CHAPTER 2

LITERATURE REVIEW

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2.1 RUBBER INDUSTRIES IN MALAYSIA

Rubber has been cultivated in Malaysia ever since the 1890's and in 1990 Malaysia supplied 30% of the world's rubber, making Malaysia one of the world's largest producer and exporter of quality natural rubber (Info Malaysia, 1990). Therefore it is of no surprise that one of the major sources of organic pollution in Malaysia is the rubber processing industry.

Rubber processing consumes a large quantity of water. About 100 million litres of effluent containing 200 tonnes of BOD which is equivalent to the population load of 4.5 million people is discharged daily from this industry into the waterways (Phang, 1987).

Two main types of rubber processing in Malaysia are latex concentrate and the Standard Malaysian Rubber (SMR). In 1990 the number of latex concentrate and SMR factories were 84 and 124 respectively (Zaid Isa, 1993). Latex concentrate factories produce the largest volume and the most polluting wastewater. Characteristics of the different kinds of rubber effluent are presented in Table 2.1.

TABLE 2.1 Effluent characteristics of two main types of rubber processing factories (Zaid Isa et al., 1988)

	LATEX CONCENTRATE	SMR	MIXED
pH	3.7	5.7	6.2
TS	7576	1915	2123
TSS	182	237	757
BOD	3192	1747	2078
COD	6201	2740	3988
TN	616	147	345
NH3–N	401	66	268
Sulphate	1610	–	–

Abbreviation: SMR = Standard Malaysian Rubber
All Parameters except pH in mgL^{-1}

A typical latex concentrate factory produces 4.17 litres of effluent per litre of crop processed (Ahmad *et al.* 1979). An average factory processing 78,000 litres per day of crop generates 325,000 litres of effluent per day. Effluent from latex concentrate factories contains high level of sulphate, causing problems during biological anaerobic treatment as H_2S is liberated to the environment causing malodour problems (Zaid Isa, 1988).

Currently Malaysia is also involved in the production of rubber items like gloves, condoms, swimming caps, catheters, finger cots, foams and toys for export. Pollution increases with the expanding rubber products industry.

2.2 CURRENT TREATMENT SYSTEMS IN MALAYSIA

The rubber and rubber products industries have adopted various effluent treatment systems (Table 2.2) developed by the Rubber Research Institute (RRIM) to comply with the Environmental Quality (Prescribed Premises) (Raw Natural Rubber) Regulation (1978) of the DOE (Table 1.1). Common treatment systems used in Malaysia are summarized in Table 2.2 while Table 2.3 compares the cost and treatment efficiencies of the treatment systems concerned.

All the four treatment systems involve biological processes which have proven to be the most economical way for treatment of rubber effluent with satisfactory pollution control.

Anaerobic-facultative ponding is the most popular treatment system used by 83% of the factories (Zaid Isa, 1990).This system consists of rubber traps, anaerobic and facultative ponds arranged in series or parallel. However malodour problems can arise due to hydrogen sulphide if latex concentrate factory effluent is being treated. In the anaerobic facultative ponding system the anaerobic step is necessary to reduce the high organic content in the effluent (Muthurajah *et al.*, 1973). The aerobic ponds help to reduce the pollution parameters through the activity of algae and bacteria in the pond. However due to the long retention time a large land area is required.

This system has however been the most cost effective due to its simple operation and low cost if land is available (Zaid Isa, 1990) [Table 2.3].

The Oxidation Ditch system is energy intensive but requires a smaller land area and is appropriate for urban areas where land is limited. Here the effluent flows through a ditch where oxygenation of organic matter is achieved through high speed paddle-wheel mixing. However it has high capital and operating costs due to high electricity consumption (Zaid Isa *et al.* 1988). Other aerobic systems include aerated lagoons, activated sludge and submerged aeration systems. The aerobic systems in general do not create any malodour problems.

Land application involves using the effluent which is rich in nutrients as fertilizer for crops like oil palm and rubber. A 22% increase in yield was observed in oil palm fresh fruit bunches (Lim and P'ng, 1990) while the yield of rubber increased 11-18% (Tayeb Dolmat *et al.* 1979). Effluent application has to be conducted in a controlled manner to avoid ground water contamination. Bacteria and fungi from the effluent are effectively trapped by the soil, with an observed increase in nitrifying bacteria. These organisms increase the soil fertility.

The land application system involves the collection of effluents in equalization ponds for distribution to the

TABLE 2.2 : A summary of the different treatment systems currently practised in Malaysia

TREATMENT	MODES	NOTES
1. Anaerobic Facultative Ponding	consists of rubber traps, anaerobic and 3 tier facultative ponding	most popular due to simplicity
2. Aerobic Ponding	a. Oxidation Ditch b. Activated Sludge c. Aerated lagoons d. Submerged aeration systems	oxidation ditch is the most common form of aerobic ponding – odour problems
3. Land Application	a. flat bed aplication b. furrow system	controlled application necessary to prevent water logging – 18% improvement in growth of rubber and 19% in palm oil
4. Enclosed Anaerobic Digestor	tank digestors at high loading rate and short retention time – possible to recover methane	methane derived from the anaerobic tank digestor can be converted to electricity to supply 56% of energy consumed in a latex concentrate factory
5. Trickling Filtration	experimental	flexible system
6. Rotating Bio – Disc	For Block Rubber Effluent	experimental

TABLE 2.3 : REVIEW OF EFFICIENCY OF TREATMENT SYSTEMS

REF.	SYSTEMS	% REMOVAL				RT	LAND	REMARKS	CAPITAL	OPERATING
		COD	BOD	NH_4–N	TSS	(days)	AREA		COST	COST/YEAR
a.	Anaerobic/facultative ponding (3 tier system)	89	96	71	56	30 (anaerobic) 9–5 days (facultative)	large	latex conc. effluent	RM850k	RM50k
b.	Anaerobic/facultative ponding (3 tier system)	92	97	38	61	30, 12, 12,10 (facultative)	large	SMR effluent		
c.	Oxidation ditch	96	97	–12	70	7	Less than facultative	some mallodour High operating cost in form of electricity to produce a flowrate of 30 to 40 ms^{-1}	RM500k	RM100k
d.	Land application	*15.5	4.2	4.1	–		None			
e.	Anaerobic–aerobic treatment	94–97	95–99	13–5.9	16–50		Minimal	Algal aerobic treatment	RM35k	RM1.8k
f.	Trickling filtration	–	–	–	–	–		High N & P remain		
f.	Rotating Bio–disc	–	–	–	–	–		Experimental		
g.	High–rate algal pond	–	–	–	–	–		BOD removal rate = $0.72d^{-1}$ TN = $0.33d^{-1}$		
h.	High–rate algal pond	94–99	–	72–97	–	5	–	75–95% PO_4–P removal	RM320k	RM85k

