

CHAPTER 3

**MATERIALS AND
METHODS**

MATERIALS AND METHODS

3.1: *Chlorella vulgaris*

Chlorella vulgaris Beijerinck (001) was obtained from the Microalgal Culture Collection at the Institute of Advanced Studies, University of Malaya (Plate 1). This algae was isolated from anaerobically digested Palm Oil Mill Effluent (POME). Axenic cultures are maintained in Bold Basal Medium (BBM) (Nichols, 1973) using agar slants under continuous illumination of $40-60 \mu\text{Em}^{-2}\text{s}^{-1}$ at 25°C . Subculturing was done every month to maintain viability of culture.

Out of five species (*Chlorella vulgaris* 001, *Chlorella vulgaris* 072, *Scenedesmus quadricauda* 039, *Scenedesmus quadricauda* 041, and *Ankistrodesmus convolutus* 101) tested, *Chlorella vulgaris* 001 had the highest specific growth rate in rubber effluent (Geetha, 1992). It also had nutritional value of 41.9% dry weight protein, 6.0% dry weight lipid and 8.1% dry weight carbohydrate making it a high grade animal feed supplement. The *Chlorella vulgaris* 001 is heterotrophic and grow well in media supplemented with organic carbon sources (Chui, 1994).

3.2: Inoculum preparation

Figure 3.1 summarizes the procedure observed when inoculum was generated for the pond studies.

All steps up till the 4L culture in BBM were maintained under sterile conditions. The 100 ml cultures were generated by taking a loopfull of culture from the agar slant and dispensing it axenically into 100ml sterile BBM solution in a 250 ml conical flask. The flasks were then shaken at 150 rpm in a New Brunswick incubator shaker, under illumination of $40-60 \mu\text{Em}^{-2}\text{s}^{-1}$ with a light-dark cycle of 12:12 hour at 25°C for 5 days or until optical density reached 0.2 at 620 nm measured with a Shimadzu UV Spectronic Spectrophotometer. This culture was then ready for use as inoculum for laboratory studies. Ten percent inoculum was used in all experiments.

For pond studies scaling-up was carried out as in the above Figure 3.1. 10L glass aspirator bottles were rinsed 3 times with boiling water before being filled with sterile BBM and inoculated. The cultures were allowed to grow for 5 days before being used as inoculum for the tank culture.

The tanks used were transparent plastic aquariums which were rinsed with chlorox (hypochlorite solution five percent). RE was filtered through gauze to sieve out any solids and nine litres were used in each tank which was

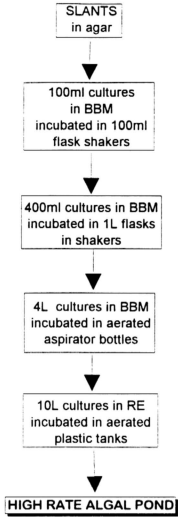


Fig: 3.1 : Inoculum scale-up for High Rate Pond Studies

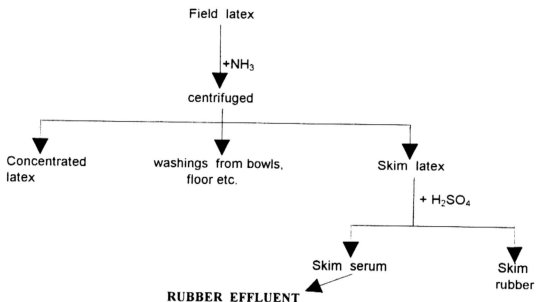
aerated overnight to increase the pH from 4.5 to 7.0. The tanks were inoculated with one L *Chlorella* culture at logarithmic phase ($OD_{620} = 0.2$) grown in BBM.

The tanks were aerated with air through silicon tubes attached to the base of the tanks. The inoculum was generated after a four day incubation period with an irradiation of $40-60\mu\text{Em}^{-2}\text{s}^{-1}$ with a 12:12 light dark cycle at room temperature (26 to 28°C).

Fifty litres of inoculum was required for each HRAP batch culture.

