THESIS APPENDIX

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APPENDIX A. PUBLISHED PAPERS FROM THIS THESIS

Published papers based on some of the results reported in this thesis are presented in this section. The folder (Appendix A in the attached CD-ROM) contains the PDF copies of the published papers based on some of the results of this thesis.

APPENDIX B. THE RBCL SEQUENCE OF THE *CHLORELLA* SPECIE USED IN THIS STUDY. [1,2]

LOCUS EF012700 1128 bp DNA linear PLN 29-OCT-2006

DEFINITION *Chlorella* sp. LCR ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast.

ACCESSION EF012700

VERSION EF012700.1 GI:116563961

KEYWORDS :

SOURCE Chloroplast *Chlorella* sp. LCR

ORGANISM *Chlorella* sp. LCR

Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae;

Chlorellales; Chlorellaceae; *Chlorella*.

REFERENCE 1 (bases 1 to 1128)

AUTHORS Novis P.M. and Halle C.

TITLE Physiology and taxonomy of unicellular green algae from New Zealand, and their potential as bioindicator taxa

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1128)

---

[1] The details of the rbcL sequence of the *Chlorella* sp presented in this appendix was obtained from Dr. Phil Novis of Landcare Research, Lincoln, New Zealand who supplied the *Chlorella* cultures used in this study.

[2] Information on the species sequence can be accessed from GenBank®, the national institute of health (NIH) genetic sequence database (a collection of all the publically available DNA sequences).

AUTHORS Novis P.M. and Halle C.

TITLE Direct Submission

JOURNAL Submitted (19-SEP-2006) Allan Herbarium, Landcare Research, P.O.Box 40, Lincoln, Canterbury 7640, New Zealand

FEATURES Location/Qualifiers

Source 1..1128

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/organelle="plastid:chloroplast"

/mol_type="genomic DNA"

/strain="LCR"

/db_xref="taxon:408119"

CDS <1..>1128

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/transl_table=11

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/protein_id="ABJ99071.1"

/db_xref="GI:116563962"

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ALRLEDLRIPPAYVKTFQGPPHGIQVERDKLNKYGRGLLGCTI
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GFVDLMRDDYIEKDRSRGIYFTQDWVSLPGTMPVASGGIHVW
H"

ORIGIN

```
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  61 ccaaaagaca ctgatattct tgcagcgttc cgtatgactc ctcaaccagg tgttccacca
 121 gaagaagcgg gtgcggcagt agcagcagaa tcatcaactg gtacttggac gactgtatgg
 181 actgatggtt taactagttt agaccgttac aaaggtcgtt gttatgacat cgagcctgtt
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//
```

APPENDIX C. DETERMINATION OF THE MICROALGAE PHOTOAUTOTROPHIC GROWTH CURVE

C.1. Introduction

The growth phase has been reported to significantly influence the triacylglycerol (TAG) and fatty acid content of the microalgae biomass (Hu et al., 2008). For example, the chlorophyte *Parietochloris incise* TAGs were demonstrated to increase from 43% (total biomass fatty acid content) in the logarithmic growth phase to 77% in the stationary phase (Bigogno et al., 2002). Also, the marine microalgae *Gymnodinium sp.* TAG cellular composition was shown to increase from 8% (for the logarithmic growth phase) to 30% with harvesting carried out after the observed stationary growth phase (Mansour et al., 2003).

With the microalgae cultivation in this study carried out using batch cultivation units, a determination of the autotrophic growth curve was carried out to facilitate knowledge of the stationary growth phase for the *Chlorella sp.* This corresponds to the period in which the biomass harvesting would ideally be carried out..

The methods and results obtained for the determination of the autotrophic growth curve is discussed in this section.

C.2. Methods

The starter cultures provided in the form of agar plates were transferred to 150 ml culture flasks containing BG-11 medium as described in Chapter 3 (main thesis). The starter cultures were then grown with the optical density (OD) of the cultures at 730 nm ($OD_{730\ nm}$) recorded using a spectrophotometer. The cultures were used when an $OD_{730\ nm}$ of 0.8 was reached.

Working in a laminar flow hood, 50 ml of the culture was poured in to a sterile Falcon tube. The cultures were spun down in an IEC refrigerated centrifuge at 2325 g at 25°C.

Using a vortex, the pellets from the centrifuged samples were re-suspended in 5 ml BG-11 solution in the Falcon tube. The Falcon tubes were topped up to 50 ml with BG-11 solution and spun again. The obtained pellets were re-suspended in 5 ml BG-11 solution.

A 1:100 dilution of the suspended pellets was carried out (to facilitate an accurate spectrophotometer reading) and the OD at 730 nm measured.

The amount of concentrated cells required for 150 ml BG-11 to obtain a starting $OD_{730\ nm}$ of 0.05, was then calculated using the dilution formula (Eq. C.1):

$$V_1, ml = \frac{C_2 \times V_2}{C_1} \tag{C.1}$$

where,

V_1 is the volume of the concentrated cells required to be added to the flask, ml

C_2 is the final concentration of the cells in the flask ($OD_{730\ nm}$ of 0.05)

V_2 is the working volume in the flask (150 ml)

C_1 is the $OD_{730\ nm}$ of the washed, concentrated cells in the Falcon tubes

The 150 ml culture flasks were prepared by pouring 150 ml of BG-11 solution with the addition of the *Chlorella* cells so that the $OD_{730\ nm}$ of the flask content is 0.05 as calculated above. The flask was carefully mixed by hand swirling.

