

Hydrolytic effects of acid and enzymatic pre-treatment on the anaerobic biodegradability of *Ascophyllum nodosum* and *Laminaria digitata* species of brown seaweed

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4

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12

13 **ABSTRACT**

14 Abundant marine biomass in coastal regions has continued to attract increasing attention in
15 recent times as a possible source of renewable energy. This study aimed to evaluate the
16 effects of hydrolytic pre-treatment for the purpose of enhancing biogas yield of *Laminaria*
17 *digitata* and *Ascophyllum nodosum* species found on the west coast of Scotland. Results
18 show that *L. digitata*, in its natural and untreated form, appears to be more readily
19 hydrolysable than *A. nodosum*. Two treatments were assessed: acid only and acid followed
20 by enzyme. Both treatments enhanced the hydrolysis of both seaweed species, with acid-
21 enzyme treatment providing a better performance.

22

23 **Keywords:** Acid hydrolysis; acidogenesis; *Ascophyllum nodosum*; enzymatic hydrolysis;
24 *Laminaria digitata*; volatile fatty acids production.

25

26 **1. INTRODUCTION**

27 The need for sustained energy security has led to the realization of the need for alternatives
28 to fossil fuels [1,2]. This coupled with the need to mitigate greenhouse gas emissions, which
29 is believed to be the major cause of climate change has simulated increased research into
30 alternative energy sources [1,3-6]. In order to achieve global reduction in greenhouse gas
31 emissions, countries across the world have set targets on the amount of energy to be
32 generated from renewable sources. For instance, the European Union has set a binding
33 target of 20% of energy use by member states to be generated from renewable sources by
34 2020 [1], while the USA plans to replace 75% of its imported oil by renewable energy by
35 2025 [7]. Scotland aims to generate 100% of its electricity needs from renewable sources by
36 2020 [8].

37 Various means of generating renewable energy including solar, wind, hydro, tidal and
38 biomass energy have been reported in literature [1,9,10]. Biomass energy has received
39 significant attention due to its availability, ease of utilisation and the relative maturity of the
40 technology involved [1,10-12]. A huge amount of scientific publications on biomass energy
41 (56%) in relation to other sources of renewable energy has been published in the last 30
42 years [13]. Of particular interest is the use of marine biomass for renewable energy
43 production [12,14,15]. Marine macroalgae have many advantages over terrestrial energy
44 crops such as lack of competition with agricultural practices for land and high growth rates.
45 It can also withstand different environmental and nutritional conditions and much is known
46 about their cultivation processes. These factors make algae biomass a promising energy crop
47 for increased energy security and greenhouse gas emission mitigation across the world and
48 in the UK in particular [1,15-17].

49 For efficient macroalgae conversion into energy, various components making up the biomass
50 must be amenable to biodegradation. Alginate is the main structural compound and the

51 most abundant polysaccharide in brown seaweed. While the intercellular matrix of the
52 brown algae is dominated by alginate, the cell walls also contain cellulose, fucoidan and
53 protein [18-20]. These polysaccharides are broken down during hydrolysis prior to biogas
54 production. Another polysaccharide is laminarin, the main storage carbohydrate in Laminaria
55 species. Fucoidan is another storage carbohydrate present in brown algae and made up of
56 sulphated fucan. The absence of lignin and low cellulose content of algae makes it more
57 suitable for microbiological conversion to energy fuels than terrestrial plants [21,22]. Since
58 seaweeds have growth and primary production rates that exceed those of most terrestrial
59 plants, the concerns over feed stock supply would be considerably reduced compared to
60 terrestrial crop, when used for energy production [16,23].

61 Seaweeds have been found to be suitable feedstocks for biogas production via anaerobic
62 digestion processes [19,21,24]. This is due to the presence of readily hydrolysable sugars
63 (e.g. alginate and laminaran) present in the seaweeds, with low amount of cellulose and zero
64 lignin content [21]. However, hydrolysis remains the rate limiting step in the anaerobic
65 digestion of biomass (including marine biomass) [11,14,25]. Different pre-treatment
66 methods have been reported in literature to enhance hydrolysis of algal biomass including;
67 mechanical chopping, grinding, ultrasonic treatment, ozone oxidation, thermal treatment,
68 alkaline treatment and Fenton pre-treatment [14]. Others include heating and milling to
69 reduce the particle size to 1-5 mm. It has been reported that the process of releasing sugars
70 from algal biomass can be enhanced by the combination of acid hydrolysis followed by
71 treatment with a cocktail of different enzymes rather a single enzyme [26]. Enzymatic pre-
72 treatment employing multienzymatic preparations containing cellulase is reportedly
73 effective in addressing the heterogeneous nature of the algal carbohydrates [27].

