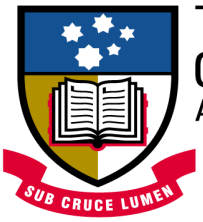

THE UNIVERSITY
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Microbial Flocculation for Large Scale Harvesting of Marine Microalgae for The Production of Biodiesel

A thesis in fulfilment of the requirements for
the degree of Master of Engineering Science

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Summary

This research project aims to develop a large scale harvesting process suitable for the production of biodiesel from the marine microalga *Pleurochrysis carterae*. The ideal process required the consideration of factors such as cost, reliability and low levels of contamination in the final product. However, a review of harvesting literatures revealed that there to be no suitable commercial technique available for the production of biodiesel and laboratory experiment showed bioflocculation by pH stress is ineffective.

Microbial flocculation is based on the principle that by stressing certain bacteria, extracellular polymeric substances (EPS) may be produced to co-flocculated the microalgae. With a dosage of 0.1 g L^{-1} of organic carbon (acetic acid, glucose or glycerine) and a 24 h mixing time, a recovery efficiency (RE) of over 90% and a concentration factor (CF) of 226 was achieved. Statistical analysis showed that both RE and CF were independent of the substrates used and that RE was positively correlated with mixing time, while CF was correlated positively to the mixing time but negatively to the interaction of substrate concentration and mixing time.

The harvested microalgae were not under stress and remained viable, with laboratory result showing that the media could be reused without further treatment. The process was observed to be reliable.

Modelling from an existing wastewater treatment plant in Bolivar showed that by incorporating 2 clarifiers and 1 baffled hydraulic flocculator in the plant design, industrial scale harvesting was feasible with a theoretical energy consumption of 2.6 kWh per 10^4 m^3 of culture media. Raw materials were the major cost, however, they could be potentially low-cost as glycerine is a by-product of biodiesel production and acetic acid is one of the major products of anaerobic digestion of the biomass residue after lipid extraction.

Further experiments are required to optimise the mixing time and the scale up.

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1 Introduction

During the last century, world anthropogenic energy consumption increased 18 fold from 25 EJ year $^{-1}$ to 450 EJ year $^{-1}$ (Demirbas 2007b), with the majority coming from fossil fuels such as crude oil, coal or natural gas. Two of the major concerns are;

1. The increase in atmospheric CO_2 concentration and its possible link to climate change;
2. The depletion of the petroleum crude reserve and the security of its future supply.

Biofuels such as biodiesel or ethanol are potentially CO_2 neutral and may offer a solution to these two concerns. Furthermore, their advantages such as portability and high energy density are essential in the transport fuels of which the majority are still fossil fuel based. Some microalgae, with their high lipid yield, appear to be attractive choices for the production of biodiesel. Much research has been conducted on the microalgal cultivation, growth conditions and selection (Sheehan 1998; Song 2008), however, their harvesting remains to be one of the most difficult areas in the production (Benemann 1996). Some of the essential criteria for the harvesting for the production of biodeisel are; a low processing cost in order to compete with other plantation crops, a low level of contamination by trivalent metals in the biomass and the process reliability.

This research project aims to develop a harvesting method that is able to satisfy the above mentioned criteria. The project is summarised as follows: A literature review on microalgal harvesting is conducted to identify the harvesting technique that is suitable for the production of biodiesel. If such a procedure is available, then further details such as recovery and cost will be investigated. Otherwise, some other methods from related industries will be considered.

The project is presented in the flow chart as shown in Figure 1.1.

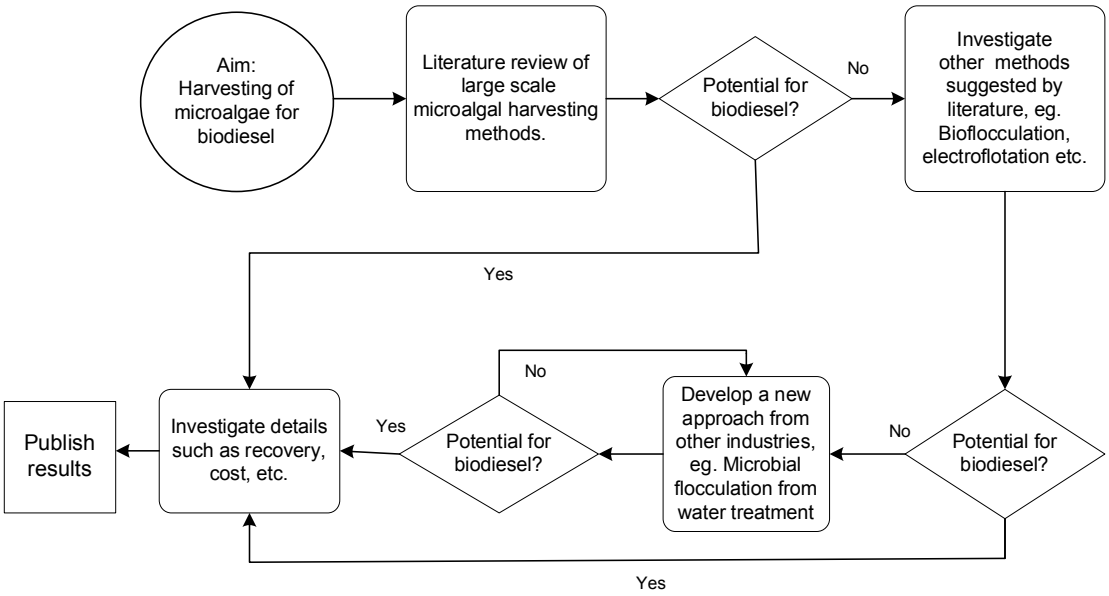

Figure 1-1 Flow Chart for this research project

This chapter provides the background information on the importance of renewable fuels, the advantages of biodiesel over other alternatives and the use of microalgae as a feedstock for the production of biodiesel.

1.1 CO_2 and climate change

Atmospheric CO_2 concentration has been steadily increasing since the beginning of the Industrial Revolution (284 ppm in 1832) to the present (384 ppm in 2007) (Geider 2001; ESRL 2008). The major cause is the burning of fossil fuels while deforestation also plays a minor role (Geist 2002). The increase has been linked to the observed climate change and its consequences, e.g. global warming, destabilised weather patterns, increased frequency of severe weather events, rising sea levels, changes in ocean current flows (for example; The Gulf Stream) and so on. The impact on the economy from climate change has been brought into focus by the Stern Review "The Economics of Climate Change" (Stern 2006) and the Intergovernmental Panel on Climate Change "AR4 Synthesis Report" (IPCC 2007). These two reports evaluate the cause and effects of climate change as well as identify the current atmospheric CO_2 level of above 450 ppm CO_2-e (equivalent) as a dangerously high level. These reports recommend a 1% expenditure of the GDP to be spent on green house gas remediation to avert a potential 20% reduction in GDP. Perhaps the more serious environmental issue is the possibility of a runaway greenhouse effect. This is where

the rate of climate change increases independently of human activities. This may occur, for example, as a result of accelerating methane release from seawater (Torre Jorgenson 2001) or the thawing of the permafrost (Waelbroeck 1997).

1.2 Crude oil reserve and transport fuel

The global energy market can broadly be divided into the power generation and transport fuel sectors, both of which will have to achieve significant emission reduction targets. Currently in USA, the electricity generation accounts for around one third of its energy demand and has developed a range of low CO_2 emission approaches, for example; solar, wind, nuclear, geothermal and hydroelectric. (The list does not include "clean coal technologies" such as carbon capture and storage (CCS), or Integrated Gasification Combined Cycle (IGCC) as they are either different forms of CO_2 sequestration or a more efficient use of coal). Many of these technologies are commercially viable and are increasing their share in the energy market. However, transport fuel, which accounts for about 30% of all fossil fuel consumption (Energy Information Administration 2008) is far less developed (Figure 1.2).

Source: Energy Information Administration, Annual Energy Review 2008

Figure 1-2 Energy consumption by sector from 1949 – 2007

The majority of transportation fuels are derived from crude oil with approximately 90 million barrels being consumed per day. A much lower amount is coming from alternative sources, for example, liquefied petroleum gas (LPG) or compressed natural gas (CNG) and even less is obtained from renewable sources such as biodiesel (Section 1.4) or ethanol (Demirbas 2007a). Together, the diminishing reserve of the world's crude oil and the increase in atmospheric carbon dioxide make the development of CO_2 neutral transport fuel one of the most urgent challenges facing humanity.

1.3 Developments in alternative transport fuel

Transport fuel is one of the major contributors to the increase in atmospheric CO_2, however, most of the low CO_2 emission transport technologies are still in various stages of development. Examples of alternative transport fuels are hydrogen, fuel cells, nuclear, hybrid, different types of biofuel or even "plug-in" batteries, but their potentials are limited by some common problems such as the high cost of developing a new motor, the lack of fuel storage and distribution infrastructures, the high cost of replacing existing technologies and the low energy densities of the carriers; Some of the problems are more specific, for example, fuel cells are expensive, have high operating temperatures and a limited membrane life. Electric plug-in cars have low range of mobility and bulky batteries; they require long recharging times and have limited rechargeable lives. Hybrid cars have similar battery problems and are generally more expensive as they require both an internal combustion engines and an electric motor. Biofuels have feedstocks which are low in photosynthetic conversion efficiencies and competes with food production. These disadvantages may out weigh their advantages of low CO_2 emission or improved fuel economy. Table 1.1 is a summary of various alternative transport fuels. Hydrogen and electricity are essentially carriers of energy as their commercial productions are mainly fossil fuel based. Hence, the rest of this chapter will focus on biofuels and in particular, biodiesels.

1.4 Renewable biofuels and their possible future directions

Photosynthetic higher plants currently produce the majority of the commercially available biofuels, for example; ethanol from starch or sugar, biodiesel from oil

Table 1-1 Advantages and disadvantages with various alternative transport fuels.

Alternative Fuel	Advantages	Disadvantages
Biodiesels (Fatty acid methyl esters, FAME)	• Have higher energy densities than other alternative fuels. • Can be incorporated into existing petroleum diesel storage and distribution network. • Do not require engine modification. • Have low S emission (Bomb 2006).	• Compete with food crops for available arable land, fresh water and other resources. • The production from available farmlands cannot satisfy demand. • High cost of feed stock when compared with petroleum crude. • Have higher cloud/pour point which affect their low temperature operations.
Ethanol	• Feedstock (sugar) is cheaper than plantation oil. • By-products from sugar ethanol are recyclable (Hahn-Hägerdal 2006). • E10 does not require engine modification.	• Corn starch ethanol has a high negative energy balance (Schmidt 2007). • Fuel system modifications will be required for E85. • Suitable only for flexi fuel vehicles (FFV). • Starch and sugar production cannot meet energy demand.
Hydrogen Fuel cells (Song 2002)	• Hydrogen may have zero net carbon emission depending on the source of production. • Fuel cells have better electrical conversion efficiency than internal combustion engine. • Energy required for hydrogen supply from solar, geothermal or wind is virtually unlimited (Hankamer 2007).	• Fuel cells are bulky, have high operating temperature, require long start up time and cell membranes have limited life, all these factors result in high cost. • Hydrogen can only be considered as a carrier of energy and its net carbon emission depends on the production methods. • Hydrogen gas is expensive to produce, difficult to compress, has low energy density and has few distribution networks. (Keith 2003)
Electric / Plug in batteries	• Can be carbon emission free depending on the source of electricity. • Energy can be conserved during braking. • Distribution network readily available.	• Batteries have low energy / mass ratio, bulky, long recharging time and limited rechargeable life. • Have low range of mobility when compared with liquid fuel.

Hybrid	• Improved fuel economy, hence lower carbon emission. • The technology is available commercially and is still being improved in terms of energy management and battery performance. • Provides an alternative transition to fully electric.	• Hybrid vehicles are more expensive as they require both electric motors and internal combustion engines. • Batteries are bulky, heavy and have limited life. • Fuel consumption is similar to turbodiesel engines which have comparable fuel economy but without the need for bulky batteries.
Syngas and GTL by Fischer-Tropsch synthesis	• Coal reserve is more abundant than oil, and syngas from coal can be sustained for a much longer period than crude oil. • Technology has been proven.	• Heat energy of syngas is less than natural gas due to presence of CO_2. • Syngas from coal did not reduce atmospheric CO_2. • Separation of pure CO from nitrogen is energy intensive, making the Fischer-Tropsch process expensive.
Compressed natural gas (CNG), liquefied natural gas, (LNG), liquidfied petroleum gas (LPG), methane.	• Have lower carbon emission than coal or crude oil distillate for the same energy output. • LPG has established distribution net work, but only limited distribution for CNG and LNG. • CH_4 can be produced by sustainable crop rotation and anaerobic digestion. (Amon 2007).	• These fuels are of fossil origin and they are not renewable. • CNG and LNG require high pressure storage and distribution. The new infrastructures are costly to construct and maintain. • Methane from anaerobic digesters is produced together with CO_2 and therefore has a lower overall heating value.

(canola, palm, tallow or soy), biodiesels from methyl or ethyl esters of vegetable oils, while lignin or cellulosic ethanol are still under research; they are all potentially important as a replacement for the world's diminishing oil reserve and as a carbon neutral option for atmospheric CO_2 remediation as most of the carbon is derived from the atmosphere. However, the production of fertilisers, farm machinery and transport etc. all require additional energy and hence the emission of CO_2. It was estimated that relative to the fossil fuel they replaced, corn starch ethanol has a net CO_2 reduction of 12% while that of the biodiesel is 41 % (Hill 2006). However, this estimation does not take into the account that some of the tropical forests are being cleared for the plantation of fuel crops, reducing their CO_2 absorption capacity (Schubert 2006).

Comparing with other alternative transport fuels such as hydrogen, fuel cells, ethanol, or electricity, biodiesel has the advantage of high energy density and can be mixed in any proportion with petroleum diesel and therefore, be able to distributed in existing infrastructure. Furthermore, biodiesel does not require new motors or modifications of the existing fuel systems; these are additional advantages over ethanol (E85) which can only be used by flexible fuel vehicles (FFV). For all these reasons, biodiesel has the best potential to be accepted by the market.

Biodiesel production from vegetable oils is a proven technology and is widely available in Europe and the United States of America (Ahmann 2007). However, traditional agricultural crops can only utilize less than 1% of the solar energy that falls on earth's surface; furthermore, only a small part of the plant is available as oil. These two factors make land plants very inefficient as a source of oil supply (Geider 2001). Other feedstock for biodiesel such as waste vegetable oil or animal fat are only available in limited amount and the diversion of food crops and various resources for the production of biofuels is a major concern (Kleiner 2007).

Currently approximately 8 % of the plant based oil is used for biodiesel production which supplies about 0.3 % of the current transport fuel consumption (BP 2008). Therefore, even dedicating all the oil productions to biodiesel would only meet fewer than 4 % of the diesel demand. Currently, biodiesel is mainly produced from canola, oil palm and soy bean, of which palm has the highest yield among the higher plants. But as shown in Table 1.2, even if all the biodiesel is sourced from oil palm, it will

still require over 40 % of the world's available arable land to meet the transport energy demand. This competition for farmland is one of the reasons to force up food prices and the clearing of forests (Kleiner 2007).

Table 1-2 Areas required for the biodiesel production from different crops

Plant source	Biodiesel production (L ha^{-1} y^{-1})	Area to produce global oil demand (ha x 10^6)	Area required as % of global land mass	Area required as % of global arable land
Cotton	325	15,002	100.7	756.9
Soybean	446	10,932	73.4	551.6
Mustard seed	572	8,524	57.2	430.1
Sunflower	952	5,121	34.4	258.4
Canola	1,190	4,097	27.5	206.7
Jatropha	1,892	2,577	17.3	130
Oil palm	5,950	819	5.5	41.3
P. carterae	12,000	406	2.7	20.5

Although renewable biofuels have great potential to provide a carbon neutral way to produce transport fuel, the present production system has been criticised with regard to their economic viability and carbon mitigation potential (Patzek 2004; Palmer 2007). Some species of microalgae, with their high growth rates and high lipid contents, appear to be attractive alternatives as a feedstock for biodiesel production (Chisti 2007). In particular, the marine microalga *Pleurochrysis carterae*, with a doubling time of 2.5 days and the ability to grow in arid areas not suitable for farming, offers good potential to address these concerns. The use of these marine microalgae as a feedstock for the production of biodiesel will be discussed in the next section.

1.5 Coccolithophorid as a feedstock for biodiesel

The microalgae coccolithophorid are a group of unicellular marine planktons with calcium carbonate platelets. In this research, the coccolithophorid *Pleurochrysis carterae* (CCMP 647) was obtained from the Murdoch University, Western Australia.

This species was first collected by Christensen from Salton Sea Salt Lake, California, USA (33.30N 115.8W). The microalga has a high lipid content of 33 % and therefore has a good potential to be a feedstock for the production of biodiesel. It also has a $CaCO_3$ content of 10 % (Moheimani 2006) which can be used as a more permanent form of carbon dioxide sequestration.

P. carterae has been shown to have a good pH tolerance of 7-11, this property enables the microalga to sustain itself as a monoculture over 13 months in an outdoor open pond (Moheimani & Borowitzka 2006). It had been found that during photosynthesis, CO_2 in the culture media had been taken up by the microalgae, increasing the pH to a maximum of 10.9 in summer and 9.9 in autumn. This high pH discouraged the growth of many other undesirable algal and biological species. In fact, the failure to reach a high pH indicated a collapse of the algal culture. Furthermore, the laboratory grown culture had a maximum photosynthetic rate at 25 ^{o}C while the outdoor culture had a maximum at 32 ^{o}C; showing this microalga had a good adaptation to grow in a wide range of temperatures. Finally, the microalga's marine origin means this species does not require fresh water of which the supply has been an issue in many parts of Australia

1.6 Commercial culturing system

The two main microalgae culturing systems are the open air high rate algal pond (HRAP) and the enclosed photobioreactors.

