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Original Research Paper

Maximising high solid loading enzymatic saccharification yield from acid-catalysed hydrothermally-pretreated brewers spent grain

Stuart Wilkinson[1], Katherine A. Smart[2], Sue James[2], David J. Cook[1,*]

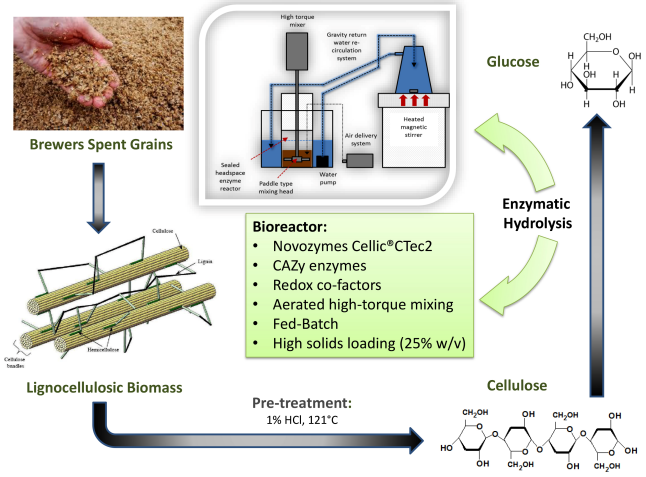
[1] *International centre for Brewing Science (ICBS), Division of Food Sciences, The University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, U.K.*

[2] *SABMiller Plc, SABMiller House, Church Street West, Woking, Surrey, GU21 6HS, U.K.*

HIGHLIGHTS

- Cellulolytic enzyme saccharification of pre-treated brewers spent grains was investigated at high solids loading.
- Aerated high-torque mixing offered enhanced glucose yields to 53 g/L.
- Fed-batch protocols enhanced glucose yields to 59 g/L.
- Supplementary carbohydrate degrading enzymes boosted achieved glucose yields to 64 g/L.
- A novel consolidated saccharification protocol further increased glucose yields to 78 g/L at 25% w/v solids loading.

GRAPHICAL ABSTRACT



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ABSTRACT

Enzyme saccharification of pretreated brewers spent grains (BSG) was investigated, aiming at maximising glucose production. Factors investigated were; variation of the solids loadings at different cellulolytic enzyme doses, reaction time, higher energy mixing methods, supplementation of the cellulolytic enzymes with additional enzymes (and cofactors) and use of fed-batch methods. Improved slurry agitation through aerated high-torque mixing offered small but significant enhancements in glucose yields (to 53 ± 2.9 g/L and 45% of theoretical yield) compared to only 41 ± 4.0 g/L and 39% of theoretical yield for standard shaking methods (at 15% w/v solids loading). Supplementation of the cellulolytic enzymes with additional enzymes (acetyl xylan esterases, ferulic acid esterases and α-L- arabinofuranosidases) also boosted achieved glucose yields to 58 – 69 ± 0.8 - 6.2 g/L which equated to 52 - 58% of theoretical yield. Fed-batch methods also enhanced glucose yields (to 58 ± 2.2 g/L and 35% of theoretical yield at 25% w/v solids loading) compared to non-fed-batch methods. From these investigations a novel enzymatic saccharification method was developed (using enhanced mixing, a fed-batch approach and additional carbohydrate degrading enzymes) which further increased glucose yields to 78 ± 4.1 g/L and 43% of theoretical yield when operating at high solids loading (25% w/v).

* Corresponding author at: Tel.: +44 (0)115 9516245
E-mail address: david.cook@nottinham.ac.uk

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1. Introduction

Production of bioethanol from brewers spent grains (BSG) is a current area of research interest as higher value uses are sought for this co-product derived from the beer brewing process. The recalcitrant nature of BSG (like other lignocellulosic biomass types) renders some forms of pre-treatment essential before saccharification enzymes can be used to liberate fermentable sugars. Numerous effective chemical and thermal pre-treatments for BSG have been developed that can enhance the subsequent enzymatic saccharification yields (Wilkinson et al., 2014a; Wilkinson, 2014b). However, large doses of expensive commercial enzymes are still usually required to achieve high conversion efficiencies of cellulose to glucose (Leathers, 2003; Chundawat et al., 2008; Qing et al., 2010). Therefore, optimisation of the enzymatic saccharification stage is a key objective in the cost-effective production of lignocellulosic biofuels.

Maximising the operational solids loading used (a high solid to liquid ratio) during the enzymatic saccharification step, whilst minimising the required enzyme dose are likely to be key factors which need to be addressed in order to produce high glucose concentrations cost effectively and minimise water usage (Hodge et al., 2008; Kristensen et al., 2009). Commercial scale bioethanol production would likely require a minimum glucose concentration of ca. 100 g/L (ca. 10% w/v) in order to realistically produce the benchmark minimum ethanol concentration of ca. 50 g/L (ca. 5% v/v) which may then facilitate economically viable distillation (Lau and Dale, 2009). Operation at high solids loading (>10% w/v) during the enzymatic saccharification step would likely be essential in order to achieve this through limiting the dilutional effect of surplus liquid water (Kristensen et al., 2009). However, operation at high solids loading would likely drop the conversion efficiency of cellulose to glucose resulting in % theoretical yields well below acceptable limits of process efficiency. This is possibly due to rheology related mass transfer limitations because of the high viscosity of the media impeding enzymatic access to all of the available substrate (cellulose) and impeding dispersal of the hydrolysis products which may feedback inhibit the cellulases (Gan et al., 2003; Chundawat et al., 2008). There is also a minimum requirement of some available water for the successful catalytic function of many lignocellulolytic enzymes due to their hydrolytic mechanism of glycosidic bond fracturing through addition of a water molecule (Horn et al., 2012). This would suggest that an upper limit exists with regards to the maximum functional solids loading that can be used during the enzyme saccharification step as going beyond this would detrimentally limit the available free water present.

Various enzymes from the CAZy database (http://www.cazy.org/) of carbohydrate degradation specific enzymes are indicated as being potentially involved in the successful degradation of lignocellulosic substrates such as BSG, many of which come from fungal enzymatic systems such as brown and white rot fungi (Evans et al., 1994; Delmas et al., 2012). These enzymes include members of the glycoside hydrolase (GH) family, carbohydrate esterase (CE) family, and polysaccharide lyase (PL) family (**Fig. 1**). Other non-CAZy classified candidates could include ferulic acid esterases (feruloyl esterases) which have been shown to release ferulic acid (and other hydroxycinnamic acids) from substrates such as BSG (Bartolome and Gómez-Cordovés, 1999; Faulds et al., 2002). Also various metal ions (in particular copper) have been suggested to bind to the active site of GH61 class enzymes and play a crucial role in their activity (Quinlan et al., 2011). In addition various redox active cofactors such as gallate and ascorbate have been suggested to function as possible enhancers of GH61 enzyme activity as they may act as electron donors or chemical reductants (Quinlan et al., 2011; Horn et al., 2012).

Most commercial enzyme preparations designed for advanced generation biofuel processes are tailored to be as broad spectrum as possible so as to be effective on the wide variety of lignocellulosic substrates of interest such as woody biomass, energy crops/grasses, and industrial waste streams etc. (Harris et al., 2010). However, the highly substrate specific nature of the format of the lignocellulose (such as the specific side chain decorations of the xylan backbone which composes hemicellulose) may render generic enzyme preparations sub-optimal in