From the prepared culture flask, 3 ml of the contents was pipetted into a test tube and the OD of the starting cultures measured at 730nm to ensure that it was close to the calculated OD of 0.05. This measurement was taken to represent the zero time for this study.

The culture growth flasks as shown in (Figure C.1) were attached to air pumps which facilitated the mixing of the flask contents via bubbling.

Figure C.1. Culture flasks used for the growth curve determination.

The $OD_{730\ nm}$ spectrophotometric readings were measured every 12 h for 8 days. Before reading the OD of the cultures, the flasks were topped up to 150 ml with sterile milli-Q water, to correct for any evaporative water losses. Also, it was ensured that the actual OD reading at 730 nm was below 0.4. This was achieved by appropriate dilutions of the samples during the course of the experiment.

A plot of the recorded $OD_{730\ nm}$ versus the cultivation time was obtained. From the plot the various growth phases were then identified.

The doubling time for the *Chlorella* species was also determined.

C.3. Results and discussions

Using a logarithmic plot, the results of the variation in the concentration of the *Chlorella* cells in the culture flasks (recorded as $OD_{730\ nm}$) versus the cultivation time (h) is shown in Figure C.2.

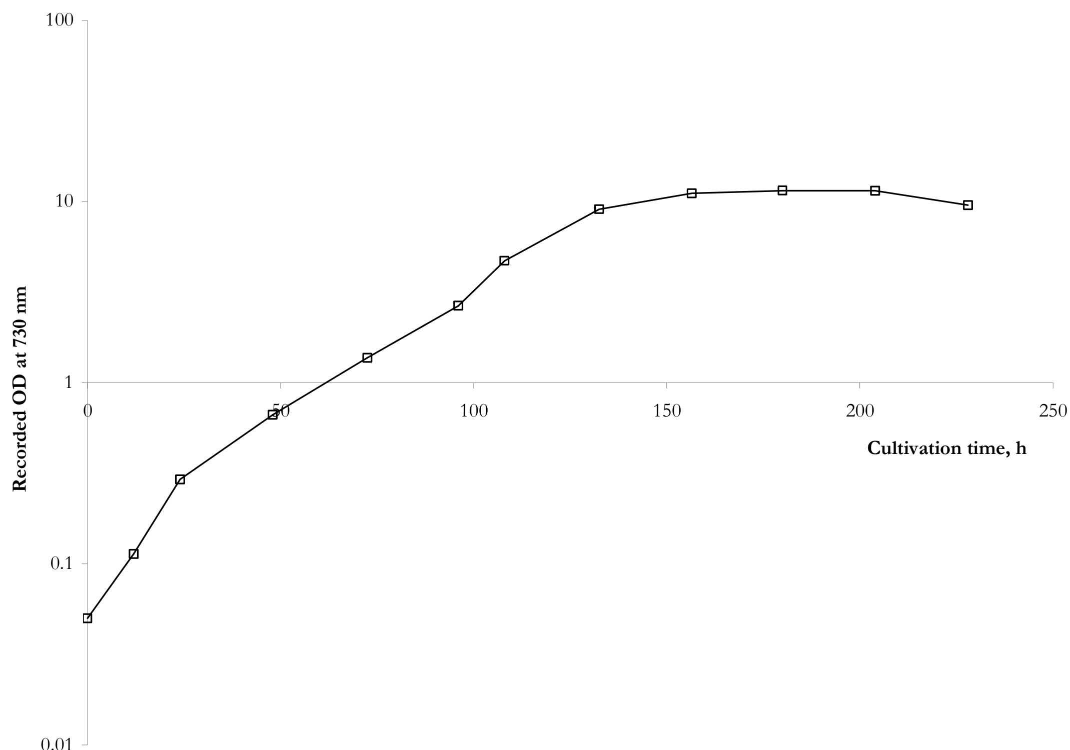

Figure C.2. Logarithmic plot of the culture OD at 730 nm versus the cultivation time, h.

From the plot (Figure C.2), it can be seen that a stationary growth phase was observed after a cultivation time of 180-204 h from the cultivation zero point.

The microalgae cells for use in this study were therefore harvested from the batch reactors after a cultivation period of 8 days as mentioned in Chapter 3.

Also, the time required for the *Chlorella* biomass to double in concentration was also calculated from Figure C.2 solely using the logarithmic phase. A doubling time of 25 h (1.04 d) was estimated.

C.4. References

Bigogno C., Khozin-Goldberg I., Boussiba S., Vonshak A., Cohen Z. 2002. Lipid and Fatty Acid Composition of the Green Oleaginous Algae *Parietochloris incise*, the Richest Plant Source of Arachidonic Acid. Phytochemistry 60, 497-503.

Hu Q., Sommerfield M., Jarvis E., Ghirardi M., Posewitz M., Seibert M., Darzins A. 2008. Microalgal Triacylglycerols as Feedstocks for Biofuel Production: Perspectives and Advances. The Plant Journal 54, 621-639.

Mansour M.P., Volkman J.K. Blackburn S.I. 2003. The Effect of Growth Phase on the Lipid Class, Fatty acid and Sterol Composition in the Marine dinoflagellate, *Gymnodinium sp*. in batch culture. Phytochemistry 63, 145-153.

APPENDIX D. THE R-VALUE CONCEPT AND ESTIMATION OF THE MICROALGAE MACROMOLECULAR COMPOSITION[3]

D.1. Introduction

All of the organic carbon content of photoautotrophic organisms is obtained from the reduction of CO_2 via the photosynthetic process. A simple means of determining the degree of reduction of the photoautotrophs carbon content could therefore be acquired from its elemental composition.

