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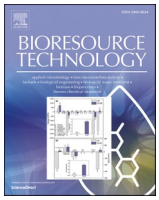

Marine bacteria associated with the green seaweed *Ulva sp*. for the production of polyhydroxyalkanoates

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HIGHLIGHTS

- Exploring Polyhydroxyalkanoats production by green seaweed associative bacteria.
- *Ulva* sp. hydrolysates as a substrate for PHA production by microbial isolates.
- New *Cobetia* isolates afforded high PHA production, 61% (w/w)

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ABSTRACT

This work aimed to isolate a series of bacterial strains associated with the green seaweed *Ulva* sp. and evaluate their capability to manufacture PHA. The effect of the type of supplemented sugars found to be in macroalgae, on the growth and PHA productivity of the strains was studied. Analysis of the 16S rRNA gene sequence of the isolated strains revealed that the PHA-producing bacteria were phylogenetically related to the genus *Cobetia*, *Bacillus*, *Pseudoaltermonas* and *Sulfitobacter,* which showed high PHA contents among the isolates. The highest PHA content was observed in the case of *Cobetia* strain, with up to 61% w/w in the presence of mannitol and 12% w/w on *Ulva* sp. acid hydrolysate as a substrate.

1. Introduction

Limited petroleum resources and their hazardous impact on the environment have led to an increase in biopolymers development from renewable resources (Li et al., 2016). Among these polymers are polyhydroxyalkanoats (PHAs) (Grigore et al., 2019).

PHAs are prospective substitutes for petrochemical-derived polymers due to their biodegradability, sustainability, and versatile thermal and mechanical properties. PHAs are intracellular microbial aliphatic polyesters synthesized by numerous organisms as carbon and energy storage in intercellular granules (Grigore et al., 2019). The PHAs are usually produced to respond to environmental stresses such as nutrient limitation (Kasan et al., 2015).

Around 150 various chemical structures of PHA were reported. Poly (3-hydroxybutyrate) (P(3HB)) gained more recognition due to its unique physio-mechanical properties. Therefore, it offers great potential for various industrial applications in agriculture, food packaging and biomedical fields (Mostafa et al., 2020). The development of desirable PHA polymers from a widespread microbial resource for industrial purposes is investigated (Kourmentza et al., 2017). Recently, marine microbial strains such as *Alteromonas, Bacillus, Pseudomonas* spp., *Cupriavidus* spp. (Możejko-Ciesielska and Kiewisz, 2016) have gained a lot of attention. They can produce superior PHA polymers because of the challenging marine conditions they live in, i.e. high salinity, low temperature and low organic matter (Gunny et al., 2014). Due to their exclusive metabolic and physiological capabilities, high salt tolerance and enzymatic stability, marine halophilic bacteria can help develop low-cost biotechnological processes to produce valuable products

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(Blandón et al., 2020).

Although many bacteria and archaea species have been identified to produce PHA, the potential to discover and identify novel marine species isolated from green macroalgae with vastly superior production capacity remains untapped. Besides, optimization of bacteria growth and PHA accumulation using various carbon sources presents an essential component for commercialising these biopolymers (Sangkharak and Prasertsan, 2012).

Marine macroalgae or seaweeds, especially *Ulva* sp. are among the most attractive biomass for exploring PHA production by their associated bacteria due to macroalgae abundance in many ecosystems on earth (Wei et al., 2013). This type of seaweeds offers many environmental and biotechnological benefits comparing to terrestrial crops. For example, they are easily accumulated in various areas around the world; they don't require harsh agronomical treatments and have high growth rates and high polysaccharide content making them a stellar for large-scale production (Gajaria et al., 2017).

Numerous studies have described the biosynthesis of a wide range of valuable materials such as biogas, butanol, and ethanol by fermentation of seaweed (Ashokkumar et al., 2017). However, only recently, seaweeds have been explored as a potential substrate for PHA production. Very recent studies have shown that bacteria accumulated PHA in a medium containing brown algae (Moriya et al., 2020), red algae (Sawant et al., 2018), and green seaweed *Ulva* sp. (Ghosh et al., 2019). Golberg's research group has demonstrated that *Ulva* sp. hydrolysate is a promising feedstock for PHA production using Haloferax *mediterranei* (Ghosh et al., 2019).

In the present study, more than one hundred strains of bacteria, isolated from green macroalgae *Ulva* sp., were evaluated for their capability to manufacture PHA using various supplemented fermentative substrates found to be in macroalgae, e.g., glucose, fructose, galactose, mannitol, mannose, arabinose, rhamnose, glucuronic acid, and xylose. A total of thirty-one bacteria were found to produce PHA. Ten strains related to genus *Cobetia, Bacillus, Pseudoaltermonas,* and *Sulfitobacter*, showed high PHA contents among the isolates, were further investigated. The effect of the type of supplemented sugars on the growth and PHA productivity of these strains were studied.

Furthermore, the effect of bacteria co-culture and mixed fermentative substrates on the production of PHA was investigated. Also, 16S rRNA sequence identification of several isolated bacteria was performed. Finally, the ability of *Cobetia* 105 to produce PHA on acid-hydrolyzed *Ulva* sp. as a carbon source was demonstrated. This study could contribute to the understanding of PHA production by diverse bacterial strains associated with marine macroalgae.

2. Materials and methods

2.1. Chemicals, instruments and media

The medium for bacterial cultivation on plates was prepared as follow: Agar powder (2% w/v) (Difco, USA) was dissolved in a medium with Marine Broth (MB) (Beit Dekel, Israel) containing (per L) 19.4 g NaCl, 3.24 g $MgSO_4{\cdot}7H_2O$, 5.0 g Peptone, 8.8 g $MgCl_2{\cdot}6H_2O$, 1.8 g $CaCl_2$, 1 g yeast extract, 0.55 g KCl and 0.16 g $NaHCO_3$ (pH 7.6). The supplemented sugars (glucose, fructose, galactose, mannitol, mannose, arabinose, rhamnose, glucuronic acid, and xylose) were purchased from Sigma-Aldrich (Israel) and used without further purification. Nile Blue A (Sigma-Aldrich, Israel) for staining of PHA was used to screen isolated bacteria. The sugar's solutions were filtered through a 0.22 μm pore membrane microfilter (CSI, Israel). Bateria in liquid cultures was grown in aerobic flask bottles (175 mL) in a shaking incubator.

