EFFECT OF TEMPERATURE AND MOISTURE ON HIGH PRESSURE LIPID/OIL EXTRACTION FROM MICROALGAE

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Abstract

Commercially viable carbon-neutral biodiesel production from microalgae has potential for replacing depleting petroleum diesel. The process of biodiesel production from microalgae involves harvesting, drying and extraction of lipids which are energy- and cost-intensive processes. The development of effective large-scale lipid extraction processes which overcome the complexity of microalgae cell structure is considered one of the most vital requirements for commercial production. Thus the aim of this work was to investigate suitable extraction methods with optimised conditions to progress opportunities for sustainable microalgal biodiesel production. In this study, the green microalgal species consortium, Tarong polyculture was used to investigate lipid extraction with hexane (solvent) under high pressure and variable temperature and biomass moisture conditions using an Accelerated Solvent Extraction (ASE) method. The performance of high pressure solvent extraction was examined over a range of different process and sample conditions (dry

biomass to water ratios (DBWRs): 100%, 75%, 50% and 25% and temperatures from 70 to 120 °C, process time 5-15 min). Maximum total lipid yields were achieved at 50% and 75% sample dryness at temperatures of 90-120 ^{0}C. We show that individual fatty acids (Palmitic acid C16:0; Stearic acid C18:0; Oleic acid C18:1; Linolenic acid C18:3) extraction optima are influenced by temperature and sample dryness, consequently affecting microalgal biodiesel quality parameters. Higher heating values and kinematic viscosity were compliant with biodiesel quality standards under all extraction conditions used. Our results indicate that biodiesel quality can be positively manipulated by selecting process extraction conditions that favour extraction of saturated and mono-unsaturated fatty acids over optimal extraction conditions for polyunsaturated fatty acids, yielding positive effects on cetane number and iodine values. Exceeding biodiesel standards for these two parameters opens blending opportunities with biodiesels that fall outside the minimal cetane and maximal iodine values.

Keywords: Microalgae; fatty acid; lipid extraction; sample dryness; Soxhlet; accelerated solvent extraction; biodiesel quality

1. **Introduction**

There is increasing global demand for renewable and carbon-neutral environmentally friendly transport fuels [1, 2]. As a new source of renewable energy in the form of biodiesel, microalgae have received much attention [3-7]. Photosynthesis and carbon assimilation mechanisms of ancient microalgae are similar to higher plants [8]. Microalgae, however, can convert solar energy, and access water, CO_2 and other nutrients more efficiently because of cultivation in aqueous suspensions [9]. Some of the main characteristics which make algae more attractive than other forms of biomass are higher yields per unit light and area, higher lipid contents, smaller land footprint, ability to grow in saline water and wastewater and ability to utilise CO_2 from combustion gas [10, 11]. Quantitative and qualitative lipid

contents and compositions are critical parameters in the selection process of microalgal species for large-scale production [12]. Furthermore, careful downstream process planning for commercial biodiesel production from microalgae is also mandatory for establishing a microalgae-based renewable fuel industry [13].

Even though microalgae offer many advantages, they are not yet commercially viable for biodiesel production because of the high cost of the production process. The most important challenges lie in the fields of biomass harvesting and lipid extraction technology, which together account for around 70-80% of the total production cost, but biomass dewatering and drying are the main hurdles for economically and energetically sustainable biodiesel production from microalgae [9, 14, 15]. Unlike terrestrial oil feedstock such as soy or canola, from which lipids can be extracted by crushing of the seed followed by solvent extraction, the small size and presence of rigid cell walls hinder the extraction of lipids from microalgae currently investigated for biofuel production[16]. Compared to other microalgal groups and a few oleaginous green algae, freshwater green microalgae typically contain low amounts of fatty acids [17, 18], with most fatty acids being membrane fatty acids during favourable growth conditions [19]. For example, only 13% of the total lipids were tri-acylglycerides in *Scenedesmus* sp. [20], a dominant species of the Tarong polyculture biomass processed here. Membrane lipids are mainly polar lipids and require extraction with polar solvents.

Optimal conditions for large-scale lipid extraction from microalgae are species-dependent. Furthermore, sample dryness plays a role in lipid extraction. Dried biomass is preferred for optimal extraction yields but wet samples can be efficiently extracted at high pressure, since water acts as a solvent due to reduced polarity under these conditions [21, 22]. Supercritical carbon dioxide (SC-CO_2) is considered one of the most promising techniques for producing solvent-free extracts [23]. However, supercritical CO_2 is less effective for

polar lipids (membrane lipids) because of its low polarity [24]. It is also reported that super critical carbon dioxide (SC-CO_2) can be used to extract lipid from wet microalgae biomass. However, the total fatty acid of extracted lipid by SC-CO_2 was lower than conventional solvent extraction [25].

Laboratory-scale lipid extraction procedures from microalgae are well developed for determining the total lipid content of a sample gravimetrically. On the other hand, parameters affecting large-scale extraction of lipids from microalgae for commercial biodiesel production are not well understood. Moreover, most of the reported research has focused on nutraceutical/maricultural application of microalgal lipids and does not assess the requirements for biodiesel production [23, 24, 26, 27]. Optimal commercial lipid extraction processes should not only consider total lipid yields and minimization of co-extraction of impurities such as pigments, but should also aim to preferentially extract specific fatty acids that provide optimal biodiesel characteristics [28-30]. Traditional hexane solvent extraction and *in-situ* transesterification [31] methods for biodiesel production may not be suitable for many microalgae because of the presence of water and cell wall barriers [32, 33]. The high levels of fat-soluble pigments in microalgae further complicate selective extraction of fatty acids and purification of the biofuel [34]. Recently one-step microwave irradiation has been reported as a faster and easier lipid extraction method compared to conventional two-step heating methods [35].

However, optimal solvent-based extraction processes for microalgae will vary based on cell construction and chemical interactions of lipids and solvents used for extraction [36, 37]. In general, non-polar lipids will dissolve optimally in non-polar solvents, while polar lipids will extract better with more polar solvents [34]. Organic solvent-based extraction processes currently in use remain largely bench-scale methods. They are considered difficult to scale up for industrial processes due to the toxicity of the solvents and quantities required resulting in

expensive operation and infrastructure requirements. The use of higher temperatures for lipid extraction under pressure has gained popularity due to two main factors: (1) the rate of mass transfer increases at higher temperatures and pressures due to enhanced solvent access to pores within the biomass matrix [38], and (2) the dielectric constants are reduced at high pressures for immiscible solvents to better match the polarity of the lipid [24, 39]. Accelerated solvent extraction (ASE) methods use organic solvents at pressures and temperatures above their standard boiling points to extract lipids quickly and efficiently with minimal solvent use [39, 40]. Laboratory-scale ASE allows for extraction of 1-100 g biomass in minutes rather than hours required for more traditional extraction techniques [39]. Programed ASE extraction procedures additionally reduce labour costs and are also time-saving. Therefore, this study, evaluated optimal lipid extraction conditions (temperature, moisture, treatment time and solvent requirements) using ASE from a green freshwater microalgal consortium cultivated in large outdoor ponds.

