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Research Article

Identification and characterization of polyhydroxyalkanoates producing *Acinetobacter* spp. isolated from contaminated soils, landfills, and waste dumping sites in the province of Baghdad, Iraq

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Abstract

Polyhydroxyalcanoates (PHAs) are well-known biodegradable polymers produced by bacteria under certain conditions. In this study, 100 different locally bacterial isolates after cultivating ecological models on Modified Solid Nutrient Medium from samples collected from hydrocarbon contaminated soil and landfills and waste dumping places in different sites of Baghdad, Iraq. By performing a microscopic examination after staining the bacterial isolates with Gram stain, it was found that sixty-seven isolates are gram-negative. All bacterial isolates were screened for PHA production by staining using Sudan black B. PHA production was detected in twenty-three bacterial isolates. Our findings indicate that five isolates accumulated a high PHA, ranging 25-56%, with an increase in the dry-weight of cells, ranging 1.20-3.53g\100ml (in a medium containing 10gm/l glucose, pH=7; incubated at 37°C after 48hrs) were *Sphingomonass paucimobilis*, *Klebsiella pneumonia*, *Citrobacter braakii*, *C. sediakii*, and *Acinetobacter lowffii.* Vitek test was done to support the characterization of *A. lowffii* as the most efficient isolate in PHA production. Extracted compound on FTIR-analysis gave characteristic C=O peak of PHA, thus, confirming the isolate to be PHA producer.

Keywords: Bioplastic, Bacterial isolates, Polyhydroxyalkanoate, FTIR.

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Introduction

Plastic has wide applications in every aspect of our life due to the advances in technology and the increase in the global population. Unfortunately, large quantities of petrochemical plastics are dumped without treatment as waste (Amal 2015; Noor 2018). Advances in technology in various fields negatively impact soil, water, and air (Noor 2018). These problems are caused by environmental pollution with these non-degradable petrochemical compounds, which are constantly increased, contributing to the raising the quantities of solid waste. This concern has led researchers to search for an alternative to synthetic plastics.

One of the important alternatives is PHA, which was considered a solution to the environmental problem arising from the damage caused by the indegradability of traditional plastic materials. PHA is a type of polyester produced inside cells by microorganisms in the form of carbon, which is fat and energy storage when it grows in an environment with limited nutrients such as nitrogen, phosphorous, magnesium, or oxygen with an excess of carbon source in the nutrient medium (Thellen et al. 2008). One of the most important features of the PHA compound is its ability to biodegrade through

fragmentation or breakdown of materials by microorganisms such as bacteria or fungi growing under aerobic or anaerobic conditions or through other biological means such as enzymes. In general, these polymers biodegrade through enzymatic and non-enzymatic hydrolysis and not necessarily by physical and chemical methods such as thermal oxidation, photoradiative decomposition (Elbahloul 2009; Noor 2018). Today, these biopolymers have become a necessity because they are of natural origin, and their applications have spread extensively for their important role in our daily life. In addition, it has important specifications for being biodegradable, biocompatible, environmentally friendly, and renewable compounds. PHA is involved in many medical applications and many areas of human life research, such as tissue engineering and various drug delivery systems (DDS) (Mandel et al. 1964). Polyhydroxyalkanes (PHAs) are polymers produced by many bacterial specious (MacFaddin 1985; Singh et al. 2011), where bacteria consume sugars and lipids in nature and is converted as a source of energy in the form of carbon stored in the cytoplasm of the cell (Muheim & Lerch 1999; Kemavongse et al. 2008).

