CHAPTER THREE

MATERIALS AND METHODS

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3.1 PHA Production

3.1.1 Bacterial strain

Pseudomonas putida PGA1 was used in this study to produce the PHA.

3.1.2 Stock culture storage

The strain was maintained on nutrient agar medium (Oxoid) slants and stored at 4°C.

3.1.3 Media and growth condition

Experiments were conducted using rotary shaking-flasks set at 240rpm and at a constant temperature of 30°C. *Pseudomonas putida* were first cultivated in 400ml of rich medium (R-medium) (Doi *et al*,1989) which was first autoclaved, to produce the biomass. The cells were harvested by centrifugation after 24 hours, washed with distilled water and transferred to E2 medium (Lageveen *et al*, 1988) containing saponified palm kernel oil (SPKO) as the carbon substrate at a concentration of 0.5% (w/v). The E2 medium, together with the carbon substrate, were autoclaved before the

cells were added. The cells were harvested after 48h by centrifugation, washed and lyophilized.

Table 3: Types of medium used in culturing

R-medium (Doi *et al*, 1989)	per 400ml of distilled water
Yeast extract	4g
Nutrient broth	6g
Ammonium sulphate	2g
E2 medium (Lageveen *et al*, 1988)	per 400 ml of distilled water
$NaNH_4HPO_4.H_2O$	0.69g
$K_2HPO_4.3H_2O$	1.50g
KH_2PO_4	0.74g
MT solution	0.40g
$MgSO_4.7H_2O$	4.0g
Saponified palm kernel oil (0.5%)	2.0g
Microelement solution (MT)	In 500ml of 1N HCl
$FeSO_4.7H_2O$	1.39g
$MnCl_2.4H_2O$	0.99g
$CoSO_4.7H_2O$	1.41g
$CaCl_2.2H_2O$	0.74g
$CuCl_2.2H_2O$	0.08g
$ZnSO_4.7H_2O$	0.14g

3.1.4 Palm kernel oil

Palm kernel oil (PKO) was given by the Southern Acid (M) Ltd, Jalan Kapar, Kelang, one of the palm oil processing factories in Malaysia. PKO is extracted from the kernel of oil palm fruit. It is composed of 16% unsaturated fatty acid mainly oleic acid (C18:1) and linoleic acid (C18:2) and 84% saturated fatty acid of which lauric (C12), myristic (C14) are the major components.

3.1.5 Saponification of PKO

Saponification of PKO was done according to the method described by PORIM (Test methods for Palm Oil Products, 1981) to get the saponified palm kernel oil (SPKO). The glycerides present are split by alcoholic alkali reaction, which releases the fatty acids. 2.8g of sodium hydroxide was dissolved in 100ml of ethanol. 8g of palm kernel oil was added to the ethanolic sodium hydroxide solution and refluxed gently for 1 hour. Excess ethanol was evaporated by rotary evaporation, leaving behind the solid sodium salt of fatty acids. The salt were then dried and stored at room temperature.

3.1.6 Extraction of PHA

1g of dried cells were put into 100ml of chloroform and refluxed for 4 hours. The extract was then cooled to room temperature and filtered through Whatman filter paper. The excess chloroform was then evaporated to about 10ml under reduced pressure, using Eyela Rotary Evaporator. The polymer in the concentrate was then precipitated by adding the solution drop-wise into 100ml of stirred methanol. The solution was left for 2 hours without stirring to allow the polymer to settle under gravity. The methanol-chloroform mixture were then decanted and the precipitated polymer was redissolved in 10ml of chloroform and poured into a glass petri-dish where the remaining solvent were allowed to evaporate. Upon evaporation, a polymer film was obtained.

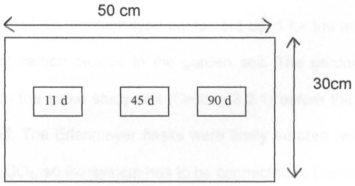
3.2.Biodegradability Testing Methods in Soil

3.2.1 Natural environment

Specimens of PHA were cut into 3 pieces with a size of 1.5cm x 1.5cm each. Specimens were measured for weight in mg. PHA was buried in garden soil with pH of 6.5 and the experiment was carried out at 205, Jalan Damansara, Kuala Lumpur, Malaysia . The burial site was located on level land. The area used for burial was approximately 50cm x 30cm. Earlier, the burial site was weeded and small stones were removed. Before

burial a mass of soil in the required place was removed out to approximately 10cm deep from the surface. The soil was then sieved. Half of the sieved soil was put back into the hole and its surface was mildly leveled. Burial of test specimens was partitioned into 3 testing periods so that digging out the specimens could easily be done without disturbing the other specimens which would be tested at other periods. The space between each specimen was approximately 8cm. Burial was carried out without bending the film and the remaining half of the sieved soil was then put back and the surface is mildly leveled so that the specimens were located around 5cm under the surface. The burial site was left under natural condition. Each of the burial film was dug out at appropriate intervals, washed to remove the adhered soil, dried in dessicator and residual weight was taken. The samples were used for the analysis studies of biodegradation.

