THE UNIVERSITY of ADELAIDE

ELUCIDATION AND ISOLATION OF SPECIFIC BIOACTIVE COMPOUND IN CYANOBACTERIA ISOLATES

by

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Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

Albert Einstein

ABSTRACT

Sulfoquinovosyldiacylglyceride (SQDG), one of the bioactive compounds isolated from *Spirulina*, has been proven to act as an inhibitor of reverse-transcriptase, and has potential for the use in the Combination Anti-Retroviral Therapy (cART). A number of researchers report that the many potential uses of *Spirulina*, with the most widely used strains being *Spirulina platensis* and *S. maxima*, require further investigation to determine their actual usefulness in medical applications.

In this work, four commercial isolates marketed as *Spirulina* and an Australian isolate, *Spirulina* sp. CS-785/01 (CSIRO), were selected to determine their bio-composition and applicability as a source of bioactive compounds. During the course of the investigation, it was determined that the four commercial isolates were in fact *Arthrospira* and the Australian isolate is *Halospirulina*. However, this insight occurred towards the end of the study and the '*Spirulina*' has been adopted as common name when referring to the isolates during the experimental programme that has been reported in the thesis. The commercial isolates used in this research were *Spirulina* (J), *Spirulina* (M), *Spirulina* (P) and *Spirulina* (S), while the Australian isolate *Spirulina* sp. was also used throughout the investigation.

The identification of these isolates was examined based on their molecular and the chemotaxonomic classification. Furthermore, the genes responsible for the production of SQDG were isolated to assess the potential therapeutic value of these isolates in treating disease, such as HIV (Human immunodeficiency virus). Since SQDG is a compound of current interest, a suitable extraction technique was developed to optimise its production. Different extraction techniques, such as microwave-assisted sonication and homogenisation, coupled with various forms of organic solvents, were examined in this study. Preliminary phytochemical analysis was also undertaken to reveal the potential use of the investigated isolates for the further development of pharmaceuticals due to the presence of specific phyto-constituents. Finally, sequential extracts and the isolated compound of SQDG were used to determine their bioactivity against a range of microbes and HIV.

The results revealed that the Australian isolate was not highly productive in respect to growth. This is due to its limited adaptability to changes in temperature and culture media type. The Australian isolate is limited to a specific temperature range and culture media type. Additionally, it has substantial biochemical variation compared to the commercial isolates. The diversity of these isolates could be explained by their molecular and chemotaxonomic classification, which revealed that the Australian isolate belongs to the genus of *Halospirulina,* while the commercial isolates belong to the genus *Arthrospira*. The carbohydrate, protein, lipid and fatty acid content of the commercial isolates also indicated that they have higher nutritional value when compared to the *Spirulina* sp. This strongly suggested that the commercial isolates belonged to a different genus to the *Spirulina* sp.

In spite of the diversity of the classification, all investigated isolates showed the presence of SQDG. Overall, the results showed that *Spirulina* (S) has a high potential for synthesis of SQDG, due to its high content of C16:0 and C18:2, while the SQDG content of Australian isolate was lowest among the investigated isolates. By applying this data, the Australia isolate is unlikely to be suitable for large-scale production. It also showed lower appreciable amounts of SQDG and gamma linolenic acid (GLA). The highest yield of SQDG from *Spirulina* (M) was from using a chloroform:methanol (2:1, v/v) extraction solvent system. However, a methanol extraction solvent system is suggested, due to its high recovery of SQDG and low toxicity. Because SQDG is a potent inhibitor of HIV-reverse transcriptase, it can be concluded that commercial isolates are good sources for drug production because of their high content of SQDG and rapid biomass production.

STATEMENT OF ORIGINALITY

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Chee Kuan Kwei

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LIST OF PUBLICATIONS

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2. C. K. Kwei, D. M. Lewis, K. D. King, W. Donohue, B. A. Neilan (2008), 'Therapeutic potential of *Spirulina* for the treatment of HIV', International Biotechnology Symposium and Exhibition, Dalian, China, 12-17oCtober 2008 (Poster Presentation).
3. C. K. Kwei, D. M. Lewis, K. D. King, W. Donohue, B. A. Neilan (2010), 'The extraction of sulfoquinovosyldiacylglyceride from *Spirulina*', CHEMECA, Adelaide, Australia, 27-30 September 2010 (Oral Presentation).
4. C. K. Kwei, D. M. Lewis, K. D. King, W. Donohue, B. A. Neilan (2011), 'Molecular classification of commercial *Spirulina* strains and identification of sulfolipid biosynthesis genes', *Journal of Microbiology and Biotechnology*, 21(4), pp. 365-371.
5. C. K. Kwei, D. M. Lewis, K. D. King, W. Donohue, B. A. Neilan (n.d), 'Preliminary phytochemical analysis of *Arthrospira maxima* and *Arthrospira seawater*', *Indian Journal of Pharmaceutical Sciences* (Submitted).

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ABBREVIATION

^{o}C	Degree Celsius
α	Alpha
AIDS	Acquired Immunodeficiency Syndrome
AFDW	Ash free dry weight
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BuOH	Butanol
C	Cytosine
$CaCl_2$	Calcium chloride
cART	Combination Anti-retroviral Therapy
$CHCl_3$	Chloroform
chl	Chlorophyll
CSIRO	Commonwealth Scientific and Industrial Research Organization
DGDG	Digalactosyldiacylglycerol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide trisphosphate
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FAME	Fatty acid methyl esters
g	Gram
G	Guanine
GC	Gas chromatography
GLA	Gamma linolenic acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus

HPLC	High pressure liquid chromatography
HSV	Herpes simplex virus
IMVS	Institute of Medical and Veterinary Science
IUPAC	International Union of Pure and Applied Chemistry
kb	Kilobases
L	Litre
m	Milli
MeOH	Methanol
mg	Milligrams
$MgCl_2$	Magnesium chloride
MGDG	Monogalactosyldiacylglycerol
min	Minute
MS	Mass spectrometry
NaAc	Sodium acetate
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse-transcriptase inhibitors
NRPS	Non-ribosomal peptide synthase
NRTIs	Nucleoside reverse-transcriptase inhibitors
PCC	Pasteur culture collection
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PUFA	Poly unsaturated fatty acids
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
rpm	Revolution per minute
s	Second
SARDI	South Australian Research and Development Institute
SDS	Sodium dodecyl sulphate
SQDG	Sulfoquinovosyldiacylglycerol
TAE	Tris-acetate-EDTA
TE	Tris-EDTA

Tris	Tris hydroxymethyl aminomethane
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UNSW	University of New South Wales
UV	Ultraviolet
V	Volt
v/v	Volume per volume
WH	Woods Hole
w/v	Weight per volume
XS	Xanthogenate-SDS

CHAPTER 1 INTRODUCTION

Many plants, including algae, produce a tremendous variety of secondary metabolites that are useful in clinical research. Interest in studying algae for medicinal applications has increased, because many bioactive compounds in algae have anti-coagulant properties and anti-platelet and anti-tuberculosis potential (Mayer & Hamann, 2002). This chapter reviews the comprehensive and up-to-date information available on natural products, and briefly describes the background of the investigation that addresses global health issues, the Acquired Immunodefiency Syndrome (AIDS) and the importance of developing better treatment for the systematic health of patients with HIV infection.

1.1 Aims

Cyanobacteria, belonging to the genus *Spirulina* and previously grouped within the genus *Arthrospira* (Vonshak, 1997), is cited in the published literature as having the potential to yield novel pharmaceutical compounds. Aside from the medical sector, *Spirulina* has been introduced as food supplement, and the market for *Spirulina* has resulted in the production of 22,490 tonnes of drymass from 2003–2004 in China alone (Belay *et al*,. 1993; Habib *et al*., 2008). *Spirulina* is rich in nutrients, and is available in tablet and powder form. However, to date, no specific strain of *Spirulina* has been proposed for anti-HIV treatment. Furthermore, classification of the genus *Spirulina* remains unclear and many products marketed as '*Spirulina*' may actually belong to the genus *Arthrospira*, and vice versa.

In an attempt to further knowledge on this topic, this PhD research project focused on investigating four commercial isolates marketed as '*Spirulina*', and an Australian *Spirulina* isolate, *Spirulina* sp. CS-785/01 Commonwealth Scientific and Industrial Research Organisation (CSIRO), for their nutritional value and potential for the treatment of HIV. The overall objective of this thesis was to contribute information to help recognising commercial *Spirulina* isolates and an Australian *Spirulina* isolate as sources for bioactive compounds. In addition, it evaluated that the identification of *Spirulina* isolates were incorrect and taxonomic confusion within *Spirulina* and *Arthrospira* was evident. Throughout the thesis, the designations of these isolates were

based primarily on the common name '*Spirulina*' to avoid confusion with names of *Spirulina* or *Arthrospira*.

The following coding system was chosen to remove any ambiguity about the source of the particular strain that was studied. The letter S, J, M and P were chosen for the individual *Spirulina* isolates, which related to: S is for South China Sea Institute Oceanology (SCSIO), J is for Jiangmen Yue Jian Biology Engineering Co. Ltd.; M is for *Spirulina maxima* and P is for *Spirulina platensis*.

The main objectives of this research were to:

1. Elucidate the growth conditions of an Australian *Spirulina* isolate to obtain large amounts biomass for extraction, as well as for screening for bioactive compounds).
2. Study the molecular biology of four commercial *Spirulina* isolates and an Australian *Spirulina* isolate to identify these *Spirulina* isolates at the genus and species levels, screen the non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) genes, and understand the biosynthesis pathway of sulfolipids from the five *Spirulina* isolates.
3. Compare the five *Spirulina* isolates for their bio-composition to identify major biologically active phyto-constituents, evaluate the nutritional value of the five isolates, for example commercial *Spirulina* isolates and Australian *Spirulina* isolate; which are potentially and highly active in producing potential bioactive compounds.
4. Determine suitable extraction techniques for the five *Spirulina* isolates to optimise the yield, with a focus on sulfolipids, which were found to have high potency as reverse-transcriptase inhibitors.
5. Test the bioactivity against a range of microbes including HIV to verify various bioactive modes of actions of sulfolipids.

1.2 Natural products

Pathogens and newly emerging infections have developed resistance to the drugs available for treatment (Cragg & Newman, 2002). A large number of potential natural products have been found, and some of them can be a cost effective inhibitor against various infections (Lee, 2003). In addition, many researchers have focused their

attention on these natural products because of their low toxicity and considerable medicinal benefit. It is also reported that more than 150,000 natural products have been screened, and over 60% of anti-cancer and anti-infective agents were approved world-wide from 1983–1994 (Cragg & Newman 2002; Yang *et al*. 2001). To evaluate the current findings in the development of drug discovery, a library containing a compilation of natural products is available. These natural products were researched and developed to produce more affordable medication, especially in developing countries.

There are 250,000 to 300,000 species of plants identified on Earth (Borris, 1996). Plants were used as medicines in ancient times because they contained particular chemical compounds that can cure and prevent infection. The rich variety of bioactive compounds from plants directed civilization to use them for traditional healing, and many of them have compiled their own pharmacopoeia fabricated from plants. Plants are one of many organisms consisting of a tremendous variety of secondary metabolites that were found to be very useful in clinical research. These substances are derived from the plants secondary metabolism, and are produced for defence against external factors such as environmental factors (Huyghebaert, 2003). Therefore, many people have used plants as a source of drugs (Koda, 2007).

Many investigations have reported potential applications of the bioactive compounds produced in plants. Many bioactive compounds have potential in anti-microbial, anti-diarrheal, anti-inflammatory and other bioactivities (Cowan, 1999; Schinella *et al*., 2002). Alkaloids, coumarins, flavonoids, lignans, phenolics, quinones, saponins, terpenes, xanthones, carbohydrates, peptides and proteins are also reported to responsible for anti-HIV activity. A list of the important bioactive components and their sources are divided into several categories, and summarised in Appendix A.

Natural products extracted from algae and cyanobacteria appeared to be very useful, and have several biological activities, such as anti-bacterial, anti-fungal, anti-coagulant, anti-platelet, anti-tuberculosis and anti-viral activity (Abed *et al*., 2009; Mayer & Hamann, 2002). Robertson and Fong (1940) conducted one of the first research activities describing the significant role of algae in the development of pharmaceuticals, such as the anti-bacterial properties of *Chlorella vulgaris*. Such findings have generated an increase in the number of research reports on algae and cyanobacteria. They also

helped scientists exploit their potential in relation to various mechanisms. Cyanobacteria are not only sources of food for humans and animals, the natural products produced are valuable for ecological application (Abed *et al*., 2009; Abed & Koster, 2005).

Algae and cyanobacteria have also been recognised as a potential source of anti-HIV agent (Gustafson *et al*., 1997). Compounds isolated from algae that inhibit anti-HIV activity include sulphated polysaccharides, diterpenes, carrageenans, fucoidan and sesquiterpene hydroquinones (Beress *et al*., 1993; Boyd *et al*., 1997; Gustafson *et al*., 1989; Gustafson *et al*., 1997; Haslin *et al*., 2001; Hayashi *et al*., 1996a; Hoshino *et al*., 1998; Khan *et al*., 2005; Lau *et al*., 1993; Loya *et al*., 1995; Loya *et al*., 1998; Nakashima *et al*., 1987; Nowothy *et al*., 1997; Ohta *et al*., 1998; Reshef *et al*., 1997; Schaeffer & Krylov, 2000; Spieler, 2002; Tziveleka *et al*., 2003). Table 1.1 presents all bioactive compounds isolated from algae and cyanobacteria that have been widely studied for their potential use as anti-HIV agent. As shown in Table 1.1, cyanobacteria have been researched for their high concentration of secondary metabolites. A total of 23 cyanobacteria strains showed positive results for the inhibition of cell fusion, transmission, viral replication, reverse-transcriptase and protease. Research focussed on red algae and brown algae has received less attention than cyanobacteria. Screening programmes have demonstrated that the occurrence of anti-HIV reverse-transcriptase inhibition can occur from the activity of extracts from microalgae.

Table 1.1 Anti-HIV products from algae and cyanobacteria

Bioactive compound	Species	Phylum	Inhibition
Cyanovirin-N	*Nostoc ellipsosporum*	Cyanobacteria	CF T
Calcium-Spirulan	*Spirulina platensis*	Cyanobacteria	VR
Sulfolipids,	*Oscillatoria raoi*	Cyanobacteria	
Sulfoglycolipids	*Scytonema spp.*	Cyanobacteria	
	Oscillatoria trichoides	Cyanobacteria	
	Phormidium tenue	Cyanobacteria	
	Oscillatoria limnetica	Cyanobacteria	
Sulfoglycolipid	*Scytonema spp.*	Cyanobacteria	RT
Acylated diglycolipids	*Oscillatoria spp.*	Cyanobacteria	
Sulfolipids	*Lyngbya lagerheimii*	Cyanobacteria	
	Phormidium tenue	Cyanobacteria	
	Phormidium cebennse	Cyanobacteria	
	Oscillatoria raciborskii	Cyanobacteria	
	Scytonema burmanicum	Cyanobacteria	
	Calothrix elenkinii	Cyanobacteria	
	Anabaena variabilis	Cyanobacteria	
Aqueous extract	*Microcystis aeruginosa*	Cyanobacteria	PI
Polysaccharide	*Schizymenia pacifica*	Red Algae	RT
Polysaccharide	*Fucus vesiculosus*	Brown Algae	RT
Fucoidans	*Adenocystis utricularis*	Brown Algae	
Sulphated polysaccharide	*Agardhiella tenera*	Red Algae	
Extract	*Aphanocapsa pulchra*	Cyanobacteria	RT
Extract	*Aphanocapsa pulchra*	Cyanobacteria	RT
Extract	*Aphanothece clathrata*	Cyanobacteria	RT
Extract	*Aphanothece nidulans*	Cyanobacteria	RT

CF: Cell Fusion; T: Transmission; VR: Viral replication; RT: Reverse-transcriptase

(**Table 1.1 Continued)**

Bioactive compound	Species	Phylum	Inhibition
Polysaccharide	*Asparagopsis armata*	Red Algae	
Diterpenes	*Dictyota dichotoma*	Brown Algae	
	Dictyota patens	Brown Algae	
Sulphated Polysaccharide	*Euchema cottonii*	Red Algae	
Extract	*Aphanothece nidulans*	Cyanobacteria	RT
Polysaccharide	*Asparagopsis armata*	Red Algae	
Diterpenes	*Dictyota dichotoma*	Brown Algae	
	Dictyota patens	Brown Algae	
Sulphated Polysaccharide	*Euchema cottonii*	Red Algae	
Sulphated Polysaccharide	*Gigardina aciculaire*	Red Algae	
Sulphated Polysaccharide	*Gigardina pistillata*	Red Algae	
Sulphated Polysaccharide	*Nothogenia fastigiata*	Red Algae	
Sesquiterpene hydroquinones	*Peyssonelia sp.*	Red Algae	RT
Extract	*Phormidium valderianum*	Cyanobacteria	RT
Polysaccharide	*Sargassum horneri*	Brown Algae	
Sulfolipids	*Spirulina platensis*	Cyanobacteria	
Carrageenan		Red Algae	
Sulfoquinovosyldiacylglycerol, KM043	*Gigartina tenella*	Red Algae	RT

CF: Cell Fusion; T: Transmission; VR: Viral replication; RT: Reverse-transcriptase

Adapted from: Beress *et al*., 1993; Boyd *et al*., 1997; Gustafson *et al*., 1989; Gustafson *et al*., 1997; Haslin *et al*., 2001; Hayashi *et al*., 1996a; Hoshino *et al*., 1998; Khan *et al*., 2005; Lau *et al*., 1993; Loya *et al*., 1995; Loya *et al*., 1998; Nakashima *et al*., 1987; Nowothy *et al*., 1997; Ohta *et al*., 1998; Reshef *et al*., 1997; Schaeffer & Krylov, 2000; Spieler, 2002; Tziveleka *et al*., 2003

1.3 Current HIV treatment used

Remarkable development has been made in the field of medicine with enhancements in science and technology, including diagnosis, treatments and pharmaceuticals. The current method of treating HIV is to suppress viral replication with drugs. This can also reduce the risk of the transmission of the virus. Combination Anti-Retroviral Therapy (cART) is the combination of several drugs used for the treatment of HIV infection.

Combining several anti-retroviral drugs can interfere in multiple stages of HIV replication. Many of these drugs have been brought to market, and most of the drugs that have been approved are protease and transcriptase inhibitors. Natural products from plants and microalgae reported to have the modes of action of nucleoside reverse-trancriptase inhibitors (NRTIs) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs) could potentially produce more affordable drugs. For instance, sulfolipids from cyanobacteria are reverse-transcriptase inhibitors, and have a high potential to act as the therapeutic agents for a remedy for HIV.

Anti-retroviral drugs inhibit in different stages of HIV replication. This treatment tends to prolong the life of the people living with HIV. To achieve better health for HIV patients, the right combination of drugs are needed. Some people living with cART treatment have been shown to have better health quality (UNAIDS, 2011). However, this treatment is expensive, particularly for patients in developing nations, and treatment services are hard to access. Some HIV patients receiving cART treatment might suffer several of the common adverse effects of the drug, such as abdominal pain, anaemia, asthenia, diarrhoea, flatulence and headache. If regimens are more complex, patients are more likely to miss doses and the virus may develop drug resistance (Teas *et al*., 2004). Current medication has been shown to increase the life expectancy of HIV patients; however, there are some side effects depending on the specified patient.

1.4 Human immunodeficiency virus

In spite of the advance in technology, the Acquired Immunodeficiency Syndrome (AIDS) remains as a major cause of mortality world-wide, and the mortality rate is still increasing. AIDS is a class of disease in which the human immune system begins to become less effective. Moreover, this disease can lead to death. HIV causes this disease, which is one of the members of the retrovirus. The two main types of HIV that infect humans are HIV-1 and HIV-2. Among these two viruses, HIV-2 is found to be less prevalent, because it is confined to West Africa and India (Tebit *et al*., 2010). The first published case of HIV was in June 1981, where five young homosexual men from the United States of America (USA) were diagnosed with *Pneumocystis carinii* pneumonia (PCP). HIV-1 and HIV-2 were originally found in chimpanzees and West African monkeys respectively. These viruses were then transmitted from chimps to humans in the Sub-Saharan region in the late nineteenth century (Gao *et al*., 1999; Huet *et al*., 1990; Peeters *et al*., 1989).

1.4.1 Epidemiology of HIV

HIV infection is an epidemic, especially in developing countries. According to the Joint United Nations Programme on HIV/AIDS, in 2010 an average of 34.0 million (31.6–35.2 million) people were living with HIV, 2.67 million (2.46–2.90 million) people had become infected with HIV and 1.76 million (1.59–1.91 million) people died of AIDS-related causes (UNAIDS, 2011). In addition, HIV affects people at all ages, and Table 1.2 shows the estimated number of AIDS cases in 2010.

Table 1.2: Global summary of the AIDS epidemic December 2010

Number of people living with HIV in 2010	
Total	34.0 million [31.6–35.2 million]
People newly infected with HIV in 2010	
Total	2.67 million [2.46–2.90 million]
Children under 15 years	390 000 [340 000–450 000]
AIDS deaths in 2010	
Total	1.76 million [1.59–1.91 million]

Adapted from UNAIDS, 2011

The ranges around the estimates in this table define the boundaries within which the actual numbers fit based on the best available information.

Although the number of new HIV infections has stabilised in some countries, HIV is still a world-wide health problem, as can be seen in Figure 1.1. This issue has attracted a lot of public attention, and there is current world-wide interest in finding a new and effective treatment to control HIV infection.

NOTE:
This figure is included on page 9 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1: Global prevalence of HIV in 2009

Adapted from UNAIDS, 2010.

1.4.2 Signs and symptoms of HIV/AIDS infection

People with primary infection are often mistakenly diagnosed as having influenza. A person who is infected might shows symptoms such as fever, headache, tiredness, diarrhoea, skin rashes, rapid weight loss, a persistent dry cough and other impacts that affect quality of life (Bowtell & Weissbort, 2010; Thaplial *et al*., 2007). HIV can lie in the host quiescently for a period of eight or more years (Guss, 1994). However, if the virus begins to activate, the immune system will be damaged, with a subsequent declination in the number of CD4 cells. HIV patients who have a low CD4 cell count increase the risk of developing an opportunistic infection, including Kaposi sarcoma and/or non-Hodgkin lymphoma. The risk of progression to AIDS is higher for haemophiliacs, who have a CD4 cell count of less than 200 cells/μl for more than one year. Furthermore, 'patients with baseline CD4 cell counts less than 100 cells/μL have much higher cumulative mortality estimates at 1 and 4 years (11.6 and 16.7%) compared with those of patients with baseline counts of at least 100 cells/μL (5.2 and 9.5%, respectively) largely because of greater cumulative person-time at CD4 cell counts less than 200 cells/μL (Lawn *et al*., 2009).

To develop new therapy for HIV treatment, an understanding of the HIV replication cycle is essential in helping to interfere with the process. Figure 1.2 depicts the process of HIV entry into the cells.

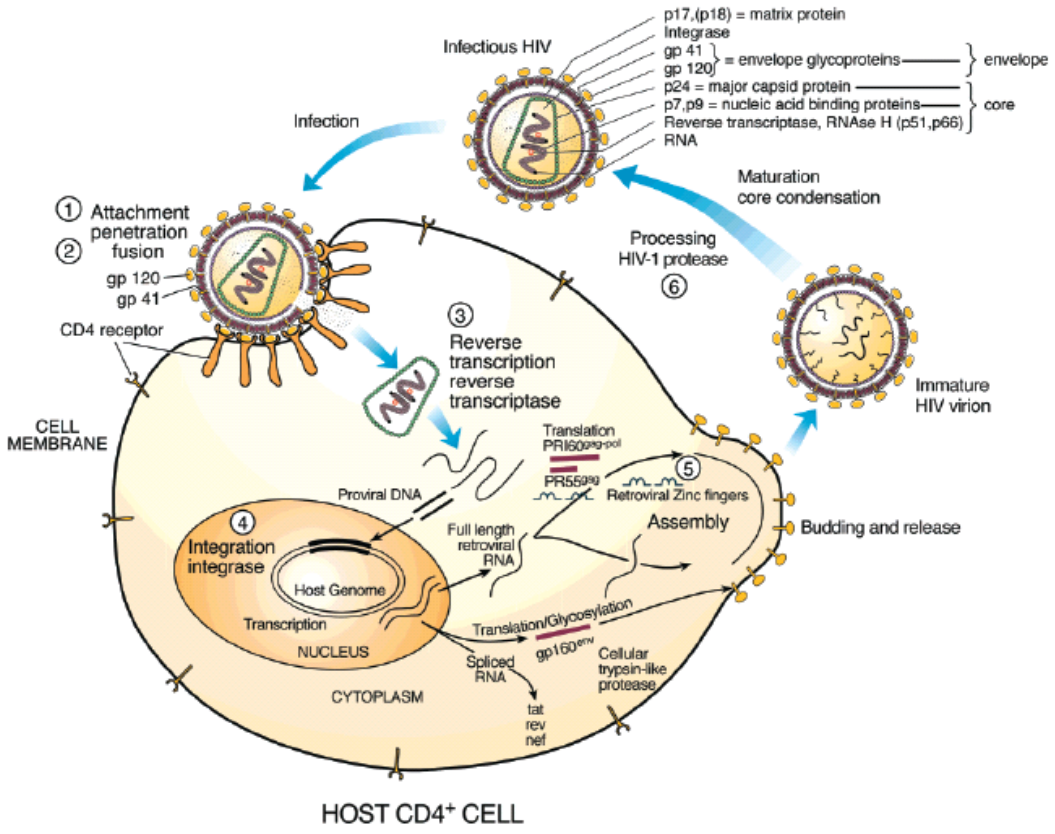


Figure 1.2: Targets within the different phases of HIV-1 viral replication cycle and infection of a T-cell, as used for anti-HIV cell-based assays

Adapted from Yang *et al*., 2001.

In the primary stage, HIV enters the immune system, specifically the CD4 cell. After targeting the cell membrane, HIV releases the capsid into the cell and liberates the single-stranded RNA to double-stranded DNA. A complementary DNA molecule enters the nucleus of the CD4 cell and inserts itself into the cell's DNA. The DNA then produces virus RNA. Virus RNA produces the virus protein and the virus protein is cleaved. A new virus, as shown in Figure 1.2, step five, is assembled and leaves the cell. This new virus, as shown in Figure 1.3, is ready to infect other CD4 cells.

NOTE:
This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3: The expression of HIV

(a) New copies of HIV bud from the surface of an infected Helper T-cell. (b) During budding, the nascent capsid of the HIV associates with the plasma membrane of the host cell and induces the extrusion of the lipid bilayer (Roingeard & Brand, 1998).

1.5 Gap statement

Acquired Immune Deficiency Syndrome is a serious illness throughout the whole world. Much research has been conducted in order to develop treatment options and affordable medication for HIV patients. Several researchers have found that the alga *Spirulina*, which is consumed by people in Lake Chad basin, Africa, has shown constantly low percentages of populations infected with HIV and thus studies in investigating the therapeutic effects of *Spirulina* have been performed. The screening of the bioactive compounds for anti-HIV properties from *Spirulina* has been implemented; however, researchers have not sought to overproduce these compounds from *Spirulina*.

It was previously mentioned that sulfolipids have the potential in inhibiting HIV activity. This bioactive compound is expressed from the photosynthetic membranes of higher plants, mosses, ferns, microalgae and most photosynthetic bacteria. Interest in the bioactive compound production from microalgae was encouraged by Babu and Rajasekaran (1991) who stated that: 'the microalgae have advantages such as high growth rate, the use of areas not suitable for agricultural and the possibility to induce the cultivation for the production of different compounds'

Spirulina is regarded as a good candidate for several biotechnological applications because of its potential in producing bioactive compounds without causing toxicity to humans. For example, the genera of cyanobacteria, such as *Microcystis*, *Nodularia*, *Oscillatoria*, *Scytonema*, *Lyngbya* and *Calothrix*, possess sulfolipids. However, these

strains cause toxicity to humans (Loya *et al*., 1998; Shirahashi *et al*., 1993). In addition, *Spirulina* does not impose a severe impediment for mass production, and the species of *Spirulina* has been commercially cultivated world-wide. The availability of the raw material is necessitated to produce sufficient amounts of biologically active compounds for preclinical evaluation.

The nutritional value of *Spirulina* is extensive, creating interest in species such as *Spirulina platensis* and *Spirulina maxima*. To date, many of the unknown species of *Spirulina* have not yet been examined for their biochemical composition and nutritional value. Furthermore, the taxonomy of *Spirulina* is confused (e.g. with *Arthrospira*), and the published data based on *Spirulina* is a doubt. Therefore, there is a need for reassessment for the biochemical composition and nutritional value of the *Spirulina* and *Arthrospira*. Four commercial *Spirulina* isolates and an Australian *Spirulina* isolate were used to assess the taxonomic classification of these isolates. In part, this research aimed to broaden the selection of the isolates of *Spirulina* to act as a source of novel pharmaceutical compounds. The five *Spirulina* isolates were screened for SQDG in particular, because this bioactive compound has ability to inhibit reverse-transcriptase, which was revealed by Gustafson *et al*. (1989). A comparison between the different extraction methods were undertaken for the five investigated isolates and was evaluated to obtain the best yield of sulfolipids. Additionally, the biosynthesis pathway of sulfolipids was investigated, and an identification of the gene responsible was identified in order to gain a better understanding of the evolution of genes within different isolates of cyanobacteria.

1.6 Thesis overview

This thesis contains eight chapters. This first chapter summarises the knowledge and work undertaken in understanding HIV, as well treatments that have been developed since the HIV epidemic commenced. A literature review of drugs that have been isolated from plants and algae, as well as cyanobacteria, has been compiled; and a gap statement and main objective of this project is stated to identify the importance of this thesis.

Since this research project also investigates isolates identified as species of *Spirulina*, the literature review in Chapter Two provides the background for this particular study. This chapter summarises the work done by culturing *Spirulina.* A literature review is

also presented in Chapter Two to give an overview of the environmental aspects of *Spirulina* cultivation. In addition, Chapter Two includes studies on the biosynthesis pathway, and the genes responsible for sulfolipid production. A review of past work in obtaining the bioactive compounds from *Spirulina* by using several methods of extraction is also investigated and reported. The following chapter details the comprehensive material and methods used for this study.

Chapter Four details prospective studies that have been conducted focusing on the optimal growth conditions of an Australian *Spirulina,* isolated from Western Australia. Several aspects, such as nutrient uptake and temperature, were considered when culturing the Australian isolate. Moreover, the classifications of four commercial isolates and the Australian isolate were identified to clarify the taxonomic confusion between the *Spirulina* and *Arthrospira*. The subsequent sequence data was submitted to the GenBank database to determine the taxonomic status of these five isolates. The sequence data in the GenBank plays a key role in prevention of inconsistencies in sequence information found in the scientific literature. The taxonomic status of these five isolates was further supported by the chemotaxonomic evidence obtained during this study.

Chapter Five emphasises the study of the biosynthesis pathway of sulfolipids production by elucidating the genes involved. A number of polymerase chain reactions were also performed to amplify NRPS and PKS, which are often associated with toxin pathways in cyanobacteria. Despite the wide use of *Spirulina* in the health food industry, the screening for the presence of genes encoding non-ribosomal peptide synthetase and polyketide synthetase was essential in order to assess the suitability of these strains for human consumption and safe therapeutic use.

Chapter Six demonstrates several methods of extraction, based on different forms of organic solvents, soxhlet and supercritical carbon dioxide extraction, which optimise the yield of sulfolipids from the investigated isolates. The reason to optimise the yield of sulfolipids is that this bioactive compound is reported to have a great inhibitory effect on HIV-reverse transcriptase. An optimisation for the extraction of sulfolipids was carried out based on literature, and the data obtained from the five *Spirulina* isolates was evaluated to determine whether genetic variation existed.

The quantification of the sulfolipids from several extraction methods follows in Chapter Seven, reporting the results analysed by different analytical techniques, such as high pressure liquid chromatography (HPLC) with ultraviolet-visible detector and evaporative light scattering detection. A preliminary anti-microbial study against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* was also performed. The mode of action of sulfolipids in different mechanisms of anti-HIV activity, such as cell-cell transmission, was also conducted by the Institute of Medical and Veterinary Science (IMVS). Final remarks and future work is proposed in Chapter Eight, and the main conclusions are summarised.

CHAPTER 2 LITERATURE REVIEW

2.1 *Spirulina*

2.1.1 History of *Spirulina*

Spirulina has been used as a nutritional supplement since 1519. It was discovered by Spanish invaders when Hernando Cortez and his conquistadors saw that the Aztecs were collecting a blue coloured 'tecuitlatl' from Lake Texcoco. The blue coloured tecuitlatl was then made into a cake and sold at a local market (Vonshak, 1997).

In 1940, Pierre Dangeard observed *Spirulina* around Lake Chad when the Kanembu population was harvesting cyanobacteria using clay pots. They drained the water and spread out the algae for drying in the sunlight. The dried algae were then made into cakes, called dihé, in Chad (Ciferri, 1983; Ciferri & Tiboni, 1985). *Spirulina* is the main source of nutritional food for the population in Chad, as these microalgae can be easily obtained from the lake, and can be eaten once it has been dried. It was found that, in the region of low levels of malnutrition, *Spirulina* comprised 70% of their meals (Abdulqader *et al*., 2000; Ciferri & Tiboni, 1985).

After re-discovery by Jean Leonard in 1960, French researchers were keen to commercialise this daily food source. In the early 1970s, they built the first large-scale *Spirulina* production plant, which drew international attention. People started to consume *Spirulina* as a food supplement, and the need for *Spirulina* rose. Although no data has been published in recent years, Belay *et al*. (1993) reported that the total annual production of *Spirulina* in 1993 reached 800 tonnes per annum. This was an eight-fold increase in annual production, compared to 40 years previously (Belay *et al*., 1993; Vonshak, 1997). Habib *et al*. (2008) also reported that the production of *Spirulina* in China alone had increased to 40,750 tonnes in 2004. Although no figures are available on present production, the growth of the production is rising dramatically (Habib *et al*., 2008).

2.1.2 Classification of *Spirulina*

Spirulina belongs to the kingdom of Monera, the division of Cyanophyta, the order of Oscillatoriales and the family of Phormidiaceae. Cyanobacteria are often referred to as cyanobacteria, even though they are a phylum of bacteria that obtain their energy through photosynthesis. Cyanobacteria can be divided into a simple or complex structure, while *Spirulina* is a multicellular and filamentous blue-green microalga. *Spirulina* has trichomes with a length of 50 to 500 μm and a width of 3 to 4 μm, where the trichome is divided into well-defined cell by cross walls. The cell wall of *Spirulina* is similar to a gram-negative bacterium that contains peptidoglucan, a lysozyme–sensitive heteropolymer. This cell wall confers shape and compatible solutes and ions to help alleviate osmotic stress (Habib *et al*., 2008).

Spirulina grows vigorously in lakes rich in salts, such as in Lake Chad in Africa, Lake Klamath in North America, Lake Texcoco in Mexico and Lake Titikaka in South America. The reproductive structure of *Spirulina* is binary fission, as it involves the splitting of a parent cell into two equal parts. It grows naturally in strong sunlight under high temperatures and high alkaline conditions. Therefore, *Spirulina* normally acts as a dominant species in alkaline lakes, as it is difficult for other organisms to thrive under these conditions. *Spirulina* sometimes form in floating mats as a consequence of the presence of gas filled vacuoles in the cells, together with the trichome (Ciferri & Tiboni, 1985). This phenomenon can be referred to as an ‘algae bloom’.

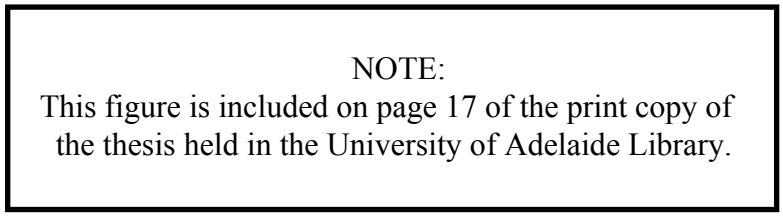
The total number of *Spirulina* species has been estimated to be 87, with 47 species currently accepted taxonomically, which include *Spirulina subsalsa*, *Spirulina labyrinthiformis*, *Spirulina laxissima, Spirulina nodosa* (Fogg *et al*., 1973; Guiry & Guiry, 2011; Habib *et al*., 2008); while the most common species used for human consumption are *Spirulina maxima* and *Spirulina platensis* (Khan *et al*., 2005).

2.1.3 Taxonomy

Spirulina are reviewed under the same genus as *Arthrospira* often, partially due to the erroneous group classification of these organisms as *Arthrospira* by Geitler in 1932 (Vonshak, 1997). These organisms were later described into two distinct genera, however the species definition is not well established (Whitton *et al*., 2002). The

identification of the *Arthrospira*, which was marketed as *Spirulina*, is the greatest barrier for the establishment for a stable classification system for these organisms.

In order to recognise the difference between these two species, characterisation of the helicity and trichome size, cell wall structure and pore pattern, gas vesicles, thylakoid pattern, trichome motility and fragmentation can be made under a light microscope. The comparison of each species of *Spirulina* was made by Spiller *et al*. (2000) under an x-ray microscopy. The photomicrographs for *Spirulina subsalsa* and *Spirulina platensis* under x-ray microscopy are illustrated to compare the spherical shapes and looping strands (Figure 2.1). However, biochemical testing such as Guanine and Cytosine (G&C) content and oligonocleotide catalogue of 16S rRNA is more convincing in making a distinction between these two species.



(a) (b)

Figure 2.1: View of Spirulina under a microscope

(a) *Spirulina platensis* and (b) *Spirulina subsalsa* (XM-1 image from beamline 6.1.2). Scale bar indicates 1 μm (Spiller *et al*., 2000).

A better understanding on the morphologies of *Spirulina* has been proposed by Nubel *et al*. (2000). The classification of an isolate cyanobacteria was easily categorised with the use of a microscope (Figure 2.2) coupled with a 16S rRNA gene sequence analysis (Nubel *et al*., 2000). 16S rRNA amplification has been used widely to identify the morphological classification between the genotypes of cyanobacteria. The universal primers for 16S rRNA sequence are used to amplify the gene as it is conserved between species of bacteria and archaea (Weisburg *et al*., 1991). In addition, the 16S rRNA gene sequencing provides a rapid identification and differentiation of bacteria and archaea. The separation of these two genera was done by a complete sequence of both 16S rDNA and this approach makes clear the expression and regulation of genes of both *Spirulina* and *Arthrospira* (Vonshak, 1997; Whitton *et al*., 2002).

NOTE:
This figure is included on page 18 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.2: Photomicrographs of cyanobacteria isolates

All organisms are shown at the same magnification and the bar shown applies to all panels. Bar: 10 µm. Adapted from Nubel *et al*., 2000.

2.1.4 Chemical composition of *Spirulina*

An insight into the exploitation of the more ideal strains of *Spirulina* for commercial use is important. An ideal strain of *Spirulina* primarily relies on its rich source of

nutrition and stability in the culture. Previous studies have only focused on the strains of *Spirulina platensis* and *Spirulina maxima*, which are sub-tropical strains of *Spirulina* and are used for commercial production. The general chemical composition of the *Spirulina platensis* and *Spirulina maxima* strains are presented in Table 2.1. The chemical composition of these strains of *Spirulina* have been investigated intensively due to their long and continuous history of use as a food supplements. Moreover, these strains are the best produced in countries such as in the USA, Mexico, Thailand, and China (Vonshak, 1997).

Table 2.1: Chemical composition of different strains of *Spirulina*

General Composition	Hawaiian *Spirulina*[1]	*Spirulina platensis*[2]	*Spirulina maxima*[2]	*Spirulina subsalsa*[3]
Protein	53-62%	67	67	41.4
Carbohydrates	17-25%	15.3	14.5	18.7
Lipids	4-6%	11.5	13.1	24.9
Minerals	8-13%			
Moisture	3-6%			

[1] (Kelly *et al*., 2006); [2] (Ciferri & Tiboni, 1985); [3](Tredici *et al*., 1988)

Many studies have been carried out to analyse the fatty acid pattern in different strains of *Spirulina* (Al-Hasan *et al*., 1989; Cohen *et al*., 1987; Ötles & Pire, 2001; Ramadan *et al*., 2008). As can be seen in Table 2.2, the data indicates that most strains of *Spirulina* have a predominant fatty acid component of palmitic acid, followed by gamma linolenic acid and linoleic acid. Only the *Spirulina subsalsa* strain has a different fatty acid distribution, which varies greatly to other *Spirulina* strains. The fatty acid composition of the *Spirulina subsalsa* from the study of Cohen *et al*. (1987) is mainly distributed in C16:0 (49%), secondly in C16:1 (35%) and C18:2ω6 (13.1%). However, these data did not comply with the results obtained from Al-Hasan *et al*. (1989), in which it was found that *Spirulina subsalsa* is dominant in C18:1 (27.6%). The variance between these studies is most likely due to different strains of *Spirulina* isolated from different geographic locations and trophic states. In the Al-Hasan *et al*. (1989) study, the lipid classes that are rich in C18:3 (non-disclosed isomer of linolenic acid) were investigated. Digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) were found to be enriched in this acid contribution, with 10% of C18:3 from 37% of DGDG, and 13% of SQDG. In addition, the main fatty acid composition of SQDG is C16:2 for the *Spirulina subsalsa* strain. For the *Spirulina platensis* strain, the

main fatty acid composition of SQDG is different, were C18:2 is dominant, while SQDG from the *Spirulina maxima* strain C16:0 (51.6%) is the predominant fatty acid (Al-Hasan *et al*., 1989; Kataoka & Misaki, 1983; Vonshak, 1997). From these studies, it seems certain that the distribution of the fatty acid is strain specific.

The fatty acid composition of the *Spirulina* can also be used as an application to classify the taxonomy of *Spirulina* (Kenyon *et al*., 1972; Romano *et al*., 2000). From the fatty acid patterns of the *Spirulina subsalsa* strain, Vonshak *et al*. (1997) suggested that it should be classified in the fifth group of cyanobacteria, and this new group of cyanobacteria are specific to the strains that only contain two double bonds in polyunsaturated fatty acids (PUFAs). Since then, chemotaxonomic studies of fatty acid composition were established. For instance, the fatty acid compositions were primarily performed to study the chemotaxonomic classification of microalgae (Franz *et al*., 2010; Marshall *et al*., 2002).

In addition, understanding of the fatty acid distribution in different strains of *Spirulina* can facilitate the selection of elite strains of *Spirulina* for the production of gamma linolenic acid. Attributable to a growing interest of SQDG, Vonshak *et al*. (1997) suggested a method to optimise the production of SQDG and gamma linolenic acid. He proposed that the isolation of SQDG can be coupled with a downstream process of concentrating the gamma linolenic acid.

Table 2.2: Fatty acid composition of different strains and species of *Spirulina*

	Strains/Species	Fatty acid composition (percentage of total fatty acids)					
		16:0	16:1	18:0	18:1	18:2ω6	18:3ω6
[1]	SB	44.6	4.4	0.5	6.4	17.1	27.0
	Mad	47.0	0.5	0.7	9.3	10.8	31.7
	Cat	47.6	2.5	1.0	8.0	15.3	25.6
	Art B	46.1	1.0	1.6	10.9	13.6	26.8
	1928	47.3	2.0	1.0	2.9	18.1	28.7
	L1	45.0	1.4	1.0	15.5	16.4	20.7
	AR	49.1	2.2	1.0	6.4	15.7	25.6
	B4	49.6	2.1	0.7	5.0	16.5	26.1
	B2	47.3	3.4	0.8	5.8	20.7	20.7
	G	49.2	2.9	0.9	8.0	15.7	23.3
	PC	52.5	2.4	0.8	7.2	14.0	23.2
	B3	52.9	2.2	1.1	7.6	13.7	22.5
	Art. A	48.5	2.4	1.3	6.0	15.8	26.0
	Eth	54.1	2.6	1.0	7.7	13.5	21.3
	L2	50.7	1.1	0.8	7.3	14.3	25.8
	Minor	46.8	1.2	1.5	12.0	18.4	20.1
	2342	47.5	1.6	0.5	9.3	21.8	19.3
	2340	49.3	2.2	1.2	8.6	30.7	8.0
	Subsalsa	49.2	35.0	1.7	1.0	13.1	nd
[2]	P	44.2±0.07	6.42±0.07	nd	0.93±0.07	18.7±0.07	23.4±0.07
[3]	PB	46.07	1.26	1.41	5.23	17.43	8.87
	P1	42.30	1.00	0.95	1.97	16.18	20.06
	P2	43.65	1.50	1.39	2.05	17.19	21.73
	M	35.82	0.85	1.49	5.03	16.34	18.16
	PO	35.34	1.20	1.47	4.51	16.87	17.49
[4]	*Subsalsa*	15.0	16.7	tr.	27.6	11.4	10.1

[1] (Cohen *et al*., 1987) (Israel); [2] (Ramadan *et al*., 2008) (Egypt); [3] (Ötles & Pire, 2001) (Turkey); [4] (Al-Hasan *et al*., 1989) (Kuwait);Nd is expressed as not detected; tr. is expressed as traces; PB: *Spirulina platensis*, bio-organic; P1: *Spirulina platensis*-1; P2 *Spirulina platensis*-2; M: *Spirulina maxima*; PO: *Spirulina pacifica*, organic.

2.2 Maintain an axenic culture

It is essential to obtain an axenic culture of microalgae, because these conditions are required for the determination of bioactive compounds from a specific species of microalgae. Maintaining an axenic culture is time consuming, and achieving a bacteria-free culture requires practice and experience. Moreover, different species of microalgae might require different treatments to achieve bacteria-free cultures. For instance, a unicellular cyanobacterium is suitable in the application of single-cell isolation, using centrifugation, rinsing (Bolch & Blackburn, 1996; Vaara *et al*., 1979), filtration or treatment with antibiotics (Rippka & Lester Packer and Alexander, 1988). To identify a better treatment for bacteria-free filamentous cyanobacteria culture, Choi *et al*. (2007) introduced different types of antibiotics, such as cefoxitin, chloramphenicol, erythromycin, imipenem and neomycin. *Spirulina* was found to be resistant to imipenem and neomycin, though no further investigation was carried out to determine the bioactive compounds of the treated *Spirulina* (Choi *et al*., 2007). Richmond and Beacker (1986) suggested subjecting the culture to a temporary extreme change of environmental conditions to decrease contaminants. As a consequence, an alternative treatment of ultraviolet (UV) irradiation is recommended in obtaining an axenic culture by Mehta and Hawxby (1977).

2.3 Cultivation of *Spirulina*

The production of *Spirulina* continues to increase annually, and the need for *Spirulina* for human consumption has increased by approximately 800 tonnes per annum (Belay *et al*., 1993). The data on the growth of the production of *Spirulina* is shown in Figure 2.3 from Borowitzka (1999). The growth of the production of *Spirulina* has risen well-beyond the predicted value, where the annual production of *Spirulina* in China for 2003 and 2004 at 19,080 and 40,750 tonnes per annum, respectively (Habib *et al*., 2008). Although there is no up to date world-wide data on the annual production of *Spirulina*, the high throughput of *Spirulina* in China has clearly shown the growing importance of *Spirulina* in the world.

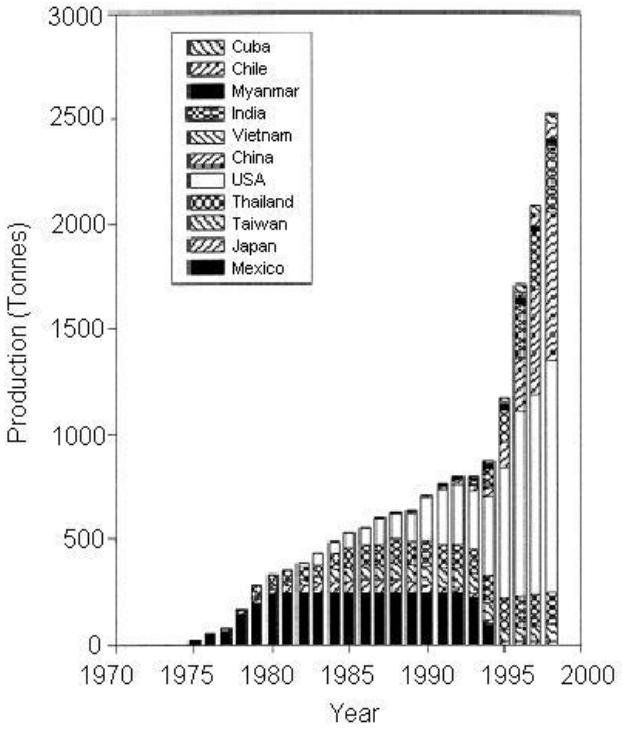


Figure 2.3: Global production figures of Spirulina by country based on literature, company and trade information

Data for 1997 and 1998 are estimates based on projected production figures were provided by the producers (Borowitzka, 1999).

Regardless of its enormous commercial importance, this has encouraged a number of researchers to study the growth condition of *Spirulina* to optimise its production. Large-scale *Spirulina* production has been established since the early 1970s, and many countries commenced establishing *Spirulina* plants, such as the USA, Mexico, Thailand, and China. *Spirulina* requires a high pH and temperature for growth, and thus *Spirulina* can be grown in an open air pond because of its highly selective environmental needs. In addition, growing in an open pond is economical and easier for scaling up (Borowitzka, 1999). Figure 2.4 shows a large commercial open pond for the cultivation of *Spirulina* in Darwin, Australia.

NOTE:
This figure is included on page 24 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.4: An existing site of production of Australian Spirulina at Darwin, Northern Territory
http://www.australian*Spirulina*.com.au/*Spirulina*/aboutus.html (Accessed 27 September 2008)

2.3.1 Effect of the environment on *Spirulina*

There are multiple factors involved in the growth of microalgae. Light, temperature, chemical composition and salinity are factors that may inhibit the growth of microalgae. *Spirulina* has important advantages as a supplement in high protein foods. Therefore, for successful *Spirulina* culture, suitable growth conditions must be selected. To optimise the growth conditions of different species of microorganisms, modification is required for different species of microorganisms in different geographical regions (MacArthur, 1972).

Illumination can affect the growth of microalgae by either the length of the photoperiod or intensity (Andersen, 2005). Some of the microalgae prefer low light intensities (<60 μmol photons m^{-2} s^{-1}), while some grow under higher light intensities (<100 μmol photons m^{-2} s^{-1}). *Spirulina* was suggested to grow under high light intensities, and it was found that the growth of *Spirulina maxima* and *Spirulina platensis* saturated at a range of 420–504 μmol photons m^{-2} s^{-1} and 150-200 μmol photons m^{-2} s^{-1}, respectively (Vonshak, 1997). Kebede and Ahlgren (1996) also confirmed that *Spirulina platensis* has an optimum growth at 330 μmol photons m^{-2} s^{-1} (Kebede & Ahlgren, 1996).

It has been observed that the influence of temperature might bring significant change to the growth of *Spirulina*. *Spirulina maxima* and *Spirulina platensis* have a temperature optimum of 30 ^{o}C (Costa *et al*., 2000; Oliveira *et al*., 1999; Tri-Panji & Suharyanto, 2001). Vonshak (1997) studied the optimum temperature of three strains of *Spirulina* in the laboratory, where they were found to have a relatively high temperature of 35–38 ^{o}C (Vonshak, 1997). It is also well-known that *Spirulina platensis* and *Spirulina maxima* preferably grow in hot climates, and the outdoor production of *Spirulina* is mostly developed in tropical regions.

It has been reported that a mini pond with slower flow rates has a better production of *Spirulina* when compared with a raceway pond at turbulent flow. These results clearly showed that high mixing rates in *Spirulina* cultures may result in harmful shearing forces and might cause physical cell damage, while under low mixing rates, *Spirulina* cultures may be limited by the oxygen transfer and the cells that sink to the base may suffer light inhibition. Therefore, a balance between mixing to obtain proper light and mass transfer regimes must be achieved to obtain a better yield of *Spirulina* (Richmond & Grobbelaar, 1986).

