MATERIALS AND METHODS

3.1 Culture

Bacillus licheniformis JF-2 (ATCC 39307), laboratory stock no. H6435, was provided by Prof. Ho Coy Choke of the Department of Genetics and Cellular Biology, University of Malaya. This culture was originally a gift from Prof. M.J. McInerney, Department of Botany and Microbiology, University of Oklahoma, U.S.A. This bacterium is rod shaped, filamentous, encapsulated, highly motile and spore forming. The volcano shaped colonies were maintained on Nutrient agar (Difco) slants which were incubated at 30°C and streaked on Nutrient agar plates (Plate 2). These stock cultures were used in this study.

3.2 Media

3.2.1 Inoculum preparation

Cultures of *B. licheniformis* JF-2 incubated at 30°C for 5 days on Nutrient agar slants were used to prepare the inoculum. Cultures on agar slants were scraped off with a flame sterilized platinum wire loop. These were diluted in sterile distilled water contained in Universal bottles. This was vortexed to obtain a uniform suspension. The cultures were standardized by making dilutions in sterile distilled water such that the absorbance at 480 nm (McInerney *et al.*, 1985) was between 0.5-0.6. The cell suspension, 1 mLof which was inoculated in 49 mLof Nutrient broth. This was used as the standardized inoculum for process optimization studies.

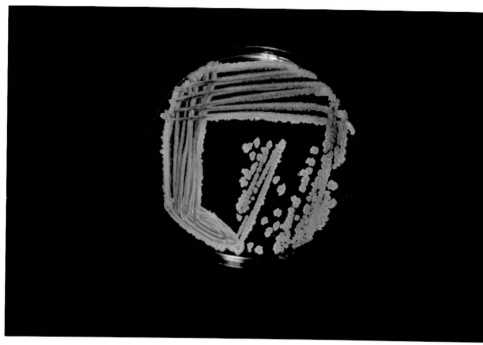

Plate 2: *Bacillus licheniformis* JF-2, volcano type colonies grown on Nutrient agar (96 hours)

3.2.2 Fermentation

For fermentation in the 1.5L fermenter, the culture was prepared as in Section 3.2.1. The inoculum, 1mL of which was transferred into 44 mL of sterile Nutrient broth and incubated at 40°C in an orbital incubator shaker at 250 rpm for 8 hours in order to achieve the log phase. Then, 45 mL of the bacterial culture was used as the inoculum for the 1.5L fermenter where the working volume was 900 mL. The initial pH of the medium was set at 7.0, the air flowrate was maintained at 1000 cm^3/min, stirring speed of the impeller was maintained at 400 rpm and the optimum temperature of 40°C was maintained throughout the studies.

3.3 Physical parameters

3.3.1 Biomass

Culture broth consisting of 50 mL was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was removed, the cells were suspended in distilled water and centrifuged again. The supernatant was removed and the cell pellets scraped into preweighed crucibles (W_1) and oven dried overnight at 105°C. The crucibles were then put into dessicator for 5 hours and weighed (W_2). The biomass (W_3) was determined.

$$W_2 - W_1 = W_3$$

3.3.2 pH

pH was measured by a pH meter (Hanna Instruments, 8417). The pH probe was immersed in 50 mL of culture broth immediately after sampling to

determine pH.

3.3.3 Surface tension

Surface tension was measured with a Fisher Autotensiomat (Model 21). The ring consisted of 6 cm diameter of platinum-irridium. It was cleaned by dipping in benzene (to remove hydrocarbons) and then in acetone (to remove the benzene). The ring was then air dried.

The cleaned platinum-irridium ring was attached to the hook on the lever arm. The culture broth, 15mL of which was poured into a disposable clean plastic Petri dish and placed on the sample table. The sample table was adjusted until it was directly beneath the platinum-irridium ring. The table was then raised until the ring immersed in the culture broth, at approximately 1/8th inch. The knob on the right side of the case was used to adjust the vernier to zero after which the height of the sample table was adjusted until the index and the image were exactly in line with the reference mark on the mirror. The switch was turned on and the surface tension was measured when the ring was pulled through the interface. When the surface film ruptured, the lever arm actuates a contact which in turn actuates a relay and stops the drive motor. The surface tension was then read from the scale on the dial.

