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Waste date seed oil extract as an alternative feedstock for Poly(3-hydroxybutyrate) synthesis

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Highlights

- Feasibility of PHB synthesis using date seed oil as a substrate is demonstrated.
- Maximum PHB concentration of 11.8 g/l attained, 82% PHB content.
- Characterisation of the PHB product gave a melting temperature of 171°C.

Abstract

The economic production and wider utilisation of poly(3-hydroxybutyrate) (PHB) is dependent on the development of processes based on alternative, low cost and renewable feedstocks. The purpose of this study is to investigate the suitability of using waste date seed oil extract as an alternative carbon source for PHB synthesis. The extraction of date seed oil using different solvents, a chloroform, methanol mixture (MCM), hexane and petroleum ether (PE) was studied. Results indicate that the MCM gave the highest oil yield of 9.3%, whilst

the oil yields obtained using hexane and PE were 5.3% and 3.4%, respectively. This date seed oil was used as the sole carbon source in a series of microbial fermentation experiments, and the results show that PHB is accumulated as a significant percentage of dry cell weight (DCW). A maximum DCW of 14.35 g/l was obtained with a PHB content of 82%, giving a PHB concentration of 11.77 g/l.

The molecular structure and thermal properties of the PHB produced were determined by ¹H NMR, DSC and GPC, with results being broadly comparable to those of a PHB standard. Our results demonstrate that date seed oil is a feasible substrate for the production of meaningful amounts of PHB.

Keywords

Poly(3-hydroxybutyrate) (PHB), date seed oil, *Cupriavidus necator*, agricultural waste, extraction.

1. Introduction

Plastics are a ubiquitous part of modern life and can be modified to meet the material requirements for almost any application. Virtually all plastics in everyday use are manufactured using petrochemical derived material of fossil oil and gas origin, with 8 % of annual petroleum production being used in the manufacture of plastics [1]. Several problems exist which make conventional plastics less desirable, mainly the lack of environmentally friendly disposal routes and the dependence on finite and depleting oil reserves in a volatile market [2].

In 2015 25 Mt of plastic waste were generated in the European Union, and about 49% of this waste ended up in landfill [3]. Not only are plastics environmentally persistent, but also plastic waste in landfill can leach harmful chemical compounds into groundwater. Incineration is another disposal option, though this method is expensive and results in the emission of harmful chemicals such as hydrogen chloride and hydrogen cyanide [4].

Consequently, there is a need to find alternative processes for the production of biodegradable plastics from renewable resources, to reduce the environmental impact of plastic manufacture, use and disposal.

Biopolymers are naturally occurring biodegradable polymers which are accumulated by living microorganisms. Poly(3-hydroxybutyrate) (PHB) is one such biodegradable polyester which has been identified as a potential alternative to conventional petroleum-based plastics. PHB is produced by microbial fermentation, utilising various carbon sources under stress conditions (typically nitrogen limitation). Substrate cost has been identified as one of the main constraints to economic PHB production, cheap and renewable carbon substrates are therefore being investigated as replacements for conventional substrates [5]. The material properties of PHB are similar to those of many synthetic thermoplastic polymers, such as poly(propylene), making PHB a promising candidate as a drop-in replacement for petroleum-based polymers in a wide range of applications, such as paper coatings, packaging and bottles [6].

In bacterial cells PHB is produced as intracellular inclusion bodies surrounded by a monolayer of phospholipid, non-unit membrane. *Cupriavidus necator* is a Gram-negative bacterium and a known model organism for biodegradable polymer production. This is because *C. necator* capable of accumulating a large number of intracellular polymer granules in the cytoplasm, which can reach up to 90% of cell dry weight under nutrient limitation (nitrogen or oxygen) in the presence of an excess of carbon [4].

