Development of a bio-refinery process for the production of speciality chemical, biofuel and bioactive compounds from *Laminaria digitata*

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Abstract

The development of cascading bio-refinery processes that are capable of producing a range of valuable products is of increasing significance and will help to ensure that mankind makes efficient usage of bioresources. Seaweed feedstocks have the potential to be refined into fractions used for biofuel production, as renewable sources of platform chemicals or for a range of potentially interesting bioactive compounds. This study describes the development of a putative bio-refinery approach using *Laminaria digitata* as feedstock. Firstly, the commercially valuable polysaccharides fucoidan and alginate were extracted. Analysis of the monosaccharide and sulphate contents of the fucoidan extract confirmed its isolation with a purity of ca. 65%. Analysis of the composite residue remaining after extraction of alginate and fucoidan from *L. digitata* showed an increase in crude fibre content, of which the predominant monosaccharide was glucose (161.9 mg glucose per g residue), making this residue a potential feedstock for bioethanol production. After dilute acid hydrothermal pre-treatment (1.5 N H_2SO_4, 24 min, 121°C, 25% [biomass / reactant] solids loading) and enzymatic saccharification of this residue, a 93.8% of theoretical glucose yield was achieved. This hydrolysate was fermented using *Saccharomyces cerevisiae* NCYC2592 and a yield of ca. 94.4% of the theoretical ethanol yield was achieved. To add value to the biorefining process, waste streams from the production of alginate, fucoidan and bioethanol were collected and screened for a range of bioactivities. Subsequently, a methanol extract prepared from the liquor waste stream which remained after polysaccharide extraction was shown to exhibit both anti-oxidant (EC_{50} 15.3 mg/mL) and anti-microbial activity against the human bacterial pathogen *Burkholderia cenocepacia*. The identification of extracts with bioactive attributes which have been recovered from a seaweed-based bio-refinery process is novel,

and offers a potential route through which added value can be derived from natural resources such as *L. digitata*.

KEYWORDS: Macroalgae, Bio-refinery, *Laminaria digitata*, Bioethanol, Bioactives, Polysaccharides

1 Introduction

Seaweeds have gained much attention in recent years as alternative renewable feedstocks due to their large biomass yields, fast growth rates and the fact that they require no terrestrial land for cultivation (1). However, many production processes of chemicals from seaweeds have focussed on a single product, for example alginic acid, carrageenan or even colourants, with the rest of the seaweed treated as a waste material (2). Furthermore, many current seaweed research programmes also focus on a single product objective, with much current research focus on biofuels (3, 4). As an alternative to developing processes based on single products from seaweeds, cascading bio-refineries are being sought in order to maximise the inherent value of all components present in the biomass (2, 5-7). Seaweeds are excellent feedstocks for such bio-refineries as they contain both high value components (such as speciality polysaccharides and bioactive molecules) and compounds which are considered to be platform chemicals for the bio-based economy such as glucose (2).

A number of seaweed bio-refinery processes have already been investigated for the production of biofuels and commodity compounds (e.g. see Kumar et al (8)). Following the extraction of agar from the red seaweed species *Gracilaria verrucosa* the residual pulp was converted into bioethanol; achieving an ethanol yield of 0.43g/g of sugar. Trivedi et al (9) developed an integrated process which was applied to the green seaweed *Ulva fascita* that sequentially recovered a mineral rich liquid extract, lipids, ulvan and cellulose; four fractions of economic importance. Additionally, various nutrients, pigments (8) and even seaweed salts (10) could also be extracted from seaweeds, thus increasing the potential value of bio-refining processes. van der Wal et al [13] generated not only bioethanol but also acetone and butanol from a hydrolysate derived from green seaweed *Ulva lactuca* using *Clostridium beijerinckii* and *Clostridium acetobuticum*. This was achieved by solubilising over 90% of the sugars found in the green seaweed into a fermentable solution (11). However, solubilisation of all

66 functional seaweed polysaccharides for biofuel conversion may in the long term jeopardise
67 the seaweed hydrocolloid industry (12). In 2016 commercial seaweed market was estimated
68 to be valued at $11.34 billion (http://www.marketsandmarkets.com/Market-
69 Reports/commercial-seaweed-market-152763701.html). The seaweed hydrocolloid market
70 was estimated to be valued at $1.1 billion (13), compared to bioethanol which at the time of
71 writing this article was valued at $23.2 million (Renewable Fuels Association;
72 http://www.ethanolrfa.org/). Therefore the fractionation and selective utilisation of biofuel
73 substrates, such as the laminarin fraction from seaweed biomass (which is a less utilised
74 material) for the production of biofuels, would prevent negative impacts on the present
75 hydrocolloid industry and associated worldwide markets. Additionally, the net worth of a
76 seaweed bio-refinery for fuels and platform chemicals would also increase.

77 Brown seaweeds are amenable to bio-refinery processing as they contain a diverse array of
78 metabolites with existing or potential applications. These include extracellular matrix
79 polysaccharides such as alginates and fucoidans, storage polysaccharides such as laminarin
80 and mannitol and bio-active polyphenolic compounds and pigments such as fucoxanthin (14).
81 Such species have been mainly exploited for the anionic polysaccharide alginate that is
82 widely used in the pharmaceutical, food, cosmetic and biotechnology industries due to its
83 favourable gelling properties (15). The interest in new sources of natural bioactives such as
84 antioxidants and antimicrobials has increased in recent years. One reason for this has been a
85 desire to reduce the use of synthetic forms of antioxidants and antimicrobials such as
86 butylated hydrolxytoluene (BHT) and propyl gallate (PG) where strict regulations have been
87 applied due to their potential health hazards. In recent years sulphated polysaccharides such
88 as fucoidan have received attention (16) due to their specific biological activities and
89 properties such as anti-inflammatory (17), anti-tumor (18) and anti-coagulant (19).

90

91 Bio-refinery processes using different species of brown seaweeds, including *Saccharina*
92 *latissimi* and *Ascophyllum nodosum* have been explored for mannitol isolation (20) and
93 fucoidan, alginate, sugars and biochar production (7), respectively. In addition, bio-refinery
94 scenarios were investigated with *Laminaria digitata* where bioethanol (21) and succinic acid
95 (22) were produced and the remaining residues analysed as potential feedstocks for biogas
96 production, biodiesel, and feed supplements (due to enriched protein and fatty acid fractions).

97 The kelp *Laminaria digitata* (Hudson) J.V. Lamouroux is found in North Atlantic waters and
98 is one of the most prevalent species of brown seaweed found around the UK coastline (23). It
99 is one of the largest growing species within the brown taxonomic group and large scale
100 mechanical harvesting of this species takes place in Brittany, France and Iceland (1). On
101 average, approximately 60,000 tonnes (wet weight) of the seaweed is harvested annually in
102 France (24); making it a suitable feedstock for the development of bio-refinery processes in
103 Europe.