2.2. Growth of the green macroalgae Ulva sp.

The growth of *Ulva* sp. was carried out by adding 20 g of fresh *Ulva* sp. in 40 mL cylindrical, sleeve-like seaweed photobioreactor (MPBR, Polytiv, Israel) (Chemodanov et al., 2017) in a seawater medium containing 3.7% w/v of dried Red Sea salt (Red Sea Inc, Israel), ammonium nitrate (NH_4NO_3, Haifa Chemicals Ltd, Israel) and phosphoric acid (H_3PO_4, Haifa Chemicals Ltd, Israel). The final concentration of nitrogen (N) and phosphorus (P) in the medium were 6.4 g m^{-3} and 0.97 g m^{-3}, respectively. The pH, temperature and flow rate were controlled as stated earlier (Chemodanov et al., 2017).

2.3. Acid hydrolysis of the green macroalgae Ulva sp.

A fresh *Ulva* sp. (500 g) was dried at a temperature of 40 °C for three days. Subsequently, the dried biomass was crushed with an electric grinder (Grinding machine, Henan Gelgoog Machinery GG9FZ-19) to obtain fine powdered *Ulva* sp. The acid hydrolysis was performed as described previously (Vitkin et al., 2020). Briefly, 45 g of dry powdered *Ulva* sp. were added to 500 mL of sulfuric acid solution (2% v/v). The mixture was autoclaved at 121 °C for 30 min. The cooled mixture's pH was adjusted to 6.7 by adding 117 mL of 3 M NaOH solution and 80.6 mL of PBS buffer (Phosphate Buffer Saline). Subsequently, 12.2 g of MB powder were dissolved in the resulting mixture to supplement minerals and nitrogen sources. The *Ulva* sp. acid hydrolysate was filtered with 0.22 μm syringe-filter (Millipore, USA) and the filtrate solution was used immediately as a cultivation medium.

2.4. Analysis of Ulva sp. Acid hydrolysate by ion chromatography

The chemical composition of the *Ulva* sp. acid hydrolysate solution was determined using high-pressure ion chromatography (HPIC) via Dionex ICS-5000 (Dionex, Thermo Fischer Scientific, MA, USA). The acid hydrolysate solution was diluted in ultrapure water to reach a ratio of 1:2. The sample was then filtered with a 0.22 μm syringe filter (Millipore, USA) and added to HPIC vials (Thermo Fischer Scientific, USA). The phase flow rate was 0.25 mL/min, and the column temperature was set to 30 °C. The standards used as a reference to identify and quantify the resulted monosaccharides were fructose, xylose, glucose, galactose, rhamnose and glucuronic acid (Sigma-Aldrich, Saint-Louis, USA).

2.5. Isolation of bacterial strains

Ulva sp., a green seaweed collected from the Mediterranean Sea, was used as a source for isolation PHA-producing bacteria. The isolation of bacteria such as *Sulfitobacter*, *Bacillus, Altermonas*, *Vibrio, Microbacteria*, *Pseudoaltermononas* and *Cobetia* was carried out by three to six isolation rounds, until achieving a homogenous single colony. The colonies were detected using a binocular microscope. The first round of bacterial isolation was done by smearing a fresh algae thalli on agar plates with a concentration of 0.7% w/v, 1% w/v and 1.5% w/v containing five different mediums. The five medium contents were: (1) natural Mediterranean Seawater (SW); (2) live *Ulva* sp. (5 g of fresh *Ulva* sp.) with double-distilled water (DDW); (3) 1.5% w/v of *Ulva* sp. dry weight (UDW) with DDW, The *Ulva* sp. was dried at 40 °C and was ground with mortar and pestle; (4) MB, 3.7 g L^{-1} in DDW; (5) DDW without any carbon source. The subsequent isolation rounds were done by streak-plating bacteria cultures up to isolate a single colony. All isolation rounds were done on MB plates (1.5% w/v of agar, 1.75% w/v of MB). Finally, 110 isolated bacteria colonies were transferred to 2 mL liquid MB (3.7 g L^{-1}) and kept overnight at 32 °C in a shaker incubator (180 RPM, Incu-Shaker Mini, Benchmark Scientific). The bacteria isolates were stored in glycerol (final concentration of 25% v/v) at −80 °C.

2.6. Screening of bacteria utilizing different sugars for PHA production

All of 110 bacterial isolates from the genus of *Sulfitobacter*, *Bacillus, Altermonas*, *Vibrio, Microbacteria*, *Pseudoaltermononas* and *Cobetia* were tested for PHA production using Nile Blue A staining method. The bacteria isolates were cultivated on agar plates containing MB and the

selected sugar (2% w/v) and incubated for four days at 32 °C. Nile Blue A (0.5 μg/mL) was directly added to a rich MB agar medium; thus, the bacterial cells were grown in the presence of Nile Blue A. Subsequently, the bacteria were exposed to UV illumination (320 nm) using the Enduro™ Gel Documentation System (Labnet International, Inc. Israel). This technique allowed rapid screening of the viable colonies for PHA production and considered a powerful tool for distinguishing between PHA-negative and PHA-positive strains. The bacteria that have shown a bright white fluorescence on irradiation with UV light were selected as potential PHA accumulators. The selected bacteria were repeatedly grown on different sugars in MB plates, and the accumulation of PHA on each sugar was also examined by Nile Blue A staining. All experiments were carried out in triplicates.

