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*Published in:*
Biomass Conversion and Biorefinery

*Publication date:*
2015

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*Citation for published version (APA):*
Schiener, P., Atack, T., Wareing, R., Kelly, M. S., & Hughes, A. D. (2015). The by-products from marine biofuels as a feed source for the aquaculture industry: a novel example of the biorefinery approach. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-015-0190-6

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# The by-products from Marine Biofuels as a feed source for the aquaculture industry: a novel example of the bio-refinery approach.

Peter Schiener[1,3], Tim Atack[2], RossAmy Wareing[2], Maeve S. Kelly[1], Adam D Hughes[1]

Affiliation:

1. Scottish Association for Marine Science, Scottish Marine Institute, Oban, PA37 1QA, United Kingdom.
2. Ardtoe Marine Laboratory, Ardtoe, Acharacle Argyll PH36 4LD, United Kingdom
3. Corresponding author: Email: schienerpeter@gmail.com; Tel. 0044-28-4272-7806; Fax 0044-28-28902

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13 # 1. ABSTRACT


14 Through increased interest in the use of macroalgae for biofuel production, the concept of a
15 biorefinery approach has been developed to improve its economic viability. This paper looks
16 at the use of seaweed residue after enzymatic saccharification of the seaweeds *Saccharina*
17 *latissima, Alaria esculenta* and *Ulva lactuca* as a novel feed component for the aquaculture
18 industry. Chemical composition analysis was used to evaluate the impact of enzymatic
19 treatment on the nutritional aspect of seaweed residue. During saccharification the relative
20 ash and carbohydrates content were reduced but total nitrogen, total carbon and lipid content
21 increased in all three species. In addition the saccharification of *S. latissima,* and *A. esculenta*
22 resulted in the elimination of polyphenols, further enhancing the nutritional value of the
23 residue. The residue supported the survival and growth of bivalve spat and commercially
24 valuable sea urchins over the course of three week preliminary trials.


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26 Keywords:

27 Enzymatic saccharification; hatchery food; aquaculture; bivalve; sea urchin; *Ostrea edulis*

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29 ## 2. INTRODUCTION

30 Although the concept of biofuels from macroalgae dates back to the 1970's [1], recently there
31 has been a resurgence in interest in the concept [2, 3]. Marine biofuels in general and
32 macroalgae in particular offer a number of advantages over terrestrial biofuels including
33 reduced competition for freshwater resources, reduced competition for land use, zero
34 fertiliser input requirement [2] and higher photosynthetic efficiencies and associated higher
35 growth rates compared to terrestrial plants [4, 5]. In addition these fast-growing macroalgae
36 are rich in unique carbohydrates, proteins, metals and bioactive components, which make
37 them not only well suited for biofuel production but also for the exploitation of high-value
38 products. The economics of a low-value product such as biofuel has been questioned [6, 7]
39 and in combination with multi-step biorefining more of seaweed biomass can be utilised and
40 as a consequence promote sustainability and profitability.

41 In this paper we describe a new biorefinery process based on the concept of ethanol
42 production from macroalgae: where the residue from ethanol production is used as a novel
43 microalgae substitute (MAS) diet for the aquaculture industry. The global aquaculture
44 industry was worth US$144.4 billion in 2012 [8] and ninety per cent of the industrial finfish
45 and shellfish aquaculture producers have juvenile or larval life stages that are micro-
46 planktivorous [9]. It is the supply of microalgae to feed larvae and juveniles that has been
47 identified as a major bottle-neck to the aquaculture industry. The culture of unicellular algae
48 is labour intensive and is often accompanied by various technical difficulties when in large
49 scale production. Due to these economic and technical constraints there are a number of
50 alternatives to growing live microalgae available to the aquaculture industry such as
51 concentrated preparations of preserved non-viable micro algae (PNVMA), yeasts and bacteria
52 [10]. However these have met with limited success either due to cost of production, their

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53 physical properties or their nutritional content. Therefore the development of a diet to replace
54 unicellular algae has a significant industrial value. The residue from enzymatic
55 saccharification of macroalgae is characterised in terms of its physical and nutritional
56 properties and its possible use as a MAS for aquaculture production.