Each time the Fisher Autotensiomat is to be used, it is first calibrated with deionized distilled water to give a reading of 72 mN/m. The reading obtained from the dial gives an accuracy of up to one decimal point. During the measurements of samples in batches, the Fisher Autotensiomat is checked

regularly against deionized distilled water to maintain its accuracy in readings.

3.3.4 Emulsification Measurement (E_{24})

Emulsification activity was measured by adding 6 mL of kerosene (commercial grade) to 4 mL of cell free culture broth aqueous sample. This was vortexed for 2 minutes. The height of the emulsion layer and total height were measured after 24 hours. The emulsion index (E_{24}) was the height of the emulsion layer, divided by the total height, multiplied by 100.

3.3.5 Thin Layer Chromatography

The lipopeptide was separately extracted in methanol, chloroform or dichloromethane which were then detected by thin layer chromatography using silica Gel-60 F_{254} precoated plates (Merck, Germany). Samples of 10 μL of 20 mg/mL solution was applied to the plates and the spots were air dried. The plates were developed in a standard developing tank, 9.5 cm x 29.0 cm x 27.5 cm, containing a developing solution consisting of chloroform-methanol and 28% NH_4OH (65:35:5, v/v) (McInerney *et al.*, 1990).

The plates were allowed to dry and stained with anisaldehyde reagent which contained a mixture of anisaldehyde/sulphuric acid/acetic acid at 0.5/1/50 (v/v/v) or were sprayed with concentrated sulphuric acid and placed at 100°C until charred to visualize the migrated spots (Sasidharan, 1995). The R_f of each spot was determined according to the following formula:

$$R_f = \frac{\text{Distance of spot from origin}}{\text{Distance of the solvent from the origin}}$$

3.4 Wastewater analysis

3.4.1 Chemical Oxygen Demand (COD)

Apparatus

500 mL Erlenmeyer flask with ground glass 24/40 neck and 300mm (12") Allihin condenser with 24/40 ground joint and a heating mantle.

Reagents

Distilled water

Low in organic matter.

Potassium dichromate standard solution

12.259g $K_2Cr_2O_7$, primary standard grade, previously dried at 103°C for 2 hours, dissolved in distilled water and made up to 1L to obtain 0.25N. This standard solution is used to determine high COD level (> 100 mg/L).

100 mL of 0.25N solution is diluted to 1L to obtain 0.25N and this standard solution is used to determine low COD levels (< 100mg/L).

Sulphuric acid reagent

23.5g argentum sulphate (Ag_2SO_4, Merck) was dissolved in 4.1 kg of concentrated sulphuric acid (Merck).

Ferrous ammonium sulphate standard solution (FAS)

0.25N of FAS was obtained by dissolving 98g Fe $(NH_4)_2$ $(SO_4)_2.6H_2O$ (BDH) in water and added with 20 mL of cencentrated sulphuric acid and later made up to 1L with distilled water.

Phenanthroline ferrous sulphate indicator solution

1.48g 1.10-(ortho)-phenanthroline and 0.70g $FeSO_4.7H_2O$ (BDH) was dissolved in distilled water and diluted to 100 mL.

Assay

1g $HgSO_4$ (BDH) was placed in reflux flasks and added with 5.0mL of concentrated H_2SO_4 (Merck) and swirled until $HgSO_4$ dissolved. The reflux flask was placed in an ice bath and slowly added with 25.0 mL 0.25N $K_2Cr_2O_7$ while swirling. 70 mL H_2SO_4-Ag_2SO_4 (sulphuric acid reagent) was added. While still in bath, 50 mL sample was pipetted in with continuous mixing. Allihin condensers were attached to the reflux flasks and the reflux mixtures were refluxed for approximately 1½ hours.

