Science of the Total Environment 737 (2020) 139716

Contents lists available at ScienceDirect

Science of the Total Environment

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

Utilizing the crop waste of date palm fruit to biosynthesize polyhydroxyalkanoate bioplastics with favorable properties

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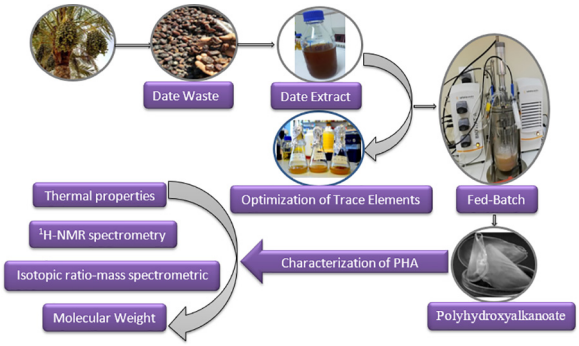
HIGHLIGHTS

- Date waste is utilized for PHBV production by halophilic archaeon *H. mediterranei*.
- *H. mediterranei* is able to accumulate PHBV with high 3-hydroxyvalerate mol % (18%).
- *H. mediterranei* feeds on date fruit waste as a sole carbon and elements sources.
- IRMS can be used to investigate biopolymer origin and biosynthesis mechanisms.
- *H. mediterranei* prefers lighter bonds to break and lighter atoms for biosynthesis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 April 2020
Received in revised form 23 May 2020
Accepted 24 May 2020
Available online 27 May 2020

Editor: Damia Barcelo

Keywords:
Polyhydroxyalkanoates
Date fruit waste
Biodegradable plastic
Fed-batch fermentation
Bioeconomy
Isotopic ratio mass spectrometry (IRMS)

ABSTRACT

Polyhydroxyalkanoate (PHA), a family of biodegradable and renewable biopolymers that could potentially play a significant role in bioeconomy. In this study we investigated the potential of date waste (DW) biomass as feedstock to produce PHA by the halophilic archaeon *Haloferax mediterranei*. The concentration of essential trace elements for *H. mediterranei* cells during growth and PHA biopolymer accumulation was optimized. A maximum cell dry mass of (CDM) (12.8 g L^{-1}) and PHA concentration of (3.20 g L^{-1}) were achieved in DW extract media that was not supplemented with trace elements, indicating that DW is a promising source for trace elements. The cultivation was scaled-up to fed-batch bioreactor fermentations under non-sterile conditions and resulted in CDM and PHA content of 18.0 g L^{-1} and 25%, respectively. The produced PHA was confirmed to be poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) with high 3-hydroxyvalerate (3 HV) content of 18.0 mol%. This 3 HV molar percent was achieved without the addition of expensive precursors. The PHBV is of high molecular weight (746.0 kDa) and narrow polydispersity (PDI = 1.5), and displayed reduced melting at 148.1 °C. The X-ray diffraction (XRD) analysis showed that the PHBV has amorphous nature which increases the degradation rates and workability of the biopolymer. The isotopic ratio $^{13}C/^{12}C$ ($\delta\ ^{13}C$) for PHBV was found to be – 19.1‰, which indicated that *H. mediterranei* prefers lighter bonds to break and uses the lighter atoms for the biosynthesis of PHBV.

1. Introduction

Nowadays, bioeconomy is considered as a new way to address a wide range of global challenges such as food security, sustainable economy and resources management, climate changes, unemployment and

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https://doi.org/10.1016/j.scitotenv.2020.139716
0048-9697/

the huge dependence on non-renewable fossil resources (Lainez et al., 2018). The bioeconomy refers to the efficient utilization of sciences and industrial technologies for the production of renewable biological resources and to convert these into food, feed, bioenergy and other added-value products (Scarlat et al., 2015). The bioeconomy has strong innovation potential to improve resource efficiency, minimize waste, improve production processes and create valuable environmentally friendly products. Recently, comprehensive life cycle assessment (LCA) studies and environmental footprint tool have shown that biodegradable polymers such as polyhydroxyalkanoate (PHA) contribute to bioeconomy concepts, reduce the greenhouse gasses emissions (by ≈200%), use less fossil fuel energy (by ≈95%) and reduce toxic wastes production (Dietrich et al., 2017). Therefore, PHA has been considered as superior alternative to petroleum-derived plastics (Xu et al., 2018).

PHA is a family of intracellular polymer accumulated as energy and carbon reserves by several archaea and bacteria. Though the major PHA is the homopolymer poly(3-hydroxybutyrate) (PHB), the copolymer which was later discovered poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) showed better mechanical properties and biocompatibility, and this makes PHBV extensively innovative for the biomedical and pharmaceutical applications such as bone scaffolds, drug delivery systems, implant coatings and tissue engineering (Thorat Gadgil et al., 2017). The current market of PHBV is very small compared to that of petroleum based plastic. The major factors that restrict the utilization of PHBV on a wider scale are the expensive highly pure substrates, the discontinuous production in batch or fed-batch; the need for the addition of expensive precursors, such as propionate and valerate; the use of considerable amounts of toxic solvents through the downstream clean-up in addition to the risk of microbial contamination (Kourmentza et al., 2017; Rodriguez-Perez et al., 2018).

The extreme halophilic microorganisms that thrive in the extreme environments of ~4.5 M salt, are considered as promising candidates for the economically feasible large scale production of PHA (Alsafadi et al., 2018; Koller, 2019; Yue et al., 2014). Halophiles have relatively rapid doubling time under simple growth requirements with a wide window of choices in carbon substrates. More importantly, the high salinity of halophilic microorganism's cultivation media minimizes the risk of microbial contamination, which allows a possible open and unsterile fermentation process for PHA production to occur (Yue et al., 2014).