57 The idea of using macroalgae as a MAS has been previously trialled, using similar
58 biotechnology to the saccharification step in biofuels production to produce a hatchery feed
59 for bivalve molluscs [11] and detrital particles of macroalgae are known to be an important
60 component of marine food webs [12, 13]. In this paper we investigated the nutritional aspects
61 of three potential seaweed candidates for mass cultivation and future biofuel production after
62 enzymatic treatment using cellulosic and hemicellulosic enzymes. This approach was used to
63 extract carbohydrates for biofuel production. Comparison of total carbohydrate, protein,
64 polyphenol, total C and N, lipid composition and ash content of *Alaria esculenta, Ulva*
65 *lactuca* and *Saccharina latissima* before and after treatment were used to determine the
66 suitability of the by-product for the production of alternate aquaculture diets, along with
67 preliminary trials to test their suitability as MAS in two economically important aquaculture
68 species, the bivalve *Ostrea edulis* and the sea urchin *Paracentrotus lividus*.

# 69 3. EXPERIMENTAL

## 70 3.1. MATERIALS

71 Small scale pre-treatment studies were carried out with freeze dried *Alaria esculenta*
72 (collected in May 2011), *Saccharina latissima* (October 2011) and *Ulva lactuca* (July 2011)
73 all harvested from Clachan Sound in Scotland (56.3N, 5.6E). Chemical characterisation

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74 studies were carried out to determine ash, polyphenol, carbohydrate, protein, lipids, carbon
75 and nitrogen content of the seaweeds before and after enzymatic treatment. The frond from
76 the macroalgae were air dried and ground to a fine powder in a twin cutting stainless steel
77 blade grinder (DeLonghi 170 W, Model KG40) until particles passed through a 1 mm
78 laboratory test sieve (Endecotts BS 410-1). Sub-samples of the seaweed powder (500 to 1000
79 mg) were removed to determine residual moisture content. Seaweed powders were stored in
80 air tight plastic or glass containers at room temperature.

81 ## 3.2. BIOFUEL TREATMENT

82 The species *U. lactuca*, *S. latissima* and *A. esculenta* were enzymatically treated using
83 cellulosic and hemicellulosic enzyme blends provided by Novozymes, Denmark.
84 Approximately 13.00 ± 0.02 g of dried seaweed was added to a 250 ml Duran glass bottles
85 with 100 ml of deionised water. The pH of the solution was adjusted to 5.2 with 10 % HCl
86 and bottles autoclaved for 15 minutes at 121°C. Once cooled to 45°C in a water bath,
87 enzymes were added at 10 % NS 22086 (w $w^{-1}$) and 1.2 % NS 22119 (w $w^{-1}$) and bottles
88 incubated at 45°C and 200 rpm in an orbital shaker (New Brunswick Scientific, Innova 4230)
89 for 2 days.

90 Digested seaweed was centrifuged for 10 minutes at 3,200 *g* and residue washed with equal
91 volumes of deionised water before re-centrifugation. Washed solids were frozen at -20°C and
92 vacuum freeze dried until dry.

93 ## 3.3. CHEMICAL ANALYSIS OF SEAWEED

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94 ### 3.3.1. ASH CONTENT

95 Determination of residual moisture of seaweeds was based on removing the moisture content
96 of organic matter by a convection oven drying procedure at 105°C until the weight remained
97 constant. A standardized method was used to determine the ash content by removing the
98 organic (volatile) content through oxidation (550°C) from oven dried (105°C) seaweed [14].

99 ### 3.3.2. TOTAL CARBOHYDRATE ANALYSIS

100 For total carbohydrate analysis 50 ± 5 mg of dried seaweeds was weighed into 10 x 100 mm
101 Pyrex® screw capped glass tubes and soaked with 0.25 ml of a 72 % (v $v^{-1}$) sulphuric acid
102 solution for 60 minutes at 30°C in a water bath. Addition of 4.2 ml of deionised water
103 resulted in a 1 M sulphuric acid solution which was autoclaved for 15 minutes at 121°C. All
104 solutions were finally diluted after autoclaving to 5 ml total volume. The total carbohydrate
105 content of seaweed biomass was determined from acid hydrolysates using a phenol/ sulphuric
106 acid method described by [15]. The results were expressed as a percentage of g of glucose
107 equivalents (GE) per g of dry weight.

108 ### 3.3.3. TOTAL POLYPHENOLICS

109 The polyphenol content was extracted using an acidified aqueous acetonitrile mix and
110 quantified using a Folin Ciocalteau colourimetric method [16]. This solvent contained an
111 equal amount of acetonitrile 50 % (v $v^{-1}$) with acidified deionised water 50 % (v $v^{-1}$) which
112 contained 0.2 % (v $v^{-1}$) formic acid. Approximately 100 ± 10 mg of dried seaweed was
113 weighed into 2 ml micro centrifuge plastic tubes to which 1 ml of solvent was added. Tubes
114 were taped on to a rotary wheel, rotating at a 45° angle and mixed at 20 rpm at room

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115 temperature for 1 hour. After completion, tubes were centrifuged for 2 minutes at 16,200 *g*
116 and supernatants transferred to 5 ml volumetric flasks. Extraction was repeated by the
117 addition of fresh solvent and re-suspension of the cell pellet on the rotary wheel for a further
118 hour. After centrifugation the cell pellet was washed once with 1 ml of solvent and all
119 supernatants and wash solvents combined in the volumetric flasks, which was made-up to 5
120 ml with solvent.

