthesis in microbes cultivated on wastewater.* Science of The Total Environment, 2021. **756**: p. 143729.
99. Dalal, J. and B. Lal, *Microbial Polyhydroxyalkanoates: Current Status and Future Prospects*, in *High Value Fermentation Products*. 2019. p. 351-387.
100. Berwig, K.H., C. Baldasso, and A. Dettmer, *Production and characterization of poly(3-hydroxybutyrate) generated by Alcaligenes latus using lactose and whey after acid protein precipitation process.* Bioresource Technology, 2016. **218**: p. 31-37.
101. Mostafa, Y.S., et al., *Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium Pseudodonghicola xiamenensis through date syrup valorization and structural assessment of the biopolymer.* Scientific Reports, 2020. **10**(1).
102. Fiorese, M.L., et al., *Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1,2-propylene carbonate.* Engineering in Life Sciences, 2009. **9**(6): p. 454-461.
103. Watanabe, Y., et al., *Development and validation of an HPLC-based screening method to acquire polyhydroxyalkanoate synthase mutants with altered substrate specificity.* Journal of Bioscience and Bioengineering, 2012. **113**(3): p. 286-292.
104. Karr, D.B., J.K. Waters, and D.W. Emerich, *Analysis of Poly-β-Hydroxybutyrate in Rhizobium japonicum Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV Detection.* Applied and Environmental Microbiology, 1983. **46**(6): p. 1339-1344.
105. Liang, S.-Y., et al., *Rapid Quantification of Polyhydroxybutyrate Polymer from Single Bacterial Cells with Mass Spectrometry.* Analytical Chemistry, 2022. **94**(34): p. 11734-11738.
106. Kučera, F., J. Petruš, and J. Jančář, *The structure-hydrolysis relationship of poly(3-hydroxybutyrate).* Polymer Testing, 2019. **80**: p. 106095.
107. Yu, J., D. Plackett, and L.X.L. Chen, *Kinetics and mechanism of the monomeric products from abiotic hydrolysis of poly[(R)-3-hydroxybutyrate] under acidic and alkaline conditions.* Polymer Degradation and Stability, 2005. **89**(2): p. 289-299.
108. Del Don, C., et al., *Biomass composition and methods for the determination of metabolic reserve polymers in phototrophic sulfur bacteria.* Aquatic Sciences, 1994. **56**(1): p. 1-15.
109. Don, C.D. and H. Brandl, *Poly-3-hydroxybutyrat (PHB)*, I.o.P.B.M. Microbial Ecology Group/ University of Zürich, Editor.: Zürich
110. Autor, R., R. Redaktion, and A. Wüst, *ionische Flüssigkeiten*. 2022, Thieme Gruppe.
111. Shiflett, M.B. and A.M. Scurto, *Ionic Liquids: Current State and Future Directions*, in *Ionic Liquids: Current State and Future Directions*. 2017, American Chemical Society. p. 1-13.
112. Kar, M., J.M. Pringle, and D.R. MacFarlane, *Fundamentals of ionic liquids*. 2017: Wiley-VCH.
113. Muller, P., *Glossary of terms used in physical organic chemistry (IUPAC Recommendations 1994).* Pure and Applied Chemistry, 1994. **66**(5): p. 1077-1184.
114. Cho, C.-W., et al., *Environmental Concerns Regarding Ionic Liquids in Biotechnological Applications*, in *Application of Ionic Liquids in Biotechnology*, T. Itoh and Y.-M. Koo, Editors. 2019, Springer International Publishing: Cham. p. 241-328.
115. Zavgorodnya, O., et al., *Translational Research from Academia to Industry: Following the Pathway of George Washington Carver*, in *Ionic Liquids: Current State and Future Directions*. 2017, American Chemical Society. p. 17-33.
116. Schubert, T.J.S., *Current and Future Ionic Liquid Markets*, in *Ionic Liquids: Current State and Future Directions*. 2017, American Chemical Society. p. 35-65.
117. Roosen, C., P. Schäfer, and L. Greiner, *Ionic liquids in biotechnology: Applications and perspectives for biotransformations.* Applied microbiology and biotechnology, 2008. **81**: p. 607-14.
118. marketsandmarkets. *Ionic Liquids - Global Market Trajectory & Analytics*. 2021; Available from: https://www.globenewswire.com/news-release/2021/10/26/2320403/28124/en/Global-Ionic-Liquids-Market-Report-2021-2026-with-Profiles-of-Major-Operators-Suppliers-BASF-Ionic-Liquid-Chemicals-Koei-Chemical-Co-Proionic-Solvay-and-Synthio-Chemicals.html.
119. Mongili, B., et al., *Novel insights in dimethyl carbonate-based extraction of polyhydroxybutyrate (PHB).* Biotechnol Biofuels, 2021. **14**(1): p. 13.
120. Hahn, S.K., et al., *The recovery of poly(3-hydroxybutyrate) by using dispersions of sodium hypochlorite solution and chloroform.* Biotechnology Techniques, 1993. **7**(3): p. 209-212.
