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1 **Chemical characterisation and analysis of the cell wall polysaccharides of**
2 **duckweed (*Lemna minor*)**
3

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23 **Abstract**

24 Duckweed is potentially an ideal biofuel feedstock due to its high proportion of
25 cellulose and starch and low lignin content. However, there is little detailed information
26 on the composition and structure of duckweed cell walls relevant to optimizing the
27 conversion of duckweed biomass to ethanol and other biorefinery products. This study
28 reports that, for the variety and batch evaluated, carbohydrates constitute 51.2 % (w/w)
29 of dry matter while starch accounts for 19.9 %. This study, for the first time, analyses
30 duckweed cell wall composition through a detailed sequential extraction. The cell wall
31 is rich in cellulose and also contains 20.3 % pectin comprising galacturonan,
32 xylogalacturonan, rhamnogalacturonan; 3.5 % hemicellulose comprising xyloglucan
33 and xylan, and 0.03% phenolics. In addition, essential fatty acids (0.6 %, α –linolenic
34 and linoleic/ linoelaidic acid) and p-coumaric acid (0.015 %) respectively are the most
35 abundant fatty acids and phenolics in whole duckweed.

36

37 **Keywords:** Duckweed, *Lemna minor*, cell-wall polysaccharides, fractionation, chemical
38 analysis

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39

40 **Abbreviations**

41	AIR	alcohol insoluble residue
42	CWM	cell wall material
43	DM	dry matter
44	FA	fatty acid
45	FDM	freeze dried and freeze milled

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48 **1. Introduction**

49 The *Lemnaceae* family, members of which are commonly known as duckweeds,
50 contains the smallest flowering plants. These plants float on the surface of water and
51 have thin leaves attached to a simple root (Landolt & Kandeler, 1987). This morphology
52 enables duckweed to obey Kleiber's 3/4-power rule ($G \propto M^{3/4}$) (Niklas & Enquist, 2001)
53 in that duckweed exhibits very much higher specific growth rates than other larger
54 aquatic or terrestrial plants, with doubling times of between 48 h to 96 h depending on
55 species (Zuberer, 1982). Consequently, duckweeds potentially can produce high
56 biomass yields, with high proportions of protein and carbohydrate (Landolt & Kandeler,
57 1987). Zhao et al. (2012) summarized earlier studies demonstrating the higher
58 productivity of duckweeds compared to other energy crops. Duckweed can also be used
59 for the decontamination of wastewater, absorbing problematic minerals as it grows
60 (Tripathia & Srivastavaa, 1991; Jayaweera, Kasturiarachchi, Kularatne, & Wijeyekoon,
61 2008).
62
63 Many studies report that duckweed is a protein rich resource for animal feed (e.g. Leng,
64 Stambolie, & Bell, 1995). However, recent research also shows that duckweed is a good
65 feedstock for bioethanol production due to its high proportion of starch (Cheng &
66 Stomp, 2009). We have shown previously (Zhao et al., 2012) that the cell wall material
67 of duckweed can be simply and efficiently saccharified. Ethanol and other biofuel
68 products, such as gas, oil and biochar, can be produced, the latter through pyrolysis
69 (Muradov, Fidalgo, Gujar, & T-Raissi, 2010) and thermolysis (Campanella et al., 2011).
70 However, the lack of information on the chemical composition of duckweed, for
71 example cell wall composition, whole duckweed fatty acid and phenolic components,

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72 hinders the exploitation of duckweed as a useful industrial feedstock of biofuel and
73 other products, potentially of high value, by biorefining.
74
75 The cell walls of plants generally consist of three main groups of polysaccharides -
76 cellulose, hemicellulose, and pectins (Brett & Waldron, 1996). Cellulose forms the main
77 structural component of the cell wall and is a relatively homogenous polymer of β-1,4-
78 linked glucose. The non-crystalline matrix phase of the cell wall consists of a variety of
79 polysaccharides, proteins and phenolic compounds (Brett & Waldron, 1996). The pectic
80 and hemicellulose polysaccharides are heterogeneous in nature and comprise polymers
81 of different sugar compositions. Golovchenko, Ovodova, Shashkov, & Ovodov (2002)
82 investigated the pectic polysaccharides of *L. minor* and analysed the monosaccharide
83 compositions of the pectin. However there is little research giving a comprehensive
84 understanding of duckweed cell wall polymer composition. This paper describes the
85 chemical composition of duckweed cell wall polymer groups, fatty acids and phenolics,
86 and assesses the proportions of cellulose, hemicelluloses and pectic polysaccharides and
87 investigates their monosaccharide composition.
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88

89 **2. Material and methods**

90 ***2.1 Preparation of plant material***

91 *L. minor* plants were collected from a pond in Norwich, UK (52.622295 N, 1.221894
92 E), cleaned with tap water and then rinsed with distilled water. The cleaned, fresh wet
93 biomass was packed in aluminium foil in long flat-thin packages and preserved at -40
94 °C. Material was treated by various means depending on the experiment being
95 conducted. The frozen duckweed material was dried using a freeze drier (Freeze Dryer
96 3.5, Birchover Instruments Ltd., UK). The freeze-dried materials were ground by
97 freeze-milling in liquid nitrogen (Spex Freezer-Mill 6700, Spex Industries Inc., USA) to
98 reduce the plant material to a powder. The freeze-dried and freeze-milled (FDM)
99 duckweed was used for general chemical analysis. For the sequential extraction (see Fig
100 1) of polysaccharides of duckweed cell wall, the original fresh biomass was treated by a
101 rotary ball-mill process (ball mill jar, Capco Test Equipment Ltd., Ipswich, Suffolk,
102 UK) to disrupt plant structure and remove starch (Parr, Waldron, Ng, & Parker, 1999).
103 The fresh duckweed (200 g) was prepared as cell wall materials (CWM) by ball-milling
104 and filtering on 25 μm nylon mesh to separate starch from the CWM. The ball-mill jar
105 was filled with ceramic beads (750 g of 2.54 cm diameter beads, 500 g of 1.91 cm
106 diameter beads and 500 g of 1.27 cm diameter beads) to break down plant tissues.

107

108 ***2.2 Moisture determination***

109 The moisture content of the original fresh materials (1 g in triplicate) was determined
110 using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd, Beaumont
111 Leys, Leicester, UK).

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112

113 ***2.3 Ash test***

114 The total mineral nutrients were established by determination using the total ash method
115 (Sluiter et al., 2008). The FDM duckweed (1g in triplicate) was placed in crucible and
116 dried in an oven (105 °C) for 16 h. The dry weights were recorded. The resulting
117 samples were then charred in a muffle furnace (Vulcan 3- 550, Jencons Scientific Ltd,
118 Leighton Buzzard, Beds., UK), starting at 200 °C with a gradual temperature increase to
119 350 °C. Finally, the samples were ashed at 500 °C for 16 h.