2.7. Molecular identification of the isolates

PHA-positive bacteria from the genus *Sulfitobacter*, *Bacillus, Pseudoaltermononas* and *Cobetia* were genomically identified to the genus level using 16S rRNA sequencing profiling. For strain identification, genomic DNA extraction was performed, a colony of each bacterial strain was transferred into a 2 mL sterile Eppendorf tube containing distilled water (100 μL). The samples were then centrifuged for 3 min at 10,000 g and heated at 100 °C for 10 min in an Eppendorf Thermomixer C (Thermo Fisher Scientific, USA) to lyse the bacterial cells. The supernatant of the sample, which contains the DNA fragments, was kept, and the cell pellet was discarded. The microbial DNA was purified using the ExoSAP DNA Clean-Up Kit (Sigma-Aldrich, Israel) using 5 μL aliquot of the supernatant. The 16S rRNA was amplified by PCR using standard protocols (Wang et al., 2011) based on the primers data shown in Table 1. The PCR product was purified by ExoSAP clean up kit. Sequencing of 16S rRNA was carried out by TAU (Tel Aviv University) genomic unit, and a homology search of the databases was performed using the BLAST. A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm using Mega 5. The resulting tree topologies were evaluated by bootstrap analysis of neighbor-joining data sets based on 100 resamplings.

2.8. Cultivating of PHA-positive isolates in liquid media with different sugars

Starters of the selected PHA-positive bacteria from the genus *Sulfitobacter*, *Bacillus, Pseudoaltermononas* and *Cobetia* were prepared by adding one bacterial colony into MB medium. The starters were incubated at 37 °C for 18 h then were poured into a single sterile bottle. 1.75% w/v of MB media (900 mL) was prepared and autoclaved. The selected carbon source (2% w/v) was dissolved in the MB medium and adjusted to afford pH 7. For each treatment, a sterile glass bottle containing 135 mL of MB media was prepared. Subsequently, 15 mL of bacterial suspension were added from the bacteria inoculum to the medium (total volume of 150 mL). The bottles' content was then appropriately mixed, and the 150 mL solutions were divided to afford three portions of 50 mL solutions. The cultures were grown under aerobic conditions in a shaking incubator at 32 °C with a rotational speed of 90 rpm for four days. The bacterial growth was examined by measuring OD 600. The resulting biomass was collected by centrifugation at 4500 g for 30 min in a swing rotor centrifuge (Rotanta 420R, Hettich Instruments LP, USA), rinsed twice with a saline solution followed by 15 min centrifugation, dried in an oven at 45 °C for 24 h until a constant weight was obtained. The dry cell weight (DCW) of the bacteria in g L^{-1} and %DCW per fermentation volume was calculated. The PHA was extracted by treating the dried biomass (~200 mg) with hot (60 °C) chloroform (10 mL) with magnetic stirring overnight. The suspension was filtered through a filter paper, and the chloroform filtrate solution was evaporated to dryness under vacuum to afford a white polymeric film. The PHA was analyzed by GC–MS, as described in section 2.11. All experiments were carried out in triplicates.

2.9. PHA production by bacterial co-cultures on sugar mixtures

The best PHA-producing bacteria were chosen to study the effect of bacterial co-cultures and sugar mixtures on PHA production. Starters of the selected PHA-positive bacteria were prepared by adding one bacterial colony into MB medium and were incubated at 37 °C for 18 h following the previously mentioned procedure (section 2.8.). The bacteria starters were poured equally (5 mL each bacteria) into a sterile bottle. MB media was prepared and autoclaved. The selected carbon

Table 1
PCR primers and conditions used for bacteria identification.

Organism Name	Primer Type	Sequence	Start	Length	Tm	GC %	Amplicon
Sulfitobacter	Forward-1	TAATACCGCATACGCCCTTC	120	20	54.6	50.0	880
	Reverse-1	ATCACGGGCAGTTTCCTTAG	1000	20	54.8	50.0	
	Forward-2	AACGCGCAGAACCTTACC	887	18	55.7	55.6	253
	Reverse-2	ATTGTAGCACGTGTGTAGCC	1140	20	55.2	50.0	
	Forward-3	AGGAAACTGCCCGTGATAAG	1060	20	54.8	50.0	960
	Reverse-3	GGCTACCTTGTTACGACTTCA	1400	21	54.4	47.6	
Pseudoaltermonas	Forward-1	GTCATGAATCACTCCGTGGTAA	30	22	54.6	45.5	808
	Reverse-1	GAGTGTGATAGAGGGTGGTAGA	838	22	55.0	50.0	
	Forward-2	CTCTGTATGTCAAGTGTAGGTAAGG	500	25	54.4	44.0	770
	Reverse-2	ATTGGCCCAAGTGGGATTAG	1270	20	55.0	50.0	
	Forward-3	GTACGCTTTACGCCCAGTAAT	930	21	55.0	47.6	520
	Reverse-3	GTCGAGCGGTAACAGAAAGTAG	1450	22	55.1	50.0	
Cobetia	Forward-1	AACTCAGGCTAATACCGCATAC	150	22	54.5	45.5	530
	Reverse-1	CTGGTATTCCTCCCGATCTCTA	700	22	54.9	50.0	
	Forward-2	GGAAGAACGCTTCGGGATTA	398	20	54.7	50.0	702
	Reverse-2	CTCCTTAGAGTTCCCGACATTAC	1100	23	54.5	47.8	
	Forward-3	CGGAATTACTGGGCGTAAAG	495	20	53.5	50.0	925
	Reverse-3	CCCTAGGGCTACCTTGTT	1420	18	53.6	55.6	
Alteromonas	Forward	TCAACCTGGGATGGTCATTTAG	589	22	62.0	45.5	765
	Reverse	GGAACGTATTCACCGCAGTAT	1353	21	62.0	47.6	
Bacillus	Forward-1	ATCCTGGCTCAGGACGAA	16	22	55.4	50.0	706
	Reverse-1	CCTCCACATCTCTACGCATTTC	722	18	55.8	55.6	
	Forward-2	TCGGATCGTAAAGCTCTGTTG	427	21	54.7	47.6	811
	Reverse-2	GTGTGTAGCCCAGGTCATAAG	1238	21	55.1	52.4	
	Forward-3	GGGAGCGAACAGGATTAGATAC	781	22	54.7	50.0	739
	Reverse-3	CGGCTACCTTGTTACGACTT	1520	20	54.6	50,0	