Once cooled, the condensers were washed with 25 mL of distilled water and the samples were diluted to 300 mL with distilled water and let to cool to room temperature. 8-10 drops of ferroin indicator solution were dropped into each flask and titrated for excess $K_2Cr_2O_7$ with 0.25N Fe $(NH_4)_2$ $(SO_4)_2$ to a sharp reddish end point (S mL). A blank consisting of 50 mL of distilled water was treated the same as the samples, including refluxing and titrating with 0.25N Fe $(NH_4)_2(SO_4)_2$ (B mL). The COD was determined using the

formula given below:

$$\text{mg COD/L} = \frac{(B\text{-}S) \times N \times 8000}{V}$$

where,

B = volume of $Fe(NH_4)_2(SO_4)_2$ titrated for blank
S = volume of $Fe(NH_4)_2(SO_4)_2$ titrated for sample
N = normality of $Fe(NH_4)_2(SO_4)_2$ solution
V = volume of sample used

3.4.2 Dissolved phosphate

Reagents

Molybdate solution

15.0g ammonium paramolybdate $[(NH_4)_6Mo_7O_{24}.4H_2O]$ (BDH) in 500 mL of distilled water and stored in a plastic bottle out of direct sunlight.

Sulphuric acid reagent

140 mL of concentrated sulphuric acid was diluted and made up to 900 mL with distilled water.

Ascorbic acid solution

5.4g of ascorbic acid was dissolved in 100 mL of distilled water. This solution was prepared on the day it was to be used.

Potassium antimonyl tartarate solution

0.34 g of high quality potassium antimonyl tartarate, $KSbO.C_4H_4O_6$, was dissolved in 250 mL of water and stored in glass bottle.

Mixed reagent

100 mL of ammonium molybdate, 250 mL sulphuric acid, 100 mL ascorbic acid and 50 mL potassium antimonyl tartarate were mixed. The mixed reagent was prepared shortly before use and discarded 6 hours later.

Standard phosphate solution (PO_4^{3-})

0.43 g of desiccated potassium dihydrogen phosphate, KH_2PO_4(BDH) was dissolved in distilled water and made up to 100 mL. A few drops of chloroform (Merck) were added and stored in a dark bottle. This prepared stock solution contained 100,000 μg PO_4^{3-}/L. 1L of PO_4^{3-} solution containing 1000 μg PO_4^{3-}/L was obtained by diluting 10 mL of the stock solution.

Apparatus

Glassware

Glassware were cleaned throughly with concentrated sulphuric acid and rinsed well in distilled water. Glassware used in dissolved phosphate analyses were not utilised for other analyses. Unused glassware were filled with 0.1% (v/v) H_2SO_4 in distilled water solution.

Assay

Reference solutions containing 0, 2, 5 and 7 μg of PO_4^{3-} by taking 0, 2, 5 and 7 mL of the 1000 μ PO_4^{3-} standard solution and diluting to 100 mL with distilled water. Samples were all warmed up to a temperature of between 15°C and 30°C with thermostatically controlled water bath and the absorbance of the samples are measured to obtain a turbidity correction. 100 mL of each sample and to the reference solution 10 mL and 0.5 mL of mixed reagents

were added from a 20 mL measuring cylinder and both were mixed at once. After 10 minutes and within 2 hours, the absorbance of the solutions in a 10 cm cuvette was measured against distilled water at a wavelength of 885 nm.

3.4.3 Determination of total sugar (Dubois *et al.*, 1956)

Reagent

5% (w/v) phenol (BDH)

96% (w/v) concentrated sulphuric acid (Merck)

Assay

1.0 mL of 5% phenol solution was mixed with 1.0 mL of appropriately diluted sample. To this was added 5.0 mLof concentrated H_2SO_4. This mixture was vortexed and allowed to stand at room temperature for 30 minutes. Optical density was read at 490 nm and total sugar was determined against a standard curve prepared from glucose at concentrations between 0-100 μg/mL (Appendix VII).

