Enhancement of Biomass and Lutein Production in *Chloroparva pannonica*

A thesis submitted for the award
Doctor of Philosophy
at Flinders University of South Australia

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2016

Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree of diploma, and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Liu Fei Tan

Acknowledgements

First and foremost, I would like to thank my supervisor Prof Chris Franco for his guidance, support and patience throughout my Ph.D study. His immense knowledge in the field and critical inputs have definitely better shaped my research direction as well as my dissertation. My sincere appreciation in also given to my co-supervisor, Prof Wei Zhang for his encouragement, inspiration and helpful feedback. Secondly, my gratitude toward the Flinders University for giving me chance to do my study through a Flinders University Research Award.

Special thanks Angela Binns, Barbara Kupke, Hanna Krysinska and all the people in the Department of Medical Biotechnology. This big book would not able to be produced without the help and support from many people. I would like to thank Tanya Bernardo for proof reading my chapter 1 and 2. Thanks to Fitri Widiantini for helping me for the polyphasic studies and for her encouragement. Thanks to Dao Swain for listening to my complaints and the drinking sessions. Thanks for the burger and fries group in what's app (Tanya Bernado, Fitri Widiantini and Dao Swain) who accompanied me all the time. Thanks to Onuma Kaewkla for teaching me how to generate phylogenetic tree, Margaret Stopa and Hao Jiang for his expertise in HPLC, Eko Sitepu for his expertise in fatty acid analysis, Sophia Zhao, Jenny Wang, Xuyen Hoang Le, Qi Yang, Abdulmajeed Almutary, Evita Chrisnayanti, Jane Keane, Peng Su, Shuang Peng, Abdullah Alnuqaydan for their friendship and their help in the lab. Not to forget my editor, Valerie Williams who has made a great effort in proofreading my chapters. Without her assistance, things will certainly be much more complicated.

Finally, I would like to thank to my family back home, my parents and brothers in Malaysia, Adelaide and Glasgow who supported me throughout my study. Thanks to my aunties and uncles in Malaysia who keep me up to date with all 'news' by whatsapp-ing everyday. Last but not least, I would like to give thanks for my husband, Che Kiong and my two beautiful kids Sophie and Lucas, for not only giving me a wonderful family, but everything.

Abbreviations

β-carotene	beta-carotene
μ	specific growth rate
μg	microgram
μg/L	microgram/litre
μM	micromolar
μmol photons $m^{-2}s^{-1}$	photosynthetic photon flux (PPF)
(6-4)PPs	pyrimidine (6-4) pyrimidine photoproducts
Å	ångström
AMA	antibiotic medium No. 1 (agar)
AMD	age-related macular degeneration
amiRNAs	artificial microRNAs
ARM	aclonifen-resistant mutant
ASDW	ash-free dry weight
ASTM	American Society for Testing Materials
ATP	adenosine triphosphate
bp	Base pair
BLAST	Basic Local Alignment Search Tool
C-P RM	chlodinafop-propargyl resistant mutant
$C_2H_5NO_2$	glycine
CA	California
$Ca(NO_3)_2$	calcium nitrate
cfu or CFU	colony-forming unit
cfu/ml	colony-forming unit per millitre
ch *a*	chlorophyll *a*
ch *b*	chlorophyll *b*

CH_4N_2O	urea
Co^{2+}	cobalt
CO_2	carbon dioxide
$CoCl_2$	cobalt (II) chloride
$CO(NH_2)_2$	urea
CPD	cyclobutane pyrimidine dimer
CRISPR	clustered regularly interspaced short palindromic repeats
cSt	centistoke
CTAB	hexadecyltrimethyl ammonium bromide
$CuSO_4$	copper sulphate
d wt	Dried weight
d^{-1} or d	day
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DOE	Department of Energy (US)
DW	dry weight
EDTA-2NA	ethylene diamine tetraacetic acid disodium salt
EIA	Energy Information Administration (US)
EMS	ethyl methanesulphonate
EPA	eicosapentaenoic acid
EST	expressed sequence tag
EU	European Union
$FeCl_3$	ferric chloride/iron (III) chloride
FU	Flinders University
g	relative centrifugal force
G+C	guanine+cytosine
GE	General Electric

GGPP	geranylgeranyl pyrophosphate
GHG	greenhouse gas/es
gL	gram per litre
GLA	γ-linolenic acid or gamma-linolenic acid
GOGAT	glutamate synthase
GS	glutamine synthetase
GtC	Guanine – cytosine
h	hour
H^+	hydrogen
H/C ratio	hydrogen–carbon ratio
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HO	high output
HPLC	high-performance liquid chromatography
HR	homologous recombination
IEA	International Energy Agency
INS	International Numbering System for Food Additives
IPP isomerase	isopentenyl pyrophosphate isomerase
IPCC	Intergovernmental Panel on Climate Change
kg L^{-1}	Kilogram per litre
KNO_3	potassium nitrate
KOH	potassium hydroxide
kPa	kilopascal
L or l	litre
MEGA	Molecular Evolutionary Genetics Analysis (software)
mg	milligram
Mg	magnesium
mg KOH/g^{-1}	milligrams of potassium hydroxide per gram (of oil sample)

mg.g	milligram to gram
mg/L	milligram/litre
mg/l/d (or mg/L/d)	milligram/litre/day
min	minute
mm	millimetre
mM	millimolar
M_nCl_2	manganese (II) chloride
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MNU	N-methyl- N-nitrosourea
$Na_2H_2PO_4$	monosodium phosphate
Na_2MoO_4	sodium molybdate
NaCl	sodium chloride
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NCRIS	National Collaborative Research Infrastructure Strategy
NH_4^+	ammonium
NH_4Cl	ammonium chloride
NH_4HCO_3	ammonium bicarbonate
NH_4NO_3	ammonium nitrate
nm	nanometre
NO_3^-	nitrate
NREL	National Renewable Energy Laboratory
NTG	1-methyl 3-nitro 1-nitrosoguanidine
–OH	hydroxyl or hydroxyl groups
•OH	hydroxyl radical
O_2^-	superoxide anion
O_2*	singlet oxygen

OD	optical density
OH^-	hydroxide
OK	Oklahoma
OSCT	one-step closed-tube (method)
OsO_4	osmium tetroxide
PBS	phosphate buffered saline
PC	parent culture
PCR	polymerase chain reaction
PGPB	plant growth-promoting bacteria
pH	acidity
PMMA	polymethyl methacrylate
PO_4^{3-}	phosphate
ppm	parts per million
PSI	photosystem I
PSII	photosystem II
PUFAs	polyunsaturated fatty acids
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RP-HPLC	reverse-phase high-performance liquid chromatography
rpm	revolutions per minute
rRNA	ribosomal RNA
SA	South Australia (or South Australian)
SARDI	South Australian Research and Development Institute
SCOs	single cell oils
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SFE	supercritical fluid extraction

sgRNAs	single chain guide RNAs
SRP	signal recognition particle
T	traditional (method)
TAGs	triacyglycerides or triacylglycerols (also called triglycerides)
TALENs	transcription activator-like effector nucleases
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TLC	thin layer chromatography
TSA	tryptic soy agar
TSB	tryptophan synthase (beta subunit)
UK	United Kingdom
US/USA	United States of America
USEPA	United States Environmental Protection Agency
UV	ultraviolet
UV/Vis	ultraviolet/visible
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
Wis	Wisconsin
wt	weight
WT	wild type
wt%	weight per cent
Z_nSO_4	zinc sulphate
ZFNs	zinc-finger nucleases

Abstract

The consumption of non-renewable fossil fuels has increased with the rapid development of modern industry, agriculture and transportation. The oil-based bio-refinery process based on photosynthetic microalgae has been the most promising for biofuel production. However, the production on a commercial scale is not yet economically viable. Biomass productivity plays an important role in achieving economical mass productivity of compounds produced by microalgae and this is a major factor for success in any new industrial scale venture. This thesis seeks to develop *Chloroparva pannonica* which has the potential for the bio-refinery process to grow rapidly, produce higher biomass and enhanced production of a type of carotenoid, lutein.

As the project involved the screening of a large number of mutants, rapid methods using relatively low levels of sample were developed. The lutein content was extracted and measured with a one-step closed-tube (OSCT) under dark conditions without the need for large amounts of samples and solvents.

The production medium was optimised via the formulation of new FU medium, to achieve high-density culture as the standard Guillard's F/2 medium resulted in low biomass productivity. In the FU medium, urea was used as the main nitrogen sources. In addition, the concentration of ferric ions was increased twofold, cobalt, sodium molybdate and zinc were increased fourfold and manganese was increased eightfold.

The exposure of *Chloroparva pannonica* to different concentrations of copper resulted in the unexpected selection of *Chloroparva pannonica* Cu40 with a higher growth and lutein content than the *Chloroparva pannonica* wild type. *Chloroparva pannonica* Cu40 was been selected for the random mutagenesis though UV-C irradiation. The mutants were further screened for resistance to one of two herbicides, aclonifen and chlodinafop-propargyl. Mutants that exhibit in high lutein content and high biomass in small scale were chosen for the short-term laboratory

experiments in batch mode using 3 L flasks with 1% carbon dioxide supplied. 10 mutants that exhibit in high lutein content and high biomass were chosen then for the short-term laboratory experiments in 11 L rectangular inter-loop airlift photobioreactor. Three (3) of these mutants (Fu4, Fu5C and Fu41A) were selected for the taxonomy study along with the *Chloroparva pannonica* Cu40 and wild type to ensure that they belong to the same species and were not contaminants.

Based on the molecular analysis of the 18S rRNA gene sequence, *Chloroparva pannonica* wild type (WT), Cu40 parent culture (PC), Fu4, Fu5c and Fu41A were close to *Chloroparva* sp. ACT 0608 within Trebouxiophyceae, Chlorophyta. The molar guanine+cytosine (G+C mol%) of the genomic DNA ranged from 56.27 – 59.06%. The spherical cells ranged in size from 2–5 μm. The lipid content ranged from 22.94–25.59%. The predominant fatty acids were oleic acid, linoleic acid, linolenic acid and palmitic acid. The pigments were chlorophyll *a* and *b*, while the carotenoid was lutein. The lutein concentration ranged from 1864–5502 mg/kg of biomass. However, the lipid and lutein content could be manipulated based on their cultivation conditions. These strains were tolerant of salinity up to 7%, a pH ranging from 6–11 and temperature from 10–30°C.

As previously mentioned, 10 mutants that showed higher growth, lutein content and total lipid content than the Cu40 parent culture and wild type were selected to evaluate their growth, lutein and lipid content in batch mode in an 11 L rectangular inter-loop airlift photobioreactor. Of these, Fu5C was identified as the strain for the semi-continuous cultivation in the 11L photobioreactor. In order to improve the yields of lutein, it was decided in the current study, based on the growth and lutein content patterns, to harvest the culture every eight days when the lutein was at its highest point while maintaining the exponential phase of the growth. Having achieved very high lutein concentration the draw-fill cycles were continued for a 94-day period. In the long-term semi-continuous system, the performance (biomass productivity, lutein productivity and lipid productivity) of *Chloroparva pannonica* Cu40 parent culture was better than that of Fu5C and wild type. The maximum total dried biomass, biomass productivity, specific growth rate, lutein content and lutein productivity were 1.678 g/l, 0.15 g/l/day, 0.482 μ/day, 5768 mg/kg of biomass and 604.32 mg/kg of biomass/day, respectively. Due to the performance (high biomass

productivity and lipid productivity) of Cu40 parent culture, the long-term semi-continuous 11 L rectangular inter-loop airlift photobioreactor system was continued for a further 96-day period to investigate the ability to produce sufficient biomass and lutein yield to be a feedstock for the microalgae based bio-refinery process. The maximum total biomass, biomass productivity and specific growth rate were 1.705 g/l, 0.1538 g/l/day and 0.4963 μ/day, respectively. The maximum lutein content was 6960 mg/kg of biomass while lutein productivity was 613.4 mg/kg of biomass/day. However, lipid content ranged from 10–22% while the lipid productivity averaged 2.5%/day. In the current study, lutein was successfully extracted and purified from *Chloroparva pannonica* Cu40 parent culture cultivated in the long-term semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor.

In conclusion, this work has demonstrated that, by using the semi-continuous system, *Chloroparva pannonica* Cu40 and Fu5C have shown improved biomass productivity and enhanced lutein accumulation when cultivated using the FU medium. These strains could be considered as promising microalgae for the production of lutein in a large-scale system

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Chapter 1 Literature Review

1.1 Sustainable energy

Climate changes and the warming of the Earth caused by excessive consumption of fossil fuels has received much attention from scientists and governments (Wood and Overland, 2010). Carbon dioxide (CO_2) is one of the most important anthropogenic greenhouse gases (GHGs) produced during the combustion of fossil fuels (IPCC, 2007). The annual emissions of carbon dioxide from fossil fuels increased from an average of 6.4 gigatonnes of carbon (GtC) per year in the 1990s, to 7.2 GtC per year in the period from 2000–2005 (IPCC, 2007). The consequences of the warming of the Earth include melting of the polar ice caps, rising sea levels, climate changes such as redistribution of rainfall and storms, and the possibility of species' extinction (Sivakumar *et al.*, 2010).

The consumption of non-renewable fossil fuels has increased with the rapid development of modern industry, agriculture and transportation. Furthermore, the extraction, processing and combustion of fossil fuels have contributed to pollution of the air, water and soil. It is estimated that, in the USA alone, transportation consumes about 450 billion litres of fossil fuels per year (EIA, 1998). The world demand is approximately 84 million barrels a day and, by 2030, it is estimated that this will rise to about 116 million barrels a day (IEA, 2007). Based on the present rate of fossil fuels' consumption, fossil fuels will deplete to the point of exhaustion (Huang *et al.*, 2010, Sheehan *et al.*, 1998). Consequently, there has been a move towards exploring alternative environmentally sustainable energy sources and seeking lipid-rich biological materials for biofuel production.

Biodiesel is an alternative diesel fuel comprised of long mono-alkyl esters produced from plant oils (e.g. soybean, sunflower, jatropha, palm oil and rapeseed) or animal-based fats (e.g. pork lard and crude beef tallow) (Ma and Hanna, 1999). The main sources of first-generation biodiesel production were rapeseed, soybeans, vegetable oils, palm oil and sunflower oil (Al-Widyan and Al-Shyoukh, 2002, Antolin *et al.*, 2002,

Bunyakiat *et al.*, 2006, Peterson *et al.*, 1996). However, plant-based biodiesel has a number of limitations. The current scale of production is unable to satisfy global fuel requirements (Buczek and Czepirski, 2004, Murugesan *et al.*, 2009). The production cost of biodiesel from these crops is high with costs of raw materials being 60–75% of the total cost of biodiesel production which is competing with the traditional use of these crops as food (Krawczyk, 1996). For example, Haas *et al.* (2006) reported that the cost of soybean oil input amounted to 88% of the total US biodiesel production cost. In addition, biodiesel production is highly dependent on the production of oil-seed crops, which are restricted geographically and by the seasons (Kalscheuer *et al.*, 2006). Moreover, the cultivation of some of the oil-seed crops, such as rapeseed and soybeans, requires a frequent crop-rotation regime to prevent soil erosion (Kalscheuer *et al.*, 2006). As a result, among the alternative sources, non-food crops, such as *Brassica napus*, *Brassica rapa*, *Jatropha curcas*, *Thlaspi arvense* and *Cuphea* spp., were considered for biodiesel production (Geller *et al.*, 1999, Iriarte *et al.* 2010*,* Kumar *et al.* 2010a*,* Moser *et al.*, 2009a, Moser *et al.*, 2009b, Moser and Vaughn, 2010, Patil *et al.* 2009*,* Zanetti *et al.*, 2009).

1.2 What are microalgae?

Microalgae are phylogenetically-diverse, photosynthetic, autotrophic eukaryotes: together with unicellular protozoa, they belong to the Domain Eukaryota. They are divided into four groups: *Cyanophyta* (blue-green algae), *Prochlorophyta, Rhodophyta* (red algae) and *Chlorophyta* (green algae). Microalgae are able to convert light energy and inorganic nutrients (e.g. carbon dioxide, nitrogen and phosphorus) into valuable biomass and high-value compounds (lipids, proteins, carotenoids, etc.). Most microalgae are free-living organisms: they are found in a variety of habitats, including soil and water (both fresh water and salt water), and in a wide range of environmental conditions (Parker *et al.*, 2008, Richmond, 2004).

Microalgae are cultured or grown commercially as an important source for use in aquaculture, cosmetics, nutraceutical and pharmaceutical industries. Extensive studies have been conducted to determine the biochemical composition of microalgae which could possibly be used as health food sources for human consumption, as feed additives in the commercial rearing of aquatic animals and as sources of phycocolloids which are

used as thickening and gelling agents for different industrial applications (Abbott, 1996, Bixler, 1996, Borowitzka, 1997, Brown and Farmer, 1994, Brown *et al.*, 1993, Deroeckholtzhauer *et al.*, 1993, Dunstan *et al.*, 1992, Mabeau and Fleurence, 1993, Volkman *et al.*, 1993, Volkman *et al.*, 1991, Volkman *et al.*, 1989, Volkman *et al.*, 1981). Humans have been consuming microalgae for health benefits and to prevent several diseases (Mares-Perlman *et al.*, 2002, Sukenik, 1991). For example, *Chlorella* sp. and *Spirulina* sp. have been sold as tablets since 1964 and 1975, respectively (Liang *et al.*, 2004).

Microalgae can be used as a renewable source to produce different types of biofuel. For instance, methane can be produced by anaerobic digestion using algae biomass (Spolaore *et al.*, 2006). Microalgal lipids are also able to convert into biodiesel through transesterification (Converti *et al.*, 2009, Pan *et al.*, 2010, Umdu *et al.*, 2009). However, as the costs of microalgal biodiesel production is still too high, other high value compounds can be produced this time.

A number of microalgae are known for their richness in high-value pigments such as lutein, zeaxanthin and β-carotene. The major photosynthetic pigments in the four groups of microalgae are listed in Table 1.1.

Table 1.1 Major photosynthetic pigments for four groups of microalgae

Microalgae groups	Major photosynthetic pigments
Cyanophyta	Chlorophyll α, β-carotene, mycoxanthin
Prochlorophyta	Chlorophyll α, chlorophyll β, β-carotene, zeaxanthin
Rhodophyta	Chlorophyll α, chlorophyll *d*, α-carotene, β-carotene, lutein
Chlorophyta	Chlorophyll α, chlorophyll β, α-carotene, β-carotene, ϒ-carotene

Source: James (1968)

The use of microalgae as a food source in the commercial rearing of aquatic animals is important as microalgae are at the beginning of the food chain. The survival, growth rates, quality and quantity of aquatic animals, especially fish, for human consumption are highly dependent on the quality of the algae supplied. The reason is that the polyunsaturated fatty acids (PUFAs) and other dietary components, such as proteins, carbohydrates, lipids and vitamins synthesized by the microalgae, are transferred from

them to the fish larvae for their growth and development (Abu-Rezq *et al.*, 1999, Gatesoupe and Robin, 1981, Hirayama and Funamoto, 1983, James and Aburezeq, 1988, Koven *et al.*, 1989, Sukenik *et al.*, 1993, Watanabe *et al.*, 1978). *Isochrysis galbana, Chaetoceros muelleri, Chaetoceros calcitrans, Skeletonema costatum, Thalassiosira pseudonana, Tetraselmis* sp., *Nannochloropsis* sp. and *Pavlova lutheri* are the microalgae species that are commonly used as feed additives in aquaculture (Borowitzka, 1997).

1.2.1 Potential of microalgae-based biodiesel

Oleaginous microalgae have been considered as one of the non-food biodiesel feedstocks. German scientists were the first to attempt to produce liquid fuel from microalgae during World War II (Cohen *et al.*, 1995). Several groups of scientists later conducted similar research on developing microalgae as a non-food biodiesel feedstock. These groups included scientists at the Carnegie Institution of Washington and at the National Renewable Energy Laboratory (NREL). In 1953, scientists at the Carnegie Institution of Washington published a book titled *Algal Culture from Laboratory to Pilot Plant* in which they suggested that the technologies for developing microalgae as a source of biodiesel had not been fully exploited. They added that there were several significant drawbacks: (1) the lipids for biodiesel production in microalgae were less known; (2) the microalgae oil production process was more focused on high-value products such as omega-3 polyunsaturated fatty acids; and (3) the prices for plant oil and animal-based fat for biodiesel production were cheaper (Burlew, 1953). The NREL was later shut down by the US Department of Energy (DOE) in the mid-1990s as a result of the low gasoline (petroleum) price (Sheehan *et al.*, 1998). It was not until 2008 that the US DOE renewed its investment in the NREL (Sheehan *et al.*, 1998).

Biofuel appears to have gained immense popularity and has emerged as an alternative to fossil fuels due to several beneficial characteristics. Firstly, biofuel is a renewable source of energy. Secondly, through the process of photosynthesis, the bio-feed stocks used for biofuel production are able to absorb carbon dioxide emitted in the biofuel combustion process from the atmosphere and, therefore, can recycle carbon dioxide back to the atmosphere (Brown *et al.*, 1998, Szulczyk and McCarl, 2010). The carbon dioxide level can be reduced from 60–90% in comparison to what is being emitted by

vehicles run on fossil fuels (Brown *et al.*, 1998). This would slow down climate changes and the warming of the Earth. Thirdly, large-scale production of biofuels can replace fossil fuels and, therefore, potentially reduce the demand and price of fossil fuels, thus preventing their depletion. Fourthly, large-scale production of biofuels can increase job opportunities within individual countries while also reducing the amount of fossil fuels imported from politically unstable countries (Szulczyk and McCarl, 2010).

Companies are attempting to commercialize microalgae biodiesel and to produce algal fuels that meet the standards for aviation petroleum used as jet fuel, with worldwide investment having reached over US$900 million (Deng *et al.*, 2009, Service, 2009). These include ExxonMobil partnering with California-based Synthetic Genomics Inc, Sapphire Energy, Solazyme, Green Fuels, Aurora Biofuels and the United Kingdom (UK)'s Carbon Trust (Deng *et al.*, 2009, Service, 2009). The first algal fuel test flights were performed in 2009 by Sapphire Energy, General Electric (GE), Continental Airlines, UOP and Boeing using a mixture of algae-, jatropha- and petroleum-based jet fuel (Deng *et al.*, 2009). In addition, according to the American Society for Testing Materials (ASTM), the biodiesel produced from microalgae, when compared to standard biodiesel, has a similar property.

Microalgae are able to utilize carbon dioxide, using the atmosphere as a carbon source, through the process of photosynthesis in a wide range of climates and environments (e.g. in ponds and wastewater) and in open (e.g. a raceway pond) or controlled conditions (e.g. a photobioreactor). By this process, they can provide several different types of renewable biofuels, such as methane, biodiesel and biohydrogen (Banerjee *et al.*, 2002, Brown *et al.*, 1998, Fedorov *et al.*, 2005, Gavrilescu and Chisti, 2005, Kapdan and Kargi, 2006, Melis, 2002, Szulczyk and McCarl, 2010, Valderrama *et al.*, 2002). For example, *Chlorella protothecoides* can grow photoautotrophically or heterotrophically under different culture conditions to produce biodiesel (Xu *et al.*, 2006). *Chlorella protothecoides* were found to be able to accumulate lipids at up to 55% of cell dry weight when fed with corn powder hydrolysate under heterotrophic conditions for 144 hours (Xu *et al.*, 2006).

Carbon dioxide provides 50% of the carbon in cell dry weight in microalgae (Miron *et al.*, 2003). As a result, approximately 180 tons of carbon dioxide can be recycled through the production of 100 tons of microalgae biomass (Converti *et al.*, 2009). Therefore, the Emissions Trading (ET)-Kyoto Protocol suggested microalgae are one

idea for plants that could be used in greenhouse gas emission (GHG) control (Converti *et al.*, 2009).

Microalgae are able to grow and produce biomass extremely rapidly (within a few days) compared to the time needed for oil crops. As a result, the biodiesel productivity and oil yield of microalgae are far in excess of what can be generated from oil crops (Table 1.2). For example, the microalgae that contain 30% oil are able to produce 51,927 kg of biodiesel per hectare per year while soybeans are only able to produce 562 kg of biodiesel per hectare per year (Table 1.2) (Deng *et al.*, 2009). Based on the current rate of biodiesel consumption in the US, 0.53 billion m^3 of biodiesel will be required if all transport fuel is to be replaced by biodiesel (Chisti, 2007). This level of demand cannot be satisfied by the large-scale cultivation of oil crops, and the use of waste cooking oil and animal fat. The average oil yield per hectare from soybeans, oil palms and microalgae (containing 30% oil [by wt] in biomass) are 172 L/ha, 136,900 L/ha and 58,700 L/ha, respectively (Table 1.2) (Chisti, 2007). In addition, the land area needed to satisfy 50% of all US transport fuel needs by using soybeans, oil palms and microalgae (containing 30% oil [by wt] in biomass) are 594 M/Ha, 45 M/Ha and 4.5 M/Ha, respectively (Chisti, 2007). Based on this calculation, the scenario would change dramatically as only 4.5 M/Ha of the total US cropping area used for microalgae biodiesel production would replace at least 50% of the supply of transport oil needs, leaving a million hectares of fertile soil in the country available for food production.

Table 1.2 Comparison of oil yield of different biodiesel feedstocks, and land area needed to meet 50% of all US transport fuel needs

Crop	Oil yield (L/Ha/year)	Biodiesel productivity (kg/Ha/year)	Land area (M/Ha) needed to meet 50% of all US transport fuel needs
Oil Palm	5950	4747	24
Corn	172	152	1540
Soybean	446	562	594
Jatropha	1892	656	140
Microalgae[a]	58700	51927	4.5
Microalgae[b]	136900	121104	2

[a]Algae containing 30% of oil (w/t) in biomass; [b]Algae containing 70% of oil (w/t) in biomass.

Source: Chisti (2007) & Deng *et al.* (2009)

The key to a successful microalgae based bio-refinery process is to have the ability to inexpensively produce large quantities of fast-growing oil-rich microalgae biomass. Therefore, it is crucial to choose the appropriate microalgae species that has high biomass productivity and is able to accumulate a high level of lipids. Lipid production can be evaluated in two ways, either by the lipid content, which is a percentage of lipids per dry biomass weight, or by lipid productivity, which is the amount of lipids produced per litre of working volume per day (Yen *et al.*, 2013). The averages of lipid composition and content vary between different types of microalgae. Triacylglycerols (TAGs) (with a fatty acid profile rich in C16 and C18) are the main lipids accumulated in microalgae and the main ingredient in the biodiesel transesterification reaction (Meireles *et al.*, 2003, Papanikolaou *et al.*, 2002). Microalgae with lipid content of 20–50% are common, with studies showing that these include *Nannochloropsis salina*, *Scenedesmus* sp., *Chlorella protothecoides*, *Choricystis minor*, *Dunaliella tertiolecta*, *Microcystis aeruginosa* and *Gymnodinium* sp. (Boussiba *et al.*, 1987, Campenni *et al.*, 2013, Chen *et al.*, 2011a, Chisti, 2007, Miao and Wu, 2004). However, at certain conditions, species such as *Botrococcus braunii* and *Schizochytrium* sp. able to accumulate up to 70% lipids in the cells (Chisti, 2007, Li *et al.*, 2010, Mansour *et al.*, 2003, Sobczuk and Chisti, 2010). In addition to TAGs, microalgae synthesize high levels of polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) (20:5ω3), in the oil, making these oils one of the sources of fatty acids for human consumption instead of being used as a feedstock for biodiesel production (Lubian *et al.*, 2000).

1.3 Microalgae based bio-refinery concepts

In summary, oleaginous microalgae have high potential as a source of feedstock in biodiesel production as they have the following advantages: (1) arable to CO_2 to generate biomass and thus to reduce greenhouse gas (GHG) emissions; (2) biodiesel produced from microalgae can be carbon-neutral; (3) higher photosynthetic efficiency; (4) shorter life cycle and higher biomass productivity than terrestrial oil crops as growth can be doubled in 24 hours; (5) able to produce high-value compounds such as carotenoids and proteins; (6) high lipid content and productivity with some microalgae species able to accumulate up to 70% lipids under certain conditions; (7) higher oil yield (L/Ha/year), higher biodiesel productivity (kg/Ha/year) and lower land area

needed (M/Ha) compared to other terrestrial oil crops; (8) microalgae are aquatic micro-organisms that can grow on non-arable land or rural areas under conditions that are unsuitable for food crops and, therefore, they do not compete for land needed for agriculture crops, while also offering new employment opportunities in rural areas; (9) microalgae are able to grow in salt water and thus do not compete with humans for fresh water; (10) microalgae are able to grow in wastewater by utilizing water contaminants such as ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}) as nutrients; and (11) biodiesel produced from microalgae is non-toxic and biodegradable.

Although systems based on photosynthetic microalgae have been the most promising for biofuel production, production on a commercial scale is not yet economically viable. Microalgae have the ability to alter the lipid, carbohydrate and protein content in their cells under stress conditions with certain microalgae species able to trigger the synthesis of high-value products such as carotenoids and vitamins. The major drawback for cultivation under stress conditions is decreased biomass productivity due to increased productivity of the compound of interest. Changes in composition in microalgae cells or the synthesis of these compounds increase the possibility of microalgae surviving under unfavourable conditions. Therefore, strategies have been applied, such as a multi-stage process in which microalgae are cultured under optimum conditions to maximize biomass production which is then followed by the application of stress factors to trigger the biosynthesis of these valuable compounds. The majority of high-value compounds from microalgae are either not established in the market or are still not commercialized. It seems that clear market opportunities for new microalgae high-value products exist. In addition, these high-value compounds have particular interest as they could become the potential source for animal feed, food additives and aquaculture, as well as being used for pharmaceuticals, nutraceuticals and cosmetics. However, the multi-stage process requires advanced technology, higher investment costs, high energy use and a detailed design process. Therefore, the optimization of the simultaneous production of biomass and high-value products during biofuel production has recently been the focus for improving the feasibility of the total production cost of the microalgae based bio-refinery process.

The definition of ‘biorefining’, based on IEA Bioenergy Task 42, is “[b]iorefining is the sustainable processing of biomass into a spectrum of marketable products and energy” (IEA). The idea of a bio-refinery involves the use of biological, chemical and physical

technologies to extract high-value compounds such as carbohydrates, proteins, lipids, oils, pigments, hormones, vitamins and proteins from biomass and the conversion of these compounds into marketable products. To date, the most successful refinery is the petroleum refinery where crude oil is separated by fractional distillation into different types of fuel and products such as liquefied petroleum gas, petrol, jet fuel, lubricants and waxes.

The microalgae based bio-refinery process to produce biomass as well as the separate desired compounds consists of five major steps: species identification and improvement; microalgae cultivation; harvesting; extraction (cell disruption and fractionation); and purification (Figure 1.1). One of the important components in the process is species identification and improvement. Careful screening, optimizing the culture conditions and understanding the metabolic pathway of the rapid-growing microalgae in order to produce lipids, secondary metabolites and high-value compounds all play an important role in achieving a cost-effective bio-refinery process. The cultivation process requires light, carbon dioxide, water and nutrients, such as nitrates, phosphate, trace metal elements and vitamins. Seawater supplemented with nutrients has generally been used to grow marine microalgae. According to Chisti (2007), the estimated production cost of microalgae biomass is about US$3000 ton^{-1} for a 100-ton per annum facility and this would decrease when the scale of the production operation scale increases. In addition to water and seawater, wastewater, with a high level of nitrogen and phosphorus, from various industries has also been used to cultivate microalgae (Hemalatha and Mohan, 2016, Molinuevo-Salces *et al.*, 2016, Olkiewicz *et al.*, 2016, Park *et al.*, 2011, Pittman *et al.*, 2011, Usha *et al.*, 2016). The microalgae based bio-refinery system is able to simultaneously produce microalgae biomass for biofuel production and to reduce pollutants in an ecologically safer way.

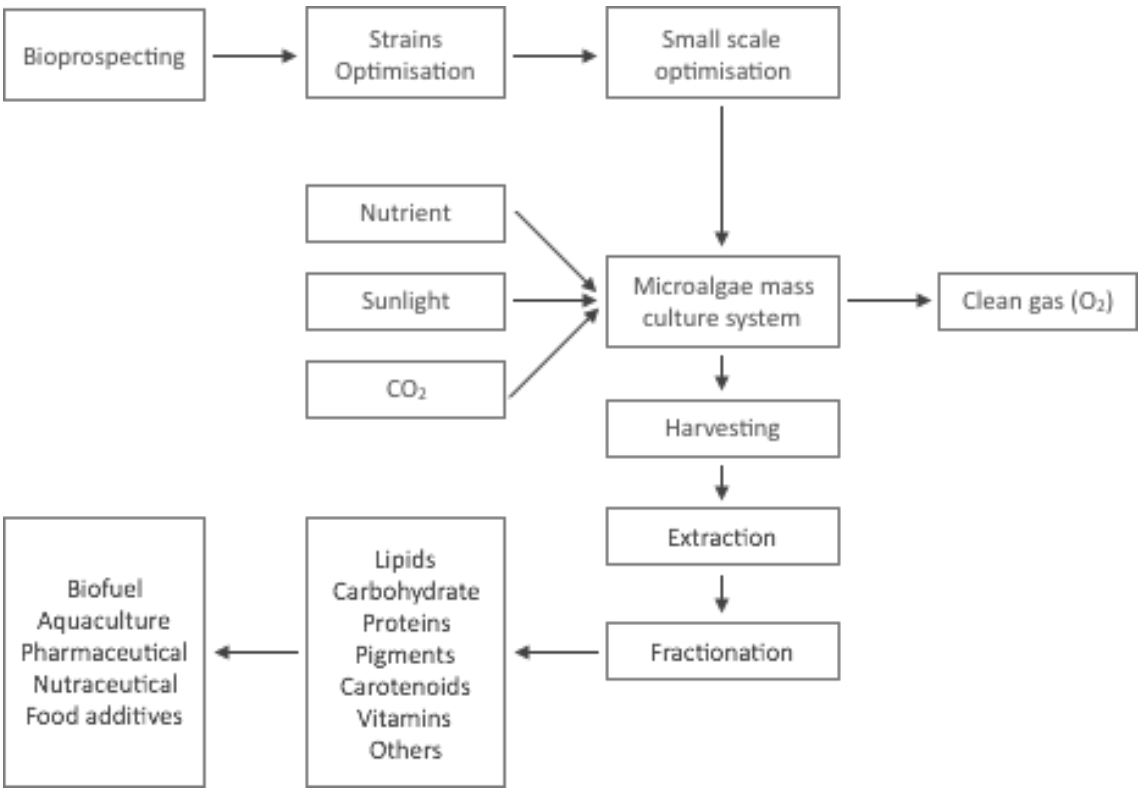


Figure 1.1 General scheme of microalgae bio-refinery concept

Currently, the steps of harvesting, extraction (cell disruption and fractionation) and purification are major bottlenecks in the bio-refinery process. An ideal harvesting process should involve low use of energy and low-cost operation and maintenance in achieving a high biomass concentration. Several biological, chemical and physical harvesting methods, such as chemical sedimentation, coagulation/flocculation, bioflocculation, gravity sedimentation, flotation, electrical-based methods, filtration and centrifugation are currently used to thicken and dewater the microalgae during the harvesting process (Barros *et al.*, 2015). These methods can be applied individually or in combination to improve effectiveness and to reduce the operation cost. The major drawbacks of these methods are: (1) chemical coagulation/flocculation may be expensive and toxic to microalgae; (2) changes in cellular composition; (3) time consuming; (4) high energy use and high cost; (5) initial high cost and maintenance of the membrane used for filtration; and (6) efficiency of the method is highly dependent on the size of the microalgae (filtration is more suitable for large microalgae such as *Coelastrum proboscideum*) (Barros *et al.*, 2015). Harvesting usually contributes 20–40% of the total cost of biomass production (Barros *et al.*, 2015, Molina *et al.*, 2003). However, to date, no single method has been found to be both economically viable and

efficient in harvesting low concentrations of microalgae biomass. This is due to the small size and low concentration of the microalgae and the large volume of water being used in the cultivation system (Barros *et al.*, 2015). For example, the average concentration of microalgae biomass in an open pond system is approximately 0.5–1 g/l with 50% TAG content; therefore, 1000 L of water is required to produce 1 kg of microalgae biomass and 0.5 kg of microalgae TAG (Chisti, 2007). The biomass achieved with microalgae is much less compared to that achieved in bacteria or yeast fermentation which is able to produce at least 100 g/l in a fermenter (Pienkos and Darzins, 2009).

The idea of a bio-refinery is to develop an extraction and purification method for a wide range of compounds of interest depending on their polarities (Nobre *et al.*, 2013, Serive *et al.*, 2012). However, the high energy use and cost-intensive extraction and purification downstream process are considered as major bottlenecks in the microalgae bio-refinery. In addition, the structure of microalgae with their rigid and thick cell walls is the biggest barrier for compound extraction. Numerous techniques have been used to disrupt the cell walls: these include homogenization, ultrasonication, microwave, solvents, acid-base disruption, Fenton's chemical reaction, hydrolytic enzymes and supercritical CO_2 (Kim *et al.*, 2016a). While these techniques have been very useful, the efficiency of extraction methods and operation conditions are highly dependent on the microalgae species and the cell wall characteristics (Kim *et al.*, 2016a, Popper *et al.*, 2011, Praveenkumar *et al.*, 2015). The toxicity of the chemicals and of the solvent used in the process should be considered as the major application of the high-value compounds, such as DHA, EPA and carotenoids, is for human consumption. In addition, it is difficult to separate more than one compound from the microalgae extracts without destroying the other compounds at the same time. To date; only a few studies have had success at simultaneously extracting and recovering different metabolites in a continuous method. Nobre *et al.* (2013) successfully extracted 4.5 g of lipids and recovered 70% of the pigments from 1 kg of dried *Nannochloropsis* sp. biomass using a supercritical CO_2 flow rate of 0.62 g/min, at 300 bar and 40°C with ethanol (20 wt%). The remaining biomass was fermented to produce biohydrogen using *Enterobacter aerogenes* with a yield of 6.06 g/kg of dried biomass. A similar study using a supercritical CO_2 flow rate of 30 g/min, at 600 bar and 60°C with 5% (w/v) ethanol successfully extracted lipids containing chlorophyll and carotenoids in *Chlorella vulgaris* (Safi *et al.*, 2014).

1.4 Fundamental roles of carotenoids

Carotenoids are a large class of isoprenoid-derived pigments that are synthesized *de novo* by both photosynthetic and non-photosynthetic organisms. In addition, carotenoids are found in heterotrophic bacteria and fungi. The global market value for carotenoids was about US$1.2 billion in 2010 and it is estimated that by 2018 it will be US$1.4 billion (Markou and Nerantzis, 2013).

Carotenoids play an important role in human health and nutrition, as well as in the production of animal feed additives. In humans, carotenoids are a major dietary source of biological antioxidants and for making retinoids (the main visual pigments), retinol (Vitamin A) and retinoic acid (a substance that controls morphogenesis). The correlation between the high consumption of carotenoids and health benefits was first reported in the 1970s. Dietary sources with high levels of carotenoids, when consumed by humans, are related to the prevention of many chronic diseases, cancer, cardiovascular diseases and age-related macular degeneration (Bone *et al.*, 2003, Kumar *et al.*, 2010b, Michaud *et al.*, 2000, Richer *et al.*, 2004, Slattery *et al.*, 2000). Moreover, carotenoids are able to induce apoptosis in T-lymphocyte cell lines (Muller *et al.*, 2002) and can protect genome stability (Collins, 2001). On the other hand, carotenoids are used as colourants in the food industry to pigment salmon and trout and to intensify the colour of egg yolks.

As humans do not have the capability to synthesize carotenoids, they depend on dietary sources of carotenoids, for example, vegetables and fruits. Approximately 60 different carotenoids consumed by humans have been identified as being from fruits and vegetables (Bauernfe, 1972, Khachik *et al.*, 1992). In addition, humans can also consume carotenoids by eating eggs, poultry and fish, but only if the poultry and fish have been fed plant or algal products.

Carotenoids are essential for plants as they play an important role in photobiological systems as light-harvesting pigments and as protective agents against chlorophyll-sensitized photo-oxidation. Furthermore, carotenoids serve as precursors for biosynthesis for the plant-growth regulator, abscisic acid.

1.4.1 Protective role of carotenoids in photosynthesis

Carotenoids can be divided into primary carotenoids and secondary carotenoids. Primary carotenoids are synthesized and accumulated in plastids. They function as accessory pigments in photosynthetic organisms (Cogdell and Frank, 1987, Grung *et al.*, 1992, Guedes *et al.*, 2011a). Secondary carotenoids are usually produced under oxidative-stressed growth conditions that cause cellular damage (Dodge, 1990, Foote *et al.*, 1968, Mathewsroth, 1987).

At high light intensity, the light-harvesting chlorophyll molecules are saturated. They receive and absorb more light energy than can be used in the electron transport system. Under these saturated conditions, the overexcited chlorophyll will undergo intersystem crossing to the triplet state (^{3}CHL*). The ^{3}CHL is able to pass its excitation energy to oxygen to produce the highly reactive singlet oxygen ($^1O_2$*) which can cause the destruction of lipids, membranes and tissues. This photodynamic effect can be prevented by carotenoid molecules that quench either the excitation energy of ^{3}CHL* or $^1O_2$* (Dodge, 1990, Krinsky, 1979).

1.4.2 Carotenoid biosynthesis pathway

Carotenoids, belonging to a class of tetraterpenoid pigments, are mainly C40 isoprenoids, which consist of a chromophore of eight or more isoprene units joined together. The carotenoid molecule extends from 3–15 conjugated double bonds. The absorption spectrum of the carotenoid molecule is determined by the length of the chromophore. The carotenoid biosynthesis pathway occurs within the chloroplasts of plants and algae, with the overall pathway shown in Figure 1.2 and having been discussed in detail by Britton (1983). The carotenoid biosynthesis pathway consists of three stages: (1) the formation of phytoene; (2) desaturation; and (3) cyclization. Carotenoids are also divided into two classes based on their backbones: carotenes (hydrocarbon carotenoids) and xanthophylls (oxygenated carotenoids) (Guedes *et al.*, 2011a).

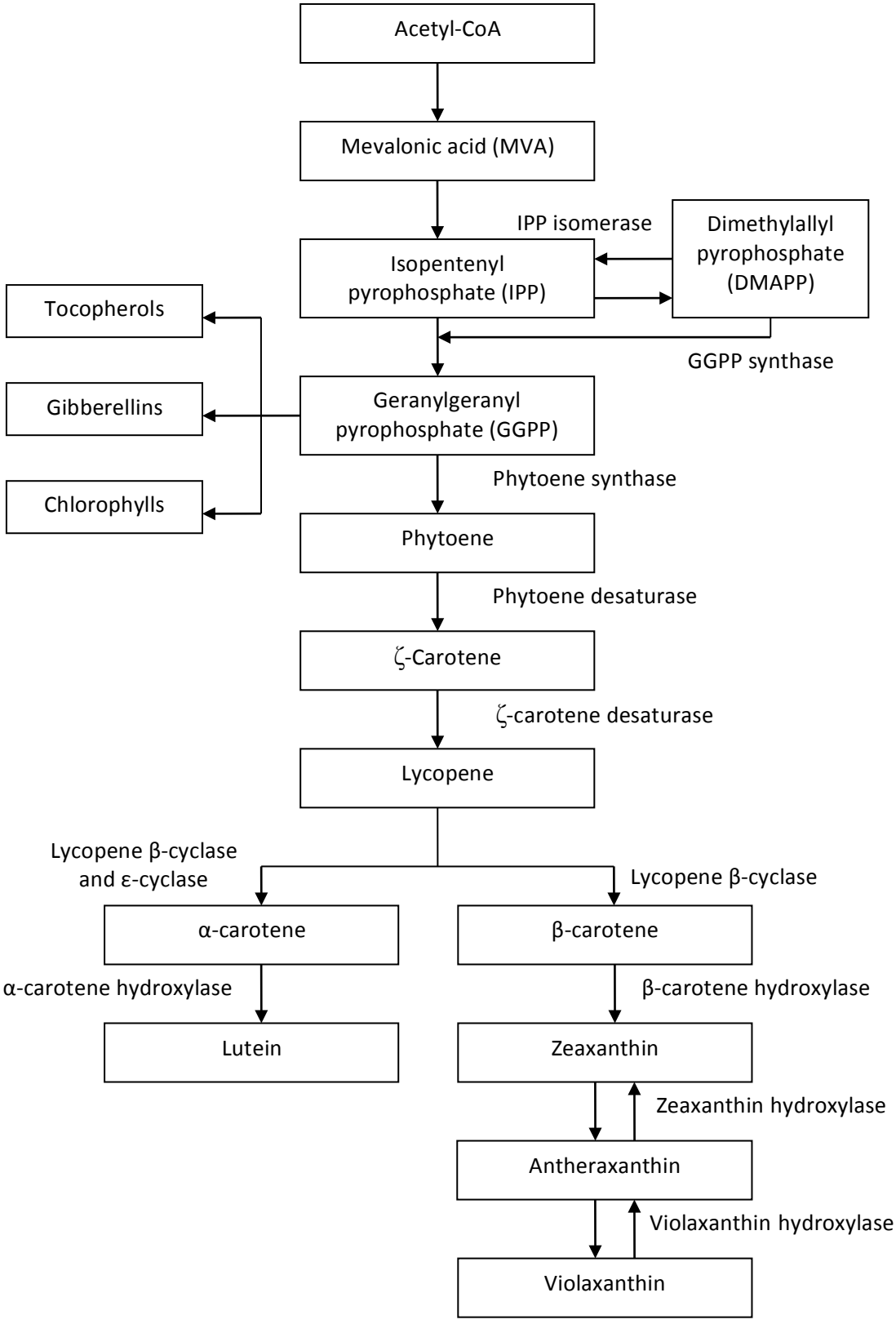


Figure 1.2 Carotenoid biosynthetic pathway

Source: Hirschberg (2001)

1.4.3 Lutein

Lutein is a type of pigment that exists in higher plants and other photoautotrophic microalgae. It belongs to a group of xanthophylls that are frequently used as food additives (e.g. to deepen the colour of egg yolks) but in which interest has recently been growing for nutraceutical and pharmaceutical applications. Most carotenoids on the market have been chemically synthesized, with the growing demand for natural additives making the production of lutein from plants and microalgae very attractive. The worldwide market for lutein, including food, nutraceutical and pharmaceutical applications, was worth US$233 million in 2010 and is expected to grow to US$308 million by 2018 (Lin *et al.*, 2015). In the EU, lutein, extracted from marigold flowers and used as a food additive, has the E number E161b (INS number 161b). Different types of commercial lutein products with lutein content ranging from 3–80% (w/w) are being sold in the market for different purposes: this includes capsules, granules, liquid, oleoresin and powders.

Lutein ([3R,3'R,6'R]-β,ε-carotene-3,3'diol) is a yellow xanthophyll, with the chemical formula $C_{40}H_{56}O_2$, a molecular weight of 568.88 and containing two cyclin end groups: β-ring and ε-ring (Figure 1.3). The synthesis of lutein starts from the liner, symmetrical lycopene, that undergoes cyclization by lycopene β-cyclase to become carotenoids that have cyclic β-ring and ε-ring end groups (Figure 1.3) (Britton *et al.*, 1998). The β-rings and ε-rings are formed separately and cannot be interconverted. The β-carotene with two β-rings is a precursor for xanthophylls such as zeaxanthin, entheraxanthin and violaxanthin, while α-carotene with one β-ring and one ε-ring is a precursor for lutein (Figure 1.3) (Williams *et al.*, 1967). Lutein is usually located in the light-harvesting complex II antenna protein and plays an important role in photosystem II (PSII) in higher plants and other photoautotrophic microalgae (Jahns and Holzwarth, 2012). In addition, lutein is able to absorb higher energy from blue light and can eliminate photo-oxidative damage from excessive illumination (Frank and Cogdell, 1996).

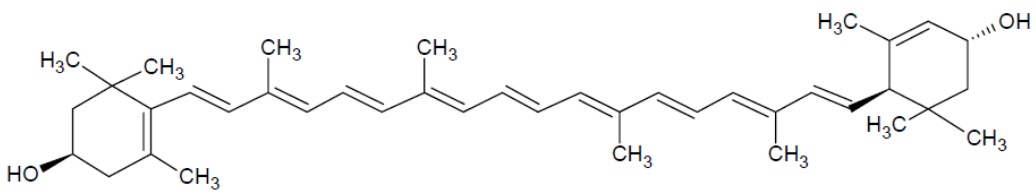


Figure 1.3 Structural formula of lutein

The use of lutein in nutritional supplements has increased in popularity in ageing societies for preventing age-related macular degeneration (AMD) that leads to blindness. Globally, 8.7% of blindness is caused by AMD (Tiwary *et al.*, 2014). Lutein are important for humans for several reasons: (1) humans are not capable of synthesizing lutein which they must obtain from food; (2) lutein is a strong antioxidant; (3) lutein is able to accumulate in the retina and absorb blue light to protect the underlying tissue from phototoxic damage; and (4) AMD can be reversed by the introduction of lutein in the diet, as long as the condition is not irreversible. Studies have shown how an adequate intake of lutein might be able to prevent AMD (Carpentier *et al.*, 2009, Gerster, 1991, Hammond *et al.*, 1996, Hammond *et al.*, 1997, Junghans *et al.*, 2001, Kamoshita *et al.*, 2016, Kijlstra *et al.*, 2012, Landrum *et al.*, 1997, Richer *et al.*, 2004, Roberts *et al.*, 2009, Sommerburg *et al.*, 1998). Hammond *et al*. (1996) were the first to recognize the correlation between dietary carotenoid intake and macular pigment density. This work was later followed by the focus on lutein by Landrum *et al.* (1997) and Hammond *et al*. (1996) in larger study groups. Recent studies by Kamoshita *et al.* (2016) evaluated lutein's effects on light-induced AMD in mice, with their results indicating that lutein usage produced the sustained reductions in oxidative stress necessary for AMD prevention.

Moreover, studies of dietary lutein have shown that there are several benefits: (1) lutein may have a role in preventing cell cancer as lutein is a strong antioxidant that is able to quench active radicals; (2) lutein might play a role in controlling the inflammatory pathway in the immune system; and (3) lutein might reduce the risk of heart disease (Chew *et al.*, 1996, Izumi-Nagai *et al.*, 2007, Mares-Perlman *et al.*, 2002, Michaud *et al.*, 2000, Muller *et al.*, 2002, Roberts *et al.*, 2009, Slattery *et al.*, 2000).

Although lutein can be consumed and incorporated into the human diet from green leafy vegetables and fruits, the recommended daily intake for lutein is 6 mg. Lutein is commonly found in fruits and vegetables but the content is very low (Sommerburg *et al.*, 1998). Furthermore, the lutein content varies between different vegetables and fruits, how they are cultivated, prepared and processed, and the presence of other factors such as fatty acids and fibre ingested in the same meal.

1.4.3.1 Lutein production from marigold flowers

Currently, the petals of marigold flowers, which are yellow to orange-red in colour, are a source of lutein for commercial production. Marigold flower petals usually contain 0.1–0.2% dry carotenoid matter of which 80% is lutein. Lutein generally exists in the form of lutein-fatty acid esters in the petals and is converted to free lutein upon saponification using an alkali solution during extraction (Gau *et al.*, 1983). Various species of marigolds including *Tagetes erecta* (African Aztec), *Tagetes patula* (French marigold) and *Calendula officianalis* (calendula or garden marigold) have been identified as opportunistic sources of lutein in China, India, Peru, Ecuador, Spain and Mexico. Currently, *Tagetes erecta* is the main flower species for commercial lutein production.

In general, commercial lutein production from marigold flowers involves cultivation, flower separation, ensilage, pressing, drying, hexane extraction, saponification and fine processing. After ensilage, dewatering and drying, lutein is extracted using hexane, followed by the removal of hexane to obtain oleoresin. The oleoresin is further purified to obtain lutein at the required level of purity in the form of crystal suspensions in oil, for use as a food additive or for human consumption (Barzana *et al.*, 2002). The fatty acid ester content of lutein in the flower petals ranges from 4 mg/g in greenish-yellow flowers to 800 mg/g in orange-brown flowers (Sowbhagya *et al.*, 2013). The most common colour of lutein ester is yellow to deep orange: with the more intense colours, lutein content increases.

However, several drawbacks of marigold-based lutein production make it infeasible to cultivate marigold flowers as a feedstock. Although the lutein content in *Tagetes erecta* can increase up to 20 g/kg of dry material, the growth rates of marigold flowers are slow. Moreover, the lutein content in marigolds is variable even though research has been conducted to optimize the lutein content in marigold petals by varietal improvement with different species. In addition, the cultivation of marigold flowers requires a large amount of land especially during the flowering season, which is from early July to the end of October in the Northern Hemisphere. Finally, the whole process from cultivation to fine processing highly relies on human labour, with extra labour required during the flower harvesting period.

1.4.3.2 Lutein production from microalgae

Lutein is found in certain strains of microalgae with most of the lutein in free lutein form. Microalgae are being considered as a potential source for commercial lutein production for several reasons: (1) higher lutein content per kilogram of dried biomass compared to marigold flowers; (2) biomass productivity; (3) able to synthesize valuable by-products such as lipids and other carotenoids; (4) can be cultivated on non-arable land; (5) require less labour involvement; (6) unlike marigold flowers in which the petals need to be separated, the whole microalgae biomass can be processed; and (7) can be cultivated in all seasons of the year in a bioreactor on a large scale.

Several species of microalgae have been identified as lutein producers, for example, *Muriellopsis* sp., *Scenedesmus almeriensis*, *Chlorella zofingiensis*, *Chlorella protothecoides*, *Chlorococcum citriforme*, *Chlorella sorokiniana*, *Chlorella vulgaris*, *Coccomyxa acidophila*, *Dunaliella salina* and *Neospongiococcus gelatinosum* (Cordero *et al.*, 2011b, Deenu *et al.*, 2013, Del Campo *et al.*, 2000, Del Campo *et al.*, 2001, Fu *et al.*, 2014, Sanchez *et al.*, 2008a, Sanchez *et al.*, 2008b, Vaquero *et al.*, 2012, Wei *et al.*, 2008). Of these lutein producers, only *Chlorella vulgaris, Muriellopsis* sp. and *Scenedesmus almeriensis* have been optimized under growth conditions and tested for large-scale lutein production (Del Campo *et al.*, 2007, Del Campo *et al.*, 2001, Fernandez-Sevilla *et al.*, 2008, Fernandez-Sevilla *et al.*, 2012, Jeon *et al.*, 2014). In a recent study, Jeon *et al.* (2014) optimized the culture medium for *Chlorella vulgaris*, focusing on magnesium sulphate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na) and a trace metal solution for lutein production in 25,000 L and 240,000 L bioreactors, and were able to increase 80% of the lutein concentration from 139 mg/l to 263.13 mg/l. In addition, Fernandez-Sevilla *et al.* (2008) designed a 10-unit 2800 L fence-type configuration bioreactor to cultivate *Scenedesmus almeriensis*. Their results indicated that, over a year, the average lutein productivity was 3.6 mg/l/day with maximum productivity of 10 mg/l/day.

1.4.3.3 Comparison of lutein content in marigold flowers and microalgae

Lutein is generally located within the cells: the lutein in marigold flowers is mainly in ester form while the lutein in microalgae is mainly free form. Cell disruption is one of the key steps during the extraction process. The molecular size of lutein is 27–28 ångströms (Å) (Lin *et al.*, 2015). Lutein ester found in marigolds is spread over the

marigold petals as a colour agent and has a linear length that is 2–3 times longer than free lutein. Even though the pore diameter in the plant cell wall is larger than the size of lutein (38–40 Å), lutein is still unable to pass through the cell walls easily (Lin *et al.*, 2015). However, microalgae have a thicker cell wall than marigold flowers and the free-form lutein in microalgae is usually bound with proteins and other pigments such as light-harvesting protein; therefore, a stronger cell disruption process is required to extract the lutein than is the case in marigold flowers.

The lutein content of *Tagetes erecta*, *Tagetes patula* and *Calendula officianalis*, based on dry weight of biomass, was from 0.829–27.946 g/kg, 0.597–12.31 g/kg and 0.04–0.301 /kg, respectively. However, the lutein content for various microalgae species was as follows: *Chlorella fusca* (4.2–4.7 g/kg of biomass); *Chlorococcum citroforme* (7.47 g/kg of biomass); *Coelastrum proboscideum* (3.4–5 g/kg of biomass); *Chlorella zofingiensis* (2.8 g/kg of biomass); *Chlorella sorokiniana* (7 g/kg of biomass); and *Muriellopsis* sp. (5.5 g/kg of biomass) (Cordero *et al.*, 2011b, Del Campo *et al.*, 2000).

Overall, lutein content (measured in g/kg) was lower in microalgae-based lutein production when compared with marigold-based lutein production. However, marigold-based lutein production requires a large amount of land and is labour-intensive, while microalgae-based lutein production requires advanced technology, higher investment costs and a detailed design process. Therefore, microalgae-based lutein production should not only focus on optimizing lutein content and extraction, but should also increase biomass and by-product productivity to compensate for the higher production cost compared to marigold-based production. In the near future, when lutein supplements are more popular and lutein is proven to reverse age-related macular degeneration (AMD), when less land is available and labour is more expensive, the best technological approach will be microalgae-based lutein production.

1.4.3.4 Improving lutein content in microalgae

The potential of microalgae as a commercial source of lutein is widely recognized (Chiu *et al.*, 2016, Del Campo *et al.*, 2007, Del Campo *et al.*, 2000, Guedes *et al.*, 2011a, Sanchez *et al.*, 2008b, Soares *et al.*, 2016, Vaquero *et al.*, 2014b, Wei *et al.*, 2008). The high capital cost due to the low lutein productivity in microalgae is a major bottleneck in the microalgae based bio-refinery process. Microalgae with improved biomass

productivity and enhanced lutein accumulation would be able to make the microalgae based bio-refinery process more economically feasible. Moreover, the synthesis of lutein in microalgae can be improved by using mutation methods focused on certain carotenoid pathways, or can be manipulated by applying stress factors, such as imposing chemical or physical stimuli on the environment during the cultivation period.

Chemically or physically induced mutations focus on the carotenoid biosynthesis gene, with mutant selection using specific inhibitors being one of the methods for obtaining mutants with a higher carotenoid content. *Chlorella sorokiniana* mutants with a twofold higher lutein content have been obtained by random mutagenesis and selection using inhibitors with this presented in a study carried out by Cordero *et al.* (2011b)

Lutein is a carotenoid for which the synthesis can be triggered by certain stress conditions or variations in cultivation conditions. A wide range of studies have been carried out on lutein induction using stress factors which have had a significant impact on microalgae, such as the use of irradiance, pH, temperature, nitrogen sources, salinity and the presence of oxidizing compounds (Table 1.3) (Del Campo *et al.*, 2001, Rao *et al.*, 2007, Sanchez *et al.*, 2008a, Sanchez *et al.*, 2008b, Vaquero *et al.*, 2012, Vaquero *et al.*, 2014b, Wei *et al.*, 2008). The effects of these factors on lutein content and productivity are summarized in Table 1.3. However, it is clear that these stress factors are considered to be species-specific. Irradiance is the most well-known factor in terms of factors influencing lutein production. High irradiance leads to damage to the photosynthetic antenna system and to the photosystem II (PSII) reaction centre (Li *et al.*, 2009). Therefore, the chlorophyll and carotenoid content decrease when irradiance is increased (Bhandari and Sharma, 2006).

To date, only two species (*Muriellopsis* sp. and *Scenedesmus almeriensis*) have been studied in relation to different stress factors and cultivation in a mass production system (Del Campo *et al.*, 2000, Del Campo *et al.*, 2001, Sanchez *et al.*, 2008a, Sanchez *et al.*, 2008b). A study conducted by Del Campo *et al.* (2000) and Del Campo *et al.* (2001) using *Muriellopsis* sp. indicated that nitrogen deprivation, increased light intensity and increased salinity resulted in a decrease in lutein content. However, increased temperature and pH were able to significantly increase lutein content in *Muriellopsis* sp. When the temperature was increased from 28–33°C, a sixfold increase in lutein content was observed. In addition, a sevenfold increase in lutein content was observed when *Muriellopsis* sp. was cultivated at between pH 6–9. On the other hand, the lutein content

of *Scenedesmus almeriensis* increased up to 0.46% when cultivated under high irradiance (1700 μmol photons $m^{-2}s^{-1}$) and at a high temperature of 30°C.

Table 1.3 Stress factors affecting lutein content in different microalgae species

Factor	Microalgae species	Effect	Changes in lutein after induction	Reference
Irradiance	*Coccomyxa onubensis*	+	Maximum lutein content 1.0 g/l	Vaquero *et al.*, 2014a
	Tetraselmis suecica	+	Higher production of lutein under UV irradiance	Borghini *et al.*, 2009
	Chlamydomonas acidophila	+	Increased lutein production to 0.2 mg/l under 1000 $LEm^{-2}s^{-1}$	Garbayo *et al.*, 2008
pH	*Scenedesmus obliquus*	+	pH 6 at 30° C increased lutein content to 203 mg/ml	Guedes *et al.*, 2011b
Temperature	*Scenedesmus almeriensis*	+	Maximum lutein of 0.54% d wt at 44°C	Sanchez *et al.*, 2008a
	Chlorella protothecoides	+	Maximum lutein yield at 35°C	Shi *et al.*, 2006
Nitrogen source availability	*Coccomyxa acidophila*	+	Lutein accumulated up to 3.55 mg/g when cultures were grown on urea at pH < 2.5	Casal *et al.*, 2011
Salinity	*Botryococcus braunii*	+	Twofold increase in lutein content under salinity at 85 mM	Rao *et al.*, 2007
	Dunaliella sp.	-	High light irradiance (480 mu mol quanta) and salinity (160 g/l and 200 g/l) decreased lutein content	Fu *et al.*, 2014
Oxidative stress	*Chlorella protothecoides*	+	Addition of H_2O_2, NaClO and Fe^{2+} increased lutein content to 1.98 mg/g	Wei *et al.*, 2008

1.5 Broad research objectives

The broad objectives of this research are as follows:

- To develop a method using small samples of microalgae which allows both rapid extraction and quantification of lutein.
- To optimize the nutrient medium composition for *Chloroparva pannonica* biomass production
- To enhance biomass yield and lutein content of *Chloroparva pannonica* using random mutagenesis by UV-C irradiation and selection using inhibitors
- To use polyphasic taxonomy to characterize the *Chloroparva pannonica* wild type, and confirm the identities of the mutants - Cu40 parent culture and strains Fu4, Fu5C and Fu41A.
- To develop a production system for enhanced biomass and lutein production by *Chloroparva pannonica* in a photobioreactor.

Chapter 2 Methodology

This chapter, which is divided into two parts, examines the research methods and materials used in this study. The first part (Section 2.1) focuses on the development of carotenoid extraction. A brief introduction of the first part is provided, followed by the presentation of methods and materials, results and discussion. The second part (Section 2.2) outlines the general methods and materials used in this research. The specialized techniques, including modifications to existing techniques, are outlined in the relevant experimental chapters.

2.1 Introduction: carotenoid extraction

Carotenoids belong to a class of tetraterpenoid pigments: they are constructed from a 40-carbon polyene chain resulting in distinctive molecular structures and chemical properties such as light harvesting for photosynthesis and scavenging of reactive oxygen species (ROS) (Fernandez-Sevilla *et al.*, 2010). Carotenoids are naturally occurring pigments that have important roles in photosynthesis and photo-protection in plants (Demmig-Adams and Adams, 2002, Demmig-Adams and Adams, 1996), and as antioxidants and blue light filters in human tissue (Alves-Rodrigues and Shao, 2004, Bendich and Olson, 1989, Kijlstra *et al.*, 2012, Krinsky, 2001, Krinsky *et al.*, 2003, Leach *et al.*, 1998). They can be divided into primary and secondary carotenoids. Primary carotenoids are the components that produce photosynthesis in cells while secondary carotenoids are usually produced under stressed growth conditions(Grung *et al.*, 1992, Guedes *et al.*, 2011a).

Based on their backbone, carotenoids are divided into two classes: carotenes (hydrocarbon) and xanthophylls (oxygenated) (Guedes *et al.*, 2011a). Xanthophylls are a type of oxygenated carotenoid containing oxygen which is present as –OH (hydroxyl) groups, oxy groups or a combination of both. Lutein is a type of xanthophyll that is frequently used as a food additive but in which interest has recently been growing for nutraceutical and pharmaceutical applications. In addition, it is a food colourant allowed by the European Union (EU) with its sales amounting to US$150 million per year in the United States (US) alone (Fernandez-Sevilla *et al.*, 2010). Studies have shown how

consumption of lutein might be beneficial to human health, preventing cell damage to eyes and also preventing age-related macular degeneration (Moeller *et al.*, 2006, Snodderly, 1995, Yildirim *et al.*, 2011). Lutein is a type of antioxidant and has the ability to solubilize in animal tissue, accumulate in the retina and filter out blue light, and also to inactivate singlet oxygen to prevent cancer (Bartlett and Eperjesi, 2007, Chasan-Taber *et al.*, 1999, Granado *et al.*, 2002). Currently, lutein is obtained from petals of marigold flowers, but the lutein content in petals is variable, with production of marigold petals being labour-intensive and requiring a large amount of land. However, the increased demand for lutein has resulted in growing interest in its extraction and analysis from different sources such as certain strains of microalgae, for example, *Chlorella zofingiensis*, *Murielopsis* sp. and *Scenedesmus almeriensis* (Del Campo *et al.*, 2007, Del Campo *et al.*, 2000, Zapata *et al.*, 2000). The main reasons that make microalgae a good lutein producer are as follows: the lutein content in the cells, the biomass productivity, it can be cultivated on non-arable land and it requires less labour involvement. For example, in a case reported by Del Campo *et al.* (2007), *Murielopsis* sp. was able to produce 4.64 g of lutein from over a kilogram of dried biomass per square metre.

In many research studies, the quantification of the composition of carotenoid content from phytoplankton is very important as it contains a wide range of molecular structures with different polarities. This allows researchers to access the chemotaxonomy of microalgae to distinguish individual species, cell physiology and also the nutrition value of the cells (Deventer. B and Heckman, 1996, Hou *et al.*, 2011, Rebolloso-Fuentes *et al.*, 2001). The analysis and quantification of carotenoid content can be carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) (Li *et al.*, 2002). This very powerful and useful tool separates individual carotenoids and quantifies the concentration of lutein extracted from microalgae. The disadvantage of RP-HPLC is that it is a very complex, expensive and time-consuming process when large amounts of pigment extracts need to be analysed. It is therefore worthwhile to consider a method that allows the rapid estimation of lutein prior to RP-HPLC analysis, especially when screening a large number of microalgae strains.

Numerous studies have been conducted on methods of extracting carotenoids from different microalgae. The common steps in extracting certain carotenoids from

microalgae include harvesting, cell disruption, and carotenoid extraction and determination.

The difficulty of extracting lutein from microalgae has inspired a number of laboratories to develop different extraction methods to extract and quantify lutein content from microalgae. The biggest challenge for the extraction of carotenoids from microalgae is the need to break the thick hard cell wall as the carotenoid is retained within the cells. The disruption of cells can usually be accomplished by using chemical, mechanical or enzymatic procedures such as ultrasonic, microwave, mortar, bead mill, bead beating, high-pressure homogenizer, French press, Hughes press, cell lysis with solvent or detergents, temperature shock and osmotic shock (Chisti and Mooyoung, 1986, Henriques *et al.*, 2007, Pasquet *et al.*, 2011). The second challenge has a direct effect on the first challenge, with this being the selection of the solvent and the percentage of solvent used during the extraction process. The reason is that solvents play an important role in the extraction ability and solubility of the carotenoid, and also in cell lysis (Leon *et al.*, 1998, Mojaat *et al.*, 2008a). Methanol was the first solvent commonly used for carotenoid extraction, but the biggest drawback of this solvent is its toxicity. Therefore, it was replaced by other types of solvent, including acetone and ethanol. The other cell disruption methods are shown in Table 2.1 (Hejazi *et al.*, 2002, Jeffrey and Humphrey, 1975, Leon *et al.*, 2003, Porra *et al.*, 1989).

Table 2.1 Methods for carotenoid extraction from microalgae

Cell disruption method	Extracting solvent
Osmotic shock	Ethanol, hexane, cyclohexane and benzene
Thermal treatment	Halogenated, aliphatic, aromatic hydrocarbon, mixture of one acid ester and oil
Homogenization	Edible oil
Osmotic shock mechanical methods	Hexane, cyclohexane and petroleum ether
Thermal treatment	Edible oil
Strong solvent	Methylene chloride, ethanol, acetone, methanol and diethyl ether

Source: Hejazi *et al.* (2002), Jeffrey and Humphrey (1975), Leon *et al.* (2003) & Porra *et al.* (1989)

The increased demand for lutein and the disadvantage of lutein production from dry marigold petals have resulted in researchers seeking alternative lutein sources. Many microalgae have been isolated and reported as lutein producers, and they can be competitive with marigold petals (Del Campo *et al.*, 2007, Miyazawa *et al.*, 2013). However, lutein production based on microalgae requires higher costs and more extensive technology for its operation. Therefore, many studies are now focusing on the improvement, cultivation and biomass productivity of different microalgae strains for lutein production (Fu *et al.*, 2014, Vaquero *et al.*, 2014a, Vaquero *et al.*, 2014b). The most important aspect of this research is to screen more strains of microalgae from the large number of microalgae populations to identify those that are high lutein producers.

The difficulty of extracting carotenoids from microalgae has inspired a number of laboratories to develop different extraction methods to extract and quantify carotenoid content from microalgae (Ceron *et al.*, 2008, Fernandez-Sevilla *et al.*, 2012, Hejazi *et al.*, 2002, Li *et al.*, 2002a, Mendes-Pinto *et al.*, 2001, Roukas and Mantzouridou, 2001). These techniques have been very informative and useful. However, the significant drawbacks in analysing a large number of microalgae strains to identify the high lutein microalgae producers are that it is a complicated, time-consuming and expensive process, requiring a large amount of samples and toxic solvents as well as many purification steps. As lutein is constructed of many conjugated double bonds and is a type of highly unsaturated molecule, it can degrade when exposed to light and temperature, and also can easily oxidize during the process (Henry *et al.*, 1998, Minguezmosquera and Jarengalan, 1995). Consequently, lutein loses its colour and function; therefore, it is important to develop a method for screening lutein-rich microalgae that is fast, cost-effective and environmentally friendly.

To achieve this aim, the current study has focused on developing a one-step closed-tube method, without the need for large amounts of samples and solvent, which allows both the rapid extraction and quantification of lutein content from microalgae samples. The method in the study utilized glass beads, a sonicator, acetone, an ultraviolet (UV) spectrophotometer and high-performance liquid chromatography (HPLC) to extract, analyse and quantify the lutein content from microalgae.

The first part of the study focused on the detection of lutein using the UV spectrophotometer and on the correlation between the UV spectrophotometer results with the HPLC analysis. The second part of the study concentrated on comparing the

lutein concentration obtained by the traditional extraction method with that obtained by the rapid extraction method. In addition, the study investigated the extraction time and solvent percentage (pure acetone [100%] and aqueous acetone [90%]) of the rapid method. The last part of the study focused on pigment stability during the traditional and one-step closed-tube extraction methods under light and dark conditions, and also the effect of light on lutein extract stability over time.

2.1.1 Methods and materials: carotenoid extraction

2.1.1.1 Microalgae culture conditions

The microalgae strain used in this study was *Chloroparva pannonica* Cu40, a copper-adapted strain from wild type *Chloroparva pannonica*, obtained from the South Australian Research and Development Institute (SARDI). Cultures of *Chloroparva pannonica* were grown photoautotrophically in Guillard's F/2 medium (Guillard and Ryther, 1962) with seawater: deionized water (1:1 v/v). The preparation of the medium was performed, based on the method described in Section 2.2.2, with 40 µg/L of copper sulphate ($CuSO_4$). The liquid medium was sterilized at 121°C at 100 kPa for 15 minutes. The *Chloroparva pannonica* strain was grown at 25±1°C for 10 days under a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod and bubbled through with CO_2-enriched air (1%).

2.1.1.2. Standard curve for lutein

Analytical standard lutein (Sigma-Aldrich: 7168) was used as the standard solution. The lutein was diluted with 90% acetone (40 mg lutein/L) as a stock standard solution. The tube was stored in the dark in an airtight container at -70°C for up to six months. Prior to HPLC analysis, serial dilutions were made from the stock standard solution. All samples were performed with triplicate independent dilutions.

2.1.1.3 Extraction method for lutein

Acetone was chosen for the extraction process. Pure acetone (100%) and aqueous acetone (90%) were tested using the traditional method (subsection 2.1.1.3.1) and the

one-step closed-tube method (subsection 2.1.1.3.2). All samples were processed again for a second and third time in a fresh portion of solvent to determine the completeness of the extraction. Extractions were performed in triplicate. Lutein concentration was determined using UV-spectrophotometer spectrum scans and RP-HPLC methods (see subsections 2.1.1.5 and 2.1.1.6). The amount of lutein from the first extraction was determined as a percentage of the total of the three extractions. This gave a measure of efficiency of the initial extraction.

For the one-step closed-tube method, extraction in the sonicator with aqueous acetone (90%) was tested at four different times: five minutes, 10 minutes, 15 minutes and 20 minutes. Extractions were performed in triplicate and were repeated three times. The method used to determine lutein concentration was UV-spectrophotometer spectrum scans (see subsection 2.1.1.5).

2.1.1.3.1 Traditional extraction method

The traditional extraction method, described below, was carried out according to Wright *et al*. (1991).

A microalga sample of 20–50 ml of culture was filtered through a pre-weight nitrocellulose membrane (Millipore™ AAWP, nominal pore size 0.8 µm) using a vacuum pump filtering system under low vacuum pressure (≤100 mm Hg) and rinsed twice with sterile seawater: deionized water (1:1 v/v). The filter was removed from the filter base and folded once with the microalga sample inside. The filter was dried at 60°C to a constant weight, cooled down in a vacuum desiccator for two hours and then weighed. If the filter was not to be processed immediately, it was wrapped in aluminium foil, thus protecting the microalga from light, and stored for up to three weeks in the desiccator in a -20°C freezer until extraction.

The workplace lighting was kept to a minimum. The filter was subsequently cut into small pieces and pushed to the bottom of the 10 ml tissue grinder (Thomas Scientific, Potter-Elvehjem, coated, 50 mm x 20 mm Teflon glass pestle). Next, the microalga sample was extracted with 10 ml of acetone: water (9:1 v/v) and the pieces were slowly ground with the Teflon rod until they dissolved. The tube was affixed to a shaker and subjected to continuous shaking at 115 rpm for two hours in the dark in a 4°C cold room. The sample was then centrifuged at 8000 rpm for five minutes at 4°C, and the supernatant containing the pigments was collected. The extraction procedure was

repeated two more times until the cell debris was almost colourless. The samples had to be protected from light and heat. A blank filter was extracted in the same way as the sample to assess the possibility of the contamination of the reagents or apparatus. All extractions were performed in triplicate.

2.1.1.3.2 One-step closed-tube method

A microalga sample of 2–6 ml of culture was harvested by centrifugation at 10,000 rpm for 10 minutes in a 2 ml microcentrifuge tube, and then washed twice with sterile seawater: deionized water (1:1 v/v). The sample was subsequently dried at 60°C to a constant weight, cooled in a vacuum desiccator for two hours and then weighed. If the dried sample was not to be processed immediately, it was protected from light and stored for up to three weeks in the desiccator in a -20°C freezer until extraction.

Workplace lighting was kept to a minimum. An amount of glass beads (0.5 g) sufficient to cover the conical part of the microcentrifuge tube was used. The pellet was resuspended in 1.5 ml of acetone: water (9:1 v/v), vortexed with the suspension then placed in the ultrasonic bath at 4°C for five minutes. The tube was then affixed to a shaker and subjected to continuous shaking at 115 rpm for one hour in the dark in a 4°C cold room. Next, the sample was centrifuged at 8000 rpm for five minutes at 4°C, and the supernatant containing the pigments was collected. The extraction procedure was repeated two more times until the cell debris was almost colourless. The samples had to be protected from light and heat. A blank tube with glass beads was extracted in the same way as the sample to assess the possibility of the contamination of the reagents or apparatus. All extractions were performed in triplicate.

2.1.1.4 Influence of light on lutein stability and degradation of pigment extracts

The lutein stability during pigment extraction was tested by performing the one-step closed-tube extraction method (subsection 2.1.1.3.2), in triplicate, in light and dark conditions with three different concentrations of cell biomass (2 mg, 4 mg and 6 mg). The lutein extracts were subsequently exposed to light intensity of approximately 20–30 μmol photons $m^{-2}s^{-1}$ illumination (which is approximately the intensity range of light indoors) for 60 minutes at room temperature. Lutein extracts were collected from the vials every 15 minutes with HPLC used to determine the lutein concentrations (subsection 2.1.1.6).

2.1.1.5 UV wavelength scan

The pigment extracts were transferred into a flat-bottom MicroWell™ 96 well plate. Each sample's spectrum was generated with wavelength scanning from 300–700 nm using a spectrophotometer (BioTek Synergy™ 4 Hybrid Microplate Reader).

2.1.1.6 High-performance liquid chromatography (HPLC) operations and analysis

In line with the description by Wright *et al*. (1991), HPLC operations and analysis were carried out. Work needed to be performed in subdued light with samples protected from light and heat in amber HPLC vials. The HPLC operation and analysis method were optimized by NCRIS Algae Biofuel Facility, South Australia.

2.1.1.6.1 Reagents and solutions

Acetone, methanol, acetonitrile and ethyl acetate were purchased from Merck (Germany) with ultrapure water used in the analysis. The solvent system contained the following as described by Wright *et al.* (1991):

- Solvent A: methanol: 0.5 M ammonium acetate (80:20 v/v) pH 7.2
- Solvent B: acetonitrile: water (90:10 v/v)
- Solvent C: ethyl acetate

The solvent was filtered through a 0.45 μm cellulose triacetate membrane (HVWP, Merck Millipore).

2.1.1.6.2 HPLC system

Analytical separations were performed by HPLC (Agilent 1100 series, Agilent Technologies, USA) using a reversed-phase 250 mm x 4 mm (5 μm) C18 column (Merck Millipore). The column temperature was set at 20°C and the auto sampler was chilled to 10°C. The pump was programmed with the gradient conditions as shown in Table 2.2. The flow was fixed at 1 mL/min with the detection wavelength at 430 nm.

Table 2.2 High-performance liquid chromatography (HPLC) gradient conditions

Time	Solvent A%	Solvent B %	Solvent C%
0.0	100	0	0
2.0	0	100	0
2.6	0	90	10
13.6	0	65	35
20.0	0	31	69
22.0	0	100	0
25.0	100	0	0
30.0	100	0	0

The lutein concentration (in mg/L) was calculated from the standard curve (Figure 2.5):

$$Lutein\ concentration\ \left(\frac{mg}{L}\right) = \frac{HPLC\ area + 80.686}{342.61}$$

The lutein concentration (in mg/Kg of biomass) was calculated using the equation below:

$$Lutein\ concentration\ \left(\frac{mg}{kg\ biomass}\right) = \frac{Extraction\ volume \times lutein\ concentration\ (\frac{mg}{L})}{Total\ cell\ pellet\ weight}$$

2.1.2 Results

2.1.2.1 Standard curve for lutein

The spectra scans of the authentic standards of lutein using the UV-spectrophotometer are shown in Figure 2.1. The spectra scans reveal two peaks at 440 nm and 479 nm, with an extended shoulder at the left-hand side of the first peak at around 423 nm, and the highest point at the first peak. The dilution in the spectra of the authentic standards of lutein ranged from concentrations of 1 ml/L to 40 mg/L for the first peak and second peak, as shown in Figure 2.2 and Figure 2.3.

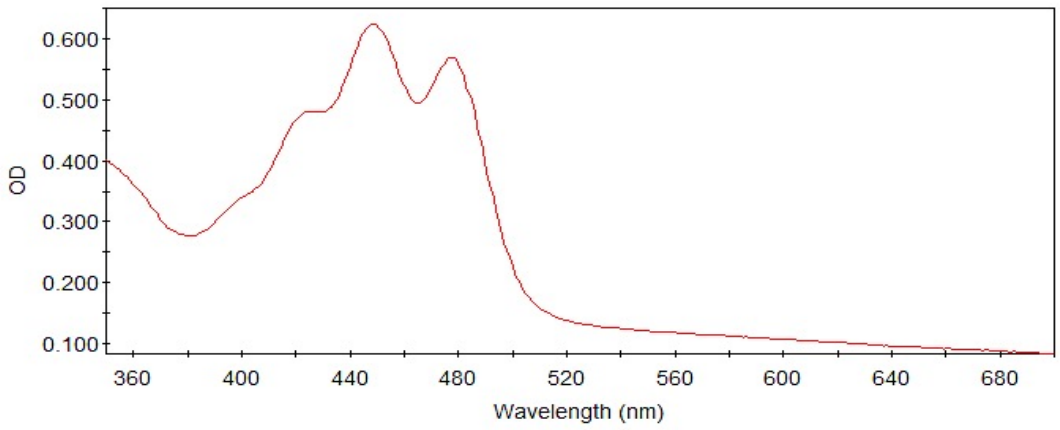


Figure 2.1 Absorption spectra of authentic lutein standards

Note: OD: optical density

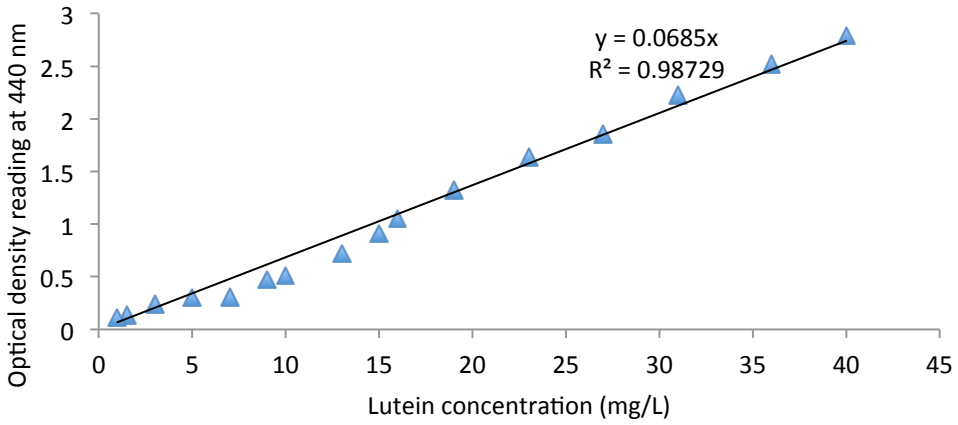


Figure 2.2 Optical density reading at 440 nm for serial dilutions of authentic lutein standards

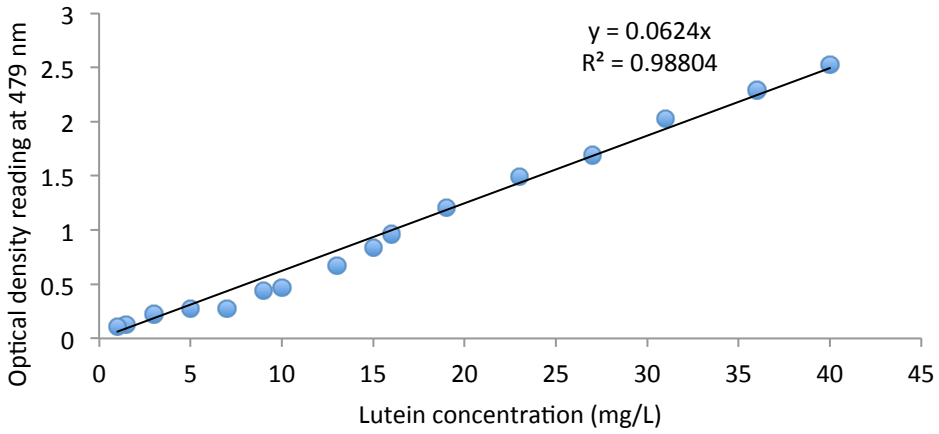


Figure 2.3 Optical density reading at 479 nm for serial dilutions of authentic lutein standards

Figure 2.4 presents the RP-HPLC chromatogram showing the authentic standards of lutein. The chromatogram consists of one peak at a retention time of 17 minutes. Figure 2.5 shows the standard curve of the authentic standards of lutein, with concentrations ranging from 1 mg/L to 40 mg/L. The relationship, described by the equation of the line Y = 342.61x-80.686, is then used to transform the measurements made on test samples into estimates of the amount of lutein present.

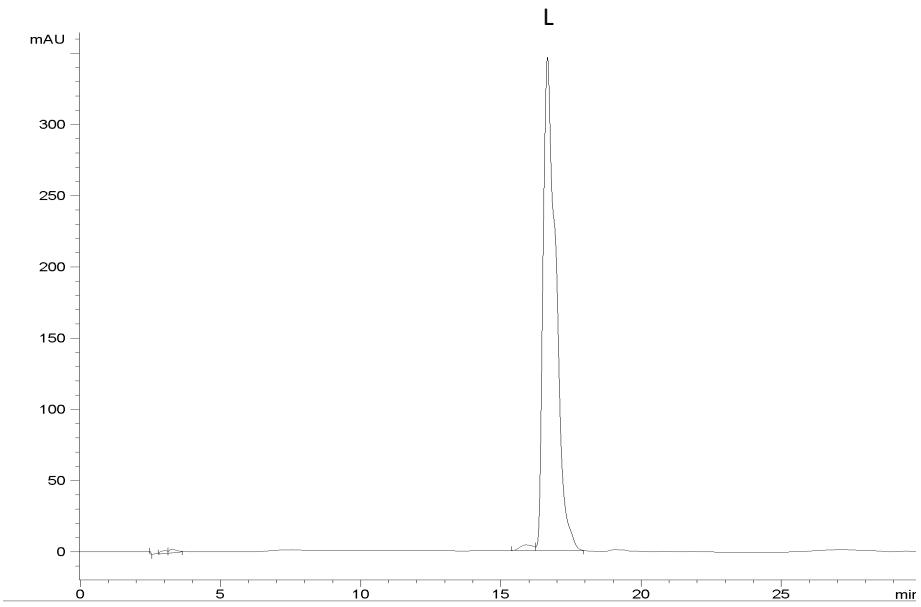


Figure 2.4 RP-HPLC chromatogram of authentic standards of lutein at a concentration of 16 mg/L with detection wavelength at 440 nm

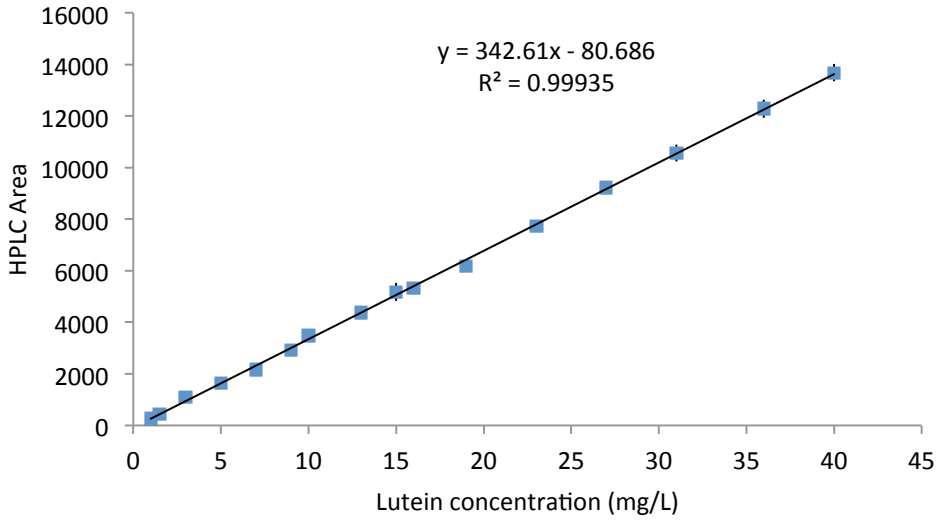


Figure 2.5 Standard curve of authentic standards of lutein

2.1.2.2 Comparison between traditional and one-step closed-tube methods of effect on extraction solvent concentration

2.1.2.2.1 Spectra scans of pigment extracts

The pigment extracts were analysed using a UV-spectrophotometer. The spectra scans of pigment extracts using the traditional method with pure acetone (100%) and aqueous acetone (90%) are shown in Figure 2.6. The spectra scans for both 100% and 90% acetone consist of two peaks at 430 nm and 660 nm, with two extended shoulders at the left-hand and right-hand sides for the first peak around 410 nm and 465 nm. The pigment extraction using 90% acetone has a higher optical density (OD) reading than the one using 100% acetone.

