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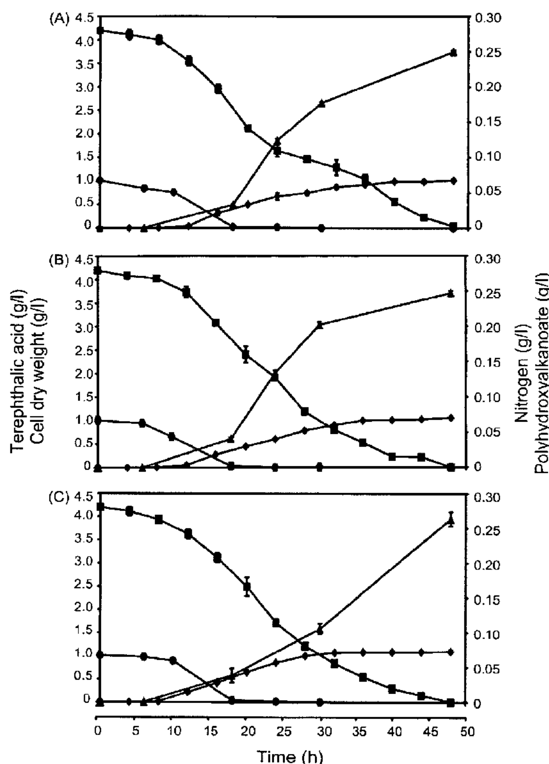


Figure 1

**(57) Abstract**: A method for producing polyhydroxyalkanoate (PHA), comprising (i) culturing in a culture medium comprising terephthalic acid and/or a salt thereof and/or an ester thereof one or more bacterial strains which are capable of accumulating PHA from terephthalic acid or a salt or ester thereof and which are selected from *Pseudomonas putida* strain GO 16 having the accession number NCIMB 41538, *Pseudomonas putida* strain GO 19 having the accession number NCIMB 41537, and *Pseudomonas frederiksbergensis* strain GO23 having the accession number NCIMB 41539; and (ii) recovering the PHA produced from the culture medium. The invention also provides *Pseudomonas putida* strain GO16 having the accession number NCIMB 41538; *Pseudomonas putida* strain GO 19 having the accession number NCIMB 41537; and *Pseudomonas frederiksbergensis* strain GO23 having the accession number NCIMB 41539.

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# Method for producing polyhydroxyalkanoate

The present invention relates to a method for producing polyhydroxyalkanoate (PHA) and to novel bacterial strains used in the method.

Polyethylene terephthalate (PET) is one of many petrochemical based plastics that contribute greatly to the convenience of everyday life. Best known worldwide for its use in plastic bottles, it is produced on a multi-million tonne scale worldwide. Due to the success of PET it has, like other plastics, become a major waste problem. Greater than 5,400 million lbs (2,400 million kg) of PET bottles were on shelves in the United States in 2006. In general, less than 25% of these bottles are recycled, and thus the vast majority of PET bottles worldwide end up in landfill. This occurs despite a variety of recycling technologies being available such as mechanical grinding for use of waste PET in the fiber industry, reprocessing of waste PET for food contact usage, and pyrolysis (thermal treatment in the absence of air) of waste PET to generate chemical feedstocks.

PET can be degraded by pyrolysis to its monomeric components, terephthalic acid and ethylene glycol. However, the resulting terephthalic acid is generally used as a chemical feedstock for the re-synthesis of PET, which is a non-biodegradable and consequently low value product. Thus, factors such as the high relative cost of sorting of waste PET and the low value of the downstream product, contribute to the poor recycling rates for PET.

PHA is the general term for a range of diverse biodegradable polymers that consist of polyesters of (*R*)-3-hydroxyalkanoic acids. These polymers are of interest due to a broad range of applications and the fact that they are completely biodegradable thus offering little or no long term waste issues.

These polymers can be accumulated by some bacteria intracellularly as carbon storage materials. It has been shown that PHA accumulation occurs in bacteria in response to a

range of environmental stress factors such as inorganic nutrient limitation. The substrates that are supplied to bacteria to accumulate PHA are divided into two groups 1) PHA related substrates, i.e. alkanoic acids (fatty acids) that resemble the monomers that make up PHA ((R)-3-hydroxyalkanoic acids) and 2) PHA unrelated substrates, which are substrates
5 that do not resemble the monomers that make up PHA e.g. glucose.

It is an object of the invention to mitigate or eliminate the disadvantages associated with the recycling of PET.

10 It is also an object of the invention to provide a method for producing PHA from the degradation product of PET, namely terephthalic acid and/or a salt thereof and/or an ester thereof.

According to the invention, there is provided a method for producing polyhydroxyalkanoate
15 (PHA), comprising (i) culturing in a culture medium comprising terephthalic acid and/or a salt thereof and/or an ester thereof one or more bacterial strains which are capable of accumulating PHA from terephthalic acid or a salt or ester thereof and which are selected from *Pseudomonas putida* strain GO16 having the accession number NCIMB 41538, *Pseudomonas putida* strain GO19 having the accession number NCIMB 41537, and *Pseudomonas frederiksbergensis* strain
20 GO23 having the accession number NCIMB 41539; and (ii) recovering the PHA produced from the culture medium.

The invention also provides *Pseudomonas putida* strain GO16 having the accession number NCIMB 41538. The 16S rDNA sequence of strain GO16, presented in SEQ ID NO. 1, shares
25 99% homology with a known *Pseudomonas putida* strain (DQ133506, see Table 2).

The invention further provides *Pseudomonas putida* strain GO19 having the accession number NCIMB 41537. The 16S rDNA sequence of strain GO19, presented in SEQ ID NO. 2, shares 99% homology with a known *Pseudomonas putida* strain (AY512611, see Table 2).

30

The invention still further provides *Pseudomonas frederiksbergensis* strain GO23 having the accession number NCIMB 41539. The 16S rDNA sequence of strain GO23, presented in SEQ ID NO. 3, shares 99% homology with a known *Pseudomonas frederiksbergensis* strain (AJ249382, see Table 2).

5

Each of the strains *Pseudomonas putida* GO16, *Pseudomonas putida* strain GO19 and *Pseudomonas frederiksbergensis* strain GO23 is capable of accumulating PHA from terephthalic acid or a salt or ester thereof.

10 As described in more detail in Example 1 (A) below, the base sequence of 16S rDNA of each of the strains of the invention was analysed and identified using the NCBI GenBank database BLAST programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and each of the strains was deposited on January 24, 2008 in the depositary institution NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, Scotland, an International Depository
15 Authority under the Budapest Treaty.

