



Recovery and characterization of polyhydroxybutyrate from *Chlorogloea fritschii* TISTR 8527 using halogenated and green solvents

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Abstract

Polyhydroxybutyrate (PHB) is a potential biopolymer and can replace petroleum-based plastic. PHB accumulated by bacteria needs a supply of carbon source whereas cyanobacteria are capable of synthesizing PHB by carbon fixation using sunlight energy. Since PHB is an intracellular product the polymer recovery cost is about 50 % of production cost. Apart from these, cyanobacterial pigments also affect the purity and recovery yield. In the present study, *Chlorogloea fritschii* TISTR 8527 was chosen as a PHB cell factory which accumulated 28 % of biopolymer. The hypochlorite-chloroform dispersion method could remove the pigments along with PHB extraction with 95.51 ± 3.164 % of the recovery in a single step. In addition to this, PHB extraction was performed with various halogenated solvents and green non-halogenated solvents with hypochlorite pretreatment. Selected solvents were unable to recover more than 90 % of polymer yield due to unsuitable operating conditions. Besides this, the green solvent dimethyl carbonate (DMC) was employed for PHB recovery with prior pigment removal from biomass using chilled methanol. DMC was capable of 83.34 ± 0.76 % biopolymer recovery with a purity of 75 %. PHB recovered using DMC has improved thermal and material characteristics.

Keywords Cyanobacteria · PHB recovery · Dispersion · Green solvent · Solvent extraction

Introduction

Global plastic production was 368 million tonnes in 2019 and is expected to double in the next two decades. Plastic is an inexpensive, nonbiodegradable, hydrophobic material that consumers may utilize in their daily routine. Plastic films are strong, impermeable to gas and moisture, and found in packaging applications. Due to a lack of infrastructure and process expenses, only 9 % of plastic packaging products are recycled in the USA despite their durability and intactness (Lebreton and Andrady 2019). Approximately 53 t of plastic waste will be entering the aquatic ecosystem by 2030 which affect the food chain and influence human health (Borrelle et al. 2020; Castro-Castellon et al. 2022). Apart from this, plastic processing generates greenhouse gases (GHGs) which contribute to global warming. The solution to these

problems is the usage of bioplastics or biodegradable plastics which are functionally similar to polyethylene.

Bioplastics may be biodegradable or partially degradable and include polymers such as polycaprolactone, polybutylene succinate, polylactic acid (PLA), starch, and cellulose-based plastics. The degradation of bioplastics depends on their composition, degree of crystallinity, and environmental factors (Tokiwa et al. 2009). Polyhydroxyalkanoates (PHAs) are esters that can be synthesized from renewable sources: plants and heterotrophic bacteria. Polyhydroxybutyrate is a short chain length PHA made of hydroxybutyric acid with a molecular weight of $10^5 - 10^6$ Da. PHB is isotactic and hence biodegradable (Wölfle et al. 2009). PHB has a high solubility in organic solvents such as chloroform, dichloromethane, and dichloroethane. Heterotrophic bacteria such as *Alcaligenes eutrophus*, recombinant *Escherichia coli*, and *Bacillus megaterium* need a sterile source of glucose for PHB synthesis (Ahn et al. 2000; Yang et al. 2010; Mohanrasu et al. 2020). Cyanobacteria can also produce PHB by CO₂ fixation using sunlight with minimal nutrients under nitrogen or phosphate limitations (Lee et al. 2022; Yashavanth and Maiti 2024). The thermoplastic,

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biocompatible, biodegradable, and non-immunogenic nature of PHB eases its use in the form of surgical mesh and surgical sutures, ocular devices, bone plating systems, cardiovascular patches, bone marrow scaffolds, tendon, and cartilage repair devices (Chen and Wu 2005; Patnaik 2007). The antimicrobial nature of PHB means it is used in aquaculture in preventing *Vibrio* epidemics (Defoirdt et al. 2007). PHB hydrogels play an important role in targeted drug delivery and sustained release of drugs (Fang et al. 2023). In the skin healing process, methyl groups of PHB help in the adhesion of cells by promoting extracellular matrix (Sangsanoh et al. 2017; Castellano et al. 2018). Applications of PHB-based supercapacitors in consumer and industrial electronics, agriculture, and environmental monitoring are numerous (Migliorini et al. 2020). Thus, various fields demand good quality polymer for its end use.

PHB has to be recovered and purified by various methods such as solvent extraction, surfactants, non-PHB cellular material (NPCM) removal using hypochlorite, dispersion of hypochlorite and chloroform, and enzymatic digestion (Yashavanth et al. 2021). Recovering PHB from cytoplasm increases production cost by up to 50 % of the cost of the polymer since PHB has to be purified in multiple steps (Samorì et al. 2015b). The molecular weight of the recovered polymer may decrease because of NPCM digestion with hypochlorite. Hypochlorite and chloroform dispersion can decrease the loss in molecular weight of recovered PHB while raising the yield and purity (Hahn et al. 1994). Solvent recovery is the most used method by industries due to its high recovery efficiency resulting in polymer of high molecular weight along with the removal of endotoxins (Lee et al. 1999).

Cyanobacteria contain pigments that affect the material properties of PHB after extraction. Therefore, pigment removal from cyanobacteria is essential before PHB recovery with a minimum number of solvents and steps involved (Troschl et al. 2017). Methanol can be used for pigment removal at 4 °C before PHB extraction (Gopi et al. 2014; Zavřel et al. 2015). The combination of hypochlorite and chloroform may result in higher recovery with pigment removal, but both are toxic to humans and the environment. Therefore, the selection of solvents based on safety and reusability has significance in the evaluation of the extraction process (Mongili et al. 2021). Dimethyl carbonate (DMC) is a solvent of industrial importance and is used as a reagent in various processes. DMC is considered a green solvent since it is biodegradable and less toxic to humans and the environment (Delledonne et al. 2001) and could be used as an alternative to halogenated solvents (Mongili et al. 2021). Previously, DMC was used for the extraction of homopolymer PHB and copolymer polyhydroxybutyrate-hydroxyvalerate (PHBV) from single strains and mixed consortia (Samorì et al. 2015a, 2015b; Elhami et al. 2022).

The present study focuses on the recovery of PHB from the cyanobacterium *Chlorogloea fritschii* TISTR 8527 using various extraction methods. PHB was extracted with pigment removal using the dispersion method. Dispersion involves a two-phase extraction procedure using hypochlorite (for biomass digestion) and chloroform (solvent for PHB extraction). Various halogenated such as chloroform and dichloroethane (DCE) and non-halogenated solvents namely propylene carbonate, dioxolane, DMC, and acetic acid were selected for PHB recovery based on the capabilities of solvents to dissolve PHB after hypochlorite pretreatment (Terada and Marchessault 1999). A suitable solvent is selected based on performance and environmental concerns. DMC is employed as a green solvent in the current investigation to recover PHB after pigment removal from cyanobacteria using methanol. The selection of a suitable biomass-to-solvent ratio, extraction temperature, and mixing time are critical factors as they affect the recovery yield. Once the PHB is extracted from the biomass, it is recovered by evaporation of the solvent. Comparison of thermal properties of recovered PHB are shown to decide the most suitable method based on polymer characteristics.

