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# Simultaneous saccharification and fermentation of steam exploded duckweed: Improvement of the ethanol yield by increasing yeast titre

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## HIGHLIGHTS

- Steam explosion of duckweed enhances SSF at low (2% w/v) substrate concentrations.
- High substrate concentrations (20% w/v) result in much lower ethanol yields.
- Ethanol yields can be considerably increased with higher yeast inoculum.
- Or by preconditioning yeasts in steam explosion liquor containing inhibitors.
- The extra/preconditioned yeast metabolise fermentation inhibitors.

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## ABSTRACT

This study investigated the conversion of *Lemna minor* biomass to bioethanol. The biomass was pre-treated by steam explosion (SE, 210 °C, 10 min) and then subjected to simultaneous saccharification and fermentation (SSF) using Cellic® CTec 2 (20 U or 0.87 FPU $g^{-1}$ substrate) cellulase plus β-glucosidase (2 U $g^{-1}$ substrate) and a yeast inoculum of 10% (v/v or $8.0 \times 10^7$ cells $mL^{-1}$). At a substrate concentration of 1% (w/v) an ethanol yield of 80% (w/w, theoretical) was achieved. However at a substrate concentration of 20% (w/v), the ethanol yield was lowered to 18.8% (w/w, theoretical). Yields were considerably improved by increasing the yeast titre in the inoculum or preconditioning the yeast on steam exploded liquor. These approaches enhanced the ethanol yield up to 70% (w/w, theoretical) at a substrate concentration of 20% (w/v) by metabolising fermentation inhibitors.


## 1. Introduction

Renewable biofuels are increasingly sought as alternatives to fossil fuels because of their higher levels of sustainability and their contribution to a reduction of greenhouse gas emissions. Currently, biofuels account for approximately 1.5% of global transportation fuels (International Energy Agency, 2010). Duckweeds (aquatic plants of family *Lemnaceae*) have been proposed as a potential feedstock for biofuel production, because of their high proportion of carbohydrate, low lignin content (Jarvis et al., 1988) and high productivity (Landolt and Kandeler, 1987), as well as their useful role in the decontamination of wastewater (Zhao et al., 2012). Research to date has demonstrated the potential for conversion of duckweed biomass to ethanol. A high conversion rate of starch to ethanol following different pretreatments and enzyme treatments has been achieved (Cheng and Stomp, 2009; Chen et al., 2012), and high glucose yields have been obtained from cell wall material (CWM) using enzyme cocktails (Zhao et al., 2012). Chen et al. (2012) reported that almost 1300 gallons ($5.9 \times 10^3$ L) $ha^{-1}$ $y^{-1}$ of ethanol based on the dry matter harvest of 30 t $ha^{-1}$ $y^{-1}$ could be achieved from duckweed (*Lemna punctate*) treated with pectinase and α-amylase, a yield higher than that for corn stover (865.6 gallons equivalent to $3.9 \times 10^3$ $ha^{-1}$ $y^{-1}$) and corn (641.9 gallons equivalent to $1.9 \times 10^3$ $ha^{-1}$ $y^{-1}$). Ethanol yield from the duckweed *Spirodela polyrrhiza* is estimated at $6.4 \times 10^3$ L $ha^{-1}$ $y^{-1}$, based on an obtained starch yield of

*Abbreviations:* BG, Novozyme® 188; CE, Celluclast® 1.5; CTec 2, Cellic® CTec 2; CWMs, cell wall materials; FDM, freeze dry and freeze mill; SE, steam explosion; SSF, simultaneous saccharification and fermentation; YM, yeast and mould broth.

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9.42 t $ha^{-1}$ $y^{-1}$, which is 50% higher than that of maize-based ethanol production (Xu et al., 2011).

The extensive application and effectiveness of steam explosion for the conversion of lignocellulosic biomass to ethanol has resulted in numerous published studies in recent years. Our previous research demonstrated that high glucose yields were easily obtained from duckweed CWM using the cocktail of Celluclast 1.5 (CE) and Novozyme 188 (BG) (Zhao et al., 2012) and this enzyme cocktail was further optimised to a considerably lower dosage in conjunction with steam explosion pretreatment (Zhao et al., 2015). The above glucose yields also indicated the hydrolysis of most of the starch content. Although steam explosion results in the formation of undesirable fermentation inhibitors (Pedersen and Meyer, 2010), it is still considered to be one of the most tractable and economic approaches to improve ethanol yield and reduce production cost.

CE, although suited for laboratory research, is relatively expensive (Sigma–Aldrich, 2009) and not suited to use at larger scale. In this study it has been replaced by Cellic® CTec 2 (CTec 2), a more advanced cellulase for industrial use, containing a mixture of cellulase and β-glucosidase (Novozymes, 2012). The high activity of cellulase and β-glucosidase on lignocellulosic biomass was demonstrated by Cannella et al. (2012) although 4% of the glucose was unexpectedly converted to gluconic acid by CTec 2. Klein-Marcuschamer et al. (2012) stated that the contribution of enzymes to the total cost of production is much higher than scientists predict. Thus, choosing the most appropriate enzyme and employing the minimal enzyme dosage would be beneficial for maximising the ethanol product and reducing the cost. Based on previous studies (Zhao et al., 2012, 2015), the optimisation of CTec 2 to low levels in the simultaneous saccharification and fermentation (SSF) on the steam exploded duckweed biomass may enhance the conversion of duckweed to ethanol as well as potentially reducing the cost of ethanol production. In this paper we describe the production of ethanol from steam exploded duckweed biomass under SSF conditions using CTec 2 and approaches to increase the yield and concentration of ethanol at higher substrate concentrations.