The PHA recovered from the culture medium advantageously comprises at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% medium chain length (mcl) PHA. MclPHA is classified as comprising repeating units derived from 3-hydroxyalkanoic
20 acid monomers containing 6 carbon atoms (C6) to 14 carbon atoms (C14). Preferably, at least 80%, more preferably 85%, even more preferably at least 90%, most preferably at least 95% of the mclPHA comprises repeating units of C8, C10 and C12 monomers. More preferably, the mclPHA comprises repeating units in a respective amount by weight of mclPHA of 15% - 25% C8, 40% - 50% C10 and 30% - 40% C12, preferably as determined using an Agilent 6890N
25 series gas chromatograph (GC) fitted with a 30m x 0.25mm x 0.25μm BP-21 column (J & W Scientific), and further preferably as confirmed using an Agilent 6890N GC fitted with a 5973 series inert mass spectrophotometer, using a 12m x 0.2mm x 0.33μm HP-1 column (Hewlett-Packard). In a preferred embodiment, the amount of C8, C10 and C12 totals 100%.

PHAs are generally classified as short chain length PHAs (sclPHAs), medium chain length PHAs (mclPHAs) or long chain length PHAs (lclPHAs), depending upon the number of carbon atoms of the constituting monomers thereof. SclPHA comprises monomers of C3 – C5, mclPHA comprises monomers of C6 – C14, and lclPHA comprises monomers of more than 14 carbons (> C14). This variation in monomer chain length gives rise to different properties in the polymer, with sclPHAs and lclPHAs both having undesirable properties, sclPHAs having a high degree of crystallinity and being rigid and brittle, and lclPHAs being sticky and very difficult to handle. The properties of sclPHAs and lclPHAs limit the range of their applications. MclPHAs, as produced by the method of the invention, and which may comprise monomers selected from C6, C8, C10, C12 and C14 monomers, have much more desirable properties, being elastomers with a low glass transition temperature and having properties being suitable to a wide range of applications including biodegradable rubber and coating materials. Therefore, the production of mclPHA according to the invention advantageously provides a polymer which has a wide range of applications and is biodegradable.

Salts of terephthalic acid useful in the invention include alkali metal and alkaline earth metal salts and mixtures thereof, such as sodium and potassium salts, and magnesium, calcium and barium salts. Mono- or di- sodium or potassium salts of terephthalic acid are preferred. Monosodium terephthalate is especially preferred. In an especially preferred embodiment, the terephthalic acid or salt or ester thereof is in the form of monosodium terephthalate, also referred to herein as sodium terephthalate or terephthalic acid (sodium salt).

Esters of terephthalic acid which can be used in the invention include mono- and di- esters of terephthalic acid and mixtures thereof. Suitable mono- and di- esters of terephthalic acid include mono- and di- C1 – C4 alkyl esters and mono- and di- glycol esters.

The terephthalic acid or salt or ester thereof may be obtained from hydrolysis or pyrolysis of polyethylene terephthalate (PET), preferably pyrolysis of PET, which produces a solid, liquid and gaseous fraction. If a terephthalate salt is required, the solid fraction is reacted with a base, preferably with an alkali metal or alkaline earth metal hydroxide. If an ester is required, the solid

fraction is reacted with a suitable alcohol. In a particularly preferred embodiment, the solid fraction obtained by pyrolysis of PET is reacted with sodium hydroxide, to form sodium terephthalate.

5 The method of the invention preferably comprises culturing the one or more strains in the culture medium for a period of from about 12 hours to about 72 hours, more preferably from about 24 hours to about 60 hours, most preferably about 48 hours, at a temperature of from about 25ºC to about 35ºC, preferably about 30ºC.

10 In the method of the invention, the culture medium preferably comprises strain GO19 and/or strain GO16.

In the method of the invention, the culture medium is preferably nitrogen limited, with a maximum nitrogen content of about 0.5 g/l, preferably 0.067 g/l culture medium. A
15 suitable nitrogen source is sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$).

In the method of the invention, recovering the PHA produced from the culture medium preferably comprises extracting the PHA produced using the following method. The cultures are harvested by centrifugation at 6000 x g/20 min, cells collected and resuspended in 1/100 of the
20 original culture volume of 50 mM phosphate buffer (pH 7). Cell suspensions are centrifuged again at 6000 x g/20 min and supernatant discarded. Cell pellets are freeze dried. Dry cell material is resuspended in 10 volumes of acetone and the suspension is stirred vigorously at 25ºC for 12-20h. Suspension is then centrifuged at 6000 rpm x g/20 min and the supernatant retained. Supernatant is filtered through 1 µm PTFE filter and the solvent evaporated in vacuo
25 to 1/10 -1/25 of the original volume. The product is a PHA suspension that is then dropwise added to chilled methanol (in the ratio 1:20). PHA polymer is harvested by centrifugation at 6000 rpm x g/20 min and dried under vacuum.

Advantages of the invention include the following:-

- It provides a clean, low cost method for producing PHA. The cost of PHA production through fermentation is inextricably linked to the cost of the starting substrate. Thus, the use of terephthalic acid (sodium salt), the degradation product of an easily sourced and inexpensive petrochemical waste plastic (PET), as a feedstock for bacteria as described
5 herein, provides a cheap, clean and reliable source for the synthesis of PHA.
- The PHA produced is a high value polymer. Specifically, in contrast to sclPHAs having a high degree of crystallinity and being rigid and brittle, and lclPHAs being sticky and very difficult to handle, the PHAs capable of being produced herein are mclPHAs having much more desirable properties, being elastomers with a low glass transition
10 temperature.
- The PHA produced is completely biodegradable and has a wide range of applications ranging from biomedical applications to packaging.

The following examples serve to illustrate the invention but it will be appreciated that the
15 invention is not limited to these examples:-

**EXAMPLE 1 (A)**

**Hydrolytic Pyrolysis of PET**

20 Virgin polyethylene terephthalate (PET) (Krupp-Formoplast) was supplied to a laboratory scale pyrolysis plant (as described in Yoshioka *et al*, 2004. Pyrolysis of poly(ethylene terephthalate) in a fluidized bed plant. Polymer Degradation and Stability. 86:499-504) at a feed rate of 1,035 kg/h. The electrically heated fluidized bed had a diameter of 130 mm. 9 kg quartz sand with diameters between 0.3 and 0.5 mm led to a height of 480 mm in the
25 fluidized bed which was maintained at a temperature of 450 °C. The PET entered the fluidized bed reactor via a screw conveyor. The hot pyrolysis products (see Table 1) passed a cyclone to be cleaned by small amounts of fillers and then were cooled down by mixing with cold water to room temperature in a precipitator (desublimator). In the precipitator, the whole solid fraction including the terephthalic acid was desublimated to generate a
30 white powder. The sand bed was fluidized by steam with a flow rate of 2,5 kg/h. The solids

were analyzed by HPLC-MS-system (HP 1100, column Multospher ® 100) using a diode array detector by 220 nm. The gas and oil fractions were characterized by gas chromatography (GC-FID, HP 5890 Machery & Nagel SE 52) and GC-MS (Fisons Instruments VG 70 SE, Machery & Nagel SE 52).

