Steam explosion pre-treatment and enzymatic saccharification of duckweed (*Lemna minor*) biomass

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Abstract

Our previous research has shown that duckweed is potentially an ideal feedstock for the production of biofuels because it can be effectively saccharified enzymatically. Here we report the results of experiments in which duckweed was pretreated by steam explosion prior to enzyme digestion. A range of temperatures, from 130 to 230 °C with a fixed retention time of 10 minutes, were employed. The best pretreatment conditions were 210 °C for 10 min; these conditions produced the highest amount of water soluble material (70 %), the greatest levels of starch solubilisation (21%) and hemicellulose and pectic polysaccharides degradation (60%). The use of these steam explosion conditions enabled large reductions in the concentrations of enzymes required for effective saccharification. The amount of Celluclast required was reduced from 100 U (4.35 FPU) g^{-1} substrate to 20 U g^{-1} substrate, and additional beta-glucosidase was reduced from 100 to 2 U g^{-1} substrate.

Keywords: duckweed, *Lemna minor*, steam explosion, enzymatic saccharification, cellulase

Abbreviations

AIR	alcohol insoluble residue
SE	steam explosion
FDM	freeze dried and freeze milled
WSM	water soluble materials
WIM	water insoluble materials
CWM	cell wall material
FWM	untreated fresh material
BG	beta-glucosidase
CE	Celluclast®

1. Introduction

Cellulose, hemicelluloses, pectin and starch are the main classes of carbohydrate present in plant biomass and as such have received attention as substrates for the production of bioethanol as a second generation biofuel [1]. Lignocellulose (which is principally composed of cellulose, hemicellulose and the aromatic polymer lignin) is a major fraction of many types of biomass and constitutes approximately w = 40 to 50% of crops such as straw and wood [2]. The exploitation of lignocellulose is, however, hindered by its recalcitrance to degradation, which means that energetically and financially expensive processes are required [3]. The emergence of novel biomass feedstock with low lignin content may represent an effective and economically feasible solution to the production of bioethanol from lignocellulosic biomass. Aquatic plants are attractive in this respect as they generally have low lignin content and more cellulosic fibres [4].

Duckweeds (family *Lemnaceae*) are common aquatic plants and possesses cell wall material which is rich in cellulose, hemicelluloses and pectin but contains little lignin [5]. Duckweed is abundant in most areas of the world, especially in the tropic and subtropic zone. It is suited to a wide range of geographic and climatic zones and will grow in still or slow-moving water at temperatures of between 6 and 33 °C [6]. Zhao et al [7] summarize earlier studies demonstrating the higher productivity of duckweeds compared to other energy crops. Duckweeds grow rapidly and yields of up to 44 t ha^{-1} y^{-1} (dry matter) have been obtained under experimental conditions.

Conversion of (lignocellulosic) biomass to bioethanol generally consists of four stages: pretreatment, enzymatic saccharification, fermentation and distillation. Various pretreatments have been developed to disrupt the cell wall structures and make the cellulosic biomass more accessible and susceptible to enzymatic saccharification to obtain a higher sugars yield for the subsequent fermentation. Employing an appropriate pretreatment is a primary and crucial step for efficient and economic bioethanol production from lignified materials [8]. Acid, alkali, thermal and combination approaches may also be applied [9]. Steam explosion is a recognized thermo hydrolytic pretreatment that has been investigated as a method for improving enzymatic saccharification and optimising bioethanol production [10]. The process explodes biomass by sudden decompression from high pressure and temperature conditions to improve accessibility of the cell wall material to hydrolysis by cellulases [11]. Pedersen and Meyer [9] compared various pretreatments and noted the high yield of glucose and xylose that results from steam explosion. However, disadvantages were seen to be the high energy input, because of the high pressures and temperatures required, and the formation of inhibitors.

We have previously reported the cell wall composition of *Lemna minor* [12] and the enzymatic saccharification of cell wall material (CWM)to fermentable sugars [7]. Here, we have explored the use of thermophysical pretreatment (steam explosion) to assess whether it can improve the efficiency of saccharification by inducing structural and chemical changes to the duckweed biomass.

2. Materials & Methods

2.1 Plant material

L. minor plants were collected from a pond located at the John Innes Centre, Norwich, UK (52.622295 N, 1.221894 E), then cleaned by rinsing in tap water followed by distilled water. The washed, wet biomass was either frozen at -40 °C until required or freeze dried (Freeze Dryer 3.5, Birchover Instruments Ltd., Hitchin, UK) and milled using a freeze mill (Spex Freezer-Mill 6700, Spex Industries Inc., USA). For analytical purposes, water-insoluble alcohol-insoluble residues were prepared [7].

2.2 Steam explosion pretreatment (SE)

L. minor biomass was treated by SE at a range of severities. The steam explosion of fresh duckweed was carried out using a Cambi™ Steam Explosion Pilot Plant (Cambi, Asker, Norway) with a sealed 30 L vessel. The severity factor [13] was calculated from the process temperature and the residence time by the following equation [13]:

$$S = \mathrm{Log10}[t \times \exp((T-100)/14.75)]$$

where: S = Severity factor, t = Residence time (min), T = temperature (ºC).

In all treatments, severity factor was controlled by changing temperature whilst maintaining a constant residence time (10 min). The pressure of six SE temperatures (130, 150, 170, 190, 210 and 230 °C) were 0.2, 0.4, 0.7, 1.2, 1.8 and 2.7 MPa respectively. Steam exploded products were obtained as slurries and the volume of the slurries was measured before freezing in a coldroom (-80 °C) until required. An aliquot (200 mL) of each SE product was stored in individual bottles with added thiomersal (0.1 kg m^{-3}) in a refrigerator (4 °C).

2.3 The subsequent treatment of steam exploded materials

Representative aliquots (7 mL, triplicates) of steam exploded slurries were transferred to Pyrex® culture tubes and centrifuged to separate the supernatant, containing water-soluble materials (WSM), from the solid residue (water-insoluble material; WIM). Supernatants were filtered using GF/C filter paper and frozen at -20 °C. The pellets were washed twice using distilled water and prepared as alcohol insoluble residues (AIR) using the following procedure. The wet residual pellets (3 mL) were homogenised twice with ethanol (100%), and the steam-exploded samples prepared at 130 °C and 150 °C were ground with a pestle and mortar for 10 min to break down plant tissues. The resulting slurry was transferred to Pyrex® culture tubes. The pestle and mortar was rinsed out, and the volume was made up to 10ml with additional pure ethanol giving a final ethanol concentration of φ_{final} = 70%. The slurries were heated at 80 °C for 15 min. After cooling and recovery by centrifugation (3000 x g, 10 min), the residue was re-extracted as before in ethanol (φ_{final} = 70 %, 80 °C, 15 min) and then once at 80 °C in pure ethanol. Finally the AIRs were extracted once in acetone at room temperature and dried overnight. The frozen residual pellets and half amounts of WSM samples were dried using a freeze dryer to recover fully dry mass. Aliquots (40 mg, duplicates) of the resulting dry mass were dried at 105 °C to test the moisture content.