## 2. Methods

### 2.1. Biomass acquisition and pretreatment

#### 2.1.1. Plant harvest

Duckweed (*Lemna minor*) plants were collected from a pond located at the John Innes Centre, Norwich, UK (52.622295N, 1.221894 E), then rinsed in tap water followed by distilled water. The cleaned fresh wet biomass was packed with aluminium foil in long flat-thin packages (1 kg each). The packed samples were stored at 4 °C for up to one week before steam explosion. The compositions of fresh and steam exploded *L. minor* have been published previously by Zhao et al. (2014).

#### 2.1.2. Steam explosion (SE)

The raw, wet *L. minor* biomass was treated by steam explosion at the Norwich Research Park Biorefinery Centre using a Cambi™ Steam explosion pilot plant at 210 °C for 10 min as described by Zhao et al. (2015). The steam exploded slurry was measured for volume and then frozen (−40 °C) until required for subsequent simultaneous saccharification and fermentation (SSF). Some freeze-dried materials were ground by freeze-milling in liquid nitrogen (Spex Freezer-Mill 6700, Spex Industries Inc., USA) to a powder for subsequent fermentation.

#### 2.1.3. Concentration of pretreated biomass

Duckweed biomass present in the SE slurry and centrifuged pellets was fermented at a range of dry matter concentrations. In initial experiments the concentrations of DM in SE slurry ranged from 2.3% to 2.8% (w/w). The dry matter concentration varied among different batches of fresh duckweed and their % DM was measured individually. For experiments employing low levels of dry matter (% DM $\leqslant$ 3%), the slurry was used directly as the fermentation substrate. To obtain higher levels of dry matter (% DM $\geqslant$ 3%), the moisture content was reduced using a rotary evaporator (Rotavapor R-114, BUCHI UK Ltd, Oldham, UK). 200 mL of the original SE slurry was transferred into a pre-weighed round-bottom flask and evaporated gently under reduced pressure at 50 °C. The SE slurries were uniformly dried to 40% of DM in batches which was monitored gravimetrically and the moisture content was finally determined using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd, Beaumont Leys, Leicester, UK).

### 2.2. Simultaneous saccharification and fermentation (SSF)

#### 2.2.1. Enzyme preparations

Commercial enzymes of Celluclast® (CE; Sigma–Aldrich Company Ltd., Dorset, UK), Cellic® CTec 2 (CTec 2, Novozymes A/S, Bagasvaerd, Denmark) and additional β-glucosidase (BG; Novozyme® 188, Sigma–Aldrich Company Ltd. Dorset, UK) were employed to hydrolyse the glucose resource that could be derived from cell wall sugars and starch. The enzyme activities are given by the supplier for CE and BG as 700 U $mL^{-1}$ (Sigma–Aldrich, 2009) and 250 U $mL^{-1}$ (Sigma–Aldrich, 2009) respectively. The FPU activity of CTec 2 was assessed as 189 FPU $ml^{-1}$, using the standard measurement for cellulase (Ghose, 1987). The enzyme preparations of "CE + BG" were made up according to Zhao et al. (2012) and were further optimised by reducing enzyme dosages to 20 U (0.87 FPU) $g^{-1}$ substrate CE plus 2 U $g^{-1}$ substrate BG based on the steam exploded duckweed materials (Zhao et al., 2015). The alternate enzyme cocktail of "CTec 2 + BG" was prepared by using the dosage of the optimised "CE + BG" as 20 U (0.87 FPU) $g^{-1}$ substrate CTec 2 plus 2 U $g^{-1}$ substrate BG.

#### 2.2.2. Yeast preparation

*Saccharomyces cerevisiae* strain (NCYC 2826) was obtained from the National Collection of Yeast Cultures (NCYC, Norwich, UK) and exhibits high ethanol tolerance of 15–20% (v/v). The strain was sub-cultured from a slope culture by inoculation into 1 L of Difco™ Yeast and Mould (YM) broth (Fisher Scientific UK Ltd., Loughborough, UK) containing 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone and 1% (w/v) dextrose (Elliston et al., 2013). Yeast was grown in this medium for 2 days at 25 °C and subsequently stored at 4 °C for up to 1 month before use (Elliston et al., 2013). Before yeast inoculation of the fermentation mix, the cultured yeast medium was centrifuged (3000 rpm, 5 min, Centrifuge 5810 R, Eppendorf UK Ltd., Stevenage, UK), the supernatant (YM media) discarded, and the yeast cells resuspended in nitrogen base (ForMedium™, Formedium Ltd, Hunstanton, UK). The total viable yeast cells were measured by using a cell count reader (NucleoCounter® YC-100™, ChemoMetec, Allerød, Denmark). The standard yeast culture contained $8.0 \times 10^7$ cells $mL^{-1}$ of *S. cerevisiae* NCYC2826.