120

121 ***2.4 Lignin determination***

122 Acid insoluble lignin was measured by the gravimetric Klason lignin method. The FDM
123 duckweed (0.1g in triplicate) was hydrolysed in 1.5 mL 72 % (w/w) H_2SO_4 at 30 °C for
124 1 h, before adding 1.5 ml water and hydrolysing at 100 °C for 2.5 h. The acid insoluble
125 lignin was recovered on a sintered glass funnel (Porosity® 4, VWR, Lutterworth, Leics.,
126 UK) and dried in an oven (50 °C). The resulting sample was burned in a muffle furnace
127 to determine Klason lignin and the weight was corrected for ash content. Phenolic acids
128 were measured by HPLC as described below.

129

130 ***2.5 Starch assessment***

131 The starch content was measured using the standard method from the Total Starch
132 Assay Procedure (Megazyme, 2012). FDM duckweed (30 mg in triplicate) was
133 dispersed in 80 % (v/v) ethanol (200 µL). After boiling for 5 min with 2 mL of dimethyl
134 sulphoxide (DMSO), the samples were hydrolysed using α-amylase (3 ml, 300 U)
135 whilst boiling for a further 6 min. The hydrolysed samples were cooled to 50 °C by

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136 adding sodium acetate buffer (4 mL, 0.2 mol L^{-1}, pH 4.5) and then hydrolysed with
137 amyloglucosidase (0.1 mL, 20 U) at 50 °C for a further 0.5 h. The resulting sample (0.1
138 mL) was assessed by colourimetric assay using glucose oxidase-peroxidase-4-
139 aminoantipyrine (GOPOD) reagent (3 mL) at 510 nm.

140

141 ***2.6 The investigation of the lipid fraction***

142 Duckweed was also cultured under lab conditions according to Zhao et al. (2012).
143 Aliquots at various time points during a 28 day starvation period were collected and
144 observed with a light microscope to understand the variation in lipid content when
145 duckweed is starved of nutrients.

146

147 ***2.7 Sequential extraction of cell wall polysaccharides***

148 The composition of the duckweed cell wall including cellulose, hemicellulose and
149 pectin was evaluated by sequentially extracting the CWM as described by Stevens &
150 Selvendran (1984). The process is illustrated in Figure 1. CWM (2 g) was stirred in cold
151 water (deionised water) at room temperature for 2 h and the supernatant containing the
152 water-soluble components was recovered. The residual pellet was then extracted with
153 CDTA (0.05 mol L^{-1}, pH 6.5, prepared in deionised water) at room temperature for 6 h
154 following by CDTA (0.05 mol L^{-1}, pH 6.5, prepared in distilled water) extraction at
155 room temperature for 2 h, recovering chelator-soluble polysaccharides. All further
156 extractants were prepared using degassed ultrapure water. Next, the pellet was extracted
157 with 0.05 mol L^{-1} Na_2CO_3 (0.02 mol L^{-1} $NaBH_4$) at 4 ºC for 16 h to solubilise pectins
158 with weak ester linkages followed by 0.05 mol L^{-1} Na_2CO_3 (0.02 mol L^{-1} $NaBH_4$) at
159 room temperature for 2 h to remove strong ester linkage pectin. Finally, the pellet was

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160 extracted using increasing concentrations (0.42, 0.85 and 3.4 mol L^{-1}) of KOH (0.02
161 mol L^{-1} $NaBH_4$) to solubilise hemicelluloses. The supernatants were filtered using GF/C
162 filter paper (Whatman® glass microfibre filter, Whatman International, Maidstone Kent,
163 UK) and neutralised to pH 5. Salts in the aqueous and solid pellets were removed by
164 dialysis: the samples were placed into Visking dialysis tubing (Scientific Instrument
165 Centre Ltd, Colden Common, Winchester, Hants., UK) in 5 L flasks of distilled water.
166 Chloroform (several drops, Fisher Scientific Ltd, Loughborough, UK) was added to
167 prevent the growth of microorganisms. The dialysis water was changed twice daily and
168 the process was carried out for 10 days. The resulting samples were freeze dried as
169 described above for subsequent analysis.

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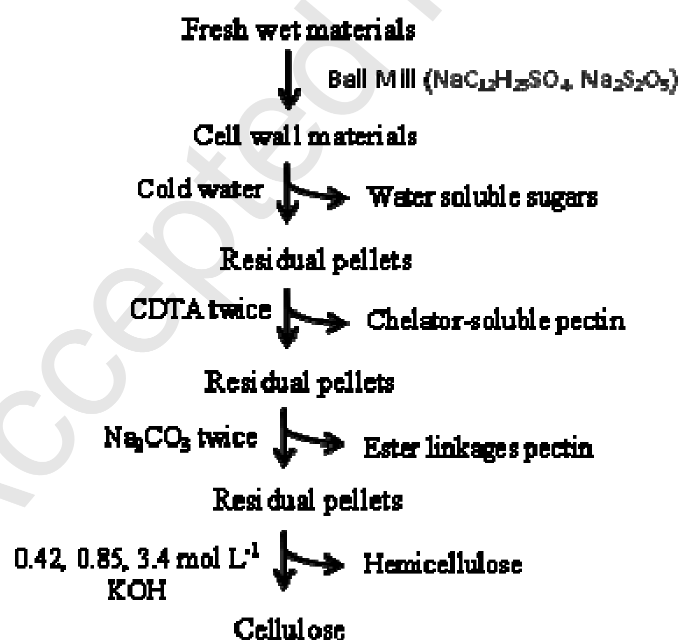

172
173
174 Figure 1.
175

176 ***2.8 Analytical methods***

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177 *2.8.1 Gas chromatography (GC) analysis of alditol acetates*

178 The monosaccharide compositions of the various samples were evaluated by GC
179 analysis of alditol acetates (Blakeney, Harris, Henry, & Stone, 1983). Ball milled and
180 distilled water washed CWM, sequentially extracted soluble materials and residual
181 pellets were freeze dried for sugars analysis. Samples were prepared as alditol acetates
182 as described in Zhao et al (2012) and then were analysed by GC on a Perkin-Elmer
183 Autosystem XL GC system with a RTX-225 (Restek, Bellefonte, USA) column and
184 flame ionization detector (Perkin-Elmer, Seer Green, Bucks., UK). 2-Deoxyglucose was
185 added as the internal standard.