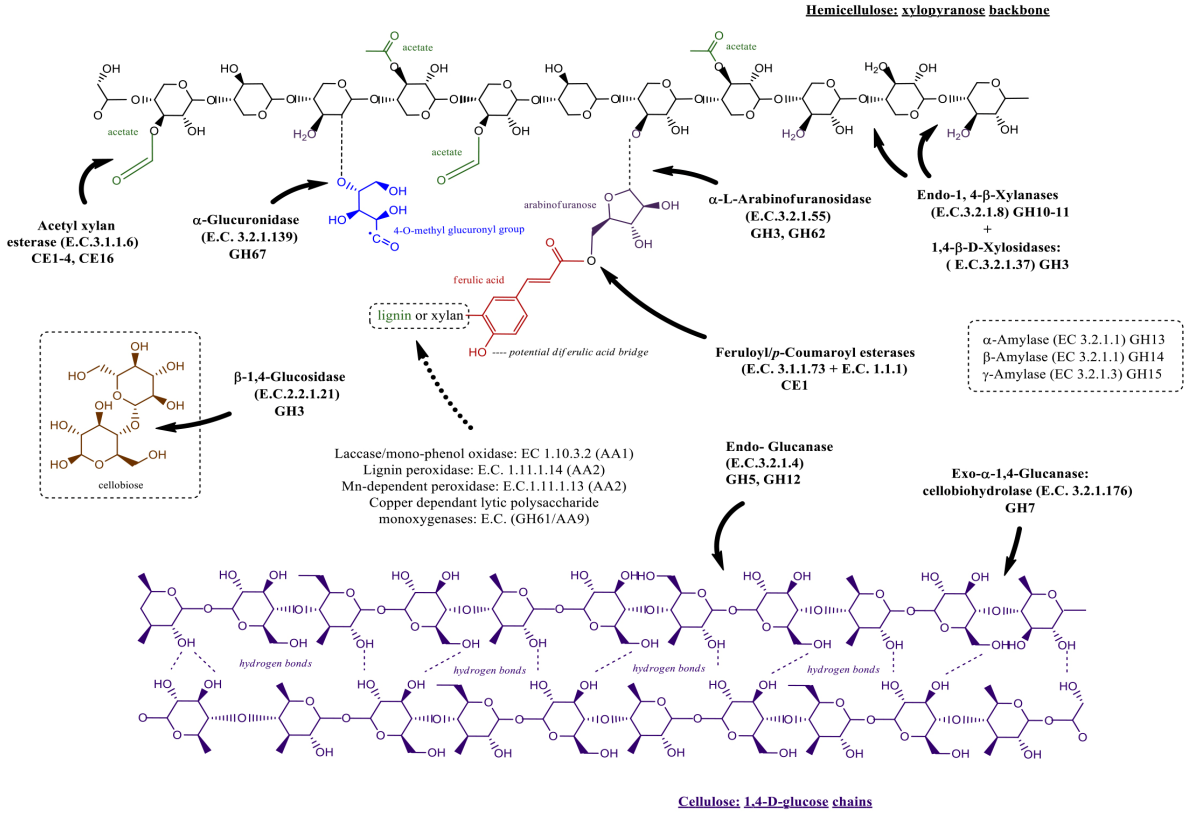

Fig.1. Schematic representation of the attack sites of various enzymes involved in deconstruction and saccharification of lignocellulosic material. (adapted from Faulds and Williamson (1995); Beg et al. (2001); Faulds et al. (2004); Dodd and Cann (2009); Rai (2009); Gupta et al. (2010); Kuhad et al. (2011), and http://www.cazy.org/).

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terms of the specific content of their constituent enzymes or the dose of any one particularly crucial enzyme for any particular substrates (Gilbert et al., 2008; Dodd and Cann, 2009). Therefore, addition of specific ancillary enzymes (used in conjunction with a broad spectrum commercial enzyme mixture) could prove effective in maximising glucose yields from a specific lignocellulosic substrate such as BSG through the cleaving of a particular linkage or bond that is critical to the recalcitrance of a particular substrate (Chundawat et al., 2008).

Here we present an investigation looking at a range of parameters associated with the efficacy of enzymatic saccharification of pretreated BSG (with regards to their significance in terms of achieved glucose yields) which has not been previously published. Factors investigated were; simultaneous effect of variation of the solids loadings at several different cellulolytic enzyme doses, the effect of extension of hydrolysis incubation time, a range of different mixing methods, the effect of supplementation of the cellulolytic enzymes with various additional enzymes and enzymatic cofactors, and also the effectiveness of fed-batch methods. Finally the development of a novel high solids loading enzymatic saccharification method is demonstrated. This was a consolidated approach of a combination of a selection of the previously optimised parameters in an attempt to further maximise achieved glucose yields. This could then provide the basis for a commercial industrial scale saccharification step as part of the process of biofuel production from BSG.

2. Materials and Methods

2.1. Reagents

All reagents were of AnalaR grade and obtained from Sigma-Aldrich (UK) and Fisher Scientific (UK). All water used was deionised reverse osmosis and of ≥18 mega-ohm purity (Purite Select Ondeo IS water system, Purite, UK). All enzymes used for the saccharification experiments are described in section 2.5.

2.2. BSG

Brewers spent grains (BSG) were sourced from the SABMiller 10 hL Research Brewery (Sutton Bonington, UK) from a high gravity brewing process using 100% malted barley (**Table 1**). The BSG was dried in an oven at 105°C overnight and ground to a particle size of less than 212 µm to ensure homogeneity prior to any sampling (KG49 grinder, Delonghi, UK).

Table 1.
Typical compositional analysis of brewers spent grains (Wilkinson et al., 2014a).

BSG component	% (w/w)
starch*	4.8 ± 0.46
protein	26.6 ± 0.38
ash	2.7 ± 0.070
lipid	5.2 ± 2.1
lignin	9.9 ± 1.4
cellulose (glucose)	19.2 ± 1.4
hemicellulose	18.4 ± 3.7
of which xylose	11.3 ± 1.2
other	7.7

Data are the mean ±SD of 3 replicate measurements.
*high residual starch present due to the use of a 2-roller mill.

2.3. Pre-treatment of BSG

All enzymatic saccharification experiments were conducted using pretreated BSG (dilute acid catalysed hydrothermal pre-treatment) according to the optimised method described by Wilkinson et al. (2014a). Pretreatment was conducted at 121°C (for 30 min) and at 25% (w/v) solids loading (with 1% HCl) using a 40-L bench-top autoclave (Priorclave, Tactrol 2; RSC/E, UK). After pre-treatment, all pretreated slurries were neutralized to pH 7.0 (±0.5) via 40% NaOH (w/w). The biomass was then centrifuged at 5000 rpm for 10 min (Heraeus Megafuge 16; Thermo Scientific, UK) and the supernatant was removed. The remaining solid residue was then exhaustively washed with water, conducted by re-suspension and centrifugation at 5000 rpm for 10 min, discarding the supernatant each time. The remaining residues were then dried in an oven overnight at 60°C prior to any enzyme saccharification. Whilst the pre-treatment step did liberate a significant quantity of sugars directly into the supernatant or hydrolysate (particularly xylose from hemicellulose depolymerisation), this was set aside as the focus of this research was more specifically on optimising the subsequent enzymatic saccharification of the remaining insoluble residue after pre-treatment.

2.4. Total glucose composition of pretreated BSG

The total glucose concentration in the remaining insoluble residue (after pre-treatment) was quantified using the method described by Wilkinson et al. (2014b). A total acid hydrolysis method (using 12M H_2SO_4 at 37°C for 1 h then diluted to 1M at 100°C for 2 h) was used to liberate glucose which was then quantified using HPAE-PAD (described in section 2.6). This was required for determination of accurate achieved % theoretical glucose yields liberated after enzymatic hydrolysis. All analyses were conducted in triplicate.

2.5. Enzymatic saccharification of pretreated BSG

All enzyme hydrolysis reactions (the saccharification of the insoluble residues after pre-treatment of the BSG as described in section 2.3) were conducted using the commercial enzyme preparation Novozymes Cellic® CTec2 (kindly supplied by Novozymes A/S, Demark) with a 50°C incubation temperature used for all saccharification reactions. The efficacy of all enzymatic saccharifications was evaluated exclusively via the achieved glucose yields (both g/L and achieved % theoretical yields). Whilst many of the experiments evaluated the effect of modification of the hemicellulose component (on the subsequent degree of cellulose saccharification achieved), the liberation of pentose sugars was not a primary objective. As such, only liberated glucose levels were quantified. All analyses were conducted in triplicate.

The Cellic® CTec2 used for saccharification had a total cellulase activity of 200 FPU/mL in undiluted format and was determined according to Ghose, 1989. An enzyme dosing range of 10 - 160 FPU/g (biomass) was used. The correct quantity of pretreated BSG (0.5 - 15 g) and 50 mM sodium citrate buffer (pH 5.0) were combined to produce a slurry and achieve the various desired solids loadings (5% - 50% w/v). After the correct incubation period (10-72 h), all enzyme saccharification preparations were centrifuged at 5000 rpm for 10 min and the supernatant was removed and sampled for HPLC based quantification of glucose concentrations. Prior to all enzymatic saccharification experiments, the glucose concentration present in the Cellic® CTec2 enzyme preparation was determined by HPLC and subtracted from final enzymatic hydrolysis glucose yields to allow calculation of accurate achieved % of theoretical yields (which were expressed as a percentage of the total glucose content of the pre-treatment generated residue prior to saccharification as described in section 2.4).

2.5.1. Evaluation of the effect of higher energy mixing methods during enzymatic saccharification

A standard shaking incubator method using agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK) was used as a benchmark with various alternative mixing methods then compared against it. All analyses were conducted in triplicate. Efficacy of each different mixing method was determined by the liberated glucose yields achieved. Roller bed mixing methods were conducted at 50 rpm using an SRT6D roller bed (Stuart Scientific, UK) whilst magnetic stirring methods were conducted at 150 rpm using a multi-plate magnetic stirrer (Variomag poly 15, Thermo Scientific, UK). Both the roller bed and multi-plate magnetic stirrers were housed within an MIR-253 incubator (Panasonic, Japan) to achieve the 50°C temperature optima. A high torque mixing (HTM) method was conducted using a custom made, 2-piece, sealed headspace reaction vessel housed within a re-circulating water bath which acted as an incubator (**Fig. 2**). A 6-bladed paddle type mixer (at

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150 rpm) was used for the actual agitation. The effect of supplemental aeration on enzymatic saccharification was investigated using the custom HTM system with an additional 2 mm air delivery line which supplied air from an external air pump (TetraTec APS100 Air Pump: 100 l/h, 2.5w). All analyses were conducted in triplicate.

