CHAPTER II

MATERIALS AND METHODS

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2.1 PHA BIOSYNTHESIS

2.1.1 Bacteria strain

Pseudomonas putida PGA1 used in this study was kindly given by G. Eggink from the Agrotechnological Research Institute, Wageningen, The Netherlands.

2.1.2 Strain maintenance

The strain was maintained on nutrient agar slants at 4°C. Testing of pure culture was done every two months on nutrient agar plates.

2.1.3 Inoculum

A single colony from nutrient agar plate (working culture) was subcultured onto a nutrient agar slant and incubated for 48 hours at 30°C. The entire slant of *Pseudomonas putida* PGA1 was then used as an inoculum.

2.1.4 Media and growth conditions

A two-stage fermentation (growth phase and production phase) was carried out using one litre Erlenmeyer flask, shaken at 240rpm and 30°C. *P. putida* PGA1 inoculum was first grown in modified Rich medium (R-medium) (Table 2.1), in which the meat extract and

peptone were replaced by nutrient broth (Doi, 1990). The cells were harvested after 20 hr by centrifugation at 3000rpm, 10 minutes at 15°C, washed with sterilised distilled water twice and transferred to the nitrogen–limiting E2 medium (Lagaveen *et al.*, 1988) (Table 2.1), containing 0.5% w/v SPKO as the sole carbon source to promote PHA production. The cells were harvested after 48 hrs by centrifugation at 3000rpm, 10 minutes at 15°C and washed with sterile distilled water twice. The washed cells were resuspended in methanol and dried in vacuum oven at 30°C to constant weight.

Table 2.1 Growth and production medium

	In 1.0 litre distilled water
Rich medium (R-medium)	
• Yeast extract	10g
• Nutrient broth (with peptone and meat extract)	15g
• Ammonium sulphate	5g
E2 medium (0.5X strength)	
• $NaNH_4HPO_4.H_2O$	1.725g
• $K_2HPO_4.3H_2O$	3.75g
• KH_2PO_4	1.85g
• $MgSO_4.7H_2O$	0.5g
• Microelement solution (MT)	1.0ml
MT solution	(In 25ml of 1M HCl)
• $FeSO_4.7H_2O$	0.0695g
• $MnCl_2.4H_2O$	0.0495g
• $CoSO_4.7H_2O$	0.0705g
• $CaCl_2.2H_20$	0.037g
• $CuCl_2.2H_2O$	0.004g
• $ZnSO_4.7H_2O$	0.007g

2.1.4.1 Palm kernel oil

Palm kernel oil (PKO) is the extract from the nut of the oil palm (*Elaeis guineensis Jacq.*) fruit. It comprises the triglycerides of the following major fatty acids: 44% saturated lauric

acid ($C_{12:0}$), 14% saturated myristic acid ($C_{14:0}$) and 17% unsaturated oleic acid ($C_{18:1}$). A small percentage of other fatty acids is also present, including caprylic ($C_{8:0}$), capric ($C_{10:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and linoleic ($C_{18:2}$) acids (Berger, 1983). The PKO was kindly given by the Southern Acid (M) Ltd., Klang.

2.1.4.2 Saponification of PKO

24g PKO was added to the ethanolic potassium hydroxide solution prepared by dissolving 8.4g sodium hydroxide in 300ml ethanol. The mixture was refluxed at 65°C for 60 minutes, to break the triglycerides into sodium salts of fatty acids. Excess ethanol was evaporated under reduced pressure in Eyela Rotary Evaporator. The remaining semi-solid sodium salts of fatty acids were further dried to constant weight in the fume hood.

2.1.5 PHA extraction and PHA film casting

1.86 g of dried cells was suspended in 186mL chloroform and refluxed at 65°C for 5 hrs to extract the PHA. The mixture was cooled to room temperature and filtered through a Whatman filter paper No. 1. The excess chloroform in the filtrate was evaporated to about 20mL under reduced pressure in Eyela Rotary Evaporator. The polymer in the concentrated filtrate was then precipitated by adding the solution drop-wise into 186ml of stirred methanol in a conical flask and stirred for 5 minutes. The solution was allowed to stand overnight. The methanol-chloroform mixture was decanted. The precipitated polymer, a gel like layer was caste into PHA film in a petri dish.