186

187 *2.8.2 Gas chromatography (GC) analysis for fatty acid methyl esters (FAME)*

188 Lipids extracted using a Soxhlet extraction were prepared for identifying FAME
189 components and assessed by a GC method. The lipid samples (in triplicate) were
190 purified with 0.5 mL dry toluene containing 0.1 mg butylated hydroxytoluene (BHT)
191 with vortex mixing. Methylation reagent (1 mol L^{-1} of methanol containing 2 % (v/v)
192 H_2SO_4) was added and the samples were vortex mixed again. The tubes were tightly
193 capped and heated at 50 °C overnight with occasional mixing. After heating, the
194 samples were cooled down to room temperature and neutralised by 1 mL mixed solution
195 of 0.25 mol L^{-1} $KHCO_3$ and 0.5 mol L^{-1} K_2CO_3. The resulting solutions were mixed
196 with 1 mL hexane and centrifuged to separate FAMEs into upper phase which were
197 transferred to clean Pyrex® culture tubes. 200 µL of prepared samples were transferred
198 into vials and evaluated using a Hewlett Packard 5890 GC system (Hewlett-Packard
199 Limited, Bracknell, England, UK) with BPX 70 column (SGE Analytical Columns,
200 New Addington, Croydon, UK). Methyl heptadecanoate (Sigma Aldrich, Gillingham,

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201 UK) was added as the internal standard and the retention time of 31 FAME components
202 had been specifically identified for the BPX 70 column by using a commercial standard
203 (SGE Analytical Columns, New Addington, Croydon, UK).
204
205 *2.8.3 FT-IR analysis of the identification of polysaccharides*
206 FTIR-ATR spectra were measured with a BioRad FTS175 Fourier (Bio-Rad
207 Laboratories Inc, USA) transform infrared spectrometer equipped with a MCT detector
208 and a GoldenGate (Specac) single reflection diamond ATR accessory. Five aliquots
209 from each sequentially extracted sample were loaded on the ATR crystal and pressed
210 down with the clamp. For each, 64 scans at a resolution of 4 cm^{-1} in the region 4000-
211 800 cm^{-1} were averaged and referenced against a spectrum of the empty crystal.
212
213 *2.8.4 Phenolic acid assessment*
214 Cell wall-bound phenolic acids (*p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde,
215 protocatechuic acid, vanillin, vanillic acid, *p*-hydroxyphenyl acetic acid, *trans*-*p*-
216 coumaric acid, caffeic acid, *trans*-ferulic acid and chlorogenic acid) of dry cell wall
217 material were evaluated by HPLC and the samples were prepared using the method
218 reported by Parr et al. (1996). *p*-hydroxybenzoic acid, protocatechuic acid, *p*-
219 hydroxybenzaldehyde, vanillin, vanillic acid, *p*-hydroxyphenyl acetic acid, *trans*-*p*-
220 coumaric acid, *trans*-ferulic acid and chlorogenic acid were obtained from Sigma
221 (Sigma-Aldrich Company Ltd. Dorset, UK); caffeic acid was obtained from Fluka
222 (Sigma-Aldrich Company Ltd. Seelze, Switzerland). The resulting samples were
223 redissolved in 50 % (v/v) methanol (1 mL) and filtered using a filter disc (0.2 µm,
224 PVDF) for HPLC analysis with a Phenomenex Luna 5 µm (250 x 4.0 mm) column and

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225 a Perkin Elmer Diode Array Detector (UV). *Trans*-cinnamic acid was added as an
226 internal standard. The phenolic acids were identified by comparing their retention time
227 relative to the internal standard and the spectra with those of the authentic standards.
228

229 *2.8.5 Quantification of lipids by using Soxhlet extraction*

230 The Soxhlet extraction apparatus was originally designed for extracting the lipid
231 fraction in solid samples (Laurence & Christopher, 1989). Sample (2 g) was contained
232 in a cellulose extraction thimble capped with glass wool. Hexane was used to extract
233 lipids from the sample in a solvent refluxing process for a 6 hour period. The solvent
234 containing the lipids was concentrated in a rotary evaporator under vacuum (Rotavapor
235 R-114, BÜCHI UK Ltd, Oldham, UK). The residual pellet was re-extracted for 6 hours
236 and solvent was concentrated and combined with the first extract. The lipid content was
237 measured gravimetrically.
238

239 *2.8.6 Microscopy*

240 Ball milled biomass was observed by microscopy (BX60, Olympus, Japan) to assess the
241 extent of starch removal during the ball mill process. The materials were stained with
242 Lugol's solution (20 kg m^{-3} KI with iodine (0.2 kg m^{-3})) to highlight the presence or
243 absence of starch. The fresh healthy plants and starved plants were immersed in CDTA
244 (50mM Na_3H CDTA and 5mM $Na_2S_2O_5$, pH7) solution for 2 days to separate the plant
245 cells, following by staining with Nile Blue (0.01% w/v, Raymond A Lamb, UK) for 5
246 min at room temperature. The samples were observed under blue Bertrand lens of a
247 fluorescent microscope (BX60, Olympus, Japan, 20×) (Wang, Chi, Song, Wang, & Chi,
248 2012).

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250 ***3 Results and Discussion***

251 *3.1 Evaluation of duckweed composition (FDM)*

252 The overall chemical composition of *L. minor* was assessed and some of them are
253 reported in the first time. The dry matter content of the total freeze-dried duckweed was
254 8.5 % (w/w DM) - which falls within the range of 3-14 % reported by Landolt &
255 Kandeler (1987). Of this dry matter, carbohydrate is the predominant component and
256 accounts for up to 51.2 % (w/w DM), of which starch contributes 19.9 % (w/w DM).
257 Ash accounts for 12.2 % – Landolt & Kandeler (1987) reported that ash equivalents
258 could constitute from 12- 27.6 % of dry matter. Zaher, Begum, Hoq, Begum, &
259 Bhuiyan (1995) give a value for ash content of 12 % of dry matter for *L. minor* cultured
260 in ponds in Bangladesh. The ash value is attributed to large amounts of calcium oxalate
261 stored in a crystal form in duckweed plants (Landolt & Kandeler, 1987). However, only
262 12.0 % of protein was present in the materials used in this study which is lower than
263 other published protein data for duckweed, such as 14 % (w/w of dry matter) in Zaher et
264 al. (1995) and 31 % (w/w of dry matter) in Shireman, Colle, & Rottmann (1977). A
265 small amount of lipid (3.1 % w/w DM) was also measured in this material. Low lipid
266 content (1.8 - 9.2 %) was also reported by Landolt & Kandeler (1987). A low level of
267 Klason lignin (2.4 % w/w DM) is present in *L. minor* while a trace of cell wall-bound
268 phenolic acids (0.03 % w/w -CWM) was detected.