104 The present study describes the development of a putative integrated bio-refinery process
105 using *L. digitata*, based around the extraction of the commercially valuable phycocolloids
106 alginate and fucoidan, the subsequent production of bioethanol and also the identification of
107 potential bioactive compounds in the waste stream liquors produced. This study was not
108 intended to represent a fully optimised process, but rather to evaluate the potential of using
109 this species of brown seaweed as a feedstock and to establish prospective processing routes
110 that could form the basis of a *L. digitata* bio-refinery.

111 # 2 Materials and Methods

112 ## 2.1 Reagents

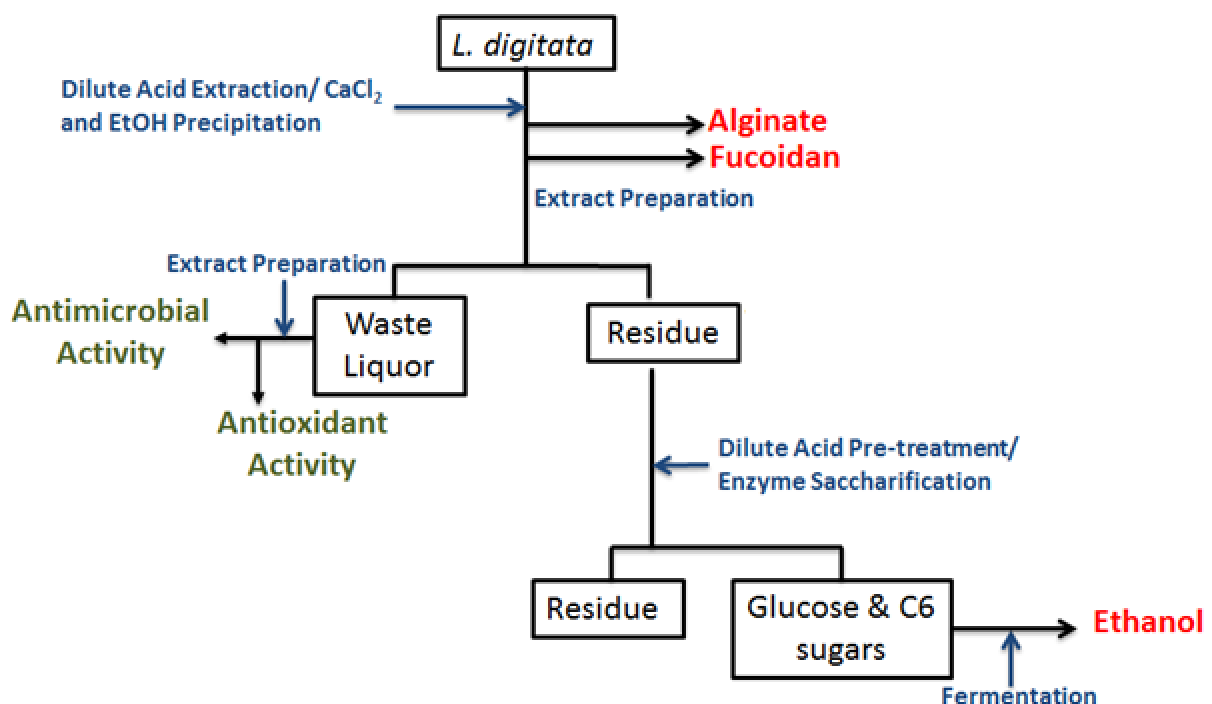
113 All reagents were of AnalAR grade and obtained from Sigma-Aldrich (UK) and Fisher
114 Scientific (UK) unless otherwise specified. All water used was subjected to deionised (DI)
115 reverse osmosis and of ≥18 mega-ohm purity.

116 ## 2.2 Seaweed collection and preparation

117 The seaweed used in this study (*L. digitata*) was collected at spring low tides in May 2013
118 near Downderry in Cornwall (GPS coordinates: 50.3623° N. 4.3687° W). The seaweed was
119 rinsed in distilled water to remove salt and debris, and then dried in a fan oven at 80 °C for a
120 minimum of 48 h until perceived to be dry. The dried seaweed was then milled using a ball
121 mill (Fritsch, Germany) to obtain a fine homogeneous powder and stored in a desiccator
122 away from direct sunlight and moisture until further analysis.

123 ## 2.3 Fucoidan (and alginate) extraction

124 A schematic of the overall bio-refinery process is summarised in Fig 1.



125

126 **Fig 1** Schematic diagram of the bio-refining process developed for *L. digitata* for the
127 extraction of value added compounds in addition to bioethanol and residues/waste streams
128 with potential bioactivity.

129 Fucoidan was extracted following the method outlined by Black at et (25). *L. digitata* (2 g)
130 was mixed with 20 mL of 0.1 M HCl (pH 2-2.5) at 70°C for 1 h. The mixture was stirred at
131 250 rpm and then centrifuged at 5000 rpm for 15 min to separate the liquid from the seaweed
132 solids. One volume of 1% (w/v) $CaCl_2$ was added to the recovered liquid, inverted and kept at
133 4°C for 72 h. The precipitate which formed (alginate) was removed from the liquid phase by
134 centrifugation at 5000 rpm for 20 mins, freeze dried and lastly weighed. Two volumes of
135 absolute ethanol (99.99%) were then added to the remaining alginate-free liquor, inverted and
136 kept at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000 rpm for 20 min
137 and the upper liquid phase (waste liquor) was stored at -4°C until further analysis. The solid
138 precipitate (fucoidan) was freeze-dried and stored at -80°C until further analysis. Seaweed
139 solids (waste residue) which remained after the extraction were dried in an oven at 60°C until
140 all water had evaporated and stored in an airtight container until further analysis.

141 **2.4 Composition analysis of native *L. digitata* biomass and waste residue generated**
142 **after extraction**

143 Native *L. digitata* and *L. digitata* waste residue generated from the process (the new
144 composite material from section 2.3) were analysed for moisture, ash, protein (total N × 6.25
145 conversion factor), lipid, crude fibre and carbohydrate contents. This analysis was conducted
146 externally by Eurofins Food Testing Ltd, UK.

147 Monosaccharide analysis of the *L. digitata* residue was determined by the method outlined by
148 Kostas et al (26) where 1 mL of 11 M H_2SO_4 was added to 30 mg of seaweed in a heat
149 resistant screw cap glass tube and incubated at 37˚C for 1 h. Water (11 mL) was added to the
150 sample to dilute the acid strength to 1 M, following which, samples were incubated at 100˚C

151 for 2 h. Liberated monosaccharides (mannitol, fucose, arabinose, galactose, glucose and
152 xylose) were analysed by HPAEC-PAD as described in section 2.8.