source was added to the media (2% w/v for each sugar type) and adjusted to afford pH 7. For each treatment, a sterile glass bottle containing 135 mL of MB media was prepared. Subsequently, 15 mL of bacteria were added from the bacteria inoculum to the MB media to yield 150 mL solutions. The 150 mL solutions were mixed and divided equally into three 50 mL solutions. The cultures were grown under aerobic conditions in a shaker (90 rpm) at 32 °C for four days. The bacterial growth was examined by measuring OD 600. The resulting biomass was collected by centrifugation at 4500 g for 30 min in a swing rotor centrifuge (Rotanta 420R, Hettich Instruments LP, USA), rinsed twice with a saline solution followed by 15 min centrifugation, dried in an oven at 45 °C for 24 h until a constant weight was obtained. The DCW of the bacteria in g L^{-1} and %DCW per fermentation volume were calculated. PHAs were extracted and analyzed using GC–MS and ^{1}H NMR. All experiments were carried out in triplicates.

2.10. PHA production by Cobetia 105 on Ulva sp. acid hydrolysate

Starters of Cobetia isolate no. 105 were prepared by adding one bacterial colony into MB medium and were incubated at 37 °C for 18 h following the procedure mentioned above. The bacteria starters were poured into a single sterile bottle. The selected carbon source was added to the *Ulva* sp. hydrolysate media (2% w/v). A sterilized glass bottle containing 135 mL of hydrolysate media was prepared. Subsequently, 15 mL of bacteria were added from the bacteria inoculum to the media to yield 150 mL solutions. The solutions were then appropriately mixed and were divided equally into three 50 mL solutions. The cultures were grown under aerobic conditions in a shaker (90 rpm) at 32 °C for four days. The bacterial growth was examined by measuring OD 600. The resulting biomass was collected by centrifugation at 4500 g for 30 min in a swing rotor centrifuge (Rotanta 420R, Hettich Instruments LP, USA), rinsed twice with a saline solution followed by 15 min centrifugation at 4500 g, dried in an oven at 45 °C for 24 h until a constant weight was obtained. The DCW of the bacteria in g L^{-1} and %DCW per fermentation volume was calculated. PHAs were extracted and analyzed using GC–MS and ^{1}H NMR. All experiments were carried out in triplicates.

2.11. Characterization and quantification of PHA by GC–MS

PHAs were analyzed after direct acid-catalyzed *trans*-esterification with methanol of the dried bacteria (DB). The tested samples of DB (10–30 mg) were added to a mixture of chloroform (1.0 mL), benzoic acid (1.0 mg, an internal standard, BA), methanol (2.0 mL) and concentrated H_2SO_4 (0.5 mL). The suspension was heated at 90 °C with magnetic stirring for overnight in a closed vial. The reaction mixture was cooled to room temperature and treated with a cooled saturated NaCl solution (15 mL) and chloroform (10 mL). Anisole (1.0 mg, an internal standard, AN) and 2,4-dimethylanisole (1.0 mg, an internal standard, DMA) were added to the mixture. The organic phase was washed twice with water, separated, dried over anhydrous sodium sulfate and concentrated under vacuum to obtain 1 mL solution. GC–MS was used to analyze the PHA methanolysis products and their chemical composition. GC–MS analysis was performed using a Thermo Trace 1310 GC, equipped with a TG-SQC GC capillary column (15 m, 0.25 mm i.d., 0.25 μm film thickness) and a mass spectrometer ISQ LT as the detector. The carrier gas was helium (He) at a flow rate of 1.2 mL/min. The column temperature was initially 50 °C for 1 min, then gradually increased to 200 °C at 10 °C/min, and finally rose to 285 °C at 20 °C/min. For GC–MS detection, an electron ionization system was used with ionization energy of 70 eV. The samples were diluted 1:1000 (v/v) with ultra-pure hexane, and 1.0 μL of the diluted samples (8 ng/1 μL) was injected automatically in split mode. Injector and detector temperatures were set at 250 °C. All experiments were carried out in triplicates.

2.12. ^{1}H NMR analysis

All samples were dissolved in deuterated $CDCl_3$ with heating at 50 °C prior analysis (5 mg mL^{-1}) and shaken vigorously until complete dissolution was achieved. About 0.5 mL of each sample was transferred into an NMR tube and analyzed by ^{1}H NMR with Pulse Program zg30 on Bruker AVANCE III 500 MHz NMR Spectrometer with 5 mm PABBO-BB probe and Topspin 3.0 software.

2.13. Statistical analysis

The results were statistically analyzed using Excel and GraphPad prism 8 for data management and quantitative analysis. One-way and two-way ANOVA using Tukey and Holm-Sidak's multiple comparison tests were performed for analyzing standard deviation, means and statistical significance for PHA concentrations, PHA content and bacteria DCW.

3. Results and discussion

3.1. PHA accumulation by Ulva sp. associated bacterial strains utilizing different sugars

A total of 110 bacteria strains from the green seaweed *Ulva* sp. were successfully isolated and tested for their ability to accumulate PHA on MB media (1.75% w/v) with various sugars (2% w/v) by using Nile Blue A staining method. All PHA-positive strains exhibited a white fluorescent emission on agar plates containing MB and different sugars under UV light. For example, *Cobetia* isolate no. 104 produced PHA in mannitol, fructose, galactose, and glucose, while no PHA was detected with *Cobetia* isolate no. 104 in the presence of arabinose, glucuronic acid, mannose, xylose and rhamnose. It is important to emphasize that all tested bacteria did not produce PHA when grown on agar with 1.75% w/v MB alone as a control. Based on fluorescence staining, the total number of bacterial isolates that accumulate PHA on particular sugar was determined. For example, 28 bacteria were found to accumulate PHA to a different extent in the presence of glucose, fructose, mannitol, or galactose; 27 different strains on glucose, 24 strains on fructose; and 17 strains on mannitol or galactose (Table 2). It was reported by Blandón et al. (2020) that most PHA producers usually afford the highest concentration of PHA when glucose is used as a substrate (Blandón et al., 2020). Adwitiya et al. (2009) and Gomez et al. (1996) described comparable results using *R. sphaeroides* N20 (Adwitiya et al., 2009) and *Alcaligenes latus* (Gomez et al., 1996), respectively. These results were later explained by Elsayed et al. (2013) that glucose is an easily assimilable carbon source which encourages bacteria to produce more P3HB (Elsayed et al., 2013).