3.4.4 Determination of reducing sugar (Miller, 1959)

Reagent

Dinitrosalicyclic solution (DNS) was prepared by mixing 10 g dinitrosalicyclic acid (BDH), 2 g phenol (BDH), 10 g sodium hydroxide (BDH) and 0.5 g sodium sulfite (BDH) in distilled water and made up to 1L.

Rochelle salt (40%) was made by dissolving 40 g potassium sodium tartarate in 100 mL of distilled water.

Assay

3 mL of appropriately diluted sample was added with 3 mL of DNS solution and vortexed. The mixed sample was heated in a boiling water bath for 15 minutes. 1 mL of Rochelle salt was added and immediately cooled. Optical density of the sample was taken at 575 nm and reducing sugar was determined against a standard curve prepared from glucose at concentrations between 100-1000μg/L (Appendix VI).

3.4.5 Total Nitrogen

Catalyst

Copper sulphate ($CuSO_4$)

Dipotassium sulphate (K_2SO_4)

Reagents

Concentrated sulphuric acid

0.2M hydrochloric acid

40% sodium hydroxide solution

Distilled water

Apparatus

Block Digest 20 (Tecator, Sweden)

Kjeltec Auto 1030 Analyzer (Tecator, Sweden)

Assay

10 mL of wastewater were pipetted into each digestion tubes with 4.5 g catalyst and to this was added 12.5 mL of concentrated sulphuric acid. The

digestion tubes were placed into Bloc digest 20 heating block and the samples were digested for between 45 minutes to 1 hour at temperature of between 420° to 450°C until a clear greenish solution appeared. Upon cooling, the solution appeared clear blue and 75 mL of distilled water was added to each tube. The nitrogen content was determined by distillation and autotitration using Kjeltec Auto 1030 Analyzer. The results displayed showed the percentage total nitrogen present in the sample.

3.5 Determination of crude lipopeptide yield (g/l)

3.5.1 Acid precipitation method for shake flask cultures

A sample of cell free culture was obtained by centrifuging 50 mLof culture broth at 10,000 rpm for 10 mins. The supernatant was acidified with 1M HCl to obtain a pH of 2 and stored overnight at 4°C for the precipitation of the crude lipopeptide. This was centrifuged at 13,000 rpm (Hermel ZK 401, Germany) for 30 minutes. The supernatant was removed, the biosurfactant in the form of a pellet was dissolved in distilled water (pH 7.0) and freeze dried to obtain the crude lipopeptide (Plate 3).

3.5.2 Foam fractionation method for isolating the biosurfactant from fermenter runs (Juwarkar, 1994; Personal Communication)

A volume of 900 mLof Nutrient broth was taken in a B. Braun Biolab fermenter, of 1.5L vessel capacity. An extra steel probe was inserted into the empty port. This was maintained at a height of about 2 inches above the

broth level in order to collect the foam. The probe was then connected by silicone tubing to a 1L sterile Erlenmeyer flask.

The vessel and its attachments were autoclaved at 121°C for 15 minutes at 15 psi. The inoculum consisted of 45 mL(5% v/v) of standardised culture. During the fermentation, foam was collected in the Erlenmeyer flask under sterile conditions (Plate 4).

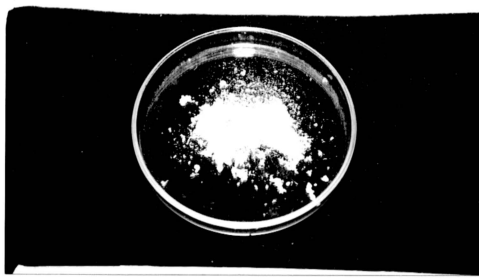

Plate 3: Crude lipopeptide produced by *B. licheniformis* JF-2 obtained from Nutrient broth during 24 hours of fermentation (Freeze dried)