74 The aim of this study is therefore to determine the effects of acid and enzymatic pre-
75 treatment methods on two of the most common types of seaweeds, *Laminaria digitata* and
76 *Ascophyllum nodosum*, using acid treatments and acid plus multienzymatic preparations.

77

78 **2. MATERIAL AND METHODS**

79 **2.1 Collection and preliminary treatment of seaweed samples**

80 The seaweed (*A. nodosum*) was collected at Broughty Ferry beach, Dundee, UK while *L.*
81 *digitata* was collected at Arbroath beach, UK in March, 2010. After collection, the seaweed
82 was placed on foil covered trays and dried in a drying cabinet at 80°C for two days. The dried
83 seaweeds were crushed and milled using a hammer mill (Retsch, fitted with a 1 mm screen)
84 to create a powder that was used for the experiments. This was done to reduce the particle
85 size to increase the surface area available for effective biodegradation [28]. The seaweed
86 powder was stored in sealed containers at room temperature until used.

87

88 **2.2 Pre-treatment methods**

89 2.2.1. Acid and heat pre-treatment

90 Powdered seaweed (10 g) for *Laminaria digitata* and *Ascophyllum nodosum*, was weighed
91 into 250 ml Erlenmyer flasks in duplicate. 100 ml of 0.2M H₂SO₄ acid was added to each of
92 the samples, then covered and autoclaved for 1 hour at 121°C and allowed to cool. After
93 cooling, the pH was adjusted to 7.5±0.4 using drops of 35% NH₄OH solution.

94

95 2.2.2. Acid, heat and enzymatic hydrolysis

96 An enzyme cocktail was added to the prepared samples from above (section 2.2.1). The
97 commercial enzyme cocktail procured from Novozyme (Denmark) was used for the algal

98 biomass hydrolysis in the following proportion: Cellulose 6% w/w, β - glucosidase 0.6% w/w,
 99 Multi-complex 0.4% w/w, Hemi-cellulase 2% w/w and Xylanase 0.25% w/w, according to
 100 manufacturer instructions (Table 1). The enzymes were added to the mixture containing acid
 101 hydrolysed seaweeds after the pH has been adjusted to 5.5 (suitable for all enzymes) using
 102 drops of 35% NH_4OH solution. After the addition of the enzymes, the samples were
 103 incubated at 50°C and 100 rpm for 18 hours. The pH dropped slightly after enzymatic
 104 hydrolysis to 4-5, but not below the required range for any of the enzymes (Table 1). After
 105 enzyme hydrolysis samples were cooled to room temperature and the pH corrected to
 106 7.5 ± 0.4 using 35% NH_4OH solution.

107

108 **Table 1. Enzyme parameters used in this study (information from Novozymes A/S)**

Enzyme	Activity	pH	Temperature (°C)	Dose (%w/w seaweed)*
Cellulase complex	700EGU ^ε g ⁻¹	4.5-6.5	45-60	6.0
B-Glucosidase	250CbU ^τ g ⁻¹	2.5-6.5	45-70	0.6
Multi-complex	500FXU ^δ g ⁻¹	4.0-6.0	40-65	0.4
Xynalase	500FXU ^δ g ⁻¹	4.5-6.0	35-55	0.5
Hemicellulose	750FXU g ⁻¹	5.0-8.0	45-70	0.4

109 *Dose values were calculated based on 10% seaweeds substrate.