Figure 3: Diagrammatic area of burial site



3.2.2 Enclosed environment

For the enclosed environment, the PHA was mainly tested for its biodegradability by the aerobic gas evolution test since in the natural environment it is difficult to monitor the carbon dioxide evolution.

Figure 4: Aerobic gas production equipment test

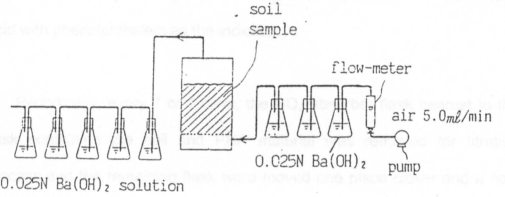


Figure above shows the aeration-type equipment used for the aerobic gas evolution test of carbon dioxide in the garden soil. The garden soil used was taken from the same study plot (Section 3.2.1) before the PHA samples were buried. The Erlenmeyer flasks were firstly aerated with air. Since air do contain CO_2, so the system has to be connected to the 0.025N barium hydroxide ($Ba(OH)_2$) to scrub out any carbon dioxide passed before

it could be connected to the system. Carbon dioxide-absorber flasks were filled with 100ml of 0.025N $Ba(OH)_2$ each and were connected to the flask containing the weighed PHA buried in soil in series as shown in Figure 4. Control system was run at the same time, with the same apparatus, except the soil do not contain any PHA. The test was started by bubbling the air through the solution at a rate of approximately 2-5 bubbles per minute. The amount of CO_2 produced in each flask will react with $Ba(OH)_2$ and precipitate as barium carbonate, $BaCO_3$. The amount of CO_2 produced was determined by titrating the remaining $Ba(OH)_2$ with 0.05N hydrochloric acid with phenolphthalein as the indicator.

Periodically, every 7 or 8 days, the CO_2 absorber flask nearest to the flask containing the soil and PHA material was removed for titration process and the remaining flask were moved one place closer and a new absorber filled with 100ml 0.025N $Ba(OH)_2$ was placed at the far end of the series. This sequence was carried out to ensure that the carbon dioxide produced do not escape from the setting. The experiment was carried out continuously for 90 days.

3.3 Studies of soil condition for biodegradation

3.3.1 Moisture content of soil

For this experiment, it is important to know the moisture content for the natural and enclosed environments because this factor will affect the degradation of PHA in the soil. The moisture content was determined by weighing an amount of soil at day 0 and day 90 for both environments. The soil was then dried in an oven at 33°C for several days. The weight of the soil was measured everyday until a constant weight was achieved. This step was taken to ensure that the soil is totally dry. The percentage of the moisture content in the soil can be calculated by equation below:

$$\text{Percentage of moisture content in soil} = \frac{\text{Weight of soil after drying}}{\text{Weight of soil before drying}} \times 100$$

3.3.2 pH of soil

pH of the garden soil was determined using pH test strips from Sigma Chemical Company. The pH test strip was dipped in the soil of the natural and enclosed environments at day 0 and day 90 to get the pH readings.

3.4 The Analytical Studies of Biodegradability

3.4.1 Gross weight loss

PHA buried in the natural environment was dug out from the soil, washed thoroughly, dried and measured for its weight loss at different intervals; 11 days, 45 days and 90 days. For enclosed environment, the gross weight loss was taken after 90 days only. Weights were measured using an electronic balance (Denver Instrument AA-200DS).

3.4.2 Surface morphology of PHA

3.4.2.1 Phase contrast microscopy

Samples, before and after burial at different time were washed and were then viewed using light microscope to see the surface morphology. The magnifications used was 200x (20x objective and 10x eyepiece). The sample was put on a piece of slaid and a drop of immersion oil was added on top of the sample before it was covered with the microscopic cover glass. The samples were then viewed using Nikon HFX-DX light microscope.

