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6 **Microstructural and carbohydrate compositional changes induced by enzymatic**
7 **saccharification of green seaweed from West Africa**
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61
62 **Abstract**
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65 The use of green seaweed as carbon source for fermentation is gaining increasing attention due to
66 their high carbohydrate content and availability. Three green seaweed species *Chaetomorpha*
67 *linum*, *Ulva fasciata* and *Caulerpa taxifolia* were investigated for their amenability to enzymatic
68 saccharification. Microstructural changes were studied in order to understand the physical changes
69 occurring in the seaweeds during saccharification and to identify structural barriers. *C. linum* had
70 highest glucan content (20%), compared to 16% in *U. fasciata* and 6% in *C. taxifolia* indicating
71 large differences in composition. Glucose yields obtained after 24 hr of enzymatic saccharification
72 were 59, 38 and 60% for *C. taxifolia*, *U. fasciata*, *C. linum*, respectively, based on the glucan
73 content. Pre-autoclaving increased the saccharification yield to 81, 99 and 71%, respectively.
74 Morphologically, *C. linum* displayed unbranched filaments, *U. fasciata* two-cell layer large sheets
75 and *C. taxifolia* featured a leaf like structure. Enzymatic saccharification resulted in cell wall
76 degradation and release of the chlorophyll content in *C. linum*, delamination of sheets in *U. fasciata*
77 and surface erosion of leaves in *C. taxifolia*. *C. taxifolia* deviated in being very rich in β -1,3 linked
78 xylan (46%), which was only hydrolysed at 1% xylose yield due to lack of β -1,3-xylanase. Based
79 on the high cellulose content and no need for pre-treatment *C. linum* was optimal as glucose source
80 in fermentation and the presence of broadly acting GH3 β -xylosidase exo-activity would
81 presumably enable xylose release.
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104 Key words: *Chaetomorpha linum*; *Ulva fasciata*; *Caulerpa taxifolia*; Cellulase, Enzymatic
105 saccharification, Scanning electron microscopy
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1 Introduction

Seaweed is receiving increasing attention for bioethanol production, and as a source of new biorefinery products and food additives [1–5]. The major advantage derived from the use of seaweeds over terrestrial lignocellulose biomass includes no agricultural land usage and no resource input including fertilizer, pesticides and water [6].

In West Africa, there is great potential for seaweed cultivation and utilization to supplement fishery income and enable bioethanol and hydrocolloid production. In the West African country Ghana, seaweeds are abundant along the 540 km shoreline [7]. Seaweed is classified into three main groups including green (*Chlorophyceae*), brown (*Phaeophyceae*) and red seaweeds (*Rhodophyceae*). Green seaweeds are particularly interesting since they are the major type present along coastal shallow waters [8], but require further characterisation of monomer sugar composition, due to specific requirements in fermenting microorganisms and enzymatic saccharification. Green seaweeds common in Ghana include *Ulva fasciata*, *Caulerpa taxifolia*, and *Chaetomorpha linum*. These species have different carbohydrate composition, which in many cases is determined as reducing sugars [9–11]. These species deviate structurally and on carbohydrate composition in that *C. linum* is filament shaped [12,13], *Ulva* sp. form thin sheets and contain Ulvan [5,14] while *Caulerpa* forms thick leaf-like structures [15].

Previous reports show a glucose content at the level of 26% in *C. linum* [16] but with uncertainty regarding xylose and arabinose content. As result of lack of lignin, enzymatic saccharification of these carbohydrates does not require prior delignification, allowing direct enzymatic saccharification. However, only limited knowledge is available on the enzymatic saccharification of these seaweeds and more precise determination of the changes in seaweed microstructure is particularly important in order to understand if there are any differences that may affect the enzymatic processability.

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180 Commercial enzymes capable of hydrolysing cellulose and hemicellulose polysaccharides present
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182 in lignocellulose biomass have been explored for saccharification of green, brown and red seaweed
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184 [11]. These enzymes can potentially hydrolyse cellulose and other carbohydrates such as laminarin
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186 containing glucose linked with $\beta(1\rightarrow4)$ bonds in cellulose and glucose linked with $\beta(1\rightarrow3)$ -bonds
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188 containing $\beta(1\rightarrow6)$ -branches in laminarin [3,11]. It is therefore of considerable interest to
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190 determine the saccharification yield on green seaweed.
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192
193 It is known that *C. taxifolia* has a high content of β -(1,3)-xylan, which requires β -1,3-xylanase for
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195 its hydrolysis [17] while β -1,4-xylanase is present in commercial cellulase products such as Cellic®
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197 CTec2. In *U. fasciata*, it is known that xylose is β -1,4 linked to c-3 sulphated rhamnose [18]. There
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199 is thereby a potential for that Cellic® CTec2 can hydrolyse that bond. While the above mentioned
200
201 studies have focused on chemical composition, there is a lack of studies of microstructural effect
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203 on saccharification and microstructural changes.
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206 Pretreatment of lignocellulose and seaweed aiming at enzymatic saccharification are done to
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208 increase yields of glucose and other fermentable sugars. Temperatures in the range 170 to 200°C
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210 have been used for lignocellulose (wheat straw) [19] and seaweed (*C. linum*) [12] resulting in
211
212 increased cellulose conversion. However, the very low lignin content in seaweed is expected to
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214 reduce the needed pretreatment severity, thus the temperature as proved for *Laminaria digitate* [3].
215
216 Biological and chemical pre-treatment has been tested on *Ulva* sp. resulting in increased biogas
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218 yields [20].
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220
221 This study explored Cellic® CTec2 treatment of the seaweeds, *U. fasciata*, *C. taxifolia* and *C.*
222
223 *linum* to determine structural and compositional differences and their effect on enzymatic
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225 saccharification yields. Requirements for pre-treatment was investigated by assessing pre-
226
227 autoclaved seaweeds (120°C for 10 min). Morphological changes in surface structure and cellular
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229 contents caused by the hydrolysis were studied on the surfaces and for release of cell contents.
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231 Seasonal effects were not investigated since temperature variations are low in tropical regions. At
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239 the same time, the Danish sample was taken late during the summer when growth conditions were
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241 optimal due to high water temperature and high solar radiation.
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246 **2 Materials and methods**

