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Extraction and characterization of polyhydroxyalkanoates from marine green alga and cyanobacteria

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ABSTRACT

The present study focusses on the extraction and characterization of polyhydroxyalkanoates from marine algal species by solvent extraction method. The following strains (i) *Chlorella* sp., (ii) *Oscillatoria salina,* (iii) *Leptolyngbya valderiana* and (iv) *Synechococcus elongatus* were used in the present research work. Algal strains were scaled up in ASN III medium and preliminary bioprospecting study was conducted to evaluate the growth profile of all algal strains in terms of dry cell weight. Of the strains studied, the higher growth was observed with *Leptolyngbya valderiana* and *Chlorella* sp. at about 2.75 g/L whereas *Oscillatoria salina* and *Synechococcus elongatus* showed low dry cell weight of 0.99 g/L and 0.32 g/L respectively. In addition, extraction of polyhydroxyalkanoates from the algal strains were carried out and characterized. Thermo Gravimetric Analysis and Differential Scanning Calorimetry studies were conducted to determine the thermal stability and thermal properties of the synthesized PHA respectively. All the PHAs were thermally stable below 260 °C. PHA obtained from *Leptolyngbya valderiana* was found to be more thermally stable compared to the PHA obtained from other algal species.

1. Introduction

In the environment, non-biodegradable plastic waste accumulation is estimated between 4.8 and 12.7 million tons per year, which creates major issues to the marine ecosystems (Löhr et al., 2017). This is due to the use of petrochemicals (non-renewable) as key sources for plastic production currently (Sabapathy et al., 2019). Hence, there is a search for degradable sources of plastics or alternatives to non-biodegradable plastics, to reduce the accumulation of plastic in the ecosystem. At this juncture, polyhydroxyalkanoates (PHA), a biopolyesters, an energy and carbon source, can be synthesized by several microorganisms. PHAs have similar characteristics to the plastics made from petrochemical origin (Martins et al., 2014). PHAs extracted from microorganisms are environmentally benign and utilized in various medical and food sectors (Cassuriaga et al., 2018). Hydroxy acid (HA) monomeric units linked together by ester bonds is the structural chemistry of PHA and are categorized based on the number of carbons in the side-chains. There are short chain length (scl) type which has less than 5 carbons, medium chain length (mcl) type having 5–14 carbon atoms and long chain length (lcl) type with more than 14 carbon atoms (Kunasundari and Sudesh, 2011). Common PHA monomers are 4-hydroxybutyrate, 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxypropionate, 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, and 3-hydroxytetradecanoate. PHAs can be used as packaging components, biodegradable printing inks, coatings and laminations. Additionally, these can be used in waxes, binders and adhesives as the PHA structures include different grades of thermoplastic elastomers and rigid thermoplastics.

Polyhydroxybutyrate is a type of PHAs. PHB is produced by numerous microorganisms from acetyl-CoA through a series of enzymatic reactions using three enzymes. β-ketothiolase (phaA) is the first key enzyme that converts acetyl-CoA in to acetoacetyl-CoA molecule; the second enzyme acetoacetyl-CoA reductase (phaB) reduces the acetoacetyl-CoA to 3-hydroxybutyrlCoA and the final enzyme PHB polymerase (phaC) catalyses the polymerization of 3-hydroxybutyrlCoA to PHB molecule. In order to keep the PHB polymerase covalently attached, PHB polymers tend to form amphipathic granules within the cell. Among the microbes used for PHA extraction, algae have gained substantial interest as they are sunlight driven cell factories that convert CO_2 into various valuable bio-products with the O_2 evolution. Also, the route of bioplastic production from algal source can be via the polymers from direct biomass or from its secondary metabolites (Abdo and Ali, 2019).

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Table 1
PHA content (%) in various algae.

Algae	PHA content (%)	References
Synechococcus subsalsus	16	Costa et al., (2018a)
Spirulina sp. LEB-18	12	Costa et al., (2018a)
Phaeodactylum tricornutum	10.6	Hempel et al., (2011)
Chlamydomonas reinhardtii	Detectable quantities	Chaogang et al., (2010)
Synechocystis PCC6803	4.1–26	(Khetkorn et al., 2016; Panda and Mallick, 2007; Wu et al., 2001)
Synechococcus MA19	55	Nishioka et al., (2001)
Arthrospira subsalsa	14.7	Shrivastav et al., (2010)
Nostoc muscorum	8.7–22	(Panda et al., 2005; Sharma and Mallick, 2005)
Phormidium sp. TISTR 8462	14.8	Kaewbai-ngam et al., (2016)
Oscillatoria jasorvensis TISTR 8980	15.7	Kaewbai-ngam et al., (2016)
Calothrix scytonemicola TISTR 8095	25.2	Kaewbai-ngam et al., (2016)
Anabaena sp.	2.3	Lama et al., (1996)
Aulosira fertilissima	10	Samantaray and Mallick (2012)
Calothrix sp.	6.4	Bhati et al., (2010)
Scytonema sp.	7.4	Bhati et al., (2010)

Algae majorly are of two types prokaryotic and eukaryotic based on well-defined nucleus. Prokaryotic algae are known as cyanobacteria or blue green algae which are capable of accumulating polyhydroxyalkanoates under photoautotrophic as well as heterotrophic conditions (Table 1).

