# CHAPTER THREE

## *MATERIALS & METHODS*

### *3.1 Sampling Site for Isolating Indigenous Bacteria*

Palm oil-mill effluent (POME) ponds are important components of palm oil mills in which effluents were degraded to permissible level before release into the common environment. Our samples were taken from ponds located within the palm oil-mill complex of Southern Acids (M) Ltd., Klang. Liquid samples were collected from the surface of three POME ponds which were at different stages of degradation. Samples were kept at 4°C before use.

### *3.2 Isolation of Bacterial Strains*

Each liquid sample was serially diluted in sterile distilled water and 0.25 ml aliquots of $10^{-3}$ and $10^{-4}$ dilutions were spread on Isolation Media A (Appendix A.4). The plates were incubated at 30°C for up to 7 days. Colonies were differentiated by colour, elevation, form and edge appearance according to Sirockin and Cullimore (1969). Pure bacterial isolates were obtained by reculturing the isolates several times on the above-mentioned solid medium and nutrient agar (NA) (Appendix A.1) to produce single colonies.

### *3.3 Screening for PHA-producing Bacteria*

Each of the bacterial isolates was suspended in 1 ml sterile distilled water. A loopful of the suspension was smeared on a glass slide and

heat fixed. Then, it was stained with Sudan Black B (Appendix B.1) and viewed under light microscope (Olympus BH-2) to detect the presence of lipidic cellular inclusions. Positively-stained isolates were considered to be potential PHA producers and were subjected to further screening. These isolates were grown in 500 ml Erlenmeyer flask containing 200 ml nitrogen-limiting E2 medium (Appendix A.3). 1% w/v palm olein (PO) or saponified palm olein (SPO) was added as carbon substrate. The cultures were shaken at 100 rpm at 30°C for 5 days (HOTECH 718 Orbital Shaker). The isolates which grew in the media were stained with PHA-specific Nile Blue A (Appendix B.2).

### *3.4 Isolation through Enrichment*

400 ml of sterile Isolation Media B (Appendix A.5) containing 1% w/v palm oil was prepared in 1000 ml Erlenmeyer flask. Then 10 ml of sample from POME was added aseptically to the media. The flask was shaken at 30°C for 4 weeks (HOTECH 718 Orbital Shaker). A loopful of culture broth was spread on solid nutrient agar (Appendix A.1) plate and incubated at 30°C. The colonies were differentiated by colour, elevation, form and edge appearance according to Sirockin and Cullimore (1969). The pure isolates were obtained through several subculturing on nutrient agar plate. The pure isolates were then cultured in 500 ml Erlenmeyer flask of 200 ml E2 medium (Appendix A.3) containing palm oil (1%w/v). The cultures were shaken at 150 rpm at 30°C (HOTECH 718 Orbital Shaker). Growth was observed daily by

judging through change in turbidity and colour of culture. Then, a loopful of bacterial suspension was smeared on glass slide and heat-fixed. The presence of PHA was confirmed by Nile Blue A staining.

### *3.5 Identification of Isolate*

Bacterial isolate which was able to grow and accumulate PHA from PO was subjected to gram staining (Appendix B.3). Then, it was identified by using Biolog Microstation System equipped with Microlog 3 programme.

### *3.6 Bacterial Strain Infomation*

*Pseudomonas oleovorans* was a kind gift from the University of Massachusets, Amherst USA. The bacteria was maintained on nutrient agar plate and slant. Stock cultures were prepared by growing the bacteria in nutrient rich medium, and while in a log phase, 250 µl aliquots was transfered into a universal bottle containing 5 ml of sterile 20% glycerol, kept at -20 $^{o}$C.

### *3.7 Carbon Substrates*

Palm Olein (PO), Palm Stearin, crude Palm Oil, Palm Kernel Oil (PKO) and Oleic Acid (OA) were kindly given by Southern Acids (M) Ltd., Klang.

