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

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TITLE RUNNING HEAD: Bio-refinery process development from seaweed *L. 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 pretreatment (1.5 N H\textsubscript{2}SO\textsubscript{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\textsubscript{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
41 1 Introduction
42 Seaweeds have gained much attention in recent years as alternative renewable feedstocks due
43 to their large biomass yields, fast growth rates and the fact that they require no terrestrial land
44 for cultivation (1). However, many production processes of chemicals from seaweeds have
45 focussed on a single product, for example alginic acid, carrageenan or even colourants, with
46 the rest of the seaweed treated as a waste material (2). Furthermore, many current seaweed
47 research programmes also focus on a single product objective, with much current research
48 focus on biofuels (3, 4). As an alternative to developing processes based on single products
49 from seaweeds, cascading bio-refineries are being sought in order to maximise the inherent
50 value of all components present in the biomass (2, 5-7). Seaweeds are excellent feedstocks
51 for such bio-refineries as they contain both high value components (such as speciality
52 polysaccharides and bioactive molecules) and compounds which are considered to be
53 platform chemicals for the bio-based economy such as glucose (2).
54 A number of seaweed bio-refinery processes have already been investigated for the
55 production of biofuels and commodity compounds (e.g. see Kumar et al (8)). Following the
56 extraction of agar from the red seaweed species Gracilaria verrucosa the residual pulp was
57 converted into bioethanol; achieving an ethanol yield of 0.43g/g of sugar. Trivedi et al (9)
58 developed an integrated process which was applied to the green seaweed Ulva fascita that
59 sequentially recovered a mineral rich liquid extract, lipids, ulvan and cellulose; four fractions
60 of economic importance. Additionally, various nutrients, pigments (8) and even seaweed salts
61 (10) could also be extracted from seaweeds, thus increasing the potential value of bio-refining
62 processes. van der Wal et al [13] generated not only bioethanol but also acetone and butanol
63 from a hydrolysate derived from green seaweed Ulva lactuca using Clostridium beijerinckii
64 and Clostridium acetobuticum. This was achieved by solubilising over 90% of the sugars
65 found in the green seaweed into a fermentable solution (11). However, solubilisation of all
functional seaweed polysaccharides for biofuel conversion may in the long term jeopardise the seaweed hydrocolloid industry (12). In 2016 commercial seaweed market was estimated to be valued at $11.34 billion (http://www.marketsandmarkets.com/Market-Reports/commercial-seaweed-market-152763701.html). The seaweed hydrocolloid market was estimated to be valued at $1.1 billion (13), compared to bioethanol which at the time of writing this article was valued at $23.2 million (Renewable Fuels Association; http://www.ethanolrfa.org/). Therefore the fractionation and selective utilisation of biofuel substrates, such as the laminarin fraction from seaweed biomass (which is a less utilised material) for the production of biofuels, would prevent negative impacts on the present hydrocolloid industry and associated worldwide markets. Additionally, the net worth of a seaweed bio-refinery for fuels and platform chemicals would also increase.

Brown seaweeds are amenable to bio-refinery processing as they contain a diverse array of metabolites with existing or potential applications. These include extracellular matrix polysaccharides such as alginates and fucoidans, storage polysaccharides such as laminarin and mannitol and bio-active polyphenolic compounds and pigments such as fucoxanthin (14). Such species have been mainly exploited for the anionic polysaccharide alginate that is widely used in the pharmaceutical, food, cosmetic and biotechnology industries due to its favourable gelling properties (15). The interest in new sources of natural bioactives such as antioxidants and antimicrobials has increased in recent years. One reason for this has been a desire to reduce the use of synthetic forms of antioxidants and antimicrobials such as butylated hydroxytoluene (BHT) and propyl gallate (PG) where strict regulations have been applied due to their potential health hazards. In recent years sulphated polysaccharides such as fucoidan have received attention (16) due to their specific biological activities and properties such as anti-inflammatory (17), anti-tumor (18) and anti-coagulant (19).
Bio-refinery processes using different species of brown seaweeds, including *Saccharina latissimi* and *Ascophyllum nodosum* have been explored for mannitol isolation (20) and fucoidan, alginate, sugars and biochar production (7), respectively. In addition, bio-refinery scenarios were investigated with *Laminaria digitata* where bioethanol (21) and succinic acid (22) were produced and the remaining residues analysed as potential feedstocks for biogas production, biodiesel, and feed supplements (due to enriched protein and fatty acid fractions).

