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Macroalgal biomass subcritical hydrolysates for the production of polyhydroxyalkanoate (PHA) by *Haloferax mediterranei*

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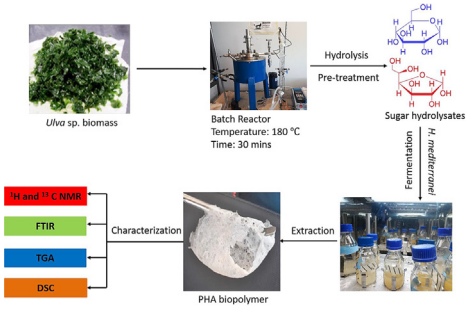
GRAPHICAL ABSTRACT



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ABSTRACT

Non-conventional carbon sources, such as macroalgae, are sustainable alternatives for large-scale production of biopolymers. The present study examined macroalgae-derived carbohydrates, as carbon sources for the production of polyhydroxyalkanoates (PHAs) by *Haloferax mediterranei*. Simulants of the hydrolysates of seven different macroalgal biomasses were prepared and the PHA production was studied. A maximum biomass concentration with maximum PHA content was detected in medium prepared from green macroalgae. The highest cell dry weight and PHA concentrations were 3.8 ± 0.2 g·L^{-1} and 2.2 ± 0.12 g·L^{-1} respectively when *Haloferax mediterranei* was grown in 25% (w/w) of *Ulva* sp. hydrolysate, at 42 °C temperature and initial pH of 7.2. Poly(3-hydroxy-butyrate-co-3-hydroxyvalerate was the major PHA constituent. The present study demonstrated that *Ulva* sp. is a promising feedstock for PHA production.

1. Introduction

The ever-rising global plastic consumption (~320 million tonnes per annum) has resulted in its accumulation into environments, including the land and the oceans (de Souza Machado et al., 2018). This has led to the problem of plastic pollution. A sustainable solution could be the use of biodegradable plastic alternatives, such as polyhydroxyalkanoates (PHAs) (Koller, 2018). PHAs have an advantage over conventional petroleum-based synthetic polymers, such as polyethylene, for being recognized as completely biosynthetic and biodegradable, having zero toxic waste, and being completely recyclable into organic waste. The variations in the structure of PHAs has allowed the development of various applications, including environmentally-friendly biodegradable plastics for packaging purposes, fibers,

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biocompatible implants, controlled drug-delivery devices and biofuels (Chen and Zhang, 2017; Zhang et al., 2009).

The two most investigated types of PHAs are poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). PHB is difficult to process into commodity goods, because it is stiff and brittle, has a high degree of crystallinity and its melting temperature (175 °C) is close to its decomposition temperature (185 °C) (Gahlawat and Soni, 2017). The introduction of 3-hydroxyvalerate (3HV) units, to form PHBV disrupts the highly crystalline PHB structure, resulting in a polymer with enhanced mechanical properties, quicker degradation rates, and improved physical properties (Ferre-Guell and Winterburn, 2018).

The challenges for PHA production include dependence on pure carbon sources, such as glucose; the requirement of organic substitutes for production of different types of PHA; the chances of contamination and the use of large amounts of solvents in downstream processing (Rodriguez-Perez et al., 2018). Extreme halophiles could provide a partial solution to the problem of carbon sources, as they can utilize various inexpensive substrates for production of PHA (Yin et al., 2015). Among several extreme halophiles proposed for PHA production, *Haloferax mediterranei* has gathered considerable attention, since it can naturally produce poly-3-(hydroxybutyrate-co-hydroxyvalerate) (PHBV), without any supplementation of structurally-similar precursors, such as valerate (Bhattacharyya et al., 2012). Furthermore, high salinity requirements of *H. mediterranei* (~15–25% salt water) decreases the chances of microbial contamination and thus reduces the requirement for media and equipment sterilization (Chen et al., 2017). Besides, the extraction of intracellular PHA does not require a high input of chemical solvents as hypotonic environments that lead to cell lysis (Moorkoth and Nampoothiri, 2016).

Recent studies on PHA production using *Haloferax mediterranei* have focused on utilization of inexpensive substrates. Starch (Chen et al., 2006), ethanol stillage (Bhattacharyya et al., 2014), vinnase (Bhattacharyya et al., 2012), molasses wastewater (Cui et al., 2017), olive mill wastewater (Alsafadi and Al-Mashaqbeh, 2017), and cheese whey (Pais et al., 2016) are some examples of the proposed substrates. However, the availability of cheap substrates hinders the production of PHA on a large scale (Koller et al., 2017). Therefore, new feedstocks are required to be explored to provide a sustainable solution for PHA production.

Macroalgal biomass can be used as an alternative and suitable feedstock for production of PHA. The various advantages of macroalgae include that it does not require land for its cultivation (Fernand et al., 2017). The growth of macroalgae also needs very low nutrient supplementation. Macroalgae do not compete with conventional food sources, thereby avoiding any conflicting applications of this feedstock (Jiang et al., 2016). Since macroalgae have a variety of carbohydrates depending on species, information on their carbohydrate compositions is necessary to utilize effectively as carbon sources for biomaterial and bioproducts (Chemodanov et al., 2017b; Ingle et al., 2018; Robin et al., 2017; Vitkin et al., 2015).