110 ^εEndoglucanase units ^τ β -Glucanase units ^δfungal xynalase units

111

112 **2.3 Anaerobic digestion**

113 2.3.1 Culture media

114 Non-growth synthetic medium was prepared for the anaerobic digestion process using the
 115 following compounds; 2.7 g/l KH_2PO_4 (strong buffer agent), 3.5 g/l K_2HPO_4 (strong buffer
 116 agent), 5 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mg/l CaCl_2 , 0.5 mg/l FeCl_3 , 0.5 mg/l KCl_3 , 0.1 mg/l CoCl_2 and
 117 0.1 mg/l NiCl_2 . The medium provided the essential nutrients required by the microorganisms

118 [29]. Anaerobically digested sludge was obtained from wastewater treatment plant in
119 Dundee (UK). The pH of the inoculum was 7.5 while the volatile solid content was 2.67 g/l.

120

121 2.3.2 Experimental design

122 Pre-treated feedstock (110 ml) was diluted with 190 ml of the non-growth medium and
123 seeded with 100 ml of anaerobically digested sludge to make up 400 ml of culture volume in
124 a 500 ml capacity culture bottle for each of the experimental condition tested. The culture
125 bottles were then purged with nitrogen gas and incubated at mesophilic temperature of
126 37°C for 25 days. Blank samples containing only the inoculum and medium were set up to
127 discount anaerobic digestion activities due to residual substrates in the inoculum. The pH of
128 the cultures was adjusted to 7.4 at the start of the experiment using drops of 35% NH₄OH
129 solution. All experimental set-ups were prepared in duplicates. Samples of about 20 ml were
130 collected at regular intervals from each culture bottle and analysed for pH, volatile fatty
131 acids concentration, total and volatile solids.

132

133 **2.4 Analytical methods**

134 The protein content of the seaweed species was analysed employing the Coomassie
135 (Bradford) protein assay. Proteins were extracted from seaweed powder using 2M NaOH in a
136 proportion of 10% seaweed powder and 90% NaOH, incubated at 65°C at 150 rpm for 60
137 minutes. Samples were centrifuged and the supernatant used for the protein assay. Total
138 carbohydrate content was determined by hydrolysis using the methods described in the
139 NREL Chemical and Testing procedure (NREL 1996). The amount of reducing sugars and the
140 specific sugars produced after acid and enzyme hydrolysis was determined using high-
141 performance liquid chromatography (HPLC) analysis. pH was evaluated using pH meter

142 Senslon 3 (HACH). Gas produced in the batch reactors was measured with gas analyser GA
 143 2000 Geotechnical Instrument (England) after which the reactor bottles were sealed with
 144 silicon to avoid gas leakages and maintain anaerobic conditions. Volatile fatty acids (VFAs)
 145 concentrations of the anaerobic cultures were determined by esterification method [30].
 146 Total and volatile solids content was determined according to standard methods [31].

147

148 **2.5 Statistical analysis**

149 Experimental error was determined for duplicate assays and expressed in standard
 150 deviation. The significance of differences in reducing sugar yields and volatile acid formation
 151 were determined by one-way analysis of variance (ANOVA). Statistical significant
 152 interactions were further analysed using post hoc test (Tukey) at 95% confidence interval.
 153 Differences between species and across treatments were also determined. All statistical
 154 analyses were performed using Minitab Statistical Software version 17.0.

155

156 **3. RESULTS AND DISCUSSION**

157 **3.1 Algal composition**

158 Table 2 shows the composition of algae used in the study.

159 **Table 2: Characterisation of experimental seaweeds prior to treatments and anaerobic**
 160 **digestion**

Component	<i>A. nodosum</i>	<i>L. digitata</i>
Total Carbohydrate (%)	57.84	64.47
Protein (%)	2.12	2.64
Others ^a (%)	20.52	13.12
Ash	19.51	19.63
VS (%)	80.49	80.33
TS (% wet solid) ^b	24.7	26.4

161 ^aother components of algae such as lipid were determined by the difference in 100% determined components.

162 ^bTotal solids in seaweeds were determined by drying wet seaweeds at 105°C for 24hours.

163 Table 2 shows significant differences in the composition of both species of seaweed
164 especially in both the protein ($P<0.032$) and carbohydrate contents ($P<0.003$), both of which
165 are greater in *L. digitata*. These characteristics seem to suggest that the *L. digitata* is likely to
166 be more readily biodegradable than *A. nodosum*. Algal biomass composition is known to
167 vary depending on the time and season of harvest [15,17,20]. It has been reported that the
168 amount of laminaran and mannitol present in *L. digitata* are lowest around March and reach
169 a peak between June and July [15]. A similar trend has also been found for *A. nodosum* [19].
170 The seaweeds used for this study were harvested in March, suggesting that the total
171 carbohydrate content shown in Table 2 may be considered as being lower than average
172 value for the species.