3.2. Identification of PHA-producing bacteria using 16S rRNA gene and phylogenetic analysis

Molecular identification of the PHA-positive isolates was carried out by the sequencing of the 16S rRNA gene. Amplification of bacterial genomic DNA by primers yielded 1400–1500 bp fragments (Fig. 1). The bacteria were found to be within the genus of *Cobetia*, *Bacillus, Sulfitobacter* and *Pseudoaltermonas.* The phylogenetic relationship among the *Cobetia* isolates is provided in Fig. 2. 16S rRNA similarity was found between *Cobetia* no. 65, *Cobetia* no. 92, and *Cobetia* no. 104, and between *Cobetia* no. 105, *Cobetia* no. 75, *Cobetia* no. 76, and *Cobetia* no. 107. All *Cobetia* isolates were found to closely relate to *Cobetia amphilecti*, *Cobetia pacifica* and *Cobetia litoralis*. Besides, Cobetia isolates were found to have a strong evolutionary relationship with *Halomonas,* as was suggested by Arahal et al. (Arahal et al., 2002), it was classified originally as *Arthrobacter marinus* (Cobet et al., 1971), then *Deleya marina* (Baumann et al., 1983) and *Halomonas marina* (Dobson and Franzmann, 1996). The genus *Cobetia* contains mainly two well-known strains,

Table 2
List of bacterial isolates which showed a white light fluorescence under UV light when grown on different sugars. The white fluorescence indicates the accumulating of PHA.

Bacteria's no.	Bacteria genus	Sugar type								
		Gal	Mat	Fru	Ara	Mas	Glu	Rha	GA	Xyl
49	*Sulfitobacter*	+		+			+			+
3	*Bacillus* sp.	+	+	+			+			
25, 26, 27	*Uncultured Altermonas*		+	+			+	+		+
28	*Altermonas*		+	+			+	+		+
52, 56	*Unclassified vibrio*		+	+			+			
41	*Vibrio* sp.		+	+			+			
6, 37	*Sulfitobacter* sp.						+			+
68, 80, 81, 85	*Unclassified Microbacteria*	+		+	+	+	+			+
86	*Unclassified Microbacteria*	+		+	+	+	+	+		+
75–76, 92, 104–105, 107	*Cobetia*	+	+	+			+			
13	*Pseudoaltermononas*	+	+	+		+	+			
71	*Pseudoaltermononas*	+		+	+	+	+	+		
14	*Pseudoaltermononas*	+	+	+			+			
65	*Cobetia*	+	+	+			+			
82	*Sulfitobacter* sp.						+			
48	*Sulfitobacter* sp.									+

+ PHA-positive. Gal-galactose; Mat-mannitol; Fru-fructose; Ara-arabinose; Mas-mannose; Glu-glucose; Rha-rhamnose; GA-glucuronic Acid; Xyl-xylose.

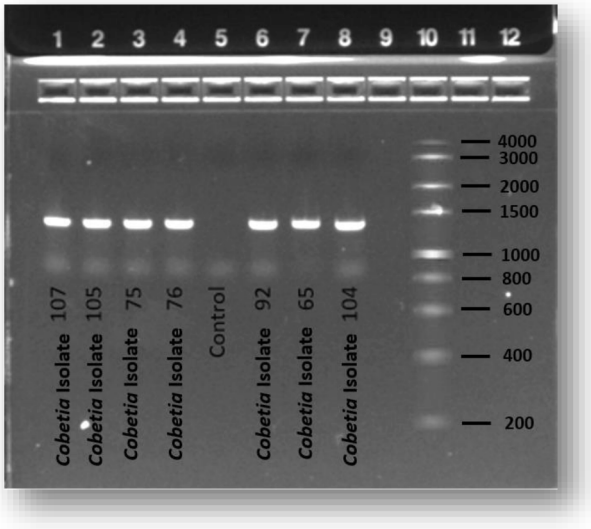

Fig. 1. Agarose gel electrophoresis represents the amplicon of 16S rRNA gene of strains isolated from seaweeds associated bacteria.

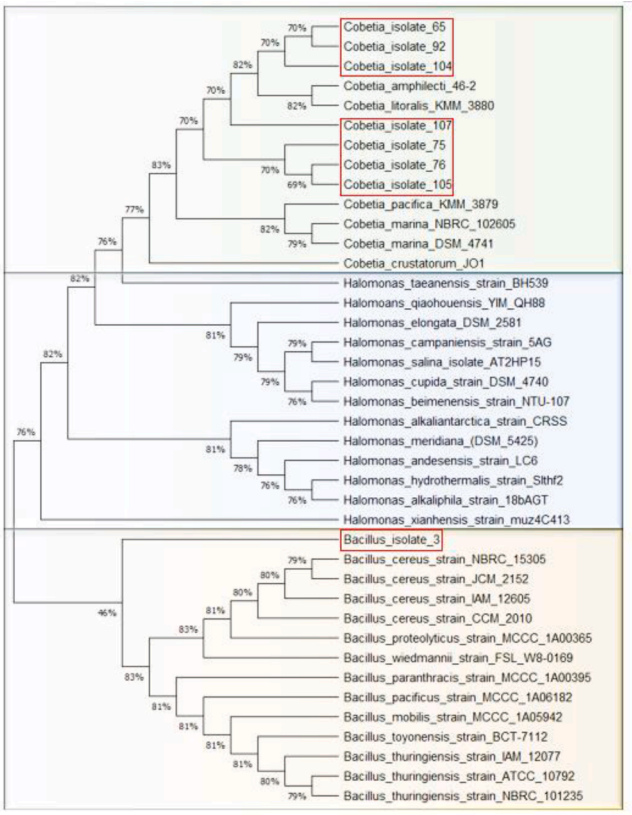

Fig. 2. Phylogenetic relationships among the isolates drawn by MEGA 5 with 100 resamplings. The bacteria in bold were isolated in the current study.