Studies on PHA production using seaweed as a substrate have focused on brown seaweed (Azizi et al., 2017) or compounds extracted from seaweed (levulinic acid), which was supplemented to the medium (Bera et al., 2015). The red algae *Gelidium amansii* has also been used as a carbon substrate for PHA production (Alkotaini et al., 2016; Sawant et al., 2017). Notably, the utilization of green macroalgae, such as *Ulva* sp. for PHA production has not been explored yet. *Ulva* sp. blooms have been reported to cause eutrophication and the treatment of these bodies have become a problem (Cui et al., 2018). PHA production using *Ulva* biomass could provide a double benefit of waste remediation as well as the production of value-added products.

Our goal was to study the production of PHA from macroalgal biomass hydrolysate using *Haloferax mediterranei*. First, the PHA production potential from 7 seaweed species was screened, using synthetic seaweed media. The composition of the green macroalgae from *Ulva* sp. had the highest potential for PHA production. Therefore, *Ulva* sp. was hydrolyzed using subcritical water (Parsa et al., 2018). The effect of different concentrations of the hydrolysate on PHA production with *H. mediterranei* was studied. The structural characteristics of the obtained PHA were determined. The results could open a new venue for the production of bioplastics and bioenergy from the offshore grown biomass (Chemodanov et al., 2017a). This could, in turn, become a sustainable and environmentally friendly alternative to produce biopolymers in the long run.

2. Materials and methods

2.1. Microorganism and culture conditions

The microorganism used for the study was wild-type *Haloferax mediterranei*. The culture was kindly provided by Prof. Uri Gophna of Faculty of Life Sciences, Tel Aviv University. The *H. mediterranei* strain were routinely grown in rich medium (Hv-YPC) containing (per L) 144 g of NaCl, 21 g of $MgSO_4{\cdot}7H_2O$, 18 g of $MgCl_2{\cdot}6H_2O$, 4.2 g of KCl, and 12 mM Tris HCl (pH 7.5). For solid media, agar (Difco, USA) was added at a concentration of 15 $g{\cdot}L^{-1}$ and was dissolved by heating the medium in a microwave oven. Yeast extract (0.5% w/v; Difco, USA), 0.1% (w/v) peptone (Oxoid) (Difco, USA), and 0.1% (w/v) Casamino Acids (Difco, USA) was added and the medium was autoclaved. After cooling, $CaCl_2$ was added to a final concentration of 3 mM (Allers et al., 2010). For the preparation of culture plates, 2% w/v of Agar powder was added to the medium. The organism was grown at 42 °C in a temperature-controlled incubator. For liquid cultures, the microorganism was grown in 250 mL Duran bottles with a working volume of 100 mL. The culture was grown in a shaking incubator (MRC Labs, Israel) at 42 °C with a rotational speed of 180 rpm. The media pH was adjusted to 7.2.

2.2. PHA production in the different cultivation medium

2.2.1. PHA production by H. Mediterranei in Hv-YPC medium

The PHA production was initially studied using the Hv-YPC medium. The medium was supplemented with 2% (w/v) glucose to maintain a higher C/N ratio. Higher C/N ratio has been reported for the enhanced production of PHA (Kucera et al., 2018). The PHA production in various pure carbon substrates was also studied. The pure substrates were supplemented to Hv-YPC medium for PHA production.

2.2.2. PHA production by H. Mediterranei in simulated seaweed hydrolysate medium

PHA production of *H. mediterranei* was also studied in synthetic media where the carbon source was equivalent to the composition of different seaweed hydrolysates. The carbohydrate compositions of different seaweed hydrolysates of the eastern Mediterranean region were studied by Robin et al. (2017). Hv-YPC media was modified by substituting with a similar carbon concentration of hydrolysates as suggested in earlier studies (Robin et al., 2017). The extracted PHA was quantified and characterized.

2.2.3. PHA production by H. Mediterranei using seaweed hydrolysate

The green macroalga *Ulva* sp. was grown in our laboratory under controlled conditions using cylindrical, sleeve-like macroalgae photobioreactors (MPBR) (Polytiv, Israel, Length 100 cm, thickness 200 mm, width 40 cm) (Chemodanov et al., 2017b) under natural irradiance in July 2017. Artificial seawater (ASW) having a total salinity of 3.7%, prepared using distilled water and dried Red Sea salt (Red Sea Inc, IS) was used as a cultivation medium. Nutrients were supplied by adding ammonium nitrate (NH_4NO_3, Haifa Chemicals Ltd, IS) and phosphoric acid (H_3PO_4, Haifa Chemicals Ltd, IS) to maintain 6.4 $g{\cdot}m^{-3}$ of nitrogen (N2) and 0.97 $g{\cdot}m^{-3}$ of phosphorus (P) in the ASW. Initially, 20 g fresh *Ulva* sp. was inoculated in each sleeve with total volume of 40.4 L in a

reactor. All other conditions such as pH, temperature and flow rate were maintained as mentioned in our previous study (Chemodanov et al., 2017b). *Ulva* sp. hydrolysate was utilized for PHA production using *Haloferax mediterranei*. The hydrolysis was performed in a customized batch reactor (Zhengzhou Keda Machinery and Instrument Equipment CJF-0.25, China) under a 180 °C for 30 min (Thiruvenkadam et al., 2015). The solid load for the hydrolysis was 8% w/v *Ulva* sp. biomass. The sugar and 5-HMF content were monitored by HPAEC-PAD (High-Pressure Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection) using a Dionex ICS-5000 platform (Dionex, Thermo Fischer Scientific, MA, USA) with an analytical column (Aminopack 10) and its corresponding guard column. An electrochemical detector with an AgCl reference electrode was used for detection. The analysis was performed using an isocratic flow of 4.8 mM KOH generated by the Eluent Generator technology (Dionex, Thermo Fischer Scientific, MA, USA). Various concentrations of seaweed hydrolysate (0 – 100% w/v) were used and the growth characteristics and PHA production were analyzed. Peptone was utilized as the nitrogen source in concentrations as used in the Hv-YPC medium. Seawater was added according to the concentrations used in the Hv-YPC medium.