153 **2.5 Characterisation of fucoidan extract**

154 **2.5.1 Fucoidan extract quantification and determination of purity**

155 Solutions of fucoidan extracts (10 g/L) were prepared with RO water. The samples were then
156 run on a HPLC using an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC
157 Pump (Jasco, Japan). The Rezex ROA Organic Acid H+ organic acid column (5 μm, 7.8
158 mm×300 mm; Phenomenex, UK) was operated at ambient temperature with a mobile phase
159 of 0.005 N H_2SO_4 at a flow rate of 0.5 mL min^{-1}. A Refractive Index cell (RI-2031 Intelligent
160 Refractive Index detector, Jasco, Japan) was used for detection, and the injection volume was
161 10 μL. Data were acquired using the Azur software package v. 4.6.0.0 (Datalys, France).
162 Prior to HPLC analysis, all samples and standards were filtered using Whatman GD/X
163 syringe filters (GF/C 25 mm filter diameter/1.2 μm pore size; Whatman, UK). Authentic
164 standards of fucoidan (Sigma-Aldrich®, USA) with concentrations within range of 10 g/L to
165 0.5 g/L were used for quantification.

166 **2.5.2 Monosaccharide profile**

167 The monosaccharide profile of the extracted fucoidan was obtained by following the method
168 of Rodriguez-Jasso et al (27). Fucoidan extract (10 mg) was hydrolysed with 2 M
169 Trifluoroacetic acid (0.5 mL) at 121°C for 2 h in N_2 sealed heat resistant screw cap glass
170 tubes. The tubes were then cooled in an iced water bath before being centrifuged at 5000 rpm
171 for 5 min. Samples were then prepared for monosaccharide quantification using the HPAEC-
172 PAD method described in Section 2.8.

2.5.3 Sulphate content

The sulphate group content of the extracted fucoidan was determined using a sulphate assay kit (Sigma-Aldrich®, USA) according to manufacturer's instructions. Proprietary reagents were mixed to induce a concentration dependant colour change which was read at 600 nm using a Jenway Spectrophotometer. Quantification was performed by comparison to proprietary standards of barium sulphate (Sigma-Aldrich®, USA) over a range of concentrations (0, 0.5, 1.0, 2.0 mM).

2.6 Bioactivity screening on selected fractions from the bio-refinery process

2.6.1 Extract preparation

Native seaweed (code: *L. digitata*), the waste residue following extraction of fucoidan and alginate (code: waste residue), waste liquor from the process (code: waste liquor), the fucoidan extract (code: fucoidan extract) and a standard of fucoidan from Sigma Aldrich (code: fucoidan standard) were investigated for selected bioactivity analysis. Extracts from the bioprocess fractions listed above were individually prepared in either methanol and/or water (in triplicate). This was done in order to investigate whether extracting in methanol or water may show any differences in the biological activities of these extracts.

The waste liquor from the process was initially prepared by rotary evaporation at 40°C under vacuum to remove ethanol. This left behind a dark yellow viscous oil which was then freeze dried. Glass beads (100 mg, 1.0 mm in diameter) were added to ca 20 mg of biomass/residue/freeze dried waste liquor in an Eppendorf tube followed by the addition of 1 mL of cold methanol (100%) or water. The samples were placed in a tissue lyser (Qiagen TissueLyser II, USA) for 10 min at 30 Hz and then centrifuged at 14,000 rpm for 1 min. The supernatant was decanted into an evaporation tube and stored on ice. A further 1 mL of methanol or water was added to the Eppendorf tube and the process was repeated one more

197 time, pooling together the relevant supernatants. In order to concentrate the extract, the
198 solvents were evaporated and then the residue re-suspended in a reduced volume (1 mL) of
199 either methanol and/or water. Extracts were then stored in amber vials at -80°C until further
200 use. Fucoidan extracts were only prepared using water due to their insolubility in methanol.

201 **2.6.2 Determination of antioxidant activity**

202 **2.6.2.1 DPPH$^{\bullet}$ scavenging capacity assay**

203 A 200 µM solution of DPPH was prepared in methanol (100%). For the assays, 100 µL of
204 DPPH (200 µM) solution was mixed with 100 µL of extract and incubated in the dark at 30°C
205 for 30 min. The reduction of the DPPH$^{\bullet}$ radical was measured by continuous monitoring of
206 decolourisation at 518 nm. The control solutions contained 100 µL of distilled water or 100
207 µL methanol (100%). DPPH$^{\bullet}$ percent of inhibition was calculated according to the following
208 equation:

$$Inhibition\ (\%) = [(1 - A_{\mathrm{sample_518}}/\mathrm{A}_{\mathrm{control_518}})] * 100$$

209 EC_{50} values were also calculated which indicates the concentration of sample required to
210 scavenge 50% DPPH radicals, according to the following equation:

211 $$\mathrm{EC}_{50} = \left[\frac{\text{sample concentration (mg/ml)}}{\text{DPPH inhibition (\%)}}\right] \times 50$$

212 **2.6.2.2 ABTS$^{\bullet+}$ scavenging capacity assay**

213 The ABTS$^{\bullet+}$ assay was carried out according to the protocol outlined in the work of
214 Martinez-Avila et al (2012). In order to generate the radical (ABTS$^{\bullet+}$), 12.5 mL of potassium
215 persulfate (2.45 mM) was mixed with 25 mL of ABTS (7 mM). The mixture was maintained
216 in the dark at room temperature for 12-16 h. The absorbance was measured at 734 nm and the
217 ABTS$^{\bullet+}$ solution was then diluted with ethanol until an absorbance value of 0.7 ± 0.01 was
218 achieved. For the assays, 950 µL of ABTS$^{\bullet+}$ solution was added to 50 µL of extract and the

219 absorbance was measured after 1 min of the reaction. The control solutions contained 50 μL
220 of distilled water or 50 μL methanol (100%). $ABTS^{\bullet+}$ percent of inhibition was calculated
221 according to the following equation:

$$Inhibition\ (\%) = [(1 - A_{\mathrm{sample_734}}/A_{\mathrm{control_734}})] \times 100$$

222 The radical-scavenging capacity of each sample was calculated according to a Trolox
223 standard curve (0 to 50 μM in 5 μM increments) and expressed as Trolox equivalent
224 antioxidant capacity (TEAC) by the extrapolation of $ABTS^{\bullet+}$ percent inhibition of each tested
225 sample. Assays were conducted in triplicate.

226 **2.6.3 Determination of antimicrobial activity**

227 **2.6.3.1 Preparation of bacterial broths, agar plates and culturing of bacterial strains**

228 Seven human pathogenic strains, one food spoilage pathogen and 3 fish pathogenic strains
229 were investigated in this study (Table 1).