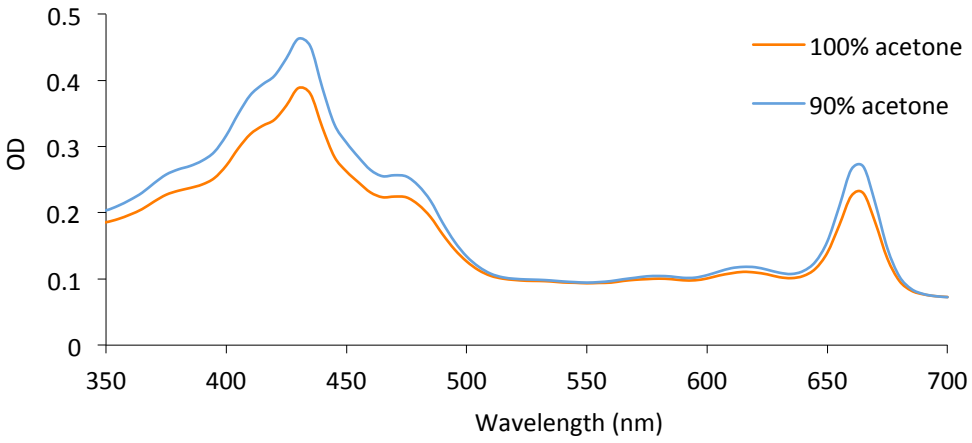


Figure 2.6 Absorption spectra scans of pigment extracts using traditional method with 100% and 90% acetone

Note: OD: optical density

The spectra scans of pigment extracts using the one-step closed-tube method with pure acetone (100%) and aqueous acetone (90%) are shown in Figure 2.7. The spectra scans for both 100% and 90% acetone consist of two peaks at 430 nm and 660 nm, with two extended shoulders at the left-hand and right-hand side for the first peak around 410 nm and 465 nm. The pigment extraction using 90% acetone has a higher OD reading than the one using 100% acetone.

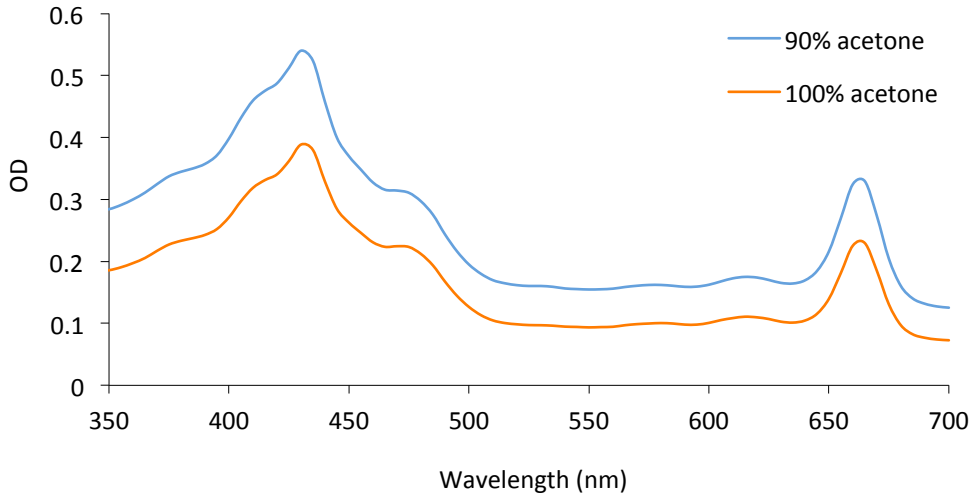


Figure 2.7 Absorption spectra scans of pigment extracts using one-step closed-tube method with 100% and 90% acetone

Note: OD: optical density

2.1.2.2.2 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram of pigments extracted

The lutein content in the pigment extracts was analysed using RP-HPLC with the chromatogram shown in Figure 2.8. The peak identified by a retention time at 17.5 minutes corresponded to lutein.

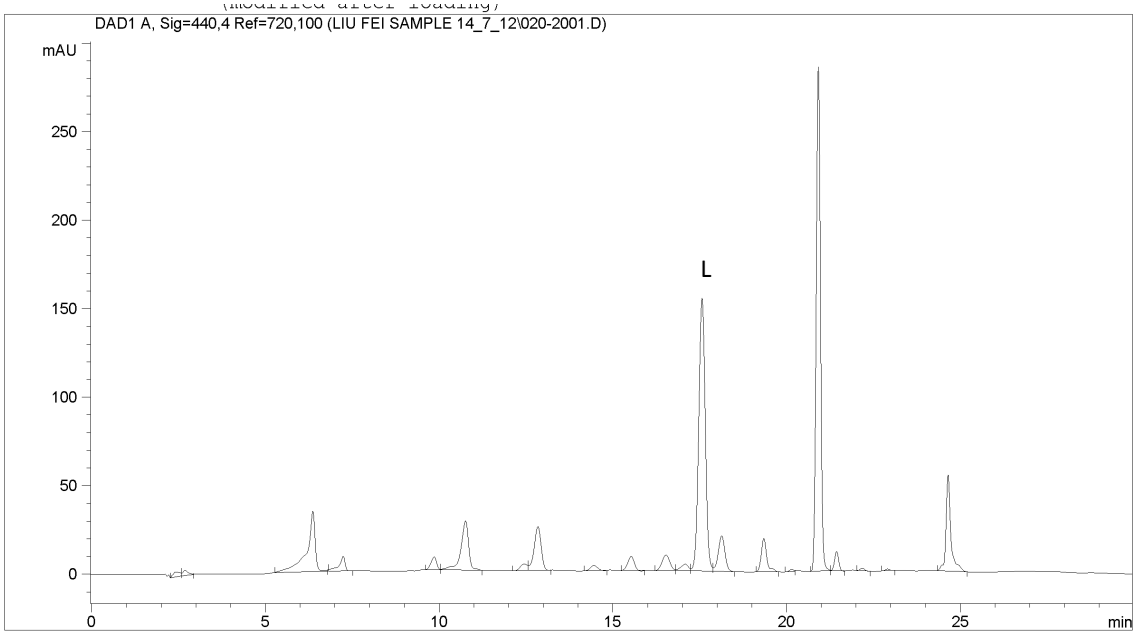


Figure 2.8 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatograms at 440 nm of pigments extracted using one-step closed-tube method with 90% acetone

Note: Peak identification: L: Lutein

2.1.2.2.3 Comparison of traditional method and one-step closed-tube method

Pigment extracts obtained by using the traditional method and the one-step closed-tube method were analysed using RP-HPLC (Figure 2.9). Lutein was released from all the samples by using the traditional one-step closed-tube method with 100% and 90% acetone, with additional lutein also released after extraction when conducted for the second and third time (Figure 2.9). After extraction was conducted three times, the lutein was fully extracted. The highest yield was obtained using the one-step closed-tube method with 90% acetone (4785 mg/kg of biomass) while the lowest yield was obtained using the traditional method with 100% acetone (2776.62 mg/kg of biomass).

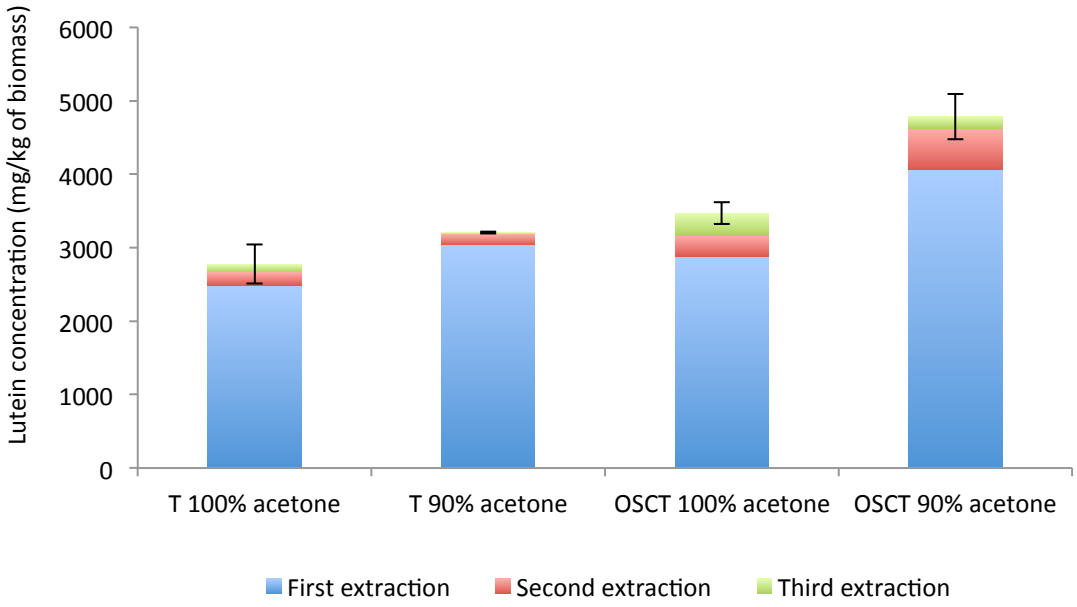


Figure 2.9 Comparison of efficiency between traditional method and one-step closed-tube method using pure acetone (100%) and aqueous acetone (90%)

Note: T: Traditional method; OSCT: One-step closed-tube method.

Both methods, using the two different concentrations of acetone, were very efficient and extracted more than 83% of lutein in the first extraction (Table 2.3). These results showed that, in the first extraction, the traditional method using 100% and 90% acetone was able to extract more than 89% of lutein while the one-step closed-tube method was only able to extract around 83% of lutein. Although the traditional method was more efficient in extraction, the concentration of lutein extracted by using the one-step closed-tube method was higher (Table 2.3). After considering pigment extraction in terms of the yield, safety and cost, the one-step closed-tube method using 90% acetone was selected as the appropriate pigment extraction procedure.

Table 2.3 Total concentration of lutein released from traditional and one-step closed-tube methods in pure (100%) or aqueous (90%) acetone

Extraction method	Percentage of acetone (%)	Total extraction (mg/kg of biomass)	Efficiency of extraction (%)		
			First	Second	Third
Traditional	100	2776.62	89.70	6.52	3.78
	90	3205.34	94.99	4.52	0.49
One-step closed-tube	100	3470.17	83.08	8.29	8.62
	90	4785.07	84.86	11.74	3.39

Note: Three sequential extractions were conducted in fresh portions of solvent. The efficiency of each extraction is expressed as a percentage of total extraction.

2.1.2.3 Extraction time effect

Two different extraction methods were investigated in the previous sections, after which the one-step closed-tube method using 90% acetone was selected for this study. This section places more attention on the effect of varying lengths of extraction time in the sonicator. The pigment extracts spent extraction times of 1, 3, 5, 8, 10, 15 and 20 minutes in the sonicator and were then analysed using a UV-spectrophotometer. The peak at 430 nm was chosen as the testing wavelength for lutein.

The spectra scans presented in Figure 2.10 show the effects of different lengths of extraction time spent by the pigment extracts in the sonicator with glass beads. An increase in sonication time was not found to increase the yield (Figure 2.11). As can be seen in Figure 2.11, an increase in sonication time resulted in a decrease in extraction yield. However, it was confirmed by this result that five minutes of sonication time is enough to achieve the maximum extraction of lutein.

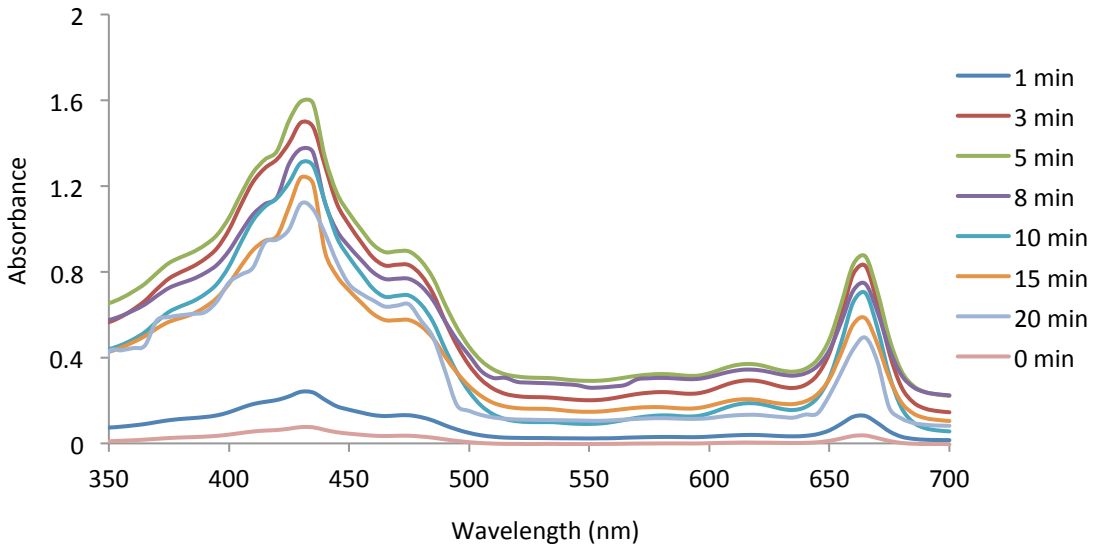


Figure 2.10 Spectra scans of pigment extracts in sonicator with glass beads for different time frames

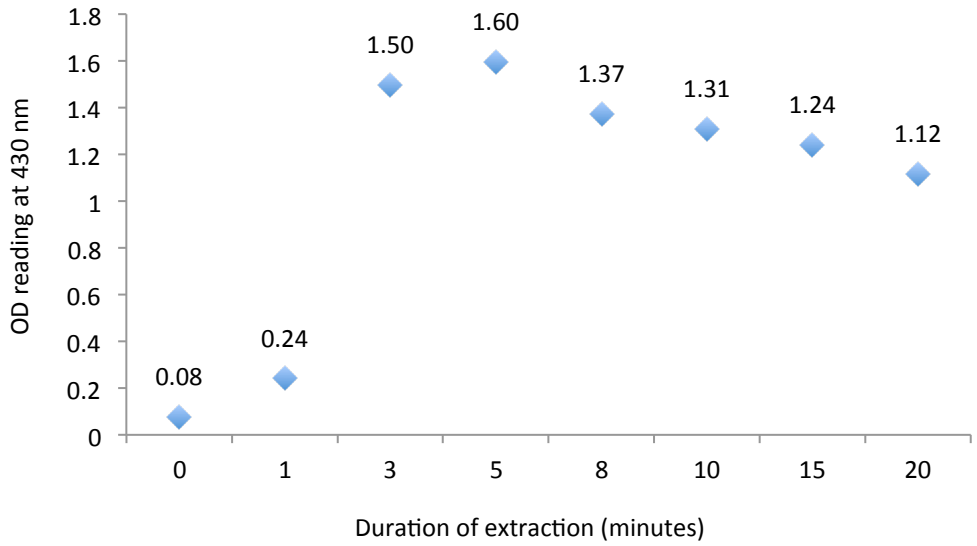


Figure 2.11 Average optical density reading at 430 nm of pigment extracts in sonicator with glass beads and 90% acetone for different time frames

2.1.2.3.1 Lutein stability and degradation

2.1.2.3.1.1 Influence of light on lutein stability during one-step closed-tube pigment extraction

Another important consideration is lutein stability during pigment extraction. The comparison of lutein concentration (mg/kg of biomass) when extracted in the light and in the dark is summarized in Figure 2.12. As shown, the pigment extracted in the light has a lutein concentration of 4664.589 mg/kg of biomass while the pigment extracted in

the dark has a lutein concentration of 6855.075 mg/kg of biomass. The concentration of lutein extracted in the dark is 32% higher than that extracted in the light.

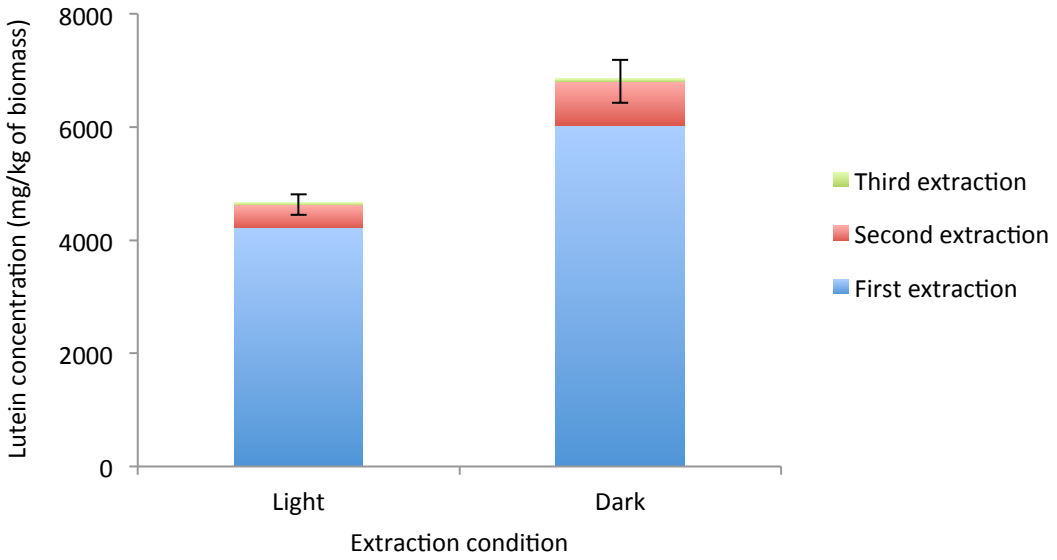


Figure 2.12 Comparison of lutein concentration in mg/kg of biomass during extraction in light and dark conditions

2.1.2.3.1.2 Influence of light and illumination on lutein stability of pigment extracts from different concentrations of biomass

Figure 2.13–Figure 2.15 show the changes of lutein concentration (mg/L) in pigment extracts extracted from different concentrations of biomass and in different light and dark conditions when exposed to the illumination condition. As can clearly be seen, the concentration of lutein decreased in relation to light exposure. This result shows that when pigment was extracted under the light condition and exposed to illumination for 60 minutes, the lutein concentration decreased from 11904 mg/L to 2052 mg/L for extraction from 2 mg of biomass; and from 35124 mg/L to 2556 mg/L and from 57407 mg/L to 13167 mg/L for extraction from 4 mg and 6 mg of biomass, respectively (Figure 2.13). However, when pigment was extracted in the dark condition and exposed to illumination for 60 minutes, the lutein concentration decreased from 21370 mg/L to 4688 mg/L for extraction from 2 mg of biomass; and from 44830 mg/L to 7037 mg/L and from 64433 mg/L to 20348 mg/L for extraction from 4 mg and 6 mg of biomass, respectively (Figure 2.14). However, when the pigment was extracted in the dark condition and kept in the dark, the differences in lutein concentration in the pigment extracts were small (Figure 2.15).

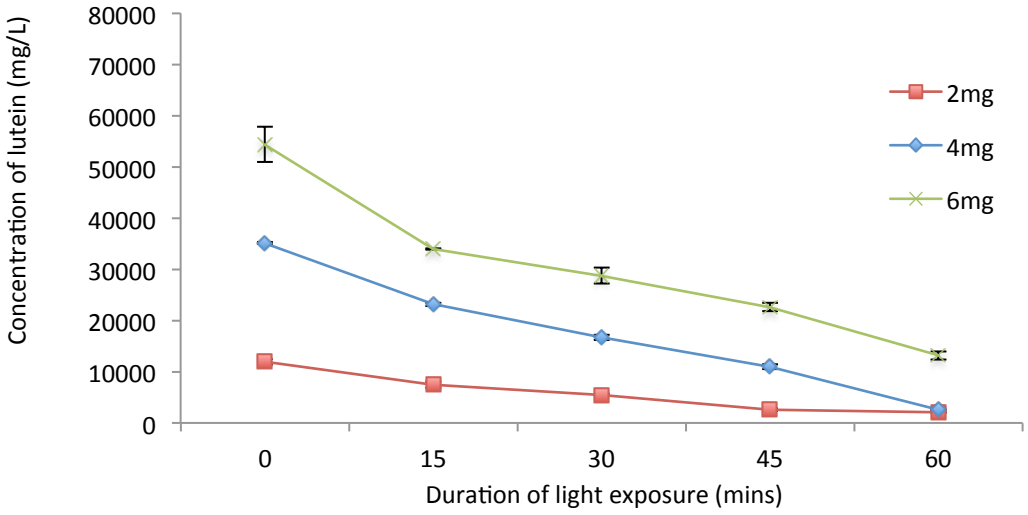


Figure 2.13 Changes of lutein concentration (mg/L) when pigment extracts from different concentrations of biomass are under light condition and exposed to illumination condition

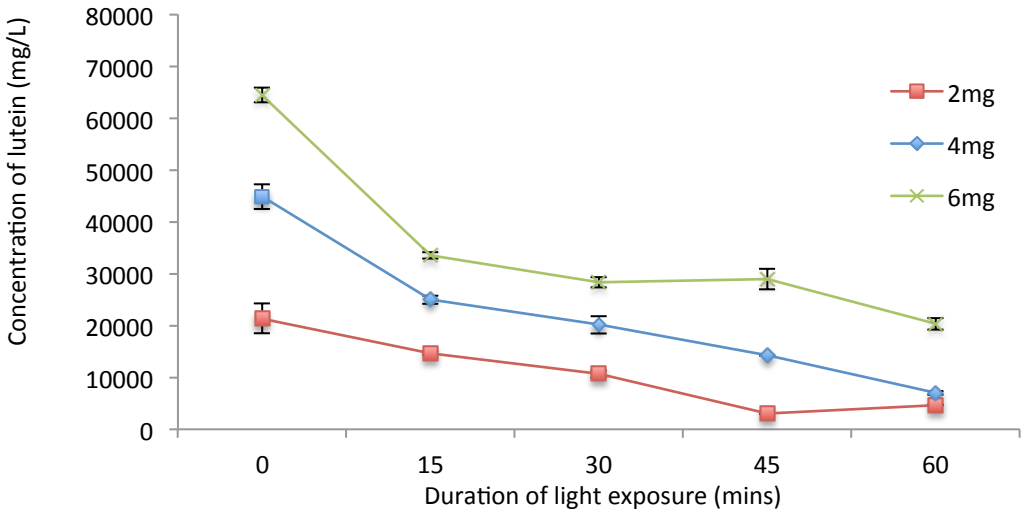


Figure 2.14 Changes of lutein concentration (mg/L) when pigment extracts from different concentrations of biomass are under dark condition and exposed to illumination condition

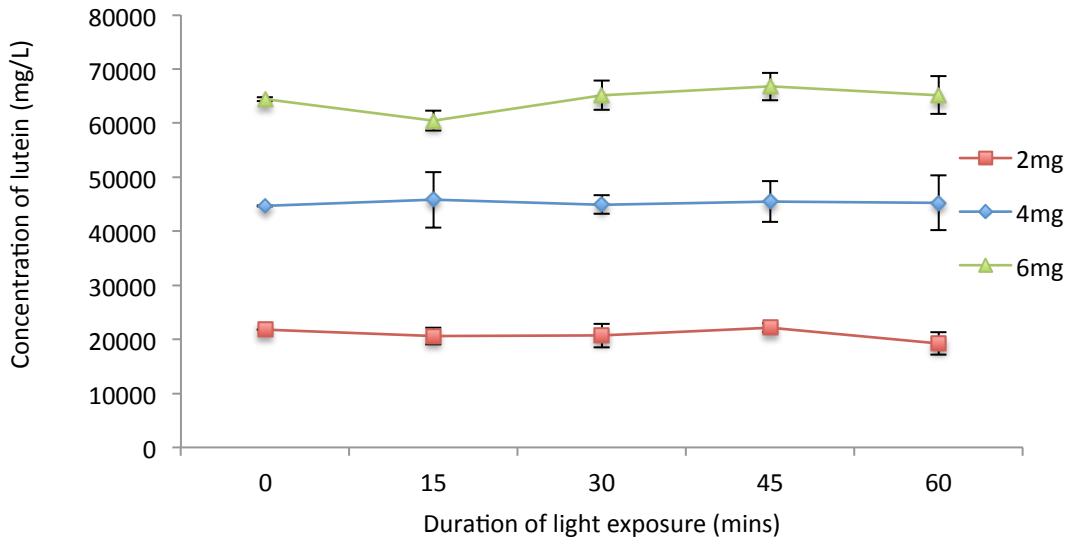


Figure 2.15 Changes of lutein concentration (mg/L) when pigment extracts from different concentrations of biomass are under dark condition and kept in the dark

2.1.3 Discussion

Microalgae, one of the oldest fundamental life forms on the planet, occupy the bottom of the food chain in aquatic systems. Isolated from various natural and artificial environments, microalgae are able to adapt to different living conditions, such as geothermal water, rice paddies and contaminated lakes (Oh and Rhee, 1991, Onay *et al.*, 2014, Stokes *et al.*, 1973). They are a type of photosynthetic organism that can utilize and convert sunlight, water and carbon dioxide to different types of complex organic compounds, as well as to bioactive secondary metabolites for pharmaceutical and food applications (Abedin and Taha, 2008, Ip *et al.*, 2004, Leon *et al.*, 2003, Lorenz and Cysewski, 2000).

In the microalgae industry, there has been a move towards microalgal production of carotenoids for use as colourants in animal feed and also as a supplement in human diets. Many microalgae are known to produce different types and concentrations of carotenoids such as β-carotene, astaxanthin, canthaxanthin and lutein (Ben-Amotz *et al.*, 1982, Grunewald *et al.*, 2001, Wei *et al.*, 2008). Two classes of carotenoids are present in microalgae: carotenes and xanthopylls. Microalgae produce two types of xanthophylls: primary xanthophylls involved in the cellular photosynthetic apparatus as light-harvesting pigments, and secondary xanthophylls which are critical for cellular survival when microalgae are exposed to specific environmental stimuli.

In order to quantify the concentration of lutein in microalgae, cells are extracted from microalgae by using either solvent extraction or supercritical fluid extraction (SFE). In solvent extraction, breaking the microalgae cells is one of the most important steps for enhancing the efficiency of pigment extraction. Different methods, such as mortar and pestle, heating, enzymatic disruption, tissue homogenizer and ultrasound, have been used to extract pigments from microalgae (Choubert and Heinrich, 1993, Hagen *et al.*, 1993, Harker *et al.*, 1996, Kobayashi *et al.*, 1993). In addition, this process involves using a solvent to penetrate the cells to dissolve the lipids and cell membrane to improve extraction efficiency. Common solvents used in microalgae pigment extraction are methanol, ethanol and acetone (Ritchie, 2006, Sartory and Grobbelaar, 1984, Simon and Helliwell, 1998). Other solvents used have included chloroform, dimethyl sulfoxide and petroleum ether (Macias-Sanchez *et al.*, 2009). Unfortunately, solvent extraction has significant drawbacks: (1) the requirement for a large amount of microalgae biomass and solvents, (2) time-consuming process and (3) complicated methods.

Different types of techniques have been used to determine and quantify lutein concentrations in microalgae. The traditional method of detecting and quantifying pigments from extracts is UV-spectrophotometer analysis (Davey *et al.*, 2006, Kimura *et al.*, 2007, Sartory and Grobbelaar,1984, Schertz, 1923, Schon, 1935). The concentration of the specific pigment can be quantified by measuring the absorbance of the pigment extracts at a particular wavelength, and calculated using a specific equation. This method is easy and allows fast identification of the high lutein producers from a large number of microalgae strains, and can quickly analyse a large amount of pigment extract samples. However, this method has often been found to be inaccurate. Alternatively, three types of chromatography techniques have been widely used to determine and quantify pigment samples, namely, paper chromatography, thin layer chromatography (TLC) and reverse-phase high-performance liquid chromatography (RP-HPLC). Of these, RP-HPLC is the most common and accurate technique to separate and quantify different types of pigments. In addition, there are different types of columns and detectors that allow the separation and quantification of specific pigments from extracts (Jeffrey *et al.*, 1997, Leeuwe *et al.*, 2006). Although this is an expensive and time-consuming method, it is more precise, requires less samples and features automatic detection systems.

In the current study, a one-step closed-tube method using glass beads, a sonicator, a small amount of samples and 90% acetone was able to rapidly extract lutein from *Chloroparva pannonica*. The lutein content in pigment extracts from *Chloroparva pannonica* was analysed and estimated using a UV-spectrophotometer prior to quantification by the RP-HPLC method.

The UV-wavelength scans and RP-HPLC chromatogram of the authentic standards of lutein are shown in Figure 2.1 and Figure 2.4, respectively. Spectrophotometric analysis showed that the authentic standards of lutein had two peaks, one at 445 nm and the other at 479 nm, with an extended shoulder on the left-hand side of the first peak at around 423 nm. The RP-HPLC chromatogram consisted of one peak at a retention time of 17 minutes (Figure 2.4). The UV-wavelength scans and RP-HPLC chromatogram of the pigment extracts are shown in Figure 2.7 and Figure 2.8, respectively. Spectrophotometric analysis showed that the pigment extracts had two peaks at 430 nm and 660 nm (Figure 2.7) while the peak identified from the RP-HPLC chromatogram at 17.5 minutes corresponded to that of lutein (Figure 2.8). Upon comparison between the UV-wavelength scans and the RP-HPLC chromatogram of the authentic standards of lutein with the pigment extracts, along with the spectrophotometric analysis, the peak at 430 nm was chosen as the testing wavelength for lutein prior to RP-HPLC analysis.

Important factors to consider are how the microalgae cells are broken and the selection of the solvent for the extraction of lutein due to its significant role in cell lysis. Different types of solvent have been used for microalgae pigment extraction, with the solvent used either alone or as a mixture. The type of microalgae species, the target pigments, extraction efficiency, and the safety and cost of the solvent are all critical for determining the best solvent for pigment extraction. Methanol (particularly 60°C methanol) is an excellent solvent for the extraction of pigments, particularly chlorophylls from microalgae (Marker, 1972, Porra *et al.*, 1989, Ritchie, 2006). It is one of the least expensive solvents, but is more toxic than other common solvents used for pigment extraction. Ethanol is also one of the solvents widely used for the extraction of pigments from microalgae (Macias-Sanchez *et al.*, 2009, Ritchie, 2006). Ethanol is much safer than methanol, but the price of ethanol is much higher than that of other solvents. Acetone is another low-cost solvent that is commonly used by studies in the literature for the extraction of microalgae pigments. Of these solvents, both methanol and acetone are commonly used in microalgae pigment extraction (Leeuwe *et al.*, 2006,

Nakamura and Watanabe, 2001). The extraction efficiency of acetone is usually lower than that of methanol. Simon and Helliwell (1998) found that methanol is able to extract three times more pigment than when acetone is used as the solvent, with the help of a sonicator. However, the extraction efficiency of using methanol or acetone for pigment extraction is also highly dependent on the microalgae species. Furthermore, the stability of the pigment in the solvent has to be considered. Several research studies have shown that pigments are more stable in acetone than in methanol (Bowles *et al.*, 1985, Brereton *et al.*, 1994, Leeuwe *et al.*, 2006). Acetone is also able to produce sharper peaks during RP-HPLC as it has greater electrophilic strength for carbon-rich substrates when compared to methanol (Stock and Rice, 1967).

Owing to safety reasons and the cost of the solvent, acetone was chosen as the extraction solvent in the current study. Complete extraction of the pigments from *Chloroparva pannonica* Cu40 necessitated comparisons between different concentrations of acetone, and between the traditional and one-step closed-tube methods. In addition to cell disruption and solvent concentration, the duration of extraction and the number of steps had to be considered as they directly influence the amount of lutein degradation in the pigment extracts.

The efficiency of extraction steps was compared between the two methods (traditional and one-step closed-tube) and between the solvent concentration (pure [100%] solvent or 90% acetone) as summarized in Figure 2.9. As shown, the complete extraction of lutein and the highest yields were obtained using the one-step closed-tube method with 90% acetone, while the lowest yield was obtained using the traditional method with 100% acetone (Figure 2.9). Although extraction efficiency for the traditional method was higher, the one-step closed-tube method resulted in a higher lutein concentration (Table 2.3). In addition to cell disruption and solvent selection, the duration of extraction of the one-step closed-tube method was investigated. Figure 2.11 shows the optical density (OD) readings at 430 nm of pigment extracts obtained by using a sonicator with glass beads and 90% acetone for different time frames. The extraction yield increased after sonicating for 0, 1, 3 and 5 minutes and decreased after sonicating for 8, 10, 15 and 20 minutes. The highest extraction yield was at five minutes of sonication, leading to the conclusion that five minutes is sufficient to achieve the maximum extraction of lutein using a sonicator with glass beads and 90% acetone, compared to the traditional method. Considering the time requirement, the yield of

pigment extraction, and the cost and toxicity of the solvent, the one-step closed-tube method with 90% acetone was chosen as the pigment extraction procedure.

The comparison of both extraction methods prior to UV-spectrophotometric analysis and RP-HPLC analysis is shown in Figure 2.16. In terms of total minimum hours for processing 10 samples, the traditional method required six hours while only 1.5 hours were required for the one-step closed-tube method to complete the extraction process. In addition, the total cost for processing 10 samples using the traditional method is A$40, while the one-step closed-tube method is only A$10. This suggests that the one-step closed-tube method requires less extraction steps and less time, and is a cheaper method for extracting pigment from microalgae samples.

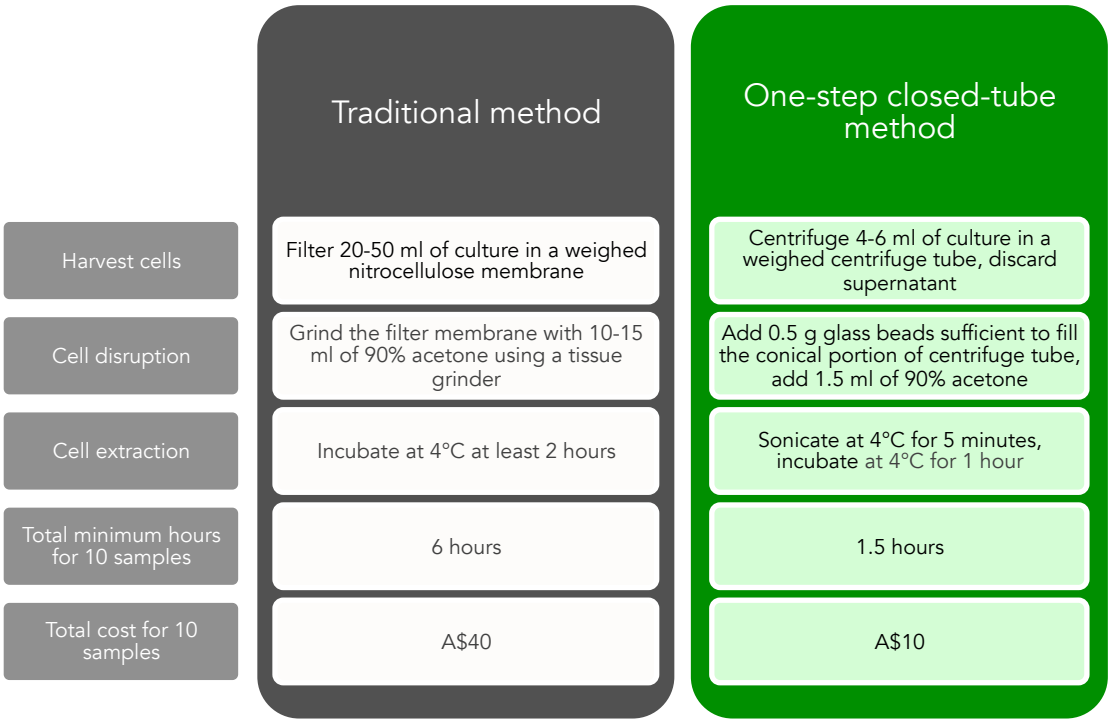


Figure 2.16 Comparison of traditional method and one-step closed-tube method for pigment extraction

The other important factors that need to be considered are lutein stability and the conditions during pigment extraction. As lutein is a type of antioxidant and is able to act as a blue light filter, degradation of lutein can occur when exposed to high temperature, light, oxygen, air, and acidic and basic conditions (Boon *et al.*, 2010, Chan *et al.*, 2013, Henry *et al.*, 1998, Jeffrey *et al.*, 1997, Minguezmosquera and Jarengalan, 1995). In the current study, pigment extraction was performed at 4°C and the influence of light on lutein stability during and after extraction was investigated. This study used light

intensity which is within the intensity range of light indoors, that is, an illumination of about 20–30 μmol photons $m^{-2}s^{-1}$.

Figure 2.12 shows the difference in lutein concentration (mg/kg of biomass) after performing the one-step closed-tube method three times each in light and dark conditions. The lutein concentration extracted in the dark condition was 32% higher than that extracted in the light condition. Furthermore, the influence of light on lutein stability in the pigment extracts was investigated. Microalgae biomass at 2 mg, 4 mg and 6 mg was used for performing pigment extraction utilizing the one-step closed-tube method in the light or dark condition. The lutein concentration from the pigment extracts was analysed and quantified using RP-HPLC after 15-minute intervals of light exposure in order to determine lutein stability in the pigment extracts. Figure 2.13–Figure 2.15 show the changes of lutein concentration (mg/L) in the pigment extracts extracted from different concentrations of biomass when exposed to illuminated conditions. A strong correlation was found between the decreasing concentrations of lutein and increasing light exposure. Pigment extracted under the light condition and exposed to illumination for 60 minutes resulted in an 83% decrease in lutein concentration from 2 mg of biomass, 90% from 4 mg of biomass and 80% from 6 mg of biomass. In contrast, pigment extracted in the dark condition and exposed to light for 60 minutes resulted in an 88% decrease in lutein concentration from 2 mg of biomass, 84% from 4 mg of biomass and 75% from 6 mg of biomass. However, the control which was extracted in the dark condition and kept in the dark only exhibited a slight decay in lutein. Thus, the results suggested that lutein is susceptible to oxidation upon exposure to light.

Lutein extracted from marigold flowers is commonly used as a food ingredient in dietary supplements. Several research studies have demonstrated that lutein becomes degraded under light illumination and at high temperature in food production systems, such as in vegetable juice, egg powder and spray-dried vegetables (Del Campo *et al.*, 2004, Junghans *et al.*, 2001, Khalil *et al.*, 2012, Kline *et al.*, 2011, Kopas-Lane and Warthesen, 1995, Lin and Chen, 2005, Lin and Chen, 2003, Pesek and Warthesen, 1987). For example, lutein in tomato juice became degraded under full light illumination, and became more degraded when the storage temperature increased (Lin and Chen, 2005). In addition, Kline *et al.* (2011) indicated that lutein concentration decreased more when exposed to 463 nm (which is around the blue light wavelength)

than when exposed to other wavelengths, and suggested that the absorption of blue light leads to degradation of lutein.

2.1.4 Conclusion: carotenoid extraction

Analysis of the data obtained from the current study presents strategies leading to the improvement of efficiency in lutein extraction from *Chloroparva pannonica* at a lower cost. The one-step closed-tube method using a small number of samples, 90% acetone, glass beads and 5-minute sonication in the sonicator in the dark condition was able to rapidly extract 72.4% more lutein from *Chloroparva pannonica* compared to what was achieved with the traditional method. Moreover, the lutein concentration can be estimated by using a UV-spectrophotometer prior to quantification by the RP-HPLC method when a large number of *Chloroparva pannonica* pigment extracts need to be analysed. Therefore, in the current study, this method was chosen to extract lutein from *Chloroparva pannonica* for the work discussed in the following chapters. Moreover, the results showed that lutein becomes degraded in the presence of light. It is suggested that pigment extracts have to be protected from light during extraction and analysis.

2.2 General method

The methods and materials established in the laboratory during the course of the current study and repeatedly referred to in other chapters are described here.

2.2.1 Microalgal strain

The microalga used in this study was obtained from the South Australian Research and Development Institute (SARDI) from a saltpan at Waikerie, South Australia, Australia.

2.2.1.1 *Chloroparva pannonica* growth conditions

Cultures of *Chloroparva pannonica* were grown photoautotrophically in Guillard's F/2 medium (Guillard and Ryther, 1962) with seawater: deionized water (1:1 v/v). Agar plates were prepared with 1.5% agar in Guillard's F/2 medium with seawater: deionized water (1:1 v/v). Natural seawater was collected by SARDI from West Beach, Adelaide,

South Australia. Both liquid and agar medium were sterilized at 121°C at 100 kPa for 15 minutes.

The composition of the medium contained the following per litre as described by Guillard and Ryther (1962):

$NaNO_3$	75 mg
$Na_2H_2PO_4$	5 mg
Trace metals:	
$Na_2EDTA.2H_2O$	8.7 mg
$FeCl_3.6H_2O$	1.3 µg
$CoCl_2.6H_2O$	10 µg
$CuSO_4.5H_2O$	9.8 µg
$MnCl_2.4H_2O$	0.18 mg
$Na_2MoO_4.2H_2O$	6.3 µg
$ZnSO_4.7H_2O$	22 µg
Vitamins (freshly made every three months, filter sterilized):	
Thiamine HCl	20 µg
Biotin (Vitamin H)	0.1 mg
Vitamin B12	0.1 mg

The pH of the trace metal solution was lowered to approximately 4.5 with 1 M of sodium hydroxide (NaOH) to aid dissolution. The nitrogen, phosphate, trace metal solution and vitamins were all prepared in a 1000x stock, sterilized separately and stored at 4°C. The nitrogen, phosphate and trace metal solution were sterilized by autoclaving. The vitamins were sterilized using a nitrocellulose filter (Sarstedt Filtropur Syringe Filter, 0.20 µm pore size).

All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 40 ml of culture medium. Both liquid and agar cultures were maintained in a plant cell culture room at 25±1°C, pH 7.5, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. All liquid cultures were transferred to a new fresh medium every 14 days.

2.2.2 Algal culture growth cell density evaluation

Culture growth was estimated on the basis of cell counts, optical density (OD) readings and dry weight (DW).

2.2.2.1 Cell counts using a Neubauer haemocytometer

The Neubauer haemocytometer can be used when cell densities are in the range $5x10^4$–10^7 cell/ml. Depending on the concentration of the sample, a suitable dilution was undertaken to prepare for cell counting.

For cell counting, 10 µl of 1% Lugol's solution was added to 1 ml of the sample. The glass cover was placed on the haemocytometer chamber's central area. Next, 10 µl of the sample was transferred into the haemocytometer chamber. The cells were allowed to settle for one minute and the grid was then checked under the microscope (x40 objective) for satisfactory distribution of cells. The cells in the given haemocytometer chambers were counted. The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 cells/ml. Therefore, to obtain the count:

$$Cell\ count\ (^{cells}/_{ml}) = \frac{The\ total\ count\ from\ 5\ haemocytometer\ chambers}{5} \times 10^4 \times Dilution\ factor$$

2.2.2.2 Cell density assessed by spectrophotometry

The maximum absorbance was examined by scanning a culture sample of from 600–800 nm using a µQuant™ UV/Vis (ultraviolet/visible) spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia). The highest absorbance value, which was 685 nm, was then used to measure the algal cell density. The samples were properly diluted to ensure the absorbance value was below 1.0. All measurements were carried out in triplicate.

2.2.3 Total dried biomass

For total biomass dry weight, a sample of 20–50 ml of culture was filtered through a pre-weight, pre-dried glass fibre filter (W1) (Whatman® Grade GF/F, 47 mm, nominal

pore size 0.7 μm) using a vacuum pump filtering system under low vacuum pressure (≤100 mm Hg), washed three times with distilled water. The filters were dried to a constant weight at 60°C in the oven and then cooled down in a vacuum desiccator for two hours. Next, the filters were weighed (W2). All measurements were carried out in triplicate. The total dried biomass and total dried biomass per ml were calculated by the following formula:

$$Total\ dried\ biomass\ (g) = W2 - W1$$

$$Total\ dried\ biomass\ per\ ml\ \left(\frac{g}{ml}\right) = \frac{W2 - W1}{Filtered\ volume}$$

2.2.4 Total lipid analysis

The total extractable lipid content was determined gravimetrically, in accordance with Bligh and Dyer (1959) with minor modification. In summary, a microalga sample was harvested by centrifugation at 8000 rpm for 15 minutes, and washed twice with sterile deionized water. The sample was subsequently dried at 60°C to a constant weight and ground with a mortar and pestle into a fine powder. Next, 200 mg of ground microalgae biomass was extracted with 22.8 mL of methanol: chloroform: water (2:1:0.8 v/v) added. The tube was affixed to a shaker and subjected to continuous shaking at 115 rpm for an hour. An additional 6 ml of chloroform and of water were each added. The sample was vortexed and centrifuged at 2500 rpm for 10 minutes to achieve the phase separation. The aqueous layer (top layer) was removed and discarded without disturbing the organic layer (bottom layer). Then, 8 ml of the organic layer was transferred into a clean tube, and centrifuged at 6000 rpm for five minutes to spin down the left-over biomass. After that, 6 ml of the organic layer was collected into a clean pre-weight glass tube (W1). The chloroform was evaporated using a vacuum evaporator and the glass tube containing lipids was put in a -80°C freezer for three hours and transferred to a freeze dryer overnight. The sample was weighed and recorded as W2.

The total lipid content was calculated by:

$$Total\ lipid\ content = \frac{Weight\ of\ total\ lipid\ extract\ (W2-W1)}{Biomass\ weight} \times 100\%$$

Chapter 3 Optimization of nutrient medium composition for *Chloroparva pannonica*

3.1 Introduction

Microalgae are considered to be a potential source of third-generation biodiesel due to three main factors: their simple cellular structure; their higher biomass productivity; and the higher lipid content in their total dry weight when compared to other energy crops such as oil palms (Brennan and Owende, 2010, Mata *et al.*, 2010, Norsker *et al.*, 2011, Razon and Raymond, 2011). Furthermore, microalgae can be cultivated using wastewater, salt water and blackish water in extreme environments on non-arable land, thus avoiding competition with food crops for fresh water and arable land.

A successful microalgae based bio-refinery process depends on the selection of suitable microalgae strains that are capable of growing fast, producing lipids and also producing a high-value desired product under optimal conditions. The production of a high-value desired product(s) such as carotenoids is essential to improve the economic feasibility of the microalgae bio-refinery process (Rodolfi *et al.*, 2009). Under certain conditions, some microalgae strains are able to accumulate up to 50–70% lipids in their total dry biomass. They can also be manipulated to accumulate more lipids during certain conditions in the medium such as nitrogen deficiency and phosphate limitations and with changes in trace element concentration, temperature, light intensity and salinity (Deng *et al.*, 2011, Gouveia and Oliveira, 2009, Sharma *et al.*, 2012, Yeesang and Cheirsilp, 2011). Lipid accumulation caused by nitrogen deficiency was first studied by Spoehr and Milner (1949) in *Chlorella*: their study demonstrated that *Chlorella* is able to accumulate up to 80% lipids in a nitrogen-deficient environment. Microalgae species such as *Nannochloropsis, Chlorella sorokiniana, Chlorella vulgaris, Chlamydomonas reinhardtii, Isochrysis galbana* and *Scenedesmus acutus* have all shown lipid accumulation in their cells under nitrogen deficiency (Campos *et al.*, 2014, Eustance *et al.*, 2016, Kamalanathan *et al.*, 2016, Paranjape *et al.*, 2016, Ramirez-Lopez *et al.*, 2016, Zhang and Liu, 2016).

However, in addition to lipid content and productivity, the biomass productivity of microalgae is one of the limitations of the microalgae based bio-refinery process.

Although microalgae have a higher photosynthetic efficiency than plants, are able to double their cell numbers every few hours and can be harvested daily, this level of productivity is not high enough to improve the economic feasibility of the microalgal bio-refinery process. Several studies have shown that there is an inverse relationship between the lipid content and growth during conditions of nutrient element deficiency, especially during nitrogen deficiency (Illman *et al.*, 2000, Yeesang and Cheirsilp,2011). Under nitrogen-deficient conditions, the growth of microalgae is inhibited and lipids start to accumulate in their cells. According to Illman *et al.* (2000), although the lipid content of *Chlorella emersonii* increased in the low nitrogen medium, the dry biomass was reduced from 28 mg/l/d to 25 mg/l/d in the normal medium. Decreased growth of *Scenedesmus obliquus* under nitrogen-deficient conditions was also seen by Mandal and Mallick (2009). Therefore, a suitable microalgal strain needs to be selected: in addition, optimization of the medium for biomass production is required.

Chloroparva pannonica is one of the potential microalgae for biofuel production due to its ability to synthesize lipids and a unique type of carotenoid, lutein (Somogyi *et al.*, 2011). Traditionally, lutein has been produced from the petals of marigold flowers which is both labour-intensive and land-intensive. Microalgae are considered as one of the sources for lutein production; however, the process is not fully commercialized. This is due to the low production of biomass and lutein; the high cost of the facility; and the complicated downstream processing required to extract and purify lutein from cells.

To achieve economical mass production of microalgae biofuel, not only is lipid content significant, but biomass productivity also plays an important role (Nigam *et al.*, 2011). The growth of microalgae can be affected by abiotic, biotic and mechanical factors. The abiotic factors include irradiance, temperature, carbon dioxide, pH (acidity), salinity and some biologically active compounds such as vitamins, while the biotic factors include cell fragility and cell density (Huerlimann *et al.*, 2010). Mechanical factors include mixing and bubbling (Fabregas *et al.*, 2000, Ivanova *et al.*, 2015, Schenk *et al.*, 2008). Among all of the factors, the nutrient medium is suggested as one factor that plays a critical role and is very strain-specific in supporting the growth of microalgae. Therefore, it is important to formulate a suitable growth medium to achieve high-density cultures as low biomass productivity is obtained with standard media. As microalgae growth is mostly affected by the medium composition, nitrogen and some trace metal elements were the medium components considered in this study.

Besides carbon, nitrogen is one of the key nutrients that support the growth of microalgae. The effects of nitrogen levels and nitrogen sources on microalgae growth have been demonstrated for *Chlorella pyrenoidosa* (Nigam *et al.*, 2011); *Neochloris oleoabundans* (Li *et al.*, 2008); *Tetraselmis suecica*, *Porphyridium cruentum* and *Chlorococcum* sp. (Rodolfi *et al.*, 2009); and *Botryococcus braunii* (Zhila *et al.*, 2005). Results reported by the above researchers have shown that microalgae are able to utilize nitrogen from different sources, and that nitrogen is one of the key elements that promote the growth of microalgae. However, during nitrogen deficiency, the growth of microalgae can be halted due to the metabolic fluxes being channeled to lipid accumulation (Courchesne *et al.*, 2009, Juneja *et al.*, 2013). During a five-day nitrogen starvation treatment for *Scenedesmus obliquus*, Ho *et al.* (2012) observed that the lipid content increased from 11.48% to 22.4%. As also shown in their results, around 90% of the lipid content was composed of C16-C18 fatty acids which are suitable for biodiesel production (Ho *et al.*, 2012). Unfortunately, the optimum conditions for producing lipids are not the optimal conditions for the growth of microalgae. Studies have shown that the growth of microalgae decreased when the concentration of nitrogen sources in the medium decreased (Nigam *et al.*, 2011, Rangel-Yagui *et al.*, 2004, Zhila *et al.*, 2005). On the other hand, careful control of nitrogen levels in the cultivation medium was able to increase carotenoid production in microalgae. In addition, studies have shown that the nitrogen concentration is critical for the synthesis of light-harvesting proteins to support lutein production in microalgae (Del Campo *et al.*, 2000, Jin *et al.*, 2003). For example, *Haematococcus pluvialis* is able to produce astaxanthin in a one-stage production system when the nitrogen level is manipulated during growth (Del Rio *et al.*, 2005).

In addition to the nitrogen level in the cultivation medium, the source of nitrogen is important in supporting the growth of microalgae. In theory, any nitrogenous compound that is able to pass through the plasma membrane through diffusion or active transport and enter the microalgae biochemical pathway to support microalgae growth could be considered as a nitrogen source. Studies have shown that among nitrogen sources, ammonium is the most preferred nitrogen source for microalgae growth and biomass productivity, followed by nitrate and then urea (Levasseur *et al.*, 1993, Syrett, 1988). The investigations have included nitrogen uptake and assimilation; preference and inhibition of different nitrogen sources; and the effect of different nitrogen sources on the growth of microalgae (Dortch, 1990, Fidalgo *et al.*, 1995, Glibert *et al.*, 1982). The

assimilation of nitrogen sources by microalgae cells can be affected by five factors: the microalgae species; the type of nitrogen sources; the nitrogen status in the cells; the presence of other nitrogenous compounds in the surroundings; the activity and concentration of enzymes; and light conditions (Antia *et al.*, 1991, Fernandez and Cardenas, 1982, Tischner, 1984, Tischner and Huttermann, 1978).

Ammonium is the favourite nitrogen source for microalgae: it can be transported into cells by ammonium protonation within the cells and is assimilated by the glutamine synthetase (GS) glutamate synthase (GOGAT) cycle (Figure 3.1) (Henderson, 1971, Klochenko *et al.*, 2003). In the study by Kim *et al.* (2012), the growth of *Chlorella* sp. and *Dunaliella* sp. increased when 20 mM of ammonium was supplied as the main nitrogen source. However, with ammonium and nitrate both present in the cultivation medium, ammonium assimilation is preferred because the nitrate reductase is strongly inhibited by ammonium (Boussiba *et al.*, 1987, Collos, 1982, Yin *et al.*, 1998).

Nitrate is one of the most abundant inorganic nitrogen compounds found in seawater. It is also the most common nitrogen source for the basic microalgal medium. Nitrate uptake occurs through an adenosine triphosphate (ATP)-driven active transport system and is then reduced to nitrite by nitrate reductase (Figure 3.1). Nitrite is then, in turn, reduced to ammonium by nitrite reductase (Figure 3.1) (Fernandez and Cardenas, 1982). The assimilation of nitrate to ammonium is regulated by the concentration and activity of nitrate reductase in microalgae cells (Fernandez and Cardenas, 1982). The effect of nitrate on the productivity of biomass and carotenoids and on microalgae lipid accumulation has been widely studied. It has been well established that there is an inverse relationship between biomass productivity and lipid accumulation when nitrate was used in the cultivation medium. For example, when grown in a medium with three-fold sodium nitrate, the dry biomass of *Tetraselmis* sp. was increased by 0.35 gL^{-1}. However, under nitrate-deplete conditions, the biomass decreased while the lipid content increased from 22.4% to 30.5% (Kim *et al.*, 2016b). Microalgae such as *Nannochloropsis*, *Chlorella vulgaris, Chlamydomonas reinhardtii*, *Neochloris oleoabundans, Nannochloropsis oculata* and *Scenedesmus bijugatus* showed high amounts of lipid content, decreased growth rate and the accumulation of secondary carotenoids under nitrogen starvation (Arumugam *et al.*, 2013, Cakmak *et al.*, 2012, Li *et al.*, 2008, Yeesang and Cheirsilp, 2011).

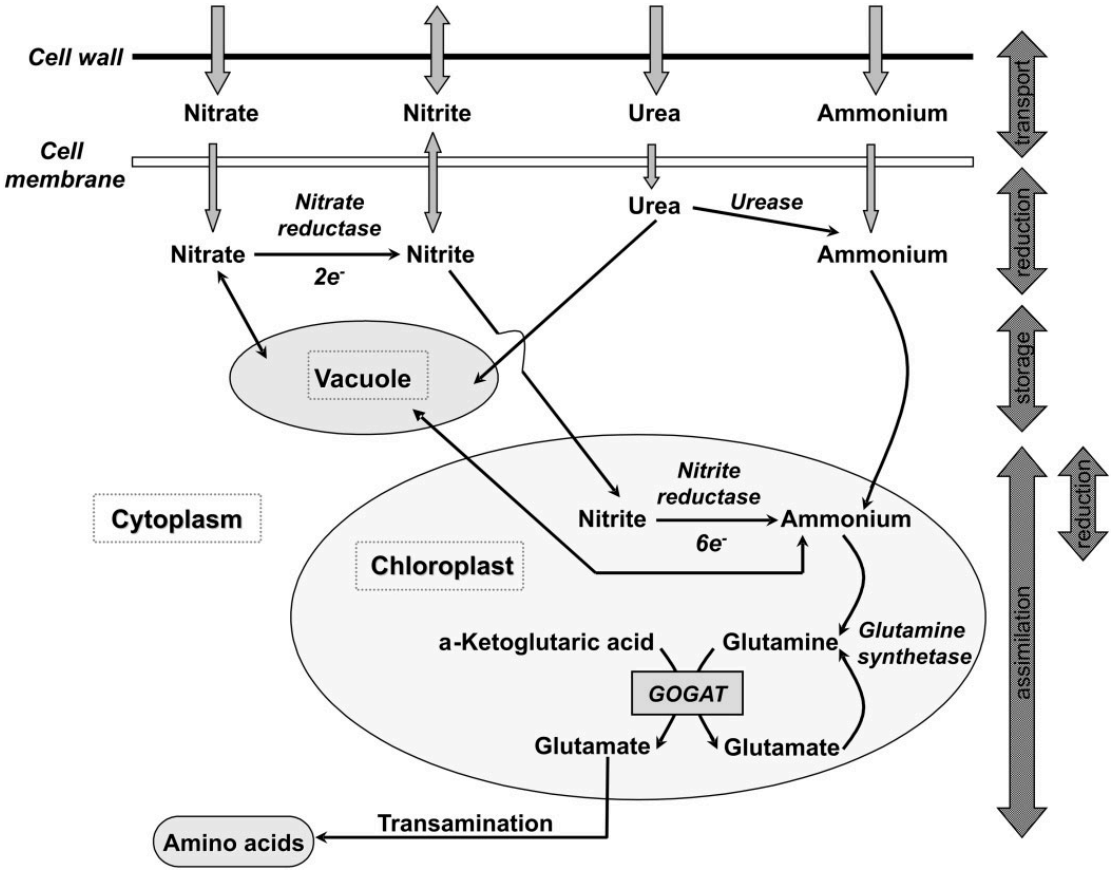


Figure 3.1 Intracellular pathways of uptake of different nitrogen sources in microalgae cells

Source: Adapted from Roopnarain *et al.* (2015).

In addition to inorganic nitrogen compounds, organic nitrogen compounds, such as urea ($CO(NH_2)_2$), have been used in the microalgae cultivation medium for growth. Urea is considered as a combined source of nitrogen and carbon compound and is commonly used as a source of nitrogen for the growth of bacteria, fungi and plants (Mobley and Hausinger, 1989). It is one of the most available and cheapest nitrogen compounds compared to other nitrogen sources. In microalgae, urea is assimilated by energy-dependent urease into bicarbonate and ammonium (Solomon and Gilbert, 2008) (Figure 3.1). *Spirulina* sp. (Rangel-Yagui *et al.*, 2004, Stanca and Popovici, 1996); *Coccomyxa acidophila* (Casal *et al.*, 2011); *Chaetoceros muelleri* (Lopez-Elias *et al.*, 2015); *Isochrysis galbana* (Roopnarain *et al.*, 2015); and *Nannochloropsis oculata* (Chaturvedi *et al.*, 2004) have been reported as being able to utilize urea for growth. The results of the study by Roopnarain *et al.* (2015) indicated that, although there was no difference in the growth parameters of *Isochrysis galbana* in urea and nitrate, the lipid accumulation

started earlier in the cells grown in urea. Interestingly, urea plays an important role in carotenoid accumulation in microalgae. The study by Stanca and Popovici (1996), in which urea was utilized to cultivate *Spirulina platensis*, demonstrated that the biomass and carotenoids, especially chlorophyll, had increased. Moreover, during mixotrophic cultivation of *Coccomyxa acidophila* using urea, cell growth increased: in addition, cells were able to accumulate lutein up to a dry weight of 6.1 $mg.g^{-1}$ (Casal *et al.*, 2011).

Trace metals, play an important role as a stress modulator and trigger in the growth and synthesis of microalgae bioproducts. They can be divided into two categories: essential and non-essential. Each trace metal plays a different role in microalgae cellular functions. Copper and ferric are essential components for the photosynthesis of electron transport proteins; manganese is responsible for photosynthetic water-oxidizing centres (Miazek *et al.*, 2015); cobalt is one of the vitamin elements; and zinc is the co-factor for carbonic anhydrase in carbon dioxide fixation (Moroney *et al.*, 2001), for RNA polymerase in DNA transcriptions and for alkaline phosphatase in phosphorus acquisitions (Sunda, 2012). Essential trace metals, such as copper, ferric, manganese and zinc, are required at very low concentrations for the growth and development of microalgae while, at high concentrations, they can be toxic. At high concentrations, the trace metals attached to the sulphydryl groups in proteins result in a disruption of the protein structure (Van Assche and Clijsters, 1990). Furthermore, high levels of trace metals are able to stimulate the formation of free radicals and reactive oxygen species (ROS) such as hydroxyl radical (•OH); superoxide anion (O_2^-); singlet oxygen (O_2*); and hydrogen peroxide (H_2O_2) in cells (Miazek *et al.*, 2015, Van Assche and Clijsters, 1990). All these compounds are able to attack the amino acids, proteins, carbohydrates, nucleic acids and lipids in the cells. For example, Lustigman *et al.* (1995) showed that when *Chlamydomonas reinhardtii* was cultivated at 20 ppm cobalt (Co^{2+}), its growth decreased, the colour of the culture changed and cells tended to clump together. Furthermore, trace metals may have an effect on the morphology of microalgae cells. For example, Andosch *et al*. (2015) showed that when *Desmidium swartzii* was cultivated in a medium with a high level of copper, the ultraculture of the cell walls changed, while mitochondria became enlarged when it was cultivated in a medium that had a high level of zinc.

Although negative effects have been observed on growth when microalgae are exposed to certain levels of trace metals, some studies have shown that growth has increased

when the levels of trace metals are appropriately manipulated in the microalgae-cultivation medium. The addition of ferric chloride ($FeCI_3$) has been shown to increase the final cell density and prolong the exponential phase in *Chlorella vulgaris* (Liu *et al.*, 2008). Similarly, growth and pigment content were increased when a low concentration of CO^{2+} was used for *Monoraphidium minutum* (El-Sheekh *et al.*, 2003) and *Prorocentrum minimum* (Segatto *et al.*, 1997).

However, microalgae have developed detoxification and tolerance defence mechanisms at the cellular level as self-protection against trace metals. Chelation of metals in microalgae cytosol is one of the protective responses to free radicals and reactive oxygen species (ROS). Microalgae are able to synthesize high levels of chelating agents such as phytochelatins and metallothioneins to create a chelate-metal complex which prevents the interaction of metals with any biological components in the cells (Monteiro *et al.*, 2012, Perales-Vela *et al.*, 2006, Rauser, 1999). Furthermore, microalgae are able to trigger the biosynthesis of chlorophyll and different types of carotenoids (a type of antioxidant) to quench free radicals and reactive oxygen species (ROS). Therefore, the presence of trace metal stress can be considered as an inducer for carotenogenesis in microalgae. Ferric at a concentration of 450 μM, with acetate at a concentration of 67.5 mM, has been found to result in a seven-fold increase in the level of β-carotene production in *Dunaliella salina* (Mojaat *et al.*, 2008b). In addition, when *C. onubenssi* was cultivated in a medium with 0.2 mM copper in semi-continuous cultivation, growth rates increased (by up to 0.50 d^{-1}) and lutein production was also enhanced (by up to 50%) (Vaquero *et al.*, 2012). Dineshkumar *et al.* (2015) optimized the medium for *Chlorella minutissima* by focusing on nitrate, phosphate, manganese and copper and were able to enhance lutein productivity by up to 3.45 $mgL^{-1}d^{-1}$.

From the economic point of view, in the microalgae based bio-refinery process, the appropriate nitrogen sources and trace metals must be chosen at the optimal level for use in the process. Therefore, there is a need to formulate a production medium that would be beneficial to biomass productivity, and to lipid and carotenoid accumulation in the cells. Most studies have focused on using the strategy of a single factor at a time, with not many studies having focused on the formulation of a production medium for biomass and lutein production in microalgae. In addition, little research has been conducted on optimizing the growth medium for *Chloroparva pannonica* to increase biomass productivity.

In the present study, the aim was to increase the growth of *Chloroparva pannonica* through its cultivation in Guillard's F/2 medium containing different types of nitrogen sources at various concentrations, as well as different types of trace metals: cobalt, ferric, manganese, sodium molybdate and zinc.

3.2 Methods and materials

3.2.1 Microalgae culture conditions

The microalga used in this study was copper adapted *Chloroparva pannonica* strain Cu40. *Chloroparva pannonica* Cu40 cultures were grown photoautotrophically in Guillard's F/2 medium (Guillard and Ryther, 1962) with 40 µg/L of copper sulphate in seawater: deionized water (1:1 v/v).

In following the description of (Guillard and Ryther, 1962) with 40 µg/L of copper sulphate, the medium was composed of the following components per litre:

$NaNO_3$	75 mg
$Na_2H_2PO_4$	5 mg
Trace metals:	
$Na_2EDTA.2H_2O$	8.7 mg
$FeCl_3.6H_2O$	1.3 µg
$CoCl_2.6H_2O$	40 µg
$CuSO_4.5H_2O$	9.8 µg
$MnCl_2.4H_2O$	0.18 mg
$Na_2MoO_4.2H_2O$	6.3 µg
$ZnSO_4.7H_2O$	22 µg
Vitamins (freshly made every three months, filter sterilized):	
Thiamine HCl	20 µg
Biotin (Vitamin H)	0.1 mg
Vitamin B12	0.1 mg

The pH of the trace metal solution was lowered to approximately 4.5 with 1 M of sodium hydroxide (NaOH) to aid dissolution. The nitrogen, phosphate, trace metal solution and vitamins were all prepared in a 1000x stock, sterilized separately and

stored at 4°C. The nitrogen, phosphate and trace metal solution were sterilized by autoclaving. The vitamins were sterilized using a nitrocellulose filter (Sarstedt Filtropur Syringe Filter, 0.20 μm pore size).

All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 40 ml of culture medium. Both liquid and agar cultures were maintained in a plant cell culture room at 25±1°C, pH 7.5, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. All liquid cultures were transferred to a new fresh medium every 14 days.

3.2.2 Medium development

3.2.2.1 Optimization of the concentration of different types of nitrogen-containing compounds

Guillard's F/2 medium was selected for the flask experiments as the starting point for the medium optimization. Eight different types of nitrogen sources at the different concentrations listed in

Table 3.1 were individually prepared in Guillard's F/2 medium (Guillard and Ryther, 1962) which resulted in a total of 40 new culture media. Three replicates were set for each culture condition which resulted in a total of 120 cultures. The pH of all growth media was adjusted to 7.5. From each of the various media, 100 ml was then sterilized at 121°C for 15 minutes in a 250 ml conical flask. The experiment was carried out under autotrophic cultivation conditions in 250 ml Erlenmeyer flasks with 10 ml of *Chloroparva pannonica* Cu40 culture with equal number of cells (0.7 at OD at 685nm) and 100 ml of various culture media and grown for 22 days. Each test was carried out in triplicate and was repeated three times.

Table 3.1 Medium composition of different nitrogen sources at different concentrations per litre in Guillard's F/2 medium

Chemical	Concentration in Guillard's F/2 medium (mg/L)				
Sodium nitrate ($NaNO_3$)	0	75	500	1000	1500
Ammonium nitrate (NH_4NO_3)					
Potassium nitrate (KNO_3)					
Calcium nitrate ($Ca(NO_3)_2$)					
Ammonium chloride (NH_4Cl)					
Ammonium bicarbonate (NH_4HCO_3)					
Glycine ($C_2H_5NO_2$)					
Urea (CH_4N_2O)					

Source: Guillard and Ryther (1962)

3.2.2.2 Optimization of the concentration of trace metals in F/2 medium

As previously mentioned, the starting point for the medium optimization was the flask experiments with Guillard's F/2 medium. Different trace metals at the different concentrations listed in Table 3.2 were individually prepared in Guillard's F/2 medium, which resulted in a total of 30 new culture media. Three replicates were set for each culture condition which resulted in a total of 90 cultures. The pH of all growth media was adjusted to 7.5 and the growth media were then sterilized at 121°C for 15 minutes in a 250 ml conical flask. The experiment was carried out under autotrophic cultivation conditions in 250 ml Erlenmeyer flasks with 10 ml of *Chloroparva pannonica* Cu40 culture with equal number of cells (0.7 at OD at 685nm) and 100 ml of various culture media and grown for 21 days. Each test was carried out in triplicate and was repeated three times.

3.2.3 *Chloroparva pannonica* Cu40 growth estimation

The maximum absorbance was examined by scanning a culture sample at between 600 nm and 800 nm using a spectrophotometer **(**μQuant microplate reader**)**. The highest absorbance value, which was 685 nm, was then used to measure the algal cell density. Samples were taken every two days of the culture period. Each algal cell culture (a 200 μL sample) was transferred aseptically into a Nunc™ 96-well plate (Thermo Scientific, Denmark) in a laminar air flow cabinet. The reference blank used was the Guillard's F/2 medium growth medium. All measurements are carried out in triplicate.

Table 3.2 Medium composition of different trace metals at different concentrations per litre in Guillard's F/2 medium

Chemical	Concentration in Guillard's F/2 medium per L					
Iron (III) chloride ($FeCl_3$)	1.30 μg (control)	2.60 μg	3.90 μg	5.20 μg	10.40 μg	20.80 μg
Sodium molybdate (Na_2MoO_4)	6.30 μg (control)	12.60 μg	18.90 μg	25.20 μg	50.40 μg	100.80 μg
Zinc sulphate (Z_nSO_4)	22.00 μg (control)	44.00 μg	66.00 μg	88.00 μg	176.00 μg	352.00 μg
Cobalt (II) chloride ($CoCl_2$)	10.00 μg (control)	20.00 μg	30.00 μg	40.00 μg	80.00 μg	160.00 μg
Manganese (II) chloride (M_nCl_2)	0.18 mg (control)	0.36 mg	0.54 mg	0.72 mg	1.44 mg	2.88 mg

Source: Guillard and Ryther (1962)

3.3 Results

3.3.1 Effect of nitrogen sources and concentration on growth

The first part of the study was aimed at choosing the appropriate nitrogen source and determining the concentration of nitrogen needed to achieve maximum growth of *Chloroparva pannonica* Cu40. *Chloroparva pannonica* Cu40 was subjected to growing with nitrogen from different sources at different concentrations for 22 days as outlined in

Table 3.1. Throughout the experiments, *Chloroparva pannonica* Cu40 was cultivated in the Guillard's F/2 growth medium with sodium nitrate at 75 mg/L and 40 μg/L of copper sulphate as the control medium.

Figure 3.2 illustrates the *Chloroparva pannonica* Cu40 cultures grown in Guillard's F/2 growth medium with sodium nitrate ($NaNO_3$) at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and the control for a period of 22 days. The optical density (OD) at 685 nm indicated the cell growth of *Chloroparva pannonica* Cu40. It can be seen that, at 685 nm, the OD increased in a consistent manner from day 2 and, at day 22, reached the highest point of growth for all cultures grown with sodium nitrate at different concentrations, except for 0 mg/L. Results indicated that cell growth of *Chloroparva pannonica* Cu40 did not increase when the sodium nitrate concentration increased in the Guillard's F/2 growth medium: cell growth decreased when the concentration of sodium nitrate increased from 500 mg/L to 1500 mg/L (Figure 3.2). *Chloroparva pannonica* Cu40 grown with sodium nitrate at concentrations of 500 mg/L, 1000 mg/L and 1500 mg/L achieved a higher OD at day 22 compared to the control. The highest OD was 3.46 obtained in the medium with 500 mg/L of sodium nitrate, followed by an OD of 3.17 obtained in the medium with 1000 mg/L of sodium nitrate and an OD of 2.7 obtained in the medium with 1500 mg/L of sodium nitrate. The control and the medium with 75 mg/L of sodium nitrate had a similar growth trend and reached the highest OD at 2.2 (control) and 2.3 (75 mg/L of sodium nitrate).

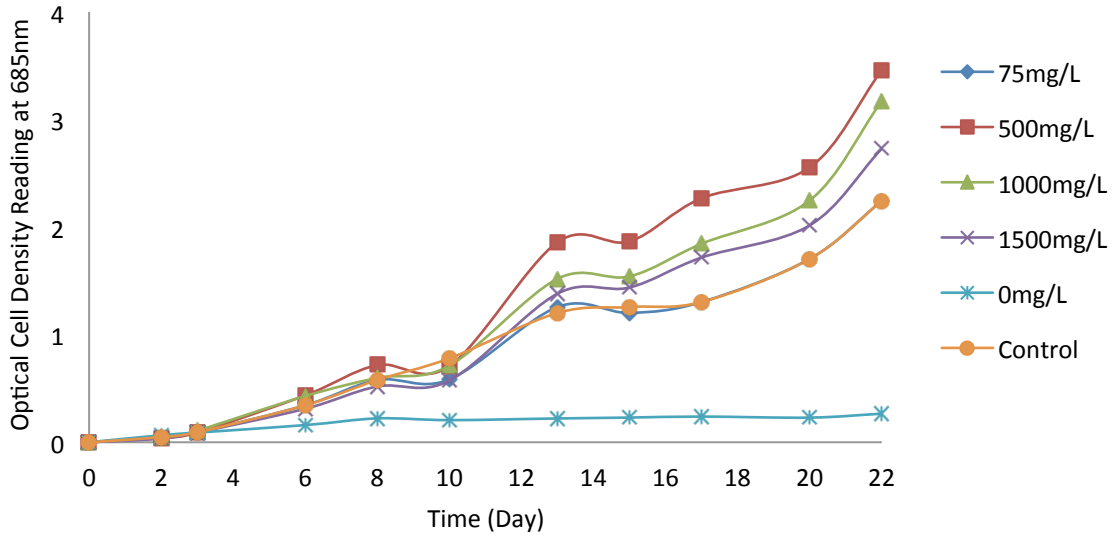


Figure 3.2 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of sodium nitrate ($NaNO_3$) over a 22-day period

Chloroparva pannonica Cu40 cultures grown in Guillard's F/2 growth medium with ammonium nitrate (NH_4NO_3) at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and the control for a period of 22 days are illustrated in Figure 3.3. The OD at 685 nm indicated that cell growth of *Chloroparva pannonica* Cu40 increased in a consistent manner from day 2 and reached the stationary phase of growth on different days depending on the concentration of ammonium nitrate being used in the medium. Cell growth of *Chloroparva pannonica* Cu40 did not increase when the concentration of ammonium nitrate increased in the Guillard's F/2 growth medium. Figure 3.3 shows that the highest OD achieved (an OD of 2.6) was when *Chloroparva pannonica* Cu40 was cultivated in the medium with 75 mg/L of ammonium nitrate, followed by the control with an OD of 2.2. However, *Chloroparva pannonica* Cu40 cultures grown with ammonium nitrate at 500 mg/L, 1000 mg/L and 1500 mg/L reached the stationary phase at day 17, with OD readings of 1.34, 1.77 and 1.49, respectively (Figure 3.3).

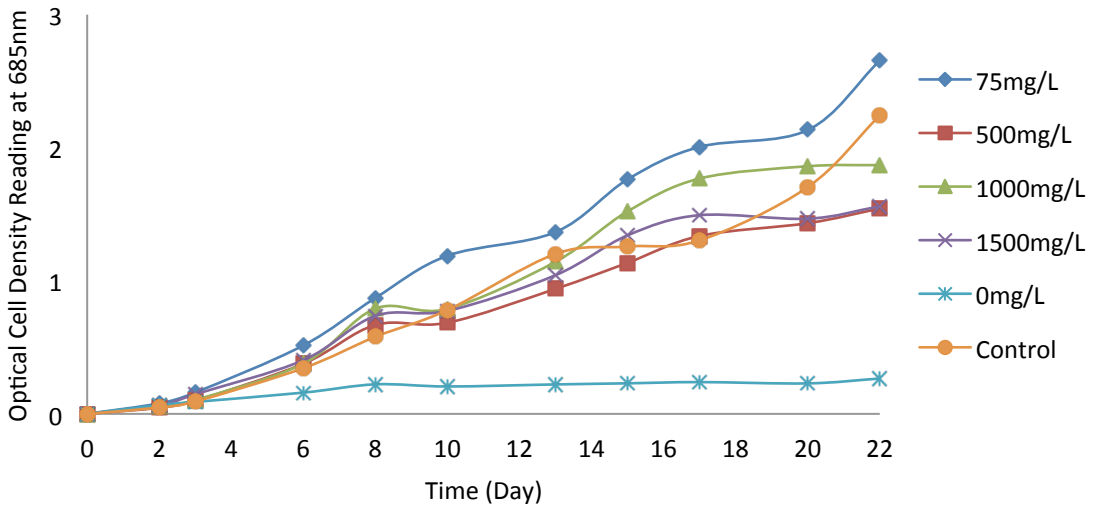


Figure 3.3 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of ammonium nitrate (NH_4NO_3) over a 22-day period

Figure 3.4 shows the *Chloroparva pannonica* Cu40 cultures cultivated in Guillard's F/2 growth medium with potassium nitrate (KNO_3) at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and the control for a period of 22 days. As indicated by OD at 685 nm, cell growth of *Chloroparva pannonica* Cu40 increased when the concentration of potassium nitrate in the F/2-Si medium increased. Cell growth of *Chloroparva pannonica* Cu40 increased exponentially from day 2 until day 22. The highest OD reading was 3.5 (1500 mg/L of potassium nitrate), followed by 3.04 (1000 mg/L of potassium nitrate), 2.85 (500 mg/L of potassium nitrate), 2.2 (control) and 1.9 (75 mg/L of potassium nitrate) (Figure 3.4).

The effect on the cell growth of *Chloroparva pannonica* Cu40 of calcium nitrate ($Ca(NO_3)_2$) supplemented at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L in Guillard's F/2 growth medium is shown in Figure 3.5. Cell growth of *Chloroparva pannonica* Cu40 increased from day 2 until day 22. The trend of cell growth with concentrations of calcium nitrate at 500 mg/L, 1000 mg/L and 1500 mg/L was similar, with cell growth showing an exponential increase and reaching its highest level at day 22 with an OD reading of 3.4. Furthermore, the trend of cell growth at 75 mg/L and in the control was similar, with both having an OD reading of 2.2 at day 22.

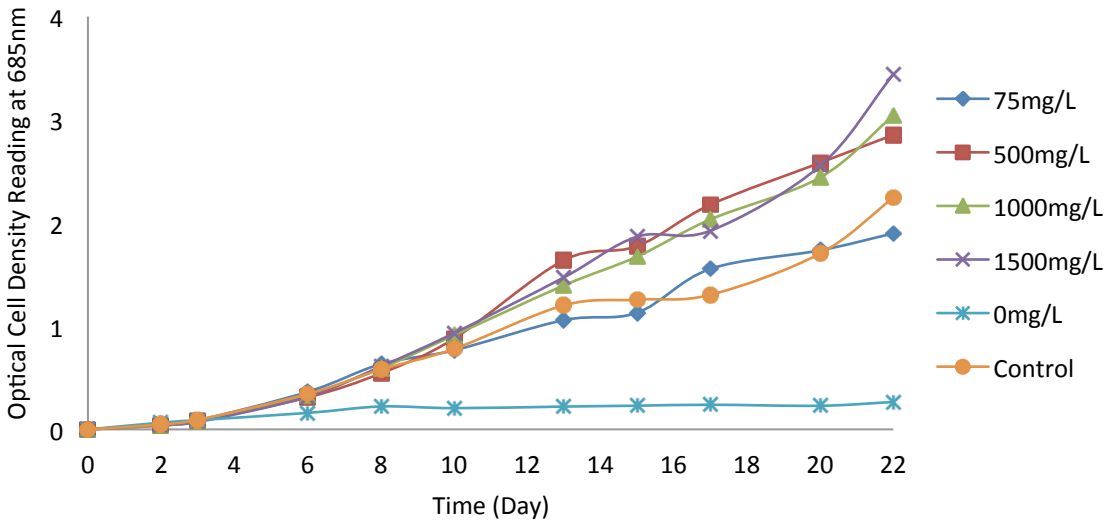


Figure 3.4 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of potassium nitrate (KNO_3) over a 22-day period

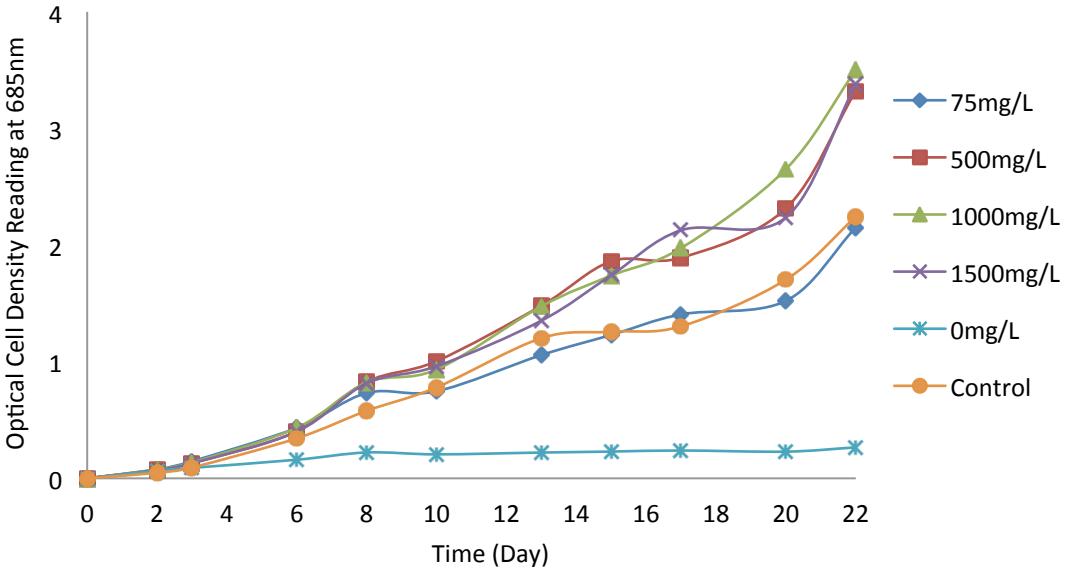


Figure 3.5 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of calcium nitrate ($Ca(NO_3)_2$) over a 22-day period

Figure 3.6 shows the cell growth of *Chloroparva pannonica* Cu40 cultivated in Guillard's F/2 growth medium media with different concentrations (0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L) of ammonium chloride (NH_4Cl). Cell growth of *Chloroparva pannonica* Cu40 decreased when the concentration of ammonium chloride in the Guillard's F/2 growth medium increased. The trend of cell growth with 75 mg/L ammonium chloride and in the control increased from day 2 to day 22 while, for concentrations of 500 mg/L, 1000 mg/L and 1500 mg/L of ammonium chloride, cell growth reached the stationary phase at day 13 (Figure 3.6). The highest growth

absorbance was in the medium with 75 mg/L of ammonium chloride with an OD reading of 2.4 at day 22.

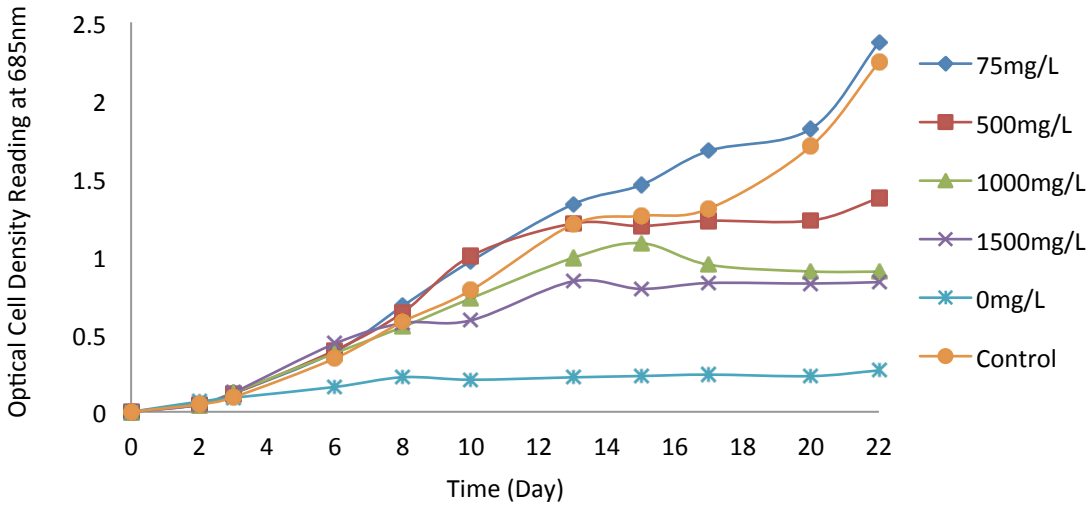


Figure 3.6 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of ammonium chloride (NH_4Cl) over a 22-day period

Figure 3.7 illustrates *Chloroparva pannonica* Cu40 cultures grown in Guillard's F/2 growth medium with ammonium bicarbonate (NH_4HCO_3) at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and the control for a period of 22 days. The cells cultivated with 500 mg/L of ammonium bicarbonate had a slightly higher OD (2.4) than the control (an OD reading of 2.2) at day 22 (Figure 3.7). When *Chloroparva pannonica* Cu40 was cultivated in Guillard's F/2 growth medium with 1000 mg/L and 1500 mg/L of ammonium bicarbonate, no difference was observed in cell growth from day 0 to day 22 (Figure 3.7).