Industrial production of PHB by fermentation has not been widely adopted because of the high costs of production. The costs of the substrate for PHB production and subsequent downstream recovery are high, making the current cost of PHB 10 times greater than that of synthetic polymers [7]. For example, the cost of petroleum based polymers, Poly(ethylene)

(PE) or Poly(propylene) (PP), is 0.25-0.5 \$/kg, whilst PHB costs 6-16 \$/kg [8]. This cost differential is the main obstacle to the replacement of traditional polymers with biodegradable, bio-based alternatives in the market [3].

Generally, the economic production of bio-based polymers requires low-cost substrates and high productivity microorganisms. The substrate type not only determines the final percentage PHB content, but also affects the final polymer properties, including molecular weight. Furthermore, 40- 50% of the total cost of biopolymer production is accounted for by the raw materials [9].

In order to reduce the cost of biopolymer synthesis, inexpensive sources of carbon and nutrients are required. Working with agricultural waste residues such as grain crops, dairy waste, date seeds or starch could therefore substantially reduce substrate and hence PHB synthesis costs. Plant oils are essential agricultural products obtained from different crops, such as rapeseed, soybean, and oil palms [10]. Depending on the plant species, the fatty acid types that are present in the oil will vary, as will their distribution. Traditionally, plant oils have been utilised not only in the food industry, but have also been processed into other chemical products such as polymers [11], surfactants [12], fine chemicals, and fuels [13]. Plant oils can be metabolised by various microorganisms through the TAGs cycle. Lipases are secreted to catalyse the release of fatty acids from the oil, which are then transported into the cell to be catabolised via the β -oxidation cycle [14].

This has led to investigation of alternative carbon sources for biopolymer production which can be used to reduce production costs by adding value to materials considered to be waste. These wastes include the seeds of vegetables or fruits, as well their peel and skin, which are usually either discarded as waste or used in secondary, low value, animal feed or soil fertiliser products.

There are few published reports on waste date palm seed valorisation, even though they contain high quantities of various nutrients such as; 20-40% dietary fibre, 5-6%, protein, 50-70% carbohydrates, 10-12% oil, minerals, including calcium, magnesium, phosphorus and potassium, and vitamins [15].

The worldwide production of date palm fruit rose from 1.8 million tons in 1961 to 9.4 million tons in 2014 [16]. Date palm fruit consists of two parts; the edible flesh and the hard seed, which is a by-product of date processing and consumption. Approximately 10-15% of the total weight of date fruit is made up by the seed. Based on this figure and annual date production, more than 1 million tons of date seed waste are produced yearly [17]. Although date seed oil is edible and hence could potentially be utilised in the manufacture of pharmaceuticals and cosmetics, these value-added routes are not available or viable because of the low oil content of date seeds compared to conventional oil crops [18]. The utilisation of date seed waste in the production of value-added products such as biopolymers, biofuel or cooking oil, dietary fibres and medicinal products has much potential [19].

Our previous study showed that *C. necator* was able to accumulate PHB up to 73% of total DCW, and DCW reached 6.3 g/l using date seed hydrolysate media which initially contained 10.8 g/l fructose [20]. The first part of this study is an evaluation of the variables influencing the oil extraction yield such as solvent type, temperature, and extraction time. Subsequently the feasibility of utilising date seed oil, extracted from Zahide type date seeds, (commonly grown in Iraq), for PHB production is investigated. The novel utilisation of date seed oil as a renewable alternative carbon source biopolymer (PHB) synthesis by *C. necator* is demonstrated.

2. Material and methods

2.1. Date seed preparation

The date seeds used in this study were obtained from a local Iraqi supermarket, free of charge, and were washed and dried overnight at 60°C. The dried seeds were milled using a heavy-duty grinder (UMA PHARMA) and the powder (≤ 4 mm) preserved at -20°C for subsequent analysis and experimental work.

2.2. Oil Extraction

Date seed oil was extracted using a Soxtec HT 1043 extraction unit (Velp Scientifica, Europe) equipped with six thimbles as described by Obruca et al., [21].