2.4 Enzymatic saccharification of steam exploded slurries

Enzymatic saccharification of the steam exploded slurries was investigated using an enzyme mixture consisting of Celluclast® (CE; Sigma Chemical Co., St. Louis, MO) and additional β-glucosidase (BG; Novozyme® 188, Sigma Chemical Co., St. Louis, MO). The enzyme activities are defined by the manufacturer for CE and BG as 700 U mL^{-1} [14] and 250 U mL^{-1} [14], respectively. The FPU activity of cellulase

(Celluclast®) was also assessed, using the standard measurement of cellulase [15]. Digestions were carried out in triplicate and contained 30 mg of dry biomass (substrate concentration is 5 kg m^{-3}) and CE (4.35 FPU g^{-1} substrate, 0.07 kg m^{-3}) and BG (100 U g^{-1} substrate, 0.2 kg m^{-3}) in 0.1 mol L^{-1} sodium acetate (pH 5.0) containing thiomersal (Sigma Chemical Co., St. Louis, MO; 0.1 kg m^{-3}) in a total volume of 6 mL. A time course of the hydrolysis reaction was carried out in Pyrex® culture tubes with time points from 0 h to 24 h at 50 ºC with continuous agitation on a Thermoshake incubator unit (C. Gerhardt GmbH & Co, Königswinter, Germany) at 120 rpm [16]. Incubations were terminated by heating to 100 °C for 5 min after which time the samples were centrifuged at 16,060 x g for 5 min. The supernatants were recovered by aspiration and frozen prior to analysis. The reducing sugars and glucose were measured subsequently using the dinitrosalicylic acid (DNS) assay.

2.5 Analytical methods

2.5.1 Moisture Assessment

The moisture contents of the resuspended samples (1 g, duplicates) of FWM and SE products were determined using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd, Beaumont Leys, Leicester, UK). Also, the density of SE slurries was assessed by the aliquot (5 ml, duplicates).

2.5.2 Starch Assessment of SE slurry

The aliquot of SE products and untreated sample (triplicates) were transferred to Pyrex® culture tubes and frozen with liquid nitrogen. The frozen samples were freeze dried and freeze milled. The starch content was measured by using the standard method for Total Starch Assay Procedure [17]. The FDM duckweed (30 mg × triplicates) was dispersed

168 in φ = 80 % ethanol (200 µL). After boiling for 5 min with 2 mL of φ = 92% dimethyl
169 sulphoxide (DMSO), samples were hydrolysed using a thermostable α-amylase in
170 MOPS buffer (3 ml, 300 U) for 6 min. After cooling, the hydrolysed samples were
171 mixed with sodium acetate buffer (4 mL, 0.2 mol L^{-1}, pH 4.5) at 50 °C followed by
172 hydrolysis with amyloglucosidase (0.1 mL, 20U, 50 °C) for 0.5 h. The resulting sample
173 (0.1 mL) was assessed by colourimetric determination with glucose oxidase-peroxidase-
174 4-aminoantipyrine (GOPOD) reagent (3 mL). Absorbance was measured using a
175 Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at 510 nm.

176 *2.5.3 DNS & GOPOD test*

177 The reducing sugars released by enzymolysis were measured by the non-specific DNS
178 test method [18] while the liberated glucose was detected by specific GOPOD test
179 method [19]. For the DNS test, 36 µL of original samples and 144 µL DNS reagent (1:
180 4 of sample : DNS reagent) were homogenised in tall- chimney 96-well plates
181 (Fisherbrand®, UK) stoppered with TPE PCR sealing mats (BRAND, Fisher Scientific
182 UK, Loughborough, UK). The solutions were heated in a Biometra® T-Gradient
183 thermocycler (Biometra, Göttingen, Germany) at 100 °C for 3 min. A cooled aliquot
184 (0.1 mL) was transferred to a 96-well flat-bottomed microtitre plate (Nunc, Roskilde,
185 Denmark) and absorbance measured in a Microplate Spectrophotometer (Benchmark
186 Plus, BioRad, CA, USA) at 580 nm. For the GOPOD test, 10 µL of original sample was
187 diluted with 10 µL sodium acetic acid buffer (0.1 mol L^{-1}, pH 5.0) and mixed with 300
188 µL GOPOD reagent. After mixing by vortexing, the samples were incubated at 50 °C
189 for 20 min after which the absorbance was measured using the microplate
190 spectrophotometer at 510 nm. The background absorbance from blank enzyme

191 preparations was subtracted and the concentration of sugars calculated from appropriate
192 standard curves.

193 *2.5.4 Microscopy of SE slurry*

194 The fresh or steam exploded plant material was immersed in cyclohexane-trans-1,2-
195 diaminetetra-acetate (CDTA) reagent including 0.05 mol L^{-1} Na_3H_1 CDTA and 5 mmol
196 L^{-1} $Na_2S_2O_5$, pH 7, for 2 - 4 days to break down tissues and separate cells [20]. The
197 tissues were stained with Lugol's solution (20 kg m^{-3} KI with iodine (0.2 kg m^{-3})) to
198 highlight the presence of starch. The samples were observed by manual fluorescence
199 microscope (BX60, Olympus, Japan).

200 *2.5.5 Sugar Analysis by Gas Chromatography (GC) Method*

201 The carbohydrates in freeze dried WSM and WIM and the whole SE slurry were
202 assessed as alditol acetates according to the gas chromatography method of Blakeney et
203 al [21]. Samples (3 mg) in triplicate were treated with 200 µL H_2SO_4 (w = 72 %; 3 h,
204 room temperature) followed by dilution to 1 mol L^{-1} H_2SO_4 and then hydrolysis (2½ h,
205 100 ºC) to their constituent monosaccharide sugars. After 1.5 h, samples were taken for
206 the colourimetric determination of galacturonic acid [22]. Samples were also hydrolysed
207 only with 1 mol L^{-1} H_2SO_4 (omitting the w = 72 % H_2SO_4 treatment), which allows
208 approximate starch content and cellulose (≤10 %) to be determined. This method
209 provides a useful estimation of the starch content [23]. After reduction and acetylation,
210 alditol acetates were analysed by GC on a Perkin-Elmer Autosystem XL GC system
211 (Perkin-Elmer, Seer Green, Bucks., UK). 2-deoxyglucose was added as the internal
212 standard.

213 *2.5.6 Inhibitor assessment*

2-furfuraldehyde (2-FA), 5-Hydroxymethylfurfural (5-HMF) and organic acids (formic, levulinic and acetic acid) were assessed as they are thought to be significant fermentation inhibitors. Aliquots of the steam-exploded slurries were centrifuged at 2465 x g and 0.2 ml of the upper clean liquor produced was filtered using a syringe filter (0.2 µm, Whatman International Ltd, Maidstone, UK), and injected into vials. The concentration of inhibitors was analysed by HPLC using a Flexar LC instrument (Perkin Elmer, Seer Green, Bucks., UK) equipped with refractive index and photo diode array detectors (reading at 210 nm wavelength) in series. The analyses were carried out using an Aminex HPX-87H carbohydrate analysis column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) operating at 65 °C with 5 mmol L^{-1} H_2SO_4 (Sigma-Aldrich) as mobile phase at a flow rate of 0.6 mL min^{-1}.