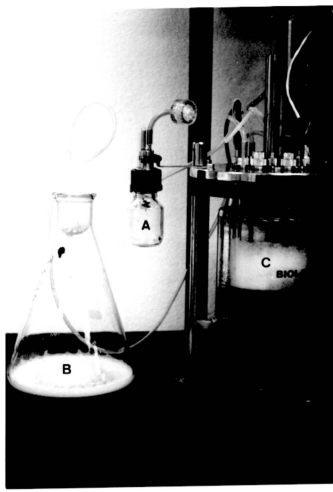

Plate 4: *Bacillus licheniformis* JF-2 grown in a 1.5L Biolab fermenter using Nutrient broth as cultivation medium (8 hours of fermentation)

A : Sampling bottle
B : Collapsed foam collected by the foam fractionation method
C : Foam

3.6 Process optimization

3.6.1 Initial pH

The seed culture consisting of 1mL was inoculated into 49 mL of Nutrient broth in 250 ml Erlenmeyer flasks adjusted to pH 3, 4, 5, 6, 7, 8 and 9 at the optimum temperature of 40°C. The cultures were incubated in an orbital shaker (Lab-Line Instruments, USA) at 250 rpm. The growth of *B. licheniformis* JF-2 was monitored hourly until the end of the log phase (8 hours) by measuring the absorbance at 480 nm (LKB Biochrom, Novaspec) and the dry weight (g/L) of the biomass was determined.

3.6.2 Temperature

B. licheniformis JF-2 was cultivated in Nutrient broth in shake flasks at the following temperatures: 35°C, 40°C and 45°C and shaken at 250 rpm in an orbital type incubator shaker. 49 mL of Nutrient broth was taken in each of 250 mL Erlenmeyer flasks. These were inoculated individually with 1 mL each of the standardized inoculum and incubated at the temperatures indicated. The growth was monitored by observing the absorbance at 480 nm (LKB Biochrom, Novaspec II) and biomass was estimated for a period of 24 hours by taking samples at hourly intervals for the first 8 hours and thereafter at 4 hourly intervals. The surface tension of the culture broth was also monitored. 2 samples were taken to determine each parameter measured and the average results are shown on graphs and tables.

3.7 Media optimization

3.7.1 The effect of different concentrations of sodium chloride on the growth and biosurfactant production by *B. licheniformis* JF-2 without glucose

Nutrient broth 49 mL of which was taken in 250 mL Erlenmeyer flasks. To each of these in duplicate, Sodium chloride (Merck, Darmstadt) was added as follows; 0.5%, 1.0%, 2.0%, 3.0%, 4.0% and 5.0% (w/v). The flasks were sterilized for 15 minutes at 15 psi. Standardised seed cultures each consisting of 1 mL of *B. licheniformis* JF-2 were introduced into each flask aseptically. These were incubated for 8 hours at 40°C at 250 rpm in an orbital incubator shaker. The crude biosurfactant yield was monitored at the end of 8 hours, at which time pH, surface tension (mN/m), optical density (A_{480}) and biomass (g/L) was determined in the broth. 2 samples were taken for each parameters measurements and the average values are represented in the graphs and tables.

3.7.2 The effect of different concentrations of sodium chloride on the growth of *B. licheniformis* JF-2 in Nutrient broth supplemented with glucose

Nutrient broth consisting of 49 mL was taken in 250 mL Erlenmeyer flasks. To each of these was added sodium chloride (MERCK, Darmstadt) at concentrations of 1%, 2%, 3%, 4% and 5% (w/v) in duplicate. The flasks were autoclaved at 15 psi for 15 minutes. Subsequently previously sterilized

glucose (autoclaved at 10 psi for 15 minutes) was added aseptically at concentrations of 5% and 10% (w/v) in each flask at the various concentrations of sodium chloride.

The seed culture consisting of 1 mL of *B. licheniformis* JF-2 was inoculated into each flask. These were incubated at 40°C in an orbital incubator at 250 rpm for 8 hours. Biomass (g/L), pH, surface tension (mN/m) and Emulsification Index (E_{24}) were determined with 2 samples and the average values are shown in graphs and tables.

3.7.3 The effect of different concentrations of yeast extract on the growth and biosurfactant production by *B. licheniformis* JF-2.

