Lipid Extraction from *Chlorella Vulgaris* & *Haematococcus Pluvialis* Using the Switchable Solvent DMCHA for Biofuel Production

Callum Russell, Cristina Rodriguez*

University of the West of Scotland, Paisley, PA1 2BE, United Kingdom

*Corresponding author: cristina.rodriguez@uws.ac.uk

Abstract

Switchable solvents (SS), also termed smart solvents, have the unique ability to change their polarity/hydrophilicity through a protonation reaction using CO_2. Recently, a novel method employing the use of SS for lipid extraction in microalgae has shown promising results. In this study the efficiency of the tertiary amine N, N-dimethyl cyclohexylamine (DMCHA) to extract lipids from the eukaryotic microalgae *Chlorella vulgaris* and *Haematococcus pluvialis*, was investigated using traditional hexane as a control under wet conditions. The results revealed that high lipid yields using DMCHA can be achieved in both species, however the extraction procedure is significantly more complex compared with hexane. This study found that lipid extraction of *H. pluvialis*, using DMCHA to be an efficient solvent, achieving a maximum lipid yield of 64.84%. Furthermore, DMCHA achieved a maximum lipid yield of 63.85% with *C. vulgaris*. The practicalities of DMCHA for lipid recovery are considerably more complex at larger volumes compared with hexane.

Keywords: Switchable solvents, lipids, microalgae, DMCHA, Biofuel

1. Introduction

The shift towards a more sustainable and renewable fuel source has increased in recent years, due to the depletion of fossil fuel reserves. Microalgae biomass provides a greener and sustainable source of energy in the form of biofuel, which is derived from lipids extracted from the biomass. Not only can microalgae produce a sustainable fuel source, but they also contribute to capturing atmospheric CO_2, which is required during the photosynthesis process, as well as acting as a wastewater treatment option, which provides the essential nutrients needed for optimal growth [1] The versatility and diversity of microalgae is what makes it a real contender in the development of alternative fuel sources [2]. Not all lipids however are suitable for biofuel production, therefore it is important to focus on the extraction of neutral lipids which consist primarily of Free Fatty Acids (FFAs), Triacylglycerols (TAGs), and sterols [3]. These compounds can be esterified into Fatty Acid Methyl Esters (FAMEs), which are the combustible compounds in biofuel [4].

Microalgae produce a mixture of polar and non-polar (neutral) lipids, each having their own unique function within the cell. Polar lipids mainly consist of glycolipids and phospholipids, whereas neutral lipids mainly consist of FFAs, TAGs, and sterols [5]. Polar lipids provide structural support to both the organelles and cell membrane/wall [6]. Whereas non-polar lipids such as TAGs store energy that can be used for cellular metabolism, repair, and reproduction [7]. The relative composition of lipids is highly dependent on the species of microalgae being investigated [8]. The eukaryotic green microalgae *Chlorella vulgaris* is one of the most researched species, due to its rapid growth rate, high value compound content, and resistance to a wide range of operating conditions. *C vulgaris* is a spherical single cell strain that measures

44 between 2 and 10 μm in diameter [9]. The large quantity of research surrounding *C. vulgaris*
45 has led to conflicting data on the lipid content. Aguoru et al, reported a 25% lipid content [10],
46 whereas Yeh et al, reports between 60-68% [11]. These discrepancies arise as a result of the
47 operating conditions and extraction method, rather than the algae itself [9] *Haematococcus*
48 *pluvialis*, however is largely associated with carotenoid extraction; namely, astaxanthin, with
49 studies shown extraction efficiencies of 87% [12]. Unlike *C. vulgaris*, there is limited data on
50 lipid extraction, however, one study reported a 34.85% lipid recovery under nitrogen-sufficient
51 conditions [13]. Furthermore, studies have shown that *H. pluvialis* produces more lipids during
52 the "red stage" compared with the "green stage" as described by Grewe et al, [14].

53 Several stages are involved in the extraction of lipids from microalgae, namely cultivation,
54 harvesting, pre-treatment, extraction, and purification [15,16]. To maximise the lipid recovery
55 yield, each stage within the process should be optimised. There is a plethora of research output
56 in recent years concerning the optimisation of the growth stage, as this is the point at which
57 lipid synthesis occurs, including other high value compounds. Despite optimised growth
58 parameters such as light intensity, temperature, nutrient deprivation, and salinity. One major
59 bottleneck remains, which is concerned with the cost-efficiency and overall lipid yield within
60 the extraction stage. This has resulted in a greater research emphasis being placed on
61 developing more sustainable, environmentally friendly extraction methods.

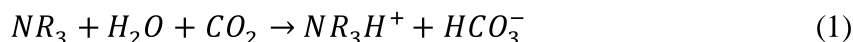
62 There are two main routes for lipid extraction namely mechanical or non-mechanical and can
63 be carried out under dry or wet conditions [17]. The key distinction between the routs is the
64 mitigation of the dewatering stage which is not required in the wet route. Conventional
65 mechanical extraction methods tend to use the dry route, whereby the cultivated cells are
66 centrifugated, filtered and then dried, leaving behind only solid dry biomass, which can be a
67 costly operation. Therefore, any extraction method that uses the wet route will inevitably be
68 more cost-effective in theory. Such methods include the use of accelerated solvents, switchable
69 solvents, and ionic liquids. There are solvent-free alternatives such as enzyme assisted,
70 supercritical CO_2, osmotic pressure, and subcritical water extraction, however these methods
71 often have very low efficiency [3].

72 One of the most common non-mechanical methods is organic solvent extraction [18]. This type
73 of extraction is based on the concept of "like dissolves like", with traditional methods such as
74 the Bligh and Dyer which uses a mixture of chloroform and methanol in ratio of 1:2 (v/v)
75 respectively [19]. Other organic solvents include, hexane, acetone, benzene, methylene
76 chloride, methanol, and cyclohexane [20]. Typically, organic solvent extraction uses a co-
77 solvent system, whereby a combination of polar and non-polar solvents is used. The most
78 common co-solvent systems include methanol/chloroform, hexane/ethanol,
79 hexane/isopropanol, butanol/methanol etc [21,22]. The presence of the polar solvent facilitates
80 the breaking of the polar hydrogen bonds that exists in the structural polar lipids and proteins,
81 causing the membrane to become porous. This allows the non-polar solvent to enter the cell
82 and bind to the neutral lipids. While pure solvents have been used Cooney et al., reported that
83 the efficiencies never exceed more than 90% of the yield obtained using the Bligh & Dyer
84 method [23]. Typically, solvent extraction that uses a single non-polar solvent such as hexane
85 is accompanied by a pre-treatment such as microwaves. This pre-treatment carries out a similar
86 function to the polar solvent the cell wall is perforated in order to expose the intracellular lipids,
87 this improves the efficiency of the solvent extraction, by reducing the mass transfer resistances
88 [24]. Pre-treatments that use radiation are often accompanied with thermal effects which may
89 contribute to the degradation of the cell membrane. Furthermore, microalgae that undergo
90 slight morphological changes during the transition from the exponential to the stationary phase,

91 almost always require the use of a pre-treatment as these changes often result in low extraction
92 yields [25,26].