In cyanobacteria, the most dominant intracellular storage compound identified is PHB and further, of the 150 polyhydroxyalkanoids studied, PHB is the prevalent bioplastic in the prokaryotic cyanobacteria (Sundaramoorthy et al., 2013). In fact, the PHAs accumulation in

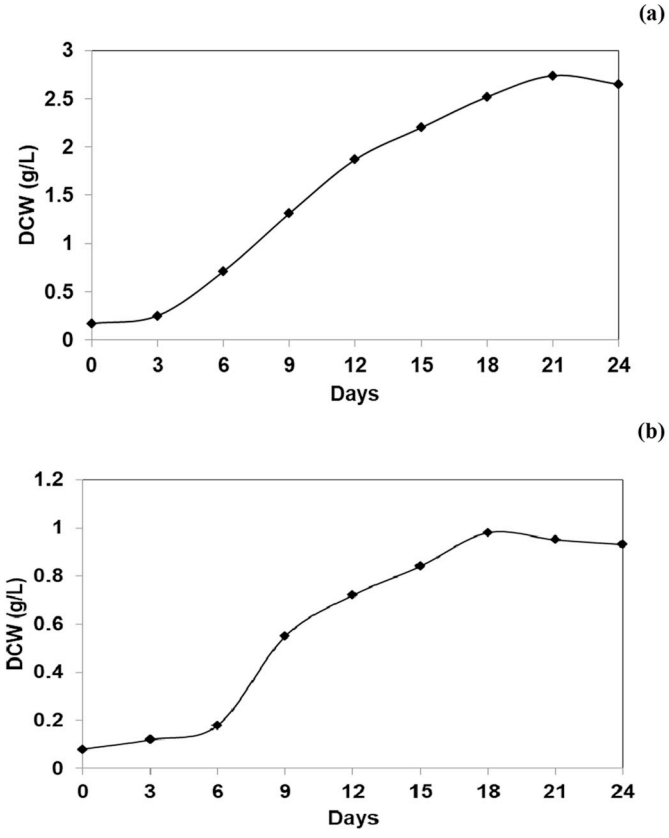

Fig. 1. Time course analysis of DCW (a) *Chlorella* sp. (b) *O. salina*.

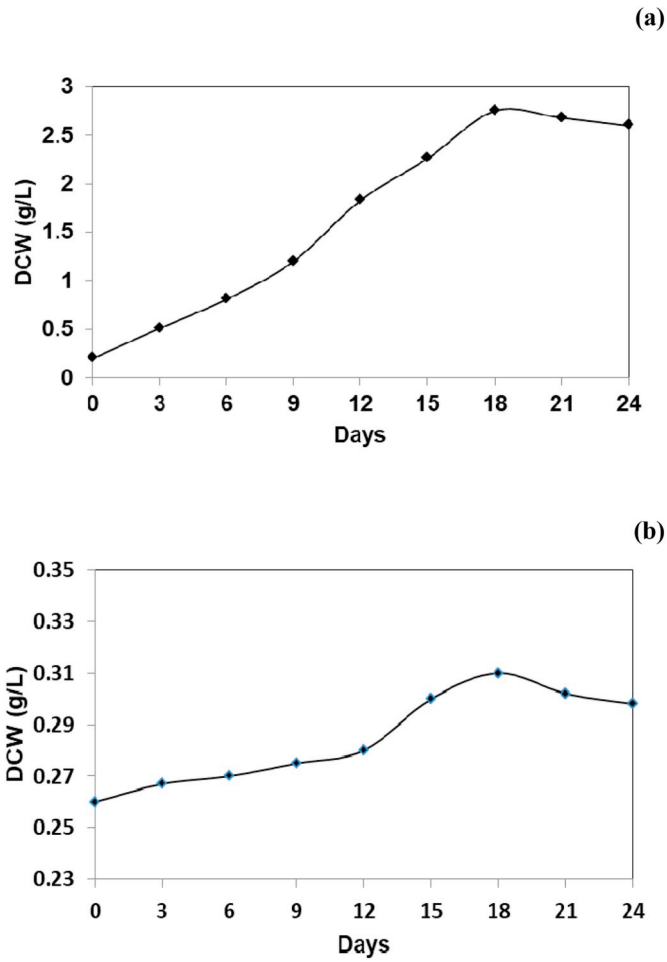

Fig. 2. Time course analysis of DCW (a) *L. valderiana* (b) *S. elongatus*.