247 **2.1 Seaweed harvest and experimental design**

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249 The seaweed samples *C. linum*, *U. fasciata* and *C. taxifolia* were collected during low tide on the
250
251 Ghanaian shore Prampram on May 5th 2017 (coordinates 5.71 °N, 0.13 °E) and rinsed with seawater
252
253 to remove impurities [21] followed by storage at -20°C. A reference sample of *C. linum* was
254
255 similarly collected in Roskilde Fjord, Denmark on Aug 21st 2015 (Risø Harbour; 55.692 °N, 12.083
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257 °E). The experimental design focused on comparing the composition of the species and *C. linum*
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259 harvested in Denmark was included for comparison; the sample treatments design is outlined in
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261 Fig. 1.
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267 **2.2 Materials and pre-autoclaving**

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269 All chemicals were purchased from Merck KGaA, Damstadt, Germany. The cellulase enzyme
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271 product used was Cellic® CTec2 (Batch VCS10008) (Novozymes A/S, Bagsværd, Denmark)
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273 containing 142 FPU/mL (filter paper activity units).
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275
276 Prior to pre-autoclaving, seaweed samples were dried at 50°C and ground to 3 mm size passing
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278 through a net. The dry matter content (DM) was adjusted to 10% (w/w) by adding distilled water
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280 and autoclaved at 121°C for 10 min. The autoclaving was done with two replicates.
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283 **2.3 Enzymatic saccharification of seaweed**

284
285 Enzymatic saccharification of seaweed was performed in 50 mM citrate buffer (pH 5.0) using
286
287 Cellic® CTec2, with 25 FPU/g DM at 50°C with a DM content of 4% (w/v). This enzyme dosage
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289 was used as it is on the same level as the one previously used for enzymatic hydrolysis of *C. linum*
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291 [12] and wheat straw [19]. Hydrolysis times tested were 0, 6, 24 and 48 hr with shaking at 1000
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298 rpm (Thermomixer Comfort; Eppendorf AG, Hamburg, Germany) in duplicate (n=2). An enzyme
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300 blank was tested in parallel. Enzymatic saccharification was terminated by heating to 96°C for 5
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302 min.
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304 **2.4 Compositional analysis of seaweed samples (solids)**

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306
307 Compositional analysis was done using two-step sulphuric acid hydrolysis adapted to seaweed
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309 samples in triplicate [22]. In step 1, the samples were hydrolysed at 30°C for 60 min in 72% (w/w)
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311 sulphuric acid using 100 mg DM per mL followed by dilution to 4% (w/w) sulphuric acid in step
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313 2 with hydrolysis at 121°C for 40 min. The hydrolysates were centrifuged at 5300 g for 10 min and
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315 analysed for monosaccharides by high-performance liquid chromatography (HPLC) outlined
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317 below. The non-extractable solid residue was analysed by drying at 105 °C and subtracting the ash
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319 content to burning at 550 °C for 3 hr (in lignocellulose samples reported as klason lignin). Algae
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321 contain considerable salts and protein and a part of it is derived from sulphated polysaccharides
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323 and protein.
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326 **2.5 Compositional analysis of extracts**

327
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329 Extracted carbohydrates were determined in duplicate after hydrolysis of the oligomer content into
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331 monosaccharides with 4% (w/w) sulphuric acid for 10 min at 121°C. Monosaccharides were
332
333 analysed using high-performance anion-exchange chromatography coupled to pulsed
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335 amperometric detection (HPAEC-PAD) [23] as stated below.
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337

338 **2.6 Chromatographic analysis**

339 *2.6.1 HPLC*

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342 Concentrations of monosaccharides were measured by HPLC (SIL-20AC; Shimadzu Corporation,
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344 Griesheim, Germany) with an Aminex HPX-87H Ion Exclusion Column (Bio-Rad Laboratories,
345
346 Copenhagen, Denmark). The temperature was 63°C, the eluent was 4 mM H₂SO₄ and the flow rate
347
348 was 0.6 mL/min. Carbohydrates were detected using a refractive index detector (RID-10A).
349
350

Standards of glucuronic acid, glucose, rhamnose and arabinose were used with retention times at 8.10, 9.22, 10.44 and 10.72 min, respectively [19].

2.6.2 HPAEC-PAD

In order to distinguish xylose and galactose, HPAEC-PAD was conducted using an ICS5000 system (Dionex; Thermo Electron A/S, Hvidovre, Denmark) equipped with a CarboPac™ PA10 column as described by Liu et al. [22].

2.7 Microscopy on seaweed

Light microscopy was carried out on small seaweed samples placed on glass slides in 50% (v/v) glycerol at 50 to 630 times magnification using a Leica DMLS bright field microscope (Leica Microsystems, Feasterville, PA, USA). Three pieces were viewed per sample resulting in 20 images.

For SEM microscopy, seaweed surface and cross sections were prepared. Samples were fixed in 3% v/v glutaraldehyde containing 2% para-formaldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 15 hr. Samples were thereafter washed in buffer and dehydrated in aqueous ethanol at 10%, 30%, 50%, 70%, 90% and 100% (15 min each). Samples were then critical point dried using an Agar E3000 critical point dryer (Agar Scientific, Stansted, Essex, UK) using liquid CO₂. Finally, samples were coated with gold using an Emitech E5000 sputter coater (Quorum Technologies Ltd, Lewes, United Kingdom) [24]. Observations were done using a Philips XL 30 ESEM (Quorum Technologies Ltd, Lewes, United Kingdom) operated at 10 kV. Three pieces were viewed per sample resulting in 20 images.

2.8 Statistical analysis

Analysis of variance (ANOVA) was performed on each direct measurement of chemical composition and enzymatic saccharification at a significance level of 5% (Minitab 19) using the Tukey multiple comparison test.