High rate algal ponds

The majority of microalgae cultivated commercially are grown in open ponds. These systems are more economical but are only suitable if the algal species can be maintained as a monoculture. The most commonly used design is the raceway pond; an area is divided into a long oval rectangular grid; a paddle wheel is used to move water around the circuit. Pond depths are usually of 15–20 cm. At these depths, a maximum daily biomass concentrations of 1 g dry weight per litre and productivities of 60–80 mg L^{-1} day^{-1} (10–25 g dry wt m^{-2} day^{-1}) are possible (Pulz 2001). Other configurations include inclined ponds and circular ponds with rotating aeration arms.

The main disadvantages of open systems are the loss of water by evaporation to the atmosphere and the susceptibility to contamination by unwanted species. From the Aquatic Species Program where a collection of over 3,000 photosynthetic organisms had been studied, none were found to be able to consistently dominate in open ponds and have high lipid contents (Sheehan 1998).

Enclosed photobioreactors

Most enclosed photobioreactors are designed as continuous stirred tank reactors, bags, tubular, plate, or bubble (air lift) column reactors (Pulz 2001). Enclosed photobioreactors can maximise light capturing efficiencies and avoid photo-bleaching (Melis 1999) while increasing the volume productivities, reduce water evaporation loss and minimise the risks of contamination. However, they are expensive to construct and difficult to maintain due to the large surface areas of the reactors with respect to their volume. Using such systems up to 47 g dry wt m^{-2} d^{-1} can be obtained (Carlozzi 2003). Higher capital costs are therefore to some extent compensated for by higher productivity (Chisti 2007).

Microalgae and some of their metabolites

High valued algal products such as astaxanthin from *Haematococcus pluvialis* Flotow NIES-144 (National Institute for environmental Studies, Tsukuba, Japan) has been produced commercially (Hata 2001) but the economical production of high volume, low valued commodities such as biodiesel still require much research (Li 2007a). Some of the desirable metabolites can be increased by manipulating certain environmental conditions, e.g. lipid content in *Botryococcus braunii* (collected by Singh from Varanasi, India) may be increased by nitrogen limitation (Singh 1992) or by heterotrophic feeding (Li 2007a) while sulphur limitation will increase H_2 production in *Chlamydomonas reinhardtii* C137 (mt^+) (Melis 2000). On the other hand the heterotrophic addition of glucose to *Chlorella pyrenoidosa* Sorokin UTEX 1663 will increase the dry mass from 0.5 g L^{-1} to 100 g L^{-1} under aerobic fermentation (Running 1994). However, the change of these environmental conditions can only be justified on economic basis.

Contaminants and extremophiles

It is difficult to maintain a contaminant free culture in an open system. Even in an enclosed system, the building up of cell debris in reactor walls and corners will attract bacteria and fungus. Therefore, maintaining a monoalgal culture is one of the major challenges (Becker 1995).

Some algal species with extreme properties (extremophiles) can be cultivated in open ponds systems and compete with other species in a particular environment (e.g. high pH or salinity) so as to maintain as a monoculture. For example, *Spirulina* survives and grows well at high pH (9 to 11) and is often grown in soda lakes (Belkin 1991) while *Cyanidium caldarium* can grow at a pH of 1.5 (De Luca 1979; Pulz 2004). Another example is *Dunaliella salina,* commercially grown in Western Australia, this unicellular microalga is well adapted to highly salinity due to its high intracellular glycerol content (Borowitzka 1984).

Therefore by selecting high oil bearing algal strains with extreme properties, an essentially monoculture of microalgae can be maintained in open ponds; however, the separation of the biomass from the media may pose a few problems. They will be discussed in the next chapter on harvesting of microalage.

2 Microalgal harvesting techniques

Commercially available solid/liquid separation techniques include filtration, centrifugation and flocculation, they are used either individually or in combination with each other. However, the harvesting of microalgae presents certain problems, they are:

- Most microalgae are under 20 μm in diameter (except filamentous species such as *Spirulina sp*), and cells are often compressible, these two factors make filtration energy consuming and ineffective.
- Microalgae exist in very dilute solutions, their dry mass concentration is usually between 200-600 mg L^{-1}, also the cells have high water content, and thus a large amount of water needs to be removed for a small amount of algal mass.
- The cell surfaces are negatively charged to keep the microalgae in suspension, making settling difficult. This, together with the high dilution, makes flocculation difficult without addition of large amount of chemicals.
- The cell densities are similar to water, together with their small sizes, these characteristics make centrifugation inefficient (Becker 1995; Benemann 1996).
- For high volume and low valued products such as biodiesel, it is important to utilise the biomass residue after lipid extraction, for example, as animal feed or co-firing in power generation. The presence of inorganic flocculants will preclude such use.

Drying by thermal energy is expensive and many processes such as waste water treatment or lipid extraction may not require dry products, therefore, a suitable level of dewatering will be determined to economise the extraction of lipid (Molina Grima 2003). According to Benemann (p. 90, PETC Final Report) an algal paste of 10-20 % solid would be sufficient for lipid extraction, alcohol fermentation or further dewatering. As different processes are required for different range of dewatering, an economical harvesting system will be a combination of different techniques.

The economy of microalgae production, to a considerable extent, depends on the economy of the harvesting technology required (Mohn 1988). Many techniques have been developed and some are applied on a commercial scale but none are economical enough to produce algal oil to compete with plantation oil. Production of microalgal

biomass is estimated to be A$ 20 kg $^{-1}$ in 2008 (Borowitzka 1992)[1] while some other plantation based oil (palm, canola) are available at around A$1 (CCC 2008). Allowing a minimum of 20~30 % of total production cost for harvesting (Molina Grima 2003), we have a target of around A$0.30 kg^{-1} for the harvesting. According to Benemann, harvesting is one of the most difficult areas in mass cultivation of microalgae (Benemann 1996),

As microalgae are physiologically and morphologically vastly different, successful economical harvesting techniques are likely to be species specific and large amount of innovations and research is required before algal oil can be harvested economically to be used as feedstock for biodiesel.

2.1 Filtration

The technical difficulties facing the large scale filtration are small sizes of most of the microalgae, compressibility of cells and gelatinous extracellular materials. As a result, cells will usually be trapped among the pores and extra pressure will cause them to squeeze through as cells are compressible. Precoatings are sometimes used to increase efficiencies but products will be contaminated with the precoat materials. Flocculants may be used to increase the flux (Al-Malack 1996), but advantages will be offset by the additional cost of chemicals and the risks of metallic contamination in the products.

The clogging of filters requires high pressure and frequent backwashing. In general, filtration is only suitable for filamentous algae such as *Spirulina sp* or large colonies such as *Micractinium sp*.

Many filtration methods have been developed, for example, belts, drums, press, gravity, vacuum, tangential flow (TFF) etc with a variety of filter materials or precoatings to increase filtration efficiency and minimise clogging. With the advances in membrane technology and the decreasing cost, small scale harvesting may find membrane filtration a suitable choice (Molina Grima 2003), especially in conjunction with TFF (Petrussevski 1994) but will not be recommended for larger operations (>

[1] Average Australian CPI 1992 is 107.7 and March CPI 2007 is 155.6. Average exchange rate is US$0.80 to one Australia dollar.

20 m^3 day^{-1}) where centrifugation may be more economical (Benemann 1996). However, for the production of biodiesel, filtration by itself is still not cost competitive. The NREL Aquatic Species Program considered the overall harvestability with microstrainer to be poor and further research on the topic was abandoned (Sheehan 1998).

2.2 Centrifugation

Centrifugation is a standard technology and the equipments are available off the shelf. A high degree of dewatering (10-20 % solid) can be obtained, the processes are reliable and the products are free from added flocculants. Capital and operating costs are determined by the particle sizes, the density difference between the medium and the particles, and the concentrations required (Mohn 1988; Becker 1995). Disadvantages are the high capital cost, high maintenance, and high-energy requirement; also, algal cells are easily damaged by the high shear forces resulting in the loss of desirable cell inclusions. However, the damages varied substantially even among closely related species (Heasman 2000). Heavy modifications of machinery may be required for individual species of microalgae, for example, in commercial harvesting of *Dunaliella*, the high salt content caused extensive corrosion and sealed types of different centrifuges were used (Mohn 1988; Becker 1995).

Many different types of centrifuges are available, for example; chambers, nozzles, tubular bowl, plate separators, perforated basket and decanters. However, due to high operating costs, they cannot be the primary means of separation for low-value commodities such as biodiesel; for example, a 76 cm commercially available centrifuge would require 3500 kWh to separate one tonne of algae from 200 mg L^{-1} suspension at a rate of 1000 L min^{-1} (Oswald 1988).

As no flocculant is required, centrifugation is the preferred microalgae harvesting method for marine hatcheries but the high cost exclude it as the choice for biodiesel production without incorporating other methods.

2.3 Flocculation –Sedimentation, flotation and bioflocculation

Flocculation produces larger particles, making sedimentation and flotation more efficient (Kim 2001). Successful separations often use flocculation as the primary

stage in combination with other methods such as centrifugation or filtration to increase the overall efficiencies. Therefore it is important to discuss flocculation in detail, as both bioflocculation and microbial flocculation are further extensions of this topic.

Mechanism

Microalgal cell surfaces are negatively charged to keep the small cells in suspension, these charges can be neutralised by the following changes (Molina Grima 2003);

- Addition of inorganic cations (eg. Fe^{3+}, Al^{3+}) either in simple or polymerised form.
- Increase in solution pH (for negatively charged particles).
- Increase in ionic strength of the solution when inorganic ions are used (this is not applicable for cationic polymers).
- Under stress by unfavourable conditions such as light or nutritional deficiencies (Lee 1998).
- Addition of organic cationic polymers such as Chitosan (Kim 2006).
- Increase in cell density (Lavoie 1987).

Various combinations of flocculation, sedimentation or flotation have been used extensively in biotechnological processes such as water treatment, food and fermentation processes. The efficiency of the overall process depends on pH, dosage and the algal culture. Costs are generally high. It was estimated to be US$ 1.39 kg^{-1} dry wt in 1994 by using simple inorganic ions for harvesting fresh water microalgae, and the cost for marine microalgae can be considerably higher. (Becker 1995)

Inorganic flocculation

Inorganic ions such as (Fe^{3+}, Al^{3+}) are effective for algal removal. The efficiency depends on pH, dosage and the characteristics of the algal culture. Costs are high as large amount of chemicals are required for small amount of biomass collected and the products collected are contaminated by the ions, therefore they are not suitable for use as animal feed or fuel extraction without further processing at additional cost.

The mechanism of cationic induced flocculation can be explained by the classical DLVO Theory or the Divalent Cation Bridging (DCB) theory; DLVO Theory suggests that the stability of a particle in solution is dependent on the total force F_T which is a balance of the attractive van der Waals force F_A, the repulsive double layer force F_R and the force from the solvent F_S, that is;

$$F_T = F_A + F_R + F_S$$

Where F_A and F_R are the main contributors and operate over a much longer distance, however, F_T is always less than F_G , the gravitational force (Chang 2005). Figure 2.1 shows the relationship between different forces F (force in N) and H (ratio of the distance between approaching particles to the diameter of approaching particles).

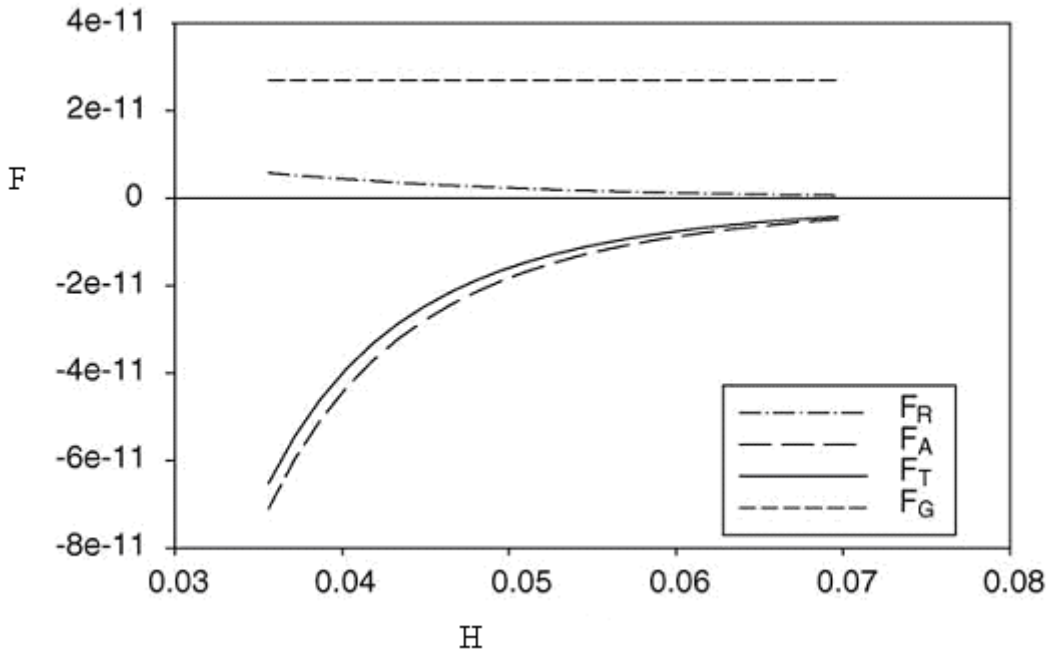

Figure 2-1 Forces predicted by DLVO Theory and gravity

This theory is applicable when the ionic strength is under 0.1 M (Zita 1994), above which flocs strength decreases with increasing ionic strength and cannot be explained by the compression of the double layer (Sobeck 2002). This theory also assumes perfectly smooth surfaces (Czarnecki 1987), whereas in microalgal harvesting, biologically active irregular bacterial and microalgal surfaces are involved (Hermansson 1999).

Tezuka explained the flocculation result by the DCB theory (Tezuka 1969) while other flocculating mechanisms were also proposed; for example, during active growth phase, microalgal cell surfaces are negatively charged to keep the cells in suspension, but as the cells mature and grow in size, their surface charge densities decrease and the cells settle (Becker 1995).

Inorganic cations are effective in solution with an ionic strength up to 5 g L^{-1} , but the high salt content (32 g L^{-1}) and the relatively high pH of around 8.2 of the sea water makes flocculation inefficient (Bilanovic 1988). Comparing with fresh water, the amount of chemicals required will be around 10 fold, making the process uneconomical (Sukenik 1988). Furthermore, the contamination of metallic ions make the microalgae collected unsuitable for a range of purposes such as food or fuel (Mohn 1988; Benemann 1996).

Dissolved air flotation

Flotation is more efficient than sedimentation for the separation for microalgae as algal cells can be made to float faster than they can settle. Tiny air bubbles of sizes under 150 μm can be generated by air supersaturation, electrolysis, photosynthesis or effervescences (Oswald 1988). The air bubbles then carry the flocs to the surface to be skimmed off. A higher solid mass of 7 % can be collected and the cells are not damaged. Flocculants are usually added to increase efficiencies. This process is effective in treating water from large open reservoirs as cells remain intact and will not impart undesirable taste (Crossley 2006)

Other variations of this process include dissolved air flocculation flotation (DAFF), countercurent (CoCoDAFF), high rate DAF, and other proprietary modifications. These processes are well established in USA, Australia, Europe and other advanced countries (Crossley 2006). However, the plants are designed for water treatment rather than microalgae harvesting, the energy required is very high when compared with the biomass collected and inorganic flocculants are still required for good separation (Sim 1988).

Autoflocculation and flotation

During daytime, the photosynthetic activities from microalgae consume carbon dioxide and raise the pH of the media to 8.5 or more, the high pH results in the precipitation of the phosphates of Ca and Mg. The microalgae are then co-flocculated. If the water is supersaturated with the oxygen produced from photosynthesis, then the gas bubbles will lift the flocs to the surface and be skimmed off. However, the process is sensitive to weather and many operational parameters (for example; flow rate, pond depth and hydrodynamics), therefore, it is not very reliable. Furthermore, it has not been studied with respect to a marine environment (Al-Shayji 1994).

When autoflocculation and flotation were used in conjunction with chemical flocculation, removal rates of 80-90 % were achieved with microalgae concentrated to over 6 % and a chemical cost of US \$0.18 kg^{-1} dry wt (Koopman 1983). The low cost may be promising but further research will be needed for the harvesting of marine algae as autoflocculation is unreliable and flotation may require energy for air compression.

Electroflocculation / flotation

Electrolysis is used to generate small gas bubbles for the flotation, the cost is high as the conductivity of fresh water is very low and a huge amount of electricity is required to split the water molecules. It is cheaper to use compressed air or photosynthesis (Oswald 1988).

Ultrasound flocculation

Standing acoustic waves of high frequencies can push suspended microalgae cells to the wave nodes, forming flocs that will settle when the ultrasounds are turned off. No chemicals are involved and cells are not subjected to shear (Bosma 2003). However power consumptions are high at 4-8 W for a flow rate of 2 - 18 L day^{-1}, furthermore, the achieved concentration factor of 20 is considered to be too low for practical purposes.

2.4 Other harvesting methods

Phototactic

Motile microalgae tend to move toward light of a certain frequency. In the laboratory, this phenomenon has been used to concentrate microalgae. In practice, a suspension of microalgae of economical concentration will block off most of the light, and only the thin layer near the light source will be attracted. The advantages are that the cells would not be subjected to shear and no chemicals are required (Oswald 1988).