REFFERENCES: a. John & Ong (1979), Zaid Isa et al.(1988); b. Ahmad Ibrahim & John (1985); d. Lim & P'ng (1990)–figure reported is water quality data 300m from the application spot in mgl^{-1}; e.Muthurajah et al. (1974), Ahmad Ibrahim et al. (1973); f. Ponniah et al. (1976); g. Muthurajah et al. (1973); Nordin Ab. Karim (1984); h. Geetha et al. (1992); i. Ahmad Ibrahim et al. (1986).

NOTE: RM2.5 = US$1
TN = Total Nitrogen

estates. Both the flatbed and furrow system that are used involve the flow of effluent by gravity. In the flatbed system the effluent flows to the top most flat bed then subsequently to the lower beds while in the furrow system the effluent flows through a series of furrows. (Zaid Isa, 1990).

The enclosed anaerobic digestion system allows the possibility of recovery of methane. Methane harvested from the tank digester in a factory at Johor provided 56% of energy consumed in the latex concentrate factory (Nordin & Ahmad, 1981). Anaerobic tank digestors were operated at high loading rate and short hydraulic retention time. No malodour problems were obtained.

The Department of Environment (DOE) Report (1990) showed that 86% of rubber factories in general discharge their waste into waterways, while 7.3% use land application and 6.8% recycle their wastes. In 1993, the natural rubber processing and rubber products industries contribute to 16.4% of the water pollution problems in the country (DOE Environment Quality Report, 1993).

2.3 Cultivation of Microalgae

Algae are efficient solar energy converters and are able to produce a great variety of metabolites. Man has taken advantage of these properties through algal mass culture.

Microalgae also play an important role as the primary producer in the aquatic foodchain. It is the natural food of fish and shellfish besides being required during certain developmental stages of crustaceans and molluscs (de Pauw and Persoone, 1988). Thus the use of microalgae in aquaculture has several potential advantages over the production of microalgae as human food. In addition to high conversion efficiencies, there is no need for harvesting, drying and storage, as animal food chains could use the algae directly.

Microalgae used in commercial mass culture have the following desirable characteristics (Borowitzka, 1992):-

1. Able to grow in extreme environment, therefore reducing problems with competing species and predators. However, only a limited number of species are available and some extreme environments are difficult to maintain.
2. Have a rapid growth rate which provides advantage over competing species and predators.
3. Morphologically have large cell size, colonial or filamenteous as this would help to reduce harvesting costs although large cells usually grow slowly.
4. Open air cultures should have wide tolerance to environmental conditions enabling less control of culture conditions required for reliable culture.
5. Possess tolerance to shear force thus allowing for use of cheaper pumping and mixing methods to be

used.

6. For production of valuable chemicals the cell should have high cell content of product giving higher value for biomass. However, these products being secondary metabolites, higher concentration may mean slower growth.

Figure 2.1 summarises the inputs and outputs involved in microalgal mass culture based on Vonshak (1990) and Borowitzka(1991).

2.3.1 Microalgal Cultivation Systems

The following are two major algal culture systems which are used for mass cultivation of microalgae, based on their engineering and hydraulic characteristics:- (i) Open system and (ii) enclosed system.

The open system, developed in the 1940's (Becker, 1981) includes shallow raceway ponds, also named the high rate algal ponds (HRAP), which are shallow circulating units (Tredici, 1992).

The most common and simplest system used today, in industrial outdoor cultivation is the open raceway where stirring is provided by a paddle wheel (Richmond, 1992). However the main drawback of the system is the lack of temperature control and low photosynthetic efficiency leading to lower productivity compared to enclosed systems.

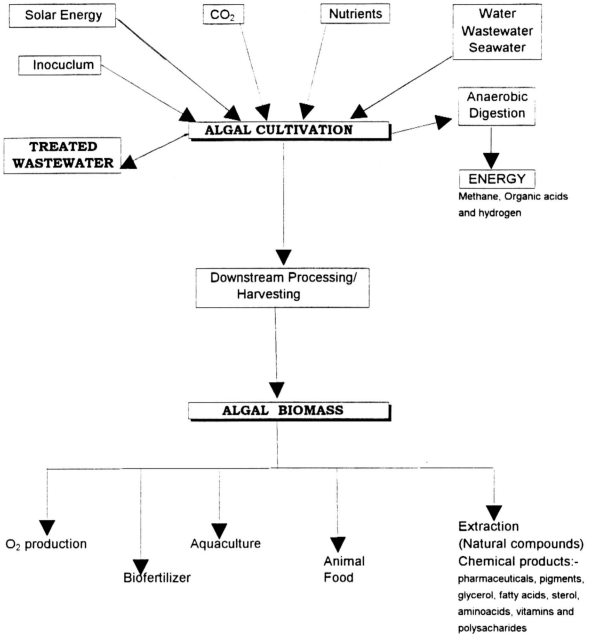

Figure 2.1: Input and Output of microalgal mass culture (Vonshak, 1990; Borowitzka, 1991)

Paddle wheel mixed raceway ponds are hydraulically well mixed and can easily be scaled up. Here mixing is inexpensive compared to the more energy-intensive circular ponds with large mixing arms. Paddle wheel mixing also provides flexibility in velocity control and direction of flow. The paddle wheel mixed system could involve a single raceway loop or series of meandering channels and can be used for "clean culture" or waste water treatment. Unmixed ponds are not satisfactory due to low yield, settling and unstable algal populations (Terry and Raymond,1985).

The Advanced Integrated Ponding system proposed by Oswald (1991) combines many functions of biological treatment. Floatation, sedimentation aeration, fermentation and disinfection are optimised in a series of fermentation pits, facultative ponds, high rate ponds, settling ponds and maturation ponds. The harvested biomass may be used as fertilizer or feed.