Many bacterial species capable of consuming oil to produce polymers have been isolated. The most important of these bacterial genera are *Sphingobacterium, Acinetobacter Brochothrix, Caulobacter, Ralstonia, Burkholderia,* and *Pseudomonas* identified from sites contaminated with hydrocarbons and their derivatives (Eman 2017). PHA is also important for bacteria by keeping their DNA damaged by helping them resist harsh environmental conditions such as heat, UV rays and osmotic shocks. (Eman 2017). They are considered materials with high potential due to their properties close to conventional petroleum elastomers and their biocompatibility (Ostle & Holt 1989). The substrate represents the largest percentage of the production cost of the compound, which may reach 30-40% if expensive materials are used. Therefore, replacing it with cheap materials such as industrial waste contributes to being economically feasible (Eman 2017). This study aimed to identify local bacterial isolates capable of producing PHA in the Baghdad region.

Materials and Methods

Samples collection and bacterial isolation: Environmental and soil samples (solid waste dumping sites, soils contaminated with hydrocarbon and oil derivatives and sewage sludge from different locations in Baghdad) were collected randomly and aseptically using a pre-sterilized spoon from the surface layer of soil (1-3cm deep), transferred into sterile plastic bags, and kept at 4°C until use (Amal 2015).

One gram of each sample was weighed and added to 10ml of sterile distilled water and mixed. Serial dilution of the samples was performed up to 10^5, then 100µl of the diluted samples were cultured on modified nutrient agar. The medium plates were incubated at 28±2°C for 48-72 hours. The components of the nutrient medium were Beef extract (0.3%), Glucose (1%), Peptone (0.5%), Sodium Chloride (0.8%), and Agar (1.5%) (Singh et al. 2011). The pure isolates were preserved by culturing them on two vials, working vials and stock vials with addition of 2% glycerol for preservation. It was kept at a low temperature under standard conditions to be reactivated before conducting the in vitro experiments. A gram stain test was conducted for all pure isolates. The 40X objective lens was used to locate the smear of bacteria. Then a drop of immersion oil was placed on the smear and examined with an oil immersion lens (100X).

Primary screening of PHA producing isolates

Qualitative screening

Microscopic examination of PHA producing isolates: Sudan black stain was used to identify microbial intracellular lipid. 0.1ml of 72hrs old cultures from each isolate was taken and spread on a clean, grease-free slide, covering an area of about 10x30mm. The smear was allowed to dry in the air and fixed by passing the slide three times over the

flame of a heat source horizontally. A few drops of Sudan black stain were placed on the fixed slide and allowed for 5-10min. For removing excess staining, the slide was immersed in xylene for 10sec and then left to dry. It was further counter-stain with 0.5% safranine solution for 10-30sec. The slide was gently rinsed with running water and allowed to dry again. After the slide was completely dried, a drop of immersion oil was added directly and examined using differential interference contrast microscopy with an oil immersion lens 100X (Timothy et al. 2009).

Detection by Sudan black B on solid medium: Using agar medium rich in nutrient carbon in Petri dishes, 67 Gram-negative bacterial isolates were cultured and incubated at 30°C for 24-72 hours to pre-screen them for their PHA yield. 60% of the Sudan black dye was spread over the colonies and the plates remained undisturbed for 30 minutes. Then they were washed with ethanol at a concentration of 98% to remove excess stains from the colonies. The blue isolates were considered PHA producers (Muheim & Lerch 1999).

Isolation and characterisation of bacterial species able to produce the highest PHA: In addition to standard microbiological and biochemical methods to determine the type of isolated bacteria, its phenotypic characteristics were observed by colour, size, cell shape and interaction with gram stain. For VITEK2 test to isolate and growth, Herellea agar, consists of the components (as g\l): 15g casein enzymic hydrolysate, 5g papaic digest of soyabean meal, 5g sodium chloride, 10g lactose, 10g maltose, 1.25g bile salts mixture, 0.020g bromocresol purple, 16g agar was used. This method was used for diagnosing *Acinetobacter* isolates. Furthermore, culture on blood and Macconkey agar, sugar utilisation test, catalase test, oxidase test, and citrate utilisation test were performed to identify the selected isolate (Mindolli et al. 2010).