3 Results and Discussions

3.1 Recovery of material following steam explosion

The recovery of dry matter following SE employing different conditions was assessed. Table 1 shows that increasing the severity of steam explosion resulted in a reduction in total dry matter recovery. Up to w = 35.4 % of biomass was lost at 230 °C. This is likely to have been due to the carriage of some of the solubilised and hydrolysed material and possibly small particles through the machine during depressurisation. Larger, insoluble particles will have been more readily recovered in the cyclone recovery system. Jacquet et al [24] reported that mass loss as a consequence of the SE of cellulose fibre starts at 70 °C and increased temperature results in increased loss. Mass loss (w = 4 – 27 %) has also been observed following SE of birch wood [25]. The same authors found that the weight loss of birch wood is positively correlated with SE retention time. At the same pressure (1.47 MPa), dry mass loss of w = 15, 25 and 26.3 % was observed at, respectively, 5, 10 and 15 min retention times.

Table 1

3.2 Visual impact of steam explosion on duckweed tissues

Steam explosion has a significant impact on duckweed tissue disruption (Fig.1). At a constant retention time of 10 min, increasing temperature resulted in increased tissue disruption observed by microscopy. Following staining with Lugol's solution, a large amount of starch granules were observed in the FWM (Fig.1a; starch granules are stained black). At the lower pretreatment temperatures (130 ^{o}C – 170 ^{o}C) the cellular structure remained visually intact. At 130 °C, fronds were slightly disrupted, but starch granules remained embedded in the cells (Fig. 1b). At 150 °C, the plant structure was

250 further disrupted and a significant amount of starch and sodium oxalate (needle shaped)
251 crystals were released from the cell (Fig. 1c). At 170 °C, the tissue was further
252 decomposed and the starch had started to gelatinise (Fig. 1d). At 190 °C, the tissue
253 began to become less well defined as cells started to separate. The starch gelatinisation
254 and the decomposition were very pronounced (Fig. 1e). At 210 °C, only a small amount
255 of residual, structured tissue was observed and intracellular contents had apparently
256 been liberated into the aqueous phase - starch granules were extensively released from
257 plant cells (Fig. 1f). At 230 °C, plant tissue integrity was completely disrupted but the
258 liberated and gelatinised starch appeared to be reduced (Fig. 1g). This is probably due to
259 degradation of polysaccharides (see below).

260 **Figure 1**

261

262 ***3.3 Chemical composition of untreated materials.***

263 Analysis of duckweed FWM was carried out to evaluate carbohydrate composition,
264 including glucose (Glc), xylose (Xyl), galactose (Gal) and galacturonic acid (GalA).
265 Dry matter accounts for w = 8.5 % of fresh wet biomass (Table 1). Of this dry material,
266 carbohydrate constitutes w = 51.2 % and glucose and xylose account for w = 33.1 % and
267 w = 4.6 % respectively (Fig. 2). Starch constitutes w = 22 % of the dry matter (Fig. 3;
268 assessed by using Total Starch Assay Procedure). These data of cell wall composition is
269 proven by Zhao et al [12] in which similar sugars compositions and the proportions of
270 monosaccharides have been reported.

271

272 ***3.4 Chemical composition of steam exploded materials.***

Figure 2a shows the relative levels of soluble (WSM) and insoluble (WIM) dry matter recovered, and shows that the severity-related increase in tissue disruption is associated with an increase in soluble material in the aqueous phase. The highest proportion of WSM (70 %) appears after pretreatment at 210 °C; SE using this temperature and these conditions would thus be an effective means of solubilising duckweed biomass. In comparison, Sun et al [26] observed that the highest WSM conversion (40 % of dry mass) by SE of wheat straw (lignocellulose) occurred at a severity factor around 4.44 (200 °C, 33 min or 220 °C, 8 min). SE with a severity factor 4.2 (233 °C, 2 min) was found to be the pretreatment that was most effective in solubilising rice straw (generating 30% WSM) [27]. Jacquet et al [24] noted that depolymerisation of cellulose fibres occurs at a severity factors in the range of 4.0 - 5.2. Based on these findings, for our work with a constant retention time of 10 min, depolymerisation would be expected to occur in a range from 200 °C to 245 °C. However, *L. minor* is poorly lignified [5], and this is likely to account for the tissue disruption at low severities which is probably initiated by the depolymerisation of pectic polymers involved in cell adhesion at 170 °C (Figure 1).

Figure 2

The carbohydrate compositions of SE slurries, WIM and WSM were quantified in order to assess variation and the impact of severity on the conversion of WIM to WSM (Fig. 2b – d respectively). In the total slurry (Fig. 2b), galactose (Gal), xylose (Xyl) and galacturonic acid (GalA) content generally decreases as temperature increases, and glucose (Glc) content as a proportion of the total generally increases with temperature although it is not a clear trend (Fig 2b). Further clarity could be obtained by evaluating

298 the WSM and WIM. In the WSM (Fig. 2c), Gal, Xyl and GalA increased from 130 –
299 170 °C, after which they all decreased to low levels also. Increase in severity up to 210
300 °C resulted in an increase in soluble glucose, after which it decreased dramatically to
301 low levels. In WIM, Gal, Xyl and GalA increased from 130 – 170 °C, after which they
302 all decreased to low levels. Glc present in the WIM increased concomitantly, then
303 decreased at high severities. Evaluation of Glc using 1mol L^{-1} hydrolysis alone (which
304 will hydrolyse non-cellulosic glucose such as starch) decreased dramatically at high
305 severities. Further information on the origin of changes in Glc was obtained by
306 measuring the levels of starch present in SE slurry, WSM and WIM (Fig. 3). The results
307 showed that in total slurry, starch levels were relatively constant as a proportion of the
308 dry matter at all pretreatment severities up to 210 °C, but dropped markedly after
309 pretreatment at 230 °C. In WIM, the level of starch decreased by about 25 % up to 190
310 °C, then dropped considerably after treatment at 210 °C and was undetectable by 230
311 °C. This was reflected in the increase in soluble starch in WSM from 130 °C up to 210
312 °C after which the starch content dropped to low levels. GOPOD analysis of the WSM
313 directly revealed a proportion of liberated glucose which comprised about w = 2.5 % of
314 the WSM DM at 130 °C, but rose to about w = 3.5 % after pretreatment at 230 °C. The
315 fact that a small amount of free glucose was produced under all SE conditions suggests
316 pretreatment solubilisation of starch may have also involved the production of di-, tri-
317 or other oligosaccharides (not evaluated).
318
319 **Figure 3**
320

The above results clearly demonstrate the solubilisation, possibly hydrolysis and breakdown of cell wall and starch polysaccharides during SE pretreatment. The movement of sugars from WIM to WSM increases as temperature rises from 130 to 210 °C. Starch analysis (Fig 3) shows clearly that starch is gelatinised and eventually completely solubilised. However, the loss of measurable starch at 230 °C suggests that the soluble starch is destroyed. Other non-cellulosic sugars follow a similar trend. The galactose and glucose decrease in WIM, and their increase in the WSM up to 170 to 190 °C suggests solubilisation. However, above 190 °C, they decrease, again suggesting that they are degraded. The degradation of non-cellulosic sugars is consistent with studies on pretreatment of lignocellulose [3, 28] and accounts for the increase in breakdown products shown in Fig. 4 (see below).