The main disadvantage of the open system is contamination due to its exposed surface, and low cell concentration. The enclosed system has been proposed as a solution to overcome these problems (Tredici and Zittelli, 1994).

The most common system used for enclosed algal cultivation is the photobioreactor (Chaumont, 1993; Borrowitzka, 1994). The examples are:-

(a) Tubular photobioreactor which are rigid or flexible - depending on their building material if made of glass or methy-polymetacrylate (rigid), silicone or polyethylene (flexible).

(b) Solar panels are made of glass or stainless steel

(c) Alveolar extended sheet (Tredicci and Matterassi, 1992).

(d) Conventional fermenters

All enclosed photobioreactors utilize natural sunlight except for fermenters where artifical light is supplied either as an internal or external source (Burges *et al.*, 1993). Some cultures in fermenters are grown heterotrophically without light.

Dialysis culture systems have also been proposed for fragile algae such as dinoflagellates. Here algae are confined in dialysis tubes which are maintained in the culture medium (Inka Dor, 1975). Dialysis culture has also been used in waste water treatment. In this way the algal biomass is not contaminated by enteric organisms like bacteria, viruses and also suspended solids.

Due to the complicated nature of enclosed systems, algal cultivation by this approach is an attractive alternative to open systems only when high value products are to be obtained (Benemann, 1989).

Studies carried out using the tubular photobioreactor

system have involved the use of inorganic media with yields varying from 0.48 $gL^{-1}day^{-1}$ (Lee, 1986); 63 mg dry *Chlorella* Lhr^{-1} (Jayavamardian and Palseen, 1991); 0.279 $gL^{-1}day^{-1}$ and 15.65 $gm^{-2}d^{-1}$ (CO_2 enriched) (Lee, 1986) and 30 $gcm^{-2}day^{-1}$ *Chlorella* (Lee and Low, 1991). Tredici and Materassi (1992) obtained 30 - 35 tons $ha^{-1}y^{-1}$ *Spirulina* production in a photobioreactor compared to 20 tons $ha^{-1}y^{-1}$ in open ponds.

2.3.2 Algal cultivation and wastewater treatment

Algae are part of the natural process in waste stabilization ponds where organic wastes are oxidized by bacteria and the oxygen is supplied by photosynthetic algae (Oswald, 1957). Waste stabilization ponds have been used for years in waste treatment systems.

Algae serve various functions in wastewater treatment including:-

(a) reduction of BOD;

(b) removal of N and/or P and

(c) removal of heavy metals (Borrowitzka, 1991).

This is achieved by recycling nitrogen, carbon and phosphorous into cellular material. By integrating field-scale algal production units with waste disposal facility, an economical system can be achieved. The algal biomass obtained can be either used as feed or biofertilizer (Lincoln and Hill, 1980).

Microalgal cultures are more favourable because conventional treatment have the following disadvantages:

i. variable efficiency depending upon the nutrient to be removed
ii. costly to operate
iii. the chemical processes often lead to secondary pollution
iv. loss of valuable potential nutrients (inorganic N and P) (Guttertan and Todd, 1990; Phang, 1990).

Microalgae on the other hand, fully utilize natural resources such as nutrients besides producing oxygen, effective disinfection due to an increase in pH during photosynthesis and concentration of xenobiotics (de la Noue and De Pauw, 1988; Mara and Pearson, 1986).

In this situation the biomass is never simply microalgal biomass, but a mixture of algae, bacteria, zooplankton and debris known as `albazod'. Bacteria may amount to one quarter of the harvested biomass (Soeder 1983; Sasson, 1991). While the biomass is most useful as animal feed, it may be a source of valuable biochemical compounds like pigments and fatty acids. Table 2.4 summarizes various products obtainable from algal biomass.

TABLE 2.4 : VARIOUS COMMERCIAL PRODUCTS FROM ALGAL BIOMASS

REF	PRODUCT	SPECIES	NOTES
1.	Biofertilizer	blue green algae of soils (commonly	Cyanotech, USA markets a biofertilizer of 8 microalgae
		used in Thailand and Vietnam)	mixture
2.	Biomass from wastewater treatment		
	a. animal feed	*Spirulina* *Chaetoceros, Dunaliella, Isochrysis, Tetraselmis*	Siam Algae (Thailand) 1990 Cell System Ltd. (UK) produce Tetraselmis powder sold as Celcys Algal
	b. Aquaculture	*Isochrysis, Nitzschia, Nannochloropsis*	sold as "Aguaro" by Martek
		Haematococcus	high in astaxanthin sold as "Algaxan Red" by Microbio
3.	Pigments for food industry		
	a. B–carotene (xantophyll)	*Dunaliella*	Western Biotechnology (Australia) Microbio (US); Nature Betacarotene Technology (Israel)
	b. phycocyanin and phycoerythrin	*Spirullina*	phycocyanin produced by Danippon Ink & Chemical Inc. under the name Lina–blue–A
4.	Polyunsaturated fatty acids	*Botryococcus braunii*	
5.	Sulfonated polysaccharide	*Porphyridium aerogineum*	ressembles carrageenan oil recovery from underground sand formation
6.	Health food	*Chlorella*	Japan & Taiwan

References: 1. Sasson, 1991 2a. Shelef & Soeder, 1980 ; Richmond, 1986 ; 2a. Tanticharoen, 1990 et.al.; 2b. Sasson, 1991; Vonshak,1990 3a. Borowitzka, L., 1991; 3b. Vonshak,1990;4&5. Sasson 1991; 6. Kawaguchi, 1980; Soong 1980.

Although algae grown in wastewater do not produce high value products that have a wide range of use, the biomass has great potential as feed for aquaculture (Sandbark and Hepher, 1980; Beneman, 1992) and poultry (Lipstein and Hurwitz, 1980; Yennai *et al.*, 1980). Table 2.5 summarizes various waste grown algal systems.

2.3.3 High Rate Algal Pond (HRAP)

The HRAP was first proposed almost fourty years ago by Oswald and Gotaas in 1957. It consists of a meandering channel type pond (Shelef *et al.*, 1980) with its contents mixed by paddle wheels. Algal growth and oxygenation of wastewater is optimized in this system (Shelef *et al.*, 1980).