Milking

Some algal metabolite can be harvested by cultivating microalgae briefly in a 2-phase photobioreactor. For example, β-carotene can be collected from a system of *Dunaliella salina* and hexane. The microalgae are continuously harvested and no new culture is added. No chemicals are required but the relation between the solvent, the extraction mechanisms and the microalgae are not properly understood. Therefore this method is only limited to a few examples (Hejazi 2004).

2.5 Summary and knowledge gap

Microalgae may be harvested commercially using centrifugation, filtration, and flocculation, either used individually or in combination. For the commercial production of biodiesel, the major requirements are the low cost, reliability of the process and the low contamination by trivalent metals of the biomass products. However, centrifugation requires high capital, high energy and running costs and is therefore only suitable for high-value products; Filtration is only suitable for filamentous or colony forming microalgae such as *Spirulina sp*. or *Micractinium sp*; Flocculation using multivalent metal salts will contaminate the algal biomass while flocculation by cationic polymers is inhibited by the high ionic strength of sea water and high cost. Therefore, currently, none of the existing large scale harvesting methods is suitable for the production of biodiesel.

An alternative is bioflocculation, and as no chemicals are required during the process, some authors suggested it may offer a cost effective and non contaminating technique for the harvesting of microalgae (Lavoie 1987; Benemann 1996).

The feasibility of bioflocculation as a microalgae harvesting technique will be discussed in the next chapter.

3 Bioflocculation

3.1 Introduction

Microalgae cells have small sizes and do not exhibit sufficient settling rate to allow economical recovery by gravity sedimentation. However, when they are under stress by some unfavourable environmental conditions, such as nutrient deficiency, low light intensities, or extreme pH, microalgae are known to flocculate. Bioflocculation refers to the spontaneous agglomeration of microbial cells under such conditions (Benemann 1996). Bioflocculation can occur in the steady or decline phase of the growth cycle, however, some authors include the addition of organic flocculants such as Chitosan (Morales 1985; Lubián 1989) in the definition of bioflocculation (Salehizadeh 2001) even though there is no distinct relationship between the flocculant and the microalgae taxonomic group (Liu 1999). For economic and practical reasons, processes involving addition of flocculants will not be discussed here.

One of the attachment mechanisms can be explained by the cell surface hydrophobicity (CSH) theory (Zita 1997). CSH can be measured by the liquid/solid intersurface contact angle but is more related to the hydrogen bonding energy of cohesion (Van Oss 1995). This thermodynamic approach accounts for the direction of the reaction but not the exact mechanism.

The proposed investigation will attempt to induce stress by unfavourable environmental factors and then determine the extent of flocculation with respect to these changes. Major factors are nutrient level, pH, light, temperature (Lindstrom 1984), salinity, mixing and carbon dioxide. Only the unfavourable environmental changes will be discussed in this chapter. Some of the considerations are the cost, reliability, the efficiency of the processes, product contaminations and the post treatment required for the harvested medium.

One of the potential advantages of bioflocculation for microalgae harvesting is the minimal use of inorganic flocculants; therefore the biomass is not contaminated and the process may have low capital and operating costs. However, bioflocculation has mainly been observed as a natural phenomenon, and as such, is neither controllable

nor reliable. Furthermore, the separation characteristic of the process may vary from species to species and the conditions for the flocculation are not fully understood.

3.2 Stress factors for bioflocculation

Nutrients

Major nutrients affecting growth of microalgae are nitrogen, phosphorus and carbon, with their optimum ranges varying from species to species (Richmond 2004a). Apart from cell growth, cell compositions also change according to the limitations of certain particular nutrients, for example, N limitation increases lipid content in some microalgae e.g. *Botryococcus braunii* (Borowitzka 1988), while P limitation in the presence of UV light decreases growth rates and chlorophyll concentration (Shelly 2005).

Nutrient limitation may stress the cells resulting in flocculation, for example, phosphorus depletion would result in higher exopolymer production in diatom *Cylindrotheca closterium* (isolated from Adriatic Sea) during the transition and stationary phase of growth (Alcoverro 2000). Also nitrogen limitation due to excess CO_2 metabolism would result in flocculation (Benemann 1996). Levels of limitation of one nutrient may depend on level of another nutrient, for example, levels of N or P limit will depend on the ratio of N : P (Molina 1991). Other trace elements (Zn, Fe, Mn etc) are also critical in growth, but since they are required in trace quantities only, limitations are unlikely to result in flocculation (Brand 1983). Also, microalgae have adapted to very low nutrient level, for example, in the ocean surface water, nitrogen is often below detectable level (Goldman 1979) and nitrogen level in the medium may not be correlated to the nitrogen status in the cells (Flynn 1990). Hence, it may be very complicated to reduce nutrients to a level low enough to induce stress on micoralgae, however, as later shown in Chapter 4, the nutrient stress can be used to produce extracellular polymeric substances (EPS) (Bossier 1996; Li 2006) from bacteria to co-flocculate microalgae.

Extreme Temperatures

For *P. carterae* within the optimal temperature range (18~26 ^{o}C), the coccolith production is temperature dependent, but 2-4 ^{o}C above this range would result in the

loss of culture (Moheimani 2006). This is especially critical for enclosed photobioreactors located under the sun and evaporative cooling is not possible (Richmond 1987; Zhang 1999). However, there seems to be no literature correlating the effects on temperature to bioflocculation in microalgae. In spirulina, low temperature together with higher solubility of oxygen induce photoinhibition and photoxidation, the reduced growth as a result of such conditions is an indication of stress (Vonshak 2004).

The change in temperature of an outdoor system will require a large amount of thermal energy entering or exiting the system. This may not be cost effective for the harvesting of microalgae in a commercial system.

Light intensity

The effective use of strong light increases CO_2 metabolism (Perner-Nochta 2007), raises pH and produces hydroxide ions which induce flocculation (Day 1999). Reduction in the light intensity can be achieved by covering the ponds, but flocculation may occur only after a number of days when the cells are in the decline phase, during which the cell lipid compositions may be changed and cells become dormant, thus affecting the yield and limiting the effectiveness (Harrison 1990; Becker 1995).

Extreme pH

A salt tolerant microalga, *Dunaliella tertiolecta* (ATCC30929) has been observed to flocculate in the pH range of 8.5 to 10.5 without the addition of flocculant (Horiuchi 2003); At pH of 11.8 and 12, extensive flocculation would occur without the addition of electrolyte (Molina Grima 2004). Some microalgae such as *Skeletonema costatum* (CS-252), *Tetraselmis pseudonana* (CS-173) can be flocculated at the pH of 10.2 with the addition of 0.5 mg L^{-1} of the flocculent Magnafloc (Knuckey 2006). The response time is in the order of minutes to tens of minutes. This may offer an alternative to the flocculation by inorganic trivalent metal ions. However, the mechanism(s) for the pH flocculation on live microalgae have not been provided by the above mentioned literature, although the rates of such responses suggested the mechanism may be physical such as charge neutralisation rather than physiological such as stress or the production of polymers.

At the lower end of the pH scale, Salehizadeh reported maximum flocculating activity of *Bacillus sp. AS 101* was at a pH of 3.7 (Salehizadeh 2000). However this is the result of a purified flocculant acting on inorganic kaolin particles; the results may not be applicable to living surfaces such as those from microalgae.

The recovery efficiencies of the harvesting process given in the above literature is in the order of 80 ~ 90 % but their concentration factors were not reported. From the result available (Horiuchi 2003), the concentration factor is estimated to be between 7 to 10. This is not very high as the typical value for the centrifugation is in the order of 100. The low value shows that while the recovery may be comparable to other processes, it may not be very effective in concentrating the products in the effluent stream.

During photosynthesis, microalgae increase water temperature by converting light into heat and metabolizing CO_2 to increase the pH, resulting in bioflocculation (Day 1999). However, in the outdoor culturing of *P. carterae*, the pH can reach as high as 11 without reports of sedimentation (Moheimani 2006). As the flocculation of *P. carterae* under extreme pH has not been studied, it is necessary to carry out some experiments to investigate the pH induced bioflocculation.

High Salinity

Salinity is essential for optimising the cell growth (Cho 2007) while high salinity induces osmotic stress, inhibits photosynthesis and decreases growth rate, but in some cases increases lipid content (Takagi 2006), or doubling the production of β-carotene (Ranga Rao 2007). After the initial decline, some of the activities may be restored (Kirst 1989). The upper and lower salinity limits on growth depend on the species and the availability of certain ions. In general, the more optimum other parameters (light, nutrient, temperature) are, the broader the range of salinity tolerance. In fact, many microalgae show remarkable salinity tolerance, for example, *Porphyra umbilicalis* survive in a salinity 6 times that of sea water (Kirst 1989). Flocculation by salinity can be induced by adding common salt, but the culture medium after harvest cannot be reused unless it is diluted with fresh water, which is a valuable resource in many parts of the world. Therefore, flocculation by salinity is not very practical.

Carbon dioxide

CO_2 is an important source of nutrient for autotrophic algae and is usually a limiting factor for growth under favourable conditions. During photosynthesis, the reduction in CO_2 concentration in the media increases pH, converting more CO_2 to HCO_3^- and increase the stress level.

$$CO_2 + H_2O \xleftarrow{pH6.5} HCO_3^- + H^+ \xleftarrow{pH9.5} CO_3^{2-} + 2H^+$$

As the plant cells require organic carbon from the fixation of atmospheric CO_2, various mechanisms have been developed to utilise the HCO_3^- instead of CO_2 (Huertas 2000).

Culture media is deficient in CO_2 in relation to their N or P content. The CO_2 enrichment will increase overall biomass production which place further demand on the N and P available. The depletion of N in the media will then act as a stress factor for the microalgae, resulting in bioflocculation. Benemann suggested CO_2 enrichment together with N limitation would result in flocculation (Benemann 2003) but again this is an indirect form of stress by nutrient depletion and still has the problem with reliability.

Hydrodynamics

Mixing is necessary in all photo-bioreactors as it make efficient use of the light/dark cycle (Horton 2004). These cycles should be in frequencies of 10 Hz or faster with the dark period being up to ten times longer than the light period (Janssen 2001), The algal cells then behave similarly to being under constant moderate light exposure (Yoshimoto 2005). Proper mixing also prevents sedimentation of the cells (Barbosa 2003) and supports an even distribution of CO_2, O_2 and other nutrients (Molina Grima 1999; Eriksen 2007).

Excessive mixing will cause structural damage such as detachments of the flagella and therefore behavioural changes (Richmond 2004b). In severe cases, cells may be damaged by bulk shear (Gudin 1991). However, the stress induced by excessive mixing will probably not result in flocculation due to the breaking up of flocs by the mechanical shear.

3.3 Experimental evaluation of pH bioflocculation

Among the various environmental stresses factor, pH is the easiest to manipulate, the quickest to respond and the flocculation has been demonstrated by some species of microalgae. Therefore, pH was chosen as the controlling factor for this experiment in bioflocculation.

Material and Methods

Pleurochrysis carterae (CCMP 647) was obtained from the Murdoch University Microalgae Culture Collection. The microalgae were grown at 22 ^{o}C on a shake table in a culture tube under a light intensity of 300 µmol photons m^{-2} s^{-1}. The growth medium was BG-11 (Rippka 1979) modified by the addition of NaCl 18 g L^{-1} as shown in Tables 3.1 and 3.2. The medium was first autoclaved at 120 ^{o}C for 30 minutes and the vitamins were added after cooling. The pH was adjusted to approximately 8.2 by the addition of sodium bicarbonate prior to the inoculation of the culture. The contents were later introduced to a 250 mL Erlenmeyer flask on a shake table and finally to a 20 L carboy photobioreactor. Mixing was provided by sparging air from the bottom of the carboy; lighting was supplied by six cool-white fluorescent tubes with an intensity 400 µmol photons m^{-2} s^{-1} with a 12/12 h light/dark cycle. Cultures near the end of log phase growth were used for the flocculating experiments.

Table 3-1 Recipe for BG-11 culture medium

Ingredient for basic medium	Concentration (g L^{-1} d H_2O)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium magnesium salt)	0.001
Na_2CO_3	0.02
Trace metal mix A5	1.0 ml
Agar (if needed)	10.0
Distilled water	Make up to 1.0 L

Table 3-2 Recipe for trace metal mix A5

Ingredient for trace metal mix A5	Concentration (g L^{-1} d H_2O)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$NaMoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	Make up to 1.0 L

All glassware, air tubes, polycarbonate containers, rubber stoppers were washed and scrubbed in mild detergent, then rinsed and soaked in 0.1 % sodium hypochlorite over night, rinsed again in deionised water and autoclaved at 120 ^{o}C before being used.

Bioflocculation of microalgae by pH stress

400 ml of the mature *P. carterae* culture sample were obtained from the photobioreactor and mixed thoroughly. The initial cell counts were recorded. The samples were then divided into 2 separate aliquots, one of 300 ml (experiment) and the other one of 100 ml (control). The pH in the experimental aliquot was adjusted to the desired level by using either 0.1 M NaOH or HCL while that of control remained unchanged. Four identical stoppered 100 ml measuring cylinders were filled exactly to the 100 ml mark, three cylinders were filled by pH adjusted culture (experimental) and one by the unadjusted media (control). All cylinders were mixed thoroughly before they were left to settle under gravity for exactly 30 min in a dark environment to prevent interference by photosynthesis. Temperature was kept constant throughout the experiment. At the end of 30 min, a clear layer of supernatant was formed at the top of the culture media; the heights of this clear column were recorded and compared with the control. Cell counts in the supernatant were recorded.

3.4 Results and discussions on bioflocculation

The settling rates for the marine microalgae *P. carterae* under various pH were fairly similar. There were also no appreciable differences between the experimental and the control samples. Table 3.3 indicates column heights of supernatant during 30 min of

settling under different pH with initial cell counts 91.5 x 10^{4} ml^{-1} and initial supernatant column height 0 mm.

Table 3-3 Column heights after 30 min of settling under different pH

pH	Height of Supernatant (mm)		Average cell counts in supernatant (x 10^{4} ml^{-1})	Remarks
	Experiment	Control		
12.52	4.5	4.5	10	Cell lyses
12.02	4	4.5	8	Cell lyses
11.5	4.5	4	15	
11.01	4.5	4	7	
9.97	4.5	4.5	9.5	
8.94	4	4.5	11	
8.2	4.5	4.5	7.5	
6.45	4	4	22	
5.08	4.5	4.5	7	
3.46	4.5	4.5	12	
2.64	4.5	4	25	Cell lyses
2.01	4	4.5	3.5	Cell lyses

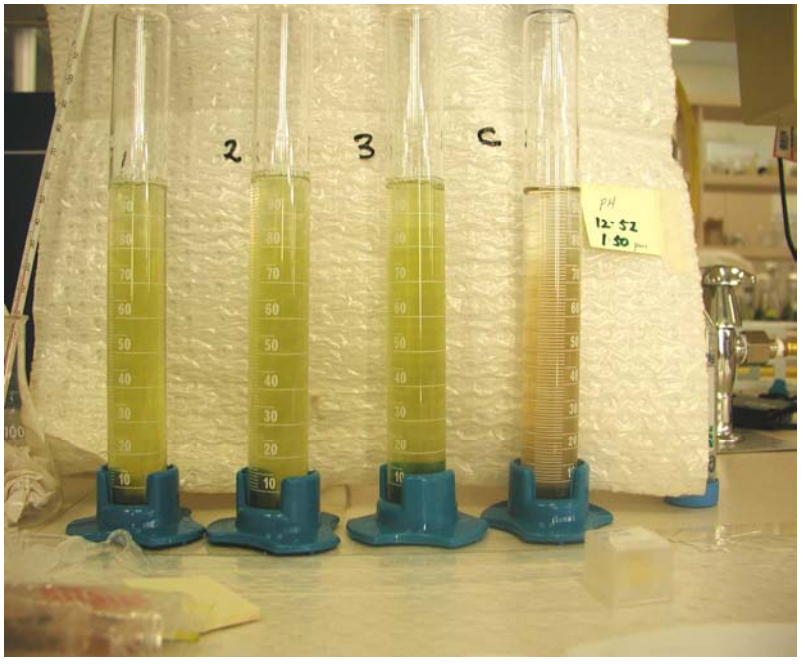

Figure 3-1 Experimental set up with *P. carterae* under a pH of 12.5.

At the extreme pH of 2.01 and 12.5, the culture media changed from brown to green after 30 min, while the control remained the normal brown colour (Figure 3.1). Under

optical microscope, moderate algal cells lyses were observed and the conditions were expected to deteriorate as time elapsed. This shows that bioflocculation of *P. carterae* by pH is not effective as a technique for harvesting.

P. carterae can survive in a medium of daily high pH of 11 and it would require 400 g (US$1.60) of NaOH to raise one m^3 of media from a pH of 11 to 12. This is uneconomical as a harvesting method for biodiesel as the dry mass concentration of the microalgae is in the order of 500 g m^{-3}. Furthermore, the treatment or disposal of a large amount of culture medium at such a high pH may impose further environmental and cost problem, thus, bioflocculation of *P. carterae* by extreme pH is not very effective.

Although Benemann (1996) considered bioflocculation might offer a low cost alternative to microalgal harvesting, the process is not reliable and ineffective for *P. carterae;* the costs associated with the environmental changes are often too high to be recovered by the biomass harvested; the time for the response from other environmental stresses may take too long. Furthermore, cell compositions and the desired metabolites may also change as a result of the stress. Therefore, harvesting by extreme pH is only suitable for a few isolated algal species and is neither low cost nor effective for *P. carterae.*

Microbial flocculation involves the production of extracellular polymeric substances (EPS) from bacteria and no addition of inorganic ions is required. Hence the process may be considered as a special case of bioflocculation. This process has not been studied as a harvesting technique for microalgae. The next chapter will investigate the feasibility of microbial flocculation for such a purpose.