121 A 20 µl centrifuged sample (2 minutes at 13,200 *g*) was diluted with 1580 µl of deionised
122 water in a 2 ml micro centrifuge tube, to which 100 µl of Folin Ciocalteau reagent was then
123 added. This was mixed by brief vortexing and incubated for up to 8 minutes at room
124 temperature. A 300 µl volume of 20 % (w $v^{-1}$) sodium carbonate solution was then added and
125 incubated for 2 hours at room temperature. The absorbance of sample and gallic acid
126 standards (100-1000 mg $l^{-1}$) were measured at 765 nm and expressed as a percentage of g of
127 gallic acid equivalents (GAE) per g of dry weight.

128 **3.3.4. PROTEIN DETERMINATION**

129 Seaweed proteins were extracted using a combined acid and alkaline extraction method
130 developed for release of proteins from microalgal biomass [17] and quantified using a Folin-
131 Ciaulteau method. 50 ± 5 mg of dried seaweed was heat treated with 2 ml of a 24 % (w $v^{-1}$)
132 trichloroacetic acid at 100°C for 15 minutes in a boiling water bath. Samples were cooled to
133 room temperature and diluted with 6 ml of deionised water and spun for 20 minutes at 3,200
134 *g* at 10°C. Supernatant was discarded and cell pellet dissolved in 4 ml of Lowry D reagent
135 and incubated for 5 minutes at 55°C in a water bath with the final volume being made-up to 5
136 ml in volumetric flasks. A 2 ml aliquot of protein extract was spun for 2 minutes at 13,200 *g*
137 to remove any residual solids. To 50 µl of supernatant, 975 µl of Lowry reagent D was added,

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138 mixed and incubated for 10 minutes at room temperature. To this 100 µl of Lowry reagent E
139 was added, mixed and incubated for 30 minutes at room temperature and absorption of
140 samples and bovine serum albumin (BSA) standards (100-1000 mg $l^{-1}$) measured at 750 nm.
141 The protein content was expressed as a percentage of g of BSA equivalents per g of dry
142 weight.

143 ### 3.3.5. Total Carbon and nitrogen

144 Seaweed samples were prepared for analysis by folding 2 ± 0.2 mg of dried material into tin-
145 foil disks. Samples were analysed with an ANCA NT prep system coupled with a 20-20
146 Stable Isotope Analyser (PDZ Europa Scientific Instruments, Northwich, UK). Calibration
147 was performed using a solution of isoleucine (L-Isoleucinie, Europa STD) at concentrations
148 of 1 µg N and 6.6 µg C. Standards were placed in tin caps (with Chromosorb W, PDZ Europa
149 ltd) and oven dried at 60°C overnight. Calibrations were performed with a series of standards
150 from 5 to 200 µg N (33– 1320 µg C) run at the beginning of each analysis. Calibration curves
151 typically gave an $r^2$ of 0.99 for both C and N (n=15).

152 The carbon and nitrogen was expressed as a percentage of g of carbon or nitrogen per g of
153 dry weight.

154 ### 3.3.6. Lipid composition

155 Direct-derivatisation of fatty acids to methyl esters (FAME) using methanolic-HCl was
156 carried out according to Methods described by [18-20]. Samples were analyzed by gas
157 chromatography (GC) using a ZB-Wax column (Phenomenex, Værløse, Denmark) with

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158 flame ionization detection (GC-2014, Shimadzu, Kyoto, Japan). The lipid content in
159 seaweeds was expressed as a percentage of g of total FAME fatty acids per g of dry weight.

## 160 3.4. Feeding trials

### 161 3.4.1. Bivalve Feeding Trial

162 The feeding trials were conducted with European Flat Oyster (*Ostrea edulis*) spat that were
163 approximately 3mm in size. Approximately 60 individuals (total weight 1 gram) each were
164 placed in 12 identical 3 litre bowls. The spat were contained within a mesh stand stand within
165 the bowl, and were fed a daily ration of the biofuels residue (*U. lactuca*, *S. latissima* and *A.*
166 *esculenta*) or as a comparison they were fed an industry standard commercially available mix
167 of non-viable microalgal cells (Reed Mariculture Instant Algae®). The water in each bowl
168 was changed daily and was circulated using an air-stone placed at the bottom of the bowl.
169 Each treatment was replicated three times. The feeding trial lasted for 15 days and the
170 specific growth rates were calculated between day 0 and 15.