2. Materials and Methodology

2.1. Sample preparation

Tarong polyculture was grown under outdoor conditions at the MBD Energy-James Cook University Research and Development Site at James Cook University, Townsville, Australia. The Tarong polyculture is a mixture of several freshwater green microalgae, including *Scenedesmus dimorphus, S. obliquus, Franceia* sp., *Mesotaenium* sp. *Chlorella* sp. and dominated by *Senedesmus obliquus*. The Tarong polyculture was grown in sterile filtered L1 medium [41] prepared in dechlorinated freshwater and omission of silicate, in 10,000 L horizontal covered aerated batch-cultures with a cultivation depth of 30 cm during the Austral autumn between March and May 2012. Outdoor temperatures did not exceed 28 °C and

photon flux densities varied from 80 to 2000 µmol photons m^{-2} s^{-1} during the day, with cultures receiving 400 µmol of photons m^{-2} s^{-1} on average during the 10 h day light period. Microalgal samples were harvested by centrifugation (Evodos Type 25) in stationary growth phase and freeze-dried. Freeze-dried biomass was posted via courier to the Queensland University of Technology for ASE-hexane extraction. The dried algae samples were mixed with water in order to produce samples of different moisture levels. Diatomaceous earth (DE) was added in different ratios depending on the dry biomass to water ratio (DBWR) as recommended by Thermo Fisher Scientific Inc. For ASE-350 (Dionex) shown in (Supplementary Table S1). While addition and recovery of DE adds additional costs to fatty acid extractions from microalgal biomass, advantages of DE are: absorbance fo some of the moisture and increase of the porosity of the sample, providing a cleaner transfer of the mixture to the ASE cell and enhancing the ASE extraction process [42]. Due to the silicate-based nature of DE, recovery is possible by oxidizing the extracted microalgal biomass under acidic conditions. In a time, however, where agricultural productivities need to be enhanced significantly due to strong population growth, it is also possible to utilize the organic nitrogen- and phosphorous containing DE as soil additives, enhancing soil structure through carbon addition and thereby water retention, whilst simultaneously providing fertilization to crops. As such, the extracted biomass containing DE could be developed as a co-product for the agricultural market, providing an additional income stream, rather than additional costs.

2.2. Experiment setup

An ASE-350 (Dionex, USA) was used for solvent extraction. The instrument contains an automated extraction control system where temperature can be selected in the range of 40 ^{0}C to 200 ^{0}C at pressures up to 11.7 MPa. In this experiment some of the parameters were fixed (cell pressure 10.3 MPa, rinse volume 40%, purge time 180 s, cell type 66 mL, solvent

saver mode off) while other parameters (sample DBWR, temperature and extraction time) were varied. For loading, hexane was pumped into the cell with the pressurized nitrogen gas (1500 PSI) until the pressure reached 10.3 MPa in the cell. The cell was then loaded into the oven for heating to the selected operation temperature with the heat-up time dependent on the operating temperature. After reaching operation temperature, fresh solvent was pumped to the cell throughout the process time. At the end of the process time, fresh solvent was pumped into the cell expelling the solvent and extracted lipids.

Extraction conditions for the ASE were focused on testing the effect of sample DBWR, temperature and process time on the extracted lipid quantity. Samples of the Tarong polyculture were extracted under four different DBWR (100, 75, 50 and 25 %) and six different temperatures (70, 80, 90, 100, 110 and 120 °C) at three different process times (5, 10 and 15 min) with 2 process cycles (Supplementary Table S2).

2.3. Determination of total lipid and fatty acid methyl ester (FAME) content

Samples of freeze-dried microalgal biomass were mixed with water to produce sub-samples of different levels of DBWR. These sub-samples were then mixed with DE, and placed in the ASE Zirconium extraction cell with extraction solvent at temperatures from 70 to 120 °C. The Tarong polyculture samples were extracted using a single solvent, hexane. Extracted lipids were collected from the ASE into vessels in the collection tray. Loading and operation of the ASE was as described above. Solvent was evaporated under a gentle stream of nitrogen in a Dionex™ SE®400 solvent evaporator (Dionex, Thermo Fisher Scientific Inc, Australia) in a fume hood after transfer to pre-weighted glass vials. After evaporation to dryness, the vial was weighed to determine lipid yield, gravimetrically. The weight of the lipid was obtained by subtracting the tare weight of the vials from the final weight of the vials.

All extracts from ASE were resuspended and simultaneously extracted and esterified in a direct transesterification method adapted from [43] and[44] as described in detail in Gosch et al. [45]. The resulting FAMEs were separated and quantified on an Agilent 7890 GC (DB-23 capillary column, 60m x 0.25 mm id x 0.15 µm) and an Agilent 5975C Electron Ionisation (EI) Turbo Mass Spectrometer (Agilent Technologies Australia Pty Ltd). The column temperature gradient was programmed following [46], ramping from 50 °C to 250 °C. The quantity and identity of fatty acids were determined using external standards (Sigma Aldrich) and NIST08 Mass Spectral Library. Total fatty acid content was determined as the sum of all FAMEs and was corrected for recovery of internal standard (C19:0).

A detailed summary of high pressure FAME extraction profiles obtained in response to temperature, sample DBWR and process time can be found in supplementary materials Tables S3-S6.

A controlled method modified from Folch *et al.* [47] and Somersalo *et al.* [48] with less toxic solvents, *i.e.*, hexane/methanol, was performed to extract lipids from the Tarong polyculture sample to confirm the extraction efficiency of ASE method. The details process is explained in [49]. FAME analysis was carried out as per [45] in scan-mode on an Agilent 7890 GC equipped with a flame ionization detector (FID) and connected to an Agilent 5975C electron ionisation (EI) turbo mass spectrometer (Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia). Detail process is given in our previous study (islam et.al. 2013) [49].

2.4. Determination of cetane number, kinematic viscosity, higher heating and iodine values

Cetane number, kinematic viscosity and higher heating value were calculated from the mass fraction of individual fatty acids using equations 1-4 as in [50], respectively.

$$CN_i = -7.8 + 0.302 \times M_i - 20 \times N \quad (1)$$

Where CN_i is the cetane number, M_i is the molecular weight and N is the number of double bond in the i^{th} FAME.

The Kinematic viscosity (υ), density (ρ) and higher heating value HHV of each FAME can be calculated by using equations (2), (3) and (4), respectively and summation of all fatty acid fuel property provides the final υ, ρ and HHV of the biodiesel.

$$\ln(\upsilon_i) = -12.503 + 2.496 \times \ln(M_i) - 0.178 \times N \quad (2)$$

$$\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118 \times N \quad (3)$$

$$HHV_i = 46.19 - \frac{1794}{M_i} - 0.21 \times N \quad (4)$$

The iodine values were determined from the molecular weights of individual fatty acids following Kalayasiri et al. [51] as shown in equation (5).

$$IV = \sum_i \frac{(254 \times D_i \times N_i)}{Molecular\ weight\ of\ ith\ fatty\ acid} \quad (5)$$

where N_i is the percentage of each component in the biomass and D_i is the number of double bonds of the i^{th} FAME.

Contour plots were chosen to evaluate the effect of ASE process temperature, sample DBWR at set process times on total lipid and FAME yields, as well as the biodiesel quality parameters described above. The interpolation process used Mathwork, MATLAB version 2012a, Function "griddata" with the 'Cubic interpolation' method of temperature(x), and sample dry biomass water ratio (y) grid. The cubic method ensures 'the interpolating surface is C2 continuous' (second order derivatives).The "griddata" function interpolates the surface at the query points specified. Cubic interpolation found with R-square =1 and Sum of Squares Due to Error (SSE) =0. Contours were plotted against temperature and sample DBWR using

a minimum to maximum mesh grid, with interpolation for every single unit of temperature and sample DBWR.

3. Result and discussion

In wet biomass extractions, water plays a significant role by creating a water layer at the cell wall which restricts access of many hydrophobic solvents. Hexane is a non-polar solvent and its hydrophobic nature restricts extraction from wet microalgal biomass. Under high pressure and temperature conditions, water changes its polarity and becomes miscible with hexane. Therefore, high pressure hexane extraction of the Tarong polyculture was chosen for this study. It was found that with higher pressure and temperature at 50% sample DBWR it extract highest amount of fatty acids mainly poly unsaturated membrane lipids Supplementary Table S7.

3.1 Impact of ASE process variables on total lipid yields as assessed through pigment content (colour saturation) of the extracts:

The effect of temperature, sample dryness and process time on extracted total lipid yields was investigated for the freshwater chlorophyte microalgal consortium (Tarong polyculture). Process time had the least effect on extraction yields. Initially, extraction performance was evaluated qualitatively by colour being representative of the amount of pigment extracted under different temperature and sample DBWR condition. Darker the colour indicating higher the amount of pigment with lipid extracted.