### *3.8 Fermentation*

#### *3.8.1 Seed Medium*

Experiments were conducted under two-stage fermentation conditions. In the first stage cells were cultivated in nutrient rich medium (Appendix A.2) for biomass production. Nutrient rich medium was prepared in 400 ml volume in 1L Erlenmeyer flask and autoclaved at 121°C for 15 minutes (TOMY Autoclave S2-325) to sterilise. The flask was innoculated with a loopful of bacteria from agar slant. Bacterial growth was carried out at 30°C and 250 rpm in HOTECH 718 Orbital Shaker for 21 hours. The cells were harvested by centrifugation at 5500 rpm for 10 minutes (Beckman J2-MI centrifuge) and washed with phosphate buffer solution (pH 7.0) and recentrifuged. The cells were then suspended into 200 ml E2 medium (Appendix A.3) before aseptically transfered into production medium for PHA accumulation, in the second stage. PHA production was carried out in nitrogen-limiting E2 medium (Appendix A.3).

#### *3.8.2 Cultivation Conditions*

Batch and Fed-batch fermentation for PHA production were carried out in a 2L baffled fermentor (B.Braun Biostat® B) containing E2 medium at a working volume of 1.5L. It is equipped with two four-disk turbine impellers for agitation, pH and $O_2$ electrodes (INGOLD). The quantity of

carbon substrate (SPO or OA) was fixed at 0.5% w/v for batch and initial fed-batch cultivation. The fermentor was autoclaved at 121°C for 15 minutes (TOMY Autoclave S2-325) to sterilise. The SPO was autoclaved together with the medium, whereas OA was autoclaved separately and added aseptically later. When autoclaved together with the medium, OA changed into a viscous and sticky dark brown substance. To reduce foam formation, antifoam (Silicone antifoaming agent, BDH) was added at 0.1% v/v. The temperature and pH of all fermentations were 30°C and 7.0, respectively. The pH was controlled with 2M NaOH and 2M $H_2SO_4$. The dissolved oxygen concentration (% saturation) was fixed at 40% by using cascade mode and thus, agitation was automatically changed during cultivation. The air flow rate was however, adjusted manually to accommodate dissolved oxygen demand.

15 ml of aliquots were withdrawn at 4, 8, 12, 24 and 48 hours for analysis. The cultivations for both batch and fed-batch were ended at 48 hours and the cells were harvested by centrifugation at 5500 rpm for 10 mins (Beckman J2-MI). The harvested cells were washed with distilled water and then dried in a vacuum oven (Napco 5831) at room temperature.

### *3.8.3 Method for Fed-Batch Culture*

The initial medium composition and cultivation conditions of the fed-batch culture were the same as those of batch culture. In fed-batch mode however, at 18, 30 and 42 hours a quantity of carbon substrate was added to

the fermentor. For cultivation with SPO, 50 ml of sterile solution containing 50mg/ml SPO was added at each of those times. This corresponded to about 2.5g of SPO was added each time. In the case of OA, 4.0 g of OA of specific gravity of 0.891 g/ml was added intermittently. From batch cultivation it was observed that SPO was more slowly utilised compared to OA. Thus, to avoid any inhibition effect to the growth the quantity of added SPO was less than that of OA during fed-batch cultivation.

### *3.9 Saponification of Palm Olein (PO)*

An 8 g sample of PO was added to the ethanolic potassium hydroxide solution prepared by dissolving 2.8 g potassium hydroxide in 100 ml ethanol. The mixture was refluxed for 1 hour and occasionally swirled. The ethanol was removed under reduced pressure in a rotary vacuum evaporator (EYELA N-I). Solid potassium salts of fatty acids formed as the concentrated solution allowed to cool at room temperature (Tan *et al.*,1997).

### *3.10 Culture Broth Analysis*

At specific times, 20 ml broth samples were taken from the fermentor. To analyse the supernatant, 15 ml of the culture broth was centrifuged (Joun C312) at 3500 rpm for 10 minutes. The remaining 5 ml of culture broth was used for Viable Cell Count and Optical Density measurements.

### *3.10.1 Ammonium*

Reagents were prepared as shown in Appendix C.1. 1 ml of phenol solution, 1 ml of sodium nitroprusside solution, 1 ml of oxidising agent was added successively into a 50 ml Elernmeyer flask containing 25 ml of diluted supernatant (1:1000 v/v). The mixture was mixed thoroughly after each addition. The colour was allowed to develop within 1 hour at room temperature. The absorbance was recorded at 640 nm in a spectrophotometer (Shimadzu UV-1601).