2.3. Extraction of PHA

The liquid culture (100 mL) was centrifuged at 2500 rpm for 15 mins in a swing rotor centrifuge (Rotanta 420R, Hettich Instruments LP, USA). The obtained cell pellet was dried in a hot air oven at 60 °C overnight. The dry weight of the pellet was determined. 15 mL of acetone (Merck) was added to the dried pellet and was kept in a refrigerator overnight to remove the pigments. The suspension was centrifuged at 2500 rpm for 15 min and dried at 60 °C to remove excess acetone. PHA was extracted with chloroform (Merck, USA) at 37 °C for 24 hrs under shaking conditions at 100 rpm (Balkrishna, 2015).

2.4. Quantification of intracellular PHA content

2.4.1. Quantification by Nile Red fluorescence method

The Nile red staining procedure was adapted from Spiekermann et al. (1999). The cell suspension (1 mL) was centrifuged at 12,000*g* for 5 min and the pellet was resuspended in 1 mL of distilled water. Subsequently, 40 µL of Nile Red (Sigma) (80 µg·mL^{-1} dissolved in dimethyl sulfoxide [DMSO]) was added to the suspension to give a final concentration of 3.1 µg Nile Red per mL suspension and was incubated at room temperature for 30 min. The stained suspension was centrifuged at 12,000*g* for 5 min and the supernatant was discarded. Distilled water (1 mL) was added and the resulting pellet was vigorously vortexed. An aliquot of the suspension was pipetted into 96-well microplate. The fluorescence was measured at excitation and emission wavelength 535 and 605 nm respectively using a Multilabel Plate Reader (Tecan, Switzerland) (Spiekermann et al., 1999). A standard curve was plotted for fluorescence intensity versus PHA concentration with known concentrations of PHA. The unknown amount of PHA was determined from the standard curve with two repeats per point. The fluorescence values were further verified by the crotonic acid method of PHA quantification (Mahansaria et al., 2018).

2.4.2. GC–MS analysis of the extracted polymer

The GC–MS analysis was performed to determine the PHA content (%) as well as its composition. Approximately 100–150 mg PHA was mixed with 10 mL butanol in presence of a solid acid catalyst (Sigma, USA). The butanolysis process was carried out for 140 min at 110 °C. After cooling, the solid residues were separated by filtration and the liquid phase was analyzed using GC (Agilent, USA). GC − MS analyses were performed on an Agilent 6890/5977A GC–MS system equipped with Agilent 30 m × 0.25 mm i.d. HP-5MS UI column (5% Phenyl/Methylpolysiloxane, 0.25 µm film thickness). The carrier gas was helium (99.999%) at a constant flow rate of 1.0 mL/min. The GC conditions were as follows: injection volume 0.2 µL (Agilent auto-sampler G4513A); injector temperature 280 °C with split ratio of 1:19; initial oven temperature 70 °C hold for 5 min and increased to 280 °C at a rate of 15 °C/min and followed by temperature rise to 320 °C at a rate of 30 °C/min with 5 min hold time. MS was performed in the EI positive ion mode, using the electron energy of 70 eV. Transfer line temperature and ion source temperature were maintained at 280 and 250 °C, respectively. MS data were collected in full-scan mode (*m/z* 50–400) and analyzed with Agilent Chemstation software.

2.5. Determination of biomass and PHA yield

2.5.1. Volumetric biomass and PHA productivity

Volumetric biomass productivity ($P_{Biomass,}$ g·L^{-1}·h^{-1}) was calculated by the following equation.

$$P_{Biomass} = \frac{X_2 - X_1}{t_2 - t_1} \quad (1)$$

where X_1 and X_2 are biomass concentrations (g·L^{-1}) at time t_1 (h) and t_2 (h) respectively.

Volumetric PHA productivity ($P_{PHA,}$ g·L^{-1}·h^{-1}) was calculated by the following equation.

$$P_{PHA} = \frac{P_2 - P_1}{t_2 - t_1} \quad (2)$$

where P_1 and P_2 are the amount of PHA accumulated (g·L^{-1}) at time t_1 (h) and t_2 (h) respectively.

2.5.2. PHA yield

The PHA yield was determined by a gravimetric method wherein the extracted polymer was dried at 70 °C till constant weight was obtained. The yield (%) was calculated as:

$$Yield = \frac{w_{PHA}}{w_{cell}} \times 100 \quad (3)$$

where w_{PHA} (g) is the amount of PHAs recovered from dry cell weight w_{cell} (g).

2.6. Characterization of PHA

2.6.1. FTIR analysis

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrum was recorded for air-dried PHA film using Bruker Platinum ATR machine from 390 to 4000 cm^{-1} and compared with the commercially available PHA (Bluepha Beijing Blue Crystal Microbial Technology Co., Ltd., China).