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Strains	Pathogen Type
Staphylococcus aureus[a]	Human pathogen
Salmonella enterica[a]	Human pathogen
Burkholderia cenocepacia[a]	Human pathogen
Burkholderia multivorans[a]	Human pathogen
Bacillus cereus[b]	Food Spoilage Pathogen
Streptococcus pyogenes[a]	Human pathogen
Acinetobacter iwofii[a]	Human pathogen
Listeria monocytogenes[a]	Human pathogen
Yersinia ruckerii[c]	Fish pathogen
Vibrio anguillerium[c]	Fish pathogen
Aeromonas hydrolphila[c]	Fish pathogen

236 **Table 1** List of pathogens included in this study. Human pathogenic[(a)], food spoilage[(b)] and
237 fish pathogenic[(c)] bacteria.

238

239 The strains were harvested from local waters around the Plymouth shoreline. Two types of
240 bacterial broth and agar plates were made depending upon bacterial strain. Luria-Bertani
241 broth (for human pathogenic strains): 5 g bactopeptone, 1 g yeast extract made up to 1L with
242 DI water. Marine LB broth (for marine pathogens): 5 g bactopeptone, 1 g yeast extract made
243 up to 1L with filtered sea water. Yeast peptone dextrose (YPD) plates were made following
244 the same recipe as mentioned above for both pathogen types, however contained 2 g agar.
245 The bacterial strains were taken from glycerol stocks stored at -80°C and spread onto
246 appropriate YPD plates and kept at 26°C for 24 h. A loop-full of cells were then inoculated
247 into 3 mL of LB/marine LB broth. Cultures were placed in a shaking incubator at 26°C for 24
248 h at 130 rpm.

249 **2.6.3.2 Agar disc diffusion assay**

250 Cells (taken from section 2.6.3.1) were subsequently diluted 1/100 into 25 mL of LB/marine
251 LB agar and the molten agar was poured into a sterile petri dish. Sterile Whatmann discs (10
252 mm diameter) were gently placed onto the dish. An aliquot of extract (20 µL) was inoculated
253 onto the disc and plates were then incubated for 24 h at 30°C. Zones of clearance around each
254 disc were indicative of antimicrobial activity and were examined by eye. Each extract was
255 tested in triplicate and water and methanol were included in this trial as controls.

256 **2.6.3.3 Validation test of antimicrobial activity**

257 Validation of the agar disc diffusion assay findings was conducted quantitatively by growing
258 the pathogen in liquid broth followed by inoculation with the selected extract. Bacterial
259 strains (from 2.6.3.1) were diluted 1/50 in LB/marine LB broth and 180 µL of the broth and
260 cell mix were subsequently pipetted into a well on a 96 well plate. Extract (20 µL) was added
261 and the plates were incubated for 24 h at 30°C. Absorbance was measured at 600 nm using a

262 plate reader and absorbance values were compared against the absorbance of wells containing
263 only LB/marine LB broth.

264 ## 2.7 Bioethanol production from the bio-refinery process residue

265 ### 2.7.1 Pre-treatment of the remaining residue

266 Processed *L. digitata* residue was subjected to a 1.5N sulphuric acid pre-treatment and an
267 entirely water-based auto-hydrolytical pre-treatment, both at a 25% (w/v) biomass to reactant
268 loading rate for 24 min at 121°C in a bench top autoclave. These protocols were previously
269 determined to be optimal for seaweed biomass (unpublished data). Residues after pre-
270 treatment were recovered and evaluated for pre-treatment efficacy by mixing subsamples (0.1
271 g) of the dried pre-treated seaweed residues with 20 mL of 50 mM sodium citrate buffer (pH
272 5) and dosed with an excess of Novozymes Cellic® CTec2 (ca. 50 FPU/g biomass). An
273 enzyme hydrolysis was also performed directly on 0.1 g of processed *L. digitata* residue
274 (without any prior pre-treatment), also with an excess of Novozymes Cellic® CTec2 (ca. 50
275 FPU/g biomass) in 20 mL of 50 mM sodium citrate buffer. Samples were then incubated at
276 50°C for 48 h in a shaking incubator set at 120 rpm. Amounts of glucose present in the
277 enzyme hydrolysate were quantified by HPAEC-PAD (Section 2.8) and calculated as the
278 amount (mg) liberated from 1 g of dried pre-treated/non-pre-treated seaweed residue.
279 Achieved percentage theoretical yields of glucose were determined by the following
280 equation:

$$Achieved\ \%\ Theoretical\ Glucose = \frac{Glucose\ released\ from\ pretreatment\ and\ enzymatic\ hydrolysis\ on\ residue}{Amount\ of\ glucose\ present\ in\ native\ L.digitata} \times 100$$

281 Higher glucose yields obtained from the enzymatic saccharification were indicative of a more
282 effective pre-treatment. All experiments were conducted in triplicate.

283 ### 2.7.2 Laboratory scale trial fermentations of residue hydrolysates for bioethanol
284 production

285 Hydrolysates (generated after enzyme saccharification and described in section 2.7.1) were
286 fermented using *S. cerevisiae* strain NCYC 2592 following the method described in Kostas et
287 al (28). Final glucose and ethanol yields were quantified by HPAEC-PAD and HPLC (section
288 2.8). All trials were conducted in triplicate.

289 ## 2.8 Quantification of monosaccharides (HPAEC-PAD) and ethanol (HPLC)

290 The monosaccharide concentrations were quantified using Dionex ICS-3000 Reagent-Free
291 Ion Chromatography, electrochemical detection using ED 40 and computer controller. A
292 CarboPacTM PA 20 column (3×150 mm) was used, with a mobile phase of 10 mM NaOH at
293 an isocratic flow rate of 0.5 mL/min. The injection volume was 10 µL and the column
294 temperature was maintained at 30°C. Authentic standards of monosaccharides (mannitol,
295 fucose, arabinose, galactose, glucose and xylose) were used to generate calibration curves
296 (0.0625-1 g/L) for monosaccharide quantification.

297 Ethanol yields were quantified by HPLC following the method outlined in Wilkinson et al
298 (29). Prior to HPLC analysis, all samples and standards were filtered using Whatman GD/X
299 syringe filters (GF/C 25 mm filter diameter/1.2 µm pore size; Whatman, UK). All
300 experiments were conducted in triplicate.

301 # 3 Results and Discussion

302 ## 3.1 Extraction of fucoidan and alginate from *L. digitata*

303 A total of 130.9 mg fucoidan with a measured purity of 65% and 98.4 mg alginate were
304 extracted from 2 g of *L. digitata* (Table 2.