3.4.2.2 Electron microscopy

Samples, before and after burial at different interval times were washed and dried. Then a small piece was cut and mounted on a specimen holder called a stub by using a special Scanning Electron Microscopy (SEM) adhesive tape. The stub is made of aluminium to provide good supports for morphological studies. The specimen was then coated with gold. Specimen coating is carried out to increase conductivity of nonconductive SEM samples to prevent surface charging which can seriously deteriorate the image. The specimens were then scanned under electron microscope (Philips 515) at 10.0kV acceleration voltage. Samples were observed at 2000 X magnification.

3.4.3 Carbon dioxide evolution

Biodegradation can also be determined by monitoring the CO_2 evolved. CO_2 evolution test was carried out for PHA from enclosed environment only. The CO_2 evolved was trapped in flask containing $Ba(OH)_2$ solution and precipitated as barium carbonate. The amount of CO_2 produced was determined by titrating the remaining $Ba(OH)_2$ with hydrochloric acid. The calculation of CO_2 produced is as follows:

Calculation for percentage carbon dioxide evolved :

A) Calculation for mg CO_2 produced theoretically:

To make the whole calculation simple, we assume that PHA only has one type of monomer which is C8 since C8 is the most dominant monomer for PHA derived from SPKO which is 82.9 % (Tan *et al* 1997).

Assume that all the carbon is converted to carbon dioxide during the degradation process.

Molecular weight of $C_8H_{14}O_2$ = (8x12) + (14 x 1) + (2 x 16)
= 142 g/mol

Molecular weight of CO_2 = 12 + (16 x 2)
= 44 g/mol

Weight of sample = 0.23 g

142.0 g ⟶ 1 mole of C8 monomer

0.23 g ⟶ $0.23 \div 142 = 1.62 \times 10^{-3}$ mole C8 monomer

In 1 mole of C8 monomer there is 96 g of carbon

So in 1.62×10^{-3} mole there is $1.62 \times 10^{-3} \times 96$ g
= 0.15552 g carbon

12 g carbon yield 44 g CO_2

0.15552 g carbon will yield $(0.15552 \div 12) \times 44$
= 0.570 g
= 570.0 mg CO_2

Theoretically, 570.0 mg carbon dioxide will be produced from the 230 mg sample

B) Calculation for mg CO_2 produced from the experiment :

$Ba(OH)_2 + CO_2 \rightarrow BaCO_3 + H_2O$ (equation 1)

$Ba(OH)_2 + 2HCl \rightarrow BaCl_2 + 2H_2O$ (equation 2)

Assume that initial solution contains n mmol $Ba(OH)_2$, after reacting with carbon dioxide to precipitate out as Ba CO_3, m mmol of $Ba(OH)_2$ is left behind for titration with HCl.

From equation 1: $n = M_1V_1$

where M_1 is the molar concentration of $Ba(OH)_2$ used and V_1 is the volume of the $Ba(OH)_2$ solution.

From equation 2 : $m = \frac{1}{2} M_2V_2$

where M_2 is the concentration of HCl and V_2 is the titer volume

$Ba(OH)_2$ that have reacted with CO_2 = n - m

= $M_1V_1 - \frac{1}{2} M_2V_2$

= $(0.025)(100) - (1/2)(0.05)(V_2)$

= $(2.5 - 0.025\ V_2)$ mmol

CO_2 produced = n - m

= $(2.5 - 0.025\ V_2)$ mmol

$\therefore$ mg CO_2 produced from expt = CO_2 produced x formula weight of CO_2

$$= (2.5 - 0.025\ V_2) \times 44$$

C) Percentage of CO_2 evolved :

$$\text{Percentage of } CO_2 \text{ evolved} = \frac{\text{mg } CO_2 \text{ produced}}{\text{mg } CO_2 \text{ theoretical}} \times 100$$

$$= \frac{(2.5 - 0.025\ V_2) \text{ mmol} \times 44 \text{ mg/mmol}}{570 \text{ mg}} \times 100$$

3.4.4 Characterization by Infrared Spectrophotometry (IR)

This method was carried out to check for any structural changes of PHA before and after the biodegradation process in the natural environment. The sample, after different period of degradation in soil, was washed to remove soil particles and dried in oven. The dried PHA was dissolved in chloroform and precipitated into excess methanol. The organic solution was decanted off and the precipitated polymer was redissolved in small amount of chloroform. One drop of the PHA solution in chloroform was placed on a sodium chloride cell which was blown with hot air to remove all solvent. After thorough drying, IR spectrum of the polymer film was recorded on a Perkin Elmer Model 6000 FTIR instrument.