Cobetia marina (Arahal et al., 2002), and *Cobetia crustatorum* (Kim et al., 2010). Several studies were conducted on microbial species genetically related to the *Halomonas* or *Cobetia* genus as PHA-producers such as *Cobetia marina*, *Halomonas salina*, *Halomonas elongate DSM 2581* (Mothes et al., 2008), *Halomonas boliviensis LC1* (Quillaguamán et al., 2005) and *Halomonas* sp. *TD01* (Tao et al., 2017).

Bacillus isolate no. 3, was found to have a close genomic relationship with 98% identity to *Bacillus cereus*, *Bacillus mobilis*, *Bacillus pacificus* and *Bacillus thuringiensis*. Two or more distinct Bacillus species may possess identical 16S rRNA sequences (Ash et al., 1991). Many studies have reported Bacillus's use for cost-effective production of P3HB (Israni and Shivakumar, 2013). *Bacillus* is predominant, and it has a very high growth proficiency even at low-cost raw materials compared to other bacteria, which make them desirable (Khiyami et al., 2011). Moreover, *Bacillus* produced various hydrolytic enzymes for P3HB using agro-industrial wastes (Israni and Shivakumar, 2013).

Additional taxonomic studies on the PHA-positive isolates showed that isolate no. 48 has a genomic relationship to *Sulfitobacter* sp. Mereuta et al. (2018) reported the isolation of *Sulfitobacter* sp. P5 from the black sea, which showed a potential high production of P3HB (Mereuta et al., 2018). Isolate no. 71 has a genomic relationship to *Pseudoaltermonas*. sp. Wang et al. (2010) reported for the first time on PHA accumulation by *Pseudoalteromonas*. They have demonstrated that *Pseudoalteromonas* sp. SM9913 could accumulate a novel kind of PHA with a composition of 3-hydroxydodecanoate (HDD) and 3-hydroxydecanoate (HD) (about 85 mol%) (Wang et al., 2010).

3.3. Chemical structure and amount of the produced P(3HB) by Cobetia, Bacillus, pseudoaltemonas and Sulfitobacter

Analysis of the produced methylated ester derivatives obtained by acid methanolysis of PHA showed mainly two large peaks corresponding to methyl-3-hydroxybutyrate (M3HB, R_t = 3.15 min), and methyl-3-methoxy-butanoate (M3MB, R_t = 3.97 min), with a small peak corresponding to levulinic acid (LA, R_t = 5.89 min) in addition to the three standards (anisole-ANS R_t = 4.67 min; methyl benzoate-MB R_t = 7.03 min and 2,4-dimethylanisole-DMA R_t = 7.49 min) as shown in a typical GC–MS chromatogram. M3HB and M3MB derivatives indicated that the PHA polymer composition is mainly P(3HB).

The DCW (g L^{-1}), P(3HB) content (%DCW w/w), and P(3HB) concentration (mg L^{-1}) values obtained with different *Ulva* sp. associated bacteria grown on various supplemented sugars are presented in Table 3. Cell growth of 1.14 g L^{-1} and 1.96 g L^{-1}, and P(3HB) content of 10.03% w/w and 13.97% w/w were obtained when *Bacillus* was grown in a medium containing fructose and glucose, respectively.

Sulfitobacter produced 7.73% w/w of P(3HB) and 2.54 g L^{-1} of DCW when it was grown in medium containing mannitol, Mereuta et al. (2018) reported similar results on the production of P(3HB) by *Sulfitobacter* genus, which was isolated for the first time from the black sea (Mereuta et al., 2018).

A DCW of 6.63 g L^{-1} and 1.06 g L^{-1} and a P(3HB) production of 17.11% w/w and 11.83% w/w were obtained with *Cobetia* isolate no. 65 grown in medium containing mannitol and galactose, respectively. *Pseudoaltermonas* produced 7.46% w/w of P(3HB) with DCW of 2.54 g L^{-1} when grown in medium containing fructose, while no PHA was produced on other sugars under similar conditions. Wang et al. (2010) have evaluated P(3HB) content obtained by *Pseudoaltermonas* sp. SM9913 when it was grown on glucose, decanoate and olive oil as a carbon source, and achieved P(3HB) accumulation of 3.10% w/w, 1.89% w/w, and 2.57% w/w of the DCW, respectively (Wang et al., 2010). The highest DCW of *Cobetia* isolate no. 75 was obtained when it was grown in a medium containing mannitol or glucose (4.72 g L^{-1} and 3.72 g L^{-1}, respectively), and the highest P(3HB) production was achieved with mannitol (18.56% w/w) and glucose (20.91% w/w). *Cobetia* isolate no. 104 produced the highest amount of P(3HB) in fructose (23.39% w/w). *Cobetia* isolate no. 105 produced 61.00% w/w of P(3HB) in mannitol with 4.58 g L^{-1} of DCW. *Cobetia* isolate no. 107 had the highest P(3HB) amount when it was grown in fructose (27.45% w/w) with a DCW of 3.53 g L^{-1}. The highest P(3HB) amount using *Cobetia* isolate no. 92 was obtained in mannitol (8.91% w/w) with 4.5 g L^{-1} of DCW. All results were statistically significant ($p < 0.04$). There are very few reports on PHA production by *Cobetia*. Moriya et al. (2020) have reported the production of P3HB used alginate as a substrate for *Cobetia* strain 5–11-6–3, which afforded 62.1% w/w of P3HB with a content of 3.11 g L^{-1}. Blandón et al. (2020) have presented a comparative study for PHA production by *Bacillus* sp. INV FIR18 and *Cobetia* sp. INV PRT122 (Blandón et al., 2020).