**Bacterial Growth Medium**

The minimal medium E2 (per litre: 3.5 g $NaNH_4HPO_4.4H_2O$, 7.5g $K_2HPO_4.3H_2O$, 3.7g $KH_2PO_4$, 0.25 g $MgSO_4.7H_2O$, 2.78g $FeSO_4.7H_2O$, 1.98 g $MnCl_2.4H_2O$, 2.81 g $CoSO_4.7H_2O$, 1.47 g $CaCl_2.2H_2O$, 0.17 g $CuCl_2.2H_2O$, 0.29 g $ZnSO_4.7H_2O$; for limited nitrogen conditions, 1.0 g/l of $NaNH_4HPO_4.4H_2O$ was used) was prepared, and used as the base media supplemented with sodium terephthalate as the sole source of carbon and energy for all culture techniques discussed herein. The sodium terephthalate was prepared by taking the whole solid fraction of PET pyrolysis (in the form of the white powder mentioned above) and dissolving it in an equimolar solution of sodium hydroxide (Sigma).

**Isolation of Bacteria from Soil**

1kg of soil was collected from PET exposed soil at an industrial site in Ireland used to mould PET granules to PET products. The granules were present in the soil adjacent to the factory setting. The soil was sieved under aseptic conditions to a particle size of 5mm, then 10g of soil were added to 90ml of sterile Ringer's solution (Sigma), this was vortexed for 5 min to homogenize the sample. 1ml of this was added to 9ml of sterile Ringer's, this was repeated to obtain a $10^{-5}$ dilution of the original soil sample. The serial dilutions were spread plated on solid E2 media containing 1.1 g/l of sodium terephthalate as the sole source of carbon and energy. 32 isolates were selected by visual differentiation of contrasting colony morphology. These 32 isolates from the soil samples were then grown in shake flask culture as described below and tested for PHA accumulation.

**Growth conditions for PHA accumulation**

The 32 soil isolates were grown in shake flask experiments, where each strain was grown in a 250ml Erlenmeyer flask containing 50 ml E2 medium (4.2 g TA/l) at 30°C with

shaking at 200rpm. To screen for organisms capable of PHA accumulation, the inorganic nitrogen source sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$) was limited to 1g/l (67 mg nitrogen/l).

5 **PHA Screening and Composition Analysis.**

The 32 soil isolates were grown in shake flasks as described above for 48 hours and tested for PHA accumulation as follows. Cell material (5-10 mg) or PHA standard isolated from *P. putida* CA-3 for which the PHA composition is known (Ward PG, de Roo G, O'Connor KE. Accumulation of polyhydroxyalkanoate from styrene and phenylacetic acid by

10 *Pseudomonas putida* CA-3. Appl Environ Microbiol. 2005 Apr; 71(4):2046-52) was resuspended in 2 ml acidified methanol (15 % $H_2SO_4$, v/v) and 2 ml of chloroform containing 6 mg/l benzoate methyl ester as an internal standard. The mixture was placed in 15 ml Pyrex test tubes and incubated at 100°C for 3h (with frequent inversions). The solution was extracted with 1 ml of water (vigorous vortex 2 min). The phases were

15 allowed to separate before removing the top layer (water). The organic phase (bottom layer) was dried with $Na_2SO_4$ before further analysis. The samples were analyzed on an Agilent 6890N series gas chromatograph (GC) fitted with a 30m x 0.25mm x 0.25µm BP-21 column (J & W Scientific) using a split mode (split ratio 10:1). An oven method was employed comprising 60ºC for 2 min, increasing by 5ºC/min to 200ºC for 1 min. For peak

20 identification, commercially obtained (*R*)-3-hydroxydodecanoic acids (Sigma) and the above-identified PHA standard (Ward *et al.*, 2005) were used. PHA monomer determination was confirmed using an Agilent 6890N GC fitted with a 5973 series inert mass spectrophotometer, using a 12m x 0.2mm x 0.33µm HP-1 column (Hewlett-Packard), with an oven method of 50ºC for 3 min, increasing by 10ºC/min to 250ºC for 1 min and

25 comparing to the above-mentioned standards. Only 3 strains exhibited high optical densities (OD540 of 4.0) and were found to produce PHA. These 3 strains were selected for further analysis, as discussed below. Additionally, 6 other strains exhibiting good growth (OD540 of 4.0) but which did not produce PHA, were randomly selected for comparison as described in more detail in Example 1 (B).

30

**Nitrogen Determination Assay**

The concentration of nitrogen in the growth media was monitored over time using the following method. The concentration of nitrogen (ammonium ion) in the media was monitored by taking 1ml samples from the culture flask at various time points and
5 centrifuging the samples at 13,200 rpm for 2min. The supernatant was retained and diluted 100 fold in deionised water. 1 ml of phenol-nitropruside buffer (per liter: 30 g $Na_3PO_4 \cdot 12H_2O$; 30 g $Na_3C_5H_5O_7$; 3 g EDTA, pH 12; 120 g phenol) was added to 2.5 ml of diluted sample and mixed. 1.5 ml of hypochloride solution (52.5 ml of sodium hypochloride solution (4% available chlorine) to 1000 ml of 1M NaOH) was added to the
10 mixture and mixed by inversion. The colour was allowed to develop for 45 min at 25 °C in dark. The absorbance at 635 nm was read using Helios delta spectrophotomer. Standard curve was also generated using known concentrations of sodium ammonium phosphate.

**Determination of Terephthalic Acid Utilization during Growth.**

15 The concentration of TA in the media was monitored by taking 1ml samples from the culture flask at various time points and centrifuging the samples at 13,200 rpm for 2min. The supernatant was retained, filtered and analyzed by HPLC. In order to analyze the sample and maintain a linear relationship between peak area on the HPLC chromatograph and TA concentration, samples had to be diluted so that the concentration of TA in the
20 final preparation did not exceed 0.63g/l. A standard curve of TA concentrations from commercially obtained sodium terephthalate (Sigma) was generated to establish the relationship between TA concentration and peak area on the HPLC. An Agilent 1100series HPLC using a C18 ODS Hypersil column (125mm x 3mm, particle size 5μm) (Thermo) was used and samples were isocratically eluted using 0.2% Formic acid and Acetonitrile
25 (ratio 80:20 respectively) at 0.5ml/min and read on a UV-Vis detector at 230nm.

