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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ABSTRACT

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

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

1. INTRODUCTION

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

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

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

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

Seaweeds have been found to be suitable feedstocks for biogas production via anaerobic digestion processes [19,21,24]. This is due to the presence of readily hydrolysable sugars (e.g. alginate and laminaran) present in the seaweeds, with low amount of cellulose and zero lignin content [21]. However, hydrolysis remains the rate limiting step in the anaerobic digestion of biomass (including marine biomass) [11,14,25]. Different pre-treatment methods have been reported in literature to enhance hydrolysis of algal biomass including; mechanical chopping, grinding, ultrasonic treatment, ozone oxidation, thermal treatment, alkaline treatment and Fenton pre-treatment [14]. Others include heating and milling to reduce the particle size to 1-5 mm. It has been reported that the process of releasing sugars from algal biomass can be enhanced by the combination of acid hydrolysis followed by treatment with a cocktail of different enzymes rather a single enzyme [26]. Enzymatic pre-treatment employing multienzymatic preparations containing cellulase is reportedly 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 H_2SO_4 acid was added to each of
92 the samples, then covered and autoclaved for 1 hour at 121^{o}C and allowed to cool. After
93 cooling, the pH was adjusted to 7.5±0.4 using drops of 35% NH_4OH 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

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

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

Enzyme	Activity	pH	Temperature (^oC)	Dose (%w/w seaweed)*
Cellulase complex	700EGU$^{\varepsilon}$ g^{-1}	4.5-6.5	45-60	6.0
B-Glucosidase	250CbU$^{\tau}$$g^{-1}$	2.5-6.5	45-70	0.6
Multi-complex	500FXU$^{\delta}$ g^{-1}	4.0-6.0	40-65	0.4
Xynalase	500FXU$^{\delta}$ g^{-1}	4.5-6.0	35-55	0.5
Hemicellulose	750FXU g^{-1}	5.0-8.0	45-70	0.4

*Dose values were calculated based on 10% seaweeds substrate.
$^{\varepsilon}$Endoglucanase units $^{\tau}$β-Glucanase units $^{\delta}$fungal xynalase units

2.3 Anaerobic digestion

2.3.1 Culture media

Non-growth synthetic medium was prepared for the anaerobic digestion process using the following compounds; 2.7 g/l KH_2PO_4 (strong buffer agent), 3.5 g/l K_2HPO_4 (strong buffer agent), 5 mg/l $MgSO_4.7H_2O$, 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 0.1 mg/l $NiCl_2$. The medium provided the essential nutrients required by the microorganisms

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

2.3.2 Experimental design

Pre-treated feedstock (110 ml) was diluted with 190 ml of the non-growth medium and seeded with 100 ml of anaerobically digested sludge to make up 400 ml of culture volume in a 500 ml capacity culture bottle for each of the experimental condition tested. The culture bottles were then purged with nitrogen gas and incubated at mesophilic temperature of 37^{o}C for 25 days. Blank samples containing only the inoculum and medium were set up to discount anaerobic digestion activities due to residual substrates in the inoculum. The pH of the cultures was adjusted to 7.4 at the start of the experiment using drops of 35% NH_4OH solution. All experimental set-ups were prepared in duplicates. Samples of about 20 ml were collected at regular intervals from each culture bottle and analysed for pH, volatile fatty acids concentration, total and volatile solids.

2.4 Analytical methods

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

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

2.5 Statistical analysis

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

3. RESULTS AND DISCUSSION

3.1 Algal composition

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

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

Component	*A. nodosum*	*L. digitata*
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

[a]other components of algae such as lipid were determined by the difference in 100% determined components.
[b]Total solids in seaweeds were determined by drying wet seaweeds at 105^{o}C for 24hours.