Sample dryness (25, 50, 75 and 100%) positively correlated with total extracted lipid yields, as judged by the increasing amounts of pigments, from the Tarong polyculture when extracted at a constant temperature 80 °C (Supplementary Fig. S1). Similarly temperature

(70 °C to 120 °C) also positively correlated with total lipid yields, as judged by a temperature-dependent increase in colour saturation, from the Tarong polyculture extracted at a sample dryness of 25% (data not shown).

3.2 Effect of temperature and sample dry biomass water ratio (DBWR) on total lipid and total FAME extraction

Temperature, sample DBWR and process time affected single-solvent (hexane) ASE lipid extraction yields from the Tarong polyculture. In general, increased temperature improved extraction yields, with 50-75% as the optimal DBWR, except for 100% DBWR sample, 5 min process time was optimal (Supplementary Table S2).

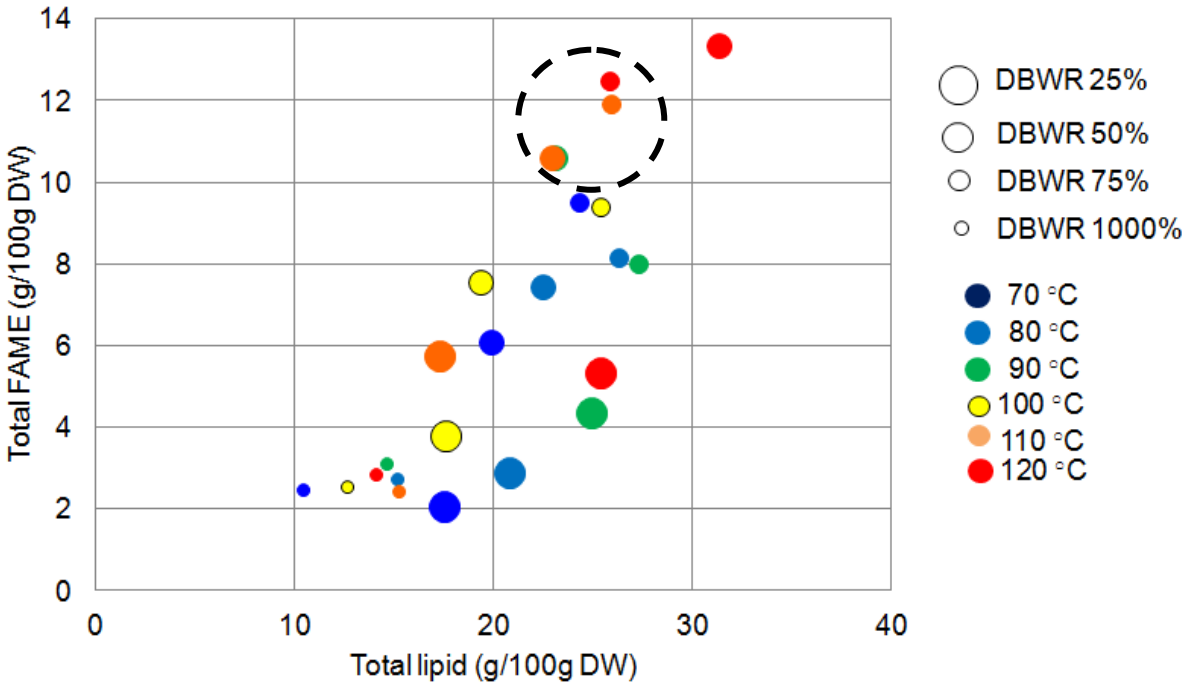

Figure 1: Effect of temperature and sample dry biomass water ratio (DBWR) on extraction performance of total lipid (g $100g^{-1}$ DW) and total FAME (g $100g^{-1}$ DW).

Figure 1 shows that maximum amount of lipid (31.3g $100g^{-1}$ DW) was extracted at 50% dryness and 120 °C, followed by 27 g $100g^{-1}$of DW at 75% DBWR and 90 °C. The lowest

percentages of total lipid (12-18 g $100g^{-1}$of DW) were extracted under three different sample DBWR and temperature conditions, 100% DBWR at all temperatures, 25 % DBWR at 70 °C and at 100 – 110 °C, confirming that optimal temperature and DBWR for maximal total lipid extraction should be between 90 °C and 120 °C for 50%, and 75% of sample DBWR.

Qualitative and quantitative analyses of FAME extractions are very important for large-scale biodiesel production. Extraction conditions for optimal total lipid yields may not be representative for conditions for optimal extraction of fatty acids (estimated based on the sum of all FAMEs). Figure 1 shows that highest total lipid yields are achieved at 50% DBWR and 120°C but total FAME yields improved only marginally. This indicates a larger contribution of pigments in the extract, which suggests that these extraction conditions are likely least suitable for ASE-hexane extract biodiesel production. However, samples with 50% DBWR at 110 °C and 90 °C and 25 DBWR at 110 °C and 120 °C seemed to be optimal for FAME extraction without too much increase in total lipid. Under these conditions, total FAME content of the total lipid fraction improved from 42% for the highest FAME and total lipid yield to 48 and 46%, respectively. All other extraction conditions with moderate total FAME yields yielded only 17% or less total FAME content of the extracted total lipids, compromising not only fatty acid extraction efficiencies, but also yielding significantly higher contributions of other non-polar cellular constituents, such as pigments. Although, as documented ASE-extraction conditions can be optimised for improved fatty acid yields within the total lipid fraction, for biodiesel production removal of co-extracted pigments will be necessary even under optimal conditions.

3.3 Effect of process time on total FAME extraction yields

To evaluate the influence of process time on extraction performance, experiments were run for three different process times 5, 10 and 15 minutes and at temperatures of 80 °C,

100 °C, and 120 °C. Process time had little effect on total lipid/FAME extraction yields at 80 °C and 100 °C. A detail contour plots visualise 120 °C with 50% sample dryness and 5 min process time are optimal conditions for high pressure total FAME extractions from the Tarong polyculture (Supplementary Figure S2).

3.4 Effect of temperature and sample dry biomass water ratio (DBWR) on individual fatty acid extraction yields

The most common fatty acids produced by chlorophytic freshwater microalgae are Palmitic - (Hexadecanoic- C16:0), Stearic - (Octadecanoic - C18:0), Oleic - (Octadecenoic - C18:1), Linoleic - (Octadecadienoic - C18:2) and Linolenic -(Octadecatrienoic - C18:3) acids, with minor quantities of some other methyl esters and other compounds [52]. The cetane number depends heavily on fatty acid composition and defines ignition quality parameters, hence, for biodiesel production, a mixture with a 5:4:1 ratio of C16:1, C18:1 and C14:0 fatty acid has been recommended [8].

The extracted amount of Myristic acid (C14:0) was very low at all temperatures and DBWR levels compared to other fatty acids. Interestingly, maximal amounts of Myristic acid (1.2 % of total FAME) were extracted at 70 °C and low DBWR levels (25%), while minimal amounts (0.4%) were extracted at 110 °C and 50% DBWR level (data not shown). Temperature and sample DBWR strongly affected extraction yields of Palmitic acid (C16:0) and Palmitoleic acid (C16:1), with increasing sample DBWR reducing yields (Fig. 2 a,b) ~~(Fig. 5a, b).~~ Highest amounts (~ 42 and 4.5 g 100 g^{-1} of FAME, respectively) were extracted at a sample DBWR of 25% and temperatures of 100 °C and 90-120 °C, respectively, while lowest amounts were obtained at a sample DBWR of 75% at all temperatures, respectively. A sample DBWR of 50% and 100% at 90 °C and 70 °C yielded intermediate quantities of C16:0 (35 g 100 g^{-1} FAME and 23 g 100 g^{-1} FAME, respectively), while the second best

yield of Palmitoleic acid (C16:1, 3.6 g 100 g^{-1} FAME) was achieved at 90 °C and 75% DBWR. Samples with a DBWR of 25% at 70 °C extracted the highest percentage (20 g 100 g^{-1} FAME) of Stearic acid (C18:0), decreasing with increasing sample DBWR and temperature. The effect of temperature (70 to 120 °C) and sample DBWR (25, 50, 75 and 100%) on percentage extraction of individual fatty acids is shown in Figure 2 (a)-(e) for the fatty acids C16:0, C16:1, C18:1, C18:2 and C18:3. Selection of these fatty acids was based on extracted quantities and importance for optimal fuel properties. Based on the results, extraction procedures aiming at optimising Palmitic and Palmitoleic acid yields from the Tarong polyculture should use a sample DBWR of 25% and temperature between 100 °C to 110 °C.