3 Results and discussion

3.1 Seaweed composition and pre-autoclaving

The monosaccharides (glucose, xylose, rhamnose and galactose) originate from polysaccharides such as cellulose, starch and ulvan. Therefore, compositional concentrations were calculated on dehydrated form using the factor 0.90 for C6-sugars (glucose, rhamnose and galactose) and 0.88 for C5 sugars (xylose and arabinose). Overall composition and fractions of DM extracted by the pre-autoclave treatment are shown in Table 1.

The carbohydrates in *C. linum* from Ghana contained less glucose (ca 22%) than the Danish variety (38%) (Table 1). The other constituents were arabinose (14 – 17%), galactose (3 – 4%) and xylose (2%). The glucose content in the Danish sample (38%) was at the same level as reported by Schultz-Jensen et al. [12] with 43% obtained at the same location in 2010. *C. linum* carbohydrates had a particularly high fraction of glucose to total carbohydrates with 54% and 62% for the Ghanaian and the Danish sample, respectively.

The carbohydrate constituents in *U. fasciata* were primarily glucose (16%) and rhamnose (13%) (Table 1) with a minor content of xylose (4%). The ulvan, present contained glucuronic acid (6%) in addition to the rhamnose content resulting in 39% carbohydrates. The fraction of glucose to total carbohydrates was lower (41%) than for *C. linum*. The non-extracted residue content was high (18%), which can be explained by the high protein content [25]. In that study a similar carbohydrate content of 43% was found.

The carbohydrate constituents in *C. taxifolia* were mainly xylose (ca 47%), galactose (13%) and glucose (only 6%) (Table 1). The xylose present in *Caulerpa spp.* is not terrestrial plant “xylan” (β -1,4-xylan backbone), but a unique type of β -1,3-xylan [17]. Nevertheless, the xylose, once released from the polymer, may be used as a source for modern ethanol fermentation with xylose-fermenting yeast *Saccharomyces cerevisiae*. The fraction of glucose to total carbohydrates was

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474
475 thereby very low (9%). Previous studies on *Caulerpa* sp. show a total carbohydrate content of 39%,
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477 10% protein and 1% lipids without specification for specific monosaccharides [26]. The non-
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479 extracted content was 15% and on the same level as the protein content of 10%.
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481
482 During pre-autoclaving, part of the DM content was extracted resulting in a solution of
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484 carbohydrates and salts. *U. fasciata* was extracted to a higher extent with 16% compared to *C.*
485
486 *linum* (10 – 11%) and *C. taxifolia* (9%). The extract composition is presented based on the total
487
488 DM extracted (Table 1). For *C. linum*, the extract contained very low glucose concentrations (2 –
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490 3%), which indicates that glucose was mainly present as cellulose that is difficult to extract and
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492 dissolve. The solubilized carbohydrates were present as sugar oligomers extracted by autoclaving
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494 and were not hydrolysed completely to monomers. Galactose and arabinose were present at 2 – 8
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496 times higher concentrations in the hydrolysate than in the solid residue indicating that they were
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498 highly extractable.
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501 For *U. fasciata*, higher glucose concentrations were extracted (i.e. 9%), showing a higher content
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503 of extractable and glucose containing carbohydrates such as starch. Starch has been observed inside
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505 the chloroplast using iodine stain (unpublished data). In *C. taxifolia*, galactose was extracted to a
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507 high extent and xylose at a low level relative to the composition. This indicates that the xylose -
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509 rich polysaccharides were difficult to extract compared to galactose. Similar pre-treatment with
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511 water / saturated steam but at elevated temperature of 170 - 200°C has frequently been used for
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513 increasing saccharification of lignocellulosic biomass such as wheat straw [19]. This results in 50
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515 – 100% extraction of the pentose sugars and increased cellulose fractions in the remaining dry
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517 matter, resulting in up to 100% enzymatic digestibility. Due to the absence of lignin in green
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519 seaweed a similar digestibility is achievable at reduced temperature.
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3.2 Enzymatic saccharification of seaweed

Fig. 2 shows the saccharification yield for glucose with and without enzyme addition based on the glucan content (Table 1) with 100% as theoretical maximum. Similarly, Fig. 3 shows the saccharification yield for xylose based on the xylose content in the polysaccharides.

Without pre-autoclaving and enzyme addition, saccharification was not observed as expected for both glucose and xylose. Pre-autoclaving (Au) increased the saccharification yield of glucose to 18.5% for *U. fasciata* (Fig. 2b) while it increased to 0.2% and 7% for *C. linum* and *C. taxifolia*, respectively (Figs. 2a and 2c). Thereby, the glucan content in *C. linum* was most resistant to hydrolysis.

For all the seaweed samples, enzymatic saccharification into glucose increased with time and levelled off after 24 hr. However, saccharification increased slightly further until 48 hr for pre-autoclaved *C. taxifolia* and *C. linum*. In general, 75% of the glucose yield was obtained during only 6 hr. Studies carried out by Trivedi et al. [25] on *U. fasciata* [27] using cellulase enzymes showed similar trends of increasing saccharification with hydrolysis time (Fig. 2).

The dry matter based enzymatic glucose saccharification of *C. linum* was 20 g glucose/100 g DM decreasing to 15.4 with pre-autoclaving. The enzymatic saccharification of *U. fasciata* resulted in 7 g/100 g DM increasing to 12 g with pre-autoclaving. The enzymatic saccharification of *C. taxifolia* resulted in 4 g/100 g DM increasing to 9 g with pre-autoclaving. Based on these results, pre-autoclaving is required for *C. taxifolia*, an advantage for *U. fasciata* and not needed for *C. linum*.