3.4.5 Characterization by Gas Chromatography

The composition of PHA samples was analysed by gas chromatographic method developed by Braunegg *et al*, 1978. Approximately 8 mg of the samples were suspended in 2ml of methanol containing 15% (v/v) concentrated sulphuric acid and put in a screw-cap test tube. After the addition of 2 ml of chloroform, the test tube was closed tightly and refluxed at 100°C in a heating block for 240 minutes. This was followed by rapid cooling in cold water. 1 ml of distilled water was added and the mixture was vortexed. It was then allowed to stand for several minutes for phase separation. The organic phase at the bottom which contained methyl esters was recovered and stored in a screw cap test tube or vial. Then 0.5% (v/v) of methyl benzoate was added to the organic phase solution as the internal standard. For the standards, β-hydroxy methyl esters ranging from C8 – C16 from Sigma Chemical Company were used.

A Shimadzu GC-14A GC system with a flame ionization detector (FID) was used along with a 30 m x 0.25 mm (ID) capillary column. 2 μl of sample was injected using a Kloehn 10 μl syringe. The column temperature was programmed as below:

Initial temperature = 68°C

Hold time at initial temperature = 2 minutes

Increase of column temperature with time	= 5.0°C/min
Final temperature	= 200°C
Run time	= 20 minutes
Injector temperature	= 170°C
Detector temperature	= 200°C

For quantitative determination of the polymer composition, the peak area ratio of the standards with internal standards (reference factor, R_f) is plotted against the concentration of the standard (standard curve for each methyl ester of 3-hydroxyalkanoic acids). These are shown on Figure 5a, b, c, d. Due to the inavailability of C6 (methyl ester of 3-hydroxyhexanoic acid) standard, the measurement for C6 was done by extrapolating the response factor of other available standards. It was shown in earlier study that only compounds containing 8 carbons or less could give a linear graph (Theanmalar, 1996). Thus, R_f of C8 at different concentration was plotted against the carbon number (Figure 5e). Based on this, a standard curve for C6 was derived (Figure 5f).

In order to get the retention time for C6, a graph of retention time versus carbon number of other standards available was plotted. This will give a linear regression line and the retention time for C6 was obtained. (Figure 6).

From the chromatogram, data to calculate the mole percentage of each monomer in a sample is obtained and calculation is as follows:

Calculations for mole percentage of each monomer in a sample:

Example: sample A = PHA before degradation

A) Find concentration of monomers

Example: C8

Reference factor is calculated from data obtained from chromatogram :

$$R_f = \frac{\text{Area of the monomer methyl ester}}{\text{Area of internal standard (methyl benzoate)}}$$

Data obtained from chromatogram of Sample A:

From chromatogram of sample A, area of internal standard = 543229

Monomer	Area of monomer methyl ester	R_f
C6	3603	0.006633
C8	41637	0.076647
C10	40470	0.074499
C12	19319	0.035563
C14	2145	0.003949

A standard graph for methyl ester of C8 is plotted using data in table below: R_f versus concentration of C8 methyl ester (Figure 5a).

Standard graphs for other monomers C10, C12, C14 and C6 are also plotted as shown in Figure 5b, c, d and e respectively.

Concentration of C8 methyl ester (mg/ml)	R_f
0.00000	0.00000
0.15625	0.00775
0.31250	0.01302
0.62500	0.01624
1.25000	0.08010

From the graph, concentration for C8 in sample A is obtained :

$y = 0.0616x$ (equation from the linear line)

$x = y \div 0.0616$

$= R_f \div 0.0616$

$= 0.076647 \div 0.0616$

$= 1.244$ mg/ml

$= 1244$ µg/ml

B) Convert the µg into µmoles

$$\mu\text{moles of C8} = \frac{\mu\text{g of monomer}}{\text{Molecular weight of C8}}$$

$$= \frac{1244}{160.18}$$

$= 7.77$ µmoles

C) Find the total µmoles

Total µmoles = µmoles of (C6 + C8 + C10 + C12 + C14)

= 14.67 μmoles

D) Calculate the mole percentage

$$\text{Mole percentage for C8} = \frac{7.77}{14.67} \times 100$$

$$= 53.0 \text{ mole \%}$$

The same calculations were carried out to get the mole % for all the monomers.