305

L. digitata IN	2 g
L. digitata OUT (residue weight)	1.54 g ± 0.02
Alginate	98.4 mg ± 0.1
Fucoidan (crude)	130.9 mg ± 1.1
Fucoidan Purity	65 % ± 2.1
Fucoidan Sulphate Content	23.8 % ± 1.6
Process losses	230.7 mg
Fucoidan Monosaccharide Profile	
Arabinose	4.6 % ± 0.3
Galactose	17.0 % ± 0.5
Glucose	30.9 % ± 1.5
Fucose	42.5 % ± 2.2
Xylose	2.6 % ± 0.1

306

307 **Table 2** Product yields and losses generated from the extraction of fucoidan and alginate
308 from *L. digitata,* including fucoidan purity, fucoidan sulphate content and fucoidan
309 monosaccharide profile.

310 From the 2 g of *L. digitata* used in the process, only 1.54 g was recovered after the extraction.
311 Therefore approximately 460 mg of material was solubilised from the starting material. The
312 main products from the extraction process (fucoidan and alginate) together yielded a total of
313 229.3 mg, leaving approximately 230.7 mg of material unaccounted for. This group of
314 unaccounted for material has been termed 'process losses' and represents other extracted
315 components of the biomass which had not been precipitated/ recovered. Other extracted
316 materials from the seaweed such as pigments, polysaccharides/monosaccharides, proteins,
317 polyphenols, minerals and salts would have comprised this solubilised matter. Furthermore,

318 products from the extraction process could have been volatilised during the first stage of the
319 extraction process, in which the contents of the reaction was heated to 70°C.

320 The fucoidan extract was predominantly composed of fucose followed by a significant
321 proportion of glucose and galactose, and minor amounts of arabinose and xylose. This is in
322 agreement with published literature which describes fucoidan as being extremely
323 heterogeneous with a branched structure (30). Besides monosaccharide content, the sulphate
324 content was determined to be 23.8% and is in broad agreement with values previously
325 reported (27). Reports have suggested that the fucoidan content of brown seaweeds is
326 typically around 10% (d/w) however this value may vary according to the species of
327 seaweed, within species population, harvesting season, region of isolation and even
328 environmental temperature (31-33). Based on this assumption, it was estimated that the
329 overall extraction efficiency was around 65.4 %, as such there is room for further
330 optimisation of the extraction process to enhance fucoidan extract yields. Interest in fucoidan
331 has increased in recent years, particularly since the polysaccharide has been shown to exhibit
332 a number of pharmaceutically interesting biological activities such as anti-cancer (34), anti-
333 inflammatory (35) and anti-viral (36) properties; making it desirable for extraction. The
334 seasonal variations in seaweed polysaccharide contents have often been a hindrance and a
335 factor which has held back the sustainable development of bio-refinery processes that are
336 based purely on speciality polysaccharide extraction. Bruhn et al (32) found that the crude
337 fucoidan content and potential harvest yields in studies performed with North Atlantic
338 *Saccharina latissima* and *L. digitata* varied by a factor of 2-2.6 over 1 year. The study also
339 found that different seasonal peaks of fucoidan exist between populations of the same
340 species; thus making it difficult to identify a general recommended harvesting time.
341 However, annual fluctuation in the levels of fucoidan from various Fucoid species was
342 recently determined by Fletcher et al (37) who identified that whilst the best time to harvest

343 (in terms of maximum fucoidan content) is late autumn/early winter, the actual range of
344 fucoidan content (minimum and maximum) was relatively small. From an industrial
345 processing perspective this is significant, since it would potentially facilitate more consistent
346 recovery of the fucoidan polysaccharide. Therefore, the selection of a suitable brown species
347 of seaweed is imperative in the design of cascading bio-refineries. Furthermore, year-round
348 use of that particular species would eliminate the requirements for drying and storage,
349 reducing overall processing costs and enhance life cycle analysis/techno-economical
350 assessment of the bio-process.

351 **3.2 Evaluation of the waste residue after fucoidan and alginate extraction**

352 The composition of the waste residue following extraction can be seen in Table 3 along with
353 the original composition of *L. digitata* seaweed for comparison.

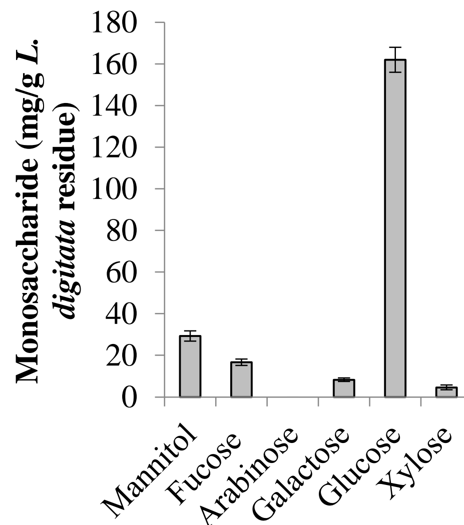
Composition (d/w %)	Native Seaweed	Residue following alginate and fucoidan extraction
Moisture	8.0	4.4
Protein	12.9	14.1
Ash	26.0	23.4
Lipid	1.0	0.9
Crude Fibre	5.5	15.5
Carbohydrate	46.6	41.4

354

355 **Table 3** Proximate composition of *L. digitata* before and after the extraction of alginate and
356 fucoidan

357 The ash decreased from a content of 26.0% (d/w) to 23.4% (d/w) and protein content
358 increased from 12.9 to 14% (d/w); suggesting a slight enrichment of the protein fraction and

359 highlighting a potential protein-based product stream that would be worth evaluating for
360 added value to the bio-refinery. Although there appeared to be a reduction in the
361 carbohydrate content, from 46.6% to 41.4% (d/w), which most likely resulted from the
362 extraction of alginate and fucoidan, there was an increase in crude fibre content from 5.5% to
363 15.5% (d/w), respectively. The authors consider that this may be the consequence of an
364 enriched cellulose fraction. When investigating the monosaccharide profile of the seaweed
365 residue, it became apparent that the predominant monosaccharide was glucose (161.9 mg/g of
366 residue; Fig 2).