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

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

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

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

2.3 Fucoidan (and alginate) extraction
A schematic of the overall bio-refinery process is summarised in Fig 1.
Fig 1 Schematic diagram of the bio-refining process developed for *L. digitata* for the
extraction of value added compounds in addition to bioethanol and residues/waste streams
with potential bioactivity.

Fucoidan was extracted following the method outlined by Black et al. (25). *L. digitata* (2 g)
was mixed with 20 mL of 0.1 M HCl (pH 2.5) at 70°C for 1 h. The mixture was stirred at
250 rpm and then centrifuged at 5000 rpm for 15 min to separate the liquid from the seaweed
solids. One volume of 1% (w/v) CaCl$_2$ was added to the recovered liquid, inverted and kept at
4°C for 72 h. The precipitate which formed (alginate) was removed from the liquid phase by
centrifugation at 5000 rpm for 20 mins, freeze dried and lastly weighed. Two volumes of
absolute ethanol (99.99%) were then added to the remaining alginate-free liquor, inverted and
kept at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000 rpm for 20 min
and the upper liquid phase (waste liquor) was stored at -4°C until further analysis. The solid
precipitate (fucoidan) was freeze-dried and stored at -80°C until further analysis. Seaweed
solids (waste residue) which remained after the extraction were dried in an oven at 60°C until
all water had evaporated and stored in an airtight container until further analysis.

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

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

Monosaccharide analysis of the *L. digitata* residue was determined by the method outlined by
Kostas et al. (26) where 1 mL of 11 M H$_2$SO$_4$ was added to 30 mg of seaweed in a heat
resistant screw cap glass tube and incubated at 37°C for 1 h. Water (11 mL) was added to the
sample to dilute the acid strength to 1 M, following which, samples were incubated at 100°C
for 2 h. Liberated monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) were analysed by HPAEC-PAD as described in section 2.8.

2.5 Characterisation of fucoidan extract

2.5.1 Fucoidan extract quantification and determination of purity

Solutions of fucoidan extracts (10 g/L) were prepared with RO water. The samples were then run on a HPLC using an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC Pump (Jasco, Japan). The Rezex ROA Organic Acid H+ organic acid column (5 μm, 7.8 mm×300 mm; Phenomenex, UK) was operated at ambient temperature with a mobile phase of 0.005 N H₂SO₄ at a flow rate of 0.5 mL min⁻¹. A Refractive Index cell (RI-2031 Intelligent Refractive Index detector, Jasco, Japan) was used for detection, and the injection volume was 10 μL. Data were acquired using the Azur software package v. 4.6.0.0 (Datalys, France).

Prior to HPLC analysis, all samples and standards were filtered using Whatman GD/X syringe filters (GF/C 25 mm filter diameter/1.2 μm pore size; Whatman, UK). Authentic standards of fucoidan (Sigma-Aldrich®, USA) with concentrations within range of 10 g/L to 0.5 g/L were used for quantification.

2.5.2 Monosaccharide profile

The monosaccharide profile of the extracted fucoidan was obtained by following the method of Rodriguez-Jasso et al (27). Fucoidan extract (10 mg) was hydrolysed with 2 M Trifluoroacetic acid (0.5 mL) at 121°C for 2 h in N₂ sealed heat resistant screw cap glass tubes. The tubes were then cooled in an iced water bath before being centrifuged at 5000 rpm for 5 min. Samples were then prepared for monosaccharide quantification using the HPAEC-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
time, pooling together the relevant supernatants. In order to concentrate the extract, the
solvents were evaporated and then the residue re-suspended in a reduced volume (1 mL) of
either methanol and/or water. Extracts were then stored in amber vials at -80°C until further
use. Fucoidan extracts were only prepared using water due to their insolubility in methanol.

