Biosynthesis of Medium Chain Length Poly-β-hydroxybutyrate by *Pseudomonas aeruginosa* Dw7

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Abstract

Polyhydroxya lkanoates (PHAs), are a promising family of bio-based polymers, which considered to be alternatives to traditional petroleum-based plastics. Poly-β - hydroxybutyrate (PHB) is the most known degradable biopolymers, produced by bacterial genera. It is generally accepted that PHB can be used instead of plastic to solve one of the greatest problems facing the environment. *Pseudomonas aeruginosa* Dw7 was grown on mineral salt medium supplemented with waste cooking of corn oil for the synthesis of a medium chain-PHB. This study is divided into three steps, at first fermentation of *P.seudomonas aeruginosa* Dw7, which was carried out aerobically at optimum temperature of 30 ˚C and 500 rpm of agitation speed. Lab scale bioreactor (5L) operated as a batch culture system for 80 hours. The highest cell dry weight (CDW) of bacteria reached to 5.3 g/l observed after 60 h of operation, which was corresponding to 62.6 % of PHB. The results suggested the efficiency of the system for production of PHB in large scale. Many solvent systems were conducted to evaluate the best solvent for PHB extraction from bacterial cell in the second step. Chloroform–hypochlorite dispersion extraction was followed by that extraction with chloroform showed the best solvent system for yielding of PHB. Relatively; it has the high rate with 63% yield of PHB. Characterization study of PHB was the last step. PHB were included chemical, physical; mechanical and solubility properties of the produced PHB have been characterized by many analyses techniques, comprising Gas Chromatography Mass Spectrometry (GC-MS), Fourier-transform Infrared Spectroscopy (FTIR), X-Ray diffraction (X-Ray) and melting point. PHB was a yellowish white crystal, soluble in chloroform and other chlorinated hydrocarbons like dichloromethane, dichlorethane and chloropropane. X-Ray diffraction (XRD) study was carried out to check if the polymer had a crystalline or amorphous structure. The increased intensity of peaks showed that the polymer had more organized packed crystalline structure. The results of the GC-MS recorded that there were 9 different active peaks predictive. The two major compounds that were produced by *P. aeruginosa* Dw7 were then identified as undecanoic acid which eluted at 15.4 min and tridecanoic acid at 17.3 min with relative abundance of 100% and 33.14% respectively. The characteristic FTIR peaks for PHB indicated that the most prominent marker band for the identification of PHB is the ester carbonyl band at C=O, OH and C-O.

Keywords: *Pseudomonas aeruginoa*, Poly-β- hydroxybutyrate, Bioreactor, Extraction with solvents.

Introduction

PHAs can be completely degraded within a year by a variety of microorganisms into CO_2 and water. Plastic materials that have been universally used in our daily lives are now causing serious

environmental problems. Millions of tons of these non-degradable plastics accumulate in the environment per year. For efficient management of used-plastic materials, recycling is one solution. Another solution is to reduce plastic residues is the use of biodegradable plastics (Yves *et al*., 1995). Hence, replacement of nonbiodegradable with biodegradable plastic from organic waste can provide multiple benefits to the environment and contribute to sustainable development (Du and Yu, 2002). In recent years, there has been significant increase in the agricultural and food processing industries. Hence, large amount of waste materials from these industries are being discarded each year. These waste materials could be used as the carbon feedstock for the PHA producing organisms. Therefore, by using waste effluents of agricultural and food processing industries would not only decrease the PHA production cost but also solve the problem of waste management (Yu and Chen, 2006).

Various bacterial strains capable of producing PHA while degrading oil have been isolated belonging to genera *Pseudomonas*, *Acinetobacter*, *Sphingobacterium*, *Brochothrix*, *Caulobacter*, Ralstonia, Burkholderia, and Yokenella from oil contaminated sites (Chee *et al*., 2010). furthermore, there are some studies which show that vegetable oil could significantly improve PHA production compared to sugars, which are normally used for the PHA accumulation by various bacteria. So the aim of this project is to produce a cheaper biodegradable plastic using oil wastes as a carbon sources by local bacterial isolates. Where it also can help in managing waste disposal in Iraq.

Material and methods:

Microorganism, preparation of vegetable oil and measurement of cell dry weight:

The microorganism was maintained at 30 °C on a solid culture medium containing (per liter): 10g trypton, 5g yeast extract, 10g NaCl and 15 agar in distilled water, and pH was adjusted to 7.

Vegetable oil was obtained from a local supermarket. Waste cooking oil was provided by deep-fries for many times in an open container at 180°C (Costa *et al*., 2001). Oils were sterilized separately in a steam autoclave and added directly to liquid medium.

After incubation, samples were centrifuged for 15min at 6000 rpm. The pellet were washed twice with sterile deionized water and dried for 24 h at 60°C. The total bacterial dry weight was determined as described by Yuksekdag *et al*., (2004). Yield of PHB accumulation (%) = Dry weight of extracted PHB (g/l / DCW (g/l) × 100 % (Indira *et al*., 2014).

Inoculum and culture medium preparation

The standard inoculum was prepared in a conical flask (250 ml) containing 50 ml of nutrient broth medium inoculated with a loop of *P aeruginosa* Dw7 and incubated at 30°C with shaking (150 rpm) for 24 h (6×10^8 cfu / ml), prior to inoculate lab scale bioreactor (Gamal *et al*.,2011).

PHB production in batch bioreactor

PHB production was carried out in 5 L lab scale bioreactor under controlled system as shown in figure (1). The standard inoculum to inoculate the bioreactor vessel to give a final working volume of 3.5 L of fermentation medium, which was prepared as in last experiment of optimization in flask and autoclaved with the bioreactor at 121°C for 15 min. Initial pH was adjusted to 7.0±0.1, which was controlled during the fermentation period. In addition, sterilized probes were inserted into ports to measure dissolved oxygen, pH and temperature. Temperature, dissolved O_2 and speed of agitation were kept at 30°C, 20 % of air saturation and 500 rpm, respectively, during 80 h of cultivation.

Samples of 25 ml were withdrawn from the liquid culture (fermentation vessel) periodically for analytical purposes. The samples were centrifuged as mentioned previously above to calculate CDW and PHB yield %.