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

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

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

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

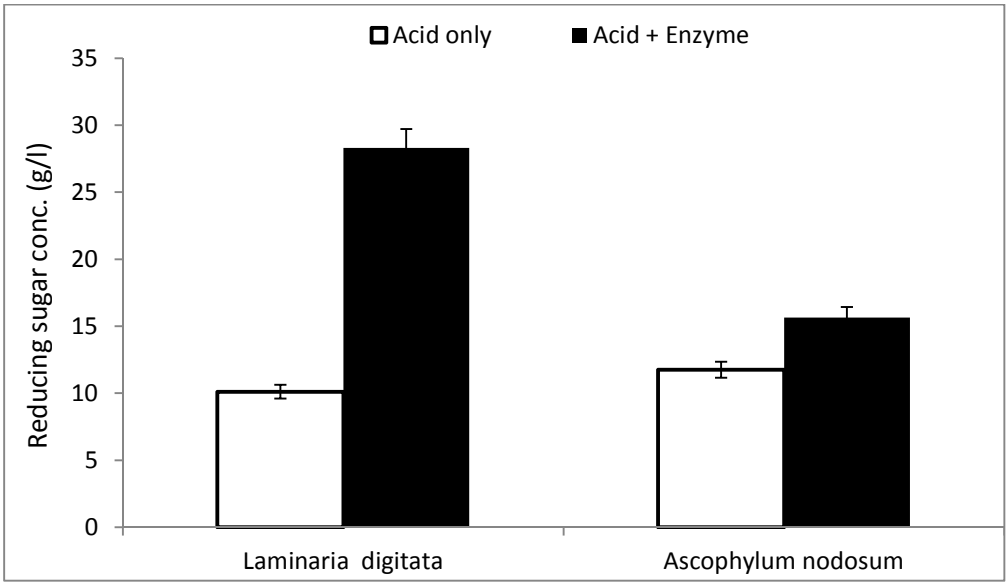


Figure 1. Reducing sugar concentration after acid and enzymatic hydrolysis of the two seaweeds.

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

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

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

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

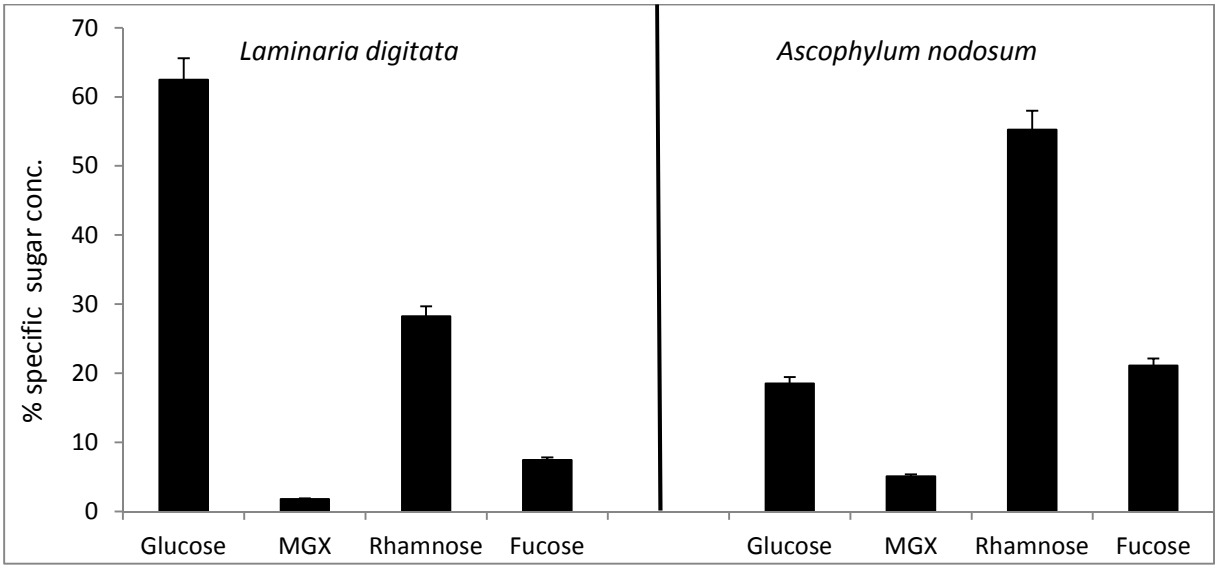


Figure 2. Percent specific sugar in *L. digitata* and *A. nodosum* after enzyme hydrolysis. (Note: MGX= Mannose + Galactose + Xylose)