It is one of the most economical and practical way to cultivate algae while contributing oxygen for the biodegradation of organic matter (Shelef *et al.*, 1980, 1978; Goldman, 1979). The cost of the system is shared by both benefits in the treatment of waste (enhancing environmental quality) and production of protein rich feedstuff. The combination of high pH and high dissolved oxygen enhances ammonia outgassing and induces precipitation of calcium phosphate (Huntley *et.al.*, 1989). It also enhances disinfection and metal precipitation.

TABLE 2.5: REVIEW OF HRAP TREATMENT SYSTEMS

REF	SUBSTRATE/WASTE	COUNTRY	BIOMASS	SPECIES	RT AND NOTES
1.	municipal waste	Israel	44.2 $gm^{-2}d^{-1}$	*Scenedesmus*	
				Euglena *Chlorella*	–
2.	Domestic wastewater	Thailand	41.0 –72.3 mgl^{-1}	–	4 days
3.	Tapioca wastewater +$NaHCO_3$ + N_2 ferterlizer	Thailand	7 – 10 $gm^{-2}d^{-1}$	*Spirullina*	Harvested when OD was between 0.9 and 1.0
4.	Anaerobic digested piggery wastewater	Singapore	5.4 – 25 $gm^{-2}d^{-1}$	–	4 – 16 days
5.	Rubber Effleunt	Malaysia	5.7 – 18.1$gm^{-2}d^{-1}$	*Chlorella*	6 – 8 days
6.	Palm Oil Mill Effleunt	Malaysia	2.7 – 12.8$gm^{-2}d^{-1}$	*Chlorella*	–
7.	Cane Molasses	India	15 – 20$gm^{-2}d^{-1}$	*Scenedesmus*	–
7.	Human Urine	India	10 – 12$gm^{-2}d$	*Spirulina*	–
7.	Bone Meal	India	–	*Spirulina*	–
8.	Livestock Manure	USA	30$gm^{-2}d^{-1}$	*Chlorella*	recycling N, P and C
9.	Pig Slurry	Northern Ireland	7.7 – 32.9$gm^{-2}d^{-1}$	*Chlorella*	–

NOTE : RT = Retention Time

Ref: 1. Shelef et al., 1980; 2. Edwards et al., 1980; 3. Tanticharoen et al., 1991; 4. Lee et al.,1980; 5. Geetha et al., 1991; 6. Phang & Ong, 1988; 7. Venkataraman et al., 1980; 8.Lincoln & Hill (1980); 9.Fallowfield & Garret (1985)

Tertiary treatment involves removal of cultured algae. This involves processes which are either purely physical (sedimentation, centrifugation, sand filtration, flotation, microstaining, electroflocculation, ultrasonic treatment and vacum filtration), physico-chemical (precipitation, pH treatment, ion exchange, chemical flocculation and auto flocculation) or biological (bioflocculation and filtration by filter feeders).

Mixing in the HRAP helps the interchange of CO_2 and O_2 in the system. The paddle wheel mixing system also requires less energy and reduces the stress forces on the algal cells (Borowitzka and Borowitzka, 1989). The slow paddle wheel mixing (15 $cm^{-1}s^{-1}$) keeps the algal cells in suspension to maximize the light capturing and dissolved nutrient assimilation by both algal and bacterial cells. The slow mixing also creates turbulence in the algal culture. This facilitates light-dark regime fluctuation as well as increase in transfer rates between the growth medium (waste) and the cultured organism (Grobbellaar, 1993). The shallow depth of the culture (0.1 - 0.3 m) prevents light attenuation.

2.4 Mircoalgal biomass - commercial products

The first microalgae to be cultivated in large scale was *Chlorella*. The attempt was made in Germany in 1942

and in the 1950's in the United States at the Carnegie Institute of Washington (Burlew, 1953). Today the commercial production of microalgae is directed towards the health food market and involves mainly three species; *Chlorella, Spirulina* and *Dunaliella* (Vonshak, 1990).

In 1957, Japan was the first country to produce and sell *Chlorella* biomass as health food or as a water soluble extract called " *Chlorella* growth factor". Commercial production of *Chlorella* was also pioneered by the Taiwan *Chlorella* Company in the late 1960's and ten years later at least thirty different companies were involved in production and marketing of various *Chlorella* based products with a total production of 1000 tons per year (Sassoon, 1991; Kawaguchi, 1980; Soong, 1980). Acetic acid is used as the carbon source for algal production in Taiwan. *Chlorella* is available in the market in the form of powder or pill. It is directed towards the health food market.

Spirulina, a blue-green alga, grows in alkaline growth media (0.2M NaH_2CO_3) with the pH maintained at 9.5 to 10.3. These two parameters allow the mainteinance of monoalgal cultures under outdoor conditions in open raceway ponds (Ciferi, 1983; Richmond, 1980; Vonshak and Richmond, 1988). *Spirulina*, besides being marketed as health food in the form of powder or tablet, is also a pigment source in feeds of koi carp and other ornamental

fish (Borowitzka, 1991). Phycocyanin, a blue-green pigment also extracted from *Spirulina* is used as a food colourant or natural dye. In Thailand *Spirulina* is cultivated in wastewater from the tapioca starch factory. Up to 36 tons of dry, low grade *Spirulina* a year, is produced and marketed mainly as animal feed (Tanchitoren *et al.*, 1990).

Dry *Spirulina* biomass contains up to 2% of τ-linolenic acid (GLA) which can help cure arthritis, heart disease, obesity and zinc deficiency (Henrikson, 1989 cited in Sasson, 1991).

Spirulina has been shown to bring rapid recovery from malnutrition, especially in infants in Mexico, Romania and China (Sassoon, 1991). 10 to 15g per day as dietary supplement mixed with millet, water and spices or with baked barley was proven to be sufficient. In Ho Chi Minh city at the Tu-Du hospital, tablets of *Spirulina* sold as "Lactogil" is used to enhance secretion of milk in mothers experiencing natural decrease of lactation due to postpartum infection. This however did not alter milk composition at a daily dose of 0.2 - 0.4g of *Spirullina*.

Studies in Japan suggested that eating *Spirulina* can increase Lactobacilli in humans and may increase absorption of vitamin B_1 and other vitamins from the entire diet (Sassoon, 1991). Researchers in Germany and Japan have also shown lower cholesterol levels

upon consumption of 4.2g of *Spirulina* daily for 8 weeks.