Nutrient broth consisting of 49 ml (pH 7.0) was taken in 250 mL Erlenmeyer flasks. Yeast extract (Difco, U.S.A.) was added at concentrations of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v). The media were sterilized and inoculated with standardized 1 mL seed culture of *B. licheniformis* JF-2. The inoculated media were incubated at 40°C in an orbital shaker at 250 rpm for 8 hours. pH Optical density (A_{480}= 480 nm), biomass (g/L), surface tension (mN/m) and crude biosurfactant yield (g/L) monitored. 2 samples were used for each measurement.

3.7.4 A time course study of *B. licheniformis* JF-2 in the final optimized growth medium

1 mL of the standardized inoculum of *B. licheniformis* JF-2 (Section 3.2.1) was added aseptically to 49 mL of the sterilized optimized growth medium

consisting of Nutrient broth with 3% (w/v) NaCl, 0.3% (w/v) yeast extract and 5% (w/v) glucose (Appendix 1). The flasks were incubated at 40°C in an orbital incubator at 250 rpm. Samples of 50 mLconsisting of whole flasks contents were removed at 2 hourly intervals for the first 8 hours and then at 4 hourly intervals for a period of 24 hours. The biomass, optical density, pH, surface tension and crude lipopeptide yield were monitored.

3.8 Fermentation

3.8.1 The growth and biosurfactant production by *B. licheniformis* JF-2 in a 1.5L capacity Biolab fermenter

The effect of agitation on the growth of *B. licheniformis* JF-2 was studied in a 1.5L Biolab fermenter with 900 mLof the Optimum Medium as cultivation medium. The inoculum consisted of 45 mLof *B. licheniformis* JF-2 grown for 8 hours. The impeller speed was maintained at 400 rpm with an air flowrate of 1000 cm^3/min. Culture growth was monitored by taking two samples and measuring the absorbance at (A_{480}) over 24 hours and the average values are used in the graphs.

The samples, 20 mLeach were taken at hourly intervals for the first 4 hours, and after that at 4 hourly intervals for 20 hours. The crude lipopeptide which had accumulated in the foam was collected by the foam fractionation method. This was acidified with 1M HCl to a pH of 2 and stored overnight at 4°C which ensured the precipitation of the crude lipopeptide. Distilled water was added to the supernatant and the pH was adjusted to 7.0. This resulted in the solubilization of neutralized crude lipopetide which was stored for 24

hours at -20°C. This was then freeze dried overnight to obtain a dried, powder of the lipopetide.

3.8.2 Production of biosurfactant by *B. licheniformis* JF-2 in selected media

Time course studies were conducted firstly by using 2 different media; Mineral Salts Medium I and Mineral Salts Medium II (Appendix II and III). The composition of the Mineral Salt Medium I is as follows in g/L; glucose, 10.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 0.25; NaCl, 5.0 and trace metal solution, 5.0 mL. This trace metal solution consisted of (g/L); EDTA, 1.0; $MnSO_4$, 3.0; $FeSO_4$, 0.1; $CaCl_2$, 0.1; $ZnSO_4$, 0.1; $CuSO_4$, 0.1; $AlK(SO_4)_2$, 0.01; H_3BO_4, 0.01 and Na_2MoO_4, 0.01.

The composition of the Mineral Salt Medium II (Juwarkar *et al.*, 1994) is as follows in g/L; $NaNO_3$, 3.0; K_2HPO_4, 1.0; KH_2PO_4, 0.5; $MgSO_4$, 0.5; KCl, 0.1; $FeSO_4$, 0.01; $CaCl_2$, 0.01 and glucose, 20.0. These were compared with the Optimized Medium. The inoculum used was 5% (v/v) in 900 mL cultivation media, with an impeller speed of 400 rpm. Growth of *B. licheniformis* JF-2 was monitored by measuring the absorbance (A480) of the sample broth at hourly intervals for the first four hours and thereafter at 4 hourly intervals. The pH of the media was also monitored in the samples. The yield of the crude lipopeptide was quantified by the foam fractionation method followed by freeze drying. 2 samples were used to determined each parameter and the average values are shown in graphs and tables.