Materials and methods

Conditions for PHB production

Chlorogloea fritschii TISTR 8527 was procured from the Thailand Institute of Scientific and Technological Research, Thailand. The inoculum was prepared in a 1 L flask containing 500 mL of modified BG-11 medium under cool light illumination of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Monshupanee et al. 2016) with a 12 h:12 h light-dark period. The composition of normal BG-11 medium is 40 mg L⁻¹ K₂HPO₄, 1.5 g L⁻¹ NaNO₃, 36 mg L⁻¹ CaCl₂·2H₂O, 75 mg L⁻¹ MgSO₄·7H₂O, 6 mg L⁻¹ citric acid, 6 mg L⁻¹ ferric ammonium citrate, 20 mg L⁻¹ Na₂CO₃, 1 mg L⁻¹ Na₂EDTA and 1 × TRACE. The composition of TRACE is as follows (1000×): 2.86 g L⁻¹ H₃BO₃, 0.222 g L⁻¹ ZnSO₄·7H₂O, 1.81 g L⁻¹ MnCl₂·4H₂O, 0.39 g L⁻¹ Na₂MoO₄·2H₂O, 0.049 g L⁻¹ Co(NO₃)₂·6H₂O and 0.079 g L⁻¹ CuSO₄·5H₂O (Rippka et al. 1979). For modified BG-11 medium citric acid was replaced with 20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH (HEPES–NaOH) and ferric ammonium citrate was avoided by usage of equimolar concentration of ferric chloride hexahydrate with final pH of 7.5 (Monshupanee et al. 2016). The mixing of inoculum was aided by a silicone rubber sparger with 1 % CO₂ and 1 vvm aeration. For experimentation, 1.8 L of optimized BG-11 medium with pH 7.5 was used (Yashavanth and Maiti 2024). The optimized BG-11 resulted from multi-objective media optimization of modified BG-11 media (Yashavanth and Maiti 2024). The composition

of optimized BG-11 medium used for experimentation was 0.1 g L⁻¹ NaNO₃, 26.24 mg L⁻¹ K₂HPO₄, 750 mg L⁻¹ MgSO₄·7H₂O, 36 mg L⁻¹ CaCl₂·2H₂O, 20 mM HEPES, 6 mg L⁻¹ FeCl₃·6H₂O, 7.11 mg L⁻¹ Na₂EDTA, 20 mg L⁻¹ Na₂CO₃ and 10× TRACE. The modified BG-11 and optimized BG-11 differ in concentrations of five components namely NaNO₃, K₂HPO₄, MgSO₄·7H₂O, Na₂EDTA and TRACE. An inoculum of 5 % (v/v) was added to the production medium to achieve an initial biomass concentration of 0.05 g L⁻¹. The experiment was performed with diurnal simulated sunlight in a flat-panel photobioreactor (PBR) (Labfors, Infors HT, 25.5 × 2 × 50 cm) with 3 % CO₂ (v/v) and 0.5 vvm air. The high cell-density biomass pre-grown under autotrophic mode was subjected to biopolymer production under the dark with 0.2 % (w/v) acetate as an inducer at the stationary phase (Yashavanth and Maiti 2024). The sampling was done at regular intervals to measure the dry cell weight (DCW) after centrifugation at 10000 rpm. The biomass harvested by centrifugation was freeze-dried and used for recovery of stored PHB (Farinacci and Laurent 2023).

PHB quantification by gas chromatography

PHB content of the lyophilized biomass was quantified by gas chromatography (GC) using a Varian 450-GC equipped with CP-Sil 8 CB (30 m × 0.25 mm i.d. 0.25 μm film thickness) column with 0.7 mL min⁻¹ helium as carrier gas with a split ratio of 100. The sample was prepared by the propanolysis method (Riis and Mai 1988) and analyzed using the thermal program by Juengert et al. (2018). The percentage of PHB present in the dried biomass was calculated using Eq. (1). Similarly, the recovered PHB was quantified by GC to calculate the percentage recovery and PHB purity using Eqs. (2) and (3).

$$PHB(\%) = \frac{\text{mg of PHB}}{\text{mg of dried biomass}} \times 100 \quad (1)$$

$$PHB \text{ recovery}(\%) = \frac{PHB \text{ recovered in g/L}}{\text{Total PHB in g/L}} \times 100 \quad (2)$$

$$PHB \text{ purity}(\%) = \frac{GC \text{ quantified of extracted PHB (g)}}{\text{extracted PHB (g)}} \times 100 \quad (3)$$

Extraction of intracellular PHB

PHB recovery by single-step hypochlorite - chloroform dispersion method

The commercially available hypochlorite (4 w/v %) was from Himedia and diluted to different concentrations (10,

20, 30, 50, 70 and 100 % (v/v)). 1 % (w/v) of lyophilized biomass (i.e., 10 mg of biomass per mL of solvent) was subjected to PHB recovery using the dispersion method with equal volumes of hypochlorite and chloroform at 30 °C for 1 h. After the dispersion the mixture was centrifuged to separate the layers i.e., the upper layer contains NPCM, the middle layer contains cell debris, and the bottom chloroform layer contains PHB. The lower bottom phase was collected to concentrate the biopolymer by evaporation of the solvent (Hahn et al. 1995) and the percentage recovery was calculated by using Eq. (2) after quantification by GC.

PHB recovery using two-step method using hypochlorite pretreatment followed by extraction with different solvents

Solvent extraction is a widely used method in the recovery of PHB due to the efficient recovery of PHB with integrity of the polymer characteristics. Various solvents used for our study were chloroform (boiling point: 61.2 °C), dichloroethane (84 °C), propylene carbonate (242 °C), acetic acid (118 °C), dioxolane (74 °C) and dimethyl carbonate (90 °C). Previous studies showed that the above solvents resulted in higher PHB recovery (Terada and Marchessault 1999). In the present study, 200 mg of lyophilized biomass was pretreated with 20 mL of 4 % (w/v) hypochlorite (Himedia) for 15 min at room temperature (Kanzariya et al. 2023). The pellet was collected by centrifugation after discarding the supernatant. PHB was recovered from the pellet at 100 °C for 1 h with the biomass-to-solvent ratio of 1 % (w/v) for the above-mentioned solvents. After extraction, the suspension was centrifuged to collect the solvent phase. The polymer was recovered by evaporation of solvents and the percentage recovery and purity of the polymer were calculated using Eqs. (2) and (3) after quantification by GC.

PHB recovery by two-step method using pigment removal by methanol followed by extraction with green solvent dimethyl carbonate (DMC)

Effect of biomass loading on PHB recovery by DMC Lyophilized biomass was used for the extraction of PHB using a green solvent DMC. First the freeze-dried biomass was subjected to pigment removal using chilled methanol at 4 °C overnight in 50 mL Falcons tubes. The biomass used for extraction varied at different concentrations. Lyophilized biomass of 50, 100, 150, and 200 mg were suspended in 4 mL of DMC to get biomass concentrations of 12.5, 25, 37.5, and 50 g L⁻¹. The extraction was performed at 90 °C while mixing at regular intervals till 4 h. After extraction, the mixture was centrifuged at 4696 ×g for 10 min to collect the DMC containing PHB. The solvent evaporated to recover the polymer (Samorì et al. 2015b; Mongili et al. 2021). The recovery percentage was calculated using Eq. (2).

