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Elghazy, E., Mohamed, S.M.D.S., Wianglor, K. et al. (2024) Large-scale cultivation of synechocystis sp. PCC6803 for the production of poly(3-hydroxybutyrate) and its potential applications in the manufacturing of bulk and medical prototypes. New Biotechnology, 83. pp. 133-141. ISSN: 1871-6784

https://doi.org/10.1016/j.nbt.2024.08.497

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New BIOTECHNOLOGY 83 (2024) 133–141

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Large-scale cultivation of *Synechocystis* sp. PCC6803 for the production of Poly(3-hydroxybutyrate) and its potential applications in the manufacturing of bulk and medical prototypes

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ARTICLE INFO

Keywords:
Cyanobacterial culture
Polyhydroxyalkanoates
Sustainable biomaterials
Large scale

ABSTRACT

Polyhydroxyalkanoates (PHAs) are biopolymers produced by microorganisms under nutrient limiting conditions and in the presence of excess carbon source. PHAs have gained popularity as a sustainable alternative to traditional plastics. However, large scale production of PHAs is economically challenging due to the relatively high costs of organic carbon. Alternative options include using organisms capable of phototrophic or mixotrophic growth. This study aimed at the production of poly(3-hydroxybutyrate) P(3HB), a type of PHA, at pilot scale using the freshwater cyanobacterium *Synechocystis* sp. PCC6803. First, to identify optimal conditions for P(3HB) production from *Synechocystis* sp. PCC6803, different supplemental carbon source concentrations and salinity levels were tested at laboratory scale. The addition of 4 g/L acetate with no added NaCl led to P(3HB) accumulation of 10.7 % dry cell weight on the 28th day of cultivation. Although acetate additions were replicated in an outdoor 400 L serpentine photobioreactor, P(3HB) content was lower, implying uncontrolled conditions impact on biopolymer production efficiency. An optimized P(3HB) extraction methodology was developed to remove pigments, and the biopolymer was characterized and subjected to 3D printing (fused deposition modelling) to confirm its processability. This study thus successfully led to the large-scale production of P(3HB) using sustainable and environmentally friendly cyanobacterial fermentation.

1. Introduction

Plastic is a commonly used material due to its versatility and affordability, however, the non-degradable nature of petroleum-based plastics contributes significantly to environmental pollution. To combat this, biodegradable and compostable bioplastics like polyhydroxyalkanoates (PHAs) have gained popularity as a sustainable alternative to traditional plastics. PHAs are biodegradable both in the soil and marine environment in addition to being highly biocompatible and hence are suitable for a large range of applications. To date, PHAs and their derivatives have been adopted in the fabrication of medical implants, drug delivery systems, printing and photographic materials, packaging and coating materials, nutritional supplements, biofuels, drugs and fine chemicals [1–3].

Poly(3-hydroxybutyrate) (P(3HB)) is a short-chain length PHA with four carbon containing monomers that accumulates inside microbial systems, usually in response to nutrient limitation (mainly nitrogen and phosphorus) and in the presence of excess carbon substrates [4]. Stress induced by the limitation of these nutrients induces microorganisms to store carbon in the form of P(3HB) granules within the cell. Previous studies have successfully produced significant amounts of P(3HB) from bacterial cultures reaching up to 90 % dry cell weight (DCW) [5,6,7], However, the relatively high cost of carbon substrates required for bacterial cultivation presents a major obstacle against large-scale economical production [8]. This has led to increasing research into alternative carbon sources, including those derived from waste [9],

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https://doi.org/10.1016/j.nbt.2024.08.497
Received 16 April 2024; Received in revised form 2 July 2024; Accepted 8 August 2024
Available online 10 August 2024
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although this has implications for downstream use of the polymer. A promising alternative is to employ photosynthetic microorganisms that consume CO_2.

The most commonly researched photosynthetic bacterial strain for P (3HB) production is the cyanobacterium, *Synechocystis* sp. PCC6803 [10]. However, the yield is relatively low compared to heterotrophic bacteria [11]. Rueda *et al.* (2022) investigated the effect of different parameters including salinity, light intensity and phosphorus content on the accumulation of P(3HB) in *Synechocystis* sp. cells. They observed that an increase in salinity with nutrient limitation stimulated the accumulation of P(3HB) [12]. In another study, Rueda *et al.* (2022) investigated the effect of organic/inorganic carbon sources, salinity, and dark/light ratio on P(3HB) production in two strains, *Synechocystis* sp. PCC6803 and *Synechococcus* sp. The authors reported P(3HB) production of 5.6 % and 26.1 % of DCW, respectively. The presence of 1.2 g/L acetate and 18 g/L NaCl enhanced P(3HB) production in *Synechocystis* sp. PCC6803 by 1.5 % and 1 %, respectively [13]. The addition of acetate is thought to provide cells with a greater pool of acetyl-CoA, which is a precursor for P (3HB) polymerization [14,15].

It should be noted that current efforts are focused on developing environmentally friendly methods for acetate production. Acetate, sourced from renewable materials such as lignocellulosic material and C1 gases, is being explored as a bio-based platform substrate for industrial biotechnology. Progress in acetate production technologies and microbial engineering holds promise for enabling its cost-effective utilization, providing a sustainable substitute for conventional carbon sources, and bolstering bioeconomy-driven industrial processes[16].

Efforts to increase P(3HB) content have also included genetic modification of strains. For instance, Orthwein *et al.* (2021) generated a mutant *Synechocystis* sp. PCC6803 strain, which during nitrogen limitation, resulted in enhanced glycogen and P(3HB) accumulation up to 50 % of DCW[17]. In another study, *Synechocystis* sp. PCC6803 lacking the *adc1* gene was reported to have more accumulation of P(3HB) up to 36.1 % of DCW after adaptation in a nitrogen and phosphorus-limited BG11 medium, containing 4 g/L acetate [15]. The most significant accumulation was achieved by cultivating an engineered strain under nitrogen and phosphorus deprivation and supplemented acetate (81 % per DCW) [18].