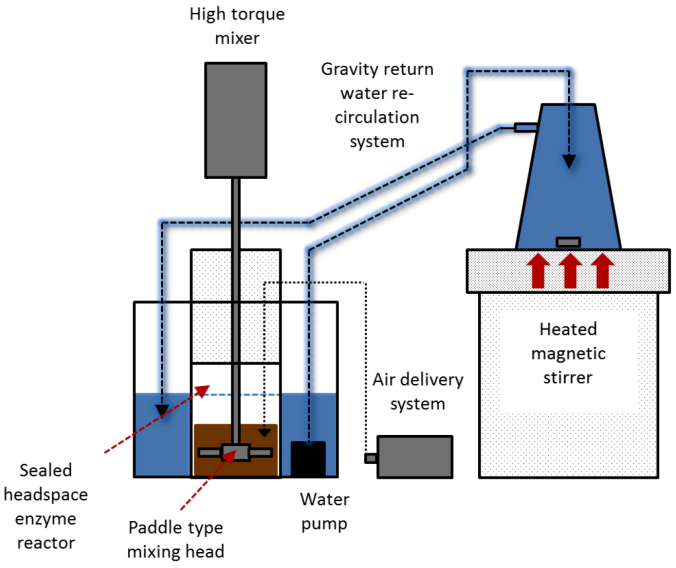

Fig.2. Schematic diagram of high-torque mixing (HTM) system and optional additional aeration.

2.5.2. Effect of supplementation of the Cellic® CTec2 with additional enzymes and cofactors on achieved glucose yields

The effectiveness of additional hydrolytic enzymes or various cofactors (in conjunction with Novozymes Cellic® CTec2) was investigated using the aerated HTM method as described in section 2.5.1. All analyses were conducted in triplicate. For preliminary proof of principle experiments, all enzymes and cofactors were dosed at levels which were considered to be in excess (100 µL aliquot of each enzyme preparation as supplied) order to ascertain any additional improvement in achieved glucose yields compared with the use of Novozymes Cellic® CTec2 alone. Additional xylanases were tested using Novozymes Cellic® HTec2 (100 µL/10 g biomass). Ferulic acid esterase was tested using Prozomix Feruloyl esterase (CAZy CE1 carbohydrate esterase family, from *Clostridium thermocellum*: 100 µL/10 g biomass; activity: 112.5 Units/mL). One unit was defined as the amount of enzyme required to release 1 µM of ferulic acid per minute from 32 µM methyl ferulate in 50 mM sodium phosphate buffer, pH 6.0, at 37°C, and at 335 nm. Acetyl xylan esterase was tested using Prozomix Acetyl xylan esterase (CAZy CE3, carbohydrate esterase family 3, from *Clostridium thermocellum ATCC 2740*, 100 µL/10 g biomass; activity: 112.5 Units/mL). One unit was defined as the amount of enzyme required to release 1 µM of p-nitrophenol per minute from p-nitrophenyl acetate (1 mM in the assay) in 50 mM phosphate buffer, pH 7, at 50°C, containing 1 mg/mL of BSA. Arabinofuranosidase was tested using Prozomix α-L-Arabinofuranosidase (CAZy GH51, glycoside hydrolase family 51, from *Streptomyces coelicolor A3*, 100 µL/10 g biomass; activity: 125 Units/mL). One unit was defined as the amount of enzyme required to release 1 µM of p-nitrophenol per minute from p-nitrophenyl α-L-arabinofuranoside (1 mM in the assay) in 50 mM phosphate buffer, pH 7, at 60°C, containing 1 mg/mL of BSA. The addition of starch degradation enzymes was tested using α-amylase and glucoamylase (Megazyme, Ireland) with each enzyme tested individually (100 µL/10 g biomass) and also both enzymes simultaneously (100 µL of each/10 g biomass). The specific activity of α-amylase was 3000 Units/ml. One Unit was defined as the amount of enzyme required to release one µM of p-nitrophenol from blocked p-nitrophenyl-maltoheptaoside per minute (in the presence of excess α-glucosidase) at pH 6.0 and 40°C. The specific activity of glucoamylase was 160 Units/mg (60°C, pH 4.5 on soluble starch). One unit of glucoamylase activity was defined as the amount of enzyme required to release one µg of β-D-glucose reducing-sugar equivalents per minute from soluble starch (10 mg/mL) in sodium acetate buffer (100 mM) at pH 4.5.

The effect of addition of proteolytic enzymes was investigated using the commercial preparations Alphalase™ NP (Danisco) and Pronase E (Sigma-Aldrich, UK). Alphalase™ NP is a commercial enzyme mixture (derived from *Bacillus amyloliquefaciens*) used within the beer brewing industry in order to assist in the hydrolysis of the protein component found within malted barley (the pre-cursor of BSG) in order to boost free amino nitrogen (FAN) levels to assist in high gravity fermentations thus rendering it potentially effective on the native protein fraction found in BSG. Alphalase™ NP was dosed at 100 µL/10 g biomass. Alphalase™ NP has a suggested dosing of 0.1-0.3 kg/MT grist when used during the mashing stage of commercial beer brewing, therefore, the dose used here was also considered to be in excess. The Alphalase™ NP was prepared in 50 mM sodium citrate buffer at pH 6.5. Pronase E is a commercial mixture of ca. 10 proteases from *Streptomyces griseus* K-1 and includes five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases, and one zinc carboxypeptidase (with an overall very broad spectrum of activity). Pronase E was prepared as a 1% w/w solution; 1 g/100 mL 50 mM sodium citrate buffer (pH 7.5). The specific activity of Pronase E was 4 Units/mg. One unit of Pronase E was defined as the amount of enzyme required to hydrolyse casein to produce colour equivalent to 1.0 µM (181 µg) of tyrosine per min at pH 7.5 at 37°C (colour by Folin-Ciocalteu reagent). For the evaluation of the effect of the supplementary protease enzyme preparations, both BSG and pretreated BSG were subjected to a 5 h pre-incubation with each of the protease solutions (150 rpm agitation; MaxQ 4358 shaking incubator, Thermo Scientific, UK) using 15% w/v solids loading (at 60°C for the Alphalase™ NP and 37°C for the Pronase E) and prior to the saccharification stage using Novozymes Cellic® CTec2 (as described in section 2.5). The effect of a protein denaturation step after the protease pre-incubation was also investigated. The denaturation was conducted by incubation at 90°C for 10 min in a water bath. This was designed to denature and inactivate the proteases prior to dosing with Novozymes Cellic® CTec2 (to minimise any possible degradation of the lignocellulolytic enzymes found within the CTec2 by the proteases). After denaturation, samples were cooled to 50°C prior to dosing with Cellic® CTec2 and then further incubated at 50°C (150 rpm agitation; MaxQ 4358 shaking incubator, Thermo Scientific, UK) for the required time period. In addition, the supplementation with the potential GH61 enzyme family cofactors, i.e., copper ($CU(NO_3)_2$) and ascorbic acid were tested (in conjunction with Cellic® CTec2) using a concentration range of 2.5 – 10 mM incorporated into the 50 mM sodium citrate buffer used for saccharification.

2.5.3. Effect of variation of solids loading and enzyme dose during hydrolysis on achieved glucose yields

The individual and combined impacts of solids-loadings (5-25% w/v) and enzyme dose (Novozymes Cellic® CTec2; 10-160 FPU/g biomass) during enzymatic saccharification of pretreated BSG were investigated using a D-optimal designed experiment (**Table 1S**). The D-optimal experimental design was created using DesignExpert (Stat-Ease, USA) using a collection of reaction combinations (solids loading and enzyme doses) from which the D-optimal algorithm chose the treatment combinations to include in the design. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided statistically valid data. Response data (glucose yields) were then modelled against these factors using Design Expert with analysis of variance (ANOVA) automatically incorporated into the modelling. For this preliminary investigation, a simplified shaking incubator method was utilised due to its high-throughput nature in comparison with the optimised aerated HTM system (described in section 2.5.1) which consisted of a 'single-shot' bioreactor.

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2.5.4. Effect of variation of solids loading and hydrolysis time during hydrolysis on achieved sugar yields

The individual and combined impacts of solids-loadings (5-25% w/v) and reaction time (10-72 h) during enzymatic saccharification of pretreated BSG (as described in section 2.3) were also investigated using a D-optimal designed experiment (**Table 2S**). Novozymes Cellic® CTec2 was used as the enzyme preparation (20 FPU/g biomass). Response data (glucose yields) were then modelled against these factors using Design Expert software v 7.0 (Stat-Ease, Mn, USA).