Figure 5a : Rf versus concentration of C8 methyl ester

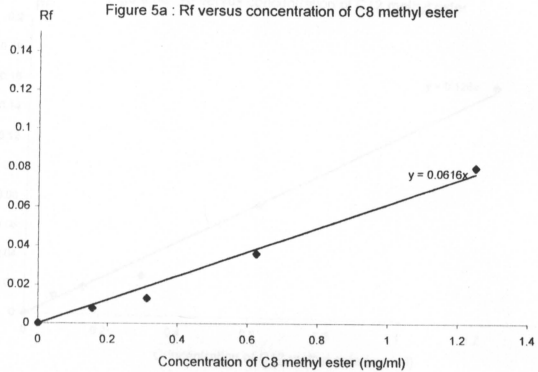


Figure 5b : Rf versus concentration of C10 methyl ester

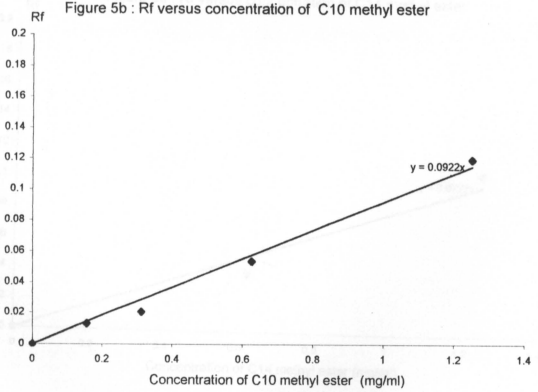


Figure 5c : Rf versus concentration of C12 methyl ester

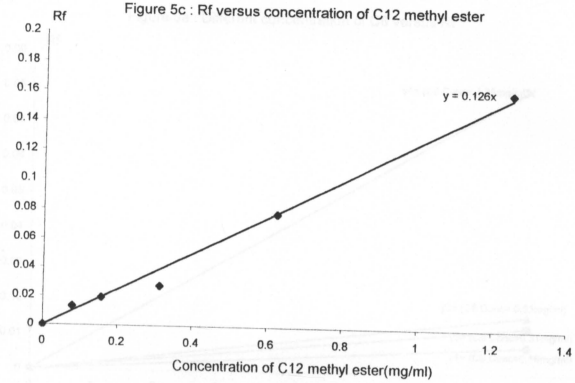


Figure 5d : Rf versus concentration of C14 methyl ester

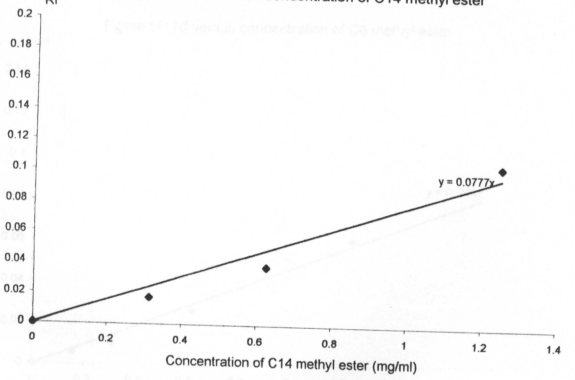


Figure 5e : Different concentration of C8 versus Rf

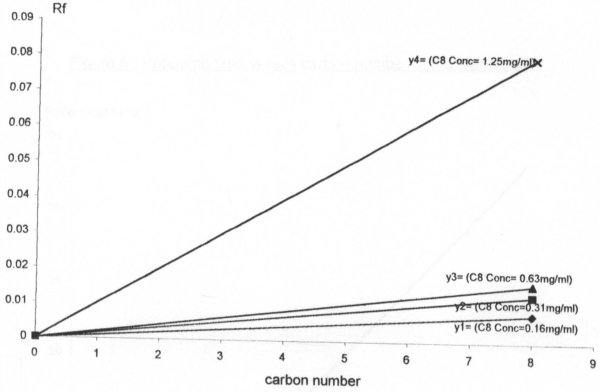


Figure 5f : Rf versus concentration of C6 methyl ester

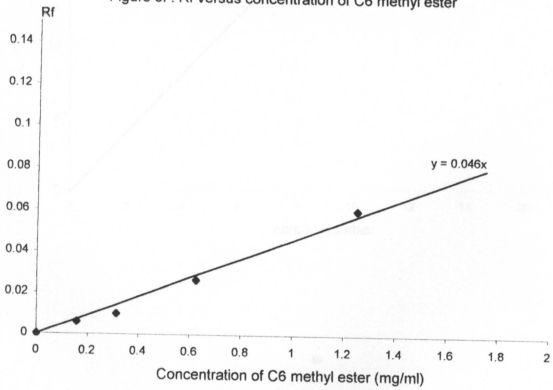


Figure 6 : Retention time versus carbon number of the standards