For untreated *C. linum*, the glucose yield was 80% based on the glucan content. The yield was slightly reduced when pre-autoclaving was applied (71%), which indicates that the carbohydrate structure is slightly sensitive to heating. For pre-autoclaved *U. fasciata*, total hydrolysis of the glucan content was achieved with 100% yield (Fig. 2 and Table 1). This is better than without pre-

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593 autoclaving yielding 38%. Pretreated *C. taxifolia* gave a glucose yield of 100% corresponding to
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595 the entire glucan content being enzymatically hydrolysed to glucose. This yield was 36% higher
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597 than without pre-autoclaving (73%). The high yield compared to wheat straw hydrolysis (72%)
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599 [12], might be related to the lack of lignin in green seaweed and increased accessibility caused by
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601 autoclaving.
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603
604 For *C. linum*, where the analysed content of xylose was extremely low (Table 4), the yield was 40
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606 – 60% of the theoretical maximum after 6 to 48 hours saccharification (Fig. 3). This yield increased
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608 to 60 – 70% with autoclave pretreatment. There is currently no evidence for presence of terrestrial
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610 plant type β -1,4-xylan in *C. linum*. Yet, we ascribe the significant enzymatic release of xylose from
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612 *C. linum* as being due to presence of broadly acting GH3 β -xylosidase exo-activity in the Cellic®
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614 CTec2 preparation. In *C. taxifolia* the xylose yield was very low (1%). The essential absence of
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616 enzymatic xylose release was due to lack of β -1,3-xylanase in Cellic® CTec2 needed for hydrolysis
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618 of the β -1,3-linked D-xylose [17]. For *U. fasciata* the xylose yield was 25% both with and without
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620 pre-autoclaving. This indicates that a part of the xylose could be hydrolytically released from the
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622 material, and we propose that the observed xylose release is due to cleavage of the β -1,4 bond
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624 between xylose and 3-sulfatated rhamnose in ulvan [18].
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627 628 **3.3 Microstructural changes of the seaweed caused by the enzymatic saccharifications** 629

630 LM microscopy and SEM microscopy were used to assess the morphological and ultrastructural
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632 changes in seaweed cell structure as a result of enzymatic saccharification and pre-autoclaving.
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634 Light microscopy images are shown in Fig. 4 and SEM images in Fig. 5. Index a, b and c show
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636 representative images from samples of *C. linum*, *U. fasciata* and *C. taxifolia*, respectively. Light
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638 microscopy shows the overall cellular structure and content of the seaweeds while SEM
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640 microscopy shows cross sections and surface micro- and ultrastructure. Key features found with
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642 SEM microscopy are shown in Fig. 6 with larger/high magnification images.
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652 *3.3.1 Structural changes in C. linum*
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654 *C. linum* forms unbranched, hair-like, uniseriate filaments with rod shaped cells of 50 – 100 µm in
655 diameter (Fig. 4a₁). Fig. 5a₁ is a representative SEM image showing the cylindrical shape of *C.*
656 *linum*. The cells began to disintegrate with enzymatic saccharification (Fig. 4a₂) with degradation
657 increasing after 24 hr (Fig. 4a₃ and 5a₃). Changes in appearance from dark green (Fig. 4a₁) to brown
658 (Figs. 4a₄₋₆) after pre-autoclaving and enzymatic saccharification was also observed. Pre-
659 autoclaving (Fig. 4a₄) resulted in some destruction of the mucilagenous like outer covering of the
660 cell wall including cracks in the cell surface allowing accessibility to the cellulose content for
661 enzymatic saccharification. Enzymatic saccharification at 6 and 24 hr after pre-autoclaving showed
662 how the closely packed filamentous cell structure became cleaved probably at cell juncture (i.e.
663 cross wall) producing separated brick-like single cells and short filaments containing a few cells in
664 addition to delaminated cell walls providing easy enzyme access and thereby improved
665 saccharification (Fig. 5a₅₋₆). Cracks in the outer cover of the cell wall surface and the fibrillar
666 structure beneath are shown in Fig. 5a. The severely disintegrated structure with some remaining
667 fibrils of 0.5 µm in thickness after enzymatic saccharification are shown in Fig. 6b.
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685 *3.3.2 Structural changes in Ulva fasciata*
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687 *U. fasciata* was identified as flat sheets of two cell layers (bi-seriate) in cross sections using SEM
688 microscopy (Fig. 6c). Each cell layer was ca 35 µm thick supporting a ca 5 µm thick outer
689 cover/cuticle. The cell size beneath the outer layer was ca 15 µm with 1 µm thick cell walls. LM
690 microscopy showed the cell walls as pale with green chlorophyll content inside cells (Fig. 4b₁ and
691 5b₁). The brown coloration of cell contents seen in Fig. 4b₂₋₃ is attributed to the high
692 saccharification temperature (50°C) and in Fig. 4b₄ to pre-autoclaving. With LM microscopy, the
693 breakdown and rupture of the cellular structure and loss of content was observed following
694 enzymatic saccharification (Fig. 4b₂₋₃). When the samples were pre-autoclaved, cellular
695 degradation was much faster during saccharification (Fig. 4b₅₋₆). Furthermore, there was a
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711 destruction to the continuous network of adjacent cell walls outlining each cell within a given area
712 of the *Ulva* thallus compared with the enzyme treated samples without autoclaving (Fig. 4b₅ cf.
713 with 4b₂).

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718 SEM microscopy of *U. fasciata* confirmed degradation and delamination of the cuticle surface
719 compared with the uncracked flat surface of untreated samples (Fig. 4b₁ and 5b₁). SEM showed
720 cavities in the cell surface resulting from enzymatic saccharification exposing the carbohydrate
721 content to hydrolysis (Fig. 4b₂, 4b₃ and 5c). Enzymatic saccharification of autoclaved seaweed
722 samples showed numerous cracks and significant erosion on the cell walls (Fig. 3b₆). Cell walls
723 connecting the bi-seriate layers were initially degraded, leaving the surface layers poorly degraded
724 (Fig. 5d). This indicates that the cellulolytic enzymes penetrated the outer layer possibly through
725 the cracks in the layer while the connecting walls of xyloglucan and cellulose [28] were degraded.
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735 3.3.3 Structural changes in *Caulerpa taxifolia*

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737 Fig. 4c₁ and 6e show the characteristic leaf-like structure of *C. taxifolia* using LM microscopy. The
738 leaf thickness was approximately 60 µm (Fig. 6f) with an outer cover (cuticle) thickness of ca 3
739 µm (Fig. 6g). The hairy like appearance observed with SEM is due to fungi and filamentous
740 bacteria living on the surface/cuticle. These structures were observed as patches and were thus not
741 a feature of the cuticle surface. The cuticle layer became only slightly distorted during enzymatic
742 saccharification (Fig. 4c₂₋₃) showing some patches of etching (Fig. 5c₂₋₃). When *C. taxifolia* was
743 autoclaved the extent of cell wall destruction was increased especially at branch tips (Fig. 4c₄). A
744 color change of green to brown (loss of chlorophyll) was observed during pre-autoclaving and
745 enzymatic saccharification (Fig. 4c₄₋₆) similar to that observed with *U. fasciata* (Figs 4b₄₋₆).