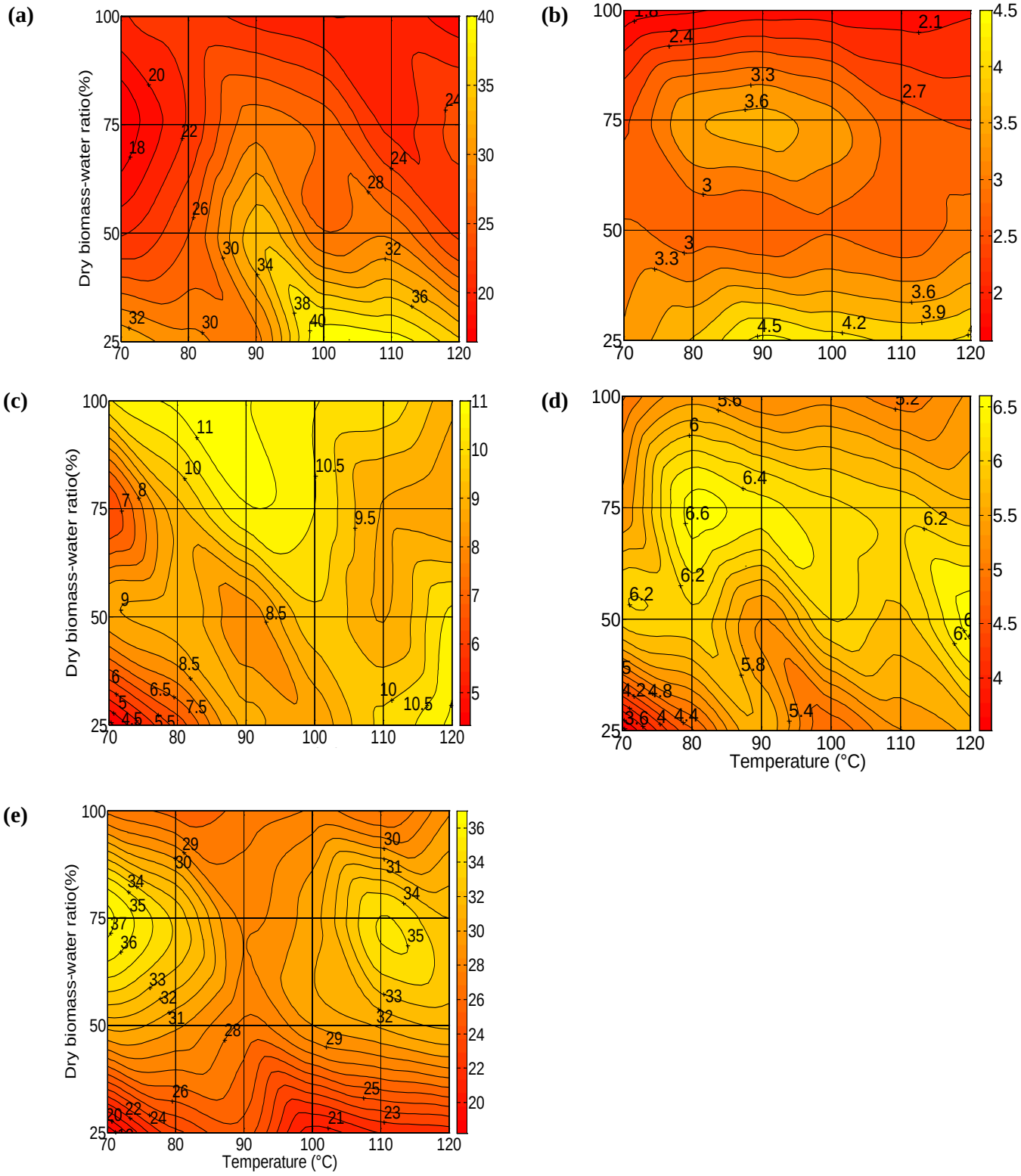

Figure 2: Effect of temperature and sample dry biomass water ratio (DBWR) on individual fatty acid extraction yields (g. 100 g^{-1} of FAME) (a) Palmitic acid C16:0 (b) Palmitoleic acid C16:1 (c) Oleic acid C18:1 (d) Linoleic acid C18:2(e) α and γ-Linolenic acid C18:3 of Tarong polyculture

In contrast, longer chain unsaturated fatty acids (C18) generally extracted better at increased levels of DBWR and temperatures showing in (Figs 2c-e). Oleic acid (C18:1) extracted best (~11 g 100 g^{-1} FAME) at either 100 % or 25 % sample DBWR at 90 to 100 °C and 120 °C, respectively (Fig. 2c). Lowest amounts were extracted at 25 and 75 % DBWR at 70 °C. In contrast, the level of DBWR rather than temperature strongly affected Linoleic acid (C18:2) yields, with highest amounts obtained at 50-75 % sample DBWR across the entire temperature range (Fig. 2d). Optimal yields of α- (37 g 100 g^{-1} FAME) and γ-Linolenic acid (36 g 100 g^{-1} FAME) (C18:3) were obtained at 75% dryness at 70 °C and 110 °C, respectively, while a sample DBWR of 25% had the lowest yields (~19 and 21 g 100 g^{-1} FAME, respectively) across the temperature range (Fig. 2e). The complete map of extraction of specific fatty acids under different temperature and DBWR levels is presented in Table 1. This table will be of value in selecting optimal operating conditions for extractions targeting fatty acid product development. Any particular combination of temperature and sample DBWR might be suitable for one purpose, such as biodiesel production but may not be suitable for other requirements.

Table 1: Extraction performance of some common fatty acids at different temperature (70 to 120 °C) and 4 different levels of sample DBWR

Sample dryness (%)	Temperature (°C)		
	Low (70-80)	Medium (80-100)	High (100-120)
25	C14:0 (max) C18:0 (max)	C16:1 (max)	C16:0 (max) C16:1 (max)
50			C14:0 (min) C18:2 (max)
75	C16:0 (min) C18:3 (max) C18:2 (max)	C16:1 (max)	C18:0 (min) C18:3 (max)
100	C16:1 (min)	C16:1 (min) C18:1 (max)	C16:0 (min) C16:1 (min)

Along with the quantity of total FAME, the composition of FAME is also important for biodiesel production. Although the highest lipid and FAME yield zones were at 70, 90 and 120°C and sample DBWR 50-75% (Supplementary Table S2), it is important to evaluate whether FAME composition is also optimal for biodiesel processing and production in a commercial facility at these settings. The fatty acid composition at the highest FAME yield zones are presented in Table 2, and detailed fatty acid compositions are presented in Supplementary Tables S3-6. Three examples are presented to demonstrate the significance of the selective extraction of individual or groups of fatty acids and their effects on fuel properties, namely: oxidation stability, cold filter plugging point and iodine value.

Table 2: Fatty acid composition at highest FAME yield zone (70, 90 and 120°C and sample DBWR 50-75%).