4 Microbial flocculation- Background and methods

4.1 Introduction

Many micro-organisms produce extracellular polymeric substances (EPS), they are made up of proteins, polysaccharides, nucleic acids and lipids. These bioflocculants form flocs by attaching onto the colloidal particle surfaces to form bridges. Some functional groups from the EPS also neutralise the surface charge density, reducing the repulsive force (Salehizadeh 2001). The effectiveness of the bridging mechanism depends on the molecular weight of the EPS, the charge between the polymer and the particle, the ionic strength of suspension, and the nature of mixing (Salehizadeh 2001). The success of this process depends on the ability of the bacteria to induce flocculation. However, the attachment of EPS onto the living surfaces of microorganisms in a complex growth media will be far more complicated (Weir 1993).

Microbial flocculation has been well researched in wastewater treatment (Al-Shahwani 1986; Noüe 1992) and fermentation (Esser 1983; Hantula 1991). This type of flocculation may be the preferred option for the primary stage of harvesting as it is potentially low cost and is free from trivalent metal contaminations. It has not been investigated as a harvesting technique for marine microalgae as their environmental conditions are vastly different. For example, wastewater is usually high in dissolved organic carbon (DOC) and bacteria, but low in salinity; while the *P. carterae* culture media is relatively low in DOC and bacteria but is high in salinity. These differences may render microbial flocculation as in wastewater treatment not applicable for the marine microalgae harvesting.

Some supporting evidences are found in other literature: for example, it has been reported that EPS produced by microalgae are indistinguishable from those produced by bacteria (Shipin 1999), and that these polymers are responsible for the adhesion of cells (Frølund 1996). Furthermore, microalgae embedded in the polymer matrix did not show signs of stress or lysis over an extended period of 20 days (Shipin 1999). Although the exact composition of the polymers may vary according to the species (Higgins 1997; Choi 1998) or extraction methods (Comte 2006), the fact that all

bacterial and microalgal surfaces are negatively charged with similar polysaccharides (Becker 1995; Liao 2002) suggests that the same flocculants may be used to aggregate microalgae.

Experiments performed by Higgins showed that deflocculation occurred when proteolytic enzymes were added, indicating that the polymers were at least partly protein. Furthermore, electrophoresis indicated only one single protein was involved. However, the effectiveness of this bounded protein decreases in the presence of high concentration of Na^+ (Higgins 1997). This may be a problem as in some countries such as Australia, where fresh water supply is in short supply and marine algae are preferred; the high ionic strength and salinity of sea water (32 g L^{-1}) often markedly change the organic polyelectrolytes' structure and reduce its effectiveness (Bilanovic 1988). Furthermore, in order to minimize contaminations by other organisms, extremophiles such as *P. carterae* and *D. salina* are chosen for their unusually high pH or salinity adaptations. These extreme conditions may further reduce the ability of the protein to flocculate the suspended solids. Therefore it is necessary to select suitable bacteria that can survive and flocculate under such conditions.

Sources and applications of bioflocculants

Soil and activated sludge samples are the best sources for EPS producing micro-organisms (Toeda 1991). His method involved isolating and culturing the bacteria, *Alcaligenes cupidus* KT201, and then purifying the bioflocculant by centrifugation and ethanol extraction. The flocculant was then applied in a purified form. Similar techniques were used by other researchers with the flocculating bacteria *Enterobacter sp.* BY-29 and *Bacillus coagulants* AS-101 (Yokoi 1997; Salehizadeh 2000). Bioflocculant has also been produced from glucose by the bacteria *Pullularia pullulans* (Zajic 1973) and *Rhodococcus erythropolis* S-1 (Kurane 1986). However, all these procedures were complex and expensive (Liu 2002; Lian 2007) and there was no direct correlation between the amount of polymer and the floc settleability (Li 2007b). For example; EPS contents and flocculation were positively correlated (Urbain 1993), negatively correlated (Goodwin 1985) or even unrelated (Liao 2001). Therefore, the methods described by these reports are not suitable for harvesting microalgae for low cost commodities such as biodiesel. Furthermore, many of the results described are also not applicable in a marine environment (Zhang 2002).

Defence mechanism and species specificity

In an aquatic environment, non-living surfaces are colonised by bacteria which eventually form biofilms with other microbes. However, living surfaces are usually relatively free from bacteria due to the surface chemistry incompatibility (Rosowski 1992), the production of repellents (Gerhart 1988), or sloughing of the surface layers (Mittelman 1996). However, when bacterial concentrations are high enough, some of the numerous functional groups from the EPS formed will be suitable for cell adhesion (Liao 2002). This attachment mechanism is a balance between the attachment of the bacteria and the repulsion of the microalgae, therefore, there is no specific level of bacterial concentration that the flocculation will occur and the process will likely be species specific.

Nutrients for the growth of bacteria

Microalgal culture media contains most of the nutrients for the growth of microbes, the major limiting factor is dissolved organic carbons as bacteria are heterotrophic. Therefore organic carbon substrates are added to stimulate the bacterial growth. Potentially cheap and readily available sources of organic carbon include glucose, acetate or glycerine. Acetates and propionates are major products of the anaerobic digestion of organic biomass (Fujita 2000) and could potentially be obtained by the anaerobic digestion of the algal residue after lipid extraction. Glycerol is a by-product of the transesterification of lipids during the production of biodiesel (Ma 1999). Glucose is commonly available and is the most utilised organic carbon by living organisms (Ukeles and Rose 1976). These three organic carbons are therefore used as substrates for the growth of the bacteria in this flocculation experiments.

Some literature reported certain organic carbon to be more effective than the others in producing flocculants. For example, it has been reported that glucose is more effective than sucrose for the bacteria *Leuconostoc mesenteroides* 13146 (Harris 1975) and is also more effective than maltose or mannose for the yeast *Saccharomyces cerevisiae* THB001 (Chang 2005). On the other hand, acetate and propionate are more effective than glucose or lactose for the bacteria *Citrobacter sp.* TKF04 (Fujita 2000) or in wastewater treatment (Li 2007b). Their effectiveness may influence the choice of the organic carbon in a commercial operation and this will be investigated during the experiments.

Characteristics of P. carterae that may affect flocculation

In *P. carterae,* the existence of the organelle haptonema whose function is to feed on other bacteria (Winter 1994) indicates only those species of bacteria that can co-exist with this microalgae will be able to flocculate, furthermore, the microalgae's surface structure and the effects of flocculating bacteria need to be investigated before the effectiveness of the microbial flocculation can be determined. These factors will be studied and discussed in Section 4.2 (Laboratory culturing of flocculating bacteria), Section 4.3 (Scan Electron Microscopy) and Section 4.4 (Axenic culture) before proceeding onto the experiments on microbial flocculation (Section 4.9).

4.2 Laboratory selection and culturing of flocculating bacteria

Some coccolithophorids such as *P. carterae* can release bactericide such as dimethyl sulphide (DMS) (Gibson 1990). Furthermore, the presence of the organelle, haptonema, indicates the coccolithophorids can capture other planktonic organisms (Winter 1994). Therefore it is essential to select the species of bacteria that are able to co-exist with and flocculate the microalgae. The procedures were performed as follow;

P. carterae from modified BG11 media was cultured as described in Section 3.3. The same culture was used to develop bacteria for the flocculation except non-sterile water was added to introduce bacterial growth. The culture was then exposed to the atmosphere for a few hours and cultivated in 1-L flasks under the same conditions as described previously in Section 3.3. Initially, *P. carterae* was the only form of organic carbon present, this ensured only those microbes that were able to co-exist with *P. carterae* would be selected. Microalgal samples that were observed to flocculate were then subcultured. A small aliquot (0.5 mL) of stock solution (100 g L^{-1} of glucose, glycerine or acetate) was added about once a week during the stationary phase to sustain the growth of various bacteria. This mixture was used as the flocculating culture for the experiments.

Results and discussion

Pale yellow flocs of around 1 mm started to appear in the media from day 2 after the exposure to air. By day 4, flocs were observed in all the samples. Flocs continued to grow in size and became dark brown in colour during the next 7~10 days while the *P.*

carterae culture also continued to grow with a normal pale brown colour. This shows that despite the presence of the bactericide, dimethyl sulphide and the organelle, haptonema, there are some species of bacteria that can co-exist and flocculate the microalgae. The characteristics of the bacteria will be discussed in further details in the next few chapters.

4.3 Scanning Electron Microscopy

Introduction

This section describes how the Scanning Electron Microscopy (SEM) was used to determine the feasibility of microbial flocculation by investigating the surface structures of the microalgae *P. carterae* and the possible mechanism(s) for flocculation. These two factors are important for successful flocculation and the results will be discussed before the experiments on microbial flocculation. The bacterial cell morphology and hence their partial identification were also investigated by the SEM.

Surface structures of P. carterae

All studied microalgae were known to produce extracellular polymeric substances (EPS) on the cell surfaces and these polymers are essential for the cells' protection against mechanical damages and bacterial attacks. In fact, as stated previously in Section 4.1, the microlagae that were embedded in the flocculating polymers did not lose their viability over an extended period of time.

Two very different images of *P. carterae* are obtained from the literatures. Figure 4.1 shows *P. carterae* surface being covered by bare calcium carbonate plates and appears to be free from any observable polymers or mucilage.

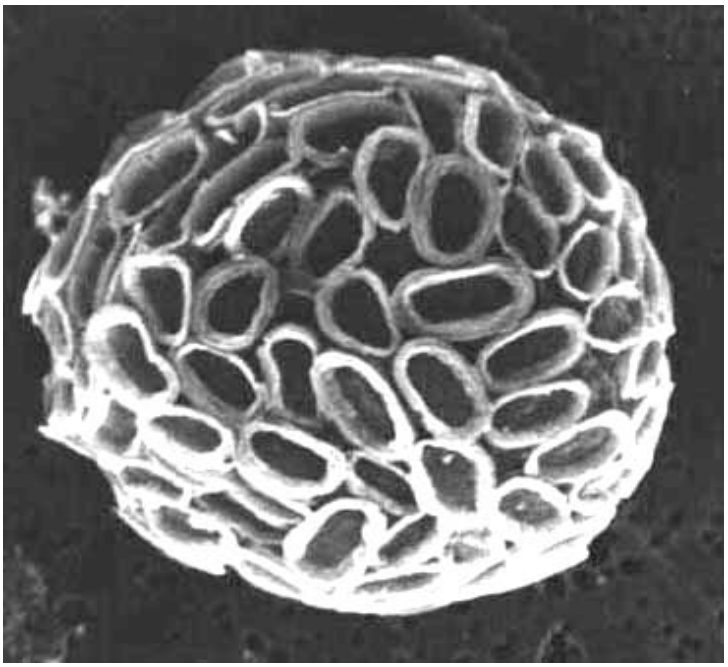

Figure 4-1 SEM images of *P. carterae* showing exposed $CaCO_3$ plates.
http://www.nhm.ac.uk/hostedsites/ina/CODENET/GuideImages

In contrast, the optical microscope image from the Culture Centre for Marine Phytoplankton (CCMP) shows the cells are covered by a thin layer of polymers (Figure 4.2).

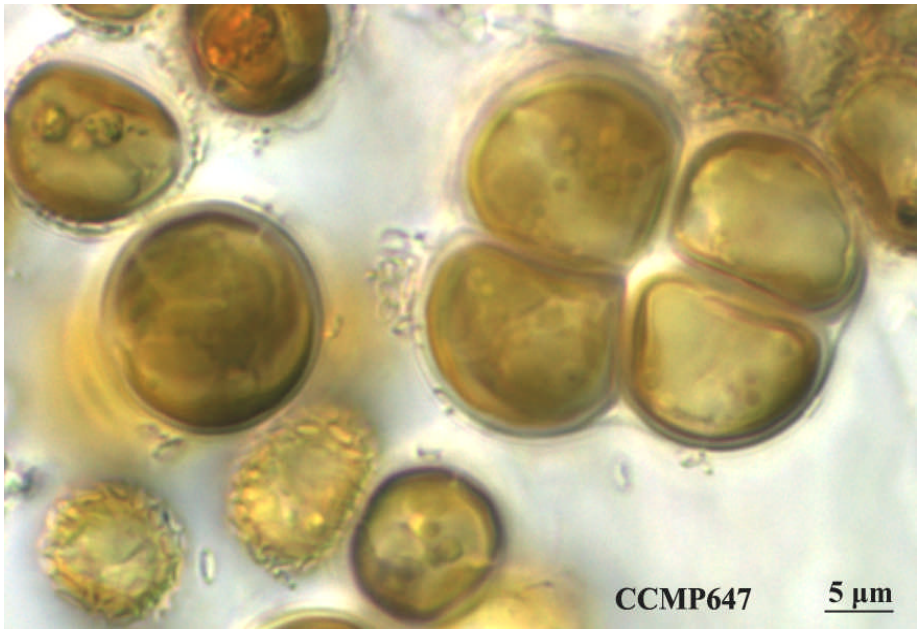

Figure 4-2 Optical microscopy images of *P. carterae* from CCMP.
(Notice the presence of a thin layer of surface polymer)

In order to reduce the stress on the microalgae during harvesting by flocculation, it is essential for the surfaces of the bacteria and microalgae to be covered by similar EPS. Therefore, the scan electron microscope is used for the investigation.

Possible mechanism for flocculation

Flocs can be produced as a result of secretion of EPS, which enhance the aggregation of bacterial cells and microalgae by the functional groups present on the polymers (Frølund 1996). On the other hand, biofilms or biogranules may also be formed by bacterial microfibrils (Hantula 1991; Veiga 1997) which can also form 2 or 3 dimensional matrixes (Liu 2004). These matrixes form open channels to allow the supply of nutrients and oxygen and are essential in maintaining the structural integrity of the flocs and the cells. The detection of microfribils will support the hypothesis of floc formation by fribil attachments.

Bacterial identification

Bacterial morphology is part of the primary identification; other means of physical identification include the colours of their colonies, the formation of endospores, motility by flagella and so on. However, their biochemical characteristics such as the staining reactions, their oxygen requirement, the catalase reaction and other biochemical behaviours will require separate treatments. Furthermore, all these tests are by no means definitive, and confirmations at the molecular level such as 16S rRNA will be required.

Sample preparation for SEM

The sample of *P. carterae* culture was diluted by a factor of 10^{-5} with modified BG11 (filtered by 0.2 µm nylon Acrodisc), then 1 ml of the diluted sample was filtered through titania coated cellulose filter paper, the filter papers together with algal cells were then fixed in 0.3 % gluteraldehyde for one hour by denaturing the protein and preserving the cell structures. The samples were then gently washed twice in phosphate buffer solution for a total of 20 min. A 2% aqueous osmium tetroxide solution was then used to stain the phospholipid bi-layer of the cell walls. The staining process took one hour at room temperature and a thin layer of metallic osmium was embedded in the cell membrane. The cells were then dehydrated as shown in Table 4.1. The dehydrated cells were then washed in acetone to prevent damages to the

delicate structures due to change in surface tension. The acetone was removed by immersing twice in supercritical carbon dioxide (31.1 ^{o}C, 1072 psi). The de-solvated samples were stored in desiccators to be metallised by platinum in a sputter coater and observed under Philips XL 30 Scan Electron Microscope

Table 4-1 Dehydration time of SEM samples

Step number	Time (min)	Reagent (Ethanol)
1	5	70%
2	10	70%
3	5	90%
4	10	90%
5	10	100%
6	10	100%
7	10	100%

Results and Discussion

Figure 4.3 shows a layer of EPS covering the entire microalgae cell; this layer is visually different from the exposed $CaCO_3$ plates observed from SEM in Figure 4.1 but is similar to the optical microscopic images from CCMP (Figure 4.2). This layer of EPS will make the flocculation less stressful to the microalgae.

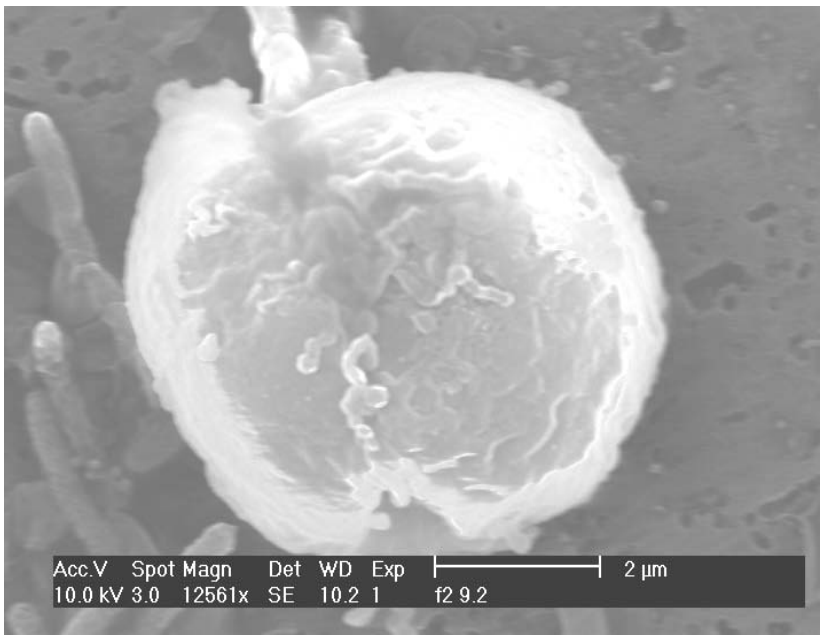

Figure 4-3 SEM image showing *P. carterae* covered in a layer of polymers.