2.6.2. TGA/DSC analysis

Thermogravimetry and differential scanning calorimetry (TG-DSC) analysis was carried out for PHA to study the thermal properties using Jupiter STA 449 F5 (NETZSCH, Germany). Dry PHA (5 mg) was subjected to a temperature range of − 30 °C to 200 °C, at a heating rate of 10 °C·min^{-1}. The empty aluminum crucible was used as the reference. Thermal analysis of *Haloferax* PHA was compared with the commercially available PHA (Bluepha Beijing Blue Crystal Microbial Technology Co., Ltd., China). Thermal stability of the PHA was investigated by thermogravimetric analysis (TGA) at a temperature range from 30 to 500 °C with a heating rate of 10 °C min^{-1} (Maheshwari et al., 2018).

2.6.3. ^{1}H and ^{13}C NMR analysis

^{1}H and ^{13}C NMR spectra were acquired by dissolving the polymer in deuterochloroform ($CDCl_3$) at a concentration of 10 mg·ml^{-1} and analyzed on a 400 MHz spectrometer (Bruker, USA).

2.6.4. SEM analysis

The lyophilized cells were subjected to SEM observation. The samples were mounted on aluminum stubs, sputter-coated with gold for 15 s prior to observation under JEOL JSM 6300 – F Field Emission SEM at an acceleration voltage of 10 kV and magnification of 1000–50,000 × (Kunasundari et al., 2017).

2.7. Statistical analysis

The experiments were conducted in triplicate to avoid any variability in results. The mean of three values obtained was used to calculate standard deviation (SD). The final values were represented as mean ± SD. The value of standard deviation obtained was referred to as an experimental error. Graphs were plotted using Sigmaplot software (Sigmaplot, USA).

3. Results and discussion

3.1. Growth and PHA production in the Hv-YPC medium

The initial studies were designed to establish the ability of *Haloferax mediterranei* for production of PHA in the growth medium. The colonies on the Petri dish were bright red due to the presence of pigments in the cells. As haloarchaea acclimatize to very high saline environments, they produce various pigments and proton pumps for osmoadaptation (Ashwini et al., 2017). The strain was screened for PHA production in plates containing 0.01% v/v Nile Red solution added to the Hv-YPC medium. A bright fluorescence was observed when the plate was illuminated with UV light in a UV transilluminator (Enduro™ GDS, Labnet, USA). This confirmed the presence of PHA in the organism. The PHA granules were visualized by Scanning Electron Microscopy. The granular shapes of PHA bodies were observed.

Further, the organism was grown in 100 mL of Hv-YPC medium in 250 mL Duran bottles. The Hv-YPC medium was supplemented with 2% w/v glucose to provide a higher C/N ratio for the accumulation of PHA. A maximum biomass concentration of 5.1 ± 0.1 $g{\cdot}L^{-1}$ was obtained after 72 hrs of cultivation. The maximum PHA concentration was calculated to be 3.4 ± 0.2 $g{\cdot}L^{-1}$ or 66.67% w/w (Fig. 1). The pH profile was also observed during fermentation. A steady decrease in pH was observed with the final pH of the medium reaching 5.0 ± 0.2. This might be due to the accumulation of acids which are a by-product of the fermentation process (Bhattacharyya et al., 2014).

The results were in parity with previous reports using various carbon sources as substrates (Bhattacharya et al., 2016, 2014, 2012). PHA production using *H. mediterranei* was confirmed by various authors with a maximum concentration of up to 75% w/w in wild-type microorganism (Han et al., 2015; Rodriguez-Valera and Lillo, 1992). Tailor-made PHA has also been produced from *Haloferax mediterranei* using modified organism (Han et al., 2015). Lillo and Rodriguez-valera (1990) reported the effects of culture conditions for PHA production in *Haloferax mediterranei*. They found that PHA accumulation starts during the logarithmic phase, increases with the biomass and reaches a peak at the beginning of the stationary phase. PHA synthesis is delayed with respect to biomass development, reaching a maximum rate of synthesis at the end of the exponential phase (Lillo and Rodriguez-valera, 1990). Similar trends were observed for our experiments in Hv-YPC medium with 2% w/v glucose substitution where the production of PHA was growth associated. The growth associated PHA accumulation could be a key observation for the production of PHA in a continuous culture thereby increasing the overall yield of PHA. The studies were further extended to the production of PHA using different sugars as a substrate.

3.1.1. PHA production utilizing different carbon sources

Another property of the organism under investigation was the ability to grow in various carbon sources. *H. mediterranei* was grown by supplementing different sugars to Hv-YPC medium and the biomass and PHA production was observed. With the exception of glucuronic acid, the organism was able to utilize all the sugars which were tested. The maximum biomass and PHA titer were observed with glucose supplementation followed by fructose, xylose, and galactose (Fig. 2a.). The highest biomass concentration of 5.2 ± 0.08 $g{\cdot}L^{-1}$ was observed with glucose as a substrate with a corresponding PHA concentration of 3.44 ± 0.11 $g{\cdot}L^{-1}$. The maximum PHA yield in the cells was estimated to be 66.15% w/w. This was followed by fructose (Biomass