Earthrise Farm in California, Cyanotech in Hawaii, Sasa Texcoco in Mexico, Siam Algae in Thailand, Nippon *Spirulina* in Japan and Blue Continent *Chlorella* in Taiwan are the various companies involved in large scale production of *Spirulina* (Vonshak, 1990).

Dunaliella salina a unicellular green algae is a source of ß-carotene. The algae is sold as natural biomass powder in tablets or mixed with vegetable oil (Borowitzka, 1994). ß-carotene, which is a provitamin A, is a natural antioxidant and has been reported to be effective against some cancers, cardiovascular and age related diseases (Borowitzka and Borowitzka, 1988). Up to 5% of the dry weight of *D. salina* can consist of ß-carotene under stress conditions.

Dunaliella salina is cultured in high salt concentration media (6-12% NaCl). A wide variety of systems are used for the cultivation ranging from concrete annular paddle-wheel stirred ponds with plastic lining in California (Microbio) and in Israel (Nature Beta Caratene Technology), to large open-air ponds mixed by wind in Whylla, South Africa (Betatene) and Australia (Western Biotechnology) (Borowitzka, 1991).

Soong (1980) suggested an integrated farming system whereby methane gas which is produced from anaerobic

digestion of hog manure can be used for generation of electricity. The digested manure is a suitable substrate for the production of algae through the high rate system to feed hogs, fish and shrimp. This will help double the income of the farmers.

2.5: Cultivation of algae in rubber effluent

Anaerobically digested rubber effluent is capable of supporting the growth of algae (Phang, 1987; Muthurajah *et al.*, 1973; Ahmad Ibrahim *et al.*, 1979). Muthurajah *et al.*(1973) observed that the algal population in facultative ponds used to treat RE was dominated by *Chlorella* species. The presence of algal was reponsible for the good reduction of pollution parameters - especially ammonia, in the final pond.

Chlorella was also successfully cultivated in RE in laboratory scale experiments (Kulkarni *et al.*, 1973; John, 1973; Ho, 1974).

The use of a high rate pond for the secondary treatment of latex concentrate effluent at the Rubber Research Institute of Malaysia(RRIM), produced good pollution removal rates (BOD = $0.72d^{-1}$; TN = $0.33d^{-1}$) (Nordin & Mohd. Zin , 1989). *Chlorella* species were dominant in the pond.

Geetha (1992) showed that *Chlorella* production of 92-360 mg DW L^{-1} with nutritional value of 37.0-55.0%

protein, 5-12.5% lipid 3.9-22.0% carbohydrates, and 0.71-2.42 mg/g carotenoids was possible using a HRAP system treating rubber effluent. In this way NH_4-N and PO_4-P in RE is recovered in the form of algal protein, suitable for animal feed. The HRAP also effectively generates photosynthetic oxygen for biodegradation of organic compounds while using less energy than the other aerobic treatment systems (Geetha, 1992). The HRAP system achieved 94.0% to 98.6% reductions in COD, 71.5% to 96.6% in NH_4^+ and 75% to 94.6% in PO_4^{3-}. The average time taken to reach maximum algal biomass ranged from 6 to 8 days. It was also observed that lower paddle-wheel speed of 20 rpm with the paddle wheel blade immersed at 0.1m below the culture surface, produced better algal yield and good reductions in the main polluting parameters. Geetha also observed that the *Chlorella vulgaris* used had heterotrophic ability.

Due to the low C:N:P ratio of RE (7:2:1) as compared to waste grown algae (50:8:1), 0.05% mollasses was supplemented as a carbon source to increase algal biomass production (Chui, 1994). Maximum biomass concentration of 1010 to 1940 mg DW L^{-1} was obtained when RE was supplemented with 0.05% molasses daily as compared to the control (RE alone; 420 to 805 mg DW L^{-1}) (Chui,1993). It was found that the best results were obtained when mollasses was supplemented in the evening, under dim light and low dissolved oxygen concentrations. Sugar

uptake may have been inhibited by the high irradiance. Another possibility is that bacterial assimilation of the organic substance was limited in the low dissolved oxygen conditions in the evening giving better opportunity to algal cells to assimilate the organic substances under low light conditions.

The increase in production also resulted in better reductions in NH_4^+ (39.0% to 89.1%) and PO_4^{3-} (64.0% to 83.2%) as compared to the control. Nevertheless addition of molasses resulted in lower COD removal in rubber effluent (17.8% to 77.3%) compared with that of the controls (78.1% to 90.3%).

Chlorella vulgaris also demonstrated a mixotrophic as well as heterotrophic growth pattern under laboratory conditions. The biomass obtained under mixotrophic (733mg dry weight L^{-1}) and heterotrophic (738mg dry weight L^{-1}) growth were much higher than autotrophic cultures (45mg dry weight L^{-1}).

Tables 2.6 and 2.7 summarise experiments carried out at the Institute of Advanced Studies.

TABLE 2.6: **Summary of initial experimental runs carried out at the Institute of Advanced Studies, University of Malaya (Geetha, 1992)**

BATCH	1	2	3	4	5	6	7
Operating Conditions							
Culture Depth	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Paddle–wheel speed	40	40	40	40	20	40	20
Paddle Immersion Depth	0.05	0.05	0.05	0.1	0.1	0.1	0.1
Culture Flowrate	25	25	25	22	17	22	17
Biomass Production							
Max. Algal Biomass (mg/l)	124.6	255.9	91.8	101.8	152.1	260.6	360.5
Estimated Gross Productivity ($gm^{-2}d^{-1}$)	4.15	6.21	2.3	3.39	5.07	6.51	12.02
Pollution Reduction (%)							
COD	98.6	98.2	84	92.2	93	91.2	95
NH_3–N	74.2	93.1	96.6	71.5	80.7	85.7	75.4
PO_4–P	81.5	79.9	94.6	83.2	85.9	81.5	77.1

TABLE 2.7: Summary of experimental runs caried out at the Institute of Advanced Studies, with molasses supplementation (Chui, 1994)

Batch	Molasses supplementation time	Maximum Algal concentration mgDWL−1	% Reduction of Pollutants		
			COD	NH_3−N	PO_4−P
IM	0.05% at 1300h and 1830h daily	1460.0	–	58.4	75.2
IIR	Control	85.6	90.3	41.4	63.0
IIM	0.05% at 1200h daily	242.5	41.1	52.8	64.7
IIIR	Control	100.6	93.1	44.7	74.2
IIIM	0.1% at 1200h alternate day	155.0	77.3	39.0	72.7
IVR	Control	52.5	93.8	62.6	47.3
IVM	0.05% at 1900h daily	126.3	17.8	86.4	83.2
VR	Control	408.0	78.1	48.6	41.3
VM	0.05% at 1900 daily	1385.0	17.8	89.1	79.8