The knowledge of this "degree of reduction" would permit an estimation of the respective proportions of the major macromolecular components of the biomass material. This section details the degree of reduction and R-value concept used in the estimation of the *Chlorella* macromolecular constituents in Chapter 6 (section 6.2.2).

D.2. Degree of reduction of carbon

Photosynthetic organisms use CO_2 (containing 72.7% oxygen (O_2), by mass) and H_2O (containing 88.9% O_2, by mass) to form a host of organic compounds, containing a lower percentage of oxygen. Energy inputs are required for the reduction process to proceed, with the formed compounds representing a form of energy storage. The level of reduction to which the carbon (C) compounds has been reduced is therefore representative of the amount of energy stored (Spoehr & Milner, 1949).

---

[3] The methods presented in this appendix were largely taken from the referenced paper on the subject by Spoehr & Milner (1949).

An expression showing the degree of reduction of C (and the energy content of the C compounds) can be derived, with these parameters in the biomass related to the percentage of C, H and O in it.

With oxidation and reduction being opposite reactions, the most oxidised C compound (in this case CO_2) may be regarded as the least reduced form of C. In this assessment, CO_2 is allocated a degree of reduction of zero (Spoehr & Milner, 1949). This is since CO_2 is a combustion product.

Conversely, methane (CH_4) represents the highest degree of C reduction, and was attributed a degree of reduction of 100%. The degree of reduction of all other organic compounds would therefore be between that of CO_2 and CH_4 (Spoehr & Milner, 1949).

With the assumption of a complete oxidation (i.e. biomass combustion), the degree of reduction of an organic compound can be determined. This is with the consideration of a complete conversion of all the biomass C and H to CO_2 and H_2O respectively, and with the energy released as the heat of combustion equal to the stored energy. The degree of reduction is representative of the weight of oxygen required for the complete combustion of the considered macromolecule, and thus is indicative of how reduced the molecule is (i.e. how much energy is stored in it).

The molar ratios of O_2 to C and O to H for the complete biomass oxidation to CO_2 and H_2O respectively are given by Eqs. D.1 and D.2.

$$\frac{O_2}{C} = \frac{(16 \times 2)}{12.01} = 2.66 \qquad \text{(D.1)}$$

$$\frac{O}{H_2} = \frac{16}{2.016} = 7.94 \qquad \text{(D.2)}$$

The degree of reduction (%) of the organic compound can be calculated using (Eq. D.3).

$$\text{Degree of reduction, \%} = (\%\text{C in compound} \times 2.66) + (\%\text{H in compound} \times 7.94) \qquad \text{(D.3)}$$

With the percentage C and H content of CH_4 of 74.85 and 25.15% respectively, a degree of reduction (%) of 398.99 was calculated for CH_4. This means that CH_4 would combine with 398.99% of its own weight of oxygen during its complete combustion to CO_2 and H_2O.

For organic compounds containing oxygen, less O_2 would be required for their complete oxidation. This is due to the fact that the compound is considered as partially oxidised (Spoehr & Milner, 1949). To determine the degree of reduction of oxygen containing organic compounds, a calculation of the additional O_2 required must be carried out. This was carried out by calculating the total O_2 required for the complete oxidation (as done with the CH_4 example) and subtracting the percentage O content of the organic compound.

The biomass nitrogen (N) content may also be considered in the degree of reduction estimation. The N content of the organic compound was predominantly dealt with in the form of amino groups. The N content would mainly affect the degree of reduction via diminishing the relative percentages of C and H in the organic compound (Spoehr & Milner, 1949).

D.3. The R-value concept

The degree of reduction for organic compounds as discussed in the preceding section (D.2) however presents a problem, since different carbon reduction values can be obtained from various organic compounds. The R-value concept was therefore derived as a suitable comparison scale by Spoehr & Milner (1984).

Since CO_2 has been previously highlighted to have a reduction level of zero, a corresponding R-value of zero was also allocated. Likewise, CH_4 would have an R-value of 100. The degree of reduction of any organic compound can be further expressed as a percentage of the degree of reduction of CH_4. This percentage value represents the R-value of the organic compound.

The general formula for the calculation of the R-value is expressed by (D.4):

$$\text{R} - \text{value} = \frac{\text{Degree of reduction of considered molecule}}{398.9} \times 100 \qquad \text{(D.4)}$$

where, 398.9 is the degree of reduction of CH_4.

With the knowledge of the biomass C, H, N and O composition, the overall energy stored during the macromolecular compounds synthesis can be estimated, with the R-value directly proportional to the heat of combustion per g of the organic compound.

Furthermore, sulphur (S) and phosphorus (P) are also components of the photosynthetic biomass that would influence the O_2 requirement and the estimated R-values. However, due to the relative small quantities of these elements in the biomass (<2% (w/w)) (Benefield & Randall, 1980), their effect on the R-value is considered negligible (Spoehr & Milner, 1949).

D.4. Determination of the microalgae macromolecular component.

The approximate lipid, protein and carbohydrate content of the microalgae biomass (*Chlorella*) was calculated using the results of the elemental analysis (Chapter 3) and R-values of the different macromolecules obtained from the literature (Spoehr & Milner).

For carbohydrates: an R-value of 28 was used (this value was between that estimated for hexose and polysaccharides (Spoehr & Milner, 1949)

For proteins: an R-value of 42 was used (Spoehr & Milner, 1949).

For lipids: calculated from published fat analysis, the R-value of plant lipids was estimated to be 67.5. This value is taken to be applicable to the total lipid content of *Chlorella* biomass (Spoehr & Milner, 1949).