cyanobacteria were first observed under ambient (photoautotrophic) conditions and it was increased under organic carbon supplements such as glucose, acetate, citrate, xylose, arabinose etc. (Cassuriaga et al., 2018; Singh et al., 2017). Cyanobacterial bioplastics can be made using biopolymers derived from two ways. They are (i) biopolymers from living organism: from cellulose, soy protein and starch, (ii) polymerizable molecules: these are made from triglycerides and lactic acid. PHB in algal cell principally acts as an energy storage molecule wherein its use comes into existence when algal cells are in shortage of macronutrients, thereby deriving energy for its survival. PHB are generally obtained in low quantity through carbon fixation or carbon assimilation process and can be significantly raised under nutrient limited conditions resulting in excessive storage of carbon compounds (Das et al., 2018). The reported microalgal strains for PHB production in the recent times are *C. reinhardtii*, *C. vulgaris*, *C. fusca* (Cassuriaga et al., 2018; Kato, 2019; Mathiot et al., 2019). The PHB production from algal sources are way better in i) recyclable property ii) biodegradability iii) biocompatibility iv) plasticizing capacity v) usability in multiple sectors such as medical, agricultural, industrial, food packing and storage materials etc than the conventional petroleum based plastics (Abdo and Ali, 2019; Das et al., 2018). One of the key challenges in PHA production from algal source is the process economization. Algal based bioplastics have innumerable benefits compared to the present era of recalcitrant petroleum based plastics in terms of degradation. Concurrently, the need of bioplastics from microbial origin has been expanded in various fields for which the present productivity of biopolymers does not suffice to achieve the commercial production process. Moreover, bioprospecting of suitable algal strain having high PHB content is of much significance

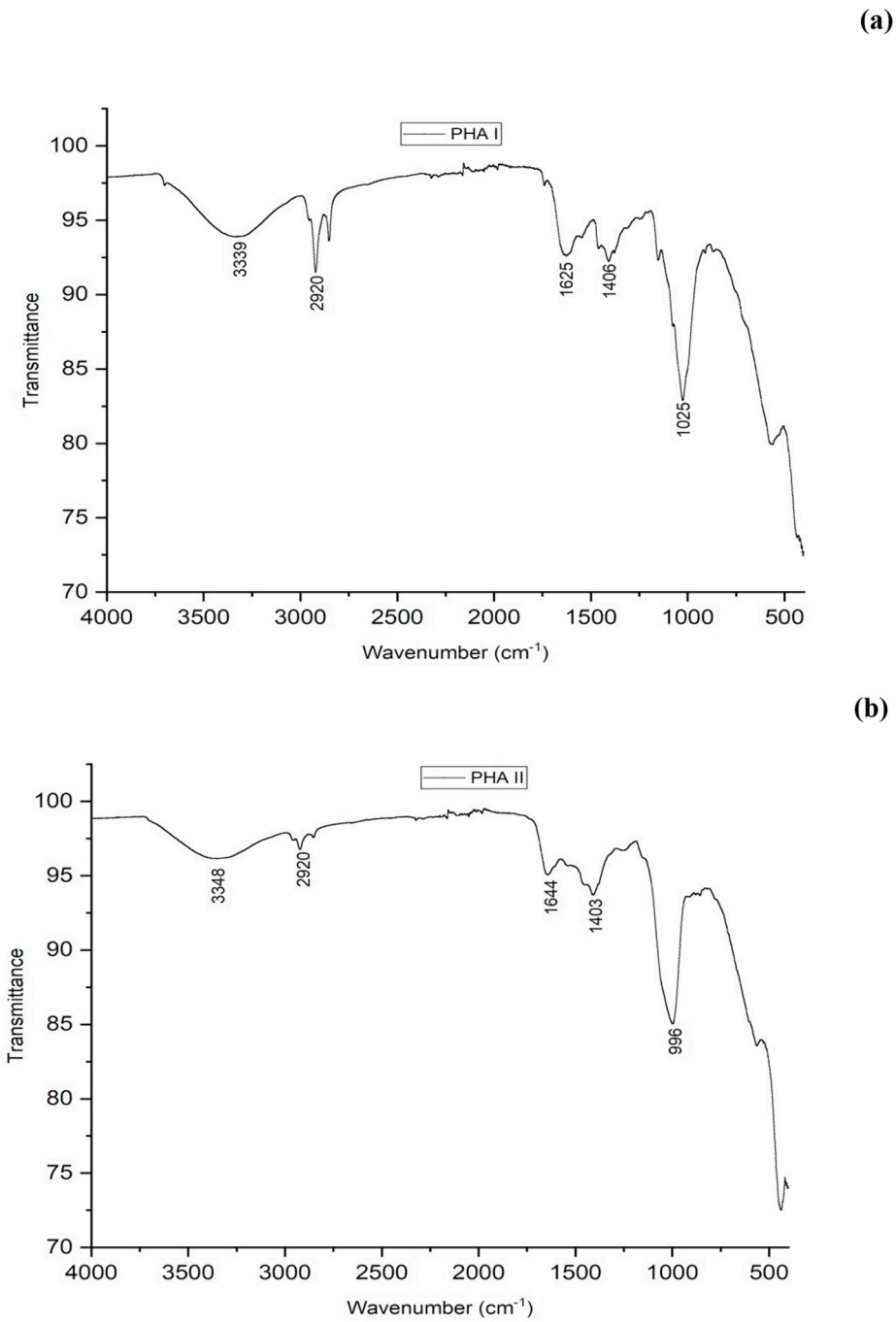

Fig. 3. FTIR Spectra of PHA (a) *Chlorella sp.* (b) *O. salina.*

and to the fact, only few microalgal strains have been reported for PHB production, thus claiming to search for highly productive cyanobacterial and microalgal strains from diverse sources. Hence, the present study initially focusses on selection of suitable algal candidate based on its growth profile. Then the further study aims at the extraction and characterization of polyhydroxyalkanoates from three cyanobacterial species and one green algal species to evaluate their potential for bioplastic.