**Determination of PHA polymer properties**

**Nuclear magnetic resonance (NMR).** Solution NMR were recorded on a Bruker DPX400 with $^{1}H$ at 400.13 MHz and $^{13}C$ at 100.62 MHz. The 400 MHz $^{1}H$ and $^{13}C$-NMR spectra of PHA
30 isolated from *P. putida* GO19 recorded at 20°C in $CDCl_3$ are shown in Figures 2 and 3

respectively. The solvent chloroform-d and tetramethylsilane (TMS) were used as internal references for chemical shifts in $^{13}C$ and $^{1}H$ NMR, respectively. $^{13}C$ NMR spectra were recorded with proton-decoupling. Typically 2200 transients were accumulated. Spectrometer peak areas were obtained directly by standard signal integration.

5 **Thermal analysis.** Differential Scanning Calorimetry (DSC) was performed with Perkin Elmer Pyris-Diamond Calorimeter calibrated to Indium standards. The samples weighing 7-8 mg were encapsulated in hermetically sealed aluminum pans and heated from -70°C to 100°C at a rate of 10°C/min. To determine the glass transition temperature (Tg), the samples were held at 100°C for 1 min and rapidly quenched to -70°C. The samples were then re-heated from -70°C to 100°C

10 at 10°C/min to determine the melting temperature (Tm) and Tg. The Tm was taken at the peak of the melting endotherm, while the Tg was taken as the mid point of heat capacity change respectively.

**Thermogravemetric analysis (TGA).** To determine the thermal stability and decomposition profile of the samples, TGA was carried out on a Perkin Elmer Pyris 1 thermogravemetric

15 analyser calibrated using Nickel and Iron standards. Each sample was weighed to c.a. 7 mg and placed in a platinum pan and heated from 30°C to 700°C at the heating rate of 10°C/min under an air atmosphere.

**Dynamic mechanical analysis (DMA).** DMA was carried out on a Perkin–Elmer Mechanical Analyzer. Dynamic measurements were made in extension mode on clamped film samples with

20 dimensions of $5 \times 2.8 \times 0.5$ mm. The experiments were performed under nitrogen atmosphere at a temperature range of -100°C to 50°C at a heating rate of 2°C/min and frequency of 0.1, 1 and 10 Hz. The Tg was identified by the sharp drop in storage modulus and the corresponding peak in the loss modulus. DMA glass transition temperature is frequency dependent and detectable at higher temperature compared to the quasistatic DSC data. The temperature at the

25 maximum point of the loss modulus ($E''$) was taken as the measure of the glass transition temperature.

**Gel permeation chromatography.** Molecular weight distribution were obtained by gel permeation chromatography (GPC) using PL gel 5 mm mixed-C +PL gel column (Perkin Elmer) with PELV 290 UV-Vis detector set at 254 nm. Spectroscopic grade chloroform was used as

30 the eluent at flow rate of 1.0 ml/min. Sample concentration of 1 wt % and injection volumes of

500 μl. A molecular weight calibration curve was generated with polystyrene standards with low polydispersity using the Turbochrom 4.0 software.

**X-ray diffraction (XRD) analysis.** XRD was performed at room temperature and diffraction patterns were collected on a Siemens D500 diffractometer fitted with a Cu -Kα radiation source.
5 The x-ray beam was Cμ-Kα (λ = 0.1514 nm) radiation operated, at 40 KV and 30 mA. Data was obtained from 2-60ºC(2θ) at a scanning speed of 0.1ºC/min.

**16S rDNA Identification.**

The three strains out of the 32 soil isolates capable of accumulating PHA with TA as the
10 sole carbon and energy source were selected and classified by sequence analysis of 16S rRNA genes. Each isolate was grown on LB agar. The genomic DNA of each bacterium was extracted as follows. Strains were cultured in 30 ml of LB medium for 24 h with shaking, 200 rpm at 30ºC. Cells were harvested by centrifugation at 4000 x g for 10 min, washed twice in 10% sucrose solution and resuspended in 10 ml of lysis solution (0.3M
15 sucrose, 25 mM EDTA, 25 mM Tris-HCl, pH 7.5 containing 2 U of RNase). Lysozyme (Sigma, 10 mg) was added and the bacterial suspension incubated at 37 ºC for 20 min. 10% SDS (1 ml) and proteinase K (Sigma, 5 mg) were added with further incubation at 55 ºC for 1 h. After addition of 5M NaCl (3.6 ml) and chloroform 15 ml, the sample was rotated end-over-end for 20 min. After centrifugation at 6000 x g for 20 min, the aqueous phase
20 was transferred with wide bore pipette into clean tube. The DNA was precipitated by addition of 1 volume of isopropanol and spooled using a sealed Pasteur pipette before being transferred into a micro-centrifuge tube and rinsed with 1 ml of 70% ethanol. Air dried DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.4 and 10 mM EDTA). The 16S rRNA genes were amplified by PCR using primers 27F (agagtttgatcmtggctcag)
25 presented as SEQ ID NO. 4, and 1392R (acgggcggtgtgtgtrc) presented as SEQ ID NO. 5, and the sequences were determined by GATC-Biotech, Germany. The resulting 16S rDNA sequences were compared to known sequences in the NCBI GenBank database by BLAST programme. The resulting sequences, presented as SEQ ID NOs 1 - 3 are discussed below with reference to Table 2.

30

**Description of Figures:**

**Figure 1.** PHA accumulation by (A) *Pseudomonas putida* GO16, (B) *Pseudomonas putida* GO19 and (C) *Pseudomonas frederiksbergensis* GO23 in shake flask containing growth medium consisting of 4.2 of sodium terephthalate and 67 mg/l of nitrogen at 30°C. CDW g/l (■), PHA accumulation g/l (▲), TA concentration (♦) and nitrogen concentration g/l (•) supplied as sodium ammonium phosphate were all monitored over a 48 h period. All data shown is the average of at least three independent determinations.

**Figure 2.** 400 MHz $^{1}H$ NMR spectrum of the mclPHA isolated from *P. putida* GO19 recorded at 20°C in $CDCl_3$.

**Figure 3.** $^{13}C$ NMR spectrum of the mclPHA isolated from *P. putida* GO19 recorded at 20°C in $CDCl_3$.

**RESULTS**

**Polyethylene Terephthalate Pyrolysis.**

The pyrolysis of PET resulted in the generation of a solid, liquid and gaseous fraction, as shown in Table 1 below.