Effect of pigment removal on PHB recovery by DMC The biomass-to-methanol ratio played an important role as pigments interfere with the recovery of biopolymer. The biomass in methanol used for pigment removal was 0.1, 0.25, 0.5, and 1 % (w/v). Biomass of 100 mg was suspended in chilled methanol of different volumes (10, 20, 40, and 100 mL) to get the above concentrations. After the incubation at 4 °C, biomass was separated by centrifugation at 8000 rpm for 10 min (Ansari and Fatma 2016). Then the pellet was suspended in 4 mL of DMC for PHB extraction at 90 °C with mixing at regular intervals up to 4 h. Then the mixture was centrifuged at $4696 \times g$ for 10 min to collect the DMC containing PHB. The solvent was evaporated to recover the polymer (Samorì et al. 2015b; Mongili et al. 2021). The recovery percentage was calculated as before using Eq. (2).

Effect of incubation time on PHB recovery To optimize the time required for extraction of PHB, 100 mg of biomass per 4 mL of DMC was selected after pigment removal with 0.5 % (w/v) and 0.25 % (w/v) biomass in methanol. The experiment was performed at different time points from 2, 4, 6, and 8 h (Samorì et al. 2015b; Mongili et al. 2021). The polymer was recovered by evaporation of DMC and the percentage recovery and purity of the polymer were calculated using Eqs. (2) and (3) after quantification by GC.

Fourier transformed infrared spectroscopy

A potassium bromide pellet was prepared for 2 mg of standard PHB (Sigma-Aldrich) and recovered PHB. Infrared spectra (IR) were recorded using a Perkin Elmer Fourier Transformed Infrared (FTIR) spectrometer with a spectral range of $3600 - 800 \text{ cm}^{-1}$ at 25 °C (Ansari and Fatma 2016).

Thermogravimetric analysis

The thermal stability of the extracted polymer of 10 mg was studied by thermogravimetric analysis (TGA) from 25 - 700 °C under the argon atmosphere (20 mL min^{-1}) at a heating rate of 10 °C min^{-1} using NETZSCH STA 449 F3 Jupiter. The decomposition temperature such as T_5 (5 % weight loss) and T_{10} (10 % weight loss) was determined using the TGA curve and maximum decomposition temperature (T_{max}) was determined by DTG analysis (Ansari and Fatma 2016).

Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) analysis was done using the NETZSCH instrument with 5 mg of sample in an aluminium pan under a nitrogen environment. The sample was heated to a temperature of -30 to 200 °C, held isothermally at 200 °C for 3 min, and cooled to -30 °C at a rate

of 10 °C min^{-1} . Similarly, a second cycle was performed (Ansari and Fatma 2016).

The degree of crystallinity $X_c(\%)$ was calculated from the Eq. (4).

$$X_c = (\Delta H_f \times 100) / \Delta H_{f_0} \quad (4)$$

where ΔH_f is the melting enthalpy of the recovered PHB (J g^{-1}), and ΔH_{f_0} is the theoretical melting enthalpy of the 100 % crystalline PHB which is assumed to be 146.6 J g^{-1} .

Powder X-Ray diffraction

X-ray diffractograms (XRD) of the standard and sample were obtained using Smartlab 9KW Powder X-Ray Diffraction System (Rigaku Technologies, Japan) for 2θ in the range of $10 - 80 \text{ °C}$ using Cu-K α radiation ($\lambda = 1.54 \text{ \AA}$). The peaks were analyzed for the crystalline nature of the recovered biopolymer (Devi et al. 2015).

Gel permeation chromatography

The molecular weight of recovered PHB was determined using gel permeation chromatography (GPC) (PL-GPC 220 High-Temperature Chromatograph, Agilent Technologies) at 40 °C with a refractive index detector. Tetrahydrofuran, HPLC grade (stabilized) was used as the mobile phase with a flow rate of 1 mL min^{-1} . PHB was dissolved in chloroform at a concentration of 1 mg mL^{-1} and a 200 μL sample was injected using an autosampler to PLgel Mixed B column ($10 \mu\text{m}$, $300 \times 7.5 \text{ mm}$) with a runtime of 25 min. Mass standards of Polystyrene in the range of $1.6 \times 10^2 - 6.5 \times 10^6 \text{ g mol}^{-1}$ were used to calibrate the system before sample injection (Lackner et al. 2019).

Mechanical properties

The solvent casting method was used to prepare PHB films to study the tensile strength and elongation at break using a 5kN Universal Testing Machine (UTM) (Zwick Roell, Z005TN). The recovered polymer was dissolved in chloroform while stirring at 60 °C for 20 min. The solution was poured into the glass Petri dish. The films resulting from the evaporation of solvent were cut into rectangular shapes (Supplementary file) and subjected to load at a rate of 1 mm min^{-1} (Ansari and Fatma 2016). The tensile strength (MPa) and elongation at break (%) were calculated by the stress-strain curve.

Statistical analysis

All experiments were conducted in duplicates with triplicate sampling measurements and average results were reported

with standard deviation. One-way ANOVA was performed followed by Tukey's post hoc tests using the OriginPro 2021 software (OriginLab). A p -value of <0.05 was considered statistically significant.

Results

PHB production from *C. fritschii* TISTR 8527

Chlorogloea fritschii was cultivated in flat-panel PBR using an optimized BG-11 medium with 3 % CO_2 under diurnal light. The initial cell density of *C. fritschii* was 0.05 g L^{-1} . After 7 days of cultivation, the culture reached the stationary phase with a DCW of 1.084 g L^{-1} . PHB was induced in the dark with 0.2 % acetate (w/v) after 7 days. PHB of 28 ± 0.08 % was obtained with DCW of $1.3 \pm 0.02 \text{ g L}^{-1}$ after 4 days of induction under dark. The biomass with a polymer content of 28 % was collected by centrifugation, freeze-dried, and used for recovery of intracellular PHB by various methods. Previously *C. fritschii* TISTR 8527 has been reported to produce 31.78 % of polymer using optimized BG-11 medium with 1 % CO_2 under diurnal light (Yashavanth and Maiti 2024). An increase in CO_2 may reduce the carbon stress required for PHB accumulation.

PHB extraction by single-step hypochlorite-chloroform dispersion method

Firstly, the lyophilized biomass was subjected to the hypochlorite-chloroform dispersion method. It was observed

that the percentage recovery of biopolymer increased with an increase in the hypochlorite percentage used for cell lysis. Use of 10 % (v/v) hypochlorite was inefficient in cell lysis and resulted in lower PHB recovery of 4.44 ± 0.574 % (w/w). Gradual increase in hypochlorite concentrations from 20 to 70 % (v/v) led to the improvement in PHB recovery from 42.88 ± 4.09 to 90.50 ± 3.26 % (w/w). Direct use of commercial hypochlorite from Himedia (100 % v/v or 4 % w/v) with an equal volume of chloroform resulted in the recovery of 95.51 ± 3.164 % PHB (Fig. 1a). The PHB recovery was found to be significant with increase in hypochlorite concentrations ($p < 0.05$).