The effect on the cell growth of *Chloroparva pannonica* Cu40 of glycine ($C_2H_5NO_2$) supplemented at concentrations of 0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L in Guillard's F/2 growth medium is shown in Figure 3.8. As indicated by OD at 685 nm, cell growth of *Chloroparva pannonica* Cu40 increased when the concentration of glycine in the Guillard's F/2 growth medium increased from day 2 to day 22. *Chloroparva pannonica* Cu40 cultivated in Guillard's F/2 growth medium containing 500 mg/L, 1000 mg/L and 1500 mg/L of glycine had a higher OD at day 22 than the control while, at a concentration of 75 mg/L and in the control, the reading for OD was quite similar (Figure 3.8).

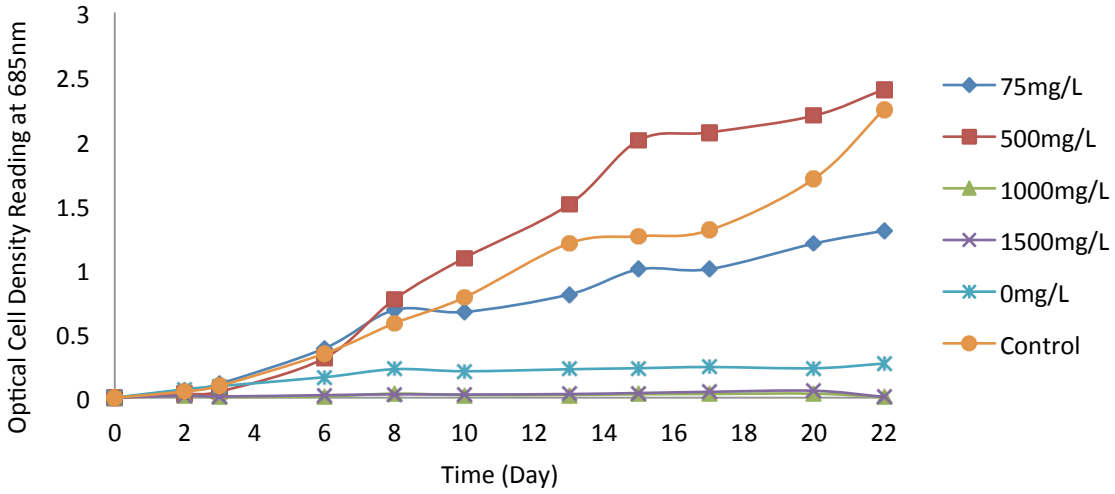


Figure 3.7 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of ammonium bicarbonate (NH_4HCO_3) over a 22-day period

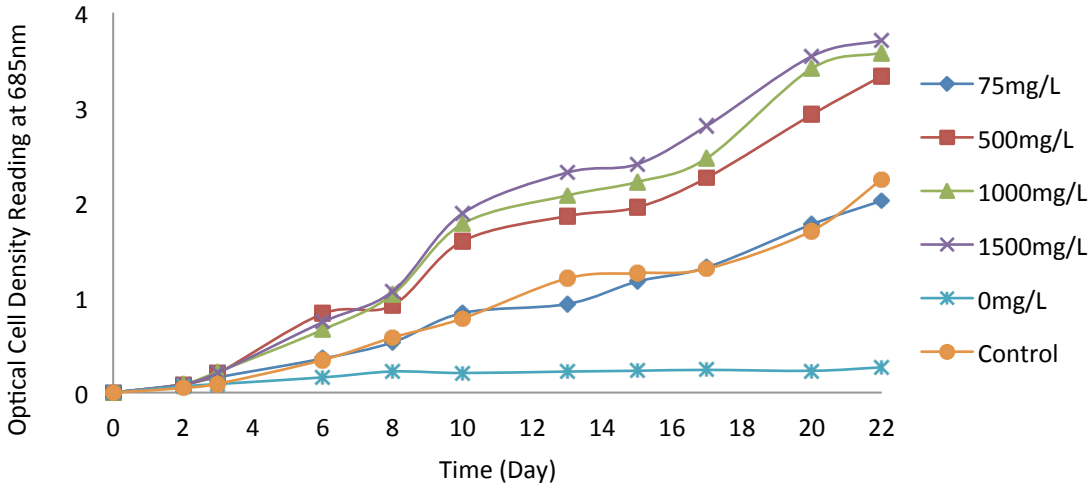


Figure 3.8 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of glycine ($C_2H_5NO_2$) over a 22-day period

Figure 3.9 shows cell growth of *Chloroparva pannonica* Cu40 cultivated in Guillard's F/2 growth medium with different concentrations (0 mg/L, 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L) of urea (CH_4N_2O). The trend of cell growth for 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L concentrations of urea was similar and was higher than the control. At day 22, the OD readings for concentrations of urea of 75 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L were between 2.5 and 2.7.

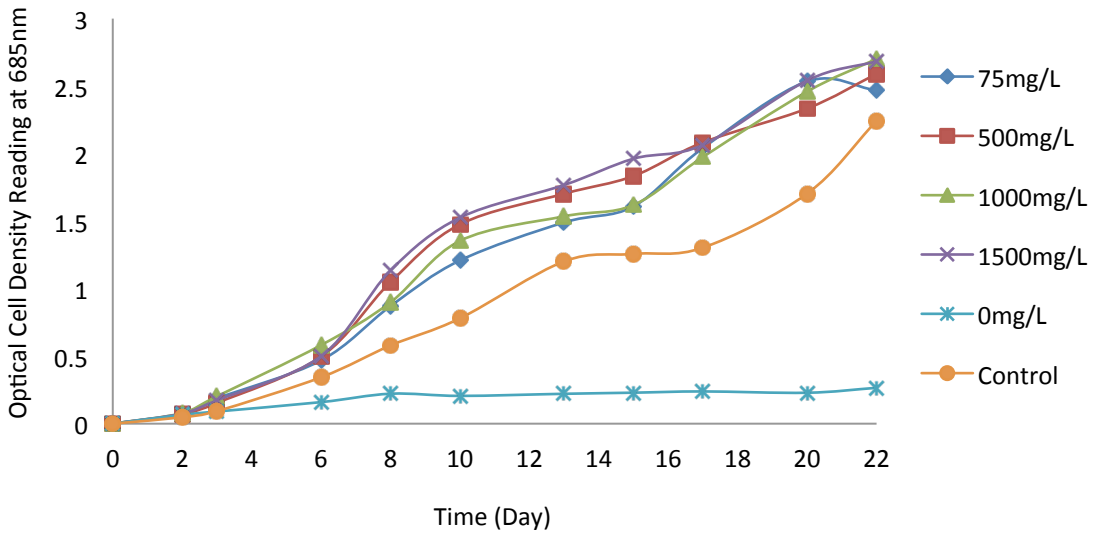


Figure 3.9 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of urea (CH_4N_2O) over a 22-day period

As shown in the figures from Figure 3.2 to Figure 3.9, results indicate that *Chloroparva pannonica* Cu40 was capable of growing in Guillard's F/2 growth medium supplemented with different types of nitrogen sources (sodium nitrate, ammonium nitrate, potassium nitrate, calcium nitrate, ammonium chloride, ammonium bicarbonate, glycine and urea) at different concentrations. Among all of the nitrogen sources, the highest cell growth level on day 22 was when 1500 mg/L of glycine (an OD reading of 3.6) was supplemented in the Guillard's F/2 growth medium (Figure 3.8), with this followed by the OD reading of 3.5 when 1500 mg/L of potassium nitrate was supplemented in the Guillard's F/2 growth medium (Figure 3.5). However, *Chloroparva pannonica* Cu40 did not grow when concentrations of 1000 mg/L and 1500 mg/L of ammonium bicarbonate were supplemented in the Guillard's F/2 growth medium. In addition, cell growth of *Chloroparva pannonica* Cu40 stopped when it was cultured in the Guillard's F/2 growth medium without any sodium nitrate, ammonium nitrate, potassium nitrate, calcium nitrate, ammonium chloride, ammonium bicarbonate, glycine or urea (Figure 3.2–Figure 3.9).

3.3.2 Effect of concentration of trace metals on growth

The second part of the study was aimed at standardizing the appropriate trace metal concentration to achieve the maximum cell growth of *Chloroparva pannonica* Cu40. Five of the seven trace metals from Guillard's F/2 growth medium were chosen in the present study (Guillard and Ryther, 1962), namely, ferric, sodium molybdate, zinc,

cobalt and manganese. *Chloroparva pannonica* Cu40 was subjected to growing with different trace metals at different concentrations for 21 days as outlined in Table 3.2. Throughout the experiments, *Chloroparva pannonica* Cu40 was cultivated in the standard Guillard's F/2 growth medium (Guillard and Ryther, 1962) with 40 µg/L of copper sulphate as the control.

The effect on the cell growth of *Chloroparva pannonica* Cu40 of cobalt chloride ($CoCl_2$) supplemented at concentrations of 10 µg/L (control), 20 µg/L, 30 µg/L, 40 µg/L, 80 µg/L and 160 µg/L in Guillard's F/2 growth medium is shown in Figure 3.10. Cell growth of *Chloroparva pannonica* Cu40 slightly increased as indicated by OD at 685 nm when the concentration of cobalt chloride in the Guillard's F/2 growth medium increased from day 2 to day 21 (Figure 3.10). Cell growth increased, reaching the highest point at day 14 with a cobalt chloride concentration of 10 µg/L and at day 18 with concentrations of 20 µg/L, 30 µg/L, 40 µg/L, 80 µg/L and 160 µg/L before it started to plateau. The highest cell growth was when *Chloroparva pannonica* Cu40 was cultivated in the Guillard's F/2 growth medium with a cobalt chloride concentration of 160 µg/L at day 18 with an OD of 2.12.

Figure 3.11 shows cell growth of *Chloroparva pannonica* Cu40 cultivated in Guillard's F/2 growth medium with different concentrations (1.3 µg/L [control)], 2.6 µg/L, 3.9 µg/L, 5.2 µg/L, 10.4 µg/L and 20.8 µg/L) of ferric chloride ($FeCI_3$). As indicated by OD at 685 nm, cell growth of *Chloroparva pannonica* Cu40 decreased when the concentration of ferric chloride in the Guillard's F/2 growth medium increased from day 2 to day 21. The trend of cell growth for a ferric chloride concentration of 2.6 µg/L, which had a slightly better growth trend than the control (1.3 µg/L), increased slowly from day 2 to day 9, increased exponentially from day 9, reached the highest growth at day 14 (an OD of 1.9) and entered the stationary phase from day 14 (Figure 3.11). However, cell growth for a ferric chloride concentration of 3.9 µg/L reached the highest growth at day 12 (an OD of 1.4), while ferric chloride concentrations of 5.2 µg/L, 10.4 µg/L and 20.8 µg/L reached the highest growth at day 18 (OD readings of 1.5, 1.48 and 1.35, respectively) before it started to plateau.

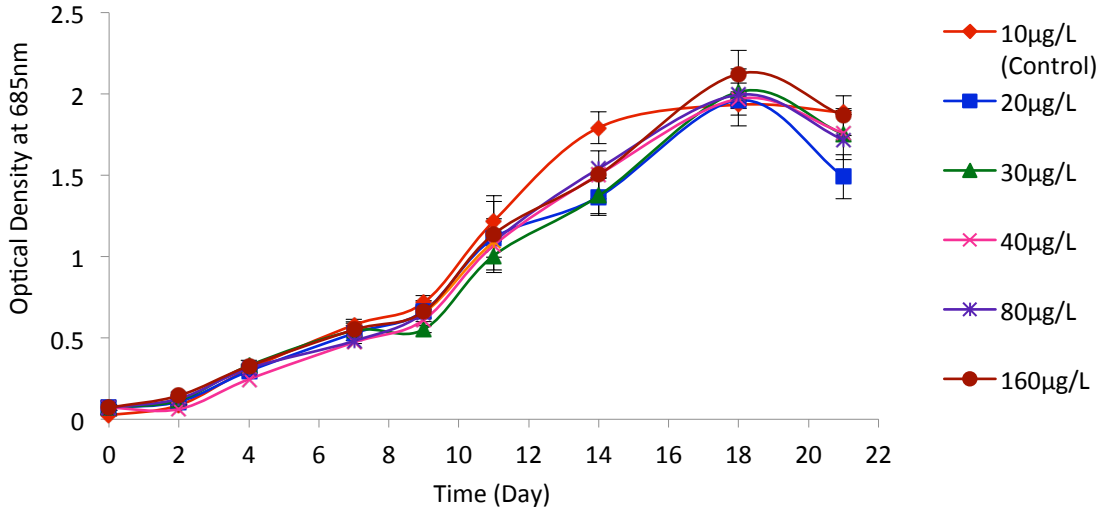


Figure 3.10 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of cobalt chloride ($CoCl_2$) over a 21-day period

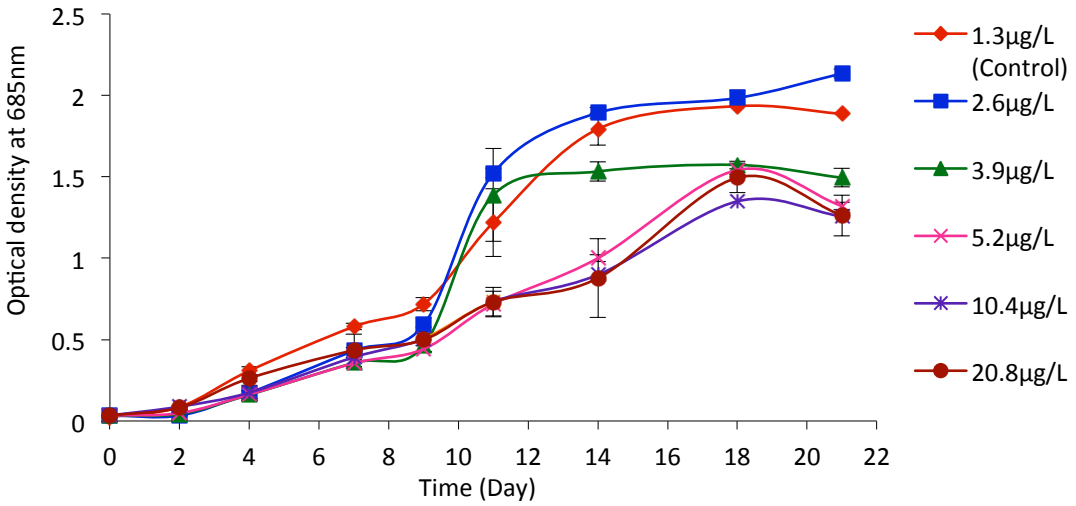


Figure 3.11 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of ferric chloride ($FeCl_3$) over a 21-day period

Chloroparva pannonica Cu40 cultures grown in Guillard's F/2 growth medium with manganese chloride ($MnCl_2$) at concentrations of 0.18 mg/L (control), 0.36 mg/L, 0.54 mg/L, 0.72 mg/L, 1.44 mg/L and 2.88 mg/L for a period of 21 days are shown in Figure 3.12. As indicated by OD at 685 nm, cell growth of *Chloroparva pannonica* Cu40 at manganese chloride concentrations of 0.36 mg/L, 0.54 mg/L, 0.72 mg/L, 1.44 mg/L and 2.88 mg/L increased slowly from day 2, then increased exponentially from day 9 and reached the highest level at day 14 (Figure 3.12). The cells entered the death phase from day 14 as the OD readings started to decline from day 14 until day 21 (Figure 3.12). As can be seen in Figure 3.12, *Chloroparva pannonica* Cu40 cultivated in Guillard's F/2

growth medium with manganese chloride concentrations of 2.88 mg/L and 1.44 mg/L had higher OD readings (2.3 and 2.06, respectively) at day 14 compared to the control (an OD reading of 1.8).

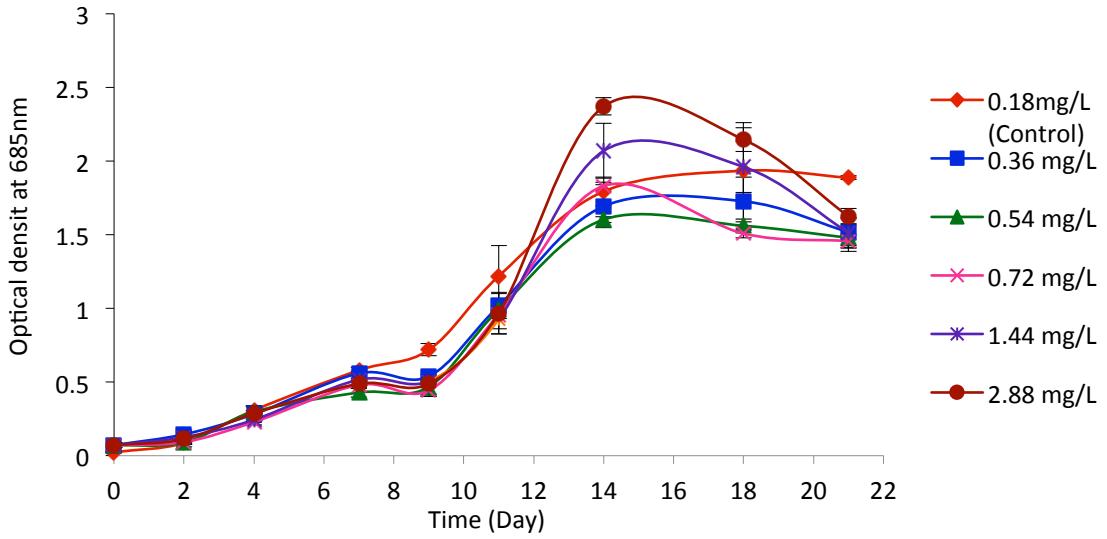


Figure 3.12 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of manganese chloride ($MnCl_2$) over a 21-day period

The effect on the cell growth of *Chloroparva pannonica* Cu40 of sodium molybdate (Na_2MoO_4) supplemented at concentrations of 6.3 µg/L (control), 12.6 µg/L, 18.9 µg/L, 25.2 µg/L, 50.4 µg/L and 100.8 µg/L in Guillard's F/2 growth medium is shown in Figure 3.13. As indicated by OD at 685 nm, the results have shown that the highest cell growth of *Chloroparva pannonica* Cu40 was when the cells were cultivated with a concentration of 100.8 µg/L sodium molybdate at day 14, with an OD reading of 2.1 (Figure 3.13).

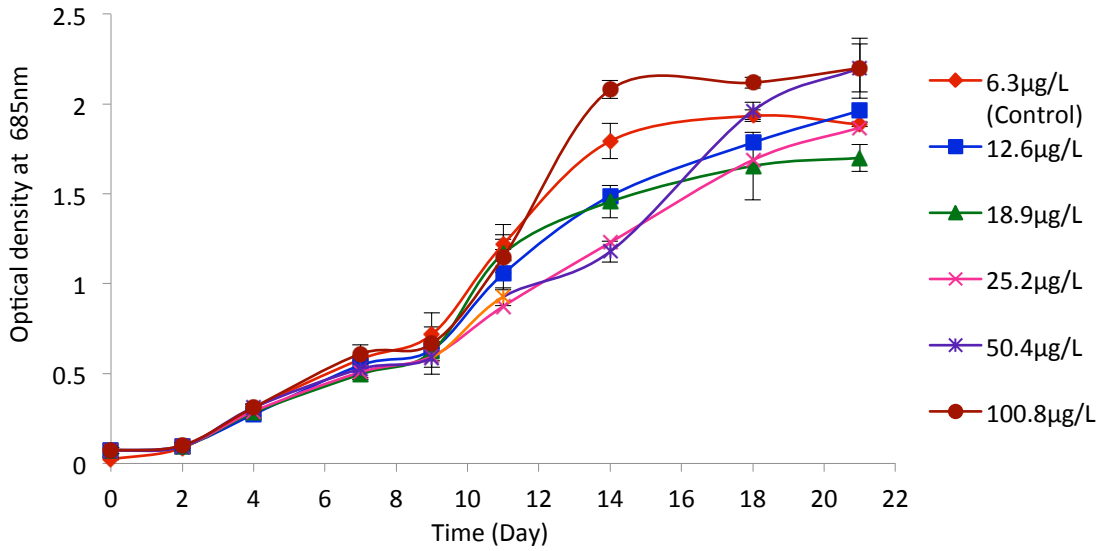


Figure 3.13 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of sodium molybdate (Na_2MoO_4) over a 21-day period

Chloroparva pannonica Cu40 cultures grown in Guillard's F/2 growth medium with zinc sulphate ($ZnSO_4$) at concentrations of 22 µg/L (control), 44 µg/L, 66 µg/L, 88 µg/L, 176 µg/L and 352 µg/L for a period of 21 days are shown in Figure 3.14. The cell growth trends of *Chloroparva pannonica* Cu40, as indicated by OD at 685 nm, were different for each zinc sulphate concentration. Cell growth with the zinc sulphate concentration of 22 µg/L (control) increased exponentially from day 2, reached the highest OD at day 18 (an OD reading of 1.87) with cell growth then declining after day 18. As indicated by the results, when the concentration of zinc sulphate increased, cell growth of *Chloroparva pannonica* Cu40 decreased.

As shown by the results from Figure 3.10– Figure 3.14 *Chloroparva pannonica* Cu40 was able to grow in Guillard's F/2 growth medium with an increased concentration of cobalt, ferric, manganese, sodium molybdate and zinc. The highest cell growth was achieved when *Chloroparva pannonica* was cultivated in Guillard's F/2 growth medium with concentrations of cobalt at 160 µg/L, ferric at 2.6 µg/L, manganese at 2.88 mg/L, sodium molybdate at 100.8 µg/L and zinc at 22 µg/L.

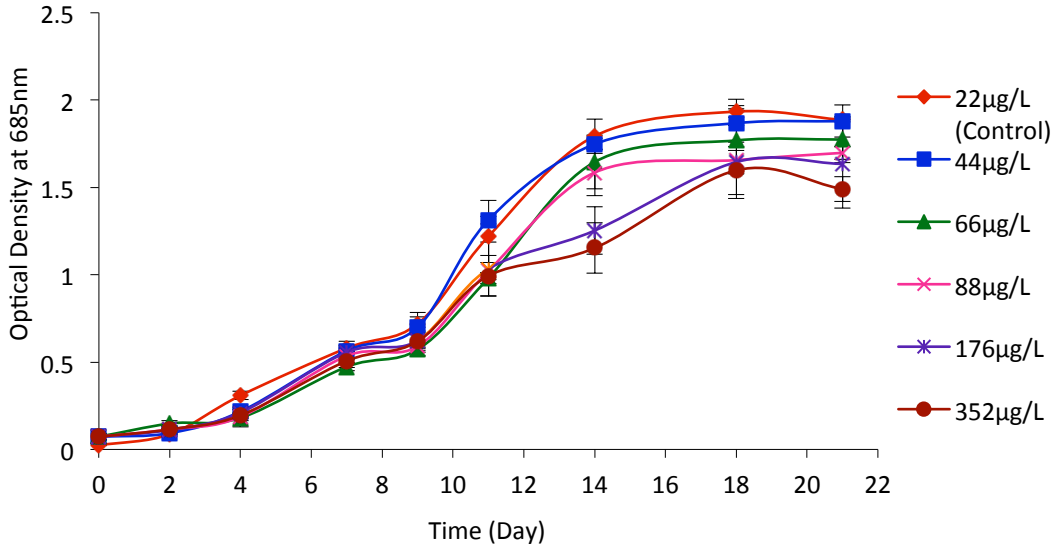


Figure 3.14 Average optical density at 685 nm of *Chloroparva pannonica* Cu40 supplied with different concentrations of zinc sulphate ($ZnSO_4$) over a 21-day period

Table 3.3 Concentration of each trace metal with the highest cell growth

Trace Metals	Concentration of trace metals with the highest cell growth
Cobalt	160.00 µg/L
Ferric	2.60 µg/L
Manganese	2.88 mg/L
Sodium molybdate	100.80 µg/L
Zinc	22.00 µg/L

3.4 Discussion

A cost-effective microalgae based bio-refinery process is highly dependent on selecting a suitable microalgae strain that has a high lipid yield (producing results from high biomass productivity and high lipid content) and is also able to produce a high-value desired product. The isolated native strains may contain high levels of lipids but do not necessarily have a high growth rate. Therefore, the first step was to identify a particular microalgae strain that is able to produce a significant amount of lipids and carotenoid(s) then optimize the culture conditions for biomass productivity to improve the economics of microalgae oil production.

Chloroparva pannonica was first discovered by Somogyi *et al.* (2011) when it was isolated from a turbid, shallow soda pan in Hungary. To date, the potential of *Chloroparva pannonica* for microalgae-based production has not been reported. In the present study's preliminary trials, this strain showed a high lipid content.

The present study was aimed at increasing the cell growth of *Chloroparva pannonica* Cu40 by manipulating the concentration and type of nitrogen sources and trace metals.

3.4.1 Effect of nitrogen sources and concentration on growth

The growth of *Chloroparva pannonica* Cu40 with different nitrogen sources at different concentrations was compared with the control in the Guillard's F/2 growth medium with 75 mg/L of sodium nitrate. The results indicated significant differences between each type of nitrogen source in its effect on the growth of *Chloroparva pannonica* Cu40 (Figure 3.2–Figure 3.9). In addition, the different concentrations of nitrogen were noted as having an effect on cell growth during the study period (Figure 3.2–Figure 3.9).

The growth of *Chloroparva pannonica* Cu40 was poor when cultured in the Guillard's F/2 growth medium without any (0 mg/L) nitrogen sources (Figure 3.2–Figure 3.9). Nitrogen, if deficient, can act as a limiting factor for biomass production. Microalgae do not have the ability to fix nitrogen from the atmosphere. In addition, studies have shown that growth was directly proportional to the amount of nitrogen in the cultivation medium with this found in *Botryococcus braunii*, *Chlorella pyrenoidosa, Chlorella protothecoides, Nannochloropsis gaditana* and *Scenedesmus bijugatus* (Arumugam *et al.*, 2013, Nigam *et al.*, 2011, Ren *et al.*, 2013, Shen *et al.*, 2010, Zhila *et al.*, 2005).

The overall experimental results indicated that *Chloroparva pannonica* Cu40 responded well to most nitrogen sources at different concentrations. Microalgae are known to have the ability to utilize different forms of nitrogen but this might vary between species. For example, no difference was found in the growth trend in *Chloroparva pannonica* Cu40 when the urea concentration increased (Figure 3.9). The highest OD readings obtained for urea concentrations between 75 mg/L and 1500 mg/L were between 2.5 and 2.7. This indicated that these concentrations of urea had no effect on the growth of *Chloroparva pannonica* Cu40. However, results also indicated that urea at the lower

concentration (75 mg/L) was able to perform better in comparison to the other nitrogen sources at the same concentration.

As indicated by OD at 685 nm, the growth of *Chloroparva pannonica* Cu40 increased with increases in the concentrations of glycine and potassium nitrate as nitrogen sources. Moreover, results from the present study indicated that glycine at 1500 mg/L (an OD reading of 3.71) and potassium nitrate at 1500 mg/L (an OD reading of 3.5) were favourable for *Chloroparva pannonica* Cu40 growth. Glycine is an amino acid which microalgae are able to utilize to enhance their growth. Shen *et al.* (2014) demonstrated that 18 mM of glycine was able to increase adhesion biomass productivity by up to 15.76 $gm^{-2}d^{-1}$ in *Nannochloropsis oculata.* Potassium ions were also able to enhance nitrogen uptake by microalgae. Cultivation of *Monoraphidium* sp. using potassium nitrate as the nitrogen source provided the highest biomass concentration (650 mgL^{-1}) when compared to ammonium chloride and ammonium nitrate (Wu *et al.*, 2013). Furthermore, their study showed that the addition of potassium ions stimulated nitrogen uptake in *Monoraphidium* sp. and increased biomass up to three-fold.

As also indicated by OD at 685 nm, the growth of *Chloroparva pannonica* Cu40 did not increase when sodium nitrate, ammonium nitrate, calcium nitrate, ammonium chloride and ammonium bicarbonate were used as the main nitrogen sources. Although studies have shown that ammonium is a preferred nitrogen source, the present study's results have shown that when the concentration of ammonium increased, the growth of *Chloroparva pannonica* Cu40 decreased. This suggested that *Chloroparva pannonica* Cu40 was only able to utilize ammonium at low concentrations. It was also noted that the growth of *Chloroparva pannonica* Cu40 was inhibited when cultivated in F/2 medium with 1000 mg/L and 1500 mg/L of ammonium bicarbonate (Figure 3.7). Ammonium is the favourite nitrogen source for microalgae as its oxidation state eliminates the need for metabolic energy and it can be utilized immediately for amino acid synthesis (Boussiba *et al.*, 1987, Collos, 1982, Yin *et al.*, 1998). However, under certain conditions, microalgae prefer nitrate because their growth also depends on factors such as genetics, irradiance, presence of carbon sources, and active and passive transport of molecules. In some cases, high levels of ammonium and nitrate in the cultivation medium can inhibit the growth of microalgae. The reason is that the consumption of ammonium during growth by microalgae can result in a decreased level of pH due to the release of H^+ ions to the medium. In contrast, during the growth of

microalgae with nitrate in the medium, the pH increases due to the release of OH^- ions (Goldman *et al.*, 1982, Raven and Smith, 1976). Moreover, when the concentration of ammonium is too high, it can be toxic and inhibit the growth of microalgae. The threshold of ammonium toxicity varies between microalgae species. Bates *et al.* (1993) reported that the growth of the diatom *Nitzschia pungens* was inhibited at ammonia concentrations above 0.2 mM. Furthermore, Taylor *et al.* (2006) reported a complete inhibition of cell growth in *Aureococcus anophagefferens* at an ammonia concentration of 500 µm. This could be due to the inability of microalgae to regulate passive diffusions of ammonium across the plasma membrane. When the level of ammonia levels is high in cells, it affects the tricarboxylic acid (TCA) cycle intermediates and disrupts cellular respiration (Norici *et al.*, 2002).

3.4.2 Effect of cobalt, ferric, manganese, sodium molybdate and zinc concentration on growth

Trace metals are considered as inducers for carotenogenesis in microalgae. Overall, the results indicated that the concentration of different trace metals had an effect on the growth of *Chloroparva pannonica* Cu40 when compared to the control (Figure 3.10– Figure 3.14). The results showed that when the concentration of cobalt, manganese and sodium molybdate increased in the F/2-Si medium, the growth, as indicated by OD, also increased. As can be seen from Table 3.3 the highest cell growth of *Chloroparva pannonica* Cu40 for concentrations of cobalt, manganese and sodium molybdate was achieved at the 16-fold concentration.

As shown by the results from Figure 3.10 – Figure 3.14 *Chloroparva pannonica* Cu40 was able to grow in Guillard's F/2 growth medium with an increased concentration of cobalt, ferric, manganese, sodium molybdate and zinc. The highest cell growth was achieved when *Chloroparva pannonica* was cultivated in Guillard's F/2 growth medium with concentrations of cobalt at 160 µg/L, ferric at 2.6 µg/L, manganese at 2.88 mg/L, sodium molybdate at 100.8 µg/L and zinc at 22 µg/L.

Conversely, the results showed that cell growth of *Chloroparva pannonica* Cu40 did not increase when the concentration of ferric chloride increased. The cell growth at a ferric chloride concentration of 2.6 µg/L was slightly more than in the control (Figure

3.11). However, when the concentration of ferric chloride increased, cell growth decreased. Iron is an important co-factor in photosystem I (PSI) of the electron transport chains in plants (Briat *et al.*, 2007). Therefore, it can affect the ability of photosynthetic apparatus to utilize light energy and fix carbon to support cell growth (Greene *et al.*, 1992, Terauchi *et al.*, 2010). Iron deficiency in the cultivation media leads to the reduction of photosynthesis activity and limits the growth of microalgae (Carmel *et al.*, 2014). Moreover, iron stress is able to change the metabolic pathway to lipid accumulation in microalgae cells (Concas *et al.*, 2014). Several studies have claimed that high biomass productivity and lipid content were achieved when *Chlorella vulgaris* (Liu *et al.*, 2008) and *Scenedesmus dimorphus* (Ruangsomboon *et al.*, 2013) were cultivated under conditions of high iron content ($1.2x10^{-5}$ $molL^{-1}$ and 45 mgL^{-1}, respectively). In addition, the study by Che *et al.* (2015) indicated that when the iron concentration increased from 0 μM to 150 μM, the dry cell weight of *Monoraphidium* sp. increased from 1.11 gL^{-1} to 1.42 gL^{-1}. However, the dry cell weight decreased to 1.17 gL^{-1} when the iron concentration was further increased to 200 μM. A similar study by Ren *et al.* (2014) indicated that the dry cell weight of *Scenedesmus* sp. increased from 2.38 gL^{-1} to 3.47 gL^{-1} when the iron concentration increased from 0 g/L to $1.2x10^{-3}$ g/L. Additional ferric to increase the concentration to $1.2x10^{-}$ g/L resulted in a decrease of biomass to 1.55 gL^{-1} (Ren *et al.*, 2014). On the other hand, studies focused on the genus *Botryococcus* have indicated that by increasing iron concentration, the growth rate decreased but the lipid content increased (Ruangsomboon, 2012, Yeesang and Cheirsilp, 2011). For example, the study by Ruangsomboon (2012) showed that when the iron concentration increased from 9 mgL^{-1} to 27 mgL^{-1}, although the growth of the cells was affected, the lipid content increased from 22% to 35%. These results indicate that the effect of iron on the growth of microalgae might be species-specific, with this still unclear.

In addition, the cell growth of *Chloroparva pannonica* Cu40 decreased when the concentration of zinc increased from 22 μg/L to 352 μg/L (Figure 3.14). Zinc is one of the essential trace metals for microalgae with zinc deficiency leading to low biomass productivity. However, increased zinc concentration in the cultivation medium can inhibit phosphate uptake into the cells and therefore limit the growth of microalgae (Kaneko *et al.*, 2004, Kuwabara, 1985, Paulsson *et al.*, 2002). Growth inhibition of microalgae in response to zinc concentration has been reported. Omar (2002) reported that *Scenedesmus obliquus* and *Scenedesmus quadricauda* required low levels of zinc

for growth (0.5 mg/L and 1.5 mg/L, respectively). However as the zinc concentration increased from 0.5 mg/L to 8 mg/L in the cultivation medium, cell growth of *Scenedesmus obliquus* and *Scenedesmus quadricauda* decreased with cell growth totally inhibited at 8 mg/L.

3.5 Conclusion

The selection of the appropriate nitrogen sources, nitrogen concentration and trace metal concentration for *Chloroparva pannonica* Cu40 is crucial for enhanced biomass and lipid productivity in the microalgae based bio-refinery process. One of the major costs in the large-scale process is nutrient supply. The process economic feasibility is improved by selecting the appropriate nitrogen sources and nitrogen and trace metals at optimum concentrations to increase biomass productivity, lipid accumulation and lutein synthesis. Results from the present study indicated that glycine at a concentration of 1500 mg/L and potassium nitrate at a concentration of 1500 mg/L provided the highest biomass yield in *Chloroparva pannonica* Cu40. However, urea and calcium nitrate were selected for the next part of the study due to their relatively low cost and their ability to achieve higher levels of growth at a lower concentration. Furthermore, the highest cell growth was achieved when *Chloroparva pannonica* Cu40 was cultivated in Guillard's F/2 growth medium with cobalt at a concentration of 160 μg/L, ferric at 2.6 μg/L, manganese at 2.88 mg/L, sodium molybdate at 100.8 μg/L and zinc at 22 μg/L.

The next part of the study was performed by Nijoy John at the Department of Medical Biotechnology, Flinders University, South Australia. This part of the study focused on the effect of biomass, lutein content, lipid content and fatty acid profiles of *Chloroparva pannonica* Cu40 when cultivated in different nutrient regimes with various concentrations of calcium nitrate, urea, sodium nitrate and trace metals (cobalt, iron, sodium molybdate, manganese and zinc) in 3L batch mode with 1% carbon dioxide. The composition of the new Flinders University (FU) medium contains the following per litre.

Urea	75 mg
$Na_2H_2PO_4$	5 mg
Trace metals:	

$Na_2EDTA.2H_2O$	8.7 mg
$FeCl_3.6H_2O$	2.6 µg
$CoCl_2.6H_2O$	40 µg
$CuSO_4.5H_2O$	9.8 µg
$MnCl_2.4H_2O$	1.44 mg
$Na_2MoO_4.2H_2O$	25.2 µg
$ZnSO_4.7H_2O$	88 µg

Vitamins (freshly made every three months, filter sterilized):

Thiamine HCl	20 µg
Biotin (Vitamin H)	0.1mg
Vitamin B12	0.1mg

By comparing the FU medium with Guillard's F/2 medium, it can be seen that urea has replaced sodium nitrate as the main nitrogen source at a concentration of 75 mg/L. In addition, the concentrations of ferric has been increased two-fold, cobalt increased four-fold, manganese increased eight-fold, sodium molybdate increased four-fold and zinc increased four-fold. The study concluded that the FU medium was able to increase the lipid content in *Chloroparva pannonica* Cu40 from 9.34% to 12.7%. The predominant fatty acid components were C16:0; C18:1 n9; C18:2 n6; and C18:3 n3 at 15.67%; 17.21%; 16.76%; and 17.75%, respectively. The dry biomass was 0.811 g/L; chlorophyll *a* content was 3.45 g/kg of dry biomass; and lutein content was 0.43 g/kg of dry biomass.

Chapter 4 Enhancement of biomass yield and lutein content of *Chloroparva pannonica* using random mutagenesis and selection

4.1 Introduction

Over 40,000 species of algae have been identified throughout the world but only a limited number of species have been studied in detail (Hu *et al.* 2008a). Many microalgae species have been identified as being able to accumulate lipids. However, the significant drawbacks for the microalgae based bio-refinery process are: (1) high capital costs and high-energy methods needed to harvest microalgae cells; (2) inconsistency in biomass, lipid and desired product production in outdoor system such as large-scale ponds; and (3) shielding of microalgae cells.

The process of using naturally occurring mutants is too slow for large-scale microalgae based bio-refinery production to achieve a quantum increase in yield required for profitable operations. Strain improvement via mutagenesis followed by strain selection of microalgae to increase biomass and high-value product production are crucial in leading to a sustainable and economically viable microalgae bio-refinery process. The two most promising methods for microalgae strain improvement are genetic engineering and random mutagenesis.

Genetic engineering of the promising microalgae strains has been used to manipulate and produce desired traits to enhance the growth, lipids and other valued products. Several research groups have used different techniques to transform the nucleus, chloroplasts and mitochondria (Cordero *et al.*, 2011a, Radakovits *et al.*, 2011, Radakovits *et al.*, 2010, Shin *et al.*, 2016, Xue *et al.*, 2015). These techniques have been very promising but the significant drawbacks are (1) limited biochemical and genetic information for most of the microalgae species; (2) limited information about the metabolic and regulatory pathways involved in the biosynthesis of the desired product and; (3) extensive technical manipulation; (4) lack of community support for GMO release. However, recent research focused on microalgae genomics, an expressed sequence tag (EST), and nuclear, mitochondrial and chloroplast genome sequencing has greatly facilitated the use of this technique to improve microalgae strains. For example, the genomes of *Chlorella* (Merchant *et al.*, 2007); *Bathycoccus prasinos* (Moreau *et al.*,

2012); *Dunaliella salina* (Smith *et al.*, 2010); *Micromonas pusilla* (Worden *et al.*, 2009); *Nannochloropsis gaditana* (Radakovits *et al.*, 2011); *Nannochloropsis oceanica* (Wang *et al.*, 2014); and *Ostreococcus lucimarinus* (Palenik *et al.*, 2007) have been sequenced to facilitate the genetic manipulation of microalgae strains for biofuel production.

One of the most common and easily achieved techniques in genetic engineering for microalgae is RNA-mediated silencing, using RNA interference (RNAi) or artificial microRNAs (amiRNA), which allows the expression of genes in microalgae to be negatively regulated or disrupted though various mechanisms, including translation inhibition, RNA degradation and transcriptional repression (Carthew and Sontheimer, 2009, Cesrutti and Casas-Mollano, 2006, Deng *et al.*, 2015). A study performed by Deng *et al.* (2015) showed that the *Chlamydomonas reinhardtii CrCO* RNAi strains were able to decrease the *CrCO* expression resulting in an increased expression of triacylglyceride (TAG) biosynthesis-related genes (*DGAT2, PAP2* and *PDAT3*) and decreased expression of *CIS* and *FBPI* genes. The result was an increase in lipid content and especially, by 24.5%, in triacyglycerides (TAGs).

On the other hand, several genome-editing technologies have been developed including homologous recombination (HR), zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) but these methods are labour-intensive and hard to use on microalgae. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein-9 nuclease (Cas9) has been developed. This method is able to target and mutate one or more genes specifically in the genome by using Cas9, guided by single chain guide RNAs (sgRNAs) (Hsu *et al.*, 2014). This has become the fastest, cheapest and most accurate method in genome editing; however, the significant drawback is that this technique is extremely difficult to use on microalgae. To date, only genomes from *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* have been successfully edited using CRISPR/Cas9 technology (Nymark *et al.*, 2016, Shin *et al.*, 2016). Shin *et al.* (2016) managed to induce mutation using CRISPR/Cas9 in *Chlamydomonas reinhardtii* on *MAA7* (the gene that encodes the beta subunit of tryptophan synthase [TSB]), *CpSRP43* (the gene that encodes chloroplast signal recognition particle [SRP]43) and *ChlM* (the gene that encodes magnesium (Mg)-protoporphyrin IX S-adenosyl methionine O-methyl transferase). In addition, Nymark *et al.* (2016) successfully targeted and disrupted *CpSRP54* (the gene that encodes

chloroplast SRP54) using CRISPR/Cas9 in the diatom *Phaeodactylum tricornutum*. However, the use of genetically manipulated microalgae outside the lab is not feasible due to concerns on the ecosystem and the environment. The public perception is negative with arguments that genetically manipulated strains might be able to transfer an antibiotic-resistant gene to the wild type and release algae-derived toxins, allergens and carcinogens to the environment (Johanningmeier and Fischer, 2010, Wolfe, 2000).

The drawbacks of genetically manipulated microalgae have inspired a number of laboratories to develop random mutation methods. These methods requires little knowledge of the pathways involved in biosynthesis of the desired product as well as needing minimal technical manipulation: the mutants pose less of a threat to the wild type strains and are of less concern to the environment. In addition, this method has experienced less difficulties with regulation as the mutants obtained from random mutagenesis are not classified as genetically modified organisms as they do not contain noon-host genetic material. Point mutations can be classified into three categories: insertions, deletions and substitutions. The error occurs in the DNA level causing the codons to be incorrectly configured.

Various mutagenic treatments are available and can be divided broadly into chemically and physically induced mutagenesis. Briefly, the random mutation method starts with the exposure of parent culture (PC) to various mutagenic treatments to produce from hundreds to thousands of independent mutants with only the potential for single nucleotide changes, with this then followed by selection and screening for the desired high-yield mutants. Once the mutants are generated, the biggest challenge is to screen and select the mutants with the desired traits. Therefore, selection strategies to screen for mutants possessing desirable traits based on rational principles is important. For example, the use of inhibitors, such as herbicides and antibiotics, are often used to facilitate the screening and selection of mutants with desired traits. Typical chemicals used for mutagenesis are ethyl methanesulphonate (EMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU) and 1-methyl 3-nitro 1-nitrosoguanidine (NTG), while typical physical mutagens are usually high-energy irradiation, such as UV irradiation, gamma rays and heavy ion beams. Different mutagens are able to induce certain mutations with their modes of action listed in Table 4.1. In most published studies, the use of chemically induced mutation has been aimed at increasing the content of fatty acids and carotenoids in microalgae (Chaturvedi and

Fujita, 2006, Chaturvedi *et al.*, 2004, Cordero *et al.*, 2011b, Cortez *et al.*, 2015, Hu *et al.*, 2008b, Patel *et al.*, 2016, Perin *et al.*, 2015). The first studies to successfully use chemically induced mutation (with EMS and MNU) to increase eicosapentaenoic acid (EPA) in *Nannochloropsis oculata* were performed by Chaturvedi *et al.* (2004) and Chaturvedi *et al.* (2006), respectively. Chaturvedi *et al.* (2004) showed that when *Nannochloropsis oculata* was subjected to MNU and selected by the herbicide, quizalofop, the mutants were rich in polyunsaturated fatty acids (PUFAs). Furthermore, when Chaturvedi *et al.* (2006), utilized EMS as a mutagen and the antibiotics, cerulenin and erythromycin, as selection agents, the *Nannochloropsis oculata* mutants contained 12–29% more EPA and had better thermotolerance compared to the wild type. In addition, Cordero *et al.* (2011a) used MNNG to generate *Chlorella sorokiniana* mutants, with their experiments and screening using nicotine and the herbicide, norflurazon, managing to increase the lutein yield twofold above that of the wild type.

Table 4.1 Mode of action of different types of chemically and physically induced mutations

Mutation	Mutagen	Mode of action	Most common mutation caused
Chemically induced mutation	EMS, MNU, MNNG, NTG	Alkylation of DNA base, particularly guanine	Point mutations
Physically induced mutation	UV irradiation	Formation of pyrimidine dimers	Point mutations, deletions
	Gamma ray irradiation	Ionization leading to double-strand breaks	Deletions
	Heavy ion beams	Ionization leading to double-strand breaks	Chromosome breaks and exchanges

Source: Hlavova *et al.*(2015)

Among the different types of mutagen for physically induced mutation, UV irradiation is the simplest method to perform as it does not require any special equipment or chemicals. This method only requires exposure of the culture under UV lamps in sterile conditions to obtain a wide variety of mutants. Ultraviolet (UV) irradiation, when used as a mutagen, has been used to successfully obtain microalgae mutants with higher fatty acid content. The microalgae in which this has been achieved include *Scenedesmus obliquus* (de Jaeger *et al.*, 2013); *Chlorella sorokiniana* (Vigeolas *et al.*, 2012);

Haematococcus pluvialis (Kamath *et al.*, 2008); *Pavlova lutheri* (Meireles *et al.*, 2003); and *Tetraselmis suecica* (Lim *et al.*, 2015). In a strain improvement program by Meireles *et al.* (2003), using *Pavlova lutheri* with UV light as the mutagen, they obtained a mutant that was 32.8% higher in EPA and 32.9% higher in docosahexaenoic acid (DHA) than the wild type.

Moreover, the combination of mutagenic treatment and various screening methods has resulted in the development of interesting mutants. This method usually begins with the exposure of microalgae strains to UV irradiation, as the physically induced mutagen, and is followed by treatment with one or two chemical mutagens. This method has been used to obtain *Haematococcus pluvialis* and *Phaffia rhodozyma* mutants with a high astaxanthin yield (Chumpolkulwong *et al.*, 1997, Kamath *et al.*, 2008, Tjahjono *et al.*, 1994, Tripathi *et al.*, 2001). For example, a study performed by Kamath *et al.* (2008) utilized UV exposure, followed by EMS and NTG treatment, and selection by the herbicide, glufosinate, on *Haematococcus pluvialis*. They found that the transcript levels of phytoene synthase, phytoene desaturase, lycopene cyclase, β-carotene ketolase and β-carotene hydroxylase enzymes in the mutants were higher than in the wild type, observing an increase of 2.2% to 3.8% (w/w) of astaxanthin in the mutants.

The main objective of the current strain improvement study is to develop *Chloroparva pannonica* with the potential to grow rapidly, and to produce higher biomass and enhanced lutein and lipid production, through random mutagenesis. Given the simplicity and potential of physically induced mutation by UV-C irradiation, it was considered worthwhile to carry out random mutagenesis using UV-C irradiation for *Chloroparva pannonica.* The published literature for *Chloroparva pannonica* is limited. The current work is a first attempt to obtain rapid-growing, lipid- and lutein-enriched strains of *Chloroparva pannonica* by applying UV-C irradiation.

This *Chloroparva pannonica* strain improvement study followed a four-stage protocol (Figure 4.1). The first stage involved purification of the parent culture (PC) by reducing the bacteria population using different combinations of antibiotics. Mutation of the PC by UV-C irradiation using a germicidal lamp placed 15 cm from the culture occurred in the second stage. The third stage followed with selection and screening using herbicide inhibitors to obtain mutants with the potential to grow fast with enriched lipids and lutein. The fourth stage involved the cultivation and comparison of biomass, and lipid

and lutein content in the selected mutant strains with the WT in 3 L flasks in batch mode with carbon dioxide (CO_2) supplied.

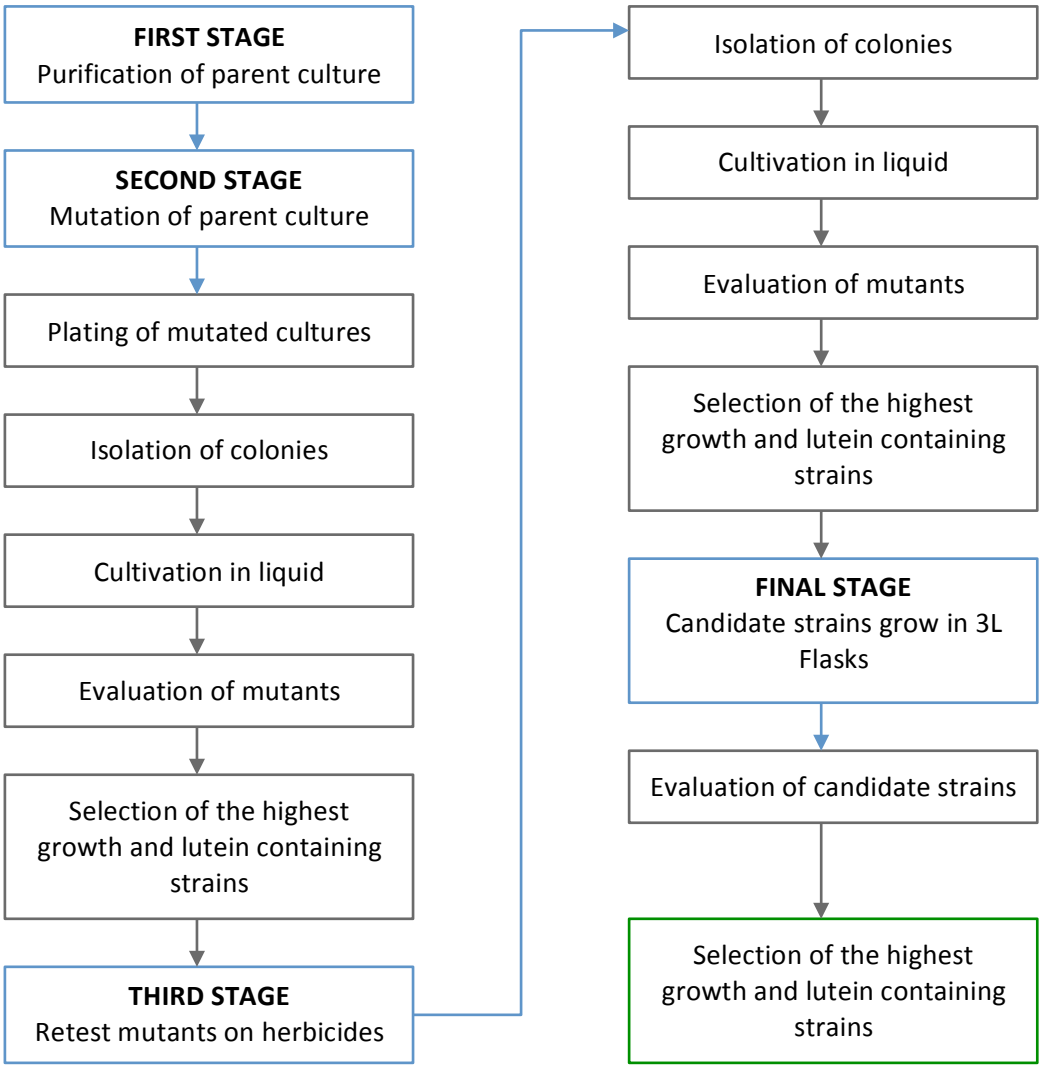


Figure 4.1 Four-stage protocol of *Chloroparva pannonica* strain improvement study

4.2 Methods and materials

4.2.1 *Chloroparva pannonica* strain

The microalga used in this study was *Chloroparva pannonica*, obtained by the Aquatic Sciences Algal Production group from the South Australian Research and Development Institute (SARDI) from a saltpan at Waikerie, South Australia, Australia.

4.2.1.1 *Chloroparva pannonica* wild type (WT) strain selection

The *Chloroparva pannonica* wild type (WT) strain underwent selection by using different concentrations of copper. For the flask experiments, Guillard's F/2 medium was selected as the starting point for copper selection of the medium. Guillard's F/2 medium consisted of 75 mg $NaNO_3$; 5 mg $Na_2H_2PO_4$; 8.7 mg $Na_2EDTA.2H_2O$; 1.3 µg $FeCl_3.6H_2O$; 10 µg $CoCl_2.6H_2O$; 9.80 µg $CuSO_4.5H_2O$; 0.18 mg $MnCl_2.4H_2O$; 6.3 µg $Na_2MoO_4.2H_2O$; 22 µg $ZnSO_4.7H_2O$; 20 µg thiamine HCl; 0.1 mg biotin (Vitamin H); and 0.1 mg Vitamin B12 in 1 L of seawater: deionized water (1:1 v/v) at a pH o 7.5. Six different concentrations of copper were selected: 9.8 µg/l (control), 10 µg/l, 20 µg/l, 30 µg/l, 40 µg/l, 50 µg/l and 100 µg/l. All media were sterilized at 121°C for 15 minutes. Three replicates were set up for each copper concentration resulting in a total of 21 cultures. The experiment was carried out under autotrophic cultivation conditions in 250 ml Erlenmeyer flasks with 10 ml of *Chloroparva pannonica* culture (optical density [OD] of 0.7 at 685 nm) and 100 ml of various culture media. Cultures were maintained at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. Cell growth in the liquid medium was analysed using a µQuant™ UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia) at an OD reading at 685 nm at day 3, 6, 9, 12 and 14, based on the method described in subsection 2.2.2.2. Each test was carried out in triplicate for each strain and was repeated three times.

The *Chloroparva pannonica* strains with high growth rates were selected and taken into grow in 3 L flasks in batch mode. The 300 ml of *Chloroparva pannonica* culture with an equal number of cells (OD of 0.7 at 685 nm) was inoculated into 2.7 ml of various sterile culture media in 3 L flasks, with 1% carbon dioxide supplied. Cultures were set up at the National Collaborative Research Infrastructure Strategy (NCRIS) Algae and Biofuel Facility, SARDI, South Australia, and, as before, were maintained at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. Cell growth in the liquid media was analysed using a µQuant UV/vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia) at an OD reading at 685 nm at day 2, 4, 7, 9, 11 and 15, based on the method described in subsection 2.2.2.2. In addition, the measurement of the total dried biomass was carried out at the end of the growth phase (day 15), based on the method described in Section 2.2.3, while the total lipids analysis was carried out, based on the method

described in Section 2.2.4. Lutein was extracted based on the method described in subsection 2.1.1.3.2. Lutein extracts were then analysed by high-performance liquid chromatography (HPLC), based on the method described in subsections 2.1.1.5 and subsections 2.1.1.6. Each analysis was carried out in triplicate for each strain and the experiment was repeated three times.

4.2.1.2 *Chloroparva pannonica* Cu40 culture conditions

The microalga used in these tests was *Chloroparva pannonica* Cu40, a copper-adapted strain from the WT. The *Chloroparva pannonica* Cu40 were cultured in the FU medium described in Chapter 3. Agar plates were prepared with 1.5% agar in a modified FU medium with seawater: deionized water (1:1 v/v). Both the liquid and agar media were sterilized at 121°C at 100 kPa for 15 minutes. All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 40 ml of the culture medium. Both liquid and agar cultures were maintained at 25±1°C, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

4.2.2 Improving *Chloroparva pannonica* growth with small bubbles using stones spargers in 3 L flasks

The *Chloroparva pannonica* was used to study biomass cultivation using various configurations (with and without bubbling with a stone spargers [Aqua Nova air stone ball 2.5 cm in diameter]) in 3 L flasks. The microalgae used in this study were *Chloroparva pannonica* Cu40 and WT strains. The *Chloroparva pannonica* Cu40 strain was cultured in an FU medium while the *Chloroparva pannonica* WT strain was cultured in Guillard's F/2medium. The 300 ml of *Chloroparva pannonica* culture with an equal number of cells (OD of 0.7 at 685 nm) was inoculated into 2.7 ml of various sterile culture media in 3 L flasks at the NCRIS Algae and Biofuel Facility, SARDI, South Australia. As shown in Figure 4.2, the flasks, connected both with and without a stone sparger, were set up with 1% carbon dioxide supplied. Cultures were maintained at 25±1°C, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. Each test was carried out in triplicate for each strain.

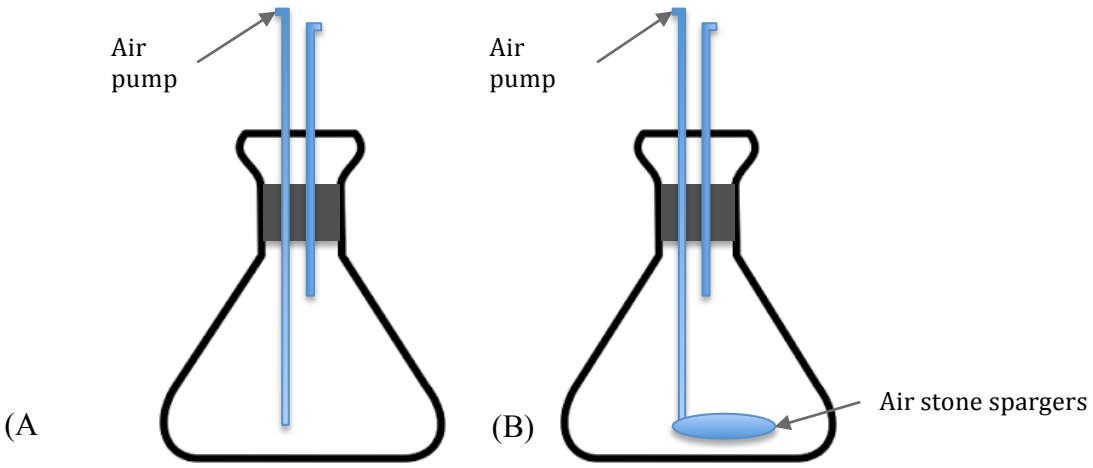


Figure 4.2 Flasks (3 L) set up (A) without and (B) with bubbling stone sparger

The measurement of the total dried biomass was carried out, based on the method described in Section 2.2.3, on day 3, 6, 9, 12 and 15. The pH was measured using an Orion™ 9102BNWP combination pH electrode (ThermoFisher Scientific) on day 3, 6, 9, 12 and 15.

4.2.3 Purification of *Chloroparva pannonica* Cu40 strains

4.2.3.1 Centrifugation and cell washing

The first step to separate bacteria from the *Chloroparva pannonica* Cu40 strains was to wash the microalgae cells by centrifugation. A 40 ml volume of day 10 *Chloroparva pannonica* Cu40 culture was transferred into a sterile 50 ml centrifuge tube and centrifuged at 3000 g (relative centrifugal force) at 20°C for 30 minutes. The supernatant was discarded and the pellet was resuspended in 40 ml of sterile liquid FU medium. This process was repeated five times. The cell pellet from the last wash was resuspended in 40 ml of sterile FU medium.

4.2.3.2 Streak plate technique

After the centrifugation and cell washing, *Chloroparva pannonica* Cu40 cultures were then purified by the streak plate technique. Inoculation was performed by dropping 50 µL of each culture onto each FU medium on 1.5% agar plates. Five equal streaks were made from the drop, followed by another five streaks made from the previous five lines. Single colonies were picked by a sterile loop and grown in a 40 ml sterile liquid

FU medium. Purification of the single strains of the species was confirmed by observation under either 40x or 100x magnification using a bright-field microscope (Olympus).

4.2.3.3 Gram's stain procedure

A loop of culture was dropped onto a clean microscope slide with the drop allowed to evaporate completely by leaving the slide on the bench for a minimum of 15 minutes. The culture on the slide was heat fixed by passing it through the blue Bunsen burner flame three times. The drop area was then covered with a crystal violet solution and left for one minute, after which the slide was washed with a gentle stream of running water from a bottle of distilled water. Next, the surface of the culture smear was covered with Gram's iodine solution, left for one minute and then washed with a gentle stream of running water. The smear was next decolourized using decolourizing solution for 10 seconds and washed with a gentle stream of running water. The surface of the culture smear was again covered, this time with safranin orange counterstain, for one minute and washed with a gentle stream of running water. Following that step, the slide was blotted gently with a tissue to remove excess stain. The slide could then be viewed under either 40X or 100X magnification using a bright-field microscope. The ingredients for crystal violet, Gram's iodine solution, decolourizing solution and safranin orange counterstain are listed in Appendix 4.1.

4.2.3.4 Kirby–Bauer antibiotic testing

Kirby–Bauer antibiotic testing was performed to determine the susceptibility of the associated bacteria in the *Chloroparva pannonica* Cu40 culture to antibiotics. Ampicillin, gentamicin, kanamycin, vancomycin, penicillin G, norfloxacin, novobiocin, tetracycline, rifampicin, nalidixic acid, colistin and streptomycin at concentrations of 0.1 mg/ml and 0.01 mg/ml were selected. All antibiotics were dissolved with distilled water, filter-sterilized (0.2 μm filter units) and stored at -20°C. Sterile water was used as the control for the Kirby–Bauer antibiotic testing. Five types of agar media were used comprising: tryptone soy agar (TSA) made up with deionized water; TSA made up with seawater: deionized water (1:1 v/v); antibiotic medium No. 1 (AMA) agar made up with seawater: deionized water (1:1 v/v); and 1.5% agar in FU medium. The media ingredients are listed in Appendix 4.1. Agar plates were divided into eight sections and labelled. Volumes of 200 μl, 400 μl and 600 μl of *Chloroparva pannonica* Cu40

inoculum were spread on each type of agar using a sterile spreader with the surface allowed to dry for five minutes. A sterile cork borer was used to make wells in the agar with 20 µl of each antibiotic transferred into each well. Plates were incubated at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. After 48 hours' incubation, the zone of inhibition was measured. Each test was carried out in triplicate.

4.2.3.5 Bacteria reduction assay

Three antibiotic combinations were selected based on the results from the Kirby–Bauer antibiotic testing. The first antibiotic combination was 0.1 mg/ml of penicillin G and 0.1 mg/ml of gentamicin sulphate in an FU medium; the second was 0.1 mg/ml of rifampicin and 0.1 mg/ml of novobiocin; while the third was 0.1 mg/ml of vancomycin and 0.1 mg/ml of ampicillin. Each test was carried out in triplicate.

After three days of treatment, the culture was transferred into a sterile 50 ml centrifuge tube and centrifuged at 3000 g at 4°C for 30 minutes. The supernatant was discarded and the pellet was resuspended in 40 ml of sterile FU medium. This process was repeated five times. The cell pellet from the last wash was resuspended in 40 ml of sterile FU medium and grown at 25±1°C, with a light intensity of 85µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

4.2.3.5.1 Chloroparva pannonica Cu40 cell density assessed by UV/Vis spectrophotometer

The effect of the antibiotic combinations on *Chloroparva pannonica* Cu40 growth was observed for a period of 17 days. The biomass concentration was determined by measuring the OD at a wavelength of 685 nm with a µQuant™ UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia), based on the method described in subsection 2.2.2.2.

4.2.3.5.2 Miles and Misra assay method

The effect of antibiotics in reducing the bacteria population in the *Chloroparva pannonica* Cu40 culture was determined by the bacterial count as colony-forming units per ml (cfu/ml) at day 15 using the Miles and Misra assay on five types of agar media. These media were: tryptone soy agar (TSA) made up with deionized water; TSA made up with seawater: deionized water (1:1 v/v); antibiotic medium No. 1 (AMA) agar made

up with seawater: deionized water (1:1 v/v); and AMA agar made up with deionized water and FU medium with 1.5% agar and 0.5% glucose in seawater: deionized water (1:1 v/v). The media ingredients are listed in Appendix 4.1. Each test was carried out in triplicate.

The day 15 *Chloroparva pannonica* Cu40 cultures from the bacteria reduction assay described in section 4.2.3.5 were diluted up to 10^{-12} by adding the cultures to the sterile FU medium in a ratio of 1: 9. Agar plates were divided into eight sections and labelled with the dilution factors. From each dilution, 10 µl was dropped onto each section of the agar's surface and left to dry. Plates were incubated in a plant cell culture room at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod. After 24 hours' incubation, colonies were counted from each sector and the cfu/ml value was calculated based on the following equation:

$$Colony - Forming\ Units\ per\ ml = \frac{No.\ of\ colonies \times dilution\ factor}{volume\ of\ culture\ plate}$$

4.2.4 Random mutagenesis and screening of *Chloroparva pannonica* Cu40 parent culture (PC)

Ultraviolet radiation (UV-C) was the mutagenic agent used in this study. The UV-C source was a 254 nm wavelength lamp placed 15 cm above the cultures. This was followed by a 24 hours dark period to prevent light repair.

4.2.4.1 Kill curve

The first step of the mutation experiment was to determine the appropriate killing times for *Chloroparva pannonica* Cu40 parent cultures (PC) when under UV-C exposure. The 100 ml samples of the cultures, at a concentration of approximately 1 x 10^7 cells/ml and undergoing continuous agitation, were exposed to UV-C light for 90 minutes at a distance of 15 cm from the cultures in an open petri dish. Every three minutes, 1 ml of culture was removed to increase the dilution up to 10^{-12} by adding 1x of culture to 9x of sterile FU medium. The sample was inoculated by dropping 50 µL of each culture onto each FU medium with 1.5% agar. Five equal streaks were made from the drop followed by another five streaks made from the previous five lines. Each sample was carried out in triplicate. The cultures were incubated in a plant cell culture room at 25±1°C, with a

light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod for 2–3 weeks. Colonies were counted to determine the viable cells. Kill curves were repeated three times with the same conditions to improve the accuracy of the appropriate killing time determined for UV-C exposure for the *Chloroparva pannonica* Cu40 PC.

4.2.4.2 Mutation of parent cultures

Preliminary analysis of several kill curves indicated an irradiation time of 63 minutes for a 98% killing rate in 100 ml samples of cultures, at a concentration of approximately 1 x 10^7 cells/ml. The 100 ml samples of cultures, at a concentration of approximately 1 x 10^7 cells/ml and undergoing continuous agitation, were exposed to UV-C light for 63 minutes, with the light located 15 cm from the cultures in an open petri dish. The exposed cultures were then plated on the FU medium with 1.5% agar with appropriate dilutions and kept in darkness for 24 hours to avoid photo-reactivation. Inoculation was performed by dropping 50 μL of each culture onto each agar plate. Five equal streaks were made from the drop followed by another five streaks made from the previous five lines. The exposed cultures were incubated at 25±1°C, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod for 2–3 weeks. Colonies were counted to determine the viable cells. The mutation of the parent cultures (PCs) was repeated four times to increase the probability of obtaining candidate microalgae strains.

4.2.4.3 Isolation of colonies of UV-C-exposed *Chloroparva pannonica* Cu40 culture

Single colonies of the exposed culture were isolated. A sterile inoculation loop was used to pick up a single colony which was resuspended into 5 ml of sterile FU medium in 6-well plates. A total of 20 colonies not exposed to UV-C light were cultivated as the control for this study to evaluate the effect of mutation on growth and lutein content.

4.2.4.4 Evaluation of mutant cell density by UV/Vis spectrophotometer

The growth of the mutants was analysed in triplicate by measuring the OD at a wavelength of 685 nm with a μQuant UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia), based on the method described in subsection 2.2.2.2.

4.2.4.5 Extraction of lutein using one-step closed-tube method and estimation of lutein by UV/Vis spectrophotometer

A known volume of the *Chloroparva pannonica* Cu40 WT and mutants was harvested, centrifuged at 10,000 rpm for 10 minutes and the cell pellet extracted with 90% acetone and then, in the dark, underwent five minutes' sonication in the sonicator with glass beads, based on the one-step closed-tube method described in subsection 2.1.1.3.2. The lutein content was estimated by analysing the pigment extract using a μQuant UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia) at 430 nm based on the method described in subsection 2.1.1.5.

4.2.4.6 Maintenance of *Chloroparva pannonica* Cu40 mutant strains

The *Chloroparva pannonica* Cu40 mutant strains with good growth, based on the OD reading at 685 nm and lutein content (mg/l), were maintained in an FU medium. All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 40 ml of culture medium and maintained in a plant cell culture room at 25±1°C with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

4.2.5 Selection of *Chloroparva pannonica* Cu40 mutant strains on herbicide inhibitors

4.2.5.1 Determination of herbicide concentration to inhibit growth of *Chloroparva pannonica* parent culture

The first step of the selection experiment was to determine the appropriate concentration that would inhibit the growth of *Chloroparva pannonica* PC. Samples of 50ul with equal numbers of cells (OD of 0.7 at 685 nm) were inoculated into an FU medium with 1.5% agar containing either of two herbicides, aclonifen (Sigma-Aldrich) or chlodinafop-propargyl (Sigma-Aldrich), ranging in concentration from 5 nm to 1000 nm. Inoculation was performed by dropping 50 μL of each culture onto each agar plate. Five equal streaks were made from the drop followed by another five streaks made from the previous five lines. The plates were then incubated at 25±1°C with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod for 2–3 weeks. Results were recorded as positive (+), negative (-) or weak (W), based on the presence or absence of growth, or weak growth. Each test was carried out in triplicate and was repeated three times.

4.2.5.2 Screening and isolation of *Chloroparva pannonica* Cu40 mutant strains on herbicide inhibitors

The *Chloroparva pannonica* Cu40 mutant strains with good growth, based on the OD reading at 685 nm and lutein content (mg/l), were allowed to grow in 40 ml of FU medium for 10 days before initiating selection. Samples of 50 ul with equal numbers of cells (OD of 0.7 at 685 nm) were inoculated into an FU medium with 1.5% agar containing either aclonifen or clodinafop-propargyl. Five equal streaks were made from the drop followed by another five streaks made from the previous five lines. Each test was carried out in triplicate. The plates were then incubated at 25±1°C with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod for 2–3 weeks. Results were recorded as positive (+), negative (-) or weak (W), based on the presence or absence of growth, or weak growth.

The herbicide-resistant mutant colonies were isolated in order to analyse growth and lutein content. A sterile inoculation loop was used to pick up a single colony which was resuspended into 40 ml of sterile FU medium in 100 ml Erlenmeyer flasks. The cultures were incubated at 25±1°C with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

4.2.6 Evaluation of *Chloroparva pannonica* Cu40 mutant strains and herbicide-resistant mutant strains

The *Chloroparva pannonica* Cu40 mutant strains with good growth, based on the OD reading at 685 nm and lutein content (mg/l), as described in subsection 4.2.4.6, and *Chloroparva pannonica* Cu40 herbicide-resistant mutant strains in subsection 4.2.5.2 were selected. The experiment was carried out under autotrophic cultivation conditions in 100 ml Erlenmeyer flasks with 40 ml of *Chloroparva pannonica* culture (OD of 0.7 at 685 nm) and 40 ml of various culture media. Cultures were maintained at 25±1°C, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

4.2.6.1 Evaluation of mutant cell density by UV/Vis spectrophotometer

The growth of the mutants was analysed, with the analysis carried out in triplicate, by measuring the OD at a wavelength of 685 nm with a μQuant UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia), based on the method described in subsection 2.2.2.2.

4.2.6.2 Extraction of lutein using one-step closed-tube method and estimation of lutein by UV/Vis spectrophotometer

A known volume of *Chloroparva pannonica* Cu40 wild type and mutants was harvested and extracted with 90% acetone and then, in the dark, underwent five minutes' sonication in the sonicator with glass beads, based on the one-step closed-tube method described in subsection 2.1.1.3.2. The lutein content was estimated by analysing the pigment extract using a μQuant UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia) at 430 nm, based on the method described in subsection 2.1.1.5, with 90% acetone and then, in the dark, by undergoing five minutes' sonication in the sonicator with glass beads,

4.2.7 Cultivation of *Chloroparva pannonica* Cu40 candidate strains in 3 L flasks in batch mode with 1% carbon dioxide supplied

The *Chloroparva pannonica* Cu40 mutant strains and the herbicide-resistant mutant strains that showed good growth and high lutein content were selected to grow in 3 L flasks in batch mode with 1% carbon dioxide supplied. The cultures were maintained in an FU medium, based on the method described in subsection 4.2.4.6, for 20 generations before being inoculated into the 3 L flasks.

For inoculation, 300 ml of day 10 pre-culture strains with equal numbers of cells (0.7 at OD at 685nm) were inoculated with 2.7 L of sterile FU medium in a 3L flask. The cultures were set up as shown in Figure 4.2A and Figure 4.3 at the NCRIS Algae and Biofuel Facility, SARDI, South Australia, at 25±1°C with a light intensity of 85 μmol photons m-2s-1 illumination, with 1% (v/v) carbon dioxide supplied, following a 12:12-h light: dark photoperiod for 14 days. Liquid samples were collected from the glass vessel under sterile conditions. *Chloroparva pannonica* Cu40 PC, WT and mutant strains were grown in triplicate with the above process repeated three times.

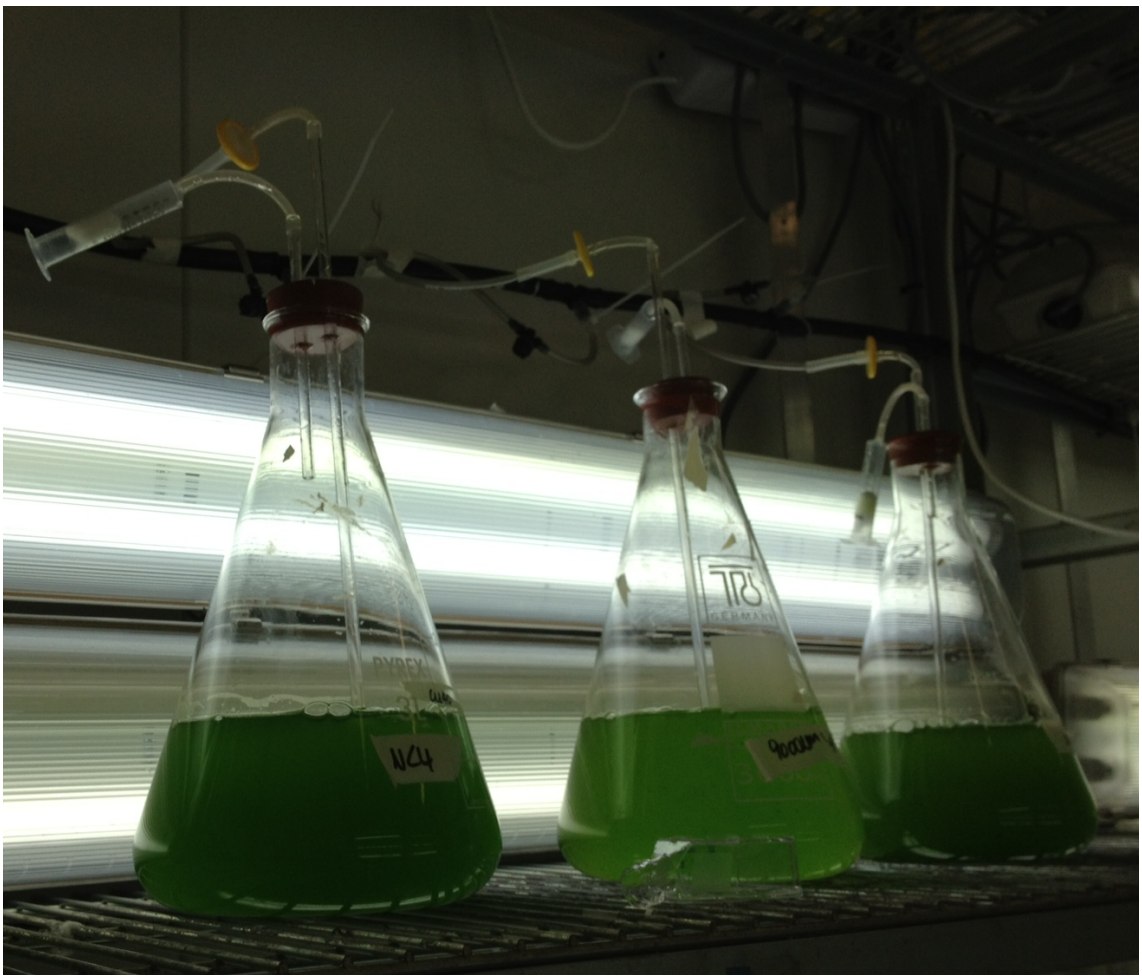


Figure 4.3 *Chloroparva pannonica* 3 L flask culture set up in plant cell culture room at NCRIS Algae and Biofuel Facility, SARDI, South Australia

4.2.7.1 Evaluation of *Chloroparva pannonica* Cu40 mutant strains and herbicide-resistant mutant strains grown in 3 L flasks

4.2.7.1.1 Cell density assessed by UV/Vis spectrophotometer

The biomass concentration was determined by measuring the OD at a wavelength of 685 nm with a UV/Vis spectrophotometer, based on the method described in Section 2.2.2.2 in triplicate.

4.2.7.1.2 Determination of total dried biomass

The total dried biomass of microalgae culture was determined based on the method described in Section 2.3.3 with this carried out in triplicate.

4.2.7.1.3 Determination of specific growth rate

The specific growth rate (μ) of the *Chloroparva pannonica* culture was calculated based on the following equation:

$$\mu = \frac{In\ \chi 2 - In\ \chi 1}{t2 - t1}$$

where $\chi 2$ and $\chi 1$ represent the dried weight (g/L) at times $t2$ and $t1$, respectively.

4.2.7.1.4 Extraction and analysis of lutein

Lutein was extracted, based on the method described in subsection 2.1.1.3.2. Lutein extract were analysed by HPLC, based on the method described in subsections 2.1.1.5 and 2.1.1.6. Each extraction and analysis were carried out in triplicate

4.2.7.1.5 Total lipids analysis

The total lipids were extracted and analysed based on the method described in Section 2.2.4, with this carried out in triplicate.

4.3 Results

4.3.1 *Chloroparva pannonica* WT strain selection

4.3.1.1 *Chloroparva pannonica* WT strain selection in different concentrations of copper

Chloroparva pannonica WT strain was grown in a wide range of copper concentrations (from 0 µg/l to 100 µg/l) for a period of 14 days. Results showed that the highest *Chloroparva pannonica* growth at day 14 was obtained from 40 µg/l copper concentration (OD of 0.91 at 685 nm) while the lowest growth was obtained from 0 µg/l of copper concentration (OD of 0.47 at 685 nm) (Figure 4.4). The trend of the growth of *Chloroparva pannonica* at copper concentrations of 9.8 µg/l (control), 10 µg/l, 20 µg/l, 30 µg/l, 50 µg/l and 100 µg/l were similar from day 2 to day 14 (Figure 4.4). Based on these results, *Chloroparva pannonica* WT at a copper concentration of 40 µg/l (Cu40) and *Chloroparva pannonica* WT at a copper concentration of 100 µg/l (Cu100) were selected to grow in 3 L flasks with 1% carbon dioxide supplied to determine the dried biomass, total lipids percentage and pigment content.

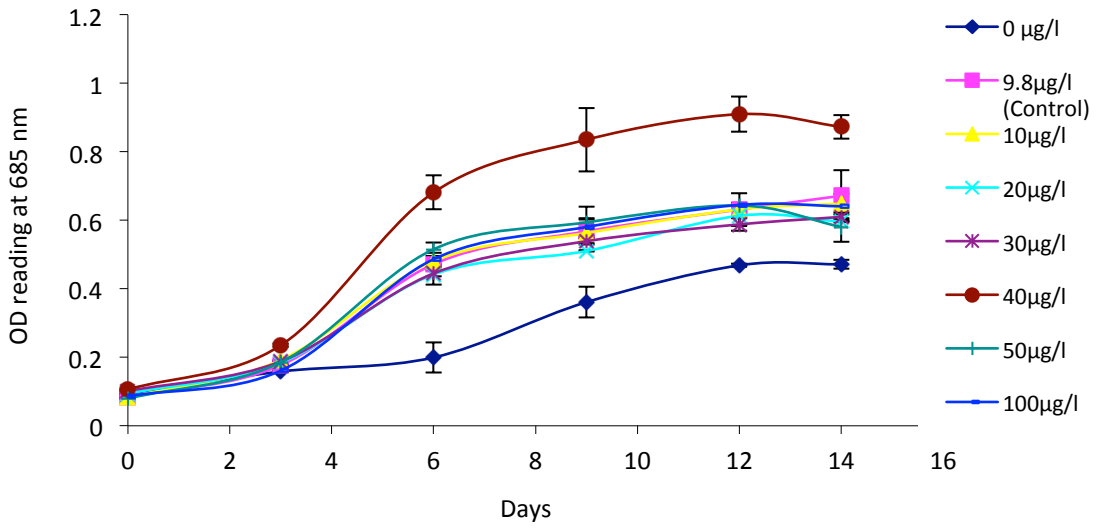


Figure 4.4 Growth of *Chloroparva pannonica* WT strains in different concentrations of copper sulphate, measured by UV/Vis spectrophotometer at a wavelength of 685 nm

4.3.1.2 Comparison of growth, biomass, lipids and lutein content for *Chloroparva pannonica* WT, Cu40 and Cu100 strains in 3 L flasks with 1% carbon dioxide supplied

Chloroparva pannonica WT, Cu40 and Cu100 were selected to grow in 3 L flasks with 1% carbon dioxide supplied to determine the dried biomass, total lipids percentage and pigment content at the end of the growth phase (day 15). The growth results based on an OD reading at 685 nm are shown in Figure 4.5. The highest *Chloroparva pannonica* growth based on an OD reading at 685 nm was Cu40 (2.99) followed by WT (2.25) at day 15 (Figure 4.5).

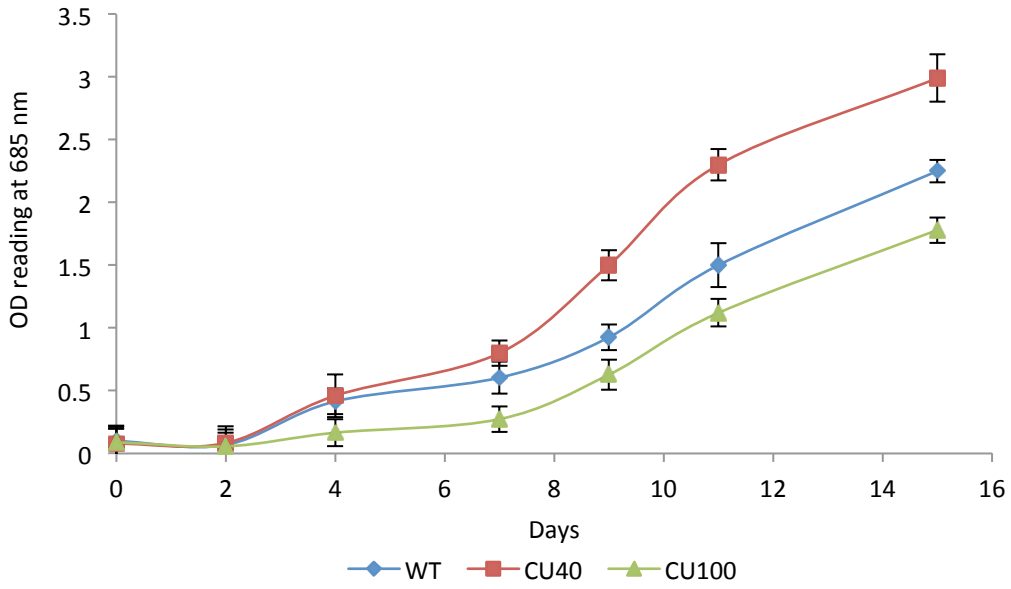


Figure 4.5 Growth of *Chloroparva pannonica* WT, Cu40 and Cu100 strains in a 3 L flask set up with 1% carbon dioxide supplied, measured by UV/Vis spectrophotometer at a wavelength of 685 nm

The total dried biomass and total lipids of *Chloroparva pannonica* WT, Cu40 and Cu100 strains were determined at the end of the growth phase (day 15) while chlorophyll *a*, chlorophyll *b* and the lutein content were determined at day 7. The total dried biomass, total lipids and pigment content at day 15 are shown in Table 4.2. The total dried biomass and total lipids of *Chloroparva pannonica* Cu40 were higher than for the *Chloroparva pannonica* WT strains. *Chloroparva pannonica* Cu40 had the highest level of total dried biomass (0.604±0.021 g/l) while the lowest was in Cu100 (0.378±0.071 g/l). In addition, the specific growth rate for Cu40 was higher than for WT and Cu100 strains. *Chloroparva pannonica* Cu40 had the highest total lipids (24.88±1.62%) while the lowest was Cu100 (19.66±2.3%) (see Table 4.2). Furthermore, chlorophyll *a*, chlorophyll *b* and the lutein content of Cu40 were higher than in the WT strains (Table 4.2). As shown in Table 4.2, chlorophyll *a*, chlorophyll *b* and the lutein content of *Chloroparva pannonica* Cu40 were increased by 52%, 158% and 132.26%, respectively, when compared to the WT strains. Therefore, *Chloroparva pannonica* Cu40 strains were selected with further studies undertaken.

Table 4.2 Growth, lipids and lutein accumulation of *Chloroparva pannonica* WT, Cu40 and Cu100 strains in a 3 L flask set up with 1% carbon dioxide supplied

Chloroparva pannonica strains	μ (day^{-1})	Dried biomass (g/l)	Pigment content (mg/kg DW)			Total lipids (%)
			Chl *a*	Chl *b*	Lutein	
WT	0.150	0.416±0.045	42701	2401	1864	23.22±2.30
Cu40	0.178	0.604±0.021	64726	6215	4333	24.88±1.62
Cu100	0.144	0.378±0.071	38673	1666	1996	19.60±0.98

Note: Biomass, lipids and pigment content data were the mean values of three independent measurements. DW = dry weight; Chl a = chlorophyll a; Chl b = chlorophyll b

4.3.2 Improving *Chloroparva pannonica* growth using stone sparger in 3L flasks with 1% carbon dioxide supplied

Chloroparva pannonica Cu40 and WT strains were used to study biomass cultivation using various configurations (with and without bubbling with an stone sparger) in 3 L flasks for a 15-day period. The effect of using the stone sparger to increase the aeration of the culture medium was investigated. The total dried biomass and pH were determined at day 3, 6, 9, 12 and 15.

The total dried biomass for *Chloroparva pannonica* Cu40 and WT strains cultured with or without stone sparger bubbling in 3 L flasks for a 15-day period are shown in Figure 4.6. For the culture without stone sparger bubbling, the total biomass increased and reached the highest 0.528 g/l for WT strains and 0.792 g/l for Cu40 strains (Figure 4.6). However, the total biomass declined and turned yellowish from day 3 when *Chloroparva pannonica* Cu40 and WT strains were cultured in 3 L flasks with stone sparger bubbling (Figure 4.6).

The pH value of *Chloroparva pannonica* Cu40 and WT strains cultured with or without stone sparger bubbling in 3 L flasks for a 15-day period are shown in Figure 4.7. The trends of the pH of *Chloroparva pannonica* Cu40 and WT strains cultured with or without stone sparger bubbling are very similar. Throughout the cultivation period, the pH was maintained at around 7 when *Chloroparva pannonica* Cu40 and WT strains were cultured without stone sparger bubbling while the pH decreased from 6 to 5 for

Chloroparva pannonica Cu40 and WT strains cultured with stone sparger bubbling (Figure 4.7).