The dried date seed powder (4g) was loaded into the thimbles and 40 ml of various solvents, petroleum ether (PE), chloroform, methanol mixture (2:1) MCM and hexane were used for extraction over range of temperature (100-180°C), and time (0.5-4 h) were tested. After oil extraction the solvent was evaporated at 60°C using a Rotavapor apparatus. The pure oil was stored in a freezer (-20°C) prior to subsequent physico-chemical analyses and PHB production. The amount of oil extracted was determined as the difference in dry weight.

2.3. Oil analysis

In order to identify the fatty acids present in the date seed oil an esterification reaction to produce fatty acid methyl esters (FAME) was carried out prior to analysis by GC-MS.

The AOCS method was followed to obtain FAME from the date seed oil extract and all samples were analysed in duplicate [22]. The GC injector port was set at 230°C and the detector temperature was 270°C, while the oven temperature was programmed to increase from 155°C to 250°C at a rate of 10°C min⁻¹. A polar capillary column, BPX 70, SGE Company (ID: 0.25 mm, capillary: 0.25 mm and length: 30m) was used for the separation of the FAME using a splitting ratio of 1:100. Peaks were identified based on their retention times compared to those of FAME standards [23].

2.4. Microorganism

The bacterial strain used in this study was *C. necator* H16; obtained from the American Type Culture Collection (ATCC17699). A master and subsequent working stocks were prepared from the lyophilised cells and stored at -80°C .

2.5. Mineral media

The composition of the mineral medium used to grow *C. necator* (per l) was prepared according to that reported by Aramvash et al., [24] and supplemented with 2.0 g/L $(\text{NH}_4)_2\text{SO}_4$ as an external source of nitrogen. Media was sterilised by autoclaving at 121°C for 20 min, whilst the oil was sterilised separately.

2.6. Culture and Inoculum preparation for *C. necator* growth and PHB production

In this study, a mineral media containing date seed oil as the carbon source was tested to examine the effect of using this oil on bacterial growth and PHB production. Fermentation experiments were carried out at 100 ml working volume in 500 ml flasks. A single colony of bacteria was used to inoculate 10 ml of nutrient broth, which was grown at 30°C and 200 rpm for 24 h. Cells were then harvested and re-suspended in 10 ml of mineral media. After a 24 h adaption stage, 5 ml of culture was used to inoculate each flask. Growth conditions for all experiments were 30°C , 200 rpm and 84 h, while pH was adjusted to 7 using NaOH and HCl (1N), before inoculation. During the first 12 h, samples were taken every 3 h, after which samples were taken every 12 h. Optical density (600nm), dry cell weight, total nitrogen and PHB concentration were measured at each point and experiments were carried out in triplicate.

2.7. Determination of Total Nitrogen (TN)

Total nitrogen concentration was determined using a total nitrogen analyser unit (TNM-1, TOC-control V). Standards of five different concentrations, 1-50 mg/l, were

prepared using KNO₃ as a source of nitrogen. For analysis 15 ml of sample was transferred to a universal tube after being filtered with a 0.2 µm syringe filter

2.8. Cell growth measurement

Optical density (OD) was measured by taking 1 ml of fermentation broth and centrifuging for 6 min. The supernatant was removed and the cell pellet washed with 0.7% NaCl and re-suspended in 1 ml of distilled water. OD was measured at 600 nm using a UV-mini1240 spectrophotometer (Shimadzu, USA).

The cell dry weight was determined gravimetrically. A 5 ml sample of fermentation broth was transferred to a dry, pre-weighed Eppendorf tube and centrifuged at 7000 rpm for 15 min at room temperature. The supernatant was decanted and refrigerated for further analysis, whilst the cell pellet was washed and re-suspended twice in distilled water and 0.7% NaCl respectively. The cell pellet was then dried at 60°C until constant weight was obtained.