2.6.2 Determination of antioxidant activity

2.6.2.1 DPPH\(^*\) scavenging capacity assay

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

\[
Inhibition \, (\%) = \left[1 - \frac{A_{sample_518}}{A_{control_518}}\right] \times 100
\]

EC\(_{50}\) values were also calculated which indicates the concentration of sample required to
scavenge 50% DPPH radicals, according to the following equation:

\[
EC_{50} = \left[\frac{sample \, concentration \, (mg/ml)}{DPPH \, inhibition \, (\%)}\right] \times 50
\]

2.6.2.2 ABTS\(^{•\,+}\) scavenging capacity assay

The ABTS\(^{•\,+}\) assay was carried out according to the protocol outlined in the work of
Martinez-Avila et al (2012). In order to generate the radical (ABTS\(^{•\,+}\)), 12.5 mL of potassium
persulfate (2.45 mM) was mixed with 25 mL of ABTS (7 mM). The mixture was maintained
in the dark at room temperature for 12-16 h. The absorbance was measured at 734 nm and the
ABTS\(^{•\,+}\) solution was then diluted with ethanol until an absorbance value of 0.7 ± 0.01 was
achieved. For the assays, 950 μL of ABTS\(^{•\,+}\) solution was added to 50 μL of extract and the
absorbance was measured after 1 min of the reaction. The control solutions contained 50 μL of distilled water or 50 μL methanol (100%). ABTS** percent of inhibition was calculated according to the following equation:

\[
\text{Inhibition} (\%) = \left[ \frac{(1 - A_{\text{sample}}/A_{\text{control}})}{} \right] \times 100
\]

The radical-scavenging capacity of each sample was calculated according to a Trolox standard curve (0 to 50 μM in 5 μM increments) and expressed as Trolox equivalent antioxidant capacity (TEAC) by the extrapolation of ABTS** percent inhibition of each tested sample. Assays were conducted in triplicate.

### 2.6.3 Determination of antimicrobial activity

#### 2.6.3.1 Preparation of bacterial broths, agar plates and culturing of bacterial strains

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Pathogen Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Burkholderia cenocepacia</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Burkholderia multivorans</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Food Spoilage Pathogen</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Acinetobacter iwoffii</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Human pathogen</td>
</tr>
<tr>
<td><em>Yersinia ruckerii</em></td>
<td>Fish pathogen</td>
</tr>
<tr>
<td><em>Vibrio anguillierum</em></td>
<td>Fish pathogen</td>
</tr>
<tr>
<td><em>Aeromonas hydrolphila</em></td>
<td>Fish pathogen</td>
</tr>
</tbody>
</table>

Table 1 List of pathogens included in this study. Human pathogenic\(^{(a)}\), food spoilage\(^{(b)}\) and fish pathogenic\(^{(c)}\) bacteria.
The strains were harvested from local waters around the Plymouth shoreline. Two types of bacterial broth and agar plates were made depending upon bacterial strain. Luria-Bertani broth (for human pathogenic strains): 5 g bactopeptone, 1 g yeast extract made up to 1L with DI water. Marine LB broth (for marine pathogens): 5 g bactopeptone, 1 g yeast extract made up to 1L with filtered sea water. Yeast peptone dextrose (YPD) plates were made following the same recipe as mentioned above for both pathogen types, however contained 2 g agar. The bacterial strains were taken from glycerol stocks stored at -80°C and spread onto appropriate YPD plates and kept at 26°C for 24 h. A loop-full of cells were then inoculated into 3 mL of LB/marine LB broth. Cultures were placed in a shaking incubator at 26°C for 24 h at 130 rpm.