Fatty Acid	50% sample DBWR			75% sample DBWR		
	70 °C	90 °C	120 °C	70 °C	90 °C	120 °C
	g 100 g^{-1} FAME					
C14:0	0.7	0.4	0.5	0.5	0.6	0.4
C14:1	0.2	0.2	0.2	0.2	0.2	0.2
C15:0	0.8	0.6	0.9	0.6	0.6	0.5
C15:1	0.3	0.1	0.2	0.2	0.3	0.2
C16:0	21.8	35.2	23.5	16.5	29.1	24.7
C16:1 (7)	2.3	2.2	2.4	2	2.9	2.1
C16:1 cis 9	0.8	0.6	0.7	0.6	0.8	0.6
C16:2 (9,12)	0.6	0.4	0.5	0.6	0.5	0.5
C17:0	0.5	0.5	0.5	0.4	0.5	0.5
C16:3 (cis 6,9,12)	0.4	0.3	0.4	0.3	0.4	0.3
C16:3 (7, 10, 13)	2.8	2	2	3.9	2.2	3
C16:4 (4,7,10,13)	9.2	6.7	7.1	12.6	5.3	8.9
C18:0	4.3	2.4	2.3	2.4	2.6	1.7
C18:1 (cis)	8.8	8.2	10.6	6.6	10.9	8.6
C18:1 (cis 7 or 8)	1.5	1.6	1.6	1.1	2	1.3
C18:2 (cis, cis- 9,12)	6.1	5.4	6.7	5.4	6.5	5.9
C18:3 (all cis 6,9,12)	1	0.8	1	1.1	0.8	0.9
C 18:3 (all cis - 9,12,15)	31.3	27.8	32.2	37.3	28.8	33
C18:4 (6,9,12,15)	4.3	3.6	4.1	5.8	3	4.5
C 20:5 (EPA)	0.9	0.9	1.8	1.3	0.8	1.8
C22:0	0.9	0.3	0.4	0.3	0.3	0.2
C22:2	0.7		0.3		0.4	0.2
C24:0	-	-	0.2	0.3	0.4	0.2
SFA	28.9	39.3	28.3	20.9	34.2	28.2
MUFA	13.8	12.9	15.8	10.7	17.2	12.9
PUFA	57.2	47.8	56	68.4	48.6	59

Oxidation stability is among one of the most important fuel properties for handling and distribution of any liquid fuel in large-scale production. In large scale biodiesel production fuels need to be stored longer period which may leads to oxidised and degrade fuel quality. In this regard, Palmitic - (C16:0) and Oleic - (C18:1) acid have a positive effect on oxidation stability, whereas Linoleic - (C18:2) and Linolenic acid (C18:3) have a negative effect [39]. If extraction conditions are to be optimised for oxidation stability, C16:0 and C18:1 must be selectively extracted, whereas the C18:2 and C18:3 groups should not be favoured. Extraction conditions that meet these opposing requirements were found at 90 °C. Extracted amounts of C16:0 were higher, and Linoleic and Linolenic acid quantities were lower at 90 °C compared to 70 °C or 120 °C. This signifies that under the optimal FAME yield settings, oxidation stability was best at 90°C.

Another important fuel property is the cold filter plug point (CFPP), which is directly related to the amount of unsaturated fatty acids in the fuel. Higher the amounts of unsaturated fatty acids yield a higher CFPP for biodiesel [39]. Iodine value is also related to unsaturated fatty acid content, and is directly proportional to the unsaturated fatty acid quantity. Ten to 20% fewer unsaturated fatty acid were extracted at 90 °C compared to 70 and 120 °C (Table 2) that means decreased CFPP and IV at this temperature. More details on fuel properties based of fatty acid profiles will be discussed in section 3.6.

3.5 Comparison of ASE with other selected extraction methods

The trialled ASE extraction technique will now be briefly discussed in context with three commonly used extraction techniques, namely: conventional organic solvent extraction, Soxhlet and super critical fluid extraction.

Conventional solvent extraction has been extensively used for many applications [53]. The selection of appropriate polar/non-polar solvents for the particular species to be extracted is important for extraction performance [21]. Using co-solvents can assist to overcoming the polar/non-polar nature of some materials [54]. This extraction process is thermodynamically limited by the lipid mass transfer equilibrium condition. Typically all polar (mainly membrane lipid) and neutral lipid can be extracted but the required large amounts of toxic solvents and the relative slowness of the process limit the application of this technique to the laboratory. This extraction process is thermodynamically limited by the lipid mass transfer equilibrium condition [55].

To evade overcome the equilibrium condition limitation, Soxhlet apparatus is used where the cell wall is continuously replenished with fresh solvent which is continuously recovered in a condenser thus reducing solvent consumption [56, 57]. The Soxhlet operation of hexane extractions is of lipids are more efficient than conventional solvent extraction in [58], extracting where it extract 0.057 g lipid g^{-1} dried microalgae biomass compared to that of the conventional solvent extraction which achieved (0.015 g lipid g^{-1} dried microalgae biomass) [58]. Despite these advantages, the Soxhlet extraction method lipid/fatty acid extraction efficiencies are limited to co-solvents which have a similar boiling temperature, thus limiting placing restrictions on the simultaneous extraction of membrane and neutral lipid resulting in a reduction of the amounts of poly poly-unsaturated fatty acids [59]. The scale up of the Soxhlet extraction method is also limited due to its high energy requirements for continuous distillation of the large amounts of solvents required [57, 60].

A modified solvent extraction, accelerated solvent extraction (ASE), using high pressures and temperatures, has been investigated and found to be highly efficient with maximal final lipid recovery of 90.21% of total lipid [22]. In the study presented here, ASE high pressure solvent extraction achieved a maximum lipid extraction of 31.5 g 100 g^{-1} dry

microalgae biomass (Supplementary Table S2) from the chlorophytic microalgal Tarong polyculture. ASE can also be used with wet biomass, reducing sample pre-treatment costs and preparation time compared to conventional hexane extraction. However, as shown here, ASE operating conditions with regards to temperature and moisture levels need to be fine-tuned to for optimal membrane lipid or poly-unsaturated fatty acid extractions, as extraction of the latter is generally unfavourable for biodiesel production. Increasing the amounts of linoleic and linolenic acid content of the extracts reduces oxidation stability of the fuel, but increases the cold filter plugging point, the latter is valuable for biodiesel applications in cold climates, while the former can cause problems if longer storage times are intended. Compared to supercritical fluid extraction (see below), the large amounts of solvents required for industrial-scale applications limits scalability somewhat, however, an advantage of ASE is that the instrumentation is readily available and that solvent use can be minimised through recycling.

Supercritical fluid extraction is believed to be the most promising extraction technique of those reviewed here due to favourable mass transfer, solvent-free (other than CO_2) and more time-efficient crude lipid extractions compared to the other techniques. In-addition, use of co-solvents can manipulate the selectivity for certain compounds in the extract [54]. However, the expensive pressure vessel installation cost and unfavourable energy requirements, as well as CO_2-demand limit the scalability of supercritical fluid extraction at present.

An optimum lipid extraction process at large-scale will be a trade-off between key factors including extraction efficiency, time taken, reactivity with lipids, capital cost, operating cost (including energy consumption), process safety and waste generation. The scale-up potential of each method is summarised in [54] and presented here supplemented with our information in Table 3.

Table 3: Comparison of four extraction techniques using key factors

Extraction Technique	Energy Consumption	Extraction time	Toxicity	Scale-up potential
Organic solvent	moderate	moderate	high	moderate
Soxhlet	high	moderate	high	Lack of scalability
Super critical fluid	high	low	low	moderate
ASE	high	low	high	moderate

Some advantages of ASE extraction have been demonstrated such as selection of process parameters for optimising FAME to lipid ratios, content and some desirable fatty acids, faster processing, and lower solvent use compared to conventional solvent extraction but further work is required to fully assess the costs of energy consumption and the capital costs for the equipment for large-scale extraction. Higher temperature and pressure processes are used in many processing industries including the refining of crude oil and the viability of using higher temperature and pressure processes for algal oil extraction will be dependent upon the relative economics of alternate processes. Such a techno-economic analysis will require a detailed investigation of relevant specialised processing equipment and co-generation technology for the combustion of process waste to provide heat to the ASE extraction. Such analysis is not within the scope of the present focus of this paper, which is on temperature and moisture effects of extraction. This study will however, assist in providing data for such future techno-economic studies

3.6 Fuel property analysis

The primary purpose of this study was to examine the sensitivity of lipid/FAME extraction yields from microalgae to sample DBWR and temperature. Biodiesel has well established standard fuel properties for use in regular diesel engines. Cetane number is one of the most significant indicators of fuel combustion ability [61]. The minimum desired cetane

number of biodiesel is 47 and 51 according to ASTM D6751 [62] and EN14214 standards, respectively [9], while a maximal iodine value of 120 is defined in the EN 14214 only.

