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Northern Icelandic Cyanobacteria Biorefinery as source of a versatile platform chemical for producing high value biodegradable plastics, pigments and residual biomass for aquafeeds

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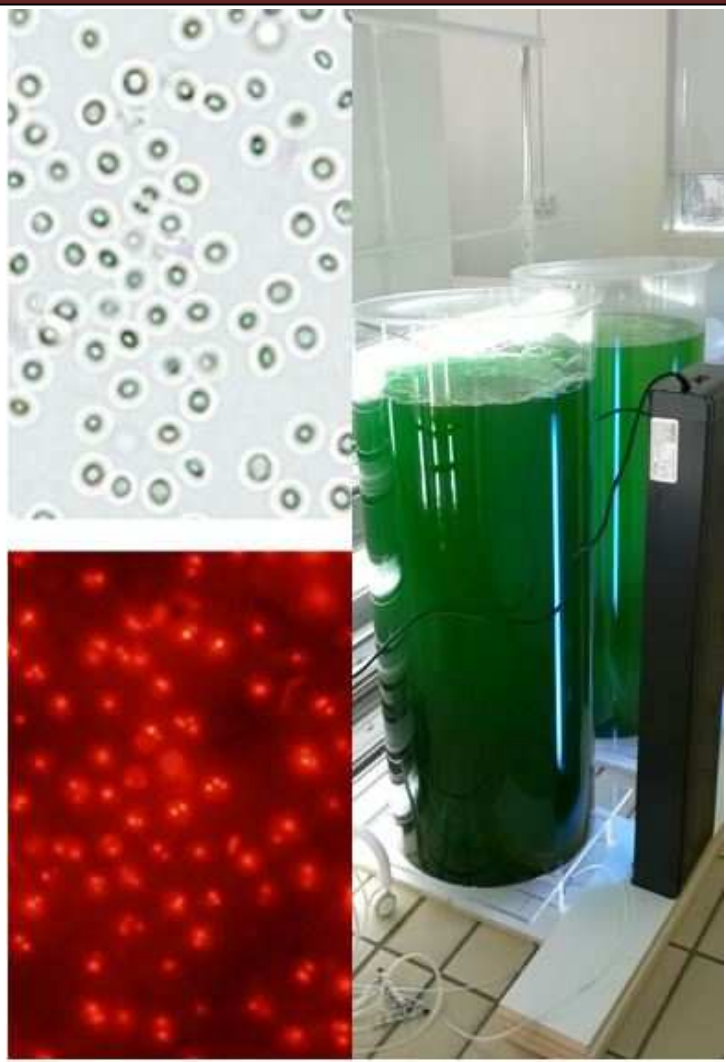


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Report Summary

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Title	Northern Icelandic Cyanobacteria Biorefinery as source of a versatile platform chemical for producing high value biodegradable plastics, pigments and residual biomass for aquafeeds	
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Summary: Synthetic polymers are potent environmental toxic pollutants which are non-degradable and are accumulating in the environment. Therefore, the efforts of scientists have been focused on alternative natural biopolymers. Among the biodegradable polymers, polyhydroxyalkanoates (PHAs), including polyhydroxy-butyrate (PHB), have been recently recognized due to their remarkable features as biodegradable thermoplastics. In the present study, three marine cyanobacteria (*Synechococcus* strain 14CY-P-SYN-SKA, *Synechocystis* strain 15CY-P-SYI-SKA and *Spirulina subsalsa* stain 14CY-P-SS-SKA) from the indoor culture collection of Icelandic protists at BioPol ehf, were tested for their potential to produce PHB under six different nutrient depletion scenarios. A biorefinery approach was applied containing two extraction steps, resulting in three product classes which are listed in the following in the order of their downstream processing: 1) photosynthetic pigments, 2) PHB, and 3) residual biomass for potential aquafeed production. While conventional extractions methods worked well for the recovery of the first two product classes, the concentration of proteins, lipids and carbohydrates in the "residual biomass" were very low. Therefore, several different alternative extraction procedures were tested with the goal to obtain a green environmentally friendly process and to gain simultaneously higher lipid and protein contents after PHB recovery. These alternative procedures comprised the use of ultrasound assisted extraction in combination with ionic liquids and dimethyl carbonate (DMC) as "environmentally friendly" solvents.

In the initial nutrient impact experiment, the highest PHB concentration was detected in *Synechocystis* (27.3% DW) at an 75% decrease of K_2HPO_4 in the growth medium followed by *Synechococcus* (21.5% DW) at an 75% decrease of $NaNO_3$. The lowest PHB content was found in *S. subsalsa* in all test assays, ranging between 0.9% and 3.6% DW at an 50% decrease of K_2HPO_4 and at an 50% decrease of $NaNO_3$, respectively. The use of alternative solvents showed best recovery results for DMC. Using FTIR, ^{1}H NMR and GC-MS, the DMC extracted polymer was identified as PHB.

Phycocyanin was the most abundant photosynthetic pigment in all tested species, being the highest in *Synechocystis* (34.94 and 32.26 µg · cell^{-1} in the control and 2-HEAF assays, respectively). Followed by phycoerythrin and allophycocyanin. Beside this, the Icelandic *Spirulina* species had also above average concentrations of chlorophyll *a* and total carotenoids (9.26 and 5.15 µg · cell^{-1} in the control assays). Phycocyanin was purified using a multistep procedure resulting in a final recovery of the eluted C-PC of 49% with a purity ratio (A_{620} /A_{280}) of 4.33. In the final step, the residual biomass was subjected to a biochemical gross composition analysis. The results showed that the highest recovery rates for protein (89±0.8%), lipids (95±2.0%) and carbohydrates (96±2.5%) were obtained after the use of the DMC/ethanol method when compared to the unpretreated control. To assess the nutritional value of the fractions of the three cyanobacteria after the final treatment with DCM, the amino acid (AA) and fatty acid (FA) compositions were evaluated on untreated biomass samples (control) *versus* residual biomass samples. The individual AA and FA concentrations obtained were still relatively low, but rather typical for picocyanobacteria. In contrast, data detected for *S. spirulina* showed high potential for the use in the tentative aquafeed formula. Overall, four aquafeeds were further developed, using already established formula for salmon feeds from a former project.

Keywords: *Marine cyanobacteria*, *bioplastic*, *multiproduct biorefinery*

Contents

1. Introduction

Aquaculture, the world's most efficient producer of edible protein, continues to grow faster than any other major food sector in the world, in response to the rapidly increasing global demand for fish and seafood (FAO 2018). Ocean-derived fishmeal (FM) and fish oil (FO) in aquafeeds has raised sustainability concerns as the supply of wild marine forage fish cannot meet the growing demand and is depleting rapidly with devasting environmental consequences globally (e.g., Pauly & Zeller 2016, Checkley et al. 2017). Thus, aquafeed manufacturers have reduced, but not fully eliminated, FMFO and are seeking cost competitive and sustainable replacements (Sarker 2018). Terrestrial crops as alternatives have been too costly for broad adoption by aquafeed manufacturers and compete with the human food production (Sarker et al. 2020). Nutritional disadvantages and poor fillet quality have prompted researchers to investigate marine microalgae including cyanobacteria as potential FMFO replacements in fish feeds (e.g., Tibaldi et al. 2015, Sarker et al. 2016, Sørensen 2017, Bélanger-Lamonde et al. 2018, Gong et al. 2018, Beal et al. 2018). Particularly, cyanobacteria are used as food supplement because of their biochemical composition and easy digestibility (Sakthivel & Kathiresan 2016). Recently lipid accumulation of up to 26% of dry weight (DW) was reported for a marine *Synechococcus* grown under nitrogen-depleted conditions, whereas protein content exceeded 50% DW (Bochenski et al. 2019). However, for aquafeed manufacturers not only the relative proportion in a certain species is important but also their composition.

In modern life petroleum-based plastic has become indispensable due to its frequent use as an easily available and a low-cost packaging and moulding material. The global contamination with non-degradable plastic is a huge environmental burden of our time (Jambeck et al. 2015, Li et al. 2016) and its rapidly growing use is causing aquatic and terrestrial pollution. Under these circumstances, research and development for biodegradable plastic (bioplastics) is inevitable.

While bioplastics have been suggested as potential solution, they still represent only a very small fraction of the plastics overall used (Geyer & Jambeck 2017) and many of these bioplastics have unsatisfying biodegradation properties. The most common bioplastic, PLA (poly-lactic-acid), is barely degraded in marine environments (Narancic et al. 2018). This has led to an increasing interest into another class of bioplastics with improved degradation properties: polyhydroxyalkanoates (PHAs). PHAs are microbial polyesters and are among the most investigated biodegradable polymers in recent years, due to their chemical and physical features that include non-toxicity, biocompatibility, and biodegradability. Particular attention has been paid to poly-β-hydroxybutyrates (PHB) because of their interesting features such as thermoplastic processing, hydrophobicity, complete biodegradability, and biocompatibility with optical purity, resembling the commodity polymer polypropylene in its properties (e.g., Lackner 2015, Koch et al. 2020).

PHB is a type of microbial polyester that accumulates as a carbon/energy storage material in various microorganisms (Fig. 1). Many gram-positive and gram-negative bacteria (*Pseudomonas* sp., *Bacillus* sp., *Methylobacterium* sp.) synthesize PHB (Doi 1990). However, cyanobacteria are the only oxygen-producing photosynthetic prokaryotes that accumulate PHB. The PHB production has been reported from cyanobacteria such as *Chloroglea fritschii* (Carr 1966), *Spirulina platensis* (Campbell et al. 1982), *Oscillatoria limosa* and *Spirulina* sp. (Stal et al. 1990, Vincenzini et al. 1990, Shrivastav et al. 2010), *Synechocystis* sp. PCC 6803 (Wu et al. 2001, Panda & Mallic 2007), *Nostoc muscorum* (Sharma & Mallic

2005a, b) and *Aulosira fertilissima* (Samantaray & Mallic 2012). Kamravanesh et al. (2018) found that a mutant strain of *Synechocystis* sp. PCC 6714 showed 2.5-fold higher productivity than their wild type under nitrogen and phosphorus starvation. Similarly, Troschl et al. (2018) produced poly-β-hydroxybutyrate from *Synechocytis* sp. CCALA192 at pilot-scale with carbon dioxide as a sole carbon source in a photobioreactor. However, studies related PHB productions by cyanobacterial members under different environmental conditions are still limited.

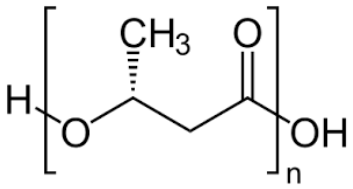

Figure 1. General structure of poly-β-hydroxybutyrate PHB.

Under conditions of nutrient limitation, for example nitrogen starvation, the cells enter into a resting state in a process that is known as chlorosis (Allen & Smith 1969). During chlorosis, cyanobacteria do not only degrade their photosynthetic apparatus, but also accumulate large quantities of glycogen as a carbon- and energy-storage (Klotz et al. 2016, Doello et al. 2018). During the late stages of chlorosis, the cells start to degrade glycogen and convert it to PHB (Koch et al. 2019, Fig. 2). However, the intracellular amount of PHB in chlorotic cells remains rather low and only represents about 10–20% of the cell dry weight (CDW). A recent economic analysis suggests that one of the factors that make the production of PHB in cyanobacteria less attractive than that in heterotrophic organisms is the low ratio of PHB/CDW in cyanobacteria (Knöttner et al. 2019). One major goal is therefore, to optimize cyanobacteria so that they accumulate higher intracellular levels of PHB.