PHB recovery by two-step method using hypochlorite pretreatment followed by extraction with different solvents

Various organic solvents used for our study were chloroform, dichloroethane (DCE), propylene carbonate, acetic acid, dioxolane, and dimethyl carbonate (DMC). These solvents were screened for recovery of PHB at 100°C for 1 h from *C. fritschii* after pretreatment of 200 mg of lyophilized biomass with 4 % (w/v) hypochlorite for 15 min at room temperature. The recovered polymer content varied with different solvents employed. is shown in Fig. 1b. The maximum recovery of 74.79 % was achieved with green solvent DMC after digestion of biomass with hypochlorite which aided in cell lysis along with pigment removal. Apart from DMC, chloroform also resulted in PHB recovery of 73.1 % but at high temperatures chloroform is highly volatile and continuous exposure to the fumes by is carcinogenic and chloroform is not

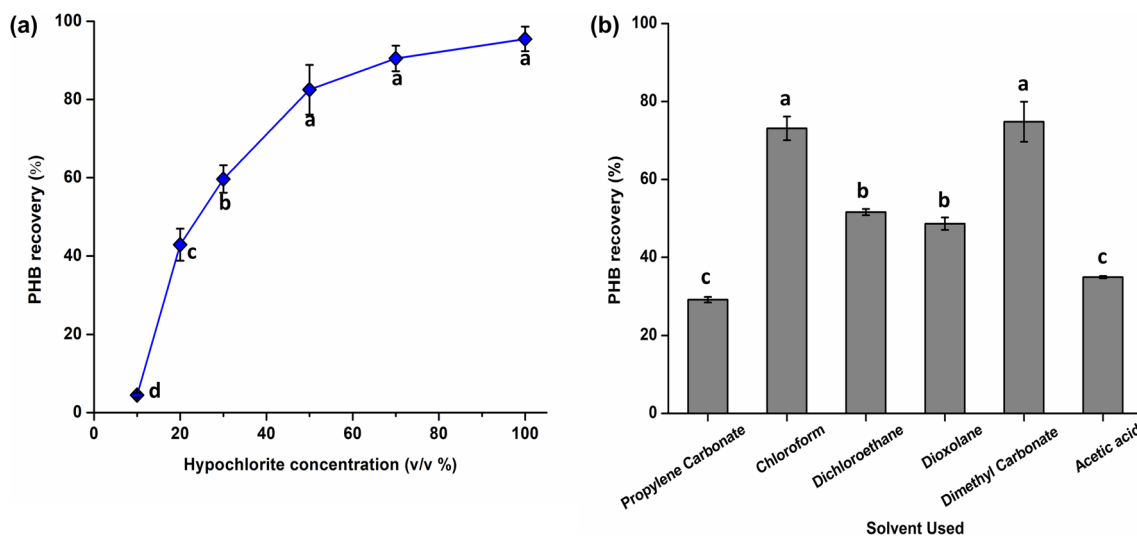


Fig. 1 PHB recovery with hypochlorite for cell lysis. **(a)** PHB recovery (%) from *C. fritschii* using hypochlorite-chloroform dispersion method with an increase in hypochlorite concentrations (v/v %), and **(b)** PHB recovery (%) from *C. fritschii* using solvent extraction with

different halogenated and non-halogenated solvents after hypochlorite pretreatment. Different letters indicate significant difference between the groups with $p \leq 0.05$

eco-friendly (Sekar et al. 2023). Hence, DMC was selected for further experiments based on its capability to solubilize PHB along with health and environmental concerns (Mongili et al. 2021).

PHB recovery by two-step method using pigment removal by methanol followed by extraction with green solvent DMC

Being an industrially important solvent, DMC recovered 19.62 % PHB directly from *C. fritschii* biomass without the removal of pigments (data not shown). Pigments were also extracted with PHB when the biomass was heated with DMC. This resulted in a lower recovery of polymer. Hence the biomass was subjected to pigment removal using chilled methanol followed by PHB extraction with DMC. The % recovery is shown in Fig. 2a. It was found that 100 mg biomass per 4 mL DMC i.e. 25 g L⁻¹ biomass resulted in maximum recovery of 53.52 % polymer in biomass variation experiment with an incubation period of 4 h. The decrease

in the % recovery with an increase in the biomass was due to the limitation of solvent. Therefore 25 g L⁻¹ of biomass in DMC was selected for further studies (Fig. 2a).

The biomass-to-methanol ratio was varied from 1, 0.5, 0.25, and 0.1 % (w/v) to study the effect of pigment removal on PHB recovery. After pigment removal, the pelleted biomass was subjected to PHB recovery with 100 mg of biomass per 4 mL of DMC (i.e. 25 g L⁻¹) for 4 h at 90 °C. Percentage recoveries of PHB was 9.98 ± 0.83, 53.63 ± 1.38, 81.88 ± 0.91, and 82.93 ± 0.37 %, respectively (Fig. 2b). Still there was scope to enhance the percentage recovery by increasing the contact time of biomass with DMC. Hence 0.5 % w/v biomass in methanol was selected for pigment removal followed by extraction with DMC resulted in the polymer recoveries of 10.77 ± 0.28, 54.42 ± 1.19, 68.87 ± 0.125, and 42.28 ± 1.11 % with an incubation times of 2, 4, 6, and 8 h respectively (Fig. 2c). Using 0.25 % w/v biomass in methanol for pigment removal and PHB recovery using DMC with 2, 4, 6, and 8 h contact time gave recovery yields of 22.97 ± 0.18, 83.34 ± 0.76, 62.2 ± 1.59 and 57.17 ± 0.54 %, respectively (Fig. 2d).