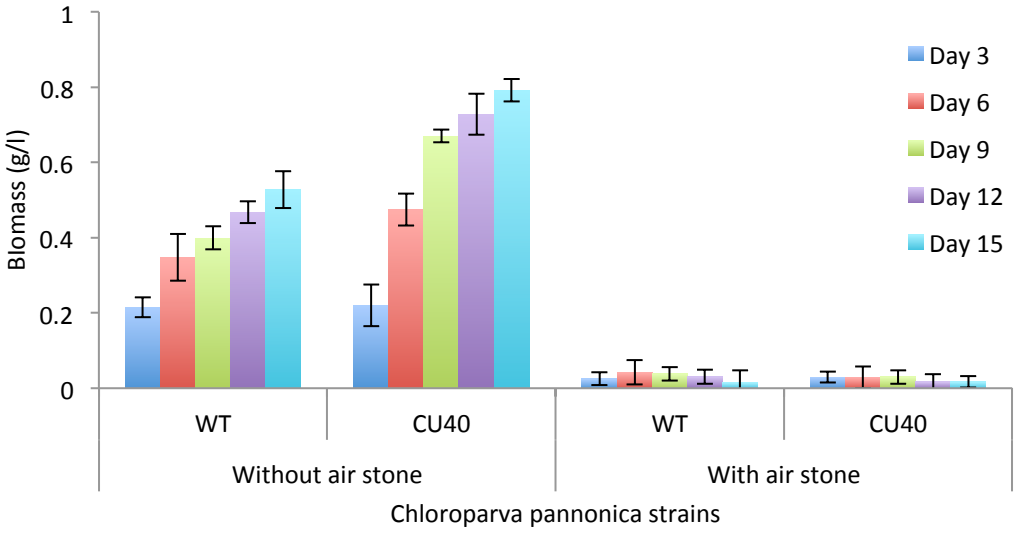


Figure 4.6 Total dried biomass of *Chloroparva pannonica* WT and Cu40 strains bubbling with and without stone sparger in a 3 L flask set up with 1% carbon dioxide supplied

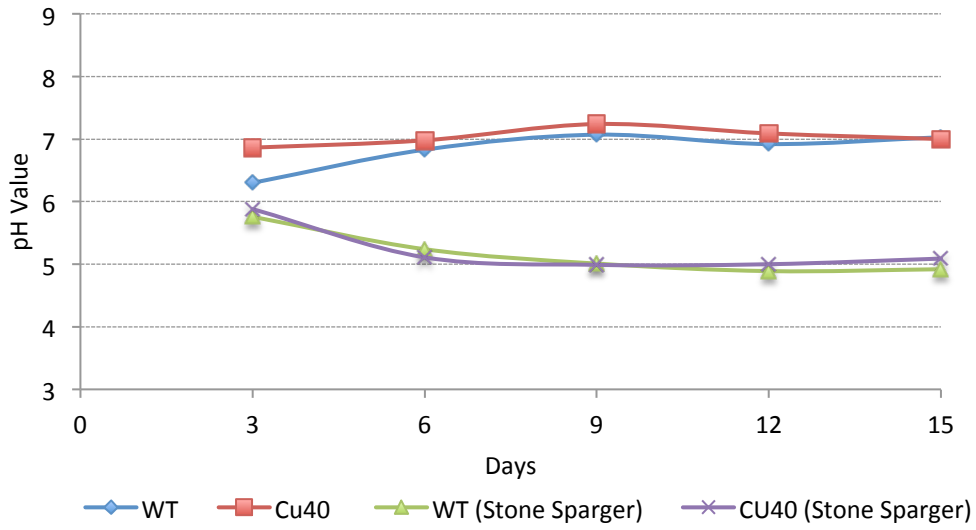


Figure 4.7 pH value of *Chloroparva pannonica* WT and Cu40 culture media bubbling with and without stone sparger in a 3 L flask set up with 1% carbon dioxide supplied

4.3.3 Purification of *Chloroparva pannonica* Cu40

4.3.3.1 Centrifugation, cell washing and plate streaking of *Chloroparva pannonica* Cu40

The bacteria populations in *Chloroparva pannonica* Cu40 were first reduced by centrifugation, cell washing and plate streaking. Green colonies were isolated and continued to streak on a new FU agar. The *Chloroparva pannonica* Cu40 strains were further confirmed based on their morphological characteristics under the light microscope. As shown in Figure 4.8A, brown and white creamy bacteria colonies and *Chloroparva pannonica* Cu40 green colonies were observed on the FU agar. Based on Gram's stain procedure, the bacteria population from the brown, yellowish creamy and white creamy colonies were mainly Gram-positive rod-shaped bacteria, Gram-negative rod-shaped bacteria and Gram-negative spherical bacteria. After the centrifugation, cell washing and plate streaking, the reduction of most of the brown, yellowish creamy and white creamy colonies could be observed on the FU agar (Figure 4.8B). The *Chloroparva pannonica* Cu40 green colonies were isolated and then routinely maintained in a liquid FU medium.

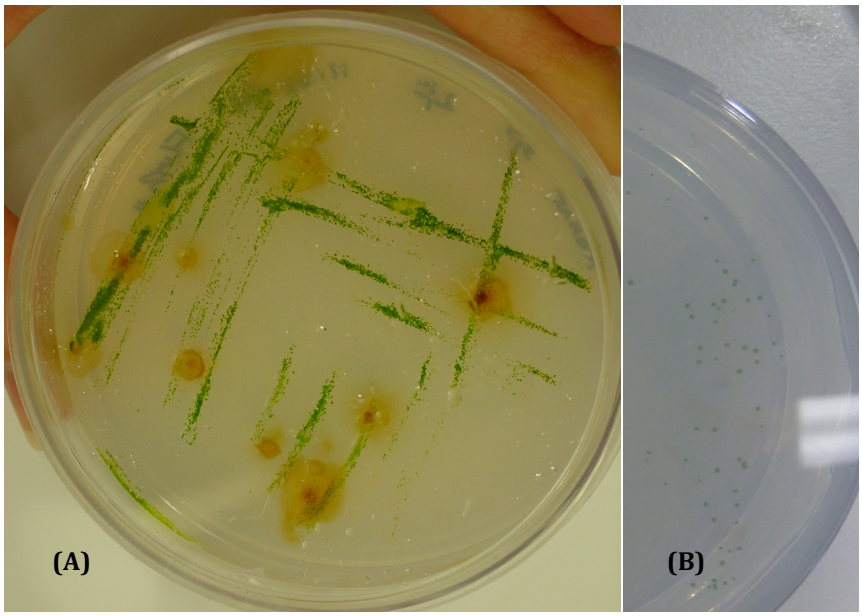


Figure 4.8 Effect of centrifugation, cell washing and continuous plate streaking of *Chloroparva pannonica* Cu40 cultures: (A) cultures contaminated with different kinds of brown, white creamy bacteria; (B) *Chloroparva pannonica* Cu40 colonies after centrifugation, cell washing and repetitive streaking on new agar plates.

Note: Contaminated cultures were centrifuged and cell washed, followed by streaking on new agar plates.

4.3.3.2 Kirby–Bauer antibiotic testing

As shown in Figure 4.9–Figure 4.14, the difference in the zone of inhibition of each antibiotic on the four different types of agar indicated that there were different bacteria populations in the *Chloroparva pannonica* Cu40 culture. The zones of inhibition of different types of antibiotics at 0.01 mg/ml using 200 µl, 400 µl and 600 µl of *Chloroparva pannonica* Cu40 as the inoculum volume are shown in Figure 4.9, Figure 4.10 and Figure 4.11, respectively. The zone of inhibition for each antibiotic did not significantly increase or decrease when the inoculation volume increased. In addition, the results indicated that there were no zones of inhibition in the control, or with ampicillin, penicillin G, norfloxacin, novobiocin, tetracycline, rifampicin, nalidixic acid, colistin and streptomycin. However, the bacteria populations in the culture were susceptible (zone of inhibition >17 mm) to gentamicin, kanamycin and vancomycin, with vancomycin the most effective antibiotic as it had the largest zone of inhibition on all four types of agar media. It was also noted that gentamicin and kanamycin were only effective on the bacteria population on the TSA agar, while vancomycin was effective on the bacteria population on all four of the agar media at the three different inoculation volumes (Figure 4.9–Figure 4.11).

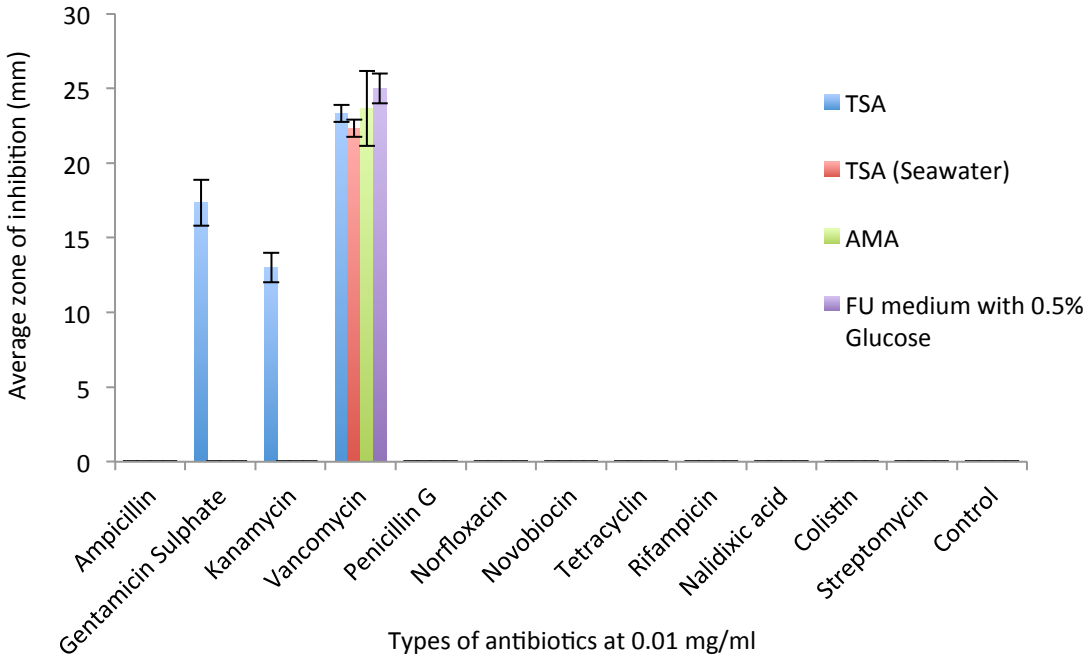


Figure 4.9 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.01 mg/ml with 200 ul as the inoculation volume

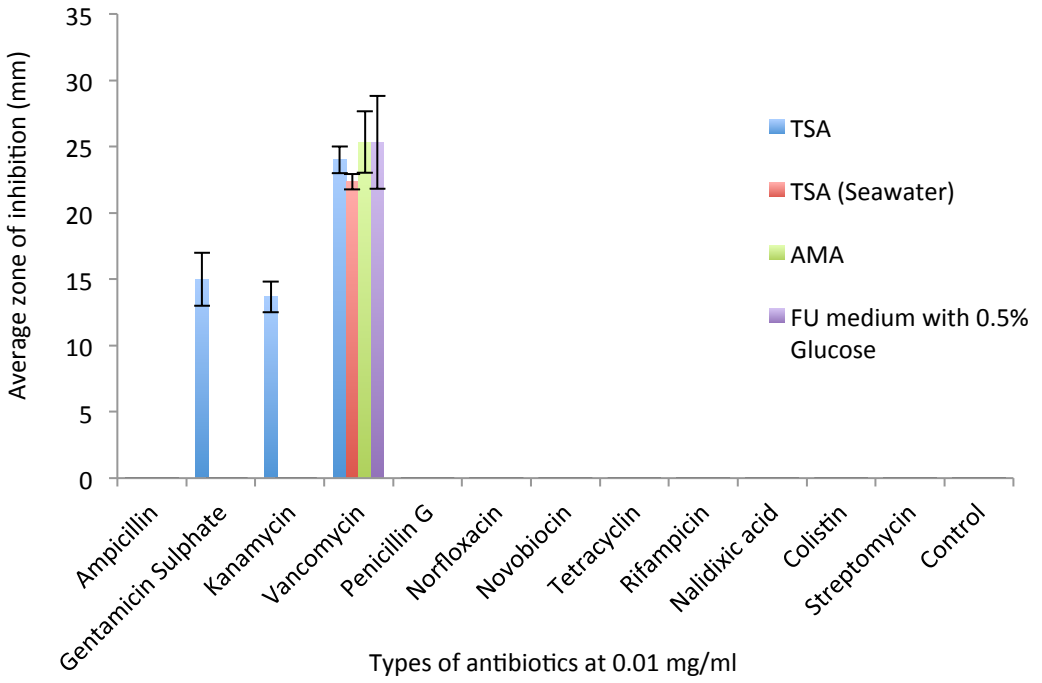


Figure 4.10 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.01 mg/ml with 400 ul as the inoculation volume

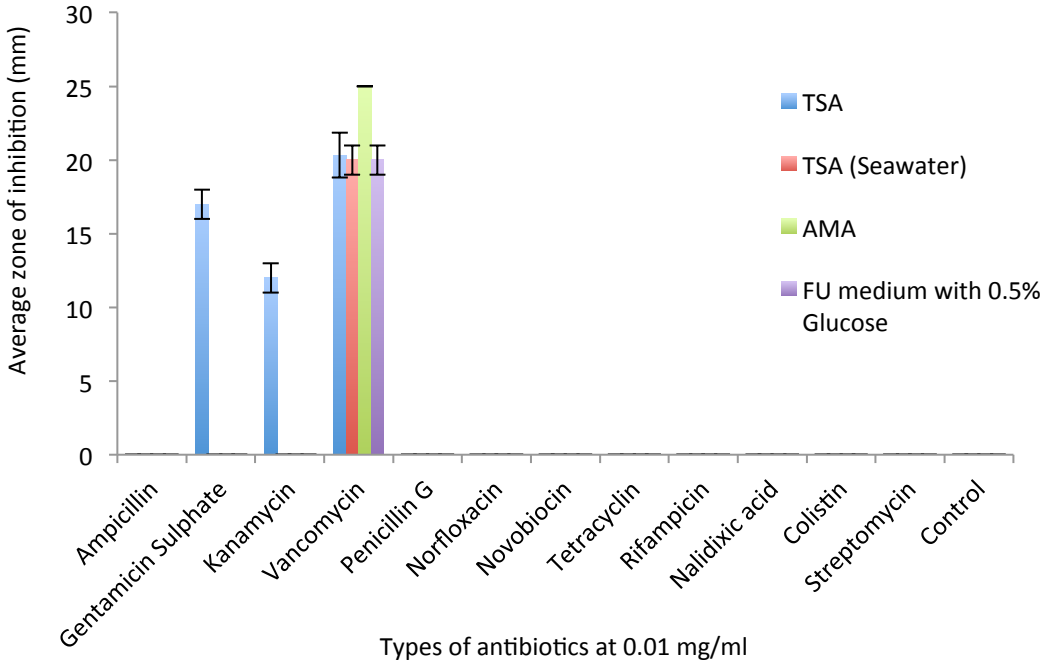


Figure 4.11 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.01 mg/ml with 600 ul as the inoculation volume

The zones of inhibition for different types of antibiotics at 0.1 mg/ml using 200 µl, 400 µl and 600 µl *Chloroparva pannonica* Cu40 as the inoculum volume are shown in

Figure 4.12, Figure 4.13 and Figure 4.14, respectively. When the inoculation volume increased, the zone of inhibition for each antibiotic did not significantly increase or decrease. Results show that there were no zones of inhibition for the control, nor with norfloxacin, nalidixic acid, colistin and streptomycin, indicating the resistance of the bacteria in the culture to these antibiotics. The most effective antibiotic on the bacteria population in the culture was penicillin G, followed by rifampicin, novobiocin, vancomycin, ampicillin, gentamicin and kanamycin. It was also noted that tetracycline was only effective on the bacteria population on the TSA agar.

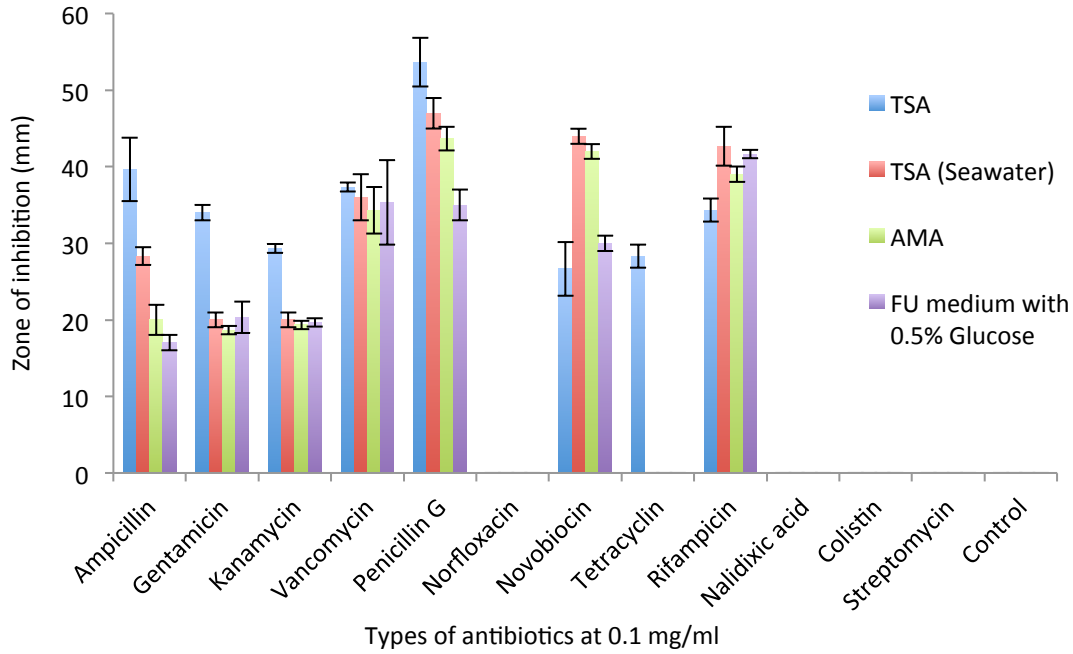


Figure 4.12 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.1 mg/ml with 200 ul as the inoculation volume

The zones of inhibition of different types of antibiotics at concentrations of 0.01 mg/ml and 0.1 mg/ml were different. Results show that the growth of bacteria was not inhibited by norfloxacin, nalidixic acid, colistin and streptomycin at 0.01 mg/ml and 0.1 mg/ml. In addition, the growth of bacteria was not inhibited at a lower concentration, as there was no zone of inhibition at 0.01 mg/ml when penicillin G, ampicillin, novobiocin, tetracycline and rifampicin were used. The only antibiotic that inhibited the growth of bacteria at both concentrations was vancomycin. Furthermore, gentamicin and kanamycin were able to inhibit the growth of bacteria, when used at concentrations of 0.01 mg/ml on TSA agar and 0.1 mg/ml on four different types of agar.

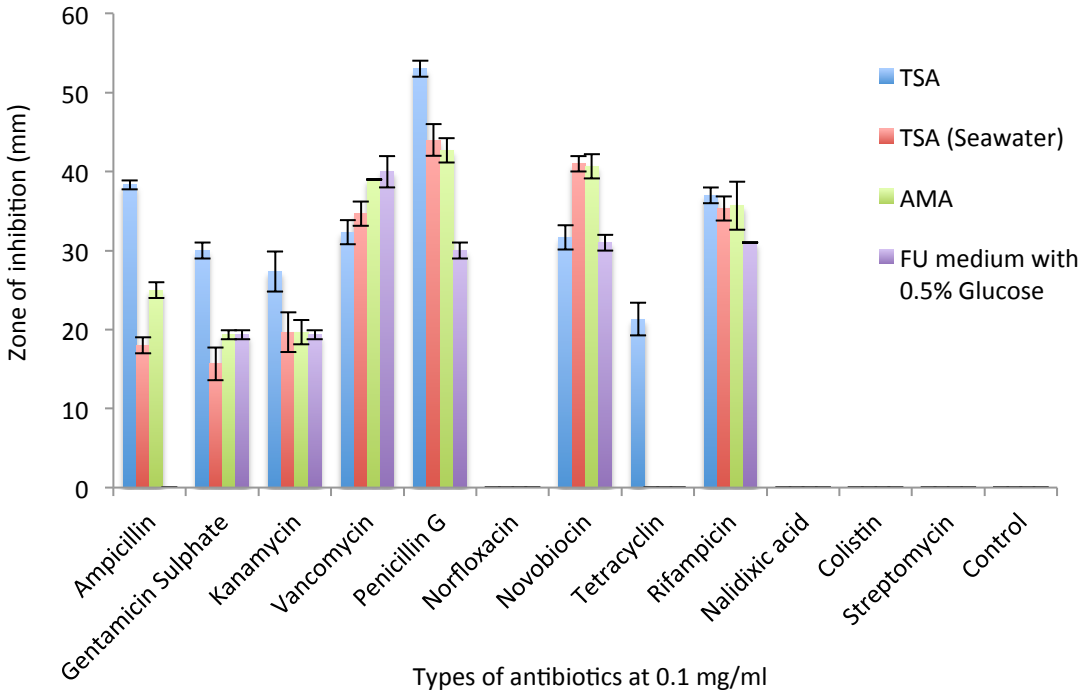


Figure 4.13 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.1 mg/ml with 400 ul as the inoculation volume

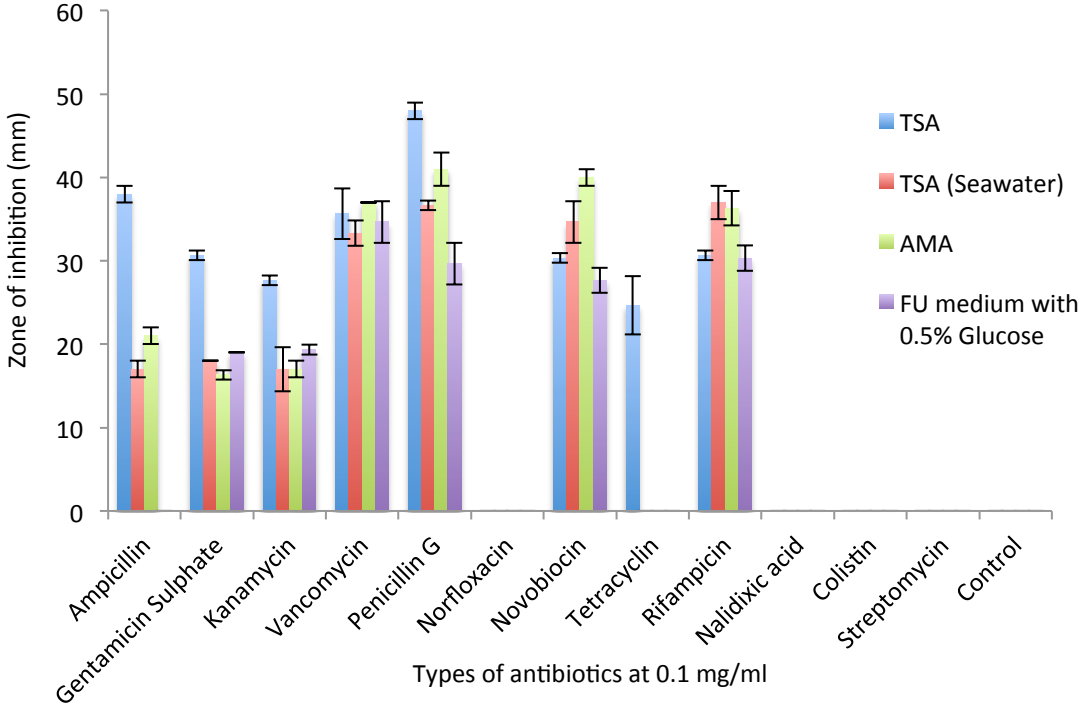


Figure 4.14 Kirby–Bauer antibiotic testing using different types of antibiotics at a concentration of 0.1 mg/ml and 600 ul as the inoculation volume

4.3.3.3 Bacteria reduction assay

Based on the results from Kirby–Bauer antibiotic testing (subsection 4.3.3.2), three antibiotic combinations at a concentration of 0.1 mg/ml were selected to reduce the bacteria population in the *Chloroparva pannonica* Cu40 cultures. The combinations were: (1) penicillin G and gentamicin, (2) rifampicin and novobiocin and (3) vancomycin and ampicillin. The *Chloroparva pannonica* Cu40 cultures were treated with different combinations of antibiotics for three days, followed by centrifugation and cell washing at day 4 and resuspended in a sterile FU medium.

The effects of the different combinations of antibiotics on the growth of *Chloroparva pannonica* Cu40 were indicated by the OD reading at 685 nm, as shown in Figure 4.15. The growth of *Chloroparva pannonica* Cu40 was slow from day 0 to day 3 and increased from day 4 until day 17. Untreated *Chloroparva pannonica* Cu40 cultures had a better growth trend than the antibiotic-treated cultures, reaching the highest growth at day 17 (an OD of 1.75), followed by penicillin G and gentamicin, and rifampicin and novobiocin. However, in the cultures treated with vancomycin and ampicillin, the growth of *Chloroparva pannonica* Cu40 was inhibited.

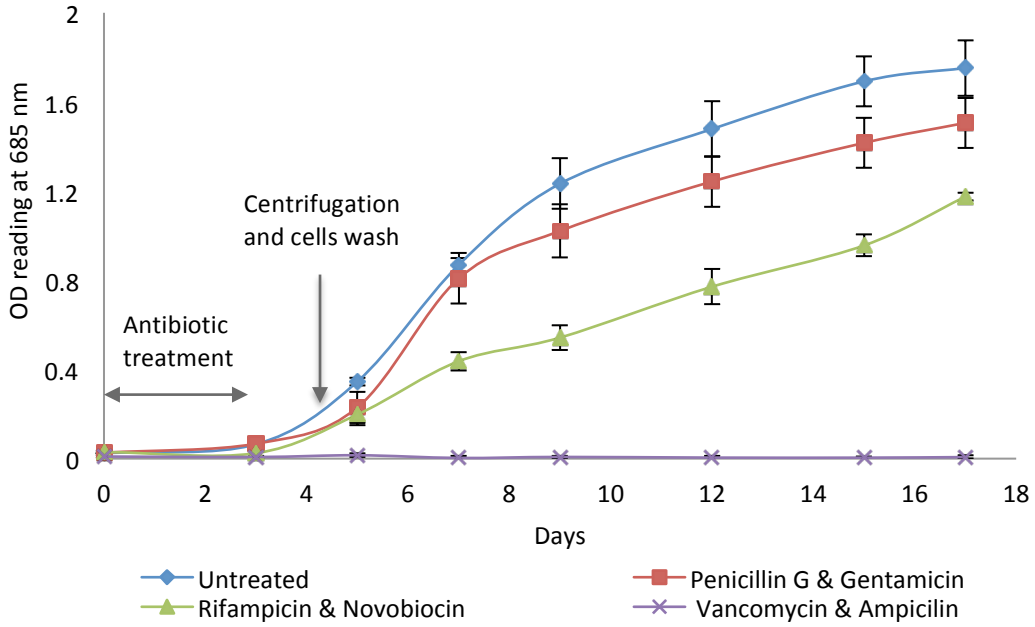


Figure 4.15 Growth of *Chloroparva pannonica* Cu40 culture treated with different combinations of antibiotics at a concentration of 0.1 mg/ml for from day 1 to day 3, followed by centrifugation and cell washing at day 4

The effects on the bacteria population in the *Chloroparva pannonica* Cu40 cultures of treatments by different combinations of antibiotics were evaluated using the Miles and Misra method (Figure 4.16). As shown on Figure 4.16 the populations of bacteria, measured in cfu/ml, significantly decreased after the microalgae cultures were treated with different combinations of antibiotics. The bacteria populations in the microalgae cultures treated with penicillin G and gentamicin had been reduced by 93%, 88%, 97%, 100% and 100% on AMA (seawater), AMA, TSA (seawater), TSA and an FU medium with 0.5% glucose agar, respectively (Figure 4.16). In addition, bacteria had been reduced by 94–100% after the microalgae cultures were treated with rifampicin and novobiocin (Figure 4.16). Furthermore, the vancomycin and ampicillin combination had totally inhibited the growth of bacteria in *Chloroparva pannonica* Cu40 (Figure 4.15 and Figure 4.16).

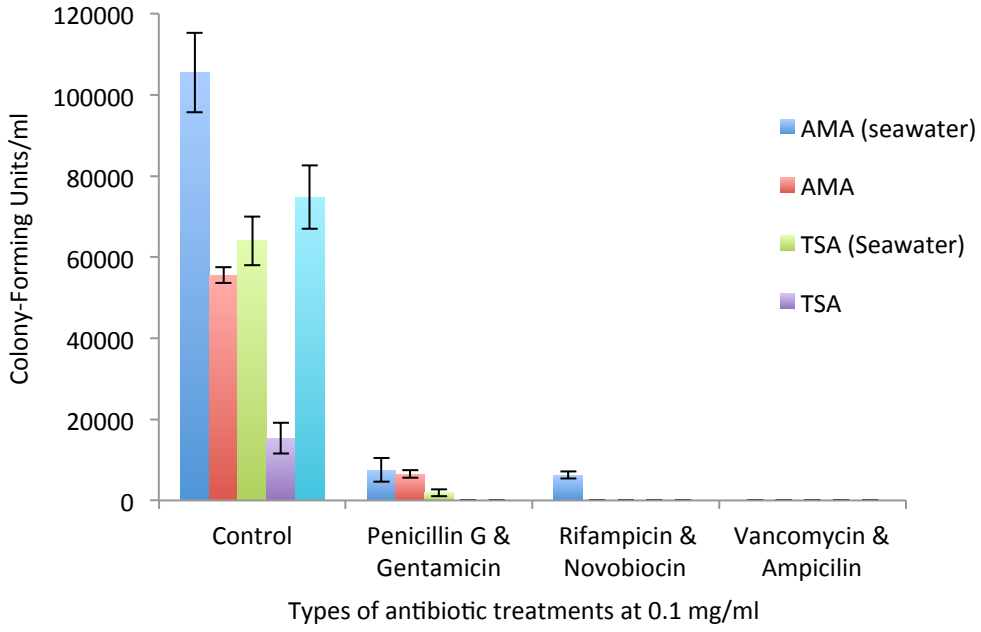


Figure 4.16 Colony-forming units per ml (cfu/ml) of bacteria at day 15 in *Chloroparva pannonica* Cu40 culture treated with different combinations of antibiotics at a concentration of 0.1 mg/ml from day 1 to day 3, followed by centrifugation and cell washing at day 4

Based on results of the growth of *Chloroparva pannonica* Cu40 cultures treated with different combinations of antibiotics (Figure 4.15) and the bacteria reduction measured in cfu/ml by the bacteria reduction assay (Figure 4.16), *Chloroparva pannonica* Cu40 cultures treated with 0.1 mg/ml of penicillin G and gentamicin were selected and studied in the next part of the current study.

4.3.4 Random mutagenesis and screening of *Chloroparva pannonica* Cu40

4.3.4.1 Kill curves

In this study, the mutagenic agent chosen was UV-C irradiation at 254 nm as it is one of the simplest ways to obtain a wide variety of mutant strains. The first step in the mutation program was to determine the appropriate length of time for UV-C exposure for *Chloroparva pannonica* Cu40 (parent culture (PC)). Kill curves for 100 ml of 1 x 10^7 cells/ml of *Chloroparva pannonica* Cu40 under UV-C light were obtained (Figure 4.17), indicating that UV-C exposure for killing rate of 50%, 96% and 98% were 36 minutes, 54 minutes and 63 minutes, respectively (Figure 4.17).

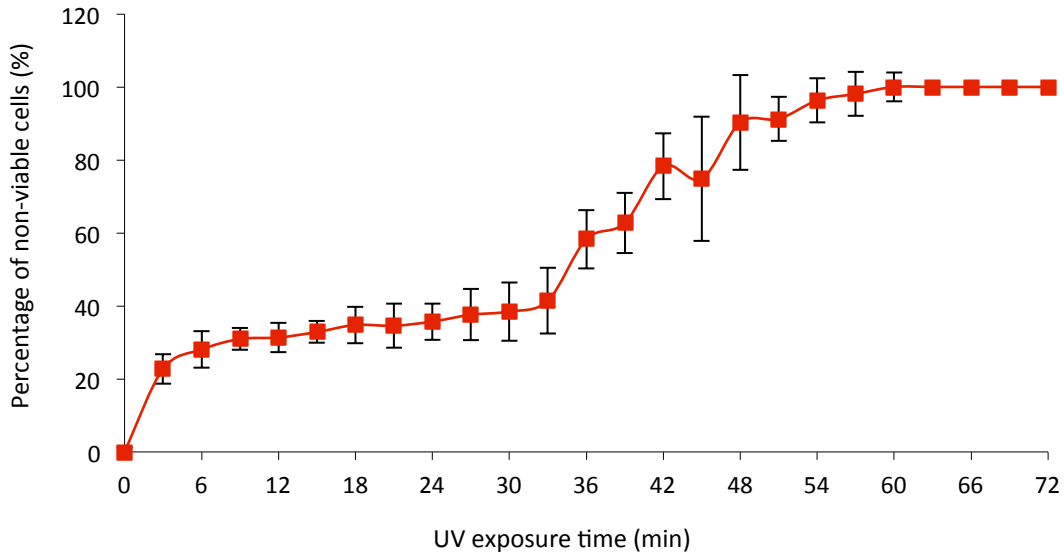


Figure 4.17 Percentage of non-viable *Chloroparva pannonica* Cu40 PC under UV-C exposure for 72 minutes

4.3.4.2 Inhibitory levels of *Chloroparva pannonica* Cu40 PC in response to herbicides: aclonifen or chlodinafop-propargyl

The sensitivity of *Chloroparva pannonica* Cu40 PC was tested by spreading equal numbers of cells (OD of 0.7 at 685 nm) on agar plates containing varying concentrations (from 5 nm to 1000 nm) of herbicides, aclonifen or chlorinafop-propargyl. Results were collected after 2–3 weeks and rated as positive (+), negative (-) or (W), based on the presence or absence of growth, or weak growth. The results showed that the PCwas more sensitive to chlodinafop-propargyl than to aclonifen. The growth was weak between 250 nm and 400 nm for aclonifen and between 150 nm and

250 nm for chlodinafop-propargyl (Table 4.3). No inhibition of growth occurred up to 600 nm for aclonifen, but at 250 nm, chlodinafop-propargyl completely inhibited growth (Table 4.3).

Table 4.3 Toxicity tests for various concentrations of herbicides, aclonifen and chlodinafop-propargyl, on *Chloroparva pannonica* Cu40 PC (parent culture)

Concentration (nm)	Aclonifen	Chlodinafop-propargyl
0	+++	+++
50	+++	+
100	+	+
150	+	(W) Light green
200	+	(W) Yellowish
250	(W) Light green	(W) Yellowish
300	(W) Light green	-
350	(W) Yellowish	-
400	(W) Yellowish	-
450	-	-
500	-	-
550	-	-
600	-	-
650	-	-
700	-	-
750	-	-
800	-	-
850	-	-
900	-	-
950	-	-
1000	-	-

** + = Positive or present; - = Negative or absent; W = Weakly positive*

4.3.5 Random mutagenesis and screening of *Chloroparva pannonica* Cu40 mutant strains

4.3.5.1 Isolation of *Chloroparva pannonica* Cu40 mutant strains

Using several cultivation media, the streaking method and with conditions tested to obtain a single colony streaked on a plate, *Chloroparva pannonica* Cu40 cultures were successfully grown on an FU medium with 1.5% (w/v) agar. Growth in a single colony was to ensure that the microalgae in the resulting liquid FU medium were all

descendants from the same single microalga instead of being a mixed culture of different strains.

Mutant isolation was undertaken by exposure of *Chloroparva pannonica* Cu40 PC to UV-C light for 63 minutes: it was kept in darkness for 24 hours to avoid photo-reactivation and then plated in FU medium agar plates as mentioned in Section 4.2 'Methods and Materials'. Green colonies were observed after 14–20 days of incubation. The colonies were selected randomly based on colour and the size of colonies. The greenest and the largest colonies were isolated and transferred into an FU medium and allowed to grow under normal growth conditions. In all, 597 mutants were isolated from four rounds of UV-C exposure of the *Chloroparva pannonica* Cu40 PC. In total, 157, 129, 185 and 126 mutants were isolated from the first, second, third and fourth rounds of UV-C exposure of the *Chloroparva pannonica* Cu40 PC, respectively.

4.3.5.2 Comparison of growth of *Chloroparva pannonica* Cu40 mutant strains in 6 ml FU medium

As mentioned above, 597 mutant strains were isolated and successfully recovered from four rounds of UV-C exposure of the *Chloroparva pannonica* Cu40 PC. A cultivation experiment was then performed in 6 ml of FU medium on tissue culture plates. The growth of the mutant strains was analysed by using a UV/Vis spectrophotometer with an OD reading at 685 nm at day 4, 7, 11 and 14. Appendix 4.5, Table A4.6 present detailed results of the growth, with an OD reading at 685 nm, of the mutant strains, *Chloroparva pannonica* WT strains and *Chloroparva pannonica* Cu40 PC in 6 ml of FU medium. In total, 366 mutant strains that showed moderate to high growth (OD at 685 nm above 1.0) were selected and taken for further studies in the next part of the current study.

4.3.5.3 Sensitivity of *Chloroparva pannonica* Cu40 mutant strains to herbicides: aclonifen or chlodinafop-propargyl

As mentioned above, 366 mutant strains that showed moderate to high growth (OD at 685 nm above 1.0) were selected and cultivated in 40 ml of FU medium and on plates with FU medium and 1.5% agar (w/v) containing either aclonifen at concentrations of 900 nm, 1 µm, 2 µm and 3 µm or chlodinafop-propargyl at concentrations of 600 nm, 1.2 µm, 1.8 µm and 2.1 µm. Results were collected after 2–3 weeks as positive (+),

negative (-) or (W), based on the presence or absence of growth, or weak growth. Appendix 4.5, Table A4.0.2 and Table A.4.0.3 present detailed results of the mutant strains on either aclonifen or chlodinafop-propargyl at different concentrations.

In total, 205 mutant strains were inhibited by aclonifen while 117 mutant strains survived at different concentrations of aclonifen (see in Appendix 4.5, Table A4.0.2). The survival rate decreased with the increasing concentration of aclonifen (Figure 4.18). Among the surviving mutant strains, 44, 39, 21 and 13 mutant strains survived exposure to aclonifen at concentrations of 900 nm, 1 µm, 2 µm and 3 µm, respectively (Figure 4.18). In addition, 27 mutant strains did not grow in the FU medium with 0 nm of aclonifen (see Appendix 4.5, Table A4.0.2). Mutant strains that survived at concentrations of 2 µm and 3 µm of aclonifen were selected, isolated and taken in the next part of the study. In addition, the mutant strains that showed positive growth at a concentration of 1 µm of aclonifen were also selected, isolated and taken for further studies in the next part of the study.

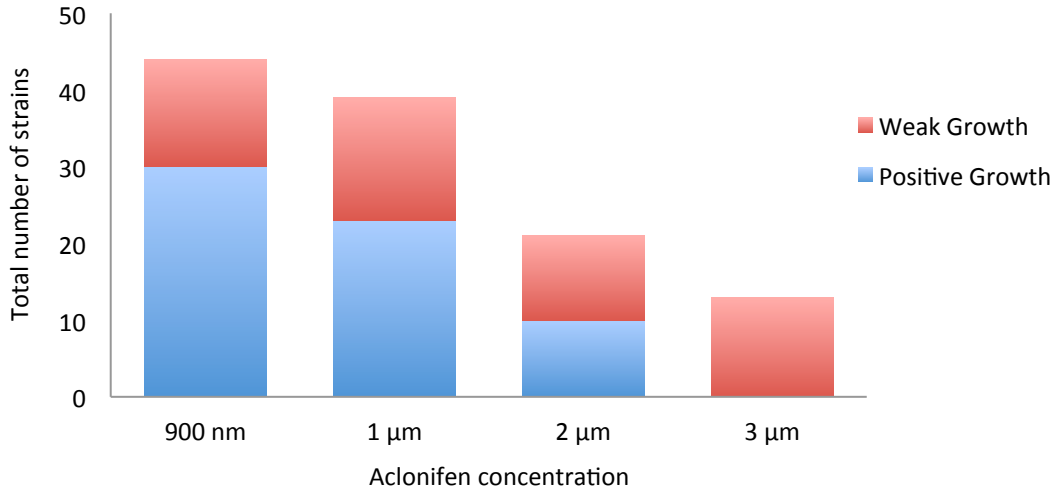


Figure 4.18 Total number of *Chloroparva pannonica* Cu40 mutant strains resistant to different concentrations of aclonifen

In total, 248 mutant strains were inhibited by chlodinafop-propargyl while 76 mutant strains survived at different concentrations of chlodinafop-propargyl. The highest total number of mutant strains survived at a concentration of 1.2 µm, with the survival rate decreasing with the increasing concentration of aclonifen from 1.2 µm to 2.1 µm (Figure 4.19). In all, 23, 28 and 14 mutant strains survived at concentrations of 600 nm, 1.2 µm and 1.8 µm, respectively (see Appendix 4.5, Table A.4.0.3). No mutants survived exposure to chlodinafop-propargyl at a concentration of 2.1 µm. In addition,

42 mutants did not grow in the FU medium with 0 nm of aclonifen (see Appendix 4.5, Table A.4.0.3). The colonies that survived at concentrations of 1.2 µm and 1.8 µm of chlodinafop-propargyl were selected, isolated and taken for further studies in the next part of the study.

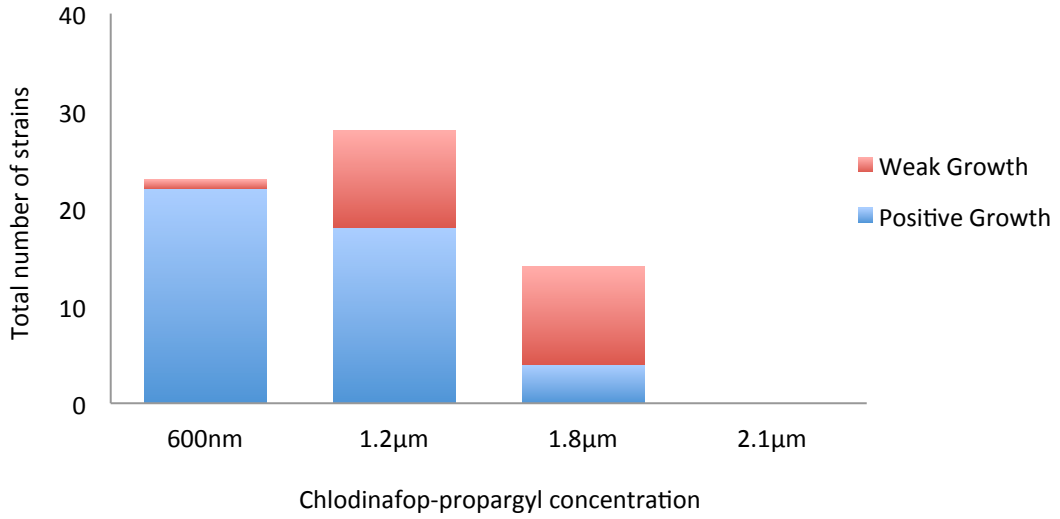


Figure 4.19 Total number of *Chloroparva pannonica* Cu40 mutant strains resistant to different concentrations of chlodinafop-propargyl

4.3.5.4 Comparison of growth and lutein content of *Chloroparva pannonica* WT strains, Cu40 PC, mutant strains and herbicide-resistant mutant strains

Among the 366 mutant strains, 50 strains with the highest growth based on an OD reading at 685 nm; 58 strains that survived aclonifen at concentrations of 1 µm, 2 µm and 3 µm; and 42 strains that survived chlodinafop-propargyl at concentrations of 1.2 µm and 1.8 µm were selected in order to compare growth and lutein content. Each test was carried out in triplicate for each strain and was repeated three times.

The growth of the *Chloroparva pannonica* PC, WT and mutants was compared by analysis using a UV/Vis spectrophotometer at an OD reading at 685 nm at day 4, 7, 9, 12, 15 and 17 after inoculation of an equal number of cells in 40 ml of FU medium (see Appendix 4.5, Table A4.0.4 – Table A4.0.7). The OD readings at 685 nm for *Chloroparva pannonica* PC and WT at day 17 were 0.585 and 0.548, respectively (Table A4.0.4). By comparing the growth at day 17, it was observed that 36% of mutants, 71% of aclonifen-resistant mutants and 21% of chlodinafop-propargyl resistant mutant isolates had higher growth than *Chloroparva pannonica* PC (Figure 4.20) while

54% of mutants, 83% of aclonifen-resistant mutants and 48% of chlodinafop-propargyl resistant mutants isolates had higher growth than *Chloroparva pannonica* WT (Figure 4.21). The results indicated that most of the aclonifen-resistant mutants had higher growth than *Chloroparva pannonica* PC and WT strains. The highest growth at day 17 between all of the mutants was 2µmA.469 with an OD reading of 0.882 at 685 nm (see Appendix 4.5, Table 4.0.6).

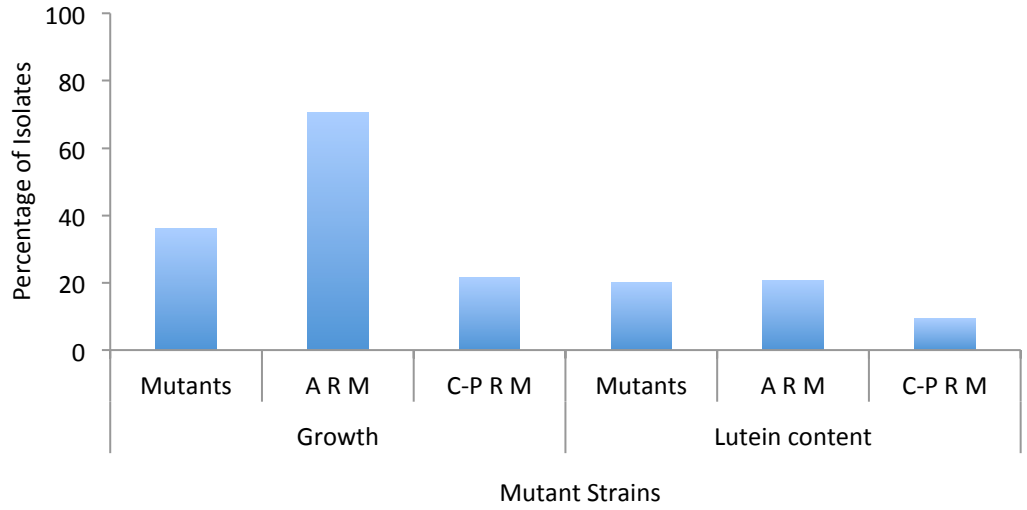


Figure 4.20 Percentage of isolates of mutants, aclonifen-resistant mutants (ARM) and chlodinafop-propargyl resistant mutants (C-P RM) with higher growth at day 17, and higher lutein concentration at day 15 compared to *Chloroparva pannonica* Cu40 PC

The lutein concentration of the mutant strains was determined by harvesting cells on day 7 and day 15. The lutein was extracted from the cells by using the one-step closed-tube method and the extracts were analysed using a UV/Vis spectrophotometer at 440 nm with the results further confirmed with HPLC (see Appendix 4.5, Table A4.0.8-Table A4.0.11). The lutein concentrations for *Chloroparva pannonica* PC for day 7 and day 15 were 3.642 mg/l and 9.489 mg/l, respectively, while for *Chloroparva pannonica* WT, they were 3.419 mg/l and 6.124 mg/l, respectively (see Appendix 4.5, Table 4.0.8). As shown in Figure 4.21, 20% of mutants, 21% of aclonifen-resistant mutants and 10% of chlodinafop-propargyl resistant mutants had higher lutein content (mg/l) than *Chloroparva pannonica* PC. In addition, 62% of mutants, 26% of aclonifen-resistant mutants and 21% of chlodinafop-propargyl resistant mutants had higher lutein content in comparison to *Chloroparva pannonica* WT (Figure 4.21). The highest lutein content among all of the mutants was 12.73 mg/l for Strain #21, at day 17 (see Appendix 4.5, Table A4.0.9).

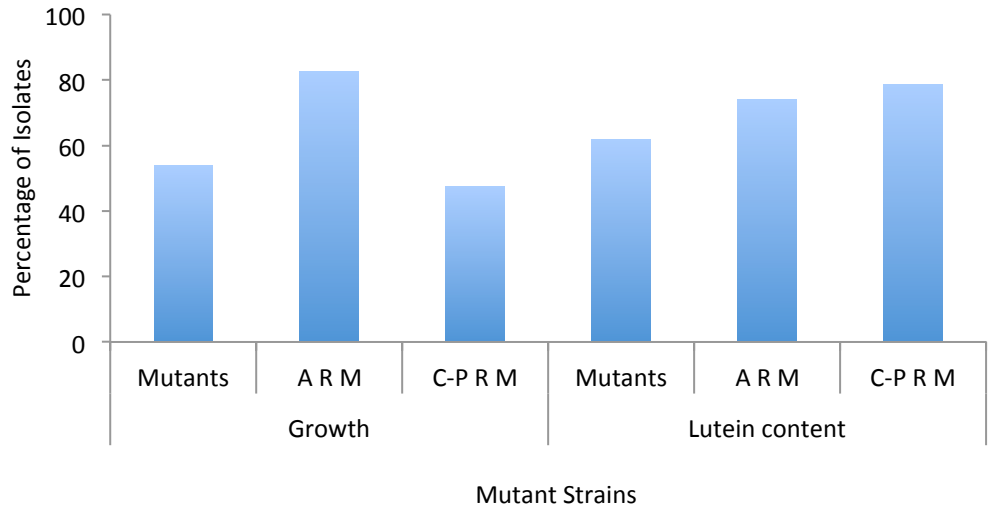


Figure 4.21 Percentage of isolates of mutants, aclonifen-resistant mutants (ARM) and chlodinafop-propargyl resistant mutants (C-P RM) with higher growth at day 17, and higher lutein at day 15 compared to *Chloroparva pannonica* WT strains

Based on the results, 39 mutants showed higher growth and lutein content than *Chloroparva pannonica* PC and WT, with these mutants selected and taken for further studies in the next part of the study (Table 4.4).

Table 4.4 *Chloroparva pannonica* Cu40 mutants selected for further studies based on growth and lutein content in 40 ml of FU medium

Mutants #	Aclonifen-resistant Mutants #	Chlodinafop-propargyl Resistant Mutants #
4	1µmA.160	1.2µmC.10
10	1µmA.210	1.2µmC.418
16	1µmA.220	1.8µmC.112
17	1µmA.85	1.8µmC.192
21	2µmA.10	1.8µmC.220
24	2µmA.114	1.8µmC.305
35	2µmA.31	1.8µmC.5
90	2µmA.4	1.8µmC.58
92	2µmA.419	
142	2µmA.469	
212	2µmA.48	
555	2µmA.523	
560	2µmA.58	
564	3µmA.41	
586	3µmA.509	
	3µmA.558	

4.3.6 Evaluation of selected *Chloroparva pannonica* Cu40 PC, and WT and mutant strains grown in 3 L flasks with 1% carbon dioxide supplied

The 39 mutants, as listed in Table 4.4 that showed higher growth and lutein content than *Chloroparva pannonica* PC and WT, were selected for cultivation in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks for a 15-day period. The cells were harvested at day 3, 6, 9, 12 and 15 to determine the total biomass (g/l) and lutein content (mg/kg of dried biomass). Total lipids were determined from the cells harvested at the end of the growth phase (day 15). Each growth and content test was carried out in triplicate for each strain and was repeated three times.

4.3.6.1 Total biomass, lutein and lipid content of *Chloroparva pannonica* Cu40 PC and WT strains

Chloroparva pannonica WT and Cu40 PC were selected as a control for this part of the study to compare with the selected mutants. Figure 4.22 shows the total biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* WT and Cu40 PC. The trend of growth based on the total dried biomass was similar and Cu40 PC was higher than *Chloroparva pannonica* WT. At day 15, the total biomass for Cu40 PC and WT was 0.884 g/l and 0.588 g/l, respectively. In addition, the lutein content for *Chloroparva pannonica* Cu40 PC, which had a higher lutein content than WT, increased from day 3, reached the highest lutein content at day 9 (4344 mg/kg of dried biomass) and decreased from day 12. The lutein content for WT increased from day 3, reached the highest at day 12 (1933 mg/kg of dried biomass) and decreased from day 15. Moreover, the percentage of total lipids for Cu40 PC and WT was 24.88% and 23.22%, respectively.

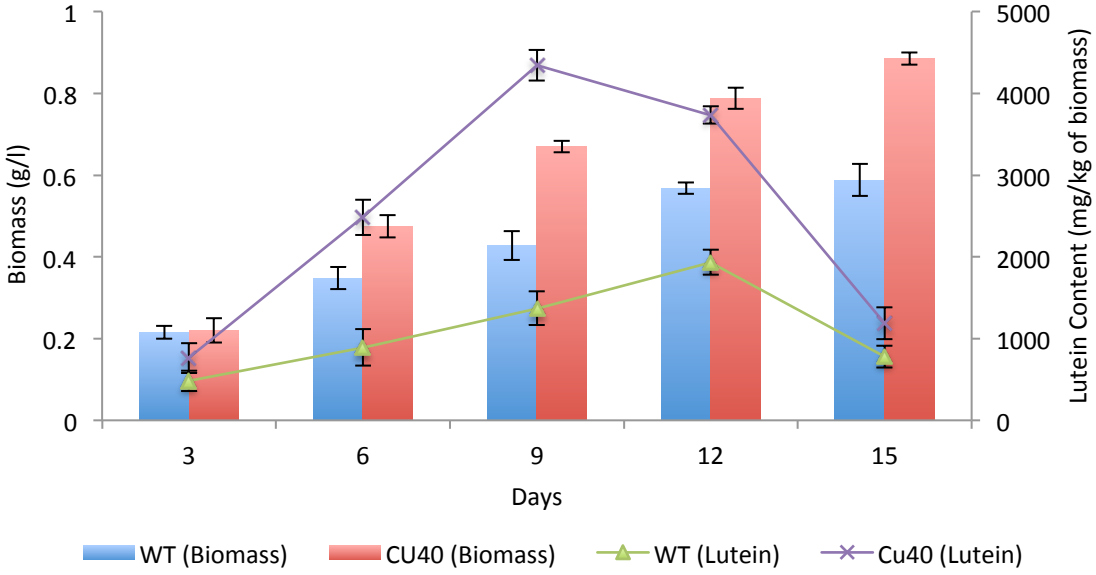


Figure 4.22 Total dried biomass (g/l) and lutein content (mg/kg of dried biomass) of *Chloroparva pannonica* WT and Cu40 PC strains in a 3 L flask set up with 1% carbon dioxide supplied for a 15-day period

4.3.6.2 Evaluation of selected *Chloroparva pannonica* Cu40 mutants

4.3.6.2.1 Total dried biomass (mg/l) of selected Chloroparva pannonica Cu40 mutants

Figure 4.23 shows the total biomass (g/l) of 15 *Chloroparva pannonica* Cu40 mutants cultivated in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks. As indicated by the total biomass (g/l), the cell growth of *Chloroparva pannonica* Cu40 mutants increased from day 3 and reached the highest biomass at day 15 (Figure 4.23). At day 15, 10 mutant strains (#4, #10, #16, #21, #24, #92, #142, #212, #555 and #586) achieved a higher total biomass than Cu40 PC while all of the mutants achieved a higher total biomass than *Chloroparva pannonica* WT. The mutant strain that achieved the highest biomass at the end of the growth phase (day 15) was #4, followed by #560, #142 and #10 with total biomass of 1.592 g/l, 1.34 g/l, 1.34 g/l and 1.264 g/l, respectively (Figure 4.23). The mutant with the lowest biomass was #586, with total biomass of 0.812 g/l at day 15.

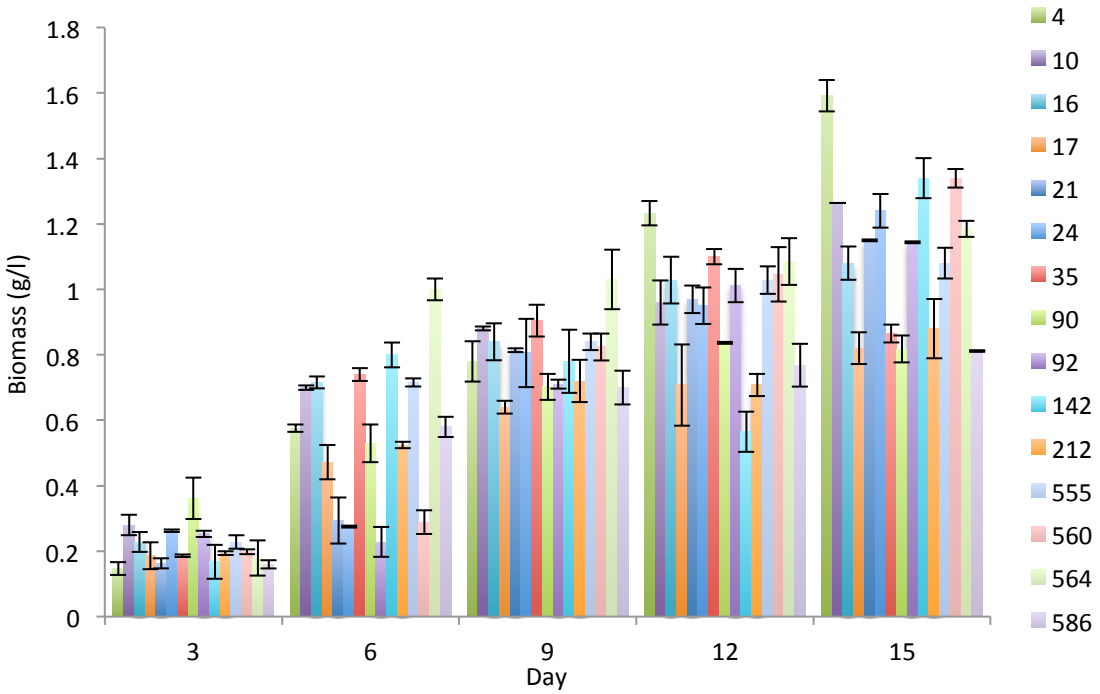


Figure 4.23 Total dried biomass (g/l) of *Chloroparva pannonica* Cu40 mutants in a 3 L flask set up with 1% carbon dioxide supplied for a 15-day period

Figure 4.24 shows the 16 aclonifen-resistant *Chloroparva pannonica* Cu40 mutants cultivated in an FU medium, with 1% carbon dioxide, in 3 L flasks for a 15-day period. As indicated by the total biomass (g/l), the trend of cell growth of aclonifen-resistant *Chloroparva pannonica* Cu40 mutants increased from day 2 and reached the highest level at day 15 (Figure 4.24). However, #1µmA.210 reached the highest growth at day 12 (0.948 g/l) and entered the death phase from day 15. There were eight aclonifen-resistant mutants (#1µmA.220, #1µmA.31, #2µmA.4, #2µmA.419, #2µmA.469, #2µmA.48, #2µmA.58, #3µmA.41, #3µmA.509 and #3µmA.558) that achieved higher total biomass (g/l) than *Chloroparva pannonica* Cu40 PC and all of the aclonifen-resistant mutants achieved higher total biomass (g/l) than *Chloroparva pannonica* WT. The aclonifen-resistant mutant that achieved the highest biomass at the end of the growth phase (day 15) was #3µmA.41, followed by #2µmA.469 and #2µmA.448, with total biomass of 1.56 g/l, 1.264 g/l and 1.244 g/l, respectively. On the other hand, the lowest biomass achieved at the end of the growth phase (day 15) was 0.796 g/l by #1µmA.85.

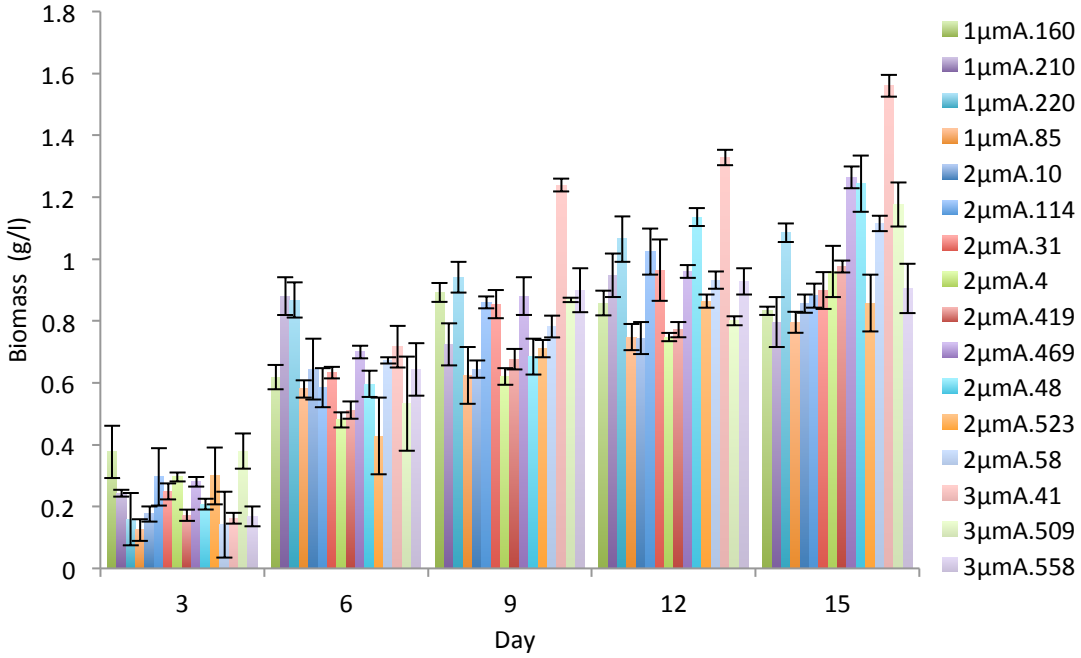


Figure 4.24 Total dried biomass (g/l) of aclonifen-resistant *Chloroparva pannonica* Cu40 mutants in a 3 L flask set up with 1% carbon dioxide supplied for a 15-day period

Figure 4.25 shows the cell growth of eight chlodinafop-propargyl resistant *Chloroparva pannonica* Cu40 mutants cultivated in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks for a 15-day period. As indicated by the total biomass (g/l), the trend of cell growth of chlodinafop-propargyl resistant *Chloroparva pannonica* Cu40 mutants increased from day 2 and reached the highest level at day 15 (Figure 4.25). Among the eight chlodinafop-propargyl resistant mutants, six (#1.2µmC.418, #1.8µmC.112, #1.8µmC.305, #1.8µmC.220, #1.8µmC.5 and #1.8µmC.58) achieved a higher total biomass (g/l) than *Chloroparva pannonica* Cu40 PC while all of them achieved a higher total biomass (g/l) than *Chloroparva pannonica* WT. The chlodinafop-propargyl resistant mutant achieving the highest biomass at the end of the growth phase (day 15) was #1.8µmC.5 (0.1444 g/l) while the lowest was #1.2µmC.10 (0.61 g/l) (Figure 4.25).

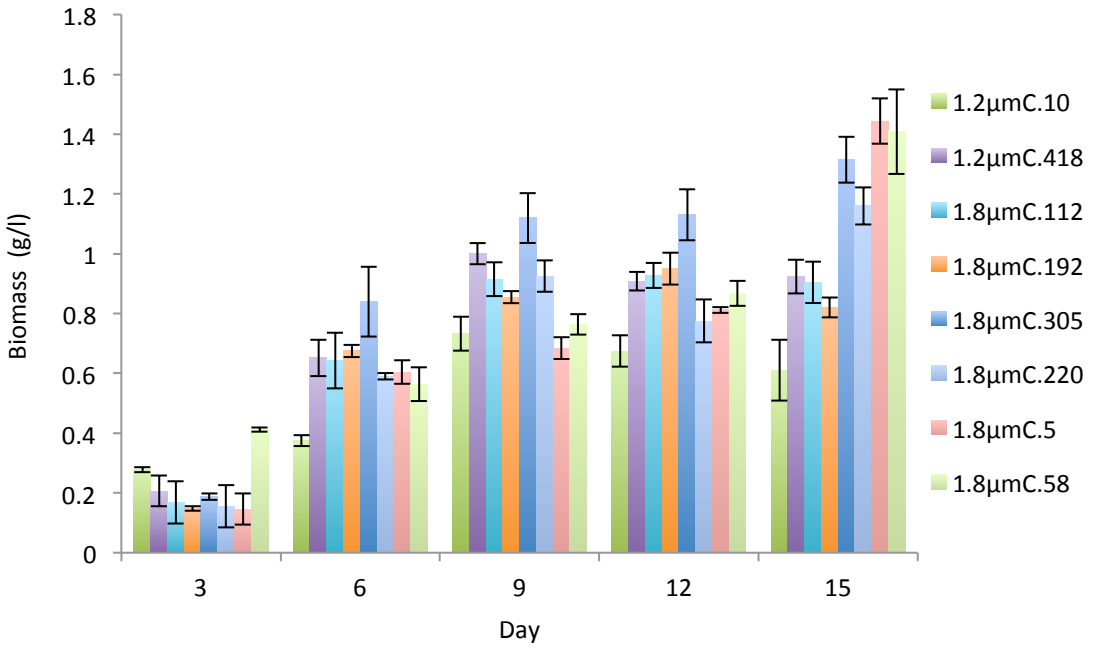


Figure 4.25 Total dried biomass (g/l) of chlodinafop-propargyl resistant *Chloroparva pannonica* Cu40 mutants in a 3 L flask set up with 1% carbon dioxide supplied for a 15-day period

The highest total biomass (g/l) achieved in the 39 *Chloroparva pannonica* Cu40 mutants compared with *Chloroparva pannonica* PC and WT are summarized in Figure 4.26. The highest total biomass (g/l) achieved in the *Chloroparva pannonica* Cu40 mutants ranged from 0.6 g/l to 1.592 g/l. In all, 26 mutants achieved a higher total biomass (g/l) than Cu40 PC while all of the mutants achieved a higher total biomass (g/l) than *Chloroparva pannonica* WT. Among all the mutants, #4 achieved the highest total biomass (g/l) with1.592 g/l, followed by #3µmA.41 with 1.56 g/l (Figure 4.26). The lowest total biomass (g/l) achieved was 0.61g/l by #1.2µmC.10 (Figure 4.26).

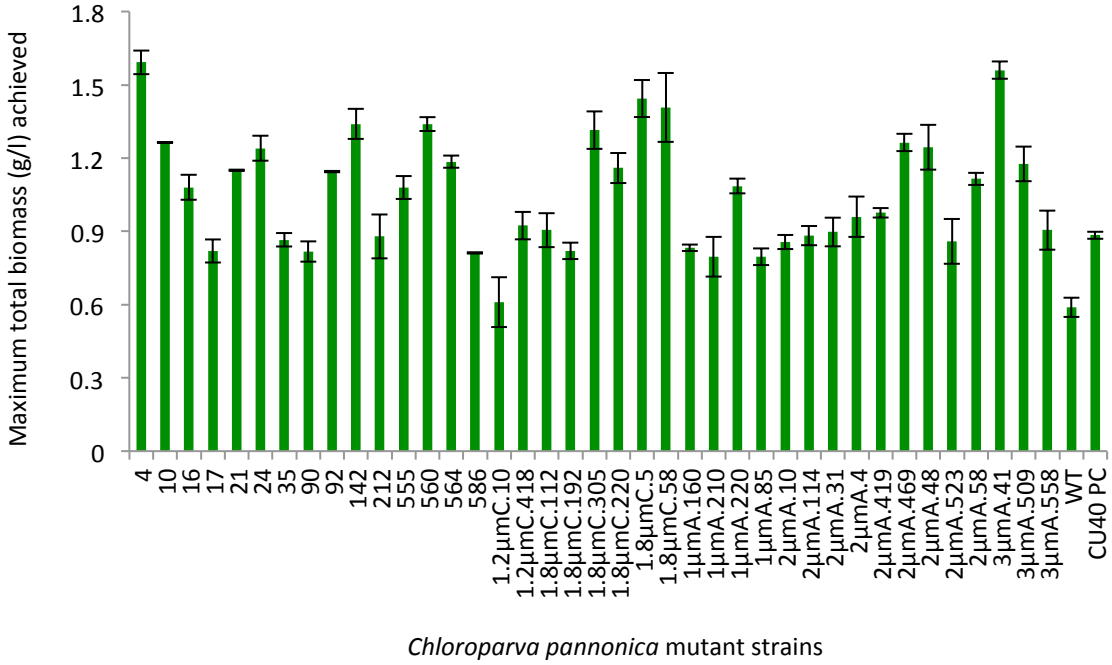


Figure 4.26 Highest total dried biomass (g/l) achieved in *Chloroparva pannonica* WT strains, Cu40 PC and Cu40 mutants in a 3 L flask set up with 1% carbon dioxide supplied

4.3.6.2.2 Lutein content (mg/kg of dried biomass) of selected Chloroparva pannonica Cu40 mutants

Figure 4.27 shows the lutein content (mg/kg of dried biomass) of 15 *Chloroparva pannonica* Cu40 mutants cultivated in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks for a 15-day period. The lutein content of *Chloroparva pannonica* Cu40 mutants increased from day 3. Among 15 Cu40 mutants, eight of them (#4, #10, #35, #92, #142, #212, #555 and #564) reached the highest lutein content at day 6; five of them (#16, #17, #24, #90 and #586) reached the highest lutein content at day 9; and two of them (#21 and #560) reached the highest lutein content at day 12 before the lutein content started to decrease (Figure 4.27). One mutant strain (#4) had a higher lutein content than *Chloroparva pannonica* Cu40 PC, while 13 mutant strains (#4, #10, #16, #17, #35, #90, #92, #142, #212, #555, #560, #564 and #586) had a higher lutein content than *Chloroparva pannonica* WT. Among the mutant strains, #4 had the highest lutein content (4386 mg/kg of dried biomass at day 6).

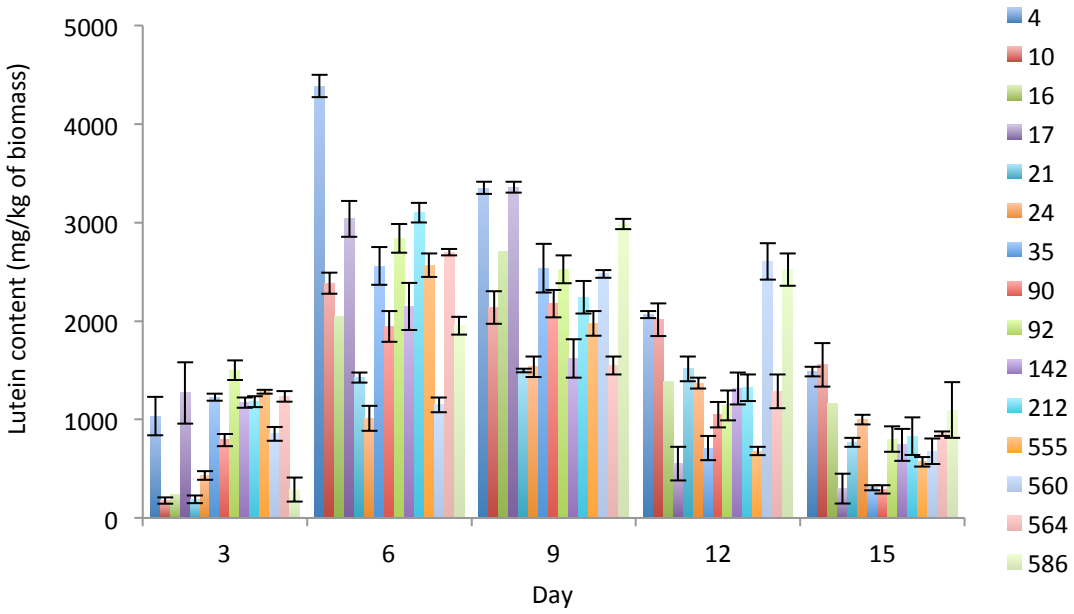


Figure 4.27 Lutein content (mg/kg of biomass) of *Chloroparva pannonica* Cu40 mutants in a 3 L bioreactor set up with 1% carbon dioxide supplied for a 15-day period

In total, 16 aclonifen-resistant *Chloroparva pannonica* Cu40 mutants were cultivated in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks for a 15-day period, with these shown in Figure 4.28. The lutein content of aclonifen-resistant *Chloroparva pannonica* Cu40 mutants increased from day 2. Of the aclonifen-resistant Cu40 mutants, 10 mutants (#2µmA.10, #2µmA.4, #2µmA.419, #2µmA.469, #2µmA.48, #2µmA.523, #2µmA.58, #3µmA.41, #3µmA.509 and #3µmA.558) had the highest lutein content at day 6 while six of them had the highest lutein content at day 9 (#1µmA.160, #1µmA.210, #1µmA.220, #1µmA.85, #1µmA.114 and #1µmA.31) before the lutein content started to decrease (Figure 4.28). There were two aclonifen-resistant Cu40 mutants (#2µmA.48 and #3µmA.41) that had a higher lutein content than Cu40 PC, while all of the aclonifen-resistant Cu40 mutants had a higher lutein content than *Chloroparva pannonica* WT. The mutant with the highest lutein content was ##3µmA.41 which achieved 5201.9 mg/kg of dried biomass at day 6 (Figure 4.28).

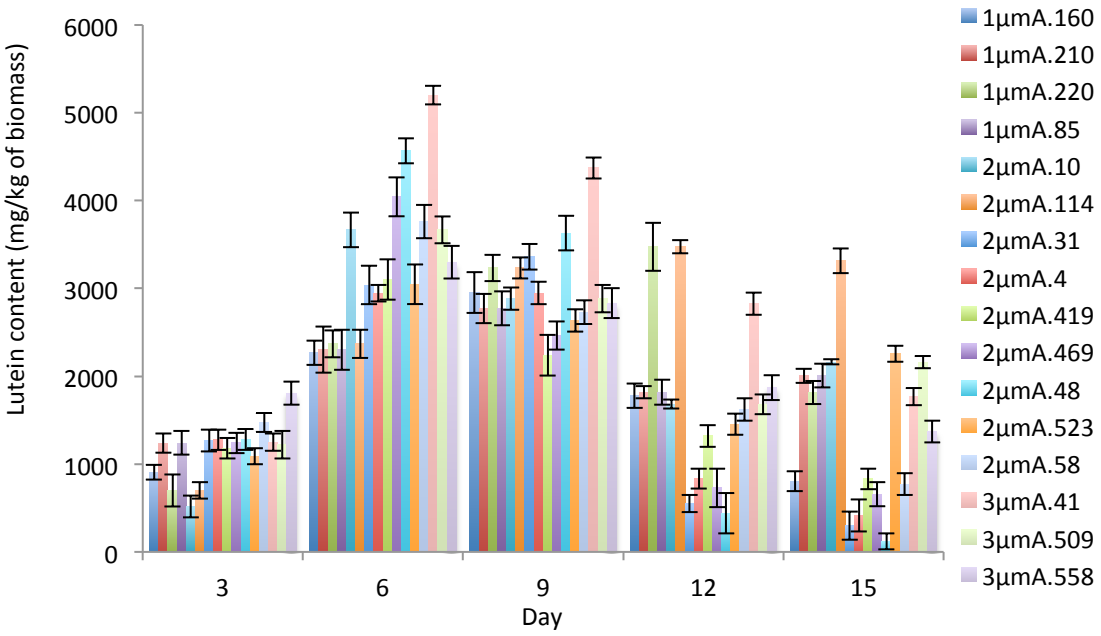


Figure 4.28 Lutein content (mg/kg of biomass) of aclonifen *Chloroparva pannonica* Cu40 mutants in a 3 L bioreactor set up with 1% carbon dioxide supplied for a 15-day period

Figure 4.29 shows the lutein content (mg/kg of dried biomass) of eight chlodinafop-propargyl resistant *Chloroparva pannonica* Cu40 mutants cultivated in an FU medium, with 1% carbon dioxide supplied, in 3 L flasks for a 15-day period. The lutein content of chlodinafop-propargyl resistant *Chloroparva pannonica* Cu40 mutants increased from day 2. Six of the chlodinafop-propargyl resistant Cu40 mutants (#1.2µmC.10, #1.2µmC.418, #1.2µmC.112, #1.8µmC.305, #1.8µmC.220 and #1.8µmC.58) had the highest lutein content at day 6 while two of them had the highest lutein content at day 9 (#1.8µmC.192 and #1.8µmC.5) before the lutein content started to decrease (Figure 4.29). When the lutein content was compared between the chlodinafop-propargyl resistant Cu40 mutants and *Chloroparva pannonica* Cu40 PC and WT, two of the chlodinafop-propargyl resistant Cu40 mutants (#1.8µmC.5 and #1.8µmC.58) had a higher lutein content than Cu40 PC while all of the chlodinafop-propargyl resistant Cu40 mutants had a higher lutein content than *Chloroparva pannonica* WT. The highest lutein content achieved was 5502.05 mg/kg of dried biomass at day 9 from mutant #1.8µmC.5.

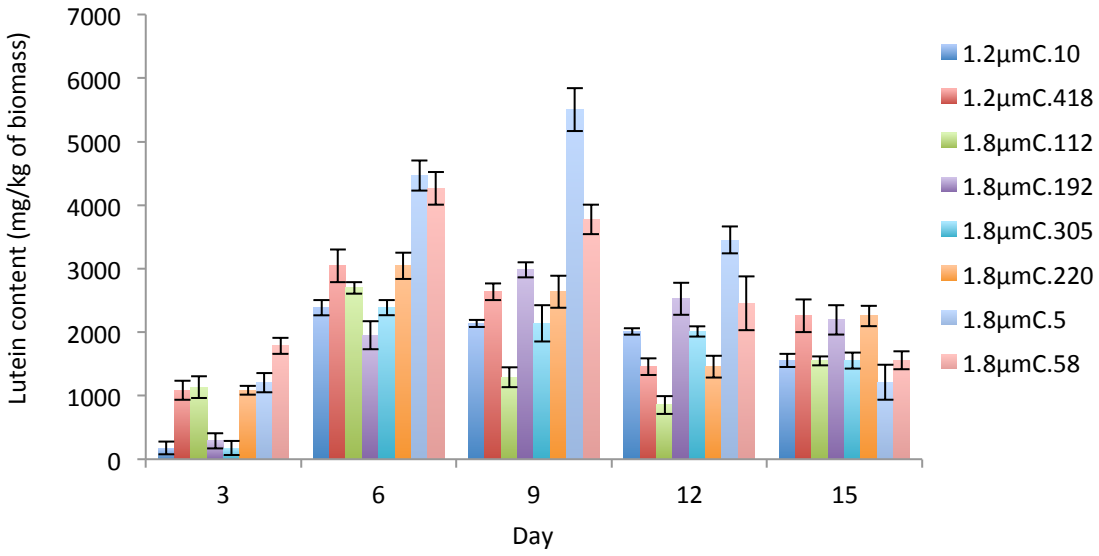


Figure 4.29 Lutein content (mg/kg of biomass) of chlodinafop-propargyl *Chloroparva pannonica* Cu40 mutants in a 3 L bioreactor set up with 1% carbon dioxide supplied for a 15-day period

The highest lutein content (mg/kg of dried biomass) achieved in all 39 *Chloroparva pannonica* Cu40 mutants compared with *Chloroparva pannonica* PC and WT are summarized in Figure 4.30. The highest lutein content achieved from *Chloroparva pannonica* Cu40 mutants at either day 6 or day 9, ranged from 1513 mg/kg of dried biomass to 5502 mg/kg of biomass. Five mutants achieved a higher lutein content than Cu40 PC while 37 mutants achieved a higher lutein content than *Chloroparva pannonica* WT. The highest lutein content achieved among all of the mutants was 5502.05 mg/kg of dried biomass by #1.8µmC.5, followed by 5201.8 mg/kg of dried biomass by #3µmA.41. The lowest lutein content achieved was 1513 mg/kg of dried biomass by #21 (Figure 4.30).

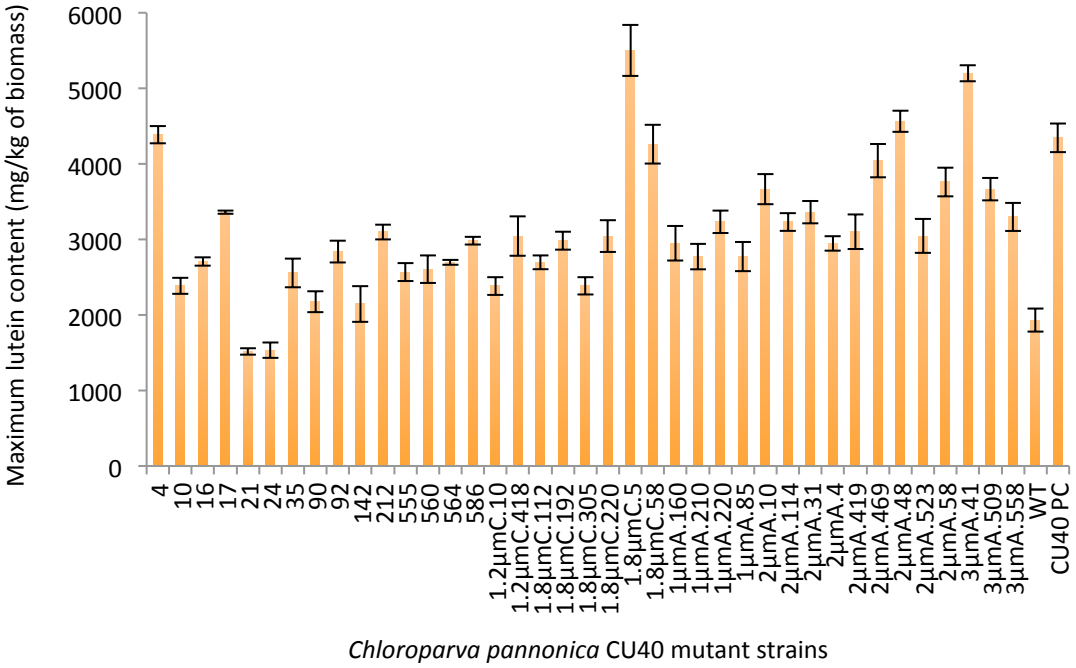


Figure 4.30 Highest lutein content (mg/kg of biomass) achieved in *Chloroparva pannonica* WT strains, Cu40 PC and Cu40 mutants in a 3 L bioreactor set up with 1% carbon dioxide supplied

4.3.6.2.3 Total lipids (%) of selected Chloroparva pannonica Cu40 mutants

Figure 4.31 shows the percentage of total lipids from the cells harvested at the end of the growth phase (day 15). The lipid content of the *Chloroparva pannonica* Cu40 mutants ranged from 10% to 26.59%. Five mutants (#1.8µmC.5, #17, #4, #142 and #1.8µmC.305) had higher total lipids than the Cu40 PC while seven mutants ((#1.8µmC.5, #17, #4, #142, #1.8µmC.305, #3µmA.41 and #555) had higher total lipids than *Chloroparva pannonica* WT. The highest total lipids achieved was 26.59% by mutant #1.8µmC.5, while the lowest total lipids was 10% by mutant #560.

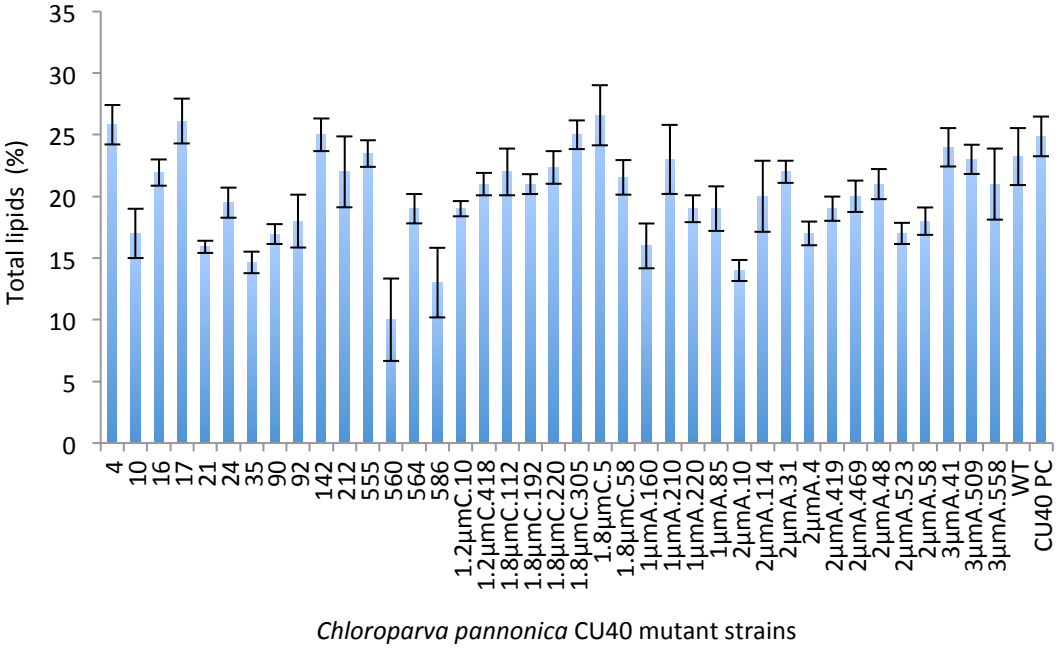


Figure 4.31 Total lipids (%) of *Chloroparva pannonica* WT strains, Cu40 PC and Cu40 mutants in a 3 L bioreactor set up with 1% carbon dioxide supplied at end of growth phase (day 15)

4.3.7 Identification of candidate biomass and lutein producer in an 11 L rectangular inter-loop airlift photobioreactor

The overall results of lutein content and total dried biomass obtained for the selected mutant strains produced by UV-C mutagenesis were followed by herbicide screening are shown in Figure 4.32. A group of mutant strains had increased growth but decreased lutein content (circled with dashed red line) in comparison to *Chloroparva pannonica* Cu40 PC. In addition, a group of mutant strains had increased growth and lutein content (circled with continuous green line) in comparison to Cu40 PC (Figure 4.32). However, another group of mutants had a lower total biomass and lower lutein content (circled with dotted purple line) in comparison to Cu40 PC (Figure 4.32).