#### 2.2.3. Subculture of yeast in SE medium

Yeast was also cultured in the liquor from steam exploded duckweed (which contains water-soluble sugars) to investigate the adaptability of yeast to SE medium. The SE medium was prepared as follows: a range of SE slurries (150, 170, 190, 210 and 230 °C) were centrifuged and supernatants were transferred into sealed bottles that were subsequently autoclaved and then cooled before yeast solution was added. With 10% (v/v) yeast inoculation, yeast was grown in the SE medium for 2 days. The total viable

yeast cells were measured using a cell count reader. The kinetics of yeast growing in the YM and SE media was established using a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA). A small scale yeast culture (200 μL) in SE medium was carried out in a 96-well flat-bottomed microtitre plate with lid (Nunc, Roskilde, Denmark). Yeast was also cultured in YM medium (200 μL) as a positive control and yeast-free media were prepared as blank controls simultaneously. The turbidity of culture solution was measured in a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at $\lambda$ = 590 nm at 30 min intervals, providing an indication of the quantity of yeast cells (Blomberg, 2011).

*2.2.4. Simultaneous saccharification and fermentation (SSF) process*

SSF of steam exploded duckweed (1, 5 10, 20% w/v) was performed in glass universal bottles. The reaction solution (10 mL) contained: substrate (1%, 5%, 10%, 20% w/v), 10% (v/v with $8.0 \times 10^7$ cells $mL^{-1}$) of *S. cerevisiae* NCYC2826, CTec 2 (20 U or 0.87 FPU $g^{-1}$ substrate) + BG (2 U $g^{-1}$ substrate) and Nitrogen base (6.9 g $L^{-1}$) (Elliston et al., 2013). The concentration of enzymes and nitrogen base were increased in proportion to the substrate concentration. The yeast inoculum comprised 10% (v/v) of final fermentation solution. The yeast titre was further concentrated by 4, 10, 20 and 50 times as part of studies into the effect of yeast inoculum. The SE slurries (20% w/w DM) were separately inoculated with concentrated YM cultured yeast: 4 times ($3.2 \times 10^8$ cells $mL^{-1}$), 10 times ($8.0 \times 10^8$ cells $mL^{-1}$), 50 times ($4.0 \times 10^9$ cells $mL^{-1}$) of the standard inoculum and SE medium cultured yeast at a basal concentration of $1.8 \times 10^7$ cells $mL^{-1}$, and concentrated 2 times ($3.6 \times 10^7$ cells $mL^{-1}$), 4 times ($7.2 \times 10^7$ cells $mL^{-1}$) and 20 times ($3.6 \times 10^8$ cells $mL^{-1}$) of the norm. When adding concentrated inocula, the enzyme cocktail and nitrogen base were proportionally added to bring the volume up to 10 mL. Substrate blanks were prepared as a control to detect fermentable sugars and ethanol from YM solution or enzyme preparations and subtracted from the sample readings. SSF samples were incubated over 72 h at 25 °C with moderate agitation (120 rpm). Strong agitation (300 rpm) was used in one experiment to assess the effect of agitation on ethanol yield from a high substrate concentration (20% w/w). Aliquots (2 mL) of fermented samples were transferred to screw-cap tubes and were heated at 100 °C for 5 min to terminate the SSF. The resulting samples were centrifuged and the supernatants were assessed for ethanol and fermentation inhibitors using HPLC methods (below).

*2.3. Analytical methods*

*2.3.1. The assessment of ethanol and residual fermentable sugars*

The ethanol product was prepared and assessed using the HPLC methods described by Elliston et al. (2013). SSF samples were centrifuged, filtered, measured using an HPLC fitted with carbohydrate analysis column with RI detector (Elliston et al., 2013). Ethanol yield (% w/w) was calculated according to the formula below.

$$\text{Ethanol yield } (\%) = \frac{\text{ethanol product (g)}}{\text{theoretical ethanol product (g)}} \times 100\%$$

The theoretical amount of ethanol product (g) was obtained from molecular weight of glucose (180.2 g $mol^{-1}$) and ethanol (46.1 g $mol^{-1}$). Thus, the theoretical ethanol product is 51.2% (w/w) of the maximum glucose content of steam exploded slurry. The maximum glucose content of steam exploded slurry have been measured in the previous research (Zhao et al., 2015).

*2.3.2. GC sugar analysis*

The carbohydrates and total glucose present in SE slurry and SSF pellets were analysed as alditol acetates according to the gas chromatography method of Blakeney et al. (1983). The sample preparation process was as described by Zhao et al. (2012). The prepared samples were analysed by GC on a PerkinElmer Autosystem XL GC system (PerkinElmer, Seer Green, Bucks., UK) containing Rtx®-225 column (Thames Restek UK Ltd, Saunderton, UK).