### *3.10.2 Fatty Acids*

The preparation of reagents was shown in Appendix C.2. 10 ml of supernatant was dissolved into 50 ml 95% ethanol:diethyl ether (1:1) solvent. The solvent contained 3 drops of phenolpthalein solution as an indicator. 0.1 N NaOH solution was added dropwise from biurette (25 ml) for titration. The volume of NaOH required to change the colour from colourless to slight pink was recorded.

### *3.10.3 Viable Cell Count*

An aliquot of the broth was serially diluted and 100 µl of the $10^{-6}$ and $10^{-7}$ dilutions were spread on nutrient agar plate by using L-shaped glass rod. The plates were incubated at 30°C and the number of cell colony was

counted and interpreted as colony forming unit (CFU) per ml. The specific growth rate ($\mu$) was calculated according to equation 1 (Pirt, 1975),

$$\ln x = \ln x_o + \mu t$$

$$\therefore \quad \mu = (\ln x - \ln x_o) / t \qquad \text{Equation 1}$$

where , x = CFU or OD at time t, $X_o$ = CFU or OD at time 0, t = time (hour)

### *3.10.4 Optical Density*

1 ml of fermentation broth was centrifuged at 3500 rpm for 10 minutes (HERMLE Z231M). The supernatant was discarded and the pellet was washed with distilled water. The pellet was resuspended in 1 ml distilled water and the suspension was diluted 1:5 with distilled water. The optical density was measured at 660 nm (Shimadzu UV-1601) where distilled water was used as the blank. Specific growth rate ($\mu$) was calculated according to equation 1(section 3.10.3)

### *3.10.5 Cell Dry Weight (CDW) Determination*

For analysis of CDW, 10 ml of cultural broth was centrifuged at 3500 rpm (Jouan C312) for 10 minutes. The supernatant was discarded and the pellet was suspended in a 0.85% saline and recentrifuged. The supernatant again discarded and the cells were washed with distilled water and centrifuged. The cells were suspended in 5 ml of distilled water and poured into a preweighed alluminium dish and dried in an oven (Memmert) at 105°C overnight. The cell dry weight (CDW) of the cells were calculated as

difference between filled and empty aluminium dish. Total cell yield from substrates ($Y_{x/s}$ ; where x = CDW and s = weight of substrate consumed) was calculated as ratio of obtained CDW over consumed substrates (g/g) (Kim *et al.*, 1993).

### *3.11 PHA Extraction*

One gram of dried cells was added into a 250 ml round bottom flask containing 100 ml chloroform. The mixture was refluxed for 4 hours (Lageveen *et al.*,1988). The chloroform solution was then filtered (Whatman No.2) to remove any cellular debris, concentrated by rotary evaporation (EYELA N-1) and added dropwise to rapidly stirrred methanol (1:10 v/v) to precipitate PHA polymer. After PHA polymer had settled, the supernatant was decanted and the PHA polymer was washed with methanol and dried at room temperature.

The dried polymer was weighed and the yield was calculated as a percentage of the cell dry weight. Before chemical characterisation, the PHA was purified by repeatedly dissolving the polymer in chloroform and precipitating it with methanol. PHA yield from substrates ($Y_{p/s}$ ; p = product and s = substrate) was calculated as ratio of PHA yield over substrate consumed (g/g) (Kim *et al.*, 1993).

### *3.12 PHA Analysis*

#### *3.12.1 Nuclear Magnetic Resonance (NMR)*

The purified PHA polymer was dissolved in deuterated chloroform ($CDCl_3$) at concentration of 40 mg/ml in a NMR tube. Then, it was analysed on a JOEL JNM-LAA400 Fourier Transformation (FT) NMR system which was operated at 400 MHz for $^1H$ and 100 MHz for $^{13}C$ analysis.