3.5 Quantification of fermentation inhibitors in steam exploded materials

Significant quantities of fermentation inhibitors were detected in the WSM and these were most prominent after the higher severity treatments of 210 °C and 230 °C (Fig. 4) coincidental with the greatest loss of carbohydrate material. 2-furfuraldehyde (2-FA) and 5-hydroxymethylfurfural (5-HMF) are known to inhibit glycolytic enzymes and thus hinder sugar fermentation by yeast [29]. Fermentation inhibition by these acid products has been reported by Pienkos and Zhang [30]; the degree of inhibition by 5-HMF, 2-FA, acetic acid and levulinic acid were reported as 50, 79, 74 and 50 % respectively when their concentrations reached 80, 40, 60 and 400 g kg^{-1}. A formic acid concentration of 27 g kg^{-1} has been reported to cause 80 % fermentation inhibition [31]. 2-furfuraldehyde is derived from xylose and 5-HMF is produced from glucose under acidic conditions [9]. Furthermore, 5-HMF and 2-FA will continue to transform to

levulinic acid and formic acid if sufficient water is present [9]. The low pH conditions of steam explosion on fresh wet duckweed are thus likely to result in the formation of 2-FA and 5-HMF and high levels of organic acids. The generation of 2-FA, 5-HMF and organic acids was assayed (Fig. 4). 2-FA and 5-HMF were detectable following SE at 190 °C and the levels increased up to 5.6 and 7.3 g kg^{-1} respectively at 230 °C. These are not high levels of 2-FA and 5-HMF and it is not expected that at these concentrations there would be a significant inhibitory effect on subsequent fermentation. However, significantly higher levels of formic acid (23.8 and 38 g kg^{-1} at 210 and 230 °C respectively) and acetic acid/ levulinic acid (53.8 and 67.7 g kg^{-1}) were produced at 210 and 230 °C respectively. Almeida et al [29] showed that monosaccharide products of hydrolysis of cellulose and hemicellulose could convert to 5-HMF and 2-FA. Acetic acid is a hydrolysis product of hemicellulose and lignin [29] but since duckweed has a low proportion of lignin [5] it is likely that the observed acetic acid, as well as the levulinic acid and formic acids, are derived from reducing sugars. In keeping with the production of acidic breakdown moieties, SE led to a reduction in pH. This was comparatively slight from 130 °C to 190 °C (pH 6.5 to 6.2) but more pronounced at higher temperatures: pH 5.6 at 210 °C and pH 4.6 at 230 °C.

Figure 4

3.6 Investigation of enzymatic saccharification on steam exploded raw slurry

To test the hypothesis that steam explosion increases the ease of hydrolysis by cellulolytic enzymes, enzymatic saccharification was carried out to hydrolyse the carbohydrate components (cellulose, hemicellulose and pectin) of the total slurry. In

keeping with the analysis of WSM (Fig 2c), the total slurry was found to contain both solid residual CWM and soluble glucose liberated by SE (Fig 5a). In addition, using DNS analysis, it was found that significant quantities of additional reducing sugars were present (Fig. 5a). The levels of SE-solubilised reducing sugars and Glc were taken into account when evaluating the potential for enzymatic saccharification of the slurry.

Figure 5

Initial studies screened for the total yield of reducing sugar released from SE slurry by enzyme treatment using Celluclast (CE, 100 U or 4.35 FPU g^{-1} substrate) and Novozyme 188 (BG, 100 U g^{-1} substrate), identified previously as optimal for digesting purified duckweed cell wall material [7]. Saccharification was found to be positively correlated with the severity of SE (Fig. 5b). Slurry treated by SE at 210 °C and 230 °C was digested completely (~100 % reducing sugar yield) within 8 h. Over the same period the SE 190 °C material exhibited a reducing sugar yield of 86.5 %. The increasing initial hydrolysis rates following the increase in SE severity imply that more carbohydrate was depolymerised at the higher temperatures. For all SE samples the bulk of the saccharification occurred in the first 2 h of incubation and was stable after 8 h. This indicated that it might be possible to improve the efficiency of the saccharification by identifying the minimum severity of pretreatment required, and the minimum levels of enzymes. Since SE at both 210 and 230 °C gave 100% saccharification, then material pretreated at 210 ^{o}C was chosen for further optimisation. Not only would this involve less energy in the process, but it would also significantly reduce the quantities of fermentation inhibitors produced (Fig. 4) [24].

To investigate the efficacy of lower enzyme doses, slurry from SE at 210 °C was treated with enzyme cocktails of CE at 100, 50, 20 and 10 U g^{-1} substrate (equivalent to 4.35 to 2.18, 0.87 and 0.44 FPU g^{-1} substrate) and BG (concentration at CE: BG of 1:1). Digestion with 100, 50 and 20 U g^{-1} substrate resulted in similar reducing sugar yields: 97.9, 93.7 and 87.3% respectively after 8 h, at which point the digestion reached completion (Fig. 6a). Digestion using CE at 10 U g^{-1} was less effective, with a maximum yield of 70.9%. Initial rates were similar at all concentrations studied. The optimum enzyme dose for slurry from SE at 210 °C is thus in the order of 20 to 50 U g^{-1} substrate, (0.87 to 2.18 FPU g^{-1} substrate).

Figure 6

To further optimize enzyme use, various CE: BG ratios were investigated. Optimal CE concentrations of 50 and 20 U g^{-1} substrate were chosen and CE: BG ratios of 1: 1 down to 1: 0.1 were employed to digest SE 210°C slurry. After a 24 h digestion, CWM was completely hydrolysed at the CE: BG ratios of 1: 1 and 1: 0.5 with a CE concentration of 50 U g^{-1} and the digestions exhibited high initial rates (Fig. 6b). When the ratio was lowered to 1: 0.5 the final yield was approximately 80%. Further optimisation involved lowering the CE concentration to 20 U g^{-1} and evaluating digestion at CE: BG ratios of 1: 1 and 1: 0.5 at CE 20 U g^{-1}. This resulted in 95.6 and 94.1% reducing sugar yields respectively. Reducing the ratio of CE: BG to 1: 0.1 again resulted in reduced yields of around 85 %. This clearly shows the important synergy between CE and BG. Under the ranges studies, CE:BG ratios of 1: 1 to 1: 0.5 achieve better digestion than the lower ratio of 1: 0.1 and the addition of BG facilitates the use of CE at lower concentrations