*2.3.3. Quantification of free glucose using the GOPOD test*

The hydrolysed glucose and unfermented glucose were detected by the specific GOPOD test method (McCleary et al., 1994). The process was proceeded according to Zhao et al. (2012). The background absorbance from blank enzyme preparations was subtracted and the concentration of sugars calculated from appropriate standard curves.

*2.3.4. Fermentation inhibitor assessment*

2-furfuraldehyde (2-FA), 5-Hydroxymethylfurfural (5-HMF) and organic acids (formic and acetic acid) were assessed to help understand their impact on ethanol yields. Steam-exploded slurry (2 mL) and SSF products were centrifuged at 2465×*g* and 200 μL of the supernatant 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 (PerkinElmer, 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 0.005 mol $L^{-1}$ $H_2SO_4$ (Sigma–Aldrich, 2009) as mobile phase at a flow rate of 0.6 mL $min^{-1}$.

## 3. Results and discussions

*3.1. Evaluation of commercial cellulase – Cellic® CTec 2*

Previously (Zhao et al., 2015) we demonstrated efficient digestion of steam exploded *L. minor* with an enzyme cocktail of Celluclast supplemented with additional β-glucosidase ("CE + BG") at a very low concentration of 20 U (0.87 FPU) $g^{-1}$ substrate of CE and 2 U $g^{-1}$ substrate of BG. Unfortunately, CE is relatively expensive and therefore inappropriate for industrial use at large scale. CTec 2, a new generation cellulase product, contains higher cellulase activity reported to vary from 119 to 132 FPU $mL^{-1}$ (Reye et al., 2011; McIntosh et al., 2012). To replace CE by using CTec 2 in the fermentation study, the appropriate CTec 2 dosage, in the form of a cocktail of "CTec 2 + BG", was chosen which gave a comparative saccharification when compared with "CE + BG". The optimised enzyme dosages of cellulase (0.87 FPU $g^{-1}$ substrate) and BG (2 U $g^{-1}$ substrate) were then used as baseline conditions against which a range of enzyme cocktails comprising "10 × CTec 2", "2 × (CTec 2 + BG)", "2 × CTec 2 + BG" were compared. In all saccharification experiments, the amount of glucose released by the steam explosion pretreatment (6%, Fig. 1) was measured using the GOPOD method and deducted from the total glucose to give the enzymatically released glucose yield. Fig. 1 shows that the enzyme cocktails of "CTec 2 + BG" and "CE + BG" resulted in comparable glucose yields over a 24 h incubation (76.6% and 79.5% respectively). Doubling the enzyme concentration to "2 × (CTec 2 + BG)" and "2 × CTec 2 + BG" gave no benefit. Enzymatic hydrolysis using only CTec 2 at 10× the concentration actually resulted in a much lower glucose yield (59.4%). It is possible that very high levels of enzyme interfered with access by the enzymes to the substrate as had been found previously in waste paper (Elliston et al., 2013). These data suggest that "CTec 2 + BG" has very similar effectiveness to "CE + BG" and that as for

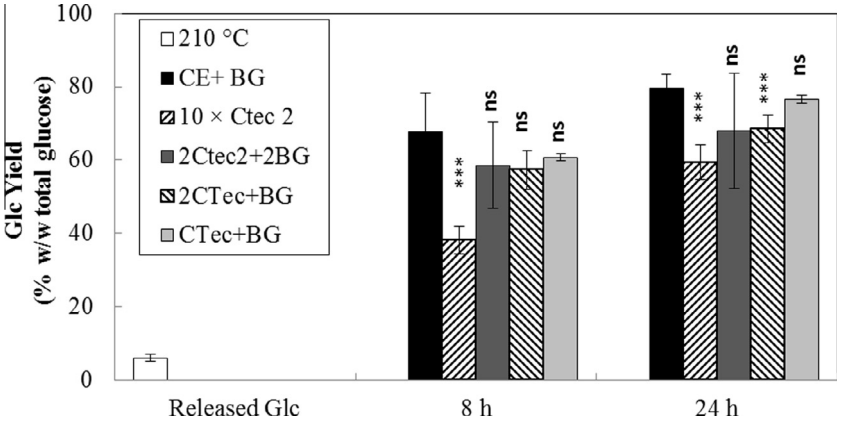


**Fig. 1.** The identification of optimum CTec 2 dosage and comparison to CE. The enzyme dosage is cellulase: 0.87 FPU $g^{-1}$ substrate, BG: 2 U $g^{-1}$ substrate. Released Glc represents the amount of glucose produced by steam explosion alone. $^{***}p < 0.001$, $^{*}p < 0.05$, ns = not significant ($p > 0.05$) compared with the sample of CE + BG.