Growth of microalgae is linked to the effect of salinity. This is because salinity may cause the enzymes to lose activity due to the adjustment of osmotic stress in the cytoplasm (Kirst, 1990). For controlling the salinity, salt or fresh water are required. Materassi *et al*. (1984) initially developed the cultivation of *Spirulina maxima* in seawater to demonstrate the salinity effect on the growth response of *Spirulina*. Under this cultivation method, *Spirulina maxima* easily adapted, and it was determined that this cultivation would be significant for economical production (Materassi *et al*., 1984). The purpose of developing this method was the unlimited availability of seawater. Moreover, Tomaselli *et al*. (1987) showed that the salinity had no effect on the cell composition of the biomass. Therefore, an outdoor culture condition in seawater was verified to be technically feasible (Tomaselli *et al*., 1987).

Oxygen concentration is one of the important physical limitations in *Spirulina* culture. High oxygen concentrations may inhibit the growth of microalgae (Tredici & Materassi, 1992). Marquez *et al*. (1995) observed that under relatively high oxygen concentration, the growth rates of *Spirulina*, as well as the contents of photosynthetic pigment

including chlorophyll-*a*, carotenoids and phycocyanin, were decreased (Marquez *et al*., 1995).

Each strain of microalgae has its own optimum media nutrient concentration (Val, 1983). The growth rate and the quality of natural products from microalgae depend on the type and the amount of nutrients supplied. Carbon, hydrogen, nitrogen, phosphorus, potassium, magnesium, iron, and manganese are essential chemicals and elements of the nutrients required for the growth of microalgae (Richmond, 2004). A study examining the effects of carbon, nitrate, phosphate, sulphate, ferric ion, magnesium and potassium on the growth of *Spirulina platensis* highlighted the effects of the nutrients on the growth of cyanobacteria. An example of this was a study conducted by Costa *et al*. (2002), who examined the effect of nitrate on the growth response of *Spirulina* in an open raceway pond.

Tri-Panji and Suharyanto (2001) found an optimum growth condition of *Spirulina* in a media from a latex factory. The latex solution found to be rich in nutrients and was good for the growth of *Spirulina* (Tri-Panji & Suharyanto, 2001). Different optimised conditions were also introduced to obtain better growth of *Spirulina* such as the use of an anaerobic effluent from digested piggery waste and waste-water from a sago starch factory were proposed by Olguin *et al*. (2001) and Phang *et al*. (2000) respectively. The quality and quantity of *Spirulina* cultured under special conditions need to be substantiated for the reason that *Spirulina* is used for human consumption (Carmichael & Gorham, 1974).

2.3.2 Effect of growth parameters on bioactive compound

Khan *et al*. (2005) studied the therapeutic properties of *Spirulina*, in which sulphated polysaccharide, sulfolipids, cyanovirin N, gamma linolenic acid, vitamin E and phycocyanin are the most important bioactive substances. The bioactive compounds were used for several scientific findings to determine their effect for anti-cancer, anti-HIV, and other therapeutic treatments (Khan *et al*., 2005). These valuable metabolites have not been investigated under different environment conditions to achieve the highest quantity of bioactive compounds, especially for the strain of *Spirulina*. Studying the effect of growth parameters for bioactive compounds is important, not only to provide insight into their functions, but to assist in optimising the growth conditions for particular pharmacologically interesting compounds.

Gamma linolenic acid (GLA) and eicosapentaenoic acid (EPA) are known as common dietary supplements, and have been studied widely by many researchers to optimise the production rate of these compounds. A study in optimising the GLA production in *Spirulina platensis* was done by regulating the light (Cohen *et al*., 1987; Olguin *et al*., 2001; Tanticharoen *et al*., 1994; Tedesco & Duerr, 1989), outdoor conditions (Qiang *et al*., 1997; Tanticharoen *et al*., 1994), growth phase (Cohen *et al*., 1987), nutrient content (Colla *et al*., 2004; Olguin *et al*., 2001; Tedesco & Duerr, 1989) and temperature (Cohen *et al*., 1987; Colla *et al*., 2004; Tedesco & Duerr, 1989). The alterations of the fatty acid compositions in varying environmental conditions might concomitantly influence particular lipids because the fatty acid composition of each lipid is frequently distinctive. It is therefore necessary to study the influence of environmental conditions on particular lipid alterations.

It has been observed that many photosynthetic plants and microalgae alter the total amount of lipids and sulfolipids when growing under salt stress (Ben Hamed *et al*., 2005), light intensities (Archer *et al*., 1997), temperature (Kleinschmidt & McMahon, 1970; Pearcy, 1978) and phosphorus starvation (Essingmann *et al*., 1998). The quantity of sulfolipids under phosphate limitation shows significant changes to maintain a net charge in the thylakoid membrane. The biochemical response of the sulfolipids under phosphate starvation was mostly studied based on the green algae (*Chlamydomonas reinhardtii* and *Chlorella kessleri*) (Elsheek & Rady, 1995; Sato *et al*., 2000), and the effect of salt stress on sulfolipids production was investigated based on the red algae (*Crithmum maritimum*) (Ben Hamed *et al*., 2005). Although researchers have addressed the effects of salt stress and phosphate starvation, the justification of the effects of salt stress and phosphate starvation might not apply to cyanobacteria, since the biochemical response of individual species is unique.

Carbohydrate changes are presumably related to the production of sulphated polysaccharide as the polysaccharides are known to have polymeric carbohydrate structures. Torzillo *et al*. (1991) conducted a five month outdoor culture of *Spirulina* and investigated the biochemical profile of *Spirulina* based on different temperatures. Olguin *et al*. (2001) focussed on food products and characterised the production of fatty acid, protein and carbohydrate related to the changes of environmental conditions. Both studies showed that when an elevated level of carbohydrate was observed, a decrease in protein evaluated (Olguin *et al*., 2001; Oliveira *et al*., 1999; Torzillo *et al*., 1991).

2.4 Application of *Spirulina*

In recent years, research in developing the use of microalgae as a biotechnological resource is increasing. Its potential use as a biotechnology resource is mainly applied to the four major industrial areas for medical, aquaculture, biofuels and environmental uses (Li *et al*., 2008; Metting, 1996; Olaizola, 2003; Singh *et al*., 2005b; Spolaore *et al*., 2006). Like other microalgae, *Spirulina* has been used widely in the application of biotechnology. There have been many investigations published that described the use of *Spirulina* in various types of biotechnology applications.

2.4.1 Use of *Spirulina* in waste-water treatment

The disposal of waste-water without proper treatment can cause serious water quality problems. The chemical constituents of industrial, municipal and agriculture waste are the main source of the water pollution (Brower, 1990). To date, many studies have investigated potential waste treatment technologies to control this pollution. Various types of waste-water treatment technologies were disseminated based on the constituents that need to be treated from the waste before disposal.

Untreated waste-water normally contains inorganic phosphorus and nitrogen from swine farming (Mezzomo *et al*., 2010) and municipal situations, chromium (VI) from textile dyeing, leather tanning, electroplating and metal finishing industries (Finocchio *et al*., 2010), vinasse from alcohol industries (Barrocal *et al*., 2010) and other heavy metals. In the field of waste-water technology, microorganisms are introduced as bio-absorbents. This treatment shows high selectivity, easiness and effectiveness in operation when compared to conventional methods, which consist of a combination of physical, chemical and biological processes. An appropriate selection of algae to remove these constituents is essential for successful waste-water application. For instance, an excessive amount of phosphorus and nitrogen can cause a serious problem, such as algae blooms and lead to an imbalance in ecosystems.

Spirulina has the potential in the waste-water treatment of swine and piggery sludge. This is because the excessive amount of metal from waste-water has become a source of nutrients for the growth of *Spirulina*. An elevated level of metal can be toxic when it is converted into biomass, which acts as a source to produce products such as pigments, carotenes, sterols, vitamins, and polyunsaturated fatty acid (Lodi *et al*., 2003). A

number of researchers established a waste-water treatment system using *Spirulina*. These studies were investigated the removal of nitrate, phosphorus, ammonium, urea and chromium (VI) from waste-water (Converti *et al*., 2006; Finocchio *et al*., 2010; Lodi *et al*., 2003).

2.4.2 Use of *Spirulina* in aquaculture

The cultivation of marine resources has drawn wide public attention because of the increasing amount of human consumption ofr aquatic animals. In addition, marine resources have reached a critical threshold, since human have harvested beyond the sustainable yields (Blaxter, 2000). To address this problem, mass production of freshwater and marine resources has been undertaken world-wide. Microalgae are at the beginning of the food chain for aquatic animals due to their rich fatty-acid content, and Omega 3 PUFAs which are an essential requirement for cultivation. (Muller-Feuga, 2000).

Microalgae are required for different growth stages of aquatic animals, depending on the species in the hatchery. For instance, microalgae are necessary in three stages of farming scallops, such as larval, juvenile and adult. The feed material used for the hatcheries is evaluated economically in the scope of the production of aquaculture. Habib *et al*. (2008) suggested that the *Spirulina* is a cheaper feed material in aqua feeds.

The demand of *Spirulina* is increasing, and not only because of its use as human food supplement. Belay *et al*. (1996) reported that 30% of the production of *Spirulina* was used for the animal feed. *Spirulina* is a rich source of protein and essential fatty acids and have been reported to increase the growth rates of fish and invertebrate species grown in the aquaculture industry. Most of this work is carried out in Japan, and many researchers have accelerated their examination of increasing the production of *Spirulina*. Belay *et al*. (1996) summarised most of the work that done on the use of *Spirulina* in the application of aquaculture.

2.5 Therapeutic potential of *Spirulina*

It has been recognised by researchers that *Spirulina* exhibit various bioactivities. Many studies depict the bioactive compounds that are possessed by *Spirulina* as *having* potential, such as anti-bacterial, anti-inflammatory, anti-oxidant, immune-stimulant,

anti-cancer and anti-HIV bioactivities. The main focus of *Spirulina* used in this thesis is on the therapeutic agents in HIV treatment. Several authors in particular have made contributions towards needs in this area.

Spirulina has been shown to be an excellent source of proteins, vitamins and mineral, with a low content of nucleic acid (Sánchez *et al*., 2003). *Spirulina* protein is superior to all standard plants and the nutritive value of a protein has higher quality of amino acids, digestibility coefficient as well as the biological value. Khan *et al*. (2005) state that *Spirulina* has three natural compounds which are potent in inhibiting HIV activity. These three natural compounds are calcium-spirulan, cyanovirin-N and sulfolipids (Khan *et al*., 2005).

2.5.1 Cyanovirin-N

Cyanovirin-N is a potential anti-HIV bioactive compound, which can be extracted from *Spirulina* (Khan *et al*., 2005). However, according to Gustafon *et al*. (1997), the isolation and determination of the primary sequence and disulfide bond structure of protein (i.e. cyanovirn-N) from the cyanobacteria *Nostoc ellipsosporum* was possible. It was a contradiction in that, although *Nostoc ellipsosporum* is the same phylum as *Spirulina*, the primary and secondary metabolites from these two different species might not be the same. Furthermore, no published paper was found to establish the potential of *Spirulina* in producing cyanovirin-N. In light of the fact that cyanovirin-N has the potential to inhibit the virus infection, as well as the replication of HIV, further investigation of the extraction of cyanovirin-N from *Spirulina* was undertaken.

2.5.2 Sulphated polysaccharides

Calcium-spirulan (Ca-SP), a novel sulphated polysaccharide which isolated from *Spirulina*, was found to have the inhibitory effects on HIV. Hayashi *et al*. (1996) successfully extracted calcium-spirulan from *Spirulina platensis* by using a hot water extraction, and tested the anti-viral effects of calcium-spirulan. Several attempts have been made to investigate the effects of calcium-spirulan by using this bioactive compound to inhibit the activity of HIV, HSV-1, human cytomegalovirus, measles virus, mumps virus, and influenza A virus, as well as Vesicular Stomatiotis Virus (Baba *et al*., 1988; Hayashi *et al*., 1996a; Hayashi *et al*., 1996c).

2.5.3 Sulfolipids

Research has focused on the potential therapeutic effects of sulfolipids. Gustafson *et al*. (1989) excreted this bioactive compound from few species of cyanobacteria including *L. lagerheimii* (strain DN-7-1) and *P. tenue* (strain CN-2-1). When the anti-viral testing of this bioactive compound was undertaken in human lymphoblastoid CEM, MT-2, LDV-7, C3-44 cell lines in the tetrazolium assay and p24 viral protein and synctium formation assay, this bioactive compound was found to have the potential for anti-HIV activity (Gustafson *et al*., 1989). This study has attracted a great number of researchers to investigate the therapeutic potential of cyanobacteria in anti-HIV activity. Blinkova *et al*. (2001) successfully proved that the sulfolipids from *Spirulina* was active against HIV.

2.6 Target natural products—Sulfoquinovosyldiacylglyceride

Sulfoquinovosyldiacylglyceride (SQDG, known as sulfolipid) is a natural product with many functions in photosynthetic organisms and in human health. Sulfolipids have shown potential in the treatment of human diseases, and also been reported to act as an inhibitor of DNA polymerases and retroviral reverse-transcriptase (Gustafson *et al*., 1989; Loya *et al*., 1998; Mizushina *et al*., 1998; Ohta *et al*., 2000; Ohta *et al*., 1998; Reshef *et al*., 1997), and an antitumor inhibitor (Sahara *et al*., 1997; Shirahashi *et al*., 1993). In recent years, sulfolipids from ferns have been found to be useful in the treatment of psoriasis and skin disorders (Vasänge, 2004). Due to its extensive biological activities, sulfolipids are strongly implicated as an attractive compound for new development.

Sulfolipids, acting either as a primary or secondary metabolite, remain unclearly defined. The debate over this compound is continues. Benning *et al*. (1993) determined that the strain of *Rhodobactor sphaeroides* did not seem to impair the photosynthetic parameters in the absence of SQDG; while Sato *et al*. (2003) observed that the absence of SQDG showed slower growth rate, and impaired the photosystem II function of *Clamydomonas reinhardtii* and *Synechocystis* sp. PCC 6803. Due to the confusion over the categorisation of sulfolipids, no research has identified the s as either a primary or a secondary metabolite since sulfolipids can be either an essential compound that aids basic growth and reproduction in photosynthetic organisms or it can be an abundant compound in the thyalokoid membrane. Instead of considering whether the compound

is exclusively a primary metabolite or a secondary metabolite, it is worthwhile to focus on elucidating the gene responsible for the bioactive metabolite, which increases the opportunities to improve the biomass production and quality.

In spite of the importance of sulfolipids, researchers reported the identification of the gene for sulfoquinovosyldiacylglycerol synthase in catalysing the final step of sulfolipids production is presented (Section 2.6.1). Studies on the biosynthesis pathway of sulfolipids of *Synechocystis* and *Synechococcus*, particularly for cyanobacteria species, have also been revealed (Aoki *et al*., 2004). The role of sulfolipids was determined, suggesting this compound is required in the photosystem II function for *Synechocystis* but not for *Synechococcus*. The role of sulfolipids differs with respect to species-specific function in cyanobacteria.

Despite the pathway for sulfolipid biosynthesis in cyanobacteria being emphasised, investigation into sulfolipid production by the cyanobacteria, such as *Gloeobacter violaceus* sp. PCC 7421, have shown it to be absent (Selstam & Campbell, 1996). This latter result showed that the strains of cyanobacteria are known to be absent in SQDG, and the production of sulfolipids may not be distinct in all strains of cyanobacteria. In order to provide untapped cyanobacteria resources for preclinical development, it must be ensured that the isolation of this novel bioactive compound can be obtained. Therefore, more attention should be paid to investigate the resources to achieve a large-scale extraction of this valuable compound.

2.6.1 Pathways for the biosynthesis of Sulfolipids

Sulfolipids are ubiquitously expressed from the photosynthetic membranes of higher plants, mosses, ferns, algae and most photosynthetic bacteria. The structure of sulfolipids is characterised with the sulfoquinovose as the head group (Benning, 1998) shown in Figure 2.5. Phospholipids and sulfolipids are known to be the only anionic lipid in the thylakoid membrane, and hence the level of sulfolipids corresponds in molar ratio with respect to phospholipid to maintain the charge balance in the thylakoid membrane. For example, when a wild type organism suffers phosphate limitation; phospholipid content in the thylakoid membrane will decrease while the level of sulfolipids will increase.

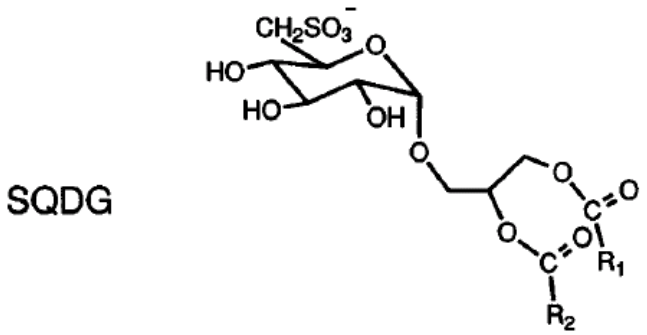


Figure 2.5: Structure of the sulfolipids sulfoquinovosyl diacylglycerol (SQDG)

A more precise designation of this compound according to the International Union of Pure and Applied Chemistry (IUPAC) is 1,2-di-*O*-acyl-3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-sn-glycerol. R_1 and R_2 indicate acyl chains of different length and degree of unsaturation (Benning, 1998).

An understanding of the biosynthesis of sulfolipids is of particular importance to produce sufficient sulfolipids in biomedical applications. With recent advances in science and technology, commercial production of metabolites with the combinatorial genetic and metabolic engineering is achievable to improve the production of metabolites. Research into metabolite production led to enormous progress in the discovery and sequencing of the gene involved in the biosynthesis of metabolites products. In the past, only the theoretical discussion on the biosynthesis of sulfolipids has been confirmed. Pugh *et al*. (1995) hypothesised the metabolic pathway of plant sulfolipids, as seen in Figure 2.6. A number of studies have been conducted on the biosynthesis of sulfolipids, revealing a great deal of information on screening the gene responsible for synthesis of sulfolipids. Due to the availability of the final elucidation of sulfolipids biosynthesis in plants and microalgae, it provides an opportunity to reveal the gene responsible for sulfolipids biosynthesis. The first characterised strain of the gene responsible for sulfolipids biosynthesis is a purple bacterium *Rhodobacter sphaeroides* (Benning & Somerville, 1992b). The biosynthesis pathway of sulfolipids has been proposed in order to improve our understanding to develop new therapeutic intervention.

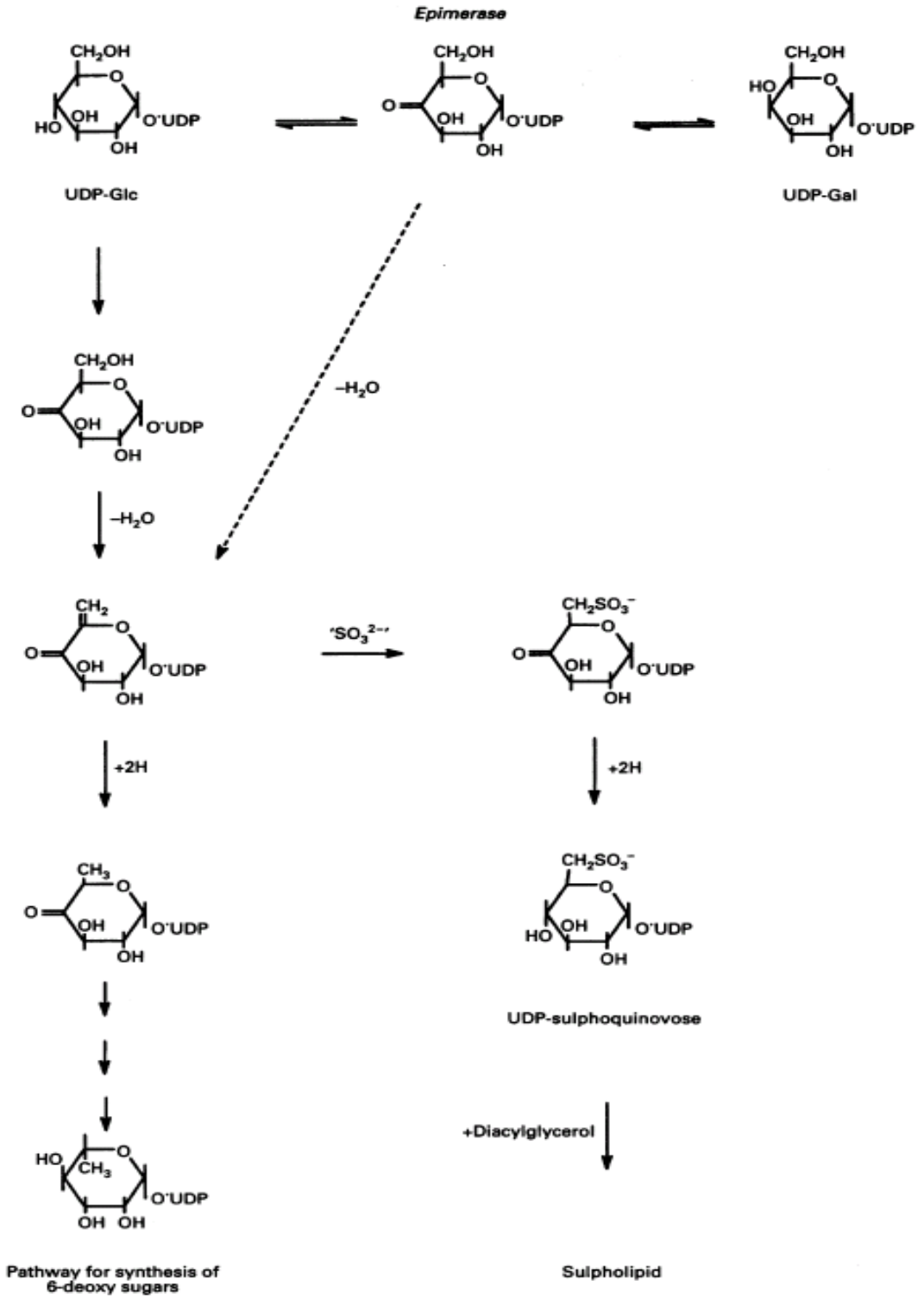


Figure 2.6: Proposed pathway for the biosynthesis of the plant sulfolipids (sulfoquinovosyldiacylglycerol)

Reproduced from Pugh *et al*., 1995

The biosynthesis of sulfolipids is a sequential, multi-step process. Several researchers, including Essingmann *et al*. (1998), Riekhof *et al*. (2003), Sato *et al*. (2003), Shimojima & Benning (2003) and Yu *et al*. (2002) have proposed that the final elucidation of sulfolipids biosynthesis involved the uridine diphosphate (UDP)-sulfoquinovose synthase for the biosynthesis of the head donor group, and the SQDG synthase responsible for the catalysed activation of the final assembly of sulfolipids. UDP-sulfoquinovose synthase was characterised to convert UDP-glucose and sulphite to UDP-sulfoquinovose for the in vitro production of *Arabidopsis* sulfolipids head group precursor (Sanda *et al*., 2001), while the SQDG synthase is found to encode with a protein (predicted glycosyltransferase) to constitute the final assembly of sulfolipids. Yu *et al*. (2002) inferred that these are the two essential steps for the biosynthesis of sulfolipids, as seen in Figure 2.7.

NOTE:
This figure is included on page 35 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.7: The final step of elucidation of sulfolipids biosynthesis

Two enzymes, SQD1 (SQDB) and SQD2 (SQDX), are specific to this process, and catalyse the reactions as indicated. DAG, diacylglycerol; R, fatty acyl groups; SQDG, sulfoquinovosyldiacylglycerol; UDP-glucose; UDP-SQ, UDP-sulfoquinovose. Modified from Yu *et al*., 2002.

Recent findings on the biosynthesis of sulfolipids indicate the importance of the initial step for the sulfolipids biosynthesis, for which the enzyme sulfotransferase is responsible, enabling the trehalose to trehalose-2-sulphate. Mougous *et al*. (2004) identified that this step is essential for sulfolipids synthesis, and results have shown the absence of sulfolipids when sulfotransferase stf0 was knocked out in the initial step of sulfolipids synthesis, that is, in the formation of the trehalose-2-sulphate (Mougous *et al*., 2004). Mougous *et al*. (2004) also provided new insight into the biosynthesis of sulfolipids and the functional role of the sulfotransferase. The latest proposal of the

sulfolipids synthesis is presumably completed in *Mycobacterium* within the cell's outer envelope as shown in Figure 2.8 from Schelle & Bertozzi (2006).

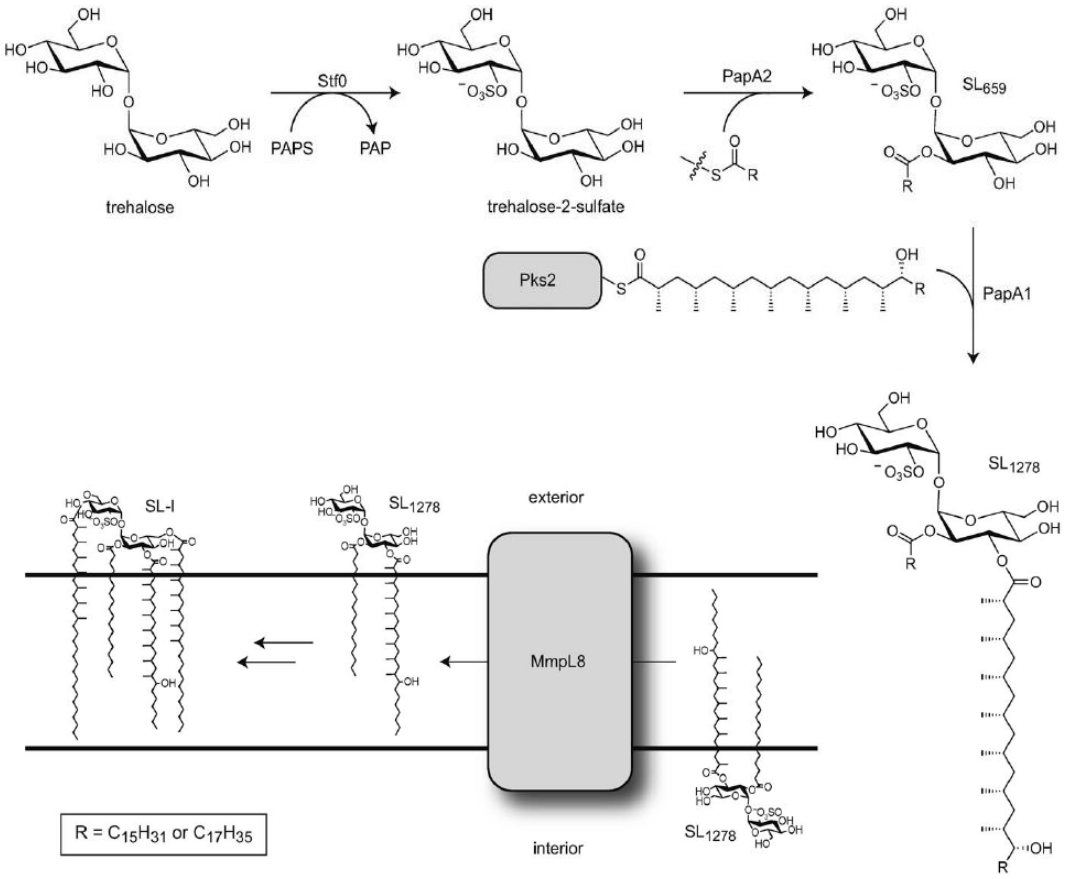


Figure 2.8: Biosynthesis of SL-1

Trehalose is sulphated by Stf0 to form trehalose-2-sulphate, which is then acylated by PapA2 at the 2' position to form SL659. This product is then acylated by PapA1 with a phthioceranoyl group synthesised by Pks2. MmpL8 transports the resulting compound, SL1279 through the cell membrane. SL-1 is presumably completed within the cell's outer envelope (Schelle & Bertozzi, 2006)

Mycobacterium tuberculosis synthesises trehalose-2-sulphate by sulfotransferase stf0 from trehalose. A PKS 2 gene is involved in the system when trehalose-2-sulphate was acylated by PapA1 with a phthioceranoyl group. Researchers discovered that the genes responsible for the synthesis of sulfolipids are the SqdX gene, however neither NRPS nor PKS genes encoded with novel domains are found. Discovery of the PKS 2 gene in *Mycobacterium tuberculosis* might lead to more availability in screening the PKS gene involved in the biosynthesis pathway of sulfolipids.

2.7 Metabolism within *Spirulina*

Metabolism is defined as: 'a set of biochemical reactions which included the uptake of nutrients from the environment, the transformation within the cells and elimination of wastes into the environment in living organisms' (Mardigan & Martinko, 2006). In each reaction, enzymes catalyse the chemical transformed and allow the organisms to reproduce. A primary metabolite is the substance that is directly involved in the reproduction of an organism; while a secondary metabolite is not. Typically, the quantity of primary metabolites exceeds much more than required, while for secondary metabolite, the production is significant less. Consequently, the study of the biosynthesis pathway of a secondary metabolite is highlighted to enhance the production of desirable metabolite.

In recent years, more than 15,000 natural products from marine unicellular bacteria, actinomycetes and fungi have been recognised as a potential source of novel pharmaceutical compounds (Füllbeck *et al*., 2006). The natural products have been chemically determined, and are significantly important to human health. Today, many of the bioactive metabolites are sourced from secondary metabolism, and this has resulted in a great interest in elucidating the metabolic pathway of the secondary metabolite. In addition, secondary metabolites attract more attention due to their insignificant production (Salomon *et al*., 2004).

2.7.1 Primary and secondary metabolism

All organisms possess their own set of biochemical reactions to survive. Primary metabolism describes the processes on the fundamental unity of all living matter, such as producing the carboxylic acids of the Krebs cycle, α-amino acids and other biochemical reactions (Torssell, 1983). A primary metabolite is the compound involved in the pathway during the growth phase of the organisms. Secondary metabolism is not necessary for normal growth or development, but only an individual expression of the species. These compounds, called secondary metabolites, are found to contribute to the species, fitness for survival (Torssell, 1983). However, the division between primary and secondary metabolism is unclear, due to the fact that these two types of metabolisms are linked closely, where the primary metabolism produces fundamental structure (e.g. glucose, phosphoenolpyruvate, pyruvate, acetyl-CoA) for the starting point of the secondary metabolism to start with, as seen in Figure 2.9.

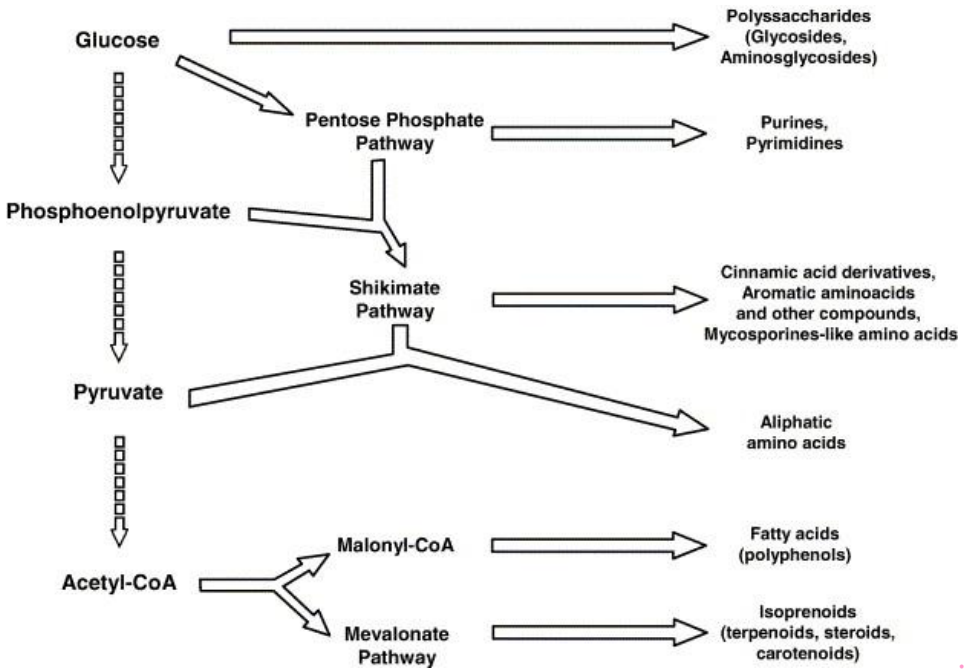


Figure 2.9: Main pathways of some secondary and primary metabolites biosynthesis

Sourced from Cardozo *et al*., 2007b

2.7.2 Metabolic pathways of the production of metabolites encoded with NRPS and PKS

The importance of studying the mechanism of the metabolic pathway by NRPS and PKS is to elucidate the biosynthetic pathways to create novel chemical structures. Many novel pharmaceutical bioactive compounds are of either polyketide, polypeptide origin or a hybrid of the two. A number of studies proved that most of the cyanobacteria are rich in non-ribosomal peptide synthase and polyketide synthase genes (Barrios-Llerena *et al*., 2007; Ehrenreich *et al*., 2005; Neilan *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000).

Ehrenreich *et al*. (2005) investigated the non-ribosomal peptide synthase and polyketide synthase by using different strains of cyanobacteria, and further performed algicide assays to determine the potential effects against *Synechococcus* and *Synechocystis*. Table 2.3 shows the screening results for the existence of NRPS and PKS from axenic and non-axenic cultures.

Table 2.3: Cyanobacteria strains analysed by (NRPS and PKS) polymerase chain reaction (PCR) and bioassay results

[a] Purities of cultures at time of study are also included. +, successful amplification for PCRs or activity observed for bioassays; -, no amplification for PCRs or no activity observed for bioassays; +/-, activity not observed in all replicate extracts of assay; ND, not determined; N, no; Y, yes. Adapted from (Ehrenreich *et al*., 2005)

The screening results from these investigations indicate that *Cyanothece* in different strains show the dissimilarity in containing the NRPS and PKS genes (Ehrenreich *et al*., 2005). Both of the NRPS and PKS genes are present in the strain of *Cyanothece* sp. WH8901, while the strain of *Cyanothece* sp. WH8904 has none of these genes. Inconsistency in the presence of NRPS and PKS genes may be due to the losses of the genes from the genome or those genes are not ancestral to the cyanobacteria lineage.

Ehrenreich *et al*. (2005) also revealed that the strain of *Spirulina* sp. PCC 6313 has both NRPS and PKS genes, with a putative NRPS A domain, and a putative PKS KS domain. Nevertheless, the strain of *Spirulina* sp. PCC 6313 has been characterised to present both of these genes. Borowitzka (1995) remarked that the natural algicides

possessed by the non-axenic culture are not inevitably precise. Therefore, the presence of NRPS and PKS in the strain of *Spirulina* needs further investigation.

There has been a steadily increasing trend in studying the biosynthesis of metabolites encoded by PKS and NRPS genes clusters in cyanobacteria. Barrios-Llerena *et al*. (2007) performed an investigation by screening 21 new cyanobacteria strains. Among these 21 strains, including fresh and marine cyanobacteria strains, 18 strains of cyanobacteria were found to have non-ribosomal peptide synthase genes; while 19 out of 21 of new strains of cyanobacteria have the polyketide synthase genes. From the results examined by Barrios-Llerena *et al*. (2007), *Leptolyngbya* sp. PCC 6703 and *Leptolyngbya* sp. PCC 7104, none were found to have either NRPS or PKS genes. However, the results from *Leptolyngbya* sp. PCC 7410 was shown to contain both of these genes, which means this strain of *Leptolyngbya* possesses secondary metabolite of nostopeptolide. Consequently, speculation that the natural product is likely to be encoded by NRPS and PKS followed this encouraging result.

Since NRPS and PKS genes play an important role in producing remarkable metabolites, the manipulation of these multifunction enzymes need to be developed in order to engineer new natural products efficiently. Zucko *et al*. (2007) made the first step to clarify the biosynthesis of natural products from *Dictyostelium discoideum* (a species of social amoeba) by screening the genes responsible. They successfully found 45 of type l iterative polyketide synthase genes that are responsible in producing the natural product from *Dictyostelium discoideum*. Furthermore, they characterised the PKS genes of *Dictyostelium discoideum* in order to provide the views on how natural products pathways evolved (Zucko *et al*., 2007). The purpose of characterising the PKS genes which underlie with the detectable activities is to expand the knowledge towards bioactive metabolites within the organisms. According to Bode and Muller (2005), the specified PKS genes that are usually possessed by cyanobacteria is a modular PKS I, which consists of the multiple sets of domains or modules (Bode & Müller, 2005).

Fungi comprise a large group of eukaryotic organisms and produce numerous secondary metabolites, and were found to include the NRPS and PKS genes. For example, Tobiasen *et al*. (2007) characterised the non-ribosomal peptide synthase genes as a producer of ferricrocin in *Fusarium graminearum*, *F. culmorum* and *F. pseudograminearium*. Although the existence of non-ribosomal peptide synthase and polyketide synthase genes can be found in cyanobacteria, the relationship between

genes and the biological activity need to be confirmed by using the fragments defined or gene expression experiments, since there is a remarkable diversity in the genes of NRPS and PKS (Tobiasen *et al*., 2007). Thus, the investigation is needed to correlate the biochemical activity to the genes.

2.8 Drying techniques

An integrated biomass production for microalgae is undertaken by three main processes: culturing, harvesting and processing of biomass. Drying, also known as 'post harvesting', is defined as an application to remove the water content after washing without affecting the quality of the biomass. Various drying methods, such as spray-drying, freeze-drying, solar-drying and convective hot-air drying have been used widely for the application of *Spirulina* (Desmorieux & Decaen, 2005; Desmorieux & Hernandez, 2004; Li & Qi, 1997; Oliveira *et al*., 2009). Typically, the most common drying processes are spray-drying and solar-drying.

There has been relatively little attention paid to the development of efficient drying methods without causing the loss of biological molecules. Due to the possible changes caused by the drying process, Desmorieux and Hernandez (2004) examined the effect of the air temperature on the drying process, and evaluated the structural changes of *Spirulina* at different oven temperatures, as shown in Figure 2.10.

Figure 2.10: Appearance of oven dried Spirulina in different air temperatures

(Desmorieux & Hernandez, 2004)

A significant change in the colour of *Spirulina* was observed when the air temperature was varied. In the aspect of the nutrition, the proteins from *Spirulina* are gradually lost from 40^{o}C–60^{o}C. Although spray-drying is the most common method used for the drying process of *Spirulina*, it has been shown that air temperature can be easily manipulated to control the quality of the biomass. Further to this, Desmorieux and Hernadez (2004) proposed a convective drying process for reducing the cost of drying, while still obtaining the minimum loss of nutritional value of *Spirulina*. Oliveira *et al*.

(2009) also attempted the drying of *Spirulina platensis* in a thin layer utilising perpendicular air flow.

2.9 Extraction

Natural products obtained from microorganisms are limited in variability and availability, inconsistent product quality, low metabolite yield; as well as the extraction process (Zhang & Furusaki, 1999). To optimise the natural products from microalgae, an appropriate extraction method is essential; especially to obtain a high recovery with low losses. The extraction of algae lipid becomes extremely complex in order to avoid the decomposition of the natural constituents. Hence, the algae extraction; lipid separation and quantification are difficult to estimate (Hagerthey *et al*., 2006; Lee *et al*., 1998b; Wiltshire *et al*., 2000).

There have been a number of researchers who have classified the advantages and disadvantages of using different types of extraction methods to excrete the natural compound from algae. Generally, an extraction method will be advantageous if it gives a shorter extraction time, less toxic solvent, environmental friendly and less capital cost as well as operating cost. The data shown in Table 2.4 is a compilation of the advantages and disadvantages of using various types of extraction methods, such as traditional solvent extraction, pressurised liquid extraction, supercritical fluid extraction, lysozyme extraction, detergent treatment extraction, microwave extraction, solid/liquid extraction and freeze-thaw. In the case of extracting the desired bioactive compounds from microalgae, some of the extraction methods used have various disadvantages. Although the extraction method used has some drawbacks, it is apparent that a better yield of the active extracts was optimised based on these extraction methods. Therefore, an improved extraction method will need to offer better extractability of the bioactive constituents.

Table 2.4: Review of advantages and disadvantages of extraction methods

Extraction Technique/s	Advantages	Disadvantages
Traditional solvent extraction (Liquid-liquid extraction)[1,2]		1. Large quantities of toxic organic solvent 2. Labour intense 3. Longer extraction time 4. Low selectivity 5. Low extraction yield 6. Expose the extracts to excessive heat, light and oxygen
Pressurized Liquid Extraction (PLE)[2]	1. less solvent 2. shorter extraction time 3. automated 4. environmental friendly	
Supercritical Fluid Extraction (SFE)[3, 4]	1. Dissolving powder of the supercritical fluid is controlled by pressure and/or temperature 2. Supercritical is easily recoverable from the extract due to its volatility 3. Non toxic solvents leave no harm residue 4. High boiling components are extracted at relatively low temperatures 5. Separations not possible by more traditional process 6. Thermally labile compounds can be extracted with minimal damage	1. Elevated pressure required 2. Compression of solvent requires elaborate recycling measure to reduces energy cost 3. High capital investment for equipment
Lysozyme extraction[5]	1. More gentle and specific for phycocyanin extraction	1. Higher lysozyme concentration, decrease the purity of phycocyanin
Extraction by detergent treatment[5]	1. Simple 2. Efficient for phycocyanin extraction	
Microwave extraction[6]	1. Shorter period time 2. Less energy consumption 3. Easily to break down by thermal means 4. Not deteriorate the products	
Solid/Liquid extraction[7]	1. Simplicity of solution phase synthesis 2. Ease of work work-up by simple filtration	
Freeze thaw[5]		1. Energy cost for large scale application is prohibited

Adapted from [1, 2](Jaime *et al*., 2005; Santoyo *et al*., 2006) [3, 4](Székely, 1986; Toschi *et al*., 2003) [5](Chauhan *et al*., 1994) [6 (]Mengal & Mompon, 1998) [7](Weller, 1998).

2.9.1 Traditional solvent extraction

Traditional extraction methods, such as liquid-liquid extraction or solid-liquid extraction, are widely used, especially for industrial applications. The philosophy of this extraction method is to use two different immiscible solvents to excrete the natural compounds from microorganisms.

Chapman *et al*. (1991) studied the cohesion of different solvents and related it to the lipids yield based on these solvents. They successfully correlated a relationship between the cohesion of different forms of solvent with the lipid yield, as shown in Figure 2.11. Due to the nature and polarity of the organic solvents, the solvents used will select the desired bioactive components accordingly based on their chemical structure; while leaving the other components behind.

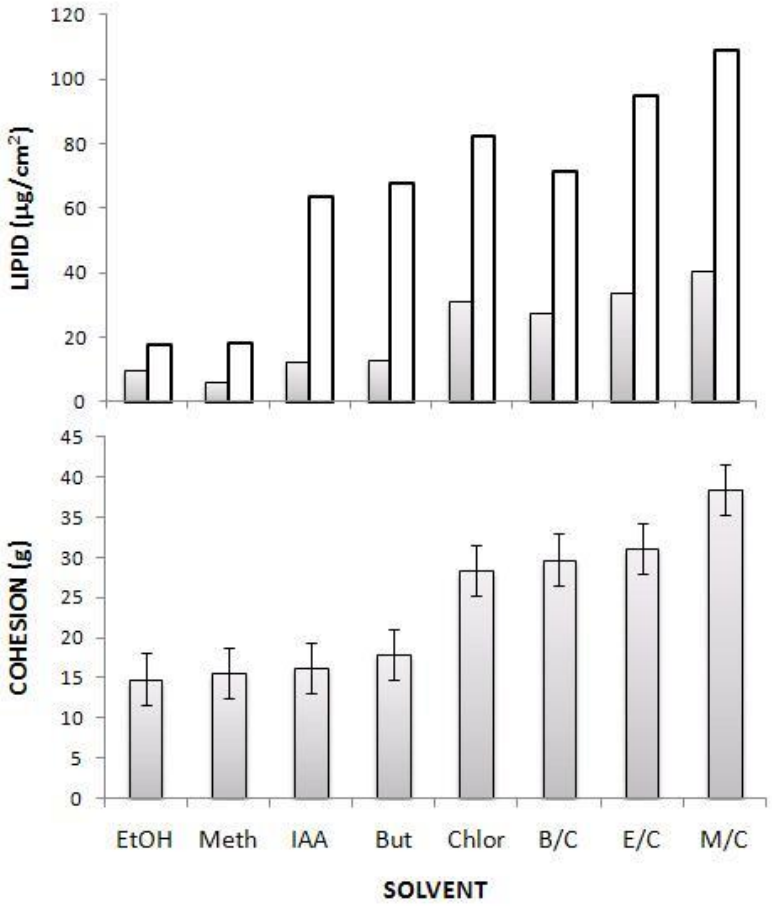


Figure 2.11: Relationship between the cohesion values with the yield of lipid based on solvent extractions of EtOH (Ethanol), Meth (Methanol), IAA (Isoamylalcohol), But (Butanol), Chlor (Chloroform), B/C (Butanol/Chloroform, 1:1, v/v), E/C (Ethanol/ Chloroform, 1:1, v/v), M/C (Methanol/Chloroform, 1:1, v/v)

Modified from Chapman *et al*. (1991)

In practice, the Bligh and Dyer method was found to be the most efficient extraction method to recover the lipids from microorganisms such as fungi (Somashekar *et al*., 2001), green algae, *Botryococcus braunii* (Lee *et al*., 1998b) and fish muscle (Lee *et al*., 1996). An ideal solvent extraction is preferred, because the desired compound is completely extracted without excreting the redundant compounds. Generally, alcohol is proposed in the extraction method because it is a relatively high polar solvent. Moreover, alcohol was found to have the ability to extract membrane-associated lipids such as SQDG.

2.9.2 Extraction techniques

Today, there are more promising extraction techniques that can be used to enhance the recovery efficiency of lipid. In order to accomplish an ideal extraction method, the extraction techniques need to be chosen wisely, depending on the functional group of the desired compound and the raw material (Shahidi, 2001).

A recent review of the selective extraction of specific biometabolite from the *Spirulina* strain is available, such as optimising the yield of GLA by supercritical fluid extraction (Mendes *et al*., 2005a), an anti-oxidant compound obtained by accelerated solvent extraction and pressurised liquid extraction (Herrero *et al*., 2004; Herrero *et al*., 2005) and an anti-viral compound obtained by hot water extraction (Hernandez-Corona *et al*., 2002). These mentioned techniques have various implementation performances for the extraction, based on the control parameters of temperature, pressure, pH and residence time. Solvent usage and energy consumption are the main issues affecting the extraction performance, especially for industrial applications. Additionally, different types of raw materials might show preference in certain extraction methods to extract the desired bioactive compound.

In order to further explore the implementation of extraction processes, a comprehensive investigation was done to compare the recovery efficiency from different raw materials (Cheung *et al*., 1998; David & Seiber, 1996; Moreno *et al*., 2003; Sporring *et al*., 2005; Szentmihályi *et al*., 2002). From their studies, the extracts from different raw materials achieved the highest recovery when different extraction methods were used. For instance, the avocado obtained a higher recovery when a microwave-hexane extraction method was used, however this application did not apply to soil.

A recent finding also showed that the supercritical fluid extraction has been used progressively for extracting specified compounds from microorganisms. This process, which involves the use of supercritical fluid, has been applied to prepare samples at an analytical scale. Carbon dioxide is one of the supercritical fluids that have been used widely in this application. Carbon dioxide is introduced as a suitable and scalable supercritical fluid because of its low cost, is a non-flammable compound and is essentially devoid of oxygen.

Mendes *et al*. (1995) stated that the yield of GLA and the other lipids from the *Spirulina maxima* was increased when it was subjected to the supercritical carbon dioxide extraction at a temperature of 50^{o}C and a pressure of 250 bars. There are various types of co-solvents that can be applied and mixed with the supercritical carbon dioxide extraction method to obtain a better yield of GLA. In their work, an addition of ethanol with supercritical carbon dioxide extraction method gave the highest yield of lipids and GLA. This implied that the supercritical fluid extraction is suitable to extract essential fatty acids, especially GLA and other lipids, such as neutral lipids, glycolipids and phospholipids (Mendes *et al*., 2003; Mendes *et al*., 2006; Mendes *et al*., 2005b).

2.9.3 Summary of studies on extraction methods applied to *Spirulina*

The published information on various types of the extraction methods for the *Spirulina* strains is summarised in Table 2.5. The bioactive compounds obtained from *Spirulina* are variable, due to the extraction techniques and solvents used. The extracts are essentially influenced by the solvents because the extraction methods are characterised differently in chemical and physical activity (Moreno *et al*., 2003). When considering the therapeutic effects of a known bioactive constituent, it was suggested that one should first consider the characteristic of the bioactive compound before the use of the extraction methods and solvents. Therefore, a large source of the bioactive compounds based on the selectivity of extraction can be achieved.

Table 2.5: Extraction methods for *Spirulina*

Compounds	Strain	Extraction Technique	Activity	References
Extracts	*Spirulina platensis*	Pressurised liquid extraction (PLE)	Anti-oxidant	(Herrero *et al*., 2004)
Extract	Cyanobacteria	Organic solvent extraction	Enzyme inhibitors, antibiotics, anti-cancer, anti-viral	(Patterson *et al*., 1993; Patterson *et al*., 1991)
Gamma linolenic acid	*Spirulina maxima*	Supercritical carbon dioxide extraction		(Mendes *et al*., 2003)
C-phycocyanin	*Spirulina platensis*	Using Klebsiella pneumoniae		(Zhu *et al*., 2007)
C-phycocyanin	*Spirulina maxima*	Inorganic salt extraction	Food colorant	(Herrera *et al*., 1989)
Phycocyanin	*Spirulina* sp.	Water extraction, homogenisation of cells, freezing and thawing, homogenisation in vortimixer, acid extraction		(Sarada *et al*., 1999)
Phycocyanin	*Spirulina platensis*	Enzymatic disintegration		(Boussiba & Richmond, 1979)
Proteins	*Spirulina platensis*	Hand-grinding		(Devi *et al*., 1981)
Polysaccharide	*Spirulina*	Hot water extraction	Anti-viral	(Hayashi *et al*., 1996b)
Lipoglucan	*Spirulina platensis*	Extract with 45% phenol		(Mikheiskaya *et al*., 1983)
Water soluble polysaccharide	*Spirulina maxima*	Ultrasonication	Anti-cancer	(Oh *et al*., 2010)

2.10 Summary

Updated information on the investigation of *Spirulina* was collated. The literature review performed on the *Spirulina* species recognised their potential as rich sources of protein, carbohydrate, fatty acids and other compounds. Even though the *Spirulina* strain is recognised, the taxonomic confusion around this strain remains unresolved.

With the advent of recent technologies, the classification of *Spirulina* can be identified by molecular tools or the chemotaxonomic classification.

From the review, it was seen that the trend of investigations with *Spirulina* was biased on the species of *Spirulina platensis* and *Spirulina maxima*. It appears there is a lack of information on other species of *Spirulina* in other locations. The use of *Spirulina* is broad, and its applications extends to waste-water treatment, aquaculture and medical applications (e.g. health). Due to its extensive use, the culture condition of *Spirulina* spp. has been well-studied. Numerous optimal growth conditions have been documented for the growth of *Spirulina*.

Cyanobacteria are well-known for producing toxic compounds because they possess the NRPS and PKS genes. Molecular screening of these genes makes rapid comparison of the possibility of producing toxin compound by cyanobacteria. In addition, the focus of the research on the SQDG has been highlighted. A number of studies that were done on the metabolic pathway of SQDG make a significant contribution to our understanding of the biosynthesis of this natural compound. Identification of the enzymes, which are responsible for the production of SQDG, also allows a rapid detection of their respective roles with other strains of cyanobacteria.

Although the biomass production of *Spirulina* has been manipulated, information about the optimisation of the extraction of target bioactive compounds (SQDG) is limited. Possible extractions for the SQDG were reviewed, and the effect that might bring about the extraction condition was examined.