Extraction and purification of PHB using different solvents:

Extraction by acetone and chloroform

A one g of dry cells powder was washed with 25 ml of hot acetone for 20 min. After drying, the cell powder was mixed with 50 volumes of chloroform for 48 h at 30°C. The clear polymer solution was recovered by centrifugation to remove the majority of the non-PHB cell material; finally, pure biopolymer was obtained by precipitation with a mixture of methanol and water 7:3 (v/v) and filtration (Hahn *et al*., 1995).

Extraction of PHB with dispersions of hypochlorite solution and chloroform

A sodium hypochlorite solution was diluted with distilled water to give a concentration of 3 % (v/v). A one g of dry cells powder was mixed with 100 ml of hypochlorite solution for 1 h at 30°C. The digested cell material was then recovered by centrifugation at 6000 rpm for 10 min. After centrifugation three phases were appeared, the upper phase represented the hypochlorite solution, the middle phase contained the non PHB cell material and undisrupted cells, while the bottom phase was chloroform containing PHB .The bottom phase was carefully removed by separating funnel and the PHB was recovered by precipitation with hexane (Hahn *et al*., 1995).

Extraction by using chloroform

A one g of dry cells powder was grounded in a mortar, and the resulting powder was extracted with 50 ml of chloroform for 4h at 50°C. The PHB-containing chloroform phase was concentrated and extracted once with water to remove residual solid particles. The organic phase was evaporated to dryness and the resulting crude extract preserved for further analyses. Purified PHB was obtained by repeated precipitations in 10 volumes of cold methanol (Simon-Colin *et al*., 2008)**.**

Extraction by sodium dodecyl sulfate (SDS)

A one g of dry cells powder treated with 10 % SDS at 100 °C for 20 min. After centrifugation, the pellets were washed, dried and extracted with chloroform at 60°C for 1 h. The non-PHB cell matter was removed by filtration and the dissolved PHB was collected from chloroform by evaporation, washed twice with methanol, filtered out and dried at 60 – 70°C (Jiang *et al.*, 2008).

Characterization of PHB

Physical characteristic of PHB

Solubility in different solvents

Solubility of PHB in different organic solvents is important in order to choose the most suitable one for recovery of the biopolymer from microorganisms. PHB with the highest purity was used as the starting material. Equal volumes of the non-halogenated solvents (Water, Diethyl ether, Ethanol, Ethyl Acetate, Methanol, and Propanol) and halogenated solvent such as (Chloroform, Dichloromethane, Dichlorethane and Chloropropane) were added to PHB in sealed test tubes, by dissolving (1 mg) of crude extract of PHB in 5 ml of each solvent.

Nature and melting point

Pure, crystalline solids have a characteristic melting point, the temperature at which the solid melts to become a liquid. The transition between the solid and the liquid is so sharp for small samples of a pure substance that melting points can be measured to 0.1^{o}C.

The melting point of PHB was determined by a melting point apparatus (Stuart /England).

Chemical characteristic of PHB

FTIR analysis of PHB

One mg of PHB was grounded well with 10 mg of spectral pure anhydrous potassium bromide crystals to prepare KBr pellet. The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400 – 4000 cm-1. IR spectra of the PHB sample was measured at ambient conditions (Reema *et al*., 2013) and recorded on a Shimadzu-IR affinity-1

Spectrophotometer. The spectra were plotted as intensity versus the wave number. This analysis was carried out in central environmental laboratory/Baghdad University.

Gas Chromatography Mass Spectroscopy (GC-MS) analysis:

The analysis of the Poly-3-hydroxybutyrate polymer extracted from the bacterial isolate was carried out in a gas chromatograph mass spectroscopy instrument using following operational conditions and steps:

1.) DB-5 Capillary column. (The length of the column is 30 meter length, the internal diameter is 0.25 mm and the column is coated with a 0.25 micrometer film with split injection ratio of 1:15).

2.) Helium was used as a carrier gas.

3.) The flame ionization detector (FID) unit at 300 ºC with an injection temperature of 250 °C.

4.) The oven temperature was set at 80ºC for 1min. Increased 10°C per minute to 120ºC, and then to 270°C by increasing 45°C per minute, then held for 3 minutes.

5.) Then the sample and standard was run to aid in identification (Oehmen *et al*., 2005).

X- Ray diffraction (XRD)

Polymer produced was made on the cover slip using 100 µl of 5 wt % polymer solutions in chloroform. Hence prepared films were then used to analyze the crystallinity of the produced polymers for X-ray diffraction (XRD) using an X-ray diffractometer (Shimadzu, model XRD-7000) equipped with a Cu tube. Cu Kα radiation (l = 1.54 A˚) operating at 40 kV and 40 mA was used for this investigations. Scans were performed with a detector step size of 0.02 over an angular range 2θ = 10-100º and counting for 1 second per step **(**Artur *et al.*, 2016). The analysis was carried out at the Directorate Laboratories of Materials Science at the Ministry of Science and Technology.

Fermentation

The main fermentation strategies used to obtain bio-products are batch culture, fed-batch culture, continuous culture and two-stage fermentation, to evaluate the performance of the isolate *P. aeruginosa* Dw7 and determine better growth parameters. The isolate was grown in controlled batch reactor. Bioreactor cultivation as a batch culture offers many advantages for production, provided that contamination is avoided and the stability of the strain is guaranteed. The advantages include simplicity of culture control, homogeneity of the production, and constancy of culture conditions (Lillo and Rodriguez-Valera, 1990).

This experiment was performed in a 5 L reactor with 3.5 L of working volume and sterile conditions were maintained (Fig 1). The medium used contained 3% (v/v) of corn cooking oil waste in MSM. The reactor was operated at 30 ºC for 80 h of incubation. Dissolved O_2^- and speed of agitation were recorded at initial reading, 20 % of air saturation and 500 rpm, respectively. The pH was maintained at 7.0±0 by the automatic addition of 2M HCl and 2M NaOH. Foam did not constitute a serious problem in this experiment and this may be due to that vegetable oils and long chain fatty acid are normally best as deaerators, which means they are best at releasing entrained air.