Notably, nearly all previous studies investigating *Synechocystis* sp. PCC6803 strains as cell factories to make P(3HB) have been undertaken at lab scale under controlled conditions. Reports of large-scale culture of *Synechocystis* sp. PCC6803 are limited. So far and to the best of our knowledge, only Troschl *et al.* (2018) have cultured *Synechocystis* sp. *CCALA192* under non-sterile conditions in a large scale 200 L tubular photobioreactor for P(3HB) production. However, the culture required controlled light and temperature conditions as well as induction of partially anoxic conditions to prevent contamination [19].

In the current study, lab-scale growth experiments using the wild-type *Synechocystis* sp. PCC6803 strain were carried out, as well as large-scale production of P(3HB). An outdoor 400 L scale serpentine photobioreactor was used to cultivate the cyanobacterial cells, inducing P(3HB) production, the largest amount reported to our knowledge under environmental conditions. The P(3HB) was subsequently extracted and characterized. In addition, the processability of the P(3HB) produced was confirmed using 3D printing (fused deposition modelling). This work thus demonstrated how sunlight, CO_2 and limited supply of simple nutrients can be successfully utilized for the large-scale production of a sustainable biobased polymer, P(3HB), which can in turn be used for both bulk and medical applications.

2. Methodology

2.1. Laboratory scale growth conditions

A series of growth experiments were conducted at lab-scale to investigate the effects of different cultivation conditions on the accumulation of P(3HB) in *Synechocystis* sp. PCC6803. The cyanobacterium was initially grown in BG11 medium for four days in an Algem photobioreactor (Algenuity, UK) under 500 μmol/s/m^2 light intensity and mixing at 160 rpm until it reached an optical density (OD) of 3 at 750 nm. This culture was then used to inoculate a total of 27 flasks, with the inoculum making up 20 % (v/v) of the media. All flasks had modified BG11 media that lacked nitrogen and phosphorus (-N,-P), with potassium phosphate replaced with potassium chloride and no sodium nitrate added. Nine different conditions with varying salinity levels (in the form of sodium chloride by adding 0 g/L, 5 g/L and 10 g/L to the media) and a carbon source (in the form of sodium acetate by adding (0 g/L, 4 g/L, 4 g/L), were prepared in triplicates to test their effects on P(3HB) accumulation. The cultures were maintained at a constant temperature of 32 °C and mixed at 120 rpm under constant lighting. Samples were collected every 7 days to quantify P(3HB) and $OD_{750\ nm}$ was measured. The condition that led to the highest P(3HB) output was used for the large-scale experiment.

2.2. Large-scale cultivation of Synechocysitis sp. PCC6803

The PhycoFlow® (Varicon Aqua, UK) is a vertical serpentine 400 L photobioreactor and was used for large scale cultivation outdoors at the Arthur Willis Environment Centre (AWEC) (53.3832000157627◦ North, 1.4995194567741421◦ West), University of Sheffield. The PhycoFlow® was enclosed in a sunlit multiwall polycarbonate unit with 83 % light transmission [20]. Cultivation included two stages, light and dark stage. The light stage involved transparent glass tubes (length: 2.5 m; outer diameter: 54 mm; wall thickness: 1.8 mm) [21]. The dark stage is a 300 L holding tank. The tank also contains a PT 100 thermocouple, a negative temperature coefficient (NTC) probe for temperature measurement, and a pH probe. The cooling system was used to maintain the temperature below a limit of 32 °C and CO_2 was manually supplied at the initial point of the light stage.

40 L inoculum was prepared in carboy bottles for 24 days until an $OD_{750\ nm}$ of 0.95 was reached under constant lighting and a maintained temperature of 32 °C. To sterilise the photobioreactor, it was filled with tap water containing sodium hypochlorite at a final concentration of 200 mg/L which was circulated through the system for 24 h. Afterwards, the water was neutralised by adding 600 mg/L sodium thiosulphate and discarded. To start the cyanobacterial culture, the system was filled with BG11 media, which was sterilised using 50 mg/L of sodium hypochlorite for 24 h, then neutralised with 150 mg/L sodium thiosulphate, before adding 40 L of the cyanobacterial inoculum. Two 50 mL samples were collected daily, centrifuged for 20 min at 4696 xg and pellets and supernatants were stored at −80 ºC, for subsequent measurements of P (3HB), nitrate and phosphate content. The pH was measured daily and adjusted using carbon dioxide. This was done by pumping pure CO_2 at a flow rate of 1 L/min for a duration of 30 min or until the pH dropped to 7. Temperature and light intensity in the greenhouse were recorded daily using a UA-002–64 Temperature/Light Weatherproof data logger (Tempcon, UK). Air was supplied using an air pump at a flow of 2 L/min. $OD_{750\ nm}$ was recorded daily until reaching a value of 2 after which stock solutions of modified media (BG11-N-P) and acetate were added to induce production of P(3HB). The Phycoflow® is a photobioreactor that operates as a closed system with a lid on top of the holding tank. It is only opened during inoculation and when adding PHB stimulating media, so it can be considered a semi-sterile growth condition. However, we monitored for bacterial and fungal contamination during the outdoor cultivation using microscope images throughout the cultivation period.

2.3. Nitrate and phosphate assays

Nitrate availability was determined in supernatant samples using the Nitrite/Nitrate Assay Kit (Sigma Aldrich, Germany) following the kit's protocol and the absorbance was measured at 540 nm in a microplate reader. Total phosphate in media was measured in supernatant samples

using the ascorbic acid method,[22] and absorbance was measured using a spectrophotometer at a wavelength of 880 nm. All samples were tested in triplicates. In addition, to ensure accuracy, all glassware and plastic bottles were acid washed.