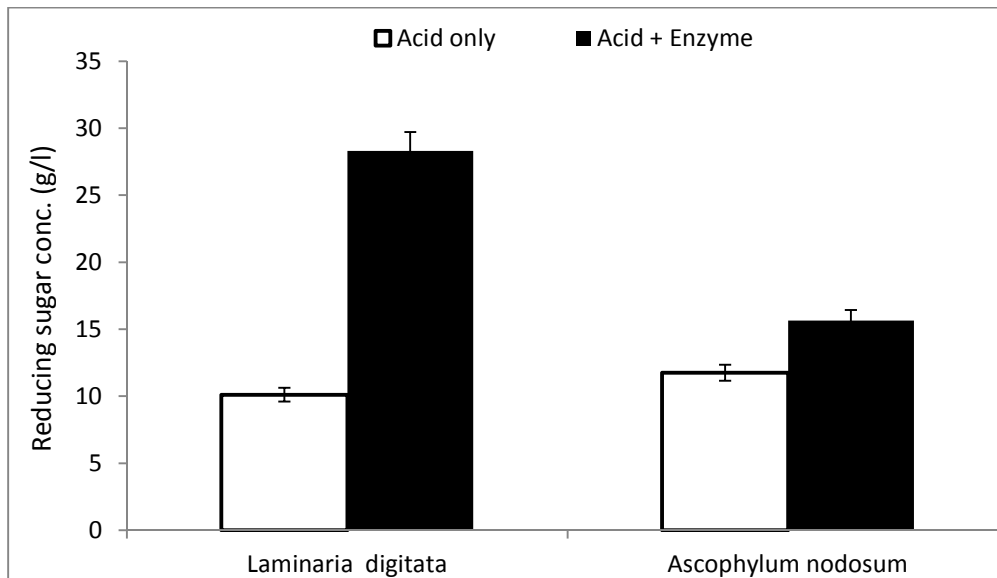
173

174 **3.2 Effect of hydrolytic pre-treatment on the production of reducing sugar**

175 The effectiveness of the hydrolysis process in this study has been assessed by the
176 determination of the amount and type of monomers produced. Figure 1 shows an increase
177 in sugar production after enzymatic hydrolysis in both seaweed cultures.

178 During acid treatment, 11.8 and 10.11g/l of sugar were produced by *A. nodosum* and *L.*
179 *digitata* respectively showing that sugars produced by *A. nodosum* was significantly higher
180 than that of *L. digitata* ($P<0.015$). However, after further enzyme treatment there was
181 significant increase ($P<0.0001$) in reducing sugar production by *L. digitata* from 10.11 to 28.3
182 g/l.