3.3 : Source of effluent

The rubber effluent (RE) for the study was obtained from two sources, Lee Latex, Gombak, Kuala Lumpur and also Sime Darby, Atherton Estate, Nilai, Negeri Sembilan. Both latex concentrate factories followed similar production procedures which is shown in the flowchart below:-



3.4 Characterization of Rubber Effluent (RE)

The Lee Latex concentrate factory, in Kuala Lumpur closed down in the middle of the study. Further samples were taken from the Atherton Latex Concentrate Factory at Negeri Sembilan. The effluent was collected from an anaerobic pond after one day's retention time. Every batch of RE collected was analysed to determine the main chemical parameters; that is Chemical Oxygen Demand (COD), ammoniacal nitrogen ($\text{NH}_3\text{-N}$), dissolved orthophosphate ($\text{PO}_4\text{-P}$) and pH.

Molasses

Molasses used for the experiments was collected from the Central Sugar Refinery at Shah Alam. All physical and chemical characteristics of the mollasses were supplied by the laboratory of the sugar refinery factory. The molasses was kept at 4°C until used.

3.5 : Laboratory studies

Laboratory studies were carried out to determine the effect of CO₂ supplementation to RE on the growth of *Chlorella*. One L flasks containing 500 ml of *Chlorella* cultures were grown in triplicates for each treatment.

The cultures were aerated at one L per minute flow-rate of either air or 5% CO₂ in air and subjected to 60 $\mu\text{Em}^{-2}\text{s}^{-1}$ light irradiation. To maximize CO₂ utilization, the CO₂ aerated cultures were aerated with CO₂ for 20 minutes with 5% CO₂ in air and with air alone for the next 40 minutes for 8 hours out of the 12 hour light phase. The cultures were subjected to 12:12 light dark cycle.

The pH of the growth medium was adjusted to 7.0 before autoclaving and checked again after autoclaving. Molasses was autoclaved separately and added to the medium after autoclaving. The pH was not controlled during the length of the experiment.

The experiments where 5% CO₂ is supplemented in experimental flasks are summarised in Table 3.1. Cell count and chl-a content were determined from daily samples for experiment I and II. pH values were also determined for both experiments daily.

3.6: High Rate Algal Pond Studies

3.6.1 : The High Rate Algal Pond (HRAP) design

Two high rate algal ponds were constructed at the experimental farm of the Institute of Advanced Studies, University of Malaya. The ponds are single-loop raceway type with dimensions of 1m x 4m x 0.2m (Figure 3.2). A light block paddle-wheel was used to mix the content of the pond (Plate 2).

To facilitate introduction of CO₂, stainless steel pipes of 20 mm diameter, were attached to the pond bottom at two locations as shown in Plate 3. The pipe placed at the pond bottom was perforated with 2 mm holes all along the surface.

The length of time for the 5%CO₂ gas to be bubbled into the pond was controlled by a regulator connected to the gas tank. The gas flowrate was controlled through a flowmeter connected to the gas tank. The gas could flow from 1L to 25L per minute at 0 to 4 atm. pressure.

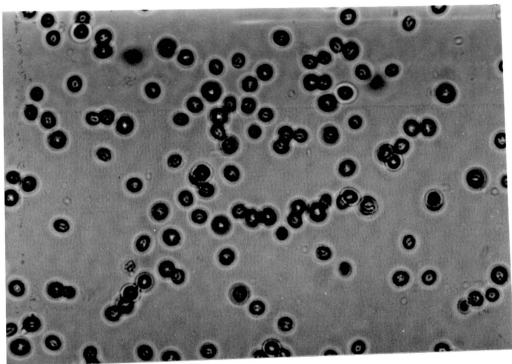


PLATE 1 : *Chlorella vulgaris* 001



PLATE 2: View of the two High Rate Algal Ponds
used in the study with gas tanks



PLATE 3: The perforated stainless steel pipes used to bubble in CO₂ and air into the pond



PLATE 4: The paddle - wheel used to mix the *Chlorella* culture

A Bonfioli-geo motor with an attached gearbox was used to operate the paddle wheel. Roofs with a height of 1.5m were fixed above the ponds to shelter them from rain. The roofing material was of transparent corrugated acrylic. Figure 3.2 and Plate 4 show a scheme of the whole construction.