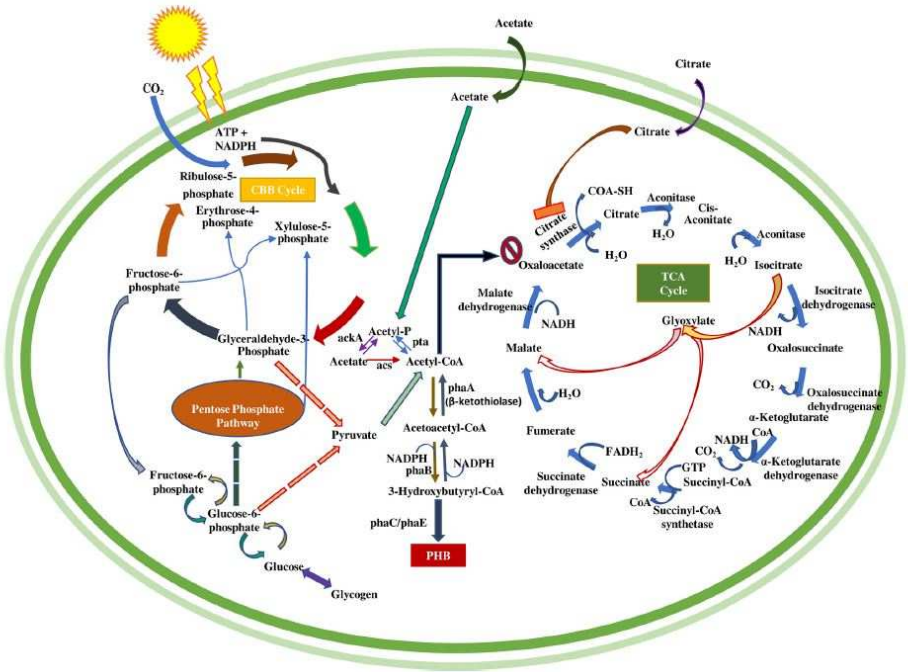

Figure 2. Schematic representation of PHB synthesis pathway. Cyanobacteria with native genes for PHB synthesis uses acetyl CoA as precursor to synthesize PHB in three steps. phaA: β-ketothiolase, phaB: acetoacetyl-CoA reductase, phaC/phaE: PHB synthase, ackA: acetate kinase, pta: Phosphate acetyltransferase, acs: acetyl-CoA synthetase (from Yashavanth et al. 2021).

For harvesting light energy, cyanobacteria as photoautotrophic organisms need pigments (Ritchie 2006, Ramos et al. 2011). The production of pigments and PHB may strongly be associated with each other, which causes problems during PHB purification (Miyake et al. 2000). These pigments include phycocyanin, carotenoids and chlorophylls, which are used in food industry, cosmetics, and biomedical research. All of them, but especially phycocyanin are sources of high valuable commercial products (Koller et al. 2014). Generating additional products (e.g., pigments, feed, fertiliser) as well as recycling nutrient streams and utilising residual biomass (Koller et al. 2014) is necessary to increase the economic efficiency of photoautotrophic PHB production. This is an important issue since the price of PHB is moderate (4–16 US$ kg^{-1}, Roland-Holst et al. 2013) and the production of PHB by cyanobacteria is, compared to heterotrophic bacteria, currently too low to have industrial potential (Drosg et al. 2015).

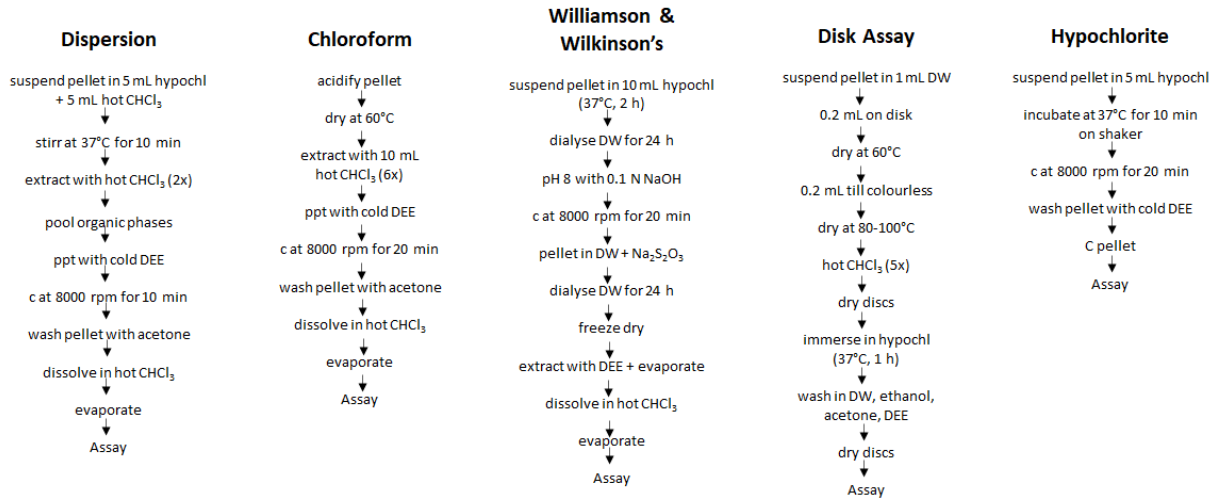

Figure 3. Overview of conventional PHB extraction methods according to Calleja (2020). Abbreviations: c= centrifugation; ppt=precipitate; DEE=Diethyl ether; DW= Distilled water; hypchl=sodium hypochlorite (2% w/v active chlorine)

Current conventional methods for PHB extraction comprise the excessive use of chlorinated solvents such as sodium hypochlorite and chloroform (Fig.3). The emission of these organic solvents to the atmosphere produces considerable damages to the environment (e.g., ozone layer degradation and/or environmental ozone formation) (e.g., Bais et al. 2018). The conventional process employed in liquid–liquid extractions consist of water-organic solvent two-phase systems and, therefore, are generally not suitable for separation of biomolecules due to problems such as protein denaturation (Klibanov 1997). Therefore, developing an environmentally and cost-effective purification process for photosynthetic pigments and PHB is important and a key step for sustainable large-scale PHB applications in biorefinery concepts (Daly et al. 2018). In this context, alternative reaction solvents such as ionic liquids, fluorous solvents, water, and renewable organics pose opportunities to create such a process.

Ionic liquids (ILs) are molten salts, composed only of ions, with melting points below 100 °C, have low vapor pressure, high solvability and better chemical and thermal stability when compared to organic solvents (Desai 2016). ILs have adjustable properties since their physical properties (polarity, hydrophobicity and viscosity) can be controlled by the exchange or combination of cations and anions (Freemantle 1998). Moreover, ILs also demonstrated a good performance in biocatalysis (Sheldon et al. 2002), while providing a non-denaturing environment for biomolecules and maintaining the protein

structure and enzymatic activity (Ventura et al. 2013). These liquids are promising alternatives for separation, and they need to be further investigated with respect of their recycling potential to decrease cost and enhance sustainability of the process. Considering their wide versatility and capacity for reuse, ILs appear as an attractive and innovative alternative for the extraction of photosynthetic pigments and PHB. In contrast, DMC is an acyclic alkyl carbonate industrially produced by catalytic oxidative carbonylation of methanol through a green process developed by Enichemand UBE Industries (JP). It receives much attention due to its versatile use, low toxicity and fast biodegradability. Recent research showed that DMC-based extraction of PHB can compete with the standard method based on chloroform (Mongili et al. 2021) and will be therefore tested in the present study.

2. Objectives and Aims of the Study

Since many decades, petroleum-based plastic has been important in day-to-day life. From simple carry bags to medical implants, such a variety and quantities of plastic means a humongous amount of plastic waste that increases every year which remains in the environment for thousands of years. These plastics are generally cheap to produce, but since their persistence has a significant environmental impact, biodegradable and biobased plastics are an essential alternative for such problems. Poly-β-hydroxybutyrate (PHB) is a biopolymer with the potential to substitute petroleum-based non-biodegradable plastics. Utilizing three Icelandic marine cyanobacteria from the indoor culture collection of protists at BioPol ehf, a biorefinery approach will be tested containing two extraction steps, resulting in three product classes which are listed in the following in the order of their downstream processing:

- **photosynthetic pigments**,
- **poly-β-hydroxybutyrate (PHB)**, and
- **residual biomass** which should still include lipids and proteins for aquafeed production.

Utilizing initially conventional extraction methods, also ultrasound assisted extraction in combination with ionic liquids and dimethyl carbonate (DMC) as environmentally friendlier and milder solvents will be tested to increase the product yield of the residual biomass.

3. Material and Methods

3.1 Cyanobacteria Strains and Culture Conditions

Overall, three marine cyanobacteria strains from the indoor culture collection of northern Icelandic and Arctic Protists were utilized in the present project: *Synechococcus* strain 14CY-P-SYN-SKA, *Synechocystis* strain 15CY-P-SYI-SKA and *Spirulina subsalsa* strain 14CY-P-SS-SKA.

Table 1. Optimum culture parameter for the tested cyanobacteria.

Culture Conditions	***Synechococcus*** 14CY-P-SYN-SKA	***Synechocystis*** 15CY-P-SYI-SKA	***S. subsalsa*** 14CY-P-SS-SKA	**Additional Remarks**
Temperature [°C]	12	10	15	
PAR (PFR) [µmol photons m^{-1} s^{-1}]	30	30	50	Use of full spectra day fluorescent light bulbs
Photoperiod (light:dark)	12:12 h	12:12 h	12:12 h	Use of timer
Salinity	30	32	30	
Seawater	TMC	TMC	TMC	Tropic Marin Classic©
Nutrient Medium	BG-11	BG-11	BG-11	

Abbreviations: PAR, photosynthetic active radiation measured in PFR, Photon fluence rates; P, phytoplankton species; SKA, Skagaströnd (Húnaflói); Growth medium: BG-11, Blue-Green Medium (Stanier et al. 1971; https://www.ccap.ac.uk/wp-content/uploads/MR_BG11.pdf)

Figure 4. Overview of upscale cultivation, comprising different volumes of 2 L and 5 L flasks and 5 L bubble columns (B, C, D) as well as experiments in the two 100 L PBRs (A).

Each axenic isolate was maintained in culture in 500 mL flasks (250 mL culture volume) under sterile conditions at 16±2°C, 12:12 h light: dark (L:D) regime and at an irradiance of 30 and 50 µmol photons m^{-2} s^{-1}, respectively, using Master TL-D 18W/840 light (Phillips, Germany). Cultures were adapted to the standard culturing conditions described in Table 1. Artificial seawater salt (Tropic Marin Classic®, GmbH Aquarientechnik, Wartenberg) dissolved in de-ionized water was used with a salinity of 30-32 and pH of 8.3, corresponding to environmental factors recorded at the sampling sites from which the species originated in northern Iceland. Salinity, pH and conductivity were measured using handheld probes (YK-31SA, YK-2001PH SI Model 33**,** Engineered Systems and Designs-Model 600, Philips W9424). Photon fluence rates (PFR, 400–700 nm) were measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A (LI-COR Lincoln, NE, USA). Cultures were maintained in bi-weekly intervals.

3.2 Experiments

The inducement of PHB was conducted by variation of culture conditions, utilizing nitrogen and phosphate starvation. Initial experiments were conducted in 5 L flasks for 504 h, utilizing a pump for mixing via air bubbling. Using the one-factor-at-a-time principle, the three cyanobacteria strains were exposed to the following nitrogen and phosphate reductions of the original BG-11 medium recipe (c.f. attachment) a) 75% $NaNO_3$, b) 50% $NaNO_3$, c) 25% $NaNO_3$, d) 75% K_2HPO_4, e) 50% K_2HPO_4 and f) 25% K_2HPO_4. As control standard cultivation conditions were used (cf. Table 1), resulting in overall 21 samples (without triplicates). Experiments were started by filling in 2500 mL fresh prepared experimental culture medium into the flasks with the cyanobacteria. The experiment which resulted the highest PHB content in the three strains was repeated in 100 L bubble column photobioreactors (PBRs) for 504 h to gain biomass for the qualitative analysis. Up-scales for the PBRs were conducted in the following steps: 5 x 1 L, 5 x 5 L and 50 L (Fig. 4). The latter step was performed in the PBR. The final experiments were started by filling in 50 L fresh prepared experimental BG-11 culture medium into the PBRs containing the test strains.