2.9. PHB Quantification

Gas chromatography (GC) was used for the quantification of PHB using the method described by Rohini et al., [25]. A 7820A Gas Chromatography system with flame ionisation detector (FID), 7679A Headspace sampler (Aligent Technologies, USA) with CP7556 Varian poraplot Q-HT 10 m x 0.32 mm x 10 µm column (Aligent Technologies, USA) was used with an injection volume of 1 µL at 230°C. The initial temperature was 120°C, increased to 230°C over 3 min and the detection temperature was 200°C, with Helium as the carrier gas. The PHB concentration was calculated by comparing peak areas to those of [(R)-3-Hydroxybutyric acid, ≥ 98%], Sigma-Aldrich, Germany, standards of known concentration.

2.10 PHB content

The percentage PHB content was determined using equation 1:

$$\% \text{ PHB} = \frac{\text{Max. PHB concentration (g/l)}}{\text{max. biomass concentration (g/l)}} \quad (1)$$

2.11. PHB extraction from the bacterial biomass

PHB was recovered following the method reported by Hahn et al.,[26]. A quantity of the fermentation broth was centrifuged at 7000 rpm for 15 min, the supernatant discarded and the pellet dried at 60°C for 48 h and subsequently ground to obtain a fine powder using liquid nitrogen. The powder was mixed with 50 volumes of chloroform and kept at 30°C for 48 h, before being centrifuged at 7000 rpm for 10 min to remove the non-PHB cell material. The clear polymer solution was recovered using 0.45 µm filters. A mixture of methanol and distilled water, 7:3 (v/v), was mixed with the clear polymer solution, using five times the volume of chloroform. Purified PHB was obtained by filtering and drying at 60°C.

2.12. Biopolymer identification and characterisation

2.12.1. Nuclear magnetic resonance spectroscopy (NMR)

PHB samples were dissolved in spectrochem grade deuteriochloroform (CDCl₃) to a concentration of 1 mg/ml. The ¹H NMR spectra of the samples were obtained at 400 MHz by using a Bruker model advance 400 NMR spectrometer [27].

2.12.2. Thermal analysis using Differential Scanning Calorimetry (DSC)

The thermal properties of PHB samples were determined by DSC (Perkin Elmer, Inc., USA) equipped with Intracooler 2P. Samples of 5 mg were placed in aluminium pans and loaded into the sampler and two scan cycles of heating and cooling conducted across a temperature range from –25 to 200°C with a scanning rate of 10°C/min. The information of enthalpy of fusion (ΔH), melting temperature (T_m) and crystallinity temperature (T_c) were determined from the second cycle. The crystallinity of PHB samples was calculated as:

$$x_c = \Delta H / \Delta H_0 \times 100$$

(2)

where: X_c is the degree of crystallinity, ΔH is the crystallization enthalpy of the PHB sample and ΔH_0 corresponds to 100% crystalline PHB, taken to be 146 J/g, [28].

2.12.3. Gel permeation chromatography (GPC)

The sample solution was prepared by adding 20 mg of PHB sample to 10 ml of CHCl_3 and filtering with a 0.2 μm polyamide membrane filter. A PL-gel guard plus two mixed bed-B columns were used with a flow-rate of 1.0 ml/min at 30 °C.

3. Results and discussion

3.1. Date seed oil extraction

The extraction of date seed oil was investigated and recovery maximised by testing various types of organic solvents, particle size, temperatures and extraction time, to give understanding of the baseline oil yield achievable and of the fatty acid content. In this paper, the feasibility of date seed waste valorisation via PHB production utilising extracted date seed oil is demonstrated. It is, however, critical to the environmental performance of the bioprocess that future scale up is based on the use of green solvents, in line with industrial trends towards reduction in organic and chlorinated solvent use.