2.6: Photosynthetic CO_2 fixation

All free energy consumed by biological systems arise from solar energy that is trapped by the process of photosynthesis. The basic requirement for photosysnthesis is water, light and CO_2, as illustrated in the following equation:

$$2H_2O + CO_2 \xrightarrow{\text{light}} (CH_2O) + O_2 + \text{chemical energy}$$

(Stryer, 1988)

Photosynthesis however can be separated into light reactions and dark reactions. The light reactions generate nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) while the dark reactions use energy rich molecules to reduce CO_2. CO_2 is known to reach algal cells through diffusion. However in algal photosynthesis there is an ongoing debate as to which carbon species is utilized.

CO_2 and HCO_3^- are the two species commonly utilized in algal photosynthesis. Miyachi *et al.* (1983) showed that 1.5% CO_2 grown cells (high CO_2 cells) utilized CO_2 while cells grown in 0.04% (low CO_2 cells) utilized HCO_3^- besides CO_2. However in 1979 Miyachi and Shiraiwa showed that both high CO_2 (2% CO_2) grown *Chlorella* cells and low CO_2 (0.04% CO_2) grown cells utilized CO_2 and were unable to utilize bicarbonate. This in accordance with the

findings of Lehman (1978) that CO_2 alone is the substrate used during carbon fixation and HCO_3^- is a vehicle for the transport of inorganic carbon into the cell. Beardall (1981) found that in *Chlorella saccharophila* cells, CO_2 is the species crossing the plasmalemma and accumulation of inorganic carbon is due to HCO_3^- and active transport of HCO_3^- at the chloroplast. Enzymic catalysis of conversion between HCO_3^- and CO_2 is also an important part of carbon fixation.

Melvin Calvin and his colleagues in 1945 determined the pathway by which CO_2 becomes fixed into carbohydrate, using the unicellular green alga *Chlorella*. Their findings proved to be pertinent in a wide variety of organisms from photosynthetic bacteria to higher plants. The pathway that they determined using radioactive compounds is called the Calvin Benson cycle (Fig.2.2) or the autotrophic CO_2 fixation pathway. Fig 2.3 summarizes the photosynthetic reactions involved during CO_2 fixation.

The rate at which algal cells fix CO_2 is conditioned by external and internal factors (Raven, 1974). External factors includes:

1. inorganic carbon supply and pH
2. the intensity and wavelength of light
3. oxygen level
4. organic and inorganic nutrient supply

The internal factors depend on:

1. algal species
2. the stage in the cell cycle for rapidly dividing cells
3. the circadian rhythms for slowly dividing cells.

CO_2 fixation is influenced by internal and external factors through the amount of enzyme present, the control of the activity of enzyme and the control of the supply of the substrate, that is CO_2, ATP and $NADPH_2$.

The influence of external pH on internal pH and photosynthesis was investigated by Lane and Burris (1981). They found that in *Euglena mutabilis* the internal pH fluctuated with changes in external pH while *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* managed to maintain their internal pH at 7.0 even at low external pH as they were acid tolerant species. However maintainace of internal pH was thought to be energy intensive. The level of external pH had no efect on photosynthesis.

Two enzymes that are important in photosynthesis are Ribulose biphosphate carboxylase (Rubisco) and carbonic anhydrase.

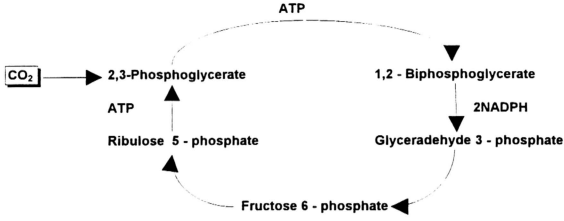

Fig 2.2: The Calvin Cycle

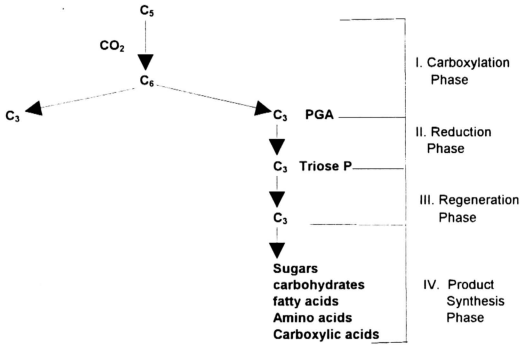

FIG 2.3 : Summary of reactions of photosynthetic CO_2 fixation
Hall & Rao, 1993

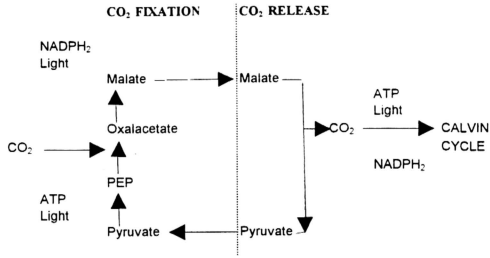

FIG 2.4 : C_4 - CO_2 Fixation

2.6.1: Role of carbonic anhydrase (CA) in algal photosynthesis

CA plays a key role in photosynthetic CO_2 fixation. The function of CA is in three modes:

(a) to convert HCO_3^- to free CO_2 for fixation by Rubisco

(b) to convert CO_2 to HCO_2^- for fixation by Phosphoenylpyruvate carboxylase and

(c) to provide rapid equilibration between CO_2 and HCO_3^- to enhance diffusion of CO_2 (Badger & Price, 1994).

In microalgae and cyanobacteria CA is required to convert the accumulated pool of HCO_3^- to CO_2. CA is also known to

play an active role in CO_2 fixation and as a carbon dioxide concentrating mechanism (CCM) which is known to exist in certain algal species. Transport of CO_2 from outside the algal cells to the site of Rubisco is accelerated by CA (Tsuzuki *et al.*, 1981; Hogetsu & Miyachi, 1977).