3.3 Determination of Growth and Microscopic Visualization of PHB

Microalgae growth was monitored by conducting cell counts and by measuring dry matter. Counting of cells during the experiments were performed under a light microscope (Axiophot, Carl Zeiss AG, Oberkochen, Germany), using a Neubauer improved counting chamber with 0.1 mm depth (LO Laboroptik GmbH, Germany). During the long-term experiments, cell counts were conducted every second day. Only cells were counted, which exhibited red fluorescence under ultraviolet light. At least 500 cells were counted in each sample at a 400× magnification. The specific growth rate (μ) was calculated with the following equation:

$$\mu = \frac{\ln_{c1} - \ln_{c0}}{t_1 - t_0}$$

where c_1 and c_0 are the number of cells at time t_1 and t_0.

Dry cell weight (DCW) or dry weight was measured in triplicates by using 0.45 µm cellulose acetate filters (WHA10404006 Whatman®). Filters were pre-dried for 10 min at 105°C to remove any moisture. Subsequently 40 mL of the algal culture was filtered and dried for 24 h at 70°C and then weighed to measure the dry weight, then expressed as grams per litre.

PHB accumulated by the tested cyanobacteria was visualised microscopically after staining with two different dyes, Sudan black B and Nile blue A. For Sudan black B staining, cells were heat fixed onto clean, grease free glass slides and few drops of Sudan black B staining solution (0.3% in 70% ethanol) were added. After 5–10 minutes, the slides were immersed in xylene until complete decolorization (about 10 s), then counterstained with safranine solution (0.5% in water) for 10 s before gently rinsing with running water. The slides were allowed to dry and then examined with an oil immersion lens (Motic, USA) (Wei et al. 2011).

Nile blue A (1 mg) was dissolved in dimethylsulphoxide (1 ml) to obtain the staining solution. Two drops of the staining solution were added to ~200µl of sterile culture which was then incubated at 55°C for 10 min. Cells were transferred to a glass slide and viewed by a fluorescent microscope (Nikon, USA) at an excitation wavelength of 450–490 nm under 1000× magnification (Shrivastav et al. 2010, Ansari & Fatma 2016).

3.4 Harvest and Initial Processing utilizing Conventional extraction Methods

After 504 h, the cyanobacteria were filter-harvested using Whatman no.1 filter paper. Subsequently the filter were dried overnight, utilizing a horizontal dryer with hot air flow at 55°C (Tray dryer, model no. FDTHQQZ). If not otherwise mentioned, all chemicals used in this study were of the highest purity from Merck© (former Sigma Aldrich).

As preparation for the extraction procedure the dried biomass was milled.

For the extraction procedure, equal amounts of the organism from all experimental cultures (50 mg) were used. In the first step, dried cyanobacterial biomass was suspended in 90 % methanol (v/v), kept overnight at 4°C for removal of the pigments and then centrifuged at 8000 rpm. The pigment concentration in the supernatant (methanolic extracts) was determined as pointed out in paragraph 3.5.1.

The pellet obtained was dried at 60°C.

In the second step, the polymer was extracted in hot chloroform ($CHCl_3$) followed by precipitation with cold diethyl ether. The precipitate was centrifuged (10,000 g for 20 min) and the pellet was washed with acetone, dissolved in hot $CHCl_3$ and then transferred to a test tube (Yellore & Desia 1998). The $CHCl_3$ was evaporated and conc. H_2SO_4 was added. The solution obtained was heated in a boiling water bath (10 min at 100 °C). After cooling and thorough mixing, the PHB content was determined as described in 3.5.2.

The residual biomass from the initial PHB precipitation step was subjected to a biochemical gross analysis (cf. 3.5.3).

3.5 Quantitative Product Screening

3.5.1 Pigment Concentrations

The concentrations of chlorophyll *a* and total carotenoids as well as of allo- and c-phycocyanin and phycoerythrin were determined by measuring the absorbance of the extracts at λ = 647 nm and λ = 664nm (chlorophylls), λ = 480 nm (carotenoids), λ = 615 nm (c-phycocyanin), λ = 652 nm (allophycocyanin) and λ = 730 nm (phytochrom). Colour corrections were carried out with cultivation media, methanol and in case of the control with acetone. Subsequently, pigment concentrations were calculated by using the following equations:

$$Chlorophyll_a\ [\mu g\ mL^{-1}] = -1.79 * A_{647} + 11.87 * A_{664}$$

Calculation of the chlorophyll *a* concentration using absorption values of λ = 647 nm and λ = 664 nm (Ritchie 2006).

$$Total\ carotenoids\ [ng\ mL^{-1}] = 4 * A_{480}$$

Calculation of the total carotenoid concentration using absorption values of λ = 480 nm (Strickland & Parsons 1972).

$$CP(g.\ L^{-1}) = \frac{OD615 - 0.474 \times OD652}{5.34}$$

Calculation of the phycocyanin (CP) concentration using absorption values of λ = 615 nm and λ = 652 nm (Rodrigues et al. 2018).

$$CAP(g.\ L^{-1}) = \frac{OD652 - 0.208 \times OD615}{5.09}$$

Calculation of the allophycocyanin (CAP) concentration using absorption values of λ = 615 nm and λ = 652 nm (Rodrigues et al. 2018).

$$CPE(g.\ L^{-1}) = \frac{OD562 - 2.41 \times CP - 0.849 \times CAP}{9.62}$$

Calculation of the phycoerythrin (CPE) concentration using absorption values of λ = 562 nm (Rodrigues et al. 2018).

All data obtained were finally transferred into pg · cell $^{-1}$. As control, the extraction was conducted with acetone 80% (acetone/water: 80/20, *v*/*v*) with subsequent sonication for 15 min.

3.5.2 PHB

For the quantification of PHB, the samples were transferred to silica cuvettes and the absorbance at 235 nm was measured against a sulfuric acid blank (Law & Slepecky 1961). Absorbance values were expressed as mg g^{-1} with the help of standard curve of commercially available PHB. In addition, the

amount of crotonic acid was calculated from the molar extinction coefficient, which is 1.55 X 101 (Slepecky & Law 1960).

3.5.3 Biochemical Gross Composition

Total protein content of the residual biomass was determined by Kjeldahl nitrogen analysis (APHA 2005) using 5.95 as Kjeldahl nitrogen-to-protein conversion factor (Gonzalez López et al. 2010).

Total lipid content was determined by extracting dry biomass (0.5–1.1 g DW) with a mixture of hot chloroform and methanol (two parts chloroform, one part methanol) for 140 min, using a unit (C. Gerhardt GmbH & Co. KG, Germany). For solvent evaporation, the lipid extract was placed beneath an extraction hood and subsequently in a desiccator until reaching a constant weight. (Gruber-Brunhumer et al. 2015).

The total carbohydrate content was measured photometrically (absorbance at λ = 485 nm) using the phenol-sulfuric acid reaction (Dubois et al. 1951, Gerchakov & Hatcher 1972). For that a phenolic solution (50 g L^{-1}, 2 mL) and concentrated sulphuric acid (96%, 10 mL) were added to this suspension. Glucose was used for the standard curve.

3.6 Application of Alternative Solvents in the Multiproduct Biorefinery Process

The two-step process utilized in the present study comprised

i) an ultrasound-assisted extraction of photosynthetic pigments using ionic liquids and
ii) the application of 1-ethyl-3-methylimidazolium diethylphosphate and dimethyl carbonate-based extraction of PHB.

3.6.1 Ultrasound-assisted extraction of photosynthetic pigments using ionic liquids

The protic ionic liquid 2-hydroxy ethylammonium formate (2-HEAF) was synthesized through an acid-base neutralization reaction. After the synthesis, 2-HEAF was stored at 10 ± 0.5 °C in dark bottles to avoid degradation by light.

Dried milled samples (cf. 3.4) were either immersed in 2-HEAF; the commercial ionic liquid, 1-butyl-3-methylimidazolium chloride (BMIMCl) or sodium phosphate buffer (SPB, 0.1 M). The extraction of pigments was conducted in an ultrasonic device with a frequency of 25 kHz at 10 ± 0.5 °C, for 15 min under dark conditions. The pH of the tested solvents as well as the solvent: biomass ratio are species and solvent specific and were tested individually prior to the multiproduct biorefinery process. The optima for each species and solvent are presented in Table 2.

Table 2. Optimum pH and solvent: biomass ratios for the different solvents used for the tested cyanobacteria.

	2-HEAF	**BMIMCl**	**sodium phosphate buffer**

Synechococcus 14CY-P-SYN-SKA			
pH [-]	6.5	5.11	8.2
solvent: biomass ratio [ml · g^{-1}]	12.6	22	19.3
Synechocystis 15CY-P-SYI-SKA			
pH [-]	6.3	5.0	8.0
solvent: biomass ratio [ml · g^{-1}]	13.9	23	20.5
S. subsalsa 14CY-P-SS-SKA			
pH [-]	6.9	5.5	8.5
solvent: biomass ratio [ml · g^{-1}]	9.2	10	8.5

After extraction, the reaction medium was centrifuged at 6000 rpm for 10 min at room temperature. The supernatant was isolated to obtain the pigment extract, while the pellet was used in 3.6.2. For the extracts' purification, 20% (w/v) of ammonium sulfate was added to each sample, homogenized, and kept overnight at 4 °C to allow the protein precipitation. On the following day, samples were centrifuged at 3000 rpm for 15 min, at room temperature. The supernatant was separated, and the pellet was re-suspended in ultrapure water. Subsequently samples were analysed in duplicate, and ultrapure water was used as a blank. The optical density of the supernatants was determined by spectrophotometry and the pigment concentrations were estimated as described in 3.5.1.

The separated supernatant from the final step, containing the ionic liquids 2-HEAF and BMIMCl, was subsequently recovered for further use as described by Rodrigues et al. (2018).

3.6.2 Application of 1-ethyl-3-methylimidazolium diethylphosphate and dimethyl carbonate-based extraction of PHB

3.6.2.1 1-Ethyl-3-methylimidazolium diethylphosphate

Following the method given by Filippi et al. (2021), the treatment with 1-Ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] was conducted at biomass/IL ratios varying between 1/5 (both picocyanobacteria) and 1/10 (w/w) (*S. subsalsa*) for the three cyanobacteria strains. The mixture was stirred at 60 °C for 24 h and was then centrifuged at 4000 rpm for 10 min to recover the insoluble PHB. The supernatant was removed and the solid was washed with methanol four times. Washing was carried out by the resuspension of the solid in methanol and the subsequent centrifugation and removal of the supernatant. Finally, the resultant solid was placed in an oven at 60 °C under vacuum for 12 h, weighed and subjected to subsequent characterizations (cf. 3.7.1). All resulting supernatants were subjected to a product screening as described in 3.5.