Measurement of cell dry-weight: Shake flask fermentation process was carried out using 250mL Erlenmeyer flasks containing 100ml of mineral salt medium containing (as g\l): 0.5g NH_4Cl, 2.8g KH_2PO_4, 3.32g Na_2HPO_4, 0.25g $MgSO_4.7H_2O$, 10g, and 1ml of trace elements. According to Kahar's formulation (Kahar et al. 2004), the trace element composition was sterilised by autoclaving at 121°C and 15lb/in^2 for 15min. Using three replicates for each isolate in the experimental design, flasks were inoculated with 24h-old bacterial culture from the original culture of each isolate. All were incubated with control at 37°C and 120rpm in a shaking incubator. Control medium and inoculated medium were taken. The samples were subjected to a centrifuge at 6000rpm for 15min. The residue was washed twice with sterile deionised water and dried for 24 h at 60°C. The total bacterial dry weight was determined based on Yuksekdag et al. (2004).

Secondary screening for production of PHA

Extraction of PHA using sodium hypochlorite method: Using the sodium hypochlorite digestion method, PHA was extracted from dried biomass after 72 hours (Ramsay et al. 1990). In 1g biomass, 10ml sodium hypochlorite (30%) was added and incubated for 90min at 37°C. The pellets were collected in clean, pre-weighted serum tubes, washed with ethanol, dissolved in chloroform, and then centrifuged (Du et al. 2001). Finally, the weight of the PHA extract was calculated after allowing the chloroform to evaporate based on Zakariaet et al. (2010).

Yield of PHA accumulation (%) = Dry weight of extracted PHA (g/l) / DCW (g/l) × 100.

Fourier transforms infrared spectroscopy (FTIR) spectroscopy: A mixture of sufficient proportions of PHA with potassium bromide (KBr) was used to form transparent granules and then ground in the motor and pestle using a hydraulic press. Infrared rays were passed through the prepared granules and placed in the sample holder at a range of 400-4000cm^{-1}. The results were analyzed to determine functional groups (Teeka 2010).

Results and discussion

Bacterial isolate: 120 bacterial isolates were

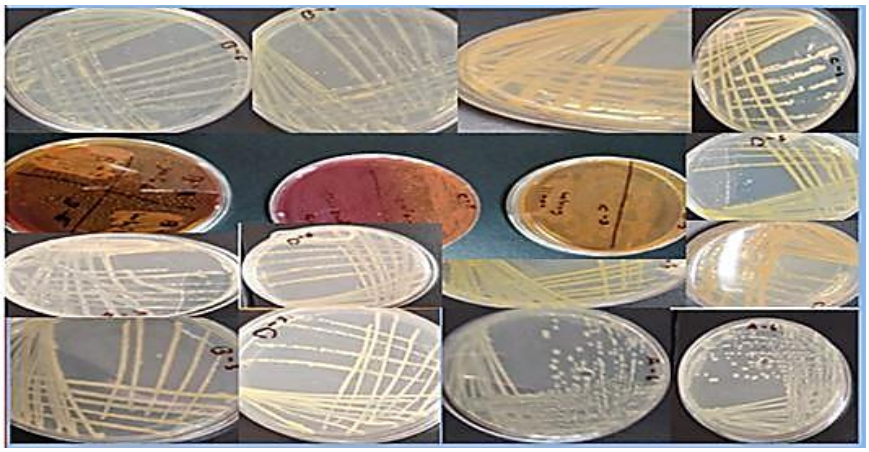


Fig.1. Various used environments for the rapid detection and isolation of PHA-producing bacteria.

Table 1. PHA production by different isolates in minimal medium broth at 30°C at 72hrs of growth.