418 (Fig. 6b). When enzyme costs are also considered, the data suggest that CE at 20 U
419 (0.87 FPU) g^{-1} substrate and BG at 2 U g^{-1} substrate is an appropriate enzyme cocktail
420 for the digestion of SE treated duckweed material.
421
422 Having identified improved CE and BG concentrations the comparative effects of a
423 number of pretreatments on the saccharification of duckweed were investigated. These
424 involved steam explosion, freeze drying, freeze drying and freeze milling, preparation
425 of a water-insoluble alcohol insoluble residue [7] and untreated (fresh) material as a
426 control. The same mass of material prepared by the different pretreatment methods was
427 hydrolysed using the optimised enzyme cocktail, CE 20 U (0.87 FPU) g^{-1} substrate, BG
428 2 U g^{-1} substrate. Here, glucose yields were specifically measured by the more accurate
429 GOPOD method since the DNS method although rapid [18] was less accurate and
430 therefore best suited to enzyme cocktail screening. The results (Fig. 6c) show that freeze
431 drying alone is a poor pretreatment, producing little if any increase in glucose yield
432 when compared to untreated material. Saccharification of blender-milled, water-
433 insoluble alcohol-insoluble residue (WIAIR) resulted in a glucose yield of only 40 %.
434 Freeze milling increased glucose yield to 55 %, 1.4 fold higher than the glucose yield of
435 WIAIR but steam explosion was by far the most effective pretreatment tested, resulting
436 in a glucose yield of 80 %. Previously Zhao et al [7] obtained similar glucose yields
437 from enzymatic saccharification of WIAIR but only by using much higher enzyme
438 concentrations (CE at 100 U (4.35 FPU) g^{-1} substrate plus additional BG at100 U g^{-1}
439 substrate). Steam explosion pretreatment thus greatly enhances the digestibility of
440 duckweed material and enables effective saccharification at reduced enzyme
441 concentrations.

An effective pretreatment is determined by following norms: it avoids the cost imposed by reducing the size of biomass particles, avoids the loss of fermentable sugars, resists the formation of fermentation inhibitors, and minimises input energy and cost [30]. SE requires energy input but, especially at higher severities, results in tissue disruption and renders duckweed biomass much more susceptible to enzymatic saccharification without further treatment. The paper of Littlewood et al [33] discussed current and prospective scenarios for the production of wheat straw bioethanol. It can be seen that steam explosion (along with liquid hot water) have the lowest minimum ethanol selling price in the prospective process scenarios outlined. In the case of duckweed, the enzyme costs are expected to be significantly lower due to low lignin content. SE pretreatment removes the requirement for physical treatments such as grinding and drying. It also greatly reduces the enzyme dosages required in the saccharification process. Enzymes are a major economic cost in conversion of biomass to bioethanol. Fermentation inhibitors were detected, but only at relatively low concentrations. The low levels of these inhibitors and the pH of the slurry provide an environment that is suitable for subsequent enzymatic saccharification and should be suitable for fermentation. It is concluded that steam explosion alone appears to be an effective pretreatment for duckweed biomass.

4. Conclusions

Steam explosion has been shown to be a suitable preteatment for maximising saccharification-derived sugar yields from *L.minor* biomass. SE (210 °C, 10 min, severity factor 4.2) results in high levels of conversion (70 %) to WSM: 21 % (by DM) of starch and 60 % of the total cell wall polysaccharides (mainly hemicellulose and pectin) are solubilised. A relatively cost-effective enzyme cocktail (Celluclast at 20 U or 0.87 FPU g^{-1} substrate plus Novozyme 188 at 2 U g^{-1} substrate) efficiently solubilises material that remains insoluble after steam explosion and results in an overall glucose yield of 70.9 %.

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565
566

567

568

569 ***Figure Captions***

570 Table 1. Dry mass recovery following SE. The retention time was 10 min for all

571 treatments.

572

573 Figure 1. Microscopy of steam exploded duckweed biomass. Tissue has been stained

574 with iodine. Bar = 100 µm.

575 a. Fresh plant tissue b. 130 °C pre-treated leaf tissue

576 c. 150 °C pre-treated leaf tissue d. 170 °C pre-treated leaf tissue

577 e. 190 °C pre-treated leaf tissue f. 210 °C pre-treated leaf tissue

578 g. 230 °C pre-treated leaf tissue

579

580 Figure 2. Solubilisation of duckweed biomass and variation in carbohydrate

581 composition in the different fractions generated following SE. Gal = galactose, Xyl =

582 xylose, GalA = galacturonic acid, Glc = glucose, Totals = all carbohydrates assayed.

583 a. Solubilisation of biomass at different SE temperatures.

584 b. Carbohydrate concentration of dry SE total slurry hydrolysed by w = 72% H_2SO_4.

585 c. Carbohydrate concentration of WSM hydrolyzed by w = 72% H_2SO_4.

586 d. Carbohydrate concentration of WIM (AIR) hydrolyzed by w = 72% H_2SO_4 and 1 mol

587 L^{-1} H_2SO_4.

588

589 Figure 3. The % of DM starch content present in FWM, SE slurry, SE WIM and SE

590 WSM, and % liberated glucose present in SE slurry. The percentages of starch were

591 calculated based on the dry matter of the original SE slurry.

592

593 Figure 4. Variation in levels of fermentation inhibitors and major acids at different SE
594 temperatures.

595

596 Figure 5. Release of sugars by steam explosion and enzymatic saccharification.

597 a. The concentration of the reducing sugars and glucose solubilised in the SE process.

598 b. The reducing sugar yield following hydrolysis of residual CWM using 100 U

599 (4.35FPU)

600 g^{-1} substrate of CE and 100 U g^{-1} substrate of BG.

601

602 Figure 6. Optimization of enzyme saccharification. Reducing sugar yields are based on

603 taking unhydrolysed carbohydrate to be 100%.

604 a. Enzymatic saccharification using reduced CE concentrations (100, 50, 20 and 10 U g^{-}

605 ^{1} substrate).

606 b. Enzymatic saccharification using reduced CE: BG ratios (1: 1, 1: 0.5 and 1: 0.1), at

607 CE concentrations of 50 and 20 U g^{-1} substrate.

608 c. Glc yield produced by hydrolysing different pretreated duckweed samples with the

609 optimised enzyme cocktail (Celluclast 20 U g^{-1} and Novozyme 188 2 U g^{-1}). 'Fresh' -

610 untreated duckweed material; 'FD' - fresh material that has been freeze dried; FDM –

611 freeze-dried and freeze-milled material; WIAIR - blender-milled, water-insoluble

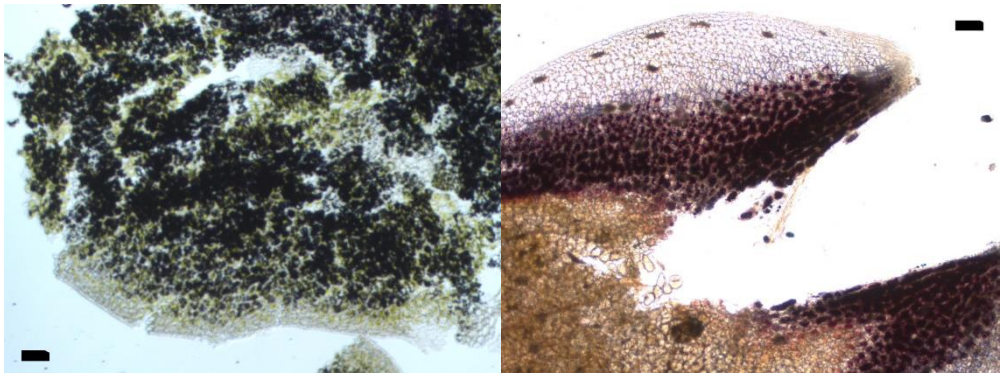
612 alcohol-insoluble residue; SE - 210°C steam exploded material.