There are two forms of CA in the algae - the CA in the periplasmic space and the internal or intracellular CA in the chloroplast and pyrenoid (Badger & Price, 1994) and cytosol (Palmqvist *et al.*, 1994).

The role of periplasmic CA is to improve the efficiency of the uptake of C_i(inorganic carbon) by the cells when grown at low concentrations of C_i while the C_i on the chloroplast envelope delivers HCO_3^- to the chloroplast stroma and the CA converts HCO_3^- to CO_2. CA activity was not detected in chloroplast of *Chlorella ellipsoidea* but there was an active uptake of HCO_3^- (Rotatore & Colman, 1990). CA however is not found in the pyrenoid of all algae (Badger & Price, 1994). *Porphyridium cruentum* and *Phaeodactylum tricormutum* do not express CA in their pyrenoids while *Chlamydomonas reinhardtii* does. However microalgae without pyrenoids for example *Cocomyxa* are known to lack a carbon dioxide concentrating mechanism (CCM) and may be more like C_3 higher plants with both CA and Rubisco distributed in the stroma.

At low CO_2 levels the periplasmic CA activity increases

to very high levels while cytosolic and chloroplast CA activity increases 5 - 20 folds. Therefore external HCO_3^- is used efficiently while the low internal CA activity allows accumulation of high CO_2 around Rubisco.

Hogetsu and Miyachi (1979) found that CA activity in cells of *Chlorella vulgaris* exposed to low CO_2 is 20 to 90 times higher than at high CO_2 levels. There is however no difference in Rubisco activity indicating that CA participates in photosynthetic carbon fixation in low CO_2 grown cells. Similar results were also reported (Palmqvist *et al.*, 1994) in *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. However in the presence of 5.1mM and 0.55mM $NaHCO_3$ at pH 8.0 (20°C), CA addition enhances photosynthetic rate under high CO_2 levels (Miyachi and Shiraiwa, 1979).

2.6.2: The Carbon Dioxide Concentrating Mechanism (CCM)

The CCM represents a means by which the overall rate of photosynthesis is increased as a result of the direct effect of CO_2 concentration and carboxylation rate of Rubisco (Badger*et al.*, 1980). The CCM is enhanced by CA (Badger *et al.*, 1980) or an active uptake mechanism (Rotatore & Colman,1992). In *Chlamydomonas reinhardtii* cells grown under low CO_2 levels were able to concentrate CO_2 up to 40 fold in relation to external medium. The mechanism was energy dependent and linked to

phosphorylation and photophosphorylation (Badger *et al.*, 1980). CA in the CCM system acts as an transporter of CO_2 located in the chloroplast membrane providing HCO_3^- externally and reconverting it back to CO_2 at the chloroplast.

However Shelp and Calvin (1980a) found that air (0.03% CO_2) adapted *Chlorella* fix carbon via the Calvin cycle and their lack of photorespiration is achieved by a CCM through utilization of HCO_3^-.

Microalgae with pyrenoids like *Cocomyxa* PA are known to lack carbon dioxide concentrating mechanism and may be more like C_3 higher plants with both CA and Rubisco distributed in the stroma (Badger & Price, 1994).

Table 2.8 gives a general overview of the interelationships between CA, CCM and species of carbon consumed in the photosynthetic process of different algal species.

TABLE 2.8: GENERAL OVERVIEW OF PHOTOSYNTHETIC PROCESS ADAPTED BY ALGAE

Ref.	Source of inorganic carbon entering cells	Species of carbon & entry mechanism	Species of carbon consumed	Role of CA	CCM	Species
a.	HCO_3^- (active transport) CO_2		CO_2	NA	present	*Chlorella emersonii*
b. c.	CO_2 HCO_3^- (indirect)	CO_2	CO_2	conversion of HCO_3^- to CO_2 in chloroplast and cytosolic membrane – high activity in low CO_2 cells	increase of CO_2 in cell	*Chlorella vulgaris*
d.	CO_2 HCO_3^- (through conversion by CA)	CO_2 active transport	CO_2	rapid supply of HCO_3^- in cell and reconvert to CO_2 in chloroplast	present – 40 fold high concentration	*Chlamydomonas reinhardtii*
e.					external CA – increase of CO_2 inside cell	*Chlorella saccharopphilla*
f.	CO_2	CO_2	–	NA	present – at chloroplast with active transport of HCO_3^-	*Chlorella saccharophila*

Ref: a. Beardall & Raven (1981); b. Miyachi and Shiraiwa (1979); c. Hogetsu & Miyachi (1979); d. Badger et al. (1980); e. Williams & Colman (1995); f. Beardall (1981)

2.6.3: CO_2 Fixation Modes

There are 2 types of CO_2 fixation modes called the C_3 and the C_4 fixation modes (Fig 2.2, 2.3 and 2.4).

There are four main phases in CO_2 fixation (Fig 2.3). The first phase is the carboxylation phase whereby CO_2 is added to the 5 carbon sugar RuBP to form two molecules of PGA. This reaction is catalyzed by ribulose biphosphate carboxylase (RuBisCo). The reduction phase converts PGA to the same energy level as sugar (Triose-P). The third phase which is the regeneration phase is where RuBP is regenerated further for CO_2 fixation through a series of reactions - Calvin cycle (Fig. 2.3). The final phase is the product synthesis phase where mainly sugars, carbohydrates but also fats, fatty acids, amino acids and organic acids are to be synthesized during photosynthetic CO_2 fixation.

Beardall in 1989 reviewed and concluded that C4 metabolism was the most probable in marine algae. C4 metabolism, also known as the Kortschak Hatch-Slack pathway, is also common among tropical grasses and plants. It is a mechanism which fixes CO_2 more efficiently (Fig 2.4). Here the CO_2 which diffuses in is fixed with phosphoenol pyruvate (PEP) to form oxaloacetate which has a greater affinity for CO_2 compared to RuBP.

2.6.4: Photorespiration - loss of CO_2

Photorespiration is the light stimulated release of CO_2. This happens because the enzyme (RuBisCo) that is responsible for CO_2 fixation in C_3 metabolism can also bind with O_2. Thus both CO_2 and O_2 compete for RuBP at the same catalytic site. High concentrations of CO_2 favour carboxylation and at low concentration of CO_2 and high O_2, oxygenation is favoured resulting in formation of phosphoglycollic acid.