**Table 1**

| Product Composition | Weight percentage (%) |
|---|---|
| **SOLIDS** | **77** |
| Terephthalic acid | 51,0 |
| Oligomers | 20,0 |
| Benzoic acid | 1,0 |
| others | 5,0 |
| **OIL** | **6,3** |
| Ethylene glycol | 0,75 |
| Acetic aldehyde | 5,10 |
| Others | 0,45 |
| **GASES** | **18** |
| $CO_2$ | 13,0 |
| CO | 3,5 |
| Hydrogen | 0,18 |
| Ethene | 1,0 |
| Others | 0,34 |

66% of the solid fraction was in the form of monomeric TA, and almost 26% of the solid
5 fraction was made up of oligomers of TA. The whole solid fraction was desublimated as described above to create a white powder, collected and used as a feedstock for PHA synthesis using the bacterial strains of the invention. The addition of the solid fraction of PET pyrolysis (in the form of the white powder) to an equimolar solution of sodium hydroxide resulted in the hydrolysis of the oligomers and increase in the proportion of TA

making up the solid fraction. The solid fraction dissolved in sodium hydroxide contained 99% TA monomer in the form of sodium terephthalate. The pyrolysis liquid (oil) fraction made up 6.3 % of the total weight of the pyrolysis products and contained predominantly acetic aldehyde and minor amounts of ethylene glycol. The gaseous fraction made up 18 % of the pyrolysis products and contained predominantly $CO_2$. The liquid and gaseous fraction were burned to provide energy for the pyrolysis of PET.

As described above, of the 32 isolates screened only three accumulated detectable levels of PHA. These three organisms were identified using 16S rDNA techniques as described above. All three strains shared 99% homology with known *Pseudomonas* species (Table 2). Two of the three *Pseudomonas* strains GO16 and GO19 were found to be from the species *putida*, and are presented as SEQ ID NOs 1 and 2 respectively. The other strain *Pseudomonas* GO23 was found to be from the species *frederiksbergensis*, and is presented as SEQ ID NO 3.

**Table 2**

| **Isolate**<br><br>**PHA accumulators** | **Closest match from GenBank: Accession number** | **Classification** | **% Homology** | **% Coverage** |
|---|---|---|---|---|
| GO16 (SEQ ID NO. 1) | DQ133506 | *Pseudomonas putida* | 99 | 97 |
| GO19 (SEQ ID NO. 2) | AY512611 | *Pseudomonas putida* | 99 | 99 |
| GO23 (SEQ ID NO. 3) | AJ249382 | *Pseudomonas frederiksbergensis* | 99 | 99 |

**PHA composition**

The $^{1}$H-NMR and $^{13}$C spectra for the PHA produced from the strain GO19 are shown in Figures 2 and 3 respectively. Peak assignments were typical of medium chain length PHA derivates.

5

**Conversion of PET derived Sodium Terephthalate to PHA in Shake Flask Experiments.**

All three strains according to the invention accumulated PHA to between 23 and 27 % of the total cell dry weight achieved, when supplied with either commercially available TA
10 (sodium salt) or TA (sodium salt) derived from the pyrolysis of PET. Each of the strains GO16, GO19 and GO23 produced high quality PHA, namely mclPHA. Specifically, the PHA accumulated included 3-hydroxyalkanoic acid monomers containing 8, 10 and 12 carbons (Table 3). The commercially available TA (sodium salt) was used as a comparison to PET derived TA. PHA levels and composition were identical from both
15 sources.

**Table 3**

| **Bacterial strain** | **PHA(% CDW)** | **3-OH-OCT*** | **3-OH-DEC**** | **3-OH-DODEC***** |
|---|---|---|---|---|
| *P. Putida* GO16 | 27 | 22 | 48 | 30 |
| *P. putida* GO19 | 23 | 24 | 45 | 31 |
| *P. frederiksbergensis* GO23 | 24 | 19 | 44 | 37 |

* 3-OH-OCT = 3-hydroxyoctanoic acid, **3-OH-DEC = 3-hydroxydecanoic acid, ***3-OH-DODEC = 3-
20 hydroxydodecanoic acid.

While the sequence homology of 16S rDNA indicated a strong similarity between these bacteria, often closely related species have differing PHA accumulation abilities. Indeed the PHA composition of all three strains looks almost identical (Table 3). However, PHA from *P. putida* GO23 contained a higher proportion of 3-hydroxydodecanoic acid (12 carbons) compared to PHA from the other two strains.

PHA accumulation by each of the three strains was monitored over time to determine when the onset of PHA occurred as well as the time course for PHA production. All three organisms were grown in shake flasks under the nitrogen limited conditions with 4.2 g/l of sodium terephthalate (generated by PET pyrolysis). Nitrogen concentration (as ammonium), TA concentration, cell dry weight and quantity of PHA accumulated were monitored (see Figure 1). All three bacteria had similar growth patterns, they showed a long lag period in growth (of between 8-12 h) which coincided with a lag in TA utilization, despite being grown in precultures overnight on TA (4.2 g/l). During the exponential phase of growth strains GO16, GO19 and GO23 consumed TA at 0.135 g/l/h, 0.157 g/l/h and 0.121 g/l/h respectively and had specific growth rates of 0.04 $h^{-1}$, 0.043 $h^{-1}$, and 0.049 $h^{-1}$. All three strains consumed TA fully within the same period of time (Figure 1).

**PHA Material Properties**

The properties of PHA polymer extracted from GO16, GO19 and GO23 are shown in Table 4.

**Table 4**

| Strain | Melting point enthalpy ΔHm | Crystalline melting temperature Tm (°C) | Glass transition temperature Tg(°C) | Molecular weight MW | Number-average molecular weight MN | Polydispersity index PD | % Crystallinity |
|---|---|---|---|---|---|---|---|
| GO 16 | 12.75 | 35.36 | -53.13 | $7.43 \times 10^4$ | $3.76 \times 10^4$ | 1.97 | 26.8 |
| GO 19 | 10.75 | 34.19 | -53.14 | $12.32 \times 10^4$ | $5.19 \times 10^4$ | 2.37 | 18.71 |
| GO 23 | 11.78 | 35.75 | -53.38 | $9.38 \times 10^4$ | $4.4 \times 10^4$ | 2.10 | 31.09 |

5

Gel permeation chromatography (GPC) analysis showed the PHA polymers ranged in molecular weight (Mw) from 74 kDa to 123 kDa (Table 4). The molecular weight distribution (Mw/Mn) of the PHAs ranged from 1.9 to 2.4 (Table 4) and these values are typical for mclPHAs. The DSC analysis showed that PHA polymers produced by the method of the invention are partially
10 crystalline, as evidenced by the presence of a melting peak. All three polymers produced with the different bacterial strains of the invention showed similar Tg, with slight changes in their Tm and ΔHm values (Table 4). X-ray diffraction (XRD) of cast films was used to calculate the crystallinity of the polymers. The strong diffraction peaks were located at the 2θ=19.58, 21.38 and 19.38 for PHA samples produced from GO16, GO19 and GO23 respectively. The calculated
15 crystallinity values for GO16, GO19 and GO23 are shown in Table 4. All three PHA products had similar thermal degradation patterns. Peak degradation maximum occurred at approximately 308°C with a high temperature shoulder most evident at approximately 350°C in

the differential thermograph. Polymer degradation was completed by 370°C with all residual carbonaceous materials produced during thermal degradation being burnt at about 600°C. Thus, the PHAs produced by all three strains were thermoplastics.