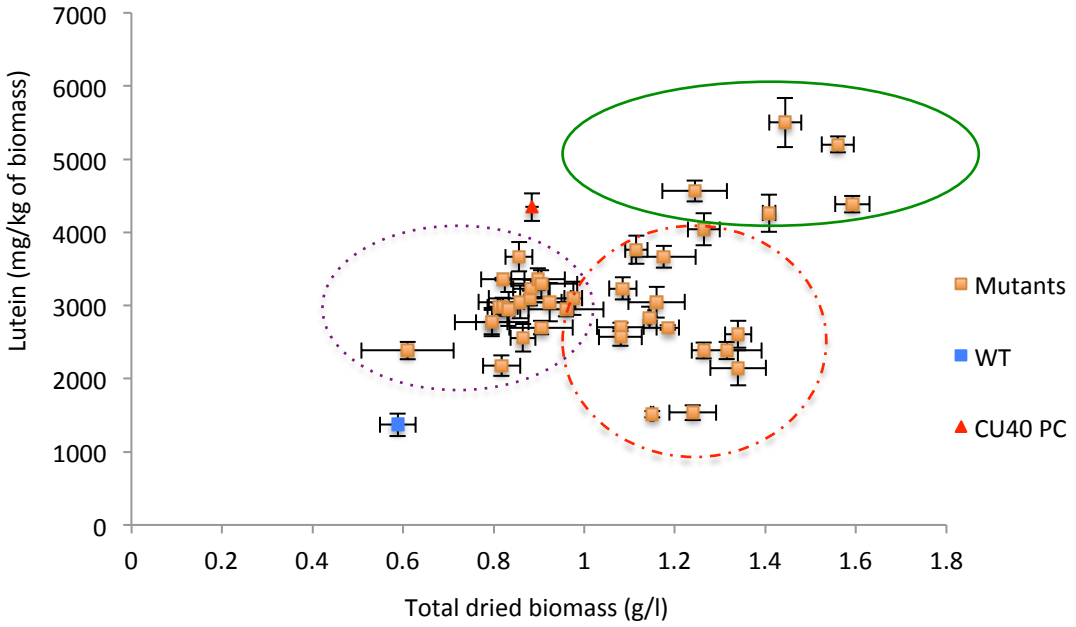


Figure 4.32 Total dried biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* WT strains, Cu40 PC and Cu40 mutant strains in a 3 L bioreactor set up with 1% carbon dioxide supplied at end of growth phase (day 15)

Figure 4.33 shows the overall results of total lipids and total dried biomass obtained for the selected mutant strains produced by UV-C mutagenesis and followed by herbicide screening. A group of mutant strains had increased growth but decreased lipid content (circled with dashed red line) while a group of mutant strains had increased growth and lipid content (circled with continuous green line) in comparison to Cu40 PC (Figure 4.33). In addition, another group of mutants had a lower total biomass and lower lipids (circled with dotted purple line) than Cu40 PC (Figure 4.33).

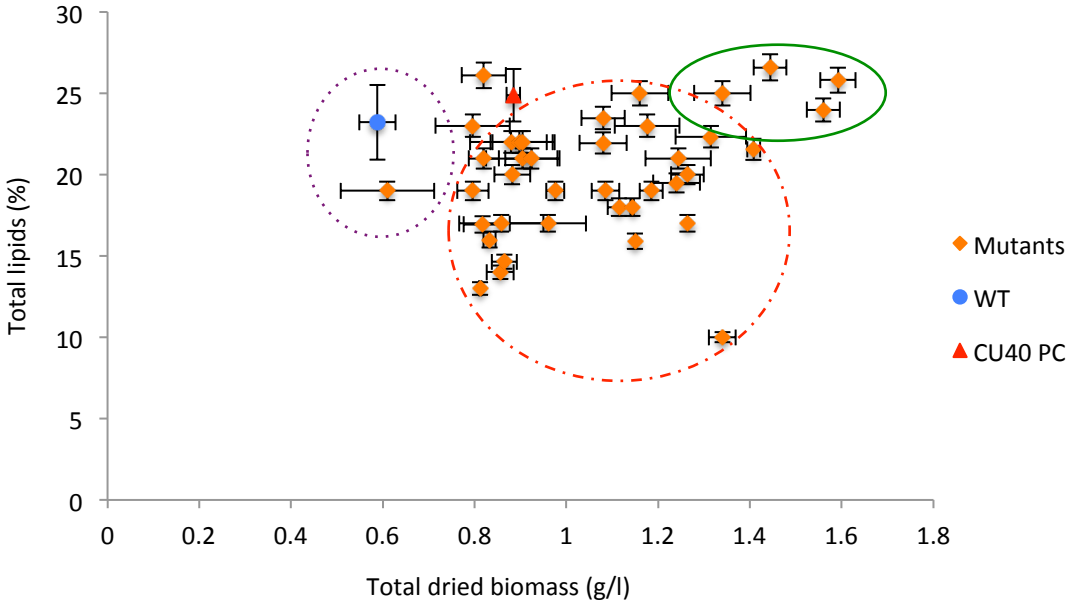


Figure 4.33 Total dried biomass (g/l) and total lipid content (%) of *Chloroparva pannonica* WT strains, Cu40 PC and Cu40 mutant strains in a 3 L bioreactor set up with 1% carbon dioxide supplied at end of growth phase (day 15)

Based on the results on total dried biomass, lutein content and total lipids content, the 10 mutants that showed higher growth, lutein content and total lipids content than *Chloroparva pannonica* Cu40 PC and WT were selected and taken for further studies.

Table 4.5 *Chloroparva pannonica* Cu40 mutants selected for further studies based on total dried biomass, lutein content and total lipids in a 3 L bioreactor set up with 1% carbon dioxide supplied

Chloroparva pannonica mutant strains (#)	Maximum total dried biomass (g/l)	µ/day	Lutein content (mg/kg of dried biomass)	Total lipid (%)
4	1.592	0.199	4386.77	25.81
142	1.340	0.174	2147.34	25.00
1.8µmC.305	1.315	0.162	2384.19	25.00
1.8µmC.5	1.444	0.191	5502.05	26.59
1.8µmC.58	1.408	0.102	4261.94	21.55
2µmA.10	0.856	0.132	3666.22	14.00
2µmA.469	1.264	0.126	4042.38	20.00
2µmA.48	1.244	0.149	4566.13	21.00
2µmA.58	1.115	0.172	3761.83	18.00
3µmA.41	1.560	0.189	5201.80	23.98

4.4 Discussion

Long-term large-scale microalgae-based production is a challenge mainly due to low productivity and the difficulty in scaling up from small-scale lab production to large-scale production. To overcome these issues, the selection and improvement of a strain of fast-growing and high-lipid-producing microalgae that is also able to synthesize high concentrations of lutein could play a very important role in achieving cost-effective biodiesel production. Random mutagenesis is one of the simplest ways to generate a high possible number of mutants. In this study, UV-C light was used as a mutagenic agent to induce mutagenesis.

Chloroparva pannonica was first discovered by Somogyi *et al.* (2011) when this species was isolated from a turbid, shallow soda pan in Hungary. To date, the potential of *Chloroparva pannonica* for microalgae based production has not been reported. In this study, a *Chloroparva pannonica* WT strain was obtained from a saltpan at Waikerie, South Australia, by the Aquatic Sciences Algal Production group from SARDI, South Australia. As this strain showed a promising lipid content, based on the current study's preliminary trials, it was therefore used as a candidate strain. Preliminary experiments with different culture media, solidifying agents and cultivation conditions were performed to determine the best conditions for the growth of *Chloroparva pannonica* in liquid and on solid media, while it was noted that microalgae face difficulties growing and forming colonies on solid media using bacteriological techniques. This was a crucial step in the current study as the microalgae in the resulting liquid culture after the random mutagenesis needed to be the descendants from the same single microalga. The solid medium, containing 1.5% (w/v) agar with Guillard's F/2 or FU medium, proved to be an effective method to produce distinct colonies of *Chloroparva pannonica*.

Recent studies have demonstrated that copper is one of the factors that influence the growth and pigment content of microalgae strains. It is known that copper is required as a cofactor of the enzymes involved in oxygen metabolism and redox reduction (Nalimova *et al.*, 2005, Nugroho and Frank, 2011). Excessive or limited amounts of copper can affect the physiological and biochemical processes in microalgae cells, such as photosynthesis, respiration, carbohydrate metabolism, ATP production, pigment synthesis, electron transport, membrane ultrastructure, DNA synthesis and cell division

(Arunakumara and Xuecheng, 2008, Debelius *et al.*, 2009, Klochenko and Medved, 1999, Markina and Aizdaicher, 2006, Ristenbil and Gerringa, 2002). Therefore, the first part of the current study focused on the selection and optimization of copper, with copper concentrations ranging from 0 µg/l to 100 µg/l, in Guillard's F/2 medium of *Chloroparva pannonica* WT for biomass and lutein production. The copper concentrations examined in this experiment did not totally suppress the growth of *Chloroparva pannonica* (Figure 4.4).

The results indicated that the growth trend of *Chloroparva pannonica* was similar for copper concentrations of 10 µg/l, 20 µg/l, 30 µg/l, 50 µg/l and 100 µg/l in Guillard's F/2 medium, highest at 40 µg/l and lowest at 0 µg/l (Figure 4.4). At the next stage of the study, *Chloroparva pannonica* with copper concentrations of 40 µg/l and 100 µg/l were cultivated in Guillard's F/2 medium in 3L flasks, with 1% carbon dioxide supplied, for a 15-day period. The *Chloroparva pannonica* selected from a copper concentration of 40 µg/l was named as Cu40, and *Chloroparva pannonica* from a copper concentration of 100 µg/l was named as Cu100 in this study. Overall, the results indicated that the copper concentration had an effect on the growth, pigment content (mg/kg of dried biomass) at day 6, total biomass (g/l) and total lipids content at day 15 in *Chloroparva pannonica* Cu40 and Cu100 when compared to *Chloroparva pannonica* WT (Figure 4.5 and Table 4.2). From day 6 of the experiment, a higher increase in cell growth was observed, based on measuring the OD reading at 685 nm, in *Chloroparva pannonica* Cu40 than in *Chloroparva pannonica* WT and Cu100. At the end of the growth phase, in the presence of the highest concentration of copper, the growth was slower. As can be seen from Table 4.2, Cu40 had the highest total dried biomass (0.604 g/l), highest lipid content (24.88%) and highest lutein content (4333 mg/kg of dried biomass). In addition, Cu100 had the lowest dried biomass (0.378 g/l) and the lowest total lipids content (19.6%). However, Cu100 had a slightly higher lutein content (1996 mg/kg of dried biomass) than the WT (1864 mg/kg of dried biomass).

It is known that copper is able to induce damage in microalgae cells as it suppresses chlorophyll *a* synthesis, increases the carotenoid content as they act as protective pigments and degrades carotenoids (Dieguez-Rojo and Gonzalez, 2003). The decrease in total dried biomass, total lipids, chlorophyll *a*, chlorophyll *b* and lutein was observed in Cu100. On the other hand, the increase in total dried biomass, total lipids, chlorophyll *a*, chlorophyll *b* and lutein in Cu40 suggested that 40 µg/l is the optimum copper

concentration for cell growth, and lipids and lutein production. However, the lutein to chlorophyll *a* ratio increased in Cu40 and Cu100 compared to the *Chloroparva pannonica* WT. The increase of the lutein to chlorophyll *a* ratio suggested that the *Chloroparva pannonica* focus on the synthesis of carotenoid rather than on growth when cultivated at a higher copper concentration, which is in agreement with similar research conducted using microalgae *Chlorella pyrenoidosa*, *Melosira varians* and *Tetraselmis suecica* (Barranguet *et al.*, 2002, Dieguez-Rojo and Gonzalez, 2003, Wang and Chang, 1991). In addition, in a study by Yan *et al.*(2002), it was shown that the growth of *Scenedesmus obliquus*, *Chlorella pyrenoidosa* and *Closterium lunula* was inhibited at copper concentrations of 50 μg/l, 70 μg/l and 200 μg/l, respectively. Based on the current study's results, the *Chloroparva pannonica* Cu40 strain was selected and taken for the strain improvement study.

In the microalgae culturing system, small-scale laboratory experiments conducted in <100 ml flasks under optimum conditions have produced promising results; however, it was crucial that the experiment be conducted on a larger scale, such as in 3–5 L flasks with carbon dioxide supplied. In the study reported here, 3 L flasks, with 1% carbon dioxide supplied, were set up as shown in Figure 4.2A and Figure 4.3. It was considered helpful to investigate the effect of bubbling with and without a stone sparger in the *Chloroparva pannonica* Cu40 and WT cultures set up in 3 L flasks for a 15-day period. Results showed that there was a massive reduction in total biomass (g/l) when *Chloroparva pannonica* Cu40 and WT strains were cultivated in 3 L flasks bubbling with a stone sparger. Moreover, the pH value of the cultivated medium bubbling with a stone sparger decreased from 6 to 5 (Figure 4.7). As shown in the results from Chapter 5 subsection 5.3.4.1, *Chloroparva pannonica* Cu40 and WT showed no growth at a pH of 5. The reason for this effect may be that when a stone sparger was used, the removal rate of carbon dioxide in the cultivated medium by the *Chloroparva pannonica* Cu40 and WT photosynthetic uptake was lower than the rate at which carbon dioxide was being supplied. Therefore, the excess carbon dioxide reacted with the water, formed carbonic acid and thus decreasing the pH of the cultivated medium. Therefore, it might be prudent to cultivate *Chloroparva pannonica* without bubbling with a stone sparger in the 3 L flasks set up in the following study.

Chloroparva pannonica Cu40 was chosen for the strain improvement random mutagenesis study. However, in the microscope observation, it was shown that bacteria

had heavily contaminated the *Chloroparva pannonica* Cu40 culture. In addition, bacteria colonies were noticed on the agar plates. A few studies have shown that microalgae–bacteria interaction can consist of mutualism, commensalism and parasitism (Droop, 2007, Kazamia *et al.*, 2012). Microalgae and bacteria often exchange micronutrients (vitamins) and macronutrients (nitrogen, oxygen and carbon) (Teplitski and Rajamani, 2011). For example, bacteria are able to produce indole-3-acetic acid and vitamin B_{12} to enhance the growth of microalgae (Cho *et al.*, 2015, Croft *et al.*, 2005, Hernandez *et al.*, 2009, Kazamia *et al.*, 2012, Teplitski and Rajamani, 2011). In addition, bioflocculation by flocculation-promoting bacteria is one of the low-cost harvesting methods that could be an alternative to conventional harvesting (Wang *et al.*, 2012). However, the establishment of an axenic culture was important to build an understanding the physiology, genetics and ecology of microalgae

Bacteria can be eliminated or reduced from the microalgae culture by different methods such as centrifugation, cell washing, plate streaking and being treated with one or more antibiotics (Kan and Pan, 2010, Lee *et al.*, 2015, Wang *et al.*, 2016b). Kan *et al.* (2010) used the streak plate method on a combination of ampicillin, cefotaxime and carbendazim agar plates to eliminate bacteria and fungi from a *Chlamydomonas reinhardtii* culture. Based on Gram's stain procedure, the bacteria populations in the *Chloroparva pannonica* Cu40 culture were mainly Gram-positive rod shape, Gram-negative rod shape and Gram-negative spherical bacteria. The bacteria populations in *Chloroparva pannonica* Cu40 were first reduced by centrifugation, cell washing and plate streaking which was further confirmed based on their morphological characteristics under the light microscope. The difference in bacteria populations before and after the process was observed on the FU agar where there was a reduction in the brown, yellowish creamy and white creamy colonies on the microalgae agar culture (Figure 4.8).

As the main aim of purification of the *Chloroparva pannonica* Cu40 culture was to reduce the bacteria populations, one or more antibiotics were used at the last steps in eliminating or reducing the growth of contaminating bacteria from the culture. The 12 types of antibiotics demonstrated different characteristics on the bacteria populations in the *Chloroparva pannonica* Cu40 culture (Figure 4.9–Figure 4.14). Based on the results from the Kirby–Bauer antibiotic testing (Section 4.3.1.2), three antibiotic combinations (penicillin G and gentamicin; rifampicin and novobiocin; and vancomycin and

ampicillin) at a concentration of 0.1 mg/ml were selected to reduce the populations of bacteria. The effect of the antibiotics treatment on the growth of *Chloroparva pannonica* Cu40 culture was also investigated. The trend of growth in the antibiotic-treated cultures was lower than in the untreated cultures, with vancomycin and ampicillin completely inhibiting the growth of *Chloroparva pannonica* Cu40 (Figure 4.15). In addition, the cfu/ml values of the bacteria at the end of the growth phase (day 15) were reduced by up to 1574% (Figure 4.16). The results showed that the reduction of bacteria populations had a negative effect on the growth of *Chloroparva pannonica* Cu40.

Traditionally, it has been crucial to obtain axenic microalgae cultures for microalgae bio-fuel production. However, the role of the microalgae–bacteria interaction in promoting growth and increasing biomass production of microalgae has recently been recognized (Cho *et al.*, 2015, Croft *et al.*, 2005, Fuentes *et al.*, 2016, Kazamia *et al.*, 2012). The Vitamin B_{12} required in functionality of the methionine synthase enzyme in microalgae is often provided by bacteria communities (Croft *et al.*, 2005, Durham *et al.*, 2014). A study by Croft *et al.* (2005) has shown that among 326 microalgae species studied, 52.5% required external supplies of vitamin B_{12} for better growth. In addition, auxotrophic *Thalassiosira pseudonana* CCMP1335's growth was slower without any vitamin B_{12} supplemented, while growth increased when *Thalassiosira pseudonana* CCMP1335 was cultured together with *Ruegeria pomeroyi* DSS-3 or supplemented with vitamin B_{12}. This suggested that the vitamin B_{12} produced by *Ruegeria pomeroyi* DSS-3 had a positive effect on the growth of *Thalassiosira pseudonana* CCMP1335 (Durham *et al.*, 2014). Based on the results from Chapter 5 subsections 5.3.4.6, the absence of vitamin B_{12} in the cultivated medium had a negative effect on growth in *Chloroparva pannonica* Cu40. This suggested that vitamin B_{12} had been produced by bacteria populations in the untreated *Chloroparva pannonica* Cu40, thus supporting the growth in comparison to the antibiotic-treated *Chloroparva pannonica* Cu40. However, the strain improvement random mutagenesis study needed to obtain an axenic microalgae monoculture. Therefore, *Chloroparva pannonica* Cu40 culture, treated with penicillin G and gentamicin, was selected for the strain improvement random mutagenesis study.

Random mutagenesis has been proven to be one of the methods for producing high carotenoid- or lipid-yielding microalgae mutants (Bougaran *et al.*, 2012, Chaturvedi and

Fujita, 2006, Chaturvedi *et al.*, 2004, Cordero *et al.*, 2011b, Cortez *et al.*, 2015, Doan and Obbard, 2012, Ishikawa *et al.*, 2004b, Kamath *et al.*, 2008, Lim *et al.*, 2015, Lopez Alonso *et al.*, 1996, Meireles *et al.*, 2003, Tillich *et al.*, 2012). The mutagenic agent, UV light (UV-A 320-400 nm, UV-B 280-320 nm and UV-C (200-280 nm), is one of the simplest ways to generate a high possible number of mutants as it induces the formation of DNA lesions (Pfeifer *et al.*, 2005). A few studies have reported that mutants have been produced through the use of UV light. A mutant *Pavlova lutheri* showing a 23.9% and 32.8% higher content of DHA and EPA, respectively was isolated by Meireles *et al.* (2003). Bougaran *et al.* (2012) managed to isolate *Isochrysis* affinis *galbana* with 52% higher total fatty acid content compared to the wild type. In addition, M5 and M25 *Tetraselmis suecica* mutants were able to produce 114% and 123% more neutral lipids compared to the wild type (Lim *et al.*, 2015). Mutants generated from microalgae exposure to UV light alone to increase the carotenoid focus on lutein content have not been previously studied. However, a few reports have focused on the isolation of high-carotenoid-yielding microalgae mutants through using chemical mutagens. Using MNNG, Cordero *et al.* (2011b) enhanced the lutein production of *Chlorella sorokiniana* by optimizing the culture condition and random mutagenesis. The lutein content of mutant *Chlorella sorokiniana* MR-16 exhibited lutein production that was twofold higher (42 mg/l) than the wild type. Obtained through MNNG mutagenesis, the Y21 *Chlorella* mutant showed a higher cellular content (Ishikawa *et al.*, 2004). Moreover, one study by Kamath *et al.* (2008), using a combination of physical and chemical mutagens (UV, followed by EMS and NTG), managed to increase 2.5% to 3.8^ of astaxanthin instead of lutein content in *Haematococcus pluvialis*.

In the current strain improvement study, *Chloroparva pannonica* Cu40 cultures were exposed to a physical mutagen, namely, UV-C light, and were subsequently screened using herbicides to obtain high growth, high lipids and high lutein yielding mutants. Ultraviolet-C (UV-C) has the highest energy per photon in comparison to UV-A and UV-B. It is able to induce a higher frequency of DNA lesions such as *cis-syn* cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidine photoproducts [(6-4)PPs] (Pfeifer *et al.*, 2005). However, a few disadvantages in using UV light as the mutagenic agent include low accuracy and time consuming to screen and characterize a high number of mutants, and to select the mutants with desired traits. Consequently, long exposure to UV-C light to achieve high killing rates has always been aimed at increasing the probability of mutation (Carlton and Brown, 1981.). For a

random mutagenesis to be successful, the highest possible killing rate at 98% is the aim to increase the mutation probability (Figure 4.17). In this study, a 98% killing rate was achieved in all four rounds of UV-C exposure, and the major goal was also to isolate the highest possible number of putative mutants. Therefore, 597 mutant strains were isolated based on the greenest colour and the largest size of colony. Subsequently, 366 mutant strains were selected based on their growth characteristics for the next part of the study.

Herbicides are able to act as enzyme inhibitors to disrupt the basic metabolic processes in plants. For example, a study conducted by Aflalo *et al.* (1999) reported that the herbicide, glufosinate, was able to inhibit the activity of glutamine synthetase, which was involved in the biosynthesis of astaxanthin in *Haematococcus pluvialis*. In the study reported here, mutants were screened using two types of herbicides, namely, aclonifen and chlodinafop-propargyl). This method has brought tremendous advances in distinguishing and selecting the mutants with desired traits. Aclonifen is a type of herbicide that inhibits protoporphyrinogen oxidase involved in the chlorophyll and carotenoid biosynthesis pathway (Figure 4.34). Chlodinafop-propargyl is a type of herbicide that inhibits the enzyme acetyl CoA carboxylase that interferes with the lipid biosynthesis (Medd *et al.*, 2001). The selection pressure of using either aclonifen or chlodinafop-propargyl was successful in generating resistant mutants. The mutants were more sensitive to chlodinafop-propargyl than to aclonifen. Out of 366 mutant strains, the number of mutant strains that were resistant to aclonifen and chlodinafop-propargyl was 117 and 76, respectively. However, 42 mutants were bleached and died during this part of the study.

To evaluate the growth and lutein content in 40 ml of herbicide-free FU medium, 50 mutant strains, 58 aclonifen-mutant strains and 42 chlodinafop-propargyl mutant strains were selected. At the end of the third stage of the study, 15 mutant strains, 16 aclonifen-resistant mutant strains and eight chlodinafop-propargyl resistant mutant strains with rapid growth (based on the OD reading) and high lutein content (mg/l) were selected to grow in 3 L flasks set up, with 1% carbon dioxide supplied to promote growth, lutein and lipid production (Table 4.4). The selected mutants were maintained for 20 generations to ensure they were stable before growing them in the 3 L flask set-up. The goal was to determine the total biomass (g/l) and lutein content (mg/kg of biomass) along the growth phase for a 15-day period (at day 3, 6, 9, 12 and 15), and the

total lipids percentage at the end of the growth phase. The pH levels in each culture were observed to ensure the cultures were cultivated at the optimum pH level of 7–8.

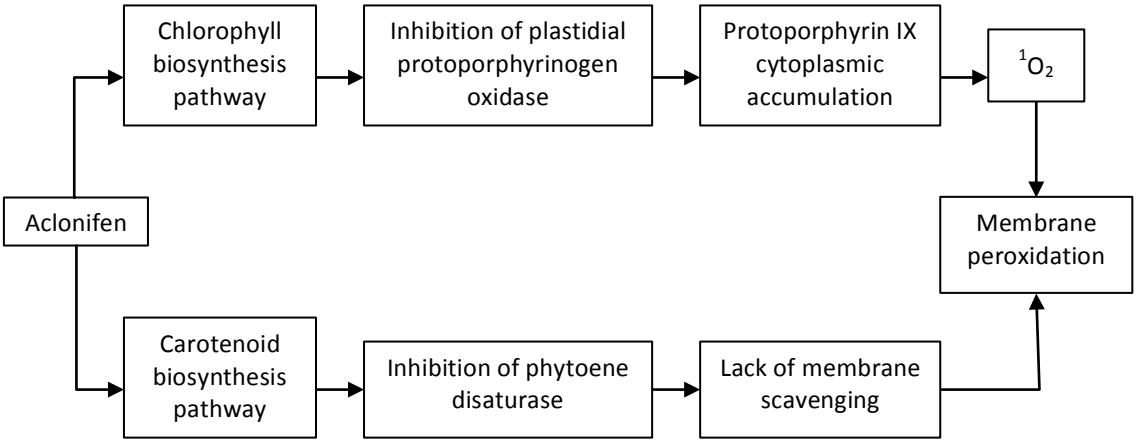


Figure 4.34 Biochemical effects of aclonifen on plants under light

$^1O_2$: Quenching of singlet molecular oxygen
Source: Klunc (2015)

The 10 mutants presented in Table 4.5 have been selected for the next chapter of the current study. The mutants were analysed, not only based on high lutein content, but also according to high biomass, high growth rate and high lipids content. The total lipids of the 10 selected mutants were only slightly higher (>1%) than the *Chloroparva pannonica* Cu40 PC and WT. However, the mutant #4 showed the highest growth with total biomass of 1.592 g/l which was 80% higher than that of Cu40 PC, while it was 171% higher than that of *Chloroparva pannonica* WT. The same strain (#4) had the highest specific growth rate (0.199) which was 72% higher than Cu40 PC and 136% higher than WT, while the lutein content was 4386 mg/kg of biomass, which was similar to that of Cu40 PC but was 127% higher than *Chloroparva pannonica* WT. In addition, another mutant, namely, #1.8µmC.5 had the highest lutein content (5502.05 mg/kg of biomass) which was 26.7% higher than Cu40 PC and 184.64% higher than *Chloroparva pannonica* WT. Furthermore, the 10 selected mutants achieved higher total dried biomass and a higher specific growth rate with respect to Cu40 PC and WT. Interestingly, the data reported here also point to lutein being produced and reaching the highest level at the earlier stage of the growth (either day 6 or day 9) while the total biomass and lipids content reached the highest level at the end of the growth phase (day 15). The higher lutein content produced at the earlier stage of the growth phase could be due to the *Chloroparva pannonica* focus on the synthesis of lutein to support its growth.

Lutein is a type of primary carotenoid which could be one of the key factors in the performance of photosynthesis. Lutein is required for the structure and function of the light-harvesting complex, and also protects microalgae from photo-oxidative damage (Alves-Rodrigues and Shao, 2004, Siefermann-Harms, 1985).

4.5 Conclusion

The *Chloroparva pannonica* Cu40 selected from the copper stress study was chosen for the strain improvement study. The *Chloroparva pannonica* Cu40 strain was heavily contaminated with different types of bacteria; therefore, the culture was purified by centrifugation, cell washing, plate streaking and treatment with different combinations of antibiotics before being subjected to the strain improvement program. After four rounds of UV-C exposure with a 98% killing rate achieved, 597 mutant strains were isolated based on the greenest colour and largest size of colony. The use of herbicide selection, based on *in vivo* sensitivity of the Cu40 PC and WT, resulted in the production of resistant mutants. After variations of the mutants were set up in 3 L flasks, with 1% carbon dioxide supplied, 10 mutants were selected for the next chapter of the study, not only based on the high lutein content, but also according to the high biomass achieved and specific growth rate.

Chapter 5 Polyphasic Taxonomy Study of *Chloroparva pannonica*

5.1 Introduction

Taxonomic classification is a method used to evaluate the diversity of all biological groups. Since the introduction of classification by Linnaeus, taxonomy classifications have changed from grouping organisms with similar morphology to a hierarchical system that represents evolutionary relationships. Green algal evolution shows that early divergence occurred into two discrete lineages: Chlorophyta and Streptophyta (Bremer, 1985). Streptophyta are comprised of charophytes which include fresh water and land plants. Chlorophyta include the majority of green algae from marine, fresh water and terrestrial environments.

Green microalgae are the phylogenetically diverse unicellular photosynthetic eukaryotes that have been playing an important role in the ecosystem for millions of years. They are often considered as the primary source of energy in the marine and fresh water food chain (Gaulke *et al.*, 2010, Stockner and Antia, 1986). Most of the green microalgae can be found in a variety of habitats ranging from the Arctic and Antarctica to oceans and lakes. These unicellular photosynthetic eukaryotes are characterized by the presence of chloroplasts enclosed by a double membrane, thylakoid groups in lamellae, chlorophyll *a* and *b*, and carotenoids.

Marine phytoplankton are one of the green microalgae that contribute up to 80% of biomass primary production in coastal ecosystems (Worden *et al.*, 2004). They were first categorized, based on their size, by Sicko-Goad and Stoermer (1984), and have been classified into six categories. These comprise net plankton (size range > 64 μm); microplankton (size range 20–500 μm); nanoplankton (2–64 μm); ultraplankton (0.5–5 μm); picoplankton (0.2–2 μm); and femtoplankton (0.02–0.2 μm) (Callieri, 2008). The category of photoautotrophic picoplankton was first introduced by Sieburth et al, (1978). As mentioned above, the size of picoplankton ranges from 0.2–2.0 μm.

Photoautotrophic picoplankton consist of prokaryotic picocyanobacteria and eukaryotic phototrophs (Callieri, 2008). Most picoplankton are free-living organisms found in a variety of habitats including soil, fresh water, salt water and shallow lakes (Gaulke *et*

al., 2010, Gaysina *et al.*, 2013, Somogyi *et al.*, 2011). In some studies, picoplankton have been referred to as 'green balls', 'Chlorophyta isolates', '*Chlorella*-like cells' or '*Nannochloris*-like algae' (Henley *et al.*, 2004, Krienitz *et al.*, 2004, Stockner, 1991).

The identification of picoplankton is formally based on biochemical, morphological and physiological traits (DaSilva and Gyllenberg, 1972, Huss *et al.*, 1999, Kessler and Huss, 1990, Krienitz *et al.*, 2004). Morphological classification allows picoplankton to be easily distinguished at class level but it is difficult to distinguish them at the genus and species levels. The reason is their size and limited morphological traits. The introduction of genetic characterization based on the 18S rRNA gene has shown that ambiguities exist in the traditional classification based on morphological characteristics and that these small cells evolved by convergent evolution (Callieri, 2008, Fawley and Fawley, 2007, Fawley and Fawley, 2004, Huss *et al.*, 1999, Krienitz *et al.*, 2004, Krienitz *et al.*, 1999, Lewin *et al.*, 2000).

However, polyphasic taxonomy analysis incorporates phenotypic and genotypic studies that are able to determine the characteristics of the species. The phenotypic study includes the comparative studies of morphological, physiological, biochemical and chemotaxonomic characteristics of the species. Huss et al.(1999) and Kessler and Huss (1990) proposed 10 ecophysiological and biochemical characteristics comprising: hydrogenase; secondary carotenoids; pH limitations; NaCl limitations; temperature limitations; nitrate reduction; thiamine requirements; Vitamin B_{12} requirements; growth on organic substances. The genotypic studies include the composition of the nucleic guanine+cytosine (G+C) content, and DNA–DNA relatedness and its taxonomic position based on either the 16S rRNA or 18S rRNA gene sequence. Several studies have demonstrated that, by using polyphasic taxonomy analysis, simple microalgae such as *Marinichlorella kaistiae*, 19 *Chlorella* spp. and *Parachlorella*, can be distinguished at the species level (Aslam *et al.*, 2007, Huss *et al.*, 1999, Krienitz *et al.*, 2004).

Somogyi *et al.* (2011) were the first to describe *Chloroparva pannonica* when it was isolated from a turbid shallow soda pan in Böddi-szék pan, Hungary by light microscopy, electron microscopy, fatty acid analysis, pigment analysis and 18S rRNA sequence analysis. Their study proved that *Chloroparva pannonica* was a new species, belonging to a new genus within Trebouxiophyceae. However, the study did not include

the ecophysiological and biochemical composition of the DNA G+C content and DNA–DNA relatedness analysis.

The aim of the current study is to use polyphasic taxonomic characterization, which is the combination of ecophysiological and biochemical ultrastructures, together with molecular data to understand the taxonomic position of our strain that has been identified on the basis of its 18S rRNA gene sequence as *Chloroparva pannonica*. In addition to *Chloroparva pannonica* WT, polyphasic taxonomic characterizations were carried out on the PC of copper-adapted *Chloroparva pannonica* strain Cu40 and three *Chloroparva pannonica* mutant strains selected not only on the basis of high lutein content and lutein productivity, but also based on high total biomass and biomass productivity (Fu4, Fu5C and Fu41A). The 3 mutants were included to ensure that they belong to the same species and were not contaminants.

5.2 Methods and materials

5.2.1 Source

The microalga used in this study was obtained from the South Australian Research and Development Institute (SARDI) from a salt pan at Waikerie, South Australia, by SARDI's Aquatic Sciences Algal Production group, South Australia.

5.2.2 Strain and culture condition

Five microalgae strains were selected: one wild strain (WT), one copper-adapted strain (Cu40 PC) and three mutant strains (Fu4, Fu5C and Fu41A). The culture information is summarized in Table 5.1.

Table 5.1 Information on *Chloroparva pannonica* isolates

Isolates	Culture history
WT	Wild type culture
Cu40 PC	Copper-adapted strain from WT, parent culture (PC) of mutants
Fu4	Cu40 PC mutant from random mutagenesis using UV-A
Fu5C	Cu40 PC mutant from random mutagenesis using UV-A and selected using herbicide inhibitor, chlodinafop-propargyl
Fu41A	Cu40 PC mutant from random mutagenesis using UV-A and selected using herbicide inhibitor, aclonifen

The WT strain was cultured in Guillard's F2 medium (ingredients are listed in Chapter 2) while Cu40 PC, Fu4, Fu5C and Fu41A were cultured in the FU medium designed as described in Chapter 3 (ingredients are listed in Chapter 4, Appendix 4.1). Agar plates were prepared with 1.5% agar in the FU medium with seawater: deionized water (1:1 v/v). Both liquid and agar media were sterilized at 121°C at 100 kPa for 15 minutes. All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 40 ml culture medium. Both liquid and agar cultures were maintained in a plant cell culture room at 25±1°C, at a pH of 7.5, with a light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod.

5.2.3 Morphological studies

Microalgal cells were cultivated, as described above, until day 10 for morphological studies.

5.2.3.1 Light microscopy observations

The microalgae morphology of *Chloroparva pannonica* WT and Cu40 PC were observed with a bright-field microscope (BX-50, Olympus, Tokyo, Japan) at Flinders Microscopy, Flinders University, South Australia.

5.2.3.2 Fluorescence microscopy observations

Chloroparva pannonica WT and Cu40 PC were selected for observation using fluorescence microscopy observation. Nile red staining and fluorescence microscopy observation were performed using the method of Chen *et al.* (2009). A Nile red dye at a concentration of 0.25 mg/L was made by dissolving 0.01 g of Nile red dye (9-diethylamino-5H-benzo [α] phenoxa-phenoxazine-5-one) (Sigma-Aldrich Pty. Ltd., Australia) in 40 ml of acetone (Sigma-Aldrich Pty. Ltd., Australia).

The cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. They were then washed three times and resuspended with sterile distilled seawater: deionized water (1:1 v/v). A 0.04 mL amount of Nile red stock solution was added to 4 mL of microalgae culture and incubated in the dark for 15 minutes. The microalgae were observed with a fluorescence microscope (BX50, Olympus, Tokyo, Japan) at Flinders

Microscopy, Flinders University, at excitation and emission wavelengths of 530 nm and 580 nm. Unstained cells and Nile red dye were used as auto fluorescence controls.

5.2.3.3 Scanning electron microscopy (SEM) observations

Chloroparva pannonica WT and Cu40 PC cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. The cells were then washed three times with sterile distilled seawater: deionized water (1:1 v/v). The cells were fixed for at least 30 minutes in a fixative solution containing 4% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffered saline (PBS) with 4% sucrose, at a pH of 7.2. They were then washed in PBS with 4% sucrose for five minutes, followed by being post-fixed in 2% osmium tetroxide (OsO_4) in PBS for one hour. The cells were dehydrated by a series of washes in 70%, 90% and 100% ethanol with two changes every two minutes for each ethanol concentration. The cell membranes were subjected to critical point drying using a Leica EM CPD300 automated critical point dryer, followed by being mounted on a stub and coated with platinum. The cell structures were observed under a Philips XL30 scanning electron microscope at Adelaide Microscopy, University of Adelaide, South Australia.

5.2.3.4 Transmission electron microscopy (TEM) observations

Chloroparva pannonica WT and Cu40 PC cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile distilled seawater: deionized water (1:1 v/v). The pellet was fixed in fixative solution containing 2.5% glutaraldehyde in 0.1 M PBS for 30 minutes. It was washed in 0.1 M PBS for 10 minutes, three times, followed by being post-fixed in 1% OsO_4 in PBS for one hour. It was dehydrated by a series of washes in 50%, 75%, 85% and 95% ethanol for two changes for each ethanol concentration every five minutes, and 100% ethanol with two changes every 10 minutes. A 1 ml amount of 100% resin was added to each sample, with the sample then embedded in a flat mould for two days. The samples were cut by a diamond knife and examined under a Philips CM200 TEM/STEM microscope at Adelaide Microscopy, University of Adelaide, South Australia.

5.2.4 Lipid profile

The microalgal cells were cultivated until day 14 for the lipid analysis, when they were harvested, followed by centrifugation at 10,000 g for 25 minutes and dried at 60°C overnight. Lipids were extracted based on the method described in Section 2.2.4, 'Total lipid analysis'.

The fatty acid profile was analysed by FOOD plus Research Centre, Waite Lipid Analysis Service, School of Agriculture, Food and Wine, University of Adelaide, South Australia, using an Agilent 5975C mass spectrometer equipped with an Agilent 7890A gas chromatograph and an Agilent 7683B automatic liquid sampler (Agilent Technologies, Santa Clara, CA, USA). An SGE Analytical Science GC BP21 capillary column (15 m x 0.25 mm with a 0.25 μm film thickness) was used.

5.2.5 Pigment analysis

The microalgae cells were cultivated until day 8 for pigment analysis. Pigment was extracted based on the method described in subsection 2.1.1.3.2. The pigment extracts were analysed by high-performance liquid chromatography (HPLC), based on the method described in subsections 2.1.1.5 and 2.1.1.6, by the NCRIS Algae and Biofuels Facility, South Australia.

5.2.6 Ecophysiological and biochemical testing

Guillard's F2 and the FU media were used as the basic media for all ecophysiological and biochemical testing in this study. The WT strain was cultured in Guillard's F2 medium while Cu40 PC, Fu4, Fu5C and Fu41A were cultured in the FU medium. Agar plates were prepared with 1.5% agar with seawater: deionized water (1:1 v/v) and sterilized at 121°C at 100 kPa for 15 minutes. Testing for sodium chloride tolerance, growth at various temperatures, nitrogen as the sole nitrogen source, and thiamine and Vitamin B12 requirements was performed in a liquid medium and a 1.5% solid medium while testing for pH tolerance was performed only on the 1.5% solid medium. *Chloroparva pannonica* cultures with equal numbers of cells (OD of 0.7 nm at 685 nm) were used as the inoculum. They were grown for a 14-day period in the usual manner.

Furthermore, the isolates were tested for microalgae chemo-organotrophic growth. Each test was carried out in triplicate for each strain and was repeated three times.

5.2.6.1 pH tolerance

The growth of *Chloroparva pannonica* at a range of pH levels was tested on 1.5% agar adjusted to levels of 1.5, 2, 2.5, 3, 5, 6, 7, 8, 8.5, 9, 10, 11 and 12 after autoclaving by using 1 M hydrogen chloride (HCl) or 1 M sodium hydroxide (NaOH). Inoculation was performed by dropping 50 µL of each culture onto each agar plate. Five equal streaks were made from the drop followed by another five streaks made from the previous five lines. The plates were incubated in the usual manner. Results were recorded as positive (+) or negative (-) based on the presence or absence of growth on days 14.

5.2.6.2 Sodium chloride tolerance

The *Chloroparva pannonica* isolates were tested for their ability to grow in different salt concentrations in a liquid medium and on a 1.5% agar medium. Sodium chloride was added to the media to obtain the following concentrations: 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% and 11% (w/v). All liquid cultures were grown in 100 ml Erlenmeyer flasks containing 4 ml of inoculum and 40 ml medium. The tests on the agar plates were performed based on the method described in the previous subsection (subsection 5.2.6.1). Results on the agar plates were recorded as positive (+) or negative (-) based on the presence or absence of growth. Cell growth in the liquid culture was analysed using a µQuant™ UV/Vis spectrophotometer 96-well microplate reader (Crown Scientific Pty Ltd, Australia) at 685 nm at day 7 and day 14, based on the method described in subsection 2.2.2.2.

5.2.6.3 Growth at various temperatures

The *Chloroparva pannonica* isolates were tested for their ability to grow at different temperatures in a liquid medium and on a 1.5% agar medium. The tests on the agar plates were performed based on the method described in subsection 5.2.6.1. . The liquid medium was inoculated as described in subsection 5.2.6.2. Microalgae were grown at 4°C, 10°C, 20°C, 25°C, 30°C, 35°C and 40°C. The results were recorded, as described in subsections 5.2.6.1 and 5.2.6.2, at day 7 and day 14.

5.2.6.4 Nitrogen requirement

For the growth tests on nitrate as the sole nitrogen source, *Chloroparva pannonica* cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile seawater: deionized water (1:1 v/v). Sodium nitrate was added to achieve the following concentrations: 0 mM, 3 mM and 0.88 nM (control). The tests on the agar plates were performed based on the method described in subsection 5.2.6.1. The liquid medium was inoculated as described in subsection 5.2.6.2. The results were recorded, as described in subsections 5.2.6.1 and 5.2.6.2, at day 7 and 14.

5.2.6.5 Thiamine requirement

For the growth tests on thiamine requirement, *Chloroparva pannonica* cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile seawater: deionized water (1:1 v/v). Thiamine was added to the liquid medium and 1.5% agar medium to achieve the following concentrations: 0 µg/L, 20 µg/L and 0.1 mg/L (control). The tests on the agar plates were performed based on the method described in subsection 5.2.6.1. The liquid medium was inoculated as described in subsection 5.2.6.2. The results were recorded, as described in subsections 5.2.6.1 and 5.2.6.2, at day 7 and day 14.

5.2.6.6. Vitamin B_{12} requirement

For the growth tests on Vitamin B_{12} requirement, *Chloroparva pannonica* cells were harvested by being centrifuged at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile seawater: deionized water (1:1 v/v). Vitamin B_{12} was added to the liquid medium and 1.5% agar medium to achieve the following concentrations: 0 µg/L, 5 µg/L and 0.1 mg/L (control). The tests on the agar plates were performed based on the method described in subsection 5.2.6.1. The liquid medium was inoculated as described in subsection 5.2.6.2. The results were recorded as described in subsections 5.2.6.1 and 5.2.6.2, at day 7 and day 14.

5.2.7 Chemo-organotrophic growth

The *Chloroparva pannonica* isolates were tested for their ability to grow in the dark when supplied with the following organic substrates: glucose, galactose, mannose, fructose, maltose, sucrose, xylose, ribose and mannitol. The final concentrations of each organic substrate were 1% (w/v). *Chloroparva pannonica* cultures with an equal number of cells (OD of 0.7 nm at 685 nm) were used as the inoculum. The WT strain was cultured in Guillard's F2 medium while Cu40 PC, Fu4, Fu5C and Fu41A were cultured in the FU medium. *Chloroparva pannonica* cells were harvested by being centrifugation at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile distilled seawater: deionized water (1:1 v/v). The tests on the agar plates were performed based on the method described in subsection 5.2.6.1. The liquid medium was inoculated as described in subsection 5.2.6.2. The results were recorded as described in subsections 5.2.6.1 and 5.2.6.2 at day 7 and day 14.

5.2.8 Identification using 18S rRNA gene sequencing analysis

5.2.8.1 Total genomic DNA extraction

The total genomic DNA extraction of the *Chloroparva pannonica* was modified and prepared according to the method from (Aslam *et al.*, 2007). The microalgae cultures were cultured in 200 ml of the FU medium (Guillard and Ryther, 1962) in 500 ml Erlenmeyer flasks in the usual manner for a 14 day-period.

A 200 ml amount of microalgae cells was harvested by centrifugation at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile distilled seawater: deionized water (1:1 v/v). The pellet was then mixed with 978 µL of sodium phosphate buffer (pH 7.5) and 0.5 g of glass beads (diameter 0.18 mm) using a Beadbeater homogenizer (Biospec Products, Bartlesville, OK, USA). A 10 µL amount of Proteinase K (1% w/v) and 70 µL of 10% sodium dodecyl sulphate (SDS) (w/v) were added and incubated at 55°C for 60 minutes. After that, 20 µL of RNase A (10 mg/ml) and 20 µL RNase T1 (2000 U/mL) were added and incubated at 37°C for 60 minutes. The mixture then received the addition of 150 µL of 5 M sodium chloride (NaCl) and 110 µL of hexadecyltrimethyl ammonium bromide (CTAB) and was incubated at 65°C for 15 minutes. The cells were precipitated, by adding an equal volume of phenol-chloroform-

isoamyl alcohol (25:24:1), and incubated at room temperature for 30 minutes with intermittent shaking every 10 minutes, followed by centrifugation at 10,000 rpm for 15 minutes. The supernatant was transferred into a fresh sterile 1.5 ml microcentrifuge tube. An equal volume of chloroform-isoamyl alcohol (24:1) was added and incubated at room temperature for 15 minutes and mixed by inversion at intervals, followed by centrifugation at 10,000 rpm for 15 minutes. The supernatant was transferred into a fresh sterile 1.5 ml microcentrifuge tube and 0.7 ml of isopropanol was added and left overnight at a temperature of -20°C. The mixture was then centrifuged at 16,000 rpm for 15 minutes: the supernatant was discarded and the pellet was washed twice with 70% ethanol. The pellet was then dried by placing the tube in a 55°C heating block for 10 minutes or until it was dried. The pellet was resuspended in 50 µL of sterile nuclease-free water and stored at 20°C for further use.

The presence of genomic DNA was confirmed by running the extracted DNA on a 1% DNA grade agarose (Quantum Scientific, Queensland, Australia) stained with GelRed™ (Biotium Inc, Fremont, CA, USA) for visualization. The size standard used was a 1 kb DNA Ladder Molecular Weight Marker (Promega™, Wis, USA). The concentration of genomic DNA was quantified by using a NanoDrop 2000C UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

5.2.8.2 Polymerase chain reaction (PCR) amplification and sequencing of 18S rRNA gene

The coding region of 18S rRNA genes was amplified by polymerase chain reaction (PCR) using primer sets of NS1-X (CCAGTAGTCATATGCTTGTC) and 18L-X (ACCTTGTTACGACTTCTCC) (Phillips and Fawley, 2000) (Fawley *et al.*, 2000). Each PCR mixture contained 2 µL of DNA samples; 5 µL of ThermoPol® reaction buffer; 1 mMol of each primer; 200 mM of each deoxynucleotide (dNTP) (dATP, dGTP, dCTP and dTTP); and 5 U of Taq DNA polymerase (New England BioLabs, Mass, USA) with injection water used to make up the final volume of 50 µL. The PCR program consisted of an initial denaturation step at 94°C for two minutes, followed by 35 cycles of denaturation steps at 94°C for one minute, annealing at 58°C for one minute, and elongation at 72°C for two minutes, with a final elongation step at 72°C for 10 minutes.

The PCR product was purified using UltraClean™ PCR Clean-Up DNA Purification Kit (MO BIO Laboratories, a Qiagen Company, Carlsbad, CA, USA). The presence of

purified PCR product was confirmed by running the extracted DNA on a 1% DNA grade agarose (Quantum Scientific, Queensland, Australia) stained with GelRed™ (Biotium Inc, Fremont, CA, USA) for visualization. The size standard used was a 1 kb DNA Ladder Molecular Weight Marker (Promega^{TM}, Wis, USA). The concentration of PCR product was quantified by using a NanoDrop 2000C UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Using the primers listed in Table 5.2 and according to the method described by Somogyi (2011) and Yamamoto *et al.* (2003), DNA sequencing was performed as part of a service provided by Macrogen (South Korea). The chromatography was checked using Chromas LITE program version 2.01 (Technelysium Pty Ltd, Queensland, Australia).

Table 5.2 List of oligonucleotide primers for sequencing

Primer name	Sequence	Position
5' PCR 1-F	5'-ACCTGGTTGATCCTGCCAGT-3'	2-21
3' PCR 1-R	5'-CGTAGGCYTGCTTTGAACAC-3'	786-767
5' PCR 2-F	5'-CMATTGGAGGGCAAGTCTGG-3'	544-563
3' PCR 2-R	5'-TAAGAACGGCCATGCACCAC-3'	1289-1270
5' PCR 3-F	5'-AAGTTRGGGGMTCGAAGACG-3'	980-999
3' PCR 3-R	5'-CCTTCYGCAGGTTCACCTAC-3'	1793-1774

Source: Somogyi *et al.* (2011) and Yamamoto *et al.* (2003)

5.2.8.3 Identification of 18S rRNA gene sequence

Chromatograms were corrected manually with Chromas 2.6 software (Technelysium Pty Ltd, Queensland, Australia). The 18S rRNA gene sequence of the 5 strains of *Chloroparva pannonica* isolates was identified by comparison against the nucleotide database collection in the NCBI (National Center for Biotechnology Information) using the BLAST (Basic Local Alignment Search Tool) program. The level of sequence similarity with the matched sequences was also calculated. Alignment was generated with ClustalW software and corrected manually using Molecular Evolutionary Genetics Analysis (MEGA) 6 software.

5.2.9 Genomic studies

5.2.9.1 Analysis for determination of guanine+cytosine (G+C) mol%

The guanine+cytosine (G+C) mol % of the *Chloroparva pannonica* strains was determined using the method described by Mesbah *et al.* (1989)

5.2.9.1.1 Total genomic DNA extraction

The total genomic DNA extraction was conducted following the method described in Section 5.2.8.1.

5.2.9.1.2 Denaturation of DNA

The total genomic extraction was then denatured by dissolving 2–25 µg of genomic DNA in 25 µl 0.01 X SSC (1 X SSC is 0.15 M NaCl plus 15 mM trisodium citrate) and then heated in a boiling water bath for two minutes and rapidly cooled in an ice water bath. Then, 50 µl of 30 mM sodium acetate buffer (pH 5.3), 5 µl of 20 mM of zinc sulphate ($ZnSO_4$) and 3 µl of P1 nuclease (1 mg/ml in sodium acerate buffer; 340 U/ml) were added and incubated at 37°C for two hours. After incubation, 5 µl of 0.1 M glycine HCl buffer (pH 10.4) and 5 µl of alkaline phosphatase (200 U/ml in glycine buffer) were added. The pH of the solution ranged between 7.5 and 8.5. The mixture was then incubated at 37°C for six hours followed by centrifugation at 10,000 g for four minutes. Samples were stored at -20°C for high performance liquid chromatography (HPLC).

5.2.9.1.3 Guanine+cytosine (G+C) mol% chromatography conditions

As mentioned above, HPLC was used to determine the guanine+cytosine (G+C) content of the *Chloroparva pannonica* cultures. Each sample contained 0.5–1.5 µg of nucleosides in 5–25 µl. The solvent system contained 12% methanol and 20 mM triethylamine phosphate (pH 5.1) (Merck, Germany) by combining 40 ml of 0.5 M triethylamine phosphate (pH 5.1), 750 ml of glass-distilled water and 120 ml of HPLC-grade methanol, with the volume being 1 L after adjustment. The 0.5 M of triethylamine phosphate was prepared by diluting triethylamine (99%) with water and adjusting the pH to 5.1 with 85% phosphoric acid, which was then brought up to its final volume. The solvent was then filtered through a 0.45 µm cellulose triacetate membrane (HVWP, Merck Millipore).

Analytical separations were performed by HPLC (Agilent 1100 series, Agilent Technologies, USA) using a reversed-phase (250 mm x 4 mm [5 μm]) C18 column (Merck Millipore). The column temperature was set at 37°C. The flow rate was fixed at 1 ml/minute. The detection wavelength was 254 nm. Calf thymus DNA (D4764, Sigma-Aldrich) and *Escherichia coli* DNA (IRMM449, Sigma-Aldrich) were used as references.

5.3. Results

5.3.1 Morphology characteristics

5.3.1.1 Morphology observations

Chloroparva pannonica strain WT, Cu40 PC, Fu4, Fu5C and Fu41A grew well and were green in the liquid FU medium (Figure 5.1).

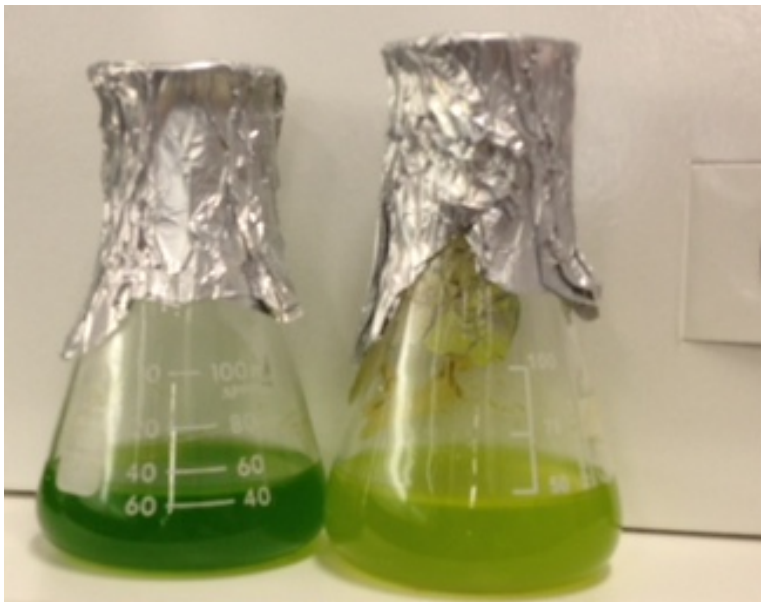


Figure 5.1 *Chloroparva pannonica* Cu40 PC (left) and WT (right) grown in liquid medium

Chloroparva pannonica WT, Cu40 PC, Fu4, Fu5C and Fu41A grew well on the 1.5% agar (Guillard's F2 medium). Figure 5.2 shows the WT strain while Figure 5.3 shows Cu40 PC colonies on 1.5% agar (Guillard's F2 medium). The colonies from all of the *Chloroparva pannonica* isolates were small, with the size of the colonies being 1–1.2 x 1–1.2 mm after a 14-day incubation period. Each colony was green and round with a smooth surface, while the edge of the colony was smooth.

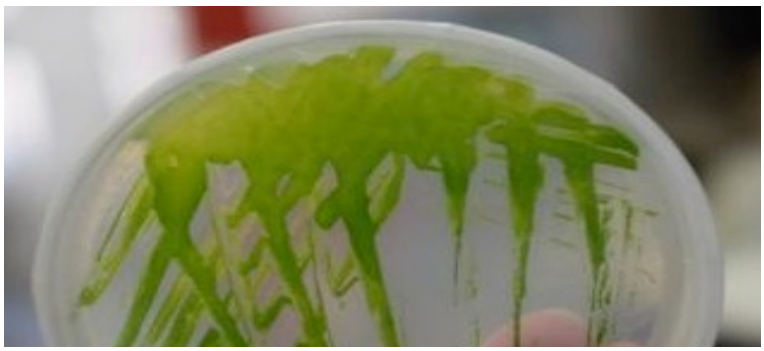


Figure 5.2 *Chloroparva pannonica* WT strain grown on 1.5% Guillard's F2 agar at day 14 at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod

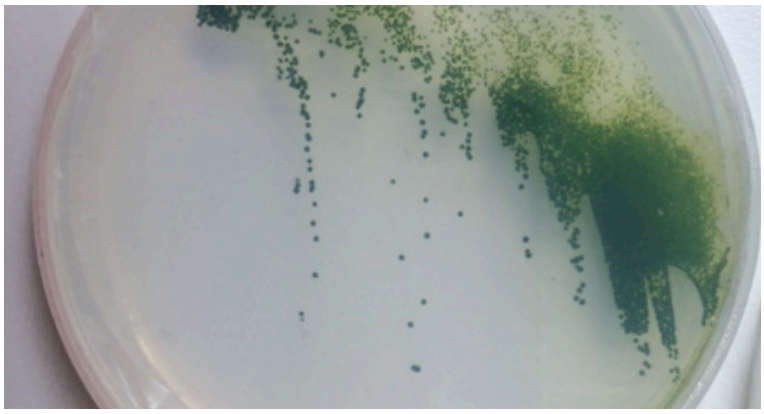


Figure 5.3 *Chloroparva pannonica* Cu40 PC strain grown on 1.5% FU medium agar at day 14 at 25±1°C, with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12:12-h light: dark photoperiod

5.3.1.2 Light microscopy observations

Light microscopy micrographs of *Chloroparva pannonica* WT and Cu40 PC cells are shown in Figure 5.4 and Figure 5.5. Little difference was observed in the morphology and cell size between WT and Cu40 PC cells. *Chloroparva pannonica* WT and Cu40 PC consisted of a single spherical cell. The cell size ranged from 2–5 µm in width and from 2–5 µm in length. Colourless oil drops may occur in the cytoplasm. Flagella were absent for both WT and Cu40 PC cells.

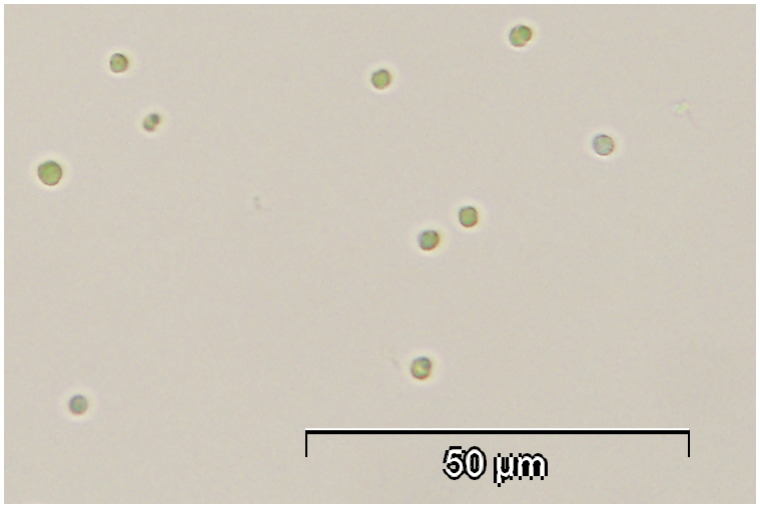


Figure 5.4 Light microscopy micrographs of *Chloroparva pannonica* WT under 100x magnification

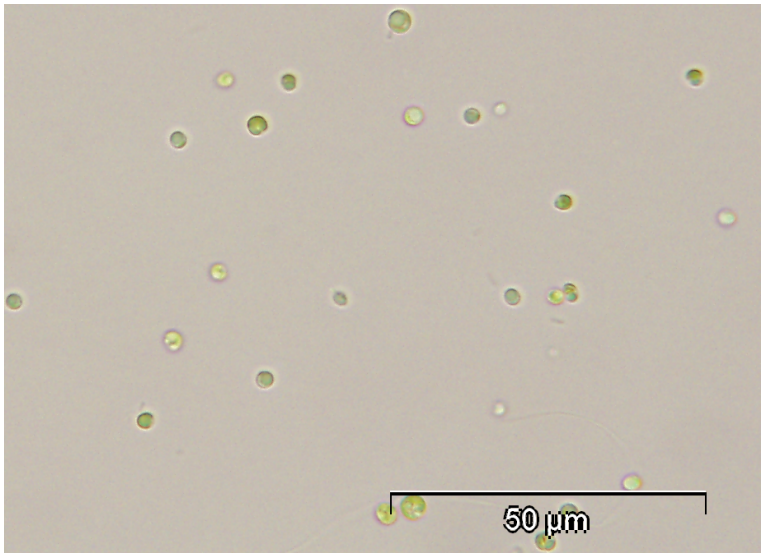


Figure 5.5 Light microscopy micrographs of *Chloroparva pannonica* Cu40 PC under 100x magnification

5.3.1.3 Fluorescence characteristics of *Chloroparva pannonica* stained with Nile red fluorescent dye

Fluorescence microscopy micrographs of *Chloroparva pannonica* WT and Cu40 PC cells are shown in Figure 5.6 and Figure 5.7. The cell size ranged from 2–4 µm in width and from 2–5 µm in length. When *Chloroparva pannonica* WT and Cu40 PC stained with Nile red fluorescent dye, the lipid droplets inside the *Chloroparva pannonica* WT and Cu40 PC cytoplasms could be seen as a yellow fluorescence under the fluorescence microscope. Due to the presence of lipid droplets inside the cytoplasms, the cell sizes appeared to be larger.

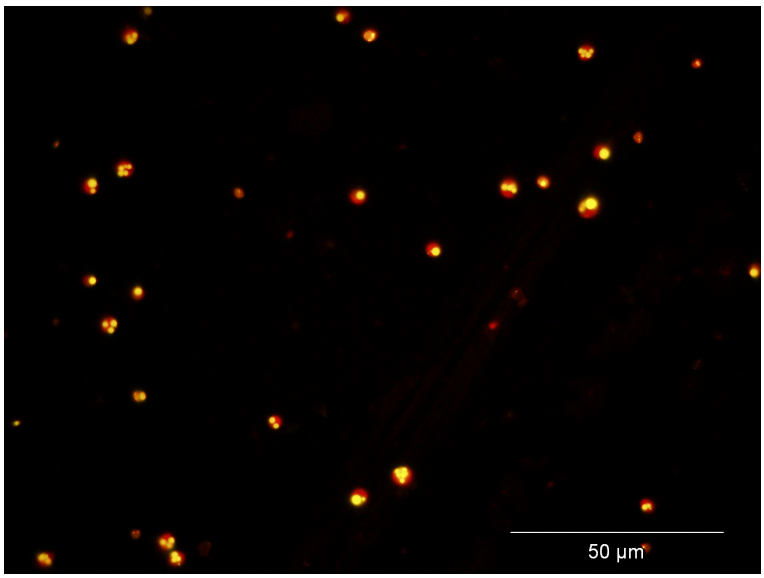


Figure 5.6 *Chloroparva pannonica* WT stained with Nile red fluorescent dye viewed under fluorescence microscope at 100x magnification

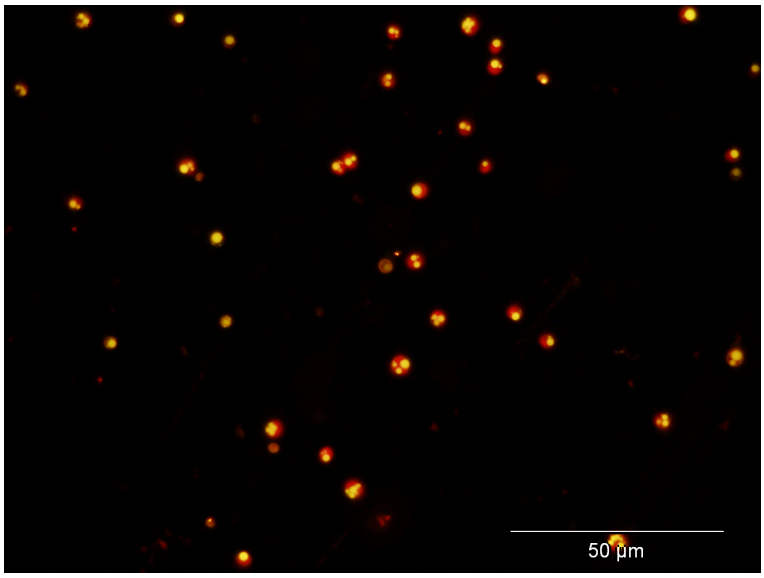


Figure 5.7 *Chloroparva pannonica* Cu40 PC stained with Nile red fluorescent dye viewed under fluorescence microscope at 100x magnification

5.3.1.4 Electron microscopy observations

5.3.1.4.1 Scanning electron microscopy (SEM) observations

The scanning electron microscopy (SEM) micrographs of *Chloroparva pannonica* WT are shown in Figure 5.8 and Figure 5.9, while those for *Chloroparva pannonica* Cu40 PC are shown in Figure 5.11 and Figure 5.12. The cell size ranged from 2–5 µm in width and from 2–5 µm in length. The SEM observations indicated that the cell surface morphology of WT and Cu40 PCcells was similar. Two types of cell surface were

observed in both groups of isolates, either smooth or wrinkled (Figure 5.9, Figure 5.11 and Figure 5.12), with flagella absent for both groups.

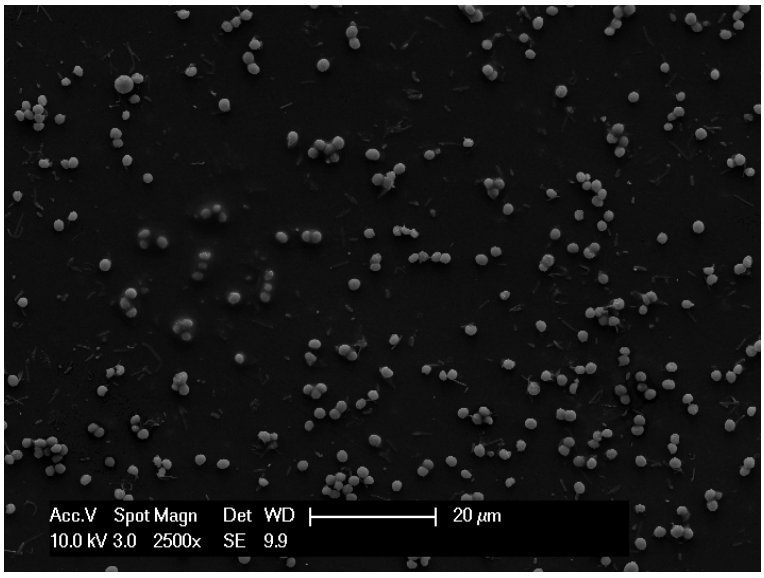


Figure 5.8 Scanning electron micrograph of *Chloroparva pannonica* WT

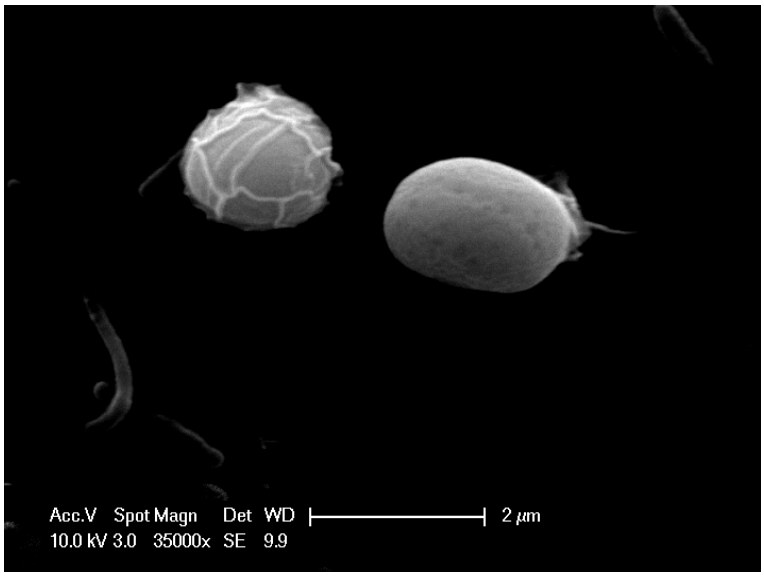


Figure 5.9 Scanning electron micrograph of *Chloroparva pannonica* WT showing different morphology of cell surface

Note: The cell on the left has a wrinkled outer surface while the cell on the right has a smooth surface.

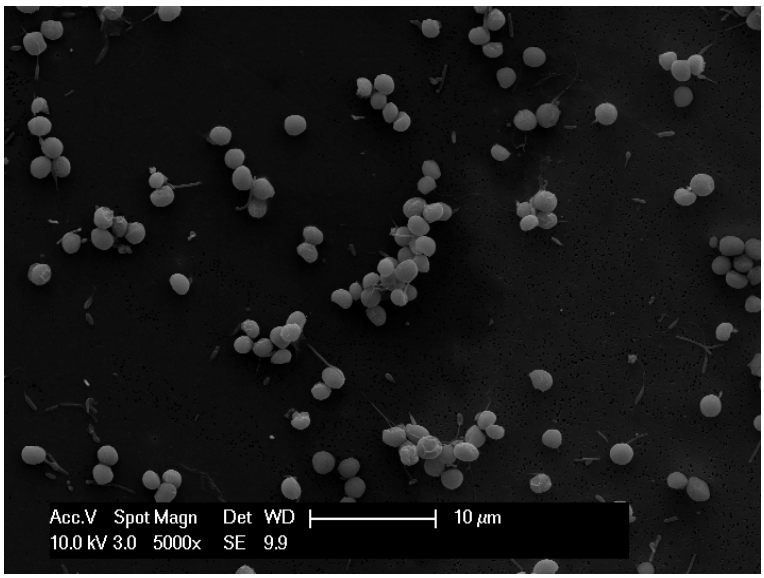


Figure 5.10 Scanning electron micrograph of *Chloroparva pannonica* Cu40 PC

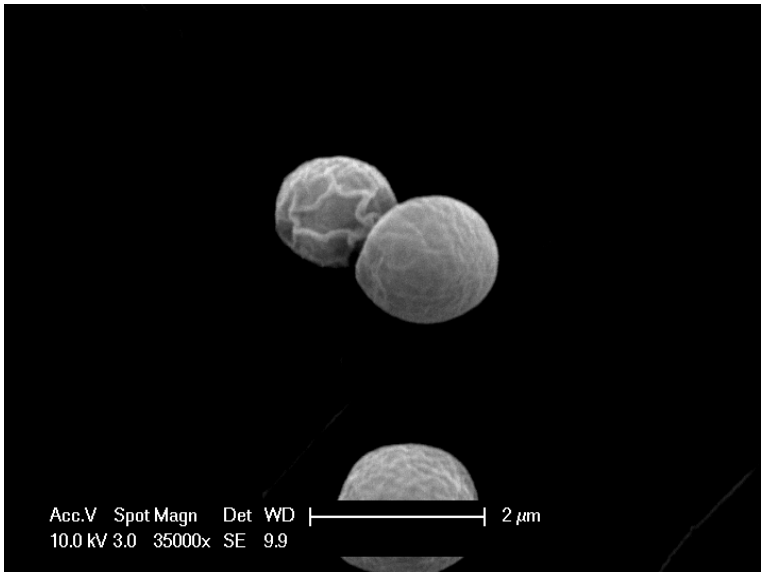


Figure 5.11 Scanning electron micrograph of *Chloroparva pannonica* Cu40 PC showing different morphology of cell surface

Note: The cell on the left has a wrinkled outer surface while the cell the right has a smooth surface.

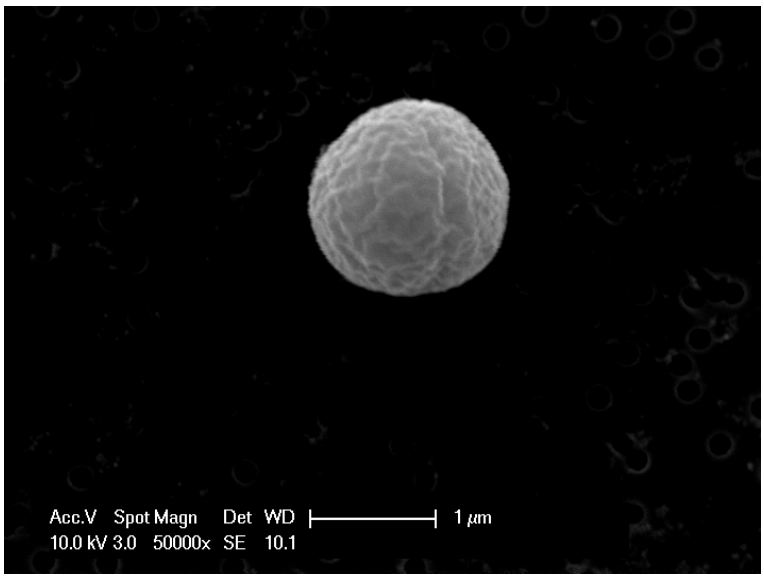


Figure 5.12 Scanning electron micrograph of *Chloroparva pannonica* Cu40 PC showing wrinkled outer surface of cell

5.3.1.4.2 Transmission electron microscopy (TEM) observations

Transmission electron microscopy (TEM) observations of *Chloroparva pannonica* WT cells are shown in Figure 5.13–Figure 5.15 while *Chloroparva pannonica* Cu40 PC cells are shown in Figure 5.16–Figure 5.18. The TEM observations indicated that the cell organization of WT and Cu40 PC cells were similar. No flagellates were observed in either of the isolates. Each WT (Figure 5.13) and Cu40 PC cell (Figure 5.16) consisted of a single nucleus, chloroplast, mitochondria, 4–6 thylakoid sacs and vacuoles.

Cell division in *Chloroparva pannonica* WT, Cu40 PC, cells occurred by autosporulation. Two daughter cells were often observed (Figure 5.15, Figure 5.17 and Figure 5.18). The remnants of the grandmother cell wall were found outside the mother cell wall around the cells during autosporulation (Figure 5.15 and Figure 5.17). A loose mother cell wall was observed during the late phase of autosporulation when the daughter cells were released (Figure 5.18).

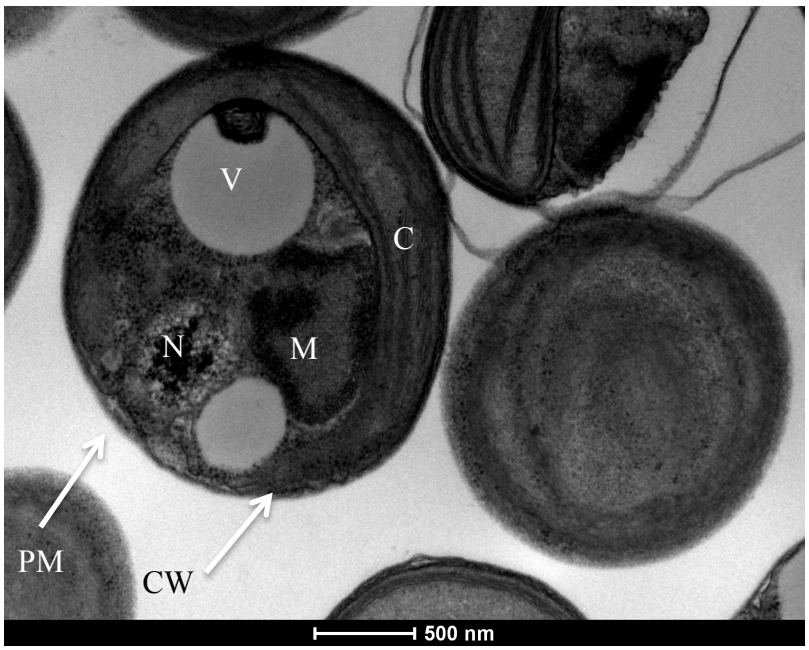


Figure 5.13 Transmission electron micrograph of *Chloroparva pannonica* WT

Note: C = chloroplast; CW = cell wall; M = mitochondrion; N = nucleus, PM = plasma membrane; V = vacuole.

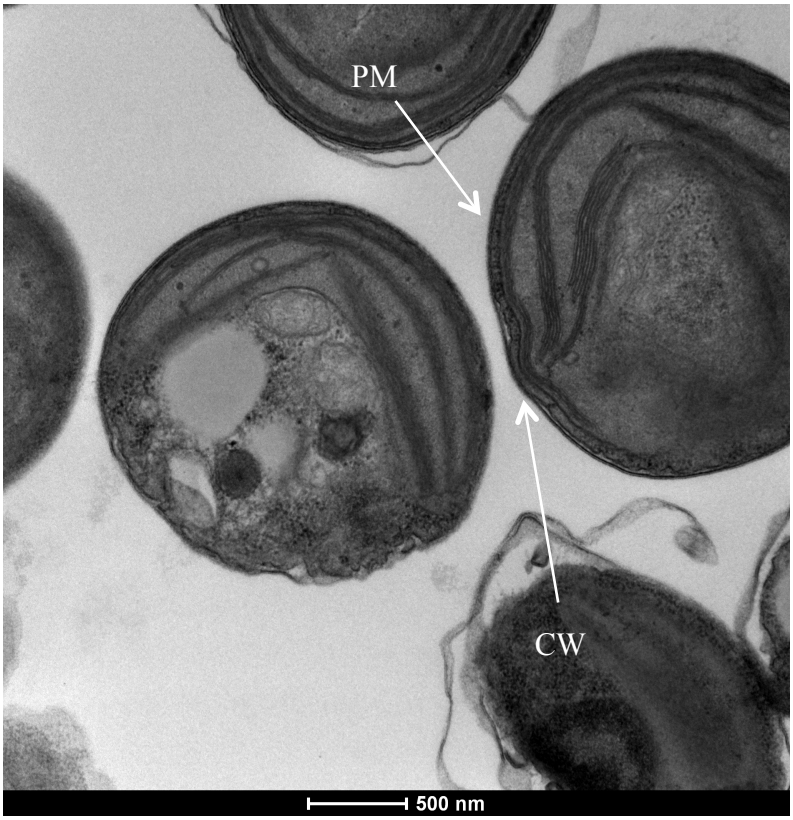


Figure 5.14 Transmission electron micrograph of *Chloroparva pannonica* WT showing cell wall structure

Note: CW = cell wall; PM = plasma membrane

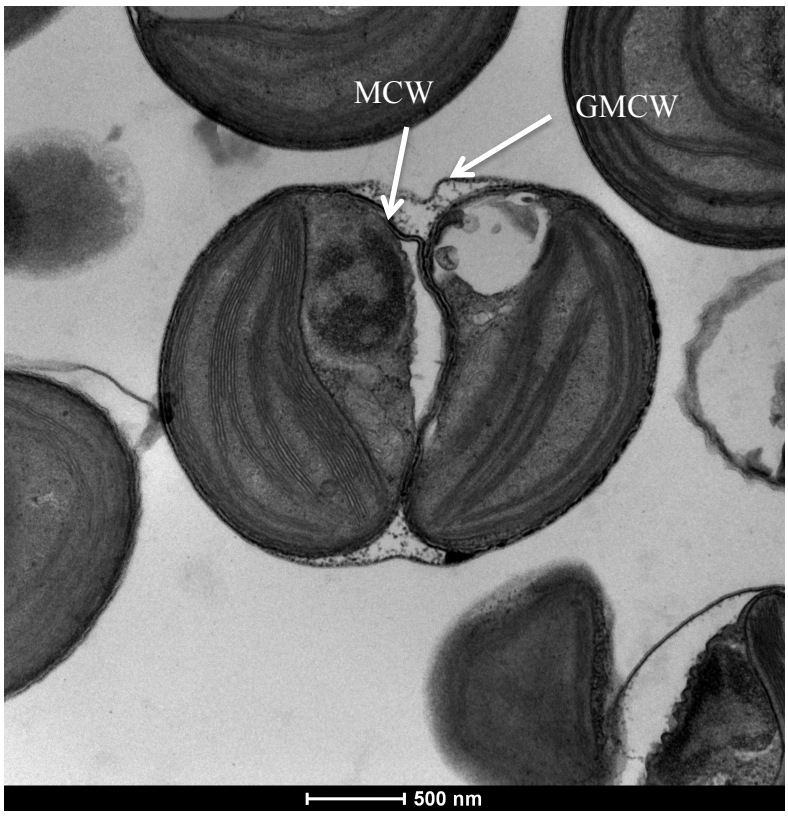


Figure 5.15 Transmission electron micrograph of *Chloroparva pannonica* WT mother cells with two autospores during autosporulation

Note: GMCW = grandmother cell wall; MCW = mother cell wall

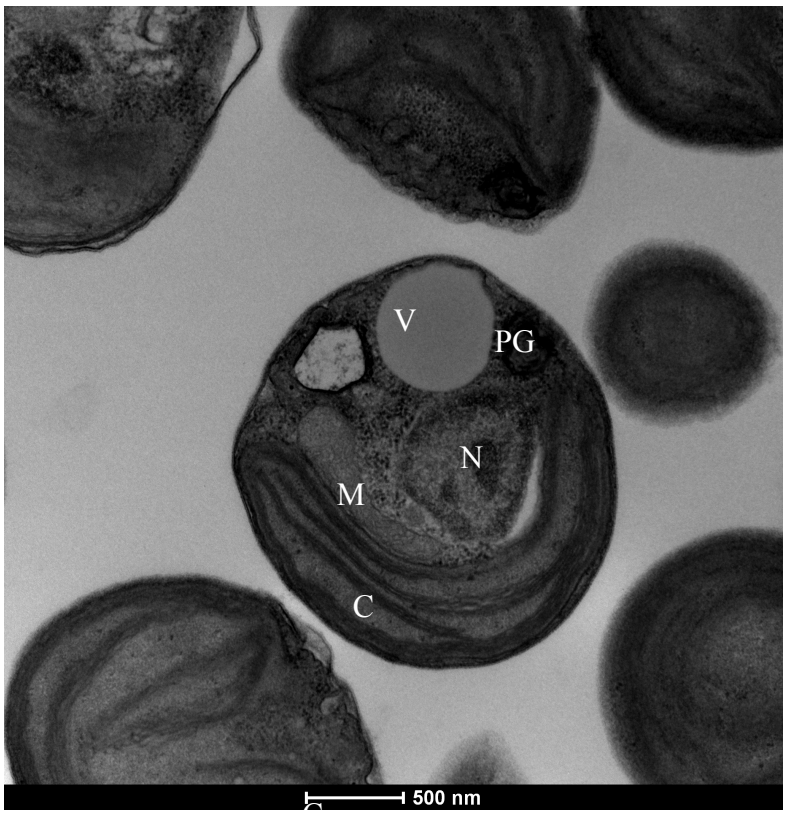


Figure 5.16 Transmission electron micrograph of *Chloroparva pannonica* Cu40 PC

Note: C = chloroplast; M = mitochondrion; N = nucleus; V = vacuole; PG = Plastoglobule; V=Vacuole

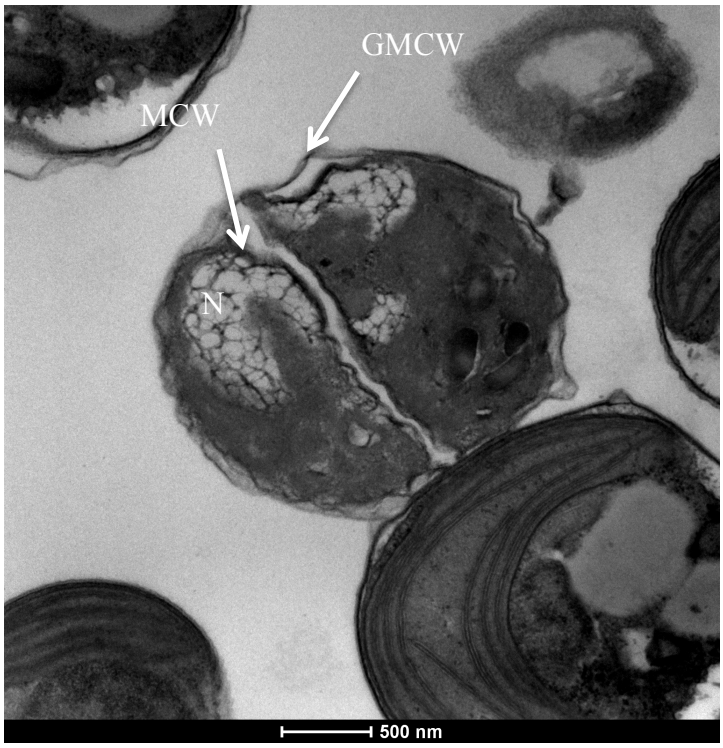


Figure 5.17 Transmission electron micrograph of *Chloroparva pannonica* Cu40 PC mother cells with two autospores during autosporulation

Note: GMCW = grandmother cell wall; MCW = mother cell wall; N = nucleus

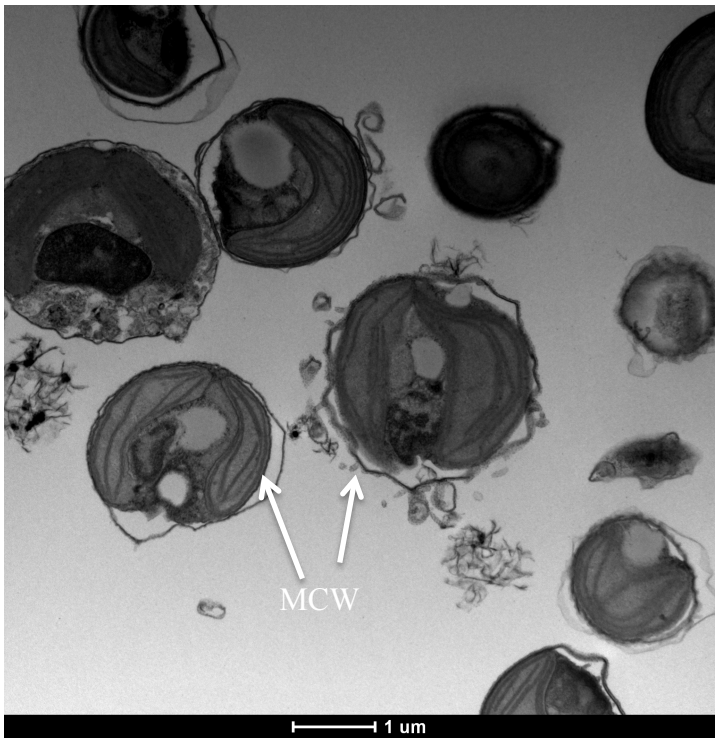


Figure 5.18 Transmission electron micrograph of two daughter *Chloroparva pannonica* Cu40 PC cells during late phase of autosporulation

Note: MCW = mother cell wall

5.3.2 Lipid profile

The total crude lipid content of *Chloroparva pannonica* isolates grown under standard conditions for a 14-day period is shown in Table 5.3.

Table 5.3 Total crude lipid (%) of *Chloroparva pannonica* isolates

***Chloroparva pannonica* isolates**	**Total crude lipid %**
WT	23.2 ± 2.3
Cu40 PC	24.8 ± 1.62
Fu4	23.8 ± 0.79
Fu5C	25.5 ± 1.44
Fu41A	22.9 ± 2.08

Chloroparva pannonica Cu40 PC was selected for fatty acid profile analysis. The fatty acid profile is shown in Table 5.4. The predominant fatty acids present in Cu40 PC when grown under standard conditions were oleic acid (25.9%), linoleic acid (24.7%), linolenic acid (20.2%) and palmitic acid (15.5%).