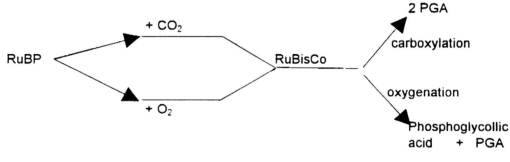

FIG 2.5: Dual Role of RuBisCo

Photorespiration involves 3 organelles in the cell, namely the chloroplast, peroxisome and mitochondria.

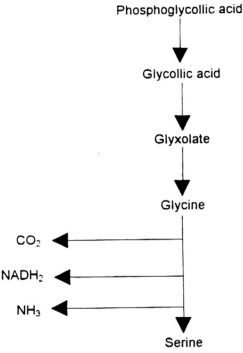

FIG 2.6 : Fate of Phosphoglycollic acid

In *Chlorella pyrenoidosa* an increase in O_2 concentration inhibited photosynthesis and the inhibition was independent of CO_2 concentration (Shelp and Canvin, 1980b). However in the algae, oxygenase activity may be supressed *in vivo* through active uptake of HCO_3^- ions. Thus in algae, sensitivity of photosynthesis to O_2 is overcome by high internal CO_2 concentrations.

Besides photorespiration, CO_2 is also lost during dark respiration in the mitochondria (Hall and Rao,1993) and also dark type light respiration (Bidwell, 1977). Dark type light respiration of *Chlorella pyrenoidosa*,

Chlamydomonas reinhardtii and *Anabaena flos-aquae*) is affected by CO_2 concentrations and rapidly declines to zero in the absence of CO_2.

2.7: Heterotrophy and Mixotrophy in algae - Utilization of organic carbon and inorganic carbon

There are many nutritional pathways in algae. Autotrophs such as already described, refer to microalgae which obtain energy from light and utilize inorganic carbon source like carbon dioxide to build cell carbon. Microalgae that utilize organic carbon are termed heterotrophs while mixotrophic microalgae obtain energy from light and utilize both organic and inorganic carbon.

In our laboratory *Chlorella vulgaris* 001 isolated from Palm Oil Mill Effluent (POME) exhibits heterotrophic and mixotrophic growth when supplemented with 0.5% glucose, sodium acetate, acetic acid and molasses (Chui, 1994). Although there was no significant difference between the mixotrophic and heterotrophic growth, however, the growth rate of the cultures supplemented with organic substrates were higher compared to the control (inorganic medium) (Chui, 1993).

Heterotrophic and mixotrophic growth was also observed by Martinez *et al.* (1987) in algae isolated from sugar refinery wastewater. Shamala *et al.* (1982a) reported that mixotrophic growth rate exceeded autotrophic and

hetetrophic growth rates in *Scenedesmus acutus*. With another strain of *Chlorella vulgaris* the autotrophic and heterotrophic growth rates were 0.073 day^{-1} and 0.209 day^{-1} respectively. Their sum equalled the mixotrophic growth rate of 0.281 day^{-1}.

Shamala *et al.* (1982a) also reported increased yield and growth rate under both indoor and outdoor mixotrophic conditions when CO_2 was supplemented to the cultures during the day and molasses during the evening. Here the presence of sugar increased photosynthetic oxygen evolution indicating enhanced photosynthetic metabolism and also heterotrophic metabolism. However, Ogawo and Aiba (1981) observed decreased photosynthetic activity in *S. acutus* in the presence of glucose.

Chlorella cells grown heterotrophically form giant cells and are partly discoloured compared to normal cells (RodrigurezLopez, 1966; Phang, 1979). This is attributed to high concentrations of starch which disorganize the chloroplasts and cause the pigments to be distributed in a larger cell volume. *Chlorella vulgaris* (Orus *et al.*, 1992) *Scenedesmus acutus* (1982a) and *Tetraselmis suicida* (Cid *et al.*, 1992) have increased carbohydrate content when grown heterotrophically or mixotrophically. Cid *et al.* (1992) also reported an increase of 22-25 times of cellular protein in *T. suicida* when grown in media consisting of yeast, glucose and peptone compared to

autotrophic cells. Mixotrophic *Scenedesmus* cultures supplemented with CO_2 and molasses had both increased growth rate and protein content (Shamala *et al.*, 1982a).

In high-rate algal pond cultures used to treat wastewaters, mixotrophy in algae would help to enhance the treatment process with organic carbon being directly assimilated and thus recovered by the algae. Since mixotrophy involves utilization of both organic and inorganic carbon sources, supplementation of both these carbon sources in the form of CO_2 and molasses would further enhance algal production in the HRAP.

2.8 : Potential Uses of Microalgae

Microalgae, besides being used as an alternate food source, health food, for wastewater treatment and aquaculture, are useful as producers of fine chemicals, fertilizers and also part of life support systems in space flights and underwater explorations (Hartig *et al.*, 1988). Of these, high valued products from algal biomass is favoured as this would justify the high cost of producing algal biomass (Borrowitzka, 1992). Extraction of fine chemical like astaxanthin, phycocyanin and canthaxanthin which fetch from US$500 kg^{-1} to US$3000 kg^{-1} would help cover costs.

Microalgae are important as aquaculture feed. Algae rich in essential fatty acids or eico-pentaenoic acid

(EPA) and docohexanaenoic acid (DHA) are currently being grown by Cell System Ltd. in the United Kingdom and Martek in the United States (Vonshak, 1990). Martek also produces *Isochrysis, Nitzschia* and *Nanochloropsis* under the brand name "Aguora". "Algaxa Red" is also another feed which contains high astaxanthin produced by Microbio in the United States (Bubrick, 1991 cited in Vonshak, 1990).

An interesting development of the enclosed system is the immobilized algal system. Although currently, immobilization technology seems to be too expensive to allow large scale operations, it however is a good alternative in waste treatment. Using *Scenedesmus obligus*, Chevalier and de la Noüe showed that removal of NH_4^+ and PO_4^{3-} proceeded with similar kinetics in both free and immobilized cells under both batch and semicontinous mode of operation (cited in Huntley *et. al.*).

A possible interesting application of algal culture in the future is to sustain human life in long periods of space flight. Here oxygen is obtained by feeding CO_2 and human wastes to algal cultures (Shelef *et al.*, 1978). It is estimated that 100L of continous culture is needed to sustain one man (Chapman and Gellenbeck, 1989, 1985).