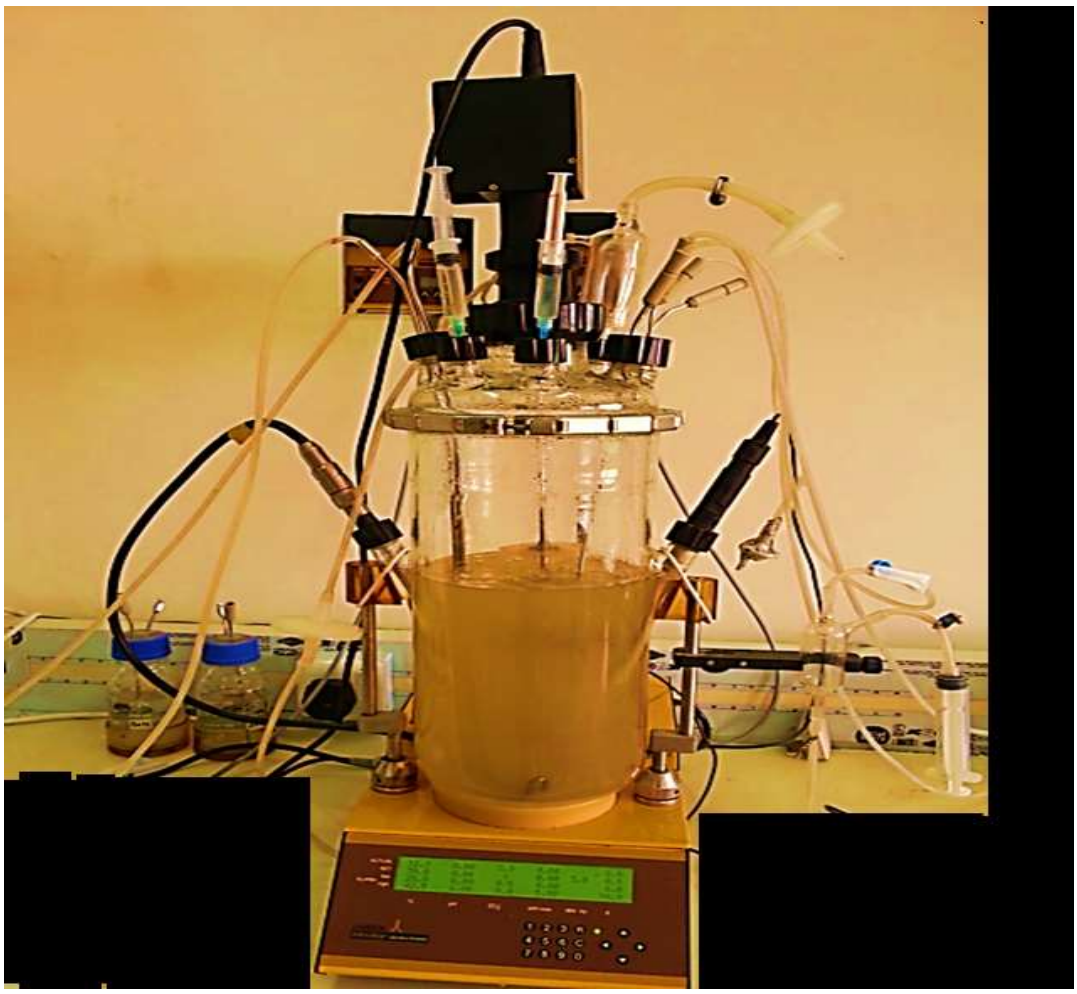

Figure 1. Bioreactor of 5 litter volume as a batch fermenter with medium growth after inoculation

Results and discussion

Bacterial growth and Biomass

Twenty-five ml sample was taken periodically during approximately 20 hours. DCW (g/l) was monitored immediately after sampling. The results obtained regarding PHB content g/l, CDW g/l and yield of PHB % are shown in (Fig 2).

Exponential phase started just first 20 hours after inoculation. There is an interesting relationship between the biomass and PHB content. Since PHB is accumulated in the cytoplasm, the biomass determines how much PHB can potentially produce. Cell growth and PHB accumulation need to be balanced, thus avoiding incomplete PHB accumulation at low cell concentration.

Cells reached already the decline phase and PHB concentration started to decrease as the isolate switches to PHB storages reserve. As shown in (Figure 1) gradual cell growth increase was observed as slow progress in the DCW and PHB was observed until 60 hours of the fermentation. A decline in the dry cell mass was noticed at the 80^{th} hour of the fermentation.

The maximum dry cell weight and PHB yield achieved were 5.34 g/l and 3.35 g/l respectively, yielding 62.2% PHB content. It is important to decide when to terminate the fermentation and in most cases, fermentation is stopped when productivity is the highest (Lee, 1996).

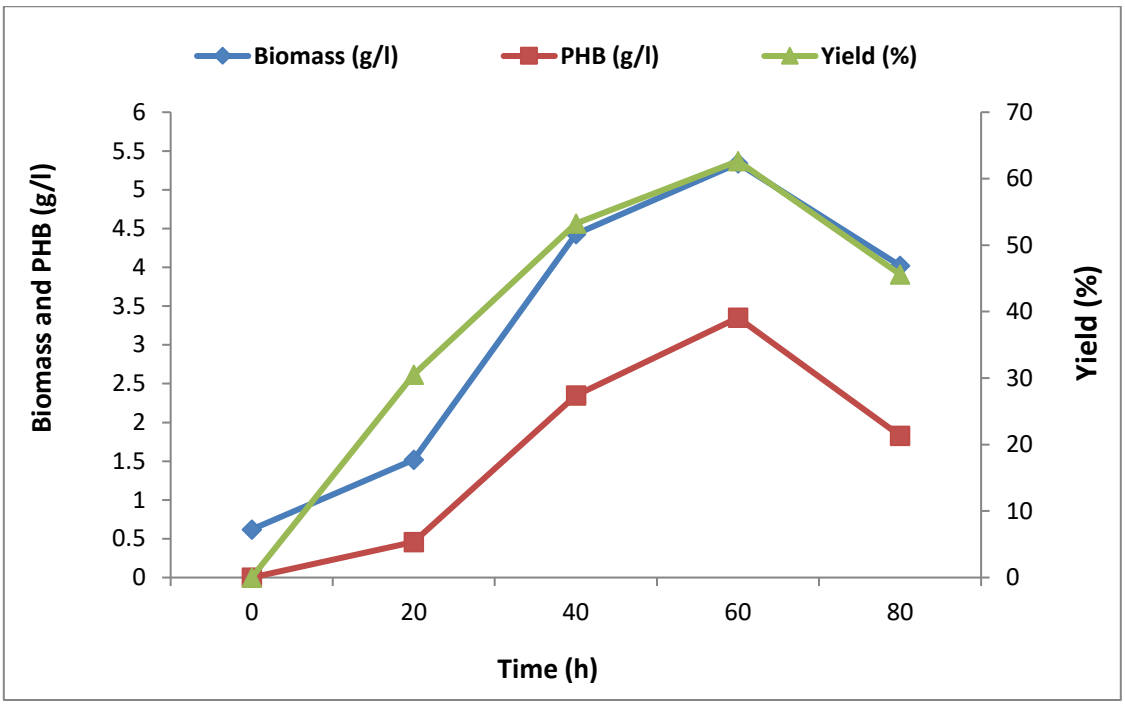

Figure 2. Growth of P.aeruginosa Dw7 isolate and PHB production during 80 hours of incubation at 30 ºC using a bioreactor as batch culture