For casting a PHA film, the precipitated polymer was redissolved in 20ml chloroform. The mixture was poured onto a glass petri dish with inner diameter of 6.6cm as a casting surface and allowed to evaporate until a polymer film was obtained. Evaporation was done in still air to prevent bubble formation on the polymer film. The polymer film was aged for 3 weeks at room temperature to reach equilibrium crystalinity prior to analysis (Shimamura *et al.*, 1994).

2.2 BIODEGRADABILITY TEST

The biodegradation test was carried out three times. First time for 10 days (Run 1) , second time for 28 days (Run 2) and third time for 86 days (Run 3). Run 1 was a trial run to test the feasibility of the experimental set up. Run 2 (short term run) was found to be too short to extrapolate the inherent biodegradability of PHA. Hence, the third run (extended run) with slight modification was performed.

2.2.1 Sampling river water

2.5 L of Kayu Ara River water was collected using sterilised screw capped Teflon bottles at 30cm below the water surface at 5 spots where the water flow was continuous. The sample was kept in an icebox during transportation to the laboratory (Reade, 1991). For Run 3, the water was collected from one spot where the flow was continuous.

2.2.2 Inoculum preparation

The river water sample was first aerated for 4 hrs to promote the oxidation of soluble organic compounds by the microorganisms and to promote microorganism growth. The

aerated sample was then left for 30 min to allow the sludge and soil debris to settle. The clear supernatant was withdrawn and stored for two days at 4°C. It was mentioned in the ASTM standard D5209-91 (1992) that the water should not be used on the day prepared and can be stored for two weeks at 4°C. This might allow the microbes to settle to lag phase as the beginning phase of the experiment. In Run 3, the supernatant was aseptically filtered through Whatman filter paper No.1 to remove suspended particles before storage.

2.2.3 Biodegradability experimental design

The biodegradation test methods were modified from the ASTM standard test methods for determining the aerobic biodegradation of plastic materials in the presence of municipal sewage sludge (D5209-91, 1992) and Doi *et al.*'s biodegradability test of polyesters in river water (1996).

225ml of the pre-treated river water, which contained the inoculum was added to a sterilised degradation reactor (500ml Erlenmeyer flask). The number of reactors used for Run 1, Run 2 and Run 3 is given in Table 2.2. In some control reactors, the river water was sterilised. 0.2mL of a sterilised mineral salt solution (Table 2.3) was then added to each of the reactor. In Run 3, the river water used was 200ml and the mineral salts were dissolved separately in four groups (Table 2.3) in a double strength concentration. Then 0.1ml of each group was added into the reactor. This step was done to ensure that all mineral salts dissolved in the water. Some difficulty in dissolving the salts was faced when all the salts were dissolved together in one flask.

Table 2.2 –Test and control reactors set up in terms of the presence of PHA/PHB film and microorganisms, and test period

Experiment	Test or control reactors	PHA/ PHB*	Microbe	Test period (days)
Run 1 without stirring (Trial run)	Test 1	+	+	10
	Control 1	-	+	10
	Control 2	+	-	10
	PHB positive control	+*	+	10
Run 2 without stirring (Short-term run)	Tests 1 – Test 5	+	+	6,10,15,21,28
	Control 1	-	+	28
	Control 2	+	-	28
	Control 3	-	-	28
	PHB positive control	+*	+	28
Run 3 with stirring (Extended run)	Test 1 – Test 5	+	+	5,20,41,60,86
	Control 1	-	+	86
	Control 2	+	-	86
	Control 3	-	-	86
	PHB positive control	+*	+	86
	PHB sterile control	+*	-	86

Note. The reactors in Run 1 were aerated with CO_2-scrubbed air at a rate of approximately 100ml/min monitored at the main supply. Every reactor of Run 2 & Run 3 were aerated with CO_2-scrubbed air at 1.0-1.5 bubbles/sec.