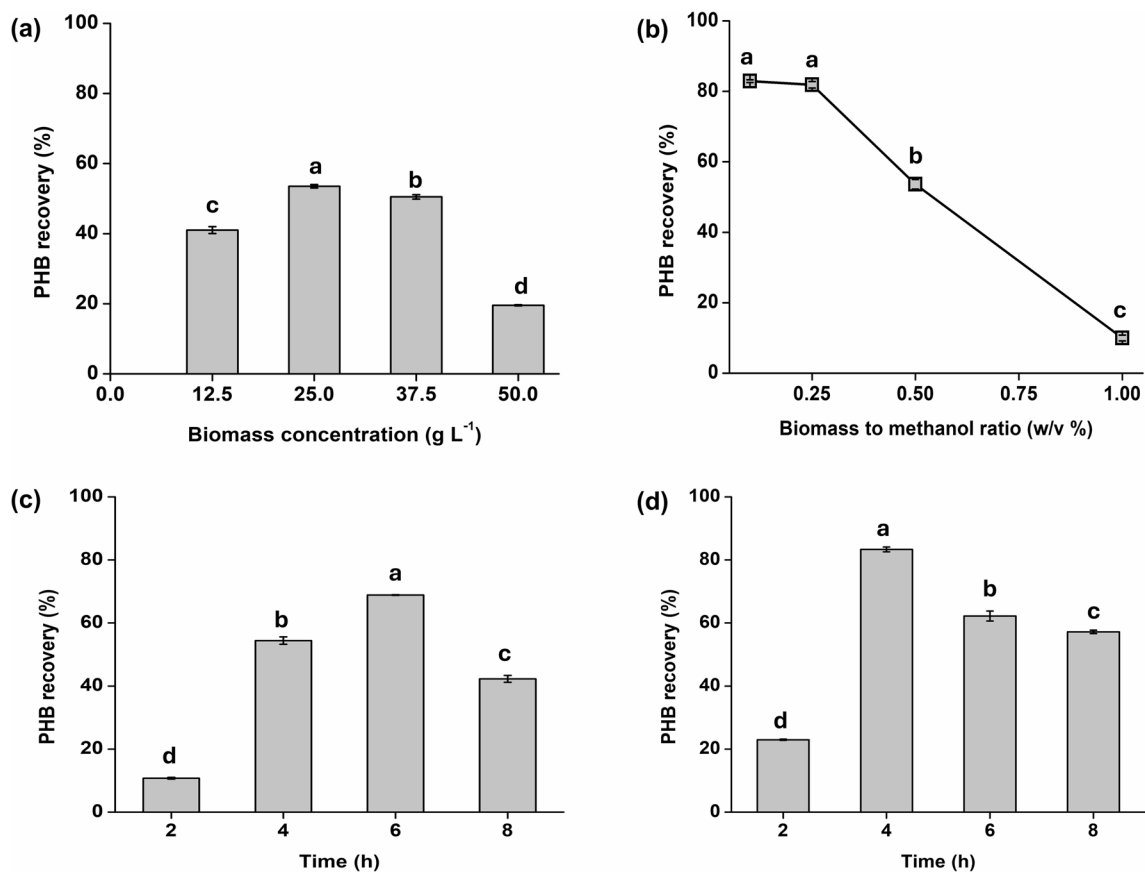


Fig. 2 PHB recovery (%) from *C. fritschii* using DMC with pigment removal with chilled methanol. **(a)** Effect of biomass amount for PHB recovery using DMC, **(b)** Effect of pigment removal by varying biomass to methanol ratio on PHB recovery by DMC, **(c)** Effect of contact time on PHB recovery with 0.5 % (w/v) biomass to methanol

and **(d)** Effect of contact time on PHB recovery with 0.25 % (w/v) biomass to methanol ratio for pigment removal. Different letters indicate significant difference between the groups with $p \leq 0.05$

PHB recovery using DMC with considering biomass variation, effect of biomass to methanol for pigment removal on PHB recovery, and contact time studies on PHB recovery with 0.25 % and 0.5 % biomass to methanol were statistically significant with $p < 0.05$ (Fig. 2c and d).

Characterization of biopolymer

Fourier Transformed Infrared Spectroscopy

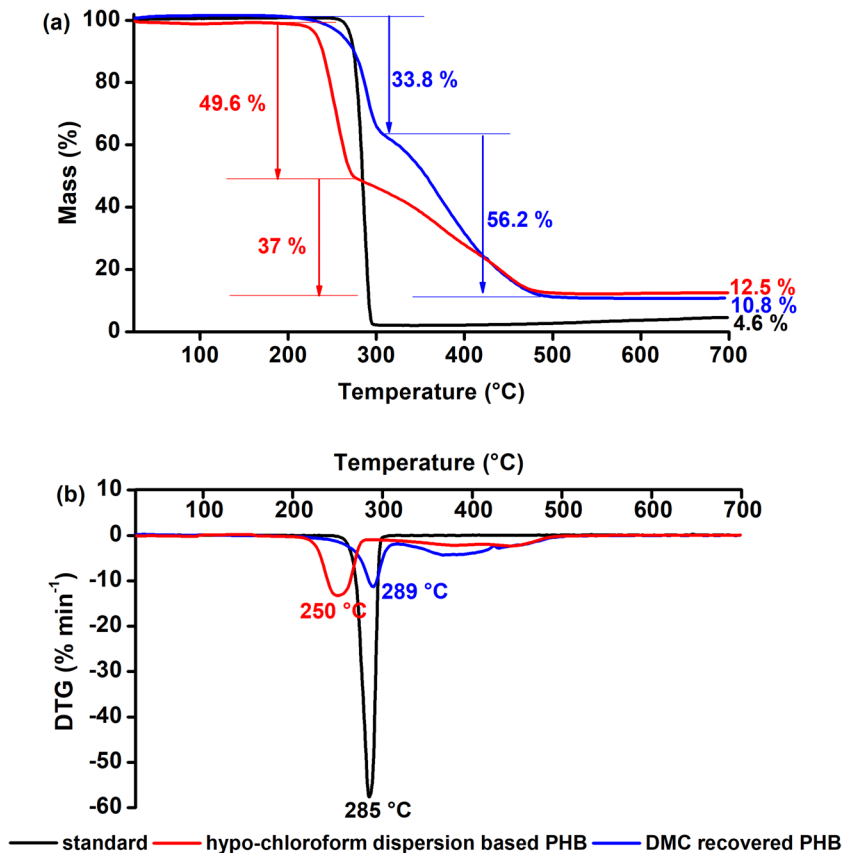
PHBs from both methods were subjected to FTIR analysis by the pellet method. The FTIR spectra of PHB from both methods were similar. The -CH group was represented by 2930 to 2975 cm^{-1} . The characteristic peak at 1728 cm^{-1} represents the -C=O group. The bands from 1058 -1281 cm^{-1} represented the C-O-C stretching vibration. The FTIR peaks of the standard and samples (Supplementary Fig. S1 and Table S1) were similar. These results were in agreement with previous studies (Ansari and Fatma 2016; Pradhan et al. 2018).

Thermogravimetric analysis PHB derived from the hypochlorite-chloroform dispersion method and DMC method were subjected to thermal degradation under an inert atmosphere. Initial mass loss corresponds to the loss of solvents adsorbed to the film (i.e. chloroform and DMC). The standard PHB was degraded in a single step since it is

highly pure. PHB derived from both methods was degraded in two steps. The loss is due to the random chain scission by β -elimination reaction and conversion of cleaved chains to crotonic acid or oligomers of crotonic acid (Ariffin et al. 2008). The former showed 49.6 % degradation in the first phase, followed by 37 % degradation in the second phase with a residual mass of 12.5 %. Later one showed a mass loss of 33.8 % in the first step and 56.2 % in the second step with a residual of 10.8 % (Fig. 3a). The characteristic decomposition temperatures i.e., temperature at 5 % (T_5) and temperature at 10 % weight loss (T_{10}) were determined using TGA curve (Fig. 3a). The maximum decomposition temperature (T_{max}) was determined by the derivative of thermogravimetry (DTG) curve (Fig. 3b). The T_5 , T_{10} , and T_{max} of PHB recovered using the hypochlorite-chloroform dispersion method were 232.7, 238, and 250 $^{\circ}\text{C}$, respectively, whereas T_5 , T_{10} , and T_{max} of PHB recovered using DMC are 261.6, 274.2, and 289 $^{\circ}\text{C}$, respectively (Fig. 3a and b). The standard PHB from Sigma-Aldrich showed T_5 , T_{10} , and T_{max} of 270, 274, and 285 $^{\circ}\text{C}$, respectively (Table 1). The characteristic decomposition temperatures of PHB recovered using DMC matched that of standard PHB.

Differential scanning calorimetry The thermal and calorimetric properties of PHB recovered from the hypochlorite-chloroform dispersion method and DMC method were

Fig. 3 Thermograms of PHB recovered from *C. fritschii* under inert atmosphere. **(a)** Comparison of TGA curve of hypochlorite-chloroform dispersion-based PHB and DMC recovered PHB with standard PHB (Sigma-Aldrich). The TGA curve depicts the step-wise degradation of PHB with a temperature range of 25 – 700 $^{\circ}\text{C}$. **(b)** Comparison of DTG curve of hypochlorite-chloroform dispersion-based PHB and DMC recovered PHB with standard PHB (Sigma-Aldrich). The DTG curve depicts the rate of degradation and maximum degradation temperature of the polymer



studied using Differential Scanning Calorimetry (DSC). The thermograms were used to determine glass transition temperature (T_g), melting temperature (T_m), crystallization temperature (T_c), and degree of crystallinity (X_c). The first heating scan (-30 °C to 200 °C) was used to eliminate the prior thermal history of the polymer. The T_c was calculated from the cooling cycle from 200 °C to -30 °C.