As the biomass increases the bacterium also starts accumulating PHB and produces maximum PHB when its biomass is at its peak level and PHB production is slowed down as the biomass is dropped, because at this phase of the growth all the nutrients are depleted leading to decrease in PHB content (Ramsay *et al.*, 1990). Different fermentation techniques were applied in order to increase the PHAs yield using waste frying oil (WFO) as the sole carbon source. In batch culture, the cell mass increased gradually leading to record the maximum values of cell dry weight and polymer concentration (2.93 and 0.92 g/L, respectively) after 72 h of cultivation. Whereas, the maximum PHAs content (33.7 %) was obtained after 60 h fermentation period (Gamal *et al.*, 2012). The production of PHB by *Pseudomonas fluorescence* was examined using bioreactor as one-stage batch. Corn oil, soybean oil (extracted from their meal) and two types of waste frying oils (WFO) were used in productive medium as a carbon source. The highest value of polymer content in the one-stage bioreactor fermentation (52 %) was obtained after 60 h on medium supplemented with extracted corn oil as a carbon source (Gamal *et al.*, 2012).

Extraction and characterization of PHB

Extraction by acetone and chloroform

Extraction with hot acetone and chloroform showed PHB yield of 40% of cell dry weight as shown in Table (1).

Extraction of PHB with dispersions of hypochlorite solution and chloroform

One of the most important prerequisites for an industrial strain for PHB production is how easy PHB can be extracted from non-PHB cell matter (Suriyamongkol *et al.*, 2007). Although several new downstream processes for the extraction of PHB have been reported as economically effective, such as the application of surfactants and the dispersions of hypochlorite solution and chloroform, solvent extraction methods are still regarded as an adequate way to gain intact polymer with high purity and recovery yield.

Extraction of PHB sample from *P. aeruginosa* Dw7 by sodium hypochlorite and chloroform was very efficient as it gave 3.5g/L, which presented 63% yield of PHB with lower melting point recorded of 175°C (Table 1).

Large scale production using solvents is harmful, and separation of insoluble cell material after extraction is difficult and 100 % extracted PHB is not possible (Choi and Lee, 1999). Sodium hypochlorite is used for the aqueous process. Although the use of sodium hypochlorite significantly increased PHA degradation, polymer purity greater than 95% is achieved (Lee, 1996). The organic solvents were investigated to determine their efficiency to recover PHB and how easy the separation of them from cells debris after extraction could be recovered.

Extraction by using chloroform

Data illustrated in Table (1) shows that the maximum efficiency of solvent recovery of PHA was attained by chloroform–hypochlorite dispersion extraction followed by that extracted with chloroform 60 % yield of PHB.

Extraction by sodium dodecyl sulfate (SDS)

SDS is a detergent that denatures proteins and solubilize lipids in membranes leading to cell lysis. But PHB recovered by SDS is expensive and it causes additive pollutions resulting in high disposal cost. However, polymer recovery by SDS gave the lowest PHB content (39 %). There is still a need to develop and improve these extraction methods further to make the entire processes much simpler and cheaper.

Table 1. Comparison of extraction methods on PHB production and yield

Solvent	PHB extract g/L	Yield of PHB %	Melting point °C
Acetone and chloroform	**2.6**	**40**	**180**
Hypochlorite solution and chloroform	**3.5**	**63**	**175**
Chloroform	**3.2**	**60**	**190**
Sodium dodecyl sulfate	**2.9**	**39**	**180**

Characteristics of extracted PHB

The produced PHB was analyzed and characterized for their chemical and physical nature, using Gas Chromatography-Mass Spectroscopy (GC-MS), and Fourier Transform Infrared Spectroscopy (FTIR), microstructural properties using X-Ray Diffraction (XRD) and melting point determination in addition the evaluation of its solubility in different solvents.

Chemical and physical Properties of PHB

The results of extraction showed that the PHB is a yellowish white crystal (Fig. 3), soluble in chloroform and other chlorinated hydrocarbons like; dichloromethane, dichlorethane and chloropropane. The higher capacity of these solvents for dissolving PHB led to their selection for PHB recovery. PHB, insoluble in water, acetone, ethanol and diethyl ether. mcl-PHAs are soluble in acetone, whereas PHB is not (Abe *et al*., 1994).

PHB exhibited several useful properties such as; moisture resistance, water insolubility and optical purity and has melting point of 175 °C (Table 2).

Figure 3. Extracted PHB crystals from *P. aeruginosa* DW7 by Sodium hypochlorite-Chloroform method

Table 2. Characterization of extracted PHB

Serial no.	Characteristic	Solvents	Results
1	Solubility in	Water	Insoluble
		Acetone	Insoluble
		Ethanol	Insoluble
		Ethyl acetate	Insoluble
		Methanol	Insoluble
		Propanol	Insoluble
		Chloroform	Soluble
		Diethyl ether	Insoluble
		Dichlorethane	Soluble
		Chloropropane	Soluble
		Dichloromethane	Soluble
2	Melting point (°C)		175°
3	Color		Yellowish white
4	Texture		Crystals

However, when the isolate grown on oils, the biopolymer produced was found to be chemically pure PHB from pure oil, heated oil and waste oil (Volova and Kalacheva, 2005). The molecular weights of polymers from waste frying were similar to those from other oils and glucose. Most of the available biodegradable plastics have certain disadvantages since they are water soluble or

moisture sensitive (Lee, 1996). P (3HB) has overcome these problems as it exhibits resistance to moisture, insoluble in water and has better optical purity (Lindsay, 1992).