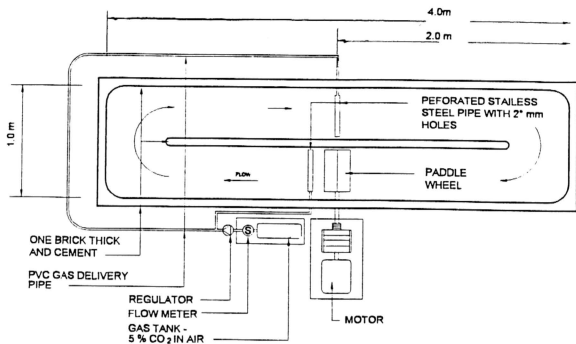
Calibration of the paddle wheel speed was done using a speedometer. The time taken by a polystyrene ball to move a measured distance on the pond culture surface was used to determine surface flow rate.

3.6.2 : Effect of CO₂ and mollasses supplementation in the HRAP

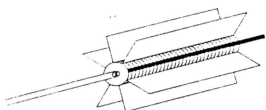
The HRAP was operated with the following conditions:-

Culture depth	:	0.15m
Paddle wheel speed (rpm)	:	20 (continuously)
Paddle immersion depth (m)	:	0.1 from the surface of culture
Flow rate (cm/s)	:	17
Total volume of culture	:	450L
Inoculum	:	40L - 46L
Total surface area	:	3.8m ²

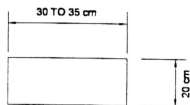
The two HRAP's were washed with hypochlorite solution before being filled with effluent. 400 - 410L of the anaerobically digested RE was aerated overnight, by



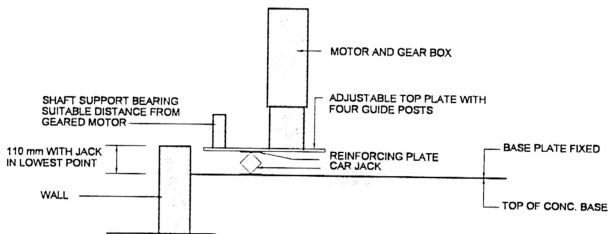
PLAN - HIGH RATE ALGAL POND



8 BLADE PADDLE WHEEL



BLADE (STAINLESS STEEL)



MOTOR MOUNTING APPARATUS

FIG 3.2 . DIMENSION OF HRAP, THE PADDLE WHEEL, GAS DISTRIBUTION SYSTEM AND MOTOR MOUNTING APPARATUS

paddle wheel mixing in the HRAP. This increases the pH of the RE to 7.0 after which the *Chlorella* was inoculated.

Aeration time of CO₂ was varied in the first three batches (Table 3.2). In the fourth batch CO₂ supplementation was compared to mollasses while in the fifth batch a combination of CO₂ and mollasses was used and compared to control. 250 ml mollasses (0.05%) was always added in the evening (1830) as this was found to be the best time (Chui, 1993). Experimental conditions are summarized below in Table 3.2.

3.6.3 : Semidiurnal Studies

Eight semidiurnal studies were carried out, one each for Batches I and II, and two each for Batches III, IV and V. The semidiurnal study was conducted for a duration of 12 hours from 0630h to 1830h. Parameters including pH, temperature, light irradiance and dissolved oxygen, cell count and chlorophyll-a content were taken hourly.

3.7: Analytical methods

3.7.1 Algal cell count

Algal cultures that were appropriately diluted and homogenized were put into a haemocytometer chamber. A magnification of 400x was used.

Table 3.1 : Summary of the laboratory experiments for CO₂ supplementation

EXPERIMENT	AIM	SUBSTRATES
I	To compare the growth of <i>Chlorella vulgaris</i> in BBM and RE supplemented with CO ₂	BBM BBM+CO ₂ RE+AIR RE+CO ₂ RE+MOL RE+MOL+CO ₂
II	To compare the growth of <i>Chlorella vulgaris</i> in RE with organic and inorganic carbon supplementation	BBM (control) RE (no aeration) RE+AIR RE+CO ₂ RE+MOL RE+CO ₂ +MOL