3.1.1. Effect of solvent

Date seed oil was extracted using three different solvents, hexane, methanol/chloroform mixture (MCM) and petroleum ether (PE), in order to study the effect of the solvent on the oil yield. For each solvent, an extraction time of 2 h and a particle size of ≤ 1 mm were selected and the percentage of oil yield was calculated, see Fig.1. A. The results indicate that a maximum oil yield of 9.3 % was obtained using MCM, followed by hexane, 5.38 %, and PE, 2.96 % at 120°C, 160°C and 180°C respectively, depending on the boiling point of each solvent. The oil yield obtained using MCM was 1.8% and 3.2% greater than the yields for hexane and PE, respectively. Sayyar et al., [29] report that the jatropha

seed oil extraction yield using hexane was 1.3% more than for PE under the same conditions, in agreement with the yields observed in this study.

3.1.2. Effect of particle size

The effect of two different particle sizes, ≤ 1 mm and 2-4 mm, on the oil extraction rate using different solvents is illustrated in Fig.1.A. The oil yields for all types of solvents are lower, by nearly half, for a particle size of 2-4 mm as compared to a particle size of ≤ 1 mm, indicating that the extraction rate from fine date seed particles is higher compared to that from coarser particles. The lower extraction rate for the larger particle size was expected, due to the lower total surface area. The solvent has the ability to penetrate into the core of the seeds to extract the oil and this is restricted in the case of the larger particles [29]. Sulaiman et al., [30] found that the rate of oil extraction from rambutan kernel seeds increases with decreasing particle size during oil extraction. The same result was obtained by Ebewe et al., [31] when they extracted oil from peanuts, who found that when the particle size was reduced from 3.35 – 4.75 to 0.86 – 1.19 mm, the total oil yield was increased from 36% to 82%. The results confirm the effect of the particle size on extraction processes for by-product materials, namely that the grinding step enhances the recovery of oil by increasing the surface area in contact with the liquid fraction.

3.1.3. Effect of temperature on oil yield

Oil extraction was carried out across a range of temperatures from 80°C to 180°C, depending on the solvent used and its boiling point. The impact of varying temperature on the date seed oil yield is presented in Fig.1.B. It was observed that the date seed oil yield increased with increasing temperature and oil yields of 9.3%, 5.38% and 2.69% were obtained at 180°C, 160°C and 120°C and using a MCM, hexane and PE respectively.

3.1.4. Effect of extraction time

Extraction of oil from the date seeds of ≤ 1 particle size was carried out at 100°C, 140°C and 160°C with MCM, hexane and PE. The extraction data are plotted in Fig. 1.C, as total oil recovery against time. Fig.1.C shows that the oil yield increases with time for all the solvents tested and that the rate of extraction is high during the first three hours. The maximum quantities of oil recovered were 9.3%, 5.4% and 3.5% with the MCM, hexane and PE respectively, when the extraction process lasted for four hours. The high initial extraction rate was due to the mass transfer driving force being large when the oil concentration in the fresh solvent was low. The extraction rate subsequently decreased with increasing oil concentration in the solvent, due to the reduction in mass transfer driving force [32].

Finally, the extraction process reached an equilibrium point when the highest amount of oil was obtained and remained constant without significant change, these results agree with Liauw et al., [33] and Sulaiman et al., [30]. In Fig.1.C a non-linear least square fit method was used to fit the oil yield data for different solvents, to determine the mass transfer coefficient, see Table 1. From the results, it can be seen that the oil yield is lower compared to oily crops, because of the lower oil content of date seeds. However, as part of a wider waste valorisation strategy it is important to maximise added value by extracting all fermentable carbon sources from the date seed waste [20]. There are also opportunities to use edible date seed oil in pharmaceutical and cosmetic products [34] and [18].