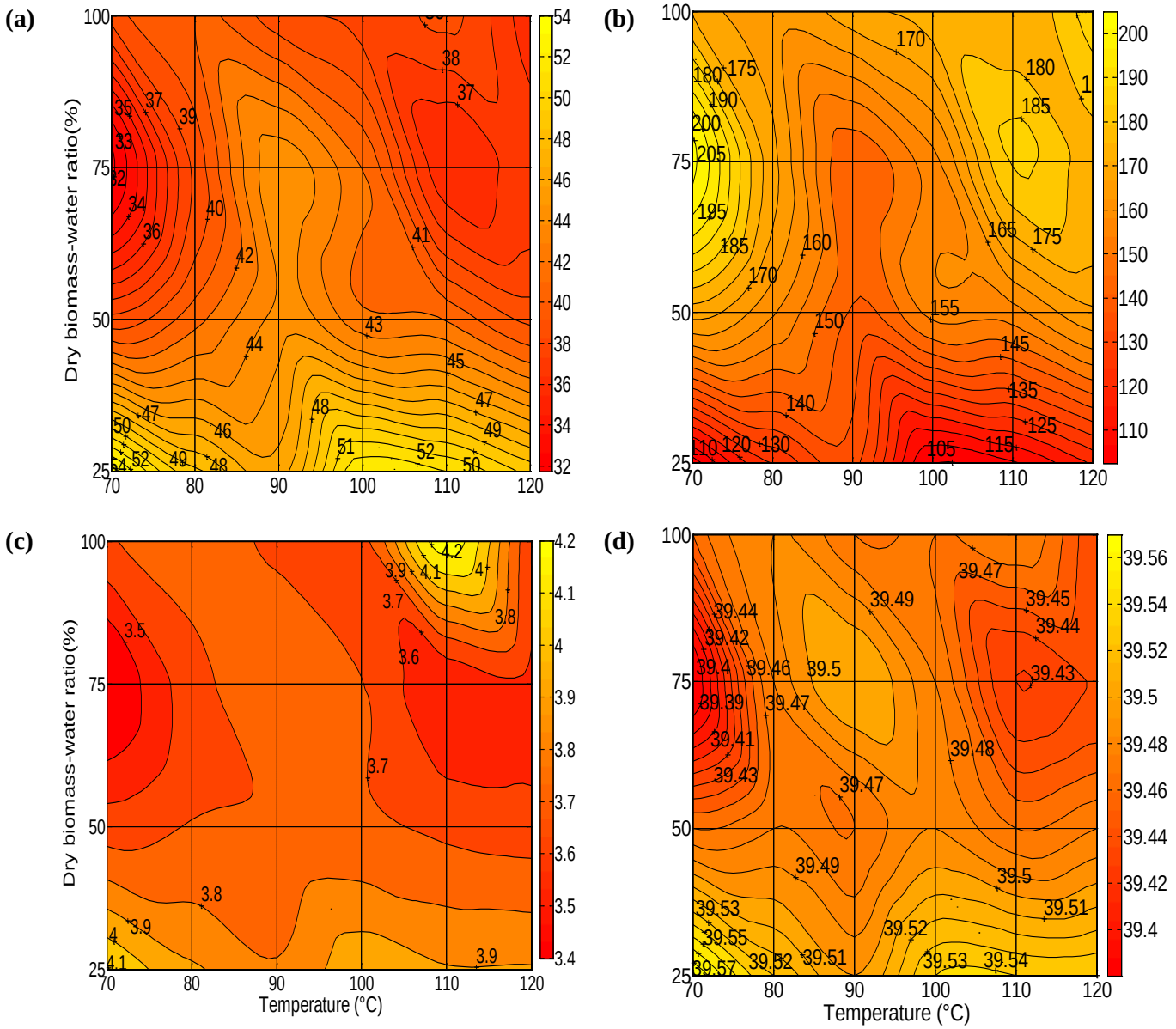

Figure 3: Effect of temperature and sample dry biomass water ratio (DBWR) on fuel properties (a) Cetane number (CN) (b) Iodine value (IV) (c) Kinematic viscosity (KV) mm^2s^{-1} and (d) Higher heating value (HHV) MJ kg^{-1} of extracted FAME of the Tarong polyculture.

Increasing sample DBWR generally negatively affected cetane numbers and iodine values obtained for FAME extracts of the Tarong polyculture (Figs 3a, b). A sample DBWR

of 25% yielded the highest cetane number (54) irrespective of temperature (Fig. 3a), positively correlating with extracted amounts of saturated fatty acids under those conditions (Fig. 4a). A sample DBWR of around 30% at temperatures between 70-75 °C and 95-120 °C was ideal for obtaining iodine values below the maximal threshold. Lowest cetane numbers were obtained at 75% dry matter content at 70 °C. Thus to obtain biodiesel with high cetane numbers and iodine levels below the maximal threshold for the Tarong polyculture, high pressure extractions should be carried out at low sample DBWR (25%) and temperatures of 70 and100 °C, because it allows for blending to improve the cetane number of lower quality biodiesel. In contrast, temperature and sample DBWR had little influence on kinematic viscosity and higher heating value of biodiesel derived from the Tarong polyculture, with kinematic viscosity staying within the set standards of 1.9 to 6.0 $mm^2 \cdot s^{-1}$ (ASTM D6751) and 3.5 to 5.0 $mm^2 \cdot s^{-1}$ (EN 14214) under all extraction conditions (Figs 3c, d).

The relative compositions of saturated and unsaturated fatty acid methyl ester are important parameters to be considered in assessing the overall quality of biodiesel. Figure 4(a) ~~Figure 7(a)~~ shows that the saturated fatty acid concentration has a similar trend to cetane number.