2.5.5. Fed-batch enzymatic saccharification experiments

Fed-batch enzymatic saccharification experiments were conducted to evaluate their effectiveness against non-fed-batch saccharification methods to determine whether the staggered addition of biomass achieved higher glucose yields. Preliminary experiments utilised the standard shaking incubator method (48 h incubation at 50°C with agitation at 150 rpm, MaxQ 4358 shaking incubator, Thermo Scientific, UK) due to its high throughput nature. The fed-batch system used the correct amount of biomass (0.5 -5 g pretreated BSG) to achieve the desired solids loading rates (5-50% w/v) but with the biomass split into two aliquots with the second aliquot added after 24 h incubation. All analyses were conducted in triplicate.

2.5.6. Consolidated (combined) enzymatic saccharification method

A consolidated enzymatic saccharification method was formulated from a combination of all of the previous individual experimental optimisations in an attempt to further maximise the achieved glucose yields when operating at high solids loading. This method was based on all the parameters that were suggested to play a significant role in enhancing the glucose yields achieved. The consolidated method involved using the aerated HTM system operating at 50°C for 72 h incubation (considered an excess reaction time and thus non rate limiting) with a fed-batch approach (tested over a final solids loading range of 5-25% w/v) using a low dose of Novozymes Cellic® CTec2 (10 FPU/g biomass; to simulate a commercially viable large scale industrial process) and with the additional enzymes; ferulic acid esterase, acetyl xylan esterase, arabinofuranosidase, Cellic® HTec2 (all dosed at 100 µL and prepared as described previously in section 2.5.4), and also with the additional enzymatic cofactors ($Cu(NO_3)_2$, and ascorbic acid (both dosed at 10 mM concentrations). All analyses were conducted in triplicate.

2.6. HPLC

Glucose concentrations were quantified using the method described by Wilkinson et al. (2015). This was then quantified using an ICS 3000 system from Dionex (fitted with a CarboPac PA20 column, 150 mm × 3.0 mm; Dionex, USA) and a high performance anion exchange pulsed amperometric electrochemical detector (HPAE-PAD; Dionex, USA). The system used isocratic elution with 10 mM NaOH at 0.5 mL/min flow rate with a subsequent column regeneration step (using 200 mM NaOH at 0.5 mL/min). Inhibitory compounds (pre-treatment generated sugar and lignin degradation products) were quantified *via* HPLC according to the method described by Wilkinson et al. (2014b). The system (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA) used UV detection at 280 nm with UV spectra for secondary confirmation of identity. A Techsphere ODS C18 column (5 µm, 4.6 mm × 250 mm; HPLC Technologies, UK) was used at ambient temperature. The mobile phase was a mixture of 1% acetic acid (solvent A) and methanol (solvent B) with a flow rate of 1.0 mL/min. Gradient elution was used ramping from 20% to 50% methanol over 30 min with a final 100% methanol column cleaning phase.

2.7. Quantification of free amino nitrogen (FAN) levels

FAN was determined using the ninhydrin colorimetric assay using glycine as the standard (European Brewery Convention, 1998).

*2.8. Measurement of the protein content of the insoluble residue following pre-treatment of the BSG*A

Thermo Flash Nitrogen Analyzer (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to determine protein content using the method described by Wilkinson et al. (2014a). The sample was heated to 900°C in the presence of an oxidation catalyst and helium carrier gas. Oxygen was then added and the temperature was increased to 1800°C. The resulting gases were then passed through a reduction reactor that converted nitrogen oxides to elemental nitrogen for detection. Protein was then calculated using the × 6.25 conversion factor. All analyses were conducted in triplicate.

2.9. Statistical analysis

Experimental design, response surface modeling, and ANOVA were all performed using Design Expert v 7.0 (Stat-Ease Inc., Minneapolis, USA). Additional ANOVA (followed by the Tukey HSD test) were performed using the software package SPSS 16.0 for Windows (SPSS, Germany). All experiments were conducted in triplicate (biological replication).

3. Results and discussion

For all subsequent experiments, the efficacy of enzymatic saccharification is presented as both % theoretical yields and g/L yields. Whilst high % theoretical yields would indicate a high degree of process efficiency (high rate of conversion of substrate to product; glucose), they do not provide a measure of the 'usability' of a feedstock. For example, a feedstock with a high % theoretical glucose yield but low g/L yield (low absolute glucose yield) would not be viable to use for biofuel production. This is due to the ca. 100 g/L minimum glucose concentration required to achieve the minimum ethanol concentration that would render distillation economically viable (Lau and Dale, 2009). Therefore, both units are included for consideration.

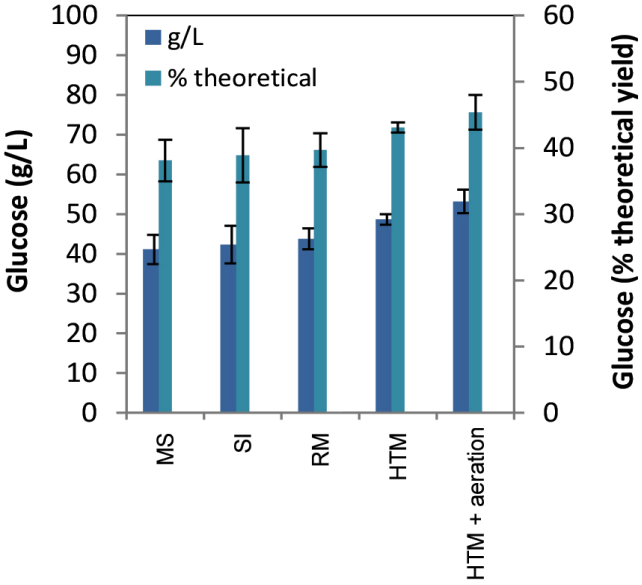

Fig.3. Impact of mixing methods during enzymatic saccharification of pretreated BSG on the resultant glucose concentrations (g/L and % theoretical yields). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Key: MS: magnetic stirring, SI: shaking incubator, RM: roller bed mixing, HTM: high torque mixing, HTM + aeration: high torque mixing with additional aeration supplied from an external air pump. Data are the mean ± SD of 3 replicate measurements.

3.1. Effect of variation of the mixing method on enzymatic saccharification yields from pretreated BSG

The use of different mixing (agitation) systems for the enzymatic saccharification revealed that magnetic stirring (MS), shaking incubation (SI), and roller mixing (RM) produced similar results with 40 - 44 (± 2.6 – 4.6) g/L final glucose concentrations and achieved 38 - 40% theoretical glucose yields after 72 h incubation when using an excess dose (160 FPU/g biomass) of Novozymes Cellic® CTec2 (**Fig. 3**). However, the use

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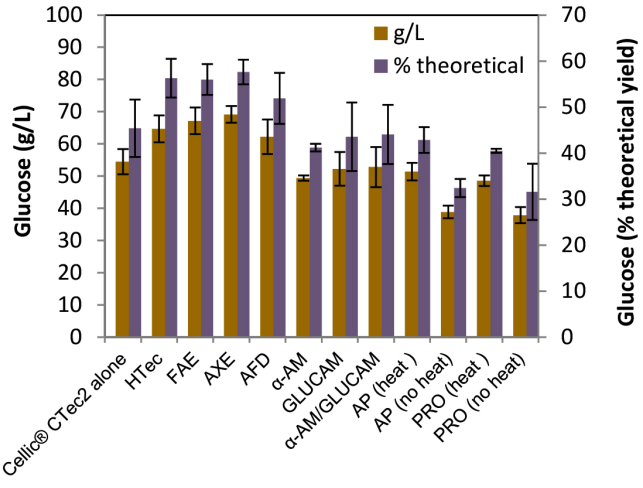
of the HTM was shown to give a small but significant boost in glucose yields (increased to 49 ± 1.3 g/L and 44% of theoretical yield; P<0.05: one way ANOVA). This suggested the other methods were sub-optimal in terms of their agitation and the enhanced mixing of the enzyme and substrate that occurred with the HTM system was beneficial. Improved mixing technology may play some part in ensuring better enzyme substrate interactions (especially at very high solids loading; >20% w/v), however, some research has suggested there may only be minimal improvements in achieved glucose yields through additional mixing (Kristensen et al., 2009).

In addition, achieving effective mixing at very high solids loadings may also prove prohibitively energy intensive due to the amount of torque likely required to agitate highly viscous slurries of the biomass. However, supplementation of the HTM system with additional aeration further increased the glucose yields (to 53 ±2.9 g/L and 45% of theoretical yield) suggesting the additional oxygen present in the air supplied externally may have enhanced the activity of some saccharification component, possibly the GH61 oxidative enzymes found within the Novozymes Cellic® CTec2. Previous published work has also suggested that the provision of supplementary oxygen or aeration may be a low cost option which could boost the catalytic activity of GH61 enzymes (Beeson et al., 2011; Horn et al., 2012).