3.9 Growth of *B. licheniformis* JF-2 in agro-industrial wastewater

B. licheniformis JF-2 was grown in locally available agro-industrial wastes such as rubber effluent obtained from coagulated latex from Atherton estate, Siliau, N. Sembilan; Palm oil mill effluent (POME) obtained from Tennammaram Estate, Klang, Selangor; Dairy wastewater from the processing of sweetened condensed milk from Premier Milk (M), Section 17, Petaling Jaya, Selangor and fish meal from the research farm at the Institute of Advanced Studies, University of Malaya.

Rubber effluent from Atherton estate consisted of untreated effluent, collected from the holding pond of the factory. Untreated POME and dairy wastewater were freshly collected before entering the wastewater treatment system. These were stored at 4°C in the cold room at the Institute of Advanced Studies, University of Malaya before use.

Fish meal as an additional nitrogen source was obtained in dried powdered form the research farm at University of Malaya. It was weighed and added indiviudally into each of the Erlenmeyer flasks containing dairy wastewater at concentrations of 1%, 2%, 3%, 4% and 5% (w/v). Molasses, a waste from the sugar-cane industry was included as an additional source of carbon. This was dispensed in concentrations of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (v/v) in dairy wastewater.

Wastewater from the processing of edible palm oil, was collected freshly from the food processing plant at Lam Soon (M) Ltd. Petaling Jaya. A bioaugmentation culture complex (consisting of *B. subtilis, B. licheniformis, P. aeruginosa, P. stutzeri, P. fluorescens and E. hermanii*) preparation from

Biotech Inc. (U.S.A) together with a seed culture (sewage sludge) was used to treat the wastewater in comparison with *B. licheniformis* JF-2. Sewage used as seed culture in the activated sludge process was obtained from Indah Water Konsortium's wastewater treatment plant at Damansara, Petaling Jaya. All the effluents were stored at 4°C before use.

3.10 Treatment of edible palm oil processing wastewater

3.10.1 Acclimatisation of seed culture

The seed culture in the activated sludge process used in the wastewater treatment was sewage sludge. For acclimatisation, 250 mLof the seed culture (from sewage) was added to the wastewater which was diluted with water at a ratio of 1:1 to a volume of 1L. The aeration was supplied by diffusing compressed air from pumps (Aqua Systems, 6000-II) with an air flowrate of approximately 1000 cm^3/min. After 24 hours, the wastewater was allowed to settle and the supernatant was removed leaving behind the activated sludge. The activated sludge was then used for the treatment of the edible palm oil processing wastewater.

3.10.2 Treatment of edible palm oil processing wastewater by the activated sludge process

1L of the wastewater containing edible palm oil processing wastewater conyaining 10 mL of the waste palm oil was added to the acclimatised seed culture and the pH was adjusted to an inital value of 7.00 with 1M HCL or 1M NaOH, and aerated. Samples were removed daily for COD analysis and

replaced with fresh material of the same volume. The treatment process, as such involved the addition of 2 mLof JAD 969P (Appendix 5) bioaugmentation culture complex in the wastewater which was then compared to the control which only contained the seed culture (from sewage). This bioaugmentation culture complex was used as a booster culture to reduce the COD and ultimately reduce the retention time (RT) of the treatment.

3.10.3 Treatment of edible palm oil processing wastewater using *B. licheniformis* JF-2

100 mLof *B. licheniformis* JF-2 grown in the Optimzed Medium (8 hours old) was added to the edible palm oil processing wastewater which was then diluted with water at a ratio of 1:1 to a volume of 1L. This was acclimatized for 24 hours. This was allowed to settle and the supernatant was removed, leaving behind the activated bacterial culture mainly consisting of *B. licheniformis* JF-2 (through microscopic examination of the sludge). 100 mL of waste palm oil and 1 L of the edible palm oil processing wastewater were then added to the activated sludge. Samples of 50 mLwere taken on alternate days for 15 days and analysed for COD reduction. Each time a 50 mL sample was taken for analysis, a fresh 50 mL of edible palm oil processing wastewater was added.