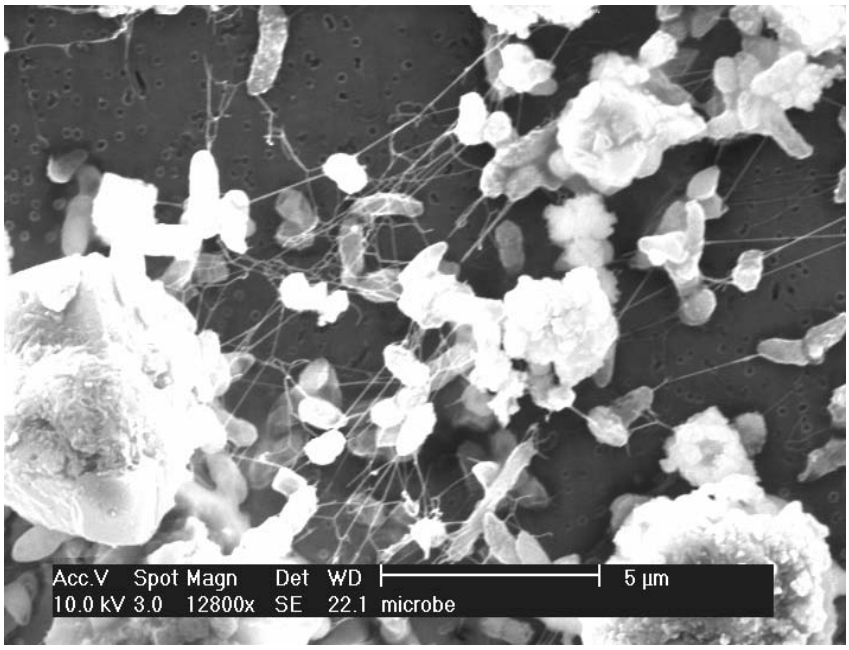

Figure 4-4 SEM images of bacteria and their microfibrils forming aggregates

Figure 4.4 shows the bacteria associated with the microalgae are of size 1-2 µm, rod, presence of flagella or microfribils but absence of endospores, further biochemical and morphological tests for their identification will be described in Section 4.5. This image also shows the presence of microfribils binding onto other solid particles, indicating that this may be one of the possible flocculation mechanisms. However this does not exclude other forms of binding such as charge neutralisation by functional groups, bridging by bivalent ions or by the back bone of the polymer structures.

The presence of a protective layer of EPS on the microalgae and the binding microfribil from the bacteria indicates a more favourable condition for the flocculation and hence better chance of success for the experiment.

4.4 Axenic culture of *P. carterae*

Introduction

The presence of bacteria in flocculating samples has been confirmed through SEM. However it remains to be demonstrated that the flocculation is caused either by the bacteria or by the microalgae themselves. One of the possible ways to demonstrate this is to compare the flocculating activities of *P. carterae* with and without the

presence of bacteria. To do this, it is necessary to prepare an axenic culture of the micoralgae and compare their flocculating results.

Andersen suggested that the differential resistance to antibiotics by the microalgae and bacteria may be used to obtain axenic samples of the microalgae cultures. The recommended doses for ampicillin and kanamycin are both 100 μg ml^{-1} (Andersen 2005; Sigma 2006), whilst for the isolation of the conchocelis from the algae *Porphyra yezoensis* is a mixture of chitosan, ampicillin, kanamycin and streptomycin (Choi 2002). However, the dosages for the axenic isolation of *P. carterae* are not available from literature. As lethal dosages for various antibiotics may be varied widely from species to species, experimental trials are required to determine the proper dosage for axenic isolation of *P. carterae.*

The trial consisted of Parts A and B; For Part A, only one type of antibiotic was used. When the treatment had been shown to be ineffective, then Part B, which combined the action of two antibiotics, would proceed. Ampicillin prevents the formation of bacterial cell walls (Singh 1954), while streptomycin or kanamycin stops the protein synthesis by binding onto the 16S rRNA (Luzzatto 1968; Waxman 1983). The combination of these two sites of action will minimise the probabilities of survival of bacteria. However, fungal contamination will require the use of fungicide such as cycloheximide but this would only be added if the antibiotics had been successful.

Procedure for Part A

The procedures for the axenic culture were as follow;

1. *P. carterae* culture was diluted 1: 1000 with sterile modified BG11 medium so that the final cell counts were in the order of 100 cells per 0.1 ml.
2. The kanamycin (100 mg ml^{-1} solution) was diluted to the required concentration in the following manner and 0.1 ml of each samples were introduced into a minimum of 3 wells in a 96 well plate and their cell counts were recorded.
    a. 1 ml of the kanamycin solution was mixed with 11.5 ml of milli Q water to give a stock solution (SS) of concentration of 8 mg ml^{-1}.
    b. 0.2 ml SS + 1.8 ml diluted culture = 800 μg ml^{-1} of kanamycin.
    c. 0.2 ml SS + 3.8 ml diluted culture = 400 μg $ml^{-1}$of kanamycin.

d. 0.2 ml SS + 7.8 ml diluted culture = 200 µg ml^{-1} of kanamycin.
e. 0.2 ml SS + 15.8 ml diluted culture = 100 µg ml^{-1} of kanamycin.
f. 0.2 ml SS + 31.8 ml diluted culture = 50 µg ml^{-1} of kanamycin.
g. Control sample was made up of diluted culture with no kanamycin added.

3. After 24 h, a minute amount of media from each well was streak on nutrient agar plates, which contained the corresponding concentration of antibiotics. The plates were then kept under the same growth conditions to observe the presence of bacteria.

4. After 7 days, the algal culture samples were examined under microscope for microalgal viability by Lugol's staining and cell counts for surviving *P. carterae* were recorded.

Results and discussions

Bacterial colonies were observed on all agar plates for concentrations up to 800 µg ml^{-1} (8 x recommended dosage) (Table 4.2); indicating kanamycin alone was not effective in producing an axenic culture of *P. carterae.* This might be due to the fact that the antibiotic could not destroy 100 % of the bacteria, and therefore only acted as a selective medium. The surviving bacteria were able to multiply and form colonies. Furthermore, it was reported that attached bacteria had higher resistance to bactericide than their free swimming counterparts (Costerton 1987).

Table 4-2 Cell counts for *P. carterae* after 7 days in kanamycin.

Group	A	B	C	D	E	F
Kanamycin (µg ml^{-1})	800	400	200	100	50	Control
Average Cell count after 7 days (x 10^{5})	5.16	5.32	3.69	3.32	4.92	4.76
Dilution factor	1.8 / 2.0	3.8 / 4.0	7.8 / 8.0	15.8 / 16.0	31.8 / 32.0	Nil
Adjusted cell counts (x 10^{5})	5.73	5.60	3.78	3.36	4.95	4.76

No cell damages were observed from all samples of *P. carterae* after 7 days and cell counts were comparable to that of control sample (See Table 4.2). This shows that *P. carterae* and some bacteria are resistant to high levels of the antiobiotic kanamycin.

Procedure for Part B

The *P. carterae* culture was diluted as described in Part A.

The stock solution (SS) of ampicillin/Streptomycin (amp/strept) was supplied at 1000 μg ml^{-1} and it was diluted to the required concentration in the following manner;

1. 0.4 ml SS + 0.6 ml diluted culture = 400 μg ml^{-1} of amp/strept
2. 0.2 ml SS + 0.8 ml diluted culture = 200 μg ml^{-1} of amp/strept
3. 0.2 ml SS + 1.8 ml diluted culture = 100 μg ml^{-1} of amp/strept
4. 0.2 ml SS + 3.8 ml diluted culture = 50 μg ml^{-1} of amp/strept
5. Control sample was made up of undiluted culture with no amp/strept added.

The rest of this experiment was similar to that described in steps 3 and 4 of Part A of this section.

Results and discussions

P. carterae cells were observed from Plates C, D and E but healthy *P. carterae* cells were only observed from plates D and E. Furthermore, number of algal counts in Plate D was 37 % less than that obtained from the control sample, showing that the growth of algal cells had been affected.

Table 4-3 Cell counts for *P. carterae* after 7 days in amp/strept

Group	A	B	C	D	E
Amp/strept (μg ml^{-1})	400	200	100	50	Control
Cell count after 7 days (x 10^5)	No viable cells	No viable cells	Under-sized cells	3.45	5.47
Dilution factor	0.6 / 1.0	0.8 / 1.0	1.8 / 2.0	3.8 / 4.0	Nil
Adjusted cell counts (x 10^5)	N/A	N/A	N/A	3.63	5.47

Bacterial colonies were observed from all samples except A, showing that some bacteria are more resistant to a combination of the amp/strept than *P. carterae.* These two tests showed that until a more suitable antibiotic could be found, it was not effective to use antibiotics to obtain an axenic culture of the microalgae. Another method to demonstrate the effects of bacteria on flocculation will be described in Section 4.10 (Experimental Design) by using non-acetate utilising bacteria.

4.5 Characterisation of flocculating bacteria

Introduction

The morphological and biochemical identification of the flocculating bacteria was carried out as described in "The shorter Bergey's manual of determinative bacteriology" (Holt 1977). The identification consisted of a series of standard procedures designed to minimise the number of irrelevant tests. They included the Gram reactions, the morphology, the reaction to atmospheric oxygen, the presence of endospores, the presence of motility organelles, reactions to hydrogen peroxide, and resistance to certain antibiotics. However, all these tests were not conclusive and confirmation tests were carried out at a molecular level.

Molecular sequencing (16S rRNA) was used as the confirmation. However, this test required equipment and expertise beyond the scope of Chemical Engineering, therefore, the test was performed by the Infectious Unit of the Institute of Medical and Veterinary Science, Adelaide and the results will be reported later in this section.

The morphology has been partly described in Section 4.3 under SEM, and the biochemical tests will be described in this section.

Methods for the bacterial characterisation

The standard bacteria media had been modified to support the growth of the maximum species of bacteria that are present in the microalgal culture, the recipe is presented in Table 4.4.

The mixed culture of microbes was diluted by a factor of 10^{-7} in sterile modified BG11 media. A small sample (0.1 mL) of the diluted microbe culture was then spread onto nutrient agar plates containing the above recipe. Observable microbial colonies

on the agar were then differentiated by their physical appearances (colour, shape of the colonies, fury or shiny surfaces etc) and aseptically transferred to different agar plates. Cultures were grown in 22 ^{o}C for a minimum of 48 h or until the colonies had reached a suitable size.

Table 4-4 Recipe for bacterial agar culture

Ingredients	g L^{-1}
Oxoid peptone	10
Oxoid lab Lemco powder	10
Agar	15
Acetic acid	0.5
Glucose	0.5
Glycerine	0.5
Modified BG 11 media (Section 3.3)	Make up to 1 L

There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), then decolorized with alcohol or acetone, and finally counterstained by safranin or basic fuchsin.

Gram-positive bacteria have cell walls with more peptidoglycan (50-90 % of cell wall) and will be stained purple by the crystal violet while Gram-negative bacteria have thinner cell walls with lipids and will be stained red or pink by the safranin.

Bacteria culture obtained from laboratory agar plates are found to be with cream coloured smooth surfaced colonies. SEM image shows microfribils holding other bacteria and algal cells together; this suggests that protein binding may be one of the possible flocculating mechanisms (Figure 4.4). Two major groups of bacteria are observed; with bacteria B outnumbering bacteria A approximately 100 to 1. Their morphology has been partially described in Section 4.3 under SEM and the laboratory tests showed that the majority of the bacteria were gram-negative, aerobic, and positive to hydrogen peroxide. The test results are tabulated in Table 4.5;

Table 4-5 Characteristics of bacteria from flocculating samples

Tests	Observations	
	Bacteria A	Bacteria B
Gram Reaction	Positive	Negative
Morphology	Size 1~2 μm, rod shaped, absence of endospores, shiny cream colour round colonies,	Size 1~2 μm, rod shaped, absence of endospores, shiny cream colour round colonies,
Reaction to oxygen	Both aerobic and anaerobic	Facultative aerobic
Motility	Absence of flagella	Absence of flagella
Catalase reaction	Positive	Positive

These results suggested that B may be a *Pseudomonas* and A may be a *Bacillus.* 16S rRNA tests further confirmed B is *Pseudomonas stutzeri* and A is *Bacillus cereus.*

4.6 Material and methods for the microbial flocculation

P. carterae was cultured in the laboratory as described in Section 3.3 and flocculating bacteria were obtained and cultured in the laboratory as described in Section 4.2.

Selection of non acetate-utilising microbial cultures

Section 4.4 shows that it is difficult to obtain an axenic culture by antibiotics, therefore an alternative method to demonstrate the effects of bacteria on flocculation will be by the use of the non acetate-utilising bacteria with acetate as the only organic carbon substrate; the lack of flocculating activities will indicate the effects of the bacteria and not the carbon substrate itself.

The procedures were carried out as follow; the flocculating cultures of *P. carterae* were selected and diluted by factors of 10^{-6}, 10^{-7} and 10^{-8} respectively. Small samples (0.2 mL) of the diluted microalgae culture were then spread onto nutrient agar plates containing 10 g L^{-1} Oxoid peptone, 10 g L^{-1} Oxoid Lab Lemco powder, and 15 g L^{-1} agar in modified BG11 media. Observable microbial colonies on the agar were then aseptically transferred to culture tubes containing Oxoid nutrient broth of the same recipe but without agar. Cultures were grown on a shake table at 22 ^{o}C and 70 rpm for

up to 48 h prior to use. The lack of acetate in the culture media will discourage the growth of acetate assimilating bacteria and those developed from this culture can assimilate nutrients such as glucose or glycerine but not acetate.

4.7 Determination of flocculation effectiveness.

The effectiveness of microbial flocculation was determined by the recovery efficiency and the concentration factor.

By definition recovery efficiency (*RE*) is defined as the ratio of the mass of cells recovered to the total original mass of cells, as the cell concentration in the flocs are very difficult to determine, the following equation is used;

$$\text{Recovery efficiency} = \frac{mass_{total} - mass_{supernatant}}{mass_{total}} \qquad \text{(Eq. 4.1)}$$

The flocculation experiments were carried out in 100 ml aliquots, and these volumes were much larger than that of the flocs which were usually under 0.5 ml. As the cell mass was the product of volume *V* and the cell concentration *C;*

$$\text{Recovery efficiency} = \frac{(100ml)(C_o) - (100ml)(C_t)}{(100ml)(C_o)}$$

$$= 1 - (\frac{C_t}{C_0}) \qquad \text{(Eq 4.2)}$$

Concentration factor (*CF*) is the ratio of the final product concentration to the initial concentration.

By mass balance;

$$C_0V_0 = C_tV_t + C_fV_f$$

Again, the cell concentration in the floc, C_f is difficult to determine, the following approximation was used;

As V_0 = 100 ml and V_f was usually under 0.5 ml, therefore Where $V_0 \sim V_t >> V_f$

$$C_0 \text{ x } 100 = C_t \text{ x } 100 + C_fV_f$$

$$C_fV_f = 100\ (C_0 - C_t)$$

$$\frac{C_f V_f}{C_0} = 100\ (1 - \frac{C_t}{C_0})$$

$$CF = \left(\frac{V_0}{V_f}\right)(RE) \qquad \text{(Eq 4.3)}$$

4.8 Determination of cell concentration

Cell concentrations C are required for the calculation of RE and CF. C can be determined by the dry mass, optical densities, or cell counts. However, the samples from the flocculation were mixtures of microalgae, flocculating bacteria and extracellular polymeric substances (EPS), all of which were presented in various amounts. Furthermore, cell concentrations were quite low in the collected supernatant and the low reading would increase the margin of error during weighing for dry mass determination. Methods for cell concentration determination will be discussed in this section.

Dry mass determination of algal cells in the culture media

Whatman GF/C (5 cm) were dried in 70 ^{o}C for 24 h and kept in desiccators until required. The filter paper was then weighed in a covered Petri dish to 4 decimal places (W_1). Culture was mixed thoroughly and 100 ml of the sample (W_o) was then placed in a filter cup on a funnel stem and dried gently by a suction pump. The paper was then gently rinsed by 5 ml of 0.5 M ammonium carbonate or formate solution to remove excessive salt. The algal cells, together with filter paper and Petri dish, were dried at 90^{o}C until a constant weight was obtained (W_2). Samples were dried in open Petri dishes but weighed in covered Petri dishes to minimise absorption of moisture from the atmosphere during weighing.

Dry weight % was obtained by $\frac{(W_2 - W_1)}{W_0}$ (g. 100ml^{-1})

During the experiment it was found that the dry mass collected from filter paper contained EPS and microbes. Furthermore, the supernatant (for Equation 4.2) had very few cells and therefore negligible cell mass, increasing the margin of error. This margin of error did not provide a satisfactory result for statistical analysis, and cell concentration as determined by dry mass was not a satisfactory method.

Determination of cell densities by optical density

The Shimadzu UV 1601 photospectrometer was used to measure the optical densities which were directly proportional to the cell concentrations. The wavelength of 670 nm was used to determine the concentration of chlorophyll (Shipman 1976), the wavelength of 440 nm was used for the bacteria (Sprenger 1993) and the wavelength of 720 nm was used for the algal cells. As the wavelengths were sufficiently far apart, measuring the optical densities could provide a quick way of measuring cell concentrations.