These differentiate PHB from other currently available biodegradable plastics which are either water soluble, or moisture sensitive (Lndsay, 1992). PHB was synthesized by the fermentation of sugar, saccharose or oil using gram negative bacteria. It is a yellowish white powder and has a molar mass MW of 220 kg/mol, a melting point of it about 173°C and a crystallinity of 55 %.

Another PHB was extracted from gram negative bacteria, was a white powder with a molar mass MW of 230 kg/mol. It has a melting point of about 175°C and a crystallinity of 60 %. The spectra are identical and have the same peaks in FT-IR. The only difference is the color (Gereco and Martuscelli, 1989). However, the less toxic non-halogenated solvents (ethanol, methanol, propanol, ethyl acetate, and butyl acetate) were chosen on PHB extraction by Terada and Marchessault, (1999), which described them as good alternative solvents to chloroform for PHB recovery.

Characterization of PHB by FTIR

Fourier transform infra-red spectroscopy (FTIR) is a routine chemical technique used to study the molecular structure. It can be both quantitative as well as qualitative

Basically, FTIR is used for determining the presence or absence of specific functional groups in reaction mixture. The accumulated PHB was extracted and the purified PHB was submitted to FT/IR analysis. The spectrum gave all the characteristic bands indicative of PHB. The IR spectrum obtained for PHB sample from *P. aeruginosa* Dw7 (Fig. 4).

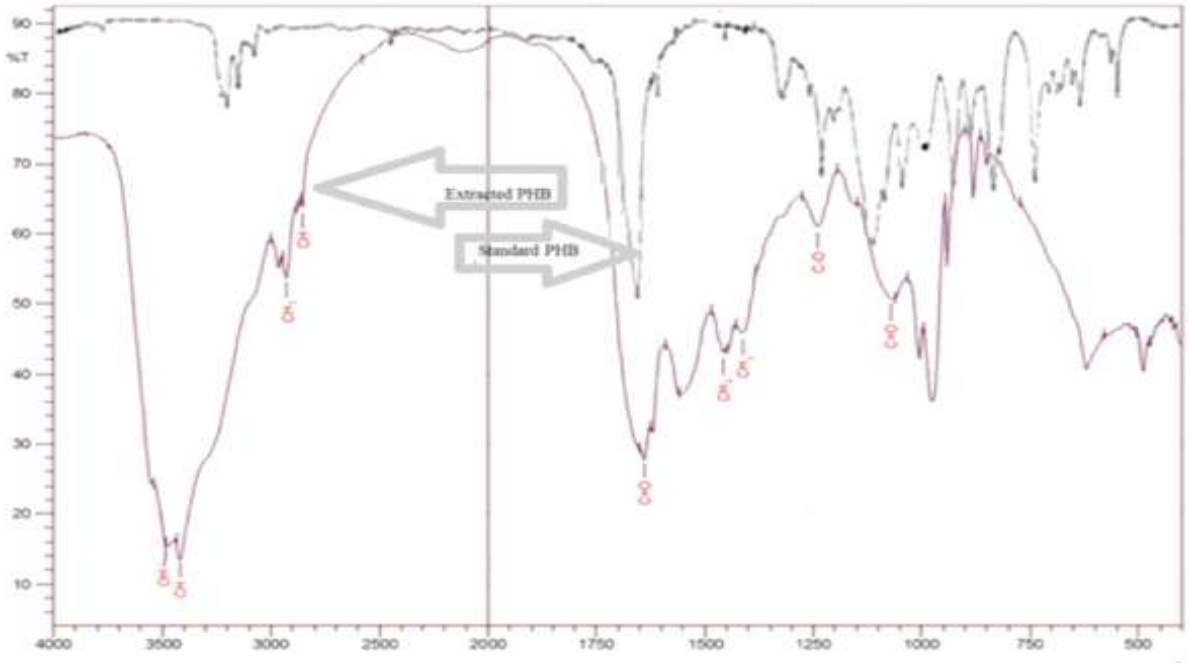

Figure 4. Finally, IR spectrum for standard and extracted PHB of the compounds showed characteristics bands for the groups CH_3, CH, C=O and C-O, and OH indicating the presence of PHB in the production medium

The IR spectra obtained showed characteristic absorption bonds for esters and the presence of C=O and C-O were obtained at 1639.49 cm^{-1} and 1238.3 cm^{-1} respectively. Apart from this a peak at 1411.8 cm^{-1} was seen, which is due to the CH_3 or methyl bending. Peak due to methyl stretching were also observed at 2924 cm^{-1}. CH_2 or methyl group was observed at 1456 cm^{-1} and methine or CH peak was at 3415 cm^{-1}. A band at 1456 cm^{-1} showed the presence of methyl group. Intensity of absorption at 3000 cm^{-1} in relation to intensity at 1720 cm^{-1} was longer.

Absorption at 3000 cm^{-1} indicates longer aliphatic chains. The absorbance band was seen at Vmax 3481, which indicate hydroxyl groups (O-H) in PHB structure; 1070.4 cm^{-1} and 1639.4 cm^{-1} carbonyl group (C=O) functional ester group, 2852.7 cm^{-1} and 2924 cm^{-1} aliphatic (C-H) groups, 1411.8 cm^{-1} and 1456.2 cm^{-1} indicate the presence of methyl (CH_3) group.

These prominent absorption bands confirm the structure of poly-β hydroxybutyrate. The analysis confirmed the presence of the characteristic marker ester carbonyl band for mcl-PHAs which occurs due to C-O stretching (Randriamahefa *et al.*, 2003) as shown at (1238.3 cm^{-1}). This observation thus indicates that these polymers were of mcl-PHB nature in currently study. The bands at 2924 cm^{-1} correspond to the aliphatic C-H group of the polymer backbone (Sánchez *et al.*, 2003). Rapid screening of scl-PHAs such as P (3HB) in lyophilised bacterial cells has been carried out using FTIR. Similarly, rapid detection of mcl-PHAs in intact cells of *Pseudomonas* has also been carried out (Hong *et al.*, 1999). For scl-PHAs the band at 1185 cm^{-1} occurs due to C-O stretching and the band at 1282 cm^{-1} corresponding to –CH group (Kansiz *et al.*, 2000). Similarly, for mcl-PHAs the characteristic marker ester carbonyl band occurs at 1742 cm^{-1} and the band at 1165 cm^{-1} which occurs due to C-O stretching (Randriamahefa *et al.*, 2003). Randriamahefa *et al.*, (2003) used FTIR for rapid qualitative and quantitative analysis of mcl- PHAs in 27 strains of *Pseudomonas* grown in sodium octanoate.