#### *3.12.2 Infrared (IR) Spectrometry*

A thin layer solution of polyester dissolved in $CHCl_3$ was smeared on the sodium chloride cell which was then blown with a hot air for drying. The IR spectrum of the polymer was recorded on Perkin Elmer FT IR Spectrometer Spectrum 2000.

#### *3.12.3 Gas Chromatography (GC)*

##### *3.12.3.1 Preparation of Sample*

To determine the polymer composition, purified PHA was subjected to methanolysis. In a tight screw-capped glass tube, a known quantity of PHA, 2 ml methanol acidified with 15% v/v $H_2SO_4$ and 2 ml chloroform was heated in a heating block at 100°C for 4 hours to break up

PHA (Huijbert *et al.*,1994) and convert the constituents to their methyl esters. To stop the reaction, the tubes were cooled immediately on ice. 1 ml of distilled water was added to the mixture to induce phase separation. The mixture was vortexed vigorously and the layers were allowed to separate. The organic layer (bottom) containing the methyl esters was removed and kept in a screw-capped vial at –20°C for future analysis. 2µL of this organic layer containing 0.1% v/v methyl benzoate as internal standard was injected for gas chromatography analysis, which was performed on a Shimadzu GC-14A system equipped with a Fused Silica Omegawax™ 250 capillary column (30 m(L) by 0.25 mm(ID)) obtained from Supelco Inc. and a flame ionisation detector. Helium was used as carrier gas. The temperature of injector and detector were 170°C and 200°C respectively. The temperature programmes are as follows; Initial Temperature=68°C, Final Temperature=200°C and Increase rate=5.0°C/min.

#### *3.12.3.2 Quantitative Determination of Polymer Composition*

Five types of standards of 3-hydroxyalkanoic acid methyl esters were used, namely, 3-hydroxyoctanoic acid methyl ester ($C_9H_{18}O_3$). 3-hydroxydecanoic acid methyl ester ($C_{11}H_{22}O_3$), 3-hydroxydodecanoic acid methyl ester ($C_{13}H_{26}O_3$), 3-hydroxytetradecanoic acid methyl ester ($C_{15}H_{30}O_3$), and 3-hydroxyhexadecanoic acid methyl ester ($C_{17}H_{34}O_3$). Each of the standards was prepared in four different concentrations (1.25, 0.63,

0.31 and 0.16 mg/ml) and each was injected into the GC (Figure 11). Three injections were run for each standard and sample. As the chromatograms obtained from the triplicate injections were highly consistent, the data shown here (Figure 11 to 17) were taken from a single representative injection. The data obtained were used to plot standard graphs as follows;

1. Retention Time was plotted against the number of carbon atom of each 3-hydroxyalkanoic acid methyl ester standard (Figure 12).

2. Response Factor (RF) (ratio of peak area of standard to internal standard) was plotted against the prepared concentrations of ech standard (Figure 13).

3. Due to inavailability of 3-hydroxyhexanoic acid methyl ester, the standard graph was derived by extrapolation method. It was assumed that the same concentration of methylated $C_4$, $C_6$, $C_8$, $C_{10}$, $C_{12}$, $C_{14}$, and $C_{16}$ would give certain response in the GC column. It was shown that the graph of RF of different concentrations of each standard against the carbon number indicated particular trend. (Theanmalar, M.Biotech.thesis,1996). Compounds containing 9 carbons or less could give a linear graph. Thus, the RF of prepared concentrations of $C_9$ was plotted against the carbon number. From the obtained graph, the RF values for each

concentration of 3-hydroxyalkanoic acid methyl ester of $C_4$ and $C_6$ could be calculated (Figure 14).

4. RF against different concentrations of $C_5$ and $C_7$ was plotted (Figure 15).

The concentrations of polymer derived from OA and SPO injected for GC analysis were 5.32 and 13.05 mg/ml, respectively. Data obtained from the prepared standard graphs were used to estimate the concentration of each monomer and hence its mole %. The mole % of each monomer was calculated as the ratio of concentration of each monomer to concentration of injected polymer.

As 3-hydroxybutyric acid methyl ester ($C_5H_8O_3$) was not available commercially, the standard was prepared by methanolysing the commercial polyhydroxybutyrate as described in section 3.12.3.1.