CHAPTER 3 MATERIALS AND METHOD

3.1 Cyanobacterial isolates

To study the potential of *Spirulina* for use in medication, five isolates of *Spirulina* from different continents were investigated. Since there is no incentive for researchers to examine the nutritional value of *Spirulina* in the Eastern Hemisphere, *Spirulina* from Australia, China and Malaysia were selected. The purpose of screening more isolates of *Spirulina* is to exploit more suitable strains of microalgae to produce bioactive compounds.

Four commercial isolates marketed as *Spirulina* and an Australian *Spirulina* isolate were studied. These five species of *Spirulina* were obtained from Western Australia, Australia (1); Queensland, Australia (2); Kuala Lumpur, Malaysia (3); Guangzhou, China (4) and Jiangmen, China (5), as shown in Figure 3.1.

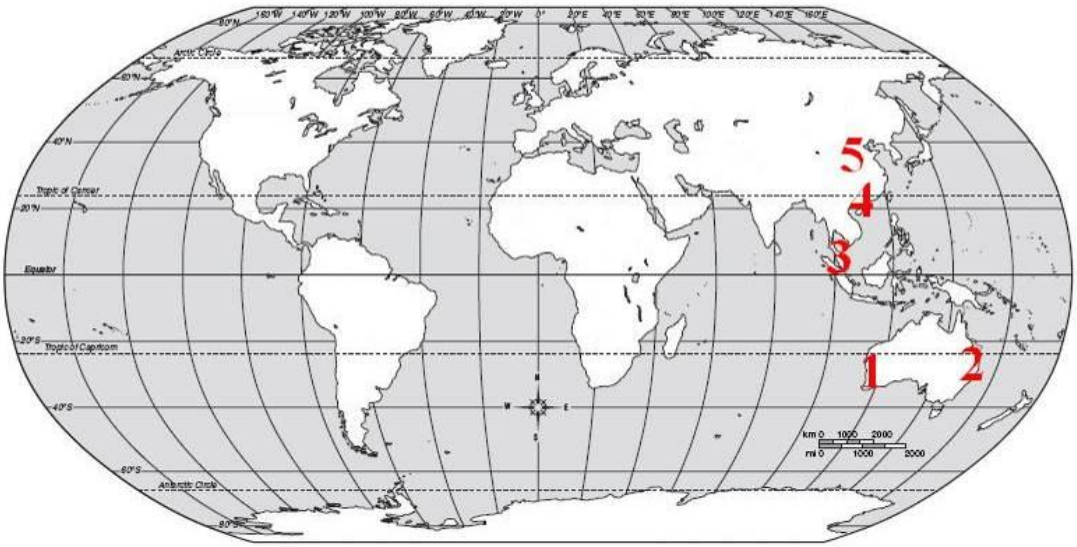


Figure 3.1: The locations of the five different isolates of Spirulina

They have been designated according to the printed label and these distinctive names were used throughout the study. Four commercial isolates, namely *Spirulina* (S), *Spirulina* (J), *Spirulina* (M) and *Spirulina* (P) were spray-dried and pelletised, while the Australia non-commercial *Spirulina* isolate, *Spirulina* sp. CS-785/01 was inoculated under laboratory conditions. *Spirulina* sp. was isolated from Pearse Lake, as shown in

Figure 3.2, Rottnest Island, Western Australia. Details of the species of cyanobacteria investigated in this research are shown in Table 3.1.

NOTE:
This figure is included on page 50 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.2: Location of Pearse Lake, Rottnest Island, Western Australia

Table 3.1: Details of algae investigated in this study

ISOLATES	DESIGNATION	ISOLATOR/MANUFACTURER	SOURCE
Spirulina sp.	*Spirulina* sp.	Ian Eliot, Commonwealth Scientific and Industrial Research Organization (CSIRO)	Western Australia, Australia[1]
Spirulina maxima	*Spirulina* (M)	OxyMin®	Queensland, Australia[2]
Spirulina platensis	*Spirulina* (P)	Elken	Kuala Lumpur, Malaysia[3]
Spirulina	*Spirulina* (J)	Jiangmen Yue Jian Biology Engineering Co. Ltd.	Jiangmen, China[4]
Spirulina	*Spirulina* (S)	South China Sea Institute Oceanology (SCSIO)	Guangzhou, China[5]

3.1.1 *Spirulina* sp.

The *Spirulina* sp. cultures were serially diluted and then spread onto the agar medium. The colonies that were free of other organisms were selected for further isolation because the second culture had lower possibility of bacterial contamination and contained more *Spirulina*. In order to check for bacterial contamination, the cultures were examined using a phase-contrast microscope after a week of culture. The non-

contaminated *Spirulina* was maintained in the culture medium, and were observed for any microbial contamination on the nutrient agar (Chem supply, Australia) and potato dextrose agar (Chem supply, Australia). This test based on the procedure carried out by Shirai (1989).

Different medium were assessed to allow the *Spirulina* isolate to be adequately maintained in the laboratory. The various media and growth conditions (e.g. temperature) were chosen from the literature related to Australian *Spirulina* based on the optimum nutrient, temperature and pH conditions from previous studies and were used to optimise the biomass productivity of an Australian *Spirulina* isolate. The Australian *Spirulina* isolate was maintained in MLA media, BG-11 media, as well as Zarrouk media (see Appendix B) at 25^{o}C with a cool light (~181.5 μmol photons m^{-2} s^{-1}) in a 12:12 light:dark cycle, in which the illumination was from beneath with light tubes (Thorn FL 26 18W/84V). Seawater was obtained from the South Australian Research and Development Institute (SARDI), and the seawater composition is presented in Appendix B. The initial pH of BG-11 media was between 7.5–8.0; the initial pH of Zarrouk media was between 9–9.5, while the initial pH of MLA media was in the range of 7.0–7.5. The isolate was cultured in larger flasks every two weeks to increase the amount of *Spirulina* biomass. The sterile conditions for cultivation were prepared by autoclaving the flasks and media that were plugged with non-adsorbent cotton wool, at 121^{o}C, 15 psi for 15 minutes. The isolate was inoculated into 10 mL culture tissue flasks, and the culture sealed up sequentially from the tissue flasks to 250 mL, 500 mL then 2L shaker flasks with a volume of 125 mL, 250 mL or 1000 mL respectively, as shown in Figure 3.3. Growth conditions for the isolate were varied with different temperature and media, as shown in Table 3.2.

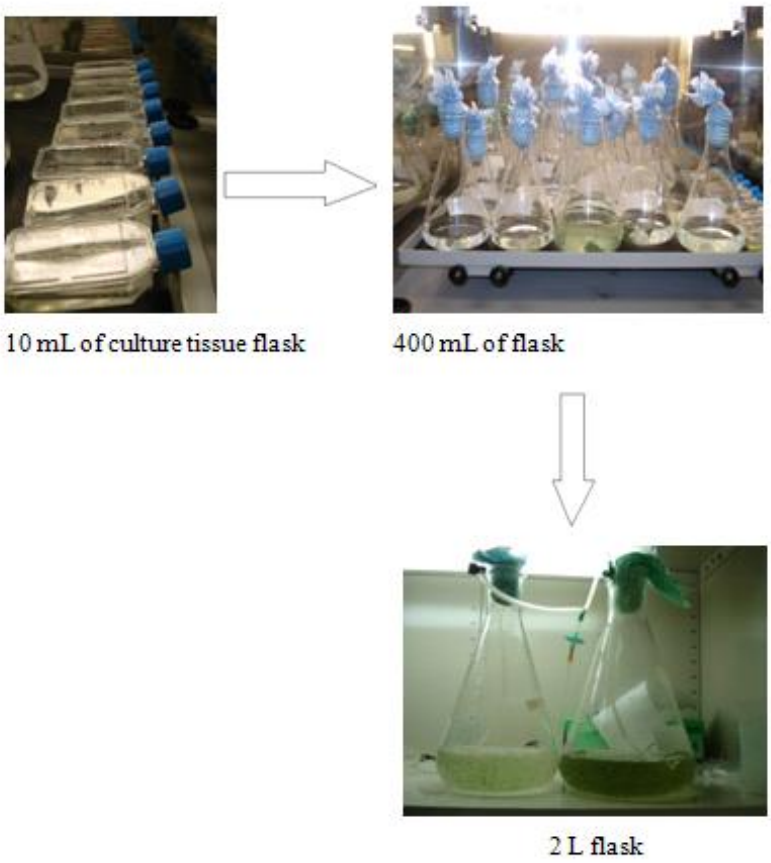


Figure 3.3: Subculture of Spirulina sp. from tissue culture flasks (small scale) to 2L flasks (large-scale)

Table 3.2: Various growth conditions for *Spirulina* sp.

Culture Media	BG-11	Zarrouk	MLA + NaCl	MLA + seawater	MLA + seawater	MLA + seawater
Initial pH	7.5-8.0	9-9.5	7.5±0.1	7.5±0.1	7.5±0.1	7.5±0.1
Temperature (^{o}C)	25	30	25	25	30	35

3.1.2 *Spirulina* (P)

For commercial products, such as pelletised *Spirulina*, information on culturing is confidential. Little information on the growth conditions of *Spirulina* was obtained. *Spirulina* (P) was cultured in a cultivation pool with cement concrete pool base, with dimensions of 8 x 40m^2. The pool is designed for water mixing, and facilitates stirring of the water evenly in the pool. *Spirulina* (P) was farmed in the artificial pools while maintaining the temperature at 30^{o}C to 35^{o}C in mineral rich water to achieve its optimal growth. *Spirulina* (P) is harvested and spray-dried. These data are divulged by Elken Sdn Bhd., Kuala Lumpur, Malaysia webpage (Reference:

www.healthsession.com/articles/elken-*Spirulina*-cultivation.html, Accessed 1/9/2008). Additional nutrients are added to the water, which includes carbon dioxide, nitrogen, potassium and iron to improve the quality of the *Spirulina* (P) cultivated.

3.1.3 *Spirulina* (M)

Spirulina (M) was purchased from OxyMin®. It is grown organically and free from contamination. *Spirulina* powder was processed under ambient temperature with a spray-drying process. The powder was subjected to a drying zone in the range of 45–55^{o}C to form fine green powder. (Reference: www.oxymin.com.au/msg10.htm, Accessed 23/12/2009)

3.1.4 *Spirulina* (S)

Spirulina (S) is a strain of *Spirulina platensis* that is isolated from Lake Chad, Africa, and grown under lab conditions with seawater. *Spirulina* (S) that is cultivated in seawater instead of freshwater was researched in the South China Sea Institute of Oceanography, Chinese Academy of Sciences, Guangzhou, China. In their study, *Spirulina* (S) possessed higher nutritional value (higher concentration of phyto-constituent) when compared to the strain cultivated in freshwater. *Spirulina* (S) was cultivated in an oval-shaped cement pond with a 15,000 m^2 area and 0.2–0.3 m depth. The pH value was always maintained at 8–9, and temperature at 26–32^{o}C. The culture media for growing *Spirulina* (S) was prepared from sodium bicarbonate (4.0~8.0 g/L), urea (0.1~0.8 g/L), sodium nitrate (1.5g/L), SSP (0.5~1.0g/L), potassium dihydrogen phosphate (0.3~0.5g/L), potassium chloride (0.5~1.0g/L), potassium sulphate (1.0g/L), salt (0.5g/L), magnesium sulphate (0.01g/L), ferrous sulphate (0.005g/L). The medium was added to reach a concentration of 8~10 g/L of sodium bicarbonate prior to use in culturing *Spirulina*. (Reference: http://www.lylzlcy.com/News_View.asp?NewsID=68, Accessed 3/6/2010)

3.1.5 *Spirulina* (J)

There is no information linking the growth conditions of *Spirulina* from Yue Jian Biology Engineering Co. Ltd. due to confidentiality. Therefore, the model that incorporates the parameters regulating the *Spirulina* (J) growth is unidentified.

3.2 Ultraviolet radiation

The culture was exposed to the ultraviolet radiation in order to maintain it in an axenic condition (Mehta & Hawxby, 1977). Experiments were carried out by varying the times irradiated under the ultraviolet radiation light Philips 30 watts/G30T8 BI-PIN Base cool light GE (General Electric Co.) germicidal tube set at 30 cm distance from the culture. The treated *Spirulina* sp. culture was subsequently inoculated on nutrient agar (Chem supply, Australia). Observation on the culture of *Spirulina* sp. and bacteria growth on agar plate was noted. The exposure times for the agar plates exposed under the ultraviolet radiation were 0, 5, 10, 15, 25, 30, 45, 60, and 120 minutes. The agar plate was checked daily for bacterial growth. The illumination time for the culture, which was shown to have the least bacteria growth without affecting the growth of *Spirulina* sp., was performed prior to subculture in this study.

3.3 Growth

The growth of *Spirulina* can be estimated by the measurement of the chlorophyll-*a* content within a certain time period and then using the data for construct a predictive scheme for the growth of *Spirulina* as a function of time (Richmond, 2004). Dry-cell weight, instead of the number of cells, was determined to obtain a better result. This is because of the difficulty in counting the filamentous *Spirulina* under the microscope. Furthermore, the accuracy of counting the number of cells is relatively low due to the different size of *Spirulina* obtained in the culture media. Firstly, 10 mL of *Spirulina* were measured and centrifuged at 3273 g for five minutes. The supernatant was decanted, and the cell weight per mL was determined. The weight of the wet paste was obtained and the paste was dried in the oven overnight until a constant weight was obtained. The cells were ground in a mortar and pestle to achieve a powder form. Then the powder form was added to a clean glass tube with addition of 0.9 mL acetone and 0.1 mL (1 g/L) of magnesium carbonate. The mixture was then homogenised and centrifuged again at 3273 g for five minutes. The absorbance of chlorophyll was carried out using the Shimadzu UV_VIS 1601 Spectrometer. Acetone was used as a blank, and the absorbance was set at zero. The extracted chlorophyll-*a* from *Spirulina* was inserted and the reading of absorbance noted. Absorbance was obtained at three different wavelengths to give a calculation of chlorophyll–*a* per mL as seen in Equation 3-1 (Jeffrey & Humphrey, 1975).

$$\mu g\ chl.a/ml = 11.85A_{664} - 1.54A_{647} - 0.08A_{630} \quad \text{Eq. 3-1}$$

3.4 Cell preparation for extraction from culture medium

The preparation for drying the cells for further experiments was required. The cells were separated from the media by using vacuum filtration through a No. 54 type Whatman filter paper. The mineral from the medium was rinsed with distilled water (volume approximately equal to three times initial culture flask volume). The resulting washed biomass was dried under silica gel.

3.5 Isolation of genomic DNA (DNA extraction)

3.5.1 XS DNA extraction

The DNA extraction was carried out using the Xanthogenate-SDS (XS) DNA extraction protocol (Tillett & Neilan, 2000). Two millilitres of cell culture was contained in an eppendorf tube and was centrifuged at 1,000 g for five minutes. After centrifuging, the supernatant was removed. Once the supernatant was removed, the cell pellet was resuspended in 500 µL XS buffer of 1% potassium ethyl xanthogenate (Fluka, Buchs, Switzerland), 100 mM Tris-HCl, pH 7, 20 mM EDTA, pH 8.1; 1% sodium dodecylsulphate, 800 mM ammonium acetate. The solution was incubated in a water bath at 65^{o}C for two hours and was then put on ice for 10 minutes. The sample was centrifuged again at 12,000 g for 10 minutes. The supernatant was removed carefully, and was transferred to a new tube. One millilitre of chloroform was added subsequently, and two layers allowed to form in the tube. The upper layer was collected and frozen with one millilitre of isopropanol overnight. The sample was centrifuged at 13,000 g for five minutes on the following day, and the supernatant was discarded. The DNA pellet was dissolved in 500 µL TE buffer solution, derived from one millilitre of Tris-HCl (pH 8.0) and 0.2 mL EDTA (0.5M), made up to 100 mL, for a few minutes. Then, the sample was added to 500 µL phenol:chloroform and the upper layer was collected. Several repeats of this step are required to obtain a clear form of DNA. Fifty millilitres of sodium acetate (NaAc) was added, followed by one volume of isopropanol. Again, the sample was frozen for another half hour. The sample was then centrifuged at 14,000 g for 10 minutes after freezing, and the pellet was washed with 70% ethanol. The pellet containing with ethanol was centrifuged at 14,000 g for three

minutes and the supernatant was disposed. The pellet was air-dried for half an hour and suspended in 50 μL of TE.

3.5.2 Improved XS DNA extraction

An improved DNA extraction method was undertaken based on the aforementioned XS DNA extraction protocol. More steps were included to achieve a better yield of DNA with less contamination. Autoclaved cryovials with silica beads were dried, a preheat XS buffer was added, and then thawed in a hot water bath at 65^{o}C. The cells from culture media were added into the XS cryovials and bead beaten for 45 seconds at six beads per second by using a FastPrep FP120 bead beater (Salvant, Scoresby, Australia). The cryovials were placed in a hot water bath for three hours and vortexed every 30 minutes. The cryovials were re-beaded and no clumps were observed. Samples were left on ice for 10 minutes, followed by a 12,000 g centrifugation for 10 minutes to allow formation of a good pellet. The supernatant was carefully discarded without disturbing the cell pellet, and phenol chloroform isoamyl alcohol solution was added to allow it to be well-mixed. The mixture was centrifuged at 12,000 g for 10 minutes at 4^{o}C. The supernatant was collected in a new tube and precipitated with 50 μL 3M NaAc and one millilitre of ice cold ethanol. This was done overnight to allow the DNA to precipitate in the cold room. Subsequently, the sample needed to spin down for 30 minutes at 14,000 g. The supernatant was drawn off and 150 μL ice cold 75% ethanol was added without dislodging the pellet. Again, the supernatant was drawn off after centrifuging for 10 minutes at 14,000 g. The tube was air-dried on the bench and the ethanol allowed to evaporate until no ethanol odour could be detected. The DNA was finally dissolved in TE buffer with a volume of 50 μL. The purity and the concentration of genomic DNA were determined by using a NanoDrop UV/VIS spectrophotometer (Biosciences, Sydney, Australia) at 260nm and 280nm.

3.6 Identification of cyanobacterial isolates

3.6.1 16S rDNA, PKS and NRPS genes PCR amplification

By using the cyanobacterial specific primers 27F/809R and 740F/1494R together with PCR reagents as described by Gehringer *et al*. (1999), 16S rDNA gene PCR amplification was performed. Degenerate primers used for the amplification of 16S rDNA, which are outlined in Table 3.3, were supplied by Sigma Genosys, Australia.

PCR was performed with a reaction mixture set up as provided in Table 3.4. The reaction mixture was vortexed gently to ensure it was well-mixed.

Thermal cycling for 16S rRNA amplification was performed using a GeneAmp PCR system 2400 Thermocycler (Perkin-Elmer, Norwalk, USA), and consisted of an initial denaturation step at 94^{o}C for two minutes, followed by 30 cycles of DNA denaturation at 94^{o}C for 30 seconds, primer annealing at 55^{o}C for 30 seconds, strand extension at 72^{o}C for one minute and a final extension step at 72^{o}C for seven minutes.

Table 3.3: Cyanobacterial 16S rRNA gene amplification and sequencing primers

Primer	Sequence	Target	T_m(^{o}C)	Reference
27Fl	AGAGTTTGATCCTGGCTCAG	16S-rDNA	56	(Neilan *et al*., 1997)
809R	GCTTCGGCACGGCTCGGGTCGATA			(Saker *et al*., 2005)
MTF2	GCNGG(C/T)GG(C/T)GCNTA(C/T)GTNCCAGGAYVP	NRPS ~1000bp	53	(Neilan *et al*., 1999)
MTR	CCNCG(AGT)AT(TC)TTNAC(T/C)TGQVKIRG		51	
DKF	GTGCCGGTNCCRTGNGYYTC	PKS ~650bp	67	(Moffitt & Neilan, 2001)
DKR	GCGATGGAYCCNCARCARMG		65	
740F	GGC(TC)(AG)(AT)A(AT)CTGACACT(GC)AGGGA	16S-rDNA		(Jungblut *et al*., 2005)
1494Rc	TACGGCTACCTTGTTACGAC		56	(Neilan *et al*., 1997)

In order to perform polyketide synthetase PCR amplification, degenerate oligonucleotide primers of DKF and DKR (Table 3.3) were used. Thermal cycling conditions were initiated with a denaturing step at 94^{o}C for two minutes, followed by 35 cycles of DNA denaturation at 94^{o}C for 10 seconds, primer annealing at 55^{o}C for 30 seconds, strand extension at 72^{o}C for one minute and DNA strand extension at 72^{o}C for seven minutes. The non-ribosomal peptide synthetase PCR amplification was performed similarly to the polyketide synthetase, with different use of degenerate oligonucleotide primers of MTF2 and MTR as shown in Table 3.3; and different primer annealing temperature of 52^{o}C for 30 seconds.

Table 3.4: Reaction mixture recipe

REAGENTS	VOLUME (μL)		
	16S rRNA	NRPS/PKS	*sqdB*/*sqdX*
10x buffer	2.0	2.0	2.0
$MgCl_2$	1.0	1.0	1.0
dNTPs	1.5	1.5	1.5
Primer (forward)	1.0	2.5	1.5
Primer (reverse)	1.0	2.5	1.5
Taq	0.2	0.2	0.2
Milli Q water	12.3	9.3	10.3
DNA	1.0	1.0	2.0

3.6.2 Identification of gene responsible for sulfolipids biosynthesis—Primer design

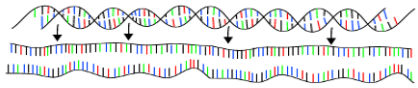
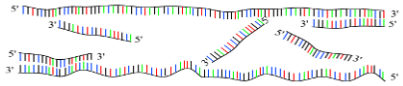
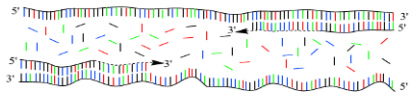
Oligonucleotide primers were designed for the amplification of cyanobacterial sqdB and sqdX fragments, as shown in Table 3.5. Refer to Appendix C for more details on designing the primer. Genomic DNA was extracted from *Spirulina* using improved XS DNA extraction method (refer to Section 3.5.2). Reaction mixture for the sqdB and sqdX PCR amplification was performed slightly differently with a smaller volume of primer, although the concentration of primer used in this case was higher.

Table 3.5: Designed degenerate primers for sqdB and sqdX genes

Primer	**Sequence**	**Target**	**T_m(^{o}C)**
d*sqdB*F	GAYGGNTAYTGYGGNTGG	*sqdB* ~900bp	55.9
d*sqdB*R	GGCGTRAAYTGRTTRAANAC		53.1
d*sqdX*F	GGTNGAYGGNATNGTNAC	*sqdX* ~900bp	49.3
d*sqdX*R	ATRTCNGGDATNCCNCC		50.6
d*sqdX*1F	GGATYCAYGTKGYBAAYCCDGC	*sqdX* ~700bp	62.0
d*sqdX*1R	CCNGCBGCCATNGCYTC		63.3

To obtain successful results (i.e. more than 60% of total sequenced templates), modification of the PCR conditions needs to be evaluated. The first trial of the cycling conditions was performed in a GeneAmp PCR system 2400 Thermocycler (Perkin-Elmer, Norwalk, USA), and the cycling conditions for amplification of either sdqX or *sqdB* genes were employed as the condition in Table 3.6.

Table 3.6: PCR cycles

PCR	Step(s)		
	Heat	94°C for 2 min	
	Denature	94°C for 5 sec	Repeat 35 cycles of 3 steps
	Annealing (forward and reverse primers)	45–60°C for 5 sec	
	Extension (only dNTP's)	72°C for 5 sec	
	Final extension	72°C for 5 min	
	Completion	20°C (Hold)	

3.7 Agarose gel electrophoresis

PCR products were loaded in the created agarose and PCR product combined with a loading buffer. The loading buffer is intended for gel electrophoresis of DNA fragments or PCR products, and contains Bromphenol Blue, Xylene Cyanol GG, and Ficoll, subsequently analysed by electrophoresis in ethidium bromide stained (0.5 μg/mL) 1% agarose gels. A 10kb ladder molecular weight standard, as shown in Figure 3.4, which comprised three reference bands at 3,000, 1,000 and 500bp (Roche), was used to estimate PCR fragment size. To visualise and identify the nucleic acid bands, ethidium bromide was used to stain for 15 minutes prior to exposure to UV light on a Gel-DOC (Bio-Rad, Gladesville, Australia) system running Quantity One 4.1R software (Bio-Rad).

Figure 3.4: Gene ruler (ladder mix 10 kb)

http://www.fermentas.com/en/products/all/dna-electrophoresis/generuler-dna-ladders/sm0313 (Accessed 15 October 2009)

3.8 Purification of DNA

3.8.1 Purification of DNA from PCR products

From the photograph taken by the transilluminator, a suitable method was selected to purify the PCR template. The PCR product that only eluted the desired band on the agarose was purified by a direct method with addition of two volumes of ice cold absolute ethanol to the PCR product and allowed to stand for 15 minutes on ice. The mixture was then centrifuged at 16,000 g for 15 minutes and the supernatant was removed without disturbing the pellet. 190 mL of 70% ethanol was added to wash the pellet followed by centrifugation at 16,000 g for 15 minutes. The supernatant was removed and the DNA pellet was allowed to dry at room temperature. The DNA was resuspended in the original volume of TE buffer.

3.8.2 Purification of DNA from agarose gels

The PCR product that amplified more than an allele was purified by a MoBio Ultra Clean^{TM} (gel purification) kit, and applied to isolate specific DNA from agarose gel. Little modification was done on the protocol to achieve better recovery of DNA. The desired DNA band was cut from a TAE agarose gel with a buffer solution made up to

Tris-acetate buffer (pH 8.0), EDTA, which sequesters divalent cations and one gram of agarose was dissolved in 100 mL of TAE solution. The gel slice weight is determined and this can be done in a spin filter or separate tube. The gel cut is approximately 0.1 g and three volumes of gel bind buffer were required to add with the gel slice in an incubation of 55^{o}C for two minutes. Once it was well-mixed, the spin filter was centrifuged at 10,000 g for 10 seconds. In order to mix the flow thoroughly, the spin filter was removed prior to vortexing the collection tube for five seconds. The liquid from the collection tube was followed by reloading back onto the spin filter. For a second time, the spin filter was centrifuged at 10,000 g for 10 seconds. The liquid from the collection tube was then discarded. To keep the DNA bound to the spin filter, a 300 μL gel wash buffer was added. The spin filter needs to centrifuge again for slightly longer (30 seconds) at the same speed. For full recovery of the DNA, the filter basket needs to be completely dried to avoid the inhibition downstream by the gel wash buffer, which contained ethanol. Once the filter basket is dried, it is transferred to a clean collection tube with addition of 50 μL elution buffer onto the centre of the spin filter membrane. The final centrifugation was taken at 10,000 g for 30 seconds, and the collection tube was ready for the use of DNA.

The PCR template needs to be clean and free of contaminants without degrading the DNA. The purified DNA was rerun on an agarose gel to confirm no unspecific products are visualised and the presence of the product is to determine the concentration size of the PCR fragments. Reaction mixture for sequencing PCR was prepared as shown in Table 3.7.

Table 3.7: Reaction mixture for sequencing PCR

Reagents	Volume (μL)
Big dye + 5X sequence buffer	10
3.2 pmol primer	1
20–50ng PCR product	1.5
Milli Q water up to 20μL	

PCR cycling conditions for sequencing is carried out with an initial denaturation of 96^{o}C for three minutes, followed by 96^{o}C for 10 seconds; 50^{o}C for five seconds; 60^{o}C for four minutes; 30 cycles and held at 20^{o}C.

3.9 Sequence clean up

After the sequencing PCR, the product was transferred to a 1.5 mL tube with the addition of 16 μL of mili Q water and 64 μL of 95% ethanol. The mixture was left for 15 minutes at room temperature and centrifuged for 20 minutes with 16,000 g speed. After centrifugation, the supernatant was removed and 190 μL of 70% ethanol was used to wash the pellet. Another spin at 16,000 g was carried out for 10 minutes, and again the supernatant was removed and dried with a Speedi-Vac vacuum pump for 10 minutes.

3.10 DNA sequence analysis

DNA sequencing was performed on the Applied Biosystems Model 373 sequencer at the Automated DNA Analysis Facility, University of New South Wales (UNSW) and automated analysis undergoes the PRISM Big Dye Terminator V3.1 cycle sequencing system (Applied Biosystems, Foster City, CA, and USA). The DNA sequence was analysed using the ABI Prism-Autoassembler programme, and multiple sequence alignments were compiled and analysed using Bioedit. A Basic Local Alignment Search Tool (BLAST) was used to identify the most closely related sequences in the NCBI database as below:
(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) (Accessed 14 September 2009)

The phylogenetic analysis was performed using CLUSTALX2 for protein alignments. The settings used in the multiple alignments were 10.0 gap opening, 0.2 gap extensions and 0.5 DNA transition weight. The phylogenetic trees were constructed using the neighbour-joining and bootstrap analysis (Larkin *et al*., 2007) and viewed using NJplot (Perrière & Gouy, 1996).

3.10.1 GenBank accession numbers

The sequences presented in this study are available under GenBank accession numbers: 16S rDNA HQ008224-HQ008228, *sqdB* HQ008229-HQ008232, *sqdX* HQ008233-HQ008236.

3.11 Ash free dry weight

The ash free dry weight (AFDW) was measured based on the standard method (ASTM, 1995). The dried sample was removed from the oven at 70^{o}C and placed in a desiccator to remove the remaining moisture. Once the dry weight of the algae cells remained constant, the sample was placed in a TGA 2950 muffle furnace (TA instruments, New Castle, DE, USA) at 575^{o}C.

3.12 Carbohydrate extraction

The total sugars were determined by using the standard phenol-H_2SO_4 (sulphuric acid) method (DuBois *et al*., 1956). A known quantity of the dried *Spirulina* biomass (1 g) was suspended in one millilitre of water and the supernatant was collected via centrifugation at 16,100 g for 10 minutes. Once the supernatant was obtained, one millilitre of 5% w/v phenol and followed by five millilitres of concentrated sulphuric acid were added. The mixtures were well-mixed and allowed to stand for 25 minutes at room temperature. The concentration of the carbohydrate was determined spectrophotometrically at 490 nm using a Shimadzu UV-VIS 1601 spectrometer. In this assay, a calibration curve was obtained by using a range of D-glucose concentration (from 0–200 μg/mL) to determine the carbohydrate content.

3.13 Protein extraction

A known quantity of the dried *Spirulina* biomass (1 g) was prepared. The sample was ground to 100 μm particle size. The crude protein was calculated by multiplying the total nitrogen with a converstion factor of 6.25, where the total nitrogen was determined by a Leco TruSpec CHN analyser (Lecon Australia, Castle Hill, NSW, Australia) (Dorsey *et al*., 1978).

3.14 Sequential extraction

A known quantity of the *Spirulina* biomass (40 g) was obtained for the use of sequential extraction prior to the preliminary phytochemistry analysis. The biomass was added to 200 mL of each of five different polarities of solvents for sequential extraction (1:5, w/v) in 250 mL beakers (Pyrex). The solvents with different polarities were selected based on the dielectric constants as shown in Table 3.8. The extraction was first carried out for a period of 48 hours by using the solvent with the least polarities. The extracts

were filtered using cotton with the philosophy of gravity filtration. With the extraction hood running, the organic layer was concentrated to dryness under a gentle stream of nitrogen gas with a warm water bath (at 50^{o}C). The extraction was repeated with the residue added in toluene, acetone, methanol and finally distilled water with the same ratio (1:5, w/v) in the same manner, except the aqueous extract was dried in sunlight. The weight of the extracts was obtained to calculate the percentage yield.

Table 3.8: Dielectric constants of solvents used for the sequential extraction

Solvent	Polarity Index
Hexane	1.9
Toluene	2.4
Acetone	21.0
Methanol	33.0
Water	80.0

3.15 Lipid extraction

3.15.1 Pre-treatment for lipid extraction

For the Australian isolate (*Spirulina* sp.), two weeks of culture was prepared from its medium via centrifugation at 2095 g for 20 minutes and washed several times with the deionised water to dissolve the salts. The supernatant was discarded and the wet mass was stored in an oven at 50^{o}C and 70^{o}C overnight. The effects of the oven temperature on the structural and lipid composition of the *Spirulina* sp. was studied. The appearance and the colour of these isolates of *Spirulina* were observed under an Olympus Microscope with available lens magnifications of 4X, 10X, 20X and 40X. To view the structure of dried *Spirulina* under the microscope, a small drop of water was placed on the microscope slide with dry samples of *Spirulina*. The addition of the water helps with viewing the sample and facilitates easier transmission of the light through the slide. For the commercial *Spirulina* (e.g. *Spirulina* (P) and *Spirulina* (M)), these *Spirulina* isolates were dried by using the spray-drying method, while the *Spirulina* (S) was dried by using the air-drying method.

The dry weight of the algal cells was checked frequently until it was constant prior to lipid extraction. Once the dried biomass was ready to be used, one millilitre of 0.9%

NaCl solution was added to the sample. The addition of 0.9% NaCl solution in the pre-treatment of lipid extraction aided the deactivation of the enzymes and the removal of the contaminants in the aqueous phase of the lipid extracts (Christie, 1993).

3.15.2 Lipid extraction techniques

The first attempt of the lipid extraction was performed by using various extraction methods with the solvent system chloroform:methanol (2:1, v/v). This solvent system was developed by Bligh & Dyer (1959). It gives relatively small losses, and has been commonly used for algae lipid extraction (Bligh & Dyer, 1959; Lee *et al*., 1998a; Pick *et al*., 1985; Siaut *et al*., 2011). For the *Spirulina* sp., the biomass was stored at 70^{o}C for oven-drying, based on previous studies (Liu *et al*., 2010; Rafiqul *et al*., 2003). The calculation of the lipid yield determination is shown below:

$$Lipid\ Content\ (\%AFDW) = \frac{Lipid\ extracted\ (g)}{Ash\ \ Free\ \ Dry\ Weight\ (g)} \times 100 \qquad \text{Eq. 3-2}$$

* The combined volume should be close to the theoretical calculated value (Lee *et al*., 1996)

3.15.2.1 Microwave extraction

A small modification of Moreno's microwave extraction method was carried out (Moreno *et al*., 2003). A known quantity of the *Spirulina* biomass (5 g) was obtained for the use of microwave extraction. The biomass was mixed with five millilitres of distilled water and the cap was securely closed before heating in a Panasonic microwave oven with 50% of 1100 watt (i.e. 550 watt) for 30 seconds. Once the mixture was cooled to room temperature, 15 mL of methanol was added and 30 mL of chloroform was subsequently added. While the mixture was allowed to stand for 45 minutes, it was vigorously shaken every 10 minutes.

3.15.2.2 Homogenisation

A known quantity of the sample (5 g) was weighed. This sample was first homogenised with five millilitres of 0.9% NaCl solution, where this process was done by using a Potter-Elvehjem homogeniser and a polytetrafluoroethylene (PTFE) pestle. The sample was subjected to repeated homogenisation, each time adding a new solvent to the sample. The sample was homogenised in 15 mL of methanol for two minutes, subsequently in 30 ml of chloroform for another two minutes. The final volume for the

extraction was made up to a total volume of 50 mL. The sample was homogenised as a solution before it was concentrated in a centrifuge.

3.15.2.3 Sonication

This is a method to improve the recovery of lipid by attempting to have sufficient cell breakage prior to the lipid extraction. Sonication was used to lyse the cell with high power. A known quantity of the samples (5 g) was mixed with five millilitres of 0.9% NaCl and 15 mL of methanol. The mixture was sonicated for 10 seconds with 30% amplitude by a Bradson Digital Sonicator. The sonication was sustained after an addition of 30 mL of chloroform.

3.15.2.4 Organic solvents extraction

A known quantity of the *Spirulina* biomass (5 g) is extracted by using various forms of the organic solvents. Different forms of the organic solvents that are mixed in a certain ratio were used, as shown in Table 3.9, for the extraction. The mixtures were then added and allowed to stand for 45 minutes.

Table 3.9: Combination system of the organic solvent used for extraction

Organic solvent	Ratio (v/v)
Butanol	
Hexane:Isopropanol	3:2
Chloroform:Ethanol	2:1
Chloroform:Butanol	2:1
Chloroform:Methanol	2:1
Chloroform:Methanol	1:1
Chloroform:Methanol	1:2
Chloroform:Methanol	1:4
Chloroform:Methanol	1:8
Chloroform:Methanol	1:16

All of the extracts were treated as follows. The cell pellet was removed via centrifugation at 3273 g for approximately 20 minutes using a Sorvall RC5-C centrifuge. The supernatant was allowed to be partitioned into two layers, where the

lower organic layer was recovered. With the extraction hood running, the organic layer was concentrated to dryness under a gentle stream of nitrogen gas.

3.15.2.5 Soxhlet extraction

Soxhlet extraction was carried out at the SARDI. A known quantity of the isolate of *Spirulina* (M) biomass (20 g) was placed into a cellulose extraction thimble and the top of the thimble was covered with glass wool to prevent floating. The lipids were extracted with 200 mL of hexane at the boiling point for seven hours in a soxhlet extractor (Figure 3.5) using a heating mantle. When the sample was cool, the solvent was removed from the extract in a rotary evaporator at 40^{o}C under reduced pressure.

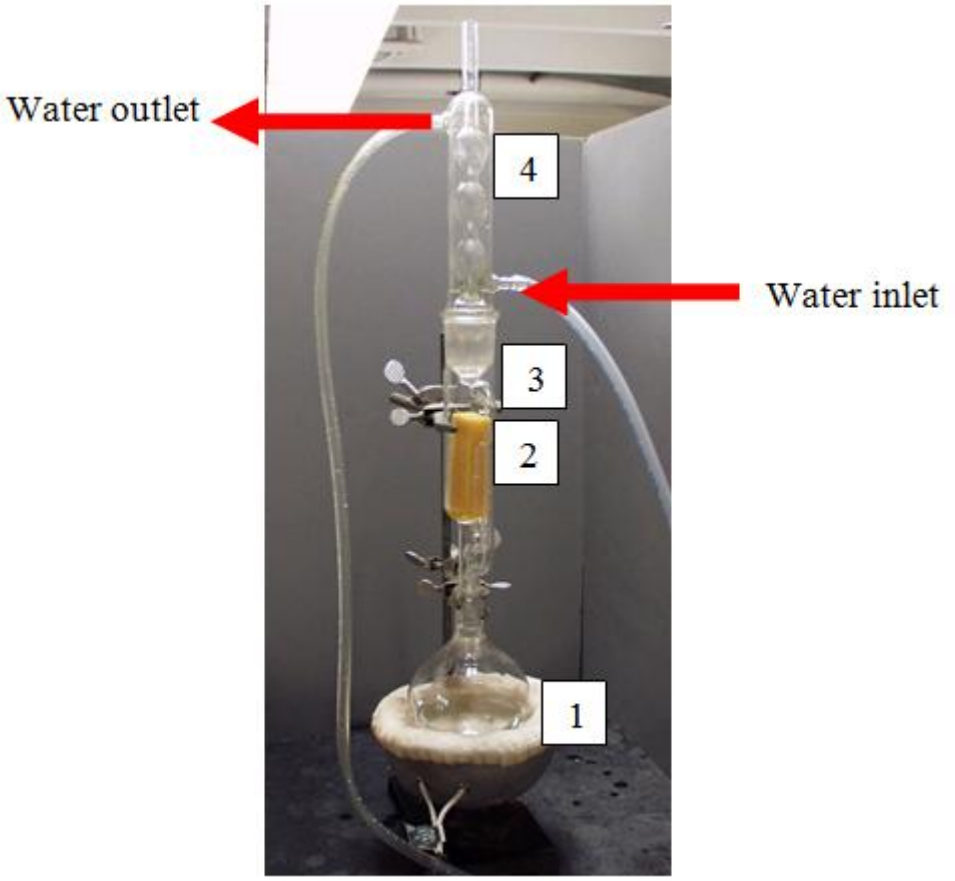


Figure 3.5: Soxhlet extraction apparatus

1: Still pot, 2: Thimble, 3: Extraction chamber, 4: Condenser

3.15.2.6 Supercritical carbon dioxide extraction

This process was started by determining the solubility parameter of the lipids under different operating conditions to achieve a better yield of lipids from the supercritical carbon dioxide. The supercritical fluid extraction was carried out by using a

Supercritical Fluid Extractor (HP7680T, Australia) at the University of Adelaide (schematic diagram is shown in Figure 3.6).

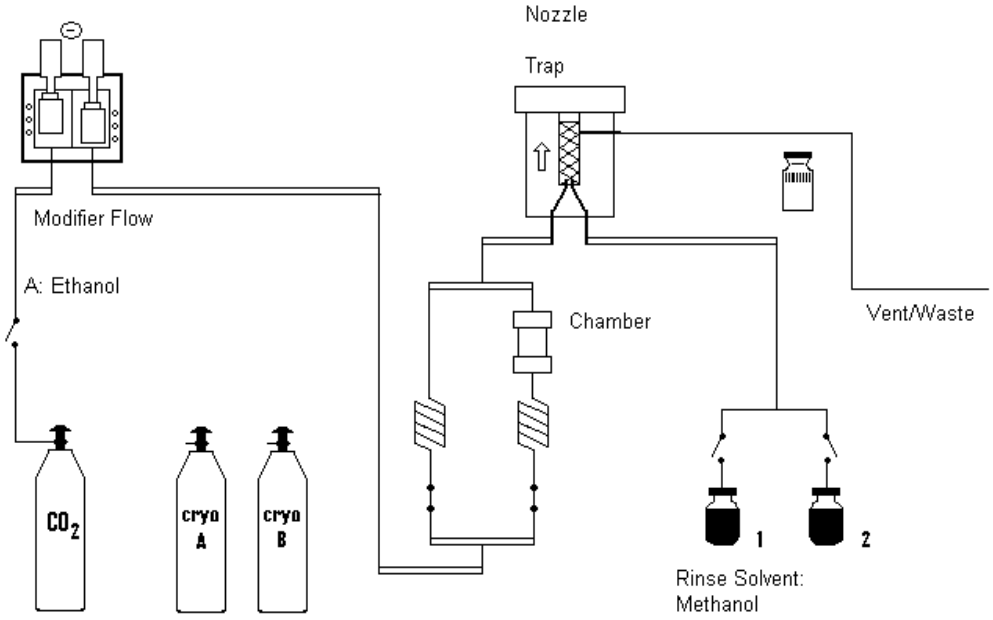


Figure 3.6: Schematic of the supercritical carbon dioxide extraction unit
(HP7680T, Australia)

In this work, a known quantity of the *Spirulina* (M) biomass (2 g) was used. The biomass was inserted into the thimble, where two pre-cut disks were placed on the lower part as well as at the top part to avoid the entrainment of the material. The operating conditions, such as temperature (T), pressure (P), density of carbon dioxide (ρ), volume percentage of co-solvent and the flow rate of carbon dioxide, were set by using the software HP ChemStation. The mass of lipid was measured following evaporation of the solvent under a gentle stream of nitrogen.

The supercritical carbon dioxide extraction was also conducted at Flinders University, South Australia. The supercritical carbon dioxide extractor unit (Spe-ed SFE-15000) is presented in Figure 3.7. This unit was able to hold a maximum mass up to one kilogram of biomass. A known quantity of the *Spirulina* (M) biomass (20 g) was inserted into the thimble. In the lower and upper part of the thimble, glass wool was used to fill up the thimble. This system can be operated up to 240^{o}C and 1000 bar, with a flow rate up to 400 mL/min. Once the extraction with supercritical carbon dioxide was completed, the extracts were collected in the sample collection unit. A reduction of temperature to five

degrees Celsius and pressure to atmospheric pressure was required in order to separate the oil from the supercritical carbon dioxide.

Figure 3.7: Supercritical carbon dioxide extractor used in present study

3.16 Chromatography

3.16.1 Thin layer chromatography

Thin Layer Chromatography (TLC) is a rapid analytical tool to determine the components of the microorganisms. This technique can be used to detect components by the phenomenon of capillary action; injecting a thin layer adsorbent material into a solvent system. The solvent travels due to the different attraction of the analytes to the stationary phase. In order to analyse a specified compound by TLC, selection of the developing system is compulsory, because the TLC system used might not separate the desired compound. Consequently, it will not be visualised from the analysis. SQDG has been investigated for many years, and several developing systems have been used to analyse this component. There are two ways of analysis to detect the SQDG. The first is to use a one-dimensional TLC, and the second is to use a two-dimensional TLC. Generally, the two-dimensional TLC was carried out only if the complex components could not be resolved in one-dimensional TLC. A number of solvent systems and methods used to visualise the spots were best introduced for the detection of SQDG, as seen in Table 3.10. The developing system chosen for this experiment, chloform:

methanol:0.02% calcium chloride ($CaCl_2$) (60:40:9, v/v) was chosen, because it consumes much less organic solvents, where only three various forms of solvent were used.

Table 3.10: TLC developing systems for SQDG

Thin Layer Chromatography	Developing systems
One-dimensional TLC	Chloroform:Methanol:0.02% $CaCl_2$ (60:40:9, v/v)
	Chloroform:Methanol:Acetic Acid:Water (68:9.6:9.6: 0.4, v/v)
	Chloroform:Acetone:Methanol:Acetic Acid:Water (50:20:10:10:5, v/v)
Two-dimensional TLC	First Direction
	Chloroform:Methanol:Ammonia 30% (75:25:2.5, v/v)
	Second Direction
	Chloroform:Methanol:Acetic Acid:Water (80:9:12:2, v/v)

The retention factor of each spot can be determined by the equation below:

$$R_f = \frac{Distance\ travelled\ by\ spot\,(cm)}{Solvent\ front\,(cm)} \qquad \text{Eq. 3-3}$$

The analysis of TLC moderately depends on the adsorbent material, temperature and pressure in the solvent tank as well as the spotting distance. The value of the retention factor is not constant in practice. Fortunately, the difficulties in obtaining a constant retention factor for a particular compound can be deciphered by running the TLC coupled with a reference standard on the sample plate (Christie, 1982). The developing system selected is for preliminary screening of SQDG from different isolates of *Spirulina*. Furthermore, the developing system is applied for verifying the purification of a specified component from different fractions, which were collected using gravity column chromatography.

The extracted lipid using various extraction methods can now be used for the separation of total lipid in TLC analysis. The lipid was directly applied to two centimetres from the edges of a silica gel 60 TLC plate (10x10cm; 0.25mm layer thickness) (MERCK). TLC was carried out by using chloroform:methanol:0.02% $CaCl_2$ (60:40:9, v/v) as the

developing reagent, and iodine vapour as the chromogenic reagent. The extracts from different isolates of *Spirulina* were lightly labelled with each mark, and the spots were carefully made on the proper mark. At the same time, a small drop of standard with a concentration of 250 μg/mL SQDG (Lipid Products, Redhill, UK) was applied onto the same plate to develop in a sealed glass TLC chamber at ambient temperature. The solvent system was kept undisturbed once the separation had started. When the separation was completed, the plates were removed from the chamber and dried for an hour. The plates were placed in sublimated iodine vapour to visualise the spots. Once the spots are coloured and visualised, the plates were immediately taken out to measure the distance the solvent drove each spot. The retention factor of each spot was then calculated to make a comparison between the lipid extract from different isolates of *Spirulina* and the standard.

3.16.2 Gas chromatography

The purpose of determining the fatty acid composition of the *Spirulina* isolate is because this alga consists of a complex mixture of fatty acids, which favours the human body's needs. The content of the fatty acid composition can be identified by gas chromatography (GC) and this technique is ubiquitous for the analysis of free fatty acid or fatty acid methyl esters (FAME). In addition, the fatty acids pattern can be used to clarify the taxonomy of the microorganisms (Kenyon *et al*., 1972).

The fatty acid patterns show up differently both physically and chemically in different classes of lipids, for example triacylglycerols consist of a glycerol moiety with each hydroxyl group esterified to a fatty acid. Therefore, the fatty acid pattern can be used as a preliminary check of the lipid content if the correlation of each lipid class has already been determined. Vonshak (1997) proposed that the production of SQDG can be coupled with the optimisation process of GLA from *Spirulina* since monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are the main source of gamma linolenic acid. To be unambiguous, the MGDG, DGDG and SQDG are branched in the same fraction (i.e. glycolipid fraction); consequently, an optimised extraction condition for glycolipid fraction will simultaneously achieve a better yield for both GLA and SQDG.

Transmethylation of lipid is a prerequisite for the fatty acid analysis by GC. Transmethylation of lipid was performed by adding five millilitres of 1% methanolic

H_2SO_4 and the reaction was undertaken in an oven at a temperature of 70^oC. The mixtures were kept in the oven for three hours with a constant shaking every 30 minutes. After baking in the oven, the mixtures were cooled in cold water and 625 μL of water, and two millilitres of heptane were added to the mixtures and shaken. The mixtures were allowed to stand for a short time until two layers formed. The upper layer was recovered and dried over a pinch of sodium sulphate. FAME is obtained and ready for the fatty acid analysis by GC.

The GC analysis of FAME is not difficult since standard methods have been developed. For the use of GC, a few parameters need to be taken into consideration; such as the column packing, the flow rate of air, the temperature and the split-ratio. FAME were separated and measured on a Perkin-Elmer Clarus 500 GC equipped with 30 m capillary column (0.32 mm internal diameter SGE) coated with polyethylene glycol (BP20) (0.25 μm film thickness), which was fitted with a flame ionization detector, using hydrogen as a carrier gas. The split-ratio was dependent on the yield of the FAME obtained and normally it was operated in 10:1. The injector temperature was set at 250^oC and an initial oven temperature of 155^oC for 10 minutes. The oven temperature was programmed to rise to 180^oC at two degrees per minute and rise to 220^oC at four degrees per minute for up to five minutes. External standard (Authentic lipid standards, Nu-Check Prep, USA) was injected and the gas chromatogram of the profile was compared in order to analyse the FAME.

3.16.3 High performance liquid chromatography

The quantification of SQDG was conducted by the School of Land, Crop and Food Sciences, University of Queensland. The method used is based on previous literature review (Yunoki *et al*., 2009).

An approximate amount of the total lipids (at least one milligram) was dissolved in chloroform:hexane (1:1, v/v) solution and gently shaken in order to mix it well. The instruments used for this analysis were the HPLC 1100 series with an Alltech evaporative light scattering detector (ELSD) 2000 and the Phenomenex, Luna 5 μm silica (column size 250 x 4.6 mm i.d). The mechanism of the ELSD is shown in Figure 3.8.

Figure 3.8: The operation of ELSD (Daniel, 2007)

The mobile phases used in this analysis were two different combinations of mobile solvent systems, which are solvent A (hexane:t-butyl ether (96:4, v/v)) and solvent B (isopropyl alcohol:chloroform:acetonitrile:acetic acid (84:8:8:0.25, v/v)). The flow rate is operated at one millilitre per minute, and the gradient was as shown in Table 3.11. The calibration curve of SQDG was obtained by using a range of concentration (from 0–0.75 mg/mL) and the ELSD provided a curvilinear response to the mass of reference standard of SQDG.

Table 3.11: Gradient elution

Time / mins	% Solvent A	% Solvent B
0	98	2
4	98	2
10	70	30
30	50	50
32	98	2
38	98	2

3.16.4 Column chromatography

An argentated silica gel column chromatography was used for the fractionation of lipids. Luer-lock glass columns (LC) were used for the fractionation of crude lipids, as shown in Figure 3.9. The column is one centimetre in diameter and a 20 cm in length. Approximately five grams of silica gel (100-200 mesh, Sigma) was added to the column to reach 15 cm in height. To assure that the silica gel is absolutely 15 cm in height, it was damped down before pouring in the solvent. A non-polar solvent would be preferable to use as a developing solvent. Hexane was added to the column slowly and allowed to flow by gravity. Once the solvent has settled, sand was added carefully to the top of the column. This is to enhance the separation efficiency by adding the sample evenly through the sand. The sample was added followed by the elution volume, which was selected in the beginning.

Figure 3.9: Luer-lock column used for the purification of SQDG

Removal of pigments

Once the silica gel column was ready for the purification, the first step was to remove the pigments in the column. The crude extracts (~0.1 g of samples) were applied. The column was eluted with the combination elution system hexane:acetone (98.5:1.5 v/v).