3.6.2.2 Dimethyl carbonate (DMC)

The PHB extraction with 99% DMC and ethanol is based on a modified protocol by Samorì et al. (2015), described by Mongili et al. (2021). In the case of direct solvent-biomass extraction, the pellet was transferred directly into 20 mL glass bottles. DMC was added to the collected biomass in a ratio of

2.5% (w/v) (both picocyanobacteria) and 5% (w/v) (*S. subsalsa*). The reaction was set under continuous stirring at 90°C, for 120 min. At the end of each extraction, the solution was transferred into 50-mL flacon and centrifuged at 4700 ×*g* for 10 min, to separate the organic phase hosting the PHB from the biomass. Then, the organic phase was recovered, and pure ethanol was added at a DMC: ethanol volumetric ratio of 1:3 to enable PHB precipitation. The mixture was stored for 12 h at 4 °C. The PHB was then filtered via vacuum pump and air-dried. In the case of filter-mediated extraction, the biomass was in a paper filter arranged as a tea-bag container (Whatman grade 1). Hence, there was no need to centrifuge the samples to separate the PHB-rich organic phase from the exhaust biomass, but just PHB precipitation was required via ethanol supplement (Mongili et al. 2021). The residual biomass was subjected to a product screening as described in 3.5.

3.7 Qualitative Product Characterisation

Samples with the highest PHB content after extraction utilizing alternative methods were subjected to a qualitative analysis of the PHB and the residual biomass as described in the following.

3.7.1 Phycocyanin

Using the first purified sample after extraction with 2-HEAF from the biomass of Synechocystis 15CY-P-SYI-SKA (3.6.1), the photosynthetic pigment C-phycocyanin was further purified according to the method given by Hazra & Saha Kesh (2017). The supernatant from the first treatment was further treated with solid ammonium sulphate until it reached 50% saturation and kept overnight at 4 °C. The resulting solution was centrifuged at 10,000 g for 30 min. The pellet mainly containing C-PC was mostly dissolved in acetate buffer (0.1 M, pH 4.5). The resulting mixture was again centrifuged at 10,000 g for 30 min. The small pellet (mainly basic proteins) was discarded, and supernatant was further brought to ammonium sulphate precipitation to achieve 50% saturation and allowed to kept overnight at 4 °C. The precipitated C-PC was dissolved in 10 mL of 20 mM Tris–HCl buffer (pH 8.1) and dialysed for 10 h at 4 °C against same buffer. The buffer was changed twice for complete removal of ammonium sulphate.

DEAE-cellulose-52 was used for anion exchange chromatography. 20 cm × 2.5 cm column was prepared for C-PC purification. The column was pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.1). The dialysed sample of C-PC was loaded on the column. Then, the column was washed with 10-bed volume of the same buffer. The column was first eluted with 10-bed volume of 0.05 M NaCl in 20 mM Tris–HCl buffer (pH 8.1). The elution was performed by subsequent addition of different concentrations of NaCl (0.1, 0.15, 0.2 and 0.25 M) in 20 mM Tris–HCl buffer (pH 8.1). The C-PC was then eluted between 0.15 and 0.25 M NaCl concentration, and sample was collected in 2-mL microcentrifuge tube. The flow rate of sample was 0.5 mL/min. Scanning of all fractions was determined in the range of 250–800 nm. Purity ratio of $A_{620}/A_{280} > 3.5$ was pooled together. The resulting solution was brought to 50% ammonium sulphate saturation. The pellet was mixed with 10 mL 20 mM Tris–HCl buffer (pH 8.1) and dialysed against water at 4 °C. The sample was freeze-dried.

3.7.2 PHB

For FTIR analysis, the potassium bromide pellet was prepared using the DCM extracted polymer from *Synechocystis* 15CY-P-SYI-SKA. Infrared spectra (IR) were recorded using Agilent Cary 630 FT-IR spectrometer with spectral range of 4000–400 cm−1 at 27°C.

For ^{1}H NMR analysis, the extracted polymer was suspended in deuterochloroform (CDCl3) at a concentration of 10 mg ml^{-1}. 1H NMR spectra were obtained on a Bruker Spectrospin DPX-400MHz NMR spectrometer at 22°C with 7.4 ms pulse width (30° pulse angle), 1 s pulse repetition, 10,330 Hz spectral width, 65,536 data points. Tetramethylsilane was used as an internal shift standard.

Samples for GC analysis were prepared by propanolysis of the isolated polymer according to the method described by Ansari & Fatma (2016). A mixture of 2 ml 1,2-dichloromethane (DCE) and 2 ml acidified isopropanol (20% v/v hydrochloric acid) was added to polymer at 100°C for 2 h. Phase separation was achieved by adding 4 ml water to the mixture. The propylated 3-hydroxybutyric acid was extracted into the 1, 2-dichloroethane phase and injected directly into the gas chromatograph. The GC-MS analysis was performed with a Shimadzu GC-MS QP 2010 Plus in electron ionization (EI) mode fitted with an RTX-5 (60 m x 0.25 mm x 0.25 µm) capillary column. The carrier gas used was helium with a flow rate of 0.7 ml min^{-1}. The injector temperature was 260°C and the initial column temperature of 80°C was held for 2 min before ramping to 250°C at 10°C min^{-1} and holding for 5 min and then finally increased to 280°C at the rate of 15°C min^{-1}. A 3.5 min solvent delay was used. Mass spectra were recorded under scan mode in the range of 40–650 m/z. Compounds were identified using NIST11 library.

3.7.3 Analysis of the residual Biomass

3.7.3.1 Fatty Acid Composition

Total lipid was extracted in a solution of dichloromethane: methanol (2 : 1, v : v), modified after Folch et al. (1957). As internal standard, a known amount of the tricosanoic acid methyl ester (23:0) was added to each sample. A 0.88% solution of KCl (potassium chloride) was added to easily differentiate the biphasic system. Transesterification of the lipid extracts was performed by heating the samples with 3% sulfuric acid (H_2SO_4) in methanol for 4 h at 80°C under nitrogen atmosphere.

Fatty acids (FA) and fatty alcohol compositions were identified according to Kattner and Fricke (1986). Subsequent analyses were done by gas liquid chromatography (HP 6890N GC) on a wall-coated open tubular column (30 × 0.25 mm internal diameter; film thickness: 0.25 µm; liquid phase: DB-FFAP) using temperature programming. Standard mixtures served to identify the FA methyl esters and the fatty alcohol derivatives. If necessary, further identification was done by gas chromatography-mass spectrometry using a comparable capillary column. Detailed FA and alcohol compositions were expressed as percent of total FA and percent of total fatty alcohols, respectively (Graeve & Greenacre 2020).

As control, untreated cyanobacteria cultures grown under standard conditions as described in 3.1 were used.

3.7.3.2 Amino Acid Composition

Extraction of soluble primary intermediates was carried out using LC-MS grade chemicals according to the method described in Reinholdt et al. (2019). Briefly, ∼50 mg sample was extracted in 500 µL of ice-cold LC-MS/MS buffer [150 µL chloroform, 350 µL methanol, 1 µL of MES as internal standard (1

mg/mL)]. Following addition of 400 µL ice-cold water, samples were vortexed thoroughly and incubated for at least 2 h at −20°C. After centrifugation (10 min, 20,000 *g*, 4°C), the aqueous phase was transferred to a new tube and 400 µL of ice-cold water again added to the extraction tube. Following stirring and centrifugation (5 min, 20,000 *g*, 4°C), supernatants were combined and lyophilized. Next, the dried extracts were dissolved in 400 µL water and filtrated through 0.2 µm filters (Omnifix-F, Braun, Germany).

The cleared supernatants were analysed using the high-performance liquid chromatograph mass spectrometer LCMS-8050 system (Shimadzu) and the incorporated LC-MS/MS method package for primary metabolites (version 2, Shimadzu). In brief, 1 µL of each extract was separated on a pentafluorophenylpropyl column (Supelco Discovery HS FS, 3 µm, 150 × 2.1 mm) with a mobile phase containing 0.1% (v/v) formic acid. The compounds were eluted at 0.25 mL min^{-1} using the following gradient: 1 min 0.1% (v/v) formic acid, 95% *Aqua destillata* (*A. dest*.), 5% acetonitrile, within 15 min linear gradient to 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile, 10 min 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile. Aliquots were continuously injected in the MS/MS part and ionized via electrospray ionization. The compounds were identified and quantified using the multiple reaction monitoring values given in the LC-MS/MS method package and the LabSolutions software package (Shimadzu). Authentic standard substances (Merck) at varying concentrations were used for calibration and peak areas normalized to signals of the internal standard. Glyoxylate, 3-HP, and glycerate were determined in the negative ion mode using selective ion monitoring for *m/z* 73, 102, and 105 corresponding to the deprotonated glyoxylate, 3-HP, and glycerate ions $[M-H]^-$. Retention time acquisition window (2 min) was verified with coelution experiments using purchased glyxoylate, 3-HP, and glycerate (Sigma-Aldrich). Varying concentrations of the three metabolites were also used for calibration curves. Data were interpreted using the Lab solution software package (Shimadzu).

3.8 Statistical Analysis

Measures were carried out in triplicate ($n = 3$), and the results are given as mean values and standard deviations. The results were statistically analysed using a one-way ANOVA with a statistical difference of 5% and the Tukey TSD test of the IBM SPSS software version for multiple comparisons.

4 Results

4.1 Impacts of different Nutrient Concentrations on the amount of accumulated PHB

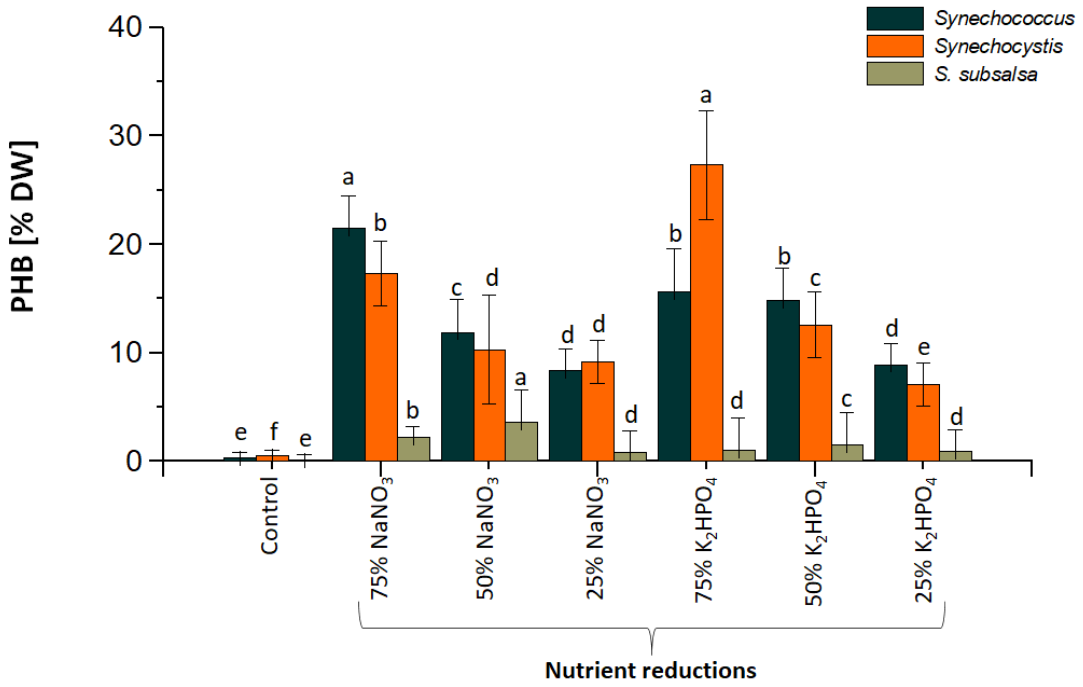

Figure 5. PHB contents in % dry weight (DW) obtained by a conventional extraction method from the three tested cyanobacteria grown under different nutrient reduced scenarios. Data are means ± SD (n=3). Different letters indicate significant difference among treatments.