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757 Fig. 5f shows the cross-section of *C. taxifolia*. A spongy-like material (probably polysaccharide
758 content) was observed between the cell layers (Fig. 6f). After 24 hrs enzymatic saccharification
759 (Fig. 5c₃), the compact spongy-like material began to disintegrate. After enzymatic saccharification
760 and pre-autoclaving, a significant decomposition of the cell structure occurred, increasingly with
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770 hydrolysis time (Fig. 4c₆). The 3 μm thick cuticle layer around *C. taxifolia* and the native low
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772 cellulose content and greater presence of other polysaccharides containing xylose and galactose
773
774 monomers, which may also inhibit glucose release may account for the low glucose yields obtained
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776 compared with the other algal species studied (Fig. 2).
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780 781 **4 Conclusion**

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783
784 The native content of glucan of importance for enzymatic saccharification in *U. fasciata*, *C.*
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786 *taxifolia* and *C. linum* were ~16%, 8% and 22%, respectively. Direct enzymatic saccharification of
787
788 these green seaweed species with a commercial cellulase preparation gave glucose yields of 37 –
789
790 80% and was highest for *C. linum*. Pre-autoclaving increased the glucose yield significantly in *U.*
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792 *fasciata* and *C. taxifolia* resulting in total saccharification, i.e. essentially 100% glucose yield while
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794 no change was found for *C. linum*. The glucose yields were higher than reported for terrestrial
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796 lignocellulosic biomass such as wheat straw (where glucose yields from cellulose are typically 60
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798 – 80% using even higher hydrothermal pretreatment temperature (180 – 200°C)).
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800
801 Microstructural investigations showed a thread like structure for *C. linum* resulting in a large
802
803 surface area for enzyme penetration reducing the need for pre-treatment. *U. fasciata*, consisting of
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805 two cell layers, had a skin layer which was not much hydrolysed and acted as a barrier explaining
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807 the need for pre-treatment. The visually apparent complete hydrolysis of the cell walls in between
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809 the layers showed that this part was hydrolysable. The structure of *C. taxifolia* deviated from that
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811 of the other species as it did not contain dividing cell walls and effects of enzymatic saccharification
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813 were less evident. The data show that green seaweeds commonly found in high amounts along the
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815 coast of West Africa hold potential for biorefining via glucan saccharification. Thus, the data
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817 provide a first step in paving the way for development of simple saccharification processing of
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819 green seaweeds, e.g. for ethanol production via glucose and/or xylose fermentation. *C. taxifolia*
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821 was shown to have a particularly high content of xylose. The xylose is likely part of complex,
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829 unique β -1,3-xylan not hydrolysed with Cellic® CTec2. Yet, the data obtained in the present study
830
831 highlight the potential of *C. taxifolia* as a source of xylose, obtainable via enzymatic
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833 saccharification with β -1,3-xylanase. However due to the high glucan content in *C. linum* and its
834
835 unique thread-like anatomy, *C. linum* is concluded to be particularly suitable for direct enzymatic
836
837 saccharification.
838

839 840 **Declaration of author contribution**

841
842
843 AT, JA, GD, DF, MM and ASM contributed to the conception, study design and data interpretation
844
845 while AT, JA, DF and JB performed the experiments. DF and GD contributed mainly by adapting
846
847 the microscopy procedures to the samples and interpretation of the obtained images. AT, JA and
848
849 ASM wrote the manuscript. All authors read and approved the final manuscript.
850
851

852 853 **Declaration of author's agreement to authorship and submission**

854
855 All authors have agreed to authorship and the submission of this manuscript for peer review.
856
857

858 859 **Declaration of competing interest**

860
861 Declaration of interest: none. No conflicts, informed consent, human or animal rights applicable to
862
863 influence the outcomes of the present research.
864

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Table

Table 1. Carbohydrate content of the seaweed samples before and after autoclaving. Values are given as dehydrated monomers, (effect of dehydration in the polysaccharides is considered for C6 sugars with 0.90 (Glu, Rha, Gal) and for C5 sugars with 0.88 (Xyl, Ara). Values with roman capital letters in each column are significantly different at $p \leq 0.05$). For the untreated samples and the autoclaved solid residues the compositional analyses were done in true triplicates (n=3), for the liquid extracts analyses were done in duplicate (true replicates, n=2). Pooled standard deviations for ANOVA of the monosaccharide analysis ranged from 0.18-0.69 for the monosaccharides, and the pooled standard deviation was 2.6 for the ash values.

Composition	Glucose	Xylose	Rhamnose	Galactose	Arabinose	GluA	Ash
Untreated samples							
g per 100 g DM							
<i>C.linum</i> , DK	38.1 ^F	2.1 ^B		4.0 ^D	17.2 ^C		16.1 ^B
<i>C.linum</i> , GH	21.9 ^D	1.5 ^{AB}		3.2 ^C	14.1 ^B		26.9 ^C
<i>U.fasciata</i> , GH	16.1 ^C	3.7 ^C	12.7 ^A	0.4 ^A		6.3 ^A	13.0 ^B
<i>C.taxifolia</i> , GH	5.9 ^A	46.8 ^E		12.9 ^F			4.6 ^A
Autoclaved samples							
g per 100 g DM							
Solid residue							
<i>C.linum</i> , DK	30.9 ^E	1.6 ^{AB}		3.1 ^C	13.7 ^B		18.1 ^B
<i>C.linum</i> , GH	19.6 ^D	0.9 ^A		1.8 ^B	7.8 ^A		22.0 ^C
<i>U.fasciata</i> , GH	10.0 ^B	4.3 ^C	14.6 ^B	0.4 ^A		7.2 ^A	12.5 ^B
<i>C.taxifolia</i> , GH	6.3 ^A	49.7 ^F		12.0 ^E			3.5 ^A
Extract fraction							
g per 100 g DM							
<i>C.linum</i> , DK	2.6 ^A	3.1 ^A		6.1 ^B	17.9 ^A		18.7 ^A
<i>C.linum</i> , GH	1.9 ^A	6.5 ^C		10.8 ^C	50.6 ^B		11.2 ^A
<i>U.fasciata</i> , GH	8.6 ^C	4.4 ^B	13.8	0.3 ^A			11.8 ^A
<i>C.taxifolia</i> , GH	5.2 ^B	9.7 ^D		17.1 ^D		5.5	13.1 ^A
DM Yields (g per 100 g treated DM)			Solid fraction		Liquid fraction		
<i>C.linum</i> , DK			89.0 ^B		11.0 ^C		
<i>C.linum</i> , GH			90.1 ^C		9.9 ^B		
<i>U.fasciata</i> , GH			84.2 ^A		15.8 ^D		
<i>C.taxifolia</i> , GH			91.1 ^D		8.9 ^A		