### 171 3.4.2. Echinoderm Feeding Trial

172 The feeding trial was conducted using the economically important purple sea urchin
173 *Paracentrotus lividus*. One year old *Paracentrotus lividus*, (20 - 27mm) horizontal test
174 diameter taken from cultured stocks produced at Scottish Association for Marine Sciences,
175 Scotland. Five individuals of approximately 110mm in test diameter were placed in each of
176 three replicated 7 litre tanks. Each urchin in three of the replicate tanks was fed one of three

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artificial, pelleted diets at a constant rate of approximately 5 % of their mean body weight every 2-3 days. Leftover uneaten food & faeces was removed carefully by siphoning around the urchins before fresh feed was introduced. The sea urchins were fed a either a standard pelleted sea urchin diet (Reed Mariculture Instant Algae®) or the standard sea urchin diet [21, 22] with a 10 % inclusion of the biofuels residue produced from *S. latissima* (Digestate ) or with a 10 % inclusion of fresh *S. latissima*. The urchins were held at 18ºC and their growth monitored for 4 weeks.

### 3.5. STATISTICAL ANALYSIS

Experimental error was determined for triplicate assays and expressed as standard deviation (SD). Associations as well as significance of differences in total nitrogen content vs protein concentration were determined by Pearson correlation analysis and 1-way Analysis of Variance (ANOVA).

## 4. RESULTS AND DISCUSSION

### 4.1. CHEMICAL ANALYSES

In all three seaweeds the ash content was lower after enzymatic saccharification (Fig. 1). In *U. lactuca* the decline accounted for 12 %, in *A. esculenta* for 32 % and in *S. latissima* for 17 %. As seaweeds are commonly rich in salts containing potassium, sodium and chloride ions [23, 24], it is likely that aqueous extraction methods such as enzymatic saccharification will extract preferably highly soluble salts [24] and therefore reduce the sodium, potassium and

Pre production Proof

196 chloride content in seaweed residue. In contrast, leaching of kelps with freshwater has also
197 shown that significant retention of di- and tri-valent cations in residue was achieved, but as
198 the predominant cations in kelps are mainly sodium and potassium, overall concentrations of
199 total metals in leached residue were lower [24]. It was also noted that a total weight loss of
200 51% in kelp meals occurred during freshwater leaching, largely due to the dissolution of
201 sodium, potassium, chloride and soluble organics such as mannitol and fucoidans [25].

202 The total carbohydrate content in treated seaweeds was found lower, were a reduction of 48,
203 32 and 18 % was seen in *U. lactuca, A. esculenta* and *S. latissima,* respectively (Fig. 1). In a
204 similar study a carbohydrate removal of 48 % was also achieved using the same enzymes on
205 the green seaweed *U. fasciata* [26]. As green seaweeds tend to contain higher cellulose levels
206 than brown or red seaweeds [27] these cellulosic enzyme blends are likely to be more suited
207 for the saccharification of cellulosic seaweeds than alginophytic algae. Leaching of brown
208 seaweeds with freshwater has shown that structural components such as alginate were almost
209 totally retained in brown seaweed residue, whilst the intracellular content containing mannitol
210 was lost [24]. Residual alginate in this experiment might have been a reason for reduced
211 enzyme accessibility to other embedded polysaccharides, leading to lower carbohydrate
212 removal rates in brown seaweeds. Despite the partial removal of carbonaceous compounds
213 such as carbohydrates, total carbon content in all three seaweed residues increased by 8% in
214 *A. esculenta*, 17% in *S. latissima* and 27% in *U. lactuca* (Fig. 1). A 21% increase in total
215 carbon content has also been observed in freshwater leached brown algae, were the increase
216 in carbon in residue was the result of the removal of mostly inorganics and the increase in
217 concentration of alginate [25]. In *U. lactuca* it remains unclear which carbohydrates increased
218 in concentration, but is also likely that structural carbohydrates such cellulose, xylans and
219 mannans which do commonly form part of the cell walls of green algae [28], increased in
220 concentration.