2. Materials and methods

2.1. Strains and culture conditions

Three cyanobacteria were obtained from National Repository for Microalgae and Cyanobacteria (NRMC), Bharathidasan University, Tiruchirappalli, Tamil Nadu for the present study. Further, algal sample was isolated from the saltpan of Kanyakumari, Tamil Nadu and it was purified for further studies. The following strains cultures were grown in ASN III medium in a temperature controlled culture room at $25 \pm 2\,^{\circ}C$. All the cultures were illuminated by cool fluorescent lamp at 1500 lux with 14:10 L/D photoperiod. Cultures used in the present study are, a. *Synechococcus elongates* (S. elongates) BDU 30312, b. *Leptolyngbya valderiana (L. valderiana)* BDU 10121, c. *Oscillatoria salina (O. salina)* BDU 10412, and d. *Chlorella* sp.

2.2. Growth profile of algal species

Growth of selected strains was determined based on dry cell weight (DCW) of the cultures. For DCW, known amount of algal suspension was centrifuged at 6000 rpm for 10 min and the pelleted algal cells were quick washed twice with water and dried at 60 °C in hot air oven till

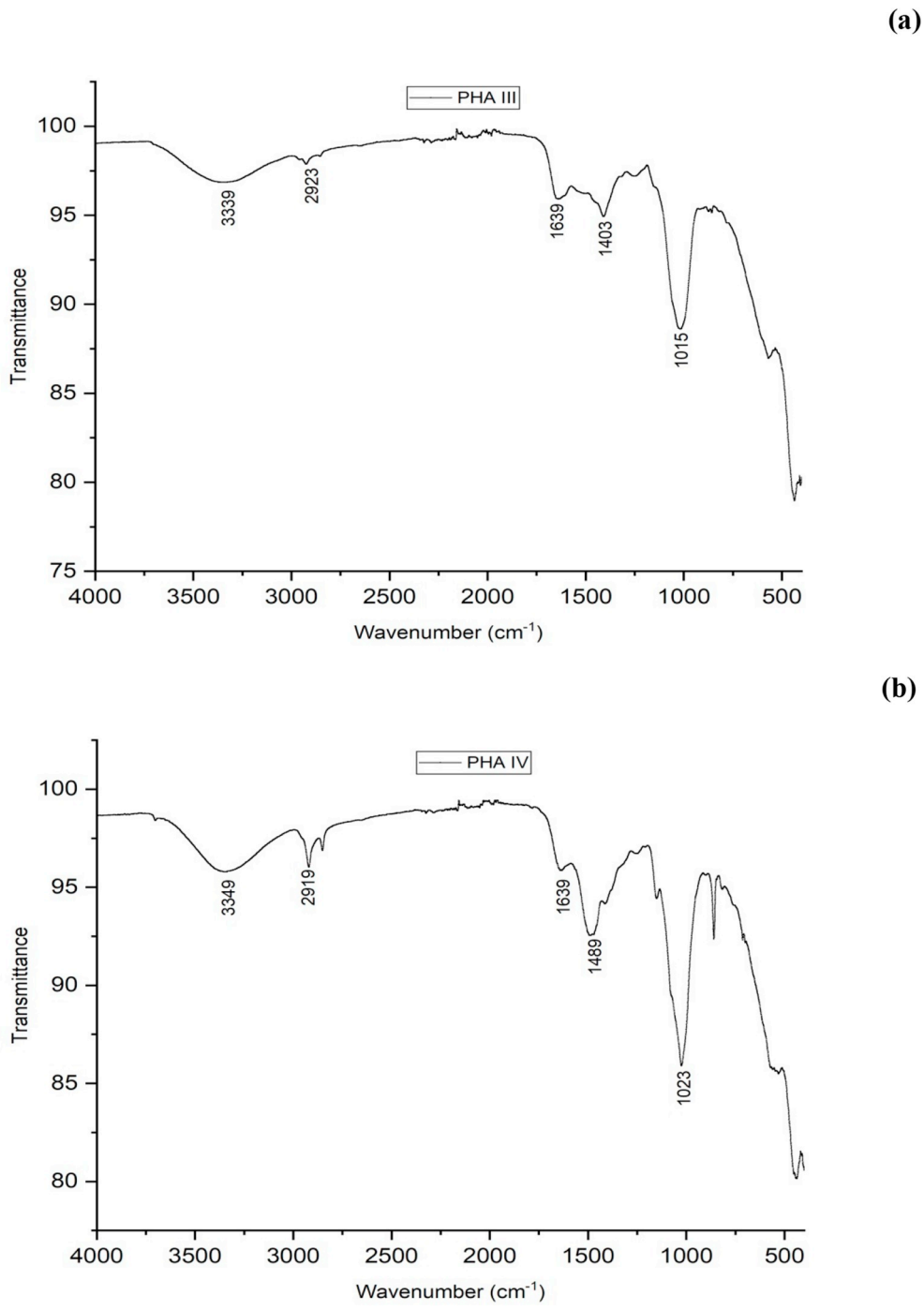

Fig. 4. FTIR spectra of PHA (a) *L. valderiana (b) S. elongates*.

consistent weight was seen. Then, DCW was calculated gravimetrically and expressed in g L^{-1}. All the DCW measurement was taken every three days interval during the course of algal growth. Based on the growth of the strains, cultures were further scaled-up in ASN III to generate biomass for PHA extraction.