The point of inflection during the second heating cycle is referred to as glass transition temperature (T_g). The T_g for standard, DMC recovered PHB and PHB recovered from the dispersion method was 4, 4.3, and 4.6 °C, respectively (Fig. 4a and Table 2). PHB from *Nostoc muscorum* has a T_g

Table 1 Comparison of degradation temperatures of PHB recovered from *C. fritschii* using hypochlorite-chloroform dispersion and DMC-based recovery with standard (Sigma-Aldrich) using TGA and DTG analysis

Sample	T_5 (°C)	T_{10} (°C)	T_{max} (°C)	Residual mass (%)
Standard PHB (Sigma-Aldrich)	270	274	285	4.6
PHB (Dimethyl Carbonate)	261.6	274.2	289	10.8
PHB (hypochlorite-chloroform dispersion)	232.7	238	250	12.5

of 6 °C (Ansari and Fatma 2016). A previous study showed that PHB from *C. fritschii* TISTR 8527 had a T_g of 3.2 °C (Monshupanee et al. 2016). The melting points (T_m) for dispersion-based PHB and DMC-recovered PHB were 169 and 174 °C, respectively, close to the T_m of the standard (174 °C) (Fig. 4a and Table 2). In an earlier study with the same strain, a T_m of 171.6 °C was reported for PHB recovered by chloroform (Monshupanee et al. 2016). The crystallization temperatures (T_c) of PHB recovered from both methods determined from the cooling curve and were 48.4 °C (DMC recovered PHB) and 48.2 °C (dispersion), respectively, which were less than T_c of the standard (84 °C) (Fig. 4b and Table 2). This was due to the lower degree of crystallinity (X_c) of 38.4 % and 35.9 %, respectively. The crystallinity (X_c) of the standard from Sigma-Aldrich is 60.3 % (Table 2). It was found that PHB recovered with chloroform from the same strain had a reported crystallinity of 45 % in previous findings (Monshupanee et al. 2016).

Powder X-ray diffraction X-ray diffractograms of standard, PHB recovered from the hypochlorite-chloroform dispersion method and DMC method were obtained using Cu- α X-ray ($\lambda = 1.54 \text{ \AA}$) in the range of $2\theta = 10\text{--}80^\circ$. Six prominent peaks were observed at 2θ : 13.6, 17, 21.6, 22.7, 25.69, and 27.3°. These peaks correspond to different planes (020, 110, 101, 111, 121, and 040) of orthorhombic unit cells of PHB crystal. Peaks at $2\theta = 21.6^\circ$ and $2\theta = 22.7^\circ$ indicated that

Fig. 4 Differential Scanning Calorimetric (DSC) curve of PHB recovered from *C. fritschii* by hypochlorite-chloroform dispersion and DMC-based recovery with comparison to standard PHB (Sigma-Aldrich) for analysis of thermal properties such as glass transition temperature (T_g), melting temperature (T_m), and crystallization temperature (T_c). (a) Heating curve and (b) Cooling curve

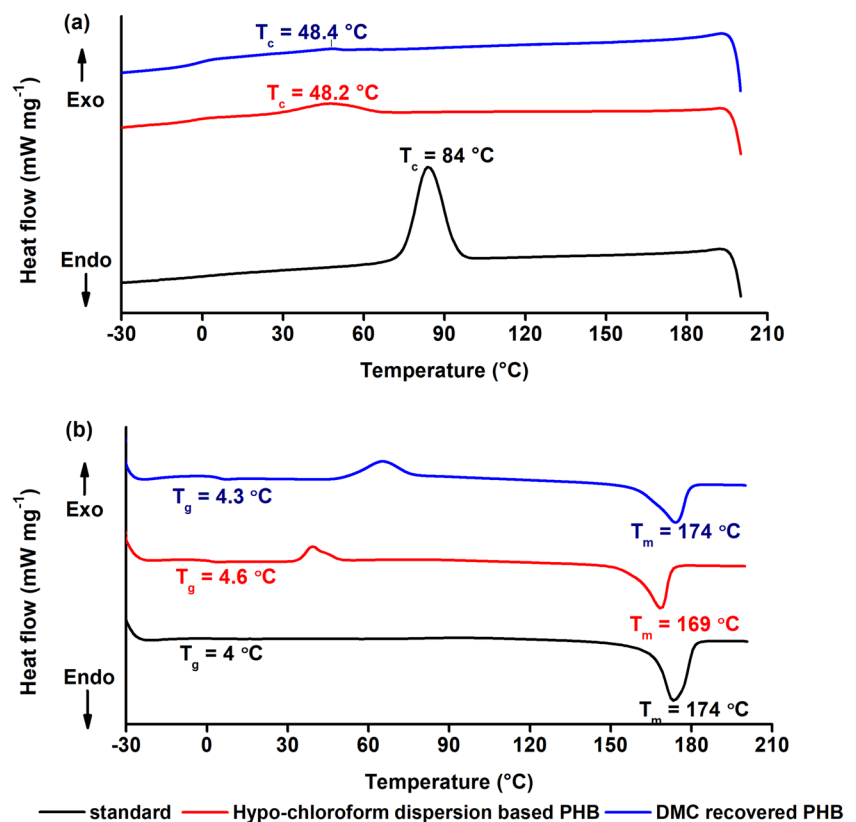


Table 2 Comparison of material properties of PHB recovered from *C. fritschii* with various sources

Sources of PHB	Thermal Properties (DSC)				Crystallinity from XRD	Mechanical properties		Molecular Weight		Reference		
	T_m (°C)	T_g (°C)	T_c (°C)	ΔH_f (J g ⁻¹)		X_c (%)	Tensile strength (MPa)	Elongation at break (%)	M_w (Da)		M_n (Da)	PDI (M_w/M_n)
						X_c (%)						
Standard (Sigma-Aldrich)	174	4	84	88.42	63.3 ± 4.8	20.47 ± 1.70	6.39 ± 1.34	570885	216638	2.63	current study	
<i>C. fritschii</i> (Dispersion method)	169	4.6	48.2	52.58	56.4 ± 1.8	11.11 ± 2.92	5.13 ± 2.66	319784	150268	2.12	current study	
<i>C. fritschii</i> (DMC)	174	4.3	48.4	56.34	56.8 ± 1.5	11.57 ± 0.35	6.42 ± 1.04	440067	155963	2.82	current study	
<i>C. fritschii</i> (Chloroform)	171.6	3.2	54	65	-	23 ± 6	5.5 ± 1.8	545000	256000	2.1	(Monshupance et al. 2016)	
<i>Nostoc muscorum</i> (chloroform)	171	6	78.8	83.25	56.78	31.1	8.6	-	-	-	(Ansari and Fatma 2016)	
PHB (Chemically synthesized)	170.7	-	82.2	78.6	53	-	-	155540	154000	1.01	(Tang and Chen 2018)	
PET (Fossil based)	260	67-81	-	-	7.9	62	230	-	-	-	(McAdam et al. 2020)	
PP (Fossil based)	160-169	-20-(-5)	-	-	42-58	31-45	50-145	-	-	-	(McAdam et al. 2020)	

the tested polymer is α -PHB crystal (Sun et al. 2011). The peaks at $2\theta = 25.69^\circ$ and $2\theta = 27.3^\circ$ indicated that the PHB from *C. fritschii* is of partial crystalline nature (Fig. 5). The % crystallinity (X_c) was calculated from analysis of XRD peaks. The X_c of PHB recovered using the hypochlorite-chloroform dispersion method and DMC from *C. fritschii* were 56.45 ± 1.81 and 56.84 ± 1.56 %, respectively (Table 2). The X_c of standard PHB is 63.33 ± 4.80 % and is in agreement with Anbukarasu et al. (2015). From the above analysis, it can be concluded that PHB from *C. fritschii* is semi-crystalline in nature.