Cobetia isolate no. 75, *Cobetia* isolate no. 92, *Cobetia* isolate no. 104 and *Cobetia* isolate no. 107 were produced P(3HB) mainly on galactose, mannitol, fructose, and glucose, while *Cobetia* isolate no. 65 on galactose and mannitol, *Cobetia* isolate no. 105 on mannitol and fructose and *Cobetia* isolate no. 76 on fructose, glucose, and mannose.

3.4. Effect of bacterial co-culture and sugar mixtures on the PHA production

The best PHA-producers, *Cobetia* isolate no. 107, Cobetia isolate no. 104, Cobetia isolate no. 92, Cobetia isolate no. 65, and Cobetia isolate no. 75, were selected to study the effect of bacterial co-culture on bacteria growth and PHA production. The selected sugars were glucose, fructose and mannitol, with a total concentration of 2% w/v. Fig. 3 presents the biomass amount, PHA content and concentration of *Cobetia* strains on a mixture of glucose, fructose and mannitol. The highest biomass, PHA concentration, and PHA content were obtained by *Cobetia* isolate no. 105 with 2.03 g·L^{-1}, 712 mg L^{-1} and 35.10% w/w respectively. The results were statistically significant ($p < 0.0001$). The results presented in Table 4 show that a mixed culture of different bacteria species afforded relatively low DCW and P(3HB) concentrations. For example, *Cobetia* isolate no. 107 and *Cobetia* isolate no. 104 independently produced 27.45% w/w and 23.29% w/w of P(3HB) in fructose, respectively. However, a mixed culture of these two bacteria in fructose afforded only 10.05% w/w of P(3HB). A similar result was observed when a bacterial co-culture of *Cobetia* isolate no. 65, *Cobetia* isolate no. 75, and *Cobetia* isolate no. 105 in mannitol was used. A content of 11.61% w/w of P(3HB) was obtained for the mixed bacteria compared to 17.11% w/w for *Cobetia* isolate no. 65, 18.56% w/w for *Cobetia* isolate no. 75, and 61.00% w/w for *Cobetia* isolate no. 105, independently. In all co-culture experiments, additional valuable fine chemicals were also exhibited in a low amount, such as hexane-2,5-dione and

Table 3
Microbial production of PHA from different supplemented sugars. A total of 10 bacteria were analyzed on different sugars for PHA production. The best bacteria with the highest PHA production were listed in the table below.

Organism Name	Sugar Type	DCW (g L^{-1})	PHA Concentration (mg L^{-1})	PHA Content (%DCW)	SD of PHA%	Monomer Composition (mol%), 3HB
Bacillus isolate no. 3	Fructose	1.14	114	10.03	0.76	100
	Glucose	1.96	221	13.97	1.13	100
Sulfitobacter Isolate no. 48	Mannitol	2.54	196	7.73	0.98	100
Cobetia isolate no. 65	Mannitol	6.36	125	17.11	1.41	100
	Galactose	1.06	573	11.83	1.45	100
Pseudoaltermonas isolate no. 71	Fructose	2.54	189	7.46	0.64	100
Cobetia isolate no. 75	Fructose	2.08	125	8.63	1.15	100
	Galactose	0.78	180	16.04	1.32	100
	Mannitol	4.72	762	18.56	0.88	100
	Glucose	3.72	876	20.91	0.82	100
Cobetia isolate no. 76	Mannose	3.68	151	1.89	0.41	100
Cobetia isolate no. 92	Fructose	3.26	206	4.45	0.92	100
	Mannitol	4.50	251	8.91	0.51	100
	Galactose	1.88	355	7.69	1.35	100
Cobetia isolate no. 104	Galactose	1.02	111	10.87	1.34	100
	Mannitol	1.02	116	11.34	0.75	100
	Glucose	0.82	131	16.01	1.12	100
	Fructose	4.44	718	23.29	0.57	100
Cobetia isolate no. 105	Mannitol	4.58	574	61.00	1.23	100
Cobetia isolate no. 107	Glucose	2.03	231	11.37	0.95	100
	Fructose	3.53	968	27.45	0.84	100

DCW represents “Dry Cell Weight”. SD represents “Standard Deviation”.

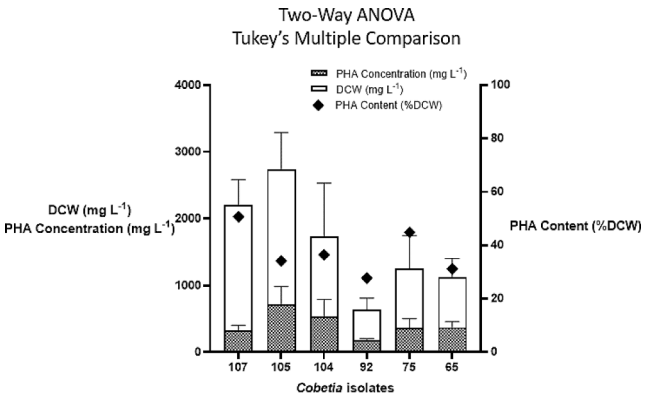

Fig. 3. P(3HB) content, P(3HB) concentration and DCW of six *Cobetia* strains (*Cobetia* isolate no. 107, *Cobetia* isolate no. 105, *Cobetia* isolate no. 104, *Cobetia* isolate no. 92, *Cobetia* isolate no. 75, and *Cobetia* isolate no. 65 grown on a mixture of carbon source, i.e. glucose, fructose and mannitol. Five replicates were obtained. Two-Way ANOVA, Tukey's multiple comparison test was performed.

Table 4
Microbial production of P(3HB) using mixed culture and mixed sugars. A Comparison between pure and mixed bacteria cultures from *Cobetia* and *Bacillus* genus are grown on single and mixture sugars substrates.