5 **EXAMPLE 1 (B)**

Out of the original 32 isolates described in Example 1 (A) above, six of the remaining 29 isolates which were found not to produce PHA were selected at random and classified by sequence analysis of 16S rRNA genes in accordance with the procedure as described in
10 Example 1 (A). The resulting 16S rDNA sequences were compared to known sequences in the NCBI GenBank database by BLAST programme, and the results are shown in Table 5.

**Table 5**

| **Isolate**<br><br>**(PHA non producers)** | **Closest match from GenBank:**<br><br>**Accession number** | **Classification** | **% Homology** | **% Coverage** |
|---|---|---|---|---|
| GO 13A (SEQ ID NO. 6) | AB008001 | *Pseudomonas putida* | 99 | 99 |
| GO 1 (SEQ ID NO. 7) | EU111737.2 | *Pseudomonas putida* | 100 | 99 |
| GO 6 (SEQ ID NO. 8) | AY823622.1 | *Pseudomonas putida* | 100 | 99 |
| GO 8 (SEQ ID NO. 9) | EF093130.1 | *Pseudomonas sp.* | 99 | 99 |
| GO 14 (SEQ ID NO. 10) | Y17052 | *Burkholderia glathei* | 99 | 99 |
| TA 1 (SEQ ID NO 11) | AM402950 | *Stenotrophomonas* sp. | 98 | 99 |

Four of the six isolates were found to be from the genus *Pseudomonas*, with three of the four *Pseudomonas* strains being specifically from the species *putida*. The utilization of terephthalic acid (sodium salt) by these six strains was monitored as described in Example 1 (A), and it was found that although sodium terephthalate was utilized, no PHA was
5 produced. Since it has been shown that some strains of *Pseudomonas putida* (e.g. the strains shown in Table 5) are incapable of accumulating PHA from sodium terephthalate, (presumably simply producing biomass instead), it is surprising that *Pseudomonas putida* strain GO16 and *Pseudomonas putida* strain GO19 were found to accumulate PHA from sodium terephthalate. Similarly, in view of the fact that a *Pseudomonas sp*. strain was
10 incapable of accumulating PHA from sodium terephthalate, it is also surprising that *Pseudomonas frederiksbergensis* strain GO23 was found to accumulate PHA from sodium terephthalate.

**EXAMPLE 2 (Comparative Example)**

15

Three conventional bacterial strains known to utilize TA as a sole source of carbon and energy, namely *Comamonas testosteroni* YZW-D, *Comamonas testosteroni* T-2 and *Comamonas testosteroni* PSB-4, identified in Table 6, were tested for PHA accumulation. These three strains are known to utilize TA, but did not produce any PHA (presumably
20 simply producing biomass instead). The utilization of terephthalic acid (sodium salt) was monitored as described in Example 1 (A), and it was found that although sodium terephthalate was utilized, no PHA was produced.

25

30

**Table 6**

| Source | Strain |
|---|---|
| Wang et al[1] | *Comamonas testosteroni* YZW-D |
| DSMZ[2] | *Comamonas testosteroni* T-2 |
| DSMZ[2] | *Comamonas testosteroni* PSB-4 |

[1]Wang Y. Z.; Zhou Y.; Zylstra G. J. Molecular analysis of isophthalate and terephthalate degradation by *Comamonas testosteroni* YZW-D. *Environ Health Perspect.* **1995**. 103 Suppl 5:9-12.

[2]DSMZ = the German resource centre for biological material. Junker F.; Saller E.; Schlafli Oppenberg H. R.; Kroneck P. M.; Leisinger T.; Cook A. Degradative pathways for p-toluenecarboxylate and p-toluenesulfonate and their multicomponent oxygenases in Comamonas testosteroni strains PSB-4 and T-2. *Microbiology.* **1996**. 142, 2419-2427.

This comparative example is further evidence of the surprising result achieved by the strains of the invention, *Pseudomonas putida* strain GO16, *Pseudomonas putida* strain GO19 and *Pseudomonas frederiksbergensis* strain GO23. Given that known TA degraders were found to be incapable of producing PHA, it is surprising that the strains of the invention were capable of accumulating PHA from TA (sodium salt).

In summary, although some strains exist which are capable of degrading TA, and some strains exist which are capable of producing PHA, no strains have existed until now which are capable of utilizing TA to produce PHA.

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| 3-3-2 | Address of depositary institution | Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, United Kingdom |
| 3-3-3 | Date of deposit | 24 January 2008 (24.01.2008) |
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**CLAIMS:**

1. A method for producing polyhydroxyalkanoate (PHA), comprising (i) culturing in a culture medium comprising terephthalic acid and/or a salt thereof and/or an ester thereof one or more bacterial strains which are capable of accumulating PHA from terephthalic acid or a salt or ester thereof and which are selected from *Pseudomonas putida* strain GO16 having the accession number NCIMB 41538, *Pseudomonas putida* strain GO19 having the accession number NCIMB 41537, and *Pseudomonas frederiksbergensis* strain GO23 having the accession number NCIMB 41539; and (ii) recovering the PHA produced from the culture medium.

2. A method as claimed in claim 1, wherein the culture medium comprises strain GO19 and/or strain GO16.

3. A method as claimed in claim 1 or claim 2, wherein the PHA recovered from the culture medium comprises at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% medium chain length (mcl) PHA.

4. A method as claimed in claim 3, wherein at least 80%, preferably 85%, more preferably at least 90%, even more preferably at least 95% of the mclPHA comprises repeating units of C8, C10 and C12 monomers.

5. A method as claimed in claim 4, wherein the mclPHA comprises repeating units of C8, C10 and C12 monomers in a respective amount by weight of mclPHA of 15% - 25% C8, 40% - 50% C10 and 30% - 40% C12.

6. A method as claimed in any preceding claim, wherein a salt of terephthalic acid is present in the culture medium, and wherein, preferably, the salt is selected from alkali metal and alkaline earth metal salts and mixtures thereof, especially sodium and potassium salts, and magnesium, calcium and barium salts.