613

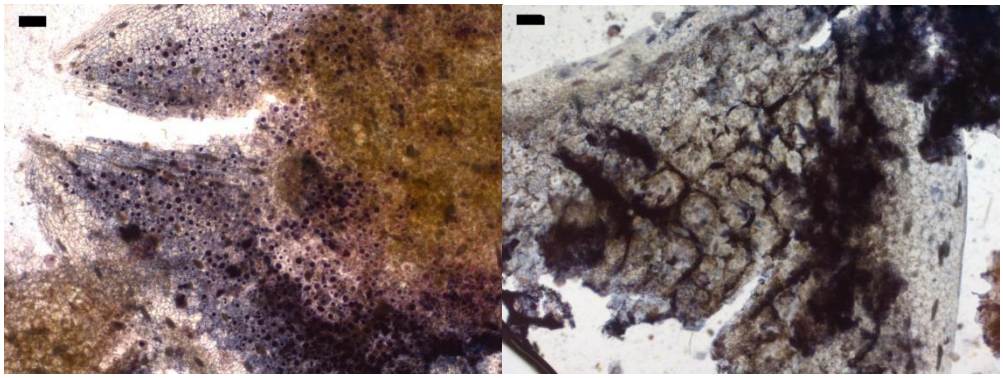
614 –Figure 1

615 –Figure 1

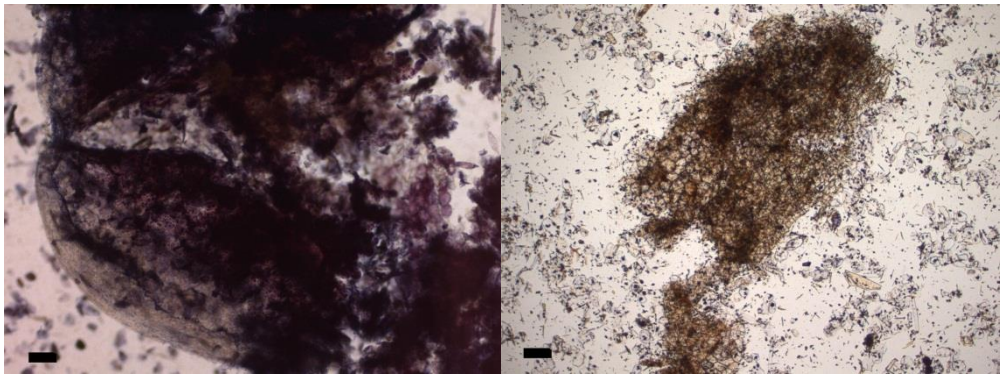
–Figure 1



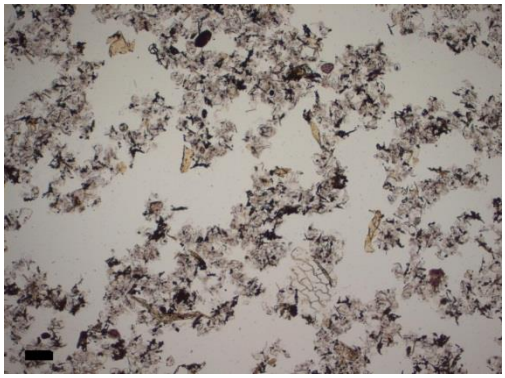
a. b.



c. d.



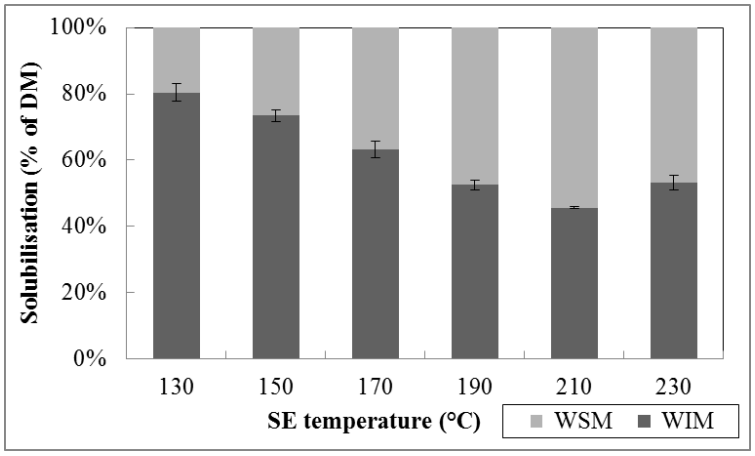
e. f.

624 

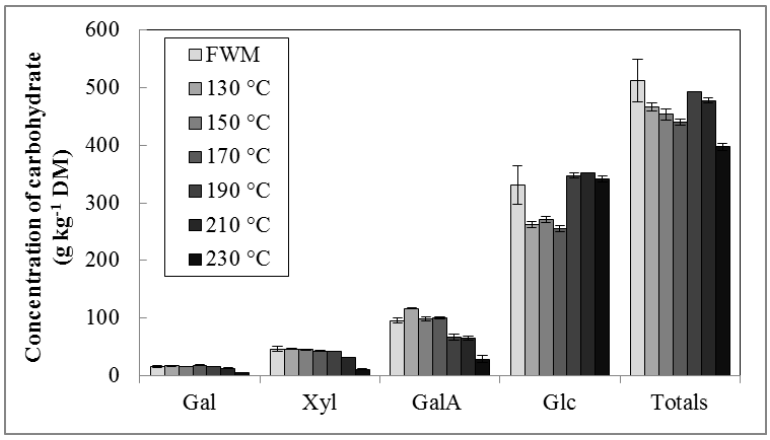
g.

625

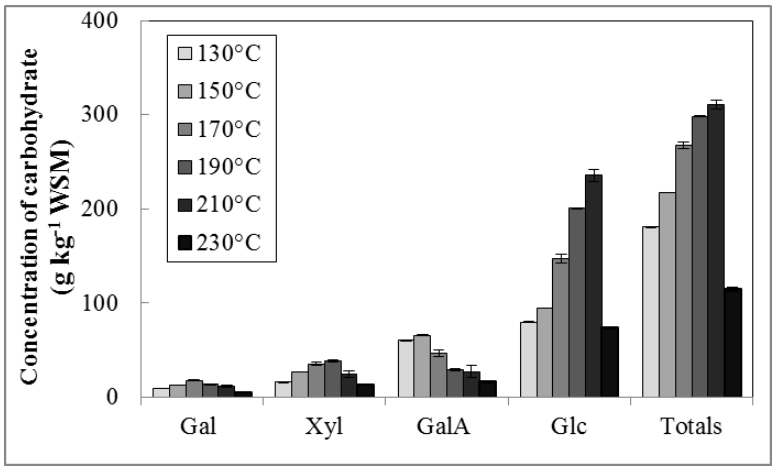
Figure 2.