269

270 *3.2 Analysis of duckweed polysaccharides*

271 The total monosaccharide composition of *L. minor* freeze dried material (FDM) was
272 investigated and the results are tabulated in Table 1. The high proportion of

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273 carbohydrate (51.2 %) indicates the potential of using duckweed as a feedstock for
274 biofuel production. The predominant monosaccharide is glucose (331.3 g kg $^{-1}$ DM)
275 followed by uronic acid (96.1 g kg $^{-1}$ DM). Other less abundant monosaccharides
276 include xylose (46.4 g kg $^{-1}$ DM), galactose (16.3 g kg $^{-1}$ DM), arabinose (8.0 g kg $^{-1}$
277 DM), mannose (7.1 g kg $^{-1}$ DM), rhamnose (4.4 g kg $^{-1}$ DM) and fucose (2.3 g kg $^{-1}$
278 DM). The large amount of uronic acid and small amount of xylose indicates that
279 duckweed biomass contains significant amounts of pectin and less hemicellulose. The
280 results of the 1 mol L^{-1} H_2SO_4 hydrolysis indicate an approximate starch content of up
281 to about 20 % (w/w of DM) in fresh duckweed although it should be noted that about 10
282 % of the cellulose is likely to have been hydrolysed by 1 mol L^{-1} H_2SO_4. Of the
283 quantifiable monosaccharides found in total duckweed, glucose, galactose and xylose
284 are reported as fermentable sugars (Delgenes, Moletta, & Navarro, 1996) and, together,
285 these three sugars account for 77.0 % of total sugars (394 g kg $^{-1}$ of DM). The high
286 proportion of fermentable sugars with a low level of lignin supports the rationale that
287 duckweed could potentially be a useful feedstock for biofuel production.

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290 Table 1.

Sample	Hydrolysis	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Totals
FDM	72% H_2SO_4	4.4±0.5	2.3±0.4	8.0±0.6	46.4±4.3	7.1±0.9	16.3±1.5	331.3±33.9	96.1±4.9	512.0±37.4
	1 mol L^{-1} H_2SO_4	4.3±0.2	2.1±0.2	6.5±1.0	38.8±1.8	3.3±0.3	15.42±0.5	199.6±6.3	44.2 ±3.5	314.3±11.1
CWM	72 % H_2SO_4	7.0±0.4	2.2±0.3	6.9±0.3	83.8±0.9	8.5±0.5	14.2±0.3	308.0±5.3	202.2±22.6	632.7±27.8
	1 mol L^{-1} H_2SO_4	6.1±0.3	2.1±0.2	6.8±0.2	75.1±3.6	3.1±0.2	13.2±0.5	31.6±0.8	86.5±10.6	224.5±6.1

291
292 Abbreviations: Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic
293 acid.
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296 *3.3 Evaluation of lipid content*

297 As noted above, total lipid (3.1% w/w of DM) was assessed by using Soxhlet extraction.
298 Lipid was further evaluated using fluorescence microscopy (see Fig 2b – e). Under blue
299 light, lipid equivalents appear yellow and chlorophyll and starch granules appear red. In
300 fresh, healthy duckweed, lipid appears to be stored mainly in epidermal cells rather than
301 the palisade cells although few lipids were observed in the healthy plant (Fig 2b and d).
302 Fig 2c shows the same palisade tissue observed under sunlight as a reference. After
303 duckweed had undergone 28 days of nutrient-starvation, more lipid equivalents were
304 formed and stored in epidermal tissues (Fig 2e). It is notable that abundant lipid
305 equivalents are stored and chlorophyll and starch granules had disappeared in epidermal
306 tissues of dead plants (Fig 2f). These images of the long term nutrient-starved
307 duckweed imply that more lipids could be released from cytoplasmic membrane or
308 chloroplasts and starch is consumed when the plant is dying due to lack of nutrition.
309 Duckweed growing in nutrient deficient conditions might be a good method for
310 biorefining lipid products from duckweed but it is not beneficial for converting
311 duckweed sugars to ethanol due to the reduced starch levels.

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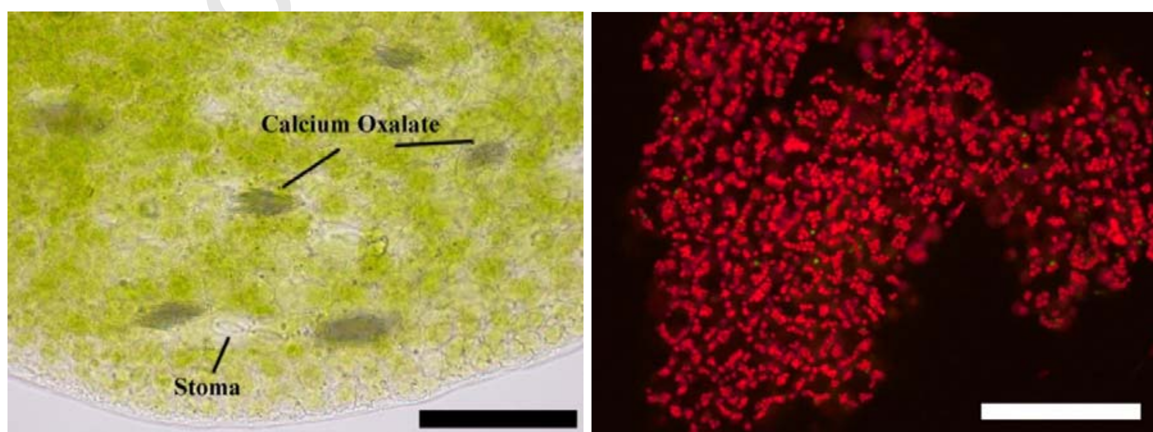
313 

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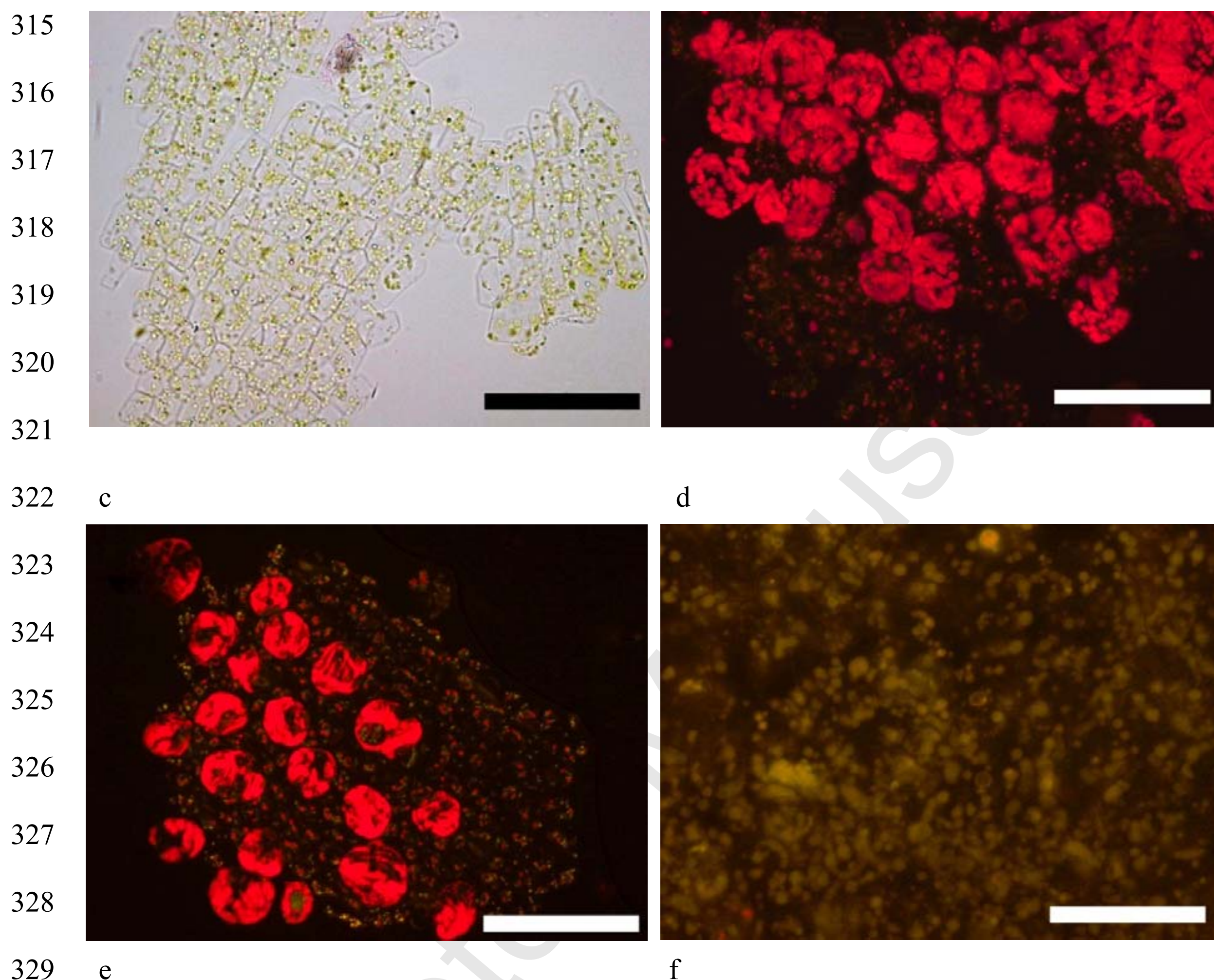