The majority of *Pseudomonas* species that have been examined are devoid of the capability of PHB synthesis. However, *Pseudomonas* species have been found to accumulate mcl-PHAs when the strains are grown in the presence of C4 to C18 fatty acids (De Smet *et al.*, 1983; He *et al.*, 1998). In *P. stutzeri*, the polymer may amount to 52% of the dry weight. After growth in minimal medium with soybean oil, the PHA contains 63% of a novel monomer (3, 6-epoxy-7-nonene-1, 9-dioic acid) and minor proportions of C8 and C10 monomers.

Characterization of PHB by GC-MS

Furthermore, the PHB production of the local *P. aeruginosa* Dw7 was further evaluated by GC-MS. Gas chromatography is very efficient method for quantitative estimation as well as characterization of PHB. Figure (5) shows the GC-MS graph of standard for PHB, which confirmed the presence of undecanoic acid, which eluted at 15.4 min and tridecanoic acid at 17.3 min with molecular formulas of $C_{11}H_{22}O_2$ and $C_{13}H_{26}O_2$. Both peaks were detected in standard and test sample.

GC-MS is a useful technique to determine and understand the molecular structure of any compound. The methyl esters obtained after methanolysis of the sample showed fragmentation patterns in GC-MS (Fig. 6) that enabled to define the structure of PHB obtained from *P. aeruginosa* Dw7.

The molecular fragments and relative abundance obtained are as shown in Tables (3 and 4), which show that the major molecular fragmentation obtained were as follows m/e: $C_5H_{10}O_2$ $C_7H_{14}O_2$, $C_{11}H_{22}O_2$, $C_{10}H_{20}O_4$, $C_4H_8NO_2$, $C_9H_{16}O_2$ $C_{12}H_{22}O_4$, $C_{13}H_{26}O_2$ $C_{16}H_{32}O_2$. According to the results observed, the compounds undecanoic acid and tridecanoic acid were the major components of produced PHB with relative abundance of 100 and 33.14 % (Table 5). All peaks with GC-MS analysis indicated the aliphatic and hydroxylated lipid nature of the PHB structure.