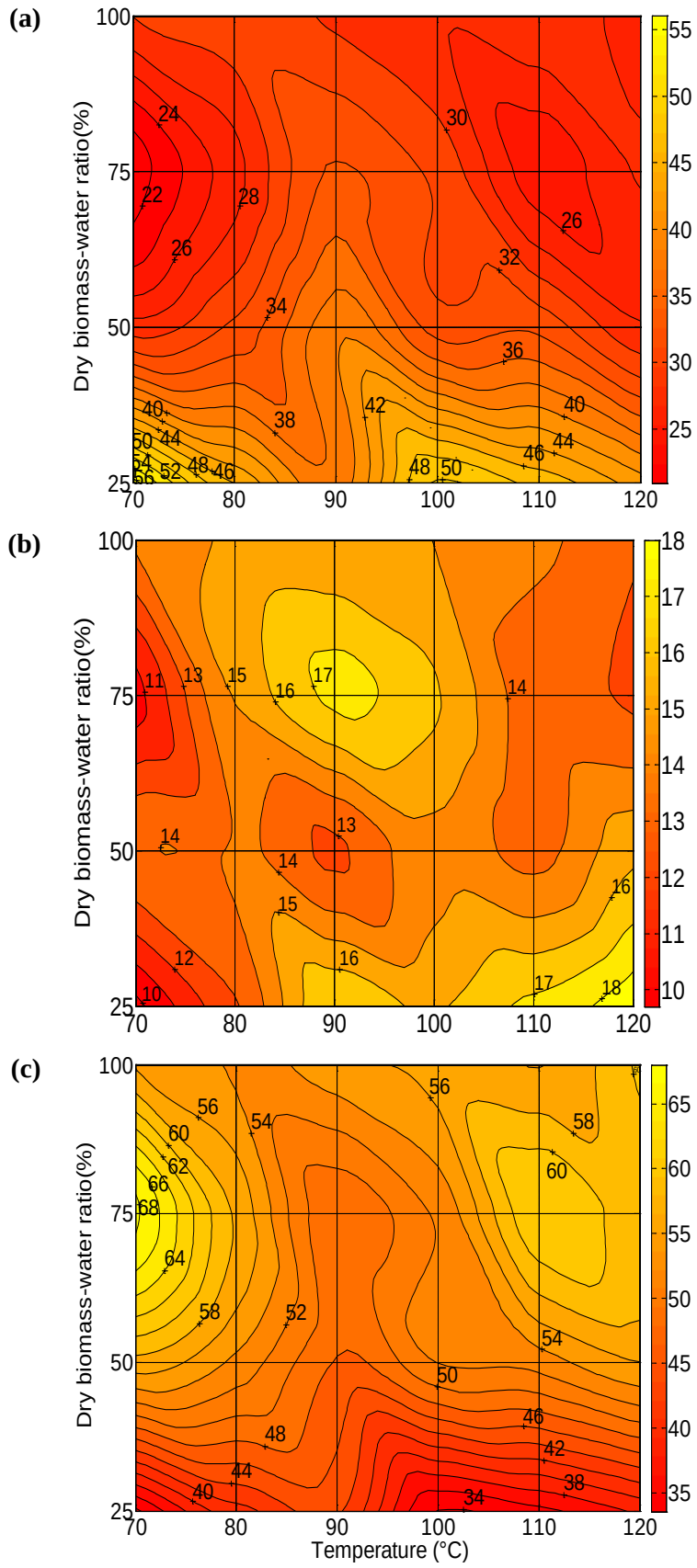

Figure 4: Effect of temperature and dry biomass-water ratio (DBWR) on percent of extracted (a) saturated, (b) mono-unsaturated and (c) polyunsaturated fatty acid methyl esters (g $100g^{-1}$ of FAME) from the Tarong polyculture

Highest amounts of mono unsaturated fatty acids were achieved at 25% dryness from 90 to 120 °C and at 75% DBWR at 90 °C (Fig. 4b), while 25% DBWR at 70 °C and 100 °C extracted minimal amounts of polyunsaturated fatty acids (Fig. 4c). Thus high pressure extraction conditions are inversely correlated for polyunsaturated fatty acid amounts and ideal cetane number. In addition; the amount of polyunsaturated fatty acid can also be used as an indicator for non-compliant biofuel iodine values.

4. Conclusion

High-pressure solvent extraction under optimised extraction conditions (level of DBWR, temperature and to a lesser extent process time) could be a critical step forward for large-scale lipid extraction for biodiesel production from microalgae. The results of this study show that the efficiency of high-pressure single solvent (hexane) extraction is strongly influenced by process temperature and sample DBWR rather than process time. Maximal total lipid yields from the Tarong polyculture were achieved at 90-120 °C at a sample DBWR of 50% and 75%. Our results show that individual fatty acids (Palmitic acid C16:0; Stearic acid C18:0; Oleic acid C18:1; Linolenic acid C18:3) extraction optima are influenced by temperature and sample DBWR. Therefore, biodiesel quality parameters of the microalgal biodiesel can be positively manipulated by selecting process extraction conditions that favour extraction of saturated and mono-unsaturated fatty acids over optimal extraction conditions for polyunsaturated fatty acids, yielding positive effects on cetane number and iodine values allowing for potential blending with biodiesels that fall outside the minimal cetane and maximal iodine values.

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Reference

1. Huerga, I.R., Zanuttini, M.S., Gross, M.S., and Querini, C.A., Biodiesel production from Jatropha curcas: Integrated process optimization. Energy conversion and management, 2014. **80**(0): p. 1-9.
2. EN 14214: 2003,"Automotive Fuels-Fatty Acid Methyl Esters (FAME) for Diesel Engines- Requirement Methods, " European Committee for Standardization, rue de Stassart, 36 B-1050 Brussels, 2008, www.novaol.it.
3. Chisti, Y., Biodiesel from microalgae beats bioethanol. Trends in biotechnology, 2008. **26**(3): p. 126-131.
4. Deng, X., Li, Y., and Fei, X., Microalgae: A promising feedstock for biodiesel. African Journal of Microbiology Research, 2009. **3**(13): p. 1008-1014.

5. Sing, S.F., Isdepsky, A., Borowitzka, M.A., and Moheimani, N.R., Production of biofuels from microalgae. Mitigation and Adaptation Strategies for Global Change, 2013. **18**(1): p. 47-72.
6. Borowitzka, M.A. and Moheimani, N.R., Sustainable biofuels from algae. Mitigation and Adaptation Strategies for Global Change, 2013. **18**(1): p. 13-25.
7. ASTM D6751-12, "Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels" ASTM- International, West Conshohocken, PA, 2012,DOI: 10.1520/D6751-12, www.astm.org.
8. Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., and Hankamer, B., Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Research, 2008. **1**(1): p. 20-43.
9. Fuel Quality Standards Act 2000,"Fuel Standard (Biodiesel) Determination 2003", Federal Register of Legislative Instruments F2009C00146, Canberra, 2009, www.comlaw.gov.au.
10. Hossain, A., Salleh, A., Boyce, A.N., Chowdhury, P., and Naqiuddin, M., Biodiesel fuel production from algae as renewable energy. American Journal of Biochemistry and Biotechnology, 2008. **4**(3): p. 250-254.
11. Hu, J., Du, Z., Li, C., and Min, E., Study on the lubrication properties of biodiesel as fuel lubricity enhancers. Fuel, 2005. **84**(12): p. 1601-1606.
12. Huerlimann, R., De Nys, R., and Heimann, K., Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. Biotechnology and Bioengineering, 2010. **107**(2): p. 245-257.
13. Rawat, I., Ranjith Kumar, R., Mutanda, T., and Bux, F., Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy, 2012.
14. Molina Grima, E., Belarbi, E.H., Acien Fernandez, F., Robles Medina, A., and Chisti, Y., Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology Advances, 2003. **20**(7): p. 491-515.
15. Klein-Marcuschamer, D., et al., Technoeconomic analysis of renewable aviation fuel from microalgae, Pongamia pinnata, and sugarcane. Biofuels, Bioproducts and Biorefining, 2013. **7**(4): p. 416-428.
16. Johnson, M.B. and Wen, Z., Production of biodiesel fuel from the microalga Schizochytrium limacinum by direct transesterification of algal biomass. Energy & Fuels, 2009. **23**(10): p. 5179-5183.

17. Lang, I., Hodac, L., Friedl, t., and Feussner, I., Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biol., 2011. **11**: p. 124-140.
18. von Alvensleben, N., Stookey, K., Magnusson, M., and Heimann, K., Salinity Tolerance of Picochlorum atomus and the Use of Salinity for Contamination Control by the Freshwater Cyanobacterium Pseudanabaena limnetica. PloS one, 2013. **8**(5): p. e63569.
19. Siaut, M., et al., Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. BMC biotechnology, 2011. **11**(1): p. 7.
20. Xin, L., Hong-ying, H., and Yu-ping, Z., Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature. Bioresource Technology, 2011. **102**(3): p. 3098-3102.
21. Mercer, P. and Armenta, R.E., Developments in oil extraction from microalgae. European Journal of Lipid Science and Technology, 2011. **113**(5): p. 539-547.
22. Herrero, M., Martín-Álvarez, P.J., Señoráns, F.J., Cifuentes, A., and Ibáñez, E., Optimization of accelerated solvent extraction of antioxidants from Spirulina platensis microalga. Food Chemistry, 2005. **93**(3): p. 417-423.
23. Sajilata, M., Singhal, R.S., and Kamat, M.Y., Supercritical CO2 extraction of c-linolenic acid (GLA) from Spirulina platensis ARM 740 using response surface methodology. Journal of Food Engineering, 2008. **84**(2): p. 321-326.
24. Herrero, M., Cifuentes, A., and Ibanez, E., Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. Food Chemistry, 2006. **98**(1): p. 136-148.
25. Taher, H., Al-Zuhair, S., Al-Marzouqi, A.H., Haik, Y., and Farid, M., Effective extraction of microalgae lipids from wet biomass for biodiesel production. Biomass and Bioenergy, 2014. **66**(0): p. 159-167.
26. De Angelis, L., Rise, P., Giavarini, F., Galli, C., Bolis, C., and Colombo, M., Marine macroalgae analyzed by mass spectrometry are rich sources of polyunsaturated fatty acids. Journal of mass spectrometry, 2005. **40**(12): p. 1605-1608.
27. Ramadan, M.F., Asker, M.M.S., and Ibrahim, Z.K., Functional bioactive compounds and biological activities of Spirulina platensis lipids. Czech journal of food sciences, 2008. **26**(3): p. 211-222.