2.6.3.2 Agar disc diffusion assay

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

2.6.3.3 Validation test of antimicrobial activity

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

2.7 Bioethanol production from the bio-refinery process residue

2.7.1 Pre-treatment of the remaining residue

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

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

Higher glucose yields obtained from the enzymatic saccharification were indicative of a more effective pre-treatment. All experiments were conducted in triplicate.
### 2.7.2 Laboratory scale trial fermentations of residue hydrolysates for bioethanol production

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

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

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

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

### 3 Results and Discussion

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

A total of 130.9 mg fucoidan with a measured purity of 65% and 98.4 mg alginate were extracted from 2 g of *L. digitata* (Table 2).
Table 2 Product yields and losses generated from the extraction of fucoidan and alginate from *L. digitata*, including fucoidan purity, fucoidan sulphate content and fucoidan monosaccharide profile.

From the 2 g of *L. digitata* used in the process, only 1.54 g was recovered after the extraction. Therefore approximately 460 mg of material was solubilised from the starting material. The main products from the extraction process (fucoidan and alginate) together yielded a total of 229.3 mg, leaving approximately 230.7 mg of material unaccounted for. This group of unaccounted for material has been termed ‘process losses’ and represents other extracted components of the biomass which had not been precipitated/ recovered. Other extracted materials from the seaweed such as pigments, polysaccharides/monosaccharides, proteins, polyphenols, minerals and salts would have comprised this solubilised matter. Furthermore,
products from the extraction process could have been volatilised during the first stage of the extraction process, in which the contents of the reaction was heated to 70°C.

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

However, annual fluctuation in the levels of fucoidan from various Fucoid species was recently determined by Fletcher et al (37) who identified that whilst the best time to harvest
(in terms of maximum fucoidan content) is late autumn/early winter, the actual range of fucoidan content (minimum and maximum) was relatively small. From an industrial processing perspective this is significant, since it would potentially facilitate more consistent recovery of the fucoidan polysaccharide. Therefore, the selection of a suitable brown species of seaweed is imperative in the design of cascading bio-refineries. Furthermore, year-round use of that particular species would eliminate the requirements for drying and storage, reducing overall processing costs and enhance life cycle analysis/techno-economical assessment of the bio-process.

3.2 Evaluation of the waste residue after fucoidan and alginate extraction

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

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

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

The ash decreased from a content of 26.0% (d/w) to 23.4% (d/w) and protein content increased from 12.9 to 14% (d/w); suggesting a slight enrichment of the protein fraction and
highlighting a potential protein-based product stream that would be worth evaluating for added value to the bio-refinery. Although there appeared to be a reduction in the carbohydrate content, from 46.6% to 41.4% (d/w), which most likely resulted from the extraction of alginate and fucoidan, there was an increase in crude fibre content from 5.5% to 15.5% (d/w), respectively. The authors consider that this may be the consequence of an enriched cellulose fraction. When investigating the monosaccharide profile of the seaweed residue, it became apparent that the predominant monosaccharide was glucose (161.9 mg/g of residue; Fig 2).

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

This of course represented a promising substrate for the subsequent production of bioethanol due to the high glucose content. However, a small fraction of fucose (16.7 mg/g) was additionally detected, suggesting that not all of the fucoidan was extracted from the *L. digitata* during the previous extraction process step and confirmed the requirement of further adjustments (optimisation) to maximise recovery of fucoidan. In addition, uronic acids such
as mannuronic and guluronic acids may have also been released (in the case that not all of the alginate was extracted) but were not quantified using the analytical method applied.

3.3 Bioethanol production from the waste residue

3.3.1 Pre-treatment and enzymatic saccharification of new residue

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

**Fig 3** Liberation of glucose after enzymatic saccharification of the waste residue. Data are the mean ± SD of three replicates.
**Ctec**: direct enzyme hydrolysis on waste residue with an excess of Novozymes Cellic® CTec2 (ca 50 FPU/g biomass) **Sulphuric acid treatment** + Ctec2: 1.5 N H₂SO₄, 25% (w/v) biomass to reactant loading rate for 24 min at 121°C in a benchtop autoclave followed by enzymatic hydrolysis with an excess of Novozymes Cellic® CTec2 (ca 50 FPU/g biomass). **Water treatment** + Ctec2: Auto-hydrolytical (entirely water based) pre-treatment at 25% (w/v) biomass to reactant loading rate for 24 min at 121°C in a benchtop autoclave followed by enzymatic hydrolysis with an excess of Novozymes Cellic® CTec2 (ca 50 FPU/g biomass).