The procedure for determination of optical densities

The cuvettes were cleaned by washing gently with 70% ethanol, rinsed in milli Q water and dried in 70 ^{o}C atmospheric air. 1 ml of BG11 media was pipetted into each of the two cuvettes, one of which was used as a reference and was put at the back slot of the photospectrometer; the other one was placed in the sample slot in the front. A suitable wavelength was chosen and the photospectrometer reading was zeroed. The cuvette in the sample slot was then emptied and filled with 1 ml of the well mixed sample to be tested and returned to the sample slot. The new reading was taken as the optical density.

In practise, both the absorption peaked at 440 nm and 670 nm were not very well defined as there had been many other interfering impurities. Furthermore, the absorption reading decreased during the measurement as some of the suspended particles readily settled, giving inconsistent readings. Therefore this method was also disregarded.

Cell counting by haemocytometer

A Neubauer haemocytometer was used to determine cell numbers, samples were observed by Olympus IX 50 microscope coupled with the Analysis LS Research software. The number of cells per ml was the total number of cells in the central grids of the haemocytometer (total area 1 mm^2) multiplied by the factor of 10^4. Another reading was taken at the opposite end of the haemocytometer and the average was used as the cell number. Therefore, only two readings were available from each 0.1 ml sample of the 100 ml culture.

The amount of media required for each haemocytometer reading was in the order of 0.1 ml, and for this experiment, it represented a sampling rate of 1 in 1000; therefore, a thorough mixing was required to minimise sampling error. However, further errors were incurred due to the distribution of random samples; typical haemocytometer readings for *P. carterae* at the steady phase were in the low 100s. As the haemocytometer would only allow 2 readings per sample, a total cell count of 200 to 250 incurred a random error of about 16 % (Andersen 2005). When Equation 4.2 was used for the calculation of RE, the margin of error would be the sum of absolute error in the numerator plus the relative error of the denominator, i.e. a total of 20 % + 16 % = 36 %. An attempt to reduce the error by increasing the number of cell counts will involve taking another sample; this showed that the cell concentration determined by the haemocytometer method would also have an error margin too large for statistical analysis. Therefore, this cell counting method was modified by using the Sedgwick Rafters cell counter as described in the following section.

Cell counts by Sedgwick Rafter counter

The Sedgwick Rafter counter is a glass slide with a rectangular chamber of area 50 mm x 20 mm and depth 1 mm, giving a total volume of 1 ml, this volume represents a sampling rate of 1 in 100 in this series of experiments (Section 4.9). Cell counts were determined by using this chamber and the Olympus IX 50 microscope coupled with Analysis LS Research software.

The chamber was filled with the well mixed sample to be counted and left to stand for 20 min or until all the microalgae cells had settled on the bottom of the chamber. A field of vision was selected randomly, the area of which was then determined from the Analysis LS Research software and the number of cells inside that area was then recorded. The counting was repeated until the readings gave a satisfactory standard deviation. In this series of experiments, an arbitrary 7% was chosen as the bench mark. As it was difficult to achieve a representative sample in counting objects of a single class when mixed with other classes (Russ 2000), a minimum of six readings were taken for each sample to achieve the desired accuracy and the means of these values were used in the calculations.

By taking a larger sample (1 ml) and increasing the number of cells counted, this method provided a much more consistent result than the three previously described, and Sedgwick Rafter counter was chosen for the purpose of determination of cell concentration.

Lugol's iodine solution

Lugol's solution is used to stain plant cells for microscopic observations, but the acidic solution may destroy the cocoliths and other calcareous structures; therefore a neutral alkaline solution was prepared as follow;

20 g KI was added to 40 ml of water, and then 10 g of I_2 crystals was added, followed by another 100 ml of water. The mixture was well shaken and filtered and was ready for use.

4.9 Experimental Procedure in microbial flocculation

A 0.1 ml aliquot of the microbe culture, or the non-acetate-utilising microbe culture, was added to 100 ml of *P. carterae* culture. The mixture was shaken vigorously and the initial microalgae concentration (C_0) determined by cell counting. Samples (100 ml) were then introduced into 250 ml flasks on a shake table at 70 rpm. An organic substrate of acetate, glucose or glycerine was added to the shake flasks with a final substrate concentration of either 0.1 or 0.5 g L^{-1}, as shown in Table 4.6. For the experiments with the non-acetate-utilising microbe culture, only acetate (at either 0.1 or 0.5 g L^{-1}) was added to the shake flask. The flasks were left on the shake table under normal growth conditions, but without illumination, for either a 6 or 24 h duration, as shown in Table 4.6. At the end of this period, 90 ml of the supernatant was collected as soon as possible to prevent settling and the concentration C_t was determined by cell counting. Any flocs, together with a small amount of media, were gently transferred to a measuring cylinder with 0.1 ml graduations and allowed to settle under gravity for 30 min. Floc volumes were thus recorded. The recovery efficiency and concentration factor for each treatment were calculated using Equations 4.2 and 4.3.

4.10 Experimental design.

Design factors

The effectiveness of the microbe culture in promoting microbial flocculation was investigated using a factorial design with three factors: organic substrate, concentration of substrate, and mixing time. The levels for each factor are shown in Table 4.6. The experimental treatments were randomised and repeated in triplicate. The effectiveness of the non acetate-utilising microbe culture was investigated similarly except that only acetate was used as the organic substrate.

Table 4-6 Factor levels in the factorial design.

Factors		Levels	
(A) Substrate	Acetate (1)	Glucose (2)	Glycerine (3)
	Low level (–)	High level (+)	
(B) Concentration of substrate (g L^{-1})	0.1	0.5	
(C) Mixing time (h)	6	24	

The factors are the substrates, their concentrations and the mixing times. Other factors that had been considered were; the amount of bacterial culture added and the bacterial incubation time. However, taking all these factors into consideration would result in 144 sample runs for each trial and the experiment would become too complex. As a result, preliminary experiments for each of the above single factors were carried out to test their potential effects on the flocculation. It was found that the last two factors, namely, the amount of bacteria added and the bacterial incubation time were not statistically significant and hence were deleted from the test runs.

5 Microbial flocculation - Results and discussions

5.1 Effectiveness of flocculation

The recovery efficiency and concentration factor for each treatment and replicate of the factorial experiment are presented in Table 5.1 for flocculation using the standard microbe culture. It is clear that microbial flocculation of *P. carterae* is substantially enhanced at long mixing times.

There was no apparent difference between the flocculation recovery efficiency with varying substrate type. Larger volumes of flocs were observed to form at higher doses of organic carbon, but the recovery efficiency remained relatively constant. Thus the concentration factor decreases with increasing substrate concentration at long mixing times.

Table 5-1 RE and CF using mixed cultures

	Substrate concentration:	0.1 g L^{-1}		0.5 g L^{-1}	
Substrate	Mixing time:	6 h	24 h	6 h	24 h
Acetate	Recovery efficiency (%)	52 (51-53)	88 (75-96)	52 (50-53)	90 (81-95)
	Concentration factor	149 (146-151)	240 (215-264)	133 (106-150)	100 (89-106)
Glucose	Recovery efficiency (%)	53 (44-70)	90 (83-96)	48 (35-60)	93 (89-97)
	Concentration factor	167 (146-182)	236 (232-239)	181 (141-242)	109 (89-121)
Glycerine	Recovery efficiency (%)	45 (36-54)	94 (91-96)	50 (38-61)	93 (93-97)
	Concentration factor	179 (143-216)	204 (192-228)	171 (152-210)	131 (100-185)

(Figures inside brackets indicate range).

The recovery efficiency and concentration factor for the flocculation of *P. carterae* using the non acetate-utilising microbe culture are presented in Table 5.2. In this case, acetate was the only substrate, while the factors of acetate concentration and mixing time were investigated using a factorial design with two factors and three replicates. When bacteria were cultured that were unable to utilise acetate, and when acetate was the only substrate provided to this culture, flocs formed were looser and paler in

colour, indicating a lack of flocculating activity. Furthermore, a significantly lower average recovery efficiency was obtained than for the standard microbe culture.

Table 5-2 RE and CF using the non acetate-utilising culture

	Substrate concentration:	0.1 g L^{-1}		0.5 g L^{-1}	
Substrate	Mixing time	6 h	24 h	6 h	24 h
Acetate	Recovery efficiency (%)	45 (38-49)	65 (43-94)	49 (47-53)	73 (44-94)
	Concentration factor	129 (110-141)	159 (144-234)	138 (131-157)	156 (125-205)

Table 5-3 ANOVA for recovery efficiency

SUMMARY OUTPUT		RE vs. Factors (A, B, C, AB, AC and BC)		
Regression Statistics				
Multiple R	0.95			
R Square	0.90			
Adjusted R Square	0.87			
Standard Error	0.08			
Observations	36.00			
ANOVA				
	df	*SS*	*MS*	*F*
Regression	6.00	1.57	0.26	41.54
Residual	29.00	0.18	0.01	
Total	35.00	1.76		
	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept #	0.45	0.09	4.87	0.00
X Variable (A)	-0.04	0.04	-1.08	0.29
X Variable (B)	-0.05	0.21	-0.22	0.83
X Variable (C) #	0.02	0.00	3.91	0.00
X Variable (AB)	0.02	0.08	0.23	0.82
X Variable (AC)	0.00	0.00	1.43	0.16
X Variable (BC)	0.00	0.01	0.23	0.82

A summary of analysis of variance (ANOVA) for recovery efficiency and concentration factor are presented in Tables 5.3 and 5.4 respectively. Only variables (indicated by # in the tables) with p-values under 0.05 are deemed to be significant. They are the mixing time from Table 5.3, and both the mixing time and the interaction of mixing time and concentration from Table 5.4.

The ANOVA showed that the nature of the organic substrates (X Variable (A)) did not have a significant effect on either the RE or the CF. Furthermore, the ANOVA demonstrated that the interactions between the substrate and both substrate concentration and mixing time were also insignificant. Thus, the recovery efficiency and concentration factor data were fitted to the following linear regression model for a 2 × 2 data set in which the data for all of the different carbon substrates were pooled:

$$Y = c_o + c_B X_B + c_C X_C + c_{BC} X_B X_C$$

Table 5-4 ANOVA for concentration factor

SUMMARY OUTPUT	CF vs. Factors (A, B, C, AB, AC and BC)			
Regression Statistics				
Multiple R	0.86			
R Sq	0.73			
Adjusted R Sq	0.68			
Standard Error	28.26			
Observations	36.00			
ANOVA				
	df	*SS*	*MS*	*F*
Regression	6.00	63735.66	10622.61	13.30
Residual	29.00	23154.55	798.43	
Total	35.00	86890.21		
	Coefficients	*Standard E*	*t Stat*	*P-value*
Intercept #	118.22	32.78	3.61	0.00
X Variable (A)	8.94	14.16	0.63	0.53
X Variable (B)	-12.03	73.63	-0.16	0.87
X Variable (C) #	6.98	1.59	4.38	0.00
X Variable (AB)	47.39	28.84	1.64	0.11
X Variable (AC)	-1.01	0.64	-1.58	0.12
X Variable (BC) #	-15.23	2.62	-5.82	0.00

The coefficients, c_o, c_i, and c_{ij}, for each linear regression model were calculated using Microsoft Excel 2003. The regression analysis for recovery efficiency gave the following relationship with an R^2 value of 0.88 (Table 5.5).

$$RE = 0.36 + 0.023\ t \qquad \text{(Eq 5.1)}$$

The regression analysis for the concentration factor gave the following relationship with an R^2 value of 0.64 (Table 5.6).

$$CF = 136 + 4.95\,t - 15.23\,t\,C \qquad \text{(Eq 5.2)}$$

Table 5-5 ANOVA of RE vs. factors (B, C & BC)

Regression Statistics		RE vs. factors (B, C & BC)		
Multiple R	0.94			
R Square	0.89			
Adjusted R Square	0.88			
Standard Error	0.08			
Observations	36.00			
ANOVA				
	df	*SS*	*MS*	*F*
Regression	3.00	1.56	0.52	84.78
Residual	32.00	0.20	0.01	
Total	35.00	1.76		
	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.36	0.05	7.94	0.00
X Variable (B)	-0.01	0.13	-0.07	0.94
X Variable (C)	0.02	0.00	8.65	0.00
X Variable (BC)	0.00	0.01	0.23	0.82

Table 5-6 ANOVA of CF vs. factors (B, C & BC)

Regression Statistics		CF vs. factors (B, C & BC)		
Multiple R	0.82			
R Square	0.67			
Adjusted R Square	0.64			
Standard Error	30.01			
Observations	36.00			
ANOVA				
	df	*SS*	*MS*	*F*
Regression	3.00	58066.35	19355.45	21.49
Residual	32.00	28823.86	900.75	
Total	35.00	86890.21		
	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	136.09	17.53	7.76	0.00
X Variable (B)	82.76	48.61	1.70	0.10
X Variable (C)	4.95	1.00	4.94	0.00
X Variable (BC)	-15.23	2.78	-5.48	0.00

The rate of settling, the recovery efficiency will be incorporated in the energy estimation of the baffled hydraulic mixer/flocculator in Section 5. 11.

5.2 Quality of polymers on flocculation

During the experiments, two main types of flocs were observed. They are shown in Figure 5.1. The flocs in the right flask are darker and denser, forming a tight algal mass that can easily be separated from the media. Figure 5.2 shows the microscopic images of the dense floc with a large number of microalgal cells embedded and relatively few microalgae cells are left in the surrounding media. This type of EPS is known as the tightly bound EPS (TB – EPS).

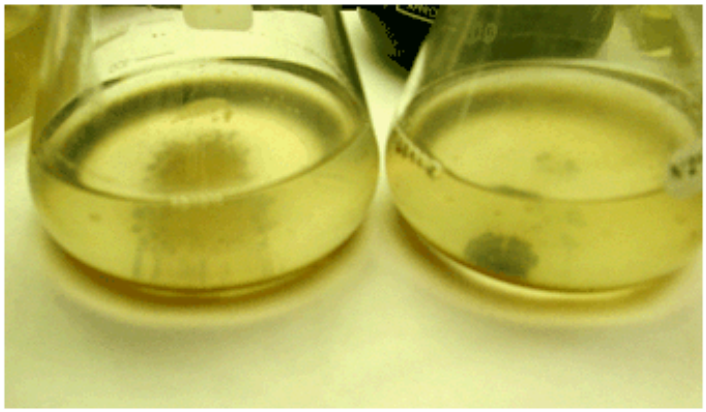

Figure 5-1 Samples showing the formation of loose and dense flocs

The flocs from the left flask in Figure 5.1 are much lighter and do not attach to each other firmly, they form a looser mass that is difficult to collect and does not dewater easily. Figure 5.3 presents the microscopic image of a loose floc from the left flask; it shows a large amount of EPS but with very few microalgal cells attached. (Some microalgal cells appear to be attached but are in fact positioned under the floc and are compressed by the glass slide). This type of EPS is known as the loosely bound EPS (LB–EPS). They have a negative effect on flocculation.

Although EPS is essential for the floc formation and the attachment of suspended particles, Figures 5.2 and 5.3 clearly demonstrate that the amount of EPS is not related to the effectiveness of the flocculation. This is due to the formation of LB- EPS which weaken the floc structure resulting in poor cell attachments, poor flocculation and inefficient dewatering. The quality of EPS, rather than the total EPS amount explains the inconsistency of many previous studies as discussed in Section 4.1 where EPS contents and flocculation can be positively correlated, negatively correlated, or even unrelated.

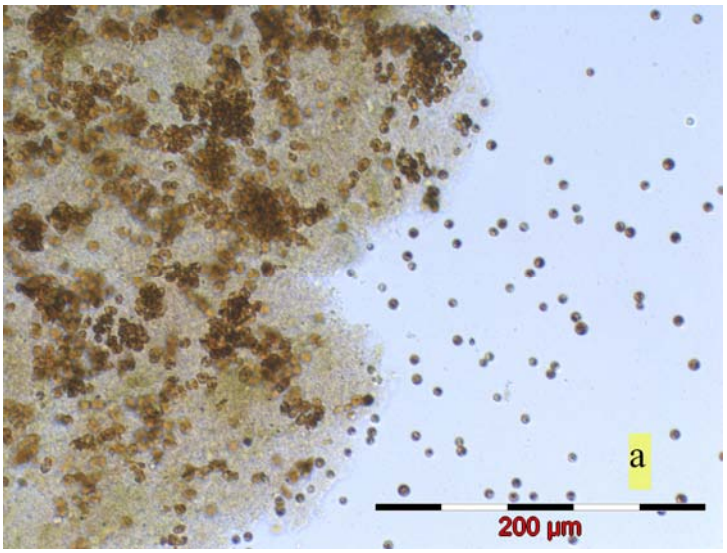

Figure 5-2 Dense flocs showing good attachment of microalgal cells

The conditions for the production of desirable TB-EPS such as those observed from the right flask will be summarised at the end of this chapter.