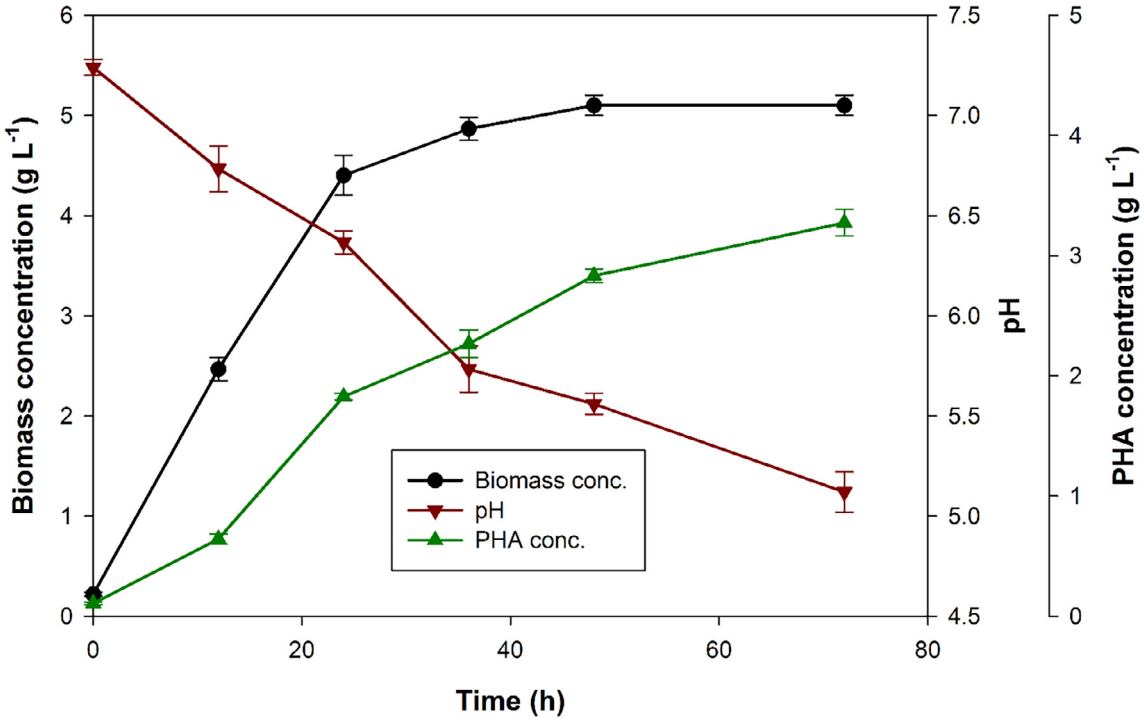

Fig. 1. Profiles of biomass, pH, and PHA concentration for the growth of *H. mediterranei* in Hv-YPC medium supplemented with 2% (w/w) glucose.

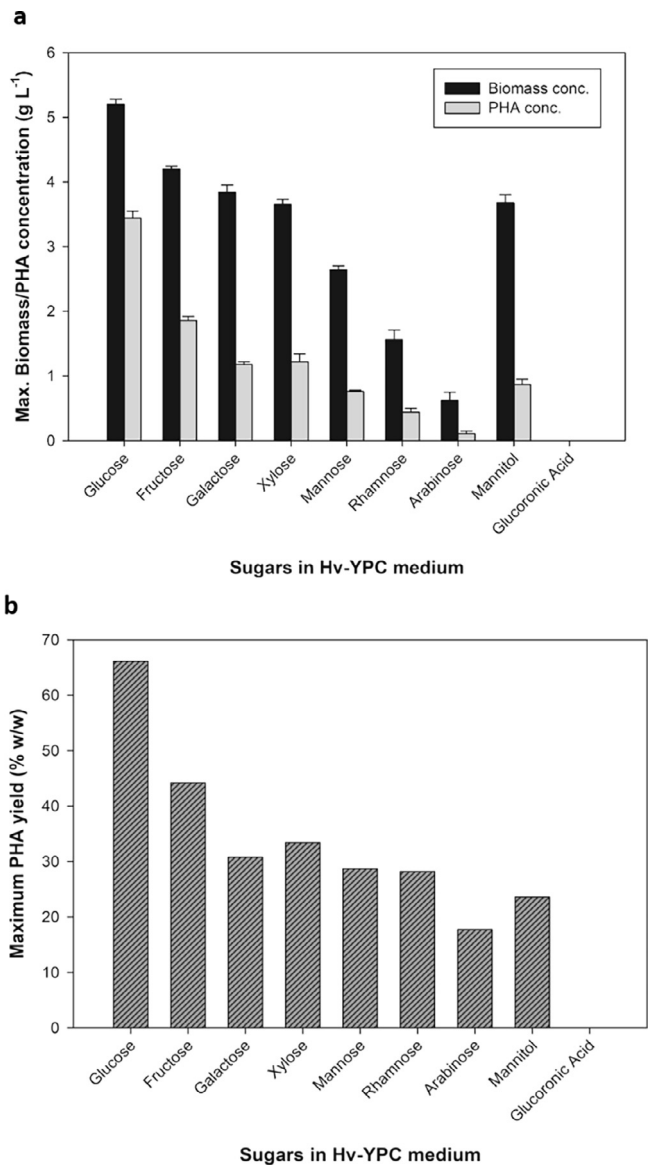

Fig. 2. (a) Maximum biomass and PHA concentrations of *H. mediterranei* for growth in different carbon sources (b) PHA content of *H. mediterranei* for growth in various carbon sources.