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1183 **Captions to figures**
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1186 **Fig. 1.** Experimental design including pre-autoclaving (Au) and enzymatic saccharification.
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1189 **Fig. 2.** Glucose yield based on total glucan content achieved by enzymatic saccharification vs. time
1190 of the seaweed samples, *C. linum* (a), *U. fasciata* (b) and *C. taxifolia* (c), with, - and without pre-
1191 autoclaving. Values with different capital letters are significantly different at a level of 5%. The
1192 data shown are average values based on duplicate enzymatic saccharifications (n=2); the coefficient
1193 of variation ranged from 6%-15% on the yield data across the different seaweed samples and times.
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1200 **Fig. 3.** Xylose yield based on total xylose content in the carbohydrate structure achieved by
1201 enzymatic saccharification vs. time of the seaweed samples, *C. linum*, *U. fasciata* and *C. taxifolia*
1202 with, - and without pre-autoclaving. The data shown are average values based on duplicate
1203 enzymatic saccharifications (n=2); the coefficient of variation ranged from 3%-12% on the yield
1204 data across the different seaweed samples and times.
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1212 **Fig. 4.** Effect of enzymatic saccharification and pre-autoclaving on the microstructure of *C. linum*
1213 (a), *U. fasciata* (b) and *C. taxifolia* (c) as observed using light microscopy. Index 1 shows the
1214 controls, index 2 and 3 the enzyme treated samples (6 and 24 hr), index 4 the autoclaved samples
1215 and index 5 and 6 the pre-autoclaved and enzyme treated samples (6 and 24 hr). For each sample,
1216 the left image is at low magnification and the right one at high magnification. Triplicates were used
1217 at each magnification level.
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1226 **Fig. 5.** Effect of enzymatic saccharification and pre-autoclaving on the microstructure of *C. linum*
1227 (a), *U. fasciata* (b) and *C. taxifolia* (c) as observed using SEM microscopy. Index 1 shows the
1228 controls, index 2 and 3 the enzyme treated samples (6 and 24 hr), index 4 the autoclaved samples
1229 and index 5 and 6 the pre-autoclaved and enzyme treated samples (6 and 24 hr). For each sample,
1230 the left image is at low magnification and the right one at high magnification. Triplicates were used
1231 at each magnification level.
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1242 the left image is at low magnification and the right one at high magnification. Triplicates were used
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1244 in achieving the images at each magnification level.
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Fig. 6. Important microstructural features of *C. linum* (a and b), *U. fasciata* (c and d) and *C. taxifolia*
1247
1248 (e, f and g) observed using SEM. *C. linum* shown before (a) and after 6 hr enzymatic
1249
1250 saccharification (b). *U. fasciata* shown as transverse section after pre-autoclaving and 6 hr
1251
1252 enzymatic saccharification (c) and after 24 hr enzymatic saccharification (d). *C. taxifolia* untreated
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1254 showing its characteristic leaf-like structure (e) and in cross section at low - (f) and high
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1256 magnification (g).
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Sample