Table 3.2 : Summary of High Rate Algal Pond Studies

Batch	Exp. Duration	CO ₂ Aeration Rate	Aeration duration	Sampling time	Semi-diurnal Studies Day	Comments
I	C	3.11.93		0630h, 1330h	Day 2	Control
	CO	TO 8.11.93	5Lmin ⁻¹ 20 min hour ⁻¹	& 1830h	Day 2	
II	C	4.12.93		0630h, 1330h	Day 1	Repetition of Batch I
	CO	TO 8.12.93	5Lmin ⁻¹ 20 min hour ⁻¹	& 1830h	Day 1	
III	C	7.4.94		0630h, 1330h	Day 5&7	Aeration time was increased
	CO	TO 17.4.94	5Lmin ⁻¹ 40 min hour ⁻¹	& 1830h	Day 5 & 7	
IV	M	7.5.94		0630h, 1330h	Day 2&4	supplemented with 0.05% molasses at 1830h on day 2,3 & 4
	CO	TO 13.5.94	5Lmin ⁻¹ 40 min hour ⁻¹	& 1830h	Day 2 & 4	
V	C	10.7.94		0630h, 1330h	Day 3 & 5	0.05% Molasses was also supplemented at 1830h on day 2,3 & 4.
	MCO	TO 17.7.94	5Lmin ⁻¹ 40 min hour ⁻¹	& 1830h	Day 3 & 5	

NOTE: C = CONTROL (compressed air was bubbled in at the same condition as the CO₂ pond)
CO = CARBON DIOXIDE
M = MOLASSES (compressed air was bubbled in at the same condition as the CO₂ pond)
MCO = MOLASSES - CARBON DIOXIDE

3.7.2 Chlorophyll-a

Determination of chl-a was done according to Strickland and Parson (1976). Filtered algal sample is mashed with a known volume of 100% acetone in a plastic centrifuge tube. The sample was then left overnight in the dark at 4°C for 24 hours to facilitate pigment extraction. The tubes were centrifuged at 3,000 rpm for 10 minutes and the O.D. of the clear supernatant obtained was determined at wave lengths 630 nm (OD_{630}), 645nm (OD_{645}) and 665nm (OD_{665}), with a Shimadzu UV 160 Spectrophotometer. The chlorophyll a concentration is calculated using the following equation.

$$\text{Chlorophyll a (mgm}^{-3}\text{)} = \frac{Y \times \text{volume of acetone (ml)}}{\text{algal culture volume}}$$

where $Y = 11.6 OD_{665} - 1.31 OD_{645} - 0.14 OD_{630}$

3.7.3 Determination of algal dry weight

A dry preweighed 0.45 μm glass fiber cellulose filter is used to filter a known volume of algal culture. The filter paper is then dried at 105°C for 1 hour and cooled in dessicator filled with silica gel and weighed. The algal dry weight is determined using the following equation:

$$\text{Dry weight (mgL}^{-1}\text{)} = \frac{\text{Weight after - weight before (mg)}}{\text{volume of algal culture (L)}}$$

3.7.4 pH

pH was determined using a portable pH meter (Hanna Instruments)

3.7.5 Light

A Gossen PANLUX Electronic 2 , Lux and Illuminance meter was used for determination of light density. The unit for irradiance was converted to $\mu\text{Em}^{-2}\text{s}^{-1}$ through the following equation,

$$1\text{kLux} = 20\mu\text{Em}^{-2}\text{s}^{-1}$$

3.7.6 Temperature and dissolved oxygen

Dissolved oxygen and temperature readings were taken using YSI model 57A. The machine was calibrated for dissolved oxygen before reading daily.

3.7.8 Daily solar radiation, sunshine hours and rainfall

The above parameters were obtained from the nearest meteorological station at Petaling Jaya (Appendix 13).

3.8 : Pollution parameters

Pollution parameters of COD, $\text{NH}_3\text{-N}$ and $\text{PO}_4\text{-P}$ were monitored during HRAP studies as a measurement of treatment efficiency. All methods are base on Standard Methods, APHA, 1989.

3.8.1 Chemical Oxygen Demand (COD)

The COD is a measurement of the oxygen equivalent required for the organic matter in a sample to be oxidized by a strong chemical oxidant (Linsky and Frazini, 1986).