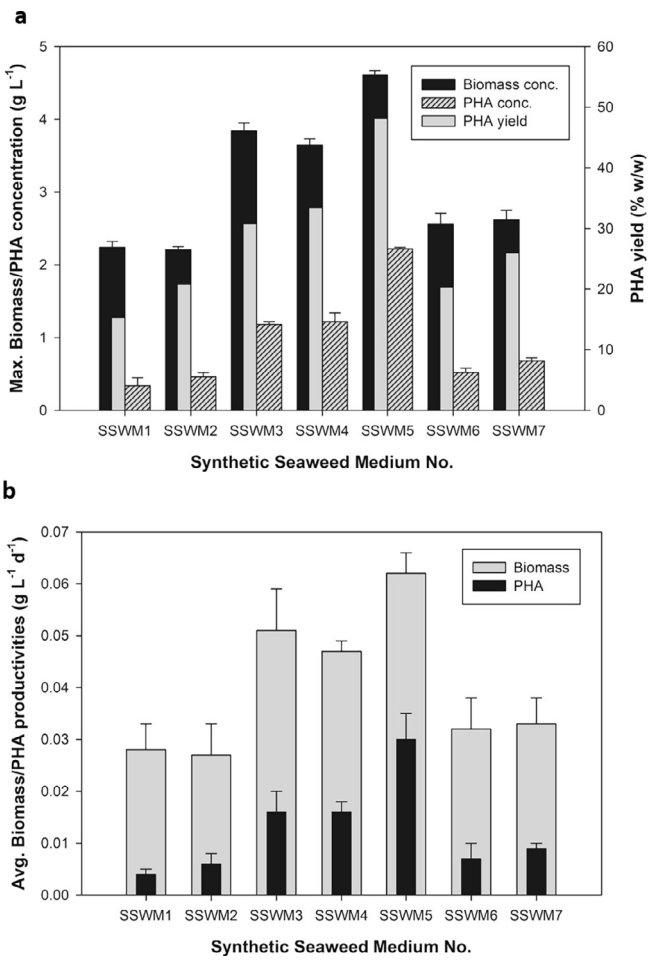

Fig. 3. (a) Maximum biomass, PHA concentrations and PHA contents of *H. mediterranei* for growth in various synthetic seaweed medium (b) Average productivities for the growth of *H. mediterranei* on various synthetic seaweed medium.

concentration: 4.2 ± 0.04 g L^{-1}; PHA concentration: 1.86 ± 0.06 g·L^{-1}; PHA yield: 44.20% w/w) and xylose (Biomass concentration: 3.65 ± 0.08 g·L^{-1}; PHA concentration: 1.22 ± 0.12 g L^{-1}; PHA yield: 33.4% w/w) (Fig. 2b.). Nevertheless, the broad spectrum of carbon sources exploitation could provide an insight on the various substrates which could be used for the inexpensive production of PHA.

An important observation was the usage of pentose sugars as generally, they are difficult to ferment as compared to their hexose counterparts (Moorkoth and Nampoothiri, 2016). *H. mediterranei* could utilize xylose and arabinose with a PHA content of 33.4% w/w and 17.34% w/w respectively with a relative biomass content.

Similar results of utilization of pentose sugars by extreme halophiles were observed by Kucera et al. (2018) when they studied the substrate consumption of *Halomonas halophila* (Kucera et al., 2018). *H. mediterranei* is known to utilize a wide array of substrates to produce PHA. Enzyme extruded starch was used as a carbon source to produce PHA by *H. mediterranei*. They could reach up to a PHA content of 50.8% w/w of dry cells (Chen et al., 2006). *H. mediterranei* also utilized extruded rice bran for production of PHA where they obtained a PHA content of 55.6% w/w of dry cell weight (Huang et al., 2006). Utilization of cellobiose and soluble starch has been reported for *H. mediterranei* (Fernandez-Castillo et al., 1986; Lillo and Rodriguez-valera, 1990). Glycerol from the biodiesel industry has also been used as a substrate for PHA production using *H. mediterranei* (Hermann-Krauss et al., 2013). After studies with pure sugars, the efficiency of the organism was investigated using a mixture of sugars. The mixture of sugars was determined according to the carbohydrate compositions of different macroalgal hydrolysates to understand the biomass accumulation and PHA production in them.

3.2. PHA production in synthetic seaweed medium (SSWM)

The diversity of monosaccharides in marine macroalgae isolated from the Eastern Mediterranean coast was studied by Robin et al. (2017) (Robin et al., 2017). This provided the basis for comparing the growth and PHA production in various hydrolysates of macroalgae. The hydrolysates were simulated according to the carbohydrate contents as provided by Robin et al., (2017). The best results were observed for hydrolysate of *Ulva* sp. (Fig. 3a.). The maximum biomass and PHA concentration were observed as 4.61 ± 0.06 g·L^{-1} and 2.22 ± 0.02 g·L^{-1}, respectively. The PHA yield was calculated to be 48.15% (w/w). The volumetric biomass and PHA productivities were also considered for a cultivation period of 72 h. The maximum volumetric biomass and PHA productivity were estimated at 0.062 ± 0.04 g·L^{-1}·h^{-1} and 0.03 ± 0.05 g·L^{-1}·h^{-1}, respectively

(Fig. 3b.).