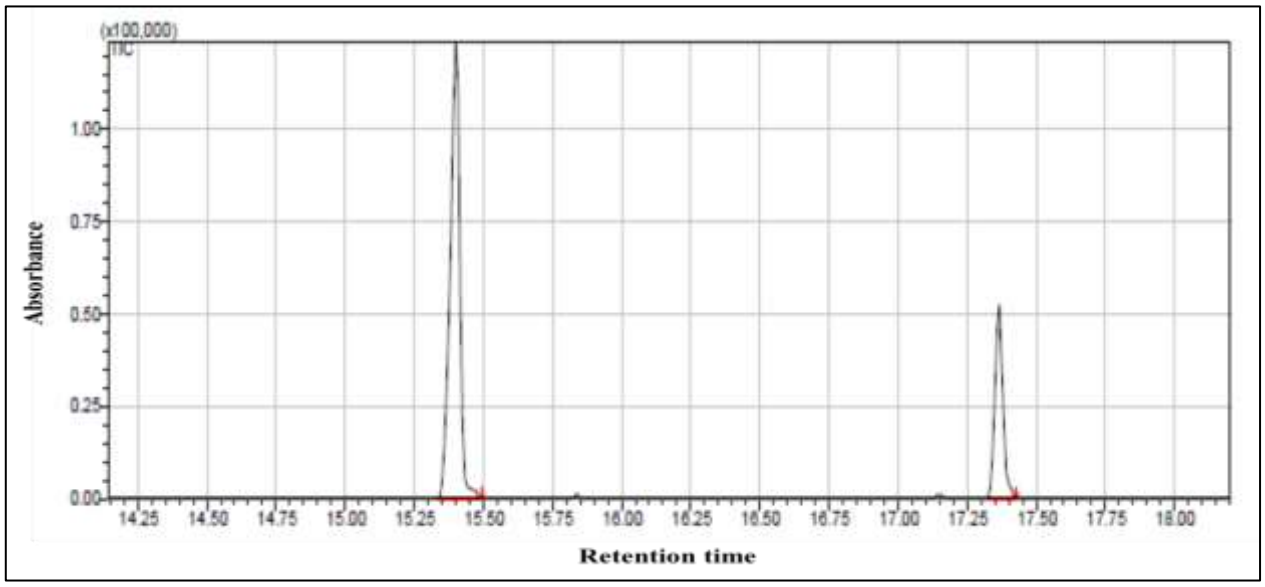

Figure 5. GC-MS graph of standard PHB

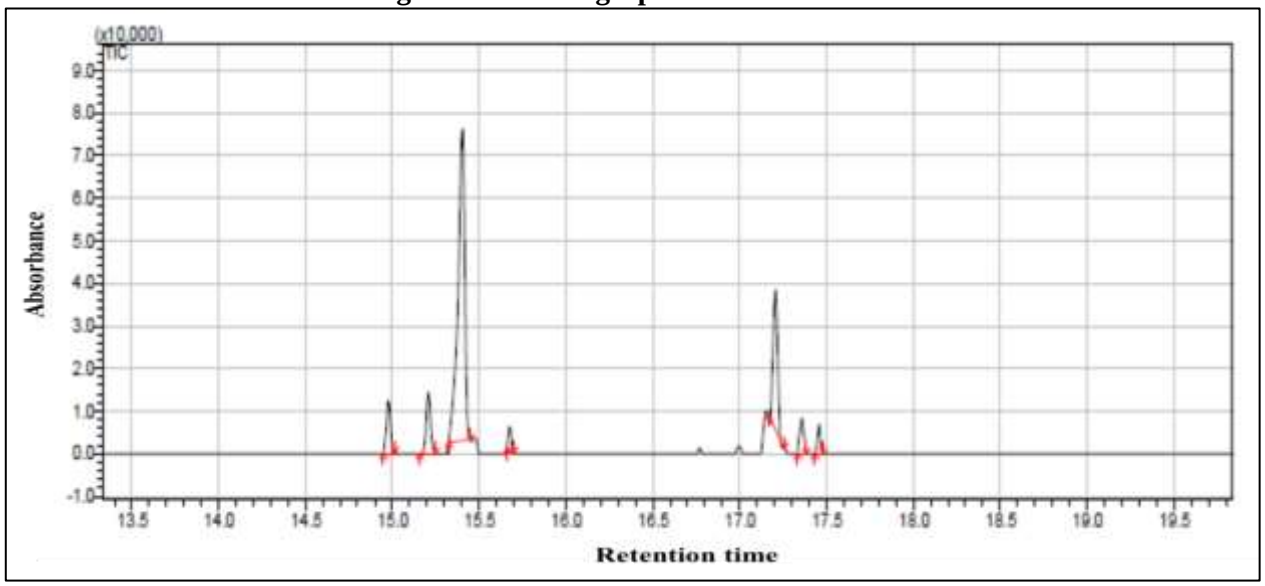

Figure 6. GCMS graph of extracted PHB produced by *P. aeruginosa* DW7

Table 3. The major molecular fragmentation of standard PHB compounds

Molecular Weight	Formula	C number	Name	A/H	Height%	Height	Area%	Area	R Time	peak
86	$C_{11}H_{22}O_2$	C11	Undecanoic acid	2.40	70.35	125040	73.77	300644	15.401	**1**
214	$C_{13}H_{26}O_2$	C13	Tridecanoic acid	2.03	29.65	52695	26.23	106879	17.365	**2**
					100.00	177735	100.00	407523		

Table 4. The major molecular fragmentation of PHB compounds obtained by P. *aeruginosa* Dw7

Mol.Weight	Formula	C Number	Name	A/H	Height%	Height	Area %	Area	R.Time	Peak
102	$C_5H_{10}O_2$	C5	3-Hydroxy-3-methyl-2-butanone	1.66	8.09	12462	6.24	20680	14.978	**1**
130	$C_7H_{14}O_2$	C7	Ethoxy-3-methyl-2-butanone	1.77	9.36	14428	7.69	25485	15.208	**2**
86	$C_{11}H_{22}O_2$	C11	Undecanoic acid	2.72	47.51	73204	52.07	198812	15.405	**3**
204	$C_{10}H_{20}O_4$	C10	4-t-Butoxy-3-hydroxy-butyric acid, ethyl ester	1.28	3.79	5844	2.12	7253	15.677	**4**
88	$C_4H_8NO_2$	C_4	Hydroxy-beta.-oxobutane	1.69	62.94	141497	6.25	23808	16.772	**5**
156	$C_9H_{16}O_2$	C_9	3,4-Hexanedione, 2,2,5-trimethyl-	2.76	2.96	6646	3.79	18317	17.10	**6**
230	$C_{12}H_{22}O_4$	C12	isobutyl hexyl ester	1.63	5.45	32787	4.13	57218	17.207	**7**
214	$C_{13}H_{26}O_2$	C13	Tridecanoic acid	1.76	21.28	8395	17.26	13700	17.358	**8**
256	$C_{16}H_{32}O_2$	C16	Hexadecanoic acid	1.20	4.51	6953	2.52	8346	17.457	**9**
					100.00	154073	100.00	331494		Total

Table 5. GC mass profile of the *Pseudomonas aeruginosa* Dw7 hypochlorite solution and chloroform.

Retention time (min)	Compounds	Molecular formula	Molecular weight (g/mole)	Area%	Relative Abundance (R A)%	Serial no.
14.978	3-Hydroxy-3-methyl-2-butanone	$C_5H_{10}O_2$	102	6.24	11.98	**1**
15.208	Ethoxy-3-methyl-2-butanone	$C_7H_{14}O_2$	130	7.69	14.76	**2**
15.405	Undecanoic acid	$C_{11}H_{22}O_2$	86	52.07	100	**3**
15.677	4-t-Butoxy-3-hydroxy-butyric acid, ethyl ester	$C_{10}H_{20}O_4$	204	2.12	4.07	**4**
16.772	Hydroxy-beta.-oxobutane	$C_4H_8NO_2$	88	6.25	12.00	**5**
17.10	3,4-Hexanedione, 2,2,5-trimethyl-	$C_9H_{16}O_2$	156	3.79	7.27	**6**
17.207	isobutyl hexyl ester	$C_{12}H_{22}O_4$	230	4.13	7.93	**7**
17.358	Tridecanoic acid	$C_{13}H_{26}O_2$	214	17.26	33.14	**8**
17.457	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.52	4.83	**9**

The fragmentation pattern of PHB showed a peak at m/z which originates from the molecule due to the cleavage between carbon atoms following McLafferty rearrangement. The peak at m/z 103 occurred due to the fragmentation ion of the hydroxyl end of the molecule following the cleavage between C3 and C4 carbon atoms; similarly, the alkyl end of this cleavage resulted in the peak at m/z 71 The peak at m/z 43 occurred due to the alkyl end of the molecule following the cleavage between C7 and C8 carbon atoms.

X-ray diffraction Crystallinity

The crystallinity % of PHB was determined using X-ray Diffraction Patterns of PHB films. The XRD studies were carried out to check if the polymer had a crystalline structure or amorphous. On looking at the diffraction patterns of PHB, it shows 2 degree values of 26.50°, 32.34°, 46.59°, 56.52° , 66.50°,75.50° and 84.10° at the intensities 300 a.u., 2800 a.u., 1000 a.u., 200 a.u, 120, 400 and 300. The increased intensity of peaks showed that the polymer have more organized packed crystalline structure (Fig. 7).