3.2. Effect of supplementation of the Cellic® CTec2 with additional enzymes and enzymatic cofactors on saccharification yields from pretreated BSG

Enzymatic saccharification experiments utilising the aerated HTM method (again using Novozymes Cellic® CTec2) indicated that supplementation with the commercial protease mixtures (Alphalase NP and Pronase E with a 5 h pre-incubation prior to the saccharification step with Novozymes Cellic® CTec2) did not result in any subsequent enhancement of the liberation of glucose from the pretreated BSG compared with when using Novozymes Cellic® CTec2 alone (yields were actually slightly lower; 49 - 51 (±1.6 – 2.7) g/L and 41 - 43% of theoretical; **Fig. 4**). It was hypothesized that proteases may have been able to hydrolyse some of the considerable protein content within the BSG which may have indirectly improved physical access of any cellulase enzymes to the crystalline cellulose present. The several kinds of endopeptidase and exopeptidase found in the Pronase has been used to digest various cattle feeds such as grasses in an attempt to produce low nitrogen feeds (Abe et al., 1979). Pronase may target dextrans (glucan) and has been shown to be more effective in doing this than even mild acid hydrolysis (Kato et al., 1991). The use of either of the protease mixtures without a heat mediated inactivation stage (denaturation of the proteases) prior to the subsequent saccharification step with Novozymes Cellic® CTec2 significantly decreased the glucose yields achieved (38 - 39 ± 1.9 – 2.5 g/L and 32 - 33% of theoretical yield) compared with when using Cellic® CTec2 alone. This suggested that some proteolytic attack of the carbohydrate degrading enzymes within the Cellic® CTec2 occurs as a direct result of the presence of the proteases. Overall, the lack of any enhancement in glucose yields indicates that the considerable protein fraction which is still present within the BSG after hydrothermal pre-treatment (ca. 22% crude protein) does not impede the activity of lignocellulolytic enzymes contained within Novozymes Cellic® CTec2. This would confirm as expected that proteinaceous tissues within the BSG are generally morphologically discreet from the lignocellulosic matrix. However, whilst glucose yields may not have been boosted by the addition of the proteases, their inclusion could still be considered as it may also be desirable to liberate free amino acids (or low molecular weight peptides) into the hydrolysate which is generated from the saccharification step (the actual feedstock subsequently fermented to bioethanol), thus increasing the free amino nitrogen (FAN) content. This could potentially improve the subsequent fermentation performance which could be particularly useful for very high gravity fermentation systems (using very high initial sugar concentrations) as it may assist in reducing the osmotic stress on the yeast (or bacterial species) used and allow for production of high ethanol titres.

Therefore, the lack of any observed improvement of the enzymatic saccharification yields with the use of additional proteases may not definitively exclude their incorporation into enzymatic cocktails used (for the saccharification step which would be required for biofuel production) due to the considerable, currently under-utilised protein fraction found within pretreated BSG. Both protease mixtures were indicated to be effective at significantly increasing FAN liberation from pretreated BSG as levels were indicated to rise from approximately 23 mg/L to >100 mg/L when using the 5 h pre-incubation. Due to this success of FAN liberation from pretreated BSG, an identical method was conducted but this time testing the protease mixtures on the starting BSG biomass (before any pre-treatment). This resulted in an even greater increase in FAN from approximately 51 mg/L to >180 mg/L. This data would suggest that hydrothermal pre-treatment does either liberate or modify a significant proportion of the protein present in the BSG, thus explaining the lower FAN concentrations liberated by the proteases on pretreated BSG. Overall, this could allow the generation of a FAN enriched solution before the saccharification step using Cellic® CTec2 and is the subject of current on-going research. This could either be used for incorporation into any subsequent feedstock after saccharification or form an additional high value product stream which could be fractionated.



Key	
HTec	Xylanases
FAE	Ferulic acid esterases
AXE	Acetyl xylan esterases
AFD	α-L-Arabinofuranosidase
α-AM	Amylase
GLUCAM	Glucoamylase
α-AM/GLUCAM	Amylase + Glucoamylase
AP (heat)	Alphalase protease with heat inactivation
AP (no heat)	Alphalase protease without heat inactivation
PRO (heat)	Pronase protease with heat inactivation
PRO (no heat)	Pronase protease without heat inactivation

Fig.4. Impact of using a range of supplementary enzymes in conjunction with Cellic® CTec2 during enzymatic saccharification of pretreated BSG on achieved glucose concentrations (g/L and achieved % theoretical yields). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) using aerated high-torque mixing (HTM) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading) with all supplementary enzymes dosed at 100 µL (all considered in excess dosages). Data are the mean ± SD of 3 replicate measurements.

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In a similar fashion, supplementation with either (or both) of the starch degrading enzymes, glucoamylase (amyloglucosidase) and α-amylase, did not significantly increase enzymatic saccharification yields (glucose yields) beyond that achieved by Novozymes Cellic® CTec2 alone. Some starch does usually remain present in BSG (although typically <5% w/w) unless a very intense and effective mashing method has been used during the initial beer brewing process such as use of mash filter brewing technology and the associated brewhouse apparatus such as a hammer mill (e.g., extra finely milled grist used initially and also possibly with additional saccharification enzymes added during mashing; both of which aid extraction efficiency of starch removal from the malted barley). However, the hydrothermal temperatures (121°C) that were used during the pre-treatment step initially would have very likely already gelatinised and solubilised any residual starch that may have been present (in the BSG) into the pre-treatment generated hydrolysate. Therefore, starch degradative enzymes did not directly boost glucose yields through starch hydrolysis or indirectly through enhanced access of the cellulases (present in the Novozymes Cellic® CTec2) to cellulose through removal of any of the very small quantity of starch grains that may have physically impeded the enzymes. In contrast to this, the supplementation with additional xylanases (Novozymes Cellic® HTec2), ferulic acid esterases, acetyl xylan esterases, and α-L-arabinofuranosidases all resulted in a significant increase ($P<0.05$: one-way ANOVA) in enzymatic saccharification glucose yields compared with Cellic® CTec2 alone. The ferulic acid esterase, acetyl xylan esterase, and xylanases (Cellic® HTec2) were suggested to achieve the greatest enhancement in glucose yields (65 - 69 ± 2.6 – 4.1 g/L and 56 - 58% of theoretical yields) whilst the α-L-arabinofuranosidase liberated ca. 62 ± 4.1 g/L glucose and 52% theoretical yield. Acetyl xylan esterase enzymes (CE1) target ester linkages (of acetate groups) on the xylose backbone of hemicellulose (Shallom and Shoham, 2003) whereas α-L-arabinofuranosidase enzymes (GH62) hydrolyse the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides that are present in various places as side chain decorations in arabinoxylans and hemicellulose (Schwarz et al., 1995; Poutanen, 1988). The successful de-branching of these residues may be crucial for complete and effective hydrolysis of branched formats of hemicellulose. This suggested the pretreated BSG still contained various lignocellulosic linkages that to a small extent impeded the function of the cellulases found within Novozymes Cellic® CTec2. It further supports the idea that specific lignocellulosic biomass such as BSG requires a tailor made enzyme cocktail in order to maximise the glucose yields achieved. Whilst Novozymes Cellic® CTec2 is a highly effective commercial enzyme preparation on its own (and a complex mixture of different lignocellulolytic enzymes), specific substrates may require larger quantities of specific enzymes, e.g., ferulic acid (feruloyl) esterases, due to the prevalence of particular linkages found within it.

An alternative hypothesis is that specific additional enzymes may be needed due to the specific format of the components present (in BSG) that may be chemically modified as a result of the specific pre-treatment step employed upstream of the enzymatic hydrolysis step. Overall, it suggested a requirement for comprehensive optimisation of the enzymatic saccharification step for each specific lignocellulosic substrate. In the specific case of BSG, it may also suggest that each different batch may also require some degree of optimisation in order to maximise the saccharification yields due to compositional differences that arise as a result of the use of differing barley cultivars used initially in the grist or different mashing conditions used during brewing (wort production).

Additionally, the supplementation of the aerated HTM method (using Novozymes Cellic® CTec2) with the GH61 specific enzymatic cofactors, i.e., copper and ascorbic acid, suggested that a small but significant increase ($P<0.05$: one-way ANOVA) in glucose yields was achieved when using a 10 mM concentration of both cofactors simultaneously (liberating 58 ± 1.7 g/L glucose and 56% of theoretical yield; **Fig. 5**). The CAZy GH61 family of glycoside hydrolases have been indicated to play an important role in the oxidative cleavage of certain linkages in lignocellulolytic substrates in nature with many GH61 genes being indicated to be present in the genome of various fungal species including the brown rot fungi *Postia placenta* (Quinlan et al., 2011). As a result, additional GH61 enzymes have been included in certain commercial enzyme products such as Novozymes Cellic® CTec2 (Cannella et al., 2012). Various other published work has suggested they may be able to boost the effectiveness of other cellulase class enzymes such as endo and exo-1,4-β-glucanases and in particular cellobiohydrolases (Harris et al., 2010; Beeson et al., 2011; Quinlan et al., 2011; Horn et al., 2012). This suggested that GH61 enzymes found within Novozymes Cellic® CTec2 may be responsive to increased concentrations of redox active cofactors and function sub-optimally without this additional supplementation and confirmed the work of Quinlan et al. (2011) and Beeson et al. (2011).