Table 5.4 Fatty acid profile of *Chloroparva pannonica* Cu40 PC at day 14 (stationary phase)

Common name	**Lipid name, number of carbons on fatty acid chain**	**Percentage (%)**
Lauric acid	12:0	0.1
Myristic acid	14:0	0.4
Pentadecylic acid	15:0	0.1
Palmitic acid	16:0	15.5
Palmitoleic acid	16:1 (n-7)	1.6
Margaric acid	17:0	7.0
Stearic acid	18:0	0.8
Vaccenic acid	18:1 (n-7)	2.3
Oleic acid	18:1 (n-9)	25.9
Linoleic acid + 19:0	18:2 (n-6) + 19:0	24.7
Linolenic acid	18:3 (n-3)	20.2
ϒ-Linolenic acid	18:3 (n-6)	0.1
Gondoic acid	20:1 (n-9)	0.2
Eicosadienoic acid	20:2 (n-6)	0.1
Erucic acid	22:1 (n-9)	0.2
Docosatetraenoic acid	22:4 (n-6) + 22:3 (n-3)	0.1
Lignoceric acid	24:0	0.1
Nervonic acid	24:1	0.7

5.3.3 Pigment composition

Chloroparva pannonica WT, Cu40 PC, Fu4, Fu5C and Fu41A contained chlorophyll *a* and chlorophyll *b*, with lutein as the dominant carotenoid. The pigment composition as follows: chlorophyll *a* (t_r = 22.577 min); chlorophyll *b* (t_r = 21.337 min); and lutein (t_r = 13.571 min) (Figure 5.19). The chlorophyll *b:a* ratio ranged from 0.056–0.099 (Table 5.5). As mentioned above, the main carotenoid for *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A was lutein which ranged from 1864–5502 mg/kg of total biomass (Table 5.5).

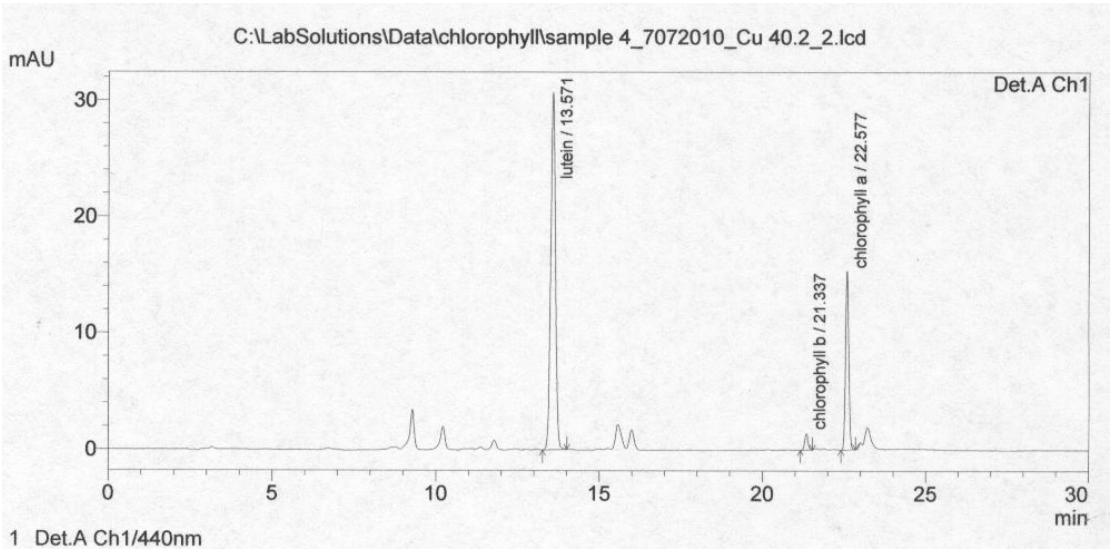


Figure 5.19 RP-HPLC chromatograms at 440 nm of pigment extracts of Cu40 PC

Table 5.5 Chlorophyll *a*, *b* and lutein content (mg/kg of biomass) for WT, Cu40 PC, Fu4, Fu5C and Fu41A

Microalgae strains	Pigment content (mg/kg of biomass)			Chlorophyll *b:a* ratio
	Chlorophyll *a*	Chlorophyll *b*	Lutein	
WT	42701	2401	1864	0.056
Cu40 PC	64726	6215	4333	0.096
Fu4	63023	3656	4387	0.058
Fu5C	55766	3560	5502	0.064
Fu41A	59891	5956	5201	0.099

5.3.4 Ecophysiological and biochemical testing

5.3.4.1 pH tolerance

The pH tolerance of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A was compared with levels of pH ranging from 1.5–12 (Table 5.6). *Chloroparva pannonica* WT, Cu40 PC and Fu5C grew well at pH levels from 6–11 while Fu4 and Fu41A grew well at pH levels from 7–11.

Table 5.6 pH tolerance of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A

pH	Growth of isolates under pH level variation (day 14)				
	WT	**Cu40 PC**	**Fu4**	**Fu5C**	**Fu41A**
1.5	-	-	-	-	-
2.0	-	-	-	-	-
2.5	-	-	-	-	-
3.0	-	-	-	-	-
5.0	-	-	-	-	-
6.0	+	+	-	+	-
7.0	+	+	+	+	+
8.0	+	+	+	+	+
8.5	+	+	+	+	+
9.0	+	+	+	+	+
10	+	+	+	+	+
11	+	+	+	+	+
12	-	-	-	-	-

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

5.3.4.2 Sodium chloride tolerance

The sodium chloride tolerance of *Chloroparva* WT, Cu40 PC, Fu4, Fu5C and Fu41A was compared at salinity levels ranging from 2–11% (w/v) (Table 5.7 and Figure 5.20). The growth of *Chloroparva pannonica* under different levels of salinity, based on the OD reading at 685 nm on day 14, is shown in Appendix A5.1.1 (Figure 5.20).

Chloroparva pannonica WT, Cu40 PC, Fu4, Fu5C and Fu41A cells all grew well at 2% (w/v) sodium chloride. At 7% (w/v) sodium chloride, WT, Cu40 PC and Fu5C cells showed weak growth while Fu4 and Fu41A cells did not grow. From 8–11% (w/v) sodium chloride, cells did not grow for any of the *Chloroparva pannonica* strains.

Table 5.7 Salt tolerance of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A from 2% to 11% (w/v) sodium chloride

Sodium chloride % (w/v)	Growth of isolates under varying sodium chloride levels (day 14) on 1.5% agar plates				
	WT	Cu40 PC	Fu4	Fu5C	Fu41A
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	W	W	-	W	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

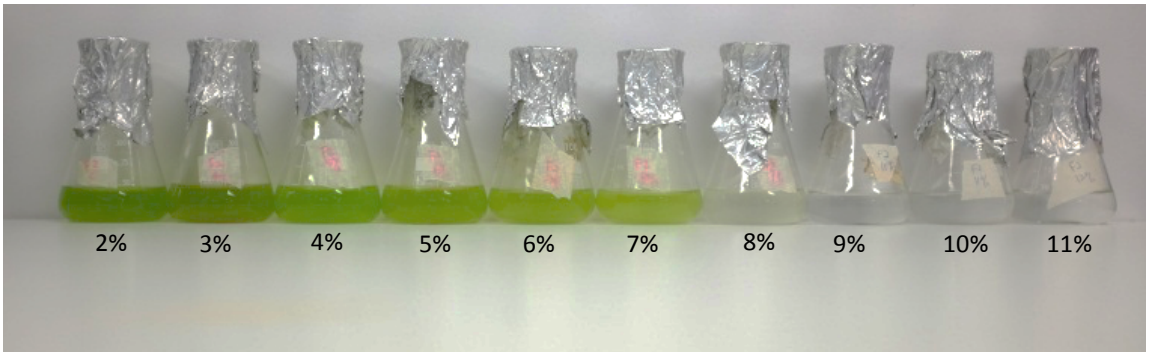


Figure 5.20 *Chloroparva pannonica* WT grown in different percentages of sodium chloride (w/v) at day 14

5.3.4.3 Growth at various temperatures

The ability of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A to grow at different temperatures was tested at 4°C, 10°C, 20°C, 25°C, 30°C, 35°C and 40°C (Table 5.8 and Figure 5.21). Results indicated that the growth of all microalgae strains was slow at 4°C and 10°C. All of the *Chloroparva pannonica* isolates grew at 20°C, 25°C and 30°C. The optimum temperature for WT, Cu40 PC, Fu5C and Fu41A was at 20°C while for Fu4, it was at 25°C (refer to Appendix A5.1.2, Figure 5.21). *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A did not grow at 35°C and 40°C.

Table 5.8 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different temperatures

Temperature	Growth of isolates at different temperatures (day 14) on 1.5% agar plates				
	WT	**Cu40 PC**	**Fu4**	**Fu5C**	**Fu41A**
4	W	W	W	W	W
10	+ (Slow)	+(Slow)	+(Slow)	+(Slow)	+(Slow)
20	+	+	+	+	+
25	+	+	+	+	+
30	+	+	+	+	+
35	-	-	-	-	-
40	-	-	-	-	-

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

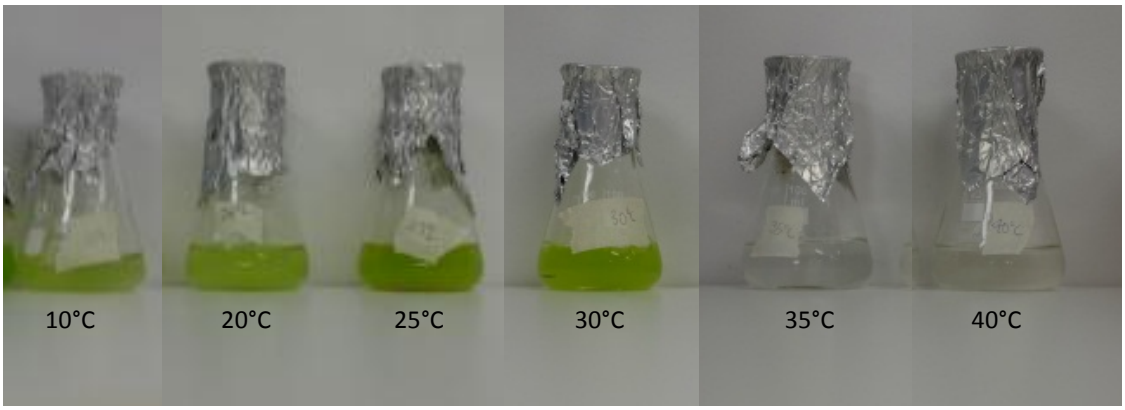


Figure 5.21 *Chloroparva pannonica* WT grown at different temperatures

5.3.4.4 Nitrogen requirement

Results of the growth test of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A with nitrate as the sole nitrogen source are shown in Table 5.9. The growth of *Chloroparva pannonica* based on OD at 685 nm with nitrate as the sole nitrogen source is shown in Appendix A5.1.3 (Figure A5.0.3). The results indicated that all of the strains required nitrogen to support their growth. The *Chloroparva pannonica* isolates did not grow when no nitrogen was present in the medium.

Table 5.9 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A with nitrate as sole nitrogen source

Nitrogen concentration	Growth of isolates with nitrate as sole nitrogen source (day 14) on 1.5% agar plates				
	WT	Cu40 PC	Fu4	Fu5C	Fu41A
0.88 nM (control)	+	+	+	+	+
0 mM	-	-	-	-	-
3 mM	+	+	+	+	+

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

5.3.4.5 Thiamine requirement

Table 5.10 shows the growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of thiamine. The growth of *Chloroparva pannonica* based on OD at 685 nm at different concentrations of thiamine is shown in Appendix A5.1.4 (Figure A5.0.4). *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A cells were able to grow without thiamine present in the medium.

Table 5.10 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of thiamine

Thiamine concentration	Growth of isolates at different concentrations of thiamine (day 14) on 1.5% agar plates				
	WT	Cu40 PC	Fu4	Fu5C	Fu41A
0.1 mg/L (Control)	+	+	+	+	+
0 mg/L	+	+	+	+	+
40 mg/L	+	+	+	+	+

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

5.3.4.6 Vitamin B_{12} requirement

Results on the growth test of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of Vitamin B_{12} are shown in Table 5.11. The growth of *Chloroparva pannonica* based on OD at 685 nm at different concentrations of Vitamin B_{12} is shown in Appendix A5.1.5 (Figure A5.0.5). Results indicated that all of the *Chloroparva pannonica* strains did not grow without Vitamin B_{12}.

Table 5.11 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of Vitamin B_{12}

Vitamin B_{12} concentration	Growth of isolates at different concentrations of Vitamin B_{12} (day 14) on 1.5% agar plates				
	WT	Cu40 PC	Fu4	Fu5C	Fu41A
0.1 mg/L (control)	+	+	+	+	+
0 µg/L	-	-	-	-	-
5 µg/L	+	+	+	+	+

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

5.3.5 Chemo-organotrophic growth

Chloroparva pannonica WT, Cu40 PC, Fu4, Fu5C and Fu41A did not grow on all of the organic substrates in darkness (Table 5.12). This included the organic substrates: glucose, galactose, mannose, fructose, maltose, sucrose, xylose, ribose and mannitol.

Table 5.12 Chemo-organotrophic growth of *Chloroparva pannonica* strains WT, Cu40 PC, Fu4, Fu5C and Fu41A) on organic substrates in darkness

Organic substrates	Growth of isolates on organic substrates in darkness (14 days)				
	WT	Cu40 PC	Fu4	Fu5C	Fu41A
Glucose	-	-	-	-	-
Galactose	-	-	-	-	-
Mannose	-	-	-	-	-
Fructose	-	-	-	-	-
Maltose	-	-	-	-	-
Sucrose	-	-	-	-	-
Xylose	-	-	-	-	-
Ribose	-	-	-	-	-
Mannitol	-	-	-	-	-
Control	-	-	-	-	-
Control with light	+	+	+	+	+

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

5.3.6 Genomic characteristics

5.3.6.1 Phylogenetic analysis of 18S rRNA gene sequences

The sequence analysis of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A resulted in 2077, 2042, 2115, 2091 and 2095 nucleotides, respectively. Based on a database search, the closest relatives were *Chloroparva* sp. ACT 0608 with 99–100% pairwise similarities.

Molecular analysis of the 18S rRNA gene sequence placed *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A together with *Chloroparva* sp. ACT 0608 within Trebouxiophyceae, Chlorophyta (Figure 5.22).

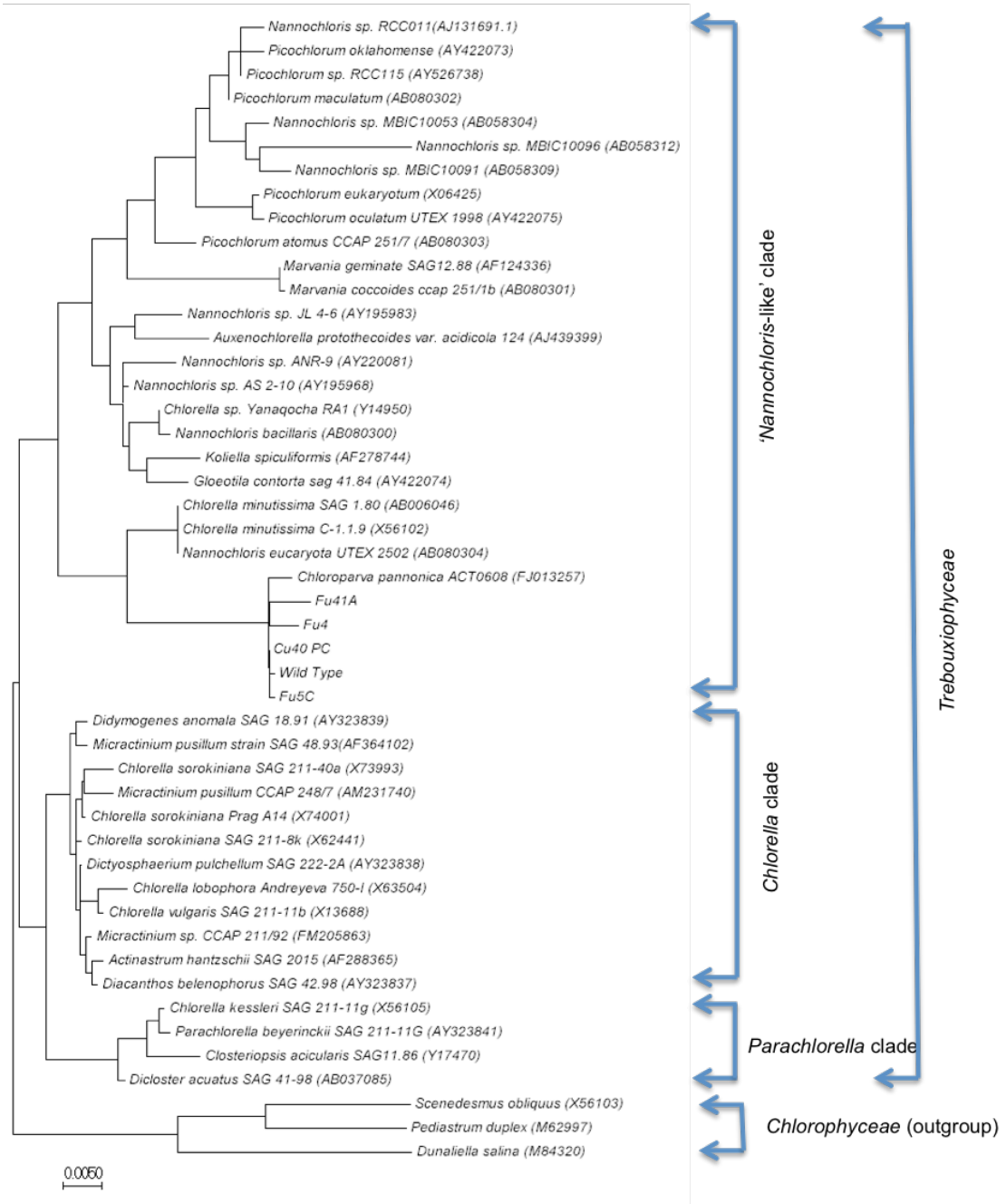


Figure 5.22 Maximum likelihood phylogenetic tree based on Tamura–Nei model using 1313 bp alignment of 18S rRNA gene sequence of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A

Note: Genetic distance was computed by Kimura's two-parameter model. Scenedesmus obliquus, Pediastrum duplex and Dunaliella salina were included as an out-group. The bar represents the position at 0.0050 substitutions per nucleotide.

5.3.6.2 Guanine+cytosine (G+C) mol% of genomic DNA

The guanine+cytosine (G+C) mol% of genomic DNA of the *Chloroparva pannonica* strains of WT, Cu40 PC, Fu4 and Fu5C was 59.01±0.76%, 57.3±1.38%, 58.67±2.47%

and 56.27±0.54% respectively (Table 5.13). Appendix 5.2, Figure A5.0.6 presents an example of an HPLC chromatogram.

5.4 Discussion

The aim of this study was to use a combination of molecular, morphological, ecophysiological and biochemical testing to understand the taxonomic position of the strains used in this study, *Chloroparva pannonica.* The results are summarised in Table 5.13.

The use of ecophysiological and biochemical characteristics has been very informative in differentiating microalgae strains at the genus level as well as being simple and easy to perform. For the classification of microalgae, Huss *et al.* (1999) and Kessler and Huss (1990) proposed 10 ecophysiological and biochemical characteristics comprising: hydrogenase; secondary carotenoids; pH limitations; NaCl limitations; temperature limitations; nitrate reduction; thiamine requirements; Vitamin B_{12} requirements; growth on organic substances (glucose, galactose, mannose, fructose, maltose, sucrose, xylose, ribose and mannitol); and guanine+cytosine (G+C)% of the genomic DNA.

In summary, *Chloroparva pannonica* cells are green and spherical with a diameter of 2–5 µm x 2–5 µm when grown in seawater. Based on the description by Somogyi *et al.* (2011), *Chloroparva pannonica* ACT 0608 cells are smaller, with sizes ranging from 1–2.3 µm in width and from 1.2–2.6 µm in length. The colonies that formed on the agar medium were 1–1.2 mm x 1–1.2 mm, and were green and round with a smooth surface after two weeks of incubation (Table 5.13). Each cell had one nucleus, mitochrondia, chloroplast, a plasma membrane and a cell wall (Figure 5.13, Figure 5.14 and Figure 5.16). The cell wall consisted of a thin (10–20 µm) trilaminar layer (Somogyi *et al.*, 2011). Each cell reproduced by autosporulation into two daughter cells (Figure 5.15, Figure 5.17 and Figure 5.18) (Somogyi *et al.*, 2011). Two types of cell surface were observed, smooth or wrinkled (Figure 5.9, Figure 5.11 and Figure 5.12). No flagella were present. According to the hypothesis by Somogyi *et al.* (2011), the wrinkled cell surface is the long persistence of the mother cell wall around the daughter cells, while the smooth cell surface is the cell after the detachment of the mother cell wall remnants (Figure 5.9, Figure 5.11 and Figure 5.12).

The total crude lipid content of *Chloroparva pannonica* isolates ranged from 22.94 – 25.59% (Table 5.3). Cu40 PC cells cultivated at 25°C were selected for fatty acid profile analysis (Table 5.4): the predominant fatty acids present were oleic acid (25.9%), linoleic acid (24.7%), linolenic acid (20.2%) and palmitic acid (15.5%). The predominant fatty acid present in *Chloroparva pannonica* ACT 0608 cultivated at 8 and 21°C was oleic acid (Somogyi *et al.*, 2011). However, several studies have suggested that the lipid content and fatty acid profile could be manipulated based on different cultivation conditions, such as light irradiation, temperature, salinity and nitrogen sources (Chen *et al.*, 2011b, Cheng *et al.*, 2016, Converti *et al.*, 2009, Srinivas and Ochs, 2012, Sukenik *et al.*, 1989). The main pigments in the cells were chlorophyll *a*, chlorophyll *b* and lutein (Figure 5.19). The chlorophyll *b:a* ratio ranged from 0.056–0.099. Lutein was the main carotenoid for *Chloroparva pannonica* isolates, ranging from 1863–5502 mg/kg of total biomass (Table 5.5). However, in addition to lutein, three carotenoids (violaxanthin, neoxanthin and β-carotene) were identified at low concentrations in *Chloroparva pannonica* ACT 0608 (Somogyi *et al.*, 2011). Many studies have been conducted on the manipulation of carotenoid content in microalgae through, for example, the use of irradiance, pH, temperature, nitrogen sources, salinity and the presence of oxidizing compounds (Del Campo *et al.*, 2001, Rao *et al.*, 2007, Sanchez *et al.*, 2008a, Sanchez *et al.*, 2008b, Vaquero *et al.*, 2012, Vaquero *et al.*, 2014b, Wei *et al.*, 2008).

Chloroparva pannonica were able to tolerate pH levels from 6–11, and to tolerate NaCl up to 7% (Table 5.13). The *Chloroparva pannonica* strains grew at 20–30°C, and also tolerated 4°C and 10°C, but with weak growth (Table 5.13). *Chloroparva pannonica* required nitrogen and Vitamin B_{12} for growth (Table 5.13). In addition, *Chloroparva pannonica* did not grow on glucose, galactose, mannose, fructose, maltose, sucrose, xylose, ribose and mannitol in darkness (Table 5.13).

Table 5.13 Biochemical and physiological characteristics of C*hloroparva pannonica* strains WT, Cu40 PC, Fu4, Fu5C and Fu41A)

Characteristics	WT	Cu40 PC	Fu4	Fu5C	Fu41A
Cell shape	Spherical	Spherical	Spherical	Spherical	Spherical
Cell size (µm)	2–5	2–5	2–5	2–5	2–5
Colony shape	Spherical	Spherical	Spherical	Spherical	Spherical
Colony colour	Green	Green	Green	Green	Green
Colony size (mm)	1–1.2	1–1.2	1–1.2	1–1.2	1–1.2
Ecophysiological and biochemical testing					
Growth at pH	6–11	6–11	7–11	6–11	7–11
Growth at %NaCl (w/v)	2–7	2–7	2–6	2–7	2–6
Growth at temperature (°C)	10–30	10–30	10–30	10-–0	10–30
Nitrogen requirement	+	+	+	+	+
Thiamine requirement	-	-	-	-	-
Vitamin B_{12} requirement	+	+	+	+	+
Chemo-organotrophic growth					
Glucose	-	-	-	-	-
Galactose	-	-	-	-	-
Mannose	-	-	-	-	-
Fructose	-	-	-	-	-
Maltose	-	-	-	-	-
Sucrose	-	-	-	-	-
Xylose	-	-	-	-	-
Ribose	-	-	-	-	-
Mannitol	-	-	-	-	-
Genomic characteristics					
G+C mol% of genomic DNA	59.06±0.76	57.3±1.38	58.67±2.47	56.27±0.54	Not done

Note: Results were recorded as positive (+) or negative (-) based on the presence or absence of growth. + = Positive or present; - = Negative or absent; W = Weakly positive

However, some differences were found between *Chloroparva pannonica* isolates. *Chloroparva pannonica* Fu4 and Fu41A were only able to tolerate pH levels from 7–11, and could tolerate NaCl up to 6% (Table 5.13). The G+C mol% of genomic DNA of *Chloroparva pannonica* strains of WT, Cu40 PC, Fu4 and Fu5C was 59.01±0.76%, 57.3±1.38%, 58.67±2.47%, 56.27±0.54%, respectively. For further verification of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A, molecular phylogenetic

analysis of their 18S rRNA gene sequence placed these strains together with *Chloroparva* sp. ACT 0608 within Trebouxiophyceae, Chlorophyta (Figure 5.22).

5.5 Conclusion

Molecular analysis of the 18S rRNA gene sequence placed *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A as being closely related to *Chloroparva* sp. ACT0608 and classified them within Trebouxiophyceae Chlorophyta.

The 5 strains were spherical in shape and the cell size ranged from 2–5 μm. The lipid content ranged from 22.94–25.59%. The predominant fatty acids were oleic acid, linoleic acid, linolenic acid and palmitic acid. The pigments were chlorophyll *a* and *b* while the carotenoid was lutein. The lutein concentration ranged from 1864–5502 mg/kg of biomass when grown in batch culture mode. These strains were tolerant of salinity at 7%, pH levels from 6–11 and temperature from 10–30°C. The molar guanine+cytosine ([G+C] mol%) of the genomic DNA ranged from 56.27 –59.06%. Molecular analysis of the 18S rRNA gene sequence placed *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A as being closely related to *Chloroparva* sp. ACT 0608 and classified them within Trebouxiophyceae, Chlorophyta.

Chapter 6 Optimization of biomass and lutein production in *Chloroparva pannonica* high-density culture cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

6.1 Introduction

The growing interest in the microalgae based bio-refinery process has focused on large-scale cultivation systems. Ideally, the microalgal oil-based bio-refinery process would be energy neutral. The photosynthetic growth of microalgae requires light, carbon dioxide, water and essential elements, such as nitrogen, phosphorus, iron and silicon for certain types of microalgae, at temperatures ranging from 20–30°C. Due to the cost of capital and the operating cost, microalgae are usually cultivated either in a closed culture system (a photobioreactor) or in an open culture system (open raceway ponds, tanks and open ponds) for large-scale biomass production.

Microalgal cultivation in a photobioreactor is recognized as the best way to achieve high production and to maintain monocultures. Several types of indoor and outdoor photobioreactors have been successfully used for cultivating microalgae. This includes the airlift photobioreactor, the horizontal and inclined tubular photobioreactor and the flat-plate bioreactor (Chiu *et al.*, 2008, Debska *et al.,*2010, Del Campo *et al.*, 2007, Del Campo *et al.*, 2001, Krichnavaruk *et al.*, 2007, Krustok, 2015, Rodolfi *et al.*, 2009, Stephenson *et al.*, 2010). Closed photobioreactors are suitable for microalgae that cannot be efficiently grown in an open system, such as *Haematococcus* (Garcia-Malea *et al.*, 2006). Although production and maintenance costs are higher, the culture conditions can be controlled and it is easier to maintain a monoculture and avoid contamination; therefore higher biomass productivity can be achieved. However, if the photobioreactor is to be used for large-scale production, further refinement in its design is needed including light provision, carbon dioxide losses, efficient mixing and the removal of oxygen.

Since the 1950s, raceway ponds have been used for the production of microalgal biomass. A raceway pond comprises a closed-loop recirculation channel with a paddle wheel that operates all the time for circulation (Chisti, 2007). The largest microalgae raceway pond for a biomass production facility is owned by Earthrise Nutritionals

(www.Earthrise.com) occupying an area of 440,000m^2 (Chisti, 2007). Unlike a photobioreactor, raceway ponds cost less to build and also have lower operating costs. On the other hand, in a raceway pond, there is little control of culture conditions and the system is more prone to biological contaminations, such as microalgae, bacteria and protozoa, and therefore biomass productivity is lower when compared to the photobioreactor system. For a long-term operation, the maintenance of the microalgae monoculture in the open raceway pond is usually conducted in an extreme environment such as high salinity. A few microalgae including *Spirulina*, *Dunaliella* and *Chlorella* have been successfully cultivated in outdoor open systems on a commercial scale (Pulz and Gross, 2004).

Chloroparva pannonica is a green microalga that is able to accumulate lipids and lutein. Although lutein, a type of xanthophyll, is frequently used as a food additive, interest has recently been growing in its use for nutraceutical and pharmaceutical applications. Studies have shown how lutein might be beneficial to human health, preventing cell damage to eyes and also preventing age-related macular degeneration (Moeller *et al.*, 2006, Snodderly, 1995, Yildirim *et al.*, 2011). The increased demand for lutein and the drawbacks of obtaining lutein from the petals of marigold flowers have resulted in growing interest in the extraction and purification of lutein from microalgae. A few microalgae have been proven to be good lutein producers including *Chlorella protothecoides*, *Murielopsis* sp. and *Scenedesmus almeriensis* (Del Campo *et al.*, 2007, Del Campo *et al.*, 2000, Shi *et al.*, 2006, Zapata *et al.*, 2000). *Murielopsis* sp. has high biomass productivity and is able to accumulate lutein when grown high photoautotrophically (Del Campo *et al.*, 2001). *Murielopsis* sp. is the only microalga to date that has been cultivated in both outdoor closed systems and outdoor open systems for biomass and lutein production (Blanco *et al.*, 2007, Del Campo *et al.*, 2001). Under the outdoor closed system conducted by Del Campo *et al.* (Del Campo *et al.*, 2001) in a 55 L tubular photobioreactor, the highest biomass productivity was 40 g dry biomass $m^{-2}day^{-1}$ while the maximal lutein yield was 180 $mg.m^{-2}day^{-1}$. However, in the semi-continuous outdoor open system study conducted by Blanco *et al.* (2007) in 1 m^2 and 3 m^2 tanks with 0.3 m maximal depth and rotation using a paddle wheel, 13 g dry biomass $m^{-2}day^{-1}$ was the biomass productivity achieved, while the maximal lutein yield was 100 $mg.m^{-2}.day^{-1}$. As can be seen, both biomass productivity and lutein yield were much lower in the outdoor open system than the closed system. In addition, microalgae, such as *Chlorella protothecoides* and *Scenedesmus almeriensis*, are also able to accumulate

lutein. A study conducted by Shi *et al.* (2006), using *Chlorella protothecoides* and carried out on a heterotrophic batch laboratory scale with glucose as the carbon source, was able to obtain cultures with a lutein yield up to 10 $mg.m^{-1}day^{-1}$ under optimized conditions. To date, the microalga with the highest lutein yield is *Scenedesmus almeriensis* which achieved up to 290 $mg.m^{-2}day^{-1}$: this was through the use of a double-layered 400 L serpentine tubular photobioreactor in a greenhouse (Del Campo *et al.*, 2007).

The lutein is generally located within cells: the lutein in marigold flowers is mainly in ester form while the lutein in microalgae is mainly free form. Lutein is able to be extracted, separated and purified from marigold flowers on a commercial scale based on a method patented by Khachik (2007). In microalgal lutein production, the process of extraction is one of the major stages and can be affected by several factors: types of solvent, concentration of the solvent, temperature and extraction techniques such as ultrasound, supercritical fluid and pressurized liquid. Although studies have successfully extracted and purified lutein from microalgal biomass, the methods used have been carried out either on a small scale or solely for analytical purposes (Farrow and Tabenkin, 1966, Hejazi *et al.*, 2002, Kimura *et al.*, 1990, Li *et al.*, 2002, Nonomura, 1987, Soares *et al.*, 2016). The extraction, separation and purification of lutein using microalgal biomass on a commercial scale have not yet been reported. However, Deenu *et al.* (2013) and Ceron *et al.* (2008) developed and optimized methods for the extraction of lutein from *Chlorella vulgaris* and *Scenedesmus almeriensis* that can be scaled up to a commercial scale. These methods comprise three major steps: cell disruption using bead milling or ultrasound; alkali treatment and saponification with the addition of potassium hydroxide (KOH); and extraction with hexane (Ceron *et al.*, 2008, Deenu *et al.*, 2013). In addition, Deenu *et al.* (2013) showed that cell disruption using ultrasound, together with enzymatic pre-treatment, was able to increase the lutein extraction yield. Therefore, the method developed by Deenu *et al.* (2013) was chosen and modified for the extraction, separation and purification of lutein from *Chloroparva pannonica* in this study.

For a feedstock in the microalgal oil-based bio-refinery process focused on lutein production, it is not only the lipid content that is of primary importance but also lutein productivity and biomass productivity. *Chloroparva pannonica*, with an improved growth rate and enhanced lutein accumulation, would increase the feasibility of lutein

production on a commercial scale. The medium optimization of *Chloroparva pannonica* (see Chapter 3) and strain improvement of *Chloroparva pannonica* using UV-C random mutagenesis and selection using herbicide inhibitors (see Chapter 4) have successfully produced mutants with increased biomass productivity and lutein productivity compared to that achieved by *Chloroparva pannonica* wild type (WT) strain. The isolated mutants exhibited an increase in biomass and lutein content when cultivated in a batch mode in 3 L flasks with 1% carbon dioxide supplied (see Chapter 4). The short-term laboratory experiments with the *Chloroparva pannonica* mutants under optimum conditions have produced promising results (see Chapter 4); however, long-term large-scale experiments with these mutants were required to determine whether they were able to produce biomass, lutein and lipids in similar or higher quantities on a larger scale.

In the microalgal oil-based bio-refinery process, maintaining a monoculture has previously been considered to be important. However, a few studies have shown that microalgae–bacteria interaction can comprise mutualism, commensalism and parasitism (Droop, 2007, Kazamia *et al.*, 2012). Microalgae and bacteria often exchange micronutrients (vitamins) and macronutrients (nitrogen, oxygen and carbon) (Croft *et al.*, 2005, Salvesen *et al.*, 2000, Teplitski and Rajamani, 2011). In addition, a group of bacteria, known as plant growth-promoting bacteria (PGPB), have been shown to enhance the growth of microalgae (Hernandez *et al.*, 2009). For example, in their study, Hernandez *et al.* (2009) showed that the growth of *Chlorella vulgaris* was enhanced by *Bacillus pumilus* ES4 when they were cultivated together. Furthermore, Cho *et al.* (2015) cultivated and ecologically engineered four different growth-enhancing bacteria and then cultivated them together with *Chlorella vulgaris*. Their study showed that the growth of *Chlorella vulgaris* was enhanced and had superior flocculation efficiency and lipid content when cultivated with the ecologically engineered bacteria. Moreover, based on the results presented in the Chapter 5 *Chloroparva pannonica* Cu40 had decreased growth if vitamin B_{12} was absent in the cultivation medium. The growth of *Chloroparva pannonica* Cu40 also decreased when bacteria were eliminated by using antibiotics (see Chapter 4). Therefore, it is essential that this study investigates the bacteria communities in the long-term semi-continuous operating strategy for biomass and lutein production conducted in an 11 L rectangular inter-loop airlift photobioreactor.

This study had four major objectives. Firstly, it was carried out in an 11 L rectangular inter-loop airlift photobioreactor designed by the Department of Medical Biotechnology, Flinders University, and SA Biomedical Engineering System Performance (Infrastructure), Flinders University, to evaluate the biomass, lutein content and lipid content of *Chloroparva pannonica* WT, Cu40 PC and 10 mutant strains in batch mode. Secondly, the selected strains with high biomass and lutein productivity were used to develop a long-term semi-continuous operating strategy for biomass and lutein production conducted in an 11 L rectangular inter-loop airlift photobioreactor. Thirdly, the harvested biomass from *Chloroparva pannonica* Cu40 PC was used for extraction and purification to obtain the final lutein extract that could be used for commercial proposes. Lastly, the bacteria diversity changes in the cultivation of *Chloroparva pannonica* Cu40 PC in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor were investigated by using pyrosequencing.

6.2 Methods and materials

6.2.1 Rectangular inter-loop airlift photobioreactor (11 L)

The bioreactor system for the experiment was composed of ten (10) 11 L bioreactors placed in a temperature-controlled (22–25°C) room (Figure 6.1) set up in the Department of Medical Biotechnology, Flinders University. The ten (10) 11 L bioreactors were designed by Flinders University's Department of Medical Biotechnology and manufactured by SA Biomedical Engineering System Performance (Infrastructure), Flinders Medical Centre. The overall dimensions of each photobioreactor are detailed in Figure 6.2. The column was made from Plexiglas (polymethyl methacrylate [PMMA], also known as Perspex or Acrylic Sheet).

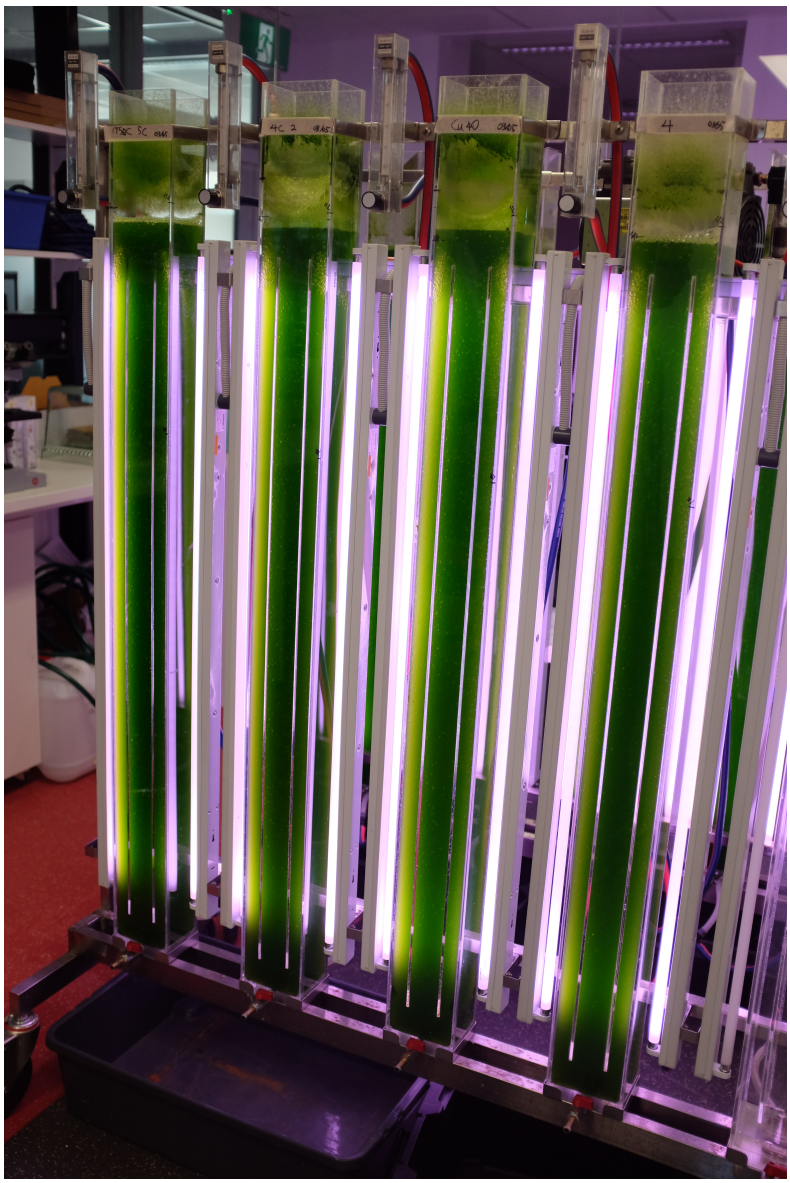


Figure 6.1 Rectangular inter-loop airlift photobioreactor (11 L) in the Department of Medical Biotechnology, Flinders University

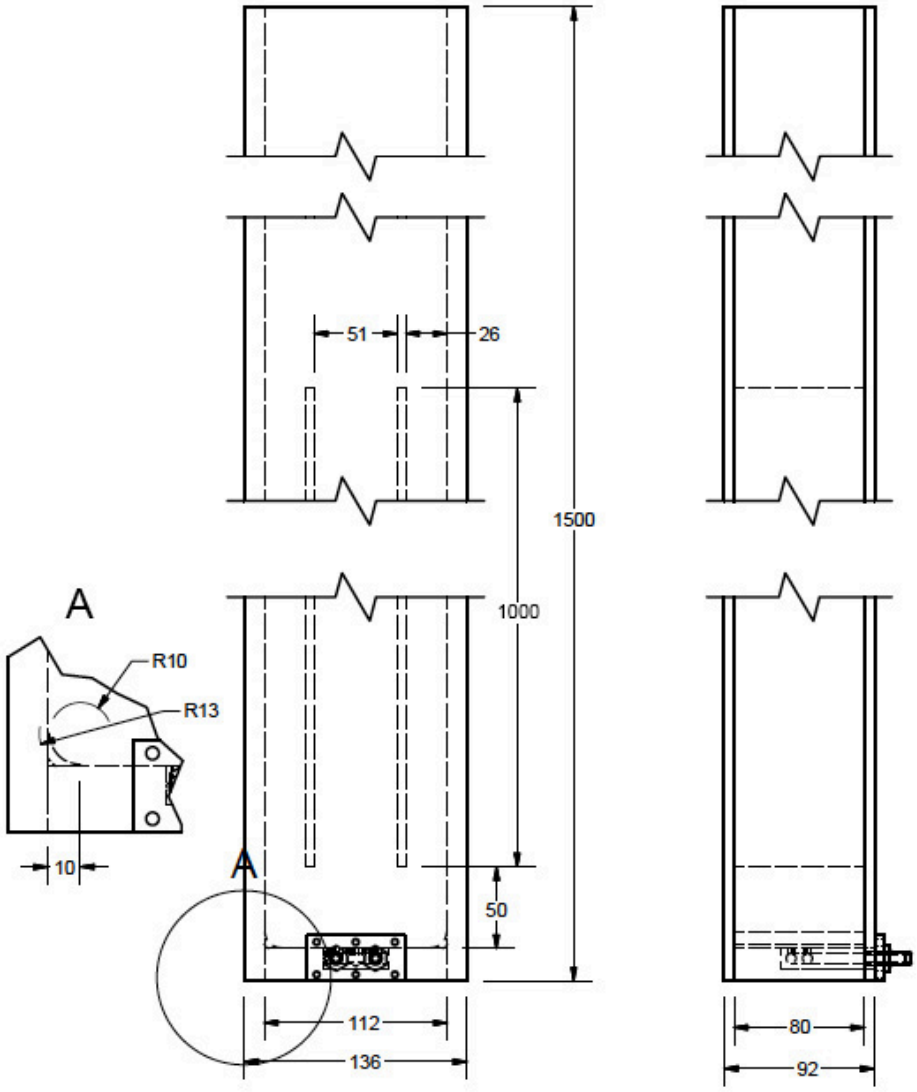


Figure 6.2 Overall dimensions (mm) of the photobioreactor, back and front views

The carbon was supplemented by an industrial carbon dioxide (CO_2) gas cylinder with a single-stage regulator. The CO_2 cylinder outlet was connected to the CO_2 inlet of the WITT dual gas mixer MM-2G (WITT-Gasetechnik, Witten, Germany) that had the ability to adjust the CO_2 level from 0% to 25%. For this study, a CO_2 level of about 1% was required for microalgal growth in a photobioreactor setting. An additional gas mixer inlet connection sourced air from the atmosphere via a low-pressure air compressor pump. The outlet of the gas mixer was then connected to a glass tube rotameter that measured and controlled the mixed air flow ranging from 0.1–1.0 m^3/h. Two T5 high output (HO) fluorescent lights were positioned about 10 cm from each side of the photobioreactor (Figure 6.3). The transparent flat surface on both sides of the

photobioreactor provided a uniform and larger surface exposure of artificial light when it was in operation. In terms of mixed air distribution, a customised sparger was placed at the bottom centre of the photobioreactor. The function of this sparger was to diffuse additional CO_2 for the microalgal cells. Two baffles created the internal-loop effect for the photobioreactor (Figure 6.3), with their function being to create internal circulation of the microalgal cells, to ensure the cells absorbed a similar rate of artificial light as well as of CO_2. When a sparger diffuses the mixed air (bubbling), into the based of the riser the dissolved gasses reduce the water pressure and the liquid starts to flow (up) in the riser. In line with water potential theory, the pressure in the riser becomes lower and the higher-pressure liquid in the downcomers on both sides loops from the bottom into the riser to normalise the pressure in the riser. If there is no restriction on the air flow, the internal loop will continue to occur until the sparger is turned off.

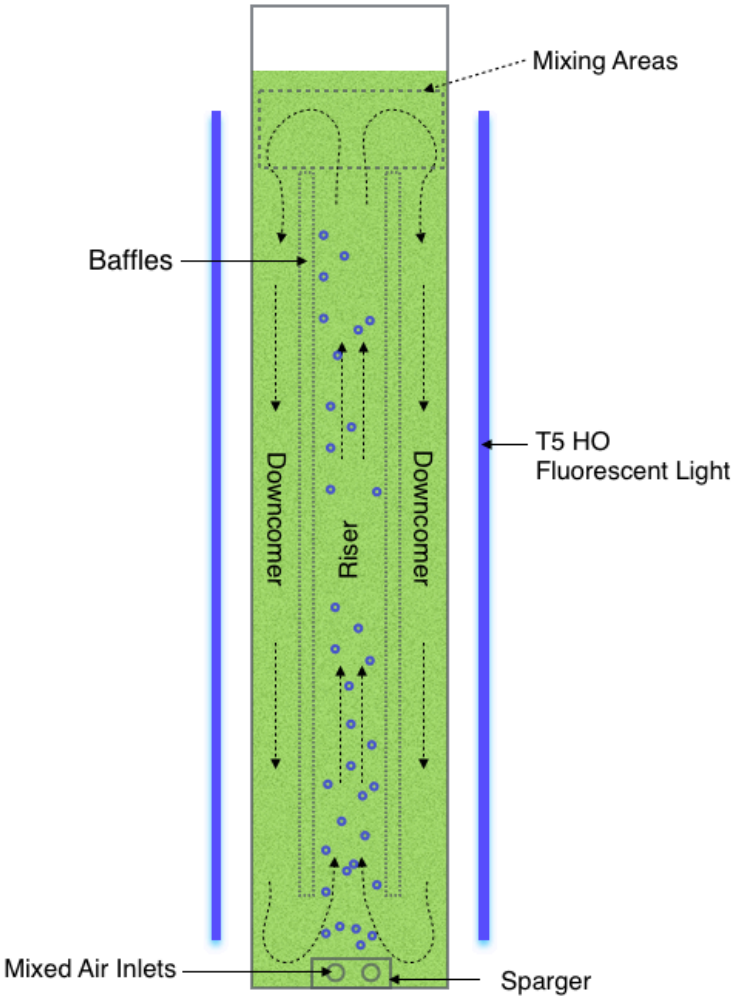


Figure 6.3 Schematic diagram of rectangular inter-loop airlift photobioreactor

6.2.2 Evaluation of growth, lipid and lutein content of *Chloroparva pannonica* Cu40 WT, Cu40 and 10 mutant strains in 11 L rectangular inter-loop airlift photobioreactor

6.2.2.1 *Chloroparva pannonica* Cu40 strains and culture conditions

The microalgae for this part of the study were *Chloroparva pannonica* Cu40, WT and mutant strains, as listed in Table 6.1. Cultures were maintained in the FU medium as described in Chapter 3 on agar plates, in liquid cultures and in 3 L flasks with 1% carbon dioxide supplied. Both liquid and agar cultures were maintained at 25±1°C with a light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination following a 12: 12-h light: dark photoperiod.

Table 6.1 *Chloroparva pannonica* Cu40 mutant strains selected from strain improvement study for this study

Chloroparva pannonica Cu40 mutant strains (#)	Renamed in this chapter
4	Fu4
142	Fu142
1.8µmC.305	Fu305C
1.8µmC.5	Fu5C
1.8µmC.58	Fu58C
2µmA.10	Fu10A
2µmA.469	Fu469A
2µmA.48	Fu48A
2µmA.58	Fu58A
3µmA.41	Fu41A

Note: See Chapter 4 for details

6.2.2.2 *Chloroparva pannonica* strain, media and inoculum preparation

Chloroparva pannonica Cu40, WT and mutant strains grown in 3 L flasks for a 10-day period, with 1% carbon dioxide supplied, were used as the inoculum. To confirm that each culture was homogeneous and free of contaminating microalgae, the cultures were examined under a microscope. A 4.4 L inoculum of day 10 culture with an equal number of cells (OD of 0.7 at 685 nm) was added into 6.6 L of sterile FU medium in the 11 L rectangular inter-loop airlift photobioreactor at 22–25°C with light intensity of 85 µmol photons $m^{-2}s^{-1}$ illumination, with 1% (v/v) carbon dioxide supplied, following a 12:12-h light: dark photoperiod. *Chloroparva pannonica* Cu40 PC, WT and the mutant strains were grown for an 18-day period in triplicate, repeated three times. The cells

were harvested from the outlet at the bottom of the column of each photobioreactor at day 3, 6, 9, 12 and 18 for analysis.

6.2.2.3 Analytical methods

6.2.2.3.1 Determination of total dried biomass

The total dried biomass of the microalgal cultures was determined based on the method described in Section 2.2.3 which was carried out in triplicate.

6.2.2.3.2 Determination of specific growth rate

The specific growth rate (μ) of the *Chloroparva pannonica* cultures were calculated based on the following equation:

$$\mu = \frac{In\ \chi 2 - In\ \chi 1}{t2 - t1}$$

where $\chi 2$ and $\chi 1$ represent the dried weight (g/L) at times $t2$ and $t1$.

6.2.2.3.3 Biomass productivity

The volumetric biomass productivity ($P_{biomass}$) (g/l/d) of the *Chloroparva pannonica* cultures was calculated based on the following equation:

$$P_{biomass} = \frac{\chi 2 - \chi 1}{t2 - t1}$$

where $\chi 2$ and $\chi 1$ represent the dried weight (g/L) at times $t2$ and $t1$.

6.2.2.3.4 pH measurements

The measurements of pH were taken using an Orion™ 9102BNWP combination pH electrode (ThermoFisher Scientific).

6.2.2.3.5 Extraction and analysis of lutein

Lutein was extracted based on the method described in subsection 2.1.1.3.2. The lutein extract was analysed by HPLC based on the method described in subsection 2.1.1.5 and subsection 2.1.1.6.

6.2.2.3.6 Lutein production rate

The lutein productivity (P_{lutein}) (mg/l/d) of the *Chloroparva pannonica* cultures was calculated based on the following equation:

$$P_{lutein} = \frac{\chi 2 - \chi 1}{t2 - t1}$$

where $\chi 2$ and $\chi 1$ represent the lutein content (mg/kg of biomass) at times $t2$ and $t1$.

6.2.2.3.7 Total lipid analysis

The total lipids were extracted and analysed based on the method described in Section 2.2.4, with this carried out in triplicate.

6.2.2.3.8 Lipid productivity

The lipid productivity (P_{lipid}) (% g/l/d) of the *Chloroparva pannonica* cultures was calculated based on the following equation:

$$P_{lipid} = P_{biomass} \times \% \, Lipid$$

where $P_{biomass}$ represents the volumetric biomass productivity and % lipid represents the total lipid content.

6.2.3 Cultivation of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor

The cultivation of *Chloroparva pannonica* Cu40 PC, WT and Fu5C in a semi-continuous system was to investigate the probability of enhancing lutein content and productivity while maintaining a high biomass concentration. The system was initially operated in an 11 L rectangular inter-loop airlift photobioreactor. *Chloroparva pannonica* Cu40 PC, WT and Fu5C were grown in 3 L flasks, with 1% carbon dioxide supplied, for 10 days. To confirm that each culture was homogeneous and free of contaminating algae, they were all examined under a microscope. A 4.4 L sample of day 10 culture with an equal number of cells (OD of 0.7 at 685 nm) was inoculated into 6.6 L of sterile FU medium in an 11 L rectangular inter-loop airlift photobioreactor at 22–25°C with light intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination, with 1% (v/v) carbon dioxide supplied, following a 12:12-h light: dark photoperiod. *Chloroparva pannonica* Cu40 PC, WT and the mutant strains were grown in triplicate.

The *Chloroparva pannonica* Cu40 PC, WT and Fu5C were grown for 14 days to achieve highest biomass concentration, then 3 L of culture was harvested and the biomass processed. The photobioreactor was topped up with 3 L of fresh sterile seawater: deionized water (1:1 v/v). The nutrient stock solutions of FU medium were then added to the system with the final nutrient concentration adjusted to the standard FU medium. Biomass and lutein levels were monitored and an 8 day draw and fill cycle was found to achieve the highest lutein levels together with high biomass levels. This was repeated over a 94-day period for WT and Fu5C and for a 190-day period for Cu40 PC. Samples were collected during the period of growth for total dried biomass, pH measurement, lutein extraction and lipid extraction.

6.2.3.1 Analytical methods

Measurement of total dried biomass, specific growth rate, pH, and lutein and lipid extraction, and analysis of lutein and total lipids were based on the method described in subsection 6.2.2.3.

6.2.3.2 Culture harvesting and drying

Three L of culture of *Chloroparva pannonica* Cu40 PC, WT and Fu5C were harvested manually every eight days from the outlet at the bottom of each column. The cells were harvested by continuous centrifugation at a force of 10,000 g for 10 minutes using a benchtop centrifuge (Allegra® X-12, Beckman Coulter). Liquid nitrogen (-196°C) was used for rapid freezing of the cells and freeze drying was undertaken using a freeze dryer (VirTis BenchTop Manifold Freeze Dryer, SP Scientific). The dried biomass was crushed using a mortar and pestle and stored in dark in a desiccator at -80°C, to avoid moisture absorption.

6.2.4 Extraction, purification and identification of lutein

The harvested and freeze-dried *Chloroparva pannonica* Cu40 cultivated in a FU medium in an 11 L rectangular inter-loop airlift photobioreactor and harvested on day 46 with a lutein content of 5702 mg/kg of biomass was used in this part of the study. The extraction of lutein was modified based on the method described by Deenu *et al.* (2013). For comparison, lutein was also extracted using a small amount of biomass,

based on the One step closed tube method described in subsection 2.1.1.3.2. All extraction and purification were carried out in triplicate in dark conditions.

6.2.4.1 Enzymatic pre-treatment and ultrasound extraction

The dried biomass was crushed using a mortar and pestle. A 4.5 g amount of *Chloroparva pannonica* Cu40 was mixed with 1.23% (v/w) of Viscozyme® (Sigma-Aldrich) and 145 ml of 0.1 M acetate buffer (pH 4.5). The mixture was then incubated at 50°C for two hours. The extract was filtered through Whatman® No. 2 paper.

The *Chloroparva pannonica* Cu40 residue on the Whatman® No. 2 paper was collected and 400 ml of 90% acetone was added. The mixture was then sonicated at 35 kHz at 4°C for two hours. The extract was again filtered through Whatman® No. 2 paper with the filtrate stored at 4°C in dark conditions for further analysis.

6.2.4.2 Purification of lutein

The *Chloroparva pannonica* Cu40 filtrate extract (400 ml) was saponified by adding 6% potassium hydroxide (KOH) (w/v) at 50°C for 30 minutes. The mixture was then evaporated using a rotary evaporator. The dried solid was dissolved in 400 ml of distilled water and partitioned with 400 ml of ethyl acetate. The 400 ml of ethyl acetate fraction was collected and evaporated using a rotary evaporator. The dried solid was redissolved with 5 ml of hexane.

A 2 ml amount of the hexane crude extract was loaded onto a silica gel column (2.0 cm x 60.0 cm) with activated silica gel 60 (#115111, Merck) and eluted with 150 ml of hexane, followed by 400 ml of hexane: acetone (7:3 v/v). The purified compound fraction was collected, evaporated by rotary evaporator, dried by nitrogen and stored at -20°C in dark conditions for further analysis.

6.2.4.3 Identification and quantification of purified compound

6.2.4.3.1 Thin layer chromatography

A volume of 2 µl of the different compound fraction was spotted onto silica gel 60 TLC sheet (Merck). One µl 1% lutein standard solution (w/v) was also spotted. The buffer system used was hexane: ethyl acetate (7:3) and equilibrated in the TLC tank for 1 hour

before developing TLC. The plate was run in the buffer system until solvent system reached 1.5cm from the top of the TLC plate in dark.

6.2.4.3.1.2 Analysis of lutein content using high-performance liquid chromatography (HPLC)

The crude extract and purified lutein from the silica gel column were analysed by high-performance liquid chromatography (HPLC) based on the method described in subsections 2.1.1.5 and 2.1.1.6.

6.2.5 Pyrosequencing-based assessment of bacteria communities in *Chloroparva pannonica* Cu40 cultivated in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor

6.2.5.1 Sample collection

Chloroparva pannonica Cu40 samples were collected manually from the 11 L rectangular inter-loop airlift photobioreactor at day 14, 15, 16, 18, 20 and 22. The samples for pyrosequencing were collected after the 3 L of cells were harvested at day 14, and the remaining culture was diluted by adding 3 L of fresh sterile seawater: deionized water (1:1 v/v).

6.2.5.2 Total genomic DNA extraction

A 200 ml amount of cells was harvested through being centrifuged at 10,000 rpm for 10 minutes. The pellet was washed three times with sterile distilled seawater: deionized water (1:1 v/v). The microbial genomic DNA was isolated using a PowerSoil DNA Isolation Kit (#12888-50, MO BIO Laboratories, a Qiagen Company, Carlsbad, CA, USA). The presence of genomic DNA was confirmed by running the extracted DNA on a 1% DNA grade agarose (Quantum Scientific, Queensland, Australia) stained with GelRed™ (Biotium Inc, Fremont, CA, USA) for visualization. The size standard used was a 1 kb DNA Ladder Molecular Weight Marker (Promega™, Wis, USA). The concentration of genomic DNA was quantified by using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

6.2.5.3 Bacteria 16S rRNA pyrosequencing

Pyrosequencing using bacterial 16S ribosomal RNA (rRNA) gene amplification, cloning and sequencing of the polymerase chain reaction (PCR) products was performed as part of a service provided by Macrogen (South Korea). To sort the raw 16S rRNA gene sequence obtained from pyrosequencing, trimBarcode.pl Perl scripts

(Macrogen, South Korea) were used to discard any that were of low quality or that had a short sequence.

6.3 Results

6.3.1 Evaluation of *Chloroparva pannonica* Cu40 PC, WT and selected mutant strains cultivated in 11 L rectangular inter-loop airlift photobioreactor

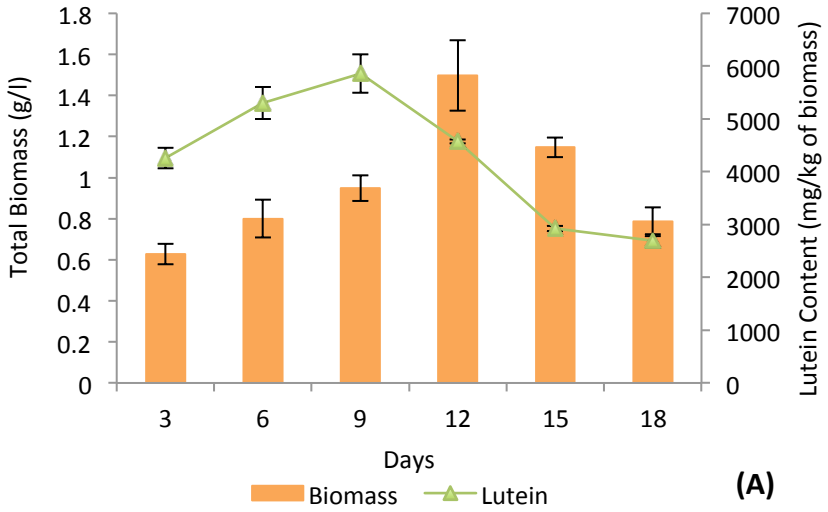
The microalgae in this part of the study were the *Chloroparva pannonica* Cu40 PC, WT and mutant strains listed in Table 6.1 which were used to select the most suitable microalgal strains for biomass, lutein and lipid production when grown in a large-scale cultivation set-up. The starting culture used for this experiment was from the exponential growth (day 10) of *Chloroparva pannonica* Cu40, WT and selected mutant strains cultivated in 3 L flasks with 1% carbon dioxide supplied. The cells were grown in an 11 L rectangular inter-loop airlift photobioreactor for an 18-day period. They were harvested at day 3, 6, 9, 12, 15 and 18 to determine the total biomass (g/l) and lutein content (mg/kg of biomass). Total lipids were determined from the cells harvested at the end of the growth phase (day 18). The culture's pH ranged between 7 and 8 during the growth phase.

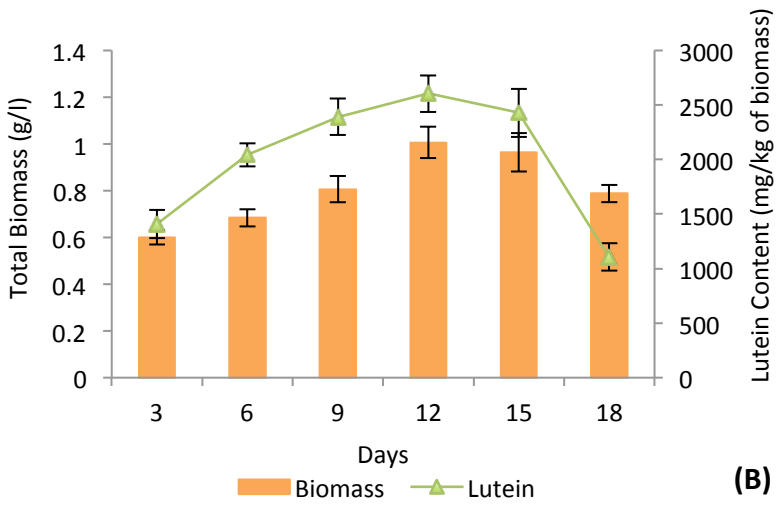
6.3.1.1 Total dried biomass and lutein content of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains

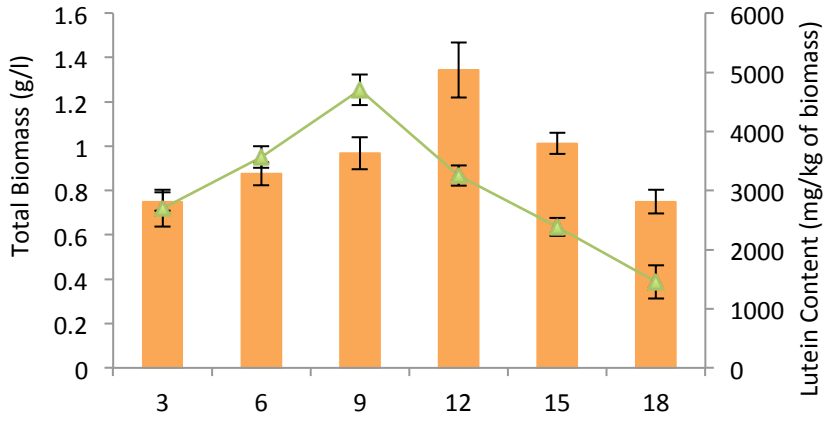
The total biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* Cu40 PC and WT were compared with the 10 selected mutants are shown in Figure 6.4 and Figure 6.5. The cell growth of *Chloroparva pannonica* Cu40 PC, WT and mutant strains increased from day 3, reaching the highest point on different days for each group before it started to enter the death phase. Eight *Chloroparva pannonica* strains (Cu40 PC, WT, Fu4, Fu5C, Fu469A, Fu48A, Fu58A and Fu41A), two strains (Fu10A and Fu58A) and two strains (Fu305C and Fu58C) reached the highest growth at day 12, day 15 and day 18, respectively, (Figure 6.4 and Figure 6.5).

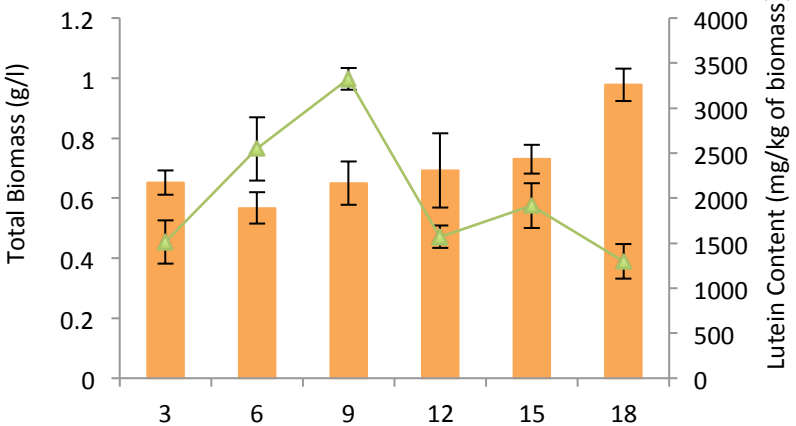
The lutein content of *Chloroparva pannonica* in general, was higher at the earlier phase of the growth, usually before the highest amount of biomass was reached. Four *Chloroparva pannonica* strains (Fu305C, Fu5C, Fu10A and Fu469A) achieved the highest point of lutein content at day 6 (Figure 6.4 and Figure 6.5). Five strains (Cu40 PC, Fu4, Fu142, Fu48A and Fu41A) and three strains (WT, Fu58C and Fu58A) reached

the highest lutein content at day 9 and day 12, respectively (Figure 6.4 and Figure 6.5). Among the *Chloroparva pannonica* strains, Fu5C was the only strain that reached the highest growth at day 12 and the highest lutein content at day 6 (Figure 6.4).


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(A)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(B)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)

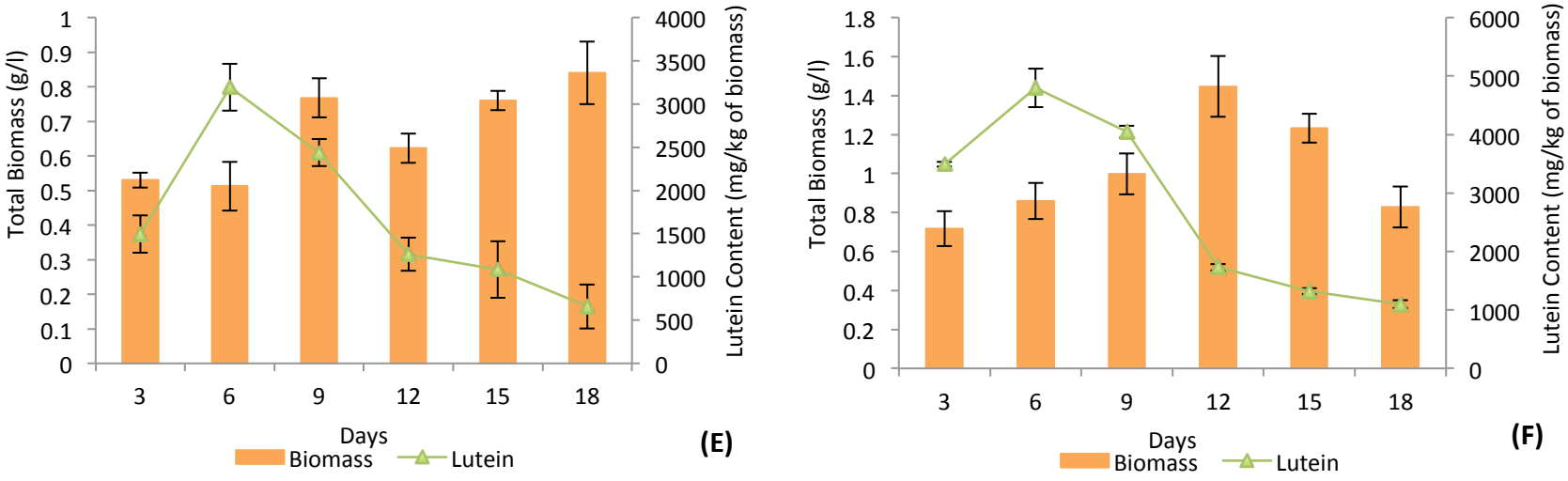
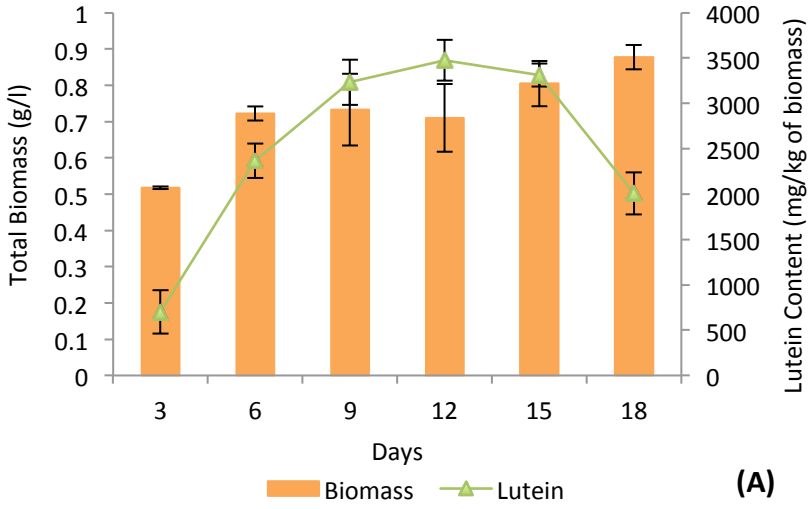
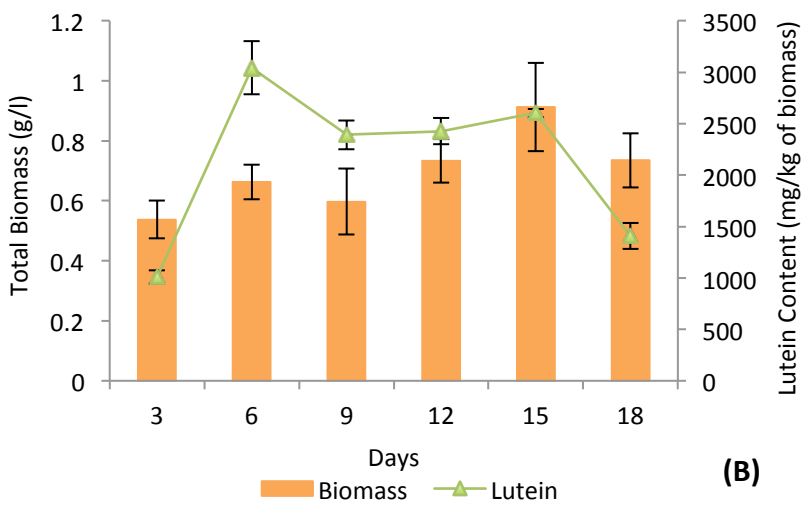
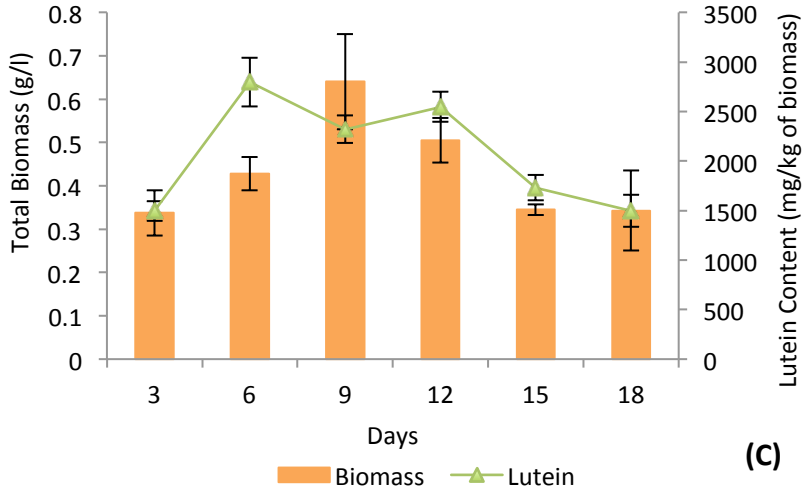
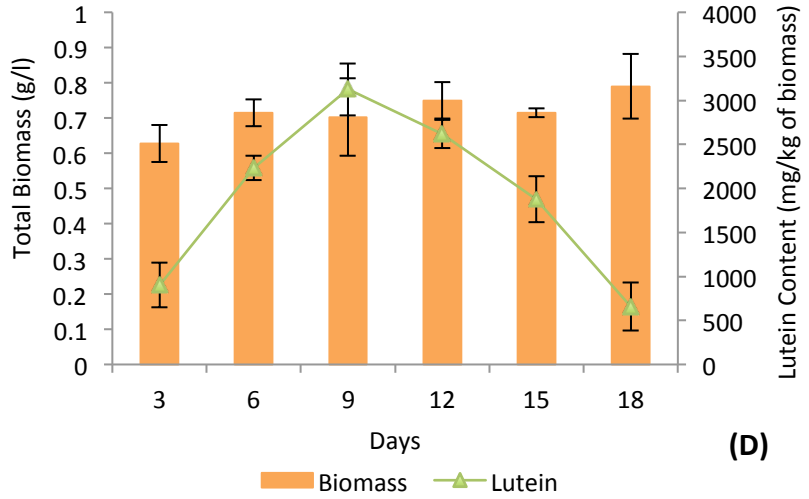


Figure 6.4 Growth and lutein content accumulation for six *Chloroparva pannonica* strains: (A) Cu40 PC; (B) WT; (C) Fu4; (D) Fu142; (E) Fu305C; and (F) Fu5C in an 18-day cultivation period in 11 L rectangular inter-loop airlift photobioreactor


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(A)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(B)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(C)


Total Biomass (g/l)
Lutein Content (mg/kg of biomass)
Days
Biomass
Lutein
(D)

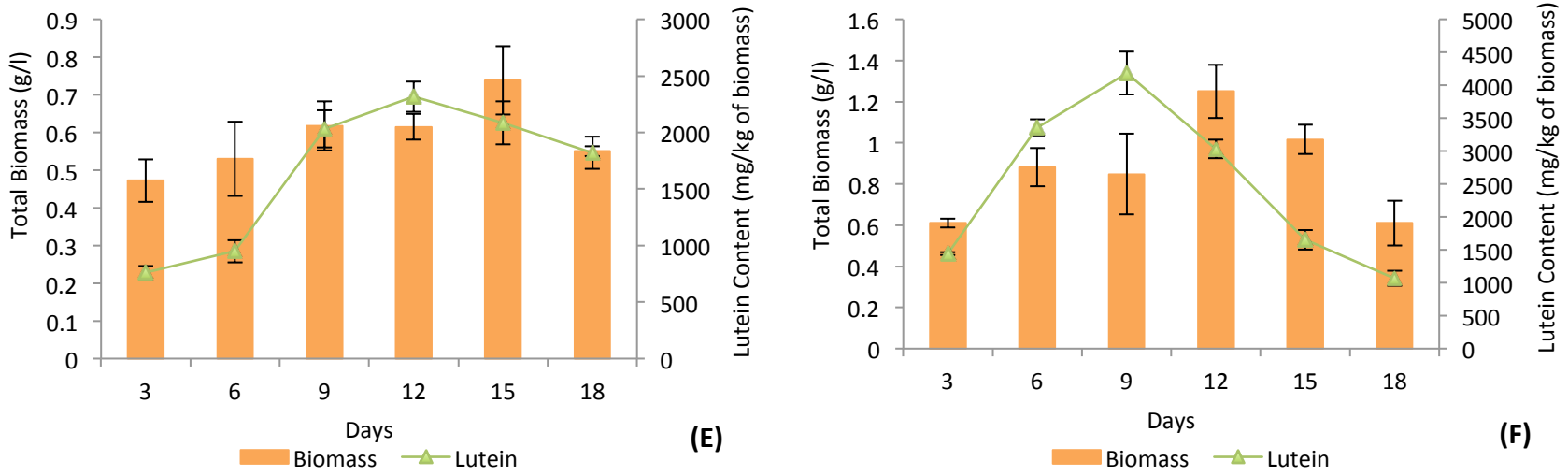


Figure 6.5 Growth and lutein content accumulation for six *Chloroparva pannonica* strains: (A) Fu58C; (B) Fu10A; (C) Fu469A; (D) Fu48A; (E) Fu55A; and (F) Fu41A in 11 L rectangular inter-loop airlift photobioreactor in an 18-day period

6.3.1.2 Maximum biomass, biomass productivity and specific growth rate

The specific growth rate (μ) of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains is shown in Figure 6.6. The specific growth rate per day ranged between 0.125 and 0.246. The specific growth rate for Cu40 PC was 0.246 per day while, for WT, it was 0.209 per day. The strains that were identified as having the highest specific growth rate were Cu40 PC, WT, Fu4, Fu5C and Fu41A.

Figure 6.7 shows the maximum total biomass (g/l) and biomass productivity (g/l/d) of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains. The maximum total biomass ranged between 0.505 g/l and 1.498 g/l while biomass productivity ranged between 0.037 g/l/d and 0.127 g/l/d. The maximum total biomass for Cu40 PC was 1.498 g/l while, for WT, it was 1 g/l: biomass productivity for Cu40 PC was 0.127 g/l/d while, for WT, it was 0.082 g/l/d.

Cu40 PC, Fu4, Fu5c and Fu41A were identified as fast-growing strains with the highest maximum total biomass and highest biomass productivity. Cu40 PC achieved the maximum total biomass of 1.498 g/l; for Fu4, it was 1.344 g/l; for Fu5C, it was 1.446 g/l; and for Fu41A, it was 1.25 g/l. Biomass productivity of Cu40 PC was calculated as 0.127 g/l/d; for Fu4, it was 0.113 g/l/d; for Fu5C, it was 0.122 g/l/d; and for Fu41A, it was 0.105 g/l/d. It was noted that WT had a high specific growth rate but the maximum total biomass and biomass productivity were low.

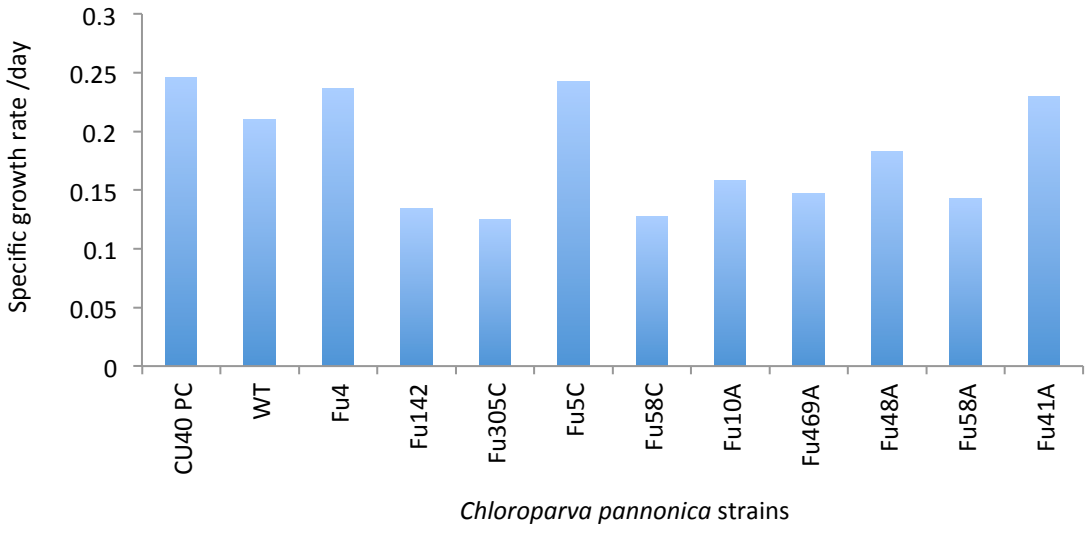


Figure 6.6 Specific growth rates per day of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains

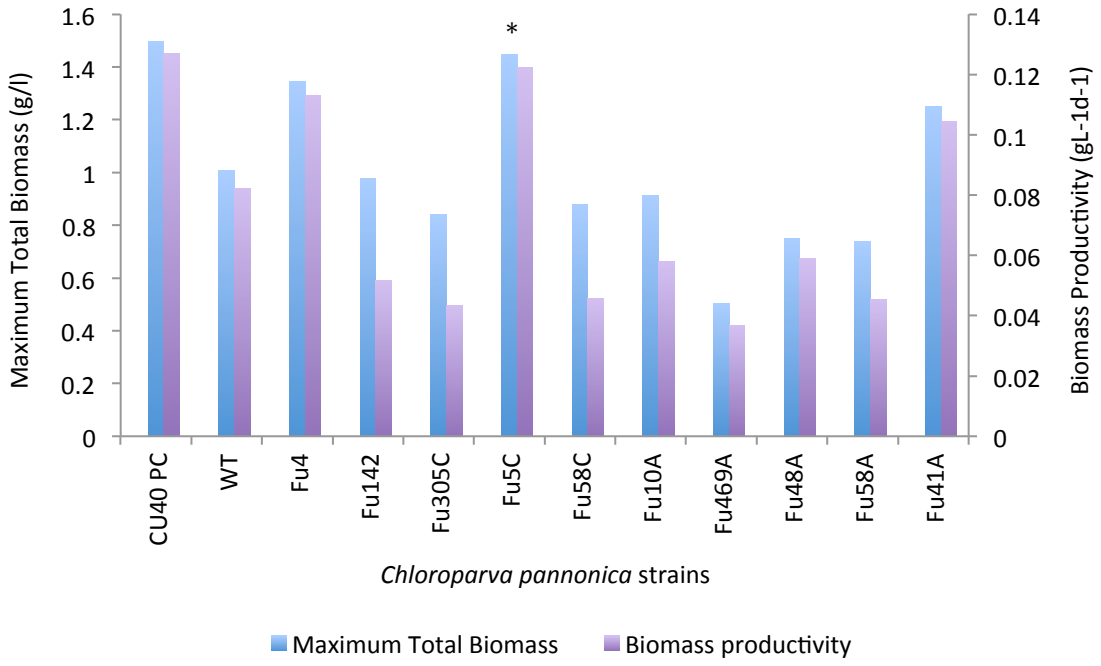


Figure 6.7 Average maximum total biomass (g/l) and biomass productivity (g/l/d) of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains

* Significant at P<0.05
Note: The maximum total biomass ranged between 0.505 g/l and 1.498 g/l while biomass productivity ranged between 0.037 g/l/d and 0.127 g/l/d.

6.3.1.3 Maximum lutein content and lutein productivity

Figure 6.8 shows the maximum lutein content (mg/kg of biomass) and lutein productivity (mg/kg of biomass/day). The maximum lutein content ranged between 2317 mg/kg and 5859 mg/kg of biomass while lutein productivity ranged between 165 mg/kg and 859 mg/kg of biomass/day. The maximum lutein content for Cu40 PC was 5859 mg/kg of biomass while, for WT, it was 2602 mg/kg of biomass: lutein productivity for Cu40 PC was 669 mg/kg of biomass/day while, for WT, it was 191 mg/kg of biomass/day.

Strains with the highest lutein content were Cu40 PC (5859 mg/kg of biomass), followed by Fu5C (4794 mg/kg of biomass), Fu4 (4701 mg/kg of biomass) and Fu41A (4185 mg/kg of biomass). In addition, the lutein productivity of Cu40 PC was calculated as being 669 mg/kg of biomass/day; for Fu4, it was 525 mg/kg of biomass/day; for Fu5C, it was 859 mg/kg of biomass/day; and for Fu305C, it was 539 mg/kg of biomass/day.

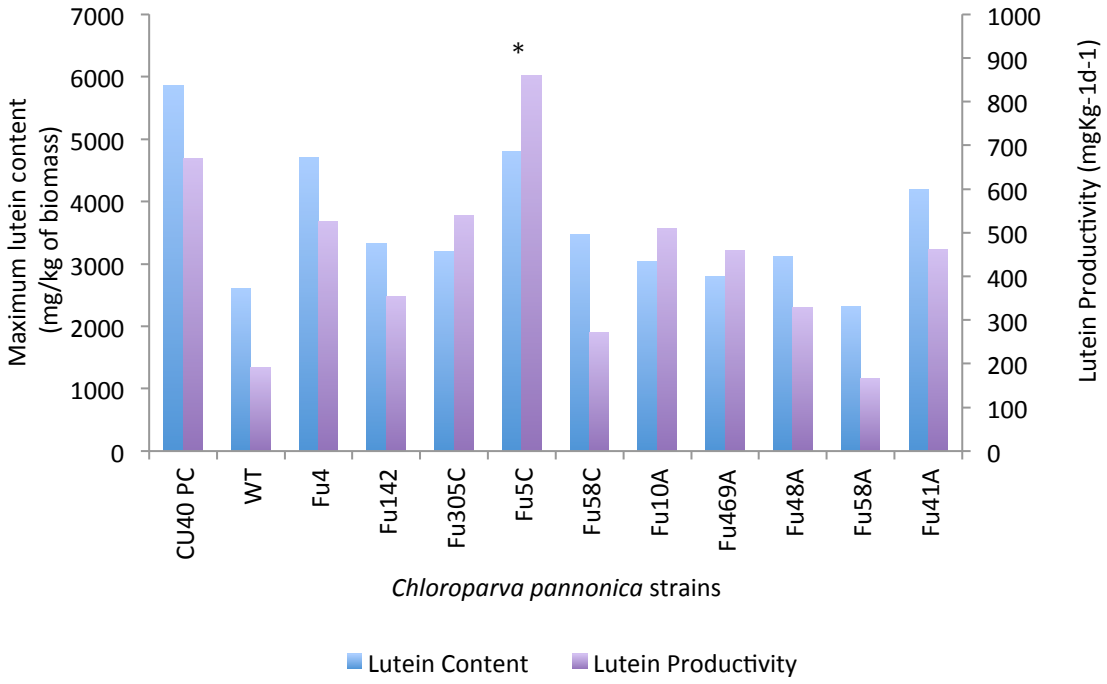


Figure 6.8 Average maximum lutein content (mg/kg of biomass) and lutein productivity (mg/kg of biomass/day) of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains

* Significant at P<0.05

Note: The maximum total lutein content ranged between 2317 mg/kg and 5859 mg/kg of biomass while lutein productivity ranged between 165 mg/kg and 859 mg/kg of biomass/day.

6.3.1.4 Total lipids and lipid productivity

Figure 6.9 presents the total lipids and lipid productivity. The total lipid percentage (%) ranged between 10% and 20% while the lutein productivity ranged between 0.5% and 2.4%/day. For Cu40 PC, the lipid content was 19% with a lipid productivity of 2.5%/day while, for WT, the lipid content was 18% with a lipid productivity of 1.48%/day. Strains with the highest lipid content were Fu5C (20%), Cu40 PC (19%) and Fu58C (19%), while strains with the highest lipid productivity were Cu40 PC (2.4%/day), Fu5C (1.9%/day) and Fu4 (1.69%/day).