The degradation reactors were then aerated with CO_2-scrubbed air at a rate of 1.0-1.5 bubbles/sec per reactor for 24 hr to purge the system free of CO_2. The airflow rate regulator to each reactor was only introduced in Run 2 and Run 3. The air pre-treatment system and

the diagrammatic set up of the degradation test are shown in Figure 2.1. All tests were performed at room temperature (28°C).

After 24hrs' purging of the test system, the CO_2-absorber and check-valve, consisting of 15 ml (Run 1), 50ml (Run 2), 50-70ml (Run 3) of 0.005 ± 0.001M $Ba(OH)_2$ in 100ml Erlenmeyer flasks were connected in series to the outlet of each degradation reactor. The aeration was continued at approximately 100ml/sec at the main supply for Run 1 and at 1.0-1.5 bubbles/sec for every degradation reactor in Run 2 and Run 3.

Table 2.3 - Mineral salt solution for biodegradability test medium

Mineral salt	In 1 liter distilled water
$MgSO_4.7H_2O$	22.5g
$(NH_4)_2SO_4$	40.0g
$CaCl_2$	27.5g
$FeCl_3.6H_2O$	0.25g
Phosphate buffer	
• KH_2PO_4	8.5g
• K_2HPO_4	21.75g
• $Na_2HPO_4.H_2O$	33.4g
• NH_4Cl	1.7g

Note. Modified from "Standard test method for determining the aerobic biodegradation of plastic materials in the presence of municipal sewage sludge (D5209-91)," by American Society for Testing and Materials (ASTM), 1992, *Annual book of ASTM standards*, (pp.815-818), Philadelphia: Author.

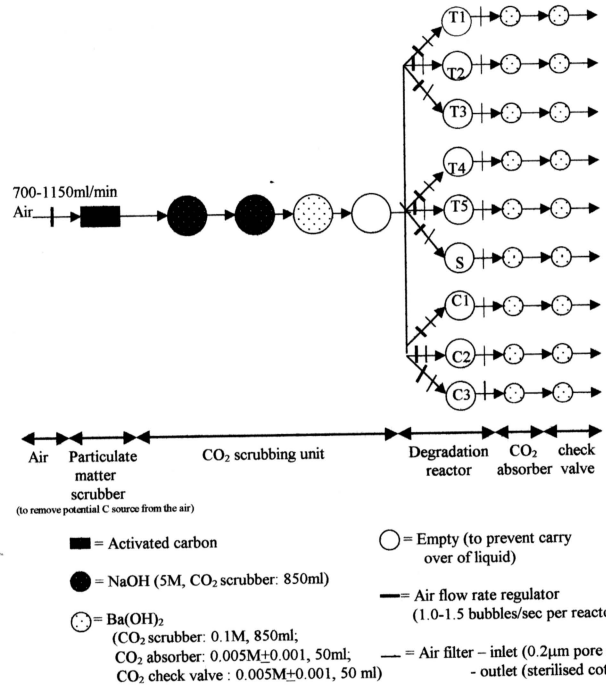

Figure 2.1 - Biodegradability test set up for Run 2. (Refer Appendix 1 for photographs of the set up).

2.2.4 Initial medium analysis -cell count, pH and DOC

25ml water sample was taken aseptically from each reactor, 5ml for initial viable cell count on nutrient agar plate and pH measurement. Another 20ml water sample for DOC measurement (Section 2.2.6.1) was acidified with concentrated HCl (35.4% v/v) to pH<2 and stored at 4°C until analysed. In Run 2 and Run 3, the samples for DOC analysis were filtered through Millipore filter paper (0.45μm & 0.22μm pore size) before acidification, storage and analysis as described in Section 2.2.6.1.

2.2.4.1 Cell count

0.1ml of river water diluted in series of 10^2, 10^3 and /or 10^4 was spread on the NA medium in triplicates and incubated at 30°C for 3-4 days. Plates with 30-300 colony forming unit (CFU) were counted and the mean CFU was calculated.