The eluent of chloroform:methanol was used to rinse the column. An air flow rate of one millilitre per minute was subjected to speed up the operation. Each millilitre of elution was collected and the pipette was filled with the solvent system for TLC. For stepwise elution, another eluent was added when the first eluent has drained. The separation stopped when the colour of sample changed to colourless. Further analysis of specified compounds from the elution volume was needed. The standard of sulfolipid

was used to run with the sample for each millilitre of elution volume. Each spot was added on TLC plate and the developing system used for polar testing. This is because the lipid classes can be determined by comparing the retention factor from each millilitre of collection with the column chromatography. The TLC plate was observed under UV lamp to spot out the lipid spots in each elution. The fraction that contained the pure samples of SQDG was combined into the same vials. The solvent was removed by a gentle nitrogen stream with a warm water bath. The mass of the SQDG can be calculated by subtracting the mass of vial without the sample and the mass of vial with the sample. A diagram of the fractionation process is shown in Figure 3.10.

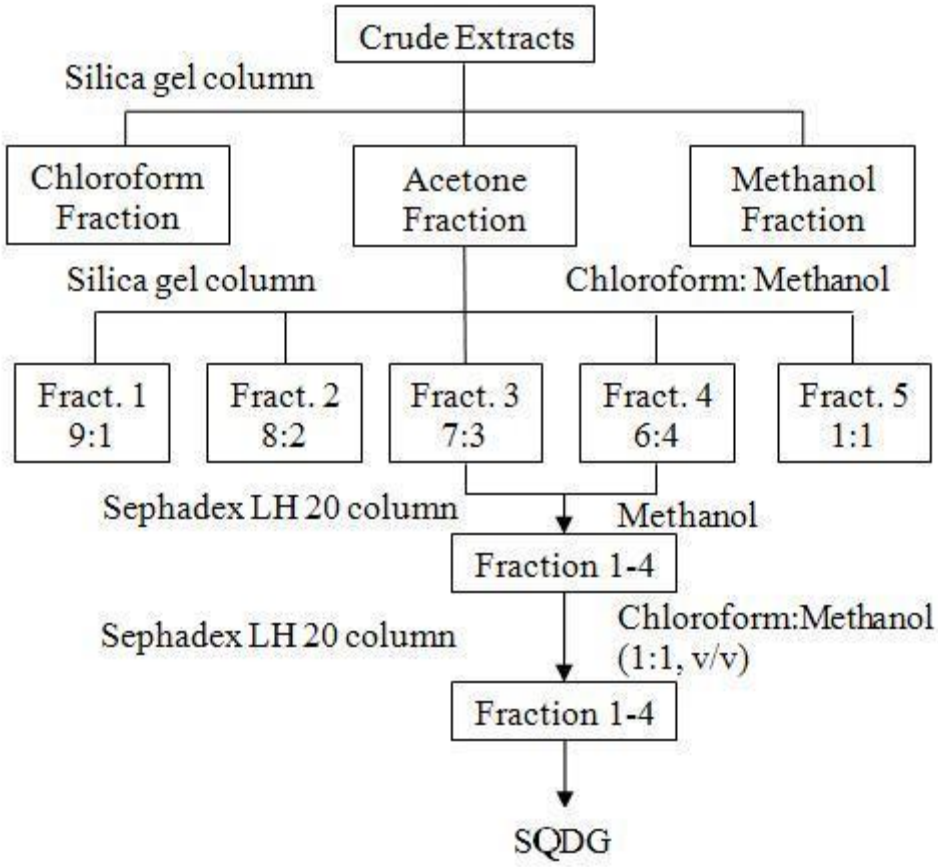


Figure 3.10: Diagram of the fractionation of SQDG from the crude extract

3.17 Phytochemical analysis

All of the sequential extracts were subjected to preliminary phytochemical screening as described by Trease & Evans (1989) and Harborne (1998). The preliminary screenings were carried out to identify alkaloids, tannins, saponins, phlobatannins, cardiac glycosides, steroids, terpenoids and flavonoids.

3.17.1 Test for tannins

The ferric chloride test was applied to test for the presence of tannins. About two millilitres of the extract was stirred with two millilitres of distilled water and a few drops of ferric chloride solution were added. The formation of a green precipitate was an indication for the presence of tannins.

3.17.2 Test for saponins

The frothing test was used for saponins. About five millilitres of extract with five millilitres of distilled water was shaken vigorously in a test tube and warmed in water bath. Saponins were found to be present when foam was produced constantly for five minutes.

3.17.3 Test for phlobatannins

The mixture of two millilitres of extract with two millilitres of 1% hydrochloric acid was boiled to check for the presence of phlobatanins. The phlobatannins were considered present when a deposition of a red precipitate was observed.

3.17.4 Test for cardiac glycosides (Keller-Kiliani test)

The Keller-Kiliani test was used for cardiac glycosides. About two millilitres of extract was added with one millilitre of glacial acetic acid and followed by a drop of ferric chloride solution. This mixture was then treated with one millilitre of concentrated sulphuric acid. A brown ring formed between the two layers, with the lower acidic layer indicating the presence of deoxy sugars, characteristic of cardenolides. If a green blue precipitate is observed, it indicates the presence of cardiac glycosides.

3.17.5 Test for steroids

The extract (2 mL) was treated with two millilitres of chloroform and two millilitres of concentrated sulphuric acid. A red colour produced in the lower chloroform layer indicated the presence of sterols.

3.17.6 Test for terpenoids

A known volume (2 mL) of the extract was dissolved in two millilitres of chloroform and evaporated to dryness. Two millilitres of concentrated sulphuric acid was added to

the dried sample and heated for about two minutes. The appearance of a greyish colour indicated the presence of terpenoids.

3.17.7 Test for flavonoids

Three drops of dilute sodium hydroxide was added to one millilitre of the extract. A colour change from an intense yellow in the plant extract to colourless, with the addition of a few drops of dilute acid, indicated the presence of flavonoids.

3.17.8 Test for alkaloids

The Wagner test was used for the detection of alkaloids. The sample with a known volume (2 mL) was treated with two drops of 1% hydrochloric acid and steamed for two minutes. Six drops of Wagner reagent (2 g of iodine, 6 g of potassium iodide and 100 mL of water) was added and a brown red precipitate indicated the presence of alkaloids.

3.18 Anti-bacterial activity

Anti-bacterial screening of the sequential extracts was conducted using the disc gel diffusion method (Collins *et al*., 1995). The bacteria and fungi used throughout this study were listed in Table 3.12.

Table 3.12: Bacteria and fungi used in the anti-microbial testing

Organisms	Strain No
Staphylococcus aureus	ATCC 25923
Candida albicans	ATCC 10231
Escherichia coli	ATCC 11775

There are a few parameters that must remain constant in testing to diminish inaccuracies. For instance, the incubation conditions (time, temperature) are held constant. The tested organisms were isolated by the IMVS. Both strains of bacteria were grown in nutrient agar (Chem supply, Australia) at 37^{o}C, and the fungi was grown in potato dextrose agar (Chem supply, Australia) at 28^{o}C. The nutrient agar and potato dextrose agar was incubated for 24 hours prior to testing. The sequential extracts were dissolved in its extracting solvent and made up to a concentration of 40 mg/mL. The tested organisms were then subcultured in the specified agar and evenly spread using sterile bent metal over the surface of an agar plate. After inoculation, the discs (6 mm in

diameter) were made and immersed in 0.2 mL of extracts, as shown in Figure 3.11. The extracting solvents, such as hexane, toluene, acetone, methanol and water; were included as controls. The petri-plates were then incubated at 37^{o}C for 24 hours for *E. coli* and *S. aureus*, while for the *C. albicans*, the plates were incubated at 28^{o}C for 48 hours. The actual growth inhibition of the test organism was examined by measuring the diameter of inhibition zones for each extract and comparing it with the diameter of inhibition zones for each control. The bioactivity assessment was conducted using three extracts from three samples of the same batch for every experiment.

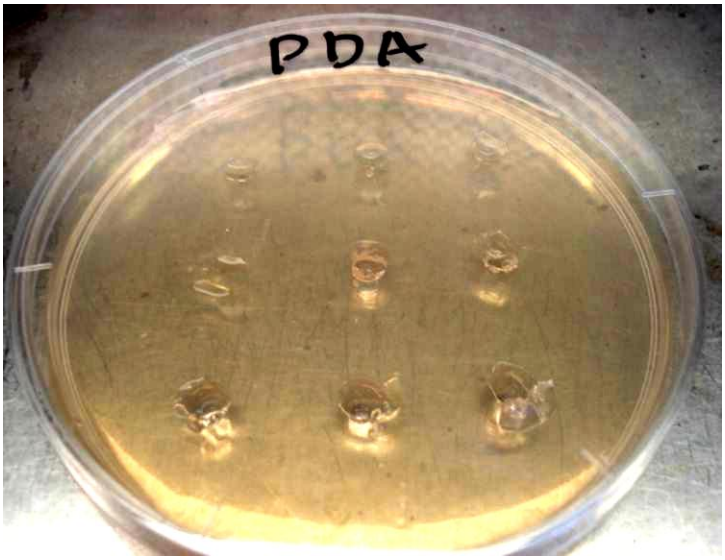


Figure 3.11: Potato dextrose agar plate with 6 mm diameter discs for anti-fungal testing

3.19 Statistical Analysis

The central tendency (mean) was used to interpret the experiment results and the standard deviation was caculated by using the 'n-1' method in Excel.

CHAPTER 4 MOLECULAR CLASSIFICATION, GROWTH CHARACTERISTICS AND CELL COMPOSITION OF *SPIRULINA* ISOLATES

4.1 Introduction

Bioactive metabolites from organisms are limited, and they have not been accessed in large quantities for the biochemical process. In addition, the limited availability of organisms is one of the constraints to achieve sufficient bioactive metabolites for medical use (Hildebrand *et al*., 2004). To solve the resource problem of natural products, the selected organisms should have a low cost of cultivation with high quantities of the bioactive metabolites. *Spirulina* is regarded as an ideal candidate in biotechnological application because of its high nutritional value. The medicinal value of *Spirulina* has therefore attracted attention in studying this species. There are many different *Spirulina* species, and *Spirulina platensis* is the most well-known and commercially produced. *Spirulina platensis* has the highest growth rate and biomass productivity when compared to the strains of *S. laxissima* and *S. lonar* (Bhattacharya & Shivaprakash, 2005).

Taxonomy is defined as: 'the science of identification, classification, and nomenclature' (Mardigan & Martinko, 2006). In clinical diagnostic microbiology, this classification is especially used to identify the bacterial taxonomy. Moreover, in order to source the biologically active natural products, an identified organism is useful, since the natural products are complex molecules that are derived from specified organism. Despite the wide use of *Spirulina* in the health food industry, classification of this genus remains unclear. Due to the taxonomic confusion within *Spirulina* and *Arthrospira*, 16S rDNA amplification for four commercial *Spirulina* isolates and an Australian isolate was investigated in this study. The designations of the investigated *Spirulina* isolates were described as in Chapter 3, and used throughout the study.

Despite the fact that survivability and local conditions are the main factors limiting the growth of *Spirulina*, it is significant to determine a local strain of *Spirulina*. In this study, an Australian isolate, *Spirulina* sp. was selected. The growth trials of *Spirulina* sp. are based on the literature under laboratory conditions. Different nutrients uptake

and temperatures were undertaken on a preliminary basis in order to maintain the stock culture of Australian *Spirulina* isolate. *Spirulina* sp. was prepared actively to permit usage for future studies. In order to avoid the microbial contamination, the culture was examined for any inconspicuous bacterial growth on the nutrient agar plate.

Many researchers have investigated the potential use of the bioactive compounds from *Spirulina* species. In spite of the rich source of nutrition from *Spirulina*, there have been very few studies on the Australian *Spirulina* isolate. *Spirulina* sp. that is native to Australia is expected to contain the same bioactive constituents as that in the reported *Spirulina* species. In this study, the chemical compositions of an Australian *Spirulina* and other commercialised *Spirulina* were analysed.

4.2 Screening of 16s rDNA

Figure 4.1 shows *Spirulina* sp. in different dilution when the XS DNA extraction method was used. As can be seen from the results, these five *Spirulina* isolates do not show any bands, even the DNA extracted by the general method in spiked PCR.

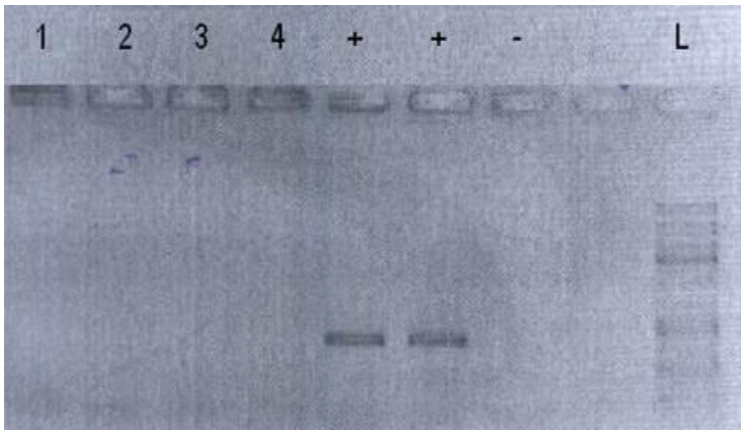


Figure 4.1: PCR amplification of 16S rRNA from Spirulina sp.

The DNA of *Spirulina* sp. was extracted using general DNA extraction Lane 1, and indicated *Spirulina* sp. in 1/10 dilution. Lane 2 indicated spiked *Spirulina* sp. in 1/10 dilution, which consisted of positive control. Lane 3 indicated *Spirulina* sp. in 1/100 dilution. Lane 4 indicated spiked *Spirulina* in 1/100 dilution, which consisted of positive control. Lane L stands for the DNA ladder, which consists of fragments, as in Figure 3.4 (Section 3.7).

Improved XS DNA extraction was used in this study. This method is practised and used repeatedly at UNSW. Results indicated that this method successfully amplified these five isolates of *Spirulina*. An approximately 700bp fragment size of 16S rDNA was amplified by PCR, using the 27F and 809R primer pairs, as seen in Figure 4.2, points

(a) and (b), and 740F and 1494R primer pairs at point (c). The purified DNA fragment of all *Spirulina* isolates is directly sequenced and has a fragment size of approximately 1,500bp of 16S rDNA, except for *Spirulina* (M).

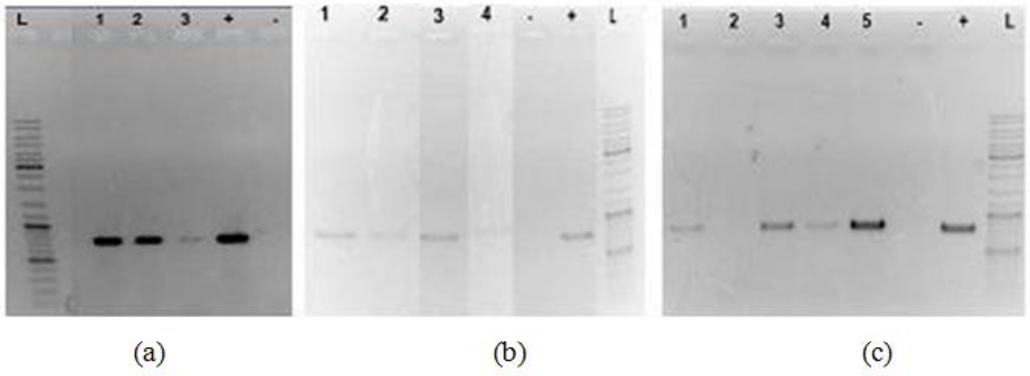


Figure 4.2: PCR amplification of 16S rRNA from five Spirulina isolates

(a) PCR DNA of *Spirulina* sp. using 27F and 809R primer pairs. Lane 1-3 contains DNA from *Spirulina* sp. 1/10 dilution, 1/100 dilution, and 1/500 dilution, respectively. (b) PCR DNA of *Spirulina* (J), *Spirulina* (M), *Spirulina* (S), *Spirulina* (P) using 27F and 809R primer pairs in Lane 1, 2, 3 and 4 respectively. (c) PCR DNA of *Spirulina* sp., *Spirulina* (M), *Spirulina* (J), *Spirulina* (P), *Spirulina* (S) using 740F and 1494R primer pairs in Lane 1, 2, 3, 4 and 5 respectively. Lane + was used as a positive control and Lane – as a negative control. Lane L stands for the DNA ladder.

The obtained DNA sequences were aligned by using the Bioedit ClustalW programme. These aligned sequences were submitted to the NCBI BLAST system to identify the closest relative in GenBank. The sequences for all *Spirulina* isolates were in the range from 640–1425 bp, as presented in Figure 4.3 and Figure 4.4. The first five highest similarities to the *Spirulina* isolates were collated in Appendix D and the taxonomic reports for each isolate of *Spirulina* were created. The fragment size of *Spirulina* sp., *Spirulina* (J), *Spirulina* (P) and *Spirulina* (S) were 1,425bp, 1,369bp, 1,363bp and 1,402bp, respectively. In this study, the fragment size of the *Spirulina* (M) was only 640bp.

>J

GAGCTAGTGGCGGACGGGTGAGTAACACGTGAGAATCTGGCTCCCGGTCGGGGACAACAGAGGGAAACTTCTGCTA
ATCCCGGATGAGCCGAAAGGTAAAAGATTTATCGCCGGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGAGGTAA
AGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCACAC
TCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAGCCCGACGGAGCAAGACCGCGTGGGGGAGGAA
GGCTCTTGGGTTGTAAACCCCTTTTCTCAAGGAAGAACACAATGACGGTACTTGAGGAATAAGCCTCGGCTAACTCC
GCGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTCCGTAGGCGGCT
GTTCAAGTCTGCTGTCAAAGACAGTGGCTTAACTACTGAAAGGCAGTGGAAACTGAACAGCTAGAGTACGGTAGGG
GCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCGGTGGCGAAAGCGCTCTGCTGG
GCCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACG
ATGGAAACTAGGTGTAGCCTGTATCGACCCGGGCTGTGCCGAAGCTAACGCGTTAAGTTTCCCGCCTGGGGAGTACG
CACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCAGGGCTTGACATGTCCGGAATCTTGGTGAAAGCCGAGAGTGCCTTCGGGAGCCGGAACACA
GGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCATTA
GTTGCCATCATTCAGTTGGGCACTTTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCA
TCATGCCCCTTACGTCCTGGGCTACACACGTACTACAATGGGGGGGACAAAGGGTAGCCAAGACGCGAGTCTGAGCC
AATCCCGTAAACCTCTCCTCAGTTCAGATTGCAGGCTGCAACTCGCCTGCATGAAGGAGGAATCGCTAGTAATCGCA
GGTCAGCATACTGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTTAGCCACGCCCG
AAGTCGTTACTCTAACCGTTCGCGGAGGAGGATGCCGAAGGCAGGGCTGATGACTGGGGTGAAGTCGTA

>W

CCTGTACGACTTCACCCCAGTCATCAGCCCTGCCTTCGGCATCCTCCTCCGCGAACGGTTAGAGTAACGACTTCGGGC
GTGGCTAACTTCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGGATTCACCGCAGTATGCTGACCTGCGA
TTACTAGCGATTCCTCCTTCATGCAGGCGAGTTGCAGCCTGCAATCTGAACTGAGGAGAGGTTTACGGGATTGGCTCA
GACTCGCGTCTTGGCTACCCTTTGTCCCCCCCCATTGTAGTACGTGTGTAGCCCAGGACGTAAGGGGCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCCTAAAGTGCCCAACTGAATGATGGCAACTAAGGACG
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGTGTTCC
GGCTCCCGAAGGCACTCTCGGCTTTCACCAAGATTCCGGACATGTCAAGCCCTGGTAAGGTTCTTCGCGTTGCATCGA
ATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACACTTGCGTGCGTACTCCCCAG
GCGGGAAACTTAACGCGTTAGCTTCGGCACAGCCCGGGTCGATACAGGCTACACCTAGTTTCCATCGTTTACGGCTA
GGACTACTGGGGTATCTAATCCCATTCGCTCCCCTAGCTTTCGTCCCTCAGTGTCAGTTACGGCCCAGCAGAGCGCTT
TCGCCACCGGTGTTCTTCCCGATATCTACGCATTTCACCGCTACACCGGGAATTCCCTCTGCCCCTACCGTACTCTAGC
TGTTCAGTTTCCACTGCCTTTCAGTAGTTAAGCCACTGTCTTTGACAGCAGACTTGAACAGCCACCTACGGACGCTTT
ACGCCCAATCATTCCGGATAACGCTTGCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTATTC
CTCAAGTACCGTCATTGTGTTCTTCCTTGAGAAAAGGGGTTTACAACCCAAGAGCCTTCCTCCCCCACGCGGTCTTGC
TCCGTCAGGCTTGCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG
TGTGGCTGATCATCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCAGAC
GCGAGCTCATCTCCCGGCGATAAATCTTTTACCTTTCGGCTCATCCGGGATTAGCAGAAGTTTCCCTCTGTTGTCCCCG
ACCGGGAGCCAGATTCTCACGTGTTACTCACCCGTCCGCCACTAGCTCCGAAGAGCCCGTTCGACTTGCATGTGTTA

>M

TAATCCCATTCGCTCCCCTAGCTTTCGTCCCTCAGTGTCAGTTACGGCCCAGCAGAGCGCTTTCGCCACCGGTGTTCTT
CCCGATATCTACGCATTTCACCGCTACACCGGGAATTCCCTCTGCCCCTACCGTACTCTAGCTGTTCAGTTTCCACTGC
CTTTCAGTAGTTAAGCCACTGTCTTTGACAGCAGACTTGAACAGCCACCTACGGACGCTTTACGCCCAATCATTCCGG
ATAACGCTTGCCTCCTCCGTATTACCGCGGCTGATGGCACGGAGTTAGCCGAGGCTTATTCCTCAAGTACCGTCATTG
TGTTCTTCCTTGAGAAAAGGGGTTTACAACCCAAGAGCCTTCCTCCCCCACGCGGTCTTGATCCGTCAGGCTCGCGCC
CATTGAGGAAAATTCCCCAATGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTC
TCAGACCAGCTACTGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCAGACGCGAGCTCATCTCCCGG
CGATAAATCTTTTACCTTTCGGCTCATCCGGGATTAGCAGAAGTTTCCCTCTTGTTGTCCCCGACCGGGAGCCAGATT
CTCACGTGTTACTC

Figure 4.3: The sequence of 16S rRNA for: J: Spirulina (J), W: Spirulina (S), M: Spirulina (M)

>P

TGCTTAACACATGCAAGTCGAACGGGCTCTTCGGAGCTAGTGGCGGACGGGTGAGTAACACGTGAGAATCTGGCTCC
CGGTCGGGGACAACAGAGGGAAACTTCTGCTAATCCCGGATGAGCCGAAAGGTAAAAGATTTATCGCCGGGAGATG
AGCTCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGAT
CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAA
GCCTGACGGAGCAAGACCGCGTGGGGGAGGAAGGCTCTTGGGTTGTAAACCCCTTTTCTCAAGGAAGAACACAATG
ACGGTACTTGAGGAATAAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGCGTTATCCG
GAATGATTGGGCGTAAAGCGTCCGTAGGTGGCTGTTCAAGTCTGCTGTCAAAGACAGTGGCTTAACTACTGAAAGGC
AGTGGAAACTGAACAGCTAGAGTACGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGG
GAAGAACACCGGTGGCGAAAGCGCTCTGCTGGGCCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGG
ATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGAAACTAGGTGTAGCCTGTATCGACCCGGGCTGTGCCGAAGC
TAACGCGTTAAGTTTCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTTGACATGTCCGGAATCTTGGTGAA
AGCCGAGAGTGCCTTCGGGAGCCGGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCCATCATTCAGTTGGGCACTTTAGGGAGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCTGGGCTACACACGTACTACAATGGGGGGG
ACAAAGGGTAGCCAAGACGCGAGTCTGAGCCAATCCCGTAAACCTCTCCTCAGTTCAGATTGCAGGCTGCAACTCGC
CTGCATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATCCGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGAAGTTAGCCACGCCCGAAGTCGTTACTCTAACCGTTCGCGGAGGAG

>S

GGTTACCGTACGACTTCACCCCACGTCACTAGACCCACCTTCGGCGCCCTCCTCTCAAAAGAGTTGGAGTAACGACTT
CGGATGTTGCCAGCTTCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGAATTCACCGCCGTATGCTGACC
GGCGATTACTAGCGATTCCTCCTTCACGCAGGCGAGTTGCAGCCTGCGATCTGAACTGAGGCCGAGTTTGATGGGAT
TCGCTGACTCTCGCGAGCTCGCTGCCCTTTGTCTCGACCATTGTAGTACGTGTGTTGCCCAGGACGTAAGGGGCATGC
TGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCCTAAAGTGCCCAACTTAATGCTGGCAACT
AAGGACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCT
GTCACGGTGCTCCCGTAGGCACTTCTCTGTTTCCAGAGAATTCACCGGATGTCAAGCCCTGGTAAGGTTCTTCGCGTT
GCATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACACTTGCGTGCGTAC
TCCCCAGGCGGGGTACTTAACGCGTTAGCTTCGGCACAGCTCGGGTCGATACAAGCTACGCCTAGTACCCATCGTTTA
CAGCTAGGACTACTGGGGTATCTAATCCCATTCG-C-
TCCCCTAGCTTTCGTCCCTCANTGTCAGTTGCGGCCTAGTAGAGCGCTTTCGCCACCGGTGT
TCTTCCCAATCTCTACGCATTTCACCGCTACACTGGGAATTCCCTCTACCCCTACCGCACTCTAGCTCTTCAGTTTCCA
CCGCCTACCCGGAGTTAAGCTCCGGTCTTTGACAGCAGACTTGACGAGCCACCTGCGAACGCTTTACGCCCAATAATT
CCGGATAACGCTTGCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTATTCCTCAGGTACCGTC
AGAACTTCTTCCCTGAGAAAAGAGGTTTACGATCCAAAGACCTTCTTCCCTCACGCGGTATTGCTCCGTCAGGCTTTC
GCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGTTCTTC
CTCTCAGAACAGCTACCGATCGCAGCCTTGGTGAGCCTTTACCCCACCAACTAGCTAATCGGACGCGAACCCCTCTCT
AGGCGATAAATCCTTTTACCCTTCGGCTCATCGGGAATTAGCAGAAGTTTCCCTCTGTTATCCCCGTCCTAGAGGCAG
GTTTTCACGTGTTACTCACCCGTCCGNCACTGATTCCTAAGAATCCGTTCGACTTGCATGTGTTAAGCATACCGCCAG
CGTT

Figure 4.4: The sequence of 16S rRNA for: P: Spirulina (P), and S: Spirulina sp.

Subsequent sequence analysis revealed a 100% similarity between the *Spirulina* (P) and *Spirulina* (S) sequences, and that of *Arthrospira platensis* PCC 9223 (Table 4.1). The *Spirulina* (M) sequence was also 99% similar to *Arthrospira platensis* MMG-9, while the *Spirulina* (J) sequence was 99% similar to *Arthrospira platensis* PCC 9223 (Table 4.1). The 16S rDNA sequence of the Australian isolate, *Spirulina* sp. was 99% similar to that of *Halospirulina* sp. (Table 4.1).

Table 4.1: Similarity to the closest relatives in GenBank of 16S rDNA

Sequence	Closest relative in GenBank	% Identity	Accession Number
Spirulina (P)	*Arthospira platensis* PCC 9223	100	DQ393285.1
Spirulina (S)	*Arthospira platensis* PCC 9223	100	DQ393285.1
Spirulina (J)	*Arthospira platensis* PCC 9223	99	DQ393285.1
Spirulina sp.	*Halospirulina* sp. 'CCC Baja-95 C1.3'	99	Y18790.1
Spirulina (M)	*Arthospira platensis* MMG-9	99	FJ839360.1

Four commercial *Spirulina* isolates (*Spirulina* (S), *Spirulina* (J), *Spirulina* (M) and *Spirulina* (P)) were closely related to *Arthrospira*; while the Australian isolate, *Spirulina* sp., was closely related to the *Halospirulina*. The categorisation of the investigated *Spirulina* isolates is shown in Table 4.2.

Table 4.2: The classification of different isolates of *Spirulina*

Classification	*Spirulina* sp.	*Spirulina* (P)	*Spirulina* (J)	*Spirulina* (S)	*Spirulina* (M)
Domain	Bacteria	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	Cyanobacteria	Cyanobacteria	Cyanobacteria	Cyanobacteria	Cyanobacteria
Class	Oscillatoriales	Oscillatoriales	Oscillatoriales	Oscillatoriales	Oscillatoriales
Family	***Halospirulina***	***Arthrospira***	***Arthrospira***	***Arthrospira***	***Arthrospira***

4.3 Exposure under ultraviolet radiation

In order to achieve monoculture growth of the Australian *Spirulina* isolate, the *Spirulina* sp. culture was exposed to ultraviolet radiation. Cultures were routinely checked for sterility by spreading a single loopful of culture medium onto a nutrient agar plate, where the plates were incubated for 48 hours after inoculation. Nutrient agar was used for the determination of bacterial growth and potato dextrose agar was used for the determination of fungal growth. This test was based on the procedure carried out

by Shirai (1989). It was observed that there is bacterial growth in the *Spirulina* sp. culture that was subjected to ultraviolet irradiation for 0, 5, 10, 15 and 25 minutes. The bacteria-alga ratio was reduced, as seen in Figure 4.5, when cultures were exposed to a longer duration under ultraviolet radiation.

Figure 4.5: Duration of exposure under ultraviolet radiation

(a) Growth of bacteria on nutrient agar plate after UV exposure for five minutes (b) the control is to note that the agar plates are prepared in a bacteria-free condition.

No bacteria were contaminated in the culture after 30, 45, 60 and 120 minutes irradiation. However, after 60 minutes exposure time under UV light, the *Spirulina* sp. lost its green pigmentation. The growth of bacteria in the treated culture after 48 hours incubation is summarised in Table 4.3.

Table 4.3: Growth of bacteria and appearance of *Spirulina* sp. after ultraviolet exposure

	Irradiation time (min)								
	0	5	10	15	25	30	45	60	120
Growth of bacteria	+	+	+	+	+	-	-	-	-
Colour of Spirulina sp	G	G	G	G	G	G	G	T	T

- No growth; + growth; G: green; T: transparent

Figure 4.6 shows the colour of the *Spirulina* sp. after 120 minutes irradiation. In this study, no bacterial growth was found in the culture medium of *Spirulina* sp. for 12 days after 30, 45, 60 and 120 minutes irradiation. However, after 60 minutes exposure time under UV light, the *Spirulina* sp. only lost its green pigmentation (see Figure 4.6) but did not cause lysis of the cells. This preliminary study indicated that 30–45 minutes exposure time did not affect the *Spirulina* sp's morphology.

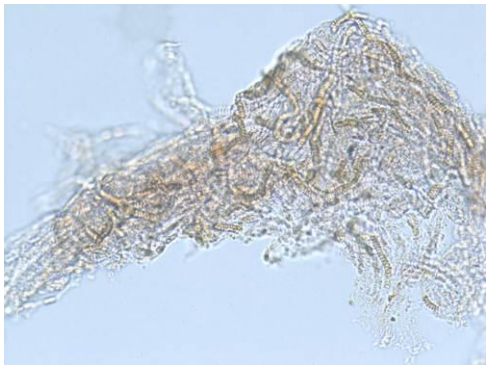
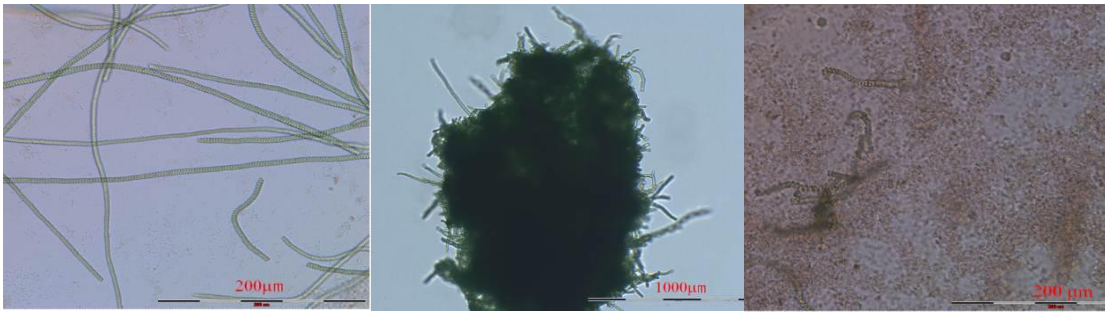


Figure 4.6: Spirulina sp. lost its green pigmentation after 120 minutes irradiation

4.4 Growth characteristics

The growth characteristic of Australian *Spirulina* sp. was observed when it was cultured in the MLA media with salt water under laboratory conditions. Figure 4.7 (a) shows *Spirulina* sp. in the growth phase (day two). The occurrence of the floating map of *Spirulina* sp. was observable at day 10, as shown in Figure 4.7(b). For the *Spirulina* sp. cultured in Zarrouk media, it changes its colour to yellowish brown at day 10, as seen in Figure 4.7 (c).



(a) (b) (c)

Figure 4.7: Growth characteristic of Spirulina sp.

(a) Day two of *Spirulina* sp.; (b) *Spirulina* sp. growing in a clump at day 10 at growth phase; (c) Death phase of *Spirulina* sp. at day 10 in Zarrouk media

4.5 Growth condition of *Spirulina* sp.

4.5.1 Effect of nutrient uptake

The growth curve of *Spirulina* sp. using the absorbance of chlorophyll-*a* at constant light irradiance is compared in Figure 4.8.

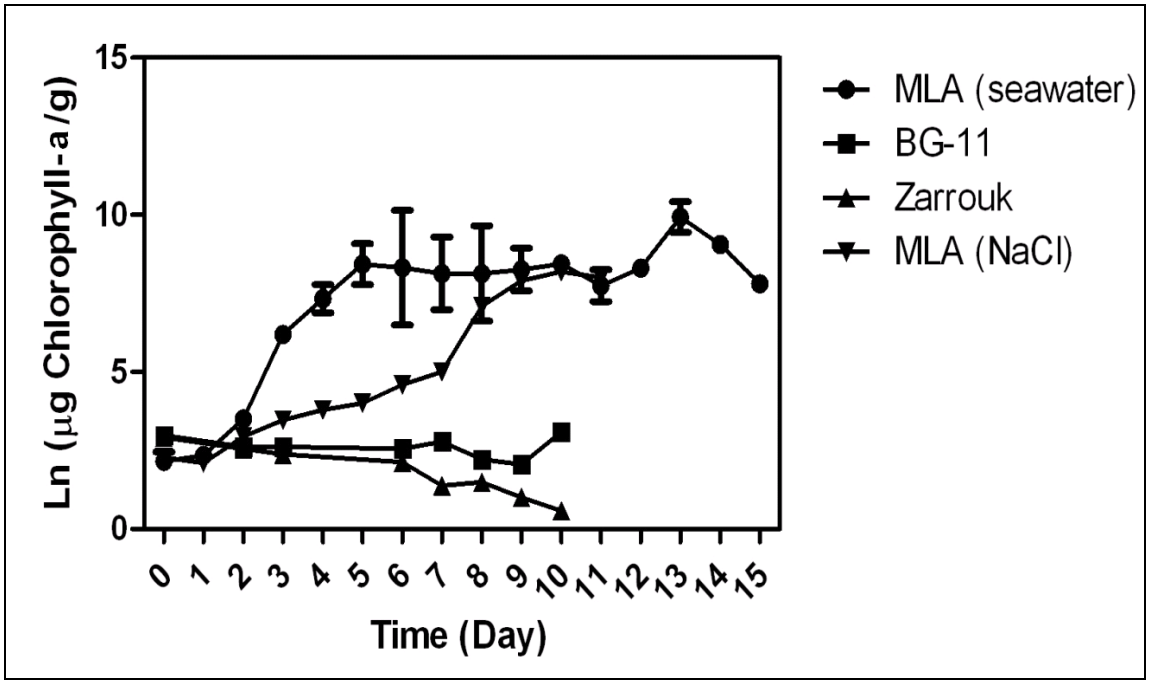


Figure 4.8: Spirulina sp. culture in different media

Each data point represents the average of three replicates, and the standard deviations of the data set are indicated as error bars.

As shown in Figure 4.8, *Spirulina* sp. cultured in MLA media with half-strength seawater demonstrated the best growth. Conversely, *Spirulina* sp. cultured in Zarrouk media showed no growth phase and the death phase occurred rapidly at day seven. The growth trends of *Spirulina* sp. in BG-11 media also showed the same response as *Spirulina* sp. cultured in Zarrouk media. In BG-11 media, *Spirulina* sp. entered senescence at around day eight. When *Spirulina* sp. cultured in the MLA media with salt water, it did not show the same response as the growth rate of *Spirulina* sp. in the MLA media with seawater. Although the growth curve of *Spirulina* sp. cultured in saltwater did not demonstrate the best growth rate, its growth curve was relatively stable.

4.5.2 Effect of temperature

One of the major factors that affect the growth of algae culture is temperature. The experimental temperatures of 25^{o}C, 30^{o}C and 35^{o}C were conducted at constant light intensities and 12:12-h light-dark cycle in MLA/seawater media (see Figure 4.9). The various temperatures showed statistically different results, and the most favourable growth occurred at a temperature of 25^{o}C. At 30^{o}C and 35^{o}C, the culture did not show growth response. It can also be observed that the green pigmentation disappeared at day 10.

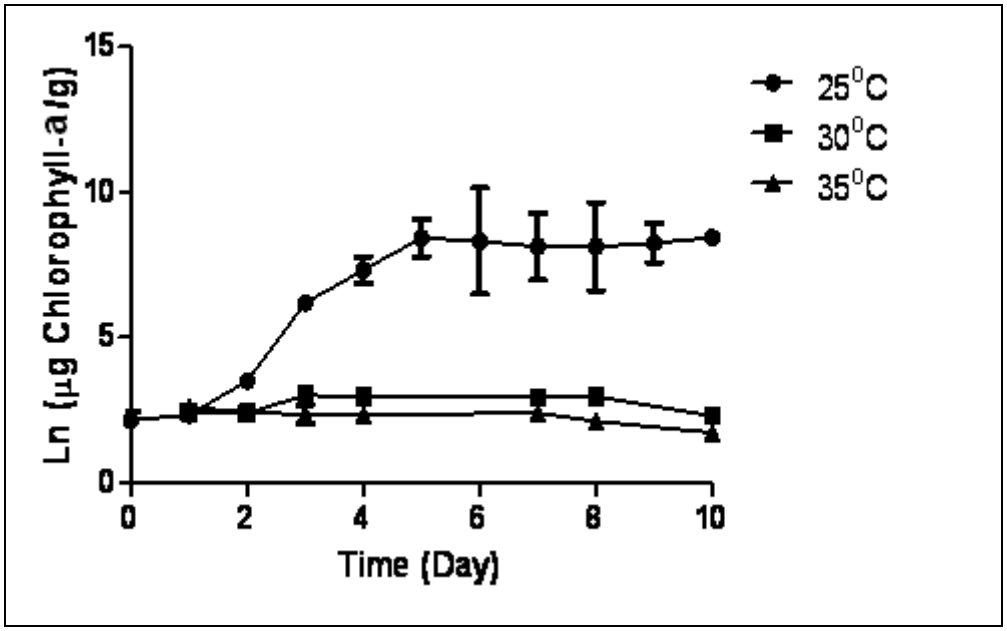


Figure 4.9: Spirulina sp. culture in MLA/seawater media at different temperatures

Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

4.6 Chemical composition of *Spirulina* isolates

The primary chemical constituents of algae are protein, carbohydrate and lipids. A comparison of these chemical constituents of the investigated *Spirulina* isolates from different continents was studied. Table 4.4 shows the chemical composition of the five *Spirulina* isolates in this study.

Table 4.4: Chemical composition of different isolates of *Spirulina*

ISOLATES	Carbohydrate (% AFDW)	Protein (% AFDW)	Lipid (% AFDW)
Spirulina (M)	9.78±2.18	65.95±0.46	6.74±0.64
Spirulina (P)	11.61±1.50	74.18±0.52	10.66±1.10
Spirulina (S)	10.02±0.93	58.55±0.33	8.67±0.83
Spirulina (J)	8.42±0.66	58.59±0.42	8.08±0.75
Spirulina sp.	18.25±0.49	57.46±0.30	9.57±0.43

Data expressed as mean ± standard deviation; n = 3

The carbohydrate content in the *Spirulina* isolates ranged from 8.42% AFDW to 18.25% AFDW. The *Spirulina* sp. contained the highest amount of carbohydrate compared to the commercial *Spirulina* isolates, while the *Spirulina* (J) contained the least carbohydrate (8.42% AFDW).

The lipid content (% AFDW) was obtained by using the lipid extraction systems introduced by Bligh & Dyer (1959). The *Spirulina* (P) yielded 10.66% AFDW of lipids; which is the highest lipid content among all investigated *Spirulina* isolates in this experiment. The *Spirulina* sp. was found to have 9.57% AFDW of lipid content.

The *Spirulina* (P) and *Spirulina* (M) showed a relatively high protein content, namely 74.18% AFDW and 65.95% AFDW, respectively. The *Spirulina* (J) and *Spirulina* (S) contained about 58.60% AFDW protein. The *Spirulina* sp. showed a fair amount of protein (57.40% AFDW). From the results obtained, most of the *Spirulina* isolates have a high protein content, which ranged from 57% AFDW to 74% AFDW.

4.7 Fatty acid profile of *Spirulina* isolates

The fatty acid profile for all *Spirulina* isolates is shown in Table 4.5. The lipids extracted for the FAME analysis were based on the extraction method described by Bligh and Dyer (1959). The GC analysis showed that all *Spirulina* isolates contain palmitic acid (C16:0), palmitoleic acid (C16:1), steraic acid (C18:0), oleic acid (C18:1n9) and linoleic acid (C18:2n6). The major fatty acid composition of all *Spirulina* isolates is palmitic acid, which is in the range of 35–48%. All investigated *Spirulina* isolates have the same fatty acid distribution pattern excluding the *Spirulina* sp. The GC analysis indicated that the *Spirulina* sp. does not possess GLA (18:3n6).

Table 4.5: Fatty acid profile for *Spirulina* isolates

Fatty Acid (%)		W	J	S	P	M
Palmitic acid	C16:0	45.68±0.57	44.86±0.39	35.32±1.18	44.29±1.18	48.1±0.21
Palmitoleic acid	C16:1n9	1.65±0.02	1.32±0.07	29.23±0.98	1.66±0.04	1.09±0.06
	C16:1n7	4.38±0.05	3.12±0.16	2.97±0.1	5.37±0.14	2.55±0.13
Steraic acid	C18:0	1.58±0.02	0.94±0.05	1.76±0.06	1.84±0.05	1.47±0.08
Oleic acid	C18:1n9	3.89±0.05	2.52±0.13	20.99±0.70	1.96±0.05	3.58±0.19
Linoleic acid	C18:2n6	22.06±0.28	22.59±1.17	2.86±0.18	15.87±0.42	22.27±1.18
GLA	C18:3n6	15.23±0.19	13.14±0.68	nd	16.61±0.44	11.63±0.62
Total		94.47±1.93	88.49±3.39	93.13±2.10	87.24±1.62	90.69±2.98

W = *Spirulina* (S); J = *Spirulina* (J); S = *Spirulina* sp.; P = *Spirulina* (P); and M = *Spirulina* (M) nd = Not Detected

Data expressed as mean ± standard deviation; n = 3

Studies in regards to the fatty acid composition and fatty acid content were analysed by GC. The compound was identified by the retention time in the gas chromatogram, in which the peak area is proportional to the amount of the compound. The peak area was used as the fatty acid content of different isolates of *Spirulina* based on a chromatogram, which was obtained under the same experimental GC conditions.

Figure 4.10 shows the fatty acid methyl ester content of the *Spirulina* isolates in the expression of the area fraction per weights. The FAME of different isolates of *Spirulina* was quantified by using the peak area in the gas chromatogram. *Spirulina* (S) possesses the highest yield of C16:0, C16:1n9, C18:0, C18:1n9 and C18:2n6, while *Spirulina* (P) possesses the highest amount of C18:3n6. The overall fatty acid content of *Spirulina* (P) resulted in the second highest amount among the *Spirulina* isolates. The results also indicated that the fatty acid content of *Spirulina* sp. was inconsistent with the commercial *Spirulina* isolates. *Spirulina* sp. has the lowest fatty acid content of C16:0. C16:1n7, C18:0 and C18:2n6.

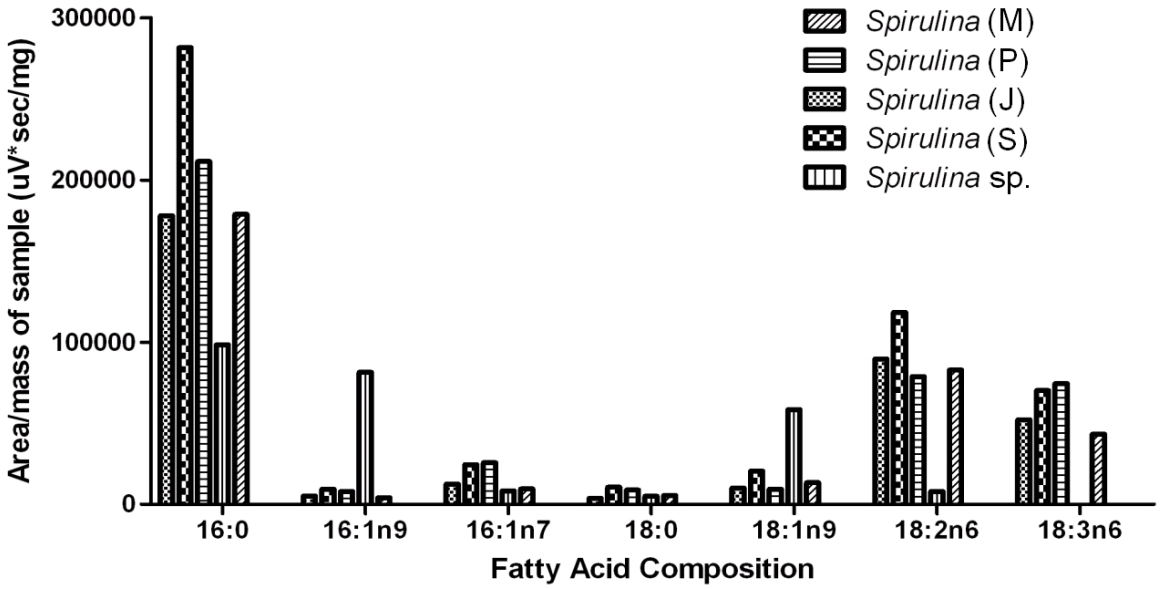


Figure 4.10: Fatty acid content in different isolates of Spirulina

A gas chromatogram for the fatty acid analysis examined in this study is shown in Figure 4.11. The gas chromatogram shows that the standards were completely separated. The fatty acids of the *Spirulina* isolates were identified by comparing the retention times of the standard and the samples; while the amounts of the samples were quantified by using a calibration curve. If the concentration of particular compound is too low or too high, the measurement of the quantity of fatty acids can be inaccurate.

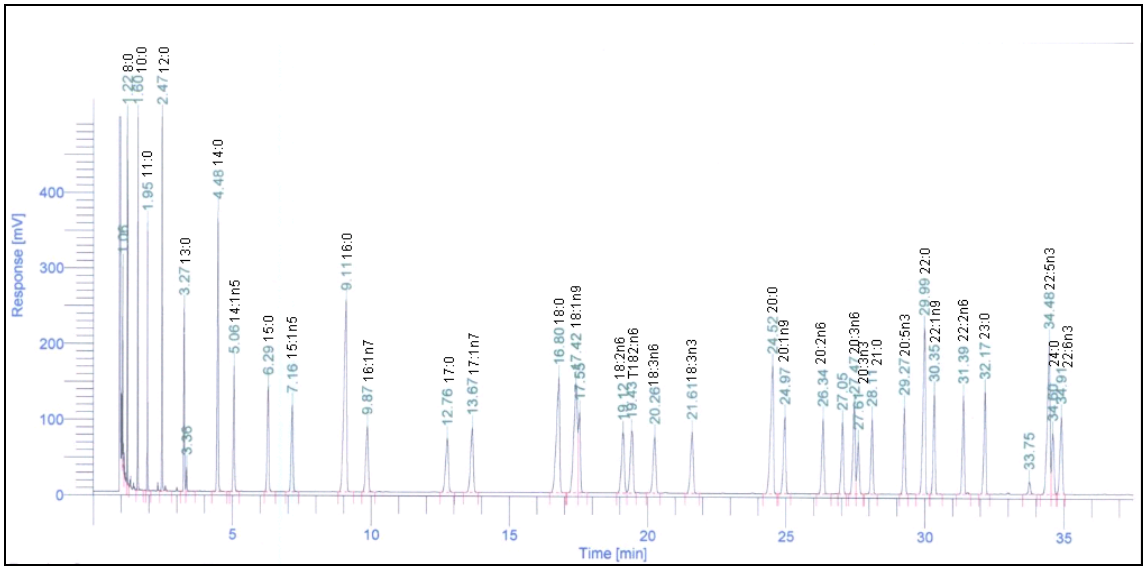


Figure 4.11: Gas chromatographic separation of the methyl ester derivatives of the fatty acids of the external standard

4.8 Discussion

It is technically difficult to amplify the 16S rDNA from the five *Spirulina* isolates in different dilution by using XS DNA extraction method. This experimental result supported the observation that the *Arthrospira* strain failed in the PCR amplification due to its inhibitory compound (Scheldeman *et al*., 1999). The failure of PCR amplification is possibly because the investigated *Spirulina* isolates possess inhibition, and thus these isolates express the inhibition to the positive control. Wu *et al*. (2000) also observed that the filamentous species of cyanobacteria have difficulty in the enzymatic manipulation of DNA due to their gelatinous sheath. The gelatinous sheaths that surround the body of the cyanobacteria are rich in polysaccharides and are particularly known to provide the interference for DNA extractions (De Philippis & Vincenzini, 1998). This phenomenon was commonly documented in the literature and a better lysis protocol for this reaction is necessary.

Normally, a successful protocol required longer lysis duration, especially for filamentous cyanobacteria. For instance, Scheldeman *et al*. (1999) successfully extracted the *Arthrospira* DNA by applying a longer lysis protocol and involving proteinase K in DNA extraction. By illustrating this assertion, the cells of *Spirulina* require a longer time to lyse. In this study, the success of the DNA extraction is based on the improved XS DNA extraction method. This protocol has a better lysis method involving the process of bead beating.

A high purity and larger sizes of the DNA fragment enhance the performance of the phylogenetic analysis (Brinkman & Leipe, 2001). Moreover, it helps to correctly identify the living organisms. From the DNA sequence chromatogram, the correct region for the molecular data was selected and the fragment sizes of the *Spirulina* isolates were determined. The different fragment sizes of the *Spirulina* isolate can be explained by the differences between the fragment lengths, which are due to an inappropriate procedure when cleaning up the DNA template prior to sequencing. Therefore, extra care must be taken when commencing the DNA clean-up stage.

In this study, all of the *Spirulina* isolates excluding *Spirulina* (M) were successfully amplified by the primer pairs of 740F and 1494R primer pairs. The fragment size of *Spirulina* (M) is smaller when compared to other investigated *Spirulina* isolates. The reason for that may be attributed to the drying process that subjected it to high

temperature and pressure, and thus has partially degraded the DNA fragments of *Spirulina* (M).

It is important to identify the taxonomy of an organism, because a correctly identified organism will work for human benefit. With an identified organism, the bioactive metabolite can be overproduced to treat specific diseases. Furthermore, an unclear taxonomic situation of *Spirulina* and *Arthrospira* was made by Geitler's mistake in 1932 (Vonshak, 1997). In order to place *Spirulina* and *Arthrospira* into a clear taxonomic status, five isolates that are identified as *Spirulina* were compared. The similarity between relatives was determined by using the sequence of 16S rDNA. Although the DNA sequence of *Spirulina* (M) is shorter, it is consistent in finding the similarity from its relative (Wolfgang *et al*., 1998).