7. A method as claimed in claim 6, wherein the salt is a mono- or di- sodium or potassium salt of terephthalic acid.

5 8. A method as claimed in claim 7, wherein the salt is monosodium terephthalate.

9. A method as claimed in any preceding claim, wherein an ester of terephthalic acid is present in the culture medium, and wherein, preferably, the ester is selected from mono- and di-esters of terephthalic acid and mixtures thereof, especially mono- and di- C1 – C4 alkyl esters
10 and mono- and di- glycol esters.

10. A method as claimed in any preceding claim, wherein the terephthalic acid or salt or ester thereof may be obtained from hydrolysis or pyrolysis of polyethylene terephthalate (PET), preferably pyrolysis of PET.
15

11. A method as claimed in any preceding claim, wherein the method comprises culturing the one or more strains in the culture medium for a period of from about 12 hours to about 72 hours, preferably from about 24 hours to about 60 hours, more preferably about 48 hours, at a temperature of from about 25°C to about 35°C, preferably about 30°C.
20

12. A method as claimed in any preceding claim, wherein the culture medium is nitrogen limited, with a maximum nitrogen content of about 0.5 g/l, preferably 0.067 g/l culture medium.

25 13. *Pseudomonas putida* strain GO16 having the accession number NCIMB 41538.

14. *Pseudomonas putida* strain GO19 having the accession number NCIMB 41537.

15. *Pseudomonas frederiksbergensis* strain GO23 having the accession number NCIMB
30 41539.