100%
80%
60%
40%
20%
0%
Solubilisation (% of DM)
130
150
170
190
210
230
SE temperature (°C)
WSM
WIM

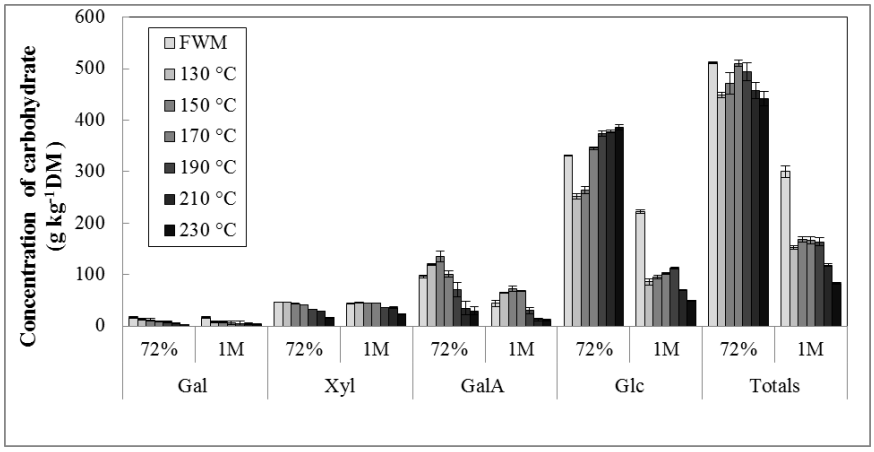
a.


600
500
400
300
200
100
0
Concentration of carbohydrate (g kg-1 DM)
FWM
130 °C
150 °C
170 °C
190 °C
210 °C
230 °C
Gal
Xyl
GalA
Glc
Totals

b.


400
300
200
100
0
Concentration of carbohydrate (g kg-1 WSM)
130°C
150°C
170°C
190°C
210°C
230°C
Gal
Xyl
GalA
Glc
Totals

c.



634

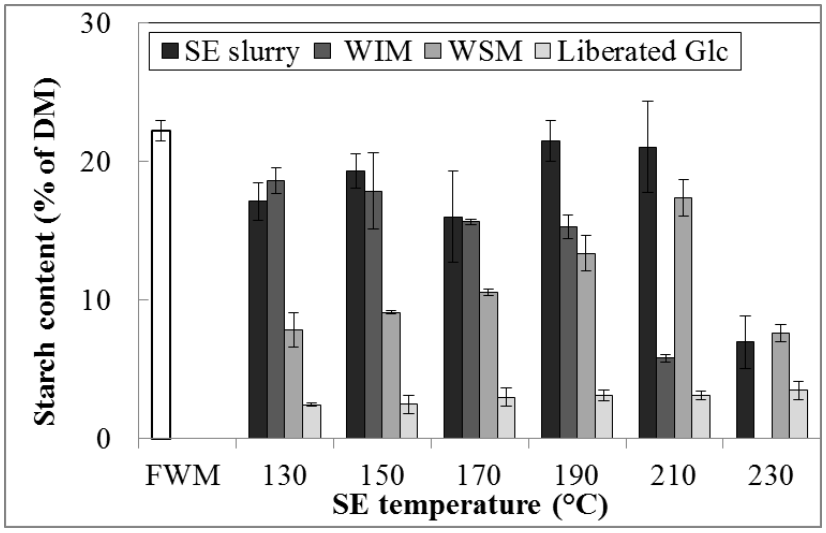
635 d.

636

637

638 Figure 3.

639



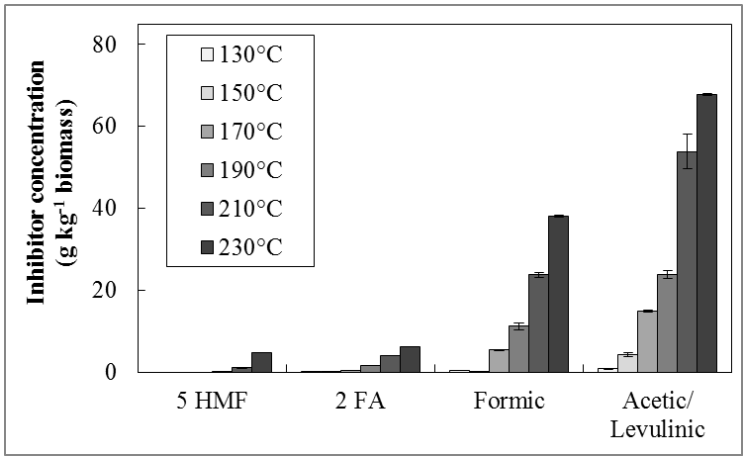
640

641

642

643 Figure 4.

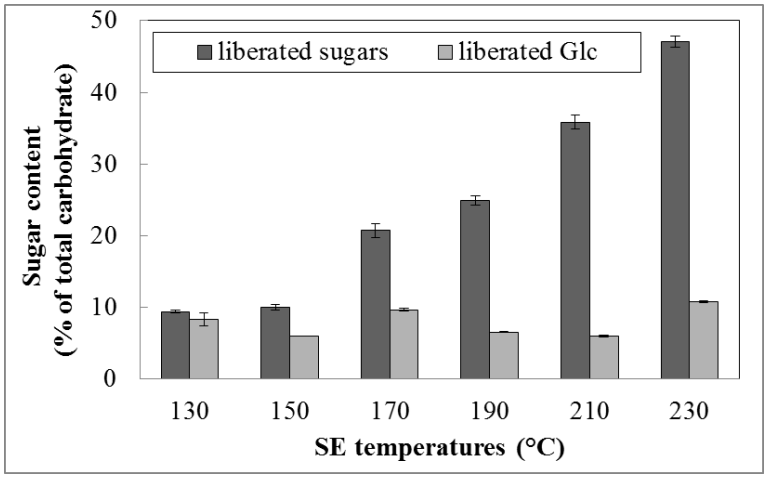
644



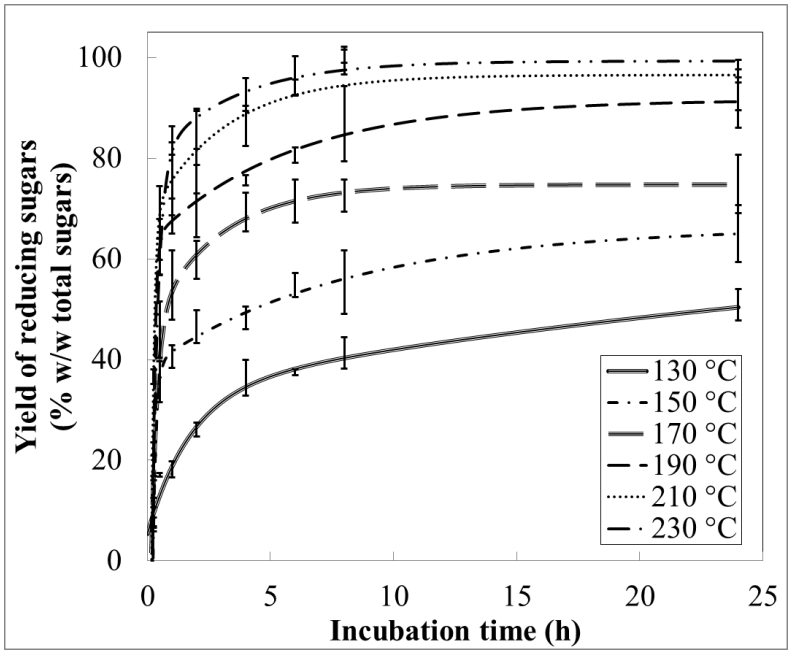
645

646

Figure 5.