2.4. P(3HB) extraction

P(3HB) was acquired using Soxhlet extraction, where the pelleted biomass was freeze-dried and 15 g were placed in a thimble in the Soxhlet apparatus [23]. 350 mL of methanol was used to remove cell contents including lipids and chlorophyll by running in the Soxhlet for two days, followed by extraction of P(3HB) by using 350 mL of chloroform for another 2 days. The P(3HB) containing chloroform solution was concentrated by drying under a fume hood and precipitated using 10 volumes of ice-cold methanol. The precipitate was re-dissolved in chloroform and precipitated using 10 volumes of ice-cold ethanol. The final precipitate was air-dried [24].

2.5. Harvesting of Cyanobacteria grown in Phycoflow®

When the stationary phase was reached, chitosan (0.1 g/L culture) was added to the culture and mixed thoroughly to harvest the biomass. It was left for 24 h to allow for flocculation and separation. This produced a more concentrated layer which was collected and reduced to 60 L. Further concentration was done through centrifugation at 4696 xg, producing 4 L of concentrated biomass.

2.6. Chemical characterization of the polymer

The functional groups of the polymer sample were confirmed using Fourier Transform Infrared-Attenuated Total Reflected (FTIR-ATR) spectroscopy analysis using a Perkin Elmer Frontier FT-IR spectrometer. Before loading the sample, the instrument was scanned empty with wavelengths between 4000 cm^{-1} to 500 cm^{-1} to set the background. The sample was prepared in the form of a solid film, fastened on the diamond probe and scanned again with a similar wavelength range. Further confirmation of the chemical structure of the polymer was carried out using ^{1}H nuclear magnetic resonance (NMR) analysis. The sample was dissolved in deuterated chloroform (Sigma-Aldrich) and measured using Bruker AVIII 400 MHz un with 5 mm solution state double resonance broadband probes. The monomer composition of the polymer sample was quantified using gas chromatography. Both dry cells and polymer samples are subjected to methanolysis, as described previously [25,26]. The amount of the methyl ester was calculated against a standard curve constructed using pure methyl ester standards. The analysis was conducted using a Perkin Elmer XL Autosampler equipped with the Zebron ZB-5plus Capillary GC column. An injection volume of 0.5 µl was used. The oven temperature was set to start at 60 °C and held for 3 min, followed by a ramp at a rate of 10 °C per minute up to 230 °C, and then held at 230 °C for 2 min. The carrier gas used was hydrogen. For molecular weight determination, gel permeation chromatography (GPC) was carried out. The polymer was dissolved in chloroform which served as the mobile phase. An injection volume of 100 µl was run using a PLgel 5 µm mixed C column with a set length of 650 mm. The flow rate was 1.0 mL min^{-1} at 40 °C. Peaks were detected using Cirrus software and set for manual analysis. The values were calculated against the polystyrene 9-point standard curve as follows:

$$y = 16.800650 - 1.727800x + 0.08949x^2 - 0.002084x^3$$

Where y is the molecular weight, M_w and x is the retention time in minutes, using narrow standard calibration with a curve fit of three.

2.7. Thermal characterization

Differential Scanning Calorimetry (DSC) analysis was conducted to determine the glass transition temperature (T_g) and melting temperature (T_m) of the samples. 10 mg of the polymer sample was placed on a DSC aluminium pan and run using the Perkin Elmer DSC4000 instrument. The samples were thermally scanned twice from −80 °C to 200 °C at a rate of 10 °C per minute, isothermal for 5 min and cooled at the same rate until −50 °C. The thermal scan ended at 30 °C. Degradation temperature (T_d) was determined by thermogravimetric analysis (TGA) using Perkin Elmer Pyris 1 TGA instrument. About 10 mg of the polymer sample was placed on a ceramic sample pan and scanned from 30 °C until 500 °C. Both DSC and TGA curves were processed using the Perkin Elmer Pyris software.

2.8. 3D printing

Fused deposition modelling (FDM) was used to create a 3D printed model. When dealing with delicate medical devices, it is crucial to ensure that the nozzle travels precisely to create filaments that form the model. This filament dictates a specific path for the slicer to follow, resulting in the generation of a G-code for the 3D printer to produce a clean and controlled print. To achieve this, first, we created a computer-aided drafting (CAD) model using AutoCAD, with dimensions of 1 cm by 1 cm and composed of parallel lines with a spacing of 2.5 mm. This model was used to generate a G-code using the Repetier Host software. Finally, for printing, we used the Cellink BioX 3D printer, utilizing bioplastic material and operating at an extrusion temperature of 185 °C, with a nozzle diameter of 0.3 mm.

3. Results and discussion

3.1. Identification of optimal conditions for P(3HB) production

We investigated the impact of varying salinity and acetate addition for P(3HB) production under both N and P-limiting conditions. In the freshwater samples, including a carbon source (4 g/L) triggered P(3HB) accumulation up to 10.7 % of DCW on the 28th day. This led to a P(3HB) titer of 73 mg/L and productivity of 0.11 mg/L/hr (Fig. 1). Increasing the acetate concentration to 8 g/L in the freshwater samples led to an overall decrease in P(3HB) content, resulting in a yield of 4.75 % of DCW. Interestingly, we found that in the absence of acetate, higher salinity was associated with a positive effect on P(3HB) accumulation, with the 10 g/L NaCl samples resulting in the highest accumulation of P(3HB) of 1.9 % of DCW on the 28th day (Fig. 1). Conversely, samples with added acetate (both 4 g/L and 8 g/L)) exhibited lower production of P(3HB) with higher salinity, i.e., up to 80 % less P(3HB) compared to fresh water containing acetate. These results suggest adverse combined effects of salinity and acetate. Given that acetate is added in the form of sodium acetate, it might be that it further enhances the sodium ion content, accentuating its adverse effects on cells. A similar outcome was observed in a previous study which found that in the presence of acetate, salinity had a negative effect on glycogen accumulation, which is a possible precursor of P(3HB) during N limitation [13]. The authors attributed this to cell lysis from high salinity, rather than high acetate.