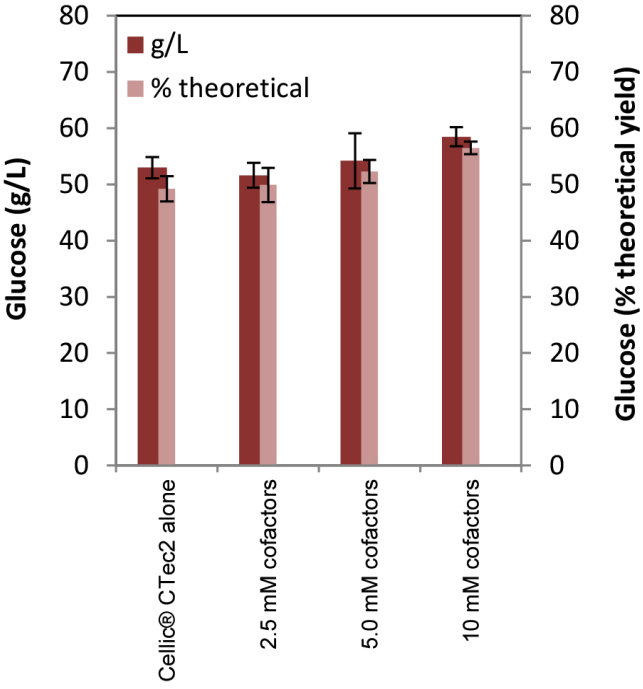

Fig.5. Impact of the simultaneous addition of both the enzymatic cofactors $Cu(NO_3)_2$ and ascorbate in conjunction with Cellic® CTec2 during enzymatic saccharification of pretreated BSG on achieved glucose concentrations (g/L and achieved % theoretical yields). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) using aerated high-torque mixing (HTM) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading) with a range of $Cu(NO_3)_2$ and ascorbic acid concentrations (2.5-10 mM with equal concentrations of both reagents). Data are the mean ± SD of 3 replicate measurements.

Further work would need to be conducted in order to ascertain whether the presence of copper ions at concentrations of around 10 mM (should those concentrations be retained in the feedstock ultimately generated) would be detrimental to any downstream fermentations conducted as various yeast species have been indicated to be sensitive to high concentrations of copper ions resulting in sub-optimal fermentation performance (Dönmez and Aksu, 1999). However, these suggested GH61 enzymatic cofactors may offer a more cost effective alternative to augmentation of generic lignocellulosic enzyme cocktails with additional expensive exotic enzymes. Whilst some members of the GH61 family exhibit weak β-1,4 endoglucanase activity, it is unlikely they act upon single cello-oligomeric chains as it has been indicated that no soluble reaction products such as cellobiose or glucose are detected when they are used alone on lignocellulosic substrates (Harris et al., 2010; Horn et al., 2012). In addition, they do not appear to enhance the activity of cellulases on pure cellulose (Harris et al., 2010). In conclusion, it would then suggest that GH61 class enzymes may act upon a rare bond found only in lignocellulosic materials (which are obviously not found in pure cellulose) that may possibly obstruct the normal function of other primary cellulolytic enzymes in some way. Alternatively, they may function *via* a non-hydrolytical based mechanism possibly similar to the suggested mechanism of carbohydrate binding modules (CBMs) in that they may penetrate micro-cracks in crystalline cellulose and cause localised

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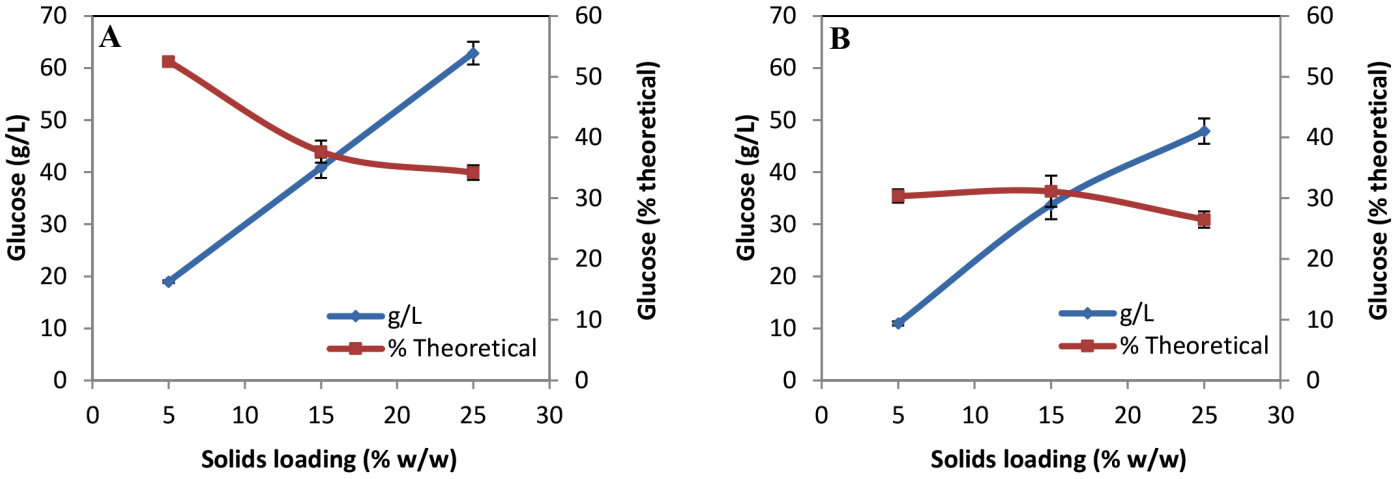

Fig.6. Effect on achieved glucose concentrations of variation of the solids loading used during enzymatic saccharification of pretreated BSG. **A:** shows the theoretical maximum yield achieved using an excess enzyme dose (160 FPU/g biomass) whereas **B:** shows the more realistic achieved yield when constraints of enzyme pricing are considered and a lower enzyme dose used (10 FPU/g biomass). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 with 72 h incubation at 50°C, 5-25% w/v solids loading) using standard shaking incubator method (SI) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Data are the mean ± SD of 3 replicate measurements.

swelling that enables free water to penetrate, thus further increasing the porosity allowing better access for additional enzymes to function (Arantes and Saddler, 2010).

3.3. Effect of variation of the solids loading and enzyme dose used during enzymatic saccharification on liberated glucose yields

Two-dimensional variants of the derived models (using a D-optimal design; see section 2.5.3 for experimental details) for glucose release (using only a selection of the data set) suggested that when using an excess of Novozymes Cellic® CTec2 (160 FPU/g biomass) increasing solids loading during enzymatic saccharification from 5% w/v to 25% w/v resulted in a linear increase in g/L glucose concentrations liberated (**Fig. 6A**). However, achieved % theoretical glucose yields dropped as solids loading was increased, particularly sharply with the increase from 5% w/v to approximately 15% w/v. Increasing solids loading beyond 15% w/v resulted in only a minor drop in achieved % theoretical glucose yields. In comparison, when shifting to using a low dose of Novozymes Cellic® CTec2 (10 FPU/g biomass), increasing solids loading from 5% w/v to 25% w/v also resulted in an almost linear increase (albeit lower ramp than when using an excess enzyme dose) in g/L glucose concentrations liberated (**Fig. 6B**). However, in contrast to when using an excess enzyme dose, with the use of a low enzyme dose (10 FPU/g biomass) there was only a very small drop in achieved % theoretical yields (from ca. 30% to 26%) as solids loading was increased from 5% to 25% w/v. This suggested that use of higher solids loading is optimal when using low enzyme doses. A direct comparison of the achieved % theoretical glucose yields at 25% w/v solids loading with both a low and an excess enzyme dose suggests that ×16 enzyme dose increase only resulted in an increase from 26% to 34% of theoretical glucose yield. This suggests that low enzyme doses at high solids loading may be optimal in terms of maximising efficiency of processing large quantities of biomass (i.e., pretreated BSG). If achieving the highest g/L glucose concentration was the primary target for a feedstock and achieved % theoretical yield a secondary objective (which could be sacrificed somewhat), then the use of very high solids loading could prove very effective as high g/L glucose concentrations could be achieved. Whilst this implies that the value of the glucose in the generated feedstock is greater than the value of the spent grains (which may not be the case), it would in addition allow a large quantity of biomass to be processed more rapidly.

Three-dimensional response surface models (3D RSM) of the glucose release data looking at the simultaneous effect of variation of solids loading and enzyme dose (using Novozymes Cellic® CTec2) suggested that increasing enzyme dose from 10 to 160 FPU/g biomass at any given solids loading between 5% and 25% w/v did not significantly increase the g/L glucose concentration liberated (**Fig. 7A**). For achieving optimal g/L glucose concentrations, a low enzyme dose - high solids loading combination was confirmed to be optimal as was previously apparent as with the 2D models (**Fig. 6B**).