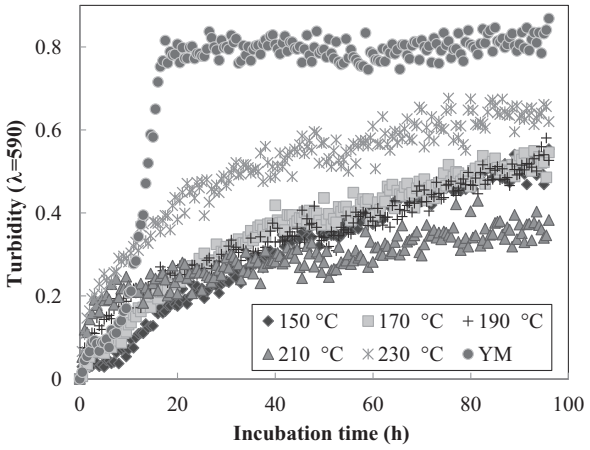


**Fig. 2.** A 96 h yeast culture in YM medium and various SE liquors pretreated for 10 min. at different temperatures. Glucose concentration in the SE liquors was 0.1 mg $mL^{-1}$ while the concentration of YM was 10 mg $mL^{-1}$. Turbidity provides an indication of the yeast cell concentration.

CE, CTec 2 exhibits synergy with BG. In addition, previous research (Zhao et al., 2012) has demonstrated the necessary addition of BG to the cellulase enzyme to hydrolyse duckweed polysaccharides to glucose. Thus "CTec 2 + BG" containing CTec 2: 20 U (0.87 FPU) $g^{-1}$ substrate and BG: 2 U $g^{-1}$ substrate was selected as the optimal enzyme preparation for SSF.

### 3.2. Yeast culture

The ability of the chosen yeast strain to proliferate in YM and media comprising liquor from steam exploded duckweed (SE media) was assessed by 96 h yeast cultures at small scale (200 μL) in a 96-well plate. Fig. 2 shows the growth kinetics of yeast in YM medium and a range of SE media. These were used "as produced" without any supplementation with fermentable sugars. The carbohydrate compositions of the SE liquors were reported previously (Zhao et al., 2015) and the concentrations of fermentable glucose were approximately 0.1 mg $mL^{-1}$. Over a 96 h incubation, yeast growing in YM medium exhibited three growth phases: a very short lag phase, a short but rapid linear growing phase (less than 24 h) and a long stationary phase (72 h). Ciani and Picciotti (1995) observed similar growth kinetics for various yeasts used in wine production. In our study, yeast growing in the SE media generally exhibited a variable lag phase then underwent a linear growth phase longer than that observed in YM medium reflecting the presence of inhibitors and the lower concentration of fermentable glucose. The linear growing phase of SE liquor-based media continued throughout the entire 96 h incubation (after the lag phase) except for that derived after SE at 230 °C. Here, the growth phase terminated after 80 h with the turbidity of 0.65 (Fig. 2). The turbidities of cultures in 150, 170 and 190 °C SE media are all close to 0.5. The results show that the yeast used will effectively proliferate in all the SE liquors with similar growth curves to those reported by Field et al. (2015). Since SE for 10 min at 210 °C was already established as the optimal severity for duckweed pretreatment, (Zhao et al., 2015) a bulk quantity of such slurry was produced for SSF investigations.

Yeast growth in YM and SE (210 °C) medium was further examined by counting viable cells. Yeast in the standard YM medium proliferated to $8 \times 10^7$ cells $mL^{-1}$ of viable yeast cells which was 4 times that seen in SE medium ($1.8 \times 10^7$ cell $mL^{-1}$; Table 1). A slightly higher number of dead cells ($2.9 \times 10^6$ cell $mL^{-1}$) was present in YM medium compared to SE medium (210 °C) ($\leqslant 6 \times 10^3$ cell $mL^{-1}$). The ratio of viable yeast cells in YM medium and SE medium (210 °C) closely reflects the differences in yeast densities measured by turbidity. The results illustrate that during 2 days of incubation, yeast growth in SE medium is likely to remain in the linear growth phase and will eventually obtain less yeast cells than YM media (Weiss et al., 2004).

### 3.3. Ethanol production by SSF of duckweed after pretreatment

The effect of pretreatments on ethanol yield was initially investigated by fermenting a range of materials including fresh raw (untreated) material, FDM and SE (210 °C) material with a freshly cultured yeast inoculum (10% v/v; $8.0 \times 10^7$ cells $mL^{-1}$). The initial

**Table 1**
Viable yeast cultured using YM and SE media. Sugars concentrations of SE medium are 1 mg $mL^{-1}$ while the concentration of YM medium is 10 mg $mL^{-1}$.

| Medium | Total cells (cells $mL^{-1}$) | Dead cells (cells $mL^{-1}$) | Viable cells (cells $mL^{-1}$) |
|---|---|---|---|
| SE liquor | $1.8 \times 10^7$ | $\leqslant 6 \times 10^3$ | $1.8 \times 10^7$ |
| YM | $8.3 \times 10^7$ | $2.9 \times 10^6$ | $8.0 \times 10^7$ |