183



184

185 Figure 1. Reducing sugar concentration after acid and enzymatic hydrolysis of the two
 186 seaweeds.

187

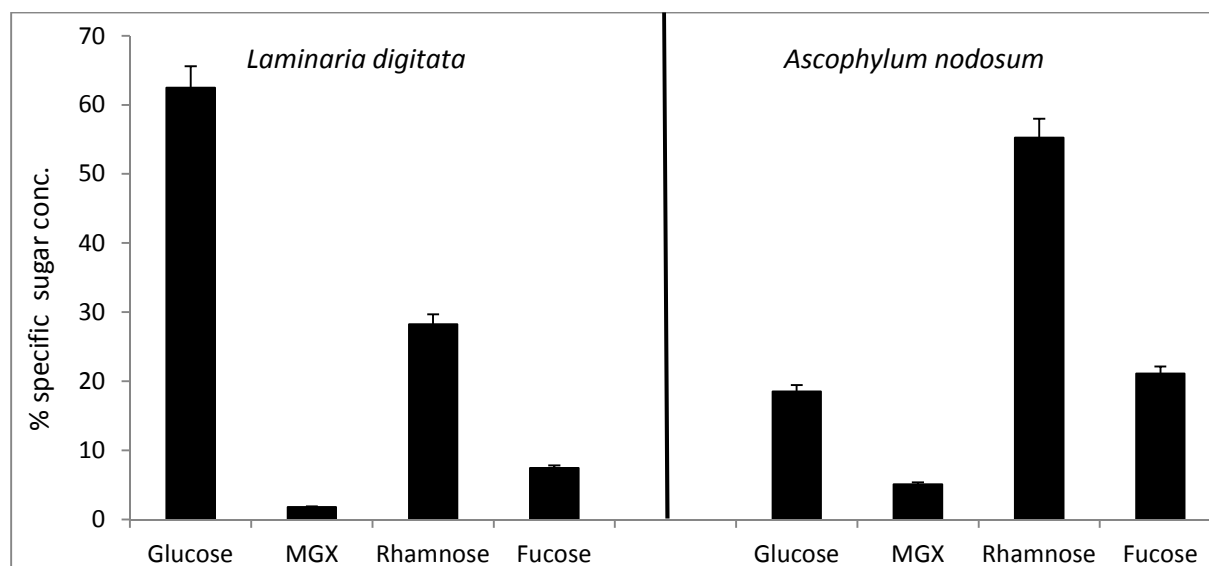
188 Similarly, addition of hydrolytic enzymes to acid treated *A. nodosum* cultures resulted in
 189 significant increase in reducing sugars production ($P < 0.002$) from 11.8 to 15.64g/l. Tukey's
 190 post hoc comparison of reducing sugar production between the two seaweeds cultures
 191 shows that significantly higher reducing sugars ($P < 0.0001$) were produced by *L. digitata* than
 192 by *A. nodosum* after enzyme hydrolysis as *L. digitata* produced 81% more sugars.

193 Analysis of the specific monomers that make up the reducing sugars showed the presence of
 194 glucose, MGX, (mannose, galactose and xylose analysed together), rhamnose and fucose as
 195 shown in Figure 2.

196 For the *L. digitata* culture, glucose accounted for most (about 63%) of the reducing sugar
 197 produced while rhamnose accounted for the highest amount (55%) of the reducing sugar
 198 produced in the *A. nodosum*. This relative abundance of glucose in *L. digitata* compared to *A.*
 199 *nodosum* is likely to have significant impact on the relative rates of biodegradation of both
 200 seaweed species. The results of this study seem to support the literature reports that

201 various bonds linking the polymers that make up algal biomass are broken during hydrolysis
202 to produce monomers (sugars), which could readily be converted to bioenergy [20,32].

203



204

205 Figure 2. Percent specific sugar in *L. digitata* and *A. nodosum* after enzyme hydrolysis. (Note:
206 MGX= Mannose + Galactose + Xylose)

207

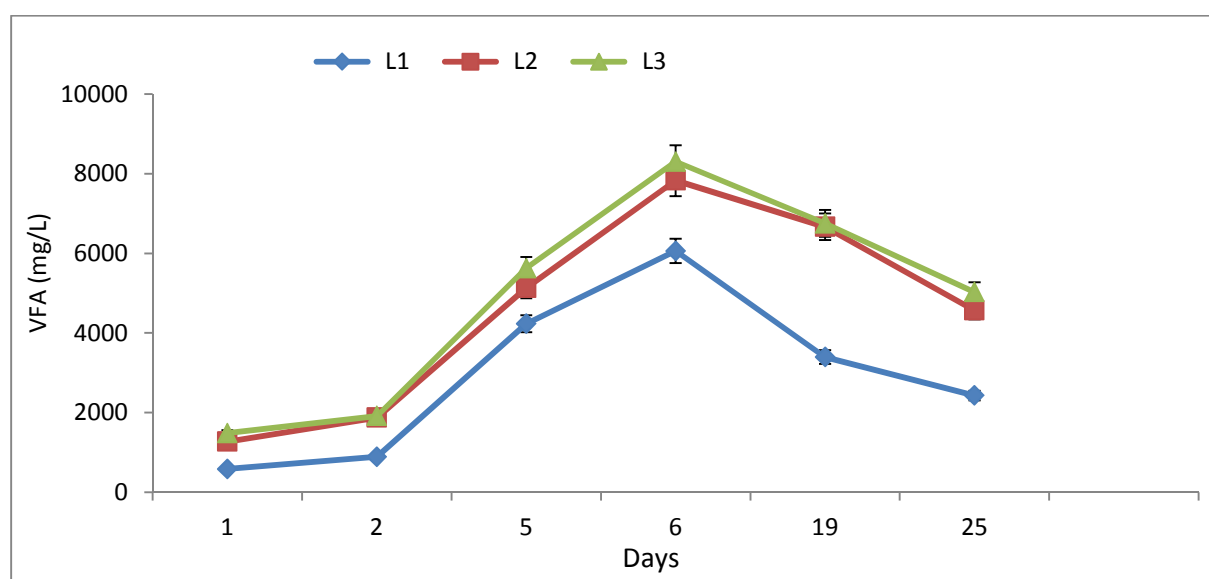
208 3.3 Effect of hydrolytic pre-treatment on anaerobic biodegradability of the seaweed 209 species