367

368 **Fig 2** Monosaccharide composition of the waste residue following extraction of alginate and
369 fucoidan from *L. digitata*

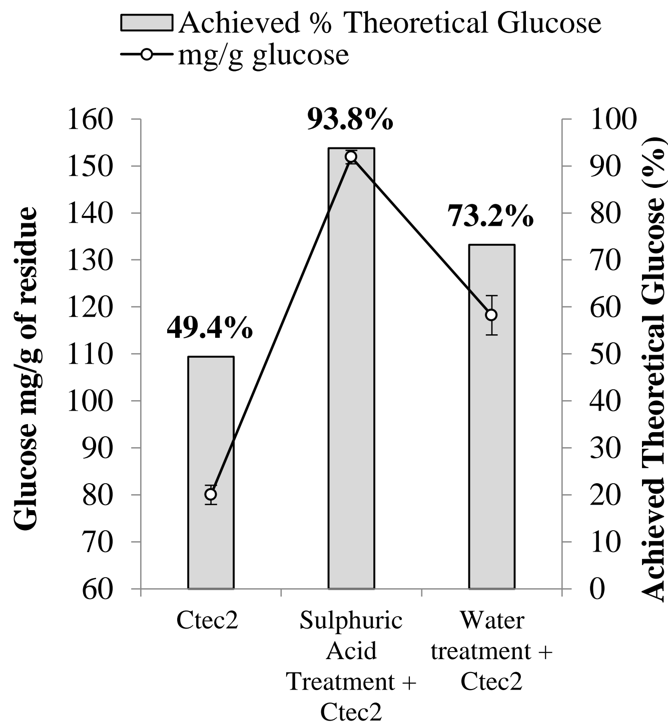
370 This of course represented a promising substrate for the subsequent production of bioethanol
371 due to the high glucose content. However, a small fraction of fucose (16.7 mg/g) was
372 additionally detected, suggesting that not all of the fucoidan was extracted from the *L.*
373 *digitata* during the previous extraction process step and confirmed the requirement of further
374 adjustments (optimisation) to maximise recovery of fucoidan. In addition, uronic acids such

375 as mannuronic and guluronic acids may have also been released (in the case that not all of the
376 alginate was extracted) but were not quantified using the analytical method applied.

377 3.3 Bioethanol production from the waste residue

378 3.3.1 Pre-treatment and enzymatic saccharification of new residue

379 The pre-treatment stage for bioethanol production from the remaining waste residue was not
380 optimised in this study. However optimum pre-treatment conditions for native *L. digitata*
381 seaweed biomass that were previously developed by the authors (unpublished data) were
382 applied as a starting point for the deconstruction of the remaining material in this study. The
383 solubilised yields of glucose can be seen in Fig 3.



384 **Fig 3** Liberation of glucose after enzymatic saccharification of the waste residue. Data are the
385 mean ± SD of three replicates

386 **Ctec:** direct enzyme hydrolysis on waste residue with an excess of Novozymes Cellic® CTec2 (ca 50 FPU/g
387 biomass) **Sulphuric acid treatment** + Ctec2: 1.5 N H_2S0_4, 25% (w/v) biomass to reactant loading rate for 24
388 min at 121°C in a benchtop autoclave followed by enzymatic hydrolysis with an excess of Novozymes Cellic®
389 CTec2 (ca 50 FPU/g biomass). **Water treatment** + Ctec2: Auto-hydrolytical (entirely water based) pre-
390 treatment at 25% (w/v) biomass to reactant loading rate for 24 min at 121°C in a benchtop autoclave followed
391 by enzymatic hydrolysis with an excess of Novozymes Cellic® CTec2 (ca 50 FPU/g biomass).

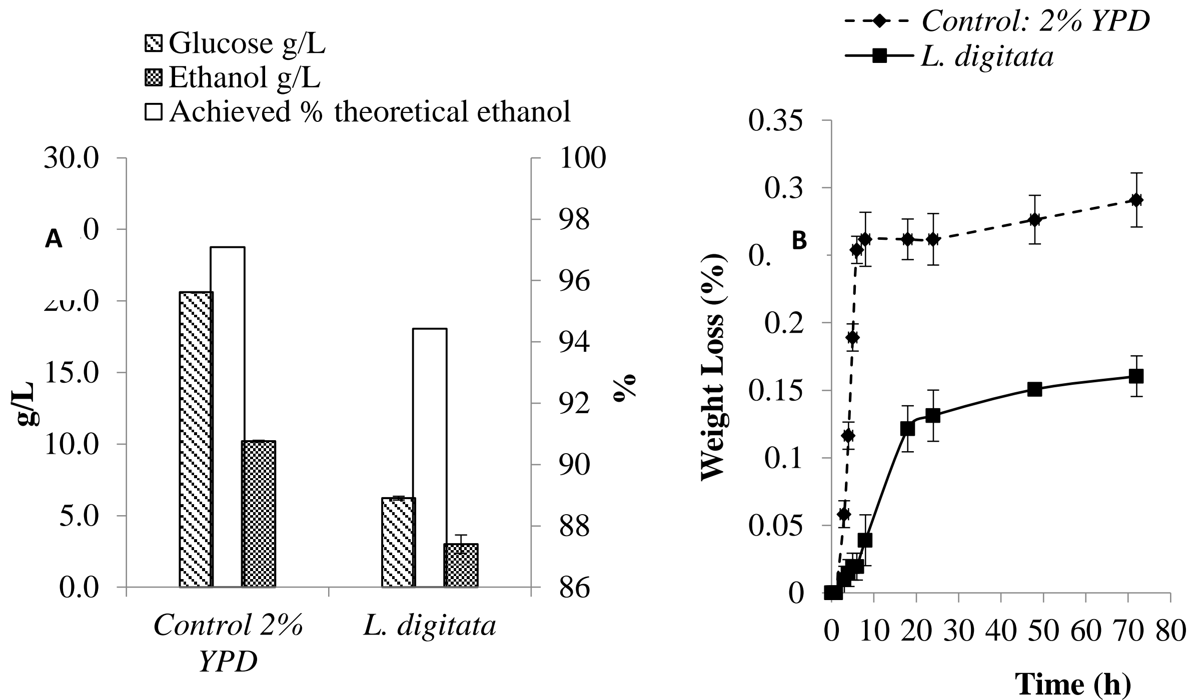
392

393 Almost 50% theoretical yield of glucose resulted from the direct saccharification (with an
394 excess of Novozymes Cellic® CTec2) of the remaining waste residue without any prior pre-
395 treatment. Pre-treating the residue with sulphuric acid liberated the greatest amount of
396 glucose (151.8 mg/g), achieving 93.8% theoretical yield of glucose following enzyme
397 hydrolysis. In contrast, the auto-hydrolytically (water-based) pre-treated residue liberated
398 118.2 mg/g glucose after enzyme saccharification which corresponded to a 73.2% theoretical
399 yield of glucose. Overall, it appeared that a dilute form of acid pre-treatment was still
400 required to achieve the maximal solubilisation of glucose from this material. This suggests that
401 the waste residue still contained recalcitrant and unexposed substrate specific surface areas, thus
402 reducing access for the cellulolytic enzymes to target. However with further optimisation, it may
403 be possible to enhance the overall yields of glucose with the application of an entirely water-
404 based pre-treatment. This would essentially make the overall bio-process more
405 environmentally friendly (avoiding the need to use acid reagents, to remove salts formed
406 from subsequent neutralisation and having to potentially discard any acid 'waste' produced)
407 and reduce the overall operation costs of the process.

408 ### 3.3.2 Bioethanol production

409 The fermentation progression and ethanol yield data using *S. cerevisiae* NCYC2592 are

410 shown in Fig 4 A and B.