Initially three marine cyanobacteria, namely *Synechococcus* (strain 14CY-P-SYN-SKA), *Synechocystis* (strain 15CY-P-SYI-SKA) and *Spirulina subsalsa* (stain 14CY-P-SS-SKA) were tested regarding the impact of reduced nutrient concentrations in the standard BG-11 growth medium (a) 75% $NaNO_3$, b) 50% $NaNO_3$, c) 25% $NaNO_3$, d) 75% K_2HPO_4, e) 50% K_2HPO_4 and f) 25% K_2HPO_4) on the PHB accumulation potential of the species (Fig. 5). As control, BG-11 with 100% $NaNO_3$ and 100% K_2HPO_4 was utilized (cf. 3.2).

While the control assays showed only low PHB contents (0.1-0.5 % DW), the results obtained for the experimental assays revealed that the highest PHB concentration was detected in *Synechocystis* (27.3% DW) at an 75% decrease of K_2HPO_4 in the growth medium followed by *Synechococcus* (21.5% DW) at an 75% decrease of $NaNO_3$ (Fig. 5). The lowest PHB content was found in *S. subsalsa* in all test assays, ranging between 0.9% and 3.6% DW at an 25% decrease of K_2HPO_4 and at an 50% decrease of $NaNO_3$, respectively. The nutrient constellations yielded in the highest PHB contents for each species were used in the following experiments, conducted in 100 L PBRs to gain biomass for further analysis and comparison of extraction methods (4.2).

4.2 Effects of different Extraction Procedures on the Product Yields

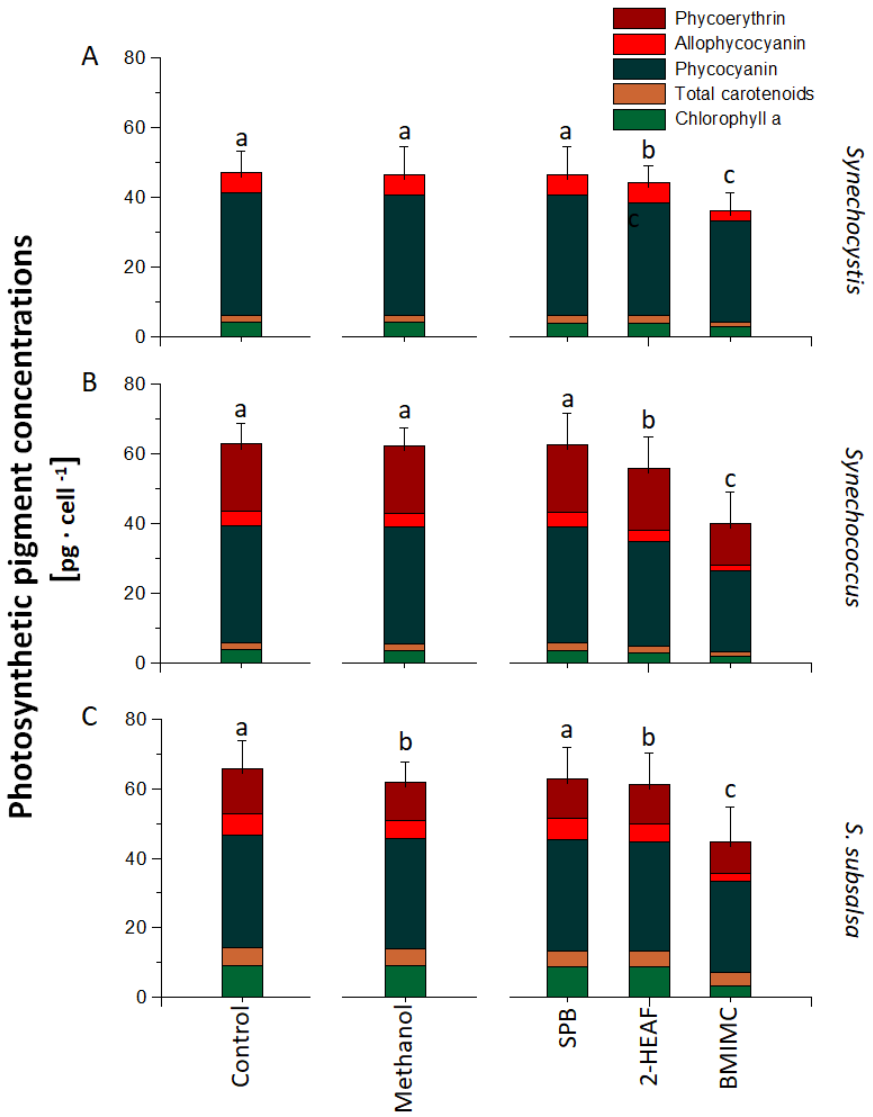

Figure 6. Photosynthetic pigment concentrations obtained by utilization of conventional (control, methanol) and alternative extraction methods. Data are means ± SD (n=3). Different letters indicate significant difference among treatments.

In the quantitative screening of the product classes obtained from the multiproduct biorefinery approach conducted in the present study, conventional and alternative extraction methods were compared (cf. 3.4 and 3.6). Photosynthetic pigments were the first product class gained from the biomass of the three cyanobacteria (Fig. 6). While the protic ionic liquid 2-hydroxy ethylammonium formate (2-HEAF), the commercial ionic liquid 1-butyl-3-methylimidazolium chloride (BMIMCl) and sodium phosphate buffer (SPB) were used as alternative solvents (3.6.1), methanol (90%) was utilized in the first step of the initial extraction procedure utilizing conventional ones. As control the pigment extraction was conducted with acetone 80% (acetone/water: 80/20, *v*/*v*). The concentrations of chlorophyll *a*, total carotenoids as well as of allo- and c-phycocyanin and phycoerythrin were determined and compared (3.5.1). The highest photosynthetic pigment concentrations were detected in the control assays, including methanol and SPB, ranging from 46.48 to 65.58 µg · cell^{-1} in the SPB

extracted *Synechocystis* biomass and the acetone extracted *Spirulina* one respectively (Figs. 6A and C). The overall recovery rates utilizing ionic liquids in combination with ultrasound were the highest for 2-HEAF in all tested species, varying from 44.13 to 61.07 65.58 µg · cell^{-1} (Figs. 6A and C).

Phycocyanin was the most abundant photosynthetic pigment in all tested species, being the highest in *Synechocystis* (34.94 and 32.26 µg · cell^{-1} in the control and 2-HEAF assays, respectively). Phycoerythrin was found in highest concentrations in *Synechococcus* (19.33 and 17.61 µg · cell^{-1} in the control and 2-HEAF assays, respectively). The third most abundant photosynthetic pigment was allophycocyanin, varying between 4.14-6.16 µg · cell^{-1} (control assay), being the highest in *S. subsalsa* (Fig. 6C). Beside this, the tested *Spirulina* species had also above average concentrations of chlorophyll *a* and total carotenoids (9.26 and 5.15 µg · cell^{-1} in the control assays). Similar concentrations of photosynthetic pigments as found in the three Icelandic cyanobacteria tested in the present study are described in the scientific literature (e.g., Śliwińska-Wilczewska et al. 2020).

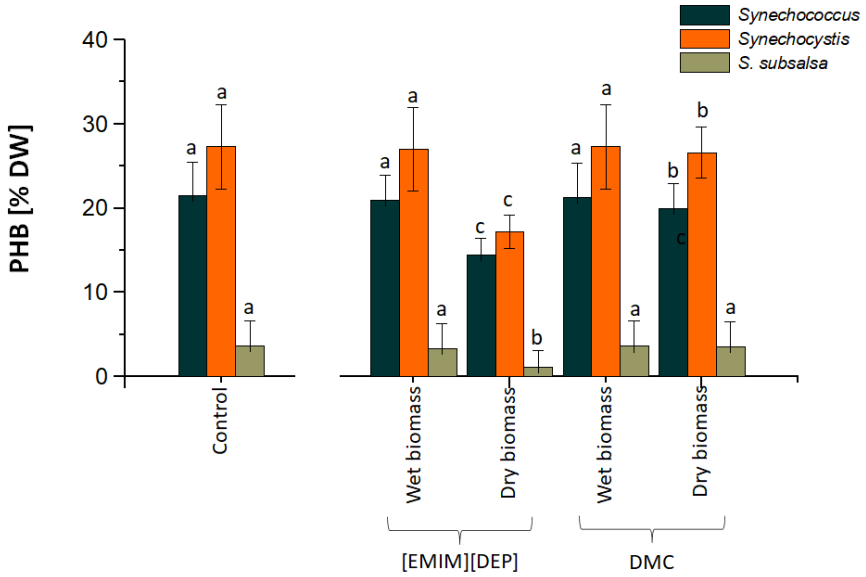

Figure 7. PHB contents (% DW) obtained by utilization of conventional (control) and alternative extraction methods. Data are means ± SD (n=3). Different letters indicate significant difference among treatments.

1-Ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] and dimethyl carbonate (DMC), as alternative extraction solvents, were tested in the present study for their potential to recover the polymer PHB from the three cyanobacteria using either wet or dry biomass (Fig. 7). As control the dry biomass was extracted using hot chloroform ($CHCl_3$) followed by precipitation with cold diethyl ether (cf. 3.4). Best results were obtained for the DMC/ethanol method for both wet and dry weight. The DMC samples from *Synechocystis* (wet biomass) were subjected to FTIR, ^{1}H NMR and GC-MS analysis to confirm whether the extracted polymer was PHB (cf. 4.3.1). In the final step the residual biomass was subjected to a biochemical gross composition analysis (Fig. 8). The results showed that the highest recovery rates for protein (89±0.8%), lipids (95±2.0%) and carbohydrates (96±2.5%) were obtained after the use of the DMC/ethanol method when compared to the unpretreated control (Figs. 8A-C). Therefore, samples obtained by the DMC method were further analysed regarding their amino and fatty acid composition to gain insight regarding the quality of proteins and lipids as well as their potential use as aquafeed (cf. 4.3.2).