In addition, the percentage N in the biomass samples (w/w) multiplied by 6.25 is used to represent the % protein content of the microalgae samples. The use of this factor (6.25) for the biomass protein content estimation was based on two assumptions; (1) that the biomass carbohydrate and lipid contents do not contain nitrogen, and (2) nearly all the biomass N is present as amino acids in proteins (AOAC, 2006). Empirical demonstrations

on the N content of proteins showed that they contained ≈16% N (w/w), which led to the use of N×6.25 (i.e.1/0.16) for the biomass protein content estimation (AOAC, 2006). This estimation is thus simplified and does not take into consideration the non-protein nitrogen compounds i.e. free amino acids and nucleotides found in the biomass sample. The use of N×6.25 was however deemed to be appropriates for microalgae (*Chlorella*) protein content determination, since the N content of microalgae proteins was experimentally shown to be ≈16% (Spoehr & Milner, 1949).

With the protein content and the R-value of the biomass sample as constants, the biomass carbohydrate and lipid contents were calculated using Eqs. D.5 and D.6. This simplified estimation was made with the assumption that the biomass was composed of the three major macromolecules: lipids, carbohydrates and proteins.

$$\text{R}-\text{value}\times 100=(\%\text{proteins}\times 42)+(\%\text{ carbohydrates}\times 28)+(\%\text{ lipids}\times 67.5) \qquad \text{(D.5)}$$

$$100=\%\text{ proteins}+\%\text{ carbohydrates}+\%\text{ lipids} \qquad \text{(D.6)}$$

For example, using the *Chlorella* sample with a biomass N composition of 7.25% (w/w) (Table 3.3) and an estimated R-value of 45.06 (calculated using Eq. D.2 and the elemental percentage composition presented in Table 3.3), we have:

$$\text{Protein content, P }(\%)=7.25\times 6.25=45.31\%$$

$$\therefore \%\text{ Carbohydrate (C)}+\%\text{ Lipids (L)}=100-\text{P}=54.69\%$$
and
$$(\text{C}\times 28)+(\text{L}\times 67.5)=2602.98$$

solving both equations, we have

$$\text{L}=27.13\%$$
$$\text{C}=27.56\%$$

The protein, carbohydrate and lipid content of the untreated *Chlorella* samples used in this study were therefore estimated to be 45.31, 27.13 and 27.56% respectively. The macromolecular estimation obtained using the R-value concept was considered to be

representative of the considered microalgae biomass and suitable for use in this thesis since the theoretically estimated lipid content was seen to be within <1.5% of the lipid content values demonstrated experimentally in Table 3.4 (chapter 3).

This R-value scheme was then applied for the estimation of the macromolecular biomass composition of the post transesterified microalgae biomass as highlighted in chapter 6 for the calculation of the potential CH_4 yield from the microalgae residues.

D.5. References

AOAC, 2006. Official Methods of Analysis of AOAC international. 18th Edition. Association of Official Agricultural Chemists. Maryland, US.

Benefield L.D., Randall C.W. 1980. Biological Process Design for Wastewater Treatment. Prentice-Hall Inc, Englewood Cliffs, New Jersey, US.

Spoehr H.A., Milner H.W. 1949. The Chemical Composition of *Chlorella*: Effect of Environmental Conditions. Plant Physiology 24, 120-149.

APPENDIX E. DETAILS OF THE ASPEN MODEL INPUTS FOR THE UP-SCALED INVESTIGATED PROCESSES (CD-ROM)[4]

E.1. Input and report files for the up-scaled mechanically stirred in-situ transesterification process

E.2. Input and report files for the up-scaled in-situ transesterification process with ultrasound agitation

E.3. Input and report files for the up-scaled mechanically stirred in-situ transesterification process with diethyl ether as a process co-solvent

E.4. Input and report files for the up-scaled ultrasound agitated in-situ transesterification process with diethyl ether as a co-solvent.

E.5. Input and report files for the up-scaled mechanically stirred conventional transesterification process

[4] The .txt report files should preferably be viewed using wordpad.

APPENDIX F. HEAT INTEGRATION ANALYSIS OF THE MINIMUM PROCESS (HOT AND COLD) UTILITIES REQUIREMENTS (CD-ROM)

F.1. Heat integration data (using pinch analysis) for the mechanically stirred in-situ transesterification process

F.2. Heat integration data (using pinch analysis) for the ultrasonically agitated in-situ transesterification process

F.3. Heat integration data (using pinch analysis) for the mechanically stirred in-situ transesterification process with diethyl ether as a co-solvent

F.4. Heat integration data (using pinch analysis) for the ultrasonically agitated in-situ transesterification process with diethyl ether as a co-solvent

F.5. Heat integration data (using pinch analysis) for the mechanically stirred conventional lipid extraction and transesterification process

F.6. Heat integration data (using pinch analysis) for the mechanically stirred in-situ transesterification process coupled with anaerobic digestion of the microalgae residues.