However, in contrast to this, increasing solids loading at any given enzyme dose was suggested to be a significant factor affecting glucose yields. 3D RSM of achieved % theoretical glucose yield data indicated that at low enzyme doses (10 FPU/g biomass), the increase in solids loading was not a significant parameter whilst at high enzyme doses (160 FPU/g biomass), increasing solids loading was a significant parameter, resulting in a considerable reduction in achieved % theoretical glucose yields (**Fig. 7B**). Increasing enzyme dose at low loadings loading (5% w/v) was suggested to have a much more significant effect on achieved glucose yield than at high solids loading (25% w/v). For optimal conversion efficiency (optimal % theoretical yields achieved), a low solids loading - high enzyme dose combination was suggested to be most suitable. Overall, the enzyme reaction kinetics seen here did not follow the traditional Michaelis–Menten model. This is likely due to the (relatively) highly insoluble slurries (with significant quantities of hydrophobic components) being used as opposed to highly soluble, dilute enzyme-substrate mixes.

3.4. Effect of variation of solids loading and reaction time of enzymatic saccharification on achieved glucose yields

As described in section 3.3, a higher throughput system using a shaking incubator was utilised for the investigation of whether the optimal hydrolysis (reaction) time varied in response to variation of the solids loading used during enzymatic saccharification.

Evaluation of the 3D RSM data suggested that liberated glucose concentrations (g/L) peaked (achieving 60 g/L) at the maximal solids loading tested (25% w/v) and when the longest reaction time (72 h) was used (**Fig. 7C**). At low solids loading (5% w/v), an extension of the reaction time beyond 6 h did not result in any significant increase in glucose liberation. However, at high solids loading (25% w/v) extension of the reaction time beyond 10 h resulted in a linear increase in glucose liberation. The data suggested that in terms of g/L glucose liberation, the optimal reaction time depends heavily on the actual solids loading used for the enzymatic hydrolysis. It was shown that increasing the solids loading from 5% to 25% w/v when using a shorter reaction time (10 h) did result in a small but significant (model R^2: 0.78) increase in liberated glucose concentrations (g/L). However, this enhancement effect was much more pronounced when using longer reaction times (>24 h).

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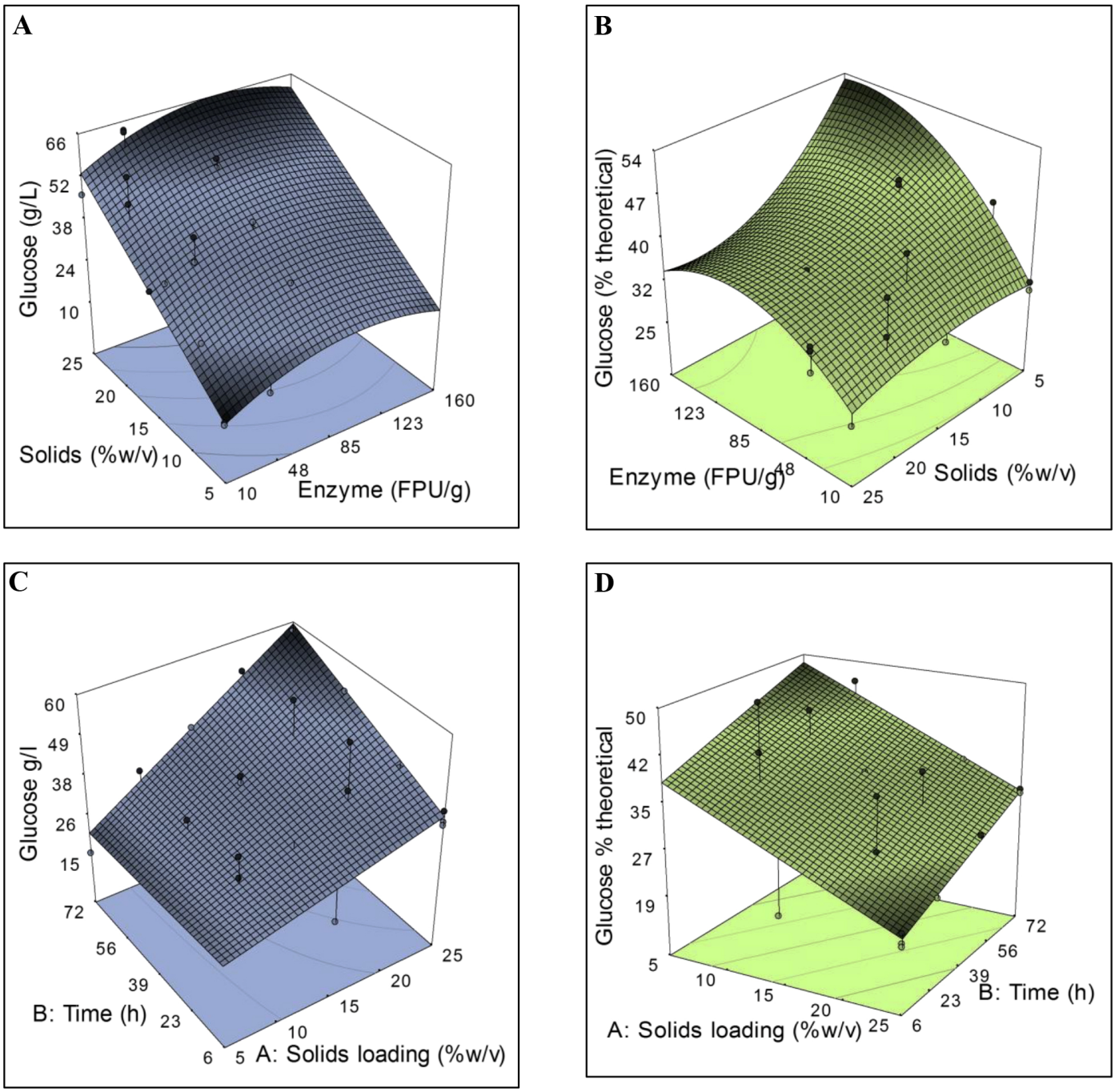

Fig.7. A-B: 3D response surface models showing the simultaneous effects of the factors (i) % solids-loading (w/v) and (ii) enzyme dose for saccharification. **A:** Glucose: g/L (model R^2: 0.94), **B:** Glucose: % theoretical yield (model R^2: 0.86). **C-D:** 3D response surface models showing the simultaneous effects of the factors (i) % solids loading (5-25% w/v) and (ii) reaction time (6-72 h) during enzymatic saccharification of pretreated BSG on the resultant glucose concentrations liberated. **A:** Glucose; g/l (R^2: 0.78), **B:** Glucose; achieved % theoretical yield (R^2: 0.65). **A-B:** Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10-160 FPU/g biomass) with 72 h incubation at 50°C, 5-25% w/v solids loading using standard shaking incubator method (SI). **C-D:** Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (20 FPU/g biomass) with 6-72 h incubation at 50°C, 5-25% w/v solids loading using standard shaking incubator method (SI). All experiments conducted using pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading).

In direct contrast to g/L glucose liberation, evaluation of the achieved % theoretical glucose yields suggested that a linear drop in conversion efficiency was observed (regardless of reaction time used) as solids loading was increased (Fig. 7D). As expected, the highest conversion efficiencies achieved (50% of theoretical yield) were observed when the lowest solids loading was used (5% w/v). At any given solids loading used, the extension of reaction time from 10 h to 72 h was shown to offer only a relatively small increase in achieved % theoretical glucose yield (from 40 - 50% theoretical yield at 5% w/v solids loading). This may suggest that extension of the reaction time from ca. 24 h to 72 h would not be economically viable when conducting an enzyme saccharification step at less than 25% (w/v) solids loading. However, this would depend heavily on the cost of the enzyme bioreactors used. Low cost vessels and the availability of space to house them could then justify this increased

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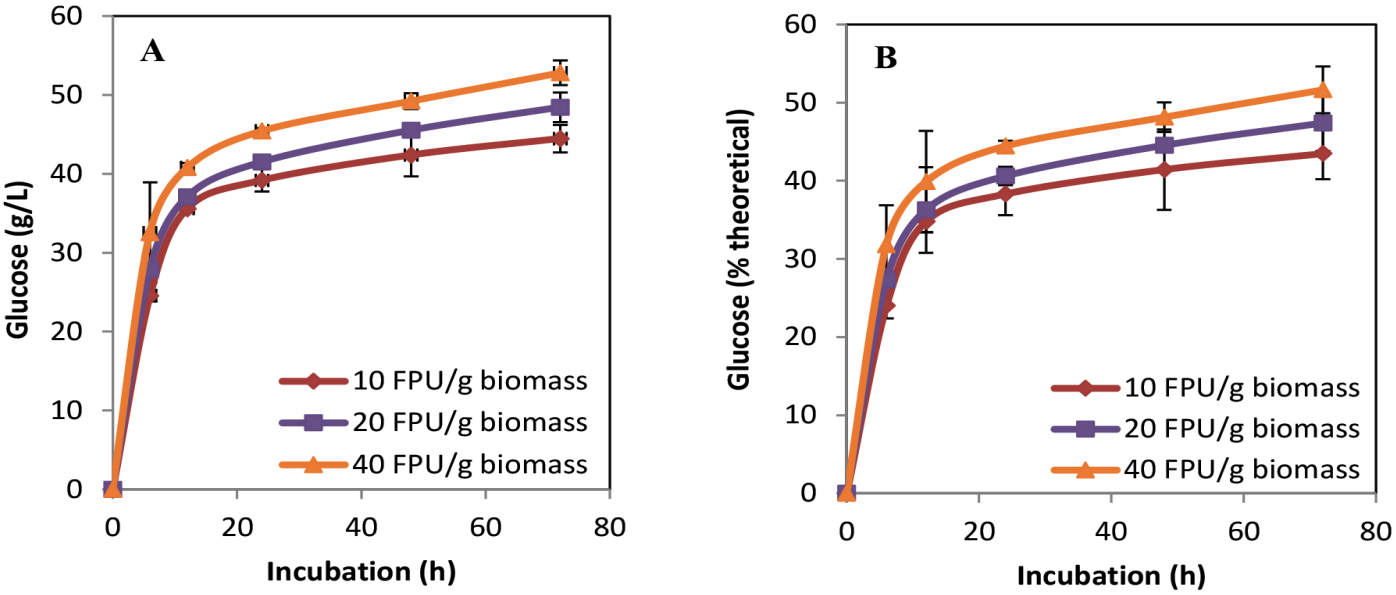