93 Switchable solvents sometimes termed "smart solvents" have the ability to switch their polarity
94 under the exposure of an inducer such as CO_2 or N_2 which shifts the polarity from polar to non-
95 polar form. This manipulation of the polarity allows for product recovery through phase
96 splitting [27]. The maximum lipid recovery for several secondary and tertiary amine systems
97 was compared and found that tertiary amines were more effective at lipid recovery, as tertiary
98 amines do not suffer as much hydrolytic instability than secondary amines [15]. Furthermore,
99 the hydrophilicity switching is mainly dependent on the chemical structure of the amine
100 compound, this includes the carbon chain length, amino group position, and number of
101 branches. The addition of CO_2 into a switchable water system causes an amine protonation
102 reaction which involves the reaction between the amino group and CO_2. This forms a
103 zwitterionic intermediate, which is followed by the formation of a water-soluble bicarbonate
104 salt [28,29]. This can be seen in equation (1).

105



$$NR_3 + H_2O + CO_2 \rightarrow NR_3H^+ + HCO_3^- \quad (1)$$

106

107 The novelty of switchable solvents is in their ability to "switch" back to their original phase
108 through decarbonization. This process can be achieved either by heating or through the use of
109 an inert trigger gas, such as Nitrogen [29]. The revers switching process is much more
110 challenging than the forward, with limited literature and data supporting the effectiveness of
111 this process. A recent study by Liu et al., investigated the separation efficiency of several
112 switchable solvents including DMCHA, and found that these solvents had the ability to be
113 recycled up to 15 times, supporting the claims of a sustainable and environmentally friendly
114 solvent for lipid extraction [30]. It is worth noting that for switchable solvents that form solid
115 bicarbonate crystals in the forward reaction, such as N, N-Diisopropyl ethanolamine (DIPEA),
116 will require the addition of solid calcium hydroxide [31].

117 For a solvent to be classed as "switchable" the *LogP* value (partition coefficient) must be
118 between 1.2 and 2.5 [32]. Solvents with a higher *LogP* values become too hydrophobic and the
119 protonation from the CO_2 is not strong enough to change the polarity. On the contrary, solvents
120 with a lower *LogP* become too hydrophilic and form a monophasic mixture rather than a
121 desired biphasic mixture. The amines must have sufficient basicity to react with the carbonated
122 water and therefore most suitable solvents have a pKa value greater than 9.5 [32].

123 The use of switchable solvents, unlike ionic liquids is more simplistic and more cost effective,
124 which has gained significant attention for lipid recovery in recent years [33]. The ability to
125 recover lipids without the need to dewater makes the process more economical, and
126 subsequently less energy intensive. Furthermore, the consumption of CO_2 as the inducer for
127 switching forward can be deemed a carbon capturing technology while simultaneously
128 extracting lipids for biofuel production; something that is not achievable with any other current
129 lipid extraction method. Switchable water technologies have been applied to lipid extraction
130 where they also consume CO_2, the aqueous CO_2-rich phase poses significant problems of high
131 corrosivity, sensible heat, heat of vaporization and heat capacities that all must be addressed
132 [29]. Switchable organic solvents mitigate almost all these negative constraints, as when
133 organic solvents are CO_2-loaded they are almost noncorrosive, and have significantly reduced

134 thermodynamic effects, making them a more energy efficient and environmentally friendly
135 option [34].

136 In this work the extractability of lipids from *Chlorella vulgaris* and *Haematococcus pluvialis*
137 using the tertiary amine N, N – Dimethyl Cyclohexylamine (DMCHA) is assessed against pure
138 hexane, using a microwave pre-treatment. This study aims to determine if lipid extraction of
139 intracellular lipid producing microalgae is possible and to assess the efficiency and lipid profile
140 for biofuel applications.

141

142 2. Materials and methods

143 2.1 Algae cultures and solvents

144 All chemicals were obtained from Merck life science UK at >99% purity, and their physical
145 properties are shown in table 1.

146 Table 1 - Physical Properties of Solvents

Compound	Hexane	DMCHA
Formula	C_6H_{14}	$C_8H_{17}N$
Molecular weight (g/ml)	86.18	127.23
LogP	3.9	2.01
Density (g/ml)	0.655	0.85
BP (°C)	68.73	162
pKa	>14	10.48

147

148 Both species of microalgae used in this study; *Chlorella vulgaris* and *Haematococcus pluvialis*
149 were obtained from Algae Research & Supply (USA). The growth medium used is the UTEX
150 recommended BG-11 which has the following composition: $NaNO_3$ 10ml/L, K_2HPO_4 10ml/L,
151 $MgSO_4.7H_2O$ 10ml/L, $CaCl_2.2H_2O$ 10ml/L, Citric Acid.H_2O 10ml/L, Ferric Ammonium
152 Citrate 10ml/L, $Na_2EDTA.2H_2O$ 10ml/L, Na_2CO_3 10ml/L, BG-11 tracer metals solution
153 1ml/L. The tracer metal recipe is as follows: H_3BO_3 2.86 g/L, $MnCl_2.4H_2O$ 1.81 g/L,
154 $ZnSO_4.7H_2O$ 0.22 g/L, $Na_2MoO_4.2H_2O$ 0.39 g/L, $CuSO_4.5H_2O$ 0.079 g/L, Co $(NO_3)_2.6H_2O$
155 49.4 mg/L, with a pH of 8.

156 All salts were of analytical grade and acquired from Merck life science UK and Fisher
157 Scientific UK. Stock solutions and sterilization were made using the recommended UTEX
158 culture procedure as a reference.

159 Both species were cultivated in 2L photobioreactors, with a working volume of 1.5L.
160 Cultivation was carried out in a specifically designed "black box", isolating each reactor to
161 maintain cultivation conditions. Each reactor was maintained at $21°C \pm 1°C$, with a pH of $7.5 \pm$
162 0.82, using cool white LEDs with a light and dark cycle of 16h:8h respectively, for 28 days
163 until the stationary phase was reached. Each reactor was inoculated with 200 ml of algae with
164 a dry weight of 0.33 mg/ml for *C. vulgaris* and 1.13 mg/ml for *H. pluvialis*. The absorbance
165 was measured every 48 hours using the Evolution 220 Thermo Scientific spectrophotometer at
166 680 nm, to monitor the concentration of the culture. Lipid extraction was carried out when the
167 stationary phase was reached.

168

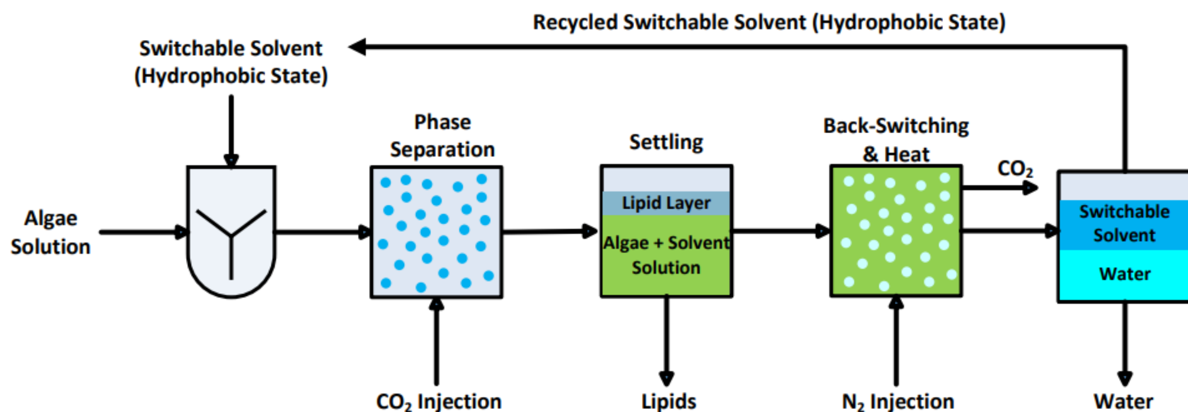
169 ## 2.2. Lipid extraction

170 Extractions were performed with varying solvent volumes and mixing times, employing a 2-
171 factorial design, which can be seen in Figure 4. Each extraction was performed in duplicates
172 whereby 25 ml of algae sample was pre-treated using 360W microwaves for 45 seconds, before
173 being left to cool for 5 minutes. Samples 1 – 4 used *C. vulgaris* and *H. pluvialis* with hexane,
174 samples 5 – 8 used *C. vulgaris* and *H. pluvialis* with DMCHA with varying mixing times and
175 solvent volumes. Details of the factorial design can be seen in section 2.4.