So far, the most preferable PHA haloarchaeal producer is *Haloferax mediterranei* (Mitra et al., 2020). *H. mediterranei* has the ability to accumulate the copolyester PHBV from unrelated and simple carbon sources such as glucose (Han et al., 2013). Additionally, the produced copolyester can be recovered easily, with less use of toxic solvents, after decreasing the external medium salinity and causing hypo-osmotic shock of the cells (Fernández-Castillo et al., 1986; Koller, 2019). Finally, *H. mediterranei* could accumulate PHA using long-lasting and continuous fed-batch fermentation (up to 3 months), with constant polymer yield (Chen et al., 2006) and regular polymer composition and quality (Ferre-Guell and Winterburn, 2019). Recent studies on *H. mediterranei* have focused on utilization of different inexpensive carbon resources for PHA production such as cheese whey (Pais et al., 2016), glycerol (Hermann-Krauss et al., 2013), macroalgal biomass (Ghosh et al., 2019) and olive mill wastewater (Alsafadi and Al-Mashaqbeh, 2017).

Although *H. mediterranei* is able to grow on simple carbon source such as glucose, other expensive nutrients and trace elements such as Cu, B, Co, Mn, Mo, Ni, Zn and Fe are included in *H. mediterranei* media to enhance the cell growth and PHA accumulation (Pais et al., 2016; Hermann-Krauss et al., 2013; Ghosh et al., 2019; Alsafadi and Al-Mashaqbeh, 2017; Koller, 2015; Koller et al., 2007b; Han et al., 2015; Koller et al., 2015; Ferre-Guell and Winterburn, 2019). For providing a sustainable and economically feasible PHA production by *H. mediterranei*, new renewable, elements-rich and inexpensive carbon feedstocks are required to be explored.

Date palm (*Phoenix dactylifera* L) is one of the most successful and vital crops in Middle East region especially in Saudi Arabia (second largest date producer), as well as in other arid and semiarid regions of the world such as southern Europe, north Africa, and parts of central and south America (Chao and Krueger, 2007). The World annual production of dates was about 8.2 million tons in 2017 (http://www.fao.org/faostat/en/#data/QC). Date palm is the main income source and essential food for local inhabitants in many countries in which they are cultivated. Therefore, date palm has been playing significant roles in the society, environment and most importantly the economy of those countries (Chao and Krueger, 2007).

A single date tree can yield around 400–600 kg/year of fruits and its productive life can extend to 60 years (Al-Alawi et al., 2017). Besides food-grade date production, large amounts of immature fruit falling, rotten and spoiled date end up as waste. In fact, the date waste (DW) is not supposed for human consumption because of either inadequate texture (too hard or soft) or being infested by insects and/or being contaminated with fungi. The global DW production is approximately two million tons/year (Besbes et al., 2009). In previous works, DW has been utilized in microbial fermentations for the production of organic acids (Chauhan et al., 2007), biofuels (Abd-Alla and Elsadek El-Enany, 2012) and enzymes (Acourene and Ammouche, 2012).

Here, we investigate DW as renewable carbon and elements source for PHA production by *H. mediterranei*. Fed-batch bioreactor cultivation process under non-sterile conditions has been developed to achieve high cell density and biopolymer production. The impact of trace elements on *H. mediterranei* growth and PHA production is also discussed. The thermoanalytical data, molecular mass determination, crystallinity, tensile strength measurements and isotopic ratio-mass spectrometric analysis were performed to characterize the produced biopolymer. This work shows -to the best of our knowledge- the first attempt to produce PHA polymer from DW.

2. Materials and methods

2.1. Strains and growth conditions

Haloferax mediterranei was obtained from Leibniz Institute DSMZ (Germany), as lyophilized sample of strain DSM1411. *H. mediterranei* was initially grown in nutrient-rich AS-168 medium (Alsafadi and Al-Mashaqbeh, 2017). The culture was incubated in a rotary shaker (230 rpm, 37 °C) for 4 days. The strain was maintained in vials containing AS-168 medium and supplemented with 20% glycerol at −80 °C. The stored strain was streaked on salt agar medium (Koller et al., 2015) and incubated at 37 °C for 48 h. The primary inoculum was cultured in a highly saline medium containing (per L) 150 g NaCl, 13 g $MgCl_2 \cdot 6H_2O$, 4 g KCl, 0.69 g $CaCl_2 \cdot 2H_2O$, 63 mg NH_4Fe(III) citrate, 20 g $MgSO_4 \cdot 7H_2O$, 0.25 g $NaHCO_3$, 0.5 g KBr, 6.25 g YE, and 10 g glucose and 1.25 mL SL-6 solution containing 100 mg $ZnSO_4 \cdot 7H_2O$, 200 mg $CoCl_2 \cdot 6H_2O$, 300 mg H_3BO_3, 6.3 g NH_4Fe(III) citrate, 6 mg $CuSO_4$, 20 mg $NiCl_2$, 30 mg $Na_2MoO_4 \cdot 2H_2O$, 25 mg $MnCl_2$. 2 H_2O. The pH of the media was adjusted at 7.2.

2.2. Pre-treatment of date waste and optimization of carbohydrates yield

Immature fruit falling, rotten, spoiled and low grade date of the Mabroom and Nabt Ali varieties were obtained from Al-Qassim area/Saudi Arabia. First, the date seeds were manually removed by slice opening of the date fruit (mechanical process), and dates were sliced to small pieces (1 cm × 1 cm × 0.5 cm). Then, 50 g of seeds-free dates were weighed and soaked in distilled water (225 mL) for 10 min. Then, the soaked dates were blended using blender (Ceado, Italy) for 5 min. The homogenized mixture was transferred to Erlenmeyer flask (500 mL) and the flask was placed on stirrer. For studying the effect of the time and temperature on extraction of carbohydrates, twelve date waste mixtures were prepared as described previously and heated at

different temperatures (40 °C, 60 °C and 70 °C) for 0.5, 1, 3 and 6 h. The resulted extracts were centrifuged at 6340 ×*g* for 25 min, and then filtered through a gauze swab (19 × 15 mesh). Sample was taken from each extract and analyzed to determine the concentration of total carbohydrates by Anthrone method (Koller et al., 2015). Finally, the date waste extract (DWE) was collected (without autoclaving) in plastic containers. The containers were then stored at −20 °C to be used for PHA production.