Serial number	Bacterial isolates	Dry cell weight (gm\100)	PHA (%)
1	A2	0.56	14
2	B1	0.40	16
3	B2	0.32	16
4	B6	0.24	13
5	T6	0.54	15
6	T8	0.34	20
7	C3	0.34	21
8	C7	0.65	12
9	C9	0.56	15
10	D9	0.52	18
11	D11	0.23	18
12	D23	0.24	21
13	G3	3.53	56
14	G5	2.30	45
15	K4	0.50	10
16	K7	0.52	15
17	R5	1.20	25
18	R8	1.53	30
19	Q3	0.32	20
20	Q6	0.82	16
21	Q8	2.01	40
22	S4	0.42	12
23	S9	0.43	20

obtained from solid waste dumping sites, soils contaminated with oil derivatives, hydrocarbons and car fuels from different locations in Baghdad, and sewage sludge amended soils. The bacterial colonies varied from small to large, round to irregular, and creamy, pinkish-red, orange or white. They were selected for obtaining isolates by culturing in a streaking method and then transferred to slant with nutrient agar (Fig. 1).

Screening of gram-negative isolates for PHA production: Cell shapes and interaction with gram stain of the isolated bacteria were studied under a light microscope. A total of 67 isolates were gram-negative (Fig. 2) that screened for PHA production and 23 of them showed positive results to Sudan Black (specific staining for detection of PHA granules production on viable) cells (Table1, Fig. 3). The lipid-containing granules were stained in blue-black or grey-blue upon microscopic observations, and the bacterial cytoplasm was light pink by anti-

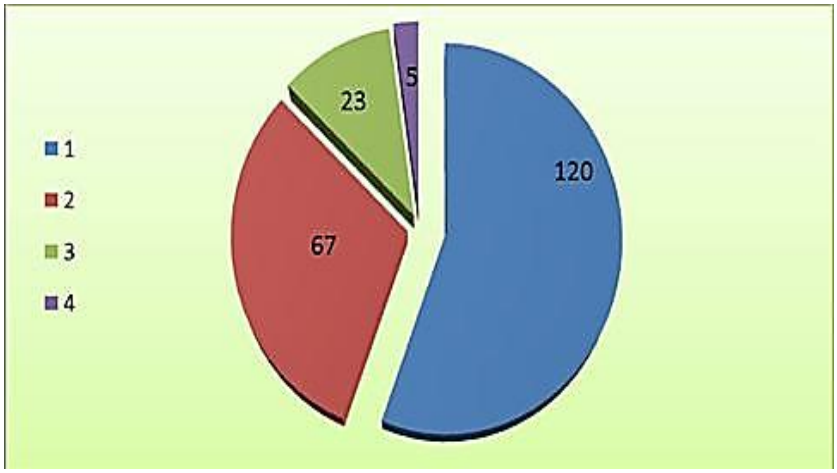


Fig.2. Screening of bacterial isolates from contaminated sites (1) bacterial isolates in general medium, (2) gram negative bacteria, (3) PHA producing bacteria, (4) the most active isolates**.**

Table 2. Characterization of G3 isolate.

Serial No.	Test	Results
1	Oxidase	Negative
2	Catalase	Positive
3	Citrate	Positive
4	Glucose	Positive
5	Galactose	Positive
6	Maltose	Negative
7	Lactose	Negative
8	Mannitol	Negative
9	Grown on Blood Agar	Heamolytic colonies
10	Grown on Macconkey Agar	Partically Lactose Fermenting Colonies.