2.2.5 Test materials

A PHA film, approximately 1.5cm X 1.5cm with an initial weight of 15-38mg or a PHB film 1.5cm X 1.5 cm with an initial weight of 36-39mg was encased in a polyester net sewed with fishing line. The net and the polymer film was sterilised by soaking in methanol for 15 min and dried with sterile air under *uv* light for 15 min in a sterile plastic petri dish. The sterilised PHA/PHB film was then added into the degradation reactor as indicated in Table 2.2 after the CO_2 scrubbing process. The PHB film used as a positive control was prepared by dissolving 0.46g PHB granules (Sigma) in 50ml chloroform at 80°C for two hours under

reflux and casted onto glass petri dish with an inner diameter of 9.4cm. The biodegradability test was then begun for the test period as specified in Table 2.2.

2.2.6 CO_2 analysis and final day river water analysis - cell count, pH, DOC and GC

The CO_2 produced in the degradation reactor reacts with $Ba(OH)_2$ and is precipitated as $BaCO_3$. The amount of CO_2 produced was determined by titrating the remaining $Ba(OH)_2$ with 0.05*M* HCl to a phenolphthalein end-point. The CO_2 produced in all degradation reactors was analysed every two days for the first ten day, and then every 5 days or as needed before $BaCO_3$ precipitate was evident in the second flask. CO_2 analysis was also done at the end of the test period of a reactor.

The CO_2 absorber nearest to the reactor was removed for titration. The second CO_2 absorber (check valve) was moved one place closer to the degradation reactor and a freshly poured CO_2 absorber was placed at the far end of the test series.

At the end of the test period for each degradation reactor, 5ml sample was taken for viable cell count on NA and pH measurement. The PHA film was removed aseptically. The net was removed and the sample film was rinsed with distilled water and dried at 30°C for several days. For Run 2 and Run 3, the sample films were washed in distilled water bath sonicator (Bandelin Sonorex RK100, 35kH) for 15 minutes twice, and then dipped in methanol and dried at 30°C for several days.

0.4 ml concentrated HCl (35.4%v/v) was added to the reactor to decompose inorganic carbonate, release the trapped CO_2 and stop microbial activities. The reactor was aerated

overnight to collect the released CO_2. The remaining CO_2 absorbers were pulled together and titrated with 0.05M HCl. The $\%CO_2$–biodegradability was calculated as shown in Appendix 2.

For Run 3, at the end of the run (86 days), the remaining river water in the test reactor was filtered with filter paper (Whatman No.1) to recover the suspended insoluble plastic.

2.2.6.1 DOC measurement for river water

30-40ml of the filtrate from the remaining river water in the reactor was filtered with 0.45μm pore-sized membrane filter for the measurement of DOC (Minear & Keith, 1984). 15-20ml of this filtrate was filtered with 0.2μm pore-sized membrane filter for the measurement of DOC without biomass. Both samples were acidified with two drops of concentrated HCl (35.4%v/v) to pH<2 and stored in the dark until they were measured with TOC analyser (Shimadzu TOC-5000A) by ALS Technichem (M) Sdn. Bhd with oxidation-infrared detection method.

2.2.6.2 Gas chromatography (GC) of river water

1 ml river water filtered with 0.45μm pore size filter was added with 1 ml acidified methanol (0.85ml methanol and 0.15ml, 2M H_2SO_4) and 1 ml chloroform in a screw cap test tube and double sealed internally with teflon tape. The mixture was then heated at 100°C for 140 minutes in a heating block and was vortexed intermittently to dissolve and depolymerise PHA into its constituent monomers, and then convert the monomers into methyl esters. The

mixture was then cooled to room temperature. After vortexing for 1 min, the mixture was allowed to stand for 10 minutes for phase separation. The organic phase at the bottom was recovered. Prior to analysis by GC, 1ml of the organic phase was spiked with methyl benzoate (0.52mg) as an internal standard to indicate the beginning of elution detected and for the determination of response factor (RF).