3.1.5. Date seed oil analysis

The fatty acid composition of date seed oil was analysed using GC-MS with the free fatty acid composition shown in Fig. 2. The results show that oleic and lauric acid are present in the highest amounts. According to Nehdi et al., [35] the date seed cultivars in United Arab Emirates can be classified as oleic-linoleic or oleic-lauric oil depending on the two major fatty acids present. Iranian date seeds cultivars yield oleic-lauric oil, whilst linoleic acid was present in the lowest quantity among the five major fatty acids. The third most abundant fatty

acid in the study by Nehdi et al., was linoleic acid, while Biglar et al., [36] reported myristic acid as a third major fatty acid. The analysis presented here is in broad agreement with studies of other varieties of date palm, such as Akbari et al., [37]. Generally, the major fatty acid composition in date seed oil is different across date varieties and climate conditions.

3.2. *C. necator* Growth and PHB production in shake flasks with date seed oil

In order to demonstrate the technical feasibility of using date seed oil as a substrate for PHB production a series of small-scale shake flask batch fermentations using *C. necator* in media containing 20 g/l of date seed oil as a carbon source were conducted. Experiments were carried out in triplicate and the average values of dry matter, PHB concentration and content production and total nitrogen are shown in Fig. 4. It was observed that *C. necator* is able to grow on non-emulsified date seed oil and produce PHB. It was also observed that the oil gradually emulsified as *C. necator* grew, though the mechanism through which this emulsification happens is unknown. Many bacteria are known to secrete surfactants [38] though there is no evidence that *C. necator* produces these types of emulsifiers. Through the breakdown of triacylglycerol (TAGs), such as free fatty acids (FFAs), monoglycerol or monoethylene glycol (MAGs) and diacylglycerol (DAGs) it is possible that polar lipids released and can themselves emulsify the date seed oil. Furthermore, some studies report that the putative lipase, encoded by gene H16-A1322 (Gene ID: 4249488), is crucial for robust growth of *C. necator* on non-emulsified media [8].

Different concentrations of date seed oil, 5 to 25 g/l, were tested and the specific growth rate, μ , was calculated for each concentration. The results show that the cells grew fastest during the first day of cultivation and gave a maximum value for μ of 0.38 h^{-1} when 20 g/l initial oil concentration was used (Fig. 3). The μ of the bacteria cell was increased by increasing the oil concentration up to 20 g/l and then a slight decrease was observed at 25 g/l. As seen in Fig.4, the initial cell growth (optical density) was similar for all oil types tested. At

48 hr the OD for the date seed oil fermentation reached 18.8, while OD in the other two fermentations were 15.8 and 12.9, for vegetable and sunflower oil, respectively. The total cell dry weight for date seed, vegetable and sunflower oils were 14.35 g/l, 12.1 g/l and 10.2 g/l, respectively at 48 hrs (Table 2). The PHB content (amount of PHB in dry pellet) for all three fermentation experiments reached a maximum value at 48 hrs. A slight decrease in PHB levels was noticed after this time, most likely due to PHB utilisation as a carbon source. The PHB production curves were found to be distinctly different for all oil types. The most PHB was produced in the fermentation with date seed oil, 11.8 g/l, achieved after 48 hrs, while only 9.1 g/l and 7.4 g/l were synthesised after 48 hrs using vegetable and sunflower oil as the carbon source. Correspondingly flasks fed date seed oil a PHB content of 82% was reached after 48 hours, while the PHB content was 75% and 72% for vegetable and sunflower oil. The higher PHB accumulation in experiments with date seed oil may be ascribed to the fatty acid composition of date seed oil, as described in section 3.1.1, which may mean that the fatty acids in date seed oil making are more accessible and simpler for the microorganism to metabolise [8].