The major monosaccharide in *Ulva* sp. hydrolysate was glucose followed by rhamnose and galactose (van der Wal et al., 2013). The higher PHA concentration using *Ulva* sp. synthetic medium might be due to the higher glucose concentration as compared to other hydrolysates. The PHA yield was relatively low in case of simulated medium for ochrophytic algae (*Padina pavonia* and *Sargassum vulagare*). This might be due to the relatively higher content of fucose and mannose present in the medium. The pH value at the end of the cultivation on simulated hydrolysate of *Ulva* sp. was observed to be 5.12, which is quite low as compared to the initial value of 6.8. Therefore, it was assumed that a drop in pH value led to unfavorable growth conditions, which ultimately resulted in growth inhibition and the nutrients consumption eventually stopped. A similar drop in pH was observed by Bhattacharya et al., (2014) for production of PHBV employing distillery stillage as a substrate (Bhattacharyya et al., 2014). When grown on some sugars and sugar alcohols, such as glycerol, glucose, fructose, maltose, and sucrose, acidification of the medium occurs; the acidic products formed during growth on glycerol were identified as D-lactate and acetate (Gurevich, 1994).

3.3. Effect of seaweed hydrolysate on PHA production

The PHA production by *Haloferax mediterranei* was also studied by utilizing the hydrolysate of *Ulva* sp. The total sugar content of the hydrolysate was 10.15 ± 0.12 mg·g^{-1} of dried macroalgal biomass with a glucose content of 6.11 mg·g^{-1}. The 5-HMF content of the hydrolysate was 1.3 mg·g^{-1} of dried macroalgal biomass. The major sugar was glucose which was followed by xylose and fructose. Glucose content of the hydrolysate was 60% w/w of the total reducing sugar content. The macroalgal hydrolysate was further utilized as a sole carbon source for production of PHA. Tryptone was supplemented as a nitrogen source along with the addition of sea water to the medium. The macroalgal hydrolysate was supplemented in different concentrations ranging from 0 to 100 % in the cultivation medium. The biomass concentration, PHA yield and PHA concentration were calculated as shown in Fig. 4.

The maximum biomass concentration and PHA yield were observed with supplementation of 25% v/v of macroalgal hydrolysate. A biomass concentration of 3.8 ± 0.2 g·L^{-1} was detected with a maximum PHA concentration of 2.2 ± 0.12 g L^{-1} after a 72 h cultivation period. The highest PHA yield was calculated as 55% (w/w) with an average volumetric productivity of 0.035 ± 0.002 g·L^{-1}h^{-1}. The average volumetric biomass productivity was calculated to be 0.052 ± 0.008 g·L^{-1}h^{-1}. The PHA obtained using 25% v/v of macroalgal hydrolysate contained 8 mol % 3-HV while the 3-hydroxybutyrate content was found to be 92 mol % as analyzed by GC–MS. The studies also showed that energy-rich butyl esters could be synthesized using solid catalysts. This might be an alternate route for the production of biofuels from PHA (Zhang et al., 2009).

Previous studies on PHB production using hydrolysates of *Gelidium amansii* also reported volumetric PHA productivities in the range of 0.002–0.900 g·L^{-1}h^{-1}. This could be attributed to the fact that most of the studies on PHA production using macroalgal hydrolysates have been carried out on laboratory scales using small setups (100–250 mL). The process parameters are to be controlled in a bioreactor in order to achieve better productivities (Cesário et al., 2018). A steady decrease in biomass and PHA content was observed when the concentration of macroalgal hydrolysate was increased. This might be due to the substrate inhibition which led to a decrease in product formation. Further optimizations of cultivation conditions (pH, temperature, salinity, inoculum strength, and C / N ratio) are required for enhancement of PHA production using macroalgal hydrolysate as substrate.

3.4. Characterization of PHA produced

3.4.1. FTIR spectroscopy

The extracted polymers were analysed by the FTIR spectrophotometer with ATR. The major absorption peaks of *Haloferax* PHA on FTIR spectrum are given in Table 1. FTIR spectra of the polymer showed absorption peak near 3289 cm^{-1} which may be attributed to the hydroxyl (O–H) groups stretching. The most important characteristics band for PHAs was observed near 1720–1740 cm^{-1} region which corresponds to the ester carbonyl bond (C = O). Relevant peaks were observed at 2924 cm^{-1} and 2871 cm^{-1}. These bands were assigned to stretching in CH_3 and CH_2 groups respectively. The bands for CH_3 bending, CH_2 wagging, C-O, C–C and C-O-C stretching were observed in the range of 1450–1000 cm^{-1}. The presence of these bands suggests the P(3HB-co-3HV) nature of the sample. The similar observations have also been described in previous literature reports for P(3HB-co-3HV) production (Alsafadi and Al-Mashaqbeh, 2017; Gahlawat and Soni, 2017).