2.3. PHA extraction

Algal species were harvested at the end of stationary phase of their growth by centrifugation as mentioned in DCW estimation. The biomasses were water washed to eliminate medium residual or salts if any present along with the biomass. For PHA extraction from *Chlorella sp., O. salina, L. valderiana* and *S. elongatus,* the following procedure was carried out. A known weight of dried biomass was added with 4 % sodium hypochlorite solution and incubated for 30 min at 45 °C. The sample was then centrifuged at 6000 rpm for 30 min and the pellet was added with hot chloroform and kept overnight and then it was precipitated with cold methanol. The precipitated polymer was then centrifuged at 6000 rpm for 30 min to obtain a pellet. The pellet was dissolved again in hot chloroform and dried at 60 °C and weighed (Costa et al., 2018b).

2.4. Fourier-transform infrared spectroscopy (FTIR) analysis of PHA samples

Qualitative analysis of PHA sample extracted from *Chlorella sp., O. salina, L. valderiana* and *S. elongatus* was carried out by FTIR. FTIR

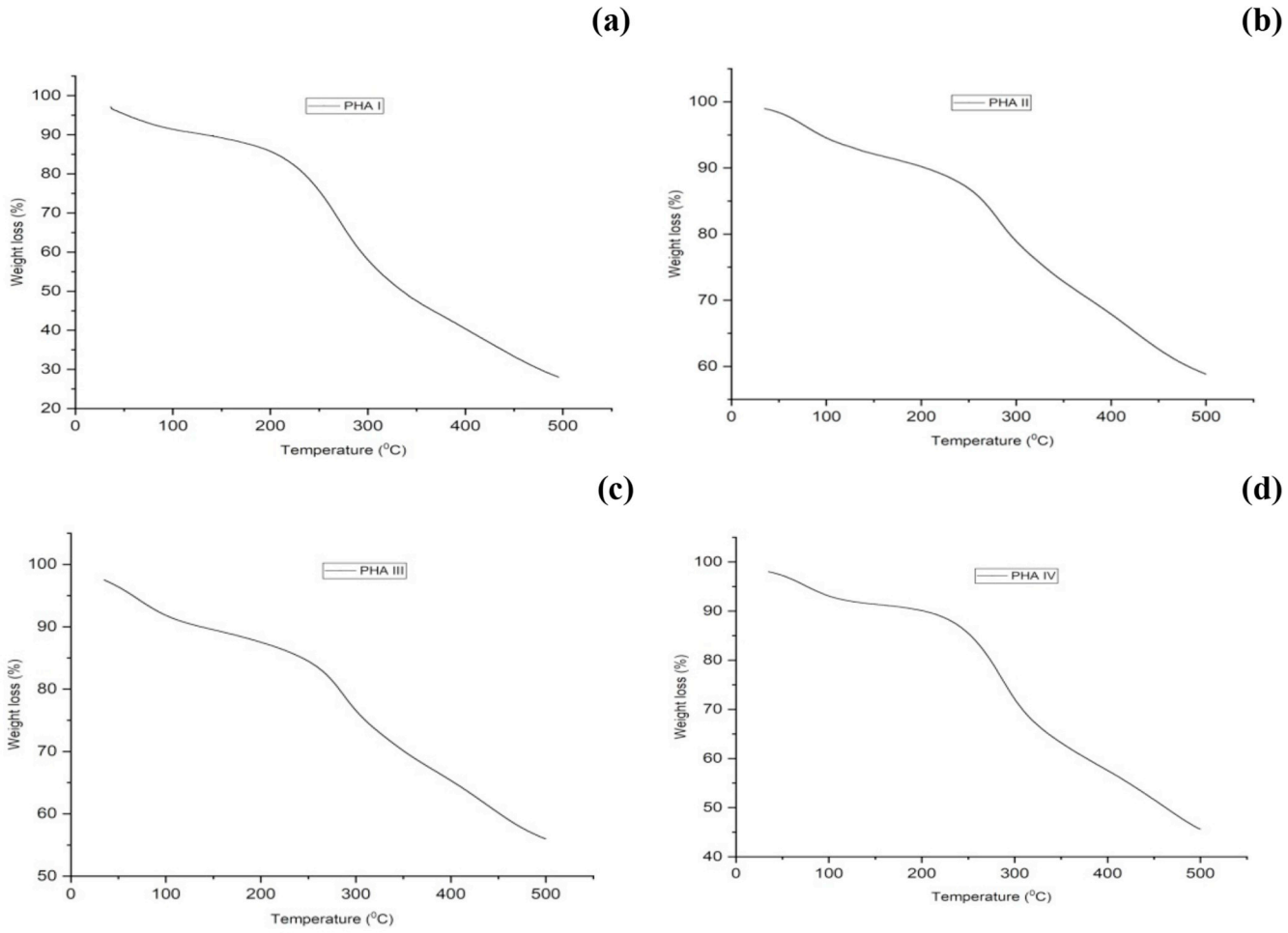

Fig. 5. TGA of PHA (a) *Chlorella sp.* (b) *O. salina* (c) *L. valderiana* (d) *S. elongatus*.