The commercial *Spirulina* isolates (that is *Spirulina* (P), *Spirulina* (M), *Spirulina* (J) and *Spirulina* (S)), which are widely available as health food supplements, were closely related to *Arthrospira* strains listed in the database. Thus, it seems that these commercial isolates have been misclassified (and mislabelled) as *Spirulina*, when they are in fact *Arthrospira*. The Australian isolate investigated in this study, *Spirulina* sp. was closely related to *Halospirulina* strains listed in the database. Previous studies have shown that this cyanobacterium has the same phenotypes as *Spirulina subsalsa*, and is characterised as low nutritional value of polyunsaturated fatty acids. Moreover, it does not seem to be economically produced in outdoor conditions due to its low productivity (Komárek, 2010; Tredici *et al*., 1988). The taxonomic results in this study varied in the above isolates, and the *Spirulina* sp. is expected to have lower potential as a food supplement.

The aim of maintaining a monoculture of Australian *Spirulina* is to further investigate the chemotaxonomic status in this study. However, to maintain bacteria-free culture without interfering with the morphology and physiology of an individual species is difficult and time consuming. To ease the preparation of a bacteria-free culture, it has been suggested to introduce antibiotics directly into the culture medium. Nonetheless, the culture medium of *Spirulina* using antibiotics did not show promising results (Choi *et al*., 2007). Most algae cultures are more resistant compared to bacteria cells when exposed to ultraviolet light (Richmond, 2004). The exposure time under ultraviolet light cannot be too long, and an appropriate exposure time for individual species needs to be

determined. As reported in a previous study, there is no significant effect of ultraviolet radiation on the *Anabaena* (Mehta & Hawxby, 1977). Moreover, no appreciable pigments of *Anabaena* were decomposed after this treatment. The Australian isolate revealed a similar reaction as in the previous report. A feasible exposure time to achieve a bacteria-free culture for *Spirulina* sp. without losing its green pigmentation is 30–45 minutes. The exposure time under the ultraviolet radiation is varied, due to the fact that different species of algae might confer different responses when they are exposed to ultraviolet radiation. Consequently, a bacteria-free culture for certain irradiation time is possible to achieve and this method is possibly the best to eliminate bacteria from the *Spirulina* sp. culture.

The culture of Australian isolate was consistently observed. The akinete formation was specifically induced during the end of the exponential growth phase in MLA media with the salt water. This occurrence might be due to several factors that initiate the akinete formation, including the light and the deficiencies in nutrients (Rother & Fay, 1977; Wyman, 1986). The *Spirulina* sp. that was cultured in the MLA media with seawater did not seem to have this phenomenon because the seawater has higher nutrient compared with the salt water. Thus, this suggested that the nutrient limitation might be the factor that initiates the akinete development. The differentiation of the akinete causes the algae to clump together and subsequently forms as a floating map when there is a large part of the gas vesicle trapped (Rother & Fay, 1977). As a consequence, it leads the floating mat to become thicker and has higher probability to cause an algae bloom.

The *Spirulina* sp. cannot be confirmed dead by only observing the changes of cell colouration. For instance, deficiencies in nitrogen led to a colour change from blue-green to a yellowish green for a cyanobacterium *Synechococcus* sp. strain PCC 7002 (Sakamoto *et al*., 1998). The *Spirulina* sp. also changed its blue-green pigmentation to transparent after long exposure time under ultraviolet radiation. To confirm that the *Spirulina* sp. had entered the death phase, the filaments and cells of the Australian isolate were examined under the microscope. The death of *Spirulina* sp. was observed when the cell of *Spirulina* sp. lyses. Moreover, the filament length of the *Spirulina* sp. was noted to become shorter when compared to the one during exponential phase.

An understanding of the Australian *Spirulina* isolate is essential for the development of cultivation for producing specified bioactive compound. The Australian *Spirulina* isolate was investigated for its adaptability to survive in different growth conditions. The molecular analysis clarified that the Australian isolate belongs to the *Halospirulina*, and therefore the growth conditions used for cultivation are based on *Halospirulina*. However, to date, the growth requirements for *Halospirulina* are unknown. To ensure the Australian isolate remained viable throughout this study, the *Spirulina* sp. cultured in four different nutrient media that are appropriate types of media for cyanobacteria.

The effect of nutrient media on the growth of *Spirulina* sp. was examined in view of the fact that the chemicals consumed for the preparation of culture media has become an important focus to optimise the biomass production. Optimised production depends on the profitability and the growth condition of an organism (Guillaume *et al*., 2003). The growth of *Spirulina* sp. was empirically compared in Zarrouk media, BG-11 media, MLA media with seawater and MLA media with salt water. The presented results illustrated that the variation in culture media influences the growth curve of *Spirulina* sp. The MLA media with an addition of seawater in the culture media increase the growth response of *Spirulina* sp. This suggested that the cyanobacteria isolates may adapt to different growth conditions and nutrient sources. Moreover, seawater is economical for the preparation of culture media due to its unlimited availability and enriched nutrients (Tomaselli *et al*., 1987). Recent studies have also shown that the *Spirulina* cultivated in supplemented seawater has higher growth rate and higher pigment production (Mary Leema *et al*., 2010).

Although the Australian *Spirulina* isolate is closely related to *Halospirulina*, *Spirulina* sp. grow preferably at 25^{o}C with MLA/seawater culture media, which is not supported by previous studies on the growth conditions of *Halospirulina*. *Halospirulina tapeticola* can tolerate temperature up to 38^{o}C (Nubel *et al*., 2000). The results obtained were not in agreement with the *Halospirulina tapeticola*, which is maybe because of the microspeciation within the *Halospirulina* genus in response to environmental condition. *Spirulina* sp., which makes an evolutionary formation of new biological species, might have different growth responses when compared to *Halospirulina tapeticola*. *Spirulina* sp. was observed to grow preferably at room temperature. This suggested that the Australian *Spirulina* have a narrow temperature optimum and could not be

economically feasible to produce since temperature will be the most limiting environmental factor in the outdoor production (Vonshak, 1997).

The overall survival of *Spirulina* sp. was found to be sensitive to the cultivation temperature. The results obtained in this study are contradictory to the previously reported *Halospirulina tapeticola*, for which temperature has no detrimental effect. The limited growth condition of *Spirulina* sp. suggested that this isolate is not a strain that is suitable for outdoor cultivation, though it might contain a high amount of bioactive metabolite that has great value in medication. Therefore, the analysis of the chemical composition of *Spirulina* sp. is necessitated and discussed in the following section.

In order to clarify the taxonomy of *Spirulina* and *Arthrospira*, Komarek (2010) suggested that: '… at least one diacritical phenotypic character (or autapomorphic set of characters) and ecological, ecophysiological, ultrastructural and biochemical characteristics …" must be done. The taxonomic classification identified that the *Spirulina* sp. does not belong to the same family as the commercial *Spirulina* (see Section 4.2.1). In order to verify this statement, the taxonomic status of the investigated *Spirulina* isolates needs to be further supported by the chemotaxonomic evidence. In this study, the chemical composition of the investigated *Spirulina* isolates was examined to obtain more detailed information about the taxonomic identification. The *Spirulina* isolates tested in this study have the carbohydrate content in the range of 8.42–18.25% AFDW. The results obtained are in reasonable agreement with those of Vonshak *et al*. (1997), who reported a carbohydrate content of 10–15%.

The results for the lipid and protein contents of the investigated *Spirulina* isolates are similar to the previous studies. All of the *Spirulina* isolates have an exceptionally high amount of protein and lipid content. Due to the high protein content of *Spirulina*, it is widely used as human food supplement and animal feed. However, the published data on the *Spirulina* genus may have been erroneously identified. The published results indicated that the '*Spirulina*' have high nutritional value and are a promising source for human foods although they may in fact belong to the genus of *Arthrospira.*

No firm conclusion can be drawn because the variation in carbohydrate, protein and lipid content of different isolates of *Spirulina* is small. This small deviation may be related to different species and growth conditions of the tested isolates. The growth conditions such as temperature, light intensity as well as the nutrients uptake of

Spirulina are highly affected by the carbohydrate, protein and lipid content of the *Spirulina* isolates. The other influences on the chemical composition maybe also attributable to the drying operation used. Although most of the commercial product is spray-dried, significant change in the drying temperature may give different characteristics of *Spirulina* (Desmorieux & Hernandez, 2004).

The nutritive value of a lipid is defined by its essential fatty acid composition. One of the essential fatty acids such as omega-3 fatty acid is considered to be 0.5% of the energy intake (World Health Organization Study Group on Diet, 2003). A distinct fatty acid distribution of *Spirulina* sp. was obtained. This fatty acid distribution is similar to the *Spirulina subsalsa* that was reported by Cohen *et al*. (1987). Although the *Spirulina* sp. does not have the same fatty acid distribution as the commercial *Spirulina* isolates, the results obtained are reasonably convincing since the molecular analysis identified the Australian *Spirulina* isolate as a totally different genus. Moreover, previous studies had indicated that the *Spirulina* strains that were cultured in the combination of non-alkaline medium with the seawater did not possess gamma linolenic acid; however it possesses a high proportion of palmitoleic acid (Cohen & Vonshak, 1991; Vonshak, 1997). Similar results also showed there is the possibility that the *Spirulina* does not contain the gamma linolenic acid (Romano *et al*., 2000).

The predominant fatty acid in the sulfolipids is C18:2n6. In this study, the *Spirulina* (S) isolate showed that it contains the highest yield of C16:0 and C18:2n6. This led to an assumption that the *Spirulina* (S) might contain the highest content of sulfolipids in this study (Vonshak, 1997). The overall fatty acid content of *Spirulina* (M) and *Spirulina* (J) resulted in them being ranked in third and fourth place. The total fatty acid content of *Spirulina* sp. was also reported. These fatty acid productivities are highly associated with SQDG and this leads to the speculation that the *Spirulina* sp. might synthesise SQDG in lower amount due to its degree of fatty acid content.

A comprehensive review indicated that the taxonomy of *Spirulina* can be classified by using the fatty acid composition of the strain (Kenyon *et al*., 1972; Romano *et al*., 2000). This application, coupled with the molecular confirmation showed that the *Spirulina* sp. isolate, which was isolated in Western Australia, is strongly designated in the family of *Halospirulina* in this study. *Spirulina* sp. displays its fatty acid composition without gamma linolenic acid and has different growth characteristics

when compared with the commercial *Spirulina* isolates. The genetic distinctiveness of *Spirulina* sp. and *Spirulina* (J), *Spirulina* (P), *Spirulina* (M) and *Spirulina* (S) has been distinguished by the 16S rDNA gene sequence analysis and the chemical composition studies. The commercial '*Spirulina*' isolates used in the human supplement product therefore belong to the strain of *Arthrospira*. The therapeutic effects of the natural products from the *Spirulina* which had been reported need to be reaffirmed with the proper scientific name of this genus.

4.9 Summary

The optimisation of the growth conditions of *Spirulina* has received much attention in the food industry. This alga contains high nutritional value and has various uses in biomedical applications. A variety of the growth conditions have been developed to produce a large biomass of *Spirulina* more economically. *Spirulina platensis* and *S. maxima* are the common strains that have been discussed generally. Both of these strains are produced economically in outdoor conditions. *Spirulina* sp., a wild species isolated in Western Australia, was the strain investigated in this study for its potential of outdoor growth. A preliminary study on different culture conditions, especially the nutrient uptake and the growth temperatures, was conducted, evaluating the potential outdoor cultivation of *Spirulina* sp. The reduction in the cost of chemicals consumed in the preparation of media is one of the main concerns for outdoor production.

Growth characteristics of each *Spirulina* strains are different, and thus an understanding of each *Spirulina* strain in relation to environmental condition is essential. In this chapter, an observation of the growth characteristics of *Spirulina* sp. was undertaken and the growth condition of *Spirulina* sp. was investigated based on previous studies. The growth condition of *Spirulina* sp. was trialled in particular medium, light irradiance, pH and temperature. MLA media with seawater at 25^{o}C yielded the best growth characteristics of *Spirulina* sp. An axenic culture was obtained after 30 minutes exposure under ultraviolet irradiation prior to subculture.

In order to overcome the confusion of the genera *Spirulina* and *Arthrospira*, a study of molecular genetic studies based on 16S rRNA sequencing was performed to classify five different isolates of *Spirulina* sourced from different locations. The ribosomal DNA of five different *Spirulina* isolates was extracted for the generic definition. The DNA extraction used for the *Spirulina* strain requires longer lysis time to remove the

contaminants from the gelatinous sheath of its filaments. An improved DNA extraction was performed and it successfully amplified the gene sequences for all *Spirulina* isolates in this study. The 16S rDNA gene sequencing amplified approximately 1500bp in size for all *Spirulina* isolates except *Spirulina* (M), where it has only 640bp sequence. The sequences of all *Spirulina* isolates confirm that the commercial '*Spirulina*, such as *Spirulina* (J), *Spirulina* (P), *Spirulina* (M) and *Spirulina* (S), has the closest similarity to the species of *Arthrospira*. Australian *Spirulina* isolate was found to have the closest similarity to *Halospirulina*.

A further study of the taxonomic classification between the investigated *Spirulina* isolates was examined by chemical analysis. The protein, lipid and carbohydrate content of the *Spirulina* isolates were studied in order to obtain an accurate taxonomic classification. The lipid compositions of five different *Spirulina* isolates were also compared; the *Spirulina* sp. contained distinctly different fatty acid composition, while the commercial *Spirulina* isolates displayed similar characteristics in fatty acid composition. The classification of the *Spirulina* isolates that included the chemotaxonomy, confirmed that the commercial *Spirulina* isolates and the Australian *Spirulina* isolate do not belong to the same genus.

In the following chapter, a study of the gene responsible for the production of sulfolipids is conducted. Since there is a limited knowledge on the identification of these genes in *Spirulina*, further research on the potential toxicity of these strains by screening for NRPS and PKS-encoding genes was examined. The phylogenetic relationship of the sqdB and sqdX of the investigated *Spirulina* isolates with several photosynthetic organisms is also discussed.

CHAPTER 5 SQDG SYNTHESIS

5.1 Introduction

The application of enzyme technologies has been used widely since 1987, when the first recombinant enzyme drug, Activase ®, was approved by the Food and Drug Administration (FDA) for treatment of heart attacks and acute massive pulmonary embolism (Michel, 2003). This technology is growing rapidly, because it is profitable in a lot of fields. However, the quantities of the bioactive compounds from the resources are limited. Moreover, the extraction and purification of the bioactive compounds are difficult processes (Balandrin *et al*., 1985; Ehrenreich *et al*., 2005; Koksharova & Wolk, 2002).

The first sulfolipids biosynthesis operon to be genetically characterised was that of the purple bacterium, *Rhodobacter sphaeroides* (Benning & Somerville, 1992a). A number of studies in photosynthetic organisms, such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, have subsequently identified other sulfolipids synthetase genes, including those responsible for the final steps in sulfolipids assembly; sqd1 (sqdB) and sqd2 (sqdX) (Sato *et al*., 2003; Yu *et al*., 2002). In 2001, Blinkova *et al*. demonstrated that sulfolipids extracted from *Spirulina platensis* inhibited the activity of HIV (Blinkova *et al*., 2001). Thus, revealing cyanobacteria as a potential source of therapeutic sulfolipids. However, the enzymes responsible for the biosynthesis of sulfolipids from *Spirulina* have not yet been thoroughly studied.

Cyanotoxins are frequently produced non-ribosomally by NRPS and PKS (Barrios-Llerena *et al*., 2007; Ehrenreich *et al*., 2005; Neilan *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000). Several studies suggested that NRP and PKS genes confer an evolutionary advantage to the cyanobacteria that possess them. However, certain species appear to lack these genes altogether and to date they have not been identified in *Spirulina*. This study screened *Spirulina* for NRPS and PKS-encoding genes to establish whether *Spirulina* has the potential to produce cyanotoxins via these enzymes.

5.2 Screening of *Spirulina* isolates with NRPS and PKS

Screening of PKS and NRPS genes were carried out by using the primer pairs of MTF2 and MTR, and DKF and DKR. None of the isolates of *Spirulina* was found to be positive for both of these genes by PCR; however smaller size fragments of amplicons were found from *Spirulina* sp., as seen in Figure 5.1.

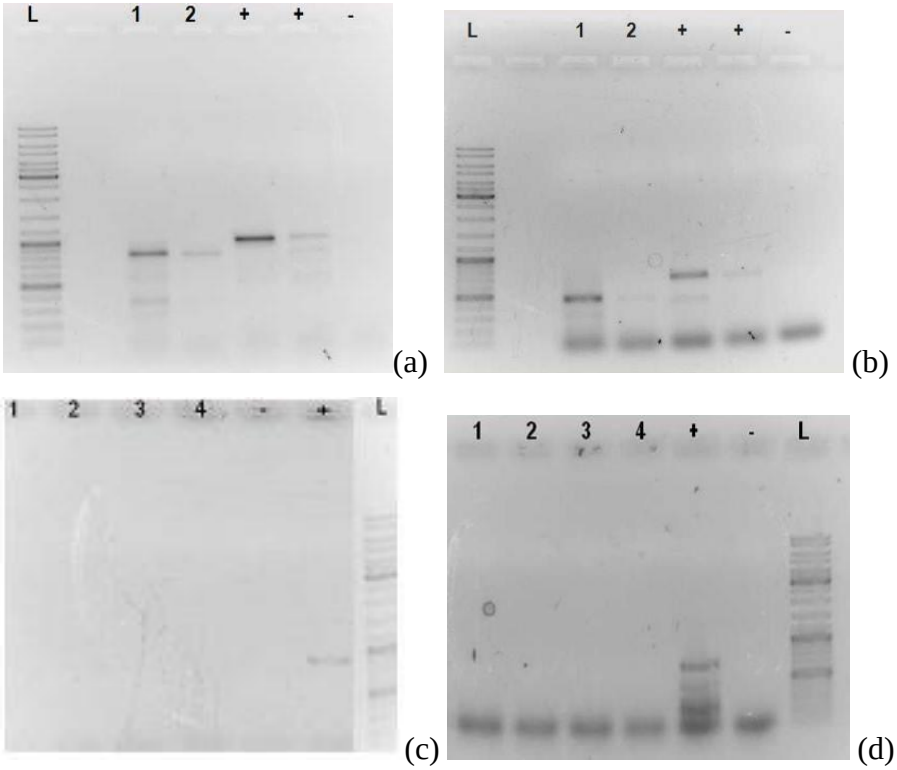


Figure 5.1: Results of NRPS and PKS PCR amplification

(a) NRPS PCR by different DNA template concentration of *Spirulina* sp. Lane 1-2 contains DNA from *Spirulina* sp. 1/10 dilution and 1/100 dilution, respectively. (b) PKS PCR by different DNA template concentration of *Spirulina* sp. Lane 1-2 contains DNA from *Spirulina* sp. 1/10 dilution and 1/100 dilution, respectively. (c) NRPS PCR for commercial *Spirulina* isolates in the order of *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* (M) (Lane 1-4). (d) PKS PCR for commercial *Spirulina* isolates in the order of *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* (M) (Lane 1-4). Lane + indicated a positive control and Lane - indicated a negative control. Lane L stands for the DNA ladder.

The DNA fragments of *Spirulina* sp. (Figure 5.2) found from the NRPS and PKS amplification were determined to be 537bp for NRPS and 419bp for PKS. A BLASTX DNA sequence analysis indicated no similarity to NRPS and PKS gene from the isolated fragment of *Spirulina* sp. The closest similarity of the DNA fragment from NRPS amplification shows only 38% similarity to the biotin-(acetyl-CoA-carboxylase) ligase of *Denitrovibrio acetiphilus*, while the similarity of the DNA fragment from PKS amplification shows 73% to an unknown protein from *Oryza sativa* Japonica Group.

a.)