2.3. PHA production at different trace elements concentration

For PHA production, *H. mediterranei* was grown in 100 mL saline medium. The culture was incubated with shaking at 230 rpm, 37 °C. When the cells had reached the late exponential phase, 2 mL of a selected preculture was transferred into 100 mL date waste extract (DWE) media containing DWE with 10 g L^{-1} final carbohydrates concentration. The DWE media was supplemented by 150 g L^{-1} NaCl, 13 g L^{-1} $MgCl_2 \cdot 6H_2O$, 4 g L^{-1} KCl, 0.69 g L^{-1} $CaCl_2 \cdot 2H_2O$, 20 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.25 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} KBr. In order to evaluate the effect of trace elements, different SL-6 solution (0.04, 0.07 and 0.13% V/V) was added, respectively. The culture was incubated in a rotary shaker (230 rpm, 37 °C) for 4 days.

2.4. Cell dry mass and PHA extraction

The cell dry mass (CDM) was calculated gravimetrically using 10 mL of broth containing *H. mediterranei*. The broth was centrifuged at 6340 ×*g* for 15 min, and then the supernatant was discarded. The remaining pellets were washed twice with isotonic NaCl solution. The resulted pellets were dried at 105 °C to constant weight. The CDM was expressed in g L^{-1}. PHA extraction was performed as described previously (Alsafadi and Al-Mashaqbeh, 2017).

2.5. Metals and carbohydrates determination

For metals determination, about 1 g of dry fruit was weighed in a crucible and ashed at 550 °C. The ash was then dissolved in 5 mL of 20% hydrochloric acid, the solution was transferred to a 50 mL volumetric flask and completed to the mark with ultra-pure water (18.2 M Ω). Metals standards (Merck) were used for calibration. Metals concentrations were quantified by inductively-coupled plasma optical emission spectrometry (ICP-OES; Shimadzu ICPS-7510). Carbohydrates such as fructose, sucrose and glucose, were determined using high performance liquid chromatography (HPLC; Thermo UltiMate 3000) equipped with RI detector and ACE-Excel amino column (5 μm, 4.6 × 250 mm). The carbohydrates were eluted using acetonitrile:water (80:20) under isocratic condition at 1 mL/min. Fructose, sucrose and glucose saccharide standards (Sigma) were used for calibration. An appropriate dilution of the date waste extract sample was prepared. 0.5 mL of the sample was filtered using syringe filter (0.45 μm, Thermo) and a sample volume of 10 μL was injected to the HPLC.

2.6. Nuclear magnetic resonance (NMR)

The chemical composition of the polymer was determined by NMR. The polymer was dissolved in deuterated chloroform ($CDCl_3$) at a concentration of 10 mg/mL with 1% tetramethyl silane (TMS) as the internal standard and analyzed on NMR spectrometer (Bruker; 500 MHz–Avance III). Chemical shifts (δ) are expressed in ppm and coupling constants (J) are expressed in Hz. The molar percentage of the 3-hydroxyvalerate (3 HV) portion in PHBV was estimated by calculating specific peak ratios in the NMR spectrum.

2.7. Gel permeation chromatography (GPC)

The biopolymer molar mass data [i.e. number average molecular weight (M_w), weight average molecular weight (M_n), and polydispersity (PDI)] was determined by Gel permeation chromatography (GPC; Tosho EcoSEC HLC-8320) equipped with a refractive index detector and a TSKgel GMHHR-M column (7.8 mm I.D. × 30 cm and 5 μm particle size). Monodisperse polystyrene standards (500 Da - 2110 kDa, PStQuick, C, TOSOH) were used for calibration. Sample preparation and chromatography conditions were performed as described previously (Alsafadi et al., 2020).

2.8. X-ray diffraction (XRD)

The X-ray powder diffraction analysis was measured by XRD diffractometer (Shimadzu XRD-6000). Radiation of wavelength 1.542 Å (Cu Kα) was employed at generator power of 30 kV and 40 mA. The X-ray diffraction pattern of PHA polymer sample was recorded in the scattering angle range $2\theta = 5°–40°$ at scan speed of 2° min^{-1}.

2.9. Tensile strength and differential scanning calorimetry (DSC) analysis

The tensile strength and differential scanning calorimetry (DSC) thermal measurements were performed as described previously (Alsafadi et al., 2020).

2.10. Isotopic ratio-mass spectrometric analysis (IRMS)

The isotopic ratio-mass spectrometric (IRMS) analysis and carbon percentage (C%) of PHBV were determined by reacting 2.89 mg of the biopolymer in a Costech ECS 4010 Elemental Combustion System (EA). The sample was weighed and loaded into tin capsules and combusted in the EA at 1100 C. The CO_2 gas emitted from the sample was introduced via a CONFLO III to the inlet of a Thermo Fisher Scientific MAT 253 IRMS system. The C% was determined with analytical precision better than 0.2% (one standard deviation). The isotopic ratio $^{13}C/^{12}C$ ($\delta^{13}C$) is reported relative to the VPDB standard with instrumental precision of ±0.35‰ (VPDB). Precision is based on 21 repeated measurements of DORM, USGS-24, IAEA-600 (caffeine), and ANU (sucrose) with known carbon content and isotopic composition.

2.11. Fed-batch bioreactor cultivation

The fed-batch bioreactor fermentation experiment was conducted in a 5 L bioreactor (BioStat A; Sartorius). First, *H. mediterranei* was grown in glucose medium and 10 mL pre-culture was taken to a bioreactor vessel containing 2 L non-sterilized DWE media with carbohydrates concentrations of 14 g L^{-1}. The cultivation process was monitored using various electrodes and automatically controlled. The temperature was set at 37 °C. The pH-value was adjusted at 7.2 ± 0.2 by the automatic addition of 1 M NaOH and 1 M H_2SO_4 solutions. A stable air flow rate was maintained in the bioreactor at 0.75 vvm during the cultivation process. The dissolved oxygen was controlled and maintained at about 20% of air saturation by varying the stirring speed (200–800 rpm), automatically. Suppression of foam was accomplished by automatic addition of 1% (w/v) antifoam A (Sigma). When the carbohydrates concentration was reduced to 2 g L^{-1}, 20 mL of DWE was added. The fed-batch cultivation process was stopped after 378 h, when the carbohydrates were almost depleted. Periodically, samples were taken in duplicates for quantification of CDM, polymer concentration and total carbohydrates.