F.7. Heat integration data (using pinch analysis) for the ultrasonically agitated in-situ transesterification process coupled with methane production from the microalgae residues

F.8. Heat integration data (using pinch analysis) for the mechanically stirred in-situ transesterification process with diethyl ether as a co-solvent coupled with anaerobic digestion of the microalgae residues

F.9. Heat integration data (using pinch analysis) for the ultrasonically agitated in-situ transesterification process with diethyl ether as a co-solvent coupled with anaerobic digestion of the microalgae residues

APPENDIX G. FUEL PROPERTIES OF THE PURIFIED FAME AND EXTRACTED CHLORELLA OIL

G.1. Preparation of the samples for analysis

The biodiesel product (containing fatty acid methyl esters (FAME) and unreacted microalgae oil) was obtained following the product purification after the in-situ transesterification processes using *Chlorella* biomass as the reaction feedstock. The biodiesel samples using the in-situ transesterification process were collected from the experiments as conducted in Chapters 4 and 5 and pooled. This was with the use of methanol volumes of ≥ 60 ml for the transesterification of 15 g of dried *Chlorella* biomass with 0.04 mol H_2SO_4 as catalyst, and reaction temperatures and times ≤ 60°C and 8 h respectively. The FAME products obtained after the use of the ultrasound assisted process for the in-situ transesterification process, as well as the integration of co-solvents in the conversion process were also collected.

The pooled *Chlorella* biodiesel samples were used for the fuel properties analysis.

Samples of the extracted *Chlorella* oil as described in Chapter 3 were also collected for analysis.

G.2. Fuel properties testing

The biodiesel product obtained from the various transesterification processes was stored in a sealed tin can. The biodiesel sample (340 ml) was then sent by courier to Independent Petroleum Laboratory Ltd (IPL), NZ Refinery, Rukaka, NZ.

The decision to use this independent fuel testing facility was based on the fact that it is listed as one of the nationally certified testing centres for the analysis of petroleum fuels and biofuels proposed for use in NZ. This laboratory was also accredited by International

Accreditation NZ (IANZ). Furthermore, due to the prohibitively expensive costs of acquiring the testing apparatus for short term use in our laboratory, carrying out the fuel tests using this accredited facility appeared to be a more practical and economical choice.

The number of fuel property tests investigated in this study was limited by the available sample size. The tests were restricted to major fuel properties or those that had not been previously described in the literature i.e. in Miao & Wu, 2006.

The heat of combustion of the extracted oil and the purified biodiesel products was carried out at the Campbell Micro-analytical Lab, University of Otago, NZ. The samples for the heat of combustion analysis were conveyed there in sealed glass tubes.

Using the same methods as described in section 3.2.6.3, the acid value of the purified biodiesel was estimated.

The testing protocols used, as well as the reasons for the different fuel properties tested are highlighted in section G.3 . The results of the fuel test are also presented in section G.3. The significance of the investigated fuel properties was also briefly discussed.

G.3. Results and discussions

The results obtained for the respective fuel properties tests, and the methods used in their determination are presented in Table G.1.

The flash point of a fuel is defined as the lowest temperature at which an introduced ignition source will result in the combustion of the vapours of the sample (Van Gerpen et al., 2004). This fuel property is important since it indicates the overall volatility as well as the flammability of the fuel. It therefore measures the tendency of the fuel to form a flammable mixture with air (Srivastsva & Prasad, 2000).

Table G.1. Fuel properties of the *Chlorella* derived biodiesel and extracted oil.

Method	Fuel property	Units	Microalgae derived biodiesel	Biodiesel standard (EN 14214)[5]
D 93-10[6]	Flash point (Pensky-martens)	°C	111	>101
IP 309A[7]	Cold filter plugging point	°C	-13	0 – -20[8]
D [9]445	Kinematic Viscosity at 40°C	cSt	4.16	3.5 – 5.0
BS EN 114111:2003[10]	Iodine value	g I/100g	102	< 120
	Acid value	mg KOH/g	<0.03	<0.5
EN 14103[11]	FAME content	% mass	97.4	96.5
D 4809[12]	Heat of combustion	kJ/g	40.70	-
Extracted *Chlorella* oil				
D 4809	Heat of combustion	kJ/g	40.15	-

[5] European standard test methods for fatty acid methyl esters (FAME)

[6] ASTM D 93-10: Standard test methods for flash point by Pensky Martens closed cup tester.

[7] IP 309: Institute of petroleum (IP) test method for cold filter plugging point.

[8] Cold filter plugging point limits vary with the season: 15 April -30 September (0°C), 1 October - 15 November (-10°C), 16 November - 28 February (-20°C) and 1 March - 14 April (-10°C).

[9] ASTM D 445-09: standard test method for kinematic viscosity of transparent and opaque liquids (and calculation of dynamic viscosity).

[10] BS EN 14111: Iodine value determination.

[11] BS EN 14103: Determination of FAME contents in biodiesel (approved alternative).

[12] ASTM D 4809-09: Standard test method for heat of combustion of liquid hydrocarbon fuels by bomb calorimeter (precision method)

The determined flash point of the *Chlorella* derived biodiesel (111°C)although satisfying the biodiesel standards (EN 14214) was observed to be significantly lower than that of other plant oil derived biodiesel products demonstrated in the literature. For example sunflower, soybean and palm oil derived biodiesel were reported to have flash points of 183, 178 and 164 °C respectively (Barnwal & Sharma, 2005).

The cold filter plugging point, CFPP (°C) specifies the lowest temperature at which the fuel will give a problem free flow. It serves as a useful indicator of the fuel under cold conditions. This parameter is especially important in environment with cold winter periods. Comparing the measured CFPP of the *Chlorella* biodiesel with that of the European biodiesel standards, it can be seen that the 100% biodiesel is suitable for use for most of the year except in the period 16 November- 28 February. With New Zealand conditions, this would correspond to the winter period between June-August. The biodiesel CFPP would be considered to be especially important in the central regions of the South Island where winter night time temperatures of < -22°C have been recorded (Walrond, 2009).The blending of the microalgae derived biodiesel with other biodiesel fuels with a suitable CFPP or with petro-diesel could therefore be considered for use for those periods and locations.