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4    QELPSTNTYAKQIVKTERFHGAIVLADNQTEGRGQHQRSWEIEAHQNLTFSIIFEPQKGD  183
184  RLSILTLACALAVSDYVEEHLKQDTQLKWPNDVLVNGKKISGLLTETVFRGDVLDRLVIG  363
364  IGFNINQRKFDEKLTETATSLAILDDSKHQREEVLARLLTRIEYLYRLWSTQDIDLLK  537
```

b.)

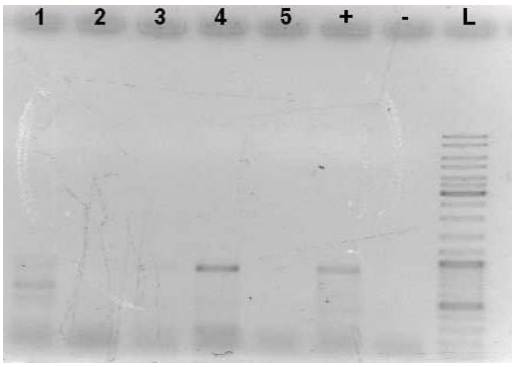
```
3    ISTHFTATPGIPSAPTVL*LFSFHCLSVVKPLSLTADLNSHLRTLYAQSFRITlassvlp  182
183  rllars*prlIPQVPSLCSSLRKGVYNPRAFLPHAVLLRQACAHCGKFPTAASRRSLGRV  362
363  SVPVWLIILSDQLLIVALV  419
```

Figure 5.2: The nucleotide sequence of unspecific band for Spirulina sp.

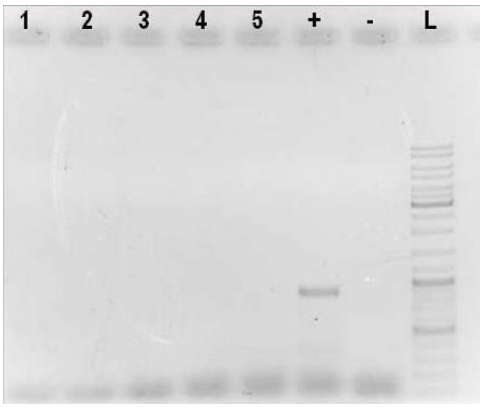
a) Isolated fragment from NRPS amplification. (b) Smaller amplicons from PKS amplification.

5.3 Screening of *Spirulina* isolates with sqdB

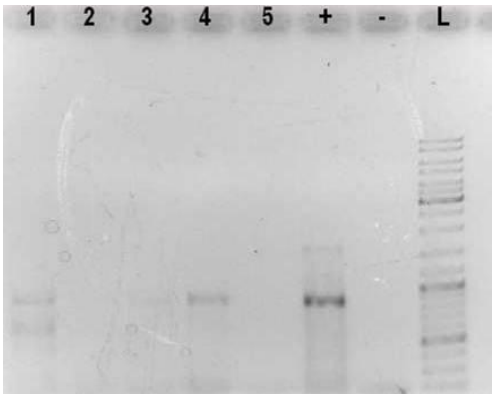
The primers for cyanobacteria were designed to amplify a 900bp fragment of the sqdB gene. Figure 5.3 (a) illustrates that one microliltre of primer concentration did not equivalently amplify all *Spirulina* isolates. The primer concentration was then adjusted to optimise the PCR conditions. By using the PCR calculator from www.biometra.com/1070.0.html (Accessed 11 September 2009), the primer annealing temperature and a suitable PCR program were determined. Figure 5.3 (b), (c), (d), (e) and (f) illustrate that an increase of annealing temperature resulted in an increased yield of specific and non-specific product. At 45^{o}C, only *Synechocystis* PCC 6803 was preferentially amplified and it showed a strong band for the specific product. The *Spirulina* isolates and the positive control were detected when the annealing temperature is at 54^{o}C. The annealing temperature at 56^{o}C showed lower specificity for all isolates of *Spirulina* and the positive control.



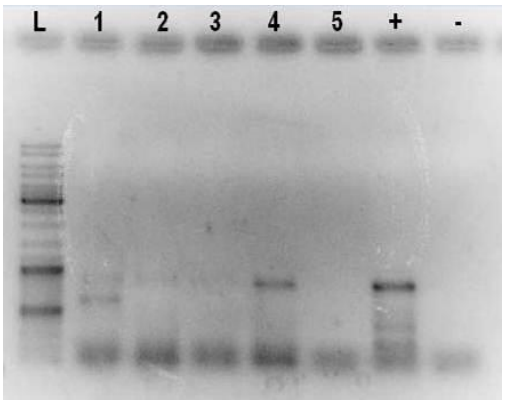
(a)



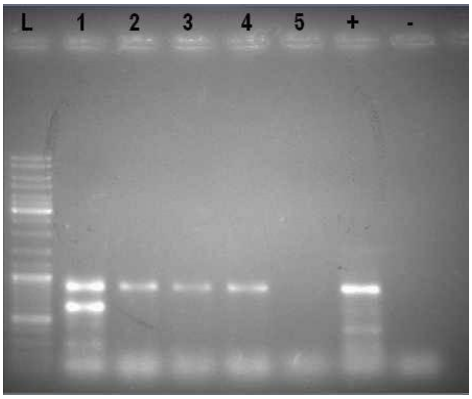
(b)



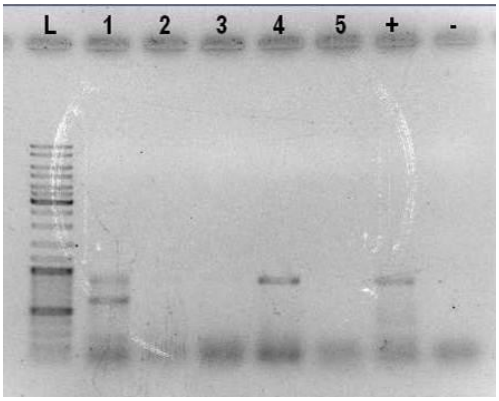
(c)



(d)



(e)



(f)

Figure 5.3: PCR in different condition for different isolates of Spirulina using sqdB

(a) Use 1 µL of primers, use annealing temperature at (b) 45^{o}C (c) 50^{o}C (d) 52^{o}C (e)54^{o}C (f) 56^{o}C PCR for *sqdB* was in the order of *Spirulina* sp., *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* (M) (Lane 1-5). Lane + indicated a positive control and Lane - indicated a negative control. Lane L stands for the DNA ladder

PCR using the primer pairs of dsqdBF/dsqdBR successfully amplified the specific product with multiple alignments for *Spirulina* sp. and *Synechocystis* PCC 6803. For the rest of the isolates of *Spirulina* except *Spirulina* (M), the amplification resulted in a single 900bp fragment. The desired gel band was cleaned and electrophoresed through a 1% (w/v) agarose gel. As can be seen in Figure 5.4, the product was purified and ready for sequencing. The consensus sequences for all isolates of *Spirulina* were in the range from 800–900bp and are presented in Figure 5.5.

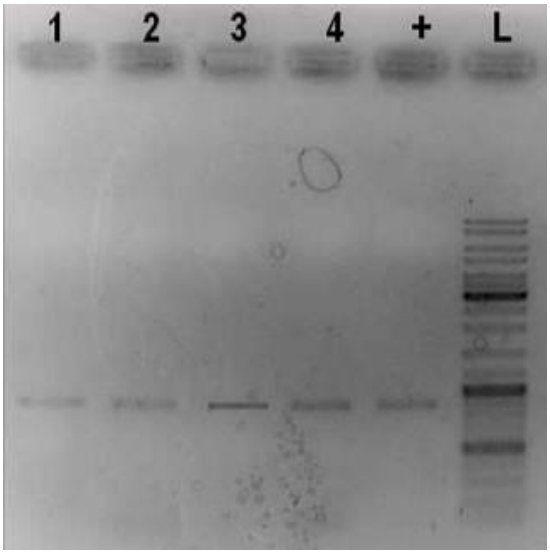


Figure 5.4: Purified sqdB product from all isolates of Spirulina

Results in the order of: *Spirulina* sp., *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) (Lane 1-4). Lane + indicated a positive control (Synhechocystis PCC6803). Lane L stands for the DNA ladder

>J
ACTTGGTGCGGCGACACTGGGATCAACAGCTAGGGAATGAGACCTTGACACCCATAGCCCCTATTCAACAAAGGATT
CAGCGCTGGCGTGATTTAACGGGTAAGTCTATTGACCTGTTTATGGGCGATATCAATAACTATAGTTTTCTGATCAAT
AGCTTGCGGGAGTTTCAGCCAGATTCTATTGTTCATTTTGGTGAACAGCGATCGGCTCCCTTTTCAATGATAGATCGT
GAACACGCAGTTCTCACCCAAAGCAATAATGTAATGGGGAATTTGAATATCCTGTATGCTATGCGGGAAGAATTCCC
TGATGCACATTTGGTCAAGTTGGGGACTATGGGTGAATATGGTACACCCAATATTGATATCGAAGAGGGCTATATTA
CCATTGAGCATAATGGCCGTAAGGATACTGTCCCGTATCCGAAACAGCCCGGAAGTTTCTATCACCTTTCTAAGGTTC
ATGACAGCCATAATATCCACTTTGCTTGTAAAATATGGGGTCTGAGGGCTACCGATTTAAACCAGGGTGTGGTTTATG
GGGTTCTCACCGAAGAGACGGGAATGGATGAGTTGTTGATCAACCGTCTGGATTATGATGGGATTTTTGGGACGGCT
TTAAATCGTTTCTGTATTCAGGCTGCCATTAATCACCCCCTGACGGTTTATGGTAAGGGAGGACAAACTAGGGCTTTC
CTGGATATCCGCGATACCGTGCGATGTGTTGAGTTGGCGATCGCTAATCCCGCTGAACCTGGTCATTTCCGCGT

>W
AGCTAGGTATTGAGACCTTGACACCCATAGCCCCTATTCAACAAAGGATTCAGCGCTGGCGAGATTTAGCGGGTAAG
TCTATTGACCTGCTTATGGGCGATATCAATAACTATAGTTTTCTGATCCATAGCTTGCGGCAGTTTCAGCCAGATTCTA
TTGTTCATTTTGGTGAACAGCGATCGGCTCCCTTTTCAATGATAGATCGTGAACACGCAGTTCTCACCCAAAGCAATA
ATGTAATGGGGAATTTGAATATCCTGTATGCTATGCGGGAAGAATTCCCTGATGCACATTTGGTCAAGTTGGGGACT
ATGGGTGAATATGGTACACCCGATATTGATATCGAAGAGGGCTATATTACCATTGAGCATAATGGCCGTAAGGATAC
TGTCCCGTATCCGAAACAGCCCGGAAGTTTCTATCACCTTTCTAAGGTTCATGACAGCCATAATATCCACTTTGCTTG
TAAAATATGGGGTCTGAGGGCTACCGATTT

>P
GTGCATTCTGGAGAACTTAGATGCGCCGACACTGGGATCAACAGCTAGGTATTGAGACCTTGACACCCATAGCCCCT
ATTCAACAAAGGATTCAGCGCTGGCGTGATTTAACGGGTAAGTCTATTGACCTGTTTATGGGCGATATCAATAACTAT
AGTTTTCTGATCAATAGCTTGCGGCAGTTTCAGCCAGATTCTATTGTTCATTTTGGTGAACAGCGATCGGCTCCCTTTT
CAATGATAGATCGTGAACACGCGGTTCTCACCCAAAGCAATAATGTGATGGGAAATTTGAATATCCTGTATGCTATG
CGGGAAGAATTCCCTGATGCACATTTGGTCAAGTTGGGGACTATGGGCGAATATGGTACACCCGATATTGATATCGA
AGAGGGTTATATTACCATTGAGCATAATGGCCGCAAGGATACTGTCCCGTATCCGAAACAGCCCGGA

>S
TGATGTCGGCATCCTCGATAACATGGTGCGTCGGCATTGGGATACGAAACTTGGAGTCAATACCCTTACCCCCATCAC
TCCCATTCAAAAACGGCTACAACGTTGGCACGATCTTACAGGCAAGCACATCGACCTGTATGTTGGCGATATTACGG
ACTACAGTTTTTTAAGTCAAACCCTACGCCAGTTTGAACCAGAGTCTGTGGTTCACTTCGGAGAACAACGGTCTGCTC
CCTATTCTATGATTGACCGGGAACACGCCGTATTTACTCAGGTGAATAATGTAGTGGGGACCTTAAATATCCTCTACG
CAATGAAAGAGGACTTCCCCGACTGTCACTTGGTTAAGTTGGGAACAATGGGAGAATACGGGACTCCCAACATTGAT
ATTGAAGAAGGTTATATCGAAATCGAACACAATGGTCGTAAAGACCTTCTCCCCTATCCTAAACAACCCGGTAGTTT
CTACCACTTATCCAAGGTTCACGATAGCCACAATATCCATTTTGCCTGCAAAATTTGGGGACTGCGAGCCACGGACTT
AAACCAAGGGGTGGTGTATGGAGTCCTCACCGAAGAAACAGGCATGGATGAGATGTTGATCAACCGTCTCGACTATG
ATGGAGTATTCGGAACCGCATTAAACCGTTTCTGTATTCAAGCCGCAGTAGGACATCCTCTCACGGTGTACGGAAAA
GGAGGCCAAACTCGCGCTTTCCTCGATATTCGGGACACGGTGCGCTGTGTGGATTTAGCGGTGCGTAATCCGGCGGA
TGCTGGACAATTCCGGGTCTTCAACAATTTAACGCCCCCAGGAGCAATTCCCAGAGTATCAGGTCTGTCCCTAGACGC
CCGATACTGCGACACGATCGCATTGTCTGTCAGTCATCTTCAGGACTACGTTTTTTATCAAACCTACGCCATTTGAAC
AGAATCTGTGGA

Figure 5.5: The sequence for sqdB for J: Spirulina (J), P: Spirulina (P), W: Spirulina (S), S: Spirulina sp.

5.4 Screening of *Spirulina* isolates with sqdX

The gene fragments did not amplify when the designed primers pairs of dsqdXF/dsqdXR were used for PCR amplification. No detectable band could be identified, as shown in Figure 5.6. However, an unexpected fragment size was found from *Synechocystis* PCC 6803 (positive control), shown in Figure 5.6. This unexpected amplicon was submitted to the BLAST system to determine the closest sequence similarity. The amplified product revealed that it did not have similarity to any of the sqdX gene in the database.

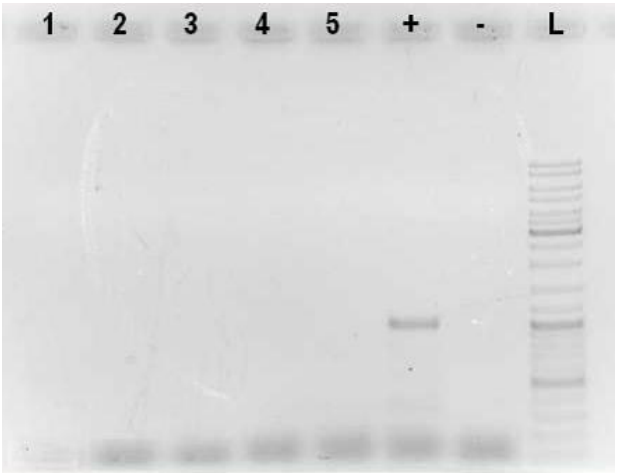


Figure 5.6: An erroneous DNA sequence amplified from Synechocystis PCC 6803

PCR for *sqdX* was in the order of *Spirulina* sp., *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* (M) (Lane 1-5). Lane + indicated a positive control and Lane - indicated a negative control. Lane L stands for the DNA ladder

New primer pairs were designed in order to amplify the correct sequence. This new primer pairs of dsqdX1F/dsqdX1R amplified a polymorphic locus, where more bands were observed on the agarose gels, as shown in Figure 5.7. To optimise the PCR condition, different annealing temperatures were performed by a PCR program. The PCR product depicted in Figure 5.7 indicates that the reaction worked more efficiently in the annealing temperature range from 56.1^{o}C to 50.0^{o}C. The first fragment is positioned at 600bp and the second fragment's position is at approximately 200bp. A slight mismatch for *Synechocystis* PCC 6803 at 56.1^{o}C, 58.0^{o}C, 59.2^{o}C and 60^{o}C was observed. It is likely that the mismatches for *Synechocystis* PCC 6803 were the results of over-amplification.

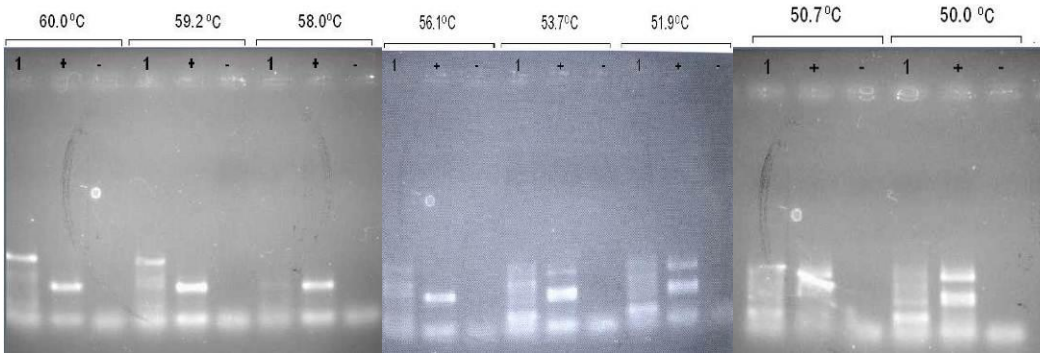


Figure 5.7: The initial trial on annealing temperature using PCR program

Results at 60.0°C, 59.2°C, 58.0°C, 56.1°C, 53.7°C, 51.9°C, 50.7°C and 50.0°C. Lane 1 indicated as PCR product of *Spirulina* sp.; Lane + indicated a positive control and Lane – indicated as a negative control.

DNA of each isolate of *Spirulina* has variable efficiency, depending on the annealing temperature. In this study, the annealing temperature ought to amplify the specific product of all isolates of *Spirulina*, as well as *Synechocystis* PCC 6803 (positive control). PCR annealing at 55°C (Figure 5.8) resulted better amplification, in which the specific product was visualised as a strong band for the positive control and *Spirulina* isolates.

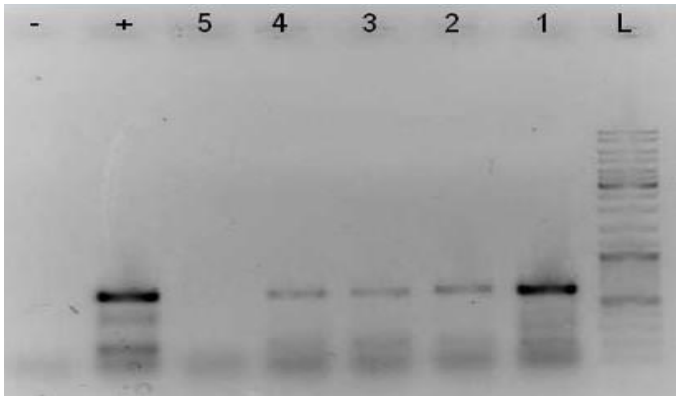


Figure 5.8: The PCR condition with annealing temperature

Results at 55°C. PCR for sqdB was in the order of *Spirulina* sp., *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* (M) (Lane 1-5). Lane + indicated a positive control and Lane - indicated a negative control. Lane L stands for the DNA ladder

The sequences for the sqdX genes obtained from *Spirulina* isolates are displayed in Figure 5.9. The sqdX yielded the fragment size of 474bp for *Spirulina* sp., 539bp for *Spirulina* (J), 445bp for *Spirulina* (S) and 446bp for *Spirulina* (P). To determine the closest similarities of these fragments with other recognised species, the sequences were submitted to the BLAST system.

>P
TATTGCGTACAGCCCATAATCAGGCTATGTTAAACCTGTGTACCTCCACAGCTATGGTTGAG
GAATTACGCAATCATGGCATTGAACGGGTAGACCTATGGCAGAGGGGGGTGGATACAGAAC
TGTTCCAGCCCCATAAAGCCACTAAAGAAATGCGCGAGAGCCTCAGTATGGGAAACCCAGA
CGATACCCTACTGTTGTATGTGGGGAGACTAGGAGCCGAAAAAGAAATCGATCGCATTAAA
CCTATTCTAGCAGCAATACCCAACGCCCGTCTCGCCCTAGTCGGAGATGGTCCGAACCGAGA
AAACCTAGAACAACATTTTGCCGGAACTCCCACCAACTTCGTGGGTTATCTGCGGGGAGAAC
AATTAGCGGCCGCTTACGCCTGCGCTGATGCCTTCATTTTCCCTTCCCGGACAGAAACCCTA
GGTTTAGTCCTCCTC

>S
TTTGTGGGAATTATTGAAAGCGGGGCATAATCAAGCCCAGTTGAATTTATGCACTTCAACGG
CCATGGTAAAGGAATTGAGCAGTCATGGCATTGAACGGGTTGATCTCTGGCAACGGGGGGT
GGATACGGAGATGTTTCAGCCTCATTTGGTGTCGGCTAAAATGCGCGATCGCTTGTCCCAAG
GCCACCCCGATGCCCCCTTATTATTGTATGTGGGTCGCGTCTCCCCCGAAAAGGAAATTGAA
CGCATTAAACCCATCCTCGAAGCCATTCCAGGAGCCAGGTTAGCCATTGTAGGGGATGGAC
CCCACCGGGCCACCTTAAAACAACATTTTCAAGACACTCCCACTAATTTTGTGGGTTACTTA
CAAGGGATGGAACTGGCCTCCGCCTTTGCCTCAGCCGATGCCTTTGTCTTCCCCTCCCAAAC
CGAAACCTTAGGACTGGTGGTATTAGAAGCCATGGCCGCCGG

>W
ATTGCGTACAGCCCATAATCAGGCTATGTTAAACCTGTGTACCTCCACAGCTATGGTTGAGG
AATTACGCAATCATGGCATTGAACGGGTAGACCTATGGCAGAGGGGGGTGGATACAGAACT
GTTCCAGCCCCACAAAGCCACTAAAGAAATGCGCGCGAGCCTCAATATGGGAAACCCAGAC
GATACCCTACTGTTGTATGTGGGGAGACTAGGAGCCGAAAAAGAAATCGATCGCATTAAAC
CTATTCTAGCAGCAATACCCAACGCCCGTCTAGCCCTAGTCGGAGATGGTCCGAACCGAGA
AAACCTAGAACAACATTTCGCCGGAACTCCCACCAACTTTGTGGGTTATCTGCGGGGAGAAC
AATTAGCGGCCGCTTACGCCTGCGCTGATGCCTTCATTTTCCCTTCCCGGACAGAAACCCTA
GGTTTAGTCCTCCTC

>J
GTCGGGTTAGTAGCCTCCTACCATACCCATCTACCCCAATACCTGCACCACTACGGCTTAGG
AATGCTGGAGGAATTCCTGTGGGGGTTATTGCGTACAGCCCATAATCAGGCTATGTTAAACC
TGTGTACCTCCACAGCTATGGTTGAGGAATTACGCAATCATGGCATTGAACGGGTAGACCTA
TGGCAGAGGGGGGTGGATACAGAACTGTTCCAGCCCCACAAAGCCACTAAAGAAATGCGCG
CGAGCCTCAGTATGGGAAACCCAGACGATACCCTACTGTTGTATGTGGGGAGACTAGGAGC
CGAAAAAGAAATCGATCGCATTAAACCTATTCTAGCAGCAATACCCAACGCCCGTCTAGCC
CTAGTCGGAGATGGTCCGAACCGAGAAAACCTAGAACAACATTTCGCCGGAACTCCCACCA
ACTTTGTGGGTTATCTGCGGGGAGAACAATTAGCGGCCGCTTACGCCTGCGCTGATGCCTTC
ATTTTCCCTTCCCGGACAGAAACCCTAGGTTTAGTCCTCCTCGAGGC

Figure 5.9: The sequence for sqdX for J: Spirulina (J), P: Spirulina (P), W: Spirulina (S), S: Spirulina sp.

5.5 Biosynthesis pathway of SQDG for *Spirulina*

It is expected that in cyanobacteria, belonging to the genus *Spirulina*, the genes essential for the biosynthesis of SQDG are closely related to *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942. In order to understand the function of these identified genes from *Spirulina*, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 were used as reference sequences. This is because the organisation of the

sulfolipids genes from these species has been well-studied. The organisation of sqd genes is shown in Figure 5.10.

Figure 5.10: Comparison between the organisation of sqd genes in R. sphaeroides, Synechococcus and Synechocystis (Benning, 1998)

The sequence similarity between the isolates of *Spirulina* and the reference sequences, that is *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 was displayed in Table 5.1. The sequence obtained for the sqdB PCR of *Spirulina* sp., *Spirulina* (J), *Spirulina* (S) and *Spirulina* (P) were 75%, 85%, 83% and 81% similar to *Synechocystis* sp. PCC 6803, and had less similarity to the *Synechococcus* sp. PCC 7942 (67%, 45%, 36% and 38%, respectively). The sqdX gene from the *Spirulina* sp., *Spirulina* (J), *Spirulina* (S) and *Spirulina* (P) has strong sequence similarity to both *Synechocystis* and *Synechococcus*, with the variation of less than 6%. The first five highest similarities to the sqdB and sqdX gene from the isolates of *Spirulina* were collated in Appendix D.

Table 5.1: Sequence analysis of the sqd genes amplified from the isolates of *Spirulina*

	Synechocystis sp. PCC 6803		*Synechococcus* sp. PCC 7942	
Isolates	*sqdB*	*sqdX*	*sqdB*	*sqdX*
Spirulina sp.	75%	77%	67%	71%
Spirulina (S)	83%	75%	36%	70%
Spirulina (J)	85%	75%	45%	72%
Spirulina (P)	81%	75%	38%	70%

Reference sequences (*Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942)

5.6 Phylogenetic studies on sqdB and sqdX

Two separate phylogenetic analyses were conducted using the sqdB and sqdX homologues identified in this study, plus several reference sequences (including cyanobacterial, archaeal, plant and algal sequences) obtained from the NCBI database. The phylogenetic analyses of sulfolipids biosynthetic pathway genes and their evolutionary rate variations were studied. The results of the sqdB-based analysis are presented in Figure 5.11, and shows that the *Spirulina* (J) sequence clustered together on the same branch as *Arthrospira maxima* sequence, while *Spirulina* (P) and *Spirulina* (S) sequences belonged to another lineage with the *Arthrospira maxima* sequence. Only the *Spirulina* sp. sequence clustered tightly with *Crocosphaera watsonii* reference sequences.

The results of the sqdX-based analysis are presented in Figure 5.12. The *Spirulina* (J), *Spirulina* (P) and *Spirulina* (S) sequences clustered together on the same branch as the *Arthrospira maxima* sequence. Once again, the *Spirulina* sp. sequence clustered tightly with *Crocosphaera watsonii* reference sequence. The *Spirulina* sequences were aligned with other cyanobacterial reference sequences and found to form a different branch within the alpha-proteobacteria, plants and algae reference sequences. This branch further justified that sqdX associated with sqdB gene might export the similar compound, SQDG. In addition, both of the phylogenetic trees show that the green algae, plants and cyanobacteria are originated from the same cyanobacterial ancestor.

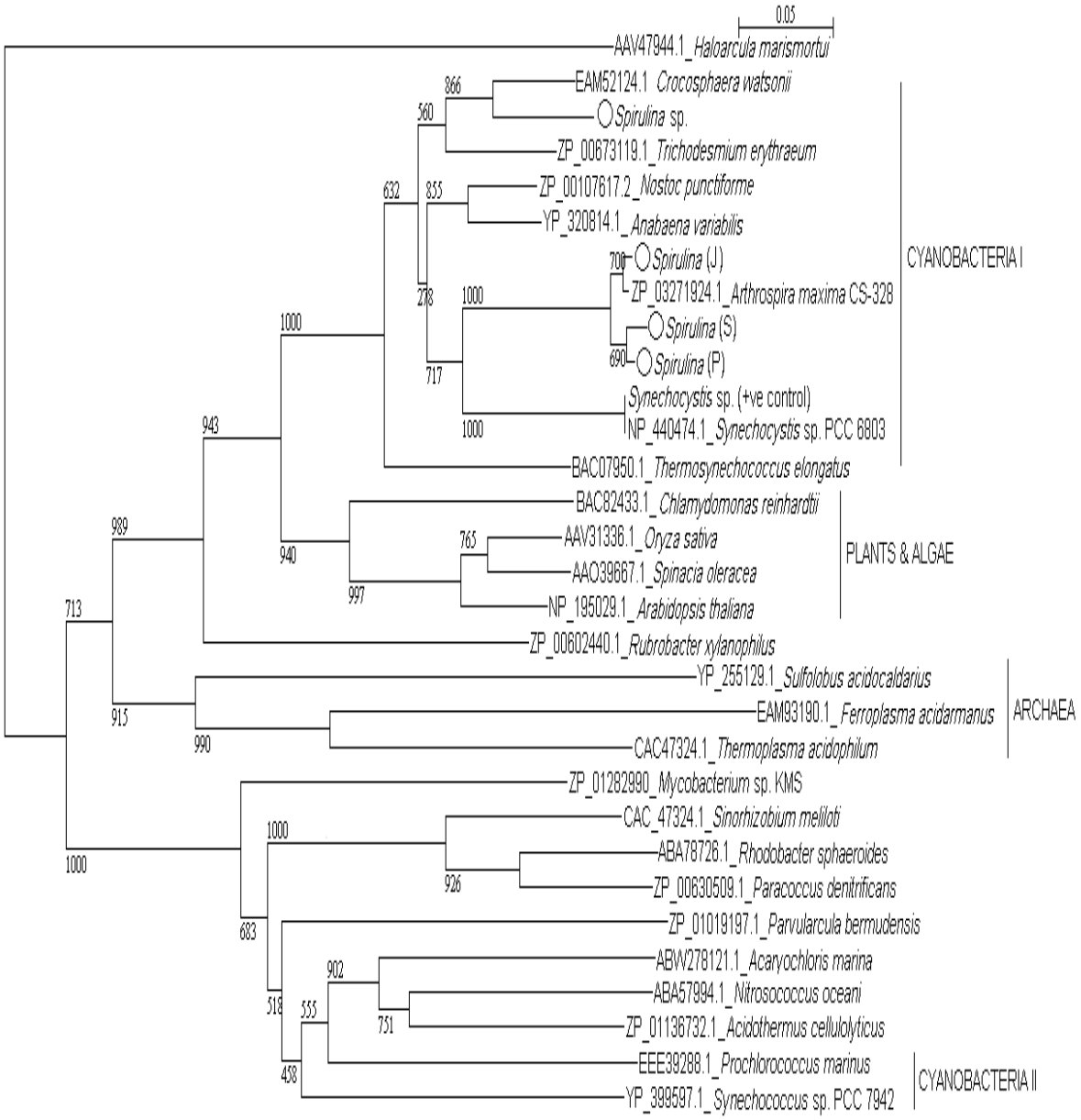


Figure 5.11: Phylogenetic tree based on sqdB homologues

Sequences determined in this study were preceded by an open circle. Bootstrap values (1000 resampling events) are shown for key branches.

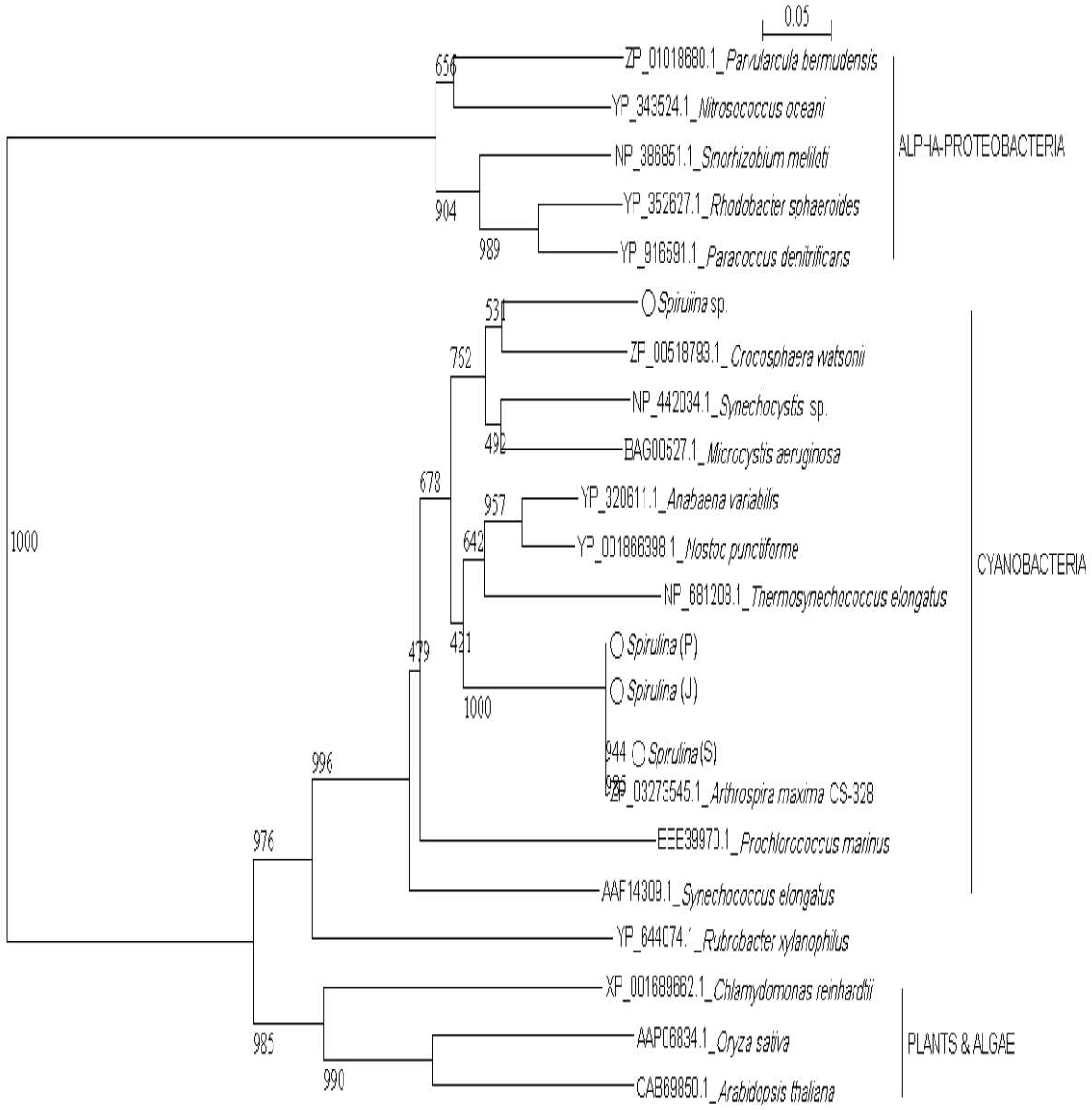


Figure 5.12: Phylogenetic tree based on sqdX homologues

Sequences determined in this study were preceded by an open circle. Bootstrap values (1000 resampling events) are shown for key branches.

5.7 Discussion

The use of cyanobacteria, such as *Spirulina*, as food supplements and drugs raise numerous health concerns, as many cyanobacteria are known to produce harmful toxins. For example, certain strains of *Aphanizomenon flos-aquae*, a popular component of health food supplements, have been shown to produce cylindrospermopsin and possess NRPS genes, putatively involved in the synthesis of the toxin (Preußel *et al*., 2006). Recent studies suggest that helically coiled cyanobacteria are more likely to possess NRPS genes than their non-spiral counterparts (Christiansen *et al*., 2001). In addition, a massive screening test on the NRPS and PKS was carried out by Ehrenreich *et al*. (2005) (discussed in Section 2.7.2) substantiated that *Spirulina* PCC 6313 shows positively in both NRPS and PKS. However, there are several exceptions to this rule such as all of the *Spirulina* isolates in this study do not shown to contain NRPS genes.

To investigate the presence of putative toxin genes in *Spirulina*, five isolates of *Spirulina* screened for NRPS and PKS genes. The results suggested that these unialgal cultures/samples do not contain NRPS/PKS genes. Although there is a smaller fragment size of amplicons found from *Spirulina* sp., this does not imply any similarity to NRPS and PKS genes. Furthermore, it is not a PCR trouble shooting issue, since the positive control yields specific product and showed a very strong expression. Therefore, the investigated *Spirulina* isolates are unlikely to produce NRP/PK toxins. However, such findings do not preclude the need for rigorous biochemical/genetic screening regimes, where bloom samples are destined for human consumption. Serious problems can arise when mixed bloom samples are used as raw materials. For example, *Aphanizomenon flos-aquae* production has contamination with microcystin which produced by *Microcystis* (Preußel *et al*., 2006). This situation called the attention of researchers to be cautious in the production of food supplement.

Different concentrations of DNA template as well as different annealing temperatures need to be employed to increase the purity and the yield of the reaction product. A successful PCR depends on the metal ion cofactors, substrate and substrate analogues, buffers and salt and co-solvents (Rochelle *et al*., 1997). Thermal cycling considerations, such as temperature, time optimisation, PCR amplification, PCR cycles, enzyme/target and hot start is essential and these variables can affect the outcome of PCR. Therefore, to achieve a successful PCR with a designed degenerate primer, the primer annealing

temperature was investigated to optimise the PCR condition for the detection of sqdB and sqdX in this case. Generally, a higher annealing temperature confers a higher specificity (Cha & Thilly, 1993). Nonetheless, the primers are not able to bind to all of the templates at 56^{o}C. In this study, an optimum annealing temperature for sqdB primers is at 54^{o}C. This shows that an optimum annealing temperature depends on the primer used.

It is important to design an appropriate primer pair in order to obtain a correct sequence. When designing the primers for PCR, the fragment size of the sequence was calculated based on the distance of the selected conserved regions as described in Appendix C. The first attempt at designing the primer pairs for sqdX genes was unsuccessful. An incorrect sequence was introduced via PCR. With the second attempt at sqdX primer design, the first fragment indicated it is an expected size. It yielded an approximately 60 bp fragment size for all *Spirulina* isolates and the positive control. It also showed that the annealing temperature at 55^{o}C coupled is the best PCR condition in this case study. Therefore, the PCR cycle condition and primer design are important in order to achieve high efficacy of PCR.

It is well-known that most photosynthetic organisms can produce sulfolipids. However, sulfolipids production is not universal among the cyanobacteria. For example, *Gleobacter violaceus* sp. PCC 7421 is unable to produce sulfolipids (Selstam & Campbell, 1996). As there is a distinct lack of information regarding the sulfolipids production in different isolates of *Spirulina*, a genetic screening study of five geographically distinct strains was undertaken. All of the isolates investigated, except *Spirulina* (M), were found to be positive for sqdB and sqdX homologues, suggesting that these isolates do in fact produce sulfolipids. A likely explanation for the lack of sqdB and sqdX amplicons obtained when using the *Spirulina* (M) template relates to the quality of the purified DNA.

The sqd genes are involved in different function and synthesis. In *Synechococcus* sp. PCC 7942 SQDG is bound specifically to the PS I complex, while in *Synechocystis* sp. PCC 6803, the compound had a deleterious effect on the photosystem II (Aoki *et al*., 2004). The sequence similarities between the sqd genes in *Synechococcus*, *Synechocystis* and five isolates of *Spirulina* was determined. The similarity analysis of sqdB and sqdX genes from *Spirulina* sp., *Spirulina* (P), *Spirulina* (J) and *Spirulina* (S)

revealed that these isolates were more closely related to *Synechocystis* sp. PCC 6803 than *Synechococcus* sp. PCC 7942.

The closer relationship between the *Spirulina* isolates and *Synechocystis* sp. PCC 6803 also agreed with the review of Wada *et al*. (2009). They reviewed the regulation of fatty acid biosynthesis in cyanobacteria and divided the cyanobacteria into four groups based on the distribution of the fatty acid composition. From their review, *Synechocystis* and *Spirulina* are in group three, where the fatty acids in MGDG and DGDG are highly unsaturated; containing mainly 18:1, 18:2 and γ18:3/16:0. Therefore, the presence and the function of sqd genes within these four isolates of *Spirulina* are more likely similar to the *Synechocystis* sp. PCC 6803.

Phylogenetic analysis of the *Spirulina* sqdB and sqdX homologues demonstrated that these putative sulfolipids biosynthesis genes are highly conserved in *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and the reference strain, *Arthrospira maxima* CS-328. The cyanobacterial reference sequences were shown to be phylogenetically separated and formed into two groups, in which the *Synechococcus* elongates reference sequence was positioned in the alpha-proteobacteria group and the *Synechocystis* sp. was compatible with the green algae and higher plants. The role of sulfolipids in both *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 was discussed in the previous section. Moreover, the similarities of the sequences between the isolates of *Spirulina* with the reference sequence (*Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803) were clarified.

The division of two phylogenetic groups for cyanobacteria corresponding to the sqdB can be explained by following the two different rates of evolutionary changes from anoxygenic to oxygenic photosynthetic prokaryotes (Sato *et al*., 2003). The information encoded in the *Synechocystis* sp. sequence exibits more genetic variation than in *Synechococcus* elongates, and therefore the *Synechocystis* sp. sequence was aligned with the higher plants and algae reference sequence. These results suggested that the *Spirulina* sequences in the branch of *Synechocystis* sp. strongly expressed the obligatory of the sqdB is necessary in the photosystem II function and the role of sqdB differs with respect to species-specific function in cyanobacteria. Interestingly, the sqdB and sqdX homologues had different phylogenetic distributions. The sqdX homologues demonstrated that this gene in these organisms partition primarily according to species

relatedness. Furthermore, the geographical origins of these isolates did not appear to influence the sequence of their putative sulfolipids biosynthesis genes.

5.8 Summary

In this chapter, studies of the molecular biology of the *Spirulina* isolates were conducted to screen the NRPS and PKS genes, design a degenerate primer for screening the gene responsible for sulfolipids production and elucidate the presence or absence of sqdB and sqdX genes.

Non-ribosomal peptide synthetase and polyketide synthetase genes are responsible for the formation of secondary metabolites, where the secondary metabolites have high potential for several biotechnological applications. In this study, the *Spirulina* isolates were examined by screening for NRPS and PKS-encoding genes. The results demonstrated that only *Spirulina* sp. is found to be positive for NRPS and PKS PCR amplification. BLAST analysis showed that the unknown fragments of *Spirulina* sp. have no significant similarity to NRPS and PKS genes in the database. Furthermore, *Spirulina* sp. gave the PCR product of 537 bp for NRPS and 419 bp for PKS; while the positive control for NRPS and PKS gave an amplicon of 700bp and 600bp, respectively. Therefore, it is presumed that these fragments are non-specific fragment amplifications. In conclusion, the investigated *Spirulina* isolates do not possess the NRPS and PKS genes.

In a review on the biosynthesis of sulfolipids, Benning *et al*. (1992) characterised the genes responsible for the biosynthesis of sulfolipids from purple bacteria. The sqdA and sqdB genes encoding the synthesis of the sulfolipids were the first operon that has been characterised. The identification of the sulfolipids genes from cyanobacteria was subsequently achieved. The sulfolipids genes from cyanobacteria are found to be relatively similar to the sqdA and sqdB gene from bacteria. In this study, degenerate primers were designed to examine the sqdB and sqdX genes involved in the final step of sulfolipids biosynthesis for cyanobacteria. The degenerate primer pairs of dsqdBF/dsqdBR successfully identified sqdB genes from *Spirulina* isolates, while dsqdXF/dsqdXR failed in this application. The elucidation of sqdX genes were successfully analysed by the identification of the primer pairs of dsqdX1F/dsqdX1R. Analysis of PCR product with specific degenerate primer pairs showed the presence of sqdB and sqdX genes in all *Spirulina* isolates except for *Spirulina* (M). The sqdB and

sqdX genes were undetectable in *Spirulina* (M) was most probably due to the quality of the purified DNA.

From the results obtained, *Spirulina* (P), *Spirulina* (J) and *Spirulina* (S) showed clearly that the DNA banding pattern of sqdB and sqdX is similar and appeared to group in the genus of *Arthrospira* even though those isolates are from different locations, while *Spirulina* sp. clustered tightly with *Crocosphaera watsonii* reference sequence in both of the sqdB- and sqdX-based phylogenetic analysis. The examination of the sequence similarity of these isolates of *Spirulina* with *Synechococcus* and *Synechocystis* indicated that the involvement of the sqd genes of all *Spirulina* isolates is highly conserved with *Synechocystis*. The results suggested that the *Spirulina* isolates are more likely to have biosynthesis pathways of sulfolipids similar to *Synechocystis*.

The phytochemical analysis is a method that forms a basis for potential extraction of bioactive compounds from the *Spirulina* isolates. The *Spirulina* isolates are screened and the sulfolipids are investigated in the following chapter. Various types of extraction methods for sulfolipid from the *Spirulina* isolates are also introduced. In addition, different extraction solvent systems are examined to determine the successfulness in extracting sulfolipids from the *Spirulina* isolates.

CHAPTER 6 EXTRACTION OF SQDG FROM *SPIRULINA* ISOLATES

6.1 Introduction

Phytochemical analysis is one of the simplest methods to detect the bioactive constituents in living organisms. This analysis is fast and the least expensive method to determine the bioactive compounds. As stated by Kumar *et al*. (2009), it is important to analyse the phytochemical constituents, because these constituents are the key to biological activities. Generally, the work on *Spirulina* extracts has been focused on the examination of their bioactivity against a wide range of microorganisms and the determination of their nutritional value. However, very little work has been done on phytochemical analysis for *Spirulina*. Owing to the wide use of *Spirulina* for the development of pharmaceuticals, the phytochemical constituents of *Spirulina* are examined in this chapter.

Due to the contribution of SQDG to the treatment of human diseases, SQDG is targeted in this study as the bioactive compound of all five isolates of *Spirulina.* As previously mentioned, SQDG is valuable because it has an inhibitory effect on HIV-reverse-transcriptase activity. Therefore, concentrating the source of SQDG from the *Spirulina* isolates is necessary. SQDG is described as a class of polar lipids and is found in the glycolipids fraction. In order to optimise the extraction of SQDG from the *Spirulina* isolates, the optimal lipid extraction is firstly determined and the extraction efficiency of SQDG is subsequently compared.

Many lipid extraction methods have been developed, the most common being the use of organic solvents. Various forms of organic solvents are utilised in this investigation, including a combination of solvent systems and a single solvent system. In this application, alcohol is viewed as an appropriate solvent in the extraction method because SQDG is more likely to be soluble in polar solvents. To investigate the factors that affect the amount of SQDG, several pre-treatment methods and different ratios of organic solvents are examined. Numerous works have proposed that the drying temperature is one of the factors affecting the extractability of lipids (Desmorieux & Decaen, 2005; Desmorieux & Hernandez, 2004). For instance, the drying temperatures

of *Chlorella vulgaris* increases, the yield decreases correspondingly. The effect of drying temperature on the Australian *Spirulina* isolate, a strain that has not been studied in this regard to date is researched. The changes in the lipid yield at different drying temperatures are observed in this study. Furthermore, there is a need to use TLC to do a preliminary screening of SQDG from different extracts of the *Spirulina* isolates. By the presence of the SQDG on the TLC plate, the availability and the proprietary of the solvent used for the extraction of SQDG is determined.

6.2 Analysis of sequential extracts

Five *Spirulina* isolates were used to determine the potential sources of phyto-constituents. The nature of the extracts and the yield of the sequential solvent extracts for all isolates of *Spirulina* were observed and are presented in Table 6.1. The yield of the sequential extracts of all isolates of *Spirulina* resulted in the same trend. The extraction using hexane yielded the least amount; while the water extraction obtained the highest amount of extracts. The amount of the extracts ranged from 8.19-77.63, 8.55-83.33, 8.61-85.73, 8.29-81.65 and 9.21-86.12 mg/g AFDW for *Spirulina* (M), *Spirulina* (S), *Spirulina* (P), *Spirulina* (J) and *Spirulina* sp., respectively.

Table 6.1: The estimation of yield of different sequential extracts from the *Spirulina* isolates

	Yield (mg/g)				
	M	W	P	J	S
H	8.19	8.55	8.61	8.29	9.21
T	16.70	28.62	15.90	17.13	16.67
A	31.33	35.09	29.19	31.13	33.34
M	54.48	64.25	45.86	49.55	46.50
Aq	77.63	83.33	85.73	81.65	86.12

M indicates *Spirulina* (M); W indicates *Spirulina* (S); P indicates *Spirulina* (P); J indicates *Spirulina* (J) and S indicates *Spirulina* sp. Subscripts H denotes hexane extracts; T denotes toluene extracts; A denotes acetone extracts; M denotes methanol extracts; Aq denotes water extracts

The appearance of each extract was similar when hexane, toluene, acetone and methanol were used for the extraction. The water extracts obtained from the *Spirulina* isolates were observed by examining the degree of greenish solid. The colour of the aqueous extract of *Spirulina* (M) was dark brownish black, while the extract of *Spirulina* sp. was light greenish in colour. The colour of the extracts from the other *Spirulina* isolates was yellowish.

Table 6.2: The characteristics of the sequential extracts of the *Spirulina* isolates

	Colour and consistency				
	M	W	P	J	S
H	Yellow-greenish, Solid	Yellow-greenish, Solid	Yellow greenish, Solid	Yellow greenish, Solid	Yellow greenish, Solid
T	Dark yellow-greenish, Solid	Greenish, Solid	Greenish, Solid	Greenish, Solid	Greenish, Solid
A	Greenish, Solid	Dark greenish, Solid	Dark greenish, Solid	Dark greenish, Solid	Dark greenish, Solid
M	Dark greenish, Solid	Dark blue greenish, Solid	Dark greenish, Solid	Dark greenish, Solid	Dark greenish, Solid
Aq	Dark brownish black, Solid	Light yellowish, Solid	Light yellowish, Solid	Light yellowish, Solid	Light greenish, Solid

M indicates *Spirulina* (M); W indicates *Spirulina* (S); P indicates *Spirulina* (P); J indicates *Spirulina* (J) and S indicates *Spirulina* sp. Subscripts H denotes hexane extracts; T denotes toluene extracts; A denotes acetone extracts; M denotes methanol extracts; Aq denotes water extracts

A preliminary screening of different sequential extracts for alkaloids, tannins, saponins, phlobatannis, cardiac glycosides, steroids, terpenoids and flavonoids are evaluated in Table 6.3. There were no phytochemical compounds revealed in *Spirulina* isolates when hexane and toluene were used for the extraction. The phytochemical screening on the aqueous and methanol extracts of *Spirulina* (M) revealed that more of the phytochemical compounds (such as tannins, cardiac glycosides, steroids, flavonoids) were obtained when compared to other isolates of *Spirulina*.

Table 6.3: Preliminary phytochemical screening of different sequential extracts

	M_Ace	M_MeOH	M_Aq	W_Ace	W_MeOH	W_Aq	P_Ace	P_MeOH	P_Aq	J_Ace	J_MeOH	J_Aq	S_Ace	S_MeOH	S_Aq
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	+	+	++	+	+	-	+	+	++	+	+	-	+	+	+
Saponins	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+
Terpenoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Observation

Tannins: Dark brown green precipitates was observed after a drop of FeCl3

Saponins: Frothing was observed

Cardiac glycosides: Dark brown ring was observed at the interphase

Steroids: Development of greenish colour was observed

Flavonoids: A light yellow solution was observed on addition of alkaline solution and turned to a colourless solution

M indicates *Spirulina* (M); W indicates *Spirulina* (S); P indicates *Spirulina* (P); J indicates *Spirulina* (J) and S indicates *Spirulina* sp. Subscripts Ace denotes acetone extracts; MeOH denotes methanol extracts; Aq denotes water extracts.

6.3 The drying condition of *Spirulina*

The tested *Spirulina* isolates in this study were dried by different types of drying method. The *Spirulina* (P) and *Spirulina* (M) are commercial spray-dried products; while the *Spirulina* (S) is an air-dried product. To ensure that the drying method did not alter the characteristics of the tested *Spirulina* isolates, the structure and the brightness of the filaments were observed.

Figure 6.1 illustrates the appearance of *Spirulina* in powder form and the microscopic view of *Spirulina*. The colour of the *Spirulina* (P) and *Spirulina* sp. (in powder form) was slightly different from the other isolates of *Spirulina*. The colour of *Spirulina* (P) and *Spirulina* sp. in the powder form appears a dark green colour. Although the colour of the *Spirulina* isolates was slightly different, no damage was observed under the microscope. The filaments of the *Spirulina* (S), *Spirulina* (M), *Spirulina* (P) and *Spirulina* (J) are smooth and appear blue-green in colour; while *Spirulina* sp. appears bright green in colour.

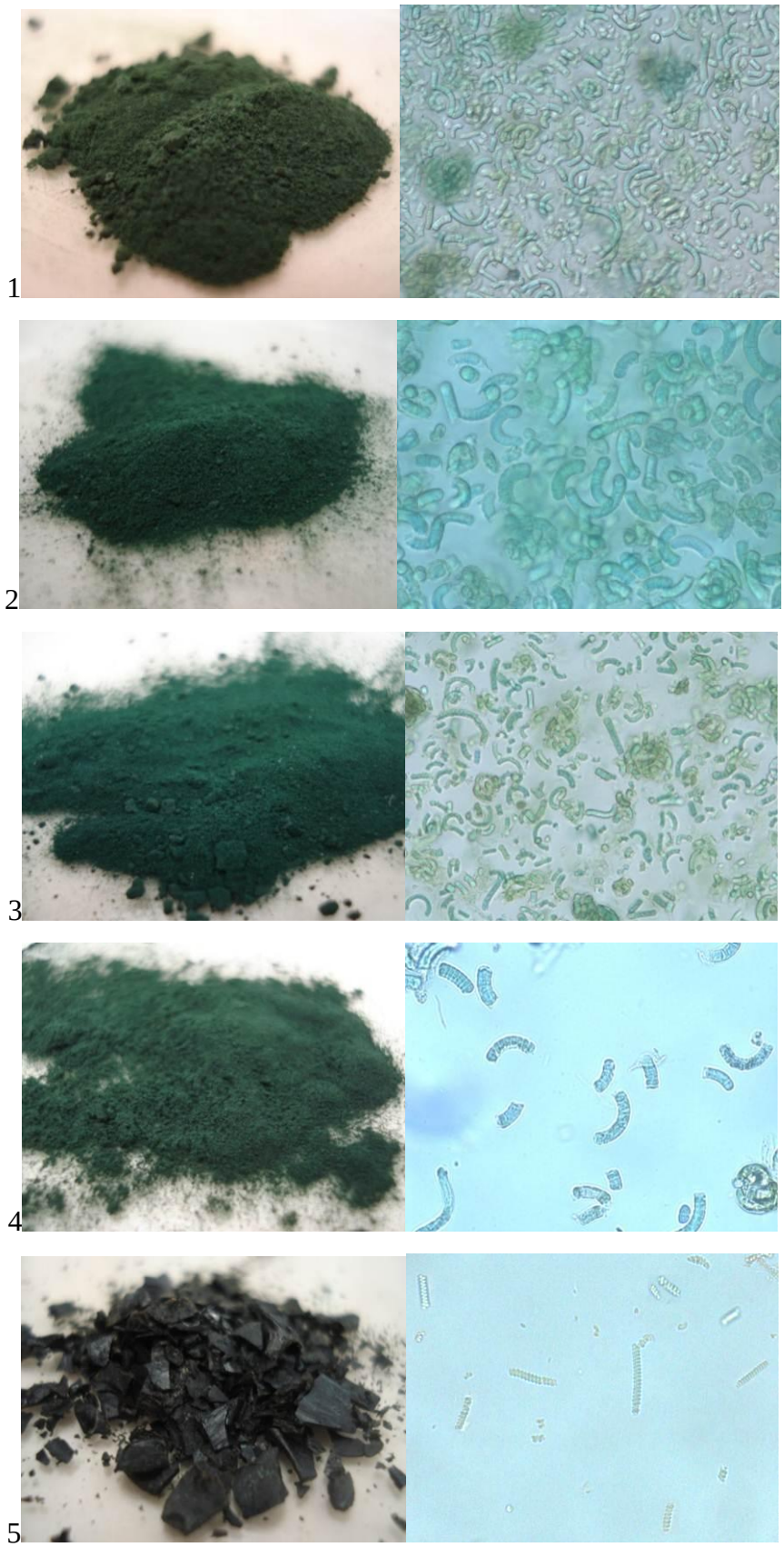


Figure 6.1: Appearance of Spirulina in powder form and view of Spirulina under a microscope

1 *Spirulina* (P); 2 *Spirulina* (S); 3 *Spirulina* (J); 4 *Spirulina* (M); 5 *Spirulina* sp. at 50^{o}C

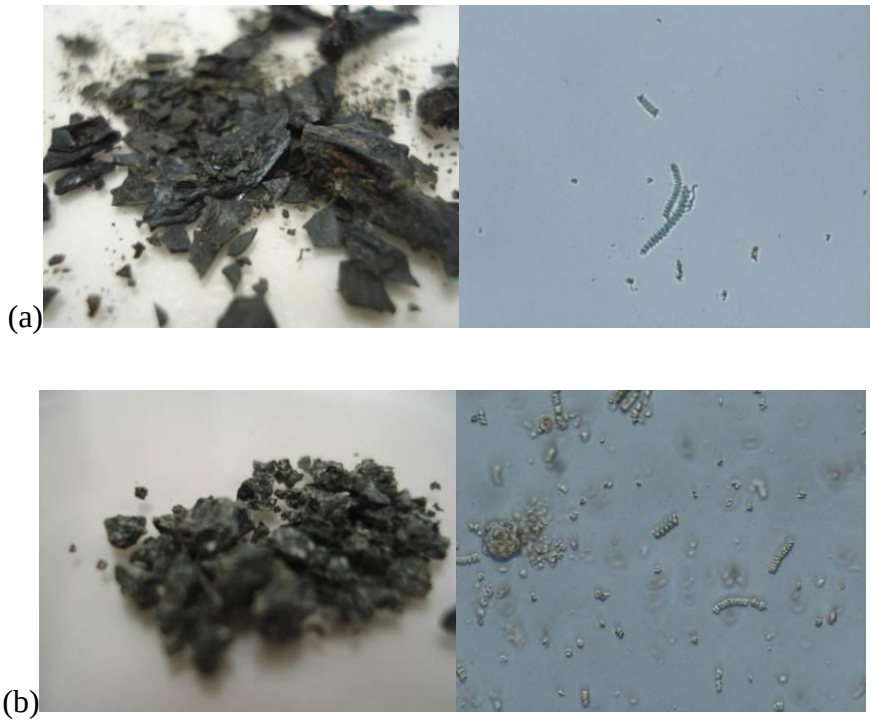


Figure 6.2: Spirulina sp. using different oven temperature for drying

(a) 60^{o}C and (b) 70^{o}C

The *Spirulina* sp. was subjected to an oven temperature between 60^{o}C and 70^{o}C and was observed under the microscope (Figure 6.2); while the effect of drying condition on the *Spirulina* (P), *Spirulina* (M), *Spirulina* (S) and *Spirulina* (J) could not be conducted. This is because these isolates of *Spirulina* are available in pelletised form and thus the form constrained the investigation.

The *Spirulina* sp. appeared in different colour when the oven temperature was at a different drying temperature. As can be seen from Figure 6.2, the filaments structure of the dried *Spirulina* sp. remained smooth except the colour of the dried *Spirulina* sp. changed. There was no damage observed in the structure of the *Spirulina* sp. filaments when the drying temperature was varied. On the other hand, approximate 5% losses of lipids were obtained from 50^{o}C to 70^{o}C for *Spirulina* sp. (as shown in Table 6.4). These results show that drying temperature is an important factor influencing the lipids content of *Spirulina* sp.

Table 6.4: Effect of drying conditions for the *Spirulina* sp.

Temperature (^{o}C)	50	60	70
Lipid yield (% AFDW)	9.57±0.98	6.47±0.60	4.63±0.43

Data expressed as mean ± standard deviation; n = 3

6.4 Lipid extraction

6.4.1 Effect of cell disruption

The lipid yield (% AFDW) obtained by using several cell disruption methods (microwave-assisted, sonication, homogenisation) with the solvent system of chloroform:methanol (2:1, v/v) is shown in Figure 6.3.

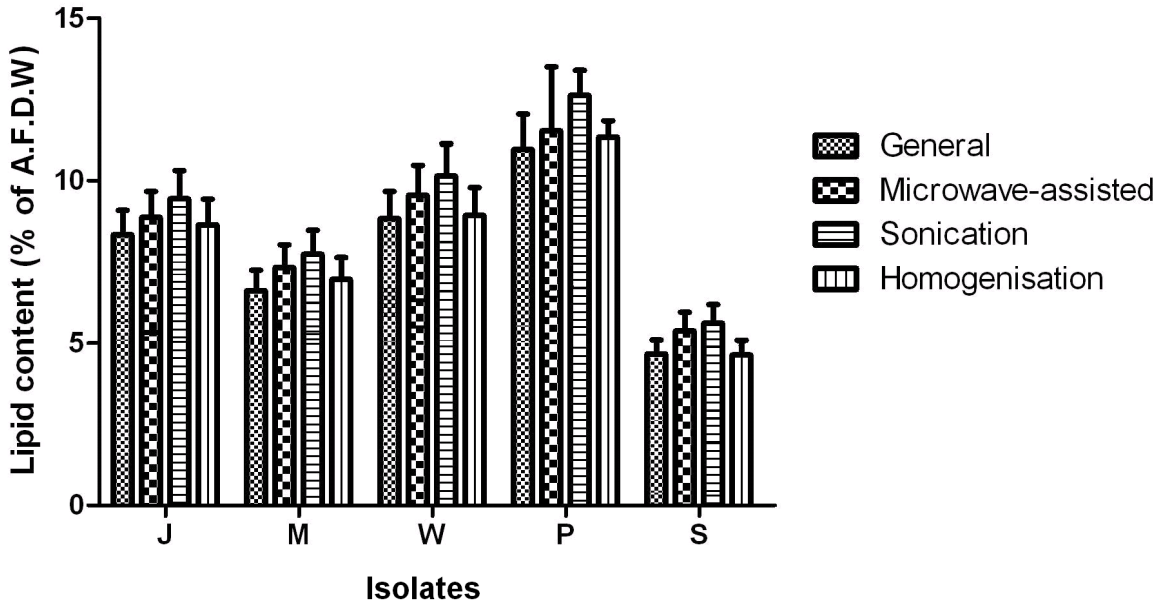


Figure 6.3: Effect of extraction systems on different isolates of Spirulina

J indicated *Spirulina* (J); M indicated *Spirulina* (M); W indicated *Spirulina* (S); P indicated *Spirulina* (P); and S indicated *Spirulina* sp. Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

The highest recovery of the lipid content of all *Spirulina* isolates was achieved through the use of sonication. The extraction coupled with the microwave-assisted and homogenisation achieved higher lipid yield when compared to the non-pre-treated extraction method. The efficiency of sonication was 1.28%, 1.05%, 1.69%, 1.24% and 1.35% higher than the non-pre-treated extraction method for the *Spirulina* (J), *Spirulina* (M), *Spirulina* (P), *Spirulina* sp. and *Spirulina* (S), respectively.

6.4.2 Effect of solvent systems

A comparison of the performance of different solvent systems was conducted to develop a high efficient lipid extraction method for the isolates of *Spirulina*. This investigation was needed because the structural resistance from different cells varies

with different isolates of *Spirulina*. Figure 6.4 shows the lipid recovered by six different solvent systems from five different isolates of *Spirulina*.

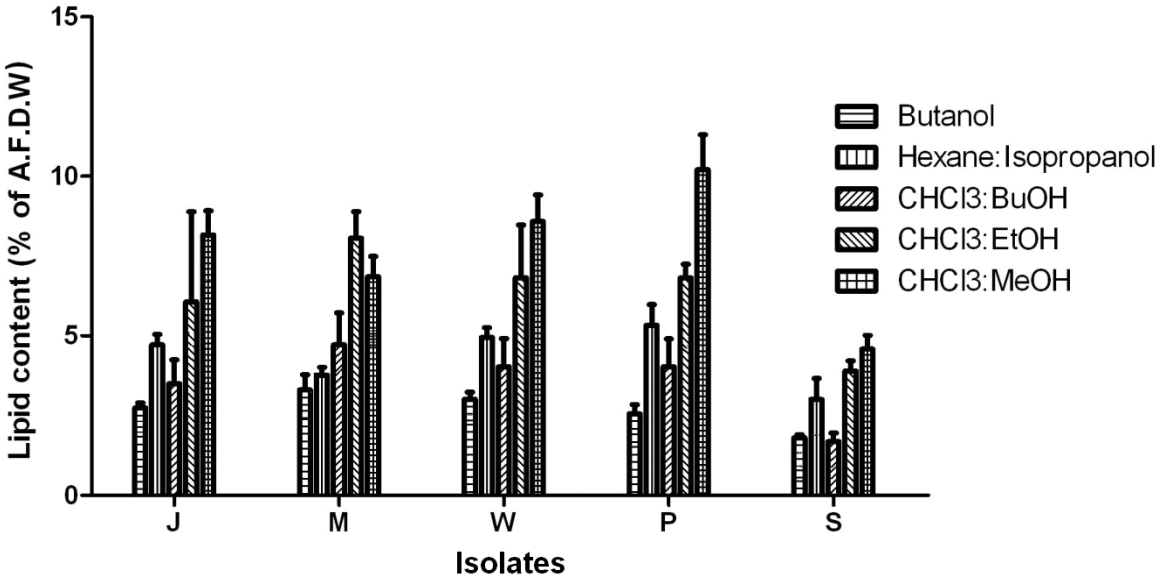


Figure 6.4: Effect of solvent systems on different isolates of Spirulina

J = *Spirulina* (J); M = *Spirulina* (M); W = *Spirulina* (S); P = *Spirulina* (P); and S = *Spirulina* sp. Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

The amounts of lipid extracted varied among the extraction solvent systems in this study. The lipids yielded the highest recovery of 11.15% AFDW when the chloroform:methanol (2:1, v/v) solvent system was used to extract *Spirulina* (P). This solvent system also provided the highest lipid recovery for the other investigated *Spirulina* isolates. The lipid for all *Spirulina* isolates recovered by using chloroform: ethanol (2:1, v/v) gave the second highest recovery of lipids, followed by hexane: isopropanol (3:2, v/v), chloroform: butanol (2:1, v/v) and butanol with the exception of *Spirulina* (M). For *Spirulina* (M), the chloroform: ethanol (2:1, v/v) is the best extraction solvent system, where it yielded 8.03 % AFDW.

Owing to the medicinal properties of gamma linolenic acid, the fatty acid composition extracted by using different forms of organic solvent in *Spirulina* isolates was studied. The fatty acid compositions of different isolates of *Spirulina* are shown in Table 6.5.

Table 6.5: Fatty acid composition of all isolates of *Spirulina* using various forms of organic solvents

	Fatty Acids (Percentage of total fatty acids)						
Isolates	16:0	16:1n9	16:1n7	18:0	18:1n9	18:2n6	18:3n6
Spirulina (J)							
1	40.98	1.19	2.83	0.82	2.85	20.86	11.28
2	31.67	0.99	2.95	1.84	2.43	20.10	11.73
3	42.20	1.25	3.33	0.72	2.50	22.42	14.10
4	37.22	1.59	3.72	0.79	1.75	19.17	17.70
5	39.44	1.30	3.41	0.67	2.18	20.97	15.50
6	44.86	1.32	3.12	0.94	2.52	22.59	13.14
7	40.66	1.43	2.90	1.75	2.93	22.08	15.50
Spirulina (S)							
1	43.47	1.57	4.17	1.50	3.70	20.99	14.49
2	27.50	1.24	3.54	2.09	3.76	19.55	14.20
3	42.92	1.57	4.12	1.21	3.86	21.64	17.52
4	37.36	1.99	4.54	1.33	2.70	19.03	21.30
5	39.30	1.63	4.47	1.13	3.36	20.76	19.20
6	45.68	1.65	4.38	1.58	3.89	22.06	15.23
7	43.28	1.75	4.66	1.84	2.56	22.23	19.27
Spirulina (P)							
1	40.17	1.54	5.64	0.94	2.04	17.78	17.90
2	30.18	1.28	4.60	3.70	2.22	16.24	15.88
3	40.07	1.56	5.64	1.31	2.05	17.56	19.26
4	35.70	1.89	6.16	1.65	1.63	12.97	23.09
5	36.53	1.60	5.73	1.20	1.77	16.23	20.23
6	44.29	1.66	5.37	1.84	1.96	15.87	16.61
7	41.12	2.09	7.04	2.91	1.98	15.92	25.12
Spirulina (M)							
1	45.77	1.11	2.72	0.95	3.21	21.18	11.49
2	25.58	0.79	1.98	2.78	3.13	18.58	8.44
3	46.60	1.05	2.62	1.23	3.45	22.33	12.08
4	39.26	1.43	2.96	1.14	2.22	20.92	16.83
5	43.03	1.11	2.65	1.18	3.02	21.80	12.99
6	48.10	1.09	2.55	1.47	3.58	22.27	11.63
7	44.74	1.57	3.19	1.93	3.23	24.29	17.06
Spirulina sp.							
1	31.91	26.53	2.71	1.36	18.99	2.59	nd
2	13.06	14.17	2.20	1.93	20.28	2.47	nd
3	31.38	24.53	3.39	1.50	20.85	2.50	nd
4	25.03	29.14	3.32	1.36	20.53	2.08	nd
5	26.98	25.47	3.59	1.39	18.13	2.49	nd
6	35.32	29.23	2.97	1.76	20.99	2.86	nd
7	32.85	31.99	3.64	2.03	19.21	2.95	nd

Extraction method was carried out using various forms of organic solvents are as below: 1: Methanol; 2: Hexane; 3: Hexane: Isopropanol (3; 2, v/v); 4: Chloroform: Butanol (2:1, v/v); 5: Chloroform: Ethanol (2:1, v/v); 6: Chloroform:methanol (2:1, v/v); and 7: Butanol; nd is expressed as not detected.

The GC analysis revealed that palmitic acid (C16:0) is the predominant fatty acid in the tested *Spirulina* extracts by using different solvent systems. Among all the extraction methods examined, the hexane extracts of the *Spirulina* isolates contained the least amount of fatty acid composition. In addition, the data showed that the neutral lipids of *Spirulina* isolates have the lowest percentage of C16:0. When chloroform: butanol (2:1, v/v) used for extraction, a higher amount of gamma linolenic acid was obtained. The fatty acid composition especially the gamma linolenic acid content in the methanol extracts is relatively high. As during the butanol extraction, *Spirulina* (P) and *Spirulina* (M) have the highest content of gamma linolenic acid. For *Spirulina* (J) and *Spirulina* (S), both of these isolates have the highest content of gamma linolenic acid when the chloroform: butanol (2:1, v/v) was used among other extraction solvent systems. No gamma linolenic acid content was found in the *Spirulina* sp. as shown in Table 6.5. There is also not much variance observed between the fatty acid composition in the hexane: isopropanol extracts and the chloroform:methanol extracts.

6.4.3 The effect of different ratio of solvent systems (Chloroform:methanol)

Theoretically, an increased amount of polar solvents in an extraction method can be attributed to a corresponding increase in polar lipids content. In order to optimise the extraction solvent system of SQDG, a variety of different ratios of chloroform:methanol (included 1:1, 1:2, 1:4, 1:8 and 1:16) was introduced. These combination solvent systems were also designed to reduce the use of toxic solvent (chloroform) by increasing the solvent ratio of methanol in this application.

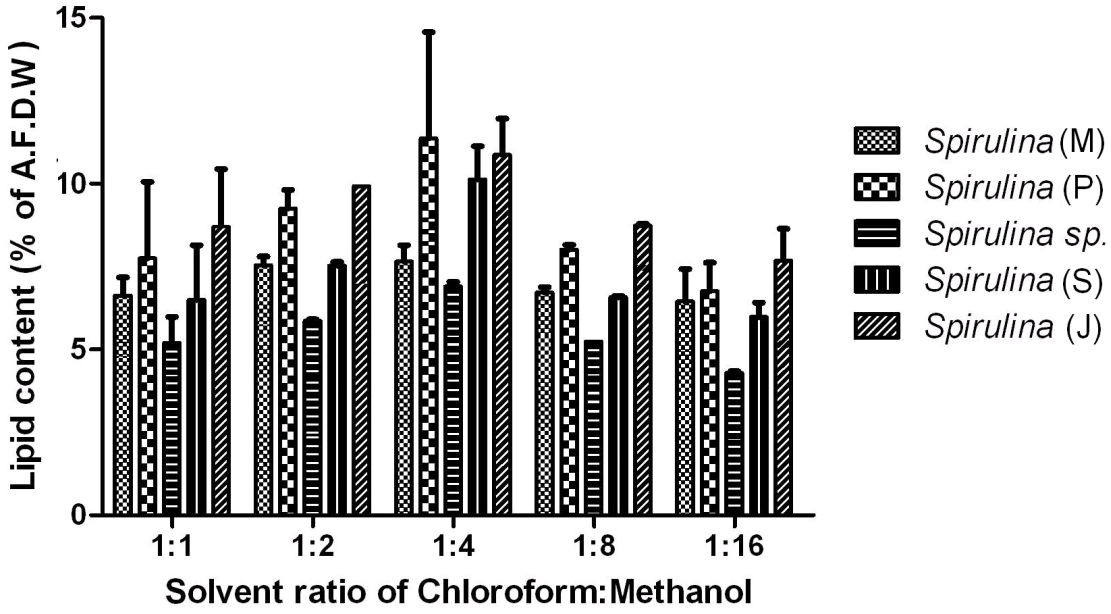


Figure 6.5: Effect of different ratio of chloroform:methanol on different isolates of Spirulina

Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

The lipids obtained from five different isolates of *Spirulina* are shown in Figure 6.5. The results showed that the chloroform:methanol in a ratio of 1:4 is prospective in extracting a high yield of SQDG. The lipid yield extracted in a ratio of 1:4 are 7.69% AFDW, 11.28% AFDW, 10.13% AFDW, 10.90% AFDW and 7.05% AFDW for *Spirulina* (M), *Spirulina* (P), *Spirulina* (J), *Spirulina* (S) and *Spirulina* sp., respectively. The data indicates that there is a small degree of divergence for the ratio of 1:1, 1:2, 1:8 and 1:16, which the difference of lipid content is less than 5% AFDW.

6.4.4 Soxhlet and supercritical carbon dioxide extraction

The lipids obtained by using soxhlet extraction coupled with hexane solvent systems was 1.4% for *Spirulina* (M). The amount of the extract by using soxhlet extraction gave a higher yield when compared to the hexane extraction, which is shown in Section 6.2.

The results obtained through the supercritical carbon dioxide extraction from the *Spirulina* (M) isolate are presented in the following section. The extraction conditions were performed at different operational parameters, including pressure, extraction time and the amount of co-solvent (ethanol). Figure 6.6 shows the yield of lipid increased when the extraction time increased.

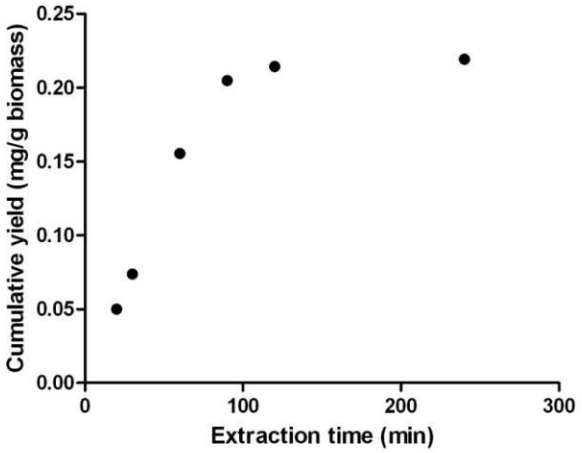


Figure 6.6: Cumulative yield of supercritical extraction of lipids from Spirulina (M) isolate

Results at 250 bar and 50^{o}C with 12 volume percentage of ethanol, as a function of carbon dioxide mass.

The lipid yield was significantly increased in the range of the extraction time at 30 minutes to 90 minutes. The cumulative yield was increasing correspondingly with the extraction time and reached a stationary value (0.22 mg/g biomass) at 120 minutes. A maximum lipid yield was achieved with the extraction time at 240 minutes for the *Spirulina* (M) isolate.

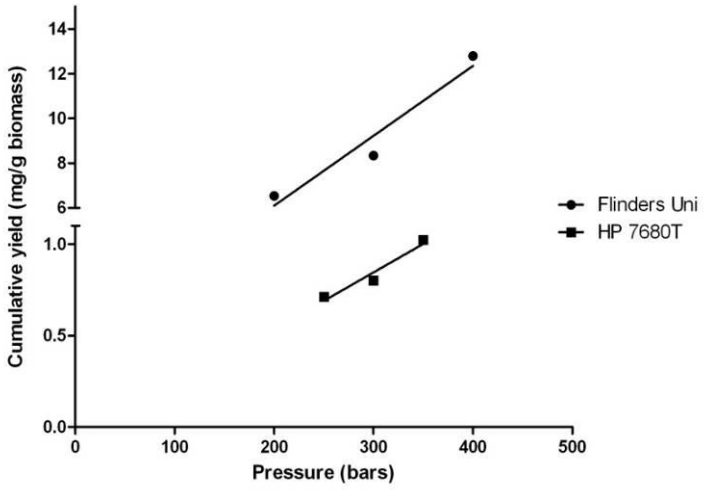


Figure 6.7: Supercritical carbon dioxide extraction conducted in two different models

Once the optimised extraction duration was achieved, *Spirulina* (M) were inserted into the supercritical carbon dioxide extraction thimble. Figure 6.7 shows the effect of pressure on the extraction yield of *Spirulina* (M) in two different models. The lipids of *Spirulina* (M) isolate was extracted at pressures of 200, 300 and 400 bars by the supercritical extractor at Flinders University and at 250, 300 and 350 bars with the HP