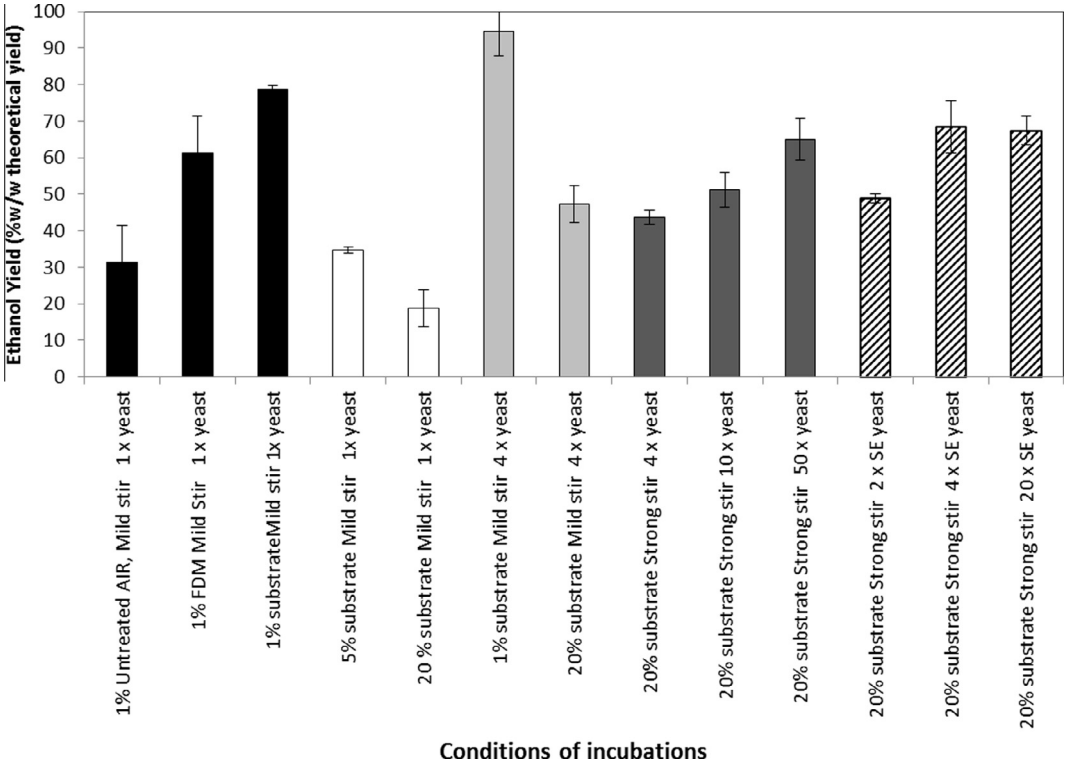


**Fig. 3.** Effect of SSF conditions on ethanol yield.

substrate was 1% (w/w) DM. SSF was carried out for 72 h and ethanol yields were assessed using HPLC. The results are shown in Fig. 3 (black bars). Ethanol yield from untreated duckweed was only 31.4% (w/w) of the theoretical maximum (based on glucose levels only). However, FD pretreatment enhanced the ethanol yield to 61.3% (w/w of theoretical maximum). Steam explosion resulted in a further increase in ethanol yield, to 78.5% (w/w of theoretical maximum). The results indicate that in spite of their simple structure and lack of lignin, thermophysical pretreatments still enhance the SSF of duckweed biomass, perhaps by helping to remove the pectic and hemicellulosic polysaccharides from around the cellulose. Pretreatments prior to enzymatic saccharification make lignocellulosic biomass more accessible to enzymes due to the removal of restricting non cellulosic components (Waldron, 2010; Chundawat et al., 2010). The results are consistent with our previous saccharification studies (Zhao et al., 2015) in which steam explosion of duckweed biomass demonstrated that SE effectively exposed cellulose to enzyme degradation and facilitated quantitative release of glucose at low enzyme loadings (0.87 FPU $g^{-1}$ substrate). In comparison, pretreated lignified biomass typically requires much higher enzyme loadings in order to achieve good saccharification (e.g. Chen et al., 2008 with wheat straw).

The extent of SSF on SE (210 °C) duckweed was reflected in the level of glucose remaining in the insoluble SSF pellets. As a function of the original substrate, the glucose level had been reduced to less than 100 g $kg^{-1}$. The amount of glucose consumed closely matched the ethanol yield (results not shown).

### 3.4. Investigation of digestibility of concentrated SE materials

A low substrate concentration (1% w/v) SSF resulted in a good ethanol yield from SE duckweed (Fig. 3). However, the low substrate loading resulted in very low ethanol concentration (from a commercial perspective), being in the order of 0.25% (v/v). Since the ethanol concentration required for distillation is generally considered to have to exceed 4% for second generation biorefining, the substrate concentration of pretreated duckweed for SSF would have to be at least 20% (w/v) biomass since it is 35.2% glucose (Zhao et al., 2015). Thus, concentrating the substrate prior to fermentation is required. This was achieved using vacuum evaporation and would be expected to potentially remove some of the volatile inhibitors (formic and acetic acids). However, SSF of 5% and 20% (w/w DM) resulted in reduced ethanol yields of 47.7% and 18.8% (w/w of theoretical) respectively (Fig. 3, white bars). For 20% (w/v) substrate, the ethanol concentrations were the order of only a few percent. These results indicate that high substrate concentrations would severely limit the ethanol yield commercially. This would increase considerably the cost of processing, particularly downstream distillation.