121. Koller, M., *Established and advanced approaches for recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from surrounding microbial biomass.* The EuroBiotech Journal, 2020. **4**(3): p. 113-126.

122. Hand, S., J. Gill, and K.H. Chu, *Phage-based extraction of polyhydroxybutyrate (PHB) produced from synthetic crude glycerol.* Sci Total Environ, 2016. **557-558**: p. 317-21.
123. Hejazi, P., E. Vasheghani-Farahani, and Y. Yamini, *Supercritical Fluid Disruption of Ralstonia eutropha for Poly(ß-hydroxybutyrate) Recovery.* Biotechnology Progress, 2003. **19**(5): p. 1519-1523.
124. Martínez-Herrera, R.E., et al., *Efficient recovery of thermostable polyhydroxybutyrate (PHB) by a rapid and solvent-free extraction protocol assisted by ultrasound.* Int J Biol Macromol, 2020. **164**: p. 771-782.
125. chempedia. *ECOENG*. 28.10.2022]; Available from: https://chempedia.info/info/ecoeng/.
126. Hecht, S.E.W.C., OH, US), Niehoff, Raymond Louis (Hamilton, OH, US), Narasimhan, Karunakaran (West Chester, OH, US), Neal, Charles William (Fairfield, OH, US), Forshey, Paul Arlen (Cincinnati, OH, US), Phan, Dean Van (West Chester, OH, US), Brooker, Anju Deepali Massey (Gosforth, GB), Combs, Katherine Helen (Cincinnati, OH, US), *Extracting biopolymers from a biomass using ionic liquids*, O. The Procter & Gamble Company (Cincinnati, US), Editor. 2006: United States.
127. Sequeira, R.A., et al., *Neoteric Solvent Systems as Sustainable Media for Dissolution and Film Preparation of Poly-[(R)-3-hydroxybutyrate].* ACS Sustainable Chemistry & Engineering, 2020. **8**(32): p. 12005-12013.
128. Fujita, K., et al., *Direct dissolution of wet and saliferous marine microalgae by polar ionic liquids without heating.* Enzyme and Microbial Technology, 2013. **52**(3): p. 199-202.
129. Kobayashi, D., et al., *A simple recovery process for biodegradable plastics accumulated in cyanobacteria treated with ionic liquids.* Applied Microbiology and Biotechnology, 2015. **99**(4): p. 1647-1653.
130. Favre, H.A. and W.H. Powell, *Nomenclature of Organic Chemistry*. 2014: The Royal Society of Chemistry.
131. Chiappe, C., et al., *Temperature effects on the viscosity and the wavelength-dependent refractive index of imidazolium-based ionic liquids with a phosphorus-containing anion.* Phys. Chem. Chem. Phys., 2017. **19**.
132. Filippi, S., et al., *Extraction of Polyhydroxyalkanoates from Purple Non-Sulfur Bacteria by Non-Chlorinated Solvents.* Polymers (Basel), 2021. **13**(23).
133. Dubey, S., et al., *1-Ethyl-3-methylimidazolium Diethylphosphate Based Extraction of Bioplastic "Polyhydroxyalkanoates" from Bacteria: Green and Sustainable Approach.* ACS Sustainable Chemistry & Engineering, 2018. **6**(1): p. 766-773.
134. (CIEL), C.f.I.E.L. *FOSSIL FUELS & PLASTIC*. 2022; Available from: https://www.ciel.org/issue/fossil-fuels-plastic/.
135. OECD. *Plastic pollution is growing relentlessly as waste management and recycling fall short, says OECD*. 2022; Available from: https://www.oecd.org/environment/plastic-pollution-is-growing-relentlessly-as-waste-management-and-recycling-fall-short.htm.
136. Gould, R. *RETHINKING THE FUTURE OF PLASTICS*. 2022; Available from: https://www.iso.org/news/ref2792-1.html.
137. Möller, D., *Chemistry of the climate system*, in *Chemistry of the Climate System*. 2014, de Gruyter.
138. Harvey, D. *Optimizing the Experimental Procedure*. 2019; Available from: https://chem.libretexts.org/Courses/Northeastern_University/14%3A_Developing_a_Standard_Method/14.1%3A_Optimizing_the_Experimental_Procedure.
139. Redaktion, R. and F. Geldsetzer, *Chlormethane*. 2010, Thieme Gruppe.
140. Hou, L., et al., *Enhanced polyhydroxybutyrate production from acid whey through determination of process and metabolic limiting factors.* Bioresource Technology, 2021. **342**: p. 125973.
141. Spanos, N., et al., *Precipitation of Calcium Phosphate from Simulated Milk Ultrafiltrate Solutions.* Crystal Growth & Design, 2007. **7**(1): p. 25-29.
142. Doppler, P., et al., *In Situ Quantification of Polyhydroxybutyrate in Photobioreactor Cultivations of Synechocystis sp. Using an Ultrasound-Enhanced ATR-FTIR Spectroscopy Probe.* Bioengineering, 2021. **8**(9): p. 129.