3. Results and discussion

3.1. Date palm fruit waste characterization

The compositional analysis of date waste (DW) sample used in this study showed it to contain 80.1% carbohydrates, 12.1% moisture, 4.9% crude fiber with the remainder composed of ash (2.2%) (Table 1). The main carbohydrates found in the sample were glucose (29.1%) and fructose (27.5%). These results were comparable to the reported carbohydrates content of fresh date fruit in the literature (Mohamed et al., 2014; Al-Shahib and Marshall, 2003). The DW was also rich in trace elements such as B, Cu, Mn, Zn and Fe, however, the concentrations of some elements were different when comparing with the reported values in previous studies (Mohamed et al., 2014; Al-Shahib and Marshall, 2003). For example, the concentration of iron (Fe) (1.9 mg/100 g) was lower than Fe content of Sudanese dates which ranged from 4.06 mg/100 g in variety Barakawi to 7.06 mg/100 g in the cultivar Jaw (Mohamed et al., 2014). The zinc (Zn) concentration (1.1 mg per 100 g) was within the range concentration (0.1–1.8 mg/100 g) of dates results from various varieties (Al-Shahib and Marshall, 2003). The variation in elements concentration could be attributed to differences in cultivars, ripening stage, and growing environment. In general, the results indicate that DW is an attractive carbohydrate source for PHA accumulators as it is composed of high content of simple sugars (glucose and fructose) which are readily converted to PHA by a broad range of bacteria and archaea (Jiang et al., 2016). More importantly, DW could be used as source of essential trace elements that support *H. mediterranei* growth and PHA production. The effect of these trace elements on *H. mediterranei* growth and PHA production will be investigated.

3.2. Effect of temperature and heating time on carbohydrates extraction

The extraction of carbohydrates from DW was investigated and recovery maximized by testing extraction conditions including temperature and extraction times (Fig. 1). At 40 °C, the carbohydrates concentration increase with time and maximum carbohydrates concentration obtained was 210 g L^{-1} after 6 h. Increasing the temperature from 40 °C to 60 °C resulted decline in the extractable carbohydrates from 210 g L^{-1} to 150 g L^{-1}. Furthermore, carbohydrates extraction was not efficient at 70 °C, only 90 g L^{-1} carbohydrates were recovered after 6 h. This low carbohydrates yield could be attributed to the formation of saturated thick layer around the date pieces at high temperature which then entrapped the soluble sugars hence reduced their extractions. This was also observed in previous work for carbohydrates extraction from carrot roots (Cazor et al., 2006), where the concentration of sugar (sucrose) increased with increasing the temperature until 75 °C, after which further increase to 100 °C resulted decreasing in sugar yield. Interestingly, the pre-treatment of DW was achieved under mild conditions and without the need for chemical and/or enzymatic treatment which usually used in the pre-treatment of many carbon sources for PHA production (Pais et al., 2016; Hermann-Krauss et al., 2013; Chen et al., 2006; Huang et al., 2006; Ghosh et al., 2019). This could help to reduce the PHA polymer production cost considerably.

Table 1
Chemical composition of the date fruit waste.

Parameter	
pH 10%	5.2 ± 0.1
Moisture at 105 °C (%)	12.1 ± 0.6
Ash (%)	2.2 ± 0.05
Crude fiber (%)	4.9 ± 0.3
Total carbohydrates (%)	80.1 ± 0.5
Glucose (%)	29.1 ± 0.1
Fructose (%)	27.5 ± 0.3
Sucrose (%)	14.3 ± 0.3
Zn (mg\100 g)	1.1 ± 0.1
Fe (mg\100 g)	1.9 ± 0.2
Cu (mg\100 g)	0.4 ± 0.05
Mn (mg\100 g)	0.3 ± 0.05
B (mg\100 g)	1.6 ± 0.1
Na (mg\100 g)	7.6 ± 0.2
K (mg\100 g)	324.1 ± 5.0
Ca (mg\100 g)	44.1 ± 1.0
Mg (mg\100 g)	54.4 ± 1.0

The results are presented as mean ± standard deviation.

3.3. Effect of trace elements concentration on H. mediterranei growth and PHA production

In this experiment, *H. mediterranei* was cultivated in batch shaking flasks supplemented with DWE and different concentrations of SL-6 trace element solutions (0.04, 0.07 and 0.13% V/V). The DWE has been prepared under optimized carbohydrate extraction conditions (40 °C and 6 h). The *H. mediterranei* culture's efficiency in terms of polymer accumulation and cell growth are presented in Table 2.

Clearly, the growth of *H. mediterranei* and PHA production were impacted by trace elements concentration. Cultures with higher trace elements concentration showed lower CDM, and subsequently less PHA production was quantified at the end of the fermentation. For example, the cultures supplemented with trace elements concentration 0.13 and 0.07% V/V were characterize by CDM of ≈9.1 g L^{-1} and r_p PHA of 0.65 g L^{-1}.day^{-1}, and these values were increased to 12.4 g L^{-1} and 0.71 g L^{-1}.day^{-1}, respectively, for the culture supplemented by 0.07% V/V of SL-6 solution. The maximum *H. mediterranei* CDM (12.8 g L^{-1}) and PHA concentration (3.20 g L^{-1}) were recorded in DWE media that was not supplemented with SL-6 solution. This result indicates that the increase in trace elements concentration had negative effect on *H. mediterranei* cell growth and polymer accumulation. The cell growth inhibition could be attributed to the toxicity that occurred to the *H. mediterranei* cells due to the high quantity of trace elements that came from both DWE and the added amount of SL-6 solution. In previous studies, several inhibitory compounds present in biomass such as polyphenols, furfurals and organic acids (e.g., acetic, formic or levulinic acid) were evaluated for effects on cell growth and PHA production (Pan et al., 2012; Kucera et al., 2017). Also, significantly decreasing the trace element concentration (SL-6) resulted in lower biomass when *H. mediterranei* cells were cultivated in batch shaking flasks experiment with glucose and galactose as the carbon source (Pais et al., 2016). Voica et al., 2016 reported the mechanisms of trace metals resistance in halophilic bacteria and archaea. In fact, the extracellular polysaccharides (EPSs) play important roles in the halophilic bacteria metal tolerance. At high metals concentration, halophilic bacteria produce EPSs which are rich in negatively charged residues that are prone to bind metal cations in a non-specific manner (Voica et al., 2016). For example, *Halomonas* strains can tolerate 5 mM of Pb^{2+} and Cd^{2+} by the EPS-mediated adsorption of the metallic ions (Manasi et al., 2015). Voica et al., 2016 showed that halotolerant cyanobacterium *Aphanothece halophytica* was capable to uptake Zn^{2+} with binding capacity of 133 mg g^{-1} by high EPSs production. It has been reported that the extreme halophilic Archaeon *H. mediterranei* also produces extracellular polymeric substances (EPSs) as byproduct simultaneously with PHA (Koller et al., 2015). The high trace metals concentration in *H. mediterranei* media promote EPSs production thereby, reduce PHA production. Table 2 also showed that *H. mediterranei* utilized the sugars (glucose, fructose and sucrose) in DW effectively for cell growth and PHA production. In order to evaluate DW biomass as carbohydrates source, *H. mediterranei* was cultivated in glucose media containing 10 g L^{-1} of glucose (same total carbohydrates concentration in DWE media) and 0.13% of SL-6 (common amounts in *H. mediterranei* media). Under these conditions, 9.3 g L^{-1} CDM and 1.9 g L^{-1} PHA