176 Each 25 ml algae sample was combined with the respective solvent in a 100 ml conical flask
177 and mixed for the allocated time, using a magnetic stirrer at 1000 rpm and 22°C. Samples were
178 transferred to a 50 ml separating funnel and left to settle for 5 minutes, allowing the
179 solvent/water biphasic layer to form. For hexane the bottom water layer was removed leaving
180 a lipid rich solvent layer. Hexane was removed by evaporation using a water bath. For DMCHA
181 each sample was subjected to CO_2 bubbling using a glass gas dispersion tube and 50ml conical
182 flask until the hydrophobic DMCHA switched to its protonated hydrophilic state, leaving a thin
183 lipid layer on top.

184 In preparation for analysis, the biphasic lipid/DMCHA mixture was transferred to a 50 ml
185 separating funnel. 10 ml of hexane was added to increase the size of the lipid layer before the
186 DMCHA was removed. The hexane carrier was removed using a water bath.

187



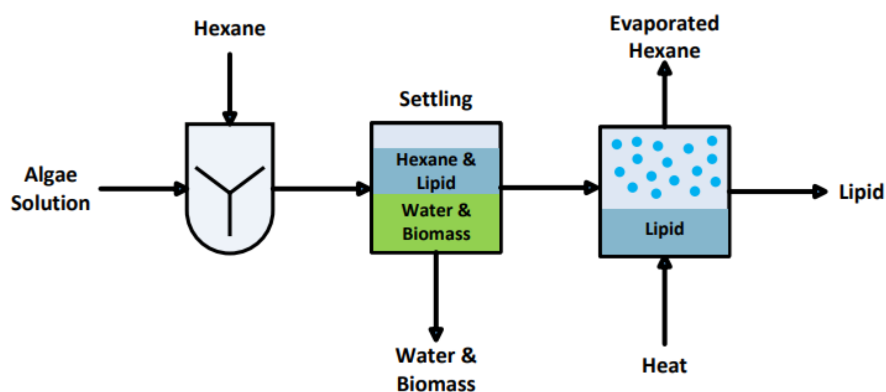
188

189 *Figure 1 - DMCHA lipid extraction process*

190

191

192



193 *Figure 2 - Hexane lipid extraction process*

194

195

196 *Figure 3 - DMCHA experimental setup*

197 It is worth noting that during the switching process, the biphasic solution changes from cloudy
198 light green to a darker clear green. Furthermore, on top of the visual conformation, once
199 DMCHA has switched to its hydrophilic state, it becomes less volatile and therefore the
200 pungent odour is no longer noticeable.

201

202 **2.3. Transesterification**

203 Before analysis of the lipid profile, each recovered sample was methylated using a standard
204 transesterification methodology. Methanol was added at each sample in a 3:1 ratio respectively.
205 Anti-bumping granules were added prior to adding sulphuric acid to the mixture, at a ratio of
206 1:12.5 with the initial sample volume. This ratio was fixed for all hexane samples, however,
207 due to the high basicity of DMCHA, the ratio was adjusted to 1:6.25.

208 The mixture was refluxed for 1.5 hours under constant heat using an electric heating mantle.
209 The solution was allowed to cool before adding individual drops of 10% NaH_2 carbonate to
210 neutralise any remaining sulphuric acid. The pH should be checked periodically to prevent any
211 salt formation. Furthermore, DMCHA samples should be carefully monitored to prevent any

212 micro-emulsification occurring. Each sample was then washed with hexane three times before
213 evaporating off any residual hexane using a water bath.

214 FAMEs were analysed on an Agile Technologies 7820A gas chromatograph, using a 30-meter
215 column, with 0.25mm ID operating at 240°C, using a standard injection and Nitrogen as a
216 carrier. The FID heater operates at 280°C.

217

218 ## 2.4. Factorial design

219 A two-level factorial design was used in the design of the experiment. Details of each factor
220 with their respective low and high limits is shown in Figure 4. Similarly, the experimental
221 configuration for each sample is illustrated in Table 2.

222

Factor	Low	High
Solvent Volume (ml) A	10	25
Mixing Time (Minute) B	5	30

223 *Figure 4 – Two factorial design*

224 Table 2 - Experimental configuration

Sample No.	Algae Species	Solvent	Solvent Volume (ml)	Mixing Time (Minute)
1	*C. vulgaris*	Hexane	10	5
	H. pluvialis	Hexane	10	5
2	*C. vulgaris*	Hexane	10	30
	H. pluvialis	Hexane	10	30
3	*C. vulgaris*	Hexane	25	5
	H. pluvialis	Hexane	25	5
4	*C. vulgaris*	Hexane	25	30
	H. pluvialis	Hexane	25	30
5	*C. vulgaris*	DMCHA	10	5
	H. pluvialis	DMCHA	10	5
6	*C. vulgaris*	DMCHA	10	30
	H. pluvialis	DMCHA	10	30
7	*C. vulgaris*	DMCHA	25	5
	H. pluvialis	DMCHA	25	5
8	*C. vulgaris*	DMCHA	25	30
	H. pluvialis	DMCHA	25	30

225

226 Statistical analysis for data processing of the two level-factorial design and ANOVA is carried
227 out using Design Expert software version 10.

228 ## 2.5. Measurement of cell growth

229 Cell growth measurement for each species was determined using a concentration calibration
230 curve, as described by [35]. Optical density (OD) readings of 1ml algae samples at 680nm were

231 taken using the Evolution 220 UV-visible spectrophotometer every 48 hours. Concentrations
232 of *C. vulgaris* and *H. pluvialis* were determined using Equations (2) and (3) respectively.

$$X_{Cv} = 0.1149OD_{680} + 0.2528 \quad (2)$$

233

$$X_{Hp} = 0.50250D_{680} + 0.1206 \quad (3)$$

234

235 Where X is the concentration of each species ($mg.ml^{-1}$) and OD_{680} is the optical density reading
236 at 680nm.

237

238 ## 3. Results and discussion

239 Lipid extraction yield is an essential indicator for evaluating the efficiency of the proposed
240 extraction method, since it represents the total amount of lipids obtained from the biomass. The
241 ability of DMCHA to extract lipids from *C. vulgaris* and *H. pluvialis* was tested under a series
242 of conditions, as discussed in section 2.4. Hexane is used as a control for determining the
243 effectiveness of DMCHA for lipid extraction. Lipid yield results can be seen in Figure 5 and
244 Figure 6 for *C. vulgaris* and *H. pluvialis* respectively. Equation (4) was used to calculate the
245 lipid yields.