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

### 3.3.2 Bioethanol production
The fermentation progression and ethanol yield data using *S. cerevisiae* NCYC2592 are shown in Fig 4 A and B.

**Fig 4** Ethanol yield data (A) and fermentation progression (B) for fermentation with *S. cerevisiae* strain NCYC2592 of the hydrolysate produced from the remaining seaweed residue after fucoidan/alginate extraction. Data are the mean ± SD of three replicates.

A: Theoretical ethanol yield based on mean glucose concentration in the three feedstocks. B: Fermentation progression monitored by weight-loss of vessels due to CO$_2$ evolution

A yield of 3.0 g/L of ethanol was produced from the fermentation of the hydrolysate generated from the new composite which equated to ca 94.4% of theoretical ethanol yield (calculated from the initial content of glucose present in the hydrolysate). Although the total
volumetric yield of ethanol produced from the hydrolysate was low (0.3% ABV), ethanol production from this hydrolysate was not fully optimised for other sugars that may be present such as galactose and xylose. Given that an apparent 94.4% theoretical ethanol yield (from glucose) was achieved from fermentation, it appeared that the ethanol titre could only be significantly improved in the present process by increasing the concentration of sugars present in the hydrolysate prior to fermentation. This may certainly be possible once pre-treatment and enzyme hydrolysis conditions on the new seaweed residue have been optimised.

3.4 Bioactivity analysis of process products/waste streams

3.4.1 Antioxidant activity

As seen in Fig 5, both assays (DPPH• and ABTS•+) revealed comparable antioxidant activity levels across all extracts investigated.

![Fig 5](image-url) Antioxidant activities (DPPH• and ABTS•+) of bio-refinery process extracts. Extract (and solvent) and corresponding extract codes: Waste liquor (methanol) - WL-M; Fucoidan extract (water) - FE-W; Fucoidan standard (water) - FS-W; *L. digitata* (methanol) - LD-M; Waste Residue (methanol) - WR-M; Waste Residue (water) - WR-W; *L. digitata* (water) - LD-W.

The fucoidan extract from the bio-refining process had a DPPH• inhibition value of 76.0% ± 5.0 at a concentration of approximately 20 mg/mL, which was 12% lower than that of the
fucoidan standard. This could be due to the fact that the fucoidan standard is of a higher purity than the extracted fucoidan. Studies have suggested that the scavenging effect of the fucoidan polysaccharide may result from the presence of the sulphate group positioned at O-2 which is close to the glycosidic bond (38). In contrast to the DPPH\(^\bullet\) assay, the extracted fucoidan was not able to interact with the ABTS\(^{\bullet+}\) radical and therefore a colour change was not detected. The DPPH\(^\bullet\) scavenging ability of native \textit{L. digitata} (water extract) had an inhibition value of 46.5\% \(\pm\) 10.4, whereas inhibition values of the waste residue extract (water extract) were lower (27.9\% \(\pm\) 2.6). Values obtained from the ABTS\(^{\bullet+}\) assay was 12.7\% \(\pm\) 0.5. A cause of these lower values may have been the prior extraction of the fucoidan polysaccharide which itself has been shown to have substantial antioxidant capacity (39). Additionally, other compounds present in the native \textit{L. digitata} that possess antioxidant properties (e.g. phenolic compounds (40)) could have likewise been removed during the extraction process. The DPPH\(^\bullet\) assay revealed the waste liquor from the process to have an inhibition value of 65.05\% \(\pm\) 3.2 (for the methanol extract). In order to put the DPPH antioxidant values of the extracts produced from the bio-refining process into perspective, EC\(_{50}\) values of the samples were calculated and compared against a known antioxidant (ascorbic acid) and also other extracts of fucoidan obtained from the literature (Table 4).
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.