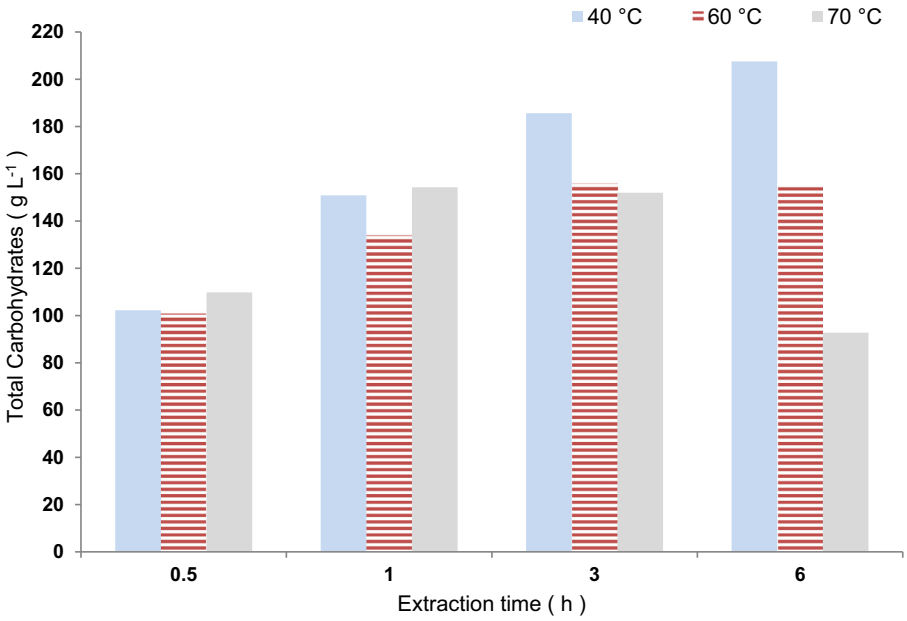

Fig. 1. Effect of heating time and temperature on carbohydrates extraction from date waste.

were obtained which were significantly lower than the all results of DWE media (Table 2).

3.4. PHA production from date waste by fed-batch fermentation

The fed-batch fermentation process was performed under non sterilized conditions and without the addition of trace elements (the best condition from shake-flask experiments). Feeding the bioreactor was accomplished by addition of DWE during the cultivation process. An increase in the carbohydrates concentration to higher than 10 g L^{-1} caused an inhibition in *H. mediterranei* cell growth (data not shown), therefore the carbohydrates concentration was maintained at level of 2–8 g L^{-1} within 335 h of the fed-batch process (Fig. 2). Initially, up to 167 h of cultivation, slow growth profile of *H. mediterranei* was observed and the PHA production follows the cell growth. In this phase, CDM reached 13 g L^{-1} containing around 12% of PHA. From 167 h to 194 h there was significant increase in *H. mediterranei* growth and the CDM increased from 12 g L^{-1} to 18 g L^{-1}. This has led to an increase in the PHA concentration from 2 g L^{-1} to 4.5 g L^{-1}. From 194 h to 331 h the PHA accumulation reached the maximum value with CDM of 18 g L^{-1} containing 25% PHA. At the last 30 h of cultivation there was a significant decrease in the CDM (14.5 g L^{-1}). However, the PHA concentration and PHA content did not change 4 g L^{-1} and 26%, respectively. Table 3 summarizes several studies that employed fed-batch fermentation for the production of PHA using *H. mediterranei*. When date waste was used as feedstock for fed-batch bioreactor cultivations of *H. mediterranei*, a maximal CDM of 18 g L^{-1} and PHA production of 4.5 g L^{-1} were obtained. These results are close to the results obtained from many carbon sources such as hydrolyzed whey permeate (7.2 g L^{-1} PHA) (Koller et al., 2015), mixes of butyric and valeric acid (4.01 g L^{-1} PHA and 6.8 g L^{-1} CDM) (Ferre-Guell and Winterburn, 2019), starch (6.5 g L^{-1} PHA) and glucose (3.5 g L^{-1} PHA) (Lillo and Rodriguez-Valera, 1990). The highest PHA production from the fed-batch culture of *H. mediterranei* was recorded for extruded rice bran feed with 140.0 g L^{-1} of PHA and 77.8 g L^{-1} CDM (Huang et al., 2006). Table 3 also shows that the salinity and trace elements supplies in the *H. mediterranei* cultivation media are important for both cell growth and PHA production. However, here we reported that *H. mediterranei* cultivated in DW does not require addition of trace elements and only requires minimum salinity of 150 g L^{-1} NaCl to grow and produce the PHA, this is expected to reduce PHA production cost.

3.5. Characterization of PHA

3.5.1. ^{1}H NMR spectrometry

The produced biopolymer by *H. mediterranei* was extracted and purified as described in the materials and methods part. The chemical structure of the biopolymer was confirmed by ^{1}H NMR spectrometry as copolyester poly(3-hydroxybutyrate-*co*-3 hydroxyvalerate) (PHBV) (Fig. 3).