330 Figure 2.

331

332 FAME components were assessed by GC and the fatty acid profile is tabulated in Table
333 2. Fatty acids only account for 0.8 % (w/w of DM), but contain a high proportion of the
334 essential fatty acids (EFA), e.g. linoleic and α-linolenic acid. Of the total fatty acid,
335 saturated FA accounts for 27.4 % (equivalent to 6.9 % of total lipid). Unsaturated FA
336 contributes 72.6 % (equivalent to 18.3 % of total lipid) – however, due to the low
337 overall amounts of fatty acids present, the quantity of unsaturated FA remains lower
338 than most other vegetables (Holland, Buss, & Unwin, 1991). Palmitic acid as the

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339 predominant saturated FA compound accounts for 22.4 % (w/w of total FA) and only
340 1.9, 1.7, 1.0 and 0.5 % (w/w of total FA) of myristic, stearic, eicosanoic and lauric acids
341 respectively were detected. Of the unsaturated FAs, α-linolenic acid was detected as the
342 primary compound (33.4 %), followed by linoleic/linoelaidic acid (25.2 %). These
343 unsaturated FAs are well known types of EFA and are potentially high-value
344 byproducts. Other unsaturated FAs with nutritional and medicinal value are relatively
345 low, e.g. stearidonic acid (3.6%), oleic acid (3.0 %), γ-linolenic acid (1.8 %) and
346 eicosatrienoic acid (1.8 %).
347
348 Table 2.

Fatty acids	C: D [a]	n-x	% (w/w of FA)	% (w/w of lipid)
Total FA			0.8 ± 0.12 (of DM)	25.2
Saturated FA			**27.4**	**6.9**
Lauric acid	12: 0		0.5 ± 0.03	0.1
Myristic acid	14: 0		1.9 ± 0.02	0.5
Palmitic acid	16: 0		22.4 ± 0.13	5.6
Stearic acid	18: 0		1.7 ± 0.1	0.4
eicosanoic acid	20: 0		1.0 ± 0.13	0.3
Unsaturated FA			**72.6**	**18.3**
Palmitoleic acid	16: 1	n-7	3.0 ± 0.03	0.8
vaccenic acid	18: 1	n-7	1.0 ± 0.12	0.3
Oleic acid (ω -9)	18: 1	n-9	3.0 ± 0.12	0.7
Linoleic/Linoelaidic acid (ω - 6)	18: 2	n-6	25.2 ± 0.04	6.4
α -linolenic acid (ω - 3)	18: 3	n-3	33.4 ± 0.21	8.4
γ-Linolenic acid (ω - 6)	18: 3	n-6	1.8 ± 0.07	0.4
Stearidonic acid (ω - 3)	18: 4	n-3	3.6 ± 0.09	0.9
Eicosadienoic acid (ω - 6)	20: 2	n-6	0.5 ± 0.04	0.1
Eicosatrienoic acid (ω - 3)	20: 3	n-3	1.0 ± 0.12	0.3

349 [a] C: D represents the ratio of carbon and double bonds while n-x represents the position
350 of the first double counted from methyl end.
351

352 *3.4 Compositional analysis of phenolics*

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353 Phenolic acids are the main low molecular weight components of lignin and also play a
354 crucial role in the linkage among hemicellulosic polysaccharides (Brett & Waldron,
355 1996). The phenolic acids are therefore worthy of investigation even though the lignin
356 content of *L. minor* is relatively low (2.4 % w/w of DM). Cell wall-bound phenolics
357 account for 0.03 % (w/w of CWM), and this mainly involves the five types of phenolic
358 acids tabulated in Table 3. The proportions of lignin and phenolic acids are much lower
359 than in other well-known monocotyledonous energy crops: lignin levels in switchgrass
360 and wheat straw are 15-29 % and 15-20 %, respectively (Harris & DeBolt, 2010;
361 Theander & Aman, 1982) and phenolics in wheat straw represent approximately 1%
362 (Waldron, 2010). *p*-coumaric acid is the most abundant (0.15 g kg^{-1} CWM), accounting
363 for 60 % of phenolics, followed by truxillic acid (0.04 g kg^{-1} DM), protocatechuic
364 aldehyde (0.02 g kg^{-1} CWM) and ferulic acid (0.02 g kg^{-1} CWM). 0.01 g kg^{-1} CWM *p*-
365 OH-benzaldehyde was also detected. Coumaric acid and ferulic acid are common
366 phenolic components– indeed, they are the most abundant phenolics present in wheat
367 straw (Merali et al., 2013).