143. Carpine, R., et al., *The Cyanobacterial Route to Produce Poly-ß-hydroxybutyrate.* Chemical Engineering Transactions, 2015. **43**: p. 289-294.
144. Rueda, E., B. Altamira-Algarra, and J. García, *Process optimization of the polyhydroxybutyrate production in the cyanobacteria Synechocystis sp. and Synechococcus sp.* Bioresource Technology, 2022. **356**: p. 127330.
145. Kanwal, S., R.P. Rastogi, and A. Incharoensakdi, *Glutamate decarboxylase activity and gamma-aminobutyric acid content in Synechocystis sp. PCC 6803 under osmotic stress and different carbon sources.* Journal of Applied Phycology, 2014. **26**(6): p. 2327-2333.
146. Wu, G., et al., *Sodium acetate stimulates PHB biosynthesis in synechocystis sp. PCC 6803.* Tsinghua Science and Technology, 2002. **7**(4): p. 435-438.
147. Laboratories, K. *Galactose metabolism - Synechocystis sp. PCC 6803* Available from: https://www.genome.jp/pathway/syn00052.
148. Palabhanvi, B., et al., *Preferential utilization of intracellular nutrients supports microalgal growth under nutrient starvation: multi-nutrient mechanistic model and experimental validation.* Bioresour Technol, 2014. **173**: p. 245-255.
149. Wang, B., et al., *Unlocking the photobiological conversion of CO2 to (R)-3-hydroxybutyrate in cyanobacteria.* Green Chemistry, 2018. **20**(16): p. 3772-3782.
150. Koch, M., K.W. Berendzen, and A.K. Forchhammer, *On the Role and Production of Polyhydroxybutyrate (PHB) in the Cyanobacterium Synechocystis sp. PCC 6803.* Life (Basel), 2020. **10**(4).
151. Ueda, S., et al., *Anionic metabolite biosynthesis enhanced by potassium under dark, anaerobic conditions in cyanobacteria.* Scientific Reports, 2016. **6**(1): p. 32354.
152. Chen, L.X.L. and J. Yu, *Abiotic Hydrolysis of Poly[(R)-3-hydroxybutyrate] in Acidic and Alkaline Media.* Macromolecular Symposia, 2005. **224**(1): p. 35-46.
153. Kang, S. and J. Yu, *Reaction routes in catalytic reforming of poly(3-hydroxybutyrate) into renewable hydrocarbon oil.* RSC Advances, 2015. **5**(38): p. 30005-30013.

154. Satoh, H., et al., *Application of the Alkaline-Digestion-HPLC Method to the Rapid Determination of Polyhydroxyalkanoate in Activated Sludge.* Journal of Water and Environment Technology, 2016. **14**(5): p. 411-421.
155. Duvigneau, S., et al., *Fast, inexpensive, and reliable HPLC method to determine monomer fractions in poly(3-hydroxybutyrate-co-3-hydroxyvalerate).* Applied Microbiology and Biotechnology, 2021. **105**(11): p. 4743-4749.
156. *Appendix A - Sulfuric acid properties*, in *Sulfuric Acid Manufacture (Second Edition)*, M.J. King, W.G. Davenport, and M.S. Moats, Editors. 2013, Elsevier: Oxford. p. 363-367.
157. Manangan, T. and S. Shawaphun, *Quantitative extraction and determination of polyhydroxyalkanoate accumulated in Alcaligenes latus dry cells.* ScienceAsia, 2010. **36**.
158. Jablonski, P., et al., *Sustainable, Highly Selective, and Metal-free Thermal Depolymerization of Poly-(3-hydroxybutyrate) to Crotonic acid in Recoverable Ionic Liquids.* Green Chemistry, 2022. **24**.
159. Proionics. *Coordinating ionic liquids - efficient solvents for dissolution and processing of biomass*. 30.10.2022; Available from: https://proionic.com/downloads/files/proionic-downloads/newsletter-30092020.html.
160. Mouret, A., et al., *Eco-friendly solvents and amphiphilic catalytic polyoxometalate nanoparticles: a winning combination for olefin epoxidation.* Green Chemistry, 2014. **16**: p. 269-278.
161. Kang, S., et al., *Tailored recycling chemicals and fuels from poly-3-hydroxybutyrate: A review.* Biofuels, Bioproducts and Biorefining, 2022. **16**(5): p. 1412-1427.
162. Girard, J.-M., et al., *FT-IR/ATR univariate and multivariate calibration models for in situ monitoring of sugars in complex microalgal culture media.* Bioresource Technology, 2013. **144**: p. 664-668.
163. Rodríguez Lorenzo, F., et al., *Monitoring PHB production in Synechocystis sp. with hyperspectral images.* Water science and technology, 2022. **86**(ISSN: 0273-1223): p. 226.
164. Lee, L.-T. and C.-T. Yang, *Investigations on Green Blends Comprising Biodegradable Polymer and Ionic Liquid.* Polymers, 2016. **8**: p. 444.