The retention time and quantity of the PHA monomer indicated in the gas chromatograph was calibrated with standard methyl ester of 3-hydroxyalkanoaic acids - C_8 (methyl ester of 3-hydroxyoctanoic acid), C_{10} (methyl ester of 3-hydroxydecanoic acid), C_{12} (methyl ester of 3-hydroxydodecanoic acid), C_{14} (methyl ester of 3-hydroxytetradecanoic acid) and C_{16} (methyl ester of 3-hydroxyhexadecanoic acid)(Sigma Aldrich) at concentration of 0mg/ml, 0.15625mg/ml, 0.3125mg/ml, 0.625mg/ml and 1.25mg/ml. A mixture of 1.0mg/ml of all the standards spiked with 0.52mg methyl benzoate /ml of the mixture was prepared to obtain a standard chromatograph containing all peaks of all standards. Due to unavailability of standard methyl ester of 3-hydroxybutyric acid (C_4) and methyl ester of 3-hydroxyhexanoic acid (C_6), their RF and retention time were derived by extrapolation based on the RF and retention time of the other available standards respectively (Figure A9 & A12, Appendix 3). This was described in detail in Appendix 3. All analysis was done using Shimadzu GC-14A equipped with a fused Silica Omegawax^{TM} 250 capillary column (30m (length) by 0.25mm (internal diameter)) obtained from Supelco Inc., and a flame ionisation detector. Helium was used as a carrier gas. The temperature of injector and detector were 170°C and 200°C respectively. The temperature programme for the capillary column was as follows: initial temperature = 68°C, final temperature = 200°C and programme rate = 5.0°C/min.

2.2.7 PHA sample analysis

2.2.7.1 Dry weight

The dried sample films and debris recovered as described in 2.2.6 were weighed, and the change in weight was calculated.

2.2.7.2 Scanning electron microscopy (SEM) & phase-contrast microscopy

A representative piece of PHA sample was cut and placed onto a stub surface with a double-sided tape as the base. The sample was gold coated (by Bio-Rad Microscience Division-SEM coating system) and viewed at 20X, 503X, 1010X, 2020X and 5000X magnification with SEM (Philips SEM 515) to see the surface morphology. Surface view of PHA samples were also done with phase-contrast microscope at 100X and 200X magnification.

2.2.7.3 Purification of incubated PHA films

The incubated PHA films were purified before the analysis by infra red-spectrometry. The weighed sample was dissolved in 5ml chloroform and filtered with sintered glass no. 3 to remove insoluble inorganic. The sintered glass was then washed with 5ml chloroform to completely remove PHA from the filter. 4ml methanol was added to the filtrate to dissolve organic impurities and to precipitate PHA. PHA was allowed to settle for 6 hrs to form a thin film. The methanol and chloroform mixture was poured off. The PHA film was dried overnight in ventilated fume hood at room temperature.

2.2.7.4 Infrared (IR) spectrometry

PHA samples weighed within 0.1 mg to 24 mg was dissolved in 1-2ml chloroform. Two to three drops of the PHA solution was put onto the sodium chloride cell. The solvent was removed completely by blowing with a hair dryer. The IR absorption spectra were recorded on a Perkin Elmer FTIR 2000.

2.2.7.5 Molecular weight by gas permeation chromatography (GPC)

It has been planned to analyze the molecular weight of the PHA films by GPC but due to the break down of the GPC instrument, the test was called off.

2.2.7.6 Monomer composition by gas chromatography (GC)

The monomer compositions of PHA sample were determined by GC by a modified method of Braunegg *et al.* (1978). 8mg of the sample film before and after the experiment was dissolved in 1ml chloroform, 0.85ml methanol and 0.15ml concentrated (2M) sulphuric acid in a screw-cap test tube. The rest of the preparation was similar to Section 2.2.6.2 except that after heating and cooling down to room temperature, 1 ml distilled water was added, followed by vortexing and phase-separation.