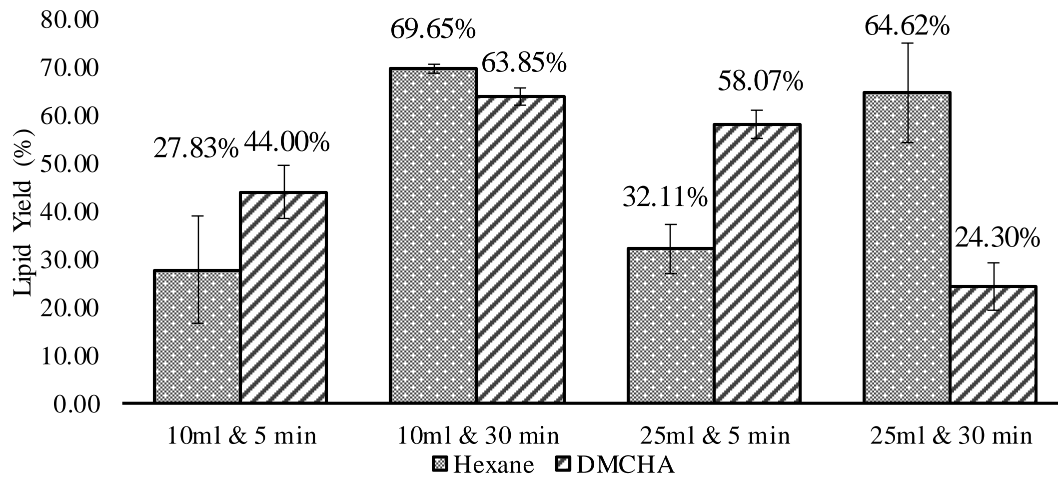
$$Lipid\,Yield\,(\%) = \frac{Weight\ of\ oil\ (mg)}{Dry\ weight\ of\ biomass\ (mg)} \times 100 \quad (4)$$

246

247 The location of lipid production is an essential element to consider. The intracellular lipid
248 producing *C. vulgaris* and *H. pluvialis* poses a key problem for lipid extraction using a single
249 hydrophobic solvent, since these solvents alone are not able to penetrate through the cell
250 membrane to extract the lipids easily. The use of the microwave pre-treatment was essential
251 for gaining access to the intracellular lipids, as generally non-polar solvent extraction efficiency
252 is reduced due to mass transfer resistances. The electromagnetic radiation from the microwave
253 agitates the polar water molecules that cause local heating, compromising the cell wall
254 structure. The cavitation mechanism that is inflicted allows easy access for lipid recovery this
255 has been extensively investigated with lipid increases of 6.79% and 15% by Hu et al., and
256 Rokicka et al., using *C. vulgaris* respectively [36,37]. It is worth noting that lipid extraction of
257 *C. vulgaris* in the context of hexane, there is little to no information regarding its performance,
258 as typically hexane is accompanied with a co-solvent to allow for both polar and non-polar
259 lipids to be extracted. Comparison of literature that uses co-solvent systems would not be valid.

260 Each solvent utilised the “like dissolves like” property, whereby the hydrophobic lipids would
261 diffuse easily into the hydrophobic solvents, and vice-versa. Furthermore, during the extraction
262 process of DMCHA the available amine functional groups interact with the polar lipids after
263 cell disruption has occurred. The large hydrocarbon neutral lipids have a tendency to form
264 globules in which the amines bond to these molecules through Van der Waal attractions [38].

265



266

267 *Figure 5 - C. vulgaris lipid yields*

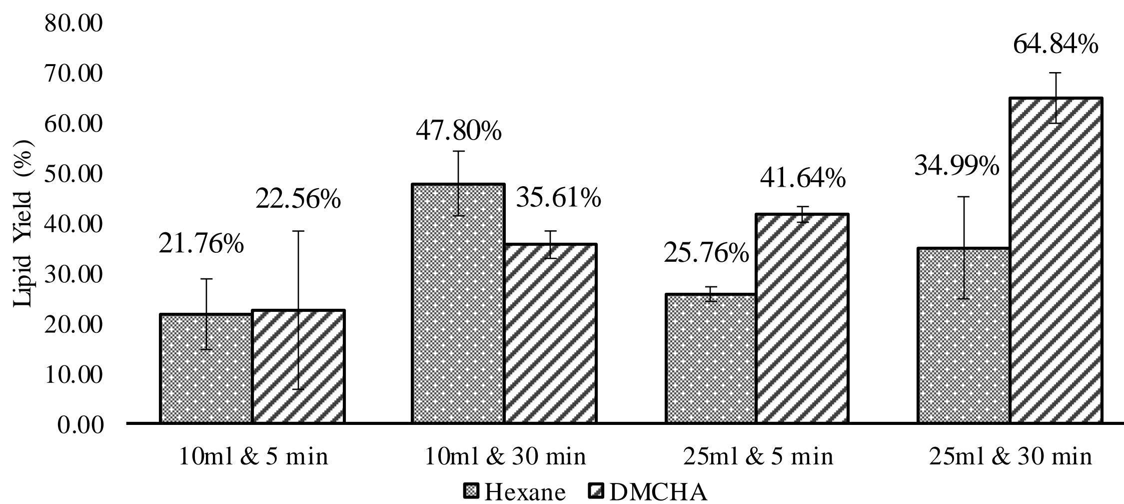


Figure 6 - H. pluvialis lipid yields

268

269 Considering *C. vulgaris*, the results from Figure 5 show that at lower mixing times DMCHA
270 is more efficient than hexane. On the contrary, hexane is more efficient at extracting algal lipids
271 at higher mixing times. This opposite behaviour between mixing times suggests that for
272 DMCHA, an increase in duration for mass transfer negatively affects either the extraction
273 efficiency or the lipid structure directly, whereby it is possible that the ester link breaks down
274 into free fatty acids. This is particularly prevalent in sample 4 whereby DMCHA recovery was
275 24.30% compared with hexane at 64.62%. Hexane, however, favours high durations for mass
276 transfer, yielding 64.62% in sample 3, and 69.65% in sample 2; the highest lipid yield achieved
277 in this study. Analysis of Figure 6 shows that longer mixing times for both hexane and DMCHA
278 favour higher lipid yields for *H. pluvialis*, with DMCHA recovering 64.84% in sample 8; the
279 highest lipid yield for this solvent in this study.

280 Compared with *C. vulgaris*, *H. pluvialis*, has a significantly thicker and rigid cell wall,
281 consisting of two layers containing polysaccharides and non-hydrolysable sporopollenins
282 which make extracting intracellular compounds a significant challenge despite the inclusion of

283 microwave pre-treatment [39]. The increased structural integrity of *H. pluvialis* would support
284 the hypothesis that solvent extraction efficiency is influenced by the morphology and rigidity
285 of the cells, given that in all instances – excluding sample 8 – both hexane and DMCHA lipid
286 yields were lower in *H. pluvialis* compared with *C.* vulgaris. Furthermore, the biocompatibility
287 of DMCHA with *H. pluvialis* has been successful in extracting large quantities of astaxanthin
288 [12]. However, to the best of our knowledge this is the first study to investigate the extraction
289 of lipids from *H. pluvialis* using DMCHA, which has shown significant positive results.
290 Research into the lipid recovery using DMCHA during the red and green phase of *H. pluvialis*
291 would provide new knowledge on the interaction of switchable solvents on both lipid and
292 carotenoid composition simultaneously.

293 While DMCHA showing promising results with relatively high lipid yields, it's important to
294 consider the additional complexity of the extraction process, when compared with hexane. A
295 macro perspective should be taken when conserving the overall extraction process, as while
296 more steps are required for DMCHA, with varying degrees of complexity, the reusability of
297 DMCHA compared with hexane makes the overall process more sustainable and
298 environmentally friendly, as discussed previously. Therefore, further investigation into the
299 efficiency of repeatably used DMCHA would determine a suitable lifecycle per batch, and
300 subsequently verify if the additional separation/purification steps are worth the investment.