The ^{1}H NMR spectrum clearly shows the prominent peaks of the PHBV as the following: the peak at 0.86 ppm corresponds to the methyl group of the valerate residue (V5). The peak at 1.2 ppm corresponds to the methyl group of the butyrate monomer (B4). The peak at 1.5 ppm is assigned to the methylene group of the valerate monomer (V4). The peaks at 2.5 ppm are correlated to the two methylene groups adjacent

Table 2
Parameters obtained from *H. mediterranei* cultivated in shake-flask and supplemented by different trace elements (SL-6) concentration after 96 h.

Carbon source	SL-6 (%,v/v)	CDM (g L^{-1})	PHA (g L^{-1})	r_p PHA (g L^{-1}.day^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{PHA/S}$ (g g^{-1})
DWE	0.13	9.2 ± 0.5	2.61 ± 0.04	0.65	0.99	0.31
DWE	0.07	9.0 ± 0.7	2.60 ± 0.05	0.65	0.58	0.42
DWE	0.04	12.4 ± 0.6	2.82 ± 0.03	0.71	1.094	0.26
DWE	0.00	12.8 ± 0.8	3.20 ± 0.07	0.80	1.32	0.38
Glucose	0.13	9.3 ± 0.5	1.90 ± 0.04	0.48	0.923	0.24

DWE: Date waste extract containing 10 g L^{-1} carbohydrates, SL-6 (%, v/v): trace elements, r_p PHA: PHA volumetric productivity, $Y_{X/S}$: biomass yield, $Y_{PHA/S}$: PHA yield.

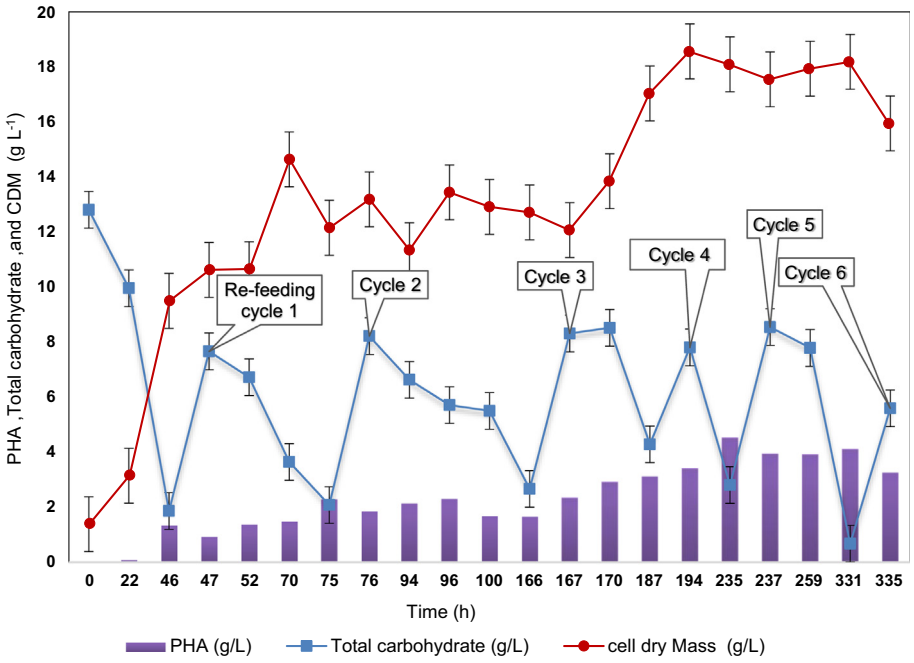

Fig. 2. Fed-batch culture of *H. mediterranei* by using a feeding stream of DWE under non sterilized condition and without the addition of trace elements. Arrows indicate the re-feeding of DWE.

to carbonyls in the two monomers which are almost identical in electronic environment (B2 and V2). The peaks at 5.2 ppm are assigned to the highly de-shielded methine groups (—CH) connected to the oxygen (carbon # 3). The molar ratio of valerate in PHBV was calculated by the area ratio of methyl group peak of the valerate at (0.86 ppm) to the sum of the two peaks of methylene groups at 2.5 ppm; i.e. V5 and (B2 + V2). Accordingly, the percentage of the 3 HV unit in the PHBV co-polymer was estimated to be 18 mol%. This 3 HV content was significantly higher than the values reported for PHBV polymers produced by *H. mediterranei* and feeding on different carbon sources such as hydrolyzed whey permeate (Koller, 2015), enzymatic extruded starch (Chen et al., 2006), glucose and yeast extract (Koller et al., 2015), extruded rice bran and extruded cornstarch (Huang et al., 2006) (Table 3). In fact, the production of PHBV with high 3 HV molar ratio is desired for improving the mechanical properties (flexibility and impact strength) and thermal properties of the polymer. Previous studies have investigated the effect of phosphorus (Melanie et al., 2018) and nitrogen (Ferre-Guell and Winterburn, 2017; Alsafadi et al., 2020) sources supply on 3 HV content in PHBV produced by *H. mediterranei*. Although more kinds of PHBV with high 3 HV content can be obtained by precursors supplying, the high cost will be another impediment to its development (Ferre-Guell and Winterburn, 2019; Han et al., 2015) (Table 3).

Table 3
PHA production from different carbon sources using fed-batch cultivations of *H. mediterranei*.