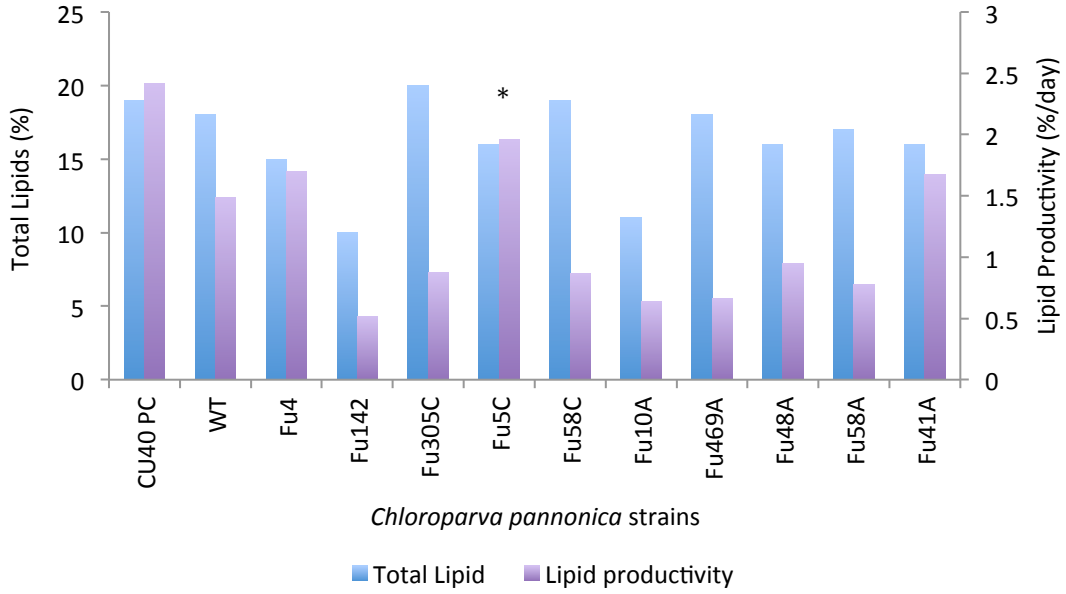


Figure 6.9 Average total lipids (%) and lipid productivity (%/d) of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains at end of growth phase

* Significant at P<0.05
Note: The total lipid percentage (%) ranged between 10% and 20% while lutein productivity ranged between 0.5%/day and 2.4%/day.

6.3.1.5 Identification of candidate biomass and lutein producers for cultivation in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

The overall results of lutein productivity and biomass productivity obtained from *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains cultivated in the 11 L rectangular inter-loop airlift photobioreactor are shown in Figure 6.10. One mutant, Fu5C (circled with green line), had a higher lutein productivity than Cu40 PC. A group of mutants (circled with purple line) had a higher lutein productivity compared to WT but lower than Cu40 PC. Two mutants, Fu4 and Fu41A (circled with orange dashed line), had higher biomass productivity and lutein productivity than WT but lower biomass productivity and lutein productivity than Cu40 PC.

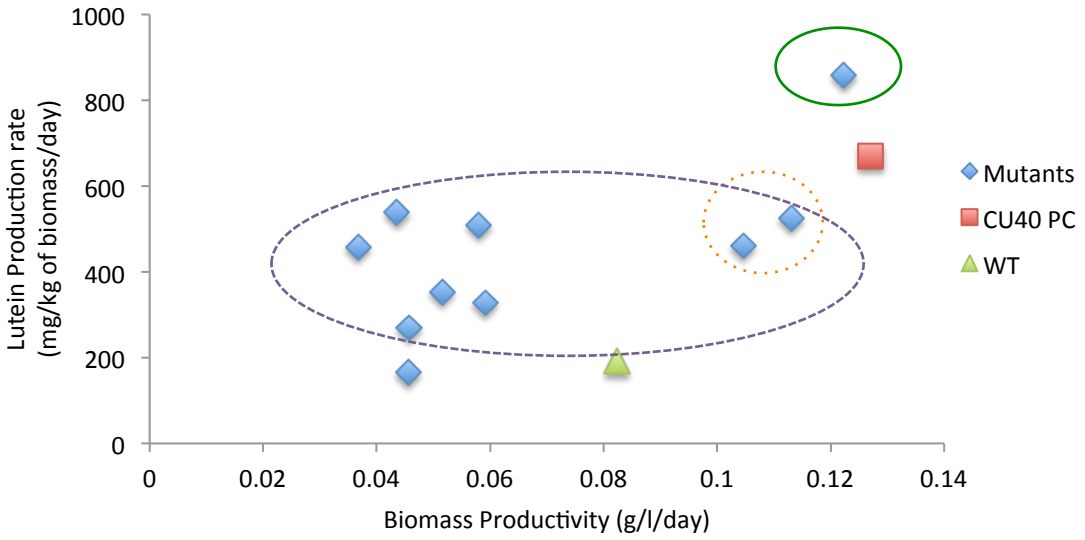


Figure 6.10 Lutein productivity and biomass productivity of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains in 11 L rectangular inter-loop airlift photobioreactor

The overall results of lipid productivity and biomass productivity obtained from *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains cultivated in an 11 L rectangular inter-loop airlift photobioreactor are shown in Figure 6.11. As shown, Cu40 PC had the highest biomass productivity and lipid productivity compared to WT and mutants. However, three mutants, Fu5C, Fu4 and Fu41A (circled with orange dashed line), had higher biomass productivity and lipid productivity than WT but lower biomass productivity and productivity than Cu40 PC. The rest of the mutants (circled with purple line) had lower biomass productivity and lipid productivity than Cu40 PC and WT. In order to determine the significance differences between the mutant strains, biomass, lipid and lutein content of each mutant are then paired for t-test. The results show that Fu5C is the only strain that show significant positive relation among the others (Figure 6.7 - Figure 6.9).

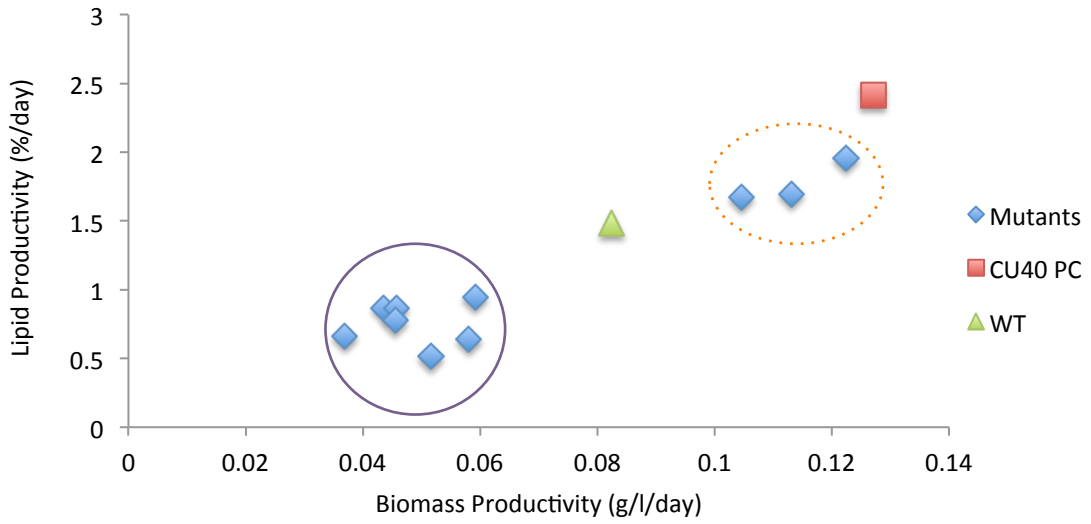


Figure 6.11 Lipid productivity and biomass productivity of *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains in 11 L rectangular inter-loop airlift photobioreactor

Based on its high lutein productivity, biomass productivity and lipid productivity, Fu5C was identified as the strain for cultivation in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor in the next part of the study. The characteristics of Fu5C, Cu40 PC and WT are summarized in Table 6.2.

Table 6.2 Characteristics of *Chloroparva pannonica* Cu40 PC, WT and Fu5C cultivated in 11 L rectangular inter-loop airlift photobioreactor for an 18-day period

Component	Cu40 PC	WT	Fu5C
Maximum total dried biomass (g/l)	1.498	1.006	1.446
Biomass productivity (g/l/day)	0.127	0.082	0.122
Specific growth rate, (μ/day)	0.246	0.210	0.243
Lutein content (mg/kg of dried biomass)	5859.031	2602.938	4794.594
Lutein productivity (mg/kg of biomass/day)	669.879	191.176	858.919
Total lipids (%)	19	18	16
Lipid productivity (%/day)	2.415	1.483	1.958

6.3.2 Cultivation of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains in semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor for a 94-day period

The batch cultivation, although it was able to provide a relatively high biomass and lutein content, often suffered from an initially slow growth regime, especially the loss

of the time that occurred during the final photobioreactor cleansing. The cultivation of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains in a semi-continuous system was to improve the lutein content and productivity while maintaining the biomass at a high concentration.

6.3.2.1 Evaluation of growth and lutein content for first 30 days of cultivation period

This section focuses on the selection of the time period for the semi-continuous mode. Figure 6.12 shows the biomass and lutein content of *Chloroparva pannonica* Fu5C for the first 14 days of growth. The highest biomass was achieved at day 12 (1.3896 g/l) which had decreased by day 14 to 1.03 g/l. The highest lutein content was achieved at day 6 (4951 mg/kg of biomass) and then decreased to 1024 mg/kg at day 14.

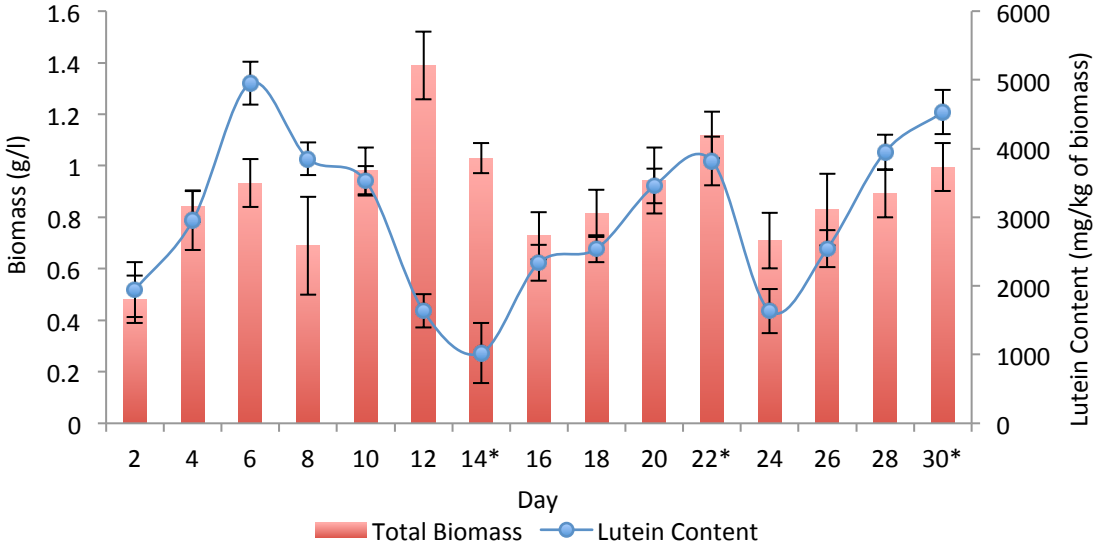


Figure 6.12 Total dried biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* Fu5C cultivated in 11 L rectangular inter-loop airlift photobioreactor for a 14-day period, then harvested every eight days

*Note: The 3 L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium. * = harvest day*

Figure 6.13 shows the biomass and lutein content of *Chloroparva pannonica* Cu40 PC for the first 14 days of growth. The highest biomass was achieved at day 12 (1.46 g/l) which had decreased at day 14 (1.29 g/l). The lutein content at day 2 was 5132 mg/kg of biomass, which increased slowly to day 6 (5356 mg/kg of biomass) and started to decrease from day 8, reaching the lowest level at day 14 (1503 mg/kg of biomass).

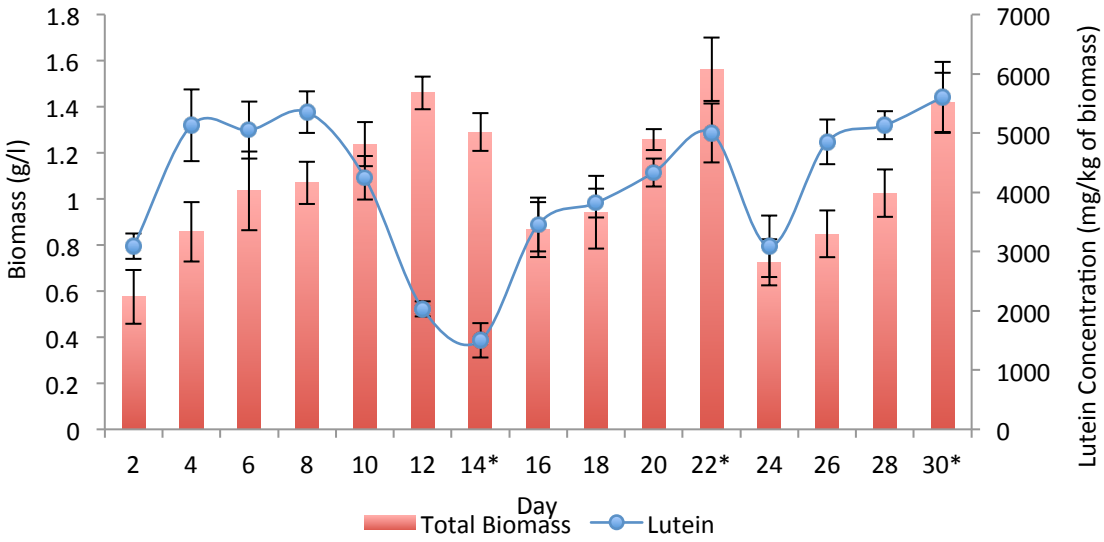


Figure 6.13 Total dried biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* Cu40 PC cultivated in 11 L rectangular inter-loop airlift photobioreactor for a 14-day period, then harvested every eight days

*Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium. * = harvest day*

Figure 6.14 shows the total biomass and lutein content of *Chloroparva pannonica* WT for the first 14 days of growth. The highest total biomass was achieved at day 12 (1.02 g/l) which had decreased at day 14 to 0.82 g/l. The lutein content at day 2 was 1744 mg/kg of biomass which had decreased to 1066 mg/kg of biomass at day 4. The lutein content then increased, reaching the highest point at day 8 (2583 mg/kg of biomass) and decreasing to 758 mg/kg of biomass at day 14.

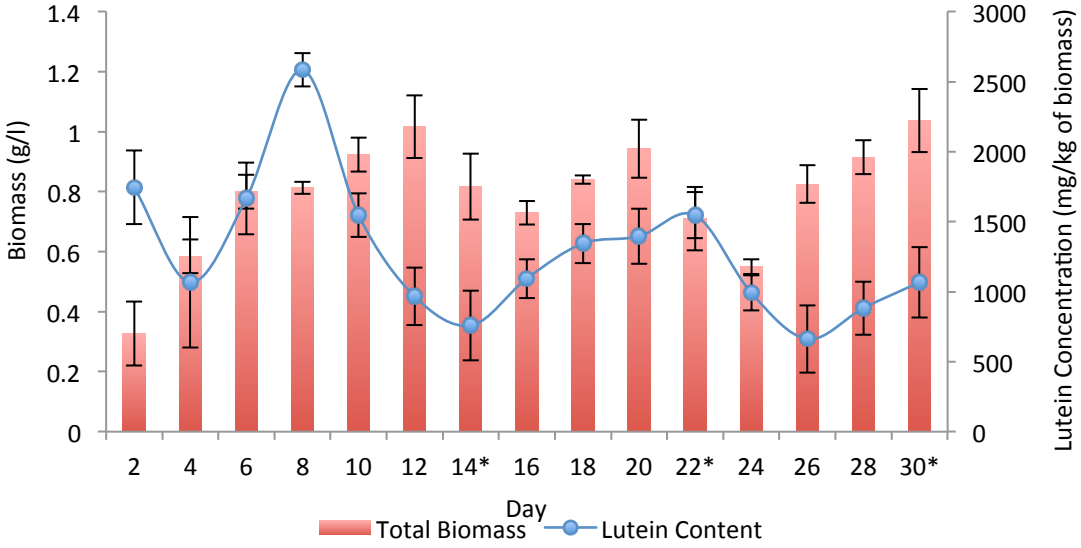


Figure 6.14 Total dried biomass (g/l) and lutein content (mg/kg of biomass) of *Chloroparva pannonica* WT cultivated in 11 L rectangular inter-loop airlift photobioreactor for a 14-day period, then harvested every eight days.

*Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium. * = harvest day*

A 3 L amount of culture was harvested at day 14 for *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains. After that, the system was switched into semi-continuous mode, in which the culture was harvested and refilled with fresh medium every eight days. As can be seen from Figure 6.12 to Figure 6.14, the total biomass decreased after cells were harvested from the culture which was then refilled with fresh medium. However, the total biomass and lutein content increased and reached the highest level on the same day, day 8, which was when the culture was ready to harvest.

6.3.2.2 Evaluation of growth and lutein content of *Chloroparva pannonica* Fu5C, Cu40 PC and WT cultivated in semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor for a 94-day period

6.3.2.2.1 Total biomass and productivity

The total biomass results when the cells were harvested at day 8 from the semi-continuous 11 L rectangular inter-loop airlift photobioreactor cultivation of *Chloroparva pannonica* Fu5C, Cu40 PC and WT are illustrated in Figure 6.15. Comparisons of the total biomass, biomass productivity and specific growth rates for the microalgae strains are shown in Table 6.3. The range of total biomass achieved during harvest for Fu5C was between 0.72 g/l and 1.125 g/l; for Cu40 PC, it was between 1.29 g/l and 1.678 g/l; and for WT, it was between 0.71 g/l and 1.193 g/l

(Figure 6.15 and Table 6.3). Cu40 PC was the strain with the highest total biomass, highest biomass productivity (0.150 g/l/day) and highest specific growth rate (0.482 µ/day).

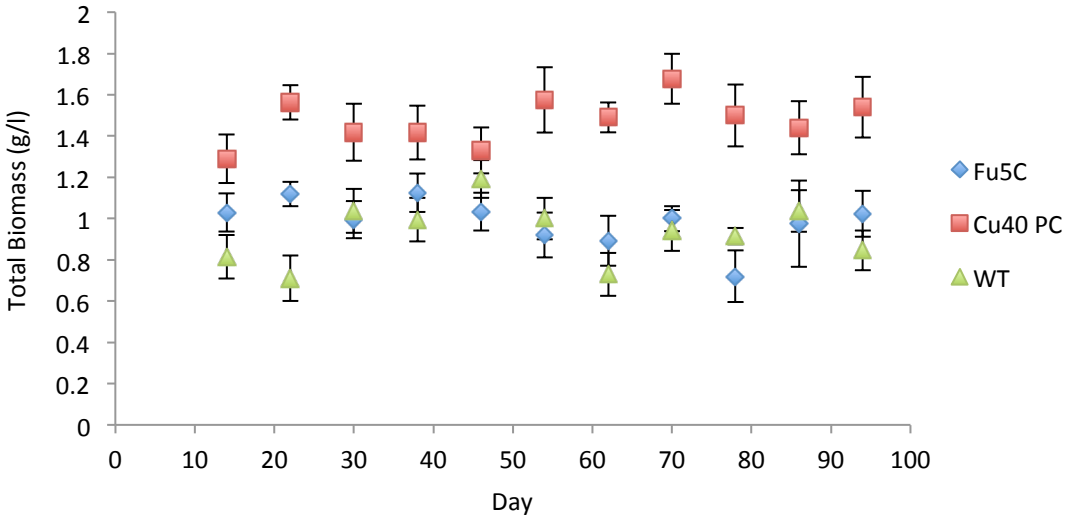


Figure 6.15 Total dried biomass (g/l) of *Chloroparva pannonica* Fu5C, Cu40 PC and WT cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 94-day period

Note The 3 L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

Table 6.3 Comparison between minimum, maximum and average total biomass, biomass productivity and specific growth rate of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains cultivated in semi-continuous system, harvested every eight days

Strains	Total biomass (g/l)			Biomass productivity (g/l/day)	Specific growth rate (µ/day)
	Minimum	Maximum	Average		
Fu5C	0.720	1.125	0.985	0.084	0.106
Cu40 PC	1.290	1.678	1.476	0.150	0.482
WT	0.710	1.193	0.929	0.100	0.113

6.3.2.2.2 Lutein content and productivity

Figure 6.16 presents the lutein content when the cells of *Chloroparva pannonica* Fu5C, Cu40 PC and WT were harvested at day 8 from the semi-continuous 11 L rectangular inter-loop airlift photobioreactor. Table 6.4 presents the comparisons of the lutein content and lutein productivity for the microalgae. For Fu5C, the lutein content ranged between 2951 mg/kg of biomass and 5001 mg/kg of biomass; for Cu40 PC, it ranged between 3990.5 mg/kg of biomass and 5048 mg/kg of biomass; and for WT, the lutein

content ranged between 1067 mg/kg of biomass and 1867 mg/kg of biomass (Figure 6.16 and Table 6.4). The strain with the highest lutein productivity was Cu40 PC (604.32 mg/kg of biomass/day), followed by Fu5C (467.6 mg/kg of biomass/day) and WT (198.4 mg/kg of biomass/day) (Table 6.4).

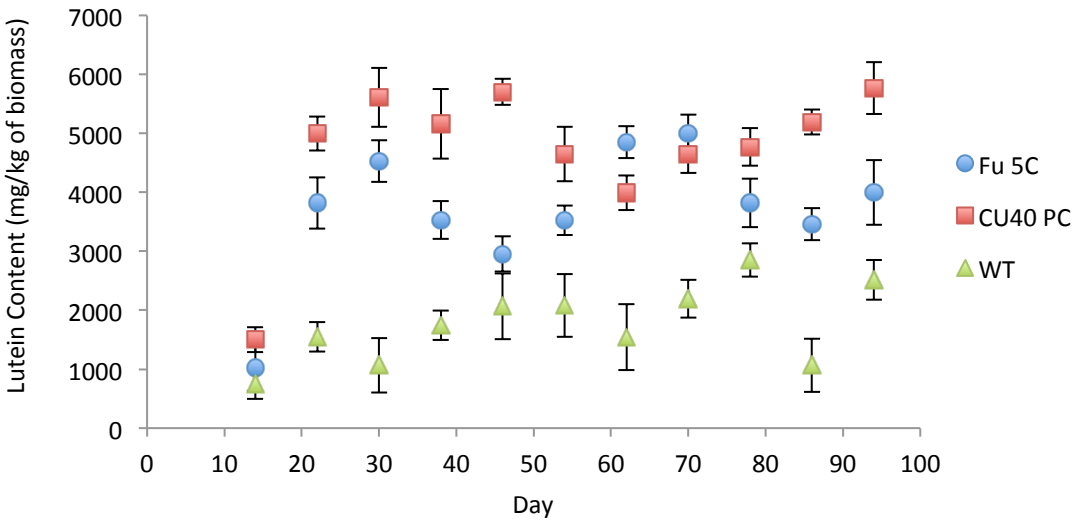


Figure 6.16 Lutein content (mg/kg of biomass) of *Chloroparva pannonica* Fu5C, Cu40 PC and WT cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 94-day period

Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

Table 6.4 Comparison between minimum, maximum and average lutein content and lutein productivity of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains cultivated in semi-continuous system, harvested every eight days

Strains	Lutein content (mg/kg of biomass)			Lutein productivity (mg/kg of biomass/day)
	Minimum	Maximum	Average	
Fu5C	2951.489	5001.290	3948.861	467.643
Cu40 PC	3990.501	5768.395	5048.919	604.320
WT	1066.745	2852.084	1867.191	198.365

6.3.2.2.3 Total lipid content and productivity

Figure 6.17 shows the lutein content of *Chloroparva pannonica* Fu5C, Cu40 PC and WT harvested at day 8 when cultivated in a semi-continuous 11 L rectangular inter-loop airlift photobioreactor. As shown in Figure 6.17 and Table 6.5, the range of lipid content for Fu5C was 11% to 19%; for Cu40 PC, it was 12% to 21%; and for WT, it

was from 11% to 19%. Cu40 PC had the highest lipid productivity at 2.4%, followed by WT (1.5%) and Fu5C (1.289%) (Table 6.5).

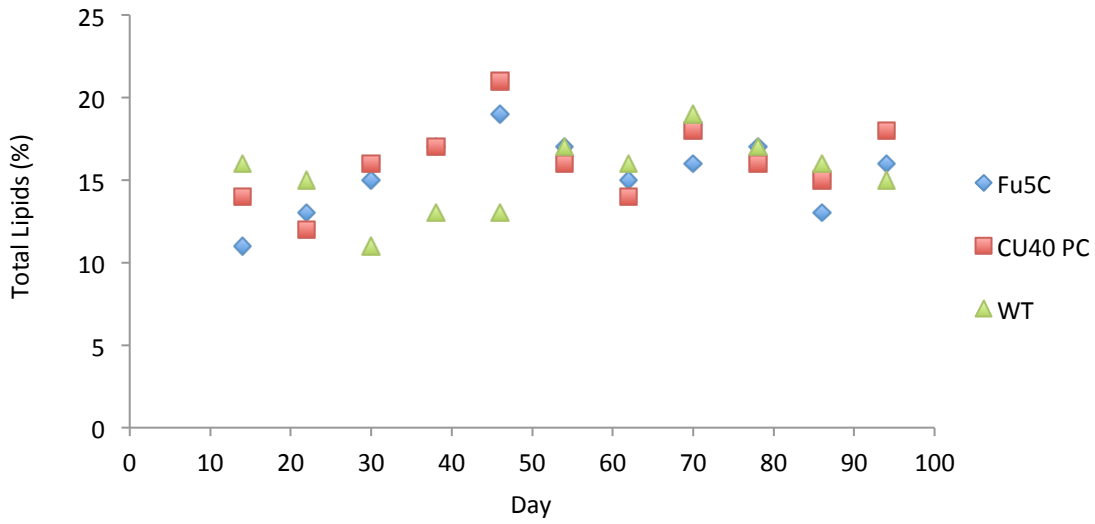


Figure 6.17 Total lipid content (%) of *Chloroparva pannonica* Fu5C, Cu40 PC and WT cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 94-day period

Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

Table 6.5 Comparison between minimum, maximum and average lipid content and lipid productivity of *Chloroparva pannonica* Cu40 PC, WT and Fu5C strains cultivated in semi-continuous system, harvested every eight days

Strains	Total lipids (%)			Total lipid productivity (%/day)
	Minimum	Maximum	Average	
Fu5C	11	19	15.364	1.289
Cu40 PC	12	21	16.091	2.418
WT	11	19	15.273	1.533

6.3.3 Cultivation of *Chloroparva pannonica* Cu40 PC in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor for a 190-day period

Chloroparva pannonica Cu40 PC was identified as the strain with the highest biomass productivity, lutein productivity and lipid productivity based on the results in these areas after cultivation in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor for 94 days (see Section 6.3.2), Therefore, the cultivation of the Cu40 PC was continued for 96 days in the semi-continuous mode, with the main reason

being to examine Cu40 PC's long-term performance in this system to see whether it was suitable for the growth, biomass production and lutein production of Cu40 PC.

6.3.3.1 Total dried biomass and biomass productivity

The results of total dried biomass from the cultivation of Cu40 PC in the semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor for a total of 190 days are presented in Figure 6.18. It was found the total biomass ranged from 1.29 g/l to 1.705 g/l (Figure 6.18). The biomass productivity was 0.1538 g/l/day while the specific growth rate was 0.4963 μ/day.

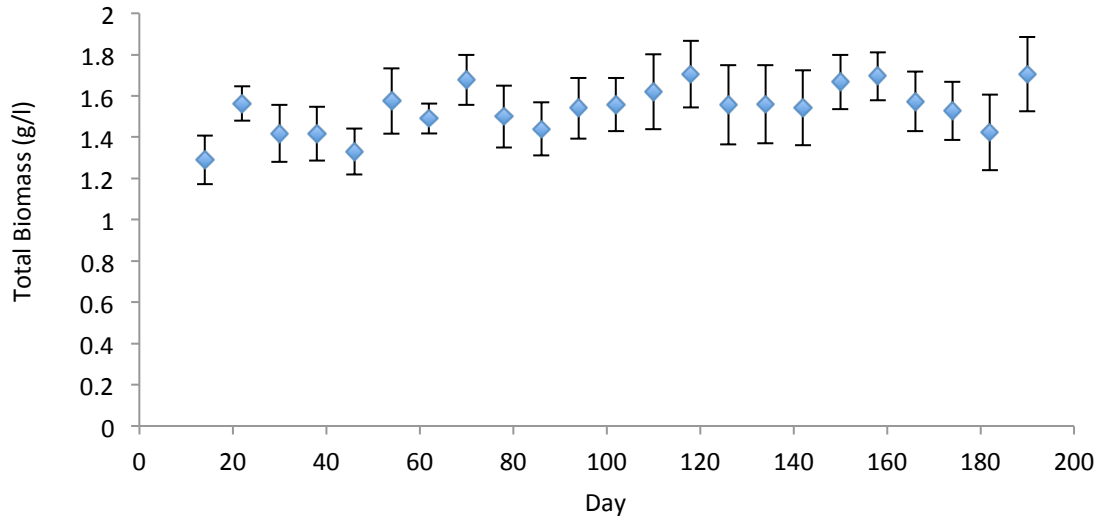


Figure 6.18 Total dried biomass (g/l) of *Chloroparva pannonica* Cu40 PC cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 190-day period

Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

6.3.3.2 Lutein content and lutein productivity

Figure 6.19 shows the lutein content from the cultivation of Cu40 PC in the semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor for a total of 190 days. The lutein content ranged from 3990 mg/kg of biomass to 6960 mg/kg of biomass. The lutein productivity was 613.4 mg/kg of biomass/day.

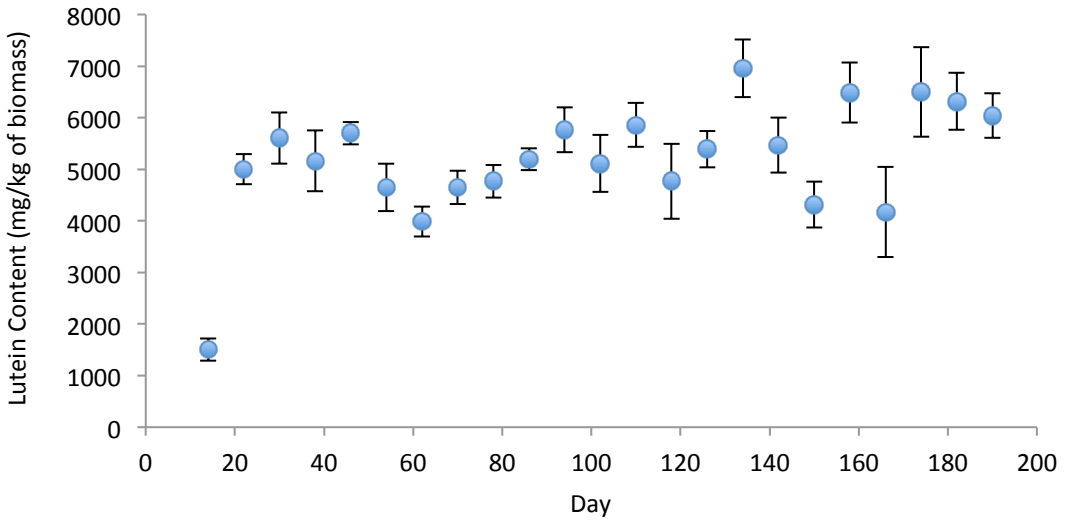


Figure 6.19 Lutein content (mg/kg of biomass) of *Chloroparva pannonica* Cu40 PC cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 190-day period

Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

6.3.3.3 Total lipids and lipid productivity

Figure 6.20 presents the total lipids of Cu40 PC cultivated in the semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor for a total of 190 days. The total lipids ranged from 10% to 22% while the lipid productivity was 2.5%/day.

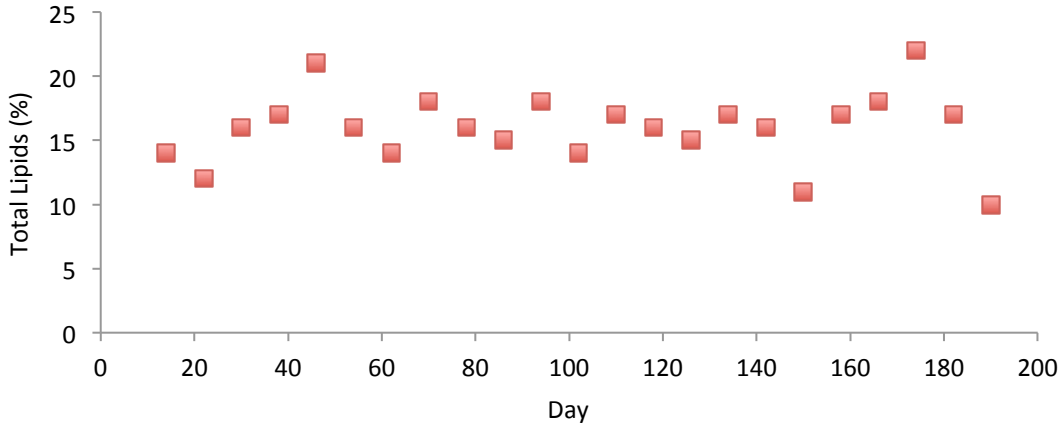


Figure 6.20 Total lipids (%) of *Chloroparva pannonica* Cu40 PC cultivated in 11 L rectangular inter-loop airlift photobioreactor and harvested every eight days for a 190-day period

Note: The 3L of fresh sterile seawater: deionized water (1:1 v/v) was replaced. The nutrient stock solution of the FU medium was then added to the system with the final nutrient concentration adjusted to the standard FU medium.

6.3.3.4 Extraction and purification of lutein

The harvested and freeze-dried *Chloroparva pannonica* Cu40 cultivated in the FU medium in an 11 L rectangular inter-loop airlift photobioreactor and harvested on day 46 with total biomass of 1.33g/l and lutein content of 5702 mg/kg of biomass was used in this part of the study. Figure 6.21 shows the elution of the lutein from the hexane crude extract loaded on the silica gel column. The fractions were collected in different tubes and analysed on a thin layer chromatography (TLC) plate. The bands for the fractions (Lanes 7–14) that match with the lutein standard (Lane 3), as shown in Figure 6.22, were combined, evaporated, dried by nitrogen and stored at -20°C for lutein analysis by high-performance liquid chromatography (HPLC) (Figure 6.23).

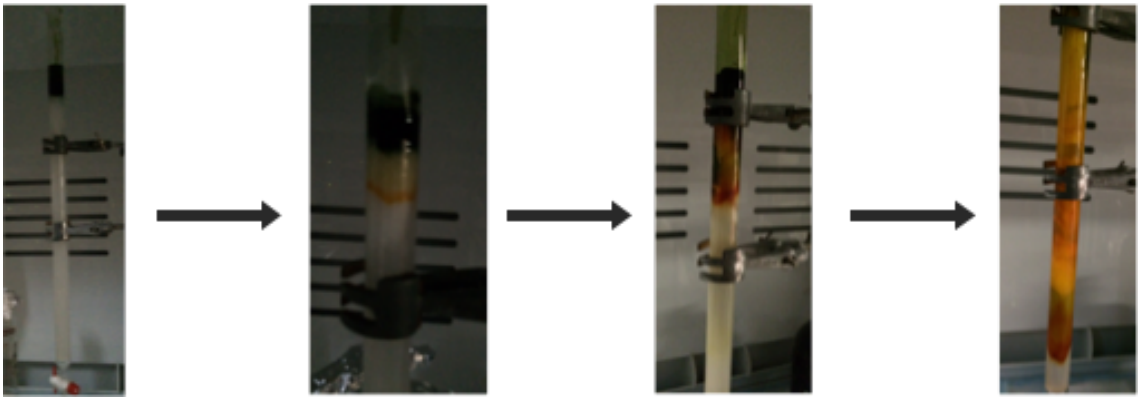


Figure 6.21 Hexane crude extract loaded on silica gel column (2.0 cm x 60 cm) and eluted with 150 ml of hexane, followed by 400 ml of hexane acetone (7:3 v/v)

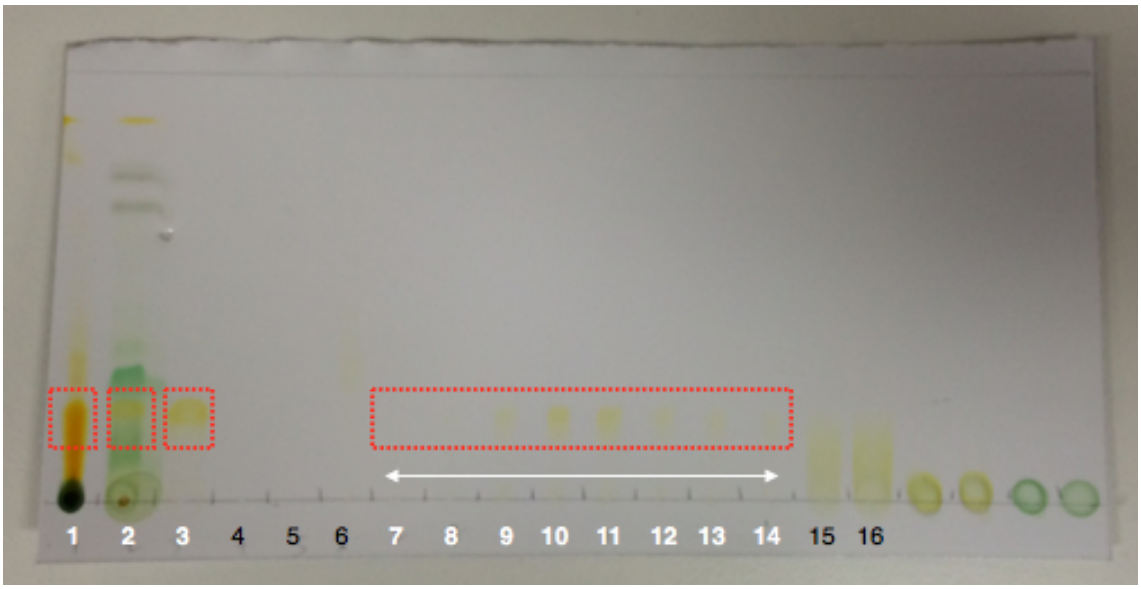


Figure 6.22 Thin layer chromatography of fractions collected from silica gel column compared to lutein standard: Lane 1, hexane crude extract; Lane 2, lutein extract extracted by one-step closed-tube (OSCT) method; Lane 3, lutein standard; and Lanes 7–14, fractions collected from silica gel column

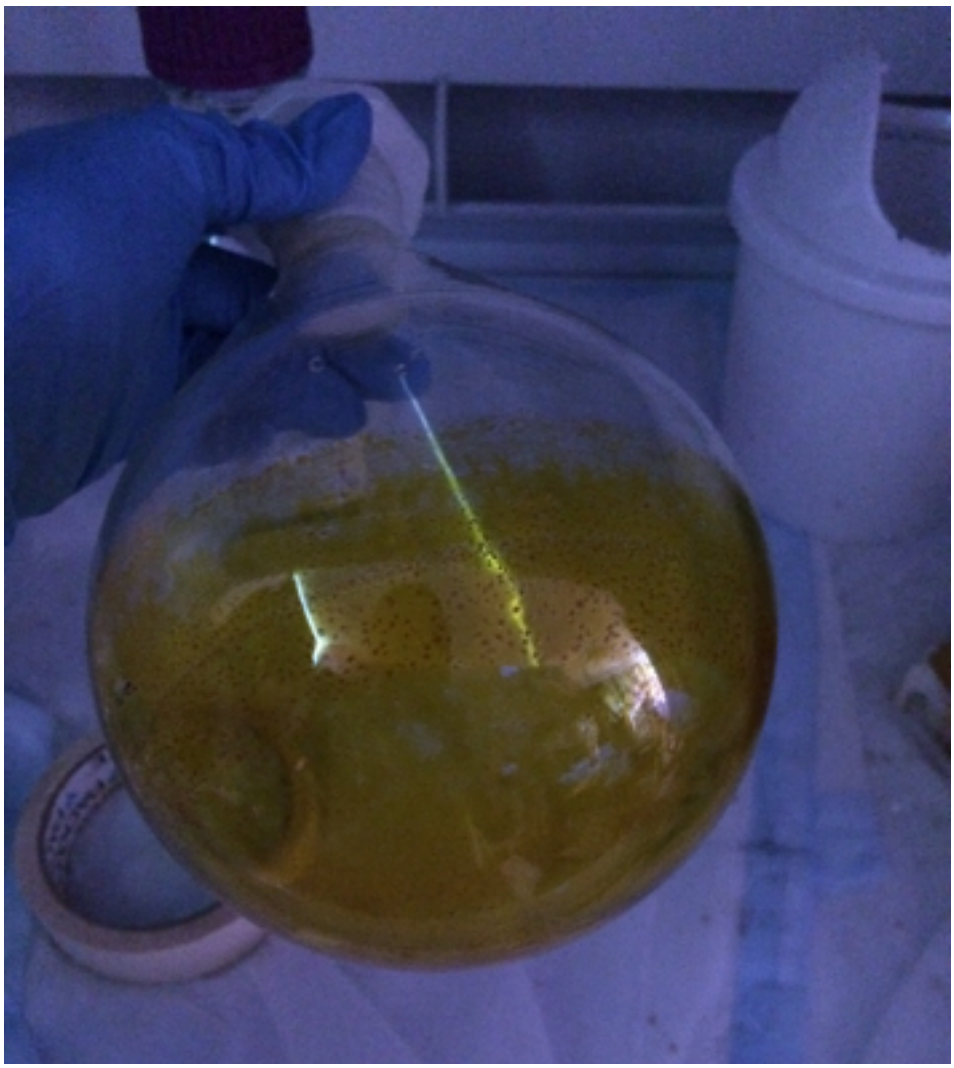


Figure 6.23 Purified compound fractions from Lanes 7–14 collected, evaporated, dried by nitrogen and stored at -20°C

The lutein extracted by using the one-step closed-tube (OSCT) method, the hexane crude extract and the purified lutein extract were analysed by using HPLC, based on the method described in subsections 2.1.1.5 and Sections 2.1.1.6, with the results shown in Figure 6.24, Figure 6.25 and Figure 6.26, respectively. The peaks identified at the retention times of 17.5 minutes and 21 minutes corresponded to lutein and chlorophyll *a*, respectively. By comparing the chromatograms of the crude extracts obtained by using the two methods (the OSCT method and modification of the method described by Deenu *et al.* (2013) (Figure 6.24 and Figure 6.25), it can be seen that the disappearance of some peaks, such as chlorophyll *a*, peaked in the hexane crude extract (Figure 6.25). This was due to the saponification process, which consisted of adding 6% potassium hydroxide (w/v) at 50°C for 30 minutes, using the method described by

Deenu *et al.* (2013). In the purified lutein extract, only one peak was observed at the retention time at 17.5 minutes which corresponded to lutein (Figure 6.26).

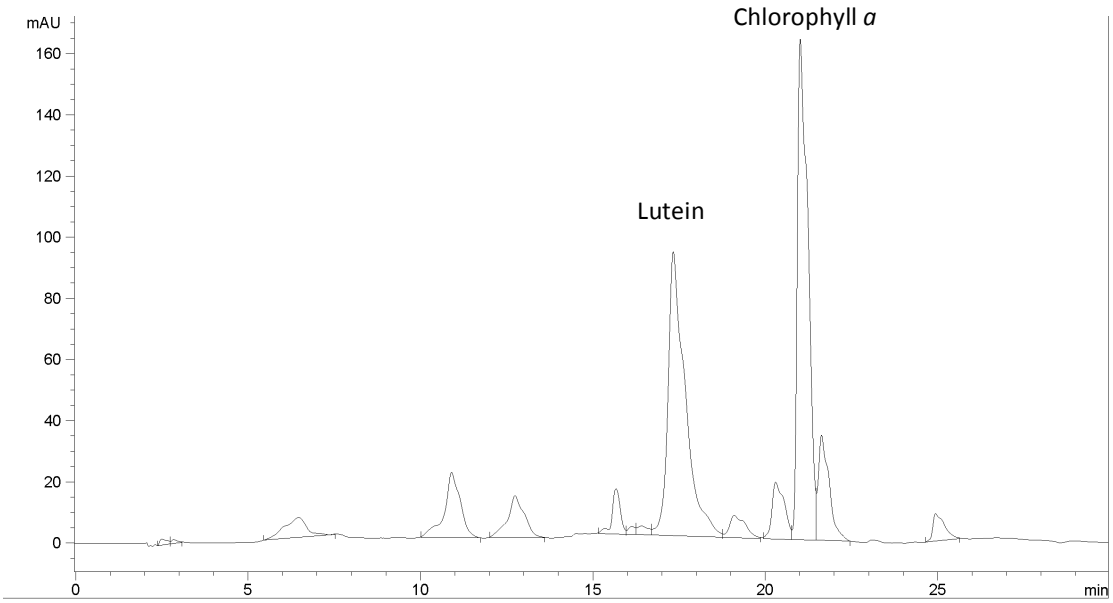


Figure 6.24 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram at 440 nm of crude extract extracted using OSCT method

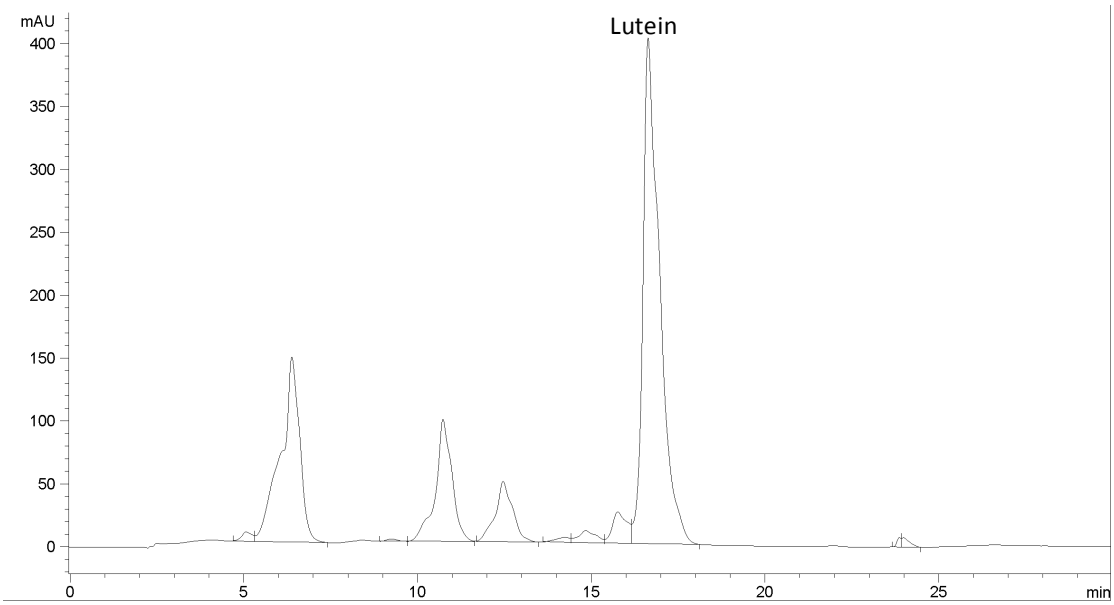


Figure 6.25 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram at 440 nm of hexane crude extract

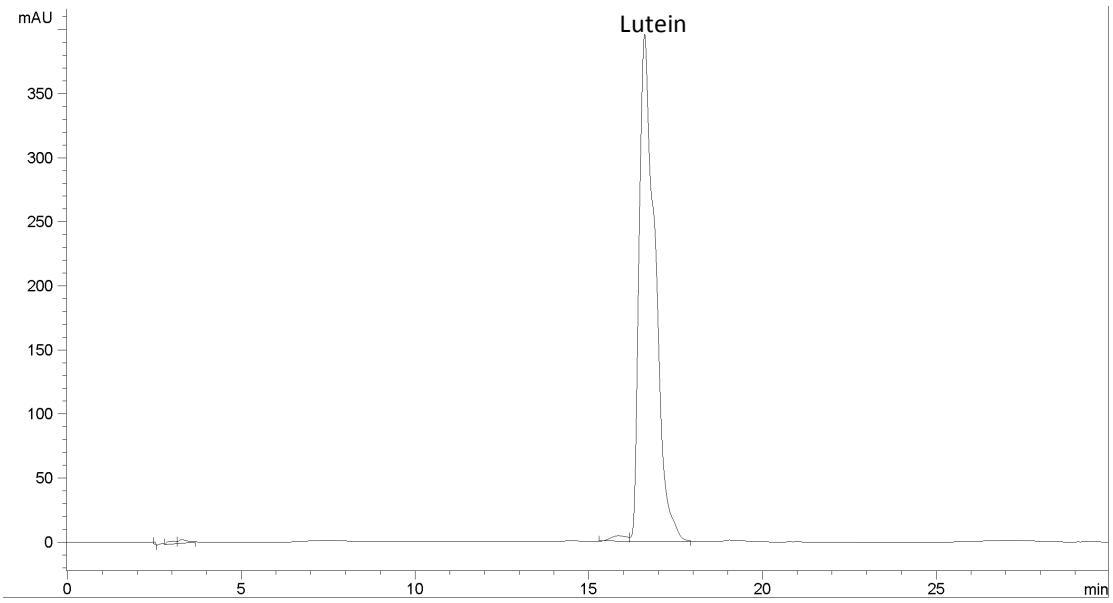


Figure 6.26 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram at 440 nm of purified lutein compound

Figure 6.27 shows the lutein content from the different extracts. The lutein content from the crude extract extracted using the OSCT method was 5678 mg/kg of biomass. The hexane crude extract was 5.4% lower at only 5386 mg/kg of biomass. However, the purified lutein from the crude extract was only 2080 mg/kg of biomass, which was a reduction of 61% from the crude extracts.

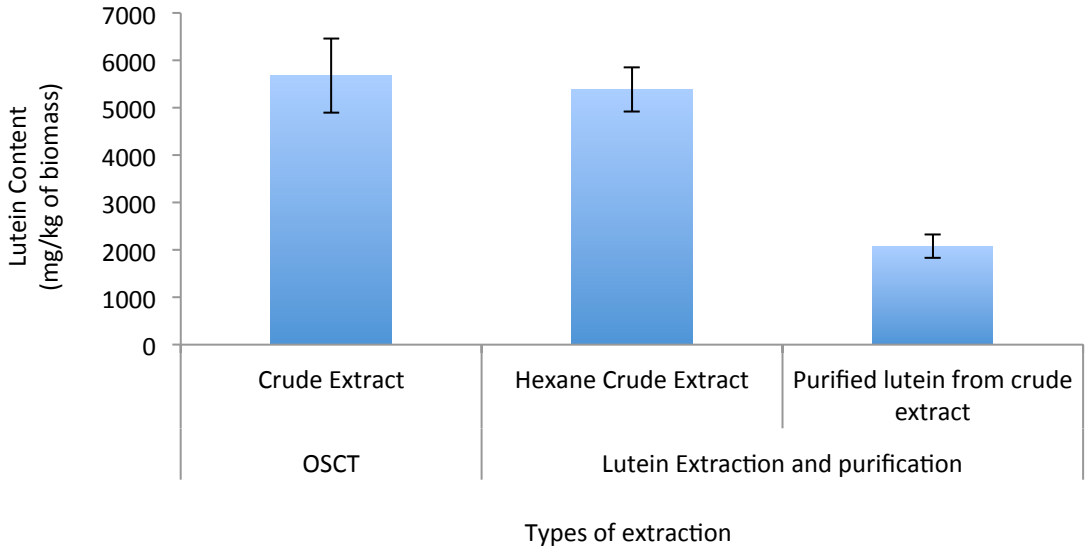


Figure 6.27 Lutein content (mg/kg of biomass) from crude extracts and purified lutein

6.3.4 Pyrosequencing-based assessment of bacteria communities in *Chloroparva pannonica* Cu40 PC cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

6.3.4.1 Changes in bacteria diversity in *Chloroparva pannonica* Cu40 PC cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor from day 14 to day 22

Chloroparva pannonica Cu40 PC samples were collected manually from the 11 L rectangular inter-loop airlift photobioreactor at day 14, 15, 16, 18, 20 and 22. At day 14, the samples for pyrosequencing were collected after the 3 L of cells were harvested, and the remaining culture was diluted by adding 3 L of fresh sterile seawater: deionized water (1:1 v/v). This goal of this part of the study was to analyse the bacterial communities during the cultivation period of the *Chloroparva pannonica* Cu40 PC in the semi-continuous system.

Over the experimental period, broad changes were observed in the bacterial communities. From the results presented in Figure 6.28, the most abundant bacteria phyla found in the photobioreactor during the growth of *Chloroparva pannonica* Cu40 PC were Chloroflexi, Proteobacteria, Bacteroidetes, Cyanobacteria and Firmicutes. As can be seen in the figure, the percentage of Chloroflexi and Firmicutes increased over the *Chloroparva pannonica* Cu40 cultivation period, whereas the percentage of Proteobacteria and Bacteroidetes decreased. However, Cyanobacteria decreased after 24 hours and increased again after 48 hours over the cultivation period.

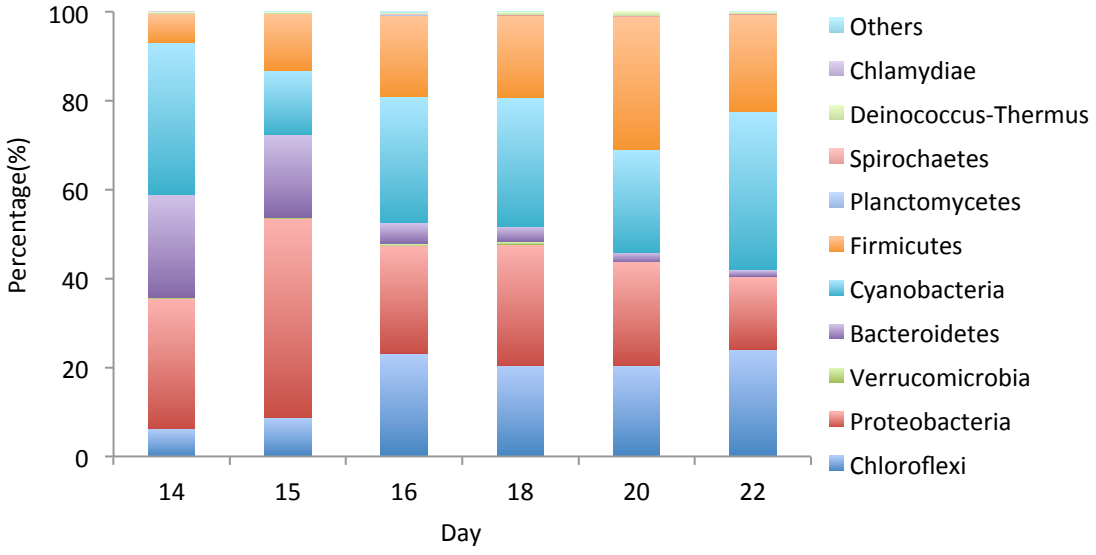


Figure 6.28 Percentage of most dominant bacteria groups based on phyla in *Chloroparva pannonica* Cu40 PC cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

The bacteria based on family found in the 11 L rectangular inter-loop airlift photobioreactor during the growth of *Chloroparva pannonica* Cu40 PC are shown in Figure 6.29. The results show that the percentage of Cytophagaceae, Parvularculaceae, Sphingomonadaceae, Caulobacteraceae and Flavobacteriaceae, Hyphomicrobiaceae decreased over the *Chloroparva pannonica* Cu40 cultivation period: on the other hand, the percentage of Thermoanaerobacteraceae and Chloroflexaceae increased.

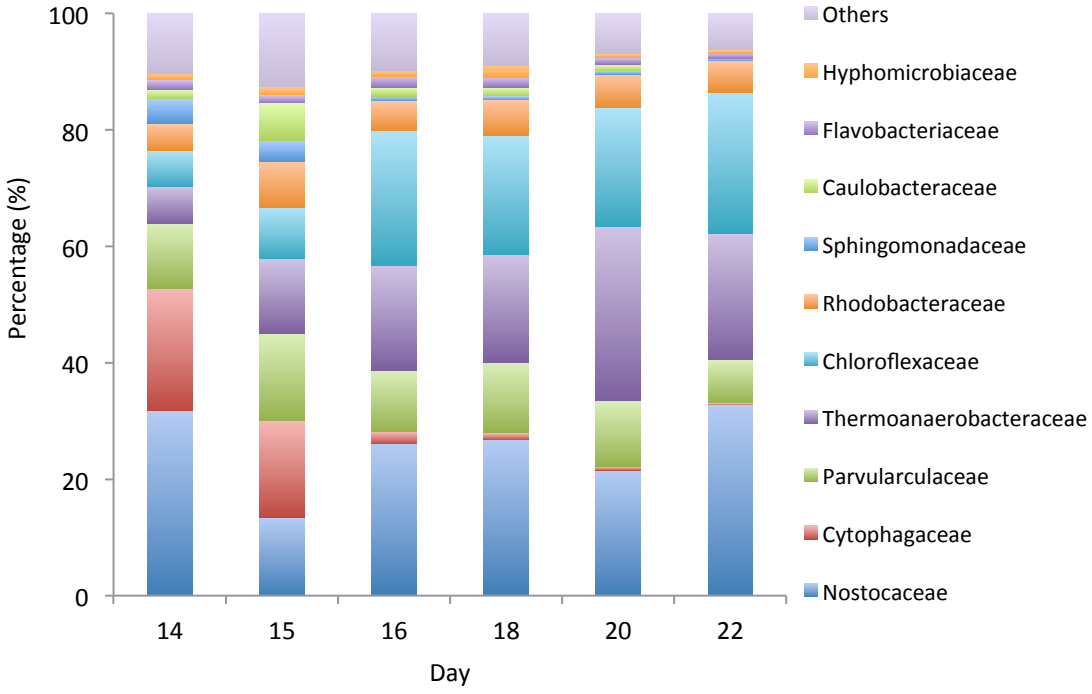


Figure 6.29 Percentage of most dominant bacteria groups based on family in *Chloroparva pannonica* Cu40 PC cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

As shown in Figure 6.30, a significant change was observed in the bacterial communities based on genus in the *Chloroparva pannonica* Cu40 PC cultivated in the semi-continuous system. The most abundant bacteria based on genus were *Anabaena*, *Cytophaga*, *Gelria*, *Chloroflexus*, *Parvularcula* and *Trichormus*. The percentage of *Cytophaga* decreased from 20.1% at day 14 to 0.005% at day 22. Little change occurred for *Parvularcula* and *Trichormus*. The percentage of *Gelri* and *Chloroflexus* increased over the cultivation period; although the percentage of *Anabena* decreased at day 15, it then increased over the cultivation period.

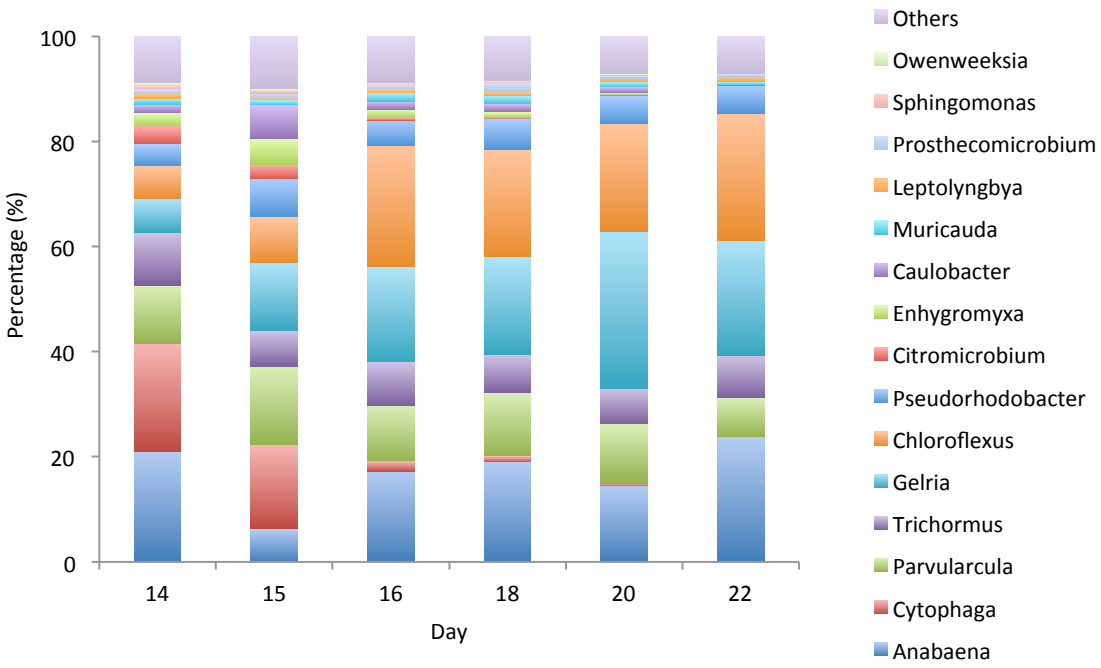


Figure 6.30 Percentage of most dominant bacteria groups based on genus in *Chloroparva pannonica* Cu40 PC cultivated in semi-continuous system in 11 L rectangular inter-loop airlift photobioreactor

6.4 Discussion

Random mutagenesis is a reliable method for improving a microalgal strain that uses non-GM methodology. For example, a study conducted by Ishikawa *et al.* (2004a) isolated a *Chlorella regularis* mutant from MNNG mutagenesis which had a higher cellular lutein content. In addition, Cordero *et al.* (2011a) managed to isolate a *Chlorella sorokiniana* mutant with a higher growth rate and also a twofold higher lutein content. However, the drawback of this method is that it requires a high level of screening to identify mutants with the desired traits.

In the current study, the mutants were selected not only on the basis of high lutein content and high lutein productivity, but also according to high total biomass and biomass productivity (see Chapter 4). The growth, lutein content and lipid content were initially evaluated in batch mode in 3 L flasks with 1% carbon dioxide supplied. *Chloroparva pannonica* Cu40 PC, WT and 10 mutant strains were selected to evaluate their growth, lutein and lipid content. The cultivation was conducted for an 18-day period in a batch mode 11 L rectangular inter-loop airlift photobioreactor. The maximum total biomass, growth rates and biomass productivity of *Chloroparva pannonica* Cu40 PC and the mutant strain, Fu5C, were higher than for *Chloroparva*

pannonica WT. The *Chloroparva pannonica* Cu40 PC achieved 50% higher total biomass and 45% higher biomass productivity than the WT while Fu5C achieved 55% higher total biomass and 49% higher biomass productivity than the WT. In addition, the lutein content of Cu40 PC and Fu5C was 125% and 84% higher, respectively, than the WT while the lutein productivity of Cu40 PC and Fu5C was 250% and 349% higher, respectively, than the WT. However, the lipid content of Cu40 PC was similar to the WT whereas, for Fu5C, it was 2% lower than the WT. The batch cultivation was able to provide a relatively high biomass and lutein content; however, this system often suffered from an initially slow growth regime, especially due to the loss of time during the final cleaning of the photobioreactor. Therefore, *Chloroparva pannonica* Fu5C with higher lutein productivity, but slightly lower biomass productivity and lipid productivity than Cu40 PC, was selected, together with Cu40 PC and WT, for this stage of the study. This involved cultivation in an 11 L rectangular inter-loop airlift photobioreactor to investigate the probability of the long-term semi-continuous system enhancing lutein content and lutein productivity while maintaining biomass at a high concentration.

In the long-term semi-continuous system, the *Chloroparva pannonica* Cu40 PC, Fu5C and WT were cultivated in an 11 L rectangular inter-loop airlift photobioreactor for a 94-day period. The culture was cultivated for 14 days to reach high density, with 3 L harvested every eight days and the culture diluted with 3 L of fresh sterile seawater: deionized water (1:1 v/v). The nutrient concentration was adjusted to the standard FU medium. Monitoring the growth and the pattern of lutein content of the *Chloroparva pannonica* Cu40 PC, Fu5C and WT revealed that lutein was produced and reached its highest point at an earlier stage of the growth phase (at either day 6 or day 8) while total biomass reached its highest point at the end of the growth phase. This could be due to the microalgal cells focusing on the synthesis of lutein to support growth as lutein is a type of primary carotenoid which is a key factor in the performance of photosynthesis and in protecting the cells from photodamage (Alves-Rodrigues and Shao, 2004, Siefermann-Harms, 1985). However, the results were not supported by the study conducted by Del Campo *et al.* (2000) and Del Campo *et al.* (2007). In these studies, when *Muriellopsis* sp. was cultivated in a batch culture and in an outdoor tabular photobioreactor, the biomass and lutein concentration increased and reached the maximum value at the early stationary phase. These results might indicate when lutein produced in the cells could be species-dependent. Moreover, the difference between and Del Campo *et al.* (2007) study and the current study was that, in the former study,

Muriellopsis sp. was cultivated in an outdoor tabular photobioreactor where natural sunlight was used. However, in the current study, two T5 high output (HO) fluorescent lights, at an intensity of 85 μmol photons $m^{-2}s^{-1}$ illumination, were positioned about 10 cm from each side of the bioreactor. The difference in light source could affect when lutein is produced in the cells as lutein is a primary carotenoid and, as previously mentioned, plays an important role in photosynthesis and also in protecting cells from photodamage (Alves-Rodrigues and Shao, 2004, Siefermann-Harms, 1985). Based on the results of the current study, it was decided to harvest 30% (3 L) of the cells every eight days, the point at which lutein content was at the highest point and biomass was being maintained at a high concentration. The results have shown a reduction of biomass and lutein content after the culture was harvested and diluted, but no lag phase was observed during growth as the exponentially growing cells were used as the inoculum for the next cycle.

As demonstrated in the current study, in the long-term semi-continuous system, Cu40 PC exhibited growth and lutein content higher than Fu5C and WT, with biomass productivity 50% higher than WT and maximum lutein content and lutein productivity 100% and 209% higher, respectively, than WT. However, biomass productivity of Fu5C was lower than Cu40 PC and WT, while lutein content and lutein productivity were 75% and 135% higher, respectively, than Cu40 PC and WT in the long-term semi-continuous system. The lipid content of Cu40 PC, Fu5C and WT was quite similar. However, Cu40 PC had the highest lipid productivity (2.418%/day), followed by WT (1.533%/day) and Fu5C (1.289%/day).

The characteristics of Cu40 PC, WT and Fu5C in batch mode and long-term semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor are summarized in Table 6.6. The performance (biomass productivity, lutein productivity and lipid productivity) of Fu5C in the long-term semi-continuous system was lower than when Fu5C was cultivated in the batch system. This result could be due to biofilm formation around the column in the photobioreactor; therefore, the efficiency of the light source delivery and distribution to the microalgae cells would be decreased. In addition, the result could be due to mutual shading where the delivery of photons into the culture was shielded by the cells near the surface (Park and Lee, 2000).

Table 6.6 Characteristics of *Chloroparva pannonica* Cu40 PC, WT and Fu5C cultivated in 11 L rectangular inter-loop airlift photobioreactor in batch mode (18-day period) or long-term semi-continuous mode (94-day period)

Component	Batch mode			Long-term semi-continuous (94 days)		
	Cu40 PC	WT	Fu5C	Cu40 PC	WT	Fu5C
Maximum total dried biomass (g/l)	1.498	1.006	1.446	1.678	1.193	1.125
Biomass productivity (g/l/day)	0.127	0.082	0.122	0.150	0.100	0.084
Specific growth rate (μ/day^{-1})	0.246	0.210	0.243	0.482	0.113	0.106
Lutein content (mg/kg of dried biomass)	5859.031	2602.938	4794.594	5768.395	2825.084	5001.290
Lutein productivity (mg/kg of biomass/day)	669.879	191.176	858.919	604.320	198.365	467.643
Total lipids (%)	19	18	16	21	19	19
Lipid productivity (%/day)	2.415	1.483	1.958	2.418	1.533	1.289

Due to Cu40 PC's performance (high biomass productivity and lipid productivity), the long-term semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor was continued for an additional 96-day period. This was to further investigate whether the selected Cu40 PC could produce, in the long-term semi-continuous mode in an 11 L photobioreactor, sufficient yield of biomass and lutein to provide feedstock for the microalgal oil-based bio-refinery process. The maximum biomass, biomass productivity and specific growth rate was 1.705 g/l, 0.1538 g/l/day and 0.4963 μ/day, respectively. The maximum lutein content was 6960 mg/kg of biomass while lutein productivity was 613.4 mg/kg of biomass/day. Lipid content ranged from 10% to 22% while the lipid productivity averaged 2.5%/day. The increased growth rate of Cu40 PC when compared to WT could be due to the higher lutein content as lutein plays an important role in function, structure and photoprotection in the photosynthesis reaction (Cordero *et al.*, 2011b).

The increase in growth and lutein content in *Chloroparva pannonica* Cu40 PC and Fu5C indicated that metabolic pathway changes had occurred caused by copper or that genetic changes had been caused by the strain improvement program (see Chapter 4). A wide range of different nitrogen sources can be used to grow microalgae. In this current

study, sodium nitrate was replaced by urea ($CO(NH_2)_2$) (see Chapter 3) with this being a combined source of nitrogen and carbon. Therefore, the extra carbon from the urea was able to support microalgae growth that had been limited by mutual shading in the photobioreactor (Cordero *et al.*, 2011b). The study conducted by Casal *et al.* (2011) showed that growth, and β-carotene and lutein content in *Coccomyxa acidophila* increased when the nitrogen source was urea. It is known that copper is an essential micronutrient for plants and microalgae involved in different metabolic pathways (Elisabetta and Gioacchino, 2004, Li *et al.*, 2006). However, copper can be toxic when in excess, and its sensitivity is species-dependent (Chang and Sibley, 1993, Soldo and Behra, 2000). Microalgae are able to trigger the synthesis of superoxide dismutase, catalase, glutathione peroxidase activities and glutathione contents as a defence mechanism when copper is in excess (Li *et al.*, 2006, Rijstenbil *et al.*, 1994, Tripathi and Gaur, 2004). Moreover, when copper is in excess, it can induce oxidative stress in microalgae cells. Therefore, chlorophyll *a* synthesis is suppressed and carotenoid synthesis is increased as carotenoids serve as protective pigments towards the oxidative stress induced by copper (Dieguez-Rojo and Gonzalez, 2003).

In addition, with lutein the main carotenoid in *Chloroparva pannonica,* the increase in the lutein to chlorophyll *a* ratio in Cu40 PC (see Chapter 4) suggested that *Chloroparva pannonica* focused on the synthesis of carotenoids rather than growth when cultivated at a higher copper concentration. On the other hand, Fu5C was a mutant strain obtained from Cu40 PC under UV-C exposure and was resistant to chlodinaflop-propargyl, a specific inhibitor of enzyme acetyl CoA carboxylase, which interfered with the lipid biosynthesis pathway and could possibly result in improved lipid production. However, the lipid content of Fu5C was lower when compared to Cu40 PC and WT while total biomass was higher than WT but lower than Cu40 PC. In addition, the mutant strains, that were resistant to aclonifen, a specific inhibitor of protoporphyrinogen oxidase involved in the chlorophyll and carotenoid biosynthesis pathway, exhibited an increase in lutein content and biomass content when cultivated in batch mode 3 L flasks with 1% carbon dioxide supplied (see Chapter 4) but did not produce promising results when cultivated in an 11 L rectangular inter-loop airlift photobioreactor. This was one example that demonstrates the challenge for microalgae research, as the short-term laboratory experiment was able to produce promising results but these were not achieved in the long-term large-scale experiment. Furthermore, it is suggested that other herbicide inhibitors, such as nicotine and norflurazon, that focus on carotenogenic

enzymes could be used in further selection studies. These herbicide inhibitors specifically inhibit lycopene β-cyclase and phytoene desaturase in the carotenoid biosynthesis pathway (Cordero *et al.*, 2011b).

The *Chlorella zofingiensis* strain CCAP 211-14, which was obtained from the Culture Collection of Algae and Protozoa and the Centre for Hydrology and Ecology, Ambleside, UK, in standard growth in batch culture exhibits lutein content of 4 mg/g dry weight (Del Campo *et al.*, 2004). In addition, *Coccomyxa acidophila* is able to accumulate up to 3.55 mg/g dry weight of lutein in mixotrophic cultures grown on urea and up to 6.1 mg/g dried weight under extreme culture conditions (Casal *et al.*, 2011). A few studies have focused on the improvement of microalgae mutants obtained from random mutagenesis and have been conducted in small-scale laboratory conditions achieving high lutein content. Ishikawa *et al.* (2004) isolated a *Chlorella regularis* mutant obtained by MNNG mutagenesis with a higher lutein content but lower growth rate than WT. In addition, a *Chlorella sorokiniana* mutant isolated by Cordero *et al.* (2011a) was reported to have shown a higher growth rate and high cellular lutein content of 7.0 mg/g dry weight. The microalga *Muriellopsis* sp. was able to accumulate lutein content up to 5.5 mg/g dried weight (Del Campo *et al.*, 2000). Although microalgae have not been used as a source of lutein on a commercial scale, the above information indicates that *Chloroparva pannonica* Cu40 PC and Fu5C using the FU medium could be considered as promising microalgae for the production of lutein.

This chapter has reported how lutein was extracted and purified from *Chloroparva pannonica* Cu40 PC cultivated in the long-term semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor to obtain the final lutein extract that could be used for commercial purposes. Extraction and purification were carried out based on the method described by Deenu *et al.* (2013). In addition and as a comparison, lutein was extracted using a small amount of biomass based on the OSCT method. In comparing the HPLC chromatogram of the crude extract using the OSCT method (Figure 6.24) with the HPLC chromatogram of the hexane crude extract (Figure 6.25), it can be seen that some peaks disappeared after a retention time of 18 minutes, including the chlorophyll α peaks at retention time of 21 minutes. This occurred due to the saponification process before the hexane extraction, which converted, esterified lutein to free form, and removed ionizable lipids and chlorophylls (Azevedo-Meleiro and Rodriguez-Amaya, 2004, Deenu *et al.*, 2013, Howe and Tanumihardjo, 2006). Major

lutein losses were not caused by the saponification step as lutein content from the OSCT method was 5.4% higher than that of the hexane extract (Figure 6.27). The HPLC chromatogram of the purified lutein extract had only one peak at 17.5 minutes which corresponded to lutein (Figure 6.26). However, a 61% reduction occurred in lutein content after the hexane extract was purified through a silica gel column, evaporated and dried by nitrogen (Figure 6.27).

The lutein found in certain strains of microalgae is mostly in free lutein form which contains many conjugated double bonds. Lutein in free form is more susceptible to photo-degradation, thermal degradation, acid exposure, autoxidation and singlet oxygen oxidation compared to lutein in the esterified form (Boon *et al.*, 2010, Ceron-Garcia *et al.*, 2010, Chan *et al.*, 2013, Davidov-Pardo *et al.*, 2016, Lim *et al.*, 2014). The massive reduction of lutein content after purification could occur either when the hexane crude extract was purified through the silica gel column in dark conditions at room temperature (22–25°C) or when the fraction was evaporated by rotary evaporator at 50°C. A study by Cerón-García *et al.* (2010) showed that the lutein extracted from *Scenedesmus almeriensis* biomass decreased by approximately 50% at ambient temperature and was only stable at 4°C or -18°C. In addition, significant lutein degradation was observed when the temperature was increased to 55°C during the ethanol extraction of lutein from *Chlorella sorokiniana* MB-1 (Chen *et al.*, 2016b). Moreover, the decay of lutein extracted from *Scenedesmus obliquus* CNW-N was fastest at ambient temperature (26°C to 28°C), with only 10% reduction of lutein when the extract was stored at temperatures of 4°C or -20°C after 80 days (Chan *et al.*, 2013). All this information indicates that lutein is only stable at the low temperatures of 4°C or -18°C. In the current study, the massive reduction of lutein could be due to the temperature during purification and evaporation; therefore, it is suggested that extraction, purification and evaporation should always be carried out in cold conditions to prevent degradation of lutein.

In the microbial oil-based bio-refinery process, most research has focused on the growth and lipid content of the microalgae. However, very little information is known about the composition of the bacterial communities in the system and the bacteria–microalgae interaction. Different algae species require different combinations of vitamins, such as Vitamin B_{12} (cobalamin), Vitamin B_1 (thiamine) and Vitamin B_7 (biotin), to support their growth (Croft *et al.*, 2005). These vitamins are often provided by the bacterial

communities (Croft *et al.*, 2005). In addition, based on the results from Chapter 5, *Chloroparva pannonica* Cu40 has no growth during the absence of vitamin B_{12} in the cultivated medium. Moreover, based on the results from Chapter 4, the growth of *Chloroparva pannonica* Cu40 PC decreased when the bacterial population was eliminated by antibiotics.

In this part of the study, the bacteria diversity in the *Chloroparva pannonica* Cu40 PC cultivated in a semi-continuous system in an 11 L rectangular inter-loop airlift photobioreactor at day 14, 15, 16, 18, 20 and 22 was investigated by using pyrosequencing. Changes occurred in the bacterial communities over the experimental period. The most abundant bacteria groups based on phyla were Chloroflexi, Proteobacteria, Bacteroidetes, Cyanobacteria and Firmicutes (Figure 6.28). Over the cultivation period, Chloroflexi and Firmicutes increased, while Proteobacteria and Bacteroidetes decreased. Cyanobacteria decreased after 24 hours of cultivation and then increased again, with Cyanobacteria one of the nitrogen-fixing bacteria able to provide nitrogen sources to microalgal cells especially for the diatoms. A study by Foster *et al.* (2011) showed that *Richelia intracellularis* was able to fix 81%–744% more nitrogen than was needed for growth, with up to 97.3% of fixed nitrogen transferred to the diatoms. The most dominant bacteria groups based on family were Nostocaceae, Parvularculaceae, Thermoanaerobacteraceae and Chloroflexaceae (Figure 6.29). The family Cytophagaceae decreased over the cultivation period. In addition, the family Rodobacteraceae was 4%–8% during the growth phase. This is one of the bacteria families that are able to provide Vitamin B_{12} (cobalamin) which is essential to support the growth of microalgae (Krustok, 2015). During the *Chloroparva pannonica* Cu40 PC cultivation in the photobioreactor, among the most dominant gennera were *Anabaena, Parvularcula, Trichromus, Gelria* and *Chloroflexus* (Figure 6.30). The results also showed a large reduction of *Cytophage*, an algicidal bacterial strain, over the cultivation period when the growth of Cu40 PC increased (Carney *et al.*, 2014). The microalgae–bacteria interaction can be through mutualism, commensalism and parasitism (Droop, 2007, Kazamia *et al.*, 2012). Research has shown that the bacteria associated with microalgae are species-dependent: different bacterial communities have been found in different microalgae cultures (Carney *et al.*, 2014, Fuentes *et al.*, 2016, Goecke *et al.*, 2013, Krustok, 2015, Nakase and Eguchi, 2007, Sapp *et al.*, 2007). For example, the most abundant bacteria phyla were Proteobacteria and Bacteroidetes in the study conducted by Krustok (2015) to evaluate the effect of lake water inoculation on the

production of algal biomass. However, in the current study, Proteobacteria and Bacteroidetes decreased when the growth of Cu40 PC increased. In addition, levels of bacteria in the culture may change depending on the stage of microalgae growth (Carney *et al.*, 2014, Krustok, 2015, Wang *et al.*, 2016a).

6.5 Conclusion

In the current study, *Chloroparva pannonica* was subjected to random mutagenesis using UV-C and mutants were selected not only on the basis of high lutein content and lutein productivity, but also according to high total biomass and biomass productivity. This work has shown that *Chloroparva pannonica* Cu40 PC and Fu5C using the FU medium can be considered as promising microalgae for the production of lutein. When Cu40 PC and Fu5C were cultivated in the long-term semi-continuous system for a 190-day period, the maximum total biomass, biomass productivity, specific growth rate, maximum lutein content and lutein productivity of Cu40 PC were 1.705 g/l, 0.1538 g/l/day, 0.4963 μ/day, 6960 mg/kg of biomass and 613.4 mg/kg of biomass/day, respectively. However, no significant difference was observed in the total lipid content between Cu40 PC and Fu5C. In addition, the long-term semi-continuous system managed to produce a high concentration of biomass with high lutein content in eight days.

Chapter 7 Major Findings and Future Directions

7.1 Summary of Major Findings and Future Directions

Microalgae, one of the oldest fundamental life forms on this planet, are a type of photosynthetic organism that can utilize and convert sunlight, water and carbon dioxide into different types of complex organic compounds, as well as into bioactive secondary metabolites. However, while oleaginous microalgae have high potential as a feedstock source for biodiesel production, production on a commercial scale is not yet economically viable. In the microalgal industry, there has been a move towards microalgal production of carotenoids for use as colourants in animal feed and also as supplements in human diets. Lutein is a type of carotenoid that exists in higher plants and phototrophic microalgae, and in which interest has recently been growing for nutraceutical and pharmaceutical applications. It has increased in popularity in ageing societies due to its potential in treating age-related macular degeneration (AMD) that leads to blindness. The potential of microalgae as a commercial source of lutein is widely recognized; however, high capital costs due to low biomass and lutein productivity in microalgae are a major bottleneck in the industry.

A successful microalgal oil-based bio-refinery process depends on the selection of microalgae strains that are capable of growing rapidly to achieve higher biomass yields, and producing a high-value desired product under optimal conditions. In this study, we have utilised the microalgae *Chloroparva pannonica* which has the potential for the bio-refinery process as it not only has the ability to synthesize lipids, but also synthesizes a unique type of carotenoid, lutein. This genus was first described by Somogyi *et al.* (2011) after it was isolated from a turbid, shallow soda pan in Hungary. In the current study, the *Chloroparva pannonica* wild type (WT) strain was obtained from a salt pan at Waikerie, South Australia, by the Aquatic Sciences Algal Production group of the South Australian Research and Development Institute (SARDI). To date, the potential of *Chloroparva pannonica* for microalgae based production has not been reported. The objective of the current study was to develop this strain to grow rapidly and produce higher biomass and enhanced lutein production.

As the project involved the screening of a large number of mutants, rapid methods using relatively low levels of sample were developed. The lutein content was measured with a one-step closed-tube (OSCT) method without the need for large amounts of samples and solvents. This method, which utilized 90% acetone with five minutes sonication with glass beads under dark conditions, was able to extract 73% more lutein compared to what was achieved with the traditional method using 100% acetone. Moreover, the lutein concentration could be estimated by using a UV-spectrophotometer at 440 nm with a strong correlation with results obtained by high-performance liquid chromatography (HPLC). In terms of the minimum time needed to process 10 samples, the traditional method required six hours while only 1.5 hours were required for the OSCT method to obtain the results. In addition, processing 10 samples using the traditional method cost a total of A$40 while with the OSCT method it was only A$10. Moreover, the shorter processing in the dark resulted in a higher lutein concentration.

Biomass productivity plays an important role in achieving economical mass productivity of compounds produced by microalgae and this is a major factor for success in any new industrial scale venture. Each microalgal strain has unique requirements in terms of nutrient intake, and environmental cultivation conditions. Therefore, the nutrients that constituted the production medium play a critical role, as well as being very strain-specific in supporting the growth of microalgae. In current study, it was important to formulate a suitable growth medium for achieving a high-density culture as the standard Guillard's F/2 medium resulted in low biomass productivity. This led to the formulation of a new medium called FU medium that resulted in increased the content of total biomass, total lipids and lutein. In the FU medium, urea replaced sodium nitrate as the main source of nitrogen. In addition, the concentration of ferric was increased twofold, cobalt was increased fourfold, manganese was increased eightfold, sodium molybdate was increased fourfold and zinc was increased fourfold. In future research, it is suggested that the effects of variations in environmental factors such as light, temperature and irradiance could be investigated as these factors were found to be critical for overall growth and lutein production in *Chloroparva pannonica*.

Recent studies have demonstrated that copper is one of the factors that influence the growth and pigment content of microalgae strains, as it is an essential co-factor of the enzymes involved in oxygen metabolism and oxidation-reduction (redox) reactions in

microalgae. The exposure of *Chloroparva pannonica* to different concentrations of copper resulted in the unexpected selection of *Chloroparva pannonica* Cu40. This strain had the highest total dried biomass (0.604 g/l), highest lipid content (24.88%) and highest lutein content (4333 mg/kg of dried biomass) when compared to other strains. Therefore, the parent culture selected was *Chloroparva pannonica* Cu40 for the strain improvement random mutagenesis study using UV-C irradiation.

Mutants of *Chloroparva pannonica* Cu40 were generated from *Chloroparva pannonica* Cu40 by random mutagenesis through UV-C irradiation, and further screened for resistance to one of two herbicides, aclonifen and chlodinafop-propargyl. The growth, and lipid and lutein content were evaluated on a small scale (40 ml) and also in batch mode in 3 L flasks with 1% carbon dioxide supplied. The mutants exhibited an 89–170% increase in total biomass and an 11–184% increase in lutein content when compared to *Chloroparva pannonica* wild type (WT). Furthermore, the mutants exhibited a 26–80% increase in total biomass and a 26% increase in lutein content when compared to *Chloroparva pannonica* Cu40 parent culture (PC). Ten (10) mutants that showed higher growth, lutein content and total lipid content than Cu40 parent culture (PC) and wild type (WT) were selected to evaluate their growth, and lutein and lipid content in an 11 L rectangular inter-loop airlift photobioreactor (Chapter 6). Three (3) of these mutants (Fu4, Fu5C and Fu41A) were selected for the taxonomy study (Chapter 5).

Based on the molecular analysis of the 18S rRNA gene sequence, *Chloroparva pannonica* wild type (WT), Cu40 parent culture (PC), Fu4, Fu5c and Fu41A were close to *Chloroparva* sp. ACT 0608 within Trebouxiophyceae, Chlorophyta. The molar guanine+cytosine (G+C mol%) of the genomic DNA ranged from 49.85–59.06%. The spherical cells ranged in size from 2–5 μm. The lipid content ranged from 22.94–25.59%. The predominant fatty acids were oleic acid, linoleic acid, linolenic acid and palmitic acid. The pigments were chlorophyll *a* and *b*, while the carotenoid was lutein. The lutein concentration ranged from 1864–5502 mg/kg of biomass. However, the lipid and lutein content could be manipulated based on their cultivation conditions. These strains were tolerant of salinity up to 7%, a pH ranging from 6–11 and a temperature from 10–30°C.