1/3

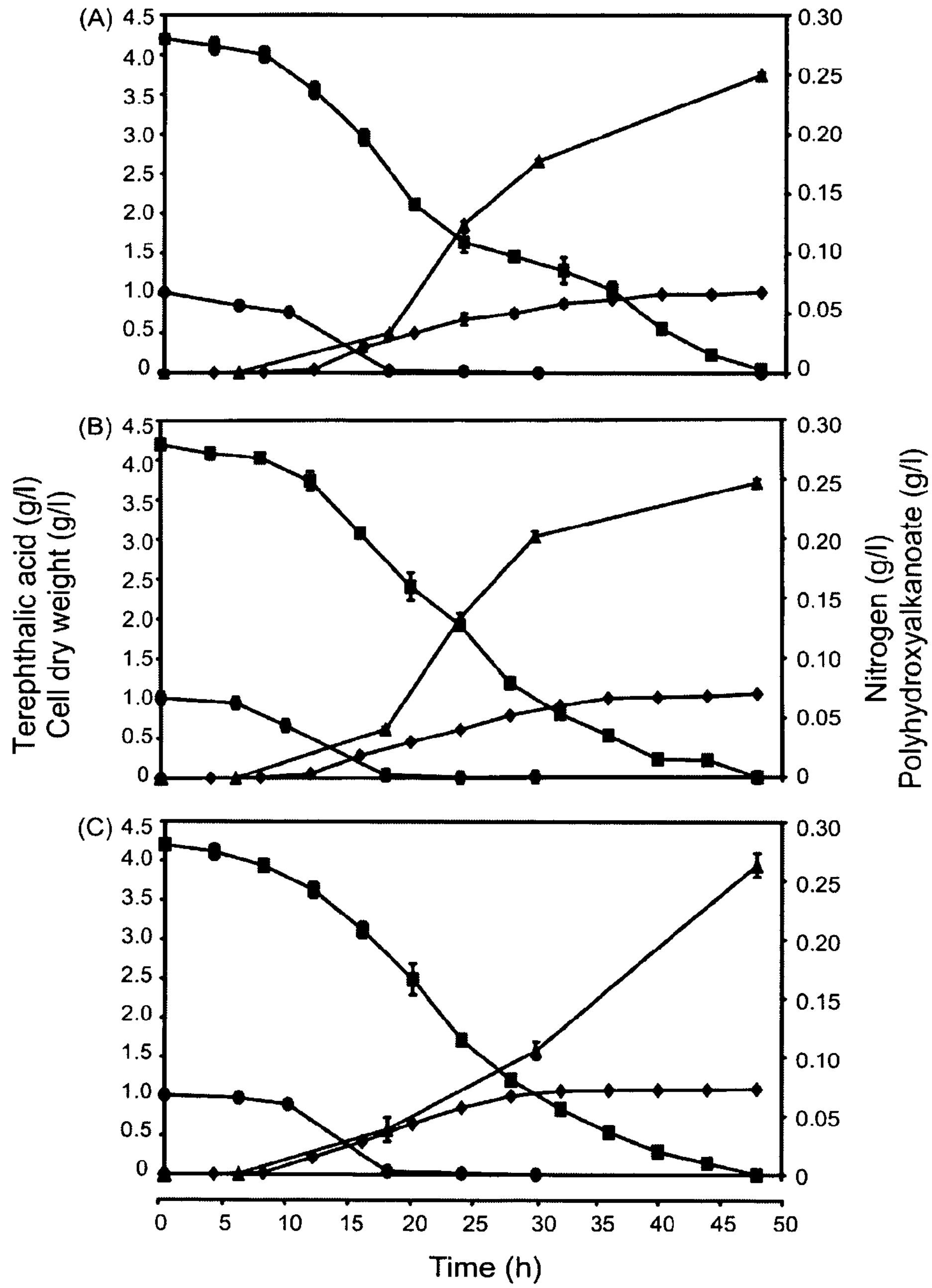


**Figure 1**

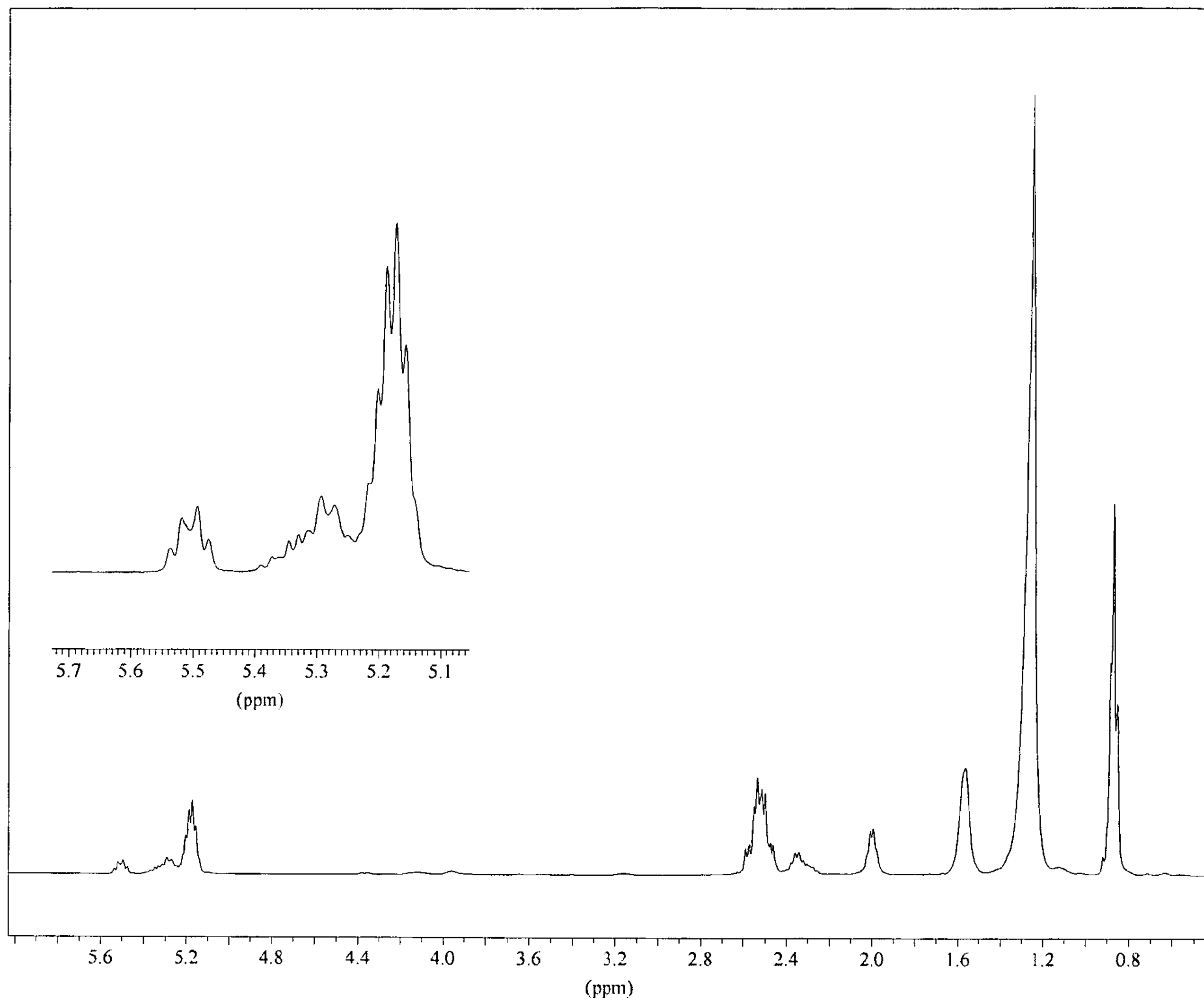


**Figure 2**

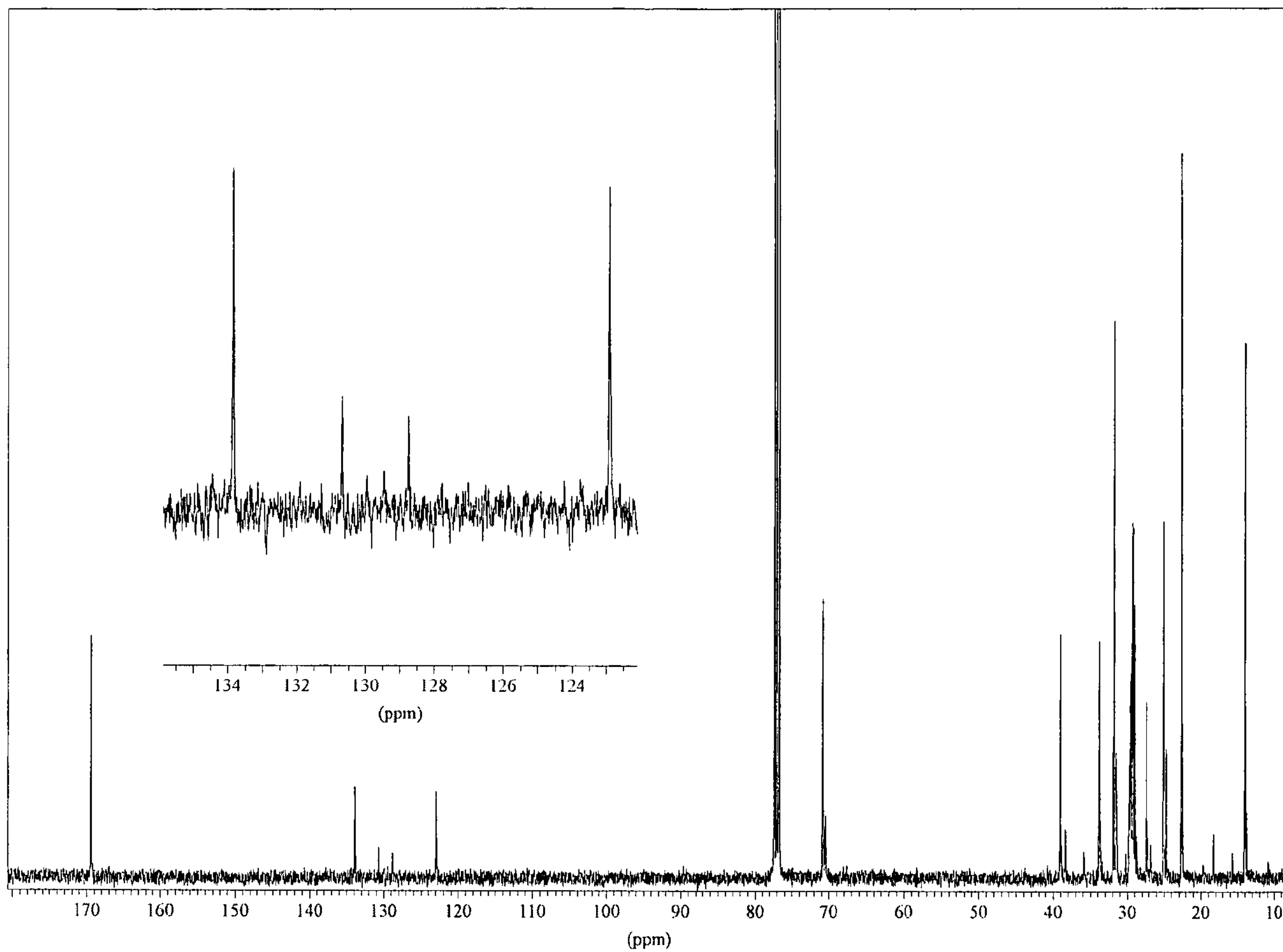


**Figure 3**