Table 2
Thermal properties of PHA samples.

PHA SAMPLES	Tg °C	Tm °C
PHA from *Chlorella sp.*	6	90
PHA from *O. salina*	9.35	116
PHA from *L. valderiana*	4	79
PHA from *S. elongatus*	10	94

analysis of the samples was done using Perkin Elmer Spectrum (Version 10.03.09) spectrophotometer and the bands were observed in the region between 4000 and 400 cm^{-1}. FTIR is used to determine the chemical groups in PHA samples.

2.5. Thermal analysis of PHA samples

The thermal properties of polyhydroxyalkanoates synthesized from *Chlorella sp., O. salina, L. valderiana* and *S. elongatus* were obtained from Thermo Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). TGA was done in Perkin Elmer Pyris 13 from 35 °C to 500 °C with a heating rate of 10 °C/min in Nitrogen atmosphere and DSC was conducted in Perkin Elmer Pyris 6 from −20 °C to 440 °C at 10 °C/min in a nitrogen atmosphere.

3. Results and discussion

3.1. Growth pattern of algae

The growth of cultures *Chlorella* sp., *O. salina, L. valderiana* and *S. elongatus* was monitored in terms of DCW. The DCW of all the cultures was presented in Fig. 1 & Fig. 2. For *S. elongatus,* the maximum growth was obtained on the 18th day with a DCW of 0.32 g/L. The freshwater *S. elongatus* UTEX 2973 and *Synechococcus* PCC 7942 yielded 0.87 g/L and 0.33 g/L dry weight within 16 h from the initial dry weight of 0.13 g/L and 0.12 g/L respectively under high light illumination and CO_2 supply (Yu et al., 2015). For L. *valderiana*, maximum growth was observed on the 18th day with DCW of 2.75 g/L, while for *O. salina* the higher growth obtained was 0.99 g/L on day 18. Reports on characterization of cyanobacterial strains for high biomass production have shown the dry biomass yields for *Synechococcus* PCC 7942, *Oscillatoria* sp., *Lyngbya* sp. as 1.09 g/L, 0.76 g/L, 0.64 g/L respectively after 19 days of batch cultivation (Patel et al., 2018). In the case of tested green alga *Chlorella* sp., higher dry weight was obtained on day 21 with 2.74 g/L. In a study by Thuy et al., (2019) for production of biomass and lipid from green alga *Chlorella sp.,* the maximum growth was observed on 16th day with a DCW of 1.1 g/L. Another study reported 0.15 g/L DCW for marine *C. vulgaris* after 48 h of cultivation in outdoor raceway pond (Mathimani et al., 2017). Colla et al., (2007) studied the growth of *Spirulina platensis* under 30 °C and 35 °C in Zarrouk's medium and the biomass concentration obtained was 0.82–0.92 g/L and 0.59–0.65 g/L respectively. Gonçalves et al., (2019) studied the biochemical composition of *Pseudoneochloris marina* under different light and temperature conditions and the highest biomass obtained was DCW of 1.8 g/L.