301 **3.1. Lipid profile**

302 Analysis of the lipid profile for each species shows that *C. vulgaris* contains higher quantities
303 of neutral lipids compared with *H. pluvialis* [40,41]. Whereas *H. pluvialis* contains higher
304 quantities of polar lipids than *C.* vulgaris. Both hexane and DMCHA extracted higher quantities
305 of lipids in *C. vulgaris* which suggests that neutral lipids have a greater efficiency for mass
306 transfer compared with polar lipids [33,39]. This is concurrent with work published by Huang
307 et al., whereby as discussed previously, non-polar solvents have an increased affinity to bind
308 to neutral lipids through Van der Waal attractions [38]. These findings would further support
309 that *C. vulgaris* contains more neutral lipids than *H. pluvialis.*

310 For biofuel production the neutral lipid yield is of greater importance. While lipid profiling is
311 out with the scope of this study, FAME data analysis for each species has been extrapolated
312 and presented in Table 3. Since biofuel is the compound of interest, only the most suitable
313 hydrocarbons have been included. The breakdown shows that C16 (Palmitic acid), and C18:1
314 (Oleic acid) is significantly more abundant in *C. vulgaris* which could suggest that these
315 hydrocarbons are more easily diffused into both solvents. Furthermore, C18:2 (Linoleic acid)
316 and C18:3 (Linolenic acid) are the only FAMEs to be more abundant in *H. pluvialis* than *C.*
317 *vulgaris* where both are detected within each species. This isn't surprising since Linolic and
318 Linolenic acid are key compounds that make up the cell membrane structure [42].

319

320

321 Table 3 - FAME composition

FAME Hydrocarbon	***C. vulgaris composition* (%)** [43]	***H. pluvialis composition (%)*** [41]
C12:0 Lauric acid	0.45	0.00

C14:0 Myristic acid	1.33	0.00
C16:0 Palmitic acid	34.37	13.70
C16:3	0.00	3.50
C16:4	0.00	3.50
C18:0 Stearic acid	4.75	0.00
C18:1 Oleic acid	44.91	4.90
C18:2 Linoleic acid	12.75	24.90
C18:3 Linolenic acid	1.40	39.7
C18:4	0.00	5.80

322

323 ## 3.2. DoE analysis

324 Statistical analysis for both *C. vulgaris* and *H. pluvialis* using DMCHA, showed significant
325 models F-test ($P < 0.05$), with values of 0.0006 and 0.0113 respectively. Figure 7 and Figure 8
326 represent the response surface for *C. vulgaris* and *H. pluvialis* respectively, in the design space.
327 Considering *C. vulgaris*, ANOVA results show that the signal to noise ratio to be 13.364,
328 implying that there is an adequate signal. Furthermore, Equation (5) represents the actual factor
329 relationship for lipid yield using the statistical analysis.

$$Lipid\ Yield\ (\%) = 24.75142 + 1.65333A + 2.224B - 0.143AB \quad (5)$$

330 Statistical analysis of *H. pluvialis* showed a signal to noise ratio of 7.712, with Equation (6)
331 representing the actual factor relationships for lipid yield.

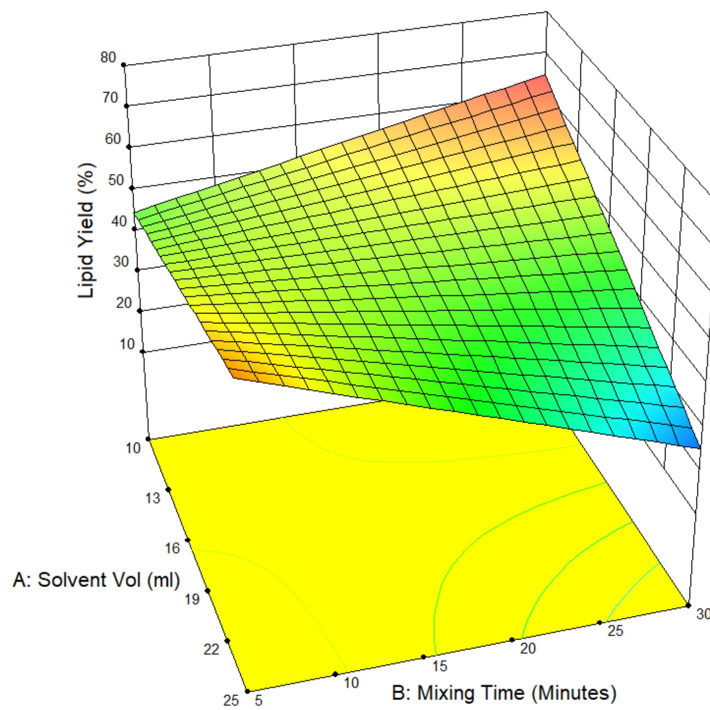
$$Lipid\ Yield\ (\%) = 2.82667 + 1.61033A - 0.725B \quad (6)$$

332 Where A is the solvent volume (ml) and B is the mixing time (minutes).

333 Reference to Figure 7, shows that at lower solvent volumes, an increase in mixing time greatly
334 increases the lipid yield recovery, supporting the hypothesis of increased mass transfer duration
335 effects. Furthermore, at higher solvent volumes the lipid yield recovery decreases with
336 increasing mixing time, further supporting the idea of a chemical breakdown and or chemical
337 inhibition occurring between the solvent and lipids; this is considering both polar and neutral
338 lipids. The response surface for *H. pluvialis* in Figure 8 shows a more linear and consistent
339 behaviour, whereby an increase of solvent volume for any fixed mixing time improves the lipid
340 yield recovery, achieving the maximum of 64.84% at 25 ml and 30 minutes. A similar
341 relationship is observed when the solvent volume is fixed, whereby longer mixing times
342 achieves higher lipid yield. This suggests that the extent of rigidity in *H. pluvialis* forms a

343 predictive linear relationship between both factors, something that hasn't been reported within
344 the literature, and would be of interest to explore in the future.