Pre-treatment

Enz. saccharification

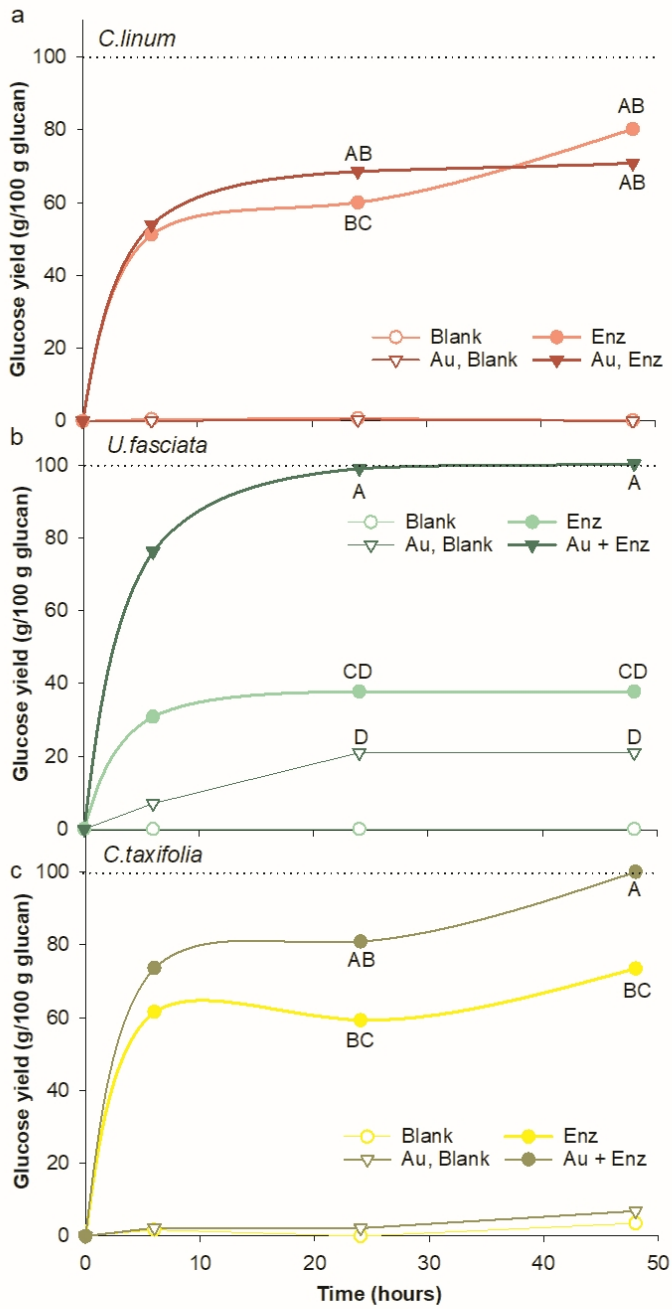
C. linum
U. fasciata
C. taxifolia

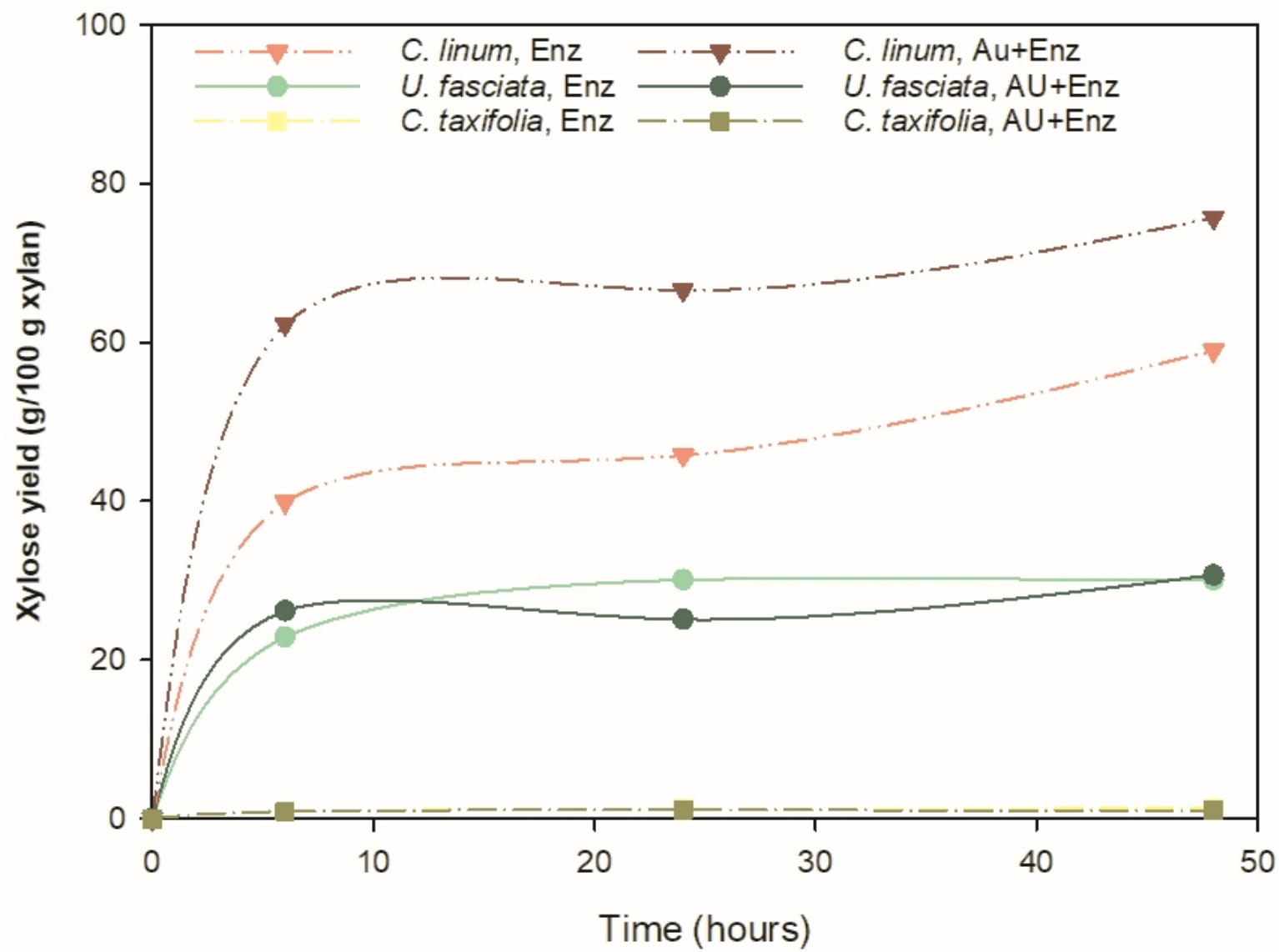
None

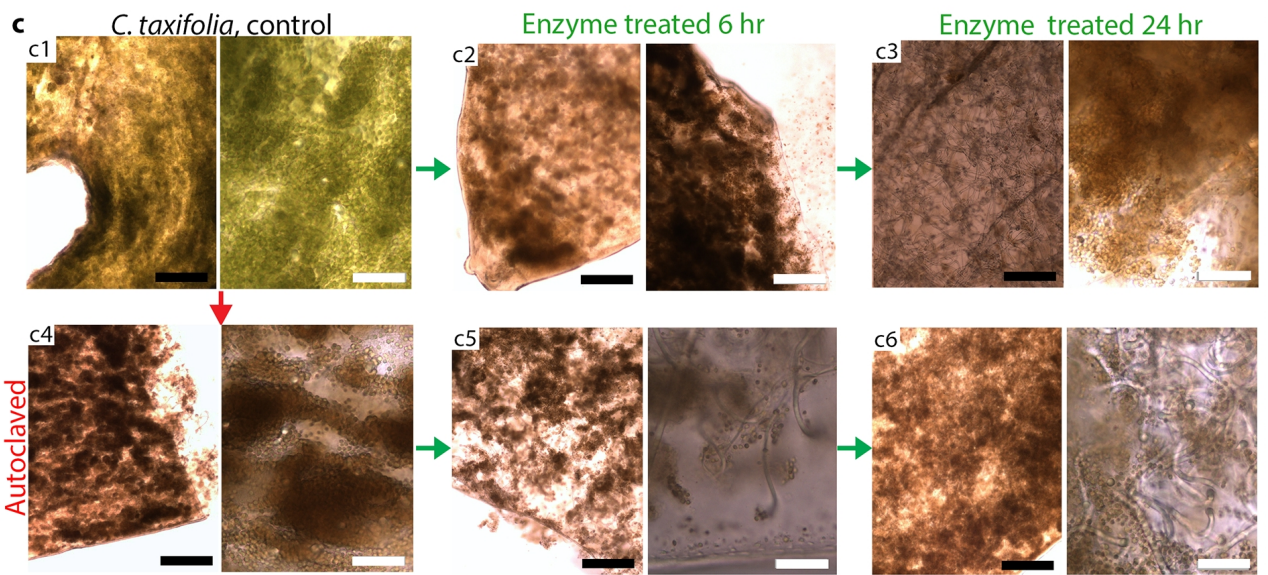