7680T model at the University of Adelaide. When using the HP 7680T for extraction, the lipid yield is insignificant. This is probably due to the malfunction of the machine. This problem was evident when the inlet fixture of the machine was lined with a large dark green material.

In this investigation, the amounts of lipids extracted by the supercritical carbon dioxide increased when the pressure increased at a constant temperature. The lipids yielded from *Spirulina* (M) at a pressure of 200, 300 and 400 bars with a constant temperature of 50^{o}C, was 6.56 mg/g biomass, 8.23 mg/g biomass and 13.10 mg/g biomass, respectively. The yield of lipids achieved by the HP 7680T was ten-fold less than the data obtained from the fully operational machine at Flinders University. The linear regression value for the model at Flinders University and the model 7680T is 0.9431 and 0.9424, respectively. This linear regression analysis showed that the model at Flinders University and the model of HP 7680T established reliable yield data.

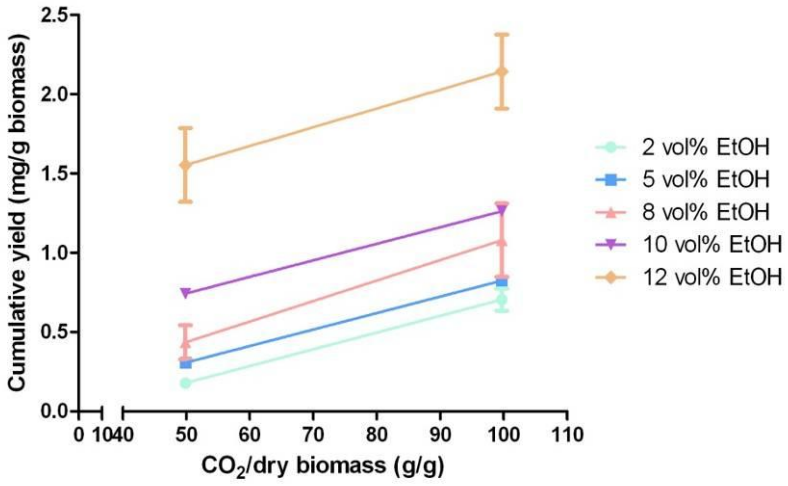


Figure 6.8: Cumulative yield of lipids from the Spirulina (M) isolate as a function of carbon dioxide mass

Results at 250 bar and 50oC, at several volume percentages of ethanol. Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

Ethanol is a polar solvent that is used to dissolve polar compounds. In order to extract polar lipids, ethanol was programmed in different volume percentage coupled with the supercritical carbon dioxide using a HP Chem station software. Figure 6.8 shows a fair presentation of the cumulative yield of lipids from the *Spirulina* (M) isolate at 250 bars and 50^{o}C with an increased volume percentage of ethanol. The yield increases with an increased amount of ethanol. Furthermore, the cumulative yield of lipids is higher when compared to the pure supercritical carbon dioxide extraction. In this investigation, the

highest cumulative yield was obtained in the supercritical carbon dioxide extraction method with an addition of 12 volume % of ethanol.

6.5 Identification of sulfolipids

It has been found that photosynthetic organisms have the potential to accumulate sulfolipids (Benning, 1998). Several strains of algae such as *Tetraselmis*, *Cytotella*, *Chlorella*, *Microcytis* and a green alga were screened for the presence of sulfolipids by TLC. Various solvent extracts from different *Spirulina* isolates also identified the presence of sulfolipids by TLC analysis. A typical result from TLC is shown in Figure 6.9.

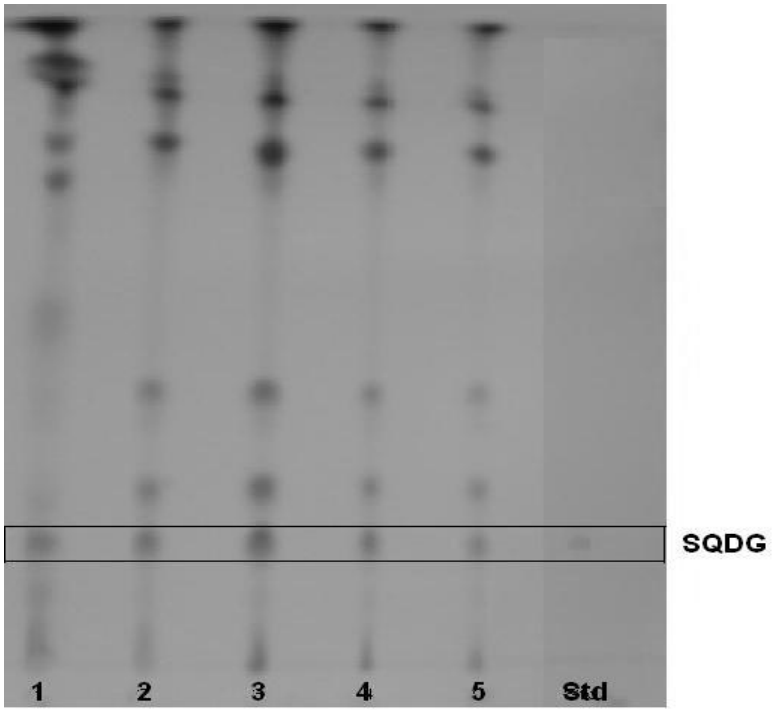


Figure 6.9: TLC for the identification of sulfolipid from different strains of algae by using the Bligh and Dyer lipid extraction method

1 = *Chlorella*; 2 = *Cytotella*, 3 = *Tetraselsmis sueica*, 4 = green algae; 5 = *Microcystis*

The retention factor was calculated and used to determine the presence of the compound of interest under the same chromatography conditions. Since it is difficult to keep the chromatography conditions constant, the reference standard was always used on the same plate to ensure the accuracy of measurement. Table 6.6 shows the determination of the SQDG from the crude extracts of all *Spirulina* isolates by using TLC for analysis, where the crude extracts were obtained by various solvent extraction methods.

Table 6.6: Sulfolipid extraction from different isolates of *Spirulina* by using various solvent systems

Organic solvents	Isolates of *Spirulina*				
	S	P	J	W	M
n-hexane	-	-	-	-	-
Butanol	+	+	+	+	+
Methanol	+	+	+	+	+
Hexane: Isopropanol (3:2)	+	+	+	+	+
Chloroform: Butanol (2:1)	+	+	+	+	+
Chloroform: Ethanol (2:1)	+	+	+	+	+
Chloroform:methanol (2:1)	+	+	+	+	+
Chloroform:methanol (1:1)	+	+	+	+	+
Chloroform:methanol (1:2)	+	+	+	+	+
Chloroform:methanol (1:4)	+	+	+	+	+
Chloroform:methanol (1:8)	+	+	+	+	+
Chloroform:methanol (1:16)	+	+	+	+	+
Supercritical CO_2					-
Standard sulfolipid = 0.22-0.29					

S: *Spirulina* sp., *P: Spirulina* (P), *J: Spirulina* (J), *W: Spirulina* (S), *M: Spirulina* (M). Standard sulfolipids were used to indicate the retention factor. (+ indicates the presence of sulfolipids; - indicates the absence of sulfolipds)

Sulfolipids are undoubtedly present in the isolates of *Spirulina* with the exception of the hexane and the supercritical CO_2 extracts. The retention factor of sulfolipids was determined and a rough estimation of the amount of SQDG was visualised by the darkness and the sizes of the spot. In this experiment, the hexane and the supercritical CO_2 extracts failed to present the sulfolipids spot on the TLC plate. Although the *Spirulina* sp. isolate does not contain gamma linolenic acid, the crude extracts showed a faint band in the sulfolipids region. This faint band demonstrates that the amount of sulfolipids is less concentrated when compared to other SQDG bands on TLC plate. Sulfolipids bands occurred in different sizes and darkness on the TLC plate which provides a rough estimate for the sulfolipids content extracted by using different solvent systems.

6.6 Discussion

The sequential extraction and analysis of five *Spirulina* isolates was performed. As can be seen in Table 6.1, the results revealed that the extraction using a higher polar solvent gave better yield, especially when water was used as an extraction solvent. Furthermore, the use of polar solvents extracted more polar compounds from the isolates of *Spirulina*. This is possibly the reason why most researchers prefer to use water and methanol extracts of algae for anti-microbial testing since the polar solvents are more capable to extract the phyto-constituents. The extracts of these five *Spirulina* isolates cannot be compared due to the fact that there are no previous studies on phytochemical analysis of *Spirulina*.

The industrial applications of the modified saponins from the source of *Quillaja* have increased significantly due to its important role as a drug for aminoglycoside antibiotics (Martín & Briones, 1999). This phyto-constituent has hypocholesterolemic, immunostimulatory, anti-inflammatory, anti-diabetic, anti-viral, anti-fungal and anti-bacterial properties. Moreover, it plays a significant role in central nervous system activities (Argal & Pathak, 2006; Lacaille-Dubois & Wagner, 1996; Milgate & Roberts, 1995; Rupasinghe *et al*., 2003; Sayyah *et al*., 2004). In the late 1980s, it was also reported that *Spirulina* has a hypocholesterolemic effect (Nakaya *et al*., 1988). The quantitative phytochemical tests revealed the presence of saponins in acetone and methanol extracts of all *Spirulina* isolates. Therefore, it is conceivable that saponins from the tested *Spirulina* isolates might exhibit various biological activities.

Cardiac glycosides have a significant effect in strengthening a failing heart. Furthermore, they can be used as a drug to treat hypertension (Adedapo *et al*., 2009). As can be seen from Table 6.3, cardiac glycosides were successfully extracted by aqueous extraction from the *Spirulina* (M) isolate. The presence of this compound indicates that the aqueous extract of *Spirulina* (M) might have the potential to lower blood pressure.

Gallotannins, ellagitannins and hamamelitannin are the best known hydrolysable tannins that have various modes of drug action such as anti-inflammatory and anti-bacterial effects (Haslam, 1996; Mackie *et al*., 1978). Wu and co-workers (2005) also showed that the water extracts of *Spirulina* (M) have anti-oxidant and anti-proliferative effects. In their study, they determined that the tannic acid (tannins) have a positive

effect on these bioactivities (Wu *et al*., 2005). In this study, the presence of tannins was only detected in the water extracts of *Spirulina* (M), *Spirulina* sp. and *Spirulina* (P), while tannins were found in the acetone and methanol extracts from all isolates of *Spirulina*. Limited tannins concentration for the isolates of *Spirulina* (S) and *Spirulina* (J) was expected because the theoretical tannin content was correlated with the colour of the precipitates. For the isolates of *Spirulina* (M), *Spirulina* sp. and *Spirulina* (P), very dark brown green precipitates were observed.

Anabolic steroids are one of the most common drugs in this class of phytochemicals, which have been used by more than 1 million people in the United States (DuRant *et al*., 1995). This class of phytochemicals are responsible for central nervous system activities (Argal & Pathak, 2006) and generally possess analgesic and anti-inflammatory effects (Lerner *et al*., 1964; Sayyah *et al*., 2004). It has also been reported that the sterols of *Spirulina maxima* are related to the anti-microbial activity (Martinez Nadal, 1971). In this study, the water extracts from all isolates of *Spirulina* possess the steroid and thus might have the potential to show anti-microbial activity.

Numerous studies have shown that flavonoids have anti-tumour, anti-HIV and anti-inflammatory effects (Al-Meshal *et al*., 1986; Wang *et al*., 1998). Flavonoids isolated from citrus fruits also exhibited anti-cancer effects both in *in vivo* and *in vitro* (Silalahi, 2002). From the results, the presence of flavonoids was only revealed from the methanol extract of *Spirulina* (M). Unfortunately, the *Spirulina* (S), *Spirulina* (P), *Spirulina* sp. and *Spirulina* (J) do not possess this phyto-constituent in their sequential extracts.

The results obtained in the preliminary phytochemical analysis revealed the presence of various chemicals components. Generally, the specific phytochemical groups are analysed by NMR or GC-MS. However, the main objective in this study was the potential use of SQDG. In the following section, several combinations of solvent systems used to optimise the SQDG production from *Spirulina* isolates are detailed. The parameters investigated to determine a suitable extraction technique for *Spirulina* are the drying conditions, the extraction method, and the solvent system.

Desmorieux and Hernandez (2007) reported that the drying conditions significantly affect the protein concentration, sugar content, the structure and the brightness of the filaments. It has been reported that the lipid content of *Chlorella vulgaris* decreases

approximately 3% when the drying temperature is from 60^{o}C to 80^{o}C (Widjaja *et al*., 2009). However, the effect of drying temperature on the lipid content of *Spirulina* remains unknown. In this study, the results revealed that the drying condition has a significant effect on the lipid content of *Spirulina* sp. When the drying temperature ranged from 50^{o}C to 70^{o}C, the lipid content of *Spirulina* sp. decreases 4.94% AFDW.

In general, the cell disruption method is used to release the biological molecules from a cell. Astaxanthin from *Haematococcus pluvialis* is an example showing that the cell disruption method is essential because the wall around the sporopollenin cell is thick. Previous study showed that a better recovery without any degradation of carotenoid was achieved with the use of a cell disruption method, where the total carotenoid content resulted in a 15.5 mg/g dry weight higher than the non-pre-treated method (Mendes-Pinto *et al*., 2001). As a result, it showed that the difficulty of extraction is not only based on the sample shape, size, and cell wall structure, understanding of the characteristics of the interested biological molecules is necessary.

The breakage of *Spirulina* is not as complex as *Haematococcus pluvialis*. As noted by Kataoka and Misake (1983), the cell wall components such as protein, carbohydrates and lipids of the *Spirulina maxima* strain readily disrupt. Due to this reason, it has been suggested that cell disruption is not necessary for *Spirulina*. However, the cell disruption methods used improved the lipid extraction efficiency of all investigated *Spirulina* isolates. The results of this study show that sonication is the most effective disruption method for all *Spirulina* isolates. Lee *et al*. (1998) and Shen *et al*. (2009) also compared various cell disruption methods such as sonication, homogenisation, French press, bead beating, microwave-assisted, autoclaving, osmotic shock and lyophilisation. In the application of *Botryococcus braunii* and *Chlorella protothecoides*, the most effective disruption was the bead beater method, while the best for Scenedesmus dimorphus was wet milling (Lee *et al*., 1998c; Shen *et al*., 2009). In summary, no standardised methods can be applied for all microorganisms. The ideal cell disruption method depends on the cell characteristics of the algal species. To verify the effect of the cell disruption method on *Spirulina*, more sophisticated applications such as wet milling, French press and bead beaters could be quantified.

All isolates of *Spirulina* achieved the highest lipid yield when chloroform:methanol (2:1, v/v) solvent system was used. This solvent system is highly recommended for the reason that it combines polar and non-polar solvents, where these solvents have been

used to extract the polar and non-polar compounds. The use of the chloroform:methanol (2:1 v/v) also showed to have relatively small losses (3% losses) of lipids when compared with the other solvent systems (Lee *et al*., 1998c). Therefore, this extraction solvent system is best to extract the investigated *Spirulina* isolates.

The extractability of the solvent systems depends on their ability to increase cohesion (Chapman *et al*., 1991). Based on the analysis done by Chapman and co-workers (1991), chloroform:methanol has the largest ability to increase cohesion followed by chloroform: ethanol, chloroform: butanol, chloroform, butanol, isoamyl alcohol, methanol and ethanol. This finding on the extractability of the solvent systems corroborated the results obtained in this study, where the highest lipid yield is the use of chloroform:methanol solvent system, followed by chloroform: ethanol, chloroform: butanol and butanol.

The optimisation of lipid extraction needs to take into account factors such as extractability, fidelity, compatibility, precision, simplicity and safety. To decrease the consumption of toxic solvents in the lipid extractions, Hara and Radin (1978) introduced the hexane: isopropanol (3:2, v/v) solvent system. This solvent system achieved the highest lipid from animal tissue (Hara & Radin, 1978). On the other hand, Lee *et al*. (1998) noted that this extraction method is less effectively to extract the lipid from *B. braunii* (Lee *et al*., 1998c). The lipids extracted by this solvent system were slightly lower when compared with the solvent system of chloroform:methanol (2:1, v/v) for all investigated *Spirulina* isolates. This has proven that the animal tissue has a different structural resistance when compared to algae.

When using a single solvent as an extraction, the selective components of lipid can be extracted due to the polarity of the organic solvents. For butanol extraction, the recovery showed lower lipid yield when compared to other solvent systems. This phenomenon is expected because as illustrated by Chapman *et al*. (1990) the extractability of butanol is lower than a combination of polar and non-polar extraction solvent systems.

As noted by Chuaprasert *et al*. (1997), the percentage of C18 fatty acid had substantial variance, while the composition of C16:0 was fairly consistent (44%) for *Spirulina* when different solvent systems were used for the extraction (Chuaprasert *et al*., 1997). The variance of C18 fatty acid from different extracts is due to an increased content of

polar lipid extracted in *Spirulina* strains (Chuaprasert *et al*., 1997). In this study, the GC analysis revealed that the investigated *Spirulina* isolates presented a consistent percentage of C16:0 when different extraction solvent systems were used. The percentage of C18 fatty acid in all *Spirulina* isolates varied is due to an increased in solvating power of the extraction solvent systems.

The GC analysis also showed that the hexane extraction is likely do not suitable in this application. The reason for that is the hexane extraction is generally used to extract low polarity compound and the amount of fatty acid content in the hexane extracts is the lowest among other extracts. The amount of fatty acid content in the extracts by using different extraction methods depends on the solvating power and the characteristics of *Spirulina*. Consequently, these results demonstrated that the different extraction methods influence the fatty acid composition of *Spirulina*. Since the effects of solvent systems are not consistent with the fatty acid composition of different isolates of *Spirulina*, no correlation was established between the solvent systems and fatty acid compositions.

Due to the fact that the extraction solvent system affects the changes in fatty acid composition, the SQDG concentrations of the investigated *Spirulina* will be varied. Apart from studying the correlation between the gamma linolenic acid with the extraction methods, the fatty acid distribution also used as a preliminary indicator for monitoring the changes of SQDG. In this study, GC analysis shows that *Spirulina* (S) possesses the highest C16 and C18:2 fatty acid composition among other *Spirulina* isolates. Therefore, it is assumed that *Spirulina* (S) may have a high SQDG content. The quantification of SQDG content is analysed by HPLC and described in chapter seven. To fully understand the correlation between the SQDG and the fatty acid composition, the isolated SQDG and fatty acid profile of *Spirulina* should be analysed by GC. However, this is beyond the scope of this research.

A single step extraction method struggles to completely extract the polar lipid concentration from the *Spirulina* isolates. The chloroform:methanol solvent system have been used and proven that the solvent systems of chloroform:methanol in a ratio of 2:1 gives the best lipid yield (Bligh & Dyer, 1959). Furthermore, Bligh and Dyer (1959) also constructed ternary phase diagrams to interpret an ideal extraction conditions for the use of chloroform:methanol. This solvent combination also achieved the best

recovery of lipids on green algae (Lee *et al*., 1998c); fungal (Somashekar *et al*., 2001) and fish tissues such as mackerel (Lee *et al*., 1996) in a ratio of 2:1. However, none of the studies researched the *Spirulina* strain in particular.

To date, the best ratio of the chloroform:methanol solvent system for *Spirulina* has not been definitively revealed. Results from this study indicated that the chloroform:methanol solvent system in the ratio of 1:4 achieved the highest lipid yield. However, it was difficult to form a two-phase system; this including the solvent system in the ratio of 1:8 and 1:16. Although these solvent systems can significantly extract the natural product from the *Spirulina* isolates; the phase separation is time consuming.

With an increased amount of methanol in this combination solvent system, it achieved a high value of lipid yield. The fatty acid compositions of lipids extracted by using different ratios of chloroform:methanol was determined and is presented in Appendix E. The lipid yield was high from all *Spirulina* isolates but the fatty acid compositions were low. This is maybe because polar solvents have broken the hydrogen bonds and the electrostatic forces in the membrane cell to release more pigments than non-polar solvents. Therefore, the pigments yield has added a significant amount to the total extraction yield. Owing to the low fatty acid content of these extracts, it is not recommended to utilise these ratios in chloroform:methanol solvent systems as the selective extraction method of sulfolipids from the *Spirulina* isolates.

In order to improve the lipid yield of *Spirulina* (M), soxhlet extraction method with hexane was introduced. The extraction yield obtained from soxhlet extractions was higher when compared to the hexane extract proposed in the previous section. There is no variance between the fatty acid compositions of the soxhlet extract and the hexane extract. This can be attributed to the fact that the soxhlet method does not change the fatty acid composition of *Spirulina* (M). On the other hand, it increases the lipid yield of *Spirulina* (M). As a result, the lipids and the SQDG content is expected to be higher if the soxhlet extraction coupled with other solvent systems used for extraction.

Many researchers have proven that supercritical carbon dioxide is used to obtain a better recovery of lipids from microorganisms (Sahena *et al*., 2009). However, an optimum extraction condition to obtain pure lipids from natural resources by supercritical fluid extraction is difficult to model because it is a complex process (Byung Soo, personal communication). One of the process parameters for supercritical

carbon dioxide extraction is the duration of extraction. In this study, the results showed that the quantity of carbon dioxide has completely extracted the lipids at 240 minutes. Mendes *et al*. (2004) also summarised the same result, where they conducted the supercritical fluid extraction for the production of gamma linolenic acid at the extraction time of 240 minutes. These results are consistent with the previous study, showing that the extraction time influences the extraction performance.

As reported by Mendes *et al*. (2004), the highest lipid yield was 0.62% when *Arthrospira maxima* were extracted by pure supercritical carbon dioxide without co-solvent at a pressure of 300 bars and temperature of 60^{o}C. A similar result of the lipid yield was observed in this investigation when *Spirulina* (M) were extracted at a pressure of 400 bars and temperature of 50^{o}C, where its highest lipid yield was 13.10 mg/g biomass. This low lipid yield clarified that this extraction method is not applicable in this study.

With an addition of organic modifier, the solvating ability of the supercritical carbon dioxide extraction is improved. Boselli and Caboni (2000) showed that there were a significant level of phospholipids in the crude extracted by neat supercritical carbon dioxide on a microscale, where the phospholipids composition in solvent extract (29% of phospholipids) has small variance when compared to 26% of phospholipids in the supercritical carbon dioxide extract (Boselli & Caboni, 2000). In this investigation, the highest lipid yield obtained was approximately 0.22% for supercritical carbon dioxide extraction coupled with 12 volume % of ethanol, while the solvent extract contained 6.18% AFDW lipid yield from *Spirulina* (M). The lipid yield has shown a large variation between the solvent and supercritical extraction. It appears that there is an incomplete lipid removal from *Spirulina* (M) when the supercritical carbon dioxde extraction method is used. Although previous studies suggested the addition of organic modifier will increase the lipid yield, the efficiency of extraction method depends on the chemical nature of microorganism (Zhang & Furusaki, 1999) Therefore, it does not suggest the use of supercritical extraction for the selective extraction of sulfolipids.

A comparison between the lipids extraction of *Spirulina* (M) using various forms of organic solvents and the supercritical fluid extraction was made. The lipid yield obtained by using the supercritical carbon dioxide extraction was extremely low when compared to the solvent extracts. Both hexane and supercritical carbon dioxide extracts,

which comprised of non-polar lipids indicated that a relatively low amount of non-polar compound was contained in *Spirulina* (M). Due to the low amount of lipid yield, supercritical carbon dioxide extraction was not recommended for the extraction of sulfolipids from *Spirulina* (M). Although the advantage of using supercritical fluid extraction is that it can alter the operating conditions to extract selectively, this extraction technique does not generate the sulfolipids from *Spirulina* (M).

TLC offers considerable precision in lipid analysis. The analysis of lipid classes by TLC showed that all of the extracts present the sulfolipids spot excluding the hexane and supercritical carbon dioxide extracts. This is because the hexane and the supercritical carbon dioxide usually extract low molecular weight and non-polar compounds. Conversely, sulfolipids are polar compounds with high molecular weight. This proves that the hexane and the supercritical CO_2 are not suitable in this application. Hence, sulfolipids bands did not occur in any of the *Spirulina* isolates when hexane and supercritical CO_2 were used for extraction. The TLC coupled with the fatty acids analysis strongly indicated that the content of SQDG varies when different solvent systems were used for extraction. The results showed that the preferred extraction method for SQDG over lipids is by the use of organic solvents.

6.7 Summary

Phytochemical analysis conducted in this study is to provide an insight into the various potential novel bioactive components among the *Spirulina* isolates from different locations. From the phytochemical analysis, the yields from using different sequential extraction method from different *Spirulina* isolates were consistent. Among the various forms of organic solvents employed in the sequential extraction method, the water extraction had the highest yield from all *Spirulina* isolates. Alkaloids, cardiac glycosides, saponins and tannins were presented in the crude extracts of the isolates of *Spirulina* in this study. The crude extracts from *Spirulina* isolates by using polar solvent for the extraction demonstrated a higher number of phyto-constituents in comparison with the non-polar solvent extraction. The phyto-constituents presented in *Spirulina* including tannins, saponins, cardiac glycosides, steroids and flavonoids are therapeutically useful compounds.

Drying temperature is the temperature used to remove the moisture of fresh *Spirulina* from a water surface to the air. In this study, the effect of drying temperature on the

lipid yield of *Spirulina* sp. was conducted. *Spirulina* (S), *Spirulina* (P), *Spirulina* (M) and *Spirulina* (J) are in pelletised forms for commercial food supplements and thus these isolates are neglected in this study. Omitting the above mentioned isolates of *Spirulina*, the effect of drying temperature on lipid yield of *Spirulina* sp. was investigated. The lipid yield of *Spirulina* sp. was highest at a drying temperature of 50^{o}C, It shows a significant decrease of the lipid yield when the drying temperature is increased, whereas drying temperature at 70^{o}C has the lowest lipid yield.

Different factors were taken into consideration to optimise the lipid extraction of *Spirulina*. Several extraction methods were used in order to obtain the highest lipid yield. From the results obtained, sonication achieved the highest lipid yield for all *Spirulina* isolates. When comparing the solvent systems used for lipids extraction, chloroform:methanol (2:1, v/v) introduced by Bligh and Dyer (1959) has the highest lipid recovery. This again suggested that different solvent extraction systems are the key to affect the lipids content in *Spirulina.*

Each lipid class is composed of a certain arrangement of fatty acids. Different fatty acid compositions might induce a change in the lipid class distribution. A preliminary screening for the extraction methods related to the yield of SQDG was analysed by GC. The results show that the fatty acid profile is different when different solvent systems were used for all *Spirulina* isolates. In order to optimise SQDG and gamma linolenic acid extraction, the fatty acid compositions are especially important.

The supercritical extraction method was conducted for *Spirulina* (M) in particular. The lipids yielded by the supercritical extraction method were 1.3%, while the lipids yielded by the use of chloroform:methanol (2:1, v/v) as extraction solvent system were 6.18%. The extraction yield by the use of organic modifier (ethanol) coupled with the supercritical extraction method was only 0.22%. Although supercritical carbon dioxide extraction with ethanol was suggested to optimise the extraction methods, this extraction method yielded a low amount of lipids from *Spirulina* (M). Therefore, supercritical carbon dioxide extraction is only suitable to extract low polarity compounds but not recommended to extract SQDG (a polar compound) in this study

CHAPTER 7 QUANTIFICATION OF SQDG

7.1 Introduction

There are several analytical techniques that can be used to identify, quantify and separate the bioactive constituents from plants and algae. These techniques are designed in different applications to meet a wide range of user needs. The most common analytical techniques used are HPLC, nuclear magnetic resonance (NMR), GC and other applications. These techniques have been researched extensively and methods used to analyse certain natural products have also been widely adopted. For instance, GC methods used to analyse the fatty acid composition are available universally.

Each substance has its own characteristic. The substance can be easily identified if a correct analytical technique is applied. SQDG, a compound which has been reported to possess several bioactivities, is of great interest in this study. In the previous chapter, various forms of organic solvents were used in order to optimise the recovery of SQDG. For quantitative work, HPLC can be used for lipid analysis coupled with an appropriate detector. Recently, this analytical technique has been used to quantify the neutral and glycolipids from plants successfully (Christie & Anne Urwin, 1995; Homan & Anderson, 1998; Yunoki *et al*., 2009). This chapter describes a suitable method for the quantitative analysis of SQDG, which was identified after a number of analytical techniques were applied. Two different types of detectors, the ultraviolet-visible (UV/VIS) detector and the ELSD are reported. In addition, comparative studies on these both detectors are detailed in this chapter.

The pure compound of SQDG from the isolate of *Spirulina* (M) was purified by using the principles of column chromatography. Prior to this research, TLC was used to identify the polarity of the compounds and to select an appropriate mobile phase used in column chromatography. This technique is very difficult to develop because multiple parameters such as the amounts of stationary phase and mobile phase, sizes of the column (diameter and length) and types of the mobile phase may affect the performance of the column. A better procedure for modifying *Spirulina* from a procedure from previous studies was necessary and more attempts were required for SQDG purification from *Spirulina* (Xue *et al*., 2002). Subsequently, a pure SQDG was isolated when the

TLC indicated a single spot. In order to study the mode of action of sulfolipids reacting with strains of bacteria, this pure compound was used to assess the bioactivity, where the bioactivity testing was conducted by using the agar disc diffusion method. The bioactivity of the sequential extracts was also investigated during the study. An assessment of SQDG for anti-HIV activity was conducted at the IMVS, Adelaide, in South Australia by Dr Tuck Weng Kok.

7.2 Quantification of SQDG

The sulfolipids concentration can be determined when sulfoquinovose is released from sulfolipids. Sulfoquinovose is no longer bonded to sulfolipids when hydrolysis is carried out with 1 mL of 2N sulphuric acid (Roughan & Batt, 1968). Sulfoquinovose is a monosaccharide sugar that is found as a building block in the sulfolipids. This means that a certain amount of sulfolipids contains a specific amount of sulfoquinovose. A calibration curve was constructed from various concentrations of sulfoquinovose, where the sulfoquinovose was released from various concentrations of sulfolipids. The calibration curve was constructed in a timely manner as required because the anthrone procedure is a very sensitive method. This procedure depends on the boiling time, hydrolysis time, anthrone standing time and so on.

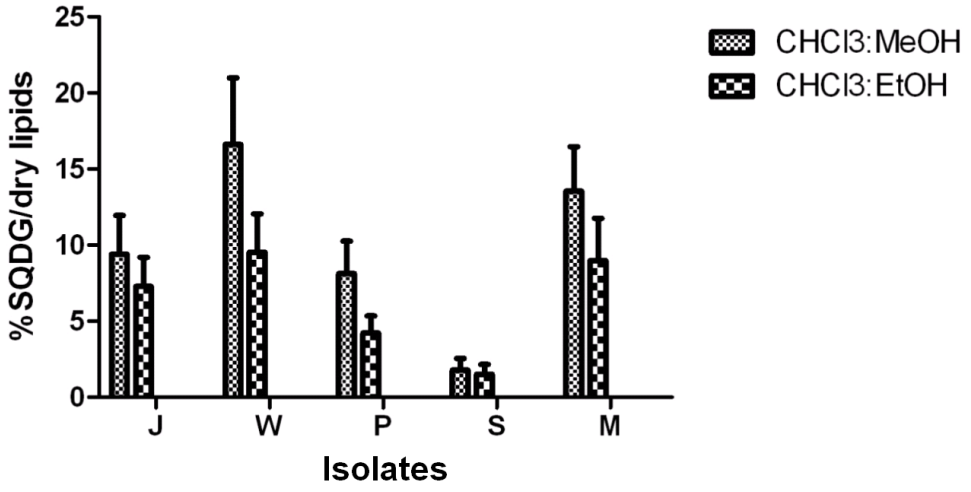


Figure 7.1: Sulfolipids recovered based on different solvent systems used

The percentage of sulfolipids recovered is based on the total lipids. J indicated *Spirulina* (J); W indicated *Spirulina* (S); P indicated *Spirulina* (P); S indicated *Spirulina* sp. and M indicated *Spirulina* (M). Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

The results presented in Figure 7.1 are an average percentage of sulfolipids per total lipids from two different extraction methods. Some of the SQDG contents extracted by using other extraction solvent systems from the isolates of *Spirulina* were omitted since the results were not consistent and difficult to interpret. Thus, the results achieved in this quantitative analysis are described as a preliminary assessment. Figure 7.1 shows that the extraction of %SQDG/total lipids using the chloroform:methanol (2:1, v/v) solvent system obtains a higher production of sulfolipids from all *Spirulina* isolates; followed by the use of chloroform: ethanol (2:1, v/v) as extraction solvent system.

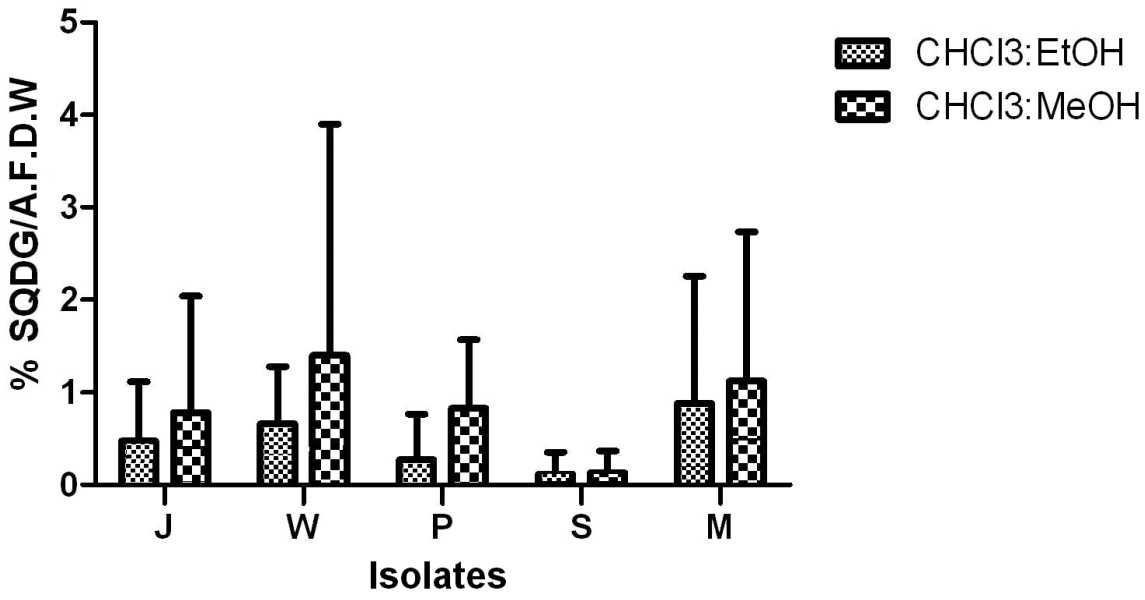


Figure 7.2: Sulfolipids recovered based on different solvent systems used

The percentage of sulfolipids recovered is based on the dry biomass. J indicated *Spirulina* (J); W indicated *Spirulina* (S); P indicated *Spirulina* (P); S indicated *Spirulina* sp. and M indicated *Spirulina* (M). Each data point represents the average of three replicates and the standard deviations of the data set are indicated as error bars.

The SQDG content of the five *Spirulina* isolates were compared by evaluating the amount of SQDG per ash free dry biomass. From the results above, it is clearly shown that the *Spirulina* (S) has the highest content of sulfolipids (1.36% AFDW) when compared to other *Spirulina* isolates. The *Spirulina* (M), *Spirulina* (P), *Spirulina* (J) and *Spirulina* sp. was in the order from the lowest to the highest of SQDG content, where the sulfolipids content are 1.13% AFDW, 0.80% AFDW, 0.79% AFDW, 0.10% AFDW, respectively.

7.2.1 HPLC-UV detector

The crude extracts obtained from the investigated *Spirulina* isolates were initially detected by TLC analysis and further quantification by using a reverse phase HPLC on a C18 column. The first attempt to develop an effective HPLC method was conducted at National Collaborative Research Infrastructure Strategy (NCRIS). The chromatograph, which illustrated in Figure 7.3 shows the elution of blank solvent, reference standard and crude extracts of *Spirulina* (M). An UV/VIS detector was operated at a wavelength of 208 nm to detect the peaks. As illustrated in Figure 7.3, there are a lot of peaks integrated on a noisy baseline and thus indicated that the failure of the UV/VIS detector.

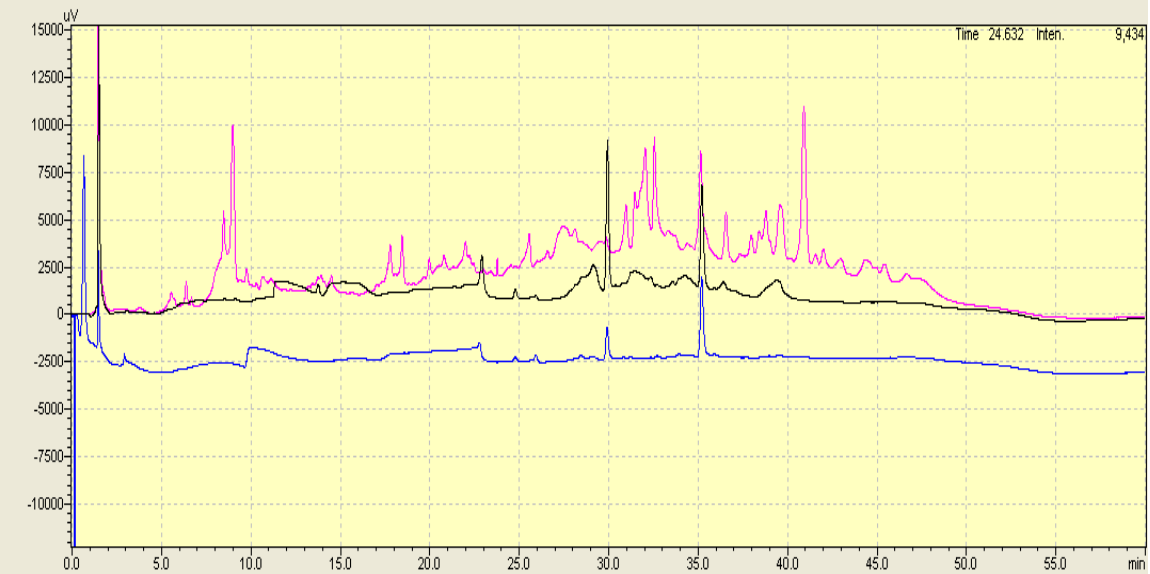


Figure 7.3: HPLC Chromatogram using UV/VIS detector at wavelength 208nm

The pink line indicates a chromatogram of the crude extract of *Spirulina* (M); the black line indicates a chromatogram of the reference standard of SQDG and blue line indicates a chromatogram of blank solvent.

7.2.2 HPLC-ELSD detector

During this study, the HPLC coupled with ELSD were employed to determine the amount of SQDG. The HPLC/ELSD method used in this application was based on a recent investigation by Yunoki *et al*. (2009). This analysis was conducted by the School of Land, Crop and Food Sciences at the University of Queensland.

From the chromatogram as shown in Figure 7.4, it was observed that the resolution of each single lipid class is not optimal. In general, an effective HPLC method was

developed based on the compound of interest as there will be no absolute end to achieve a perfect analytical system. Therefore, an optimised elution scheme of SQDG is recognisable wherein a shape peak of this particular compound was observed in the chromatogram with a stable baseline. With this improved and stable baseline, the concentration of an analyte was determined. The calibration standard ranging from 0.1 to 0.75 mg/mL was used and a graph of a squared concentration of the reference standard against the peak size area as measured in the ELSD was plotted via regression analysis. The coefficient of R^2 (0.999) from the graph (Appendix F) indicates that the linear regression is a reasonable representation of the data.

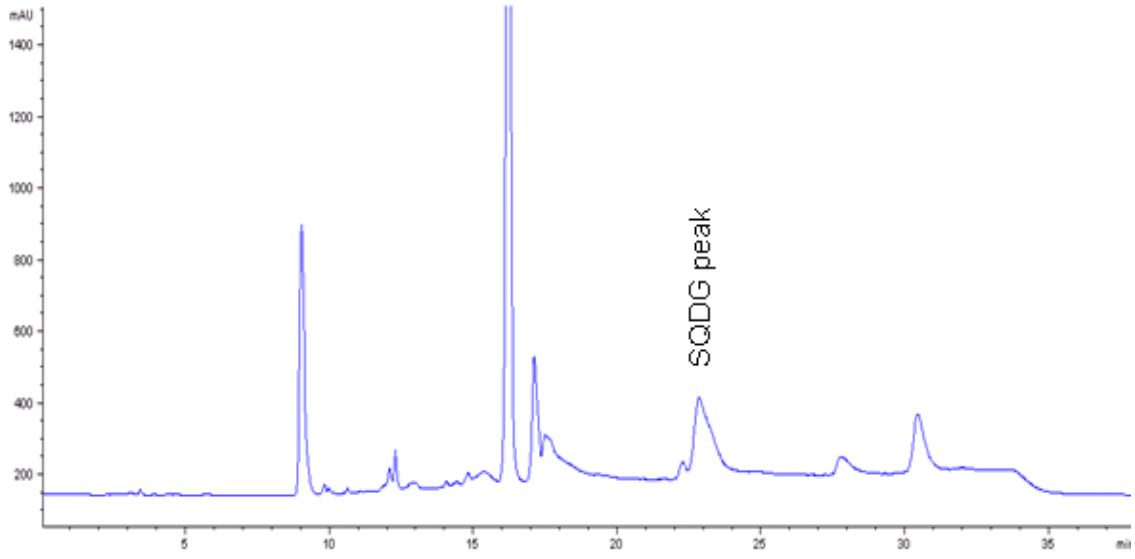


Figure 7.4: HPLC/ELSD chromatogram for the chloroform: ethanol (2:1, v/v) extract of Spirulina (M)

The extracts (using chloroform:methanol, 2:1, v/v) from different isolates of *Spirulina* was analysed (as shown in Table 7.1). The amounts of SQDG from all *Spirulina* isolates were different. The *Spirulina* (S) contained the highest amount of SQDG (1.41±0.09%) among the other *Spirulina* isolates investigated in this study. This analysis shows that the second highest amount of the sulfolipids is in *Spirulina* (M), followed by *Spirulina* (P), *Spirulina* (J) and *Spirulina* sp.

Table 7.1: Quantity of SQDG from different isolates of *Spirulina*

Isolates	% SQDG content (g/g AFDW)
Spirulina (P)	0.97±0.06
Spirulina (M)	1.34±0.09
Spirulina (S)	1.41±0.09
Spirulina (J)	0.69±0.05
Spirulina sp.	0.19±0.01

Data expressed as mean ± standard deviation; n = 3

Further comparative analysis was done by evaluating a suitable extraction method for SQDG. The extraction method for *Spirulina* (M) by using various combinations of solvents systems were investigated in this study (as shown in Table 7.2). Although *Spirulina* (S) has the highest content of SQDG, *Spirulina* (M) may be commercially significant due to its low cost production in Australia. The solvent systems of chloroform:methanol (2:1, v/v) has proved to be a valuable method for sulfolipids extraction. By using this solvent system, 13.43 mg of SQDG content/g AFDW was obtained from *Spirulina* (M). The other combination solvent systems such as chloroform: ethanol (2:1, v/v) and chloroform: butanol (2:1, v/v) have lower extract yields, where the extract yields are 12.62 mg/g AFDW and 3.66 mg/g AFDW, respectively. The extraction method proposed by Hara and Radin (1978) was less successful to extract SQDG since this solvent system only provides a small amount of extract yield (1.46 mg/g AFDW) for *Spirulina* (M). The use of HPLC/ELSD in this application showed that the hexane extract of *Spirulina* (M) has 0.57 mg of SQDG content/g AFDW. The toluene, acetone and methanol extracts of *Spirulina* (M) yielded 0.71 mg/g AFDW, 7.02 mg/g AFDW and 9.59 mg/g AFDW, respectively.

Table 7.2: Quantity and recovery of SQDG by using various forms of organic solvents for the *Spirulina* (M)

Extractions	SQDG content (mg/g AFDW)	Recovery
Hexane	0.57±0.03	4.24%
Toluene	0.71±0.58	5.29%
Acetone	7.02±0.46	52.27%
Methanol	9.59±0.62	71.41%
Butanol	2.75±0.17	20.48%
Chloroform:methanol (2:1, v/v)	13.43±0.87	100.00%
Chloroform: ethanol (2:1 v/v)	12.62±0.16	93.97%
Chloroform: butanol (2:1, v/v)	3.66±0.23	27.25%
Hexane: Isopropanol (3:2, v/v)	1.46±0.01	10.87%

Data expressed as mean ± standard deviation; n=3

The recovery was calculated under the assumption that the lipids and SQDG, which extracted with the solvent system of chloroform:methanol (2:1, v/v) has 100% recovery in the *Spirulina* (M). The SQDG recoveries are high (94%) when chloroform: ethanol (2:1, v/v) was used as an extraction method for *Spirulina* (M). On the other hand, the SQDG recoveries are the lowest when hexane extraction was used.

7.3 Purification of SQDG

The success of the separation depended on the colour vision based on the polarity of the compound. The TLC analysis illustrated that the polarity of the compound influences the colour appearing on the plate. The high polar compounds do not travel through the column very quickly (as shown in Figure 7.5). The TLC plate revealed that the green component is the least polar, followed by the yellow-greenish component, orange brown component and pink component. After putting the plate in an iodine chamber, the product of interest is visualised below the pink spot indicating that the SQDG is colourless. With such phenomenon, the SQDG should elute once the pink compound passes through the column. In order to identify the desired product, the colourless sample was collected with one millilitre per sample. The samples were then used for further justification with the TLC plate.

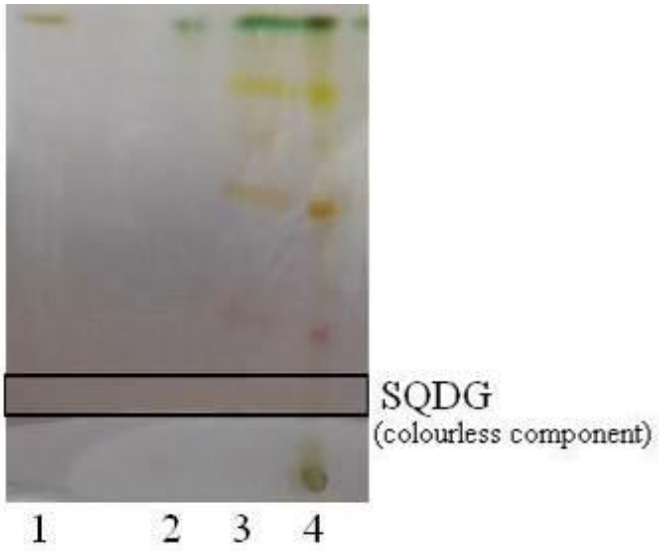


Figure 7.5: Sequential extracts in developing system

Lane 1 indicates hexane extracts; Lane 2 indicates as toluene extracts; Lane 3 indicates as acetone extracts; Lane 4 indicates as methanol extracts.

Different fractions using different elute systems were collected and observed. Table 7.3 shows the colours of the samples that are eluted from the column. The colours of the chloroform, acetone and methanol fractions were observed and subsequently applied to the TLC plates to verify the existence of the SQDG. Acetone and methanol fractions were observed to possess SQDG spot on the TLC plates. The methanol fraction revealed a higher concentration of SQDG by the size of the spot confirming that the work done by Harboune (1973) in view of the fact that the concentration of particular compound can be estimated by the size and intensity of the spot on the TLC plate. The acetone and methanol fractions were recollected and injected for another separation. SQDG component, which was observed in the fraction three, four and five on the TLC plate were further purified using the Sephadex LH 20 column. The pure compound is obtained when there is only a single spot with the same retention factor as the standard was appeared on a TLC plate.

Table 7.3: Collection of different fraction through the column

Fractions		Elute systems	Colour
		Chloroform	Yellow
		Acetone	Green, Yellow-greenish
		Methanol	Orange to colourless
Acetone	Fraction 1	Chloroform:methanol (9:1, v/v)	Green, Yellow, Orange
and	Fraction 2	Chloroform:methanol (8:2, v/v)	Orange
Methanol	Fraction 3	Chloroform:methanol (7:3, v/v)	Pink to colourless
collection	Fraction 4	Chloroform:methanol (6:4, v/v)	Colourless
	Fraction 5	Chloroform:methanol (1:1, v/v)	Colourless

7.4 Preliminary antibacterial assay

The anti-bacterial effects of *Spirulina platensis* have been investigated by researchers and are summarised in Table 7.4. These data show a varying degree of effects against the bacteria and fungi. The data showed that most of the research only concerned in researching the *Spirulina platensis* strain because this strain is highly effective in anti-microbial activity. However, there are small variances in the inhibitory effects against the bacteria and fungi. The results obtained in this study revealed that the investigated *Spirulina* isolates did not possess inhibition effects against *E. coli*, *S. aureus* and *C. albicans*. No zone of inhibition was observed.

Table 7.4: Anti-bacterial activity of sequential extracts of *Spirulina*

	E. coli	*S. aureus*	*C. albicans*
Hexane	-	11.24[c], 8.29[d]	-
Chloroform	11.87[c]	12.43[c]	-
Acetone	10[b], 10[e]	10[b], 12.43[c]	25[e]
Methanol	7[a], 12.42[d]	7[a], 15.20[c], 15.21[d], 10[e]	13[a], 15[e]
Water	12[b]	12[b]	

Diameter of inhibition zone in mm. Adapted from a (Ozdemir *et al*., 2004), b (Mala *et al*., 2009), c (Rao *et al*., 2010), d (Kaushik & Chauhan, 2008), e (Abedin & Taha, 2008).

7.5 Anti-HIV assay

The SQDG compound was successfully isolated from *Spirulina* (M) with a limited amount (14.36 mg) obtained. In order to access new biomaterials, cytotoxicity testing is always the first step in the biocompatibility testing. The data obtained from the

cytotoxicity testing can be used for *in vivo* or *in vitro* testing. Both cytotoxicity and anti-HIV testing were conducted at IMVS by Dr Tuck Weng Kok.

The cytotoxicity testing was performed by diluting the SQDG with 100 μL of dimethyl sulphoxide (DMSO) and the solution is used as 28.72mg/mL final concentration in the cell culture medium. Serial dilutions of the SQDG (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160) were used in uninfected Hut78 cells culture for cytotoxicity testing. SQDG with 1:5 and 1:10 dilution presents very strong cytotoxicity, however no cytotoxicity was observed with higher dilutions for the mixture (i.e. 1:20, 1:40, 1:80 and 1:160). This indicates that the cytotoxic effects varied with the extract concentration. The highest concentration of SQDG that did not cause detectable toxic effect was used to assess the anti-HIV testing. The cells lines exposed to serial dilutions of the mixture of SQDG and DMSO were examined and are displayed in Figure 7.6.

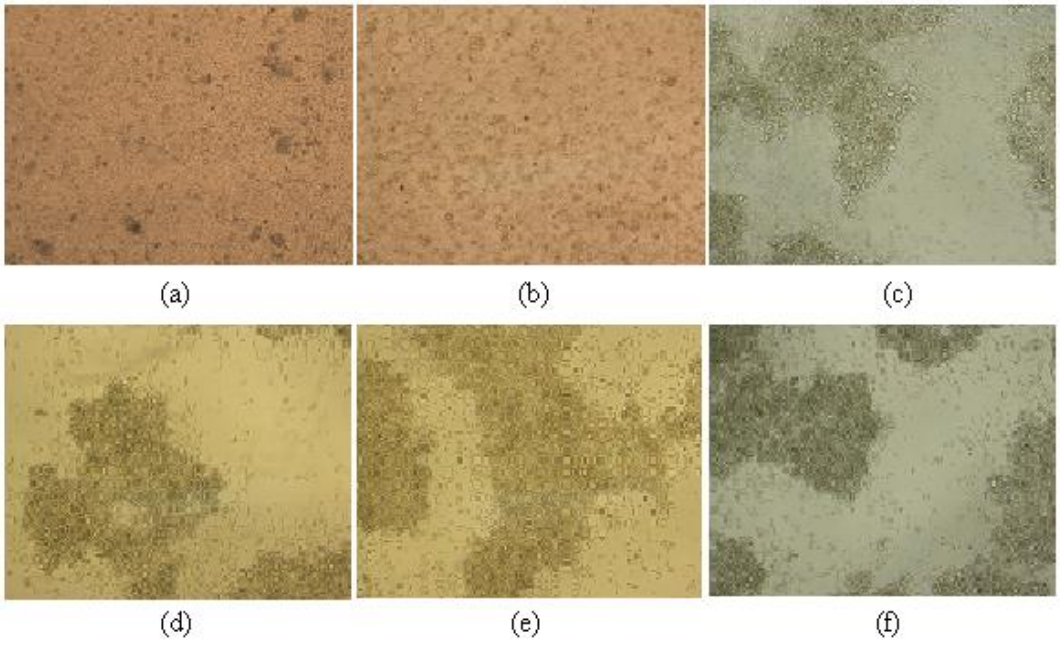


Figure 7.6: Serial dilution of the SQDG was observed under microscope for cytotoxicity effect

(a) 1:5 dilution, (b) 1:10 dilution, (c) 1:20 dilution; compared with the serial dilution of the cell culture (d) 1:5 dilution, (e)1:10 dilution and (f) 1:20 dilution.

To perform this testing, the culture medium was prepared in the mixture of a HIV producer cell line (H3B) in the ratio of 1:10 with a recipient CD4+ lymphoid cell line (Hut78) (Li & Burrell, 1992). The non-cytotoxic concentration of the pure compound (SQDG) was combined in the mix of H3B and Hut78 cells and observed by comparing with the positive control. The cultures were observed daily for three days post

inoculation. Viral cytopathic effects (formation of multinucleated giant cells) were noted on day 1 post inoculation, at a dilution of 1:20 of the compound. The results indicated that at a dilution of 1:20 and a subsequent dilution of the compound did not prevent cell-to-cell transmission.

7.6 Discussion