345

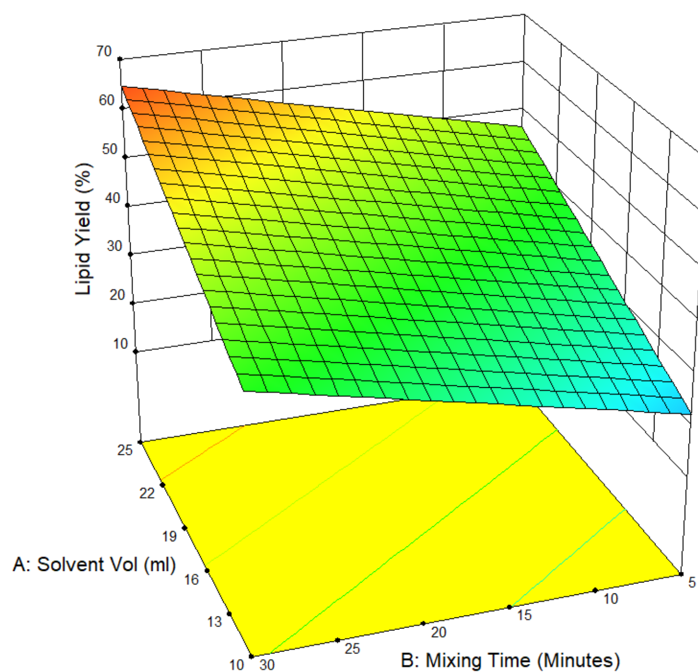


346

347 *Figure 7 - C. vulgaris response surface*

348

349

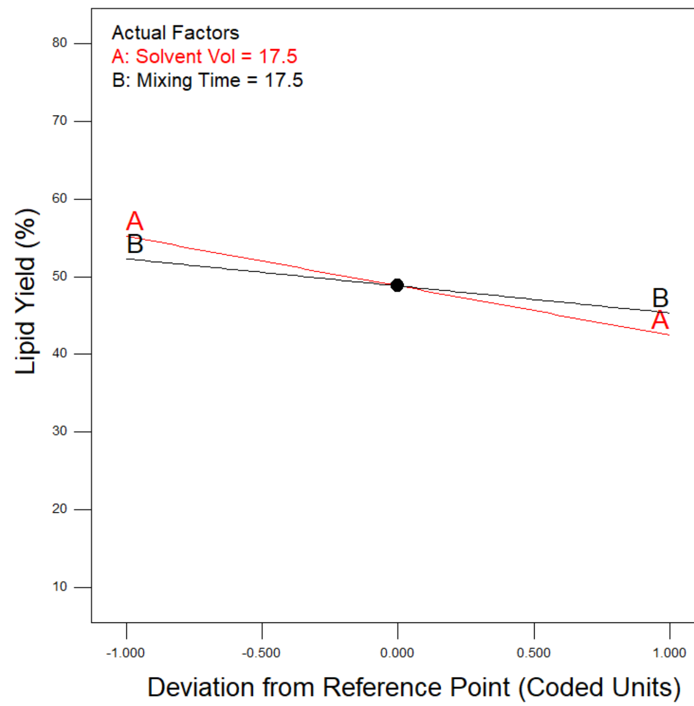


350

351 *Figure 8 - H. pluvialis response surface*

352

353 The perturbation plots for *C. vulgaris* and *H. pluvialis* seen in Figure 9 and Figure 10
354 respectively, show that the lipid yield response to each factor is reversed for each species. As
355 mentioned previously, *H. pluvialis* contains significantly more polar and less neutral lipids
356 compared to *C. vulgaris*, however two main hypotheses can be drawn from this. Firstly, the
357 efficiency of DMCHA is dependent upon the quantity of neutral lipids present rather than polar
358 lipids. Secondly, the strong alkalinity of DMCHA may cause hydrocarbon degradation
359 whereby the lipids are broken into their respective amino acids and therefore not accounted for
360 in the GC results. This offers a new potential direction for switchable solvent research.

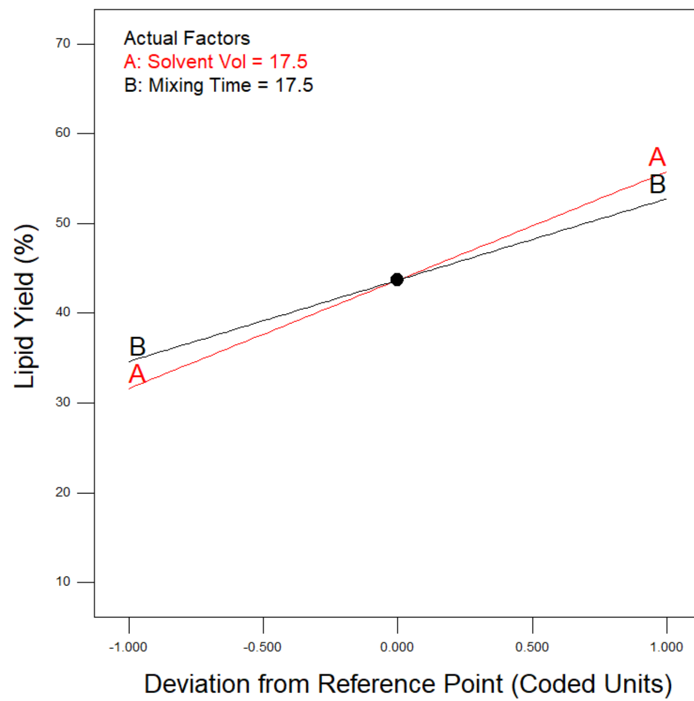


361

362 *Figure 9 - C. vulgaris perturbation plot*

363

364



365

366 *Figure 10 - H. pluvialis perturbation plot*

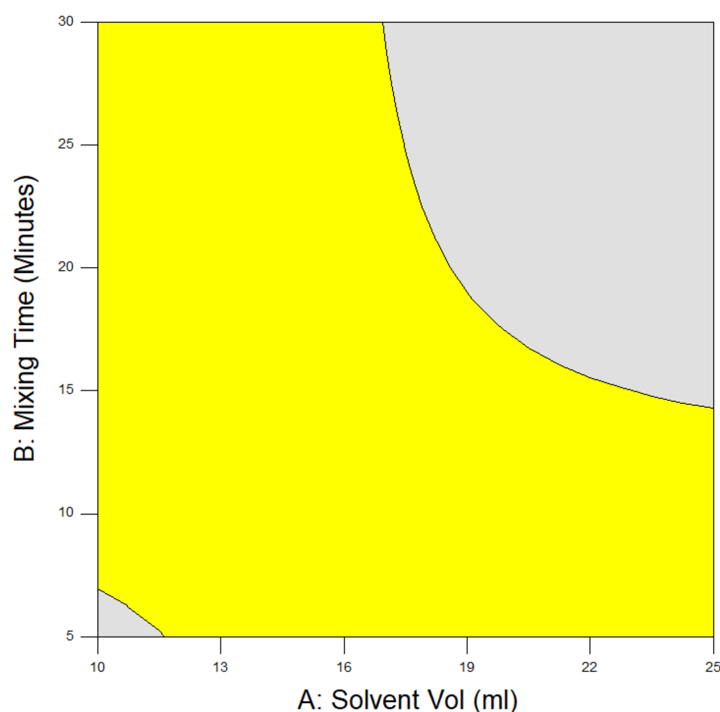
367

368

369 ### 3.3. Model optimisation

370 An optimisation criterion was applied to maximise the lipid yield (importance 3), while
371 minimising the solvent volume (importance 4). The mixing time was kept to within range as
372 the economic analysis was not considered in this study. Overlay plots for *C. vulgaris* and *H.*
373 *pluvialis* can be seen in Figure 11 and Figure 12 respectively. Based on the criteria the optimal
374 conditions for *C. vulgaris* were found to be 10 ml solvent and 30-minute mixing time yielding
375 65.11% lipids. Applying the same criteria, *H. pluvialis* achieved an optimal lipid yield of
376 42.08% using 10.87 ml of solvent and 30-minute mixing time. This further supports the
377 findings that DMCHA is more efficient at lipid extraction of *C. vulgaris* compared with *H.*
378 *pluvialis*, for reasons discussed previously.