In this study, no chemical pretreatment was used to increase the PHB production However research has been done to identify chemicals that are capable of enhancing PHB accumulation and production in Synechocystis. The study states that using (2-phenylethanol and phenoxyethanol) stimulates PHB [27] another study stated that Erythromycin treatment is capable of increasing the accumulation of glycogen and PHB in Synechocystis [28].

Based on our findings, P(3HB) accumulation was highest in freshwater with 4 g/L added acetate, and after 28 days of nitrogen and phosphorus limitation, where the growth rate was (0.044 d^{-1}). These conditions were selected to cultivate *Synechocystis* sp. PCC6803 outdoors at 400 L scale.

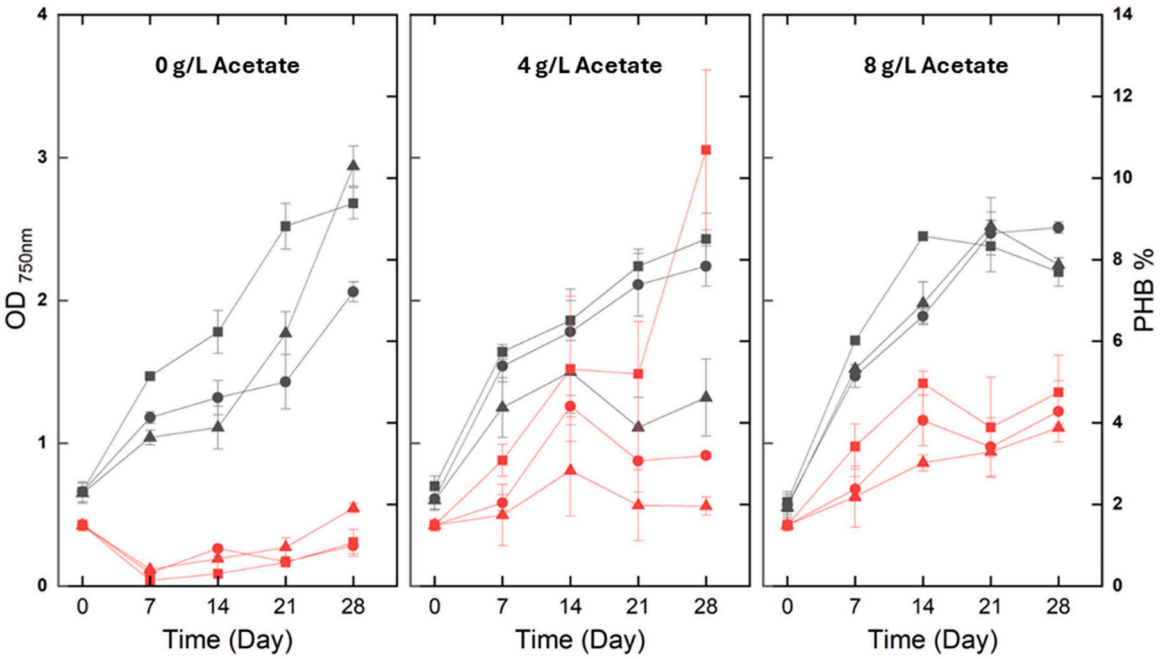

Fig. 1. Effect of sodium acetate and salinity on growth (optical density) and P(3HB) % of DCW yield. Black lines indicate OD750nm.Red lines indicate PHB% of DCW. Squares indicate freshwater samples; Circles indicate 5 g/L salinity, Triangles indicate 10 g/L salinity. Bars show mean ± standard error.

3.2. Large scale cultivation of Synechocystis sp. PCC6803

The outdoor cultivation involved a two-step process, whereby a high concentration of cells was first achieved ($OD_{750\ nm}$=2 at day 10) in the photobioreactor, followed by stimulating P(3HB) accumulation through N/P limitation along with addition of 4 g/L acetate. Fig. 2 shows all the parameters characterized in the culture of *Synechocystis* sp. PCC6803 in the Phycoflow®. The strain did not show an adaptive lag phase during growth. No change in growth rate was observed after the addition of the BG11 -N -P media at day 10 and the cyanobacteria grew exponentially up to 23 days reaching an $OD_{750\ nm}$ of 5 with a growth rate of (0.172 d^{-1}), and accumulating 560 g of dry biomass. The pH of the culture increased each day to an average value of 9.5 (with a maximum of 11) and was adjusted to pH 7 by dosing with CO_2. Since the cultivation was carried out in a natural outdoor setting, lighting and temperature were affected by varying weather conditions in Sheffield, UK, where light intensities in the greenhouse ranged from 0 lm/m^2 at night to a maximum of 172,223 lm/m^2 during the day. The average duration of daylight was 14 h. The temperature in the greenhouse ranged from 23 °C to 46 °C. Cooling was undertaken by means of a water sprinkler when the