The choice of the use of analytical techniques depends on the material that needs to be analysed. To quantify the amount of sulfolipids, an anthrone procedure was used to determine the mass of sulfoquinovose in the aforementioned isolates in conjunction with the standard sulfoquinovose determined as the analysis of the sugar content. Both procedures have the same sensitivity (Russell, 1966). By using this quantitative method, it demonstrated that the *Spirulina* (S) results in the highest quantity of SQDG when compared to other isolates of *Spirulina*. The lipid yield and the fatty acid composition of the *Spirulina* isolates which were previously discussed in chapter six, was hypothesised that *Spirulina* (S) has the highest quantity of SQDG. Although *Spirulina* (P) has the highest lipid yield, none of the extraction solvent systems were able to extract a greater value of SQDG from *Spirulina* (P) when compared to *Spirulina* (S). The extract of *Spirulina* (P) might contain more nonlipid material or pigments and is a possible cause of the highest value of lipid yield.

The values of the standard deviation obtained in the quantification of SQDG by using anthrone procedure are relatively high, ranging from 0.65 to 4.38. The high standard deviation indicates that the error is attributed to the losses during the deacylation and hydrolysis procedures. Moreover, the sulfolipids determination depends on the calibration curve, which is constructed by using the anthrone procedure. The value of the linear regression of the calibration curve is low and thus provides a prediction within a wide range. In this analysis, the crude extracts are coloured and perceive a more severe impediment for the quantification since the coloured co-extractives might produce spot streaking on the TLC plate (Scussel, 2003). Although the standard deviations vary greatly, they remained comparable. This is because when using chloroform:methanol (2:1, v/v) as an extraction solvent system, the sulfolipids content of all *Spirulina* isolates were always at the highest. The precision in the determination of sulfolipids essentially depends on the initial lipid amounts and the structural resistance from different isolates of *Spirulina.* Due to high variations of results

obtained, HPLC method is developed to effectively and accurately quantify the SQDG from the *Spirulina* isolates.

Many studies have demonstrated that HPLC can be used to separate proteins, lipids and carbohydrates (Reuhs & Rounds, 2010). This technique has several advantages when compared to the traditional column liquid chromatography. HPLC is not time consuming, has more variety of the stationary phases, better resolution is obtained, there is greater sensitivity and a higher recovery of each sample (Reuhs & Rounds, 2010). Recently, HPLC has also been used for high throughput screening in drug development (Kassel, 2007).

The development of a method for SQDG in HPLC is vital. Without sufficient understanding of the application of HPLC technique, the method development is unlikely to be successful. The key factors that affect the performance of the HPLC are the column, detector, mobile phase, sample preparation and flow rate. There are several detectors available on the market to translate the analytes into electrical signals. The use of UV detector using ultraviolet light to absorb the chromophore in the analyte is the most common use for the detection of analytes. Other detectors such as electrochemical detectors, refraction index detectors and more recently an ELSD have been used to improve the performance of HPLC.

To validate the method for the analysis of certain product, relevant specifications such as the limit of quantitation (LOQ), analytes, resolution, precision, analysis time, and adaptability for automations are likely to be considered in the application. In this approach, a HPLC method was developed. This method is mainly aimed to provide a good resolution for the quantitation analysis of SQDG. Norman *et al*. (1996) have reported the quantification method of SQDG by using HPLC coupled with an UV/VIS detector. In their analysis, reverse phase HPLC was introduced because SQDG is a high polar ionic component. Furthermore, this mechanism is the most common mode for the separation of high polar analytes. This mechanism is able to attract the less polar analytes and allow the high polar analytes to elute earlier.

The amount of SQDG cannot be accurately determined by HPLC-UV detector because of the baseline noise as shown in Figure 7.3. The baseline noise made it more difficult to locate the peak width. Moreover, it decreased the precision and accuracy for the quantitation analysis. Poole (2003) also stated that: '…a rising baseline may have a

more complex baseline structure resulting in both gains and losses in the true peak height and area depending on the shape of the real baseline'. Furthermore, lipids and organic solvents used in HPLC show high absorbances at a low wavelength, where it is extremely difficult to differentiate the lipids peaks from solvent peaks (Nissen & Kreysel, 1990). Therefore, the method validation by using HPLC coupled with UV/VIS detector is unsuccessful in this study.

To quantify the lipid compounds in a sample, it is advisable to run with the HPLC coupled with the ELSD (Lutzke & Braughler, 1990). This technique has proven to be useful for the determination of lipid classes within different polarities classes (Homan & Anderson, 1998). ELSD evaluated all the non-volatile components and the outputs signal are proportional to the SQDG contained in the sample. The mechanism of the ELSD is that the mobile phase is atomised into a small droplet by an inert gas. The droplets was vaporised and the particulate is left suspending in the atomising gas (Daniel, 2007). For this reason, this detector provides high sensitivity in the quantitation of each lipid classes. However, the disadvantage of using the ELSD in this instance is that the compound will be vaporised during the detection.

Since the advent of modern techniques, direct lipid analysis by using HPLC has become very common. A chromatograph with two different detectors was used and observed. From the results obtained, the UV/VIS detector was not reliable when compared to the ELSD. It was found that a stable baseline was achieved throughout the run when ELDS was used. HPLC also perform better than traditional method (anthrone method), where it showed a much consistent results to analyse SQDG content from *Spirulina* isolates. In addition, the linear regression value for the HPLC method coupled with ELSD is considerably more reliable than that of the anthrone method.

HPLC analysis revealed that the hypothesis stated in the previous section was correct, where *Spirulina* (S) has the highest content of SQDG. For the other commercial isolates of *Spirulina*, it is difficult to decide which isolates of *Spirulina* play a significant role in producing higher amounts of SQDG because there is no clear observation on the fatty acid contents of *Spirulina*. The SQDG content from the Australian isolates of *Spirulina* was also quantified by HPLC analysis. The data showed that the *Spirulina* sp. has the least amount of SQDG, which is expected based on its low fatty acid content.

Belay *et al*. (1993) reported that there are about 1% sulfolipids contained in an unidentified *Spirulina* strain in their lab. The SQDG content of *Spirulina* (S), *Spirulina* (P) and *Spirulina* (M) was similar to the reported value, however for *Spirulina* (J) and *Spirulina* sp., the SQDG content was relatively low. Despite the extensive uses of SQDG in the pharmaceutical application, no investigation on the analysis of SQDG in different *Spirulina* has been reported. In this study, the *Spirulina* isolates are selected based on the SQDG amount extracted and it will be used to develop a cost effective remedy. An alteration of the amounts of SQDG demonstrates that the environmental condition for the growth of *Spirulina* is very important in the bioactive constituents' production. *Spirulina* (S) and *Spirulina* (M), which have been cultivated commercially are the best candidates to meet the demand of this drug because these isolates have domesticated and available on the market.

The crude extracts of *Spirulina* (M) from different sequential extractions were also measured. Hexane extracts, which do not detect the SQDG band on the TLC revealed a minimum amount of SQDG content. This is possibly because the TLC was not able to detect on the amount used for the testing. However, it responds well to the HPLC analysis. In this regard, the TLC application is less sensitive than HPLC. This again proved that it is not possible to quantify the amount of SQDG by using the anthrone procedure. In spite of the toxicity effects of chloroform, the chloroform:methanol (2:1, v/v) extraction method is not recommended. The methanol extraction system compares well with the chloroform:methanol (2:1, v/v) and achieved a high recovery of SQDG (71%). The lipid and SQDG yield of *Spirulina* (M) resulted that the methanol extraction solvent system is practical and economical. It shows that this single solvent system is good for the use to extract SQDG. Furthermore, the fractionation process will be easier to conduct since the sequential extracts have already extracted the non-polar components in the first place.

Few organisms were used to screen the *Spirulina* extracts for anti-bacterial activity by using the disc diffusion method (Section 3.18). *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were selected because they have been shown to have chronic affect on HIV patients' health (Galit *et al*., 2001; Jose, 2010; Mathewson *et al*., 1995).

The potential anti-microbial active compounds are mostly found from the organic solvent extracts (Geissman, 1963). Previous studies also showed that the organic

solvent extracts from *Spirulina platensis* have a better anti-microbial effect (Mala *et al*., 2009). In order to identify a better solvent extraction for *Spirulina*, a sequence of different polarity solvents was used for the extraction. In this study, none of the extracts displayed any activity against *E. coli*, *S. aureus* and *C. albicans*. This finding in this study is not in agreement with previous studies. This is probably due to the influence of genetic and environmental conditions. For instance, the environmental conditions for *Arthrospira* are the key factors that influence the fatty acid compositions (Mühling *et al*., 2005). Purine alkaloid is also another example of the phyto-constituent that varied under various environmental conditions. It showed a significant effect on the growth parameter of *Ilex paraguariensis* (Cardozo *et al*., 2007a).

As can be seen in Table 7.4, the inhibition effect of the *Spirulina platensis* does not seem to be consistent. These slight inconsistencies might be due to the genetic variation since the *Spirulina platensis* were obtained from different locations. Furthermore, the bioactive compound responsible for the anti-microbial activity in *Spirulina platensis* was not identified. This phenomenon can also be explained by the investigation undertaken by Bougatsos *et al*. (2004). In their study, the results showed that two *Helichrysum* have different anti-microbial effects. The *Helichrysum cymosum* shows no anti-microbial effect, while the *Helichrysum fulgidum* have a broad spectrum of anti-microbial effects against the tested organisms (Bougatsosa *et al*., 2004). Both of the *Helichrysum* strains showed varying anti-microbial effects because these two strains of *Helichrysum* do not possess similar chemical compositions. Therefore, it is suspected that the bioactive compound which possesses the anti-microbial effect from *Spirulina platensis* may be different from the isolates of *Spirulina* in this study.

HIV cell-to-cell transmission occurs rapidly, where the infected cells transmit the virus to uninfected CD4+ cells (Phillips, 1994). This mechanism has been reported to be the predominant mode of HIV-1 spread in T lymphocyte cultures (Emerson *et al*., 2010). Moreover, Li and Burrell (1992) stated that the: 'cell-to-cell spread is a common mode of virus transmission'. Therefore, it is important to research the potential of SQDG as an HIV transmission inhibitor.

There have been a number of reviews published on the SQDG revealing that this compound has the potential to act as a reverse-transcriptase inhibitor. Teas *et al*. (2004) also hypothesised that the consumption of *Spirulina* might reduce viral replication once

infection has taken place. Anti-HIV assays were restricted to cell-to-cell transmission testing. Due to the scope of the research, RNA analysis was not undertaken. The dilutions of SQDG (1:20, 1:40, 1:80 and 1:160) were studied in a cell-to-cell transmission model to evaluate their potential use in preventing transmission.

The anti-HIV experiment demonstrated that SQDG, which was isolated from *Spirulina* (M), was not a potent inhibitor of cell-cell transmission. A possible reason for this is because the isolated compound is not as effective as the crude product found in whole plants/microorganisms (Jana & Shekhawat, 2010). This isolated compound may interact synergistically with other natural phytochemicals in *Spirulina*. For consideration in the use of *Spirulina* as a therapeutic agent, it is necessary that further tests be conducted to more accurately determine the bioactivity properties of SQDG .

7.7 Summary

The crude extracts of the *Spirulina* isolates were analysed by several types of analytical techniques to quantify the amount of SQDG. Data obtained by using Russell's method (anthrone method) for the quantification of SQDG was inconclusive due to the instability of anthrone in sulphuric acid. This traditional quantification seems to be obsolete and therefore a new analytical technique (that is HPLC) was employed. The extracts of all isolates of *Spirulina* were successfully analysed by HPLC/ELSD and revealed to have different amount of SQDG content. *Spirulina* (S) HAD the highest amount of SQDG (1.34% SQDG), followed by *Spirulina* (M) (1.34%), *Spirulina* (P) (0.97%), *Spirulina* (J) (0.69%) and *Spirulina* sp. (0.19%) when using chloroform:methanol (2:1, v/v) were used as the extraction solvents system.

The application of a suitable extraction protocol for *Spirulina* to attain better recovery of SQDG is essential. As can be seen from the results, the recovery of SQDG varied when different solvent systems were utilised. The solvent system of chloroform: ethanol (2:1, v/v), chloroform: butanol (2:1, v/v), hexane: isopropanol (3:2, v/v), hexane, toluene, acetone, methanol and butanol achieve 94%, 27%, 11%, 4%, 5%, 52%, 71% and 20% recovery of SQDG, respectively. The solvent used in the extraction of food products have increasingly displaced the use of chloroform. This is attributable to the toxicity effects of chloroform. Therefore, in this study, the methanol extraction appears to be a promising solvent and it can be used to optimise SQDG extraction from

Spirulina. The recovery of SQDG obtained by using methanol extraction was approximately 71% and the extract yield of SQDG was 9.59 mg/g.

The extracts of different isolates of *Spirulina* were employed for the anti-microbial testing. The extracts were dissolved in its mother solvents and made up to a concentration of 40 mg/mL to test the bioactivity against *E. coli*, *S. aureus* and *C. albicans* plated on the agar. However, none of the extracts were shown to have bioactivity against tested microorganisms. Thus, the results are different from previous studies. The genetic differences may act as the cause of variation, and would possibly make the differences in phytochemical pathways of nutritional importance in *Spirulina*. SQDG, which has been reported to act as an inhibitor of reverse-transcriptase was also employed to determine its bioactivity in the cell-to-cell transmission model. However, it does not prevent transmission in this study. No comparison can be made since there was no research reporting the effect of SQDG on cell-to-cell transmission.

Although no active metabolite was yielded in this study, the presence of the phyto-constituents mentioned in the previous chapter, suggests that the *Spirulina* isolates could be useful in biomedical applications.

CHAPTER 8 CONCLUSION

Cyanobacteria are more developed than other marine algae and rich in secondary metabolites, of which *Spirulina* is a well-known species among all microscopic plants. Therefore, a number of researchers have been interested in studying *Spirulina* as a promising source of pharmaceuticals. *Spirulina* contains high nutritional value including nutrients such as protein, indispensable amino acid, vitamins, beta-carotene, mineral substances, essential fatty acid, polysaccharides, glycolipids and sulfolipids (Khan *et al*., 2005), while sulfolipids are a high potency inhibitor of reverse-transcriptase-HIV.

There are a number of uses of *Spirulina* because of its unusual nutritional profile and extremely high bioavailability of various nutrients. It is used as pigments, (Richmond, 2004) food colouring, (Belay *et al*., 1993; Yoshida *et al*., 1996) food additives, (Miranda *et al*., 1998) and oxidative for stress-induced diseases (Bhat & Madyastha, 2001; Romay *et al*., 1998). Furthermore, *Spirulina*'s primary and secondary metabolites provide health benefits. The primary and secondary metabolites of *Spirulina* have the potential to play a role as a source of powerful anti-viral agents (Singh *et al*., 2005b), antineoplastic agents (Patterson *et al*., 1991), anti-bacterial agents (Singh *et al*., 2005b), anti-HIV agents (Singh *et al*., 2005b), anti-inflammatory agents (Romay *et al*., 1998), anti-tumour agents (Santoyo *et al*., 2006) as well as anti-anaphylactic agents (Yang *et al*., 1997).

Spirulina is a good candidate for several biotechnological applications. However, cyanobacteria such as Microcystis, Nodularia, Oscillatoria, Scytonema, Lyngbya and Calothrix can produce cyanotoxin and cause toxicity to humans (Loya *et al*., 1998; Shirahashi *et al*., 1993). Despite the fact that some cyanobacteria produce cyanotoxins, the potential toxicity of *Spirulina* strains remains unclear.

In this thesis, the main objectives were to elucidate and isolate specific bioactive compound in few isolates of *Spirulina*. Five isolates of *Spirulina* from different continents were investigated for the supply of the sulfolipids. A suitable extraction technique was also determined to optimise the yield of sulfolipids. Furthermore, the molecular biology of these five isolates of *Spirulina* was studied and a comparison of

the bio-composition of these isolates of *Spirulina* was evaluated. An assessment of the bioactivity of the *Spirulina* isolates was also conducted against a range of microbes.

Five isolates of *Spirulina* were explored in this study and were identified as *Spirulina* sp., *Spirulina* (J), *Spirulina* (P), *Spirulina* (M) and *Spirulina* (S). In particular, *Spirulina* sp. is a wild isolate, which was obtained from Western Australia, Australia, while the commercial isolates were provided as lyophilised pellets by Yue Jian Biology Engineering Co. Ltd. (Jiangmen, China), Elken (Malaysia), OxyMin ® (Australia), and the South China Sea Institute of Oceanology (SCSIO) (Guangzhou, China), respectively (Section 3.1). In this research, the *Spirulina* sp. isolate preferred to culture in MLA medium plus seawater at pH 7-7.5. Culturing was performed in one litre Erlenmeyer flasks, which contained 400 mL of culture medium. Cultures were grown in an orbital mixer incubator (70 rpm, 25^{o}C) under cool white light (ca. 1500 lux) on a 12:12 light: dark cycle.

The classification of the *Spirulina* isolates was also confirmed by comparing the 16S rRNA sequence. 16S rDNA gene fragments, which were successfully amplified from the five *Spirulina* isolates, were submitted for sequence classification. 16S rRNA sequencing then classified that the four commercial *Spirulina* isolates (*Spirulina* (S), *Spirulina* (J), *Spirulina* (M) and *Spirulina* (P)) were closely related to *Arthrospira* strain; while *Spirulina* sp. was closely related *Halospirulina* strain. The taxonomic status of these isolates of *Spirulina* was further supported by the chemotaxonomic evidence. The results indicate that there is remarkable variation in the fatty acid contents of these isolates of *Spirulina*. The *Spirulina* sp. isolate was observed to possess a high portion of palmitoleic acid without gamma linolenic acid. Thus, the commercial isolates may be misclassified as *Spirulina* when they are in fact *Arthrospira* and the *Spirulina* sp. does not belong to the same genus as the commercial isolates. The sequences of these five isolates were also given accession numbers and to be used for future references.

The study of the biosynthesis pathway of sulfolipids was reviewed. The presence of sqdB and sqdX genes that were involved in the biosynthesis of sulfolipids was examined. In this study, the presence of sqdB and sqdX genes in all isolates of *Spirulina* except *Spirulina* (M) was detected. The absence of sqdB and sqdX homologues in *Spirulina* (M) was possibly due to the quality of the purified DNA. Aoki

et al. (2004) remarked that the sqd genes may be involved in different functions. The presence of the sqd genes within four other isolates of *Spirulina* revealed that they are more likely similar to the *Synechocystis* sp. PCC 6803, where the sulfolipids have a deleterious effect on the photosystem II. From the phylogenetic studies, the results of the sqdB- and sqdX-based analysis also show that the tested *Spirulina* sequences are clustered in the same lineage as the *Synechocystis* sp. PCC 6803. The examination of the potential toxicity of these isolates was also conducted by screening for NRPS and PKS-encoding genes since cyanotoxins are frequently produced non-ribosomally by NRPSs and PKSs (Barrios-Llerena *et al*., 2007; Ehrenreich *et al*., 2005; Neilan *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000). These isolates appear to lack of NRPS/PKS genes and therefore; do not produce NRP and PK toxins.

Preliminary phytochemical analysis of different sequential extracts from different isolates of *Spirulina* was also performed. The yield and the characteristics of the sequential extracts varied between these five isolates of *Spirulina*. Conversely, it was observed that the water extracts exhibited the highest percentage yield for all isolates of *Spirulina*, with a percentage yield ranging from 77.63 to 86.12%. The presence of more phytochemical constituents of these *Spirulina* isolates suggested that the polar solvent extracts are better when compared to the non-polar solvents. The presence of these phytochemical constituents also supports the use of the investigated *Spirulina* isolates as a nutritional supplement.

Natural products obtained from marine microorganisms are limited by the variability and availability of microorganisms, inconsistent product quality, low metabolite yield, as well as the extraction process (Zhang & Furusaki, 1999). In order to optimise sulfolipids from the *Spirulina* isolates, an appropriate extraction method is essential, especially in obtaining high recovery with low losses. To avoid the decomposition of natural constituents, the extraction of algal lipid becomes extremely complex. Hence, the algal extraction; lipid separation and quantification are difficult to estimate (Hagerthey *et al*., 2006; Lee *et al*., 1998b; Wiltshire *et al*., 2000). Due to the fact that the efficiency of lipid yield is generally increased by the extraction methods utilised, the lipid extraction methods such as using various forms of solvent extraction and different extraction techniques were investigated. The extraction efficiencies of butanol, hexane: isopraponal (3:2, v/v), chloroform: butanol (2:1, v/v) and chloroform: ethanol (2:1, v/v) were much lower than that of chloroform:methanol (2:1, v/v).

A different ratio of the solvent system of chloroform:methanol was developed and the results indicate that using chloroform:methanol (2:1, v/v) is the most practical method. Other extraction techniques such as supercritical carbon dioxide extraction and soxhlet extraction were also conducted. However the results reveal that these extraction methods were not suitable in this application. An addition of ethanol was also programmed in different volume percentages with supercritical carbon dioxide extraction to increase the lipid yield. The rise in the ethanol volume percentage indicates a slightly increased amount of lipids; however, the lipid yield is relatively low when compared to the solvent extraction. Comparison of these extraction methods suggested that the production of sulfolipids from the *Spirulina* isolates is highly depending on the solvent extraction used.

Various forms of organic solvents have an important influence on the production of both lipids and the fatty acid compositions. When the different structural classes of lipid changed, the fatty acids composition also changed. Hence, sulfolipids from *Spirulina* varied when extracted using different forms of organic solvents. Quantification of sulfolipids was initially achieved by anthrone procedure coupled with the TLC method. In this investigation, the anthrone procedure is less applicable due to its high value of standard deviation. Techniques for analysing and identifying sulfolipids were subsequently conducted by HPLC-UV and HPLC-ELSD. It was shown that HPLC-ELSD is a suitable technique for the quantification of sulfolipids. The HPLC-ELSD analysis revealed that the *Spirulina* (S) has the highest content of SQDG (1.41% AFDW ± 0.09), followed by *Spirulina* (M) (1.34% AFDW ± 0.09), *Spirulina* (P) (0.97% AFDW ± 0.06), *Spirulina* (J) (0.69% AFDW ± 0.05) and *Spirulina* sp. (0.19% AFDW ± 0.01). On the other hand, the quantification of SQDG was shown to be the highest when chloroform:methanol (2:1, v/v) was used as an extraction system (13.43mg/g AFDW ± 0.87). However, SQDG extracted by using methanol as solvent system is suggested in this study. This is because this is a single solvent system and less toxicity effect when compared to the use of chloroform. Furthermore, the recovery of SQDG is high (that is 71%) when using this single solvent system.

Although there was no bioactivity found in the studied isolates of *Spirulina*, the presence of the phyto-constituents and the high nutritional value of the isolates (including proteins and essential fatty acids) imply that the isolates of *Spirulina* (S), *Spirulina* (M), *Spirulina* (P) and *Spirulina* (J) are useful in biomedical applications. In

this study, the Australian isolate *Spirulina* does not possess gamma linolenic acid content. Therefore, it might not be a good candidate for the production of gamma linolenic acid and SQDG as proposed by Vonshak *et al*. (1997) when compared to other commercialised isolates of *Spirulina*. This investigation provided an insight into the therapeutic value of different isolates of *Spirulina* from different locations. Moreover, an optimisation extraction method was developed to achieve a better yield of sulfolipids from the *Spirulina* isolates. A major contribution of the classification of the commercial isolates and the Australia isolate also helps to clarify the taxonomic confusion and correctly identified the five *Spirulina* isolates in this investigation.

8.1 Further direction

The establishment of the algal cultivation systems is based on the knowledge of a species' ecology and biology. The taxonomic confusion of the *Spirulina* and *Arthrospira* still remains unsolved and thus leads to a more complex research. The previous studies in regards to the *Spirulina* have the effect of confusing the experiments and observation. Furthermore, there is no evidence showing that the previously identified *Spirulina* is accurately identified species. Therefore, researches in conducting the *Spirulina* or *Arthrospira* need to be classified including the criteria such as the morphological and life cycle information before further investigation.

Further investigation is required to evaluate the effectiveness of using whole *Spirulina* or the isolated component as conjunctive therapy since, as noted by Jana and Shekhawat (2010): 'crude extract from plants are more effective than isolated components due to their synergistic effect'. Moreover, the classification of the *Spirulina* and *Arthrospira* strains is still pending due to lack of information on the morphological and life cycle of *Spirulina*. In addition, the kinetics measurement and mechanism determination of sqdB and sqdX can be used to elucidate the reaction pathway and catalytic mechanism of promising enzymatic systems.

CHAPTER 9 REFERENCES

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Appendix A

Comprehensive information of natural product from plants

Table A.1: Anti HIV product from plants (Singh *et al*., 2005)

NOTE:
This appendix is included on pages 193-195 of the print copy of the thesis held in the University of Adelaide Library.

Appendix B

The preparation of the media used in ***Section 3.1*** is shown in the following tables.

Table B.1: Composition of MLA media (Bolch & Blackburn, 1996)

Constituents	**Per Litre deionised distilled water**
Salts/Nutrients	
$MgSO_4.7H_2O$	49.4 g
$NaNO_3$	85.0 g
K_2HPO_4	6.96 g
H_3BO_3	2.47 g
H_2SeO_3	1.29 mg
Vitamins	
Primary stock: Biotin and Vitamin B_{12}	10.0 mg/ 100mL H_2O
Working stock solution: Add 100mL of distilled water to the following:	
Biotin	0.05 mL primary stock
Cyanocobalamin B_{12}	0.05 mL primary stock
Thiamine HCl	10.0 mg
Micronutrients	
Primary stock: Add 1 L of distilled water to the following separately:	
$CuSO_4.5H_2O$	1.0 g
$ZnSO_4.7H_2O$	2.2 g
$CoCl_2.6H_2O$	1.0 g
$Na_2MoO_4.2H_2O$	0.6 g
Working stock solution: To 800mL of distilled water add each of the following constituents separately:	
Na_2EDTA	4.36 g (add first until fully dissolve)
$FeCl_3.6H_20$	1.58 g
$NaHCO_3$	0.60 g
$MnCl_2.4H_2O$	0.36 g
$CuSO_4.5H_2O$	10 mL primary stock
$ZnSO_4.7H_2O$	10 mL primary stock
$CoCl_2.6H_2O$	10 mL primary stock
$Na_2MoO_4.2H_2O$	10 mL primary stock
Additional nutrients/ trace metals	
$NaHCO_3$	16.9 g
$CaCl_2.2H_2O$	29.4 g
Na_2SO_3	12.9 g

Table B.2: Composition of seawater

Constituents	Per kg of solution
Gravimetric salts	
NaCl	23.926 g
Na_2SO_4	4.008 g
KCl	0.677 g
$NaHCO_3$	0.196 g
KBr	0.098 g
H_3BO_3	0.026 g
NaF	0.003 g
Volumetric salts	
$MgCl_2.6H_2O$	0.05327 mol
$CaCl_2.2H_2O$	0.01033 mol
$SrCl_2.6H_2O$	0.00009 mol

The salinity of seawater is 3.5%.

Table B.3: Composition of Zarrouk media (Brauns *et al*., 2004)

Compounds	***Amount (g/L)***
Macro-Nutrient	
$NaNO_3$	2.50
$MgSO_4 . 7H_2O$	0.20
K_2HPO_4	0.50
K_2SO_4	1.00
NaCl	1.00
Micro-Nutrient	
$NaHCO_3$	13.60
$CaCl_2.H_2O$	0.04
$FeSO_4.7H_2O$	0.01
Na_2EDTA	0.08
Na_2CO_3	7.60
Solution A5	
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
MoO_3	0.015
Solution B6	
$KCr(SO_4)_2.12H_2O$	0.096
$NiSO_4.7H_2O$	0.048
$(NO_3)_2Co.6H_2O$	0.049
$Na_2MoO_4.2H_2O$	0.018

Table B.4: Composition of BG-11 media (‘Culture Collection of Algae and protozoa Catalogue of Strains,’ 1988)

	Stock solution (g/100ml)	Nutrient solution
$CaCl_2.2H_2O$	5.6	1ml
$MgSO_4.\ 7H_2O$	7.5	1ml
$K_2HPO_4.\ 3H_2O$	3.05	1ml
$NaNO_3$		1.5g
NaCl (Salt)		18g
Trace metal solution		1ml
Trace metal (g/500 ml)		
H_3BO_3	2.86	Add each constituent separately to ~ 800ml of deionised water and fully dissolve between each solution. Then make up to 1L.
$MnCl_2.4H_2O$	1.81	
$ZnSO_4.\ 7H_2O$	0.222	
$Na_2MoO_4.2H_2O$	0.390	
$CuSO_4.5H_2O$	0.079	
$Co(NO_3)_2.6H_2O$	0.049	

Appendix C

Degenerate primer is a mixture of primers with similar sequences. Degenerate primers are used normally because of their convenience in amplifying the same gene from different organisms. In addition, degenerate primer is introduced to increase the chances of getting specific target sequence. Few rules are applied when degenerate primer was designed such as GC content should be in the range of 40-60% and the melting temperature for both primers should not vary more than 5 ^{o}C.

The procedure of the design of degenerate primers was performed following the method described previously (Wei *et al*., 2003). Degenerate primers were designed based on a collection of sequences from a large range of organisms as most genes from a family share structural similarities. Based on the alignments, determination of the conserved regions can be made. If the conserved regions from the sequences are hardly identified, multiple alignments of sequences were clustered into smaller groups. In smaller group, the conserved regions are possible to be determined and the degenerate primers are then designed. For instance, a collection of a specific sequence (*sqdX*) was composed by using BLAST searches from GenBank.

In this experiment, conserved regions are detected in the group of cyanobacteria is due to the fact that the *Spirulina* strain is one of the organisms in the family of cyanobacteria. Therefore, to provide primers with low degeneracy and high coverage, degenerate primers are designed to match an amino acid sequence of cyanobacteria.

After composed the alignment, two conserved regions are determined for locating the forward and reverse primers (as highlighted in Figure C.1 and Figure C.2, respectively). In this case, protein sequences were used for the design of degenerate primer. Few considerations need to take into account when designing the degenerate primers such as the feasible of the annealing temperature, an appropriate GC content, reasonably sticky ends, low degeneracy, and reasonable distance between two conserved region (Wei *et al*., 2003). As several different codons can code for amino acid, (International Union of Pure and Applied Chemistry) IUPAC degeneracy (shown in Table C.1) for individual bases was used.

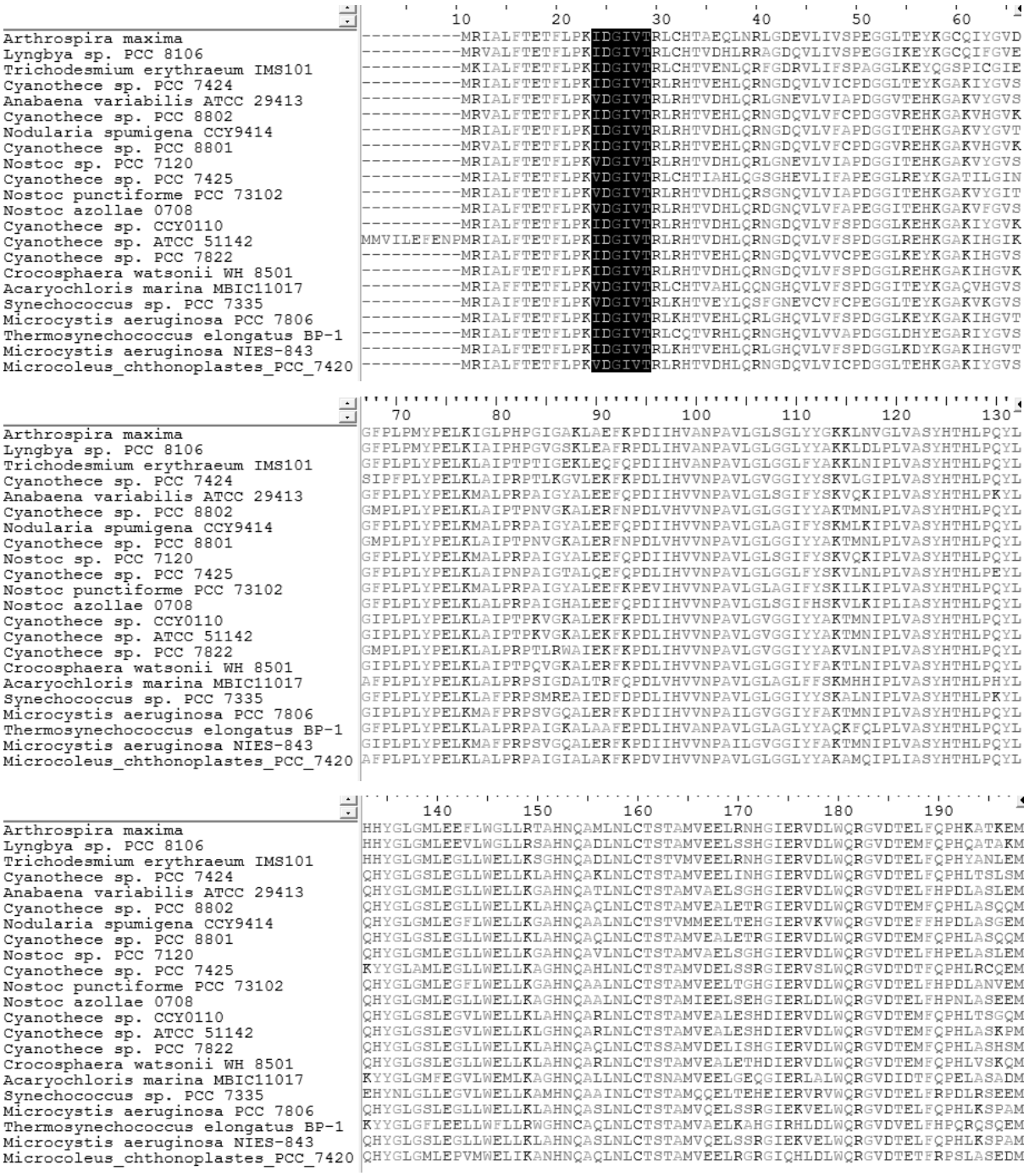

Figure C.1: The conserved regions are determined for locating the forward primers as highlighted.

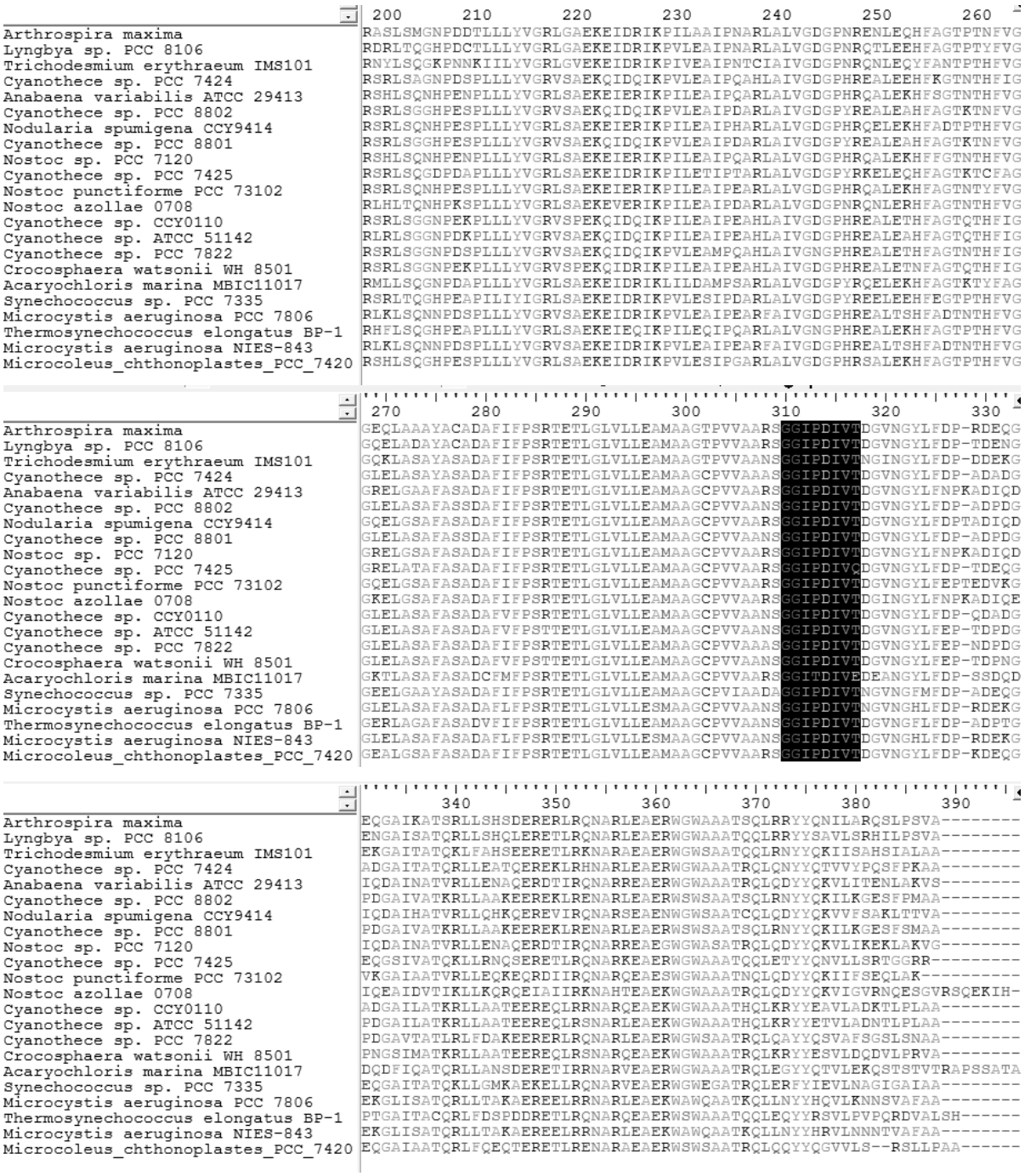

Figure C.2: The conserved regions are determined for locating the reverse primers as highlighted.

Table C.1: IUPAC Ambiguity Codes (Wei *et al*., 2003)

NOTE:
This table is included on page 203 of the print copy of the thesis held in the University of Adelaide Library.

Table C.2: Genetic Code Table (Wei *et al*., 2003)

NOTE:
This table is included on page 203 of the print copy of the thesis held in the University of Adelaide Library.

Table C.3: Genetic Code Tables with IUPAC codes (Wei *et al*., 2003)

NOTE:
This table is included on page 204 of the print copy of the thesis held in the University of Adelaide Library.

The changing of the codons will introduce much degeneracy into the designed primers and result in losing lots of bands. Consequently, a designed degenerate primer used is the one correspond to a minimum degeneracy. For example, the degenerate primer designed for the *sqdX* forward and reverse primers are as below:

Forward: I D G I V T
Reverse: G G I P D I V

These two conserved protein regions are then translated into an amino acid sequences.

Forward: T N G A Y G G N A T N G T N A C (Length: 16 mers)
Reverse: A T R T C N G G D A T N C C N C C (Length: 17 mers)

Table C.4: The degeneracy value of different amino acids (Rouchka & Rudraraju, 2006)

NOTE:
This table is included on page 204 of the print copy of the thesis held in the University of Adelaide Library.

The degeneracy of both primers; forward and reverse were calculated following the Table C.4.

Forward: 3 x 2 x 4 x 3 x 4 x 4 = 1152

Reverse: 4 x 4 x 3 x 4 x 2 x 3 x 4 = 4608

The degenerate primers were then submitted to the DNA calculator from Sigma (http://www.sigmaaldrich.com/life-science/custom-oligos.html) to calculate the melting temperature as well as the GC percent of each primer contained.

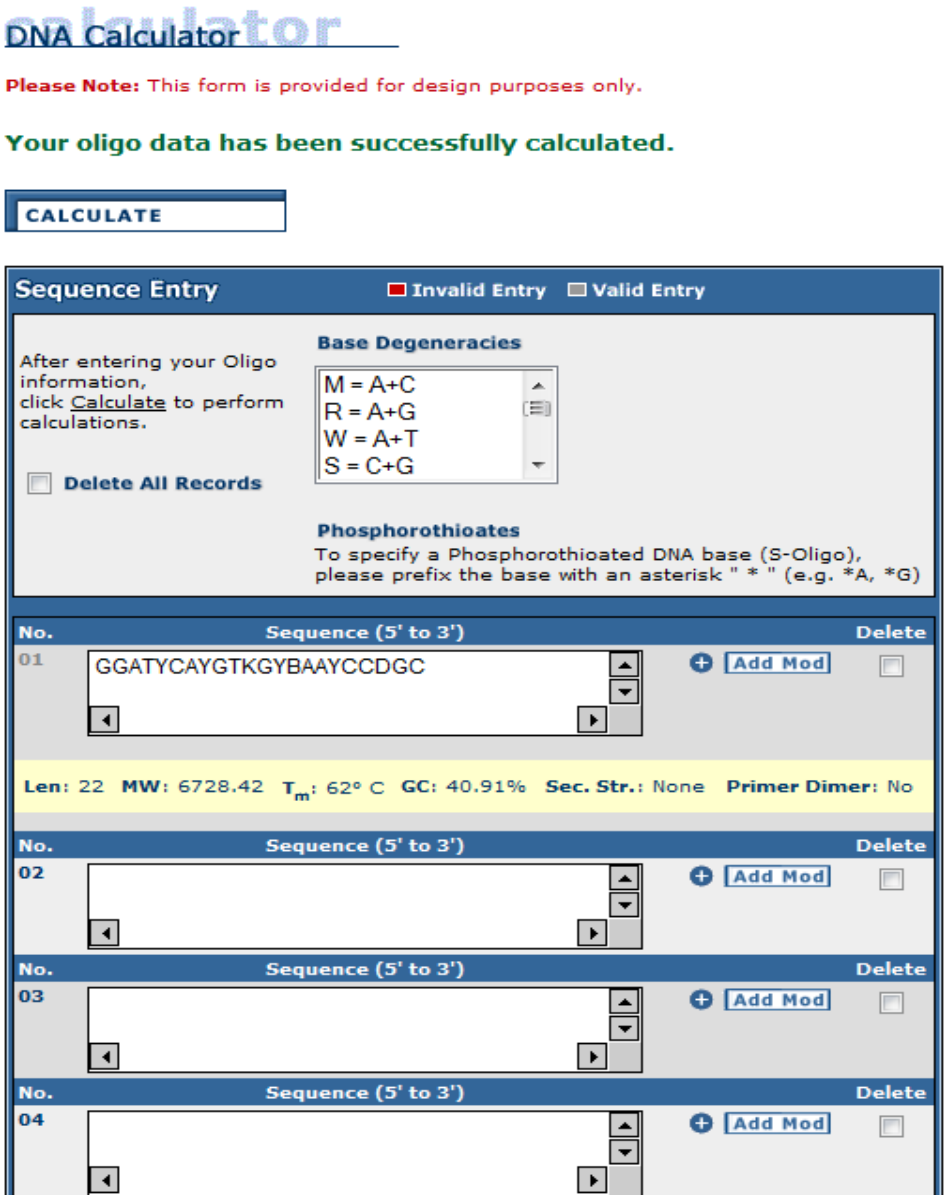

Figure C.4: Calculation for the melting temperature of the primer designed.

Both forward and reverse primers should have contained the similar percentage of GC content. G or C was added to achieve the similar GC contained for both primers. Resubmission of the primer and calculate to obtain the best sequence of the degenerate primer. As can be seen in Figure C.4, the GC content for the reverse primer shows

lower percentage. Therefore, adding GG in front to achieve the better percentage for the primer.

Appendix D

Results of BLAST DNA sequence analysis of 16S rDNA with the highest homology to the *Spirulina* strains in this study.

Table D.1: The five highest sequence similarities to strains of *Spirulina sp.*

Accession	***Description***	***Max ident***
Y18790.1	*Halospirulina* sp.'CCC Baja-95 Cl.3', 16S rRNA gene	99%
Y18789.1	*Halospirulina* sp.'MPI S3', 16S rRNA gene	98%
NR_026510.1	*Halospirulina* tapeticola strain CCC Baja-95 Cl.2 16S ribosomal RNA, partial sequence >emb|Y18791.1| *Halospirulina* tapeticola, 16S rRNA gene, strain CCC Baja-95 Cl.2	98%
AB003166.1	*Spirulina* subsalsa IAM M-223 gene for 16S ribosomal RNA	92%
AF329394.1	*Spirulina* subsalsa 16S ribosomal RNA gene, complete sequence; tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence	92%

Table D.2: The five highest sequence similarities to strains of *Spirulina* seawater.

Accession	***Description***	***Max ident***
EF432320.1	Arthrospira platensis Sp-18 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	100%
DQ393285.1	Arthrospira platensis strain PCC 9223 16S ribosomal RNA gene, partial sequence	100%
DQ393283.1	Arthrospira platensis strain SAG 21.99 16S ribosomal RNA gene, partial sequence	100%
DQ393281.1	Arthrospira maxima strain KCTC AG30054 16S ribosomal RNA gene, partial sequence	100%
DQ279770.1	Arthrospira platensis strain Sp-1 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	100%

Table D.3: The five highest sequence similarities to strains of *Spirulina platensis.*

Accession	***Description***	***Max ident***
EF432320.1	Arthrospira platensis Sp-18 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	100%
DQ393285.1	Arthrospira platensis strain PCC 9223 16S ribosomal RNA gene, partial sequence	100%
DQ393283.1	Arthrospira platensis strain SAG 21.99 16S ribosomal RNA gene, partial sequence	100%
DQ393281.1	Arthrospira maxima strain KCTC AG30054 16S ribosomal RNA gene, partial sequence	100%
DQ279770.1	Arthrospira platensis strain Sp-1 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	100%

Table D.4: The five highest sequence similarities to strains of *Spirulina* (Jiangmen).

Accession	***Description***	***Max ident***
FJ826624.1	Arthrospira sp. NMG 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer, tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	99%
EF432320.1	Arthrospira platensis Sp-18 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	99%
DQ393285.1	Arthrospira platensis strain PCC 9223 16S ribosomal RNA gene, partial sequence	99%
DQ393283.1	Arthrospira platensis strain SAG 21.99 16S ribosomal RNA gene, partial sequence	99%
DQ393281.1	Arthrospira maxima strain KCTC AG30054 16S ribosomal RNA gene, partial sequence	99%

Table D.5: The five highest sequence similarities to strains of *Spirulina maxima*.

Accession	***Description***	***Max ident***
FJ839360.1	Arthrospira platensis MMG-9 16S ribosomal RNA gene, partial sequence	99%
FJ826624.1	Arthrospira sp. NMG 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer, tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	99%
EF432320.1	Arthrospira platensis Sp-18 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	99%
EF432312.1	Arthrospira platensis Sp-6 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	99%
EF222475.1	Arthrospira platensis CG590 16S ribosomal RNA gene, partial sequence	99%

Results of BLASTx sequence analysis of *sqdB* and *sqdX* genes with the highest homology to the *Spirulina* strains in this study.

Table D.6: The five highest sequence similarities of *sqdB* gene to the strains of *Spirulina platensis.*

Accession	***Description***	***Max ident***
ZP_03271924.1	NAD-dependent epimerase/dehydratase [Arthrospira maxima CS-328]	97%
BAI88486.1	sulfolipid (UDP-sulfoquinovose) biosynthesis protein [Arthrospira platensis NIES-39]	96%
ZP_06380417.1	sulfolipid biosynthesis protein [Arthrospira platensis str. Paraca]	96%
CAO88798.1	unnamed protein product [Microcystis aeruginosa PCC 7806]	87%
YP_001659939.1	sulfolipid biosynthesis protein [Microcystis aeruginosa NIES-843]	86%

Table D.7: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina* sp.

Accession	***Description***	***Max ident***
ZP_00514286.1	sulfolipid biosynthesis protein [Crocosphaera watsonii WH 8501]	89%
ZP_01731239.1	NAD-dependent epimerase/dehydratase [Cyanothece sp. CCY0110]	89%
YP_001805985.1	sulfolipid biosynthesis protein [Cyanothece sp. ATCC 51142]	88%
YP_003421324.1	UDP-sulfoquinovose synthase [cyanobacterium UCYN-A]	85%
ZP_01620426.1	NAD-dependent epimerase/dehydratase [Lyngbya sp. PCC 8106]	85%

Table D.8: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina* seawater.

Accession	***Description***	***Max ident***
ZP_00514286.1	sulfolipid biosynthesis protein [Crocosphaera watsonii WH 8501]	91%
YP_001805985.1	sulfolipid biosynthesis protein [Cyanothece sp. ATCC 51142]	91%
ZP_01731239.1	NAD-dependent epimerase/dehydratase [Cyanothece sp. CCY0110]	91%
YP_003421324.1	UDP-sulfoquinovose synthase [cyanobacterium UCYN-A]	89%
ZP_01620426.1	NAD-dependent epimerase/dehydratase [Lyngbya sp. PCC 8106]	89%

Table D.9: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina* (Jiangmen).

Accession	***Description***	***Max ident***
ZP_03271924.1	NAD-dependent epimerase/dehydratase [Arthrospira maxima CS-328]	99%
BAI88486.1	sulfolipid (UDP-sulfoquinovose) biosynthesis protein [Arthrospira platensis NIES-39]	98%
ZP_06380417.1	sulfolipid biosynthesis protein [Arthrospira platensis str. Paraca]	98%
ZP_01620426.1	NAD-dependent epimerase/dehydratase [Lyngbya sp. PCC 8106]	90%
ZP_05026496.1	NAD dependent epimerase/dehydratase family [Microcoleus chthonoplastes PCC 7420]	88%

Table D.10: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina platensis.*

Accession	***Description***	***Max ident***
ZP_03273545.1	Glycosyl transferase group 1 [Arthrospira maxima CS-328]	99%
ZP_06380212.1	Glycosyl transferase, group 1 [Arthrospira platensis str. Paraca]	97%
ZP_01623513.1	Glycosyl transferase, group 1 [Lyngbya sp. PCC 8106]	84%
ZP_07111788.1	Glycosyl transferase, group 1 [Oscillatoria sp. PCC 6506]	82%
YP_720338.1	Glycosyl transferase, group 1 [Trichodesmium erythraeum IMS101]	78%

Table D.11: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina sp.*

Accession	***Description***	***Max ident***
YP_001735707.1	Glycosyl transferase group 1 [Synechococcus sp. PCC 7002]	82%
YP_001802985.1	Sulfolipid sulfoquinovosyldiacylglycerol biosynthesis protein [Cyanothece sp. ATCC 51142]	80%
ZP_01730205.1	Hypothetical protein CY0110_04628 [Cyanothece sp. CCY0110]	79%
ZP_00518793.1	Glycosyl transferase, group 1 [Crocosphaera watsonii WH 8501]	79%
YP_003138204.1	Glycosyl transferase group 1 [Cyanothece sp. PCC 8802]	78%

Table D.12: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina* seawater.

Accession	***Description***	***Max ident***
ZP_03273545.1	Glycosyl transferase group 1 [Arthrospira maxima CS-328]	99%
ZP_06380212.1	Glycosyl transferase, group 1 [Arthrospira platensis str. Paraca]	97%
ZP_01623513.1	Glycosyl transferase, group 1 [Lyngbya sp. PCC 8106]	84%
ZP_07111788.1	Glycosyl transferase, group 1 [Oscillatoria sp. PCC 6506]	81%
YP_720338.1	Glycosyl transferase, group 1 [Trichodesmium erythraeum IMS101]	77%

Table D.13: The five highest sequence similarities of *sqdX* gene to the strains of *Spirulina* (Jiangmen).

Accession	***Description***	***Max ident***
ZP_03273545.1	Glycosyl transferase group 1 [Arthrospira maxima CS-328]	100%
ZP_06380212.1	Glycosyl transferase, group 1 [Arthrospira platensis str. Paraca]	98%
ZP_01623513.1	Glycosyl transferase, group 1 [Lyngbya sp. PCC 8106]	86%
ZP_07111788.1	Glycosyl transferase, group 1 [Oscillatoria sp. PCC 6506]	81%
YP_720338.1	Glycosyl transferase, group 1 [Trichodesmium erythraeum IMS101]	79%

Appendix E

The fatty acid composition of different ratio of chloroform:methanol is shown in Table E.1.

Table E.1: Fatty acid composition of all strains of *Spirulina* using different ratio of chloroform:methanol

	Fatty Acid						
	16:0	16:1n9	16:1n7	18:0	18:1n9	18:2n6	18:3n6
Spirulina (Jiangmen)							
1:1	51.67	nd	3.45	nd	2.93	23.89	15.20
1:2	50.00	nd	3.27	0.98	2.62	22.85	14.70
1:4	50.69	0.79	3.15	0.94	2.48	22.29	13.11
1:8	51.60	0.80	3.23	nd	2.58	23.30	14.70
1:16	53.30	0.81	3.18	nd	2.34	21.76	13.86
Spirulina seawater							
1:1	50.40	nd	4.85	nd	nd	25.50	15.95
1:2	46.71	0.65	4.59	1.65	3.65	23.64	17.41
1:4	48.40	0.98	4.42	1.57	3.48	22.87	16.08
1:8	49.27	1.00	4.53	nd	3.74	23.52	17.04
1:16	51.22	0.82	4.46	1.09	3.19	20.95	16.07
Spirulina platensis							
1:1	48.67	nd	6.01	nd	nd	21.32	21.39
1:2	44.31	1.61	5.56	1.70	2.41	19.34	19.42
1:4	44.61	1.64	5.70	1.56	2.40	19.62	19.43
1:8	45.16	1.66	5.77	nd	2.42	19.93	19.66
1:16	45.00	1.66	5.81	1.35	2.40	19.93	19.72
Spirulina maxima							
1:1	57.61	nd	2.53	nd	3.65	22.78	11.91
1:2	55.32	nd	2.44	1.75	3.67	22.36	11.25
1:4	59.62	nd	2.23	1.74	3.33	20.10	10.10
1:8	60.90	nd	2.32	nd	3.44	21.07	10.81
1:16	62.25	nd	2.19	1.57	3.10	18.34	9.02
Spirulina sp.							
1:1	35.79	24.73	2.98	nd	20.11	2.15	nd
1:2	32.63	26.76	2.92	1.61	20.06	1.96	nd
1:4	35.26	23.59	2.57	1.52	19.86	nd	nd
1:8	35.79	25.79	2.68	1.55	19.89	1.76	nd
1:16	35.50	28.78	2.60	1.45	14.97	nd	nd

Appendix F

The quantification of the SQDG was calculated based on the calibration graph below.

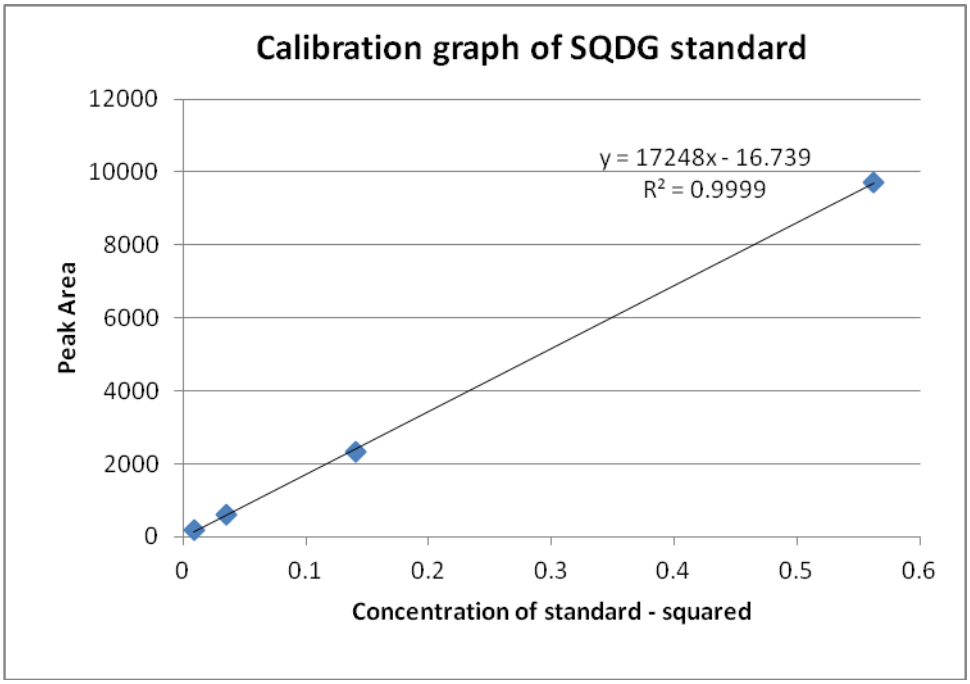

Figure F.1: Calibration chart for the quantification of SQDG by using HPLC-ELSD, which conducted by University of Queensland.