It was hypothesised that vacuum evaporation might theoretically have caused the hornification of concentrated SE materials which would reduce the ease of saccharification and consequently affect the ethanol yield (Luo and Zhu, 2011). Therefore the impact of evaporation on the digestibility of concentrated SE slurry was assessed by enzymatic saccharification (see Fig. 4a and b). Initially steam exploded slurry (210 °C, 10 min) at a range of concentrations was digested using the standard enzyme cocktail of CTec 2 (0.87 FPU $g^{-1}$) + BG (2 U $g^{-1}$). The results (Fig. 4a) showed that at a low substrate concentration of 2.3% (w/v), the glucose yield was about 80–90% theoretical, but dropped at high substrate concentrations of 20% (w/v) to only 47.7% theoretical. At the higher substrate concentration, the mix was also highly viscous. However, if the highly concentrated solids were diluted back to 2.3%, and then digested with the original enzyme cocktail of CTec 2 (0.87 FPU $g^{-1}$) + BG (2 U $g^{-1}$) under the standard conditions, similar glucose yields (2.5 mg $mL^{-1}$) were obtained for all of the samples (Fig. 4b). This indicated that the vacuum evaporation had not resulted in hornification.

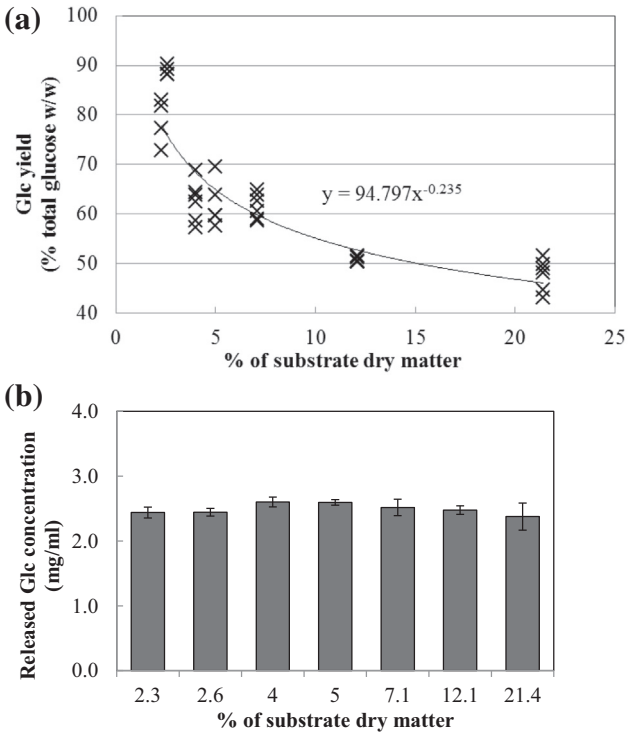


**Fig. 4.** The investigation of the digestibility of concentrated materials and their fermentation inhibition. (a) Enzymatic saccharification at different substrate concentrations. (b) Enzymatic saccharification on concentrated materials after dilution to the same dry matter contents. The samples show no significant differences ($F(6,35) = 1.77$, $p = 0.133$).

### 3.5. Improvement of ethanol yield of SSF at 20% substrate concentration

Two other possible reasons for the decrease in ethanol yield at higher concentration were (a) an increase in the concentration of inhibitors in the fermenting broth and (b) the high viscosity of the material. The former could reduce yeast performance whilst the latter could reduce diffusion of enzymes and products of hydrolysis and fermentation resulting in locally high concentrations and possibly negative feedback inhibition. The level of inhibitors present in concentrated material was certainly of an order which would be expected to impact on fermentation by yeast (Fig. 5; Elliston et al., 2015). Previously, we demonstrated that yeast could metabolise the inhibitors and adapt to their presence (Field et al., 2015). Furthermore, a previous study on enzymatic saccharification (Elliston et al., 2014) indicated that increased agitation may be beneficial for enhancing the ethanol yield from SSF of concentrated cellulosic biomass. Therefore the effects of adding larger quantities of yeast, preconditioning yeast on SE liquor, and stirring the reaction mixture more vigorously (300 rpm) were assessed.

Initially, the sole addition of yeast at 4 times the base level to 1% and 20% substrate was investigated, keeping other conditions the same (Fig. 3, light grey bars). At 1% substrate concentration the yield of ethanol was improved from 78% to over 92%. At 20% substrate concentration the yield was more than doubled from the low level of circa 20% to over 45% theoretical yield. However this was still unacceptable. Therefore the intensity of stirring was increased, and the effect of adding higher titres of yeast evaluated (dark grey bars). Extra stirring had no significant effect. However, addition of yeast at up to 50× the normal level increased the final ethanol yield to 65% of the theoretical maximum. Finally, the effect of adding preconditioned yeast at different concentrations was evaluated (Fig. 5, hatched bars). The addition of extra "SE" yeast at 4× the normal titre increased ethanol yield to nearly 70% (theoretical maximum) or 13.5% (g/g DM). Addition of "SE" yeast at 50× had no additional benefit. The enhanced levels of ethanol caused by addition of larger titres of yeast also reduced the levels of unfermented glucose (results not shown).