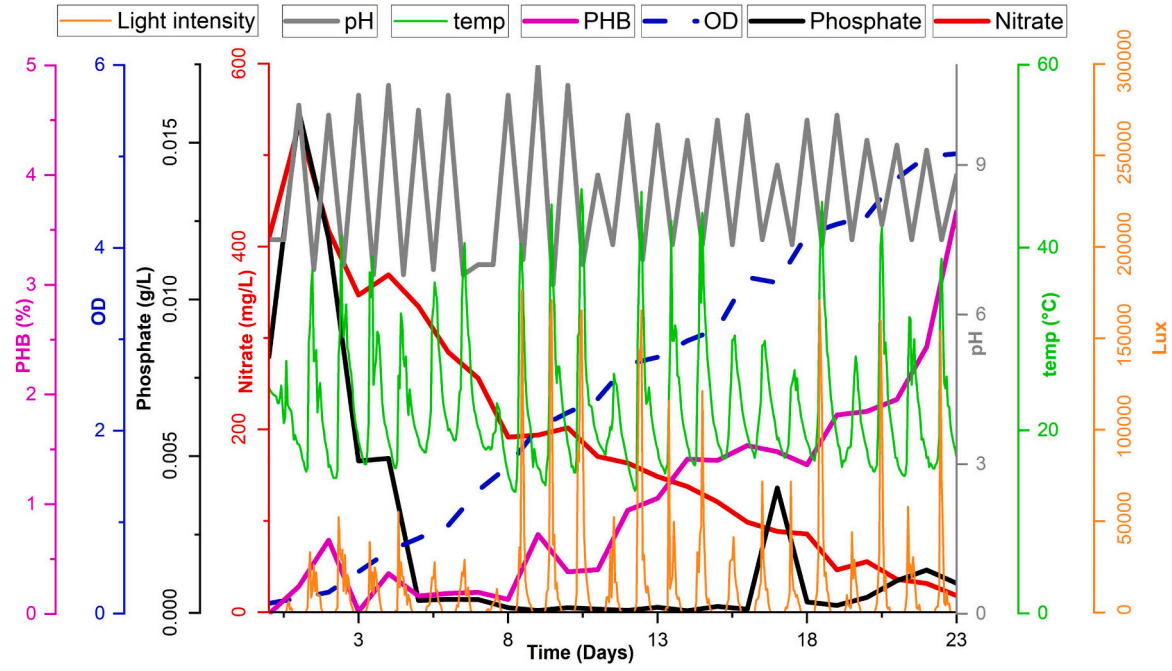

Fig. 2. Cultivation characteristics of *Synechocystis* sp. PCC6803 in an outdoor 400 L Phycoflow® in BG11 media using fresh water with sodium acetate and CO_2 as carbon sources. Optical density was used to monitor growth rates. Temperature, light, pH and nutrients (PO_3^- and NO_3^-) were quantified throughout as well as P (3HB) content.

temperature exceeded 32 ºC inside the greenhouse.

The nitrate and phosphate concentrations appeared to increase after the first day, but this was due to improved mixing after concentrated media was added to the system (Fig. 2). The amount of nitrate decreased sharply until the 8th day followed by a less steep decrease towards the end of the cultivation. In parallel, P(3HB) accumulation started increasing after nitrate had dropped to 200 mg/L at approximately day 8, highlighting the effect of nitrogen deprivation on P(3HB) accumulation, reaching approximately 4 % of DCW on day 23. Similar to nitrate, phosphate concentrations decreased steeply until day 5, and then remained at a constant low value, except for an unexpected small peak around day 17. *Synechocystis* sp. PCC6803 cells are able to accumulate phosphate when it is depleted [29]. As a result of the reported decrease in nutrients, the cells utilize the acetate, which was introduced on day 10, and consequently accumulated P(3HB). The P(3HB) content started increasing steadily from the 8th day of culture till the 23rd to reach a value of 3.5 % of DCW. This is equivalent to a PHB titer of 49 mg/L, and productivity of 0.09 mg/L/hr. The lower P(3HB) amount in the outdoor experiment compared to the 10.7 % achieved in the laboratory experiments is likely due to the uncontrolled environment in the large-scale culture, as opposed to the continuous lighting, optimum temperature and shaking conditions in the laboratory. However, P(3HB) productivity was similar.

Although reports of cultivating *Synechocystis* sp. PCC6803 at large scale are not common, a previous study in 2018 grew 200 L of *Synechocystis* sp. CCALA192 for producing P(3HB). 12 % DCW P(3HB) was achieved using non-sterile conditions, while controlling light, temperature and dissolved oxygen as a contamination prevention measure [19]. Similarly, using outdoor conditions is even less common. In 2021, Roh *et al.* cultured a genetically modified *Synechococcus* 2973 strain for the purpose of producing P(3HB) in an outdoor 3 L bioreactor. They reported P(3HB) accumulation of 10.5 % of DCW. They also utilized industrial flue gas as the sole carbon source. We suggest conducting additional research to explore methods for boosting PHB yields, as well as considering the production of co-products that could potentially mitigate the overall environmental and economic costs associated with cultivation and downstream processing.

It is worth noting that P(3HB) production under different salinity and acetate conditions were different between small and large-scale experiments under N and P limiting conditions. In the small-scale experiment, using freshwater samples with an added carbon source (4 g/L), P(3HB) accumulation reached 10.7 % of DCW by the 28th day, with a resulting concentration of 73 mg/L and a productivity of 0.11 mg/L/hr. The growth rate was relatively slow, at 0.044 d^{-1} . In contrast, the large-scale setup involved cyanobacteria grown in BG11 -N -P media, which was introduced on the 10th day. Here, the cyanobacteria grew exponentially up to the 23rd day, achieving P(3HB) concentration of 49 mg/L and a productivity of 0.09 mg/L/hr, with a significantly higher growth rate of 0.172 d^{-1} . These results suggest that while the small-scale setup produced higher P(3HB) accumulation and productivity, the large-scale system supported faster growth and substantial biomass production, albeit with a slightly lower P(3HB) concentration.

3.3. Extraction of P(3HB) from Synechocystis sp. PCC6803

The 400 L culture was utilized for P(3HB) extraction. To our knowledge, this is the first study to extract printable quantities of this polymer from a photosynthetic organism. The polymer was extracted using a Soxhlet extraction method which resulted in a polymer with a deep red colour (Fig. 3b). This red pigment was removed through washing by cold ethanol (Fig. 3b, c & d). The final modified protocol developed in this work resulted in the production of a fine solvent cast plastic film (Fig. 3e).

3.4. Chemical characterization of the produced PHAs

There are no reports characterizing the P(3HB) polymer produced by *Synechocystis* sp. PCC6803 due to inadequate sample size. Due to the scale up process successfully achieved in this work, sufficient amounts of biomass and hence P(3HB) enabled a thorough chemical and physical characterization, prior to 3D printing.

3.4.1. Fourier-transform Infrared spectroscopy (FTIR)

To confirm this is a short chain length PHA, the dried polymer sample was subjected to the Fourier-Transform Infrared spectroscopy to determine the P(3HB) critical functional groups within the polymer produced by the *Synechocystis* sp. PCC6803. The sole terminal methyl group of the side chain was detected at 2976 cm^{-1} of wavelength, and the carbonyl group was detected at 1277 cm^{-1} of the ester bond in the polymer (Fig. 4a).