368

369 Table 3.

Fractionation	Protocatechuic aldehyde	*p*-OH-benzaldehyde	Truxillic acid (CA)	Ferulic acid	*p*-coumaric acid	Total
CWM	22.5 ± 10.6	10.7 ±7.1	43.8±5.4	20.1±10.4	153.0±21.2	250.1 ±20.6
Cold water	0	13.7±0.2	51.3±0.1	26.4±0.3	33.4±0.2	125.2±0.7
CDTA 1st	0	9.5±1.9	44.5±1.5	39.2±3.5	23.8±0.9	117.0±6.7
CDTA 2nd	0	6.6±0.1	44.7±0.2	29.6±0.2	8.6±0.1	89.5±0.4
Na_2CO_3 1st	10.0 ±0.3	23.2±0.1	44.6±0.5	113.4±0.6	79.5±0.2	284.1±3.7
Na_2CO_3 2nd	45.8 ±0.5	32.1±0.1	36.6±0.1	34.4±0.1	123.0±0.1	271.8±0.9
0.42 mol L^{-1} KOH	12.8 ±0.2	21.0±0.1	27.3±0.2	55.1±0.6	334.6±1	450.8±3.8
0.85 mol L^{-1} KOH	11.1 ±0.2	23.1±0.1	27.6±0.2	36.7 ±0.1	361.2±0.4	460.0±0.6
3.4 mol L^{-1} KOH	0	17.0±0.1	47.6±0.1	86.4±0.2	97.2±0.1	248.2±0.3
Pellets	17.0 ±0.5	4.6±5.0	21.2±6.1	0	17.0±6.0	42.8±12.7

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371 *3.5 Analysis of sequentially-extracted polysaccharides*

372 The materials used for the sequential extraction were prepared using a ball milling
373 method to rupture cells and permit the release and removal of non-cell wall
374 carbohydrate such as starch. The extent of starch removal was evaluated by microscopy
375 (see Fig 3a-e). Starch granules are conspicuously present in duckweed cells (Fig 3a). In
376 the ball milling process, starch granules were gradually released from disrupted tissues
377 (Fig 3b-d) and dispersed into the liquor phase during the distilled water wash and
378 filtration (Fig 3e). Thus, the majority of the starch was washed away after 3.5 h of ball
379 milling and washing with distilled water. The monosaccharide composition of CWM
380 analysed by GC (Table 1) also demonstrated the extensive starch removal and this was
381 further demonstrated by the changes in the FTIR spectrum (Fig 4), Spectra 2 (CWM)
382 has much less intensity around 1150,1080 and 990 cm^{-1} compared with spectra 1
383 (FDM). On a dry matter basis, the levels of all of the monosaccharides except glucose
384 and the overall carbohydrate increased in CWM. This is in contrast to the
385 monosaccharide concentrations in FWM and is probably due to the removal of
386 intracellular proteins as well as starch. Only 31.2 g kg^{-1} DM glucose that could be
387 released by hydrolysis in 1M sulphuric acid was detected in CWM. This would suggest
388 that any glucose detected in the fractions from the sequential extraction is likely to be of
389 cell wall origin.

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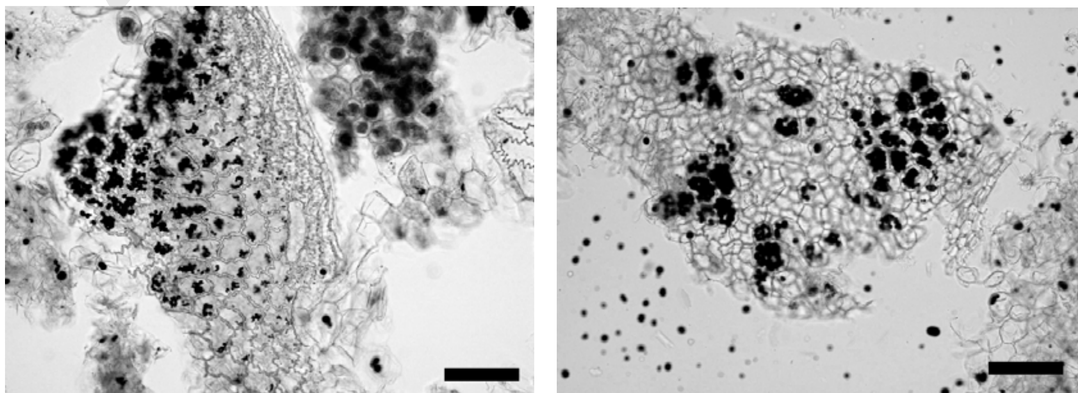

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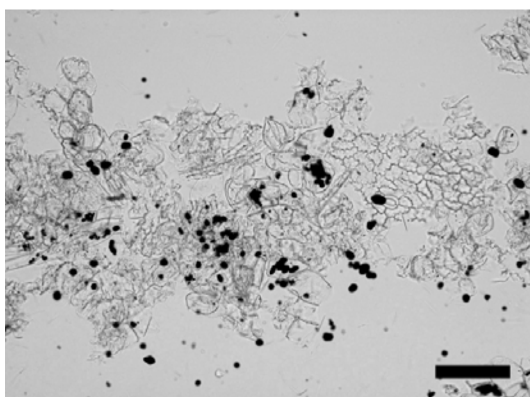

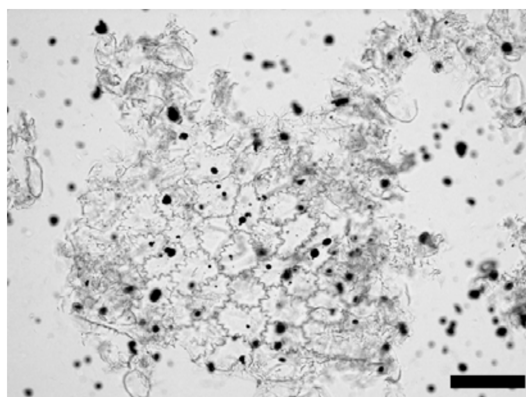

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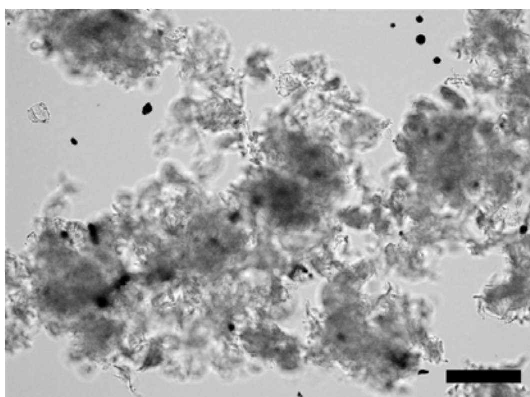

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410 Figure 3.