Carbon source	Product	Production scale	Salinity and trace elements	CDM ($g\ L^{-1}$)	PHA ($g\ L^{-1}$)	PHA/CDM (%)	References
Date waste	PHBV (18.0 mol% 3 HV)	2 L	150 $g\ L^{-1}$ NaCl, 0% SL6	18	4.5	25.0	This study
Hydrolyzed whey permeate	PHBV (10 mol% 3 HV)	200 L	150 $g\ L^{-1}$ NaCl, 0.13% SL6	–	7.2	66.0	(Koller, 2015)
Enzymatic extruded starch	PHBV (10.4 mol% 3 HV)	6.0 L	200.0–230.0 $g\ L^{-1}$ NaCl	39.4	20.0	50.8	(Chen et al., 2006)
Mixes of butyric and valeric acid	PHBV (43 mol% 3 HV at butyric/valeric acid = 56/44)	2 L	156 $g\ L^{-1}$ NaCl, 1.0% SL6	6.8	4.01	59.0	(Ferre-Guell and Winterburn, 2019)
Starch and glucose	PHB	1.5 L	250 $g\ L^{-1}$ marine salts	–	6.5 $g\ L^{-1}$ (starch) 3.5 $g\ L^{-1}$ (glucose)	–	(Lillo and Rodriguez-Valera, 1990)
Extruded rice bran and extruded constarch	PHBV (11 mol% 3 HV)	5.0 L	234 $g\ L^{-1}$ NaCl	140.0	77.8	55.6	(Huang et al., 2006)
Glucose and yeast extract	PHBV (10 mol% 3 HV)	10.0 L	150 $g\ L^{-1}$ NaCl, 0.13% SL6	–	13.0	70	(Koller et al., 2015)
Glucose and valerate	PHBV (50 mol% 3 HV)	7.0 L	140 $g\ L^{-1}$ total salt (110 $g\ L^{-1}$ NaCl), 0.13% SL6	12.3	5.1 (shaking flask)	41.3 (shaking flask)	(Han et al., 2015)
Whey permeate supplemented with sodium valerate and butyrolactone	P(3HB-co-3 HV-co-4HB) (21.8 mol% 3 HV, 5.1 mol% 4HB)	42 L	200 $g\ L^{-1}$ NaCl, 0.13% SL6	–	14.7	87.5	(Koller et al., 2007b)

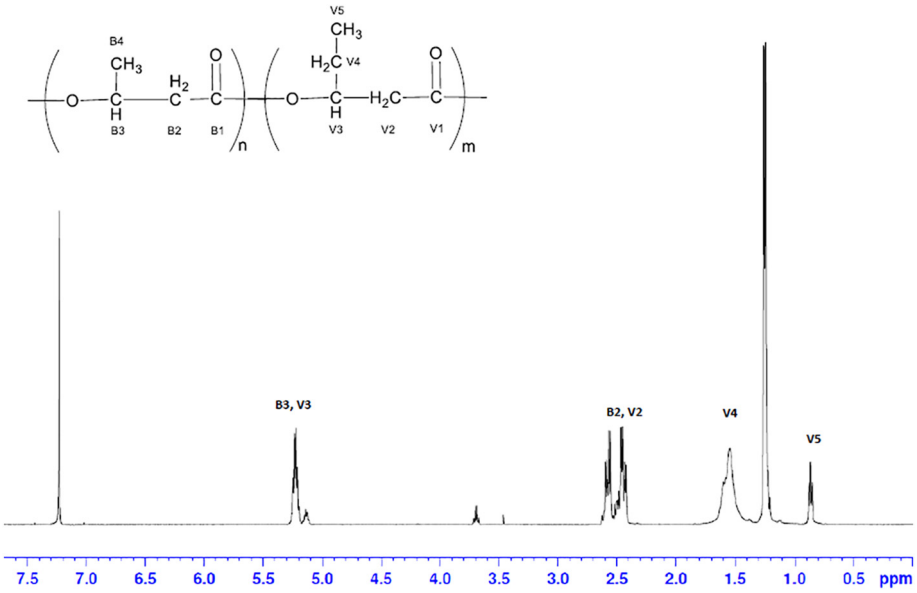

Fig. 3. ^{1}H NMR spectrum and chemical structure of PHBV co-polymer. B2, methylene (CH_2); B3, methine (CH); B4, methyl (CH_3) in the PHB unit; V2, methylene (CH_2); V3, methine (CH); V4, methylene (CH_2); V5, methyl (CH_3) in the PHV unit.

3.5.2. Thermal properties and crystallinity

Thermal properties of PHBV sample was determined by differential scanning calorimetry (DSC). The DSC analysis showed the melting temperature (T_m) of the PHBV at 148.1 °C (Fig. 4). This temperature was lower than the values recorded for PHBV with 3 HV composition of 5 mol% (T_m = 164.7 °C) (Koller et al., 2007a) and PHBV with 3 HV composition of 9.1 mol% (T_m = 151.2 °C) (Han et al., 2015). These results confirmed that the T_m decreases with increasing 3 HV fraction in the PHBV copolymer backbone. The low melting point of PHBV could improve polymer processing ability and impact strength. Du et al., 2012 reported that the T_g value correlates with the crystallization of PHBV polymer chains. For example, amorphous PHBV has T_g value range from −9 °C to 17 °C. So, PHBV recovered from *H. mediterranei* (T_g = 10.5 °C) is expected to be amorphous (Fig. 4). The degree of crystallinity of PHBV was studied by X-ray diffraction (XRD) analysis (Fig. 5). The XRD pattern shows characteristic peaks at 2θ values of 13.37, 16.82, 21.54, 25, 25.44, 27.14 and 30.1, respectively (Fig. 5). These values considerably similar to values reported values for PHBV (21 mol% 3 HV) produced by *A. eutrophus* (Kunioka et al., 1989). The XRD pattern revealed the amorphous nature of PHBV (26.5% crystallinity), which increases the degradation rates and workability of the biopolymer (Shang et al., 2012).

3.5.3. Molecular weight and mechanical properties

The molecular weight of PHBV copolymer was obtained by GPC. The weight-average molecular weight (*Mw*) of the copolymer was 746.0 kDa. This value was higher than that for PHBV produced by the recombinant *Halomonas neptunia* (345 kDa) and *Halomonas hydrothermalis* (381 kDa) (Pernicova et al., 2019). The PDI was narrow (1.5) which indicates that the polymer chains length are homogeneous.