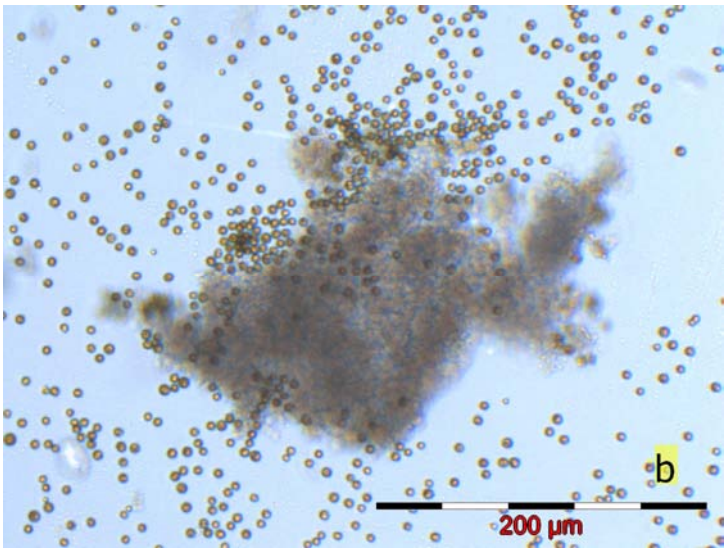

Figure 5-3 Loose flocs showing poor attachment of cells

5.3 Effects of bacteria on microalgae flocculation

It was clear from these experiments that the addition of an organic carbon substrate to a culture of *P. carterae* that was previously dosed with the bacteria culture promoted the flocculation of the microalgae. However, it cannot be concluded whether the flocculation of the microalgae is promoted by the presence of bacteria or by the effect

of carbon substrate addition on the microalgae cells themselves. Further experiments using the non-acetate-utilising bacteria culture, with the addition of acetate, demonstrated that the microalgae cultures flocculated with lower recovery efficiency than cultures containing the standard mixed bacteria culture. Thus, it was concluded that the action of the microbes in the culture enhanced the flocculation and was not an effect of the carbon substrate acting on the microalgae cells.

The bacteria isolated were identified by their morphology, biochemistry and 16S rRNA sequences (Murray 2003). The majority were found to be *Pseudomonas stutzeri* and *Bacillus cereus.* However, as a mixed culture had been used through out the experiments, the bacteria isolated from agar plates may not be a true representation of those presented in the culture.

The choice between a pure strain and a mixed culture may give rise to the difference in the observed behaviour from other literature such as the preference of one organic carbon over the other (Section 5.4); or the rate and the effectiveness of flocculation. In a commercial production, especially in open ponds, results obtained from a mixed culture would be more appropriate.

5.4 Effects of the nature of substrates

The three organic substrates had similar flocculating activities when the appropriate bacterial cultures were used (Table 5.1). The apparent differences in recovery efficiency and concentration factor were not of statistical significance as their p-values of 0.29 and 0.53 (x variables A, Tables 5.3 and 5.4 respectively) were much greater than 0.05. This observation may contradict some previous literature, which reported some organic carbon was more effective in producing flocculants than others (Section 4.1, Subsection: *Nutrients for the growth of bacteria*).

The apparent difference could be due to the fact that mixed cultures had been used throughout the flocculation experiments and the bacteria had been grown in media with the addition of different organic carbon. Furthermore, the bacteria in this study were purposely put under stress by limiting the organic carbon supplies so as to maximise flocculating efficiency. On the contrary, other authors used an excess of

nutrients so as to maximise the bacterial growth and their EPS production, for example, 10 g L^{-1} of glucose or mannose was used in Chang's bacterial culture (Chang 2005), 20 g L^{-1} of glucose was used by Yokoi (Yokoi 1997) and 10 g L^{-1} of carbon was used by Kurane (Kurane 1986). Their dosages were in the order of 2 higher than the 0.1 g L^{-1} used in this experiment.

In an open pond environment, it is reasonable to assume a small amount of mixed microbes will co-exist with the microalgal culture; hence the observation that flocculation effectiveness is independent of the nature of organic carbon is more relevant from a practical point of view.

5.5 Effects of substrate concentration

There was no appreciable increase in the recovery efficiency when excess carbon substrates were added (Table 5.1) and their p-values (x variables (B), Table 5.3 and 5.4) of 0.83 and 0.87 indicated their coefficient were insignificant. Their concentration factors were reduced as the excess substrate produced loose polymers, adding bulk to the flocs. This showed that while a supply of organic carbon was essential for the growth of bacteria, it was the depletion of nutrients that was essential for the aggregation of cells. The lack of nutrients is the major stress factor inducing the bacteria to form extracellular polymeric substances (EPS).

As the aggregation was dependent upon the amount of flocculating bacteria and their stress level, the optimum dosage occurred when the bacteria were growing actively but the nutrient depleted soon afterward, stressing the bacteria to produce TB-EPS similar to that in the right flask in Figure 5.1 (Section 3.2). This observation agreed well with previous work on production of yeast, *Saccharomyces cerevisiae*, where an increase in glucose concentration did not increase flocculating activities (Chang 2005).

The lower level for the organic carbons required during this microbial flocculation was in the order of 0.1 g L^{-1} , this compared favourably with other organic flocculants such as chitosan at 100 mg L^{-1} in sea water (Lubián 1989; Strand 2003) or even inorganic ions such as Al^{3+} at 60 mg L^{-1}. But as the maximum dry mass concentration of *P. carterae* is about 0.5 g L^{-1} , the organic carbon still represents 20% of the total

dry mass or USD 0.20 per kg if glycerine is used. This still represents a large amount of material used and needs to be further optimised.

The emphasis on stress, the mixed culture of bacteria used and the much lower concentrations of organic carbon substrate, all contributed to the discrepancies in the observation between other authors and this series of experiments.

5.6 Effects of the mixing time

The recovery efficiency increased with mixing time, from an average of 0.5 for 6 h to 0.9 for 24 h. Increasing the mixing time allowed the bacteria concentration to grow up to a level which was limited by the substrates available and also increased the probability of binding contact between the microalgal cells and the EPS. However, the recovery efficiency was expected to have a non-linear response to mixing time, this is because in the beginning, the rate of recovery was slow as bacteria needed the time to assimilate the organic carbon and grew in number, then the rate would increase as more bacteria were available while the nutrient was being depleted. Finally, the recovery rate levelled off due to the decreasing concentration of cells in the media. Hence an S-shaped response curve was expected and the efficiency may have peaked before the 24 h limit.

In practice, shorter mixing times may be necessary to optimise energy consumption and reactor size. A theoretical analysis will be demonstrated in Section 5.11 and further scaling-up trials will be necessary to allow the balance between recovery and cost to be determined.

5.7 Effects of hydrodynamics

Proper mixing is expected to increase the frequency of contact between microalgae and the EPS, and hence improves the recovery efficiency and concentration factor. Thus, too little mixing will likely to result in poor flocculation, while over-mixing will cause the flocs to break up into smaller fragments, again, resulting in poor flocculation.

In the controlled experiment, where *P. carterae* was allowed to settle under gravity without mixing (with settling condition similar to that described under Section 3.3: experimental evaluation of pH bioflocculation) the flocs formed were extremely loose and difficult to separate properly from the culture media. The concentration factor was estimated to only be in the order of 10

During this experiment, the mixing was achieved by shake tables, and this method of mixing was clearly inefficient in terms of energy usage. A more energy efficient method to promote mixing is to use baffled channels, which are widely practiced in flocculation and mixing during wastewater management (McConnachie 1993). Proper mixing together with an optimised mixing time will minimise energy usage in larger scale processes (Section 5.11).

5.8 Effects of gravity

The regression analysis showed considerable positive values of c_o for each of the predicted responses. This indicated the effect that some other forces have on the settling behaviour of *P. carterae*. From the DLVO Theory (Section 2.3), the forces involved are the Van der Waals attractive forces (F_A), the electrostatic repulsive force (F_R) and the gravitational force (F_G), and that F_G is always greater than the combined effects of F_A and F_R. However, this analysis assumes the particles have inert and smooth surfaces (Section 2.3) whereas in microalgal harvesting, flocculation occurs between live bacteria and microalgae. In this case, F_G might not be greater than the biological forces, and also it had been observed that without proper mixing to supply the nutrients to the bacteria and to provide the collision required among the suspended particles, the flocs formed were loose, difficult to collect and the settling behaviour was unreliable and unpredictable.

This shows that although F_G may be a significant force as suggested by the ANOVA, it still has to be combined with other conditions such as nutrient level and mixing for maximum efficiency.

5.9 Other flocculation factors

During the experiments, there were some environmental conditions which had been kept constant but could still affect microbial activities and hence flocculating results. The major ones were; the temperature, the pH and the divalent ions concentration; temperature would affect the growth rate and the metabolic activities of the bacteria; pH would change the Zeta potential and the cell metabolism while divalent ions would affect the bridging mechanism. Optimising these conditions might improve the flocculation results, but as the RE achieved had already been over 90 % and the CF of over 200, any further improvement will likely to be marginal and might not be cost effective (see Section 3.2 Stress factors for bioflocculation).

The lack of room for further improvement in the efficiency, the costs associate with those changes and the treatments require to recycle the media have to be taken into consideration to ensure these changes can be justified by the algal mass collected.

5.10 Possible flocculation mechanism

The biological factors are very important in floc formation but their mechanisms are poorly understood; none of the theoretical models can satisfactorily explain the adhesion of the marine microalgae by the bacteria to form flocs that had been observed. For example, the classical DLVO theory assumes perfectly smooth surfaces and an ionic strength between 0 to about 0.1 M (Section 2.3). Clearly this theory cannot be applied to the flocculation of the marine microalgae as the surfaces of the algal cells are irregular with various functional groups and the ionic strength of the sea water is about 0.6 M. The alternative is the cell surface hydrophobicity (CSH) theory (Section 3.1), which measures the liquid/solid intersurface contact angle and the hydrogen bonding energy of cohesion. However, none of these theories can explain the effect of bacterial stress on the extracellular change of the EPS from LB-EPS to TB-EPS. One possible flocculation mechanism is by the growth of microfribils from the bacteria as evident from the SEM images from Figure 4.4; however, it still does not explain how the microfribils can be attached to the microalgae and therefore other mechanisms may still be possible.

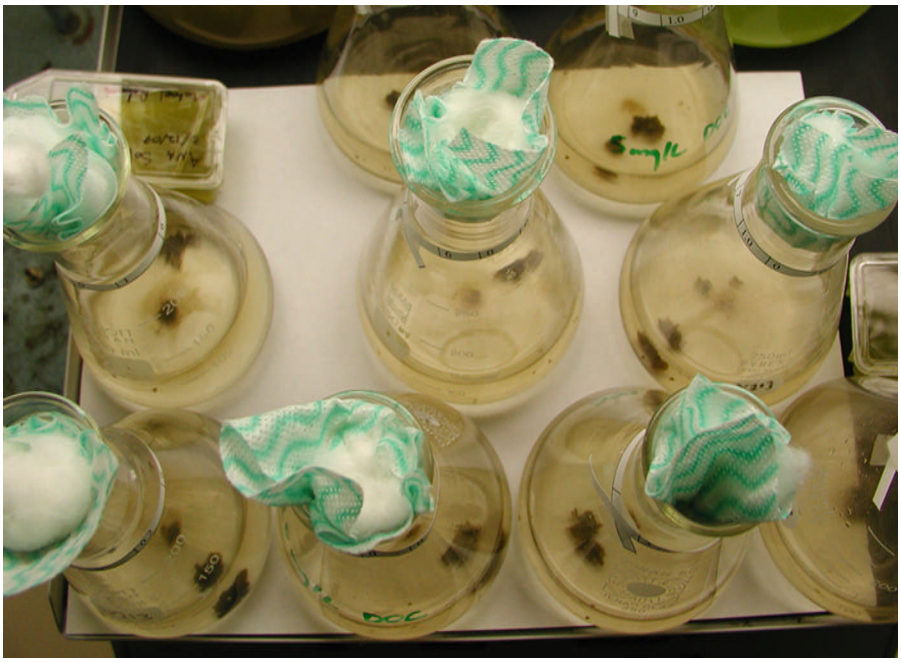

Figure 5-4 Dense flocculation achieved in all samples under proper conditions

From an engineering point of view, it is the results such as the recovery efficiency, the rate of recovery and the total cost that are important. The understanding of the mechanism and the species of the bacteria may have some effects on the flocculation but many wastewater treatment plants have been operating successfully without fully explaining the exact attachment mechanism of various colloidal particles, living or inorganic, to form the activated sludge. By controlling the mixing time, the correct culture of bacteria, proper mixing and the substrates' concentration, good flocculation can reliably be achieved (Figure 5.4).

Similar to the comments made at the end of Section 5.9, understanding the mechanism may not improve the recovery or concentration factor.

5.11 Estimation of energy required for flocculation

Laboratory bench top shake tables are very inefficient in mixing in terms of energy usage and the energy require by the shake table over a 24 h harvesting period will probably exceed that can be recovered from the algal mass. In large scale wastewater treatment, baffled hydraulic flocculating tanks are often used as they are simpler to construct and are more energy efficient.

This section will demonstrate theoretically the energy require for the flocculation of a HRAP of surface area 1 km^2 and hence the feasibility of a commercial scale operation.

Design parameter for the flocculator

Cell culture doubling time = 2.5 days (Moheimani 2006)

Depth of open HRAP = 0.20 m (Moheimani 2006)

HRAP mixing speed = 0.20 m s^{-1}(Moheimani 2006)

Lipid yield = 21.9 t ha^{-1} y^{-1} or 2190 t km^2 y^{-1}(Moheimani 2006)

Flocculation mixing speed = 0.075 m s^{-1} (McConnachie 1993)

Average recovery efficiency at 24 h = 90% (experimental result)

Average recovery efficiency at t hours = 0.36 + 0.023 t (experimental result)

Optimum recovery

The recovery at 24 h is 90 %, and from Table 5.7, it is estimated that it will take 8.75 days for the culture to complete the growth cycle for the next harvest. However, in the case of 80 % recovery, it only takes 6.25 days for the next harvest. The reduction in length of growth cycle is = $\frac{(8.75-6.25)days}{8.75days}$ or 28.6 %

This more than compensates for the 10% reduction in recovery. Also, as the media and cell culture can be recycled without any treatment or extra cost, a shorter growth / harvesting cycle correspond to similar reduction in pond size and mixings energy required.

Table 5-7 Number of days to reach the steady phase

Cell concentration (as % of steady phase)		10	20	40	80	100
Number of days to reach the required concentration	10 % start culture	0	2.5	5.0	7.5	8.75
	20 % start culture		0	2.5	5.0	6.25

(From 10 % & 20 % starting concentrations).

The higher starting cell concentrations and the more frequent harvestings will discourage the growth of other contaminating algae. Therefore, for the design for the flocculating tank, 80 % recovery is chosen as a design parameter.

For a HRAP of surface area 1 km^2, average annual turnover = (365 days / 6.25 days) = 58.4 times

The total volume of media treated annually
= 58.4 x (surface area x depth of pond) $m^3 y^{-1}$
= 58.40 x 10^6 m^2 x 0.2 m y^{-1}
= 1.168 x 10^7 m^3 y^{-1}
= 3.20 x 10^4 m^3 d^{-1}
= 0.37 m^3 s^{-1}
In practice, consideration will be given to seasonal variations, and excess capacity will be allowed during summer where algal growth rates are higher.

For a dry mass concentration of 0.05% and 80 % recovery,
Annual biomass production
= 1.168 x 10^7 m^3 x 0.05 % x 80 %
= 4.67 x 10^3 tonne per annum

For 80% recovery, from Equation 4.3, the mixing time required is
0.8 = 0.36 + 0.023 t
t = 19.13 h

The depth of the flocculation tank is determined by the gravitational settling rate of the flocs. However, they are difficult to estimate as the flows are necessarily turbulent for proper mixing, furthermore, both the flocs sizes and shapes will change during the settling due to the turbulent flow and the attachment with other microalgal cells. All these changing parameters make theoretical estimation very unreliable. A simpler and more practical way is to model from an existing water treatment plant that has been shown to be operating successfully.

The Bolivar Wastewater Treatment plant is situated 20 km north of Adelaide, South Australia, it has a design capacity of 1.35 x10^5 m^3 d^{-1} and consists of 8 circular settler / clarifiers of 5278 m^3 each and 4 sedimentation tanks of 6693 m^3 each. One quarter of the plant design capacity (3.375 x 10^4 m^3 d^{-1}) will be sufficient to meet the loading of the hydraulic flocculator for the harvesting.

The 8 circular settlers/clarifiers are of the same dimensions; 40 m in diameter, height of tank at the perimeter is 3 m while at the centre is 6.6 m. the rectangular sedimentation tanks are of length 68.4 m x width 23.3 m x height 4.2 m. For demonstration purposes in this chapter, a sedimentation tank of length 70 m x width 24 m x height 4 m is used.

Total Residence time
= resident time in clarifier + resident time in sedimentation tank
= 5278 m^3 / (0.5 x 1.33 x 10^3 m^3 h^{-1}) + (6720 m^3 / 1.33 x 10^3 m^3 h^{-1})
= 12.98 h

This residence time is still about 6 h under the 19.13 h required. As it is more economical to build a circular clarifier than a baffled pond, one of the possible solutions is to increase pond diameter from 40m to 52m while the depth remains the same. The total volume will be 2.456 x 10^4 m^3 and the total mixing time will be 19 h.

For a mixing speed of 0.075 m s^{-1}, the number of channels
= (residence time x mixing speed) / pond length
= [(6720 m^3 / 1.33 x 10^3 m^3 h^{-1}) x 3600 x 0.075] / 24 m
= 56.7 or 57 channels (i.e. 56 baffles)

Channel width
= 70 m / 57
=1.23 m (less width of baffles)

The new configuration consists of 2 circular clarifiers each of 52 m in diameter and 1 baffled hydraulic flocculator of 70 m in length by 24 m in width by 4 m in depth with 56 baffles of internal channel width 1.23 m. A sketch is shown in Figure 5.5.