Standard methods (APHA, 1989) was used to determine the COD. The suitably diluted or raw sample was refluxed for 2 hours with 10 ml of potassium dichromate and 10 ml of concentrated sulphuric acid. 0.1 M ferrous ammonium sulphate was used to titrate the excess dichromate. Feroin solution was used as an indicator. The end point was obtained when there is a sharp colour change from blue green to reddish brown. The amount of organic matter that is measured as oxygen equivalent is proportional to the potassium dichromate used. COD is calculated based on the following formula:

$$\text{COD (mgL}^{-1}\text{)} = \frac{800 (A - B)}{\text{sample volume}} \times D$$

where,

A = volume (ml) of ferrous ammonium sulphate used for dilution of blank.

B = volume (ml) of ferrous ammonium sulphate used for sample titration.

D = dilution factor used as per dilution table provided in APHA (1989).

The carbon content can also be calculated as below (Edwards et al., 1980).¹

$$\text{Carbon (mgL}^{-1}\text{)} = \text{COD (mgL}^{-1}\text{)} \times \frac{12}{32}$$

3.8.2 Ammonical-nitrogen (NH₃-N) assay

A modified phenate method from APHA (1989) was used. Fifty ml of sample (suitably diluted) was measured in 100 ml stoppered flask. A series of standard solution made up to 50 ml was also prepared together with 50 ml distilled water for blank. To this 2 ml of phenol alcohol, 2 ml of sodium nitropruside and 5 ml of oxidizing solution (sodium hypochlorite : alkaline nitrate in 1:4) was added. The blue colour of indophenol was allowed to develop. Absorbance of standards and sample were read at 640nm using a Shimadzu UV 160 Spectrophotometer.

3.8.3 Dissolved orthophate ($\text{PO}_4\text{-P}$) assay

The assay is a colorimetric test based on molybdenum blue colour development from phosphomolybdic acid (Wang and Ong, 1978; APHA, 1989). It is referred to as the ascorbic acid method. This method does not require prior hydrolysis or oxidative digestion.

The 50 ml sample suitably diluted was matched with 10ml of mixed reagent containing 100ml ammonium molybdate, 250ml sulphuric acid, 100ml ascorbic acid (fresh prepared) and 50ml potassium antimonyl titrate solutions). The absorbance reading taken at 885nm using a UV 160 Shimadzu Spectrophotometer between 10minutes and two hours from the time the mixture is prepared.

3.8.4 Total solids (TS)

100 ml of effluent was evaporated in a preweighed dry crucible using a steam bath. The evaporated sample was then further dried in an oven at 100°C to a constant weight. The weight of the dried sample is recorded after cooling in a desiccator. The following equation is used to obtain the weight.

$$\text{Total solid (mg }^{-1}\text{)} = \frac{(A - B) \times 1000}{C}$$

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$$\text{Total solid (mg }^{-1}\text{)} = \frac{(A - B) \times 1000}{C}$$

where,

A = weight of crucible + solids (mg)

B = weight of clean dried crucible alone (mg)

C = sample volume

3.8.5 Total suspended solids (TSS)

The same procedure that is used for determine dry weight is used.

3.9 : BIOMASS ANALYSIS

This is necessary to determine the nutritional value of the algae harvested using various treatment systems.

3.9.1. Total crude protein

The Tecator Kjelttec Auto 1020 Analysis was used to determine total crude protein. The protein value is obtained indirectly through total nitrogen determination.

Filtered algal-sample with known dry weight are mashed in a Tecator digestion tube in triplicates. 4.0 to 5.0 g of catalyst (a mixture of finely ground, 100g K_2SO_4 and 7g of $CuSO_4$) are added to the sample together with 12.5 ml concentrated sulphuric acid (H_2SO_4). An empty filter paper was used as blank. The samples were then digested in a Tecator Digestion system at 450°C for 45-60 minutes or till greenish colour was observed. Digested samples were than allowed to cool for one hour and 75ml of

distilled water is added. The samples are then filtrated in the autoanalyses.

$$\text{Percentage protein} = \frac{14.01 \times m \times f \times 100}{c} \times (\text{ml titrant} - \text{ml blank})$$

where,

14.01 = atomic weight of nitrogen

m = the molarity of the acid, 0.5M

f = standard Kjeldahl factor, 6.26

c = sample weight, g

3.9.2. Total dissolved carbohydrate

The Kochert (1978) method was used to determine the total carbohydrate content which is based on colorimetric measurement.