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412 Starch-free CWM (12.8 % w/w of fresh wet material; 2 g) was sequentially extracted. In
413 the fractionation process (see Table 4), 18.8 % (w/w) of dry mass was extracted by
414 CDTA solution and only 1.7 % (w/w) was extracted by Na_2CO_3 solution. It is notable
415 that KOH extracted only a small amount (3.5 % w/w) of dry mass (see Table 4). Over
416 62 % (w/w) of the dry mass remained in the residual pellets (see Table 4). As Brett &
417 Waldron (1996) described, CDTA and Na_2CO_3 extraction remove pectic
418 polysaccharides by chelation of calcium and de-esterification respectively whilst
419 alkaline extraction removes hemicellulose by breaking strong ester linkages and

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420 hydrogen bonds. The residual matter is predominantly cellulose in conjunction with a
421 significant quantity of highly cross-linked pectic polysaccharides and xylan-containing
422 hemicelluloses. Looking at the GC sugar results in conjunction with the weights of the
423 recovered fractions, it can be seen that cellulose is the major component of CWM and
424 accounts for 43.7 % of cell wall polysaccharide. Approximately 20 % of the cell wall
425 polysaccharide is pectin which is lower than the 30.1 % of pectin in *L. minor* CWM
426 reported by Kindel, Cheng, & Ade (1996). The major pectic polysaccharides were
427 extracted by CDTA and 1st Na_2CO_3 extraction, which suggests pectic polysaccharides
428 are predominantly bound with weak ester linkages (Brett & Waldron, 1996). Only a
429 small amount of hemicellulose (approximately 3.5 %) was found in *L. minor* CWM. We
430 found no literature reporting hemicellulose in duckweed cell wall. These results together
431 are consistent with duckweed cell walls as being predominantly primary walls.
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433 Table 4.

434

Fraction	Rha (g/kg)	Fuc (g/kg)	Ara (g/kg)	Xyl (g/kg)	Man (g/kg)	Gal (g/kg)	Glc (g/kg)	GalA (g/kg)	Total sugars (g/kg)	Recovered mass (g)	% of extracted mass
CWM 72%	7.0±0.4	2.2±0.3	6.9±0.3	83.8±0.9	8.5±0.5	14.2±0.3	308.0±5.3	202.2±22.6	632.7±27.8	2000	100
CWM 1mol L^{-1}	6.1±0.3	2.1±0.2	6.8±0.2	75.1±3.6	3.1±0.2	13.2±0.5	31.6±0.8	86.5±10.6	224.5±6.1	/	/
Cold water	2.7±0.3	3.4±0.4	7.8±1.2	315.9±26.9	1.6±0.3	22.3±2.6	13.6±1.6	44.5±8.4	411.7±26.1	23.9 ±13	1.2 ± 0.7
CDTA 1st	7.9±0.3	1.7±0.1	5.5±0.2	77.5±10.5	6.0±0.9	7.8±0.8	1.1±0.1	562.1±28.8	669.5±32.9	337.6 ±38	16.9 ± 1.9
CDTA 2nd	6.7±1.3	2.0±0.4	6.1±2.2	63.8±16.9	3.5±0.6	7.2±3.1	2.9±1.5	282.0±60.4	374.3±81.7	37.4 ±18	1.9 ±0.9
Na_2CO_3 1st	7.6±0.2	4.2±0.3	15.1±0.3	27.7±0.1	9.2±1.5	20.0±0.1	5.5±0.4	516.2±1.1	605.7±1.2	27.2 ±8	1.4 ± 0.4
Na_2CO_3 2nd	7.2±0.4	5.1±0.5	20.1±1.2	14.5±3.2	12.6±0.4	21.3±1.1	21.9±1.3	274.8±3.4	377.5±0.2	6.3 ±1	0.3 ± 0.1
0.42 mol L^{-1} KOH	3.3±0.1	1.9±0.3	15.3±1.5	15.0±2.1	3.0±0.2	10.8±2.0	559.2±14.5	32.1±2.9	640.7±9.1	39.7 ±6	2.0 ± 0.3
0.85 mol L^{-1} KOH	2.4±0.1	8.6±0.5	35.8±0.9	253.6±12.2	4.4±0.2	84.5±4.2	398.9±20.7	24.4±1.3	812.6±38.2	19.3 ±3	1.0 ± 0.1
3.4 mol L^{-1} KOH	2.5±0.3	5.6±0.4	48.1±2.4	115.9±8.9	11.3±0.6	56.4±0.9	213.2±5.9	32.6±2.1	485.5±7.2	10.6 ±7	0.5 ± 0.3
Insoluble residue	7.5±0.4	2.4±0.1	6.8±0.2	94.4±5.3	9.6±0.3	15.0±0.8	457.2±25.3	145.4±7.1	738.2±30.6	1241.0 ±3	62.1 ± 0.2
Total recovery	/	/	/	/	/	/	/	/	/	1742.8 ±4	87.1 ± 0.2

435 Abbreviations: Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic

436 acid.

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438 FT-IR provides more specific profiles of cell wall polysaccharides as strong auxiliary
439 evidence to identify the pectic and hemicellulosic polysaccharides. Even after the
440 extensive washing to remove starch during the milling, further small amounts of
441 material are solubilised with distilled water. The cold water extracted fraction (Table 4)
442 contained 411.7 g kg^{-1} of carbohydrate which is high in xylose (315.9 g kg^{-1}) suggesting
443 the presence of xylan polysaccharides, possibly complexed with pectic polymers as
444 found in asparagus (Waldron and Selvendran, 1991). The presence of fucose implies the
445 presence of water soluble xyloglucans (Jacobs, Palm, Zacchi, & Dahlman, 2003). FT-
446 IR (Fig 4, Spectrum 3) also shows peaks at 1078 and 1043 cm^{-1} corresponding to
447 xyloglucan as described by Kacuráková, Capeka, Sasinkováa, Wellner, & Ebringerová
448 (2000). The major carbohydrates from the CDTA and Na_2CO_3 extractions consisted of
449 galacturonic acid - the hydrolysed residues of galacturonan that form the back bone of
450 pectin. Traces of xylose, galactose, arabinose and mannose were also extracted in the
451 CDTA and Na_2CO_3 extractions, which are the main components of side chains of
452 xylogalacturonan and rhamnogalacturonan. Ovodova, Golovchenko, Shashkov, Popov,
453 & Ovodov (2000) noted a similar proportion of pectic monosaccharides in *L. minor*.
454 Accordingly, FT-IR Spectra 4-7 (Fig 4) had a characteristic shape which contains many
455 specific band peaks corresponding to pectic polysaccharides. Homogalacturonan is
456 represented by strong specific bands at 1010 and 1090 cm^{-1} (Kacuráková et al., 2000).
457 The specific bands at 1045 and 1074 cm^{-1} indicate pectic polysaccharide mixtures
458 including rhamnogalacturonan, arabinan and arabinogalactans (Kacuráková et al.,
459 2000). In addition, Hart & Kindel (1970) reported that apiogalacturonan was identified
460 in *Lemna minor* and apiose are the major components of duckweed pectin that would be
461 measured in the further study. Glucose and xylose were the major monosaccharide