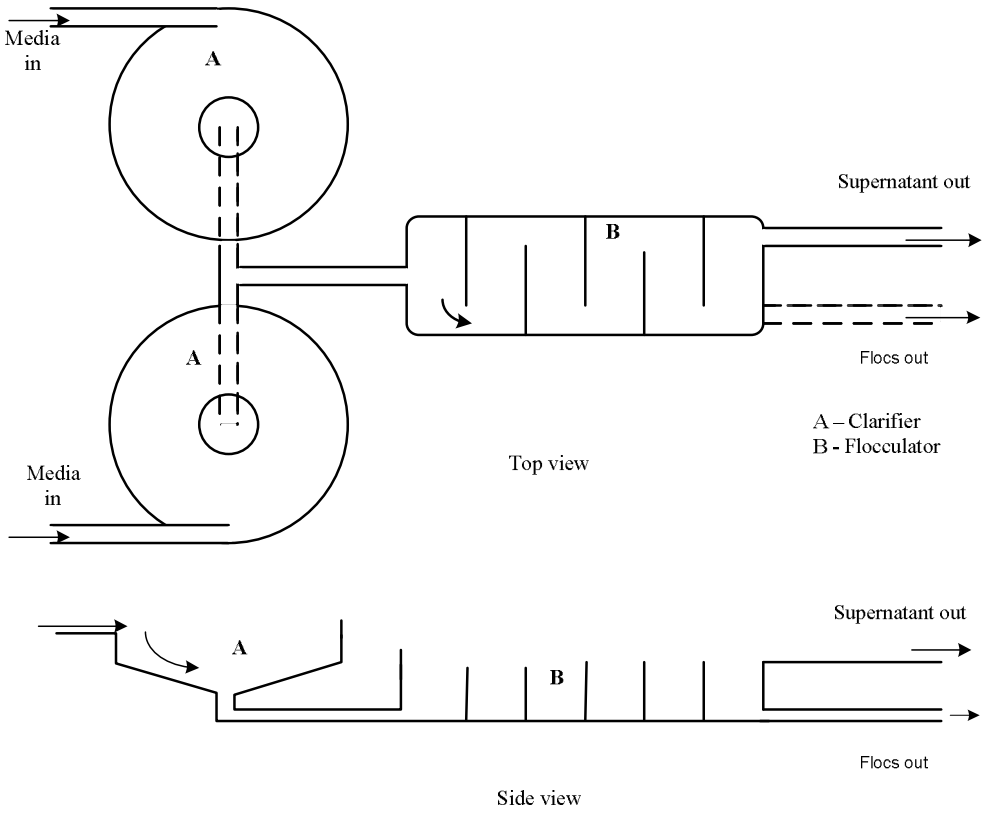

Figure 5-5 sketch showing the top and side view of the clarifier and flocculator

Energy consumption

Manning's Equation for open channels is used to estimate the head loss and hence the power requirement in the flocculator (assuming smooth surface of the baffles) (Perry 1997).

$$h_l = f\frac{L}{D}.\frac{v^2}{2g}$$

Where; h_l head loss, m

f Manning's constant

L length of total passage, m

D equivalent hydraulic diameter m

v flow velocity, m s^{-1}

G gravitational constant

L = 57 baffles x 24 m = 1368 m

R_h = hydraulic radius = $\dfrac{dw_c}{2d + w_c}$

Where; w_c channel width

d depth

Hydraulic radius = $\frac{(4m)(1.23m)}{2(4m)+1.23m}$ = 0.533 m

For open channels;

Equivalent diameter = D_{eq} = 4 x R_h = 4 x 0.533 m = 2.132 m

For open channels, a Reynold's number (Re) of over 2000 indicates turbulent flow;

Re = (ρ D_{eq} v) / μ

Where; ρ = density of media, kg m^{-3}

μ = viscosity of media, 1.31 x 10^{-3} Pa s

Therefore;

Re = $\frac{(1030)(2.132)(0.075)}{1.31x10^{-3}}$ = 1.258 x 10^5

The Reynold's number shows that the media flow in the flocculating channels are turbulent and therefore have good mixing. Manning's equation becomes

$$h_l = f\frac{L}{D}.\frac{v^2}{2g} = \frac{(0.019)(57x24)0.075^2}{(2.12)(2)(9.8)} = 0.0035\text{m}$$

Head loss due to bends = h_l = n $\frac{v^2 N_l}{2g}$

Where; n number of bends

N_l head loss coefficient

Head loss due to bends, h_l

= 56 $\frac{(0.075)^2}{(2)(9.8)}$ 3.1

= 0.0535 m

Total head loss = (h_1 + h_2)

= (0.0535 m +0.0035 m)

= 0.057 m

Total power = QΔP

= Qρg (h_1+h_2)

= (0.37 m^3 s^{-1}) (1030 kg m^{-3}) (9.8 m s^{-2}) (0.0535 m +0.0035 m)

= 213 W

Energy consumed for each batch of 2.456 x 10^4 m^3

= power consumption x time

= (213 W) (19 h)

= 4.05 kWh

Assuming 80% motor efficiency and 80% pump efficiency

Total energy

$$= \frac{4.05\ kWh}{0.8x0.8}$$

= 6.32 kWh or 2.58 kWh per 10^4 m^3 of algal suspension

= 0.52 kWh per 10^3 kg dry mass equivalent

The net surface area of the 2 clarifier and one flocculator

= 2π (radius)^2 + length x width of flocculator

= 2π (26m)^2 + 24m x 70 m

= 5927 m^2

This represents approximately 0.6% of the total open pond area for the microalgae culturing.

The physical dimension and the power consumption are within reasonable limit, therefore microalgal harvesting by microbial flocculation is feasible on a theoretical basis. All parameters used in the calculation have to be confirmed by laboratory trials as much as practicable and scaling up factors need to be incorporated in the actual design.

Summary

Based on a HRAP of an area 1 km^2, one of the feasible configurations is as follow;

The incoming media of 0.37 m^3 s^{-1} is divided into two equal stream of 0.185 m^3 s^{-1} each entering tangentially into a circular clarifier to maximise mixing. The clarifiers are 52 m in diameter, 3 m at the side and 6.6 m at the centre. After a resident time of 8 h, the media exit at the centre at the bottom of the clarifier to a rectangular baffled hydraulic flocculator of 24 m x 70 m, consists of 57 channels (56 baffles) of

separation 1.23 m for proper floc settlement and separation. The power required for the mixing in the flocculator is equivalent to a hydraulic head of 0.057 m; therefore by constructing the top of the circular clarifier 0.06 m above the top of the flocculator, the hydrostatic pressure will be sufficient for the mixing.

This design is based on the existing wastewater treatment plant at Bolivar, 20 km north of Adelaide, South Australia. The main purpose is to give an indication of the total energy required for the flocculation and the physical dimensions have to be optimised by laboratory studies and scaling up.

5.12 Recycling of the media

One of the apparent advantages for using the marine microalga *P. carterae* as a feedstock for biodiesel is that seawater can be available in unlimited quantities; however, process treatments to make the sea water suitable for culturing such as pumping, filtration, and the addition of fertilisers will all add to the cost, making it necessary to recycle the media to economise the operation. Furthermore, the nutrients in the original media recipes are usually presented in excess quantities and the recycling of the media will minimise the residual fertilisers to be discharged into the environments. Hence in terms of the cost saving and the environmental management, it is essential to recycle as much media as possible.

The test for the recycling of media is relatively straight forward; 300 ml of the supernatant from the flocculation experiments was collected and cultured under normal growth condition as in Section 3.3 without any treatment or starting culture. The cell concentration was recorded daily and a growth curve was constructed.

Results and discussion

P. carterae was observed to grow normally in the recycled media and the cells were not under stress, the growth curve shows no signs of a lag phase (Figure 5.6), but a larger amount of flocs than those found in the original media were observed. The EPS did not seem to have any apparent effects on the cell counts. This could be due to the fact that most of the bacteria were concentrated in the flocs, but some were left in the media as free swimming bodies.

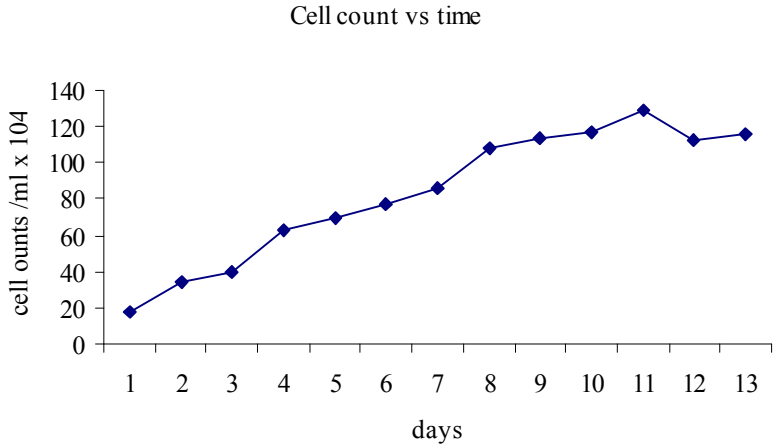

Figure 5-6 *P. carterae* cell counts using only recycled media.

The normal growth curve also shows that there is sufficient nutrient remained in the media and this will not be a growth limiting factor; it is rather the availability of irradiation, the space, or CO_2 that are essential.

5.13 Comparison with other harvesting methods.

As explained in Chapter 2, the traditional harvesting methods, apart from the high costs, suffer from a few other major drawbacks; centrifugations, especially the continuous discharge types, cause cell damages and the loss of cell inclusions; flocculations require inorganic chemicals and will contaminate the biomass; filtration alone is ineffective due to the small sizes of the microalgae and bioflocculation is too unreliable.

For the production of biodiesel, microbial flocculation is potentially a significant improvement over other commercial harvesting methods. In these experiments the microalgal cells were not damaged, and the metabolites are not lost. The cells' integrity and vitality are maintained and is able to continue growing in the recycled media after harvesting; cells remained viable among the flocs and hence no apparent change in cell composition; the flocculation and settling behaviour was observed to be predictable and reliable. All these factors are essential in commercial harvesting. At larger scale operations, media could be reused to minimise the cost of fertilisers and the demand for water; the organic carbon substrates used in the process are relatively low-cost and potentially readily available since glycerol may be obtained as a by-

product of the transesterification during the production of biodiesel, acetic acid is one of the major products during the anaerobic digestion of residue after lipid extraction. Since no metallic flocculants were used, the products and the residues are not contaminated by trivalent metallic flocculants.

Finally, the minimum concentration of organic carbon substrate used in these experiments (0.1 g L^{-1}) is still high in comparison to the dry mass concentration of the microalgal suspension, which is in the order of 0.5 g L^{-1}. Therefore, further research is required to reduce the level of substrates and to minimise the mixing energy required for this process.

Table 5-8 Comparison with other harvesting methods

Commercial harvesting Methods	Advantages	Disadvantages	Energy/cost
Filtration	Cost effective for filamentous species, products can be chemical free.	Not suitable for most species to their small and compressible cells, filtration efficiency decreases due to clogging of the membrane.	1 kWh per kg dry mass at a rate of 2-3 m^3 h^{-1} of algal suspension and membrane pore size of 5 μm
Centrifugation	Products are not contaminated	Cells may be ruptured by shear. Marine species will require modifications due to high corrosion.	3.5 kWh kg^{-1} from 0.2 kg m^{-3} algal suspension at 1 m^3 min^{-1} USD 1.71 kg^{-1} in 1995 (AUD 3.18 in 2009)*
Inorganic Flocculation	Well practised in large scale wastewater treatment	Products are contaminated by metallic ions such as Al^{3+}	USD 1.39 kg^{-1} in 1995 (AUD 2.58 in 2009)*
Microbial flocculation	The flocculation was predictable, cells remained viable among the flocs and raw materials are potentially low cost.	Flocculating bacteria are species specific	0.52 kWh per 10^3 kg or USD 0.20 per kg dry mass equivalent

Summary of microbial flocculation

The volume of EPS produced was not related to the efficiency of the flocculation and separation processes; it is the quality of EPS and the mixing time that determine the attachment and aggregation of cells. For maximum cell recovery, it is desirable for all the organic carbon added to be converted into TB-EPS.

A successful microalagal harvesting by microbial flocculation will have the following requirements;

- Long mixing time of 24 h.
- Flocculating bacteria and the corresponding substrate (s).
- Low substrate concentration of 0.1 g L^{-1}
- Mixing for contact between EPS and microalgal cells.
- Stress on the bacteria but not the microalgae.

Further optimisation of these conditions is essential for the scale up and success in commercial harvesting.

6 Conclusion

Microalgae can be harvested commercially using centrifugation, filtration, and flocculation, either used individually or in combination. However, for the commercial production of biodiesel, the processes need to be low cost, reliable and free from trivalent metal contaminations. Currently, none of the existing mass harvesting methods can satisfy all of the above criteria. Furthermore, bioflocculation by high pH or other environmental stress, a flocculation method which has been suggested by some literature, had been shown to be ineffective for *P. carterae*.

The prime objective of this project was to develop a harvesting method for the production of biodiesel from the marine microalgae *P. carterae.*

The experiments on microbial flocculation had demonstrated that *P. carterae* could be flocculated in the laboratory by first stimulating the growth of flocculating bacteria followed by stressing the same bacteria under nutrient depletion. The secretion of EPS by the stressed bacteria resulted in the flocculation of the microalgae. This process was effective in terms of solid/liquid separation; with a recovery efficiency (RE) of over 90% and a concentration factor (CF) of over 200, these results compared favourably with other traditional methods such as centrifugation or filtration.

The RE was correlated to the mixing time, t in hours by;
$RE\ (\%) = 36 + 2.3\ t$
While CF was correlated to mixing time t and substrate concentration, C (g L^{-1}) by
$CF = 136 + 4.95\ t - 15.23\ t\ C$
Both RE and CF were independent of the nature of the substrate.

This flocculation process was potentially low cost as evident from the following experimental facts; Firstly, the raw materials were only required in low concentrations (0.1 g L^{-1}); Secondly, the raw materials used (acetate or glycerine) might be obtained as by-products from the biodiesel production. Thirdly, as no inorganic flocculants were used, the media could be reused, reducing costs of purifying ordinary sea water to media and fertilisers. Finally, the biomass residue was not contaminated by Al^{3+}

and therefore had the potential for other uses such as animal feed, composting for fertilisers or co-firing in power plants.

By incorporating a circular clarifier and a baffled flocculator in a commercial plant design, this process had a low theoretical energy requirement of 2.8 kWh for every 10^4 m^3 of culture media; the high efficiency together with a low theoretical energy requirement makes this process attractive and warrants further investigation.

Recommendations for future research include further optimisation of the mixing time which will result in a reduction in the volume and construction cost of the flocculation tank and the mixing energy required.

Although the current dose of organic carbon at 0.1 g L^{-1} , compares favourably with other flocculants, this raw material still represents a significant part of the operating cost, and has the potential to be reduced. Future research will include the selection of more efficient flocculating bacteria strains to increase the efficiency, further reduction in the amount of raw material required and the substitution of acetate or glycerine by other cheaper form of organic carbon to lower the material cost.

Further experiments on scaling up will be required to optimise the plant dimensions and design. Some of the more important aspects to be investigated include; the settling characteristics of the flocs in a turbulent flow; and the possible flocculating mechanisms.

7 Nomenclatures

Abbreviations

ANOVA	Analysis of variance
Amp/strept	Ampicillin and streptomycin
CCMP	Culture Centre for Marine Phytoplankton
CCS	Carbon capture and storage
CF	Concentration factor
CNG	Compressed natural gas
CPI	Consumer price index
CSH	Cell surface hydrophobicity
DAFF	Dissolved air flotation and flocculation
DCB	Divalent Cation Bridging
DOC	Dissolved organic carbon
DLVO	Derjaguin, Landau, Verwey and Overbeek Theory
DMS	Dimethyl sulphide
E85	85% ethanol
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
ESRL	Earth System Research Laboratory
FAME	Fatty acid methyl esters
FFV	Flexible fuel vehicles
GDP	Gross domestic product
GTL	Gas to liquid
HRAP	High rate algal pond
IGCC	Integrated Gasification Combined Cycle
IPCC	Intergovernmental Panel on Climate Change
LB-EPS	Loosely bound EPS
LNG	Liquefied natural gas
LPG	Liquefied petroleum gas
NREL	The National Renewable Energy Laboratory
RE	Recovery efficiency
rRNA	Ribosomal RNA

SEM	Scan Electron Microscopy
SS	Stock solution
TB-EPS	Tightly bound EPS
TFF	Tangential flow filtration

Symbols

(A)	Factor (A)
(B)	Factor (B), g L^{-1}
(C)	Factor (C), h
C	Concentration, g L^{-1}
c	Coefficient
df	Degrees of freedom
e	Equivalent
D	Equivalent hydraulic diameter, m
d	Depth, m
f	Manning's constant
F	Force, Newton
F_A	Attractive force, Newton
F_G	Gravitational force, Newton
F_R	Repulsive force, Newton
F_S	Solvent force, Newton
F_T	Total force, Newton
G	gravitational constant, m s^{-2}
H	Ratio of the distance between approaching particles to the diameter of approaching particles
h_l	Head loss, m of media
L	Length of total passage, m
MS	Mean square
N	Hydraulic head coefficient
P	Hydraulic pressure, m of media
Q	Flow rate, m^3 s^{-1}
R	Radius, m

Re	Reynold's number
R^2	Correlation factor
SS	Sum of squares
t	Time, h
V	Volume, ml
v	flow rate, $m^3\ s^{-1}$
W	Weight, g
w	Width, m
ρ	density of media, kg m^{-3}
μ	Viscosity, Pa s

Subscripts

0	Initial
A	Attractive
A	Factor A
B	Factor B
C	Factor C
c	Channel
eq	Equivalent
f	Floc
G	Gravitational
h	Hydraulic
l	Loss
R	Repulsive
S	Solvent
T	Total
t	At time *t*

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