Filtered algal samples of known weight were mashed with five ml of 2N HCl and sonicated in a B. Braun Labsonic 2000 for four minutes. The mixture was then placed in a waterbath at 80°C for 20 minutes. The samples were then cooled and centrifuged at 3000 rpm for 10 minutes. The supernatant was kept aside while the residue was reextracted as above. The supernatant obtained from the 1st and 2nd extraction were then pooled to make 10ml.

For the colorimetric reaction 1.9 ml of phenol reagent (80%) was added to 100 μ l of supernatant in a boiling tube and to this 5 ml of concentrated sulphuric acid (H_2SO_4) was added. H_2SO_4 had to be delivered within 15 to 20 seconds as the speed of delivery was critical for colour development. The samples were cooled for 30 minutes and absorbance was read at 485 nm with UV spectrophotometer. A standard curve was prepared using a series of glucose solution.

3.9.3 Total lipids

The total lipid content of the algal biomass was determined using the Bligh and Dryer method (1959). This method involves dry weight determination of algal lipids.

Filtered algal cells of known weight was washed with 10ml of freshly prepared mixture of chloroform : methanol : distilled water ($CH_2Cl:CH_3OH:H_2O$) in 1:2:0.8 proportions and sonicated for four minutes, and centrifuged for 15 minutes at 300 rpm. The supernatant was kept aside and the residues again reextracted as above. The supernatant was then pooled to make up to 25 ml.

6ml of CH_2Cl and 6ml of distilled wasd then added to the extract and transferred into a separating funnel and shaken thoroughly. This well mixed colloidal mixture was then centrifuged at 3000 rpm for 15 minutes. The bottom

fraction was then drawn out with a specially drawn out Pasteur pipettes in 100 ml and bottom flasks and evaporated to dryers in a Rotary evaporator (Rotavapor RG III). The dried fraction was dissolved in a small volume of CH_3Cl and transferred to a preweighed vial. The fraction is then blown dry with nitrogen gas and the vials are dried overnight in a dessicator. The weight of the vial is then recorded and calculated as follows:

$$A - B$$

$$\text{Total lipid (\%)} = \frac{\text{A} - \text{B}}{\text{Dry weight of algae (g)}} \times 100$$

where

A = weight of dried sample and vial (g)

B = weight of vial (g)

3.9.4 Total carotenoids

The Liaaen-Jensen and Jensen Method (1973) based on absorbance of the carotenoid pigment extract was used to determine carotenoid content in algal biomass.

Filtered algal cells of known weight was washed with 5 ml of acetone and sonicated for four minutes followed by centrifuged at 3000 rpm for 10 minutes. The residues were reextracted as above while the supernatant were pooled and transferred to round-bottom flasks and evaporated to dryness in a rotary evaporator. To this 5

ml of diethyl ether followed by 5 ml of freshly prepared 10% KOH was added. The mixture is then left in the dark for 1 hour at room temperature to sapority. 10ml of 5% NaCl is the added and the mixture to the transfered to a separating funnel and to this then 10ml of diethyl ether is added. The lower fraction (B) is reatined and the upper fraction (A) is kept aside. 10ml of diethyethel is again added to fraction (B) and the bottom layer is then discarded. Both the upper layers are pooled in the separating funnel.

The mixture is washed with 10ml of 5% NaCl 3 times (bottom layer discarded for all was water). 0.5g of sodium sulphate anhydrous is the added and the remaining sample is evaporated to dryness in a Rotary Evaporator. The dried sample is then dissolved in a small volume of acetone and made up to 5ml. The pigment spectrum absorbance was the scanned in a UV visible recording spectrophotometer (Shimadzu UV-160) wave length 350 to 850nm.

The total amount of carotenoid is then calculated according to the following equation:

$$\text{Total carotenoid mg/g} = \frac{\text{D.v.f. } 10}{\text{dry weight of sample (g)} \times 2500}$$

where,

D = optical density at the maximum absorption of the

sample (within 440-450nm)

v = dilution fractor of the sample

2500 = average extinction coefficient for carotenoid

3.10 Growth Rate

Specific growth rate was calculated based on the following formula:

$$\mu = \frac{\ln (t_2 - t_1)}{\text{no. of days}}$$

where,

μ = Specific growth rate

t = Cell Count or chl-a concentration

where $t_2 \geq t_{21}$

3.10.1 Heterotrophic and Autotrophic Growth Rate

In the HRAP batch studies growth rate is divided into day time and night time growth. Algal growth in the day time is considered as autotrophic growth rate while algal growth during the night is referred to as the hetetrophic growth.