Pre production Proof

221

222 Polyphenol levels were highest in *A. esculenta,* followed by *S. latissima* which accounted for
223 0.83 % and 0.30 % of the biomass, respectively (Fig. 2). In both cases, polyphenol levels
224 were below detection level after enzymatic treatment. In contrast polyphenolic content in
225 treated *U. lactuca* increased threefold to 0.18 %. Despite the increase, these polyphenol levels
226 were still well below the initial concentration of both kelp species (Fig. 2). It remains unclear
227 why polyphenols in *U. lactuca* increased during enzymatic treatment, but it is likely that
228 polyphenols remained in insoluble form, bound to the seaweed matrix. As a consequence of
229 reducing soluble or solubilised seaweed components during enzymatic extraction, residual
230 polyphenols and other insoluble components increased therefore in concentration.

231 Differences between carbohydrates and total carbon as well as protein and total nitrogen
232 content was significant ($p$<0.001) in all three species, where total nitrogen content increased
233 in all three seaweeds after treatment (Fig. 3), which was not matched by its protein content
234 (Fig. 2). Protein enrichment in enzymatically treated seaweed was only seen in the green
235 seaweed *U. lactuca*, were protein concentration increased by 41%. In contrast, total protein in
236 the brown species *A. esculenta* and *S. latissima* declined by 38 and 50%, respectively. Other
237 research has shown that aqueous extraction of kelp meals increased the total nitrogen content
238 in residue by 56% as a consequence of removing its inorganic content [25]. In this study, a
239 positive correlation between protein and total nitrogen was only found in *U. lactuca* ($r$=0.91,
240 $p$<0.0001). As nitrogenous compounds such as peptides, amino acids, nucleotides and amines
241 can contribute towards 20% of the total nitrogen content in seaweeds [29] it remains unclear
242 which non-protein nitrogenous compounds in both brown seaweed residues increased in
243 concentration in this study, despite the nitrogen loss through the decline in total proteins.

Pre production Proof

244 The total fatty acid content increased in all three species after enzyme treatment. The largest
245 increase in fatty acids was seen in *A. esculenta* where fatty acids increased 8 fold, followed
246 by a threefold increase in *U. lactuca* and a 38 % increase in *S. latissima* (Fig. 4). Since algal
247 cell walls and membranes persist permeation by extraction solvents, it has been demonstrated
248 on micro- and macroalgae through enzyme-assisted extraction processes, that lipid extraction
249 efficiencies can be enhanced by more than 4 fold [30, 31]. The 8 fold increase in total fatty
250 acids in residue of *A. esculenta* is likely to be the result of a high inorganic removal rate in
251 combination with enhanced lipid extraction efficiencies using enzymatic saccharification.

252 ## 4.2. FEEDING TRIALS

253 The feeding trial showed that the biofuels residue was capable of supporting both oyster spat
254 and sea urchins, confirming the value of the residue as a potential aquaculture diet
255 replacement or substitute. In the oyster spat trial, there was an initial lag of growth over the
256 first five days, however by day 15 the performance of the MAS was comparable to the
257 currently used industry alternative Reeds Paste (Fig. 5), with the exception of the MAS
258 derived from *Ulva* which continued to perform poorly. In the echinoderm trial the MAS
259 produced growth rates equivalent to those produced from diets that included fresh *S. latissima*
260 (Fig. 6).

261 Results from these feeding trials confirmed other findings where seaweed detritus was
262 successfully trialled as a feedstock in aquaculture using red seaweeds on tropical oyster spat
263 [32], brown seaweeds on brine shrimps [33] and green seaweeds on freshwater shrimps [34].
264 Substitution of up to 30 to 50% of the dietary composition with seaweed detritus has been
265 achieved without compromising on growth rates, viability and quality [32, 34].

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## 5. CONCLUSIONS

Seaweed residue has shown to possess a high nutritional value after a simulated biofuel treatment using cellulosic and hemicellulosic enzymes. In the two kelp species *A. esculenta* and *S. latissima* the nutritional aspect of seaweed residue was enhanced through the total removal of polyphenols, the partial removal of ash and carbohydrate and the increase in total nitrogen and lipid content. The nutritional value of *U. lactuca* was also enhanced through the Biofuel Method, as ash and carbohydrates also declined, while protein, total nitrogen and lipid content increased. This study highlighted the nutritional value of seaweed residue, a likely left-over product after biofuel refining. This enhanced residue has the potential to be applied in aquaculture as an inexpensive feedstock material, which can also be easily produced at large scale.

## 6. ACKNOWLEDGEMENTS

This project was funded under the Genomia Fund award to ADH and from the European Union's Seventh Framework Programme for research; technological development and demonstration under grant agreement no 606042. Thanks also go to Mr Lars Brunner for his help in the seaweed collection.

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