Bacteria/bacterial mixture	Sugar/sugar mixture	DCW (mg L^{-1})	P(3HB) in DCW (%)	SD of PHA%
Cob. 75, *Cob*. 107	Glucose	97	1.45	1.12
Cob. 104, *Cob*. 107	Fructose	98	10.05	0.87
Bac. 3, *Cob*. 75 *Cob*. 107	Glucose	62	2.27	0.91
Cob. 65, *Cob*. 75, *Cob*. 105	Mannitol	39	11.61	1.24
Cob. 65, *Cob*. 75, Cob. 92	Galactose	32	5.15	1.56
Cob. 92, *Cob*. 104. *Cob*. 107	Fructose	45	0.70	0.76

DCW represents "dry cell weight", and P(3HB) presents poly(3-hydroxybutyrate). *Cobetia* presented as *Cob*. and *Bacillus* presented as *Bac*.

levulinic acid.

The PHA productivity reported for mixed cultures was lower than pure cultures' productivity (Serafim et al., 2008). The maximum cell concentration reported for aerobic dynamic feeding (ADF) operated systems was 6.1 g L^{-1} (Dionisi et al., 2006), which is much lower than those obtained by pure cultures, usually above 80 g L^{-1} (Lee et al., 1999). The reason for this result could be the apparent difficulty in reaching high biomass concentrations in the mixed-culture process (Oehmen et al., 2014), probably due to bacterial competition on the carbon source. On the other hand, mixed culture systems were shown to produce large amounts of PHAs in a wide range of low-cost substrates (Shalin et al., 2014). Pakalapati et al. (2018) have reported in their review that mixed bacteria cultures are advantageous over pure bacteria cultures (Pakalapati et al., 2018). Ashby et al. (2005) reported high PHA content and maximum utilisation of glycerol when *Pseudomonas corrugate* and *Pseudomonas oleovorans* were used as mixed bacteria cultures (Ashby et al., 2005). Kourmentza et al. (2009) performed a comparative study and stated that co-cultures are more efficient than pure bacteria cultures for PHA production (Kourmentza et al., 2009).

3.5. PHA production by Cobetia isolate no. 105 on Ulva sp. Hydrolysate

A mixture of monosaccharides was obtained by acid hydrolysis of *Ulva* sp. which were quantified using HPIC. The hydrolysate composed of glucose (16.1±0.8 mg g^{-1} UDW), rhamnose (6.2±0.45 mg g^{-1} UDW), fructose (2.8±0.41 mg g^{-1} UDW), xylose (1.6±0.22 mg g^{-1} UDW), galactose (1.0±0.11 mg g^{-1} UDW) and glucuronic acid (1.3±0.11 mg g^{-1} UDW). PHA production by *Cobetia* no. 105 on *Ulva* sp. hydrolysate was investigated. The results showed a biomass concentration of 1.4 ± 0.12 g L^{-1}, 167 ± 0.23 mg L^{-1} of PHA concentration and PHA content (% DCW) of 12%. The results were statistically significant ($p < 0.0001$). Similar results were reported very recently on the production of P3HB (13.5%) by *Cobetia* strain (5–11-6–3) in a medium containing crushed waste brown seaweed *Laminaria* sp. (Moriya et al., 2020). The ^{1}H NMR spectra of the PHA extracted from *Cobetia* isolate no. 105 grown on *Ulva* sp. hydrolysate compared to that produced on sugar mixture (i.e. glucose, fructose and mannitol) were investigated. The ^{1}H NMR spectral data matched with the ^{1}H NMR spectrum of P(3HBV) acquired (Ghosh et al., 2019). From the calculated peak integration, it can be concluded that the PHA produced by *Cobetia* isolate no. 105 grown on sugar mixture contains mainly (99.06%) 3HB monomers with 0.94 mol% 3HV monomers while 3.29 mol% 3HV was obtained when *Cobetia* isolate no. 105 was grown on *Ulva* sp. acid hydrolysate. Similar observations have been described for P(3HB-co-3HV) production by *H. mediterranei* when grown on *Ulva* sp. hydrolysate (Ghosh et al., 2019), on olive mill wastewater (Alsafadi and Al-mashaqbeh, 2017) and by *Cupriavidus necator* when grown on waste glycerol (Gahlawat and Soni, 2017). Numerous studies have demonstrated P(3HB) production by various marine bacteria (Mostafa et al., 2020; Pu et al., 2020). For example, Moriya et al. (2020) reported the production of P3HB on waste Laminaria sp., brown algae, as a sole carbon source by *Cobetia*. Tu et al. (2020) had reported P3HB accumulation by the halotolerant bacteria, *Halomonas boliviensis* when was grown on red algae (*Gelidium sesquipedale*) hydrolysate (Tu et al., 2020). A very recent study showed that under optimized conditions, Bacillus megaterium strain CAM12 used finger millet straw hydrolysates as the sole carbon source for their growth and produced 8.31 g L^{-1} of PHB (Pugazhendhi et al., 2020).

4. Conclusions

In this study, different P(3HB) and P(3HBV)-producing bacteria strains were isolated from seaweed *Ulva* sp. designated *Cobetia*, *Sulfitobacter* and *Pseudoaltermonas* from various sugars. The highest PHA content was observed in the case of *Cobetia* strains with up to 61% w/w in the presence of mannitol and 12% w/w on *Ulva* sp. acid hydrolysate as a substrate. To maximize P(3HB) productivity using the isolated marine bacteria strains and seaweed *Ulva* sp., it will be necessary to optimize the experiments' culture conditions.

CRediT authorship contribution statement

Rima Gnaim: Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Mark Polikovsky:** Methodology, Writing - review & editing. **Razan Unis:** Methodology, Formal analysis. **Julia Sheviryov:** Methodology. **Michael Gozin:** Supervision, Conceptualization, Writing - review & editing. **Alexander Golberg:** Supervision, Conceptualization, Writing - review & editing, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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