411

412 **Fig 4** Ethanol yield data (A) and fermentation progression (B) for fermentation with *S.*

413 *cerevisiae* strain NCYC2592 of the hydrolysate produced from the remaining seaweed

414 residue after fucoidan/alginate extraction. Data are the mean ± SD of three replicates.

415 A: Theoretical ethanol yield based on based on mean glucose concentration in the three feedstocks. B:

416 Fermentation progression monitored by weight-loss of vessels due to CO_2 evolution

417

418 A yield of 3.0 g/L of ethanol was produced from the fermentation of the hydrolysate

419 generated from the new composite which equated to ca 94.4% of theoretical ethanol yield

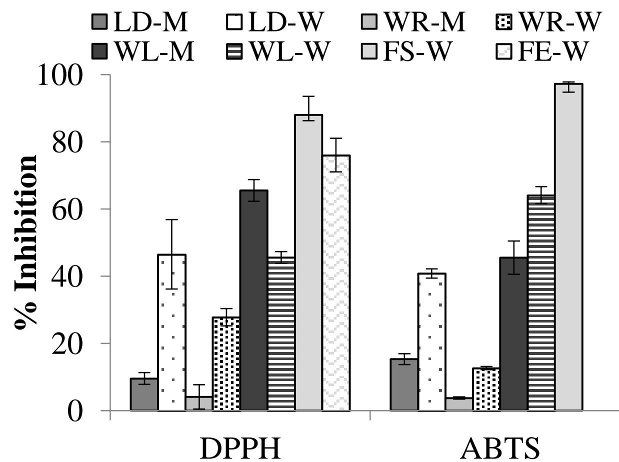
420 (calculated from the initial content of glucose present in the hydrolysate). Although the total

421 volumetric yield of ethanol produced from the hydrolysate was low (0.3% ABV), ethanol
422 production from this hydrolysate was not fully optimised for other sugars that may be present
423 such as galactose and xylose. Given that an apparent 94.4% theoretical ethanol yield (from
424 glucose) was achieved from fermentation, it appeared that the ethanol titre could only be
425 significantly improved in the present process by increasing the concentration of sugars
426 present in the hydrolysate prior to fermentation. This may certainly be possible once pre-
427 treatment and enzyme hydrolysis conditions on the new seaweed residue have been
428 optimised.

429 **3.4 Bioactivity analysis of process products/waste streams**

430 **3.4.1 Antioxidant activity**

431 As seen in Fig 5, both assays (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) revealed comparable antioxidant activity
432 levels across all extracts investigated.



433

434 **Fig 5** Antioxidant activities (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) of bio-refinery process extracts. Extract
435 (and solvent) and corresponding extract codes: Waste liquor (methanol) - WL-M; Fucoidan
436 extract (water) - FE-W; Fucoidan standard (water) - FS-W; *L. digitata* (methanol) - LD-M;
437 Waste Residue (methanol) - WR-M; Waste Residue (water) - WR-W; *L. digitata* (water) -
438 LD-W.

439 The fucoidan extract from the bio-refining process had a DPPH$^{\bullet}$ inhibition value of 76.0% ±
440 5.0 at a concentration of approximately 20 mg/mL, which was 12% lower than that of the

441 fucoidan standard. This could be due to the fact that the fucoidan standard is of a higher
442 purity than the extracted fucoidan. Studies have suggested that the scavenging effect of the
443 fucoidan polysaccharide may result from the presence of the sulphate group positioned at O-2
444 which is close to the glycosidic bond (38). In contrast to the $DPPH^{\bullet}$ assay, the extracted
445 fucoidan was not able to interact with the $ABTS^{\bullet+}$ radical and therefore a colour change was
446 not detected. The $DPPH^{\bullet}$ scavenging ability of native *L. digitata* (water extract) had an
447 inhibition value of 46.5% ± 10.4, whereas inhibition values of the waste residue extract
448 (water extract) were lower (27.9% ± 2.6). Values obtained from the $ABTS^{\bullet+}$ assay was 12.7%
449 ± 0.5. A cause of these lower values may have been the prior extraction of the fucoidan
450 polysaccharide which itself has been shown to have substantial antioxidant capacity (39).
451 Additionally, other compounds present in the native *L. digitata* that possess antioxidant
452 properties (e.g. phenolic compounds (40)) could have likewise been removed during the
453 extraction process. The $DPPH^{\bullet}$ assay revealed the waste liquor from the process to have an
454 inhibition value of 65.05% ± 3.2 (for the methanol extract). In order to put the DPPH
455 antioxidant values of the extracts produced from the bio-refining process into perspective,
456 EC_{50} values of the samples were calculated and compared against a known antioxidant
457 (ascorbic acid) and also other extracts of fucoidan obtained from the literature (Table 4).

Compound/Extract	DPPH Inhibition (%)	Concentration (mg/mL)	DPPH *EC_{50} (mg/mL)	Reference
Ascorbic acid	95.6	3.0	1.6	(41)
Fucoidan extract from *L. digitata*	76.0	20.0	13.2	Present study
Native *L. digitata* extract (water)	46.5	20.0	21.5	Present study
L. digitata residue (water)	27.9	20.0	35.8	Present study
Waste liquor extract (methanol)	65.1	20.0	15.3	Present study
Fucoidan from *A. nodosum*	30.4	10.0	16.5	(41)
Fucoidan from *S. vulgare*	22.0	3.0	6.8	(42)
Fucoidan from *S. pallidum*	19.1	3.8	10.0	(43)

Table 4 Comparison of DPPH antioxidant inhibition activity (%) of the extracts produced from the bio-refining process compared against the reference compound ascorbic acid and also extracts of fucoidan from other studies.

EC_{50} values for the $DPPH^{\bullet}$ radical scavenging activity are also included as a means to compare antioxidant capacities.* EC_{50} denoted as the concentration of sample required to scavenge 50% DPPH radicals (lower the value, the higher the antioxidant capacity).

472 The fucoidan extract from the bio-refining process (with an EC_{50} value of 13.2 mg/mL) was
473 within the same range of activity as fucoidan extracted from alternative species of
474 *Ascophyllum nodosum* (16.5 mg/mL; (41)) and *Sargassum pallidum* (10.0 mg/mL; (43). In
475 contrast fucoidan extracted from *S. vulgare* exhibited stronger DPPH radical scavenging
476 abilities than the fucoidan extracts from the literature, as only 6.8 mg/mL of the extract is
477 required to scavenge 50% DPPH radicals (42); confirming that levels may be influenced by
478 fucoidan source of origin. Interestingly, the waste liquor extract from the bio-process
479 exhibited an EC_{50} value that was in a similar range with the fucoidan extract (15.3 mg/mL
480 and 13.2 mg/mL, respectively). This highlights an opportunity for another potential stream of
481 added value from the putative process. It is speculated that algal polyphenols (including
482 tannins and flavonoids) are the principal constituents responsible for the antioxidant
483 properties of the waste liquor from the process; this is certainly worth further investigation by
484 way of developing a potential application in either the health and/or nutraceuticals industries.