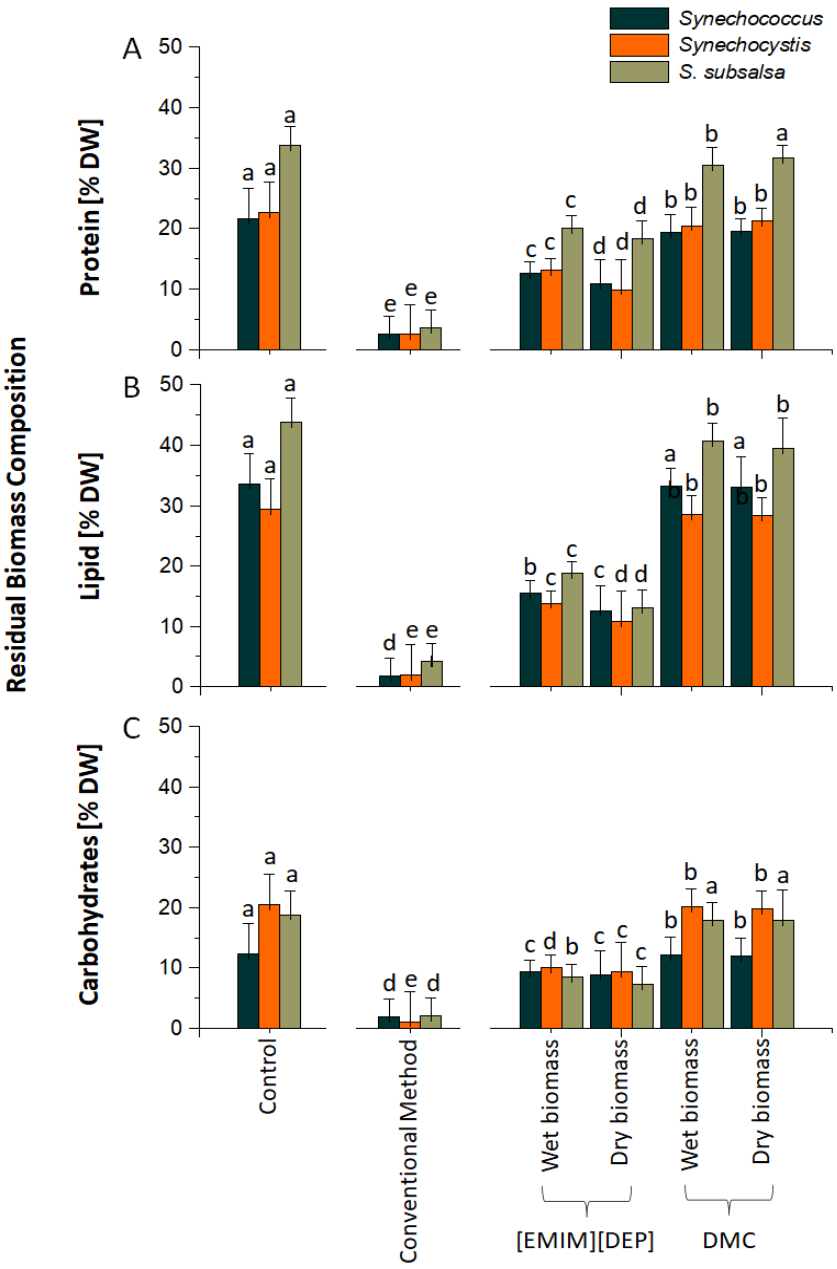

Figure 8. Composition of the residual biomass (% DW) after extraction with conventional and alternative extraction methods. As control untreated dried biomass was used, without initial pigment and PHB extraction. Data are means ± SD (n=3). Different letters indicate significant difference among treatments.

4.3 Qualitative Product Characterisation after use of Alternative Extraction Methods

4.3.1 Phycocyanin

Table 3. Data of the purification and separation of C-PC from Synechocystis 15CY-P-SYI-SKA.

Purification step	Purity ratio A_{620}/A_{280}	Separation factor A_{620}/A_{652}	C-PC (mg ml $^{-1}$)	Recovery of C-PC (%)
Crude extract	0.8	2.4	0.3	100
Ammonium sulphate precipitation with 25% saturation	0.9	2.4	0.4	93
Ammonium sulphate precipitation with 50% saturation	2.4	2.9	0.4	83
DEAE-cellulose-52	4.3	4.6	0.5	49

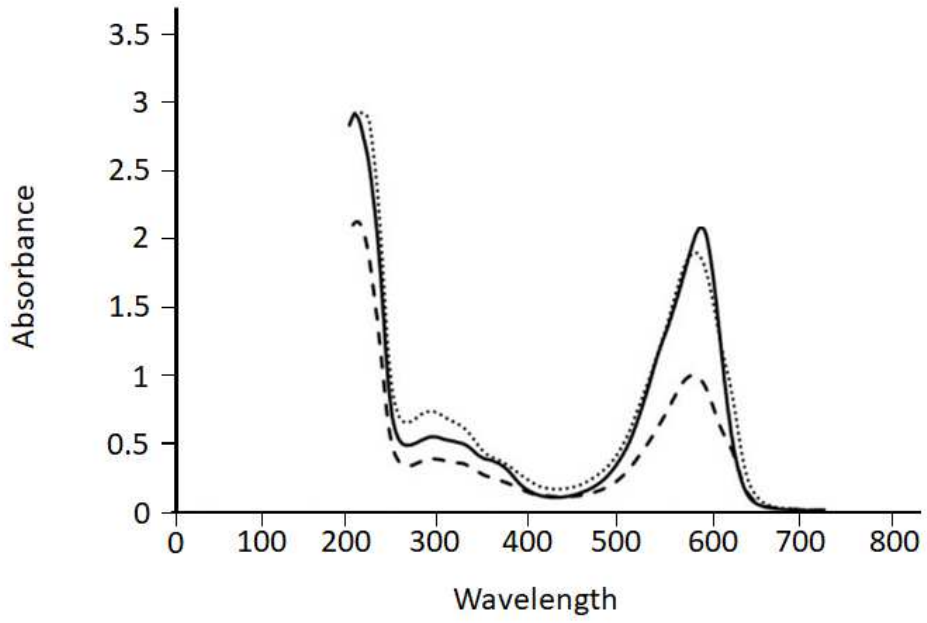

Figure 9. Overlay absorption spectra of C-phycocyanin at each stage of the purification.
……. First extract; **- - - -** After fractional precipitation with ammonium sulphate; **---------** After DEAE-cellulose

The main photosynthetic pigment detected during the first screening of the three cyanobacteria was C-phycocyanin (C-PC) (cf. Fig. 6). Thus, the sample of the species with the highest amount of this pigment was chosen for further purification: Synechocystis 15CY-P-SYI-SKA. The quantitative evaluation of the phycocyanin content is summarized in Table 3, whereas the UV-visible absorption spectra of each step of purification are shown in Figure 9. The Purity ratio (A_{620}/A_{280}) and the absorption spectrum of C-PC increased in every step of the purification (Fig. 9), as well as the separation factor (A_{620}/A_{652}). The high separation factor points to a low contamination with allophycocyanin. The final recovery of the eluted C-PC was 49% with a purity ratio (A_{620}/A_{280}) of 4.33.

4.3.2 PHB

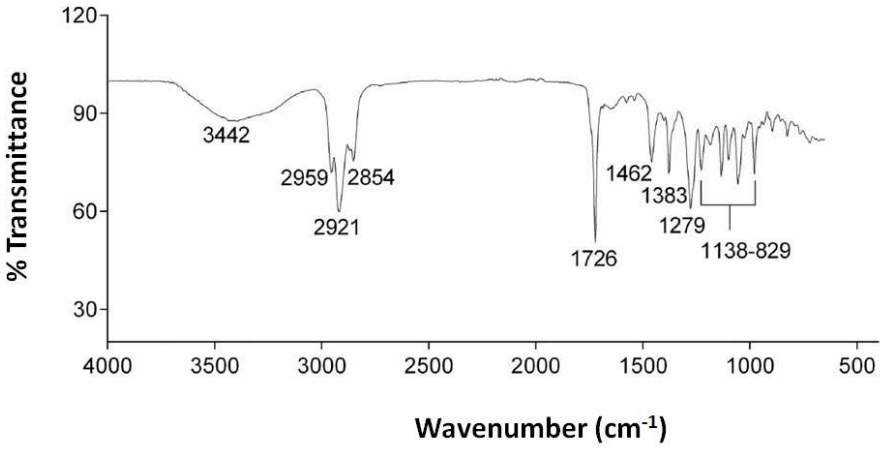

Figure 10. FTIR spectrum of the DCM isolated PHB from *Synechocystis* 15CY-P-SYI-SKA.

During the characterization of the DCM isolated PHB from *Synechocystis* 15CY-P-SYI-SKA, the FTIR spectrum showed prominent peaks at 1726 cm^{-1} and 1279 cm^{-1} (Fig. 10). These peaks denote carbonyl (C = O) and asymmetric C-O-C stretching vibration, respectively, characteristic for ester bonding found in a PHB molecule. Other adsorption bands obtained at 1383, 1462, 2959–2854, and 3442 cm^{-1} denote the -CH3, -CH2, -CH, and O-H groups respectively. The absorption bands at 1138 cm^{-1} to 829 cm^{-1} were assigned to C-O and C-C stretching vibration which could be attained by amorphous PHB (Gumel et al. 2014). Almost identical peaks at 1382, 1726, 2978, 2934, 3439 cm^{-1}, denoting the various functional groups of PHB in a marine cyanobacterium, *Spirulina subsalsa* were observed by Shrivastav et al. (2010) and for the freshwater species *Nostoc muscorum* by Ansari & Fatma (2016).

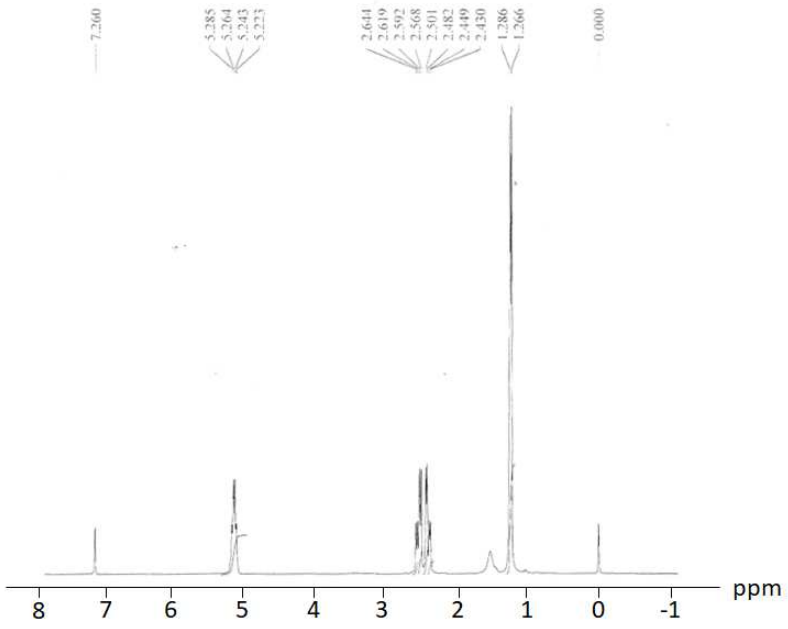

Figure 11. ^{1}H NMR spectrum of the DCM isolated PHB from *Synechocystis* 15CY-P-SYI-SKA.

^{1}H NMR spectrum of the isolated polymer dissolved in deuterochloroform showed a multiplet between 5.22 and 5.28 ppm denoting the methine proton (-CH) having the chiral carbon (Fig. 11). Diastereotopic methylene (-CH2) protons were assigned to the double quadruplet having the resonance value 2.43–2.50 ppm and 2.57–2.64 ppm. Finally, the methyl protons (-CH3) gave doublet signals at 1.27–1.29 ppm. These chemical shift signals with respect to internal standard tetramethylsilane (peak at 0 ppm) are almost identical with the chemical shift signals obtained for PHB produced by *Cupriavidus necator* (Oliveira et al. 2007).

The gas chromatogram of the isolated polymer showed a major peak with retention time 10.297 min (Peak 2) with two minor peaks at 9.227 (Peak 1) and 12.505 min (Peak 3) (Fig. 12). As identified by comparing molecules in the GC database, the major peak represents isopropyl ester of 2-butenoic acid confirming the polymer as PHB (Fig. 13). The first minor peak at 9.227 min represents the solvents used during sample preparation, while the second minor peak at 12.505 denotes benzoic acid isopropyl ester. Same sequence of these components as represented by the 3 peaks in our study was also obtained by earlier workers (Riis & Mai 1988, Ansari & Fatma 2016).