Determination of molecular weight by gel permeation chromatography (GPC) The weight average molecular weight (M_w) and number average molecular weight (M_n) was determined using GPC. The hypochlorite-chloroform dispersion-based PHB had a M_w of 319784 Da and M_n of 150268, whereas DMC recovered PHB had M_w of 440067 and M_n of 155963. The M_w and M_n of standard PHB (Sigma-Aldrich) were 570885 and 216638 Da, respectively. The polydispersity index (PDI) of PHB recovered using both methods agreed with that of the standard (Table 2). Monshupanee et al. (2016) reported a M_w of 545000 Da of PHB

recovered with chloroform as an extraction solvent from the same strain in previous studies.

Mechanical properties of PHB Mechanical properties were studied to characterize the biopolymer films (Supplementary Fig. S2). The tensile strength (σ) and elongation at break (ϵ) of PHB recovered using hypochlorite-chloroform dispersion were 11.11 ± 2.92 MPa and 5.13 ± 2.66 %. Similarly, PHB recovered using DMC resulted in tensile strength and elongation at break of 11.57 ± 0.35 MPa and 6.42 ± 1.04 %, respectively. The standard PHB has a tensile strength of 20.47 ± 1.70 MPa and elongation at break value of 6.39 ± 1.34 % (Fig. 6 and Table 2).

Discussion

Chlorogloea fritschii TISTR 8527 could produce 28 % PHB in flat panel PBR under diurnal light with single-stage cultivation using optimized BG-11 medium and 3 % CO_2 . Previously *C. fritschii* produced PHB of 31.67 % under the same condition except 1 % CO_2 was utilized (Yashavanth and Maiti 2024). The lyophilized biomass containing PHB was subjected to PHB recovery by the single-step hypochlorite-chloroform dispersion method. The dispersion method resulted in a maximum recovery of 95.51 % PHB with direct usage of an equal volume of commercial hypochlorite and chloroform. Hahn et al. (1994) also showed that an increase in the hypochlorite concentration increases the percentage recovery of the polymer up to 91 % with a polymer purity of 97 %. Solvent extraction of PHB with hypochlorite pretreatment was performed with solvents such as chloroform, DCE, propylene carbonate, acetic acid, dioxolane, and DMC. Generally, extraction of PHB using solvent depends on the solubility of PHB and the combination of the boiling point of the solvent and operating temperature. In the current extraction process, the operating temperature was 100°C . The boiling points of propylene carbonate and acetic acid are 242°C and 118°C respectively which were much higher than the operating temperature. The boiling points of chloroform, DCE, dioxolane, and DMC are lower than the operating temperature. Therefore, the PHB recovery was lesser with propylene carbonate and acetic acid (Aramvash et al. 2018). According to the Hansen solubility parameter the distance of DMC from the center of solubility radius of PHB ($R_{\text{PHB}} = 8.5$) is lowest (5.12) compared to other solvents used in this study (Terada and Marchessault 1999). As the solubility of PHB in DMC is very high and the operating temperature is close to the boiling point, the recovery using DMC is higher. When *Cupriavidus necator* biomass was subjected to PHB recovery using propylene carbonate at 100°C for 45 min it resulted in a percentage recovery of 45 % (Fiorese et al. 2009). In the present study, a low operating temperature of

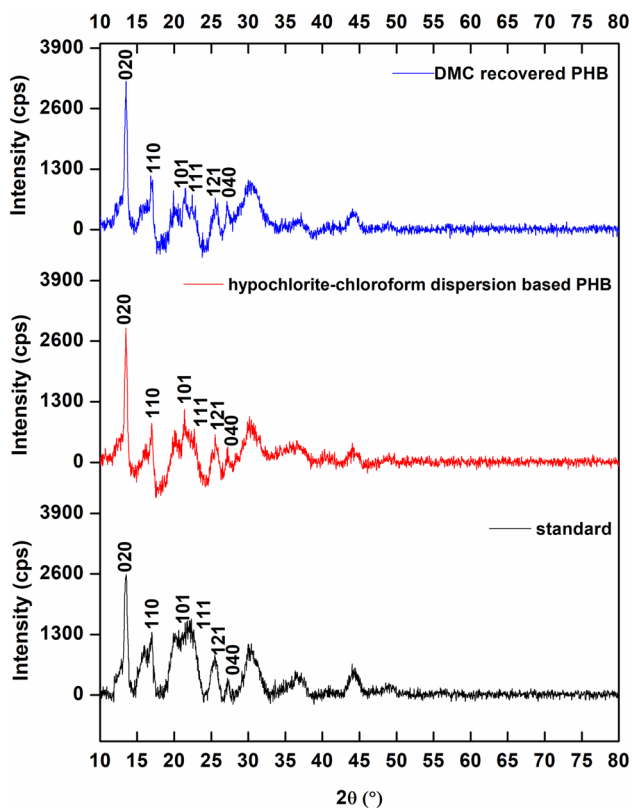
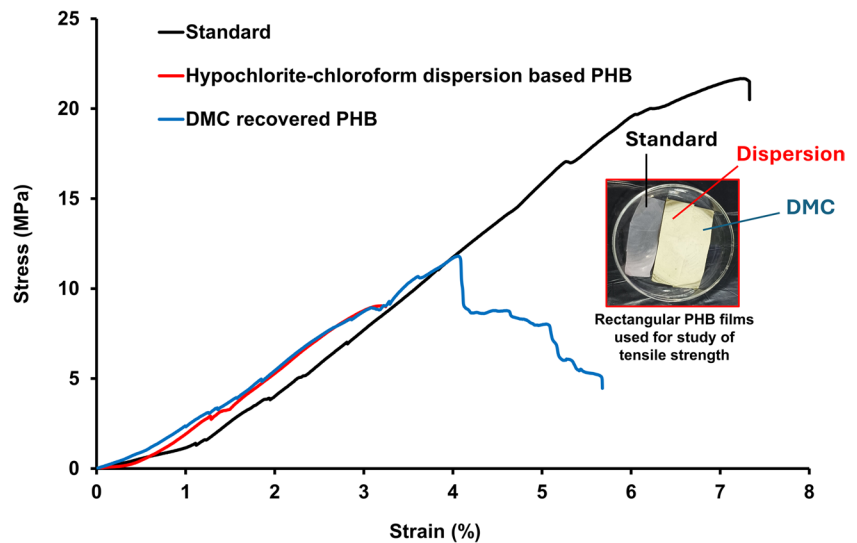


Fig. 5 X-ray diffraction (XRD) characterization of PHB recovered from *C. fritschii* by hypochlorite-chloroform dispersion and DMC-based recovery with comparison to standard PHB (Sigma-Aldrich)

Fig. 6 Stress-strain behavior of PHB films cut into rectangular film of dimensions (5 cm x 2 cm): standard PHB (Sigma-Aldrich), hypochlorite-chloroform dispersion based PHB, and DMC recovered PHB studied under 5kN Universal testing machine at a rate of 1 mm min⁻¹