Autotrophic Growth is the logarithmic increase in cell number from 06:30hr to 18:30hr

Heterotrophic Growth is the logarithmic cell increase from 18:30hr to 06:30hr the following day.

3.11: Algal Growth Potential

Assuming that maximum algal concentration will be obtained on a given concentration of carbon, nitrogen and phosphate when all other factors are in excess and not limiting (Azov et al., 1977). It is calculated as follows:

- 1.0g C yields 1.91g algal biomass
- 1.0g N yields 10.97g algal biomass and
- 1.0g P yields 76.92g algal biomass.

RESULTS

4.1 Characteristics of rubber effluent (RE) used in the study

Each batch of RE that was used was characterized prior to the experiment. Since the effluent source came from two different locations there is some variation in the RE quality as shown in the table below:

TABLE 4.1: Average Characteristics of RE collected from Lee Latex (June 1993 – Dec. 1993) and Atherton Estate, Nillai (Feb. 1994– July 1994).

PARAMETERS	AVERAGE VALUES	
	LEE LATEX	ATHERTON ESTATE
pH	5.5	6.3
COD (mgL ⁻¹)	1607.0	3903.0
NH ₄ -N(mgL ⁻¹)	149.6	362.4
PO ₄ -P (mgL ⁻¹)	14.4	38.9
TSS (mgL ⁻¹)	207.0	657.2
TS (mgL ⁻¹)	1948.0	1974.0
Total carbon (12/32 X COD)	602.4	1426.1
C:N:P ratio	41.8:10.4:1	36.7 : 9.3 : 1
Colour	White	Grey

Due to the acidic nature of the RE, ranging from 5.3 - 6.3, the pH of the RE had to be adjusted to approximately 7.0 by aerating it overnight in the HRAP. For the RE from the Atherton Estate the black grey(probably due to sulphide in the effluent) turned whitish after aeration

overnight. Therefore before inoculation the RE in both cases had an almost neutral pH and were white.

The TSS value in the RE from Atherton Estate was much higher than the one from Lee Latex, making it difficult to sieve away the solids. The RE from the Atherton Estate was allowed to settle and the supernatant was used for the experiment.

Another difference is the C:N:P ratio of the RE used here which is approximately 40:10:1 compared to the RE used by Chui(1994) which had a C:N:P of 7:5:1. The variation in the RE quality for the five batches is illustrated in the following graph.

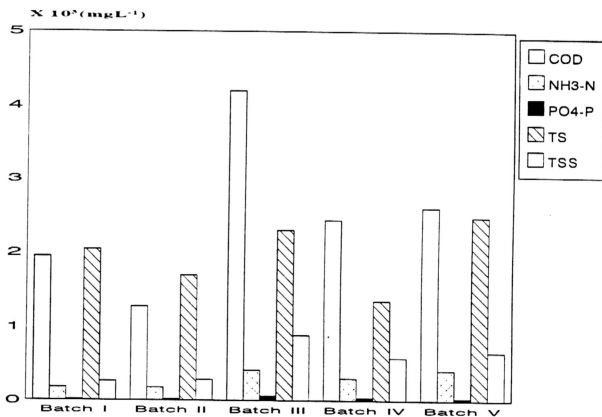


FIG 4.1: Variation in the RE quality used in the HRAP batches

4.2: Characteristics of Molasses

Molasses which is a byproduct of sugar refinery is a viscous dark brown liquid. Table 4.2 reveals that it has high total carbohydrate concentration. Total reducing sugars are only 29% of the total volume.

Trace amounts of nitrogen and minerals enhance the nutritional value of molasses making it useful as a supplement in growth media. The dark brown recalcitrant substances in molasses is due to the caramelization of sugars during the heat treatment of the refinery process.

TABLE 4.2 : Characteristics of molasses
(obtained from the Central Sugar Refinery)

Parameters	Average values
pH	5.3
Total carbohydrates	60.0%
Total reducing sugars	29.0%
Sucrose	36.5%
Ash	7.3%
Nitrogenous compounds	4.5%
Colour	Dark brown