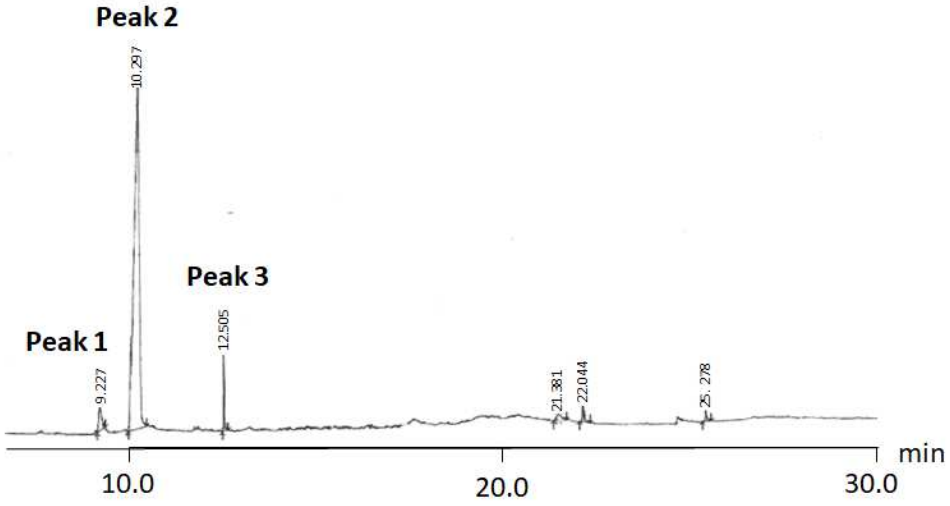

Figure 12. GC spectra of DCM isolated PHB from *Synechocystis* 15CY-P-SYI-SKA.

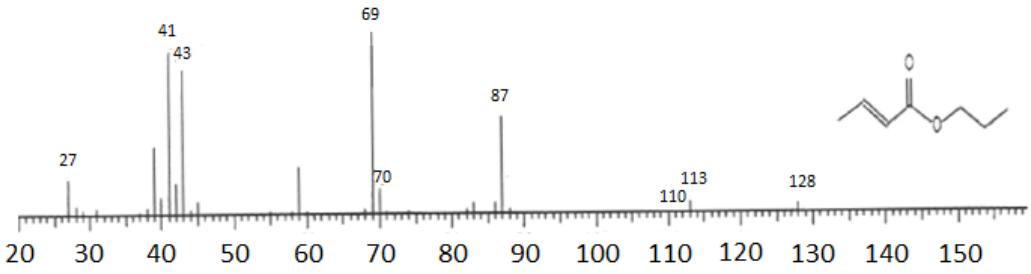

Figure 13. Comparison of the second peak (Rt-10.297) with a mass spectra MS library (NIST 11)

4.3.3 Residual Biomass

Table 4. Amino acid composition of the three tested cyanobacteria either not pre-treated (control) or after PHB extraction via DMC in the multiproduct biorefinery process (n.d. = not detected).

	Synechococcus 14CY-P-SYN-SKA		***Synechocystis*** 15CY-P-SYI-SKA		***S. subsalsa*** 14CY-P-SS-SKA	
Amino acid (ng · mg DW^{-1})	**control**	**DMC**	**control**	**DMC**	**control**	**DMC**
Asparagine	0.104	0.1	0.547	0.23	3.599	2.621
Aspartate	0.698	0.06	3.942	1.38	42.264	39.83
Serine	0.518	0.176	1.922	0.97	15.402	11.522
Alanine	298.524	252.14	245.57	214.16	424.683	386.04
Glycine	0.195	0.039	1.158	0.89	9.736	6.861
Glutamine	19.413	15.151	18.117	17.58	7.368	3.883
Threonine	1.083	1.033	3.139	2.23	19.529	16.82
Cysteine	n.d.	n.d.	n.d.	n.d.	19.664	15.38
Glutamate	33.31	30.12	49.785	41.8	58.471	53.96
Proline	0.100	n.d.	2.823	1.06	13.672	10.06
Lysine	30.529	25.14	24.11	23.93	9.322	6.168
Histidine	0.176	0.123	0.154	0.108	1.399	1.095
Arginine	0.225	0.119	7.569	5.09	13.349	10.993
Valine	0.667	0.503	8.259	6.17	16.584	13.865
Methionine	0.962	0.511	1.263	1.15	8.025	5.30
Tyrosine	1.027	1.03	5.745	3.74	10.199	8.49
Isoleucine	1.842	1.59	21.201	19.44	14.300	12.013
Leucine	2.994	1.800	30.309	25.37	15.462	11.135
Phenylalanine	3.246	2.06	27.544	15.92	15.582	11.959
Tryptophan	0.649	0.32	1.79	0.55	2.973	1.53
Malate	n.d.	n.d.	n.d.	n.d.	48.958	33.266
Succinate	n.d.	n.d.	n.d.	n.d.	51.441	48.831
Total	**396.262**	**332.015**	**454.947**	**381.768**	**821.982**	**711.622**

To assess the nutritional value of the protein fractions of the selected species grown under the culture conditions pointed out in 4.1, the amino acid (AA) compositions were evaluated on untreated biomass samples (control) *versus* residual biomass samples after the final treatment with DCM (Table 4). The overall amount of recovered AA after PHB extraction varied between 80-86% compared to the control assays, being the lowest for *Synechocystis*. The AA compositions were highly heterogenous and varied from species to species. According to Wilson (1986) qualitative AA requirements for normal growth and metabolism of, for instance, salmonids the presence of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential. All these essential AAs were present in the species analysed and were still detectable in the DMC assays, although in lower concentrations. In the following only the results of the DMC assays will be considered.

Table 5. Fatty acid composition of the three tested species/culture condition constellations (a.t. = after treatment, n.d. = not detected)

Fatty acid ($\mu g \cdot g\ DW^{-1}$)	***Synechococcus*** 14CY-P-SYN-SKA		***Synechocystis*** 15CY-P-SYI-SKA		***S. subsalsa*** 14CY-P-SS-SKA	
	control	**DMC**	**control**	**DMC**	**control**	**DMC**
14:0	273.081	157.26	81.788	39.382	120.531	109.610
Phytol	4.141	3.217	0.299	n.d.	24.944	19.042
i-15:0	73.393	66.526	63.188	58.472	314.060	282.116
15:0	8.192	5.132	1.315	1.109	n.d.	n.d.
14:0	25.386	20.109	29.876	25.528	155.885	137.510
16:0	135.092	123.447	54.593	33.271	499.989	252.315
16:1n7	122.907	97.519	140.891	118.616	642.936	559.834
16:1n5	4.478	3.108	3.041	2.577	n.d.	n.d.
i-17:0	7.811	6.371	n.d.	n.d.	n.d.	n.d.
16:2n4	n.d.	n.d.	n.d.	n.d.	48.891	36.759
16:3n4	5.977	4.199	1.347	0.994	57.535	51.414
16:4n1	n.d.	n.d.	n.d.	n.d.	42.942	38.661
18:0	56.128	45.329	48.073	40.816	257.925	204.333
18:1n9	69.339	60.115	55.027	41.925	443.622	274.59
18:1n7	184.492	157.1	109.672	98.763	198.823	139.103
18:1n5	2.976	1.59	n.d.	n.d.	n.d.	n.d.
18:2n6	3.927	2.861	n.d.	n.d.	1968.989	927.352
18:3n6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3n3	16.846	15.313	0.493	n.d.	n.d.	n.d.
18:4n3	6.876	4.27	n.d.	n.d.	n.d.	n.d.
20:0	n.d.	n.d.	n.d.	n.d.	29.001	14.302
20:1n11	n.d.	n.d.	1.705	0.989	n.d.	n.d.
20:1n9	2.344	2.115	1.728	1.351	n.d.	n.d.
20:1n7	4.601	4.038	n.d.	n.d.	n.d.	n.d.
20:2n6	n.d.	n.d.	n.d.	n.d.	31.938	22.681
20:3n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:1n11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:1n9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:1n11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:1n9	3.892	2.116	n.d.	n.d.	n.d.	n.d.
22:6n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	**1011.879**	**781.735**	**593.036**	**463.793**	**4838.011**	**3069.622**

Only lysine was the highest in residual biomass from *Synechococcus* after PHB extraction by DMC (25.14 ng · mg DW^{-1}, Table 3), whereas the residual biomass analysis of *Synechocystis* showed the presence of the highest isoleucine, leucine and phenylalanine values when compared to the other species (19.44, 25.37 and 15.92 ng · mg DW^{-1}). The residual biomass of the Icelandic isolate of *Spirulina subsalsa*, grown under nitrogen depletion (50% from the normal growth medium), showed the highest concentrations of most of the essential AAs when compared to the other two species. Specifically, threonine, histidine, arginine, valine, methionine and tryptophan were present, ranging from 1.53 (tryptophan) to 16.82 ng · mg DW^{-1} (threonine).

Dietary lipids are important in fish nutrition as energy sources, as carriers of lipid-soluble vitamins and minerals, and as sources of essential fatty acids (EFAs). To assess the nutritional value of the lipid fractions of the selected species grown under the culture conditions pointed out in 4.1, the fatty acid (FA) compositions were evaluated on untreated biomass samples (control) *versus* residual biomass samples after the final treatment with DCM (Table 5). The overall amount of recovered FAs after PHB extraction varied between 63-78% compared to the control assays, being the lowest for *Spirulina subsalsa*. The total n-3 PUFA dietary requirement of, for instance, salmonids, including ALA, EPA and DHA, has been reported to range from 1 to 2.5 % of the diet, depending on the species and experimental condition (Bou et al. 2017). In the analysed residual biomass, linoleic acid (18:2n6) was present in two out of the three cyanobacteria tested, with the highest levels in *Spirulina subsalsa* (927.352 µg · g DW^{-1}). Furthermore, α-linolenic acid (ALA, 18:3n3) was only detected in residual biomass from *Synechococcus* (15.313 µg · g DW^{-1}), whereas eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) were not detected at all (Table 5).

4.4. Tentative Aquafeed Formula

Table 6. List of the selected components (%) in the developed microalgae-based aquafeeds (MAF) for salmons.

Components	**Developed Aquafeeds**			
	I	**II**	**III**	**IV**
RB *S. salsa*	10	12	15	15
RB Synechocystis	5	2	2	2
RB Synechococcus	5	3	2	2
Whole-cell-microalgae Mix	53	61	57.7	55.8
Fishmeal (Talipia)	15	10	10	5
PHP	10		-	-
SPH	-	10		-
ASAT			11.3	18.4
others	2	2	2	1.8
Proximate composition (%)				
Moisture	21.5	19.4	20.1	18.3
Protein	34.1	36.2	50.4	56.3
Lipid	5.2	5.5	4.9	5.4
Fiber	1.1	1.0	1.2	1.3
Ash	5.0	5.1	5.0	4.8
Carbohydrates	31.4	32.3	34.4	32.0
Energy (kJ g^{-1})	11.3	11.5	11.7	11.9

Abbreviations: RB, residual biomass; PHP, insoluble partly hydrolysed protein fraction; SPH, water-soluble protein hydrolysate; ASAT, *Artemia salina* and *Acartia tonsa* meal. As others were pregelatinized starch and wheat flour designated.

Based on the results from 4.3.2 overall four tentative microalgae-aquafeeds (MAF) were further developed from already tested fish feed formula (Scholz et al. 2021). The new formulation and proximate composition of the experimental diets are shown in Table 6. Since biomasses of picocyanobacteria are usually not sufficient material for aquafeeds and the recovery rate of certain essential amino acids and lipids are still relatively low after the multiproduct biorefinery extractions presented in this study, additionally a whole-cell microalgae mix will be included in the tentative feeds. The mix will comprise selected chlorophytes and diatoms which were tested in a prior project focussing on the development of health-promoting aquafeeds for salmon (Scholz et al. 2021). Besides the high protein and lipid containing whole-cell-microalgae powder, commercially available feed ingredients are used (designated as "others" in Table 5), and diets are formulated to meet the dietary requirements of salmon, including vitamin and mineral requirements (NRC 2011). Specifically, small and balanced amounts of pregelatinized starch and wheat flour are used to compensate for the resulting modification of the overall nutritional value of the experimental diets due to algal addition. The four experimental diets are high in algae protein (>70%) and low in fish-derived protein:

- Diet MAF I contained 73% disrupted microalgae biomass, 15% fishmeal (from *Oreochromis niloticus*) and 10% partly hydrolysed fish protein (PHP),
- Diet MAF II included 78.9% disrupted microalgae biomass, 10% fishmeal and 10% soluble protein hydrolysate (SPH),
- Diet MAF III consisted of 76.7% disrupted microalgae biomass, 10% fishmeal and 11.3% mix of *Artemia salina* and *Acartia tonsa* (ASAT) and
- Diet MAF IV contained 74.8% disrupted microalgae biomass, 5% fishmeal and 18.4% ASAT.