In this study, the PHB production was low in comparison to *C. necator* batch fermentations with using different oils, performed by [39] and [40]. They achieved PHB concentrations between 6 to 11 g/l in 25 hrs using mineral medium and the conditions described by [41]. The medium used contained both extracted nutrient components and salts, which may explain the increased PHB production. The amount of nitrogen available in the media plays an important role in the trade-off between cell growth and PHB synthesis [42]. For all three substrates used, date seed, vegetable and sunflower oil, the initial total nitrogen concentration was around 65 mg/l and about 70% of the total amount was consumed to reach the steady state at 48 hrs of 23 ± 0.8 mg/l for both vegetable and sunflower oil, while being slightly lower for the date seed media of 16 mg/l. The PHB accumulation mechanism has been described as a

de novo (production of complex molecules from simple ones) route in that fatty acids will be transformed into acetyl-CoA by oxidation cycles [43].

According to the fatty acid analysis for both vegetable and sunflower oil, it was observed that unsaturated fatty acids are present in both, much more so than in date seed oil. Therefore; it is possible that saturated fatty acids are more readily taken up and converted to acetyl-CoA than unsaturated fatty acids. Generally, cell growth and PHB accumulation were in the expected range and the %PHB for date seed oil is greater than that achieved by Khan et al., [44] using Jatropha oil and also compares favourably to those reported by Kamilah et al., [9] using waste cooking oil.

3.3. Biopolymer Characterisation

3.3.1. NMR analysis

NMR was used to determine the structural composition of the PHB synthesised from date seed oil as a carbon source. In Fig.5, the ^1H NMR spectra shows chemical shifts at 1.2, 2.4, 2.7 and 5.3 indicating the presence of a proton in position 3, 2 and 1 respectively, which represent the methyl (CH_3), methylene (CH_2) and methine (CH) groups, while the chemical shift at 7.3 is due to chloroform. The molecular composition of the PHB as indicated by the chemical shifts, generates a backbone structure ($\text{CH}_2\text{-CH}$) attached to a CH_3 group [45], [46]. The results in the present study match results reported by Pal et al., [47] who found that the spectra of CH_3 at 1.2 ppm, CH_2 at 2.6 ppm and 5.3 ppm for CH group. Saruul et al., [48] identified that the peak values are at 1.2, 2.5 and 5.2 ppm for CH_3 , CH_2 , CH groups, respectively.

3.3.2. Thermal analysis using Differential Scanning Calorimetry

The PHB samples obtained from date seed oil were analysed using differential scanning calorimetry (DSC) to study the thermal properties. The PHB tested was a crude

product obtained via a single chloroform solvent extraction. The PHB extraction step gives purity in the range 80-90%, with little degradation [49] and [24].

DSC curves of PHB samples were obtained from two scan cycles of heating and cooling, conducted across a temperature range of -25 to 200°C at a scanning rate of $10^{\circ}\text{C}/\text{min}$ (Fig.6). During the first heating cycle, the melting peak for the PHB sample was at 171°C and no glass transition point was observed. During the cooling process, a crystallisation peak appeared at 95°C . During the second heating run the PHB sample shows a double melting peak, corresponding to the melting of “reformed” and re-crystallised PHB. Furthermore, there is a significant decrease in melting point compared with that of the first heating run, likely due to chain scission degradation of PHB. The peak representing the PHB melting point in the second scan appeared at 163°C , the corresponding crystallisation temperature was 88°C and the degree of crystallinity was 65%. These results agree with Rohini et al., [25] who reported that the melting temperature of pure PHB is around 160 - 180°C whilst the crystallization temperature and degree of crystallinity are 90 - 120°C and 50 - 70% , respectively. The results for PHB produced from date seed oil in this study agree with those reported for standard PHB.

3.3.3. *Molecular weight*

The weight average molecular weight and polydispersity of PHB produced using date seed oil as the sole carbon source were 2.157×10^6 Da and 8.813, respectively. As there exist a large variety of PHA structures the weight average molecular weight can vary from 10×10^4 to 10×10^6 [52]. This variation is a result of the microorganism type, growth conditions and PHB extraction method. The polydispersity of PHA samples is usually in the range 1.2 – 6.0. The higher value here indicates an increased degree in the variation in the PHB sample, likely due to the solvent extraction procedure used for recovery of the intracellular PHB.