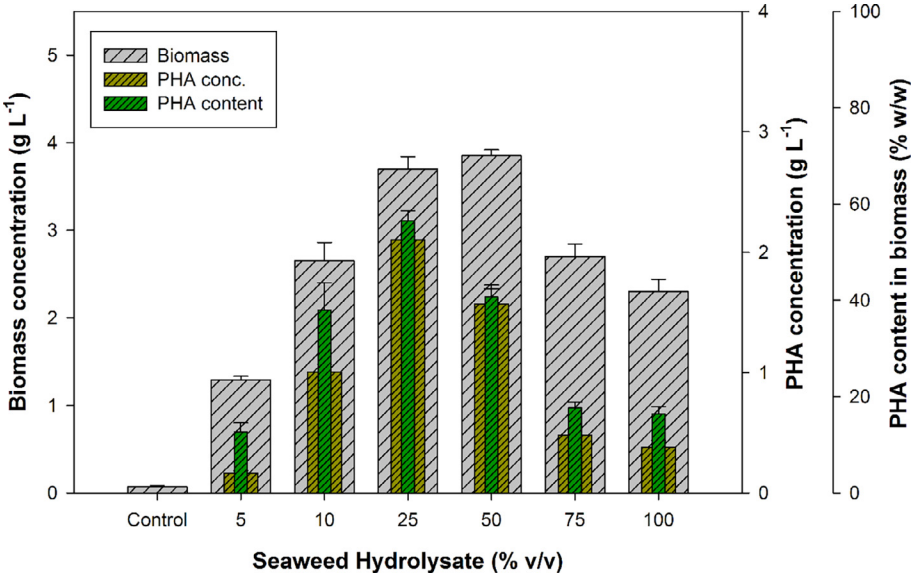

Fig. 4. Biomass concentrations, PHA concentrations and PHA content in biomass for the growth of *Haloferax mediterranei* in different concentrations of *Ulva* sp. hydrolysate.

Table 1
Major absorption peaks of *Haloferax* PHA on ATR-FTIR spectrum.

cm^{-1}	Type of vibration in the functional group
3289	O-H stretching
2924	CH_3 stretching
2871	CH_2 stretching
1724	(C = O) stretching
1181, 976	C-O-C Vibration
1056	C-O-C, C-C stretching and C-O-H bending
861	C-C-H, C-O-C bending

Table 2
Chemical shift values of various H and C atoms in P(3HB-*co*-3HV) co-polymer.

	Chemical shift δ (ppm)								
	B1	B2	B3	B4	V1	V2	V3	V4	V5
^{1}H NMR	–	2.58	5.25	1.26	–	2.48	5.25	1.57	0.83
^{13}C NMR	169.3	40.9	67.7	19.8	169.3	41.1	77.3	19.8	9.3

B1, carbonyl (C = O); B2, methylene (CH_2); B3, methane (CH); B4, methyl (CH_3) in the P(3HB) unit; V1, carbonyl (C = O); V2, methylene (CH_2); V3, methane (CH); V4, methylene (CH_2); V5, methyl (CH_3) in the P(3HV) unit.

3.4.2. TGA/DSC thermal analyses

TGA thermogram analysis provided the thermal characteristics of the P (3HB-co-3HV) produced by *Haloferax mediterranei*. The decomposition temperature minima (T_d) of the P (3HB-co-3HV) from *Haloferax mediterranei* was found to be 241 °C. A weight loss of ~40% was observed in the TGA thermogram. DSC analysis showed the melting temperature of the polymer. The melting temperature was estimated to be 150 °C. Similar studies have also been reported in previous literature (Bhattacharyya et al., 2012; Sawant et al., 2017).

3.5. 3 ^{1}H and ^{13}C NMR spectrometry

The chemical structure of our polymer was further analysed by ^{1}H NMR and ^{13}C NMR spectrometry. The assignments of various peaks are listed in Table 2. The molar composition of the repeating unit in P (3HB-*co*-3HV) was determined by the area ratio of methyl resonance peaks from 3HV unit (δ = 0.83 ppm) and 3HB unit (δ = 1.26 ppm) in the ^{1}H NMR spectrum, i.e., V5/ (B4 + V5), since they were well separated. Similar observations have also been described in previous literature reports for P(3HB-co-3HV) production (Don et al., 2006).

A comparison regarding various biomass and PHA yields reported in the literature using various organic substrates was done. In the present study, a yield of 58.1 ± 0.8% (w/w) PHA has been found to be comparable with the earlier studies done with *Haloferax mediterranei* using the hydrolysate of *Sargassum* sp. as substrate. The volumetric PHA productivity of 0.035 ± 0.002 $g{\cdot}L^{-1}{\cdot}h^{-1}$ was comparable to other studies, but conditions for fermentation are to be optimized to increase the yield and productivity of PHA production. The highest PHA production was found by culturing *C. necator* on Brown seaweed hydrolysate. But there are several advantages of *Haloferax* cultivation system. *Haloferax mediterranei* naturally produces PHBV, unlike *C. necator*, which produces PHB. Mechanically, PHB polymer is more brittle than PHBV polymer, which limits PHB's use for various purposes. Also, *H. mediterranei* is known to utilize a wide variety of inexpensive substrates, ranging from starch to other complex sugars. So, these facts could be two additional advantages of our culturing system of *H. mediterranei* over *C. necator*. Studies on levels of salinity and nitrogen source might help in increase of the PHA and biomass content. The studies suggested that *H. mediterranei* could produce PHA using seaweed hydrolysate.

4. Conclusions

PHA is an emerging biopolymer that can be used for multiple applications. Yet, a sustainable carbon source for microorganisms that accumulate PHA is not yet found. The present study evaluated the carbohydrate composition of 7 seaweeds to provide a carbon source for PHA produced by *Haloferax mediterranei*. Green macroalgae *Ulva sp*. had the best composition from the tested combinations for the maximum yields of PHA. The demonstrated processes led to PHA production without use of fresh water, suggesting the possibility for sustainable process scale up. This work is an additional step for the development of halophyte biotechnology, bioprocessing and biorefinery.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.09.108.

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