<table>
<thead>
<tr>
<th>Compound/Extract</th>
<th>DPPH Inhibition (%)</th>
<th>Concentration (mg/mL)</th>
<th>DPPH *EC₅₀ (mg/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>95.6</td>
<td>3.0</td>
<td>1.6</td>
<td>(41)</td>
</tr>
<tr>
<td>Fucoidan extract from <em>L. digitata</em></td>
<td>76.0</td>
<td>20.0</td>
<td>13.2</td>
<td>Present study</td>
</tr>
<tr>
<td>Native <em>L. digitata</em> extract (water)</td>
<td>46.5</td>
<td>20.0</td>
<td>21.5</td>
<td>Present study</td>
</tr>
<tr>
<td><em>L. digitata</em> residue (water)</td>
<td>27.9</td>
<td>20.0</td>
<td>35.8</td>
<td>Present study</td>
</tr>
<tr>
<td>Waste liquor extract (methanol)</td>
<td>65.1</td>
<td>20.0</td>
<td>15.3</td>
<td>Present study</td>
</tr>
<tr>
<td>Fucoidan from <em>A. nodosum</em></td>
<td>30.4</td>
<td>10.0</td>
<td>16.5</td>
<td>(41)</td>
</tr>
<tr>
<td>Fucoidan from <em>S. vulgare</em></td>
<td>22.0</td>
<td>3.0</td>
<td>6.8</td>
<td>(42)</td>
</tr>
<tr>
<td>Fucoidan from <em>S. pallidum</em></td>
<td>19.1</td>
<td>3.8</td>
<td>10.0</td>
<td>(43)</td>
</tr>
</tbody>
</table>

EC₅₀ values for the DPPH* radical scavenging activity are also included as a means to compare antioxidant capacities.* EC₅₀ denoted as the concentration of sample required to scavenge 50% DPPH radicals (lower the value, the higher the antioxidant capacity).
The fucoidan extract from the bio-refining process (with an EC$_{50}$ value of 13.2 mg/mL) was within the same range of activity as fucoidan extracted from alternative species of *Ascophyllum nodosum* (16.5 mg/mL; (41)) and *Sargassum pallidum* (10.0 mg/mL; (43)). In contrast fucoidan extracted from *S. vulgare* exhibited stronger DPPH radical scavenging abilities than the fucoidan extracts from the literature, as only 6.8 mg/mL of the extract is required to scavenge 50% DPPH radicals (42); confirming that levels may be influenced by fucoidan source of origin. Interestingly, the waste liquor extract from the bio-process exhibited an EC$_{50}$ value that was in a similar range with the fucoidan extract (15.3 mg/mL and 13.2 mg/mL, respectively). This highlights an opportunity for another potential stream of added value from the putative process. It is speculated that algal polyphenols (including tannins and flavonoids) are the principal constituents responsible for the antioxidant properties of the waste liquor from the process; this is certainly worth further investigation by way of developing a potential application in either the health and/or nutraceuticals industries.