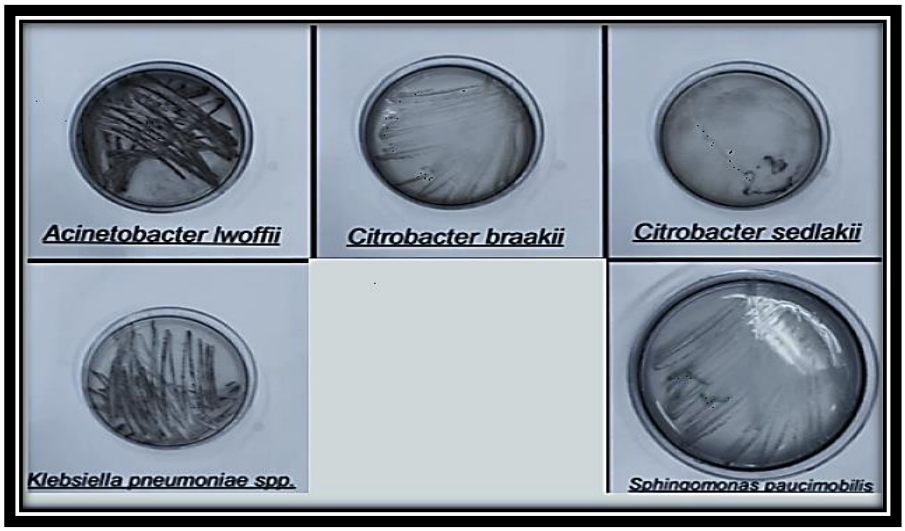
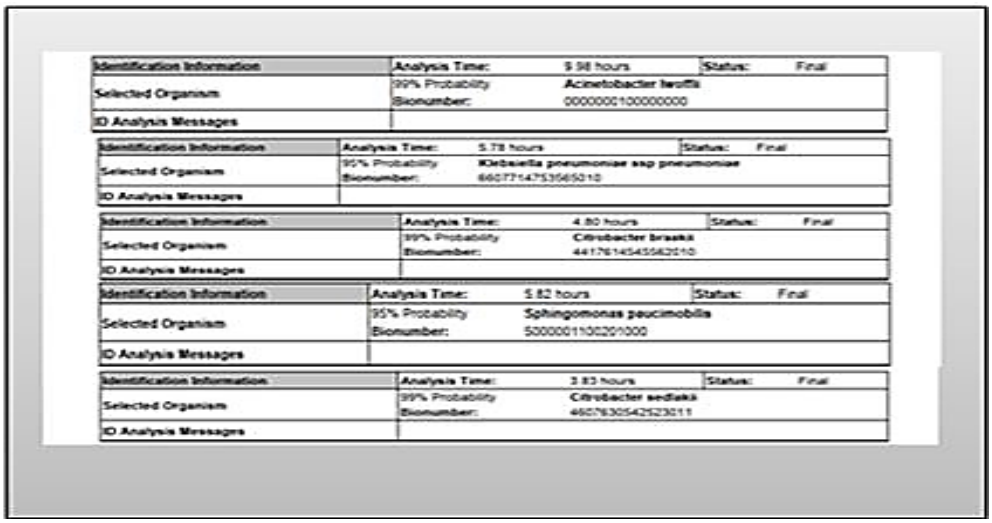


Fig.3. Sudan Black plate assay result on bacterial isolates.

Saferin staining. Bacterial isolates showed a difference in the level of blue-black granules that appeared as droplets in the cells, evidence of a difference in production efficiency. Figure 4 shows PHA lipid granules with a diameter of 0.2-0.5µm located in the cell's cytoplasm.

Gerhardt et al. (1981) demonstrated that bacteria's lipid contents and lipid inclusions can be monitored

Table 3. Vitek2 test for diagnosis of efficient bacterial isolates (G3, G5, R5, R8 and Q8).