liberated sugars
liberated Glc
Sugar content
(% of total carbohydrate)
50
40
30
20
10
0
130
150
170
190
210
230
SE temperatures (°C)

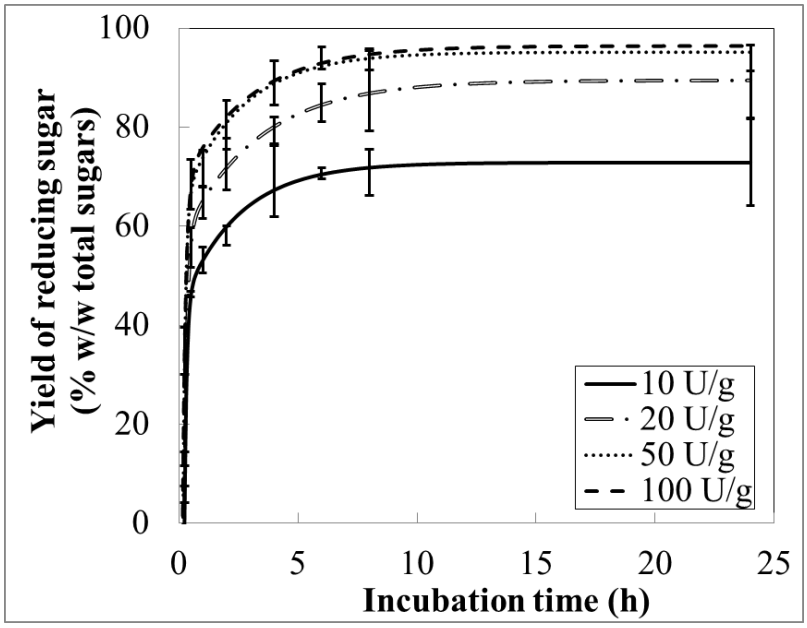
a.


Yield of reducing sugars
(% w/w total sugars)
100
80
60
40
20
0
0
5
10
15
20
25
Incubation time (h)
130 °C
150 °C
170 °C
190 °C
210 °C
230 °C

b.

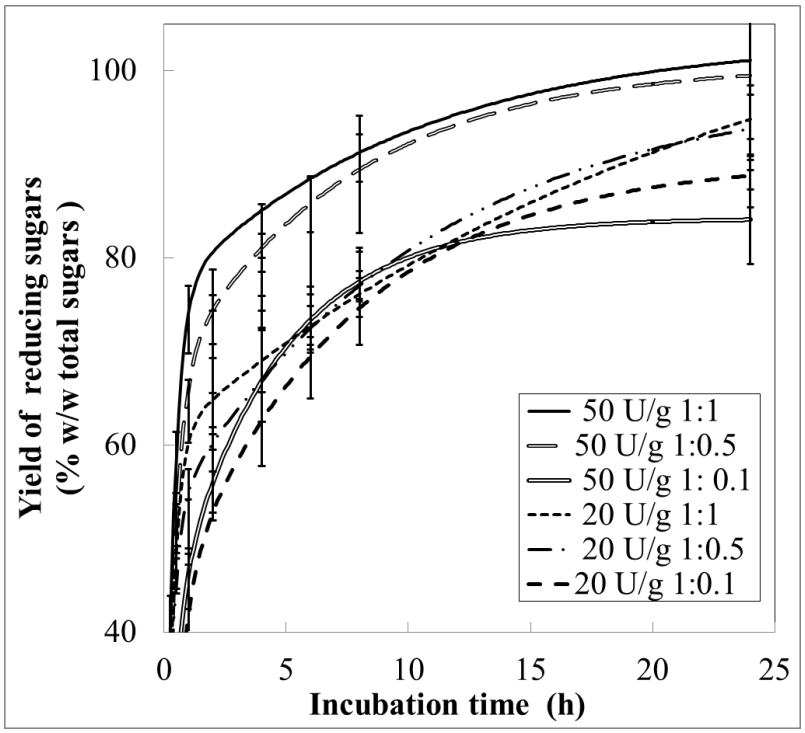
658 Figure 6.

659



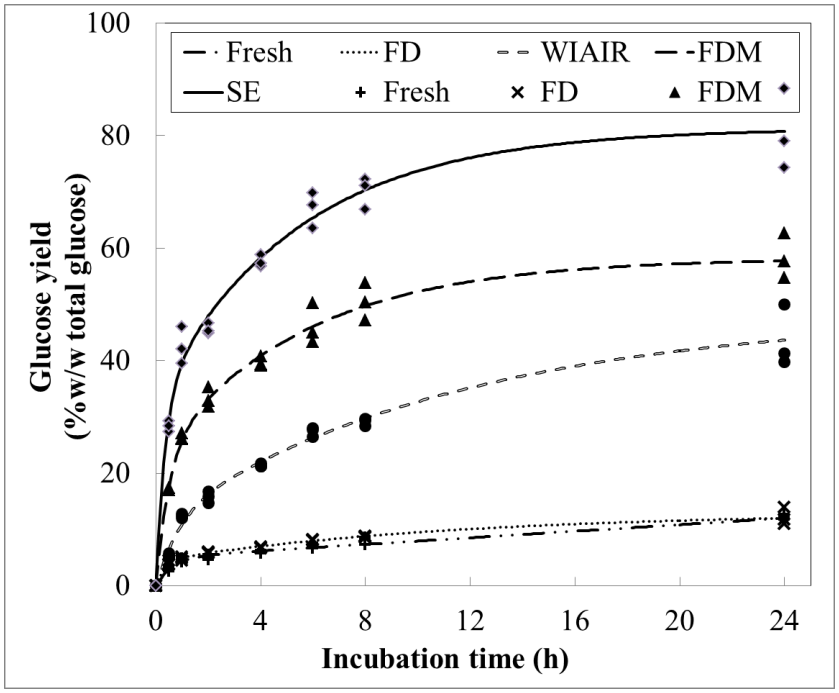
660

661 a.



662

663 b.


Fresh
FD
WIAIR
FDM
SE
Fresh
FD
FDM
Glucose yield (%w/w total glucose)
Incubation time (h)
0
20
40
60
80
100
0
4
8
12
16
20
24

c.

667

668

669 Table 1

SE temperature	Severity factor	slurry volume (ml)	slurry density (g ml^{-1})	slurry mass (g)	% DM	dry mass (g)	Recovery (%)
Untreated	/	1000	1.00	1000.0	8.5 ± 0.69	84.7	100
130 °C	1.9	1450	0.97 ± 0.01	1405.2	4.9 ± 0.05	69.3	81.8
150 °C	2.5	1860	0.95 ± 0.05	1762.9	3.6 ± 0.34	62.6	73.9
170 °C	3.1	2293	0.92 ± 0.08	2116.0	2.8 ± 0.25	59.0	69.7
190 °C	3.7	2080	0.96 ± 0.05	2003.4	3.4 ± 0.07	66.9	79.0
210 °C	4.2	2070	0.97 ± 0.05	2002.0	3.1 ± 0.05	60.9	71.9
230 °C	4.8	2310	0.94 ± 0.01	2170.8	2.5 ± 0.01	54.1	63.8

670

671