3.2. FTIR spectra of PHA samples

FTIR spectra of four PHA samples were shown in Fig. 3 & Fig. 4. The

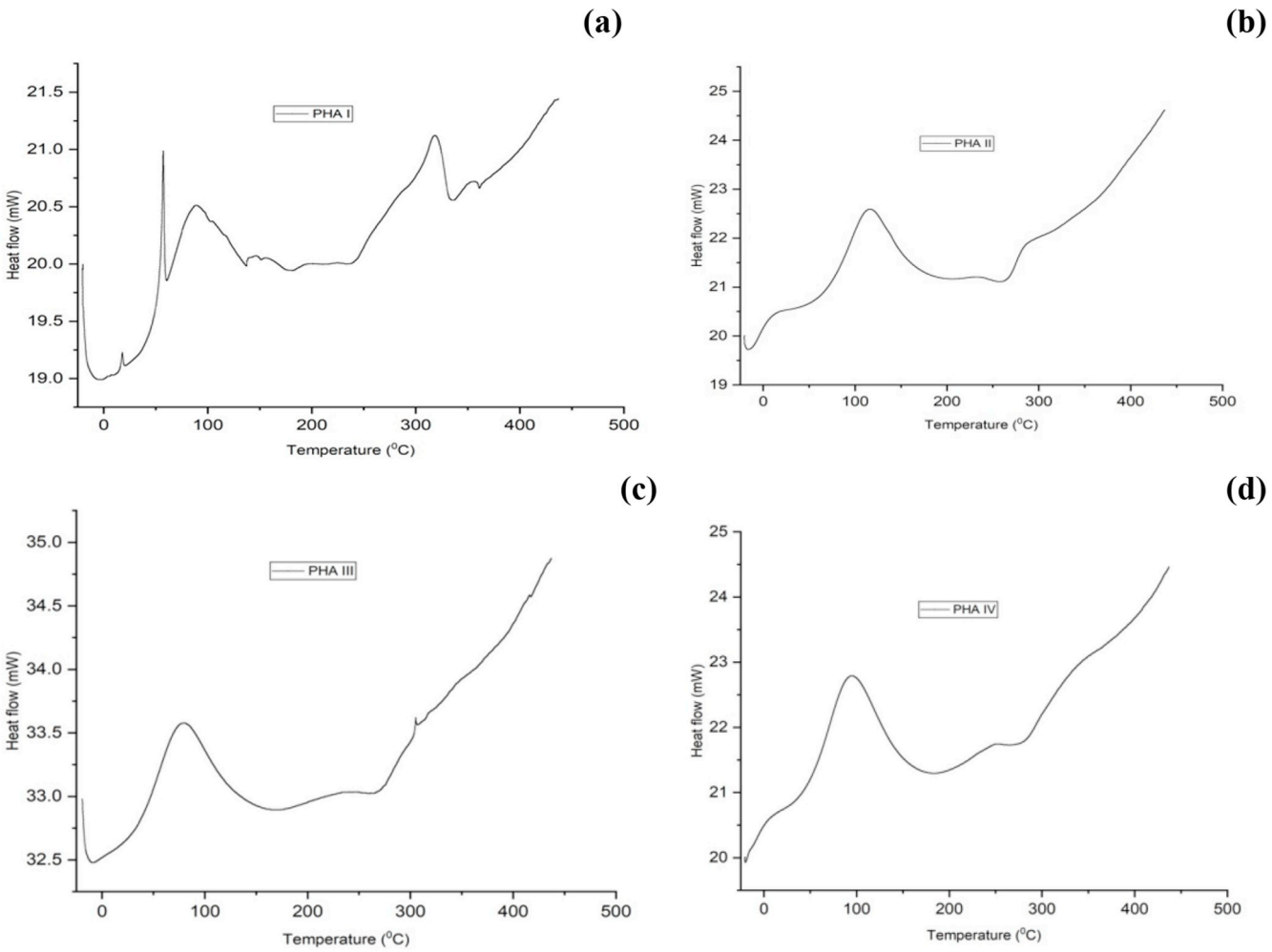

Fig. 6. DSC of PHA (a) *Chlorella sp.* (b) *O. salina* (c) *L. valderiana* (d) *S. elongatus*.

PHA samples were extracted from *Chlorella sp., O. salina, L. valderiana* and *S. elongates* were designated as PHA I, PHA II, PHA III and PHA IV respectively. There is a prominent and broad signal at 3339 cm^{-1} for PHA I and III, 3348 cm^{-1} for PHA II and 3349 cm^{-1} for PHA IV which represents the stretching of –OH within the carboxyl groups. The presence of peak around 2900 cm^{-1} ie., 2921 cm^{-1} for PHA I and II, 2923 cm^{-1} for PHA III and 2919 for cm^{-1} for PHA IV represents C–H stretching vibration of methyl and the methylene groups in the polymer. The stretching of C═O group was seen from the signals 1625 cm^{-1} for PHA I, 1644 cm^{-1} for PHA II and 1639 cm^{-1} for PHA III and IV. Absorbance in the region of 1400 cm^{-1} represents the asymmetric and symmetric stretching of methyl group present in the PHA samples. The peaks at 1025 cm^{-1}, 1015 cm^{-1} and 1023 cm^{-1} indicates the C═O stretching in ester group. Characteristic signals observed above confirm the presence of PHA in all the algal samples. Further, data of this present study is further supported by the various research works carried out by other researchers mentioned below. The FTIR studies conducted for the PHA sample synthesized from *Halogeometricum borinquense* strain E3 by Salgaonkar and Bragança (2015) showed peak at 3585 cm^{-1} which represents the *O*–H stretching and a peak around 2900 cm^{-1} corresponds to C–H stretching vibration. Extraction of PHA from the microalgae *Botryococcus braunii* was conducted (Kavitha et al., 2016b). FTIR analysis and transmittance bands were identified at: 2933 cm^{-1}, which represents the vibration of $–CH_3$ group; C═O stretching at of ester-carboxylic group obtained at 1637 cm^{-1}, which confirmed the presence of scl-PHA. Characterization using FTIR was performed on the PHA obtained from *Synechocystis salina* (Meixner et al., 2018). The spectra obtained showed signals 2975 cm^{-1} and 2932 cm^{-1} which represents the presence of methyl and methylene groups, 1453 cm^{-1} confirmed the presence of asymmetric and symmetric stretching of the methyl groups and a signal at 1721 cm^{-1} confirmed the presence of carbonyl stretching of an ester group. Samantaray et al. (2011) studied the FTIR spectrum of the PHA extracted from *Aulosira fertilissima*. The signal obtained around 3000 cm^{-1} represents the –OH group. The peak at 1460 cm^{-1} corresponded to the asymmetrical stretching in the CH_3 group. The band at 1720 cm^{-1} indicated carbonyl stretching of the ester bond. FTIR spectra of the PHA extracted from *Spirulina sp. LEB-18* and *S. subsalsus* was studied (Costa et al., 2018a). The band region around 1710-1750 cm^{-1} confirmed the presence of ester carbonyl group. Kavitha et al. (2016a) studied the FT-IR spectrum of *Arthrospira platensis* in which the peak at 3278 cm^{-1} represented the hydroxyl group, signals at 2920 cm^{-1} and 2294 cm^{-1} corresponds to the C–H stretching vibration of methyl and the methylene groups in the polymer around 1700 cm^{-1} indicated the CO stretching in ester bond and at 1415 cm^{-1} represented the asymmetrical stretching vibration in CH_3 group.