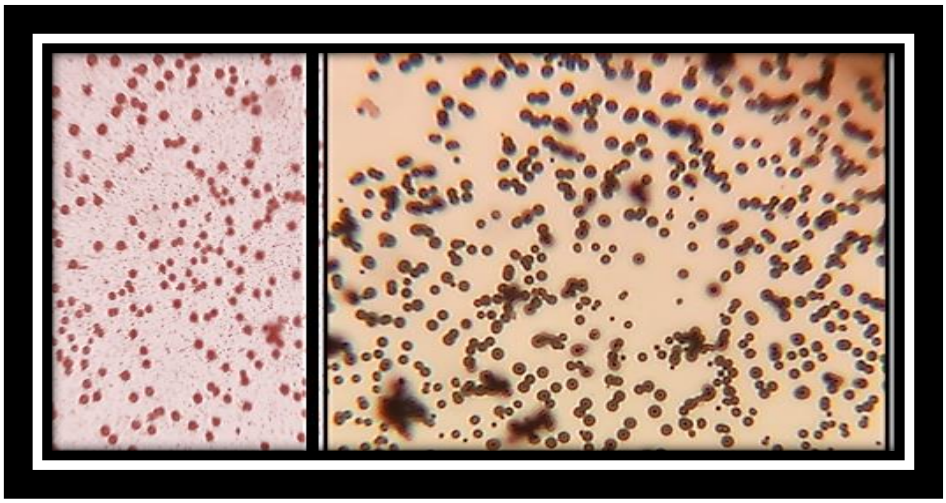


Fig.4. Formation of endocellular PHA granules under phase-contrast after staining with Sudan Black.

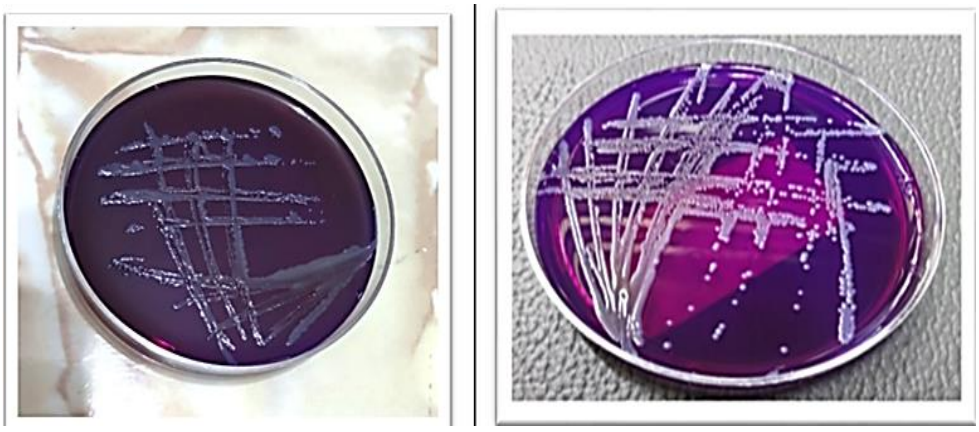


Fig.5. *Acinetobacter lowffii* (G3) on Herellea agar.

using Sudan Black B staining. PHA-stained granules appear as blue-black droplets within the pink cytoplasm of cells after 24 hours of growth (Page & Corniche 1993). It is well known to serve as an internal carbon and energy reserve under nutrient limiting conditions during log lag and steady growth