210 To evaluate the effect of the various pre-treatment methods used in the study on the
211 anaerobic biodegradability of each of the seaweed species, anaerobic digestion of both
212 treated and untreated seaweed samples was carried out over a period of 25 days. Gas was
213 analysed (%) and released during the digestion process (data not included). Negligible
214 amounts of methane (<1%) were recorded in all batches in the first 6 days of digestion. The
215 acidogenic activity was used as a measure of the biodegradability potential of the various
216 untreated and pre-treated seaweed species. Figures 3 and 4 show the volatile fatty acids
217 (VFAs) production and accumulation obtained in each of the cultures during the

218 experimental period. Both figures indicate that acidogenesis was more dominant up to Day
219 6. Thereafter, a gradual decrease of the accumulated volatile fatty acids (VFAs)
220 concentration was observed, most likely due to their conversion to methane gas. The levels
221 of VFAs production up to Day 6 were used to evaluate the immediate impact of the various
222 pre-treatment methods used in this study on the substrates' level of biodegradability.

223

224



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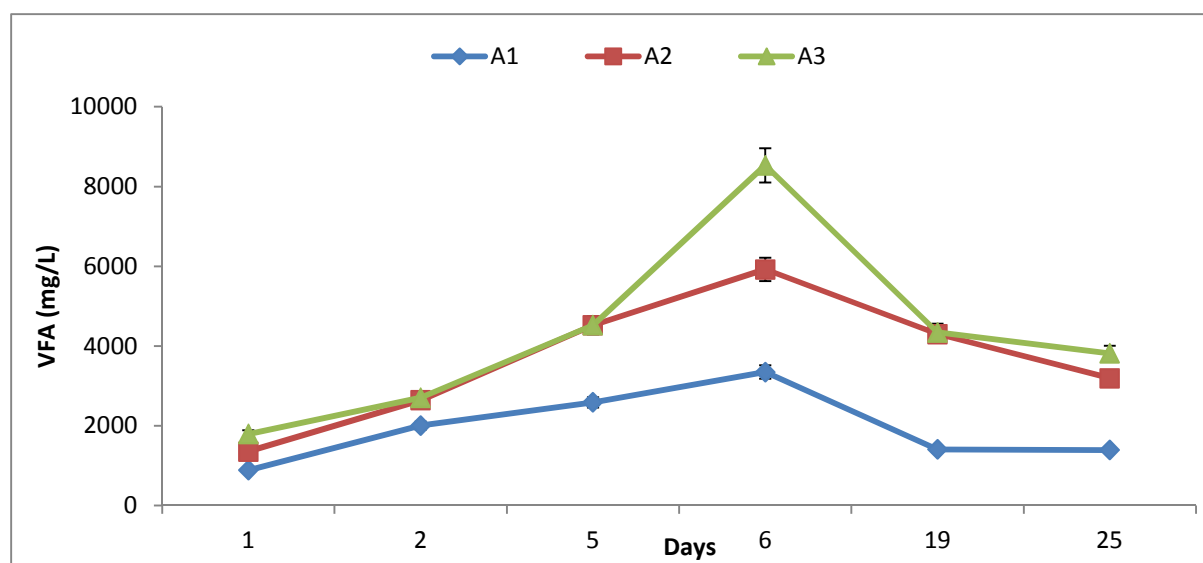
226 Figure 3. Volatile fatty acids accumulation in *L. digitata* cultures: L1: Untreated; L2: Acid
227 treated; L3: Acid/enzyme treated