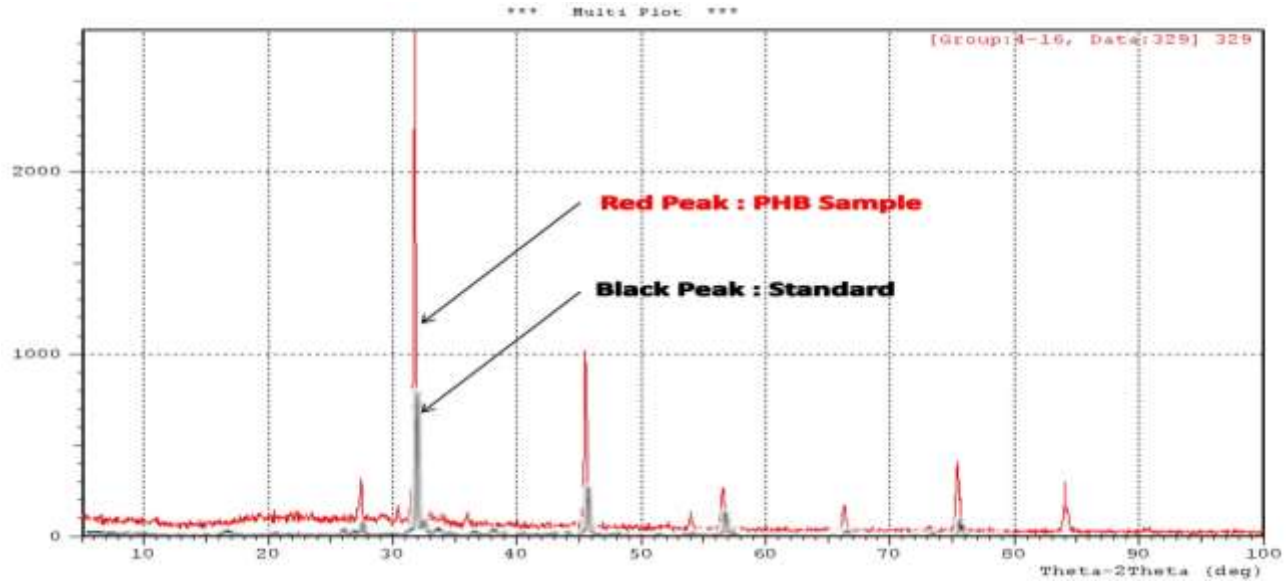

Figure 7. X-Ray Diffraction of standard and PHB *produced by P. aeruginosa* DW7

Conclusion

1-In conclusion, this work established, that vegetable oils exactly so waste cooking oil are a good economical source of carbon for production of PHB polymers effectively by *P. aeruginosa* Dw7.

2 -Infra-Red spectra study indicated the presence of CH, C=O, CH_3, OH and C-O groups in the structure of PHB.

3- GC-MS technique showed that the produced PHB contained nine compounds, of which two major separable components undodecanoic acid and tridecanoic acid with the molecular formulas $C_{11}H_{22}O_2$ and $C_{13}H_{26}O_2$ with relative abundance of 100 and 33.14% respectively. All data indicated that the results match with the researches, which confirm the affiliation of the product to PHB.

4- Optimization of other culture and operational conditions in the bioreactor could be suitable to increase the production of PHB.

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التخليق الحيوي للبولي هيدروكسي بيوتايريت ذا السّلسلة متوسطة الطول بواسطة العزلة البكتيرية *Pseudomonas aeruginoa* Dw7

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الملخص

تعد عائلة البولي هيدروكسي الكانويت Polyhydroxy alkenoates (PHAs) والمصنّعة حيوياً من المواد الواعدة كبديل للبلاستك التقليدي المصنع من البترول. البولي هيدروكسي بيوتاريت (PHB) Poly–β – hydroxy butyrate هواكثرالبوليمرات الحيوية المعروفة، القابلة للتفكك، والمنتجة من قبل أجناس بكتيرية. وبصورة عامة فقد أصبح استخدام البولي هيدروكسي بيوتاريت كبديل للبلاستك من الأمور المسلم بها، لكونه يساهم في في حل واحدة من المشكلات البيئية. نميت العزلة البكتيرية Pseudomonas aeruginosa Dw7 على مستنبت الأملاح المدعم بمخلفات الطهي من زيت الذرة. قسّمت هذه الدّراسة إلى ثلاث مراحل، حيث اختبرت في المرحلة الأولى قدرة العزلة *P.* aeruginosa Dw على النمو وإنتاج PHB في مفاعل حيوي بحجم 5 لترات، وبحجم تشغيلي 3.5 لتر، بعد مراقبة النمو لمدة 80 ساعة. أظهرت النتائج كفاءة المفاعل الحيوي في زيادة الإنتاجية، وكان معدل الإنتاج 62.6% بعد 60 ساعة من النمو وقدّرت كمية الوزن الجاف للخلايا 5.3 غ/ل، وعليه فقد أكّدت هذه النتائج إمكانية إنتاج كميات كبيرة من هذه المركبات على نطاق واسع. وفي المرحلة الثانية تم اختبار عملية الاستخلاص للمنتج البكتيري بعدة طرائق وباستخدام العديد من المذيبات لغرض تقويم أفضل نظام للإذابة للمنتج في الخلايا البكتيرية. بينت النتائج أن استخدام هايبوكلوريت الصوديوم لحل الجدر الخلوية، ومن ثمّ استخلاص المنتوج بالكلوروفورم هي الطريقة المثلى للحصول على المنتج البكتيري بأكبر إنتاجية، والتي بلغت 63% وبصورة أنقى بالمقارنة مع بقية الطرائق. تضمنت المرحلة الأخيرة دراسة الخواص الميكانيكية والفيزيائية والكيميائية للمركب الناتج. كما استخدمت عدة تقنيات لدراسة خصائصه كتقنية الكروموتوغرافيا الغازي والأشعة تحت الحمراء، والأشعة السينية. بينت النتائج أنّ PHB مادة بللورية ذات لون أبيض مصفر، ذوابة في الكلوروفورم، الداي كلوروميثان، دايكلوروايثان وكلوروبروبان. أظهرت نتائج التشخيص بالأشعة السينية بأن المركب ذو تركيب هيكلي يميل للتكوين البلوري. سجلت نتائج الكروموتوغرافيا الغازي ظهور تسعة مركبات ناتجة عن نمو العزلة P. aeruginosa Dw7 وكان الأبرز بينها المركب Undecanoic acid عند الدقيقة 15.4 والمركب Tridecanoic acid عند الدقيقة 17.3 وبوفرة نسبية بلغت 100 و14.33%، على التوالي. بينما أظهرت نتائج الفحص باستخدام الأشعة تحت الحمراء أنّ أبرز المجاميع الفعالة هي مجموعة C =O و C–O و OH.

الكلمات المفتاحية: بسيدوموناس ايروجنوزا، بولي هيدروكسي بيوتاريت، المفاعل الحيوي، الاستخلاص بالمذيبات.