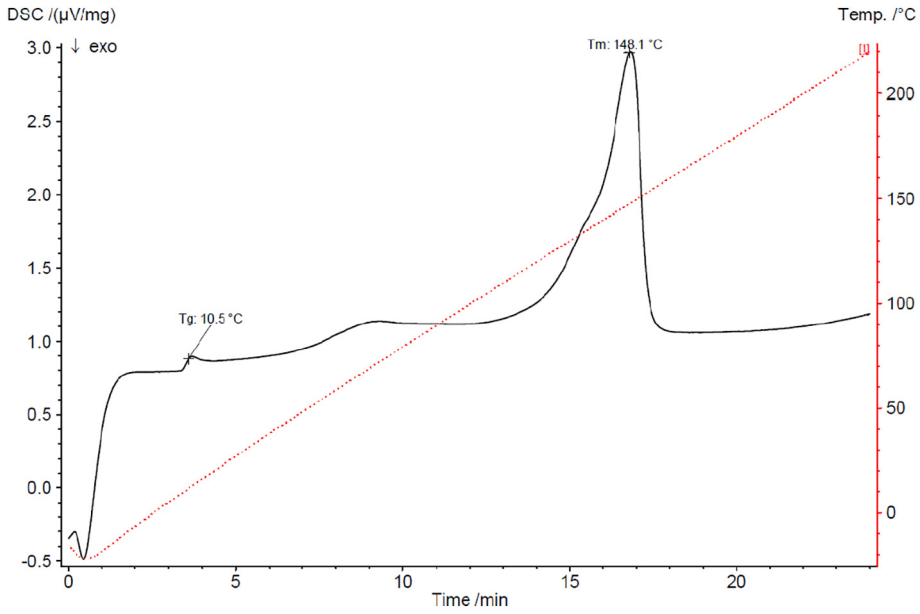

Fig. 4. DSC curve of PHBV produced by *H. mediterranei* cultivated in DW.

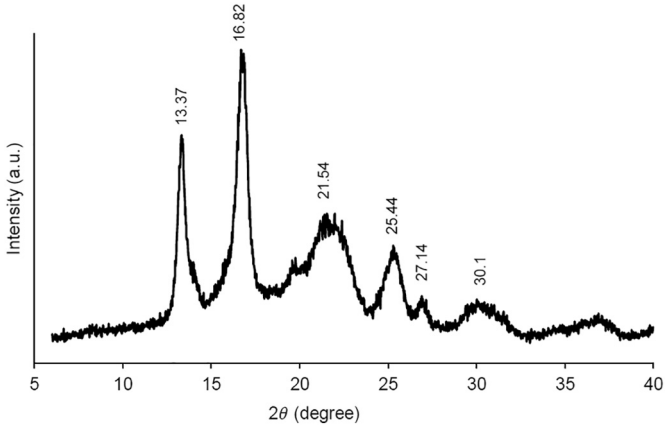

Fig. 5. X-ray diffraction pattern of PHBV produced by *H. mediterranei* cultivated in DW.

The recorded PDI is comparable to that of the biosynthesized PHBV by *H. mediterranei* from hydrolyzed whey (Koller et al., 2007b) and glucose (Don et al., 2006). In a further experiment, the tensile strength and elongation at break (%) were found to be 10.7 MPa and 1%, respectively. This value of tensile strength was close to that of the polymer produced by *Comamonas* sp. EB172 (~13 MPa) (Zakaria et al., 2013).

3.5.4. Isotopic ratio-mass spectrometric analysis and carbon percentage composition

Isotopic ratio-mass spectrometric (IRMS) analysis is an important tool in many real-life applications including food authenticity (Van Leeuwen et al., 2014), climate change studies (Ghosh and Brand, 2003), origin tracing of organic matters (Boschker and Middelburg, 2002) and many others. In the field of polymers, IRMS has been used as a tool to distinguish between polymers based on their origin (plant- and petroleum-derived plastics) (Suzuki et al., 2010). Here, the IRMS analysis of carbon isotopes was performed for the isolated PHBV from *H. mediterranei* as a novel method to find out if the microorganism has any fractionation (discrimination) towards either of the two carbon isotopes (^{13}C and ^{12}C) throughout the biosynthesis of PHBV from DW. The isotopic ratio $^{13}C/^{12}C$ ($\delta\ ^{13}C$) for PHBV was found to be – 19.1‰, the reported $\delta\ ^{13}C$ value for date fruit is (−10 to −12‰) (Carr, 2014), this shift is attributed to the well-known usual preference of microorganisms in biochemical processes towards lighter isotopes in the carbon source, as they generally have weaker bonds, which are preferentially broken by the mediating microorganisms (Hayes, 2001). The result shows that the used microorganism prefers lighter bonds to break and use the lighter atoms for the biosynthesis of PHBV. The carbon percentage (C%) in the PHBV sample was measured to be 54.2%, which is consistent with the expected (theoretically calculated) value of 56%, the difference between expected and found is negligible if we consider that the reading was out of the previously set calibration curve on the instrument.

4. Conclusion

The current work showed a promising way of utilizing agricultural crop waste from date fruit to produce PHA bioplastics. This is considered as one of many steps that are required nowadays to respond to the planet threats and environmental challenges such as global warming and accumulation of non-biodegradable plastics. We have shown for the first time that DW can be utilized as renewable carbon and elements source for PHBV production by *H. mediterranei*. Because of their high carbohydrates content and absence of lignin, the DW allows a mild pretreatment of the biomass without the production of toxic compounds which may result from the chemical treatment. No contamination was detected during the fed-batch fermentation despite the fact that equipment and DW were not sterilized prior to use. *H. mediterranei* was able to accumulate PHBV with 18 mol% of 3 HV. Though there are very little published materials in the utilization of IRMS technology in the field of polymer biodegradation and biosynthesis research, it is expected to have a greater attention in the future as it might provide answers and more understanding of the polymer degradation and synthesis mechanisms by microorganisms, it could also serve as a tool to trace the origin of the biopolymer. All of the above could contribute towards a reduction of the production cost of high added value bioplastics for industrial applications. For commercial and large-scale PHA production, the processes will be further scaled up in a large volume pilot scale (e.g., 1000 L fermenter).

CRediT authorship contribution statement

Diya Alsafadi: Conceptualization, Funding acquisition, Methodology, Validation, Writing - original draft. **Mohammad I. Ibrahim:** Data curation, Investigation, Methodology, Writing - original draft. **Khalid A. Alamry:** Data curation, Formal analysis, Investigation, Methodology, Resources. **Mahmoud A. Hussein:** Data curation, Investigation, Methodology. **Aya Mansour:** Data curation, Investigation, Methodology.

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.

Acknowledgments

This work was supported by the Scientific Research and Innovation Support Fund in Jordan (project No. WE/2/12/2016). We would like to thank professor Luis A. González for helping in IRMS analysis and the informative discussions.

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