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462 constituents of polymers extracted in KOH. A trace of arabinose, galactose and
463 galacturonic acid were also present in the KOH fractions. The presence of large
464 amounts of glucose in both the 0.42 and 0.85 mol L^{-1} KOH extracts were probably due
465 to the gelatinisation and solubilisation of small amounts of residual starch in KOH. Han
466 & Lim (2003) reported that corn starch was dissolved extensively in 1 mol L^{-1} NaOH
467 with vigorous agitation at room temperature. The FT-IR spectra (Fig 4, Spectra 8-9) of
468 these fractions were dominated by the solubilised starch. The overall band pattern is
469 very similar to that of the initial starch (Fig 4, Spectrum 1), but the increased peak at
470 1022 cm^{-1} and the smaller shoulder at 1045 cm^{-1} clearly indicate a much more
471 amorphous structure (van Soest, Tournois, de Wit, & Vliegenthart, 1995). The 3.4 mol
472 L^{-1} KOH extract mainly contained xyloglucan and xylan and might include glucan, as
473 indicated by the FT-IR spectrum (Fig 4, Spectrum 10). The bands at 1130-60 cm^{-1} were
474 dominated by the glycosidic linkage (C-O-C) from xylan, xyloglucan and glucan
475 (Kacuráková et al., 2000). Moreover, the small bands at 930-40 cm^{-1} are likely to be
476 from glucomannan and galactoglucomannan (Kacuráková et al., 2000).
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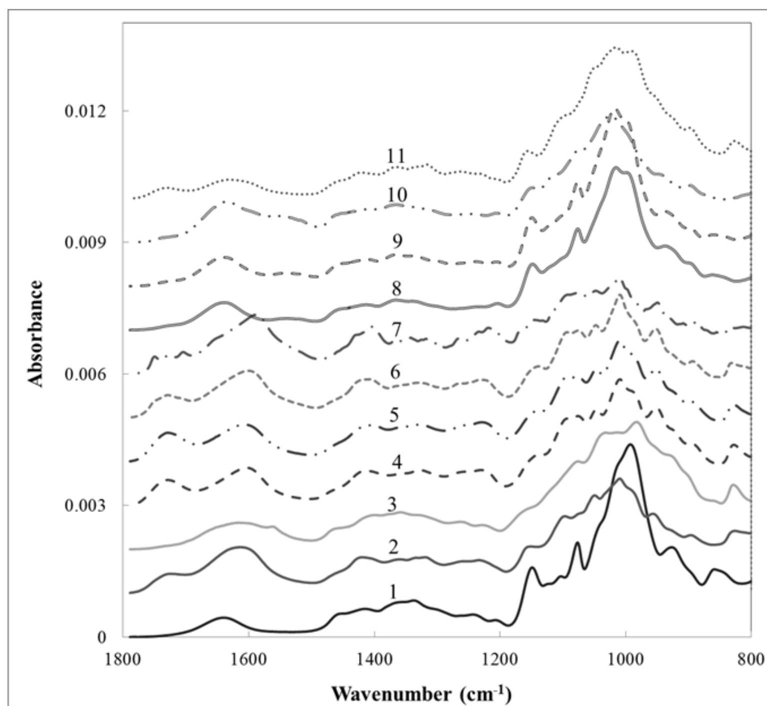
478 

479 Figure 4.

480 *3.6 Assessment of sequentially extracted phenolics*

481 Phenolics play an important role in the linkage of hemicellulosic and pectic
482 polysaccharides. Phenolic acids were therefore extracted along with cell-wall
483 polysaccharides in the sequential extraction (see Table 3). Their distribution could be
484 determined by combining the phenolic acids mass recovery from the HPLC phenolic
485 results (Table 3) with the weights of recovered fractions (Table 4). *p*-coumaric acid was
486 mainly extracted by alkali extraction (Na_2CO_3 and KOH) while truxillic and ferulic
487 acids were only extensively extracted by CDTA solution. In contrast, protocatechuic
488 aldehyde was only solubilised in Na_2CO_3 and low concentration KOH solution.
489 However, *p*-OH- benzaldehyde was found in all fractions. These data indicate that
490 protocatechuic aldehyde and *p*-coumaric acid are the important linkage compounds in *L.*
491 *minor* hemicelluloses while truxillic acid, ferulic acid and *p*-coumaric acid were proven
492 to have a role in the linkage of pectic polysaccharides. Ferulic and *p*-coumaric acids

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493 were found to contribute to xyloglucan and arabinoxylan oligosaccharides respectively
494 (Ishii & Hiroi, 1990). In addition, phenolic compounds were detected in the residual
495 pellets of sequentially extracted duckweed. Merali et al. (2013) also detected large
496 amounts of several phenolic acids retained in the residual pellet of sequentially
497 extracted wheat straw.
498
499 *3.7 Further discussion*
500 The high proportion of fermentable sugars (394 g kg^{-1} of DM) in combination with the
501 high productivity of duckweed suggests that duckweed is an ideal feedstock for biofuel
502 production. Detailed investigation of the cell wall composition implies that duckweed
503 cell wall comprises of cellulose (43.7 %), pectin (20 %) and small amounts of
504 hemicellulose (3.5 %) and lignin, which further reduce the cost of bioethanol production
505 from duckweed. Waldron (2010) stated that the difficulty of pretreating highly lignified
506 biomass. It should be noted that starch and cell wall composition may show variation
507 between duckweed species and batches cultured under different conditions. This case
508 study provides some fundamental background of duckweed sugars compositions using
509 *Lemna minor* as a model system and indicates that duckweed is potentially an ideal
510 biofuel resource. The selection of the preferred duckweed species for biofuel production
511 requires a further screening of all duckweed species and optimisation of their individual
512 growing conditions. In addition, the identification of fatty acids and pectic
513 polysaccharides as high value by-products of bioethanol production potentially
514 reinforce the nutritional and medicinal value of duckweed. However, the high moisture
515 content of fresh duckweed is an unavoidable issue in the biorefining of duckweed.

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517 ***4 Conclusions***

518 This study provides a detailed examination of the chemical constituents of duckweed (*L.*
519 *minor*). Duckweed biomass contains a high proportion of fermentable sugars (including
520 glucose, 33.1 % w/w DM) and a low amount of lignin (3 % w/w DM). The results of the
521 fractionation shows that 20 % pectin is extracted by CDTA and Na_2CO_3 solutions
522 consisting of galacturonan with small amounts of xylogalacturonan,
523 rhamnogalacturonan, only 3.5 % hemicellulose is extracted by KOH solution
524 predominantly consisting of xyloglucan and xylan, and the insoluble residue is rich in
525 cellulose. EFA (α –linolenic and linoleic/ linoelaidic acid) and *p*-coumaric acid are the
526 most abundant fatty acids and phenolics of *L.minor* respectively. The profiles of cell
527 wall structure will play an important role in the enzymatic saccharification and
528 fermentation of duckweed biomass to ethanol, such as, the selection of enzymes
529 (cellulase) and yeast (*S. cerevisiae*).

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653 **“Chemical characterisation and analysis of the cell wall polysaccharides of**
654 **duckweed (*Lemna minor*)”.**
655 **Zhao et al.**
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657 HIGHLIGHTS
658
659 1. Detailed composition of duckweed cell wall reported
660 2. High proportion of fermentable sugars was determined
661 3. Sequential fractionation of cell wall polysaccharides reported
662 4. Major phenolic compounds identified as *p*-coumaric acid and truxillic acid
663 5. Analysis of fatty acids reported for first time
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