4. Conclusions

The wider use of PHB is hampered by the high cost of PHB synthesis, which is mostly due to the expensive substrates which are used. In general, oil derived from waste materials is lower in price compared to pure substrates, such as edible vegetable oils, though both can be used for microbial growth and PHB production. Therefore, in this study, oil extraction from date seeds under various conditions and the potential of using the recovered oil as alternative carbon source were investigated. The results demonstrate that *C. necator* is capable of utilising date seed oil as a sole carbon source and synthesising PHB to a concentration of 11.77 g/l, an accumulation of 82%. Having demonstrated that oil recovered from date seeds can be metabolised by *C. necator* and PHB produced, there is a need to identify oil extraction routes based on green solvents, such as ionic liquids or deep eutectic solvents, to avoid the use of large amounts of organic, chlorinated solvents at scale. From the thermal analysis, it was observed that the biodegradable PHB produced from date seed oil, is highly crystalline with a melting temperature of 171°C. The feasibility of using date seed waste oil as an alternative carbon source to produce meaningful amounts of PHB has been demonstrated.

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Figure captions

Fig.1: Kinetic study of date seed oil extraction (A) effect of date seed particle size (≤ 1 and 2-4 mm) on the oil yield using three different types of solvents. (B) and (C) effect of temperature and extraction time, respectively, on oil yield using particle size ≤ 1 mm,

Fig.2: The fatty acid composition of date seed oil extracted using three different solvents.

Fig.3: Variation of specific growth rate as function of initial of date seed oil concentration.

Fig.4: Time profiles for *C.necator* shake flask experiments with 20 g/l of (A) date seed oil, (B) vegetable oil, (C) sunflower oil. Incubation conditions were 30°C, 200 rpm. ■ OD (600 nm), ● Residual biomass, ◆ PHB content, ▲ PHB concentration, ▼ TN concentration

Fig.5: ^1H NMR of PHB accumulated by *C. necator* grown on date seed oil.

Fig.6: DSC heating and cooling curves for a PHB sample produced from date seed oil, two scan cycles were conducted within a temperature range -25 to 200°C , scanning rate $10^\circ\text{C}/\text{min}$.

Table 1. Parameters fitted and comparison of mass transfer coefficient for oil extraction from different materials using soxhlet extraction.

<i>Raw material</i>	<i>Solvent types</i>	<i>T</i> (°C)	<i>Yield</i> (%)	<i>K.a</i> (s ⁻¹)	<i>Reference</i>
Solid coconut waste	Hexane	80	21.9	0.386×10^{-3}	[30]
Jatropha seeds	Acidic Hexane Solutions	60	21.24	0.13×10^{-3}	[43]
Sunflower seed	n-Hexane	60	25.42	0.107×10^{-3}	[51]
Neem	n-Hexane	50	44.29	7.29×10^{-3}	[33]
Date seeds	MCM	160	9.3	0.78×10^{-3}	This work
	Hexane	140	5.3	0.59×10^{-3}	This work
	PE	100	3.4	0.46×10^{-3}	This work

Table 2. Overview of PHB production by *C.necator* in shake flasks batch fermentation includes oil types, key results for polymer (PHB) accumulation and reference.

<i>Reference</i>	<i>Carbon source</i>	<i>Maximum Biomass concentration</i> (g/l)	<i>PHB concentration</i> (g/l)	<i>PHB content</i> (% wt/wt)	<i>Fermentation type</i>
[44]	Jatropha oil	11.6	8.6	74	Shake flask
[50]	Soybean oil	15	13	86	Shake flask
[9]	Waste cooking oil	15	10.7	72	Shake flask
This work	Date seeds oil	14.35	11.8	82	Shake flask
	Vegetable oil	12.1	9.1	75	
	Sunflower oil	10.2	7.4	72	