28. Fajardo, A.R., Cerdan, L.E., Medina, A.R., Fernández, F.G.A., Moreno, P.A.G., and Grima, E.M., Lipid extraction from the microalga Phaeodactylum tricornutum. European Journal of Lipid Science and Technology, 2007. **109**(2): p. 120-126.
29. Ibánez González, M., Robles Medina, A., Grima, E.M., Giménez, A.G., Carstens, M., and Cerdán, L.E., Optimization of fatty acid extraction from Phaeodactylum tricornutum UTEX 640 biomass. Journal of the American Oil Chemists' Society, 1998. **75**(12): p. 1735-1740.
30. Mittelbach, M. and Remschmidt, C., Biodiesel: the comprehensive handbook. 2004: Martin Mittelbach.
31. Haas, M.J., Scott, K.M., Marmer, W.N., and Foglia, T.A., In situ alkaline transesterification: an effective method for the production of fatty acid esters from vegetable oils. Journal of the American Oil Chemists' Society, 2004. **81**(1): p. 83-89.
32. Haas, M.J. and Scott, K.M., Moisture removal substantially improves the efficiency of in situ biodiesel production from soybeans. Journal of the American Oil Chemists' Society, 2007. **84**(2): p. 197-204.
33. Haas, M.J., Scott, K.M., Foglia, T.A., and Marmer, W.N., The general applicability of in situ transesterification for the production of fatty acid esters from a variety of feedstocks. Journal of the American Oil Chemists' Society, 2007. **84**(10): p. 963-970.
34. Cooney, M., Young, G., and Nagle, N., Extraction of Bio-oils from Microalgae. Separation & Purification Reviews, 2009. **38**(4): p. 291-325.
35. Cheng, J., Yu, T., Li, T., Zhou, J., and Cen, K., Using wet microalgae for direct biodiesel production via microwave irradiation. Bioresource Technology, 2013. **131**(0): p. 531-535.
36. Wahidin, S., Idris, A., and Shaleh, S.R.M., Rapid biodiesel production using wet microalgae via microwave irradiation. Energy conversion and management, 2014. **84**(0): p. 227-233.
37. Im, H., Lee, H., Park, M.S., Yang, J.-W., and Lee, J.W., Concurrent extraction and reaction for the production of biodiesel from wet microalgae. Bioresource Technology, 2014. **152**(0): p. 534-537.
38. Shin, H.-Y., Ryu, J.-H., Bae, S.-Y., Crofcheck, C., and Crocker, M., Lipid extraction from Scenedesmus sp. microalgae for biodiesel production using hot compressed hexane. Fuel, 2014. **130**(0): p. 66-69.

39. Richter, B.E., Jones, B.A., Ezzell, J.L., Porter, N.L., Avdalovic, N., and Pohl, C., Accelerated solvent extraction: a technique for sample preparation. Analytical Chemistry, 1996. **68**(6): p. 1033-1039.
40. Romanik, G., Gilgenast, E., Przyjazny, A., and Kamiński, M., Techniques of preparing plant material for chromatographic separation and analysis. Journal of biochemical and biophysical methods, 2007. **70**(2): p. 253-261.
41. Guillard, R. and Hargraves, P., Stichochrysis immobilis is a diatom, not a chrysophyte. Phycologia, 1993. **32**(3): p. 234-236.
42. Dionex ASE 350, Accelerated Solvent Extractor Operator's Manual, Revision 04, Revision 04., Thermo Fisher Scientific Inc, United States, 2011.
43. Carvalho, A.P. and Malcata, F.X., Preparation of fatty acid methyl esters for gas-chromatographic analysis of marine lipids: insight studies. Journal of agricultural and food chemistry, 2005. **53**(13): p. 5049-5059.
44. Cohen, Z., Vonshak, A., and Richmond, A., Effect of environmental conditions on fatty acid composition of the red alga porphyridium cruentum: Correlation to growth rate. Journal of Phycology, 1988. **24**(3): p. 328-332.
45. Gosch, B.J., Magnusson, M., Paul, N.A., and Nys, R., Total lipid and fatty acid composition of seaweeds for the selection of species for oil-based biofuel and bioproducts. GCB Bioenergy, 2012. **4**(6): p. 919-930.
46. David, F., Sandra, P., and Wylie, P.L., Improving the analysis of fatty acid methyl esters using retention time locked methods and retention time databases. Agilent Technologies-Application. Agilent Technologies, Palo Alto, CA, 2002.
47. Folch J, L.M., Stanley GHS, A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem, 1957. **226**: p. 497-509.
48. Somersalo, S., Karunen, P., and Aro, E.-M., The acyl lipid composition of wheat leaves and moss protonemata using a new, non-carcinogenic extraction solvent system. Physiologia Plantarum, 1986. **68**(3): p. 467-470.
49. Islam, M., Magnusson, M., Brown, R., Ayoko, G., Nabi, M., and Heimann, K., Microalgal Species Selection for Biodiesel Production Based on Fuel Properties Derived from Fatty Acid Profiles. Energies, 2013. **6**(11): p. 5676-5702.
50. Ramírez-Verduzco, L.F., Rodríguez-Rodríguez, J.E., and Jaramillo-Jacob, A.d.R., Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. Fuel, 2012. **91**(1): p. 102-111.

51. Kalayasiri, P.J., N.Krisnangkura, K., Survey of seed oils for use as diesel fuels. Journal of the American Oil Chemists' Society, 1996. **73**(4): p. 471-474.
52. Knothe, G., Improving biodiesel fuel properties by modifying fatty ester composition. Energy Environ. Sci., 2009. **2**(7): p. 759-766.
53. Pragya, N., Pandey, K.K., and Sahoo, P., A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renewable and Sustainable Energy Reviews, 2013. **24**: p. 159-171.
54. Halim, R., Danquah, M.K., and Webley, P.A., Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 2012.
55. Drapcho, C.M., Nhuan, N.P., and Walker, T.H., Biofuels engineering process technology. 2008: McGraw-Hill New York, NY.
56. Rajasekar, E., Murugesan, A., Subramanian, R., and Nedunchezhian, N., Review of NOx reduction technologies in CI engines fuelled with oxygenated biomass fuels. Renewable and Sustainable Energy Reviews, 2010. **14**(7): p. 2113-2121.
57. Agarwal, A.K., Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. Progress in energy and combustion science, 2007. **33**(3): p. 233-271.
58. Halim, R., Gladman, B., Danquah, M.K., and Webley, P.A., Oil extraction from microalgae for biodiesel production. Bioresource Technology, 2011. **102**(1): p. 178-185.
59. An, H., Yang, W., Chou, S., and Chua, K., Combustion and emissions characteristics of diesel engine fueled by biodiesel at partial load conditions. Applied Energy, 2012. **99**: p. 363-371.
60. Shah, A.N., Ge, Y., Tan, J., Liu, Z., He, C., and Zeng, T., Characterization of polycyclic aromatic hydrocarbon emissions from diesel engine retrofitted with selective catalytic reduction and continuously regenerating trap. Journal of Environmental Sciences, 2012. **24**(8): p. 1449-1456.
61. Lei Zhu a, b., C.S. Cheung b, W.G. Zhang a, Zhen Huang a, Combustion, performance and emission characteristics of a DI diesel engine fueled with ethanol–biodiesel blends. Fuel, 2011. **90**: p. 1743–1750.
62. Hoekman, S.K., Broch, A., Robbins, C., Ceniceros, E., and Natarajan, M., Review of biodiesel composition, properties, and specifications. Renewable and Sustainable Energy Reviews, 2012. **16**(1): p. 143-169.