### 3.4.2 Antimicrobial activity

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

### A

<table>
<thead>
<tr>
<th>Extract</th>
<th>Extract Code</th>
<th>Pathogen growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste liquor (methanol)</td>
<td>WL-M</td>
<td><em>S. enterica, B. cenocepacia, B. cereus</em></td>
</tr>
<tr>
<td>Fucoidan extract (water)</td>
<td>FE-W</td>
<td><em>B. cenocepacia, S. enterica</em></td>
</tr>
<tr>
<td>Fucoidan standard (water)</td>
<td>FS-W</td>
<td><em>S. pyogenes, L. monocytogenes, V. anguillerium</em></td>
</tr>
<tr>
<td><em>L. digitata</em> (methanol)</td>
<td>LD-M</td>
<td><em>B. cenocepacia</em></td>
</tr>
<tr>
<td>Waste Residue (methanol)</td>
<td>WR-M</td>
<td><em>B. cenocepacia</em></td>
</tr>
<tr>
<td>Waste Residue (water)</td>
<td>WR-W</td>
<td><em>V. anguillerium, Y. ruckerii,</em></td>
</tr>
<tr>
<td><em>L. digitata</em> (water)</td>
<td>LD-W</td>
<td><em>A. iwofii, A. hydrolphila, V. anguillerium</em></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>ABS at 600 nm</th>
<th>Broth &amp; Cells + Extract (ABS at 600 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WL-M</td>
</tr>
<tr>
<td><strong>Salmonella enterica(^{(a)})</strong></td>
<td>1.13±0.05</td>
<td>1.15±0.29</td>
</tr>
<tr>
<td><strong>Burkholderia cenocepacia(^{(a)})</strong></td>
<td>1.21±0.14</td>
<td>1.06±0.19</td>
</tr>
<tr>
<td><strong>Burkholderia multivorans(^{(a)})</strong></td>
<td>1.10±0.52</td>
<td>-</td>
</tr>
<tr>
<td><strong>Bacillus cereus(^{(b)})</strong></td>
<td>1.08±0.23</td>
<td>1.02±0.32</td>
</tr>
<tr>
<td><strong>Streptococcus pyogenes(^{(a)})</strong></td>
<td>0.73±0.85</td>
<td>-</td>
</tr>
<tr>
<td><strong>Acinetobacter iwofii(^{(a)})</strong></td>
<td>1.23±0.03</td>
<td>-</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes(^{(a)})</strong></td>
<td>1.04±0.56</td>
<td>-</td>
</tr>
<tr>
<td><strong>Yersinia ruckerii(^{(c)})</strong></td>
<td>0.55±0.36</td>
<td>-</td>
</tr>
<tr>
<td><strong>Vibrio anguillerium(^{(c)})</strong></td>
<td>0.73±0.25</td>
<td>-</td>
</tr>
<tr>
<td><strong>Aeromonas hydrolphila(^{(c)})</strong></td>
<td>0.85±0.16</td>
<td>-</td>
</tr>
</tbody>
</table>

**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.
Experiments were performed in triplicate and each value is presented as mean ± SD. Waste liquor (methanol) - WL-M; Fucoidan extract (water) - FE-W; Fucoidan standard (water) - FS-W; *L. digitata* (methanol) - LD-M; Waste Residue (methanol) - WR-M; Waste Residue (water) - WR-W; *L. digitata* (water) - LD-W.
Inhibition of pathogenic bacterial growth was confirmed for three out of the 10 selected pathogenic strains. Growth of *B. cenocepacia*, originally known as a plant pathogen which has now emerged as a life-threatening multi-resistant pathogen in cystic fibrosis patients (44), appeared to be inhibited by extracts prepared from the process waste liquor and both native and residue *L. digitata* extracts (methanol extracts). Absorbance readings after 24 h of growth appeared to be lower than the reading at 0 h therefore suggesting inhibition; the 0 h absorbance reading at 600 nm was 1.21 however after 24 h absorbance values were 1.06, 1.17 and 1.10 for the waste liquor, native *L. digitata* and residue *L. digitata* extracts, respectively.

Likewise, *B. multivorans* growth was suppressed after 24 h incubation with the *L. digitata* residue extract, as was *B. cereus*; however the waste liquor extract additionally inhibited *B. cereus* growth in the liquid media. It has been documented that *Burkholderia* bacteria are resistant to a number of clinically used antimicrobial agents, such as polymyxins and aminoglycosidases (45) and there is an increasing need to identify novel antimicrobial compounds for activity against *Burkholderia* species (46). Research on natural antimicrobial compound isolation from medicinal plants however is looking promising and extracts prepared from *Echinacea purpurea* (47) and allicin-containing garlic extracts (48) have shown antimicrobial effects. However, this is the first study to show antimicrobial inhibition from extracts prepared from a *L. digitata* bio-process. It appeared that the inhibition of pathogenic growth was selective to certain strains and extracts in a liquid medium, and not all of the combinations that had been identified from the agar disc diffusion assay displayed inhibitory activity. The reasons behind this are unclear; however the agar disc diffusion assay did serve as an effective and rapid screening tool. Additionally it appeared that methanol was a suitable solvent for the extraction of functional antimicrobials from process products, and 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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