Due to time limitations of the current study, a side-by-side comparison with the type strain was not done but a comparison of the chemical composition of cell walls as a

taxonomic marker and on DNA–DNA hybridization should be carried out. The chemical composition of microalgae cell walls is often species-specific and diverse. The first study on the cell wall sugar composition of *Chlorella* was reported by Northcote *et al.* (1958). A study conducted by Takeda and Hirokawa (1984) showed that the cell wall sugar composition was different in four *Chlorella ellipsoidea* strains (*Chlorella ellipsoids* C-27, C-87, C-102 and C-183). As a result, the suggestion was made that cell wall composition would be useful for the identification of strains.

The growing interest in the microalgae based bio-refinery process has focused on the large-scale cultivation system. Short-term laboratory experiments in batch mode using 3 L flasks with 1% carbon dioxide supplied and optimum conditions have provided promising results. As previously mentioned, 10 mutants that showed higher growth, lutein content and total lipid content than the Cu40 parent culture (PC) and wild type (WT) were selected to evaluate their growth, lutein and lipid content in an 11 L rectangular inter-loop airlift photobioreactor (Chapter 6). Of these, Fu5C was identified as the strain for the semi-continuous cultivation in the 11L photobioreactor.

Monitoring the growth and lutein patterns of *Chloroparva pannonica* Cu40 PC, WT and Fu5C revealed that in batch mode lutein reached it's highest point at the earlier stage of the growth phase (at either day 6 or day 8) while the total biomass reached its highest point at the end of the growth phase in about 14 to 18 days. Therefore in order to improve the yields of lutein the culture was cultivated for 14 days to reach high density with 3 L harvested every eight days and then dilute with 3 L of fresh sterile seawater: deionized water (1:1 v/v). The nutrient concentrations were adjusted to the standard FU medium. Having achieved very high lutein concentration the draw-fill cycles were continued for a 94-day period.

It was decided in the current study, based on the growth and lutein content patterns, to harvest the culture every eight days when the lutein was at its highest point while maintaining the exponential phase of the growth. The performance (biomass productivity, lutein productivity and lipid productivity) of *Chloroparva pannonica* Cu40 parent culture (PC) was better than that of Fu5C and wild type (WT). The maximum total dried biomass, biomass productivity, specific growth rate, lutein content and lutein productivity were 1.678 g/l, 0.15 g/l/day, 0.482 μ/day, 5768 mg/kg of biomass and 604.32 mg/kg of biomass/day, respectively. However, the performance of Fu5C was lower in the long-term semi-continuous system than when cultivated in the

batch system. This result could be due to biofilm formation around the column in the photobioreactor; as a consequence, the efficiency of light source delivery and distribution to the microalgae cells could have been decreased. Due to the performance (high biomass productivity and lipid productivity) of Cu40 parent culture (PC), the long-term semi-continuous 11 L rectangular inter-loop airlift photobioreactor system was continued for a further 96-day period. This further investigated the ability of the selected Cu40 parent culture (PC) to produce, in the long-term semi-continuous mode in an 11 L photobioreactor, sufficient biomass and lutein yield to be a feedstock for the microalgae based bio-refinery process. The maximum total biomass, biomass productivity and specific growth rate were 1.705 g/l, 0.1538 g/l/day and 0.4963 μ/day, respectively. The maximum lutein content was 6960 mg/kg of biomass while lutein productivity was 613.4 mg/kg of biomass/day. However, lipid content ranged from 10–22% while the lipid productivity averaged 2.5%/day.

In the current study, lutein was extracted and purified from *Chloroparva pannonica* Cu40 parent culture (PC) cultivated in the long-term semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor. A 61% reduction of lutein content occurred after the hexane extract was purified though a silica gel column, evaporated and dried by nitrogen. Lutein, in certain strains of microalgae, is mostly in free form which is more susceptible to photodegradation, thermal degradation, acid exposure, autoxidation and singlet oxygen oxidation compared to lutein in ester form. This massive reduction could be due either to the process when the hexane crude extract was purified through the silica gel column in dark conditions at room temperature or to the process when the fraction was evaporated by rotary evaporator at 50°C. Therefore, it is suggested that the extraction, purification and evaporation processes should always be carried out in cold and dark conditions to prevent degradation of the lutein. In addition, the study investigated the bacteria diversity in *Chloroparva pannonica* Cu40 parent culture (PC) grown in semi-continuous mode in an 11 L rectangular inter-loop airlift photobioreactor. The results indicated that the bacteria communities changed over the experimental period.

In conclusion, this work has demonstrated that, by using the semi-continuous system, *Chloroparva pannonica* Cu40 and Fu5C have shown improved biomass productivity and enhanced lutein accumulation when cultivated using the FU medium. These strains could be considered as promising microalgae for the production of lutein in a large-

scale system. For future research directions, further evaluation of these strains could be carried out in open raceway pond trials.

7.2 Publication arising from the study

Some findings of the current study were published at the following conference:

- **Tan, L. F,** Franco, C. & Zhang, W. (2012). Rapid quantification of carotenoids from marine microalga. In: 8th Asia-Pacific Conference on Algal Biotechnology and First International Conference on Coastal Biotechnology, Adelaide, South Australia.

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Appendices

Appendices: Chapter 4

Appendix 4.1 Medium used in the experiment

Medium used in the experiment are as follows:

1. FU medium

The composition of the new FU medium contains the following per litre (seawater: deionized water [1:1 v/v]).

Urea	75 mg
$Na_2H_2PO_4$	5 mg
Trace metals:	
$Na_2EDTA.2H_2O$	8.7 mg
$FeCl_3.6H_2O$	2.6 µg
$CoCl_2.6H_2O$	40 µg
$CuSO_4.5H_2O$	9.8 µg
$MnCl_2.4H_2O$	1.44 mg
$Na_2MoO_4.2H_2O$	25.2 µg
$ZnSO_4.7H_2O$	88 µg
Vitamins (freshly made every three months, filter sterilized):	
Thiamine HCl	20 µg
Biotin (Vitamin H)	0.1 mg
Vitamin B12	0.1 mg

2. Tryptic soy agar (TSA)

Per litre deionized water or seawater: deionized water (1:1 v/v)

Tryptic soy broth (Oxoid)	17 g
Agar	15 g

3. Antibiotic medium No. 1 agar (AMA)

Per litre deionized water or seawater: deionized water (1:1 v/v)

Antibiotic medium No. 1 (Oxoid) seed agar	27 g

4. 1.5% agar in FU medium with 0.5% glucose

Per litre FU medium

Agar	15 g
Glucose	5 g

5. Gram's stain solution

a. Crystal violet solution

20 g of crystal violet was dissolved in 100 ml of ethanol to make a 95% crystal violet stock solution. Similarly, 1 g of ammonium oxalate was dissolved in 100 ml of water to make an oxalate stock solution. A crystal violet solution was obtained by mixing 1 ml of crystal violet stock solution, 40 ml of oxalate stock solution and 10 ml of distilled water.

b. Gram's iodine solution

1 g of iodine, 2 g of potassium iodide and 3 g of sodium bicarbonate were dissolved in 300 ml of distilled water.

c. Decolourize solution

Equal volumes of 95% ethanol and acetone were mixed.

d. Safranin orange counterstain

2.5 g of safranin orange was dissolved in 100 ml of 95% ethanol to make a stock solution. One part of the stock solution was diluted with five parts of distilled water to make the safranin orange working solution.

Appendix 4.2 Growth of *Chloroparva pannonica* Cu40 mutants

Table A0.1 Growth of *Chloroparva pannonica* Cu40 mutants (OD reading at 685 nm) in 6 ml FU medium isolated from single colony of FU agar plates at days 4, 7, 11 and 14

Strains	Day 4	Day 7	Day 11	Day 14
1	0.079	0.3	0.697	1.005
2	0.044	0.231	0.828	0.876
3	0.056	0.19	0.658	0.749
4	0.059	0.863	1.734	3.213
5	0.057	0.227	1.721	2.744
6	0.078	0.225	0.852	0.975
7	0.066	0.455	1.078	0.379
8	0.105	0.363	1.066	1.416
9	0.197	0.584	1.466	2.548
10	0.074	0.394	1.18	2.732
11	0.09	0.411	1.000	1.335
12	0.063	0.339	0.92	1.157
13	0.169	0.582	0.731	1.078
14	0.08	0.232	0.708	0.919
15	0.165	0.732	1.115	0.215
16	0.09	0.621	2.04	3.108
17	0.086	1.175	1.708	2.241
18	0.194	0.426	0.979	1.327
19	0.095	0.412	0.971	1.224
20	0.294	0.003	0.004	0.005
21	0.111	0.691	1.543	2.532
22	0.195	0.363	0.801	0.195
23	0.115	0.443	1.391	1.878
24	0.096	1.07	2.196	2.033
25	0.096	0.379	1.013	1.208
26	0.099	0.401	1.077	1.293
27	0.124	0.438	1.138	0.894
28	0.114	0.878	1.072	1.578
29	0.237	0.883	1.436	0.244
30	0.133	0.631	1.709	1.772
31	0.094	0.768	2.238	1.633
32	0.012	0.247	0.622	0.913
33	0.098	0.326	1.151	1.197
34	0.075	0.358	0.757	1.335
35	0.129	1.126	1.745	2.739
36	0.079	0.455	1.509	0.324
37	0.059	0.627	1.56	1.7
38	0.072	0.489	0.772	0.743
39	0.045	0.291	0.583	0.996
40	0.16	0.385	0.873	0.989
41	0.124	1.184	1.385	1.477
42	0.094	0.239	1.829	1.681

Strains	Day 4	Day 7	Day 11	Day 14
43	0.07	0.405	0.713	0.846
44	0.055	0.616	0.729	1.123
45	0.127	0.641	1.978	1.155
46	0.099	0.284	0.621	0.795
47	0.074	0.283	0.854	1.075
48	0.06	0.297	1.144	1.114
49	0.108	0.446	0.963	0.785
50	0.049	0.403	1.01	0.855
51	0.065	1.114	2.027	1.88
52	0.08	0.578	1.504	1.057
53	0.121	0.147	0.699	0.622
54	0.097	0.319	0.795	0.811
55	0.065	0.856	2.227	2.513
56	0.088	0.691	1.548	1.271
57	0.143	0.684	0.534	0.654
58	0.141	0.323	1.287	1.649
59	0.063	0.851	2.275	1.164
60	0.083	0.348	0.937	1.416
61	0.122	0.397	1.077	0.561
62	0.597	0.351	0.825	1.189
63	0.092	0.513	1.443	1.683
64	0.403	0.32	0.892	1.15
65	0.1	0.352	1.601	0.577
66	0.141	0.759	2.052	0.917
67	0.063	0.212	0.665	0.895
68	0.313	0.262	0.775	1.22
69	0.16	0.607	1.106	0.398
70	0.075	1.136	1.258	1.14
71	0.44	0.524	1.509	1.432
72	0.045	0.665	1.957	1.656
73	0.254	1.279	1.943	1.285
74	0.078	0.345	0.837	1.03
75	0.201	0.481	1.298	1.574
76	0.28	0.949	1.132	1.705
77	0.271	0.509	0.968	2.34
78	0.344	0.445	1.618	1.627
79	0.19	0.571	1.963	1.065
80	0.313	1.564	2.159	0.589
81	0.2	1.064	1.588	2.08
82	0.218	1.187	1.673	1.321
83	0.06	1.185	1.212	1.164
84	0.251	1.638	1.912	1.063
85	0.21	1.21	1.693	1.85
86	0.188	0.562	1.505	1.487
87	0.24	1.285	1.604	1.21
88	0.175	0.774	1.802	1.933
89	0.48	1.491	2.068	1.829
90	0.288	1.174	2.111	2.493

Strains	Day 4	Day 7	Day 11	Day 14
91	0.127	0.645	1.548	1.423
92	0.174	1.813	2.202	2.027
93	0.253	0.537	1.52	1.679
94	0.493	0.604	1.738	1.382
95	0.495	0.585	1.401	1.002
96	0.192	0.364	1.129	1.754
97	0.478	0.708	1.186	1.215
98	1.27	0.759	1.274	1.657
99	0.129	1.624	2.242	1.978
100	0.343	1.605	2.23	1.98
101	0.694	0.422	1.215	1.706
102	0.319	0.968	1.36	2.636
103	0.182	0.562	1.322	1.893
104	0.433	0.545	1.243	1.49
105	0.481	1.195	1.847	2.178
106	0.411	0.42	1.146	0.675
107	0.359	0.628	1.121	0.392
108	0.122	0.455	1.467	1.819
109	0.02	0.535	0.868	0.91
110	0.102	0.096	0.47	0.128
111	0.241	0.506	1.876	1.153
112	0.259	1.572	2.264	1.941
113	0.077	1.782	2.07	1.028
114	0.26	1.412	2.568	2.232
115	0.06	0.451	1.643	1.451
116	0.107	0.602	1.601	1.432
117	0.129	0.575	1.161	1.63
118	0.478	0.548	1.241	1.36
119	0.146	2.113	1.939	1.984
120	0.262	1.861	2.473	2
121	0.124	0.253	0.8	0.842
122	0.199	0.379	1.425	0.879
123	0.156	0.328	1.038	0.977
124	0.2	0.655	2.13	2.605
125	0.184	0.463	1.263	1.507
126	0.256	0.992	1.769	1.915
127	0.199	2.367	2.271	1.111
128	0.205	0.339	1.224	1.703
129	0.17	0.693	1.726	1.369
130	0.178	0.613	1.208	0.295
131	0.185	0.584	2.025	2.143
132	0.235	0.32	1.066	1.401
133	0.256	1.789	2.51	2.904
134	0.136	1.06	1.709	2.095
135	0.222	0.872	1.37	1.457
136	0.121	0.53	1.777	1.812
137	0.186	0.422	1.141	0.393
138	0.126	0.314	1.313	1.589

Strains	Day 4	Day 7	Day 11	Day 14
139	0.155	0.369	0.986	0.896
140	0.201	2.087	1.911	2.213
141	0.084	0.51	1.086	0.909
142	0.321	0.93	2.248	2.478
143	0.135	0.431	1.423	1.415
144	0.218	0.416	0.932	0.666
145	0.003	0.801	2.004	1.599
146	0.195	0.636	1.379	1.953
147	0.136	0.68	0.988	0.964
148	0.266	0.436	1.658	1.378
149	0.158	0.706	2.013	2.264
150	0.146	0.29	1.426	1.269
151	0.079	0.409	1.186	1.71
152	0.124	0.931	2.215	1.672
153	0.156	0.365	1.094	1.249
154	0.193	1.484	1.194	0.983
155	0.129	0.88	1.58	1.742
156	0.339	1.02	2.395	2.429
157	0.168	0.553	1.185	1.465
158	0.143	0.279	0.59	1.112
159	0.196	0.433	1.347	1.217
160	0.063	0.289	0.932	1.149
161	0.087	0.338	1.021	0.862
162	0.227	0.757	1.915	1.635
163	0.095	0.428	1.004	1.022
164	0.255	0.751	1.51	1.263
165	0.163	0.464	0.989	0.622
166	0.143	0.412	1.83	1.293
167	0.251	1.437	2.611	1.509
168	0.228	0.545	0.952	0.903
169	0.657	1.114	2.534	2.58
170	0.223	0.822	1.568	1.536
171	0.446	1.151	1.75	1.915
172	-0.007	0.366	0.673	1.101
173	0.197	0.285	1.173	1.484
174	0.213	0.58	1.356	1.147
175	0.281	0.395	1.406	1.028
176	0.183	0.558	0.975	1.226
177	0.351	0.58	1.009	0.869
178	0.142	0.003	0.043	0.168
179	0.114	0.311	0.824	0.957
180	0.331	0.173	0.569	0.483
181	0.144	0.482	1.01	0.885
182	0.136	0.487	1.328	1.46
183	0.485	0.522	0.903	1.267
184	0.172	0.451	0.762	0.912
185	0.236	0.829	2.154	2.088
186	0.292	0.577	1.435	1.549

Strains	Day 4	Day 7	Day 11	Day 14
187	0.095	0.356	0.713	0.809
188	0.178	0.649	1.371	1.212
189	-0.007	0.262	1.006	1.201
190	0.191	0.408	1.052	1.072
191	0.112	0.353	0.762	1.454
192	0.023	0.723	1.423	0.677
193	0.157	0.407	1.353	1.045
194	0.106	0.368	0.908	0.974
195	0.203	0.602	1.412	1.222
196	0.194	0.635	2.092	1.84
197	0.251	0.297	0.574	0.762
198	0.38	0.512	0.935	1.679
199	0.233	0.916	1.587	1.976
200	0.276	0.878	2.234	2.04
201	0.233	0.599	1.454	1.699
202	0.165	0.677	1.019	0.913
203	0.249	0.44	1.61	0.967
204	0.211	0.287	0.52	0.918
205	0.205	0.567	1.284	3.02
206	0.122	0.704	1.499	1.734
207	0.19	0.569	0.917	0.958
208	0.191	0.481	0.701	0.834
209	0.294	1.312	2.19	2.108
210	0.086	0.563	1.566	1.941
211	0.238	0.519	0.958	0.866
212	0.224	0.578	0.951	2.408
213	0.313	0.722	1.47	1.579
214	0.277	0.326	1.188	1.611
215	0.276	0.496	1.36	1.454
216	0.138	1.135	0.819	0.41
217	0.325	0.772	0.829	1.419
218	0.083	0.673	0.831	0.69
219	0.206	0.5	1.181	2.555
220	0.147	0.529	1.452	1.235
221	0.139	0.508	1.41	1.682
222	0.228	0.671	1.453	1.76
223	0.158	1.134	0.648	0.761
224	0.183	0.472	0.95	1.229
225	0.228	0.954	1.567	1.466
226	0.153	0.625	1.549	2.748
227	0.185	0.377	0.678	0.694
228	0.221	-0.005	0.006	0.133
229	0.228	0.646	1.199	1.497
230	0.275	1.238	1.087	0.878
231	0.129	0.208	0.905	1.238
232	0.413	0.188	0.422	0.746
233	0.298	0.442	1.37	1.253
234	0.171	0.662	1.159	1.303

Strains	Day 4	Day 7	Day 11	Day 14
235	0.434	0.43	1.052	1.451
236	0.362	0.538	1.279	1.02
237	0.074	0.921	1.641	1.717
238	0.223	0.454	1.353	1.251
239	0.294	0.461	0.863	0.714
240	0.243	0.55	1.415	1.952
241	0.246	0.397	1.121	1.914
242	0.282	0.246	0.456	1.339
243	0.14	0.165	0.296	0.928
244	0.122	0.245	0.601	1.282
245	0.26	0.32	0.781	1.731
246	0.37	0.572	1.135	2.185
247	0.219	0.387	0.64	1.186
248	0.335	0.615	1.357	1.164
249	0.174	0.351	1.385	1.753
250	0.292	0.495	1.426	1.648
251	0.099	0.318	1.006	1.459
252	0.21	0.333	0.734	1.058
253	0.153	0.31	0.54	0.824
254	0.12	0.378	0.752	1.264
255	0.111	0.234	0.51	0.788
256	0.27	0.324	0.935	1.198
257	0.34	0.267	0.643	0.839
258	-0.004	0.342	0.687	1.745
259	0.166	0.369	0.749	1.006
260	0.085	0.277	0.572	0.831
261	0.158	0.346	0.834	1.389
262	0.232	0.3	0.719	1.104
263	0.114	0.337	0.722	1.034
264	0.102	0.291	0.485	0.9
265	0.146	0.432	1.114	2.034
266	0.125	0.324	1.019	1.22
267	0.17	0.28	0.615	0.768
268	0.081	0.256	0.718	0.913
269	0.092	0.368	0.918	1.208
270	0.067	0.516	0.86	1.525
271	0.105	0.523	0.707	1.293
272	0.108	0.372	0.917	1.933
273	0.12	0.282	0.606	0.86
274	0.163	0.43	0.759	1.061
275	0.129	0.128	0.315	0.416
276	0.139	0.387	0.705	1.196
277	0.089	0.201	0.474	0.663
278	0.127	0.178	0.327	0.698
279	0.153	0.302	1.342	1.796
280	0.105	0.344	0.577	0.87
281	0.152	0.336	0.564	0.875
282	0.092	0.313	0.514	0.864

Strains	Day 4	Day 7	Day 11	Day 14
283	0.179	0.253	0.466	0.597
284	0.145	0.334	0.63	1.098
285	0.114	0.164	0.411	0.789
286	0.158	0.481	1.194	2.592
287	0.059	0.25	0.494	0.843
288	0.172	0.454	0.659	0.995
289	0.189	0.363	0.746	1.097
290	0.115	0.431	0.541	0.904
291	0.172	0.435	0.913	1.505
292	0.184	0.099	0.257	0.593
293	0.124	0.614	1.249	2.161
294	0.154	0.359	0.762	1.191
295	0.141	0.268	0.407	0.784
296	0.044	0.348	0.648	1.401
297	0.146	0.371	0.525	0.858
298	0.139	0.359	1.057	1.675
299	0.156	0.242	0.361	0.684
300	0.097	0.392	0.759	1.155
301	0.146	0.258	0.554	0.789
302	0.109	0.386	0.631	1.051
303	0.137	0.284	0.67	1.014
304	0.09	0.141	0.261	0.475
305	0.238	0.327	0.819	1.265
306	0.267	0.124	0.465	0.771
307	0.088	0.347	0.701	1.038
308	0.12	0.211	0.449	0.595
309	0.23	0.437	0.727	1.104
310	0.078	0.306	0.486	0.992
311	0.185	0.24	0.551	0.95
312	0.032	0.208	0.705	1.602
313	0.171	0.276	0.601	0.918
314	0.148	0.358	0.825	1.361
315	0.206	0.217	0.641	0.848
316	0.056	0.368	0.572	0.838
317	0.086	0.411	0.739	1.481
318	0.158	0.192	0.567	1
319	0.197	0.232	0.615	1.166
320	0.107	0.252	0.558	0.769
321	0.105	0.185	0.474	0.781
322	0.092	0.227	0.538	0.869
323	0.332	0.487	0.904	1.253
324	0.263	0.458	0.797	0.998
325	0.143	0.234	0.546	0.676
326	0.026	0.093	0.193	0.544
327	0.191	0.248	0.833	1.53
328	0.176	0.374	0.619	0.89
329	0.091	0.31	0.618	0.775
330	0.132	0.28	0.451	0.7

Strains	Day 4	Day 7	Day 11	Day 14
331	0.027	0.116	0.421	0.815
332	0.104	0.255	0.626	0.835
333	0.131	0.219	0.562	0.685
334	0.132	0.59	0.701	0.874
335	0.077	0.234	0.362	0.69
336	0.055	0.159	0.356	0.79
337	0.19	0.404	0.574	1.036
338	0.041	0.289	0.477	0.702
339	0.085	0.484	0.906	1.165
340	0.21	0.32	0.715	1.045
341	0.081	0.3	0.47	0.812
342	0.158	0.356	0.537	0.945
343	0.186	0.391	0.689	0.988
344	0.136	0.368	0.713	1.09
345	0.121	0.383	0.688	1.003
346	0.037	0.37	0.748	1.181
347	0.125	0.207	0.442	0.848
348	0.131	0.146	0.29	0.767
349	0.075	0.322	0.579	0.693
350	0.123	0.294	0.593	0.867
351	0.166	0.43	0.823	1.424
352	0.119	0.298	0.616	0.866
353	0.043	0.327	0.552	0.754
354	0.095	0.357	0.499	1.063
355	0.131	0.306	0.55	0.972
356	0.094	0.419	0.745	1.042
357	0.046	0.303	0.656	0.935
358	0.079	0.306	0.435	0.745
359	0.058	0.204	0.438	0.686
360	0.132	0.353	0.631	0.859
361	0.14	0.416	0.755	1.021
362	0.088	0.219	0.413	0.758
363	0.129	0.23	0.512	0.854
364	0.071	0.266	0.56	0.866
365	0.096	0.188	0.479	0.871
366	0.077	0.245	0.676	1.232
367	0.155	0.124	0.462	0.571
368	0.163	0.253	0.541	0.846
369	0.12	0.25	0.416	0.593
370	0.142	0.321	0.63	0.859
371	0.176	0.306	0.584	0.808
372	0.148	0.25	0.452	0.594
373	0.109	0.289	0.607	0.846
374	0.224	0.321	0.735	0.906
375	0.143	0.122	0.392	0.87
376	0.086	0.215	0.296	0.68
377	0.156	0.35	0.63	1.008
378	0.079	0.275	0.544	0.973

Strains	Day 4	Day 7	Day 11	Day 14
379	0.1	0.152	0.418	0.673
380	0.097	0.168	0.446	0.664
381	0.028	0.377	0.654	0.939
382	0.367	0.202	0.392	0.599
383	0.107	0.369	0.714	1.042
384	0.142	0.246	0.495	0.76
385	0.122	0.301	0.688	0.849
386	0.199	0.191	0.45	0.796
387	0.107	0.125	0.304	0.621
388	0.034	0.278	0.522	0.725
389	0.137	0.145	0.348	0.603
390	0.211	0.296	0.413	0.904
391	0.157	0.147	0.436	0.73
392	0.094	0.368	0.646	0.856
393	0.13	0.238	0.493	0.976
394	0.072	0.284	0.654	0.836
395	0.129	0.146	0.492	0.746
396	0.099	0.318	0.561	0.862
397	0.166	0.24	0.434	0.933
398	0.092	0.196	0.376	0.657
399	0.113	0.341	0.64	0.768
400	0.082	0.2	0.474	0.845
401	0.16	0.4	0.751	1.078
402	0.256	0.497	0.887	1.258
403	0.124	0.31	0.532	1.055
404	0.183	0.282	0.664	1.119
405	0.086	0.164	0.538	0.977
406	0.149	0.052	0.14	0.407
407	0.126	0.276	0.659	1.316
408	0.116	0.32	0.667	1.074
409	0.16	0.397	0.792	1.361
410	0.113	0.319	0.84	1.625
411	0.132	0.41	0.723	1.067
412	0.19	0.153	0.96	1.017
413	0.156	0.336	0.659	1.083
414	0.198	0.101	0.33	0.868
415	0.112	0.096	0.344	0.607
416	0.206	0.199	0.499	1.018
417	0.099	0.22	0.502	1.215
418	0.061	0.512	0.863	1.445
419	0.16	0.403	0.735	1.644
420	0.075	0.241	0.525	0.819
421	0.099	0.172	0.384	0.806
422	0.186	0.228	0.549	0.889
423	0.166	0.334	0.525	0.853
424	0.261	0.195	0.217	0.967
425	0.139	0.283	0.336	0.943
426	0.102	0.333	0.577	0.794

Strains	Day 4	Day 7	Day 11	Day 14
427	0.11	0.256	0.659	1.029
428	0.202	0.183	0.46	0.75
429	0.067	0.319	0.585	0.982
430	0.19	0.176	0.352	0.683
431	0.152	0.19	0.222	0.944
432	0.055	0.332	0.671	1.302
433	0.127	0.403	0.835	1.25
434	0.116	0.215	0.335	0.696
435	0.114	0.267	0.42	0.982
436	0.166	0.166	0.469	0.869
437	0.095	0.324	0.626	0.983
438	0.158	0.183	0.204	1.323
439	0.233	0.263	0.604	1.21
440	0.091	0.291	0.699	1.219
441	0.08	0.319	0.623	1.196
442	0.03	0.269	0.446	1.055
443	0.071	0.185	0.652	1.013
444	0.128	0.388	0.714	1.223
445	0.065	0.209	0.302	1.305
446	0.109	0.421	0.764	1.374
447	0.097	0.363	0.761	1.423
448	0.131	0.212	0.409	0.776
449	0.084	0.225	0.468	0.888
450	0.084	0.212	0.472	0.802
451	0.093	0.33	0.728	1.092
452	0.061	0.241	0.552	0.594
453	0.104	0.159	0.512	1.062
454	0.041	0.349	0.639	1.297
455	0.087	0.329	0.549	1.266
456	0.132	0.264	0.44	1.105
457	0.148	0.132	0.42	0.724
458	0.118	0.287	0.713	1.189
459	0.151	0.266	0.879	0.629
460	0.14	0.172	0.452	0.83
461	0.075	0.378	0.701	1.366
462	0.236	0.298	0.477	0.812
463	0.134	0.253	0.48	1.223
464	0.098	0.378	0.728	1.116
465	0.095	0.289	0.788	1.635
466	0.18	0.204	0.378	1.708
467	0.187	0.36	0.874	1.435
468	0.12	0.248	0.645	1.29
469	0.158	0.46	0.985	1.755
470	0.038	0.324	0.535	1.226
471	0.121	0.226	0.459	0.803
472	0.114	0.402	0.917	1.451
473	0.076	0.332	0.73	0.794
474	0.129	0.212	0.634	1.301

Strains	Day 4	Day 7	Day 11	Day 14
475	0.054	0.437	0.833	1.787
476	0.111	0.394	0.641	1.571
477	0.173	0.33	0.551	1.43
478	0.186	0.167	0.505	0.838
479	0.148	0.356	0.861	1.574
480	0.196	0.362	1.177	0.84
481	0.183	0.207	0.535	1.024
482	0.096	0.468	0.91	1.848
483	0.284	0.353	0.56	0.972
484	0.17	0.314	0.588	1.531
485	0.115	0.488	0.951	1.501
486	0.124	0.354	0.953	2.147
487	0.236	0.289	0.516	2.25
488	0.238	0.471	1.083	1.893
489	0.132	0.323	0.79	1.046
490	0.158	0.285	0.71	0.896
491	0.159	0.717	0.861	1.126
492	0.092	0.311	0.466	0.919
493	0.061	0.199	0.422	0.945
494	0.221	0.511	0.767	1.411
495	0.049	0.362	0.577	0.884
496	0.097	0.604	1.147	1.534
497	0.246	0.422	0.92	1.408
498	0.106	0.381	0.578	1.045
499	0.202	0.429	0.678	1.256
500	0.228	0.492	0.876	1.297
501	0.178	0.47	0.942	1.46
502	0.156	0.48	0.888	1.311
503	0.048	0.453	0.934	1.588
504	0.16	0.259	0.542	1.05
505	0.17	0.179	0.357	0.942
506	0.096	0.403	0.741	0.914
507	0.153	0.38	0.729	1.134
508	0.209	0.536	1.055	1.954
509	0.156	0.378	0.769	1.12
510	0.057	0.424	0.711	1.001
511	0.109	0.467	0.608	1.321
512	0.164	0.382	0.688	1.211
513	0.111	0.524	0.934	1.395
514	0.056	0.395	0.871	1.273
515	0.097	0.43	0.562	1.015
516	0.064	0.247	0.529	0.815
517	0.174	0.452	0.797	1.12
518	0.174	0.522	0.965	1.298
519	0.121	0.266	0.506	0.935
520	0.159	0.291	0.632	1.096
521	0.088	0.346	0.704	1.156
522	0.113	0.232	0.585	1.103

Strains	Day 4	Day 7	Day 11	Day 14
523	0.096	0.31	0.854	1.609
524	0.191	0.157	0.553	0.699
525	0.203	0.314	0.672	1.069
526	0.144	0.307	0.504	0.746
527	0.169	0.407	0.787	1.133
528	0.22	0.382	0.72	0.989
529	0.185	0.305	0.553	0.728
530	0.133	0.358	0.805	1.085
531	0.28	0.406	0.916	1.191
532	0.175	0.148	0.469	1.058
533	0.114	0.262	0.359	0.853
534	0.194	0.431	0.802	1.329
535	0.097	0.348	0.676	1.24
536	0.123	0.19	0.511	0.81
537	0.114	0.202	0.539	0.797
538	0.034	0.482	0.831	1.253
539	0.456	0.262	0.493	0.752
540	0.134	0.464	0.927	1.362
541	0.175	0.324	0.627	0.969
542	0.156	0.389	0.856	1.106
543	0.25	0.237	0.552	0.986
544	0.137	0.147	0.356	0.743
545	0.044	0.353	0.661	0.954
546	0.173	0.171	0.429	0.729
547	0.255	0.355	0.491	1.145
548	0.196	0.195	0.532	0.948
549	0.112	0.474	0.827	1.123
550	0.16	0.293	0.61	1.193
551	0.087	0.352	0.821	1.047
552	0.161	0.187	0.608	0.928
553	0.118	0.411	0.727	1.138
554	0.209	0.301	0.54	1.255
555	0.318	0.538	1.569	2.52
556	0.384	0.349	0.711	1.948
557	0.193	0.234	0.485	1.443
558	0.162	0.33	0.913	1.878
559	0.343	0.426	1.183	2.436
560	0.479	0.763	1.604	2.916
561	0.293	0.509	0.945	1.764
562	0.435	0.866	1.923	1.628
563	0.227	0.461	1.953	2.43
564	0.377	0.645	1.963	2.215
565	0.128	0.431	1.465	2.06
566	0.292	0.425	0.954	1.403
567	0.196	0.401	0.68	1.054
568	0.163	0.468	0.955	1.67
569	0.153	0.298	0.645	1.01
570	0.356	0.433	1.228	1.555

Strains	Day 4	Day 7	Day 11	Day 14
571	0.442	0.352	0.806	1.069
572	-0.007	0.483	1.079	2.487
573	0.216	0.458	0.972	1.305
574	0.114	0.367	0.717	1.042
575	0.209	0.454	1.118	1.851
576	0.3	0.39	0.906	1.438
577	0.151	0.433	0.924	1.358
578	0.133	0.379	0.641	1.159
579	0.18	0.568	1.593	2.726
580	0.152	0.43	1.515	1.629
581	0.216	0.342	0.74	0.966
582	0.11	0.339	0.937	1.206
583	0.111	0.472	0.935	1.602
584	0.083	0.681	1.15	2.026
585	0.149	0.675	0.903	1.712
586	0.151	0.503	1.289	2.588
587	0.143	0.363	0.764	1.126
588	0.216	0.543	0.964	1.361
589	0.178	0.191	0.438	0.581
590	0.191	0.504	0.553	0.624
591	0.119	0.242	0.594	0.808
592	0.166	0.23	0.422	0.845
593	0.196	0.431	1.946	2.544
594	0.136	0.427	0.717	1.127
595	0.193	0.439	0.737	1.145
596	0.115	0.415	0.668	1.132
597	0.238	0.327	0.606	0.766
Wild type	0.089	0.339	0.569	0.811
Cu40 parent culture	0.113	0.304	0.696	1.217

Appendix 4.3 Toxicity tests for herbicide, aclonifen, on *Chloroparva pannonica* Cu40 mutants

Table A4.0.2 Toxicity tests for various concentrations of herbicide, aclonifen, on *Chloroparva pannonica* Cu40 mutants

Strains	Aclonifen concentrations				
	0 nm (Control)	900 nm	1 µm	2 µm	3 µm
1	+	-	-	-	-
4	+	+	+	+	-
5	+	+	+	-	-
8	+	+	W	-	-
9	+	+	+	+	-
10	+	+	+	W	-
11	-	-	-	-	-
12	+	W	-	-	-
13	+	+	-	-	-
16	+	-	-	-	-
17	+	-	-	-	-
18	+	-	-	-	-
19	+	-	-	-	-
21	+	+	+	+	-
23	+	-	-	-	-
24	+	+	W	-	-
25	+	-	-	-	-
26	+	+	-	-	-
28	+	-	-	-	-
30	+	-	-	-	-
31	+	+	+	W	-
33	+	-	-	-	-
34	+	-	-	-	-
35	+	W	-	-	-
41	+	+	+	+	W
42	+	+	+	+	W
45	+	+	-	-	-
47	+	-	-	-	-
48	+	+	+	+	-
51	+	-	-	-	-
52	+	-	-	-	-
55	+	+	W	-	-
56	+	-	-	-	-
58	+	+	-	-	-
59	+	+	+	+	-
60	+	-	-	-	-
62	+	-	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
63	+	-	-	-	-
64	+	-	-	-	-
68	+	-	-	-	-
71	+	-	-	-	-
72	+	-	-	-	-
73	+	+	+	-	-
74	+	-	-	-	-
75	-	-	-	-	-
76	+	-	-	-	-
77	+	-	-	-	-
78	+	-	-	-	-
79	+	-	-	-	-
81	+	+	+	-	-
82	+	-	-	-	-
83	+	-	-	-	-
84	+	+	W	-	-
85	+	+	+	-	-
86	+	-	-	-	-
87	+	-	-	-	-
88	-	-	-	-	-
89	-	-	-	-	-
90	+	-	-	-	-
91	+	-	-	-	-
92	+	+	+	+	W
93	+	-	-	-	-
94	+	-	-	-	-
95	+	+	+	-	-
96	+	+	+	-	-
97	+	-	-	-	-
98	+	-	-	-	-
99	+	+	-	-	-
101	+	-	-	-	-
102	+	-	-	-	-
103	+	+	W	-	-
104	+	-	-	-	-
105	+	W	-	-	-
108	+	-	-	-	-
111	+	-	-	-	-
112	+	+	-	-	-
113	+	-	-	-	-
114	+	+	+	W	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
115	+	-	-	-	-
116	+	-	-	-	-
117	+	-	-	-	-
118	+	-	-	-	-
119	+	-	-	-	-
120	-	-	-	-	-
124	+	-	-	-	-
125	+	-	-	-	-
126	+	-	-	-	-
127	+	+	-	-	-
128	+	-	-	-	-
129	+	+	+	+	-
131	+	-	-	-	-
132	+	-	-	-	-
133	+	-	-	-	-
134	+	-	-	-	-
135	+	+	+	+	W
136	+	-	-	-	-
138	+	-	-	-	-
140	+	+	W	-	-
142	+	-	-	-	-
143	+	+	-	-	-
145	+	W	-	-	-
146	+	-	-	-	-
148	+	-	-	-	-
149	+	-	-	-	-
150	+	-	-	-	-
151	+	+	+	-	-
152	+	-	-	-	-
153	+	-	-	-	-
155	+	-	-	-	-
156	+	+	+	+	W
157	+	+	+	-	-
158	-	-	-	-	-
159	+	-	-	-	-
160	+	+	+	-	-
162	+	+	+	-	-
163	+	-	-	-	-
164	+	-	-	-	-
166	+	-	-	-	-
167	+	-	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
169	+	-	-	-	-
170	+	+	+	-	-
171	+	-	-	-	-
172	-	-	-	-	-
173	-	-	-	-	-
174	-	-	-	-	-
175	+	+	+	-	-
176	+	-	-	-	-
182	+	W	-	-	-
183	+	-	-	-	-
185	+	+	-	-	-
186	+	-	-	-	-
188	+	-	-	-	-
189	+	+	+	-	-
190	+	-	-	-	-
191	+	-	-	-	-
192	+	+	+	+	-
195	+	-	-	-	-
196	+	-	-	-	-
198	+	+	-	-	-
199	+	+	-	-	-
200	+	-	-	-	-
201	+	-	-	-	-
205	+	+	W	-	-
206	+	-	-	-	-
209	+	-	-	-	-
210	+	+	+	-	-
212	+	-	-	-	-
213	+	+	-	-	-
214	-	-	-	-	-
215	+	-	-	-	-
217	+	W	-	-	-
219	+	W	-	-	-
220	+	+	+	-	-
221	+	+	-	-	-
222	+	-	-	-	-
224	+	-	-	-	-
225	+	-	-	-	-
226	+	-	-	-	-
229	+	+	W	-	-
231	+	+	+	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
233	-	-	-	-	-
234	+	+	-	-	-
235	+	-	-	-	-
236	+	-	-	-	-
237	+	-	-	-	-
238	+	-	-	-	-
240	+	+	W	-	-
241	+	-	-	-	-
242	+	+	-	-	-
244	+	-	-	-	-
245	-	-	-	-	-
246	-	-	-	-	-
247	+	-	-	-	-
248	+	-	-	-	-
249	+	+	-	-	-
250	+	+	+	W	W
251	+	W	-	-	-
252	+	-	-	-	-
254	+	+	+	-	-
256	+	-	-	-	-
258	+	-	-	-	-
259	-	-	-	-	-
261	-	-	-	-	-
262	+	-	-	-	-
263	+	-	-	-	-
265	+	+	+	+	-
266	+	-	-	-	-
269	+	+	W	-	-
270	-	-	-	-	-
271	+	+	-	-	-
272	+	-	-	-	-
274	+	-	-	-	-
276	+	-	-	-	-
279	+	+	+	+	W
284	+	-	-	-	-
286	+	+	+	W	-
289	+	-	-	-	-
291	+	-	-	-	-
293	+	-	-	-	-
294	+	+	W	-	-
296	+	W	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
298	+	-	-	-	-
300	-	-	-	-	-
302	+	-	-	-	-
303	+	-	-	-	-
305	+	+	W	-	-
307	+	-	-	-	-
309	+	-	-	-	-
312	+	-	-	-	-
314	+	-	-	-	-
317	+	+	+	-	-
318	+	-	-	-	-
319	+	+	+	W	-
323	+	-	-	-	-
327	+	+	+	+	-
337	+	-	-	-	-
339	-	-	-	-	-
340	+	-	-	-	-
344	+	-	-	-	-
345	-	-	-	-	-
346	+	+	+	W	-
351	+	+	-	-	-
354	+	-	-	-	-
356	+	-	-	-	-
361	+	-	-	-	-
366	-	-	-	-	-
377	+	-	-	-	-
383	+	-	-	-	-
400	+	+	+	+	-
402	-	-	-	-	-
403	+	+	-	-	-
404	+	+	-	-	-
406	+	+	+	W	-
408	+	-	-	-	-
409	+	-	-	-	-
410	+	+	-	-	-
411	+	-	-	-	-
412	+	-	-	-	-
413	+	+	W	-	-
416	+	-	-	-	-
417	+	+	-	-	-
418	+	+	+	W	W

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
419	+	+	+	+	-
427	+	W	-	-	-
432	+	-	-	-	-
433	+	-	-	-	-
438	+	+	-	-	-
439	+	-	-	-	-
440	+	-	-	-	-
441	+	+	-	-	-
442	+	-	-	-	-
443	-	-	-	-	-
444	+	-	-	-	-
445	+	+	+	+	W
446	+	+	W	-	-
447	+	+	-	-	-
451	-	-	-	-	-
453	+	W	-	-	-
454	+	+	-	-	-
455	+	-	-	-	-
456	+	-	-	-	-
458	+	+	+	-	-
461	+	-	-	-	-
463	+	-	-	-	-
464	+	-	-	-	-
465	+	-	-	-	-
466	+	-	-	-	-
467	+	-	-	-	-
468	+	-	-	-	-
469	+	+	+	W	-
470	+	-	-	-	-
472	+	-	-	-	-
474	+	+	-	-	-
475	+	+	-	-	-
476	+	-	-	-	-
477	+	-	-	-	-
479	+	+	-	-	-
481	+	-	-	-	-
482	+	W	-	-	-
484	-	-	-	-	-
485	+	-	-	-	-
486	+	+	+	-	-
487	+	-	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
488	+	+	-	-	-
489	+	+	-	-	-
491	+	-	-	-	-
494	+	+	-	-	-
496	+	-	-	-	-
497	+	-	-	-	-
498	+	+	W	-	-
499	+	-	-	-	-
500	+	-	-	-	-
501	+	+	+	+	W
502	+	-	-	-	-
503	+	-	-	-	-
504	+	+	+	-	-
507	+	-	-	-	-
508	+	-	-	-	-
509	+	+	+	+	W
510	+	-	-	-	-
511	+	-	-	-	-
512	+	-	-	-	-
513	+	-	-	-	-
514	+	-	-	-	-
515	+	W	-	-	-
517	+	W	-	-	-
518	+	-	-	-	-
520	+	+	-	-	-
521	-	-	-	-	-
522	+	-	-	-	-
523	+	+	+	W	-
525	+	-	-	-	-
527	+	-	-	-	-
530	+	-	-	-	-
531	+	-	-	-	-
532	+	-	-	-	-
534	+	-	-	-	-
535	+	-	-	-	-
538	-	-	-	-	-
540	+	-	-	-	-
542	+	-	-	-	-
547	+	+	+	-	-
549	+	-	-	-	-
550	+	-	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
551	+	+	-	-	-
553	+	-	-	-	-
554	+	-	-	-	-
555	+	-	-	-	-
556	+	-	-	-	-
557	+	W	-	-	-
558	+	+	+	W	W
559	+	+	-	-	-
560	+	-	-	-	-
561	+	-	-	-	-
562	+	-	-	-	-
563	+	-	-	-	-
564	+	-	-	-	-
565	+	+	+	W	-
566	+	+	-	-	-
567	+	-	-	-	-
568	+	-	-	-	-
569	+	+	+	+	W
570	+	-	-	-	-
571	+	-	-	-	-
572	+	W	-	-	-
573	+	+	-	-	-
574	+	-	-	-	-
575	+	-	-	-	-
576	+	-	-	-	-
577	+	+	W	-	-
578	+	-	-	-	-
579	+	-	-	-	-
580	+	+	-	-	-
582	+	W	-	-	-
583	+	W	-	-	-
584	+	-	-	-	-
585	+	-	-	-	-
586	+	-	-	-	-
587	+	+	+	-	-
588	+	-	-	-	-
591	+	-	-	-	-
593	+	-	-	-	-
594	+	-	-	-	-
595	+	+	+	W	-
596	+	+	-	-	-

Strains	Aclonifen concentrations				
	0 nm (control)	900 nm	1 µm	2 µm	3 µm
Wild type	+	-	-	-	-
Cu40 parent culture	+	-	-	-	-

* + = Positive or present; - = Negative or absent; W = Weakly positive

Appendix 4.4 Toxicity tests for herbicide, chlodinafop-propargyl, on *Chloroparva pannonica* Cu40 mutants

Table A.4.0.3 Toxicity tests for various concentrations of herbicide, chlodinafop-propargyl, on *Chloroparva pannonica* Cu40 mutants

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
1	+	-	-	-	-
4	+	+	-	-	-
5	+	+	+	W	-
8	+	-	-	-	-
9	+	-	-	-	-
10	+	+	+	-	-
11	-	-	-	-	-
12	+	-	-	-	-
13	+	-	-	-	-
16	+	+	+	-	-
17	+	-	-	-	-
18	+	-	-	-	-
19	+	-	-	-	-
21	+	-	-	-	-
23	+	W	-	-	-
24	+	+	W	-	-
25	+	-	-	-	-
26	+	-	-	-	-
28	+	-	-	-	-
30	+	-	-	-	-
31	+	+	+	-	-
33	+	-	-	-	-
34	+	-	-	-	-
35	+	-	-	-	-
41	+	+	+	W	-
42	+	+	+	W	-
45	+	-	-	-	-
47	+	-	-	-	-
48	-	-	-	-	-
51	+	-	-	-	-
52	+	+	+	-	-
55	+	+	-	-	-
56	+	-	-	-	-
58	+	+	+	+	-
59	+	-	-	-	-
60	-	-	-	-	-
62	+	+	+	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
63	+	-	-	-	-
64	+	-	-	-	-
68	+	-	-	-	-
71	+	-	-	-	-
72	+	-	-	-	-
73	+	+	-	-	-
74	+	+	-	-	-
75	-	-	-	-	-
76	+	+	+	-	-
77	+	-	-	-	-
78	+	-	-	-	-
79	+	-	-	-	-
81	+	+	W	-	-
82	+	-	-	-	-
83	+	-	-	-	-
84	+	-	-	-	-
85	+	+	-	-	-
86	+	+	+	+	-
87	+	-	-	-	-
88	-	-	-	-	-
89	-	-	-	-	-
90	+	-	-	-	-
91	+	-	-	-	-
92	+	-	-	-	-
93	+	-	-	-	-
94	+	-	-	-	-
95	+	-	-	-	-
96	+	-	-	-	-
97	+	-	-	-	-
98	+	-	-	-	-
99	+	+	-	-	-
101	+	-	-	-	-
102	+	-	-	-	-
103	+	-	-	-	-
104	+	-	-	-	-
105	+	+	+	-	-
108	+	-	-	-	-
111	+	-	-	-	-
112	+	+	+	W	-
113	+	-	-	-	-
114	+	+	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
115	-	-	-	-	-
116	+	-	-	-	-
117	+	+	W	-	-
118	+	-	-	-	-
119	+	-	-	-	-
120	-	-	-	-	-
124	+	+	-	-	-
125	+	-	-	-	-
126	+	-	-	-	-
127	+	+	+	-	-
128	+	-	-	-	-
129	+	-	-	-	-
131	+	-	-	-	-
132	+	-	-	-	-
133	+	-	-	-	-
134	+	-	-	-	-
135	+	-	-	-	-
136	+	-	-	-	-
138	+	-	-	-	-
140	+	+	-	-	-
142	+	-	-	-	-
143	+	-	-	-	-
145	+	-	-	-	-
146	+	-	-	-	-
148	+	-	-	-	-
149	+	-	-	-	-
150	+	-	-	-	-
151	+	-	-	-	-
152	+	-	-	-	-
153	+	-	-	-	-
155	+	-	-	-	-
156	+	-	-	-	-
157	+	+	-	-	-
158	-	-	-	-	-
159	+	-	-	-	-
160	+	-	-	-	-
162	+	+	W	-	-
163	+	-	-	-	-
164	+	-	-	-	-
166	+	-	-	-	-
167	+	-	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
169	+	-	-	-	-
170	+	+	-	-	-
171	+	-	-	-	-
172	-	-	-	-	-
173	-	-	-	-	-
174	-	-	-	-	-
175	+	-	-	-	-
176	+	-	-	-	-
182	+	+	W	-	-
183	+	-	-	-	-
185	+	+	-	-	-
186	+	-	-	-	-
188	+	-	-	-	-
189	+	-	-	-	-
190	+	-	-	-	-
191	+	-	-	-	-
192	+	+	+	+	-
195	+	-	-	-	-
196	-	-	-	-	-
198	+	+	+	-	-
199	+	-	-	-	-
200	-	-	-	-	-
201	+	-	-	-	-
205	+	-	-	-	-
206	+	-	-	-	-
209	+	-	-	-	-
210	+	-	-	-	-
212	+	-	-	-	-
213	+	+	-	-	-
214	-	-	-	-	-
215	+	-	-	-	-
217	+	-	-	-	-
219	+	-	-	-	-
220	+	+	+	W	-
221	+	-	-	-	-
222	+	-	-	-	-
224	+	-	-	-	-
225	+	-	-	-	-
226	+	+	+	-	-
229	+	-	-	-	-
231	+	-	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
233	-	-	-	-	-
234	+	-	-	-	-
235	+	-	-	-	-
236	-	-	-	-	-
237	+	-	-	-	-
238	+	-	-	-	-
240	+	+	-	-	-
241	+	-	-	-	-
242	+	-	-	-	-
244	+	-	-	-	-
245	-	-	-	-	-
246	-	-	-	-	-
247	+	-	-	-	-
248	+	-	-	-	-
249	+	+	+	W	-
250	+	+	-	-	-
251	+	-	-	-	-
252	+	-	-	-	-
254	+	-	-	-	-
256	-	-	-	-	-
258	+	-	-	-	-
259	-	-	-	-	-
261	-	-	-	-	-
262	+	-	-	-	-
263	+	-	-	-	-
265	+	+	-	-	-
266	+	-	-	-	-
269	+	+	W	-	-
270	-	-	-	-	-
271	+	-	-	-	-
272	+	-	-	-	-
274	+	-	-	-	-
276	+	-	-	-	-
279	+	+	+	-	-
284	+	-	-	-	-
286	+	-	-	-	-
289	-	-	-	-	-
291	-	-	-	-	-
293	+	+	+	-	-
294	+	-	-	-	-
296	+	+	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
298	+	-	-	-	-
300	-	-	-	-	-
302	+	-	-	-	-
303	+	-	-	-	-
305	+	+	+	W	-
307	+	-	-	-	-
309	+	-	-	-	-
312	+	-	-	-	-
314	+	-	-	-	-
317	+	+	+	W	-
318	+	-	-	-	-
319	+	+	W	-	-
323	+	-	-	-	-
327	+	-	-	-	-
337	+	-	-	-	-
339	+	-	-	-	-
340	-	-	-	-	-
344	-	-	-	-	-
345	-	-	-	-	-
346	+	+	-	-	-
351	+	+	+	-	-
354	+	-	-	-	-
356	+	-	-	-	-
361	+	-	-	-	-
366	+	-	-	-	-
377	+	-	-	-	-
383	+	-	-	-	-
400	+	-	-	-	-
402	+	-	-	-	-
403	+	-	-	-	-
404	+	-	-	-	-
406	+	-	-	-	-
408	-	-	-	-	-
409	+	-	-	-	-
410	+	+	-	-	-
411	+	-	-	-	-
412	+	-	-	-	-
413	+	-	-	-	-
416	-	-	-	-	-
417	+	+	-	-	-
418	+	+	+	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
419	+	-	-	-	-
427	+	-	-	-	-
432	+	-	-	-	-
433	+	-	-	-	-
438	+	+	-	-	-
439	-	-	-	-	-
440	+	-	-	-	-
441	+	-	-	-	-
442	+	-	-	-	-
443	+	-	-	-	-
444	+	-	-	-	-
445	+	+	-	-	-
446	+	+	W	-	-
447	+	-	-	-	-
451	+	-	-	-	-
453	+	-	-	-	-
454	+	-	-	-	-
455	+	-	-	-	-
456	+	-	-	-	-
458	+	+	+	-	-
461	+	-	-	-	-
463	-	-	-	-	-
464	+	+	-	-	-
465	+	-	-	-	-
466	+	-	-	-	-
467	+	-	-	-	-
468	+	-	-	-	-
469	+	+	W	-	-
470	+	-	-	-	-
472	+	-	-	-	-
474	+	-	-	-	-
475	+	+	-	-	-
476	+	-	-	-	-
477	+	-	-	-	-
479	-	-	-	-	-
481	-	-	-	-	-
482	+	+	+	W	-
484	+	-	-	-	-
485	+	-	-	-	-
486	+	-	-	-	-
487	+	-	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
488	+	-	-	-	-
489	-	-	-	-	-
491	+	-	-	-	-
494	+	+	W	-	-
496	+	-	-	-	-
497	+	-	-	-	-
498	+	-	-	-	-
499	+	-	-	-	-
500	+	-	-	-	-
501	+	-	-	-	-
502	+	-	-	-	-
503	-	-	-	-	-
504	+	+	-	-	-
507	+	-	-	-	-
508	+	-	-	-	-
509	+	+	-	-	-
510	+	-	-	-	-
511	+	-	-	-	-
512	+	-	-	-	-
513	-	-	-	-	-
514	+	-	-	-	-
515	+	-	-	-	-
517	+	-	-	-	-
518	+	-	-	-	-
520	+	-	-	-	-
521	-	-	-	-	-
522	+	-	-	-	-
523	+	-	-	-	-
525	+	-	-	-	-
527	+	-	-	-	-
530	+	-	-	-	-
531	-	-	-	-	-
532	+	-	-	-	-
534	+	-	-	-	-
535	+	-	-	-	-
538	+	-	-	-	-
540	+	-	-	-	-
542	+	-	-	-	-
547	+	-	-	-	-
549	+	-	-	-	-
550	+	-	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
551	-	-	-	-	-
553	+	-	-	-	-
554	+	-	-	-	-
555	+	+	+	W	-
556	+	-	-	-	-
557	+	+	+	-	-
558	+	-	-	-	-
559	-	-	-	-	-
560	+	-	-	-	-
561	+	-	-	-	-
562	-	-	-	-	-
563	+	-	-	-	-
564	-	-	-	-	-
565	+	-	-	-	-
566	+	+	+	+	-
567	-	-	-	-	-
568	+	-	-	-	-
569	+	-	-	-	-
570	+	-	-	-	-
571	+	-	-	-	-
572	+	+	+	-	-
573	-	-	-	-	-
574	-	-	-	-	-
575	+	-	-	-	-
576	+	-	-	-	-
577	+	+	-	-	-
578	+	-	-	-	-
579	+	-	-	-	-
580	+	-	-	-	-
582	+	-	-	-	-
583	+	-	-	-	-
584	+	-	-	-	-
585	+	-	-	-	-
586	+	-	-	-	-
587	+	+	+	-	-
588	+	-	-	-	-
591	-	-	-	-	-
593	+	-	-	-	-
594	+	-	-	-	-
595	+	+	-	-	-
596	-	-	-	-	-

Strains	Chlodinafop-propargyl concentrations				
	0 nm (Control)	600 nm	1.2 µm	1.8 µm	2.1 µm
Wild type	+	-	-	-	-
Cu40 parent culture	+	-	-	-	-

* + = Positive or present; - = Negative or absent; W = Weakly positive

Appendix 4.5 Growth and lutein concentration of selected *Chloroparva pannonica* Cu40 PC, WT and resistant mutants

Table A4.0.4 Growth of selected *Chloroparva pannonica* Cu40 PC and WT strains (OD reading at 685 nm) in 40 ml of FU medium at days 4, 7, 9, 12, 15 and 17

Strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
Cu40 parent culture	0.139	0.211	0.257	0.339	0.458	0.585
Wild type	0.118	0.218	0.243	0.321	0.421	0.548

Table A4.0.5 Growth of selected *Chloroparva pannonica* Cu40 mutants (OD reading at 685 nm) in 40 ml of FU medium at days 4, 7, 9, 12, 15 and 17

Strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
4	0.139	0.234	0.239	0.324	0.670	0.779
5	0.123	0.304	0.268	0.459	0.504	0.703
9	0.157	0.285	0.290	0.405	0.440	0.599
10	0.153	0.282	0.397	0.299	0.611	0.853
16	0.123	0.186	0.188	0.377	0.396	0.599
17	0.141	0.276	0.234	0.349	0.405	0.541
21	0.129	0.201	0.175	0.260	0.446	0.552
24	0.126	0.180	0.164	0.295	0.291	0.442
35	0.117	0.198	0.175	0.293	0.409	0.553
55	0.135	0.196	0.195	0.298	0.330	0.464
77	0.125	0.201	0.194	0.310	0.294	0.472
81	0.131	0.196	0.223	0.319	0.346	0.555
90	0.100	0.191	0.204	0.410	0.313	0.510
92	0.156	0.272	0.283	0.346	0.440	0.557
102	0.138	0.225	0.246	0.293	0.385	0.559
105	0.155	0.222	0.181	0.312	0.378	0.538
114	0.156	0.219	0.195	0.350	0.327	0.500
124	0.185	0.255	0.269	0.338	0.396	0.528
131	0.113	0.181	0.219	0.395	0.503	0.694
133	0.121	0.196	0.216	0.340	0.374	0.552
134	0.146	0.250	0.274	0.380	0.512	0.712
140	0.159	0.255	0.255	0.355	0.463	0.649
142	0.110	0.154	0.160	0.280	0.366	0.635
149	0.093	0.137	0.160	0.287	0.359	0.531
156	0.172	0.234	0.148	0.338	0.375	0.603
169	0.097	0.105	0.135	0.243	0.300	0.466
185	0.129	0.238	0.210	0.332	0.363	0.534
200	0.137	0.262	0.248	0.360	0.421	0.653
205	0.152	0.211	0.229	0.330	0.323	0.535
209	0.156	0.224	0.196	0.327	0.324	0.514
212	0.138	0.292	0.274	0.410	0.479	0.708
219	0.073	0.088	0.191	0.240	0.287	0.505
226	0.127	0.244	0.230	0.441	0.449	0.655
246	0.089	0.114	0.205	0.263	0.310	0.450
265	0.142	0.214	0.261	0.269	0.362	0.496
286	0.158	0.270	0.292	0.315	0.410	0.544
293	0.157	0.271	0.297	0.533	0.473	0.789
486	0.137	0.198	0.267	0.367	0.420	0.572
487	0.114	0.185	0.262	0.343	0.470	0.582

Strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
555	0.135	0.193	0.328	0.347	0.389	0.501
559	0.154	0.237	0.247	0.285	0.507	0.590
560	0.127	0.193	0.248	0.357	0.395	0.625
563	0.130	0.195	0.222	0.299	0.415	0.509
564	0.109	0.192	0.294	0.289	0.351	0.453
565	0.170	0.258	0.270	0.295	0.361	0.539
572	0.148	0.229	0.279	0.347	0.365	0.504
579	0.190	0.239	0.244	0.291	0.411	0.517
584	0.184	0.331	0.377	0.327	0.693	0.587
586	0.151	0.333	0.397	0.453	0.782	0.809
593	0.162	0.352	0.374	0.458	0.501	0.582

Table A4.0.6 Growth of selected *Chloroparva pannonica* Cu40 aclonifen-resistant mutants (OD reading at 685 nm) in 40 ml of FU medium at days 4, 7, 9, 12, 15 and 17

Aclonifen-resistant strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
3µmA.41	0.076	0.179	0.380	0.406	0.512	0.827
3µmA.42	0.029	0.125	0.334	0.415	0.562	0.732
3µmA.92	0.061	0.196	0.375	0.464	0.631	0.801
3µmA.135	0.048	0.144	0.367	0.463	0.519	0.761
3µmA.156	0.021	0.088	0.207	0.428	0.468	0.609
3µmA.250	0.023	0.107	0.331	0.283	0.541	0.742
3µmA.279	0.084	0.174	0.308	0.355	0.471	0.721
3µmA.418	0.058	0.141	0.305	0.350	0.442	0.664
3µmA.445	0.014	0.082	0.227	0.372	0.488	0.615
3µmA.501	0.046	0.116	0.254	0.331	0.463	0.676
3µmA.509	0.058	0.198	0.318	0.428	0.499	0.578
3µmA.558	0.065	0.156	0.304	0.451	0.438	0.696
3µmA.569	0.092	0.274	0.518	0.472	0.554	0.639
2µmA.4	0.071	0.171	0.443	0.393	0.595	0.796
2µmA.9	0.037	0.162	0.331	0.336	0.478	0.730
2µmA.10	0.096	0.197	0.388	0.486	0.606	0.703
2µmA.21	0.057	0.151	0.303	0.407	0.520	0.807
2µmA.31	0.111	0.225	0.500	0.545	0.560	0.763
2µmA.48	0.087	0.211	0.320	0.421	0.490	0.722
2µmA.58	0.060	0.122	0.215	0.428	0.511	0.708
2µmA.114	0.100	0.180	0.270	0.563	0.645	0.869
2µmA.129	0.079	0.190	0.342	0.513	0.662	0.880
2µmA.192	0.033	0.098	0.176	0.396	0.485	0.699
2µmA.265	0.035	0.191	0.319	0.288	0.376	0.581
2µmA.286	0.104	0.093	0.224	0.487	0.601	0.876
2µmA.319	0.057	0.136	0.264	0.446	0.513	0.704
2µmA.327	0.079	0.012	0.030	0.554	0.586	0.721
2µmA.346	0.176	0.278	0.449	0.580	0.593	0.745
2µmA.400	0.092	0.232	0.360	0.366	0.443	0.785
2µmA.406	0.129	0.417	0.569	0.612	0.569	0.525
2µmA.419	0.109	0.164	0.237	0.534	0.625	0.767
2µmA.469	0.040	0.172	0.319	0.451	0.547	0.882
2µmA.523	0.068	0.160	0.219	0.430	0.406	0.797
2µmA.565	0.103	0.223	0.301	0.501	0.566	0.699
2µmA.595	0.088	0.222	0.323	0.492	0.569	0.792
1µmA.5	0.030	0.185	0.331	0.387	0.588	0.734
1µmA.73	0.059	0.276	0.546	0.449	0.630	0.757
1µmA.81	0.073	0.231	0.402	0.479	0.588	0.787
1µmA.85	0.030	0.125	0.267	0.382	0.557	0.693
1µmA.95	0.063	0.089	0.176	0.518	0.627	0.757
1µmA.96	0.022	0.250	0.396	0.500	0.681	0.699

Aclonifen-resistant strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
1µmA.151	0.042	0.173	0.326	0.447	0.584	0.792
1µmA.157	0.139	0.195	0.285	0.368	0.431	0.594
1µmA.160	0.143	0.216	0.286	0.333	0.487	0.550
1µmA.162	0.136	0.187	0.268	0.291	0.432	0.546
1µmA.170	0.093	0.139	0.220	0.265	0.362	0.477
1µmA.175	0.146	0.219	0.286	0.339	0.378	0.558
1µmA.189	0.126	0.169	0.249	0.288	0.457	0.520
1µmA.210	0.132	0.184	0.280	0.263	0.398	0.523
1µmA.220	0.083	0.055	0.108	0.170	0.262	0.420
1µmA.231	0.153	0.230	0.394	0.530	0.556	0.658
1µmA.254	0.155	0.217	0.287	0.348	0.404	0.565
1µmA.317	0.185	0.296	0.308	0.342	0.425	0.523
1µmA.458	0.167	0.241	0.308	0.418	0.428	0.546
1µmA.486	0.228	0.282	0.283	0.344	0.472	0.538
1µmA.504	0.212	0.390	0.440	0.377	0.880	0.521
1µmA.547	0.161	0.346	0.435	0.519	0.952	0.548
1µmA.587	0.189	0.395	0.435	0.501	0.570	0.521

Table A4.0.7 Growth of selected *Chloroparva pannonica* Cu40 chlodinafop-propargyl resistant mutants (OD reading at 685 nm) in 40 ml of FU medium at days 4, 7, 9, 12, 15 and 17

Chlodinafop-propargyl resistant strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
1.8µmC.5	0.117	0.158	0.241	0.386	0.669	0.817
1.8µmC.41	0.117	0.149	0.218	0.280	0.327	0.580
1.8µmC.42	0.140	0.197	0.263	0.278	0.367	0.571
1.8µmC.58	0.133	0.179	0.262	0.299	0.360	0.539
1.8µmC.86	0.141	0.176	0.247	0.298	0.399	0.542
1.8µmC.112	0.130	0.316	0.349	0.439	0.434	0.571
1.8µmC.192	0.126	0.191	0.295	0.327	0.347	0.575
1.8µmC.220	0.142	0.179	0.262	0.298	0.483	0.548
1.8µmC.249	0.126	0.175	0.243	0.335	0.389	0.568
1.8µmC.305	0.167	0.259	0.371	0.453	0.486	0.687
1.8µmC.317	0.077	0.096	0.144	0.228	0.368	0.464
1.8µmC.482	0.151	0.245	0.268	0.303	0.408	0.523
1.8µmC.555	0.128	0.188	0.291	0.358	0.385	0.464
1.8µmC.566	0.141	0.225	0.280	0.323	0.418	0.521
1.2µmC.10	0.117	0.155	0.239	0.294	0.339	0.498
1.2µmC.16	0.133	0.203	0.307	0.358	0.471	0.572
1.2µmC.24	0.099	0.138	0.184	0.270	0.362	0.528
1.2µmC.31	0.140	0.209	0.280	0.363	0.411	0.595
1.2µmC.52	0.124	0.171	0.272	0.265	0.411	0.476
1.2µmC.62	0.109	0.159	0.239	0.298	0.340	0.497
1.2µmC.76	0.106	0.169	0.240	0.268	0.544	0.585
1.2µmC.81	0.120	0.169	0.241	0.254	0.441	0.536
1.2µmC.105	0.142	0.236	0.312	0.402	0.434	0.595
1.2µmC.117	0.127	0.202	0.337	0.451	0.475	0.602
1.2µmC.127	0.161	0.238	0.313	0.505	0.486	0.729
1.2µmC.162	0.125	0.208	0.284	0.304	0.406	0.541
1.2µmC.182	0.110	0.165	0.238	0.302	0.443	0.548
1.2µmC.198	0.140	0.207	0.281	0.345	0.467	0.538
1.2µmC.226	0.152	0.204	0.282	0.360	0.414	0.568
1.2µmC.269	0.112	0.184	0.266	0.280	0.544	0.619
1.2µmC.279	0.154	0.212	0.288	0.356	0.395	0.532
1.2µmC.293	0.129	0.190	0.300	0.272	0.353	0.541
1.2µmC.319	0.145	0.189	0.271	0.312	0.377	0.534
1.2µmC.351	0.148	0.216	0.263	0.335	0.431	0.502
1.2µmC.418	0.162	0.194	0.271	0.310	0.453	0.521
1.2µmC.446	0.103	0.171	0.247	0.228	0.371	0.559
1.2µmC.458	0.117	0.157	0.228	0.243	0.398	0.443
1.2µmC.469	0.108	0.172	0.276	0.276	0.422	0.448
1.2µmC.494	0.149	0.212	0.262	0.380	0.391	0.544
1.2µmC.557	0.146	0.231	0.275	0.399	0.426	0.543

Chlodinafop-propargyl resistant strains	OD reading at 685 nm					
	Day 4	Day 7	Day 9	Day 12	Day 15	Day 17
1.2µmC.572	0.118	0.173	0.250	0.317	0.345	0.516
1.2µmC.587	0.159	0.220	0.301	0.400	0.444	0.594

Table A4.0.8 Lutein concentration (mg/l) of *Chloroparva pannonica* Cu40 PC and WT grown in 40 ml of FU medium at days 7 and 15

Strains	Lutein concentration (mg/l)	
	Day 7	Day 15
Cu40 parent culture	3.642	9.489
Wild type	3.419	6.124

Table A4.0.9 Lutein concentration (mg/l) of *Chloroparva pannonica* Cu40 mutants grown in 40 ml of FU medium at days 7 and 15

Strains	Lutein concentration (mg/l)	
	Day 7	Day 15
4	3.358	10.934
5	3.343	5.562
9	3.080	7.378
10	1.533	10.299
16	4.555	9.825
17	4.577	11.818
21	4.723	12.737
24	6.109	9.124
35	2.883	12.569
55	4.701	8.124
77	3.920	7.416
81	4.496	7.861
90	6.584	9.657
92	4.336	9.883
102	4.095	5.912
105	3.562	4.555
114	3.642	1.255
124	4.307	6.533
131	3.788	7.409
133	2.175	9.058
134	8.314	6.095
140	3.642	7.409
142	1.708	9.248
149	4.000	6.080
156	5.715	6.080
169	4.620	5.292
185	9.679	4.788
200	1.839	5.832
205	3.431	6.109
209	2.993	4.336
212	4.869	8.307
219	1.818	8.701
226	5.044	4.672
246	3.241	8.781
265	2.847	5.956
286	2.781	6.635
293	4.657	5.876
486	1.197	8.533
487	4.277	5.839

Strains	Lutein concentration (mg/l)	
	Day 7	Day 15
555	3.686	9.715
559	2.277	5.577
560	1.562	9.314
563	5.628	7.985
564	2.854	10.898
565	4.212	8.204
572	2.949	5.029
579	4.701	7.226
584	3.526	4.365
586	5.964	8.409
593	1.584	7.095

Table A4.0.10 Lutein concentration (mg/l) of *Chloroparva pannonica* Cu40 aclonifen-resistant mutants grown in 40 ml of FU medium at days 7 and 15

Aclonifen-resistant strains	Lutein concentration (mg/l)	
	Day 7	Day 15
3µmA.41	4.212	9.788
3µmA.42	2.949	5.029
3µmA.92	4.701	5.182
3µmA.135	3.526	4.663
3µmA.156	5.964	2.985
3µmA.250	4.168	5.591
3µmA.279	3.883	7.898
3µmA.418	3.818	6.737
3µmA.445	3.350	4.832
3µmA.501	3.796	8.007
3µmA.509	2.774	9.423
3µmA.558	4.321	10.131
3µmA.569	2.891	9.664
2µmA.4	3.255	8.321
2µmA.9	3.847	8.745
2µmA.10	2.803	11.591
2µmA.21	5.336	6.107
2µmA.31	0.847	9.606
2µmA.48	0.380	9.905
2µmA.58	1.898	10.496
2µmA.114	3.161	8.839
2µmA.129	3.066	6.547
2µmA.192	1.876	6.949
2µmA.265	0.730	7.504
2µmA.286	1.562	5.606
2µmA.319	2.474	8.058
2µmA.327	1.044	7.387
2µmA.346	1.504	4.642
2µmA.400	0.234	5.518
2µmA.406	1.044	8.467
2µmA.419	1.504	9.891
2µmA.469	0.234	9.869
2µmA.523	1.117	9.650
2µmA.565	0.599	7.307
2µmA.595	4.168	8.599
1µmA.5	4.044	7.788
1µmA.73	3.635	6.920
1µmA.81	2.241	8.350
1µmA.85	5.051	9.876
1µmA.95	5.033	6.109
1µmA.96	3.818	5.657

Aclonifen-resistant strains	Lutein concentration (mg/l)	
	Day 7	Day 15
1µmA.151	2.847	8.153
1µmA.157	2.204	7.489
1µmA.160	7.547	9.022
1µmA.162	5.197	8.217
1µmA.170	4.949	6.832
1µmA.175	5.993	8.387
1µmA.189	2.175	6.832
1µmA.210	5.642	11.752
1µmA.220	3.022	9.226
1µmA.231	6.511	6.285
1µmA.254	3.810	7.803
1µmA.317	6.599	7.971
1µmA.458	5.562	6.861
1µmA.486	3.693	5.876
1µmA.504	2.489	2.657
1µmA.547	2.934	4.599
1µmA.587	5.453	5.825

Table A4.0.11 Lutein concentration (mg/l) of *Chloroparva pannonica* Cu40 chlodinafop-propargyl resistant mutants grown in 40 ml of FU medium at days 7 and 15

Chlodinafop-propargyl resistant strains	Lutein concentration (mg/l)	
	7	15
1.8µmC.5	3.080	10.015
1.8µmC.41	6.445	7.577
1.8µmC.42	1.730	6.905
1.8µmC.58	4.066	8.942
1.8µmC.86	0.810	6.745
1.8µmC.112	0.292	8.876
1.8µmC.192	3.650	8.387
1.8µmC.220	1.496	10.321
1.8µmC.249	2.482	7.715
1.8µmC.305	3.423	9.066
1.8µmC.317	2.774	7.226
1.8µmC.482	3.248	6.934
1.8µmC.555	2.796	7.876
1.8µmC.566	1.759	6.088
1.2µmC.10	4.277	11.606
1.2µmC.16	1.781	6.241
1.2µmC.24	2.102	5.226
1.2µmC.31	1.416	7.698
1.2µmC.52	1.781	6.985
1.2µmC.62	2.102	7.044
1.2µmC.76	0.423	6.599
1.2µmC.81	2.978	4.774
1.2µmC.105	0.423	5.328
1.2µmC.117	2.978	5.412
1.2µmC.127	3.277	6.255
1.2µmC.162	3.254	7.146
1.2µmC.182	2.035	5.270
1.2µmC.198	2.555	7.255
1.2µmC.226	2.270	8.073
1.2µmC.269	3.299	6.905
1.2µmC.279	4.212	6.547
1.2µmC.293	3.526	8.321
1.2µmC.319	3.299	6.889
1.2µmC.351	2.788	6.258
1.2µmC.418	2.978	8.540
1.2µmC.446	5.022	6.737
1.2µmC.458	2.788	7.898
1.2µmC.469	3.299	4.832
1.2µmC.494	2.810	8.321
1.2µmC.557	4.095	6.927
1.2µmC.572	3.380	5.358

Chlodinafop-propargyl resistant strains	Lutein concentration (mg/l)	
	Day 7	Day 15
1.2µmC.587	4.832	5.759

Appendices: Chapter 5

Appendix 5.1 Ecophysiological and biochemical testing

A5.1.1 Sodium chloride tolerance

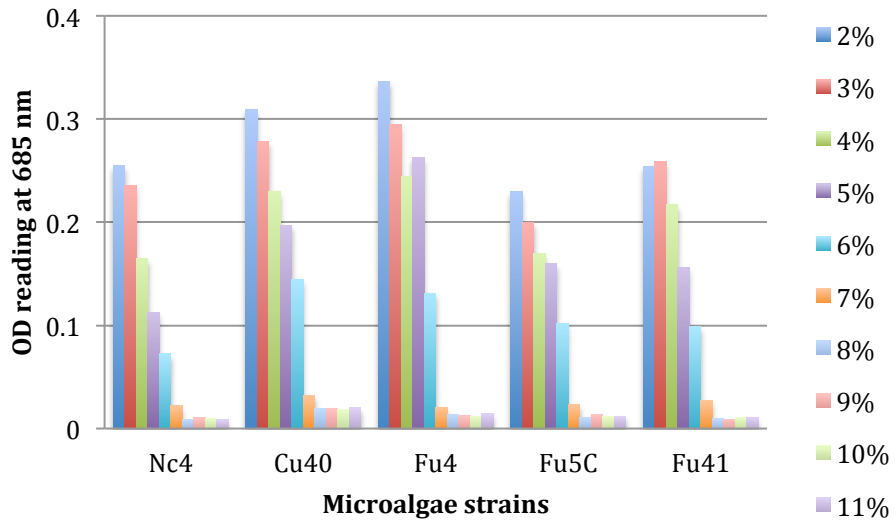

Figure A5.0.1 Salt tolerance of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A from 2–11% (w/v) sodium chloride

A5.1.2 Growth at various temperatures

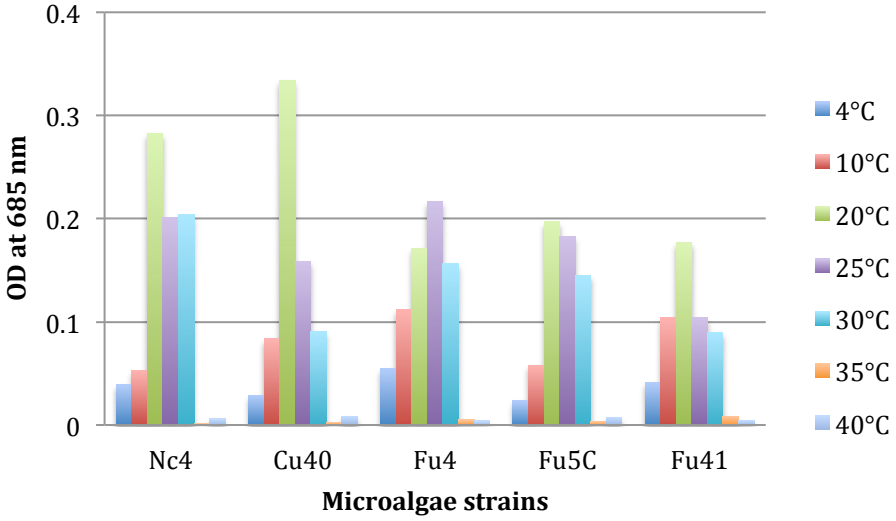

Figure A5.0.2 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different temperatures

A5.1.3 Nitrogen as sole nitrogen source

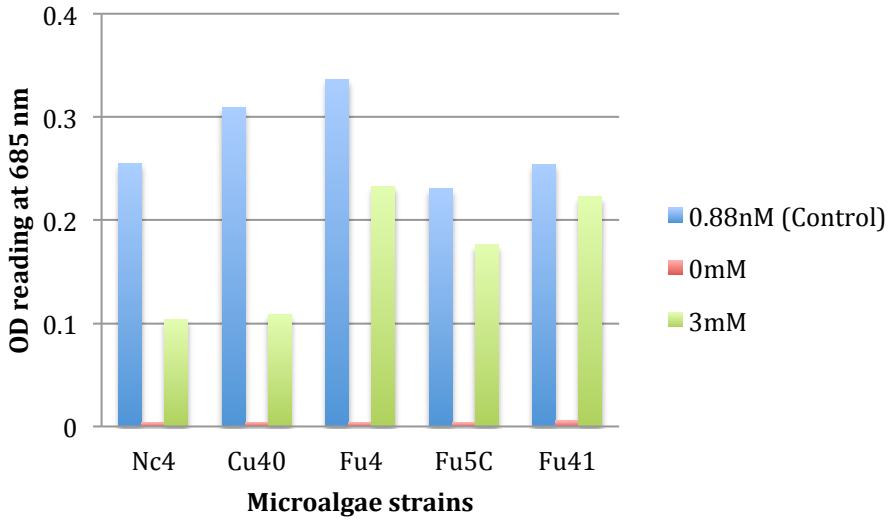

Figure A5.0.3 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A with nitrate as sole nitrogen source

A5.1.4 Thiamine requirement

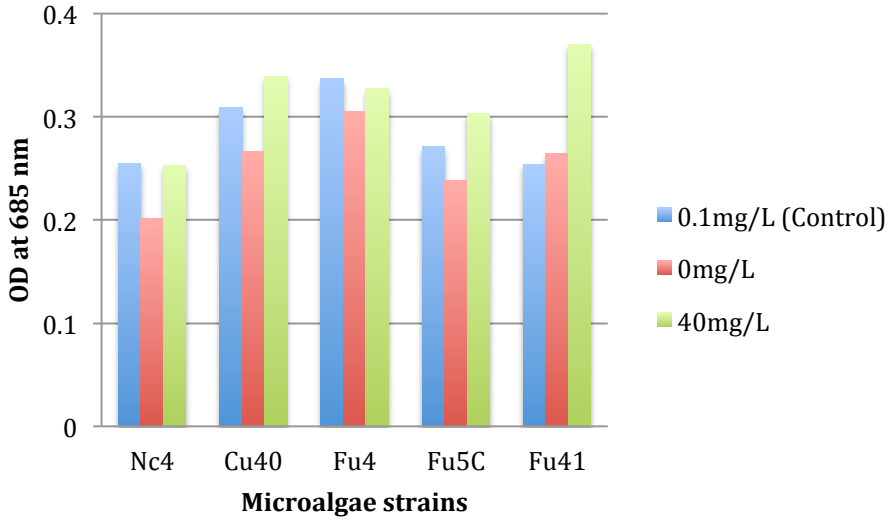

Figure A5.0.4 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of thiamine

A5.1.5 Vitamin B12 requirement

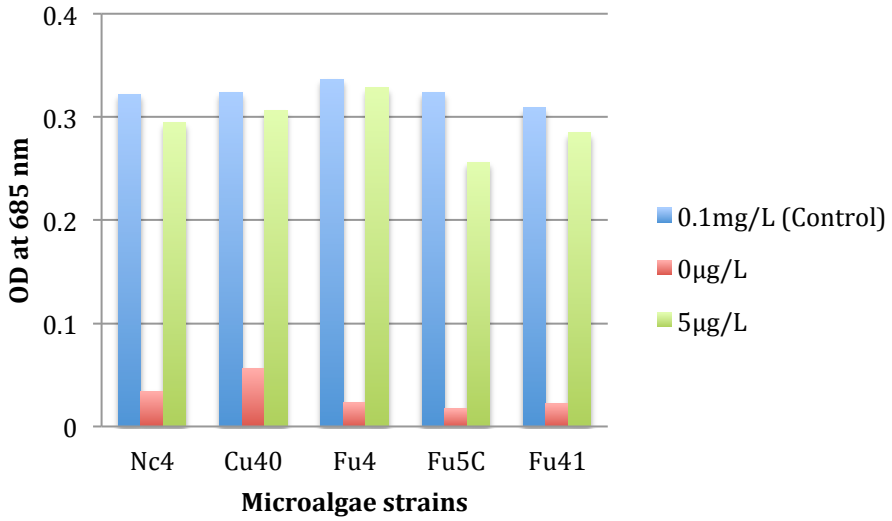

Figure A5.0.5 Growth of *Chloroparva pannonica* WT, Cu40 PC, Fu4, Fu5C and Fu41A at different concentrations of Vitamin B12

Appendix 5.2 HPLC chromatogram of guanine+cytosine (G+C) mol% of genomic DNA

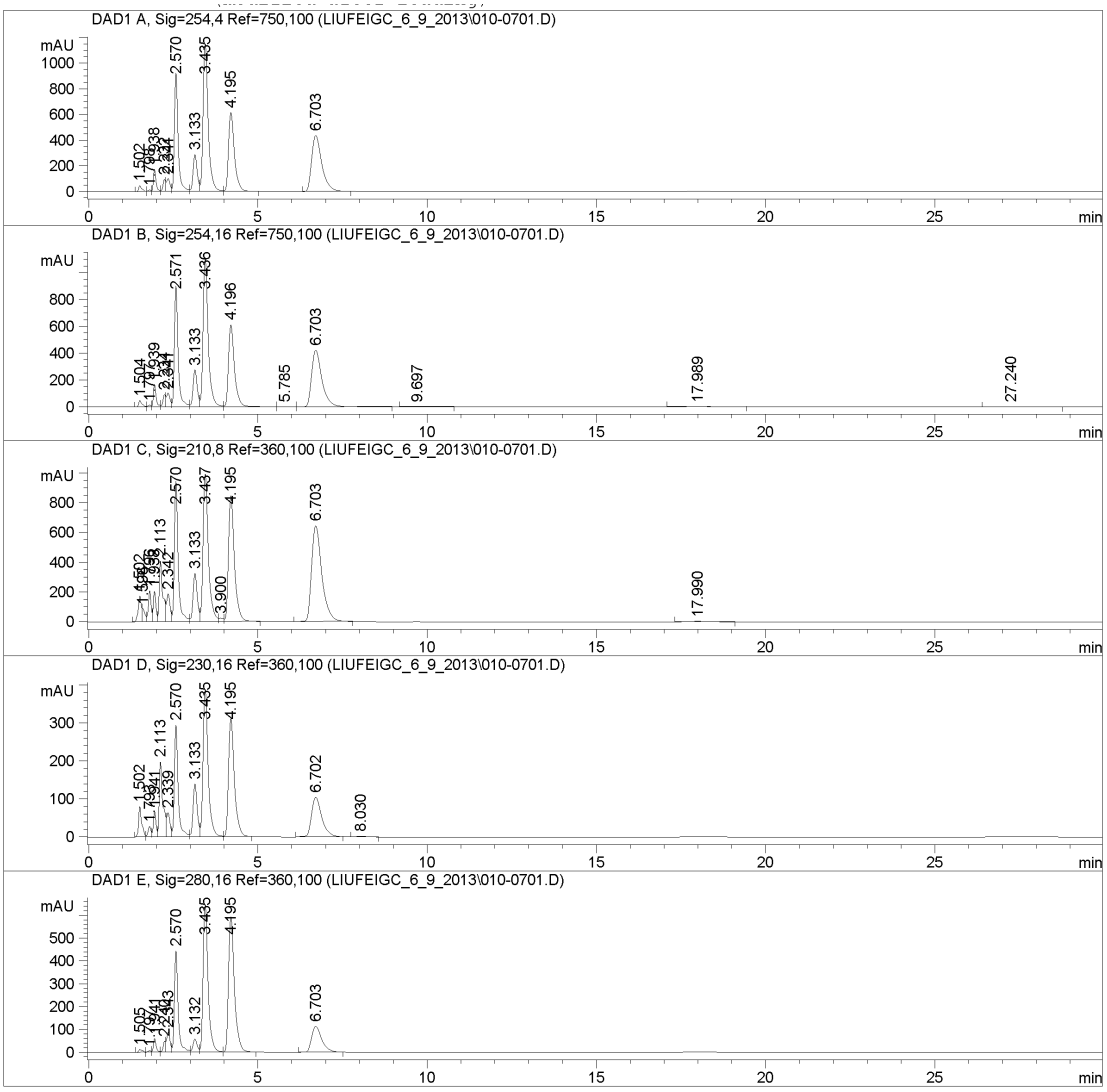

Figure A5.0.6 HPLC chromatogram of *Chloroparva pannonica* Fu5C for guanine+cytosine (G+C) mol% of genomic DNA

Appendix 5.3 18S rRNA sequence

A5.3.1 WT

TGCTTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATT
TGATGGTACCTACTACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGC
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GGCGAGTCCCTGGAGGGTGACCTCCAGATGACCGTCGTAACGCACGGAAAG
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A5.3.2 Cu40 PC

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A5.3.3 Fu4

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A5.3.3 Fu5C

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