3.4.2. Nuclear Magnetic Resonance (NMR)

Proton nuclear magnetic resonance analysis or ^{1}H NMR further confirmed the molecular structure of the homopolymer of P(3HB). The terminal methyl group of the P(3HB) side chain is known to be the most shielded with three hydrogen molecules, appearing at 1.2 ppm of the spectrum and labelled as number 1. Meanwhile, a couple of hydrogen atoms from the α carbon were detected at 2.5 ppm and labelled number 3 (Fig. 4b). Though these hydrogen atoms are intermediary with respect to shielding, they have the most extensive interaction with the neighboring and geminal hydrogen atoms and linkages and hence exhibit multiplet signals. Ultimately, the single hydrogen atom of the β-chiral carbon attached to both the methyl group and α-carbon gives another multiplet signal on the spectrum at 5.2 ppm and is labelled with number 2 (Fig. 4b), indicating the most deshielded hydrogen atom due to a direct bond with the oxygen atom of the polymer ester linkage. The presence of

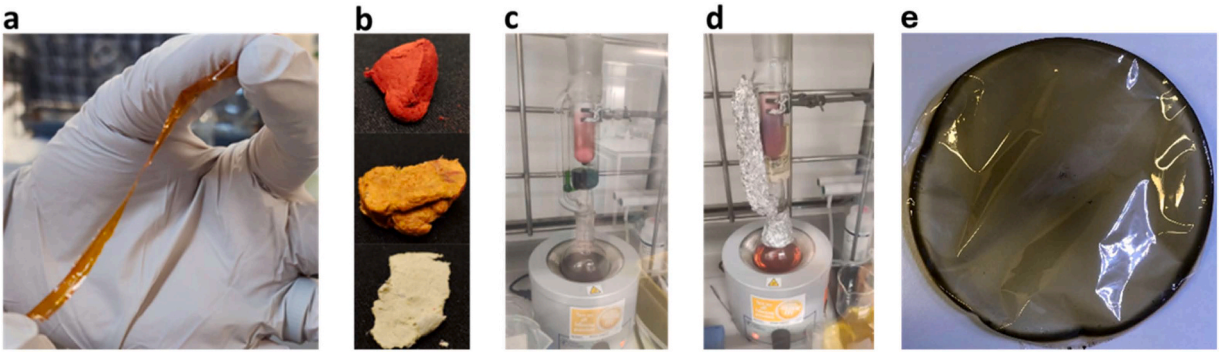

Fig. 3. The polymer extracted from *Synechocystis* sp. PCC6803 a) A piece of bioplastic (P(3HB)) extract b) Extracted P(3HB) with red pigment fading after each ethanol wash c) First stage soxhlet extraction using methanol, showing green chlorophyll in the extracted solvent. d) Second-stage soxhlet extraction using ethanol showing red pigment in chloroform solvent. e) A solvent cast of P(3HB) Film.

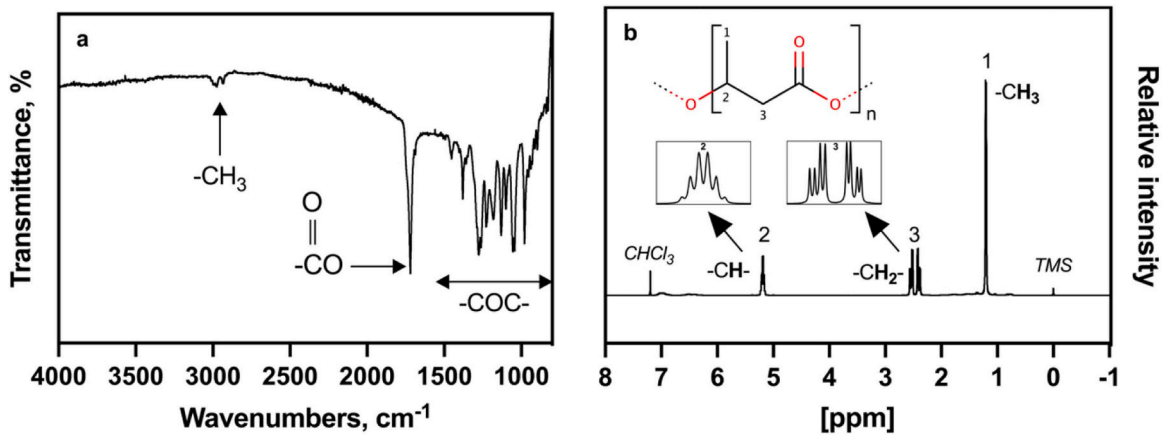

Fig. 4. : a) Fourier Transform Infrared (FTIR) spectrum for P(3HB) b) Proton Nuclear Magnetic Resonance (^{1}H NMR) spectrum with P(3HB) molecular structure (inset). TMS is tetramethyl silane, which serves as an internal standard with a 0 ppm value, and $CHCl_3$ is a trace of chloroform from the deuterated solvent used for this analysis.

the peak at 1.2 ppm and the absence of any peaks until the α-carbon at 2.5 ppm indicates no alkyl group along the side chain, confirming that the PHA is indeed the homopolymer P(3HB).

3.4.3. Gel permeation chromatography (GPC)

The P(3HB) produced was also subjected to the gel permeation chromatography analysis to determine the molecular weight (Fig. 5). This enables determination of the molecular weight, which directly influences the physical and chemical properties of the polymer. In the context of polymeric materials, this information provides a view regarding the polymer's molecular size, which is directly related to the polymer chain length that influences the mechanical strength and thermal properties, in which higher molecular weight gives higher sturdiness to the polymer. The molecular mass of the P(3HB) produced in this study was similar compared to the production by other types of microalgae and cyanobacteria (Table 1). The number average molecular weight, M_n, is 108 ± 9 kDA; meanwhile, the weight average molecular weight, M_w, is 154 ± 13 kDa. These values indicate a relatively narrow molecular weight distribution with a 1.4 polydispersity index which is consistent with other studies. The fact that the PDI is almost 1 indicates that the polymer produced is of very high quality with not much difference in M_w and M_n, resulting in homogenous and repeatable polymer properties, crucial for future applications of the P(3HB) both in the bulk and medical applications.