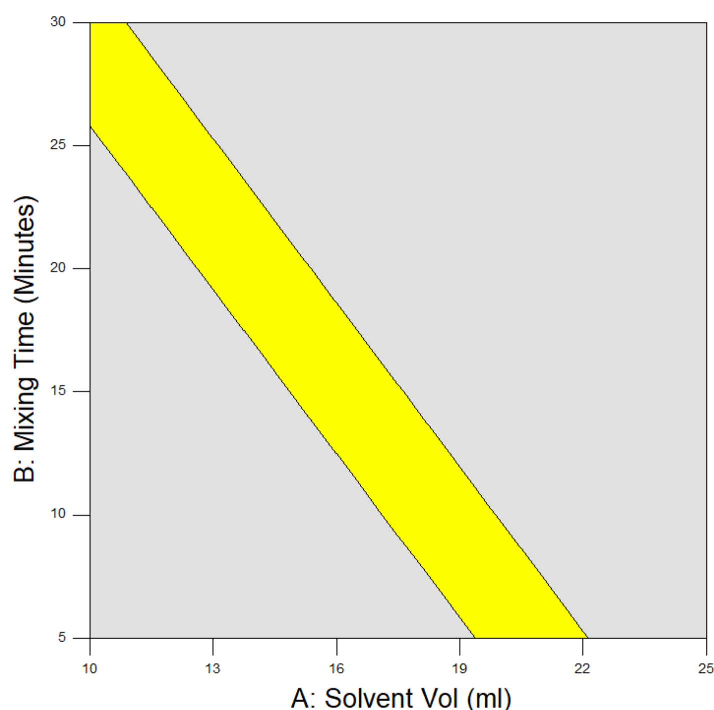
379



380

381 *Figure 11 - C. vulgaris overlay plot*

382



383 *Figure 12 - H. pluvialis overlay plot*

384

385 The overlay plots suggest that *C. vulgaris* has a greater tolerance for optimal operating
386 conditions industrially compared with *H. pluvialis* however, the extent of this plot is
387 significantly greater than deemed acceptable. This can be attributed to fact that duplicates were
388 used instead of triplets, and that samples 1 and 4 had significantly higher standard deviations.
389 Therefore, further experimentation using the same method using triplets would be
390 recommended to validate the results.

391

392 # 4. Conclusion

393 DMCHA has been shown to be an effective solvent for the extraction of lipids from both *C.*
394 *vulgaris* and *H. pluvialis* achieving maximum lipid yields of 63.85% and 64.84% respectively.
395 It's clear that the chemical structure of the cell wall greatly influences the lipid extraction
396 efficiency, despite exposure to microwave pre-treatment. Hexane extraction performed as
397 expected for both species with longer mixing times achieving the greatest lipid yield of 69.65%.
398 However, DMCHA efficiency appeared to be influenced more by differences in cell wall
399 structure/morphology and internal lipid compositions, compared with hexane. The increased
400 cell wall rigidity and reduced neutral lipid composition in *H. pluvialis* compared with *C.*
401 *vulgaris* may explain the difference in lipid recovery, through physical/chemical inhibition and
402 resistances. Statistical analysis of DMCHA has shown significant F-test models ($P < 0.05$),
403 with values of 0.0006 for *C. vulgaris* and 0.0113 *H. pluvialis*. This study has shown DMCHA
404 to be biocompatible for lipid extraction of *H. pluvialis*. Furthermore, the study has also shown
405 that hexane can be used independently for lipid extraction with assisted microwave pre-
406 treatment; something that hasn't fully been explored yet within the literature. The overall
407 findings show that DMCHA has potential to efficiently extract high quantities of neutral lipids

408 from intracellular lipid producing microalgae and is believed to be the first to report
409 experimental data on *H. pluvialis* lipids using DMCHA explicitly.

410

411 # 5. References

412 [1] Markou G, Vandamme D, Muylaert K. Microalgal and cyanobacterial cultivation: The
413 supply of nutrients. Water Res 2014;65:186–202.
414 https://doi.org/10.1016/j.watres.2014.07.025.

415 [2] Olabi AG, Shehata N, Sayed ET, Rodriguez C, Anyanwu RC, Russell C, et al. Role of
416 microalgae in achieving sustainable development goals and circular economy. Science
417 of The Total Environment 2022:158689.
418 https://doi.org/10.1016/j.scitotenv.2022.158689.

419 [3] Russell C, Rodriguez C, Yaseen M. Microalgae for lipid production: Cultivation,
420 extraction & detection. Algal Res 2022;66:102765.
421 https://doi.org/https://doi.org/10.1016/j.algal.2022.102765.

422 [4] Maity JP, Bundschuh J, Chen C-Y, Bhattacharya P. Microalgae for third generation
423 biofuel production, mitigation of greenhouse gas emissions and wastewater treatment:
424 Present and future perspectives – A mini review. Energy 2014;78:104–13.
425 https://doi.org/10.1016/j.energy.2014.04.003.

426 [5] Mimouni V, Couzinet-Mossion A, Ulmann L, Wielgosz-Collin G. Lipids From
427 Microalgae. Microalgae in Health and Disease Prevention, Elsevier; 2018, p. 109–31.
428 https://doi.org/10.1016/B978-0-12-811405-6.00005-0.

429 [6] Morales M, Aflalo C, Bernard O. Microalgal lipids: A review of lipids potential and
430 quantification for 95 phytoplankton species. Biomass Bioenergy 2021;150:106108.
431 https://doi.org/10.1016/j.biombioe.2021.106108.

432 [7] Ördög V, Stirk WA, Bálint P, Aremu AO, Okem A, Lovász C, et al. Effect of
433 temperature and nitrogen concentration on lipid productivity and fatty acid
434 composition in three Chlorella strains. Algal Res 2016;16:141–9.
435 https://doi.org/10.1016/j.algal.2016.03.001.

436 [8] Han Y, Wen Q, Chen Z, Li P. Review of methods used for microalgal lipid-content
437 analysis. Energy Procedia, vol. 12, Elsevier Ltd; 2011, p. 944–50.
438 https://doi.org/10.1016/j.egypro.2011.10.124.

439 [9] Safi C, Zebib B, Merah O, Pontalier P-Y, Vaca-Garcia C. Morphology, composition,
440 production, processing and applications of Chlorella vulgaris: A review. Renewable
441 and Sustainable Energy Reviews 2014;35:265–78.
442 https://doi.org/10.1016/j.rser.2014.04.007.

443 [10] Aguoru CU, Okibe PO. Content and Composition of Lipid Produced by Chlorella
444 vulgaris for Biodiesel Production. vol. 36. Online; 2015.

445 [11] Yeh K-L, Chang J-S. Effects of cultivation conditions and media composition on cell
446 growth and lipid productivity of indigenous microalga Chlorella vulgaris ESP-31.
447 Bioresour Technol 2012;105:120–7. https://doi.org/10.1016/j.biortech.2011.11.103.

[12] Huang W-C, Liu H, Sun W, Xue C, Mao X. Effective Astaxanthin Extraction from Wet Haematococcus pluvialis Using Switchable Hydrophilicity Solvents. ACS Sustain Chem Eng 2018;6:1560–3. https://doi.org/10.1021/acssuschemeng.7b04624.

[13] Damiani MC, Popovich CA, Constenla D, Leonardi PI. Lipid analysis in Haematococcus pluvialis to assess its potential use as a biodiesel feedstock. Bioresour Technol 2010;101:3801–7. https://doi.org/10.1016/j.biortech.2009.12.136.

[14] Grewe CB, Griehl C. 8 The carotenoid astaxanthin from Haematococcus pluvialis. Microalgal Biotechnology: Integration and Economy, DE GRUYTER; 2012, p. 129–44. https://doi.org/10.1515/9783110298321.129.

[15] Russell C, Rodriguez C, Yaseen M. Microalgae for lipid production: Cultivation, extraction & detection. Algal Res 2022;66:102765. https://doi.org/10.1016/j.algal.2022.102765.