The kinematic viscosity (ν) refers to the resistance to flow of a fluid under gravity, with this parameter related to the dynamic viscosity (η) of a fluid via its density (ϱ) (Van Gerpen et al., 2004) as seen in Eq. G.1.

$$\text{Kinematic viscosity, } \nu\,(\text{cSt}) = \frac{\text{dynamic viscosity, } \eta}{\text{density, } \varrho} \qquad \text{(G.1)}$$

Information on this fuel property is important since a proper viscosity range is required for a proper operation of the engine, flow through pipes, injector nozzles and orifices (Srivastava & Prasad, 2000). It is also important for the effective atomisation of the biodiesel fuel in the cylinder (Srivastava & Prasad, 2000).

As shown in Table G.1, the observed kinematic viscosity[13] of the produced biodiesel falls within the limits specified for use in conventional vehicle engines.

Compared with the acid value of the extracted microalgae lipids (10.21 mg KOH/g *Chlorella* oil) (section 3.3.5), a reduction in the estimated acid value of the microalgae derived biodiesel was observed. This result indicates that the acid catalysed in-situ transesterification reaction used in this study led to an almost complete conversion (via esterification) of the free fatty acid contents of the *Chlorella* oil to FAME.

The specific energy (or heat of combustion) of the fuel is the quantity of heat released by the combustion of a specified quantity of the fuel with oxygen. The heat of combustion of the fuel product was reported on the basis of the FAME content of the purified biodiesel. The transesterification of the *Chlorella* oil was observed to improve the energy content of the fuel by 1.4% when compared to that of the extracted oil.

G.4. References

Barnwal B.K., Sharma M.P. 2005. Prospects of Biodiesel Production from Vegetable Oils in India. Renewable and Sustainable Reviews 9, 363-378.

Srivastava A.., Prasad R. 2000. Triglycerides-Based Diesel Fuels. Renewable and Sustainable Energy Reviews 4, 111-133.

Van Gerpen J., Shanks B., Pruszko R., Clements D., Knothe G. 2004. Biodiesel Production Technology. National Renewable Energy Laboratory. Golden, Colorado. US. NREL/SR-510-36244.

Walrond C. 2009. Natural Environment- Climate. Te-Ara – The Encyclopedia of New Zealand. URL:http://www.teara.govt.nz/en/natural-environment/3. Accessed on the 18 September 2010.

[13] Kinematic viscosity reported in centistokes (cSt), where, 1 cSt= 10^{-6} m^2/ s

APPENDIX H. REGRESSION ANALYSIS FILES FOR THE ANAEROBIC DIGESTION EXPERIMENTS

The data contained in this appendix folder is presented mostly in a form (i.e. .mat) which is convenient for manipulation using MATLAB v.2007 or later.

A MS Excel sheet containing the data obtained during the anaerobic digestion experiments is also contained in the folder

APPENDIX I. ERRORS ESTIMATIONS USED IN CHAPTERS 3 AND 4 (MAIN THESIS)

I.1. Estimation of uncertainties associated with the determined oil content and SG

The standard error in the mean of the experimental measurements was used to estimate the uncertainties associated with the determination of the oil content and specific gravity of the *Chlorella* biomass. The standard mean error (e) of the measurement with a value x_i was estimated from the residual (d_i) of the mean ($\overline{x}$) of the number of repeated measurement (n) used in the experiment as shown in Eqs. I.1-I.3

$$d_i = x_i - \overline{x} \tag{I.1}$$

The obtained residual is used to estimate the variance, which is equal to the standard deviation (s^2), i.e.

$$s = \sqrt{\frac{1}{n-1}\sum d_i^2} \tag{I.2}$$

The standard error of the mean was then estimated from the standard deviation using:

$$e_i = \frac{s}{\sqrt{n}} \tag{I.3}$$

The estimation of the standard mean errors for the oil content and SG measurements were then obtained. The oil content and SG determination experiments were carried out in triplicates (i.e. n=3).

Although the number of the independent experimental runs (n) was not considerably large, this method was deemed to be suited for the estimation of the errors associated with the triplicate SG and oil content measurements (Squires, 2001).

For the determination of the errors associated with the estimated SG of the extracted oil, only the contributions from the extracted oil density were taken into account. This was used because only instrumental errors were observed with the measurements water density at 25°C. For example, Table I.1 shows the recorded experimental values for the water density determined at 25°C.

Table I.1. Measured values for the water density after three independent runs

Pycnometer (empty) (g)	Pycnometer +water (g)	Density water (g/ml)
4.2479	5.2443	0.9964
4.2481	5.2444	0.9963
4.2480	5.2444	0.9964

For the subsequent uncertainties calculations for the measured SG in Chapters 3 and 4, the errors attributable to the pycnometer mass and water density were hence treated as negligible.

The mean empty pycnometer mass and water density of 4.2480 and 0.9964 were used for the estimation of the errors associated with the oil SG as shown in Table I.2 in section 3.3.The standard mean error presented was calculated on the basis of the mean oil SG obtained after 3 runs as shown in Table I.2 and using Eq. I.3.

Table I.2. Estimation of the uncertainties associated with the extracted Chlorella oil SG

Run no (n)	Pycnometer (empty) (g)	Pycnometer + oil (g)	Oil density at 25°C (g/ml)	Water density at 25°C (g/ml)	SG	Standard error, $e = \frac{s}{\sqrt{n}}$
1	4.2480	5.1592	0.9112	0.9964	0.9145	0.001
2	4.2480	5.1601	0.9121	0.9964	0.9154	
3	4.2480	5.1569	0.9089	0.9964	0.9122	

I.2. Estimation of the uncertainties associated with the calibration curve for the estimation of the percentage FAME conversion.