3.5. Thermal characterisation

3.5.1. Differential scanning calorimetry (DSC)

Differential scanning calorimetry analysis reveals information regarding the thermal properties of a sample, especially for thermoplastic materials. P(3HB) from this study was subjected to multiple temperature scans between −80 °C and 200 °C. The glass transition temperature, T_g of the P(3HB), was clearly detected as a baseline shift at around 8 °C in the second heating cycle in the thermograph. The crystallisation temperature, T_c, was also detected at around 63 °C through cold crystallization (Fig. 6b) a phenomenon where crystalline structures emerge during an exothermic process before the melting temperature, T_m.[30,31] This T_c is comparable to the crystallization T_c of P(3HB),

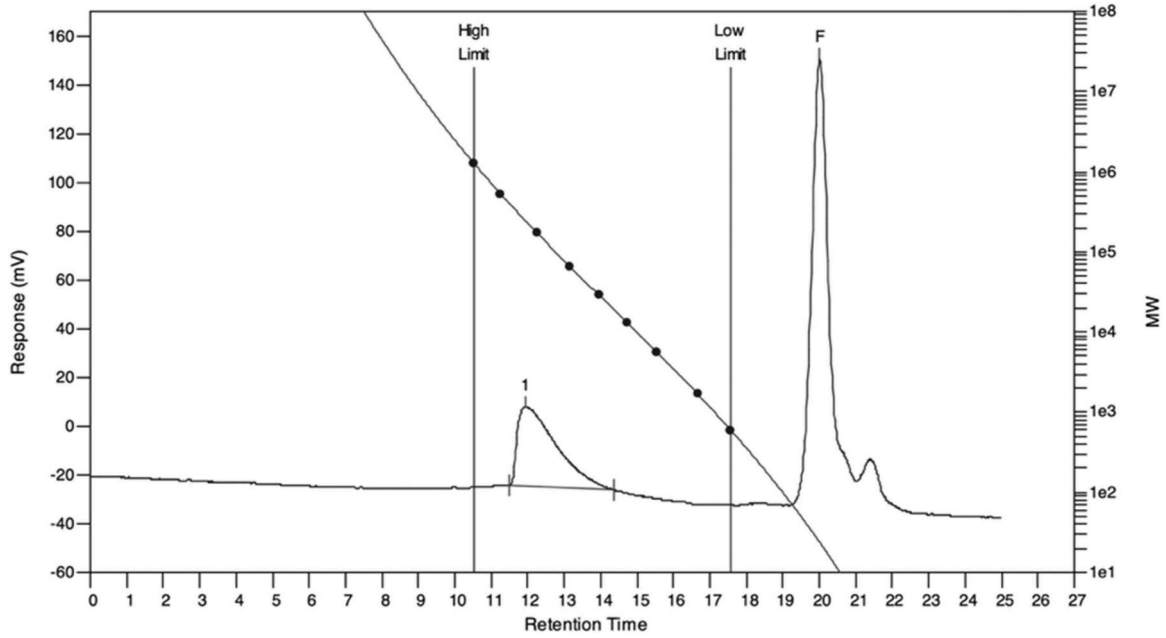

Fig. 5. Gel Permeation Chromatography (GPC) chromatogram for P(3HB), using polystyrene standard. The retention time of the polymer was detected to be around 12 min, labelled as 1. F is mobile phase of the run, which is chloroform.

Table 1
Molecular weight of P(3HB) and thermal properties of P(3HB) produced by different types of algae and cyanobacteria.

Producer	M_n, kDa	M_w, kDa	PDI	Thermal Properties				PHB %	Cultivation conditions	Reference
				T_g	T_c	T_m	T_d			
Synechocystis sp. PCC6803								10.70 %	Lab scale	This study
Synechocystis sp. PCC6803	108 ± 9	154 ± 13	1.4	8	63	171	307.8	3.50 %	Large-scale 400 L serpentine bioreactor	This study
Synechocystis sp. PCC6803	246	573	2.3	-	-	-	-	32 %	Lab scale	(Koch et al., 2020)
Synechocystis sp. PCC6803	503	875	1.7	-	-	-	-	81%	Lab scale	(Koch et al., 2020)
Synechocystis PCC 6714	316	1052	3.3	-	63	160	-	NA	Lab scale	(Lackner et al., 2019)
Spirulina sp. LEB−18	-	59 – 62	-	-	-	-	-	8.14 ± 0.30	Lab scale	(Costa et al., 2018b)
Spirulina sp. LEB−18	-	163 ± 8	-	-	-	172	278.4 – 325.5	9.56 ± 0.76	Lab scale	(Costa et al., 2018a)
Synechococcus subsalsus	-	180 ± 2	-	-	-	174	296.9 – 370.0	7.87 ± 0.71	Lab scale	(Costa et al., 2018a)
Chlorella minutissima	-	-	-	-	-	-	255.7 – 351.6	NA	Lab scale	(Costa et al., 2018a)
Spirulina subsalsa	-	-	-	5	-	~150	261	7.45%	Lab scale	(Shrivastav et al., 2010)
Spirulina sp. LEB−18	-	-	-	-	-	172	240.9	30.1 ± 2%	Lab scale	(Morais et al., 2015)
Synechocytis sp. CCALA192		930	4.4	30		174	265	12.50%	Large-Scale 200-L tubular photobioreactor,	(Troschl et al., 2018)