[16] Anyanwu RC, Rodriguez C, Durrant A, Olabi AG. Microalgae Cultivation Technologies. Reference Module in Materials Science and Materials Engineering, Elsevier; 2018. https://doi.org/10.1016/B978-0-12-803581-8.09258-4.

[17] Roux JM, Lamotte H, Achard JL. An Overview of Microalgae Lipid Extraction in a Biorefinery Framework. Energy Procedia, vol. 112, Elsevier Ltd; 2017, p. 680–8. https://doi.org/10.1016/j.egypro.2017.03.1137.

[18] Al-Ameri M, Al-Zuhair S. Using switchable solvents for enhanced, simultaneous microalgae oil extraction-reaction for biodiesel production. Biochem Eng J 2019;141:217–24. https://doi.org/10.1016/j.bej.2018.10.017.

[19] Breil C, Abert Vian M, Zemb T, Kunz W, Chemat F. “Bligh and Dyer” and Folch Methods for Solid–Liquid–Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. Int J Mol Sci 2017;18:708. https://doi.org/10.3390/ijms18040708.

[20] Chen IS, Shen CSJ, Sheppard AJ. Comparison of methylene chloride and chloroform for the extraction of fats from food products. J Am Oil Chem Soc 1981;58. https://doi.org/10.1007/BF02672373.

[21] Du Y, Schuur B, Kersten SRA, Brilman DWF. Opportunities for switchable solvents for lipid extraction from wet algal biomass: An energy evaluation. Algal Res 2015;11:271–83. https://doi.org/10.1016/j.algal.2015.07.004.

[22] Saini RK, Prasad P, Shang X, Keum Y-S. Advances in Lipid Extraction Methods—A Review. Int J Mol Sci 2021;22:13643. https://doi.org/10.3390/ijms222413643.

[23] Cooney M, Young G, Nagle N. Extraction of Bio-oils from Microalgae. Separation & Purification Reviews 2009;38. https://doi.org/10.1080/15422110903327919.

[24] Zhang Y, Soldatov S, Papachristou I, Nazarova N, Link G, Frey W, et al. Pulsed microwave pretreatment of fresh microalgae for enhanced lipid extraction. Energy 2022;248:123555. https://doi.org/10.1016/j.energy.2022.123555.

[25] Xia A, Sun C, Fu Q, Liao Q, Huang Y, Zhu X, et al. Biofuel production from wet microalgae biomass: Comparison of physicochemical properties and extraction performance. Energy 2020;212:118581. https://doi.org/10.1016/j.energy.2020.118581.

[26] Bai X, Schenk PM, Yuan Z, Lant PA, Pratt S. Enhanced triacylglyceride extraction from microalgae using free nitrous acid pre-treatment. Appl Energy 2015;154:183–9. https://doi.org/10.1016/j.apenergy.2015.04.045.

[27] Lee SY, Khoiroh I, Vo D-VN, Senthil Kumar P, Show PL. Techniques of lipid extraction from microalgae for biofuel production: a review. Environ Chem Lett 2021;19. https://doi.org/10.1007/s10311-020-01088-5.

[28] Alshana U, Hassan M, Al-Nidawi M, Yilmaz E, Soylak M. Switchable-hydrophilicity solvent liquid-liquid microextraction. TrAC Trends in Analytical Chemistry 2020;131:116025. https://doi.org/10.1016/j.trac.2020.116025.

[29] Zhang S, Shen Y, Wang L, Chen J, Lu Y. Phase change solvents for post-combustion CO2 capture: Principle, advances, and challenges. Appl Energy 2019;239:876–97. https://doi.org/10.1016/j.apenergy.2019.01.242.

[30] Liu Y, Qiu Z, Zhong H, Zhao X, Huang W, Xing X. Nitrogen-containing switchable solvents for separation of hydrocarbons and their derivatives. RSC Adv 2020;10:12953–61. https://doi.org/10.1039/D0RA00615G.

[31] Carrera M, Gómez-Díaz D, Navaza JM. Switchable hydrophilicity solvents for carbon dioxide chemical absorption. Journal of Industrial and Engineering Chemistry 2018;59:304–9. https://doi.org/10.1016/j.jiec.2017.10.036.

[32] Durelle J. Designing Switchable-Hydrophilicity Solvents and Modelling their Behaviour. 2014.

[33] Jessop PG, Heldebrant DJ, Li X, Eckert CA, Liotta CL. Reversible nonpolar-to-polar solvent. Nature 2005;436. https://doi.org/10.1038/4361102a.

[34] Zhou X, Liu C, Fan Y, Zhang L, Tang S, Mo S, et al. Energy-efficient carbon dioxide capture using a novel low-viscous secondary amine-based nonaqueous biphasic solvent: Performance, mechanism, and thermodynamics. Energy 2022;255. https://doi.org/10.1016/j.energy.2022.124570.

[35] Singh SK, Rahman A, Dixit K, Nath A, Sundaram S. Evaluation of promising algal strains for sustainable exploitation coupled with CO2 fixation. Environ Technol 2016;37:613–22. https://doi.org/10.1080/09593330.2015.1075599.

[36] Rokicka M, Zieliński M, Dudek M, Dębowski M. Effects of Ultrasonic and Microwave Pretreatment on Lipid Extraction of Microalgae and Methane Production from the Residual Extracted Biomass. Bioenergy Res 2021;14:752–60. https://doi.org/10.1007/s12155-020-10202-y.

[37] Hu Z, Jiang E, Ma X. Microwave pretreatment on microalgae: Effect on thermo-gravimetric analysis and kinetic characteristics in chemical looping gasification. Energy Convers Manag 2018;160:375–83. https://doi.org/10.1016/j.enconman.2018.01.057.

[38] Huang W-C, Kim J-D. Simultaneous cell disruption and lipid extraction in a microalgal biomass using a nonpolar tertiary amine. Bioresour Technol 2017;232:142–5. https://doi.org/10.1016/j.biortech.2017.02.037.

529 [39] Ye Z, Tan X, Liu Z, Aadil RM, Tan Y, Inam-ur-Raheem M. Mechanisms of
530 breakdown of Haematococcus pluvialis cell wall by ionic liquids, hydrochloric acid
531 and multi-enzyme treatment. Int J Food Sci Technol 2020;55:3182–9.
532 https://doi.org/10.1111/ijfs.14582.

533 [40] Yang F, Cheng C, Long L, Hu Q, Jia Q, Wu H, et al. Extracting Lipids from Several
534 Species of Wet Microalgae Using Ethanol at Room Temperature. Energy & Fuels
535 2015;29:2380–6. https://doi.org/10.1021/ef5023576.

536 [41] Mularczyk M, Michalak I, Marycz K. Astaxanthin and other Nutrients from
537 Haematococcus pluvialis—Multifunctional Applications. Mar Drugs 2020;18:459.
538 https://doi.org/10.3390/md18090459.

539 [42] Blasio M, Balzano S. Fatty Acids Derivatives From Eukaryotic Microalgae, Pathways
540 and Potential Applications. Front Microbiol 2021;12.
541 https://doi.org/10.3389/fmicb.2021.718933.

542 [43] El-Sheekh MM, Gheda SF, El-Sayed AEKB, Abo Shady AM, El-Sheikh ME, Schagerl
543 M. Outdoor cultivation of the green microalga Chlorella vulgaris under stress
544 conditions as a feedstock for biofuel. Environmental Science and Pollution Research
545 2019;26:18520–32. https://doi.org/10.1007/s11356-019-05108-y.

546