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

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

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

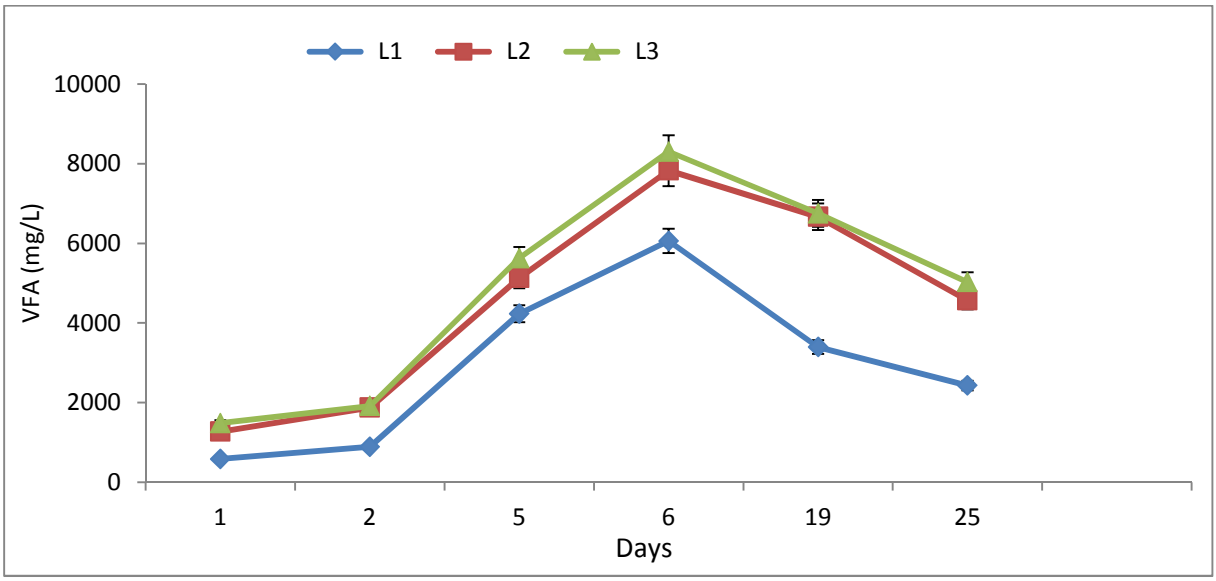
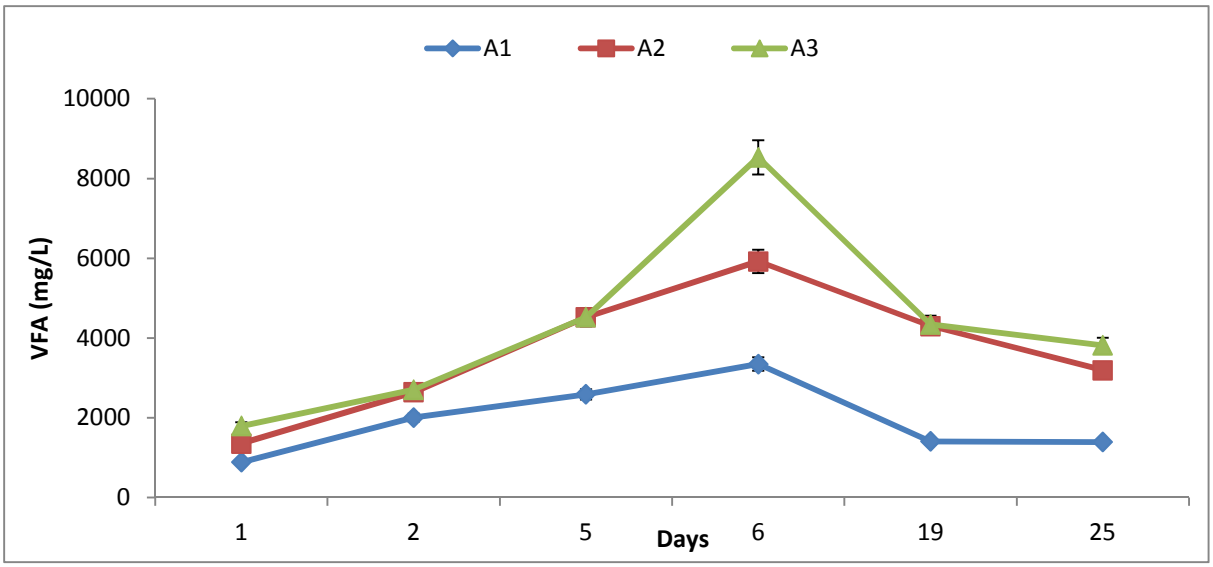


Figure 3. Volatile fatty acids accumulation in *L. digitata* cultures: L1: Untreated; L2: Acid treated; L3: Acid/enzyme treated

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

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

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

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

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

4 CONCLUSIONS

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

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

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