phase. shaking conditions varied 0.23-3.53g.l^{-1} at 30°C

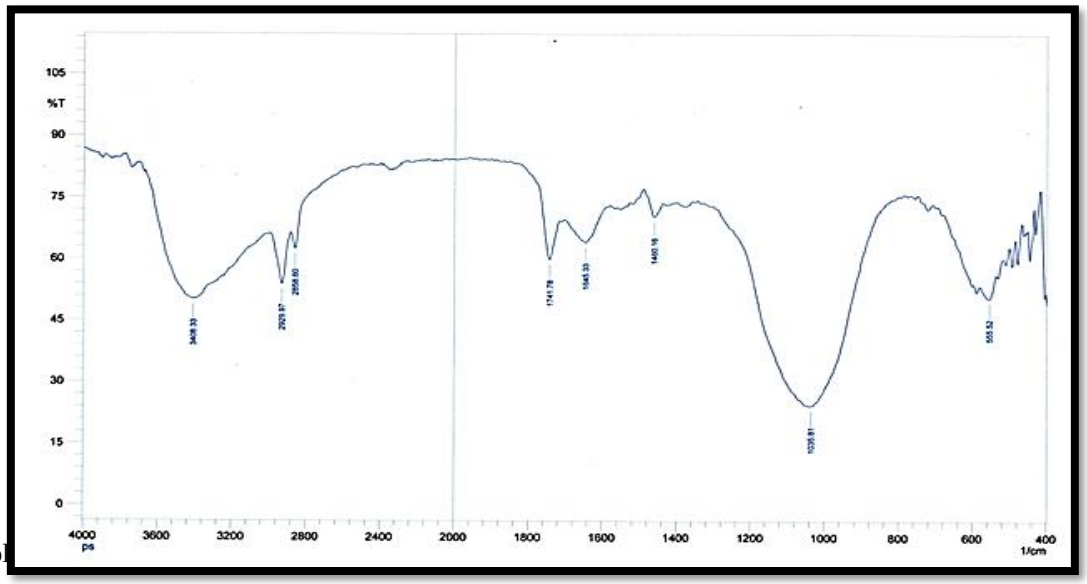


Fig.6. PHA pol

Some biochemical and phenotypic tests were carried out for the most efficient bacterial isolate (G3) using the Herellea agar medium (Fig. 5). *Acinetobacter* is a no fermenting gram negative rod bacilli, oxidase-negative, catalase and citrate-positive, glucose and galactose fermenter, but non fermenter for mannitol, maltose and lactose (Table 2). Indeed, the most notable feature of this bacterium is its ability to produce a violate pigment on Herellea agar.

PHA production in minimal medium broth: The amount of cell dry-weight at 72hrs of growth under (Table 1). Cell biomass correlated with PHA production ranging 16-56%. Five isolates exhibited high yield and accumulation of PHA with maximum biomass identified *Sphingomonass paucimobilis, Klebsiella pneumoni, Citrobacter braakii, C. sediakii* and *Acinetobacter lowffii* with a maximum of 56% of PHA in 3.53g.l^{-1}of biomass.

Extraction and characteristics of PHA: One of the most basic requirements for PHA production at the industrial level is the easiest way to extract and separate it (Suriyamongkol et al. 2007). Economically, solvent extraction methods have

Fig.7. Flourier Transforms Infrared spectroscopy (FTIR) of extracted PHA.

remained an appropriate way to obtain the polymer with good specifications, high purity and high efficiency. However, other methods, such as hypochlorite and chloroform solutions, are dispersants or surfactants (Ramsay et al. 1990). Separation of insoluble cell material after extraction is difficult, and 100% extracted PHA is impossible (Choi & Lee 1999). Using sodium hypochlorite in the extraction process, the purity is better, up to more than 95%, so this method is preferred, despite the possibility of the material being affected and disintegrated (Lee 1996). A wide variety of PHAs has been discovered in various bacteria. So far, approximately 150 different PHAs have been identified (Steinbüchel & Valentin 1995).

Over 250 different bacteria, including gram-negative and gram-positive species, produce various PHAs (Steinbüchel 1991; Steinbüchel et al. 1993). The produced PHA was yellowish-white crystals in our study (Fig. 6). The chemical and physical nature of the extracted PHA (microstructural properties and melting point, and its texture and appearance) was comparable with conventional plastic like polypropylene in terms of melting point, which was recorded at 177°C. Figure 7 shows the functional groups identified by FTI of the extracted PHA. FTIR performed for characterisation of the extracted PHA from *A. lowffii* resulted in peaks showing the presence of groups like CH3, CH and C=O, which are present in PHA structure at bands of 1454, and 3750cm^{-1}, respectively (Fig. 7).

Conclusions

In the current study, 120 bacterial isolates were obtained from sites contaminated with several different environmental pollutants, and 23 were PHA-producer. The isolate *A. lowfii* produced the highest percentage of PHA with the best growth in terms of dry-weight of bacterial cells. It was able to produce 56.6% PHA per dry weight of 3.4g after 48 hours.

Acknowledgements

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