3.3. Thermal analysis of PHA samples

Thermal degradation of PHA samples extracted from *Chlorella sp., O. salina, L. valderiana* and *S. elongatus* was studied. Fig. 5 shows the TGA graph of PHA samples. There is an initial weight loss up to a temperature of °150 °C for all the four samples due to presence of water molecules and also presence of impurities during the PHA extraction process. The thermal degradation of polymer started at 217 °C, 238 °C, 261 °C and

249 °C for PHA I, PHA II, PHA III and PHA IV respectively. The degradation rate of PHA depends upon different monomer units. Costa et al. (2018a) studied the thermal degradation of the PHA extracted from *Spirulina sp. LEB-18* and *S. subsalsus* and it was found that the onset of degradation of the polymer was around 250 °C and 287 °C respectively and it was concluded that the polymers obtained was thermally stable below a temperature of 250 °C and the melting temperature obtained from DSC was around 171 and 173 °C for *Spirulina sp. LEB-18* and *S. subsalsus* respectively. The thermal degradation of PHA extracted from diazototrophic cyanobacterium *Nostoc muscorum Agardh* was identified at a temperature of 223 °C (Bhati and Mallick, 2015). The Tg and Tm of the PHA polymers obtained was around 0.8 °C and 178 °C. There was a decrease in both temperatures as there was change in the polymer composition. The degradation of the polymer was recorded at 251 °C and at 284 °C, the complete degradation of PHA obtained from *Natrinema altunense* strain RM-G10 was occurred. In another study by Salgaonkar and Bragança (2015) the observed weight loss was around 241 °C and the total weight loss was observed in the proximity of 305 °C. DSC was conducted to determine the melting temperature (Tm) and the glass transition temperature (Tg). The melting temperature and glass transition temperature are given in Table 2. Fig. 6 shows the DSC graphs of PHA extracted the algal samples. The glass transition and melting temperatures depend on the different monomer and its composition in the PHA polymer. Samantaray and Mallick (2012) had reported the Tm and Tg of PHB from cyanobacteria in the range of 174–175 °C and 0.6–0.9 °C whereas for P(3HB-co-3HV), the temperatures were around 148.8–168 °C and −5.5 to −2.2 °C. In another study conducted by Saito, (1994). The temperatures ranged around 53 °C and - 48 °C for P(4HB).

Thermal characterization of PHA polymer synthesized from *P. putida* LS46123 was conducted by Sharma et al. (2017). The thermal degradation of the different PHA polymers was obtained at a range of 248–259 °C. From DSC studies, Tg was found in the range of −28.7 to −34.7 °C and Tm in the range of 138–166 °C. Venkateswar Reddy et al. (2017) used the bacteria *Pseudomonas pseudoflava* for the production of PHA using various carbon sources. Complete thermal degradation was not observed even after 540 °C due to the presence of inorganic material. Tm of the PHA polymer from DSC studies ranged between 140–165 °C. Mohapatra et al. (2016) studied the thermal characteristics of PHAs produced from *Lysinibacillus sp*. via submerged fermentation process. Tm and Tg of the PHA was found to be 112 °C and −11 °C respectively. Thermal degradation of PHA polymer was determined at 114 °C, 489 °C and 831 °C. This was due to the crosslinking and isomerization of the extracted PHA.

4. Conclusions

The growth study on the algal species showed the maximum growth on 18th day for *S. elongatus* BDU 30312, *O. salina* BDU 10142 and *L. valderiana* BDU 10121 and 21st day for *Chlorella sp.,* with a DCW of 0.32 g/L, 0.99 g/L, 2.74 g/L and 2.74 g/L respectively. PHAs were successfully extracted from all the strains via solvent extraction method. FTIR studies confirmed the presence of functional groups in PHA. TGA study revealed that the complete degradation took place below 270 °C for all the PHA samples and the PHA extracted from *L. valderiana* has highest thermal stability compared to other PHA samples from other algal strains. The glass transition and melting temperature of synthesized bioplastics ranged between 4 and 10 °C and 79–116 °C respectively. In this study, algal strains were cultured under photoautotrophic conditions to extract PHA, but algal strains can be cultured in heterotrophic conditions to augment the yield and quality of PHA. Induction strategies i.e., nitrogen and phosphorus repletion or depletion in medium can also be an option to study the capability of the algal strains to accumulate PHA in both photoautotrophic and heterotrophic conditions. Optimization of physical and chemical factors for algae could augment the quality and the quantity of PHA. Assessment of mechanical and biodegradability characteristics of algal PHA samples would deliver real time value of PHA for application studies. In future studies, pathway engineering or metabolic system studies of algae can be undertaken to overexpress the gene to accumulate PHA. In order to put the algal bioplastic in a sustainable path, meticulous and myriad R & D attempts need to be pursued in the avenues of feedstock improvement, lucrative extraction, and PHA productivity.

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