228

229 In general, VFAs production and accumulation were greater in cultures containing pre-
230 treated seaweeds. One-way analysis of variance of peak VFAs production by *L. digitata* on
231 Day 6 shows that VFA production in acid treated (L2) was significantly higher ($P < 0.001$) than
232 untreated (L1) cultures. This is an indication that hydrolysis was enhanced by the addition of
233 acid. Further statistical analysis of results obtained from treated cultures highlighted a
234 significantly higher VFAs production ($P < 0.008$) in enzyme treated *L. digitata* (L3) than acid
235 only treated cultures (fig 3). This shows that the combination of acid and enzyme hydrolysis

236 is better than acid only treatment and further improves the digestibility of the seaweed
237 substrates.

238



239

240 Figure 4. Volatile fatty acids production in *A. nodosum* cultures: A1: Untreated; A2: Acid
241 treated; A3: Acid/enzyme treated

242

243 Analysis of VFAs production in *A. nodosum* cultures produced 3346 mg/l in untreated (A1)
244 which increased to 5921 mg/l after acid hydrolysis (A2). One-way analysis of variance of that
245 increase in fatty acids productions shows that it is statistically significant and suggests that
246 addition of acids to *A. nodosum* biomass enhances its biodegradation. ANOVA and Tukey's
247 pairwise post hoc comparison between VFAs produced in the acid only and the enzyme
248 treated *A. nodosum* cultures indicated that a significant increase occurred ($P < 0.001$) after
249 the addition of enzymes.

250 Comparison of VFAs production by untreated seaweeds (L1 and A1) on Day 6 shows that
251 VFAs produced by untreated *L. digitata* (L1) was 45% higher than VFAs obtained from
252 untreated *A. nodosum* (A1). Statistical analysis showed that this difference in VFA production
253 was significant ($P > 0.001$). This result indicates that untreated *L. digitata* is more readily

254 biodegradable than *A. nodosum*. This result is consistent with the observations shown in
255 Figures 1 and 2.

256 One-way ANOVA and post hoc analysis of VFAs production in acid treated cultures between
257 the two seaweeds showed that significantly higher (24%) levels of VFAs was recorded in acid
258 treated *L. digitata* (L2) than in acid treated *A. nodosum* (A2) ($P>0.002$). This suggests that
259 acid pre-treatment of *A. nodosum* increases its biodegradability to a level comparable to
260 that on untreated *L. digitata* (fig.3).

261 One-way ANOVA carried out on enzyme treated seaweeds (A3 and L3) shows that there no
262 significant difference in the amount of VFAs produced between *L. digitata* and *A. nodosum*
263 ($P>0.63$). Tukey pairwise post hoc analysis at 95% confidence interval also confirmed that
264 there's no significant difference between the means of VFAs produced by both seaweeds
265 when treated with enzyme. Although significant differences were observed when both
266 seaweeds were treated with acids, the differences observed diminished when the seaweeds
267 were further subjected to enzymatic hydrolysis. This is despite the fact that *L. digitata*
268 produced significantly higher concentration of reducing sugars. This might be due to the
269 production of other fatty acids not detected by the esterification methods employed in their
270 estimation.

271 In general, it can be seen that there is significant benefit in combining acid and enzyme pre-
272 treatment for both seaweed species.

273

274 **4 CONCLUSIONS**

275 This study has shown that acid and enzymatic pre-treatment of seaweed prior to anaerobic
276 digestion can enhance their hydrolysis, with the level of impact dependent on the seaweed
277 species. *L. digitata*, in its natural and untreated form, appears to be more readily

278 hydrolysable than *A. nodosum*. The pre-treatments used in this study have been shown to
279 have a greater effect on hydrolysis of *A. nodosum* than on *L. digitata*. Acid pre-treatment
280 alone can significantly enhance the hydrolysis of seaweed species. Enzymatic treatment
281 following acid-pre-treatment can further significantly improve on the hydrolysis of both
282 species of seaweed.

283

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286

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