The protein hydrolysates are produced from whole aquacultured Nile tilapia (*Oreochromis niloticus*) frozen directly after harvest. Following thawing, lipids and bone are removed from the blue whiting and the remaining raw material is enzymatically hydrolysed. The water-soluble protein hydrolysate fraction (SPH) and the insoluble partly hydrolysed protein fraction (PHP) are separated and spray-dried to prevent thermal damage to the protein. The SPH hydrolysate is composed of lower molecular weight peptides and single amino acids and contains 91% protein, of which 96% is soluble. The PHP hydrolysate is composed of low and medium molecular weight peptides and contains 68% protein, of which 18% is soluble (appendix Table A6). As final feed components hatched and reared *Artemia salina* and *Acartia tonsa* will be harvested, freeze dried and milled (ASAT). The brine shrimp *A. salina* is cultured according to the methods given by Tiro (1980) and fed with *Nannochloropsis*, *Isochrysis* and *Pavlova*, whereas the copepod *A. tonsa* is reared as described by Støttrup et al. (1986) and are fed with *Rhodomonas*, *Dunaliella* and *Tetraselmis* (cf. Scholz et al. 2021).

All ingredients will be combined in a commercial baker's mixer and mixed thoroughly before addition of 2–3 L of water (at 80 °C) per 20 kg of diet and further mixed. The experimental diets will be extruded (1-2- and 5-mm pellets) in a single screw extruder, dried at 40 °C in a dehumidifying oven over a 24-h period and stored at -20° C in airtight bags until use. Physical quality and sinking properties of the four diets have to be tested in the future, besides the detailed analysis of the biochemical composition.

5 Discussion

In the present study three marine cyanobacteria were tested for their potential to produce PHB under nutrient stress conditions in a multiproduct biorefinery approach. While the first two steps of the biorefinery, the extraction of the photosynthetic pigments and PHB, went without interference, the final step, the recovery of the residual biomass for aquafeed production, failed (Fig. 8). Therefore, the extraction procedure was changed from using conventional methods involving organic solvents to alternative ones. Unlike hydrophobic pigments, the hydrophilic compounds will denature, precipitate, or degrade in some of the most used organic solvents under the harsh conditions they require, namely of temperature (e.g., >40°C). Proteins are particularly fragile, having a compact 3D structure in which hydrogen bounding, ionic interactions combined with the secondary structure elements as β-sheets and α-helices define their native tertiary and/or quaternary structure in aqueous solutions. Organic solvents distort these protein structures irreversibly in a way that these high-value products can no longer be used as active ingredients. In this context, more sustainable and milder (compatible with the target compounds in terms of media conditions) separation techniques are needed to recover the hydrophilic components without compromising the chemical structure and main activities of the target compounds. In the present study, the protic ionic liquid 2-hydroxy ethylammonium formate (2-HEAF) and the commercial ionic liquid (IL) 1-butyl-3-methylimidazolium chloride (BMIMCl) in combination with ultrasound treatments were used for the extraction of photosynthetic pigments from the biomass. 2-HEAF showed the highest recovery rate when compared to BMIMCl. In the following, 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] and dimethyl carbonate (DMC), as alternative extraction solvents, were tested for their potential to recover the polymer PHB from the three cyanobacteria using either wet or dry biomass. Best results were recorded for DMC (wet biomass) and using FTIR, ^{1}H NMR and GC-MS, the polymer was identified as PHB. A quality analysis of the PHB was not conducted in the present project due to limited time but from the literature it is known that pre-treating the biomass can affect the physical properties of the polymer as for chemical oxidation, which is well known to decrease the molecular weight of the polymer as well as for lyophilization and heating which cause polymer chains rearrangement with a consequent variation of its crystallinity. In this context, the use of ultrasound was adapted during the extraction of the photosynthetic pigments but still needs to be further investigated.

The recovered amount of the PHB in the present study varied between 21.5 and 27.3% DW for *Synechococcus* and *Synechocystis*, respectively (Fig. 5). The values were reached at an 75% decrease of $NaNO_3$ in the case of *Synechococcus* and at an 75% decrease of K_2HPO_4 in the case of *Synechocystis*. In the literature, PHB accumulation has been reported reaching 38, 43 and 55% of dry cell weight (DCW), respectively, in the cyanobacteria *Synechocystis* sp. PCC6803 (Nishioka et al. 2001), *Nostoc muscorum* (Sharma & Mallick 2005) and *Synechococcus* sp. MA19 (Panda & Mallick 2007) under various specific culture conditions. In these reports, the cyanobacteria accumulated PHB under photoautotrophic conditions in combination with nutrient deficiency, such as phosphate and nitrate starvation, but also reductions of magnesium, and calcium, as well as microminerals, such as ferrous, manganese, zinc, cobalt, and copper have been shown to increase the amount of PHB in the cells (e.g., Bhati et al. 2010). The above enumeration of growth media components affecting the PHB amount in

picocyanobacteria makes clear that a high number of optimizations are possible and can lead potentially to a higher PHB amount in the Icelandic species.

Photosynthetic pigments were a further product of the multiproduct biorefinery process conducted in the present study. The highest fraction of the first step of the process were phycobiliproteins (Fig. 6). Phycobiliproteins are coloured proteins that play a role in light-harvesting and energy transfer to chlorophyll in the cyanobacteria photosynthetic apparatus (Pagels et al. 2019). These are broadly classified into three primary types: phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) (Chakdar & Pabbi 2016, Pagels et al. 2019). Phycobiliproteins are widely studied as bioactive compounds with antioxidant, anticancer, anti-inflammatory, and antiviral activities that promote human health (Pagels et al. 2019, Li et al. 2019). Furthermore, they are used as analytical reagents to develop fluorescent probes that can be adapted for immunohistochemistry, flow cytometry, and confocal laser microscopy (Li et al. 2019). There is a demand for increased phycobiliprotein production owing to their valuable properties in diverse research and industry fields (Noh et al. 2021). In the present study, C-phycocyanin was the dominant photosynthetic pigment in all three tested cyanobacteria, being the highest in *Synechocystis* 15CY-P-SYI-SKA. The successful purification of this phycobiliprotein resulted in a final recovery of the eluted C-PC of 49% with a purity ratio (A_{620} /A_{280}) of 4.33 (cf. Table 3, Fig. 9). Further purifications of PE and APC were not conducted in the present study but are planned for future investigations.

The residual biomass from all three cyanobacteria after DMC extraction were subjected to amino (AA) and fatty acid (FA) analysis to verify their nutritional value. When compared to the control assays, the recovery rates after PHB extraction varied between 80-86% for AAs and 63-78% for FAs (Tables 4 and 5). Essential AAs were still present in the residual biomass, but in lower concentrations when compared to the control. As essential FA linoleic acid (18:2n6) was present in two out of the three cyanobacteria tested, with the highest levels in *Spirulina subsalsa* and α-linolenic acid (ALA, 18:3n3) was only detected in residual biomass from *Synechococcus*. Eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) were not detected at all. In principle, picocyanobacteria such as *Synechococcus* and *Synechocystis*, are not known to contain high concentrations of essential AAs or FAs. In contrast, the Icelandic *S. subsalsa* was already tested in the past and found suitable as ingredient in a salmonid aquafeed (cf. Scholz et al. 2021), but also in the present study the values of the essential AAs and FAs were comparatively low. Still an attempt was made in the present study to formulate four tentative microalgae-based aquafeeds (4.4) which contain a relative low amount of the residual biomass and in any case >50% whole-cell microalgae powder (Chlorophyceae, diatoms) besides other components to compensate for the resulting modification of the overall nutritional value of the experimental diets due to algal addition. Although our multiproduct biorefinery approach using alternative extraction solvents worked, further studies are needed testing different ILs, biomass concentrations and pH values. Despite the various studies that emphasize on the benefits of ILs, for the extraction of microalgae derived bio-compounds, ILs are still to this date not being used commercially. This may be attributed to the toxicity and non-biodegradability of some traditional ILs such as imidazolium based ILs, which may pose as an environmental risk when they are not properly treated prior to discharge to the environment. In fact, several toxicology studies have reported that conventional ILs are able to disrupt cell membrane and increase reactive oxygen species production in various organisms such as bacteria, fungus, plants, and animal cell lines *in-vitro* and ultimately lead to death. Moreover, due to the complexity of ILs' molecular structure, they tend to be much harder to be synthesized, and therefore contribute to high production cost and are generally 5–20 times more

expensive than conventional solvents. On-going efforts need to be continued to lower down the production cost of ILs, and also optimize their usage (Tan et al. 2019).

6 Conclusions and Perspectives

The increasing impact of plastic materials on the environment is a growing global concern. Biodegradable bioplastics offer a solution to this, but it is a major challenge to find economic sources obtain platform natural products for their production. Poly-3-hydroxybutyrate (PHB) is characterized by interesting features that drew research and commercial ventures. Bacterial fermentation is a known route to produce PHB but the production by chemoheterotrophic bacteria is expensive due to the high costs of the carbon sources needed. However, the production of PHB through cyanobacteria is an attractive but underexplored alternative for a lower-cost PHB production. Until now, cyanobacteria have been underexploited in Iceland, also due to a lack of integration of harvesting and processing with potential downstream monetary revenues. Shifting this imbalance by refining the biomass into multiple high value products, leads to an economically and environmentally sustainable production chain. Therefore, in the present investigation a multiproduct biorefinery process consisting of two extraction steps was tested using in the final trails alternative solvents (e.g., ionic liquids and dimethyl carbonate/ethanol) and three Icelandic marine cyanobacteria as test subjects. The process yielded in photosynthetic pigments (particularly phycocyanin which was further purified) and PHB (up to 27.3% DW) as well as proteins and lipids in the residual biomass of *Synechocystis* 15CY-P-SYI-SKA (up to 20.5 and 28.6% DW). The residual biomass from all three cyanobacteria after dimethyl carbonate extraction were subjected to amino (AA) and fatty acid (FA) analysis to verify their nutritional value. Values of the essential AAs and FAs were relatively low compared to the control assays. Still an attempt was made to formulate four tentative microalgae-based aquafeeds. The results obtained for all products show high potential for industrial application of particularly *Synechocystis* 15CY-P-SYI-SKA after further optimizations and clarifications considering, for instance, the purity of the obtained PHB.

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8 Appendix

BG-11 growth medium composition

Stocks		**per 500ml**
(1)	$NaNO_3$	75.0 g
		per 500 ml
(2)	K_2HPO_4	2.0 g
(3)	$MgSO_4.7H_2O$	3.75 g
(4)	$CaCl_2.2H_2O$	1.80 g
(5)	Citric acid	0.30 g
(6)	Ammonium ferric citrate green	0.30 g
(7)	$EDTANa_2$	0.05 g
(8)	Na_2CO_3	1.00 g
(9)	Trace metal solution:	**per litre**
	H_3BO_3	2.86 g
	$MnCl_2.4H_2O$	1.81 g
	$ZnSO_4.7H_2O$	0.22 g
	$Na_2MoO_4.2H_2O$	0.39 g
	$CuSO_4.5H_2O$	0.08 g
	$Co(NO_3)2.6H_2O$	0.05 g

Medium	**per litre**
Stock solutions 1 - 8	10.0 ml each
Stock solution 9	1.0 ml