485 **3.4.2 Antimicrobial activity**

486 Eleven different bacterial strains, ranging from common food pathogenic bacteria, food
487 spoilage bacteria and fish pathogenic bacteria were investigated in this study to identify
488 whether any of the generated extracts from the bio-process could inhibit their growth. A
489 summary of the positive pathogen and extract combination results (specific combinations that
490 produced clearance zones indicating inhibition) can be seen in Tables 5A+B.

A

Extract	Extract Code	Pathogen growth inhibition
Waste liquor (methanol)	WL-M	*S. enterica*, *B. cenocepacia*, *B. cereus*
Fucoidan extract (water)	FE-W	*B. cenocepacia*, *S. enterica*
Fucoidan standard (water)	FS-W	*S. pyogenes*, *L. monocytogenes*, *V. anguillerium*
L. digitata (methanol)	LD-M	*B. cenocepacia*
Waste Residue (methanol)	WR-M	*B. cenocepacia*
Waste Residue (water)	WR-W	*V. anguillerium*, *Y. ruckerii*,
L. digitata (water)	LD-W	*A. iwofii*, *A. hydrolphila*, *V. anguillerium*

B

	ABS at 600 nm 0 h	Broth & Cells + Extract (ABS at 600 nm) 24 h						
Strain Name	Broth&Cells	WL-M	FE-W	WR-W	LD-M	FS-W	LD-W	WR-M
Salmonella enterica[a]	1.13±0.05	1.15±0.29	1.21±0.99	-	-	-	-	-
Burkholderia cenocepacia[a]	1.21±0.14	1.06±0.19	1.24±0.32	-	1.17±0.59	-	-	1.10±0.06
Burkholderia multivorans[a]	1.10±0.52	-	-	1.02±0.06	-	-	-	-
Bacillus cereus[b]	1.08±0.23	1.02±0.32	-	-	1.03±0.09	-	-	-
Streptococcus pyogenes[a]	0.73±0.85	-	-	-	-	0.94±0.36	-	-
Acinetobacter iwofii[a]	1.23±0.03	-	-	-	-	-	1.30±0.36	-
Listeria monocytogenes[a]	1.04±0.56	-	-	-	-	1.21±0.12	-	-
Yersinia ruckerii[c]	0.55±0.36	-	-	0.54±0.09	-	-	-	-
Vibrio anguillerium[c]	0.73±0.25	-	-	0.76±0.19	-	0.79±0.32	0.74±0.09	-
Aeromonas hydrolphila[c]	0.85±0.16	-	-	-	-	-	1.00±0.04	-

Table 5 (A) Summary of extracts that produced clearance zones (inhibition of pathogen growth) on the standard agar disc diffusion assay and **(B)** growth inhibition after 24 h of selected process extracts against selected human pathogenic[(a)], food spoilage[(b)] and fish pathogenic[(c)] bacteria.

501 Experiments were performed in triplicate and each value is presented as mean ± SD. Waste liquor (methanol) - WL-M; Fucoidan extract (water)
502 - FE-W; Fucoidan standard (water) - FS-W; *L. digitata* (methanol) - LD-M; Waste Residue (methanol) - WR-M; Waste Residue (water) - WR-
503 W; *L. digitata* (water) - LD-W.

504 Inhibition of pathogenic bacterial growth was confirmed for three out of the 10 selected
505 pathogenic strains. Growth of *B. cenocepacia*, originally known as a plant pathogen which
506 has now emerged as a life-threatening multi-resistant pathogen in cystic fibrosis patients (44),
507 appeared to be inhibited by extracts prepared from the process waste liquor and both native
508 and residue *L. digitata* extracts (methanol extracts). Absorbance readings after 24 h of growth
509 appeared to be lower than the reading at 0 h therefore suggesting inhibition; the 0 h
510 absorbance reading at 600 nm was 1.21 however after 24 h absorbance values were 1.06, 1.17
511 and 1.10 for the waste liquor, native *L. digitata* and residue *L. digitata* extracts, respectively.
512 Likewise, *B. multivorans* growth was supressed after 24 h incubation with the *L. digitata*
513 residue extract, as was *B. cereus*; however the waste liquor extract additionally inhibited *B.*
514 *cereus* growth in the liquid media. It has been documented that *Burkholderia* bacteria are
515 resistant to a number of clinically used antimicrobial agents, such as polymyxins and
516 aminoglycosidases (45) and there is an increasing need to identify novel antimicrobial
517 compounds for activity against *Burkholderia* species (46). Research on natural antimicrobial
518 compound isolation from medicinal plants however is looking promising and extracts
519 prepared from *Echinacea purpunea* (47) and allicin-containing garlic extracts (48) have
520 shown antimicrobial effects. However, this is the first study to show antimicrobial inhibition
521 from extracts prepared from a *L. digitata* bio-process. It appeared that the inhibition of
522 pathogenic growth was selective to certain strains and extracts in a liquid medium, and not all
523 of the combinations that had been identified from the agar disc diffusion assay displayed
524 inhibitory activity. The reasons behind this are unclear; however the agar disc diffusion assay
525 did serve as an effective and rapid screening tool. Additionally it appeared that methanol was
526 a suitable solvent for the extraction of functional antimicrobials from process products, and
527 further research is needed to validate these findings.

4 Conclusion

The research presented in this study described the development of a feasible seaweed bio-refining process based on the abundant UK brown seaweed *L. digitata.* Overall, this study demonstrated that there is great potential for further exploratory work with regards further development of this particular bio-refinery process. Two valuable brown seaweed polysaccharides were extracted, one of which displayed interesting biological activities. Bioethanol was then successfully produced from the residue which remained after the extraction. In addition, extracts that were generated from various streams of the process (including the waste streams) displayed antimicrobial and antioxidant activities. As such, characterisation of the extracts to specifically identify the bioactive compounds would be of great interest to further develop this process.

While this study identifies significant pathways to enable the development of a *L. digitata*-based bio-refinery, there is still much more research that is required to optimise and enhance the overall process efficiency. In particular, the ethanol yields in the present study were too low (3 g/L) to be economically viable on a commercial scale. Furthermore, the identification of other valuable by-products with interesting bioactivities or the screening for potential platform chemicals in waste streams may also contribute to the development of a cost efficient bio-refining process for *L. digitata*.

Contributions

ETK: designed and performed experiments; analysed data; wrote the paper; DAW; performed experiments; analysed data; wrote paper; obtained funding; DJC; obtained funding; analysed data; wrote paper; critically revised article for intellectual content.

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Competing financial interests

The authors declare no competing financial interests.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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