The method of least squares (Squires, 2001) was used to assess the uncertainties associated with the estimation of the percentage mass conversion of oil to FAME from the measured SG using the straight line function shown in Figure 4.7 (in Chapter 4).

The straight line function ($y = mx + c$) was obtained for the fitted curve, where y is the measured SG, m is the slope and c is a constant. The estimation of the accuracy of the predicted percentage biodiesel FAME mass content (%) values incorporated the estimation of the errors associated with m, c, and y.

Assuming the errors obtained in n pairs of measurements (x_1,y_1), (x_2,y_2),…, (x_n,y_n) are entirely in the y values (measured S.G), for a given pair of values for m and c, the deviation (d) of the ith reading is (Squires, 2001):

$$d_i = y_i - mx_i - c \qquad \text{(I.4)}$$

Eqs. 4.5 and 4.6 were then used to estimate the standard errors in m and c i.e. Δm and Δc respectively:

$$(\Delta m)^2 \approx \frac{1}{D}\frac{\sum d_i^2}{n-2} \qquad \text{(I.5)}$$

Where, $D = \sum\left(x_i - \bar{x}\right)^2$, and $\bar{x}$ represents the calculated mean.

$$(\Delta c)^2 \approx \left(\frac{1}{n} + \frac{\bar{x}^2}{D}\right)\frac{\sum d_i^2}{n\text{-}2} \qquad \text{(I.6)}$$

Rearranging the fitted straight line equation, we have

$$x_i = f\left(y_i, c, m\right) = \frac{y_i - c}{m} \qquad \text{(I.7)}$$

The errors associated with the estimated percentage mass content of FAME in the biodiesel samples (Δx_i) was thus estimated using.

$$(\Delta x_i)^2 = \left(\frac{\partial f}{\partial m}\Delta m\right)^2 + \left(\frac{\Delta f}{\Delta y}\Delta y\right)^2 + \left(\frac{\Delta f}{\Delta c}\Delta c\right)^2 \qquad \text{(I.8)}$$

It must be noted that the large uncertainties (> 0.001) as seen in Table 4.2, associated with the SG of the biodiesel product arise from variations in the triplicate measurements.

I.3. Calculation of the uncertainties associated with the estimated FAME yield (g FAME/ g dry microalgae)

The uncertainties associated with the FAME yields as presented in section 4.3.7.1 (Chapter 4) were obtained on the basis of the sum of the percentage error contributions of the measured SG, corresponding mass percentage FAME content in the biodiesel and biodiesel yields.

This was calculated using:

$$\frac{\Delta F}{F}\% = \sqrt{\left(\left(\frac{\Delta SG}{SG}\%\right)^2 + \left(\frac{\Delta FC}{FC}\%\right)^2 + \left(\frac{\Delta B}{B}\%\right)^2\right)} \qquad \text{(I.9)}$$

where, $\Delta F/F$= the percentage error associated with the estimated FAME yield.

ΔSG= the standard mean error of the measured biodiesel SG

SG= the measured average SG of the biodiesel product

$\Delta FC/FC$= the percentage mean error associated with the estimated mass percentage FAME in the biodiesel (FC= the estimated % FAME mass content in the biodiesel; ΔFC= the uncertainty of the estimated FC)

$\Delta B/B$= the percentage mean error associated with the measured purified biodiesel yield. (B= the average measured biodiesel yield, g; ΔB= the measured mean errors of the biodiesel yields, g)

Samples of the MATLAB files used in the calculation of the errors are presented in the attached CD-ROM in the file Appendix I.

I.4. References

Squires G.L. 2001. Practical Physics. 4th edition. Cambridge University Press. Cambridge, UK.

APPENDIX J. CONSTITUENT FATTY ACID METHYL ESTERS IN THE REFERENCE STANDARD (FAMQ-005)

J.1. List of FAMEs in FAMQ005 (1 mL) in CH_2Cl_2 (total of 10 mg/ml)

Butyric acid methyl ester (C4:0) (0.4 mg/ml)

Caproic acid methyl ester (C6:0) (0.4 mg/ml)

Caprylic acid methyl ester (C8:0) (0.4 mg/ml)

Capric acid methyl ester (C10:0) (0.4 mg/ml)

Undecanoic acid methyl ester (C11:0) (0.2 mg/ml)

Lauric acid methyl ester (C12:0) (0.4 mg/ml)

Tridecanoic acid methyl ester (C13:0) (0.2 mg/ml)

Myristic acid methyl ester (C14:0) (0.4 mg/ml)

Myristoleic acid methyl ester (C14:1) (0.2 mg/ml)

Pentadecanoic acid methyl ester (C15:0) (0.2 mg/ml)

cis-10-Pentadecenoic acid methyl ester (C15:1) (0.2 mg/ml)

Palmitic acid methyl ester (C16:0) (0.6 mg/ml)

Palmitoleic acid methyl ester (C16:1) (0.2 mg/ml)

Heptadecanoic acid methyl ester (C17:0) (0.2 mg/ml)

cis-10-Heptadecenoic acid methyl ester (C17:1) (0.2 mg/ml)

Stearic acid methyl ester (C18:0) (0.4 mg/ml)

Elaidic acid methyl ester (C18:1n9t) (0.2 mg/ml)

Oleic acid methyl ester (C18:1n9c) (0.4 mg/ml)