100 °C resulted in PHB recovery of 29.2 % using propylene carbonate in *C. fritschii*. Aramvash et al. (2018) showed that acetic acid can recover the PHB to 36.71 % with 97 % purity using *C. necator*. In the current study, acetic acid demonstrated a similar recovery of 34.9 % from *C. fritschii* with a PHB purity of 90 %. Previously, Samori et al. (2015b) reported PHB recovery of 49 % from mixed microbial culture (MMC) using DMC along with purity of 98 % without biomass pretreatment. Hypochlorite pretreatment of MMC up to 15 min resulted in PHB recovery of 62 % with 98 % polymer purity. In the present study, DMC could recover PHB from *C. fritschii* with a high recovery yield of 74.79 % after hypochlorite pretreatment with a polymer purity of 80 %. The lower purity of the recovered polymer may be due to polymer recovery using solvent evaporation. Dioxolane was capable of PHB recovery of 60 % from *C. necator* at 100 °C for 4 h (Yabueng and Napathorn 2018). However, in this work dioxolane resulted in a recovery of 48.62 % of PHB which was due to lower contact time (1 h) of solvent with biomass at 100 °C. Chloroform and DCE recovered PHB of 73.1 % and 51.61 % at 100 °C after hypochlorite pretreatment. Chloroform and DCE are well known for good recovery of the polymer due to the high solubility of PHB (Hahn et al. 1995; Terada and Marchessault 1999; Monshupanee et al. 2016) but are not environment and human-friendly in nature (Sekar et al. 2023). Hence, DMC was selected as a green solvent for PHB recovery due to its high solubility for PHB (Terada and Marchessault 1999).

Chlorogloea fritschii biomass resulted in PHB recovery of 63.87 ± 0.13 % and 83.34 ± 0.76 % with biomass-to-methanol ratios of 0.5 % and 0.25 % (w/v), respectively. Previously *C. necator* biomass was used and DMC resulted in a recovery yield of 88 % with a purity of 95 % (Samori et al. 2015b). It is interesting to note that in the current work with *C. fritschii*, pigment removal employing a biomass-to-methanol ratio of

0.25 % (w/v) achieved a maximum recovery of 83.34 % of PHB and purity of 75 % with the same incubation period of 4 h. DMC, being an industrially important solvent, could be employed for PHB recovery from various cyanobacteria. PHB recovered from *C. fritschii* using dispersion and DMC was characterized using FTIR, TGA, DSC, and tensile strength studies. The characteristic FTIR peaks of PHB from *C. fritschii* matched with the PHB recovered from *Nostoc muscorum* NCCU- 442 (Ansari and Fatma 2016). In a study with *Nostoc muscorum*, a T_{max} of 284 °C was reported (Ansari and Fatma 2016) which is close to the T_{max} of PHB recovered from *C. fritschii* using DMC (289 °C) in the current study. Other fossil-based biopolymers such as PLA have a T_{max} of 366 °C which is higher than the T_{max} of PHB (285 °C) (Arrieta et al. 2014). Previously, PHB recovered from *Nostoc muscorum* had a T_m of 171 °C (Ansari and Fatma 2016) which is close to the T_m of PHB recovered from *C. fritschii* using dispersion and DMC in the present study. Chemical synthesis of PHB by ring open polymerization of racemic diolide (rac-DL) resulted in complete isotactic PHB with T_m of 170.7 °C (Tang and Chen 2018). DSC data of PHB from *C. fritschii* was also compared with fossil-based polymers such as polypropylene (PP) and polyethylene terephthalate (PET) (Table 2). Among the fossil-based polymers, PET was highly amorphous with a high melting temperature of 260 °C (McAdam et al. 2020). PLA had a higher T_g of 60.4 °C whereas it was highly amorphous in nature with X_c of 5.1 %. Irrespective of crystallinity the solubility parameter (δ) of PLA (19.5-20.5 MPa^{1/2}) was close to that of PHB (18.5 - 20.1 MPa^{1/2}), which aided the blending of both materials in the manufacturing of food packaging material while enhancing the crystallinity of packing material (Arrieta et al. 2014). Apart from these high concentrations of amorphous PHB has been blended with PAZO to form a photonic material while enhancing the miscibility of materials with different

solubility parameters (Sharma et al. 2005). Hence DSC helps in manufacturing materials of interest with enhanced solubility along with improved crystallinity. XRD data of PHB from *C. fritschii* was similar to the PHB obtained from *C. necator*, *Bacillus megaterium*, and *Bacillus cereus* (Devi et al. 2015; Pradhan et al. 2018). X-ray diffractograms also suggest that PHB from *C. fritschii* was semi-crystalline in nature. Monshupanee et al. (2016) reported M_w of 545000 in *C. fritschii* with extraction using a methanol-chloroform combination (Monshupanee et al. 2016). In the current study, PHB from *C. fritschii* has been reported with M_w of 319784 by hypochlorite-chloroform dispersion method and M_w of 440067 by DMC extraction method. As the extraction reported by Monshupanee et al. (2016) and current PHB extraction conditions are different the M_w can be affected. In a study of PHB recovery from *Alcaligenes eutrophus* by the hypochlorite-chloroform dispersion method, M_w has reduced drastically due to severe degradation of PHB molecules (Hahn et al. 1994). In the current study, similar phenomena have been observed with PHB recovered by dispersion. But M_w of PHB with DMC solvent has not decreased that much. Hence, with the same species molecular weight of PHB can be different (M_w and M_n) based on recovery conditions. PHB which was isotactic in nature and synthesized by ring opening polymerization of rac-DL had a PDI of 1.01 with M_n of 154000 (Tang and Chen 2018). Even though the chemical synthesis of PHB resulted in an isotactic PHB but had a lower molecular weight than PHB from biological origin (Table 2). In conclusion, the molecular weight of the polymer depends on the selected species and upstream and downstream conditions that affect material properties.

The mechanical properties of PHB recovered from *C. fritschii* depend on its molecular weight. In a study of PHB production with *Zobellella tiwanensis* using banana peel as a feed source, tensile strength of 10.344 MPa was reported with hypochlorite digestion followed by solvent extraction (Maity et al. 2020). The tensile strength of PHB from *C. fritschii* was well agreed with the polymer of *Z. tiwanensis*. Even though higher crystallinity was present, the low strength of recovered polymer films may be caused by the loose bonding of polymer chains and eased mobility at lower molecular weights due to weak interactions.

Conclusion

In the current work, PHB recovery from the cyanobacterium *C. fritschii* was performed with different methods. The dispersion method and solvent extraction were the main focus. The dispersion method achieved 95.51 % PHB recovery along with pigment removal. Various solvents showed lower recovery of PHB due to unsuitable operating conditions for PHB recovery. Among such solvents, the selection of a green solvent was critical. Optimization of the recovery process using DMC

resulted in a recovery yield of 83.31 % with pigment removal using methanol. DSC and XRD analysis showed that PHB from *C. fritschii* was partially crystalline. Characterization of recovered PHB suggested that *C. fritschii* can accumulate biopolymer similar to commercial PHB. Overall, biopolymer recovery using DMC is an eco-friendly process and can be employed for bioplastic recovery from other cyanobacteria.

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Author contributions Yashavanth P R: Conceptualization, Experimentation, Analysis, Writing-Original draft preparation

Soumen K Maiti: Conceptualization, Supervision, Writing- Reviewing and Editing

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Availability of data The data supporting the findings of this study are available upon request.

Declarations

Competing interests The authors declare no competing interests.

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