It has been reported previously that the negative impact of certain fermentation inhibitors may be mitigated by using a larger yeast inoculum (Navarro, 1994). In the current study, the ethanol production is further improved by using a precultured yeast. The beneficial effect of additional and/or precultured yeast was further evaluated by measuring the levels of inhibitors remaining after SSF (Fig. 5). The results show that additional, and pre-conditioned (SE) yeast demonstrated significant decreases in the levels of inhibitors. Compared with the costs of wheat straw biorefining to produce ethanol (Littlewood et al., 2013), the lower levels of enzyme and inoculum required for duckweed biorefining indicate that the cost of ethanol production are likely to be lower. In addition, duckweed-derived protein in the co-product may also create an economic advantage as in first generation biorefining.

Apart from the works of Zhao et al. (2015, 2014, 2012) other research on converting duckweed to bioethanol has focused on exploiting the intracellular starch alone. Xu et al. (2011) unveiled that 97.8% (theoretical ethanol) of ethanol yield was achieved by fermenting *S. polyrrhiza* containing 31% (w/w DM) of starch hydrolysed by α-amylase, pullulanase, and amyloglucosidase. Chen et al. (2012) reported that over 90% ethanol yield (3.9% v/v) could be

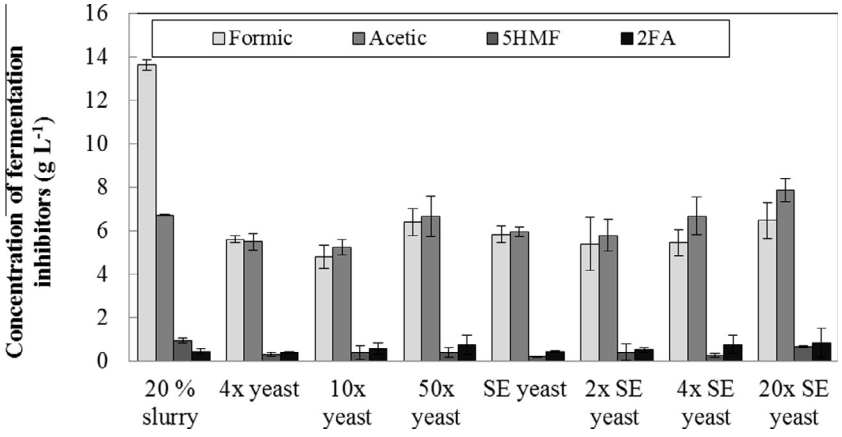


**Fig. 5.** Levels of fermentation inhibitors remaining after SSF using larger titres of yeast and/or preconditioned yeast.

obtained by fermenting pectinase (26.5 PECTU $g^{-1}$ mash) pretreated duckweed (*Landoltia punctata*) which contains 75% (w/w DM) of starch. In the present study 70% of theoretical ethanol yield (3.5% v/v) was obtained using a very low enzyme cocktail of CTec 2 (0.87 FPU $g^{-1}$ substrate) and BG (2 U $g^{-1}$ substrate) and high yeast inoculum. The study demonstrates the effectiveness of steam explosion on ethanol yield during SSF of duckweed biomass and the potential for cost reduction in the production of ethanol from duckweed feedstock. The enzyme loading is much lower than that required for lignified biomass which depends very much on thermophysical pretreatments. In research on wheat straw reported by Luo et al. (2008): 65.8% (theoretical ethanol) ethanol yield was achieved using a SSF approach on steam exploded wheat straw (substrate: 10% w/v loading) with an enzyme cocktail of Celluclast 1.5 (30 FPU $g^{-1}$ substrate) and additional BG under similar fermentation conditions. The total of the recovered glucose calculated from the ethanol yield (68.4%) and unfermented glucose (9.6%) in the SSF process is similar to the maximum glucose yields (80% w/w) obtained by hydrolysing steam exploded duckweed (Fig. 1), but does not match the glucose concentration in the substrate. This indicates that 20% of glucose was not saccharified in the SSF process or some of the glucose might be oxidised (Cannella et al., 2012). The 10% of the glucose that was unfermented may probably be attributed to inhibitory effects.

## 4. Conclusions

Using an enzyme cocktail of Cellic® CTec 2 (0.87 FPU/g substrate) together with Novozyme® 188 (2 U $g^{-1}$ substrate), 94.7% (w/w of theoretical) yield of ethanol was obtained from steam exploded duckweed substrate (1% w/w). However increasing substrate concentration lowered the yield substantially. Improved ethanol yields at the higher substrate concentration could be achieved using higher yeast titres, and/or preconditioning the yeast in pretreatment liquor so that the yeast could metabolise fermentation inhibitors. Nearly 70% (w/w of theoretical ethanol) or 3.5% (v/v) (equivalent to 13.5% g/g DM) was achieved from concentrated substrate (20% w/v).

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