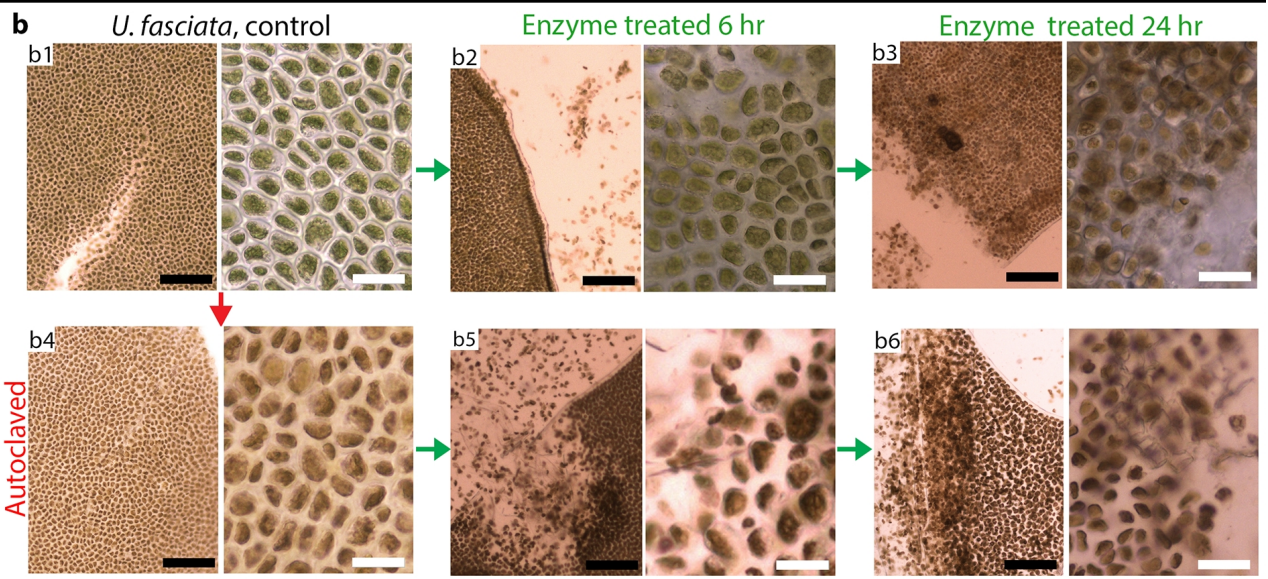
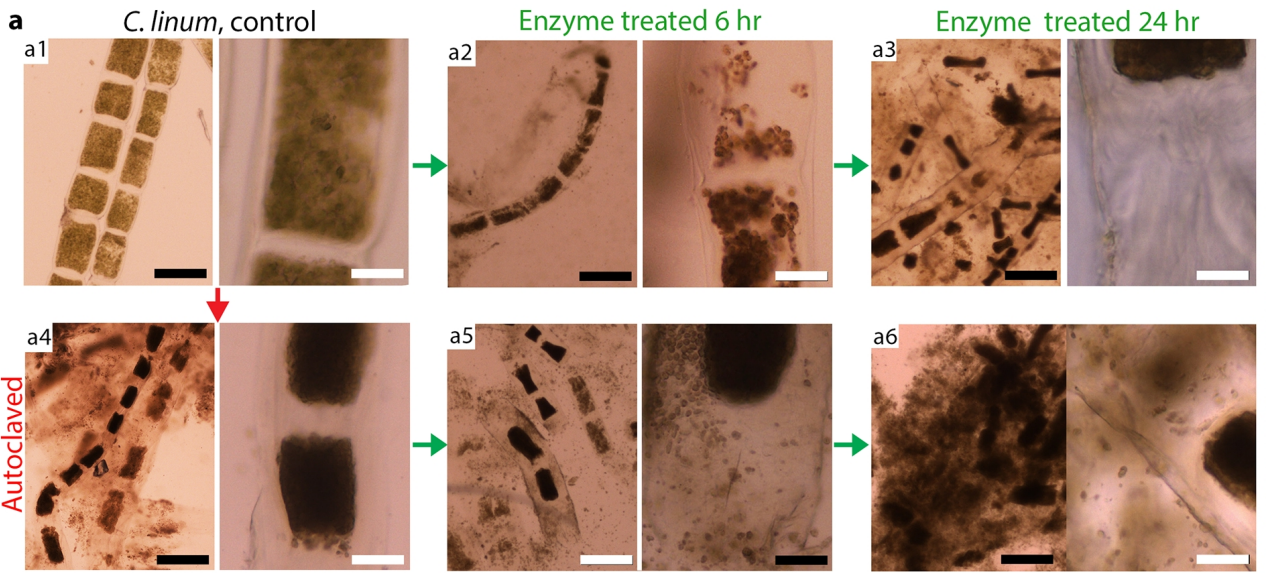
Au: 121°C, 10 min

0 hr
6 hr
24 hr
48 hr

0 hr
6 hr
24 hr
48 hr







Scale bars: 100 μ m 20 μ m; Arrows: Enzyme treated Autoclave pre-treated