Linolelaidic acid methyl ester (C18:2n6t) (0.2 mg/ml)

Linoleic acid methyl ester (C18:2n6c) (0.2 mg/ml)

Arachidic acid methyl ester (C20:0) (0.4 mg/ml)

γ-Linolenic acid methyl ester (C18:3n6) (0.2 mg/ml)

cis-11-Eicosenoic acid methyl ester (C20:1) (0.2 mg/ml)

Linolenic acid methyl ester (C18:3n3) (0.2 mg/ml)

Heneicosanoic acid methyl ester (C21:0) (0.2 mg/ml)

cis-11,14-Eicosadienoic acid methyl ester (C20:2) (0.2 mg/ml)

Behenic acid methyl ester (C22:0) (0.4 mg/ml)

cis-8,11,14-Eicosatrienoic acid methyl ester (C20:3n6) (0.2 mg/ml)

Erucic acid methyl ester (C22:1n9) (0.2 mg/ml)

cis-11,14,17-Eicosatrienoic acid methyl ester (C20:3n3) (0.2 mg/ml)

Arachidonic acid methyl ester (C20:4n6) (0.2 mg/ml)

Tricosanoic acid methyl ester (C23:0) (0.2 mg/ml)

cis-13,16-Docosadienoic acid methyl ester (C22:2) (0.2 mg/ml)

Lignoceric acid methyl ester (C24:0) (0.4 mg/ml)

cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5n3) (0.2 mg/ml)

Nervonic acid methyl ester (C24:1) (0.2 mg/ml)

cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester (C22:6n3) (0.2 mg/ml)

APPENDIX K. A GC PROFILE OF THE MICROALGAE FAME AND REFERENCE STANDARD

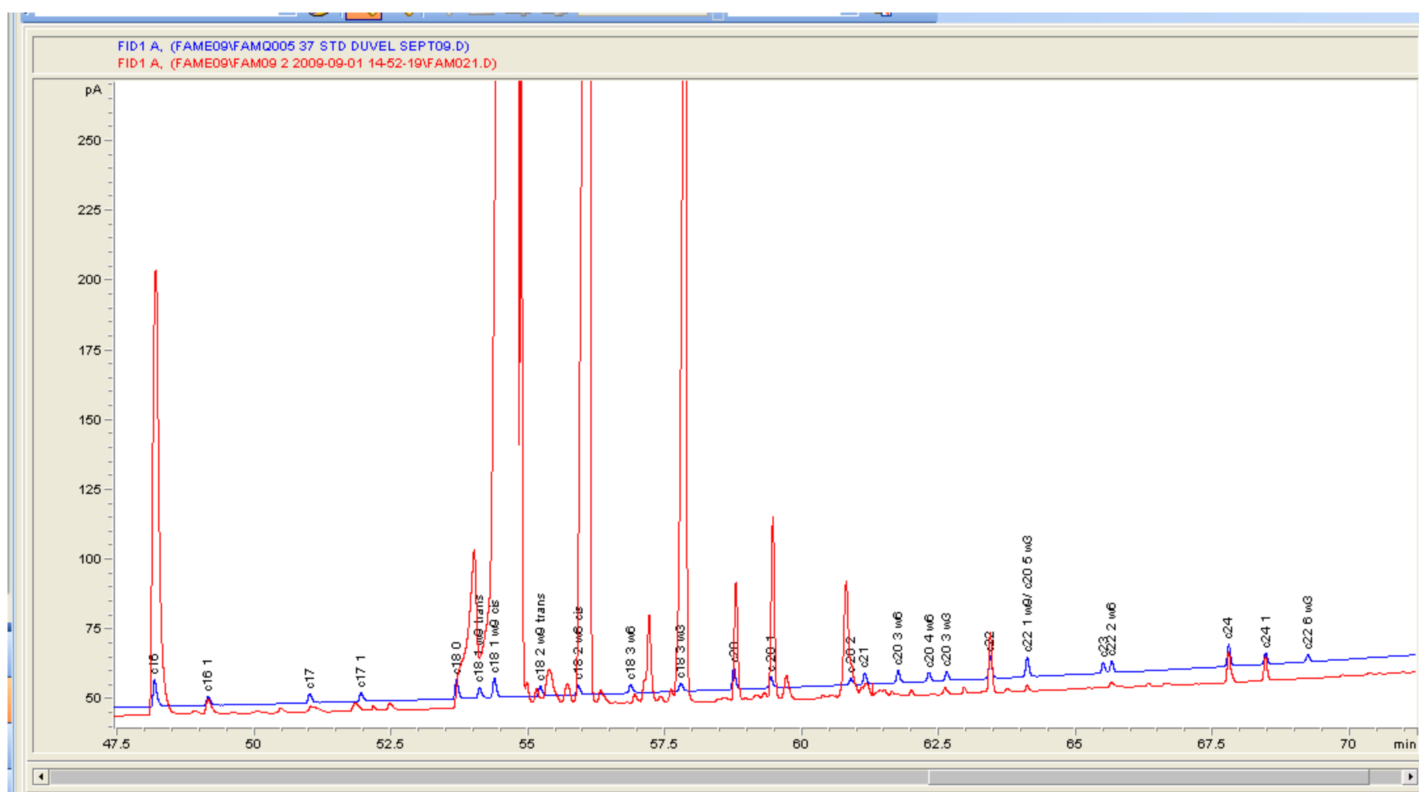

Figure K.1. Sample of GC profile showing the relationship between the area (y axis, pA) and retention time (x axis, min) for the microalgae FAME (red) and reference FAMQ005 (blue).