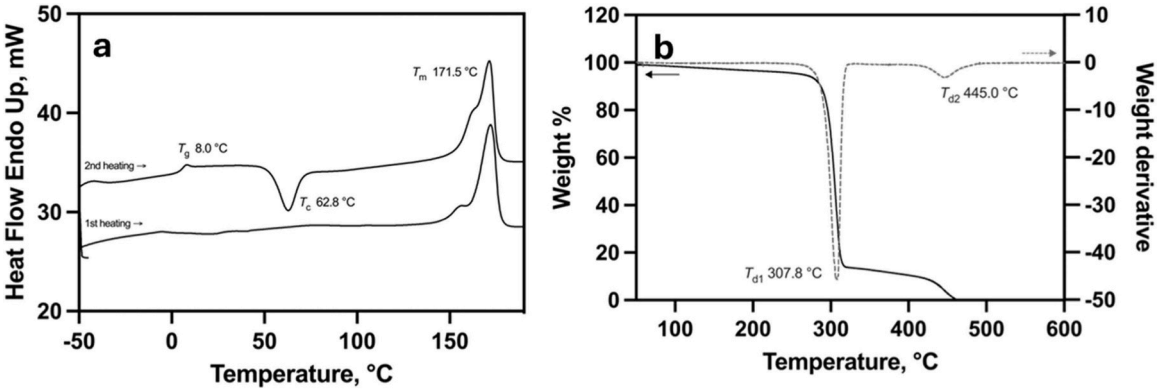

Fig. 6. : a) Thermogram from Differential Scanning Calorimetry (DSC) plot for P(3HB) produced by *Synechocystis* sp. PCC6803. This confirmed the thermal properties of the polymer) Thermogram from Thermogravimetric Analysis (TGA) for P(3HB) produced by *Synechocystis* sp. PCC6803.

62.9 °C,produced by *Synechocystis* sp. PCC 6714.[32].

As a thermoplastic material, the analysis detected the melting temperature, T_m, of the P(3HB). The T_m was around 172 °C from the second heating cycle and 175 °C from the first, consistent with the typical T_m of P(3HB) produced by other microalgae and cyanobacteria, which are found to be between 170 °C and 175 °C (Table1).

3.5.2. *Thermogravimetric analysis (TGA)*

The degradation of the polymer was detected using thermogravimetric analysis. P(3HB) has a T_d, of 308 °C, where the maximum rate of degradation was observed, comparable to P(3HB) produced from other microalgae (Table1). However, another material degradation peak was observed at higher temperatures of 445.0 °C (Fig. 6b), indicating the presence other components co-produced from the microalgae, such as lipids and pigments. This extra T_d was also observed in *Spirulina* sp. LEB-18, *Synechococcus subsalsus* and *Chlorella minutissima*, with temperature range between 364.4 °C to 623.2 °C.[33] Overall, the thermal analysis data demonstrated that the polymer is highly suitable for melt processing techniques including fused deposition modelling, melt extrusion, injection moulding and melt electrowriting, given that the degradation temperature is significantly higher than the melting temperature.

3.6. *3D printing*

A 3D printing test was undertaken using the extracted P(3HB) (Fig. 7) as a proof of concept. The P(3HB) extracted from *Synechocystis* sp. PCC6803 successfully produced a 1 cm by 1 cm print. This model serves as proof of the potential of P(3HB) from *Synechocystis* sp. PCC6803 to be used in the future for manufacturing delicate medical devices. However, further optimization, especially in terms of

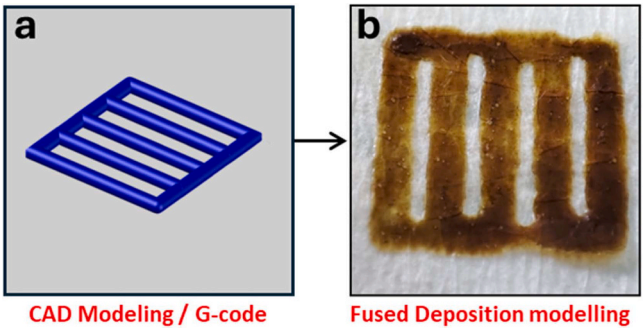

Fig. 7. : a)Digital design (CAD/G-code) to physical object through FDM via 3D printing b) 3D printed model.

purification and printing parameters need an extensive study for a feasible prototype.

4. Conclusion

The optimal conditions for P(3HB) production from *Synechocystis* sp. PCC6803 were established using freshwater with 4 g/L acetate after 28 days of dual nutrient (nitrogen and phosphorus) limitation conditions. The large-scale (400 L) cultivation of *Synechocystis* sp. PCC6803 was carried out successfully for the first time, producing promising results in terms of growth rates (0.172 d^{-1}), although P(3HB) accumulation was lower than that achieved at lab scale, possibly due to the complexity and variability of the environmental factors that impacted on polymer synthesis and the time of harvesting. However, this work has proven that large-scale culture of *Synechocystis* sp. PCC6803 is feasible for P(3HB) production utilizing sunlight and CO_2, as a sustainable process. Further research is required to increase the yield and optimize purification and extraction methods. By focusing on the extraction processes, the strain could be explored for the co-generation of multiple products, such as pigments. Overall, this study provides invaluable insights into the potential use of cyanobacteria for sustainable P(3HB) production.

Funding

We acknowledge our funders EPSRC (EP/X016951/1) and NERC (NE/P016820/1).

CRediT authorship contribution statement

Elbaraa Elghazy: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Kamonchanok Wianglor:** Formal analysis. **Syed Mohamed Daniel Syed Mohamed:** Writing – review & editing, Formal analysis. **Santosh Tetali:** Formal analysis. **Jagroop Pandhal:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ipsita Roy:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Mahendra Raut:** Formal analysis.

Declaration of Competing Interest

None.

Aknowledgments

We acknowledge the team at the Arthur Willis Environment Centre, Maggi Killion and Ben Palmer (School of Biosciences, The University of Sheffield). We would also like to thank Dr. Esther Karunakaran for provision of the *Synechocystis* sp. PCC6803 strain.

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