Fig.8. Effect of simultaneous variation of the enzyme dose and solids loading used during enzymatic saccharification on achieved glucose concentrations.**A:** Glucose (g/L) and **B:** Glucose (achieved % theoretical yield). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10-40 FPU/g biomass) with 6-72 h incubation at 50°C, 15% w/v solids loading using standard shaking incubator method (SI) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Data are the mean ± SD of 3 replicate measurements.

residence time in order to achieve higher glucose yields.

3.5. Effect of variation of reaction time of enzymatic saccharification at various low enzyme doses on achieved glucose yields

Using a 6-72 h time course (again with a standard SI based method for higher throughput than the aerated HTM system), a very similar trend in liberated g/L glucose concentrations was observed across a low range of enzyme doses (10-40 FPU/g biomass; Novozymes Cellic® CTec2) when operating at a static 15% w/v solids loading (Fig. 8A). The predominant bulk of the total final saccharification yields were achieved by ca. 24 h (86% of the final glucose yields that were achieved when using a 72 h reaction time). Regardless of the enzyme dose used, any extension of the reaction time beyond ca. 24 h resulted in only a small increase in achieved glucose yields.

A very similar trend was observed with achieved % theoretical yields (Fig. 8B). Overall, the data suggested that at 15% (w/v) solids loading and when using low enzyme doses, reaction times of ca. 24 h would be optimal as doubling the reaction time to 48 h only liberated 9% more glucose (g/L).

3.6. Investigation of fed-batch methods on enzymatic saccharification glucose yields

The preliminary screening of fed-batch methods (in which the required amount of pretreated BSG to achieve the desired solids loading was added in two aliquots with the second aliquot added after 24 h incubation) was also initially conducted using a high-throughput SI method as opposed to the optimised aerated HTM system. The use of this fed-batch method resulted in small but significant ($P<0.05$: one-way ANOVA) increases in

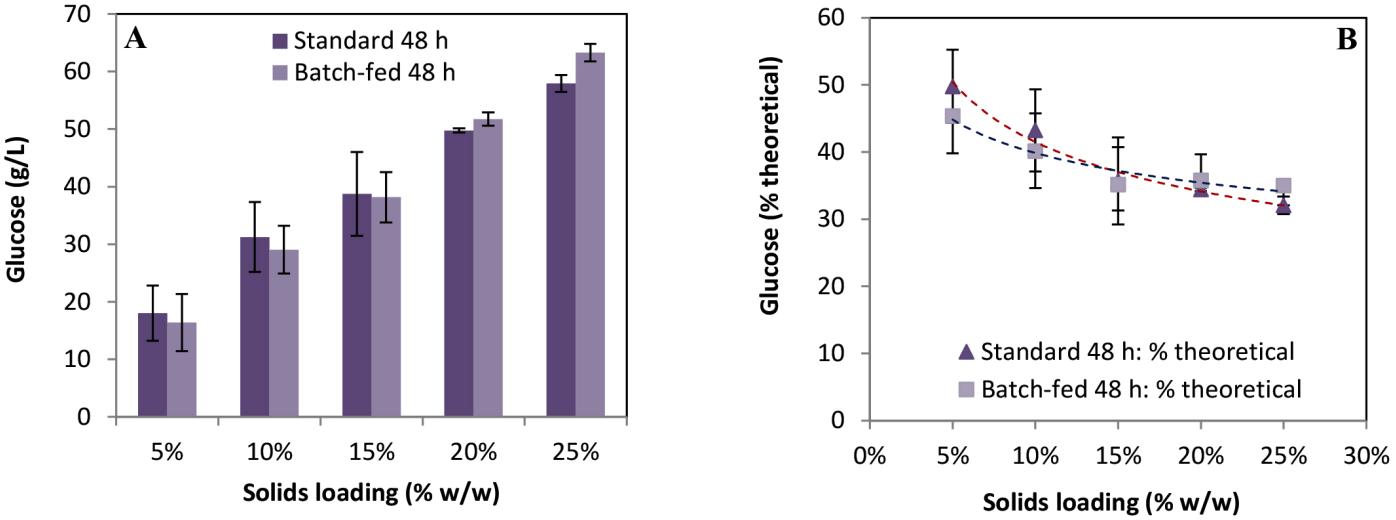

Fig.9. Efficacy of fed-batch enzymatic saccharification methods compared with non-fed-batch methods. **A:** Glucose (g/L) and **B:** Glucose (% theoretical yield). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (50 FPU/g biomass) with 48 h incubation at 50°C, 5-25% w/v final solids loading using standard shaking incubator method (SI) on pretreated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading. Fed-batch method had the required biomass quantity split into two equal portions with the second aliquot added after 24 h. Data are the mean ± SD of 3 replicate measurements.

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final g/L glucose concentrations (ca. 12% more glucose) when solids loading exceeded 20% (w/v) in comparison with a standard, non-fed-batch method using both an identical enzyme dose and identical incubation period but with all the biomass added entirely at the start of the incubation period (**Fig. 9A**). Whilst the degree of relative enhancement of the fed-batch method at both 20% and 25% w/v solids loadings was similar (12% increases in achieved % theoretical glucose yield compared with the standard non-fed-batch method at similar final solids loading), the effect on final g/L glucose concentrations was more pronounced as solids loading increased obviously due to the presence of ever greater quantities of biomass. The achieved % theoretical glucose yields followed a similar trend with fed-batch methods only functioning slightly more effectively (than non-fed-batch methods) at very high solids loading (≥20% w/v; **Fig. 9B**). Overall, this suggested that when operating at ≥20% w/v solids loading, splitting the biomass into two equal aliquots whilst using an identical enzyme dose boosted both final g/L glucose concentrations and % theoretical yields through effectively halving the operational solids loading for the first 24 h of a 48 h saccharification period. The initial 24 h saccharification period facilitated a considerable degree of liquefaction of the slurry which likely assisted in some reduction of any mass transfer limitations possibly enabling better enzyme mobility and thus better enzyme-substrate linkages.

3.7. Comparison of the effect of increasing solids loading during enzymatic saccharification using both an optimised aerated HTM method and a standard SI method

Both g/L glucose concentrations and achieved % theoretical glucose yields were suggested to be improved through the use of the aerated HTM method compared with the standard SI method with both responses ranging from 10% to 25% higher across all solids loadings tested (**Fig. 10A**). However, the degree of improvement (achieved using the aerated HTM method) for both response parameters increased as solids loading increased with the greatest and only statistically significant improvement observed at the highest solids loading tested (25% improvement in glucose yields at 25% w/v solids loading; $P<0.05$: one-way ANOVA). This suggested the HTM method overcame some of the substrate insolubility issues which limited mass transfer at very high solids loadings. Overall, it suggested higher energy mixing regimes may still play some part in facilitating effective operation of high solids loading enzymatic saccharification of pretreated lignocellulosic biomass such as BSG if very high solids loadings are to be used.

3.8. Consolidated (combined) enzymatic saccharification method

Due to the variability of enzyme activity across numerous batches of experiments, only small relative differences in glucose yields were often observed in response to variation of many of the experimental parameters. As such, a consolidated saccharification method was derived using a combination of the previously suggested marginal enhancements, all together in one single step in an attempt to maximise the achieved glucose yields when operating at high solids loading. The combination of the aerated HTM system but also simultaneously incorporating a fed-batch approach when using a low cellulolytic enzyme dose (Novozymes Cellic® CTec2 dosed at 10 FPU/g biomass) and a synergistic combination of various additional carbohydrate degrading enzymes and enzymatic cofactors previously shown to enhance the saccharification yields (ferulic acid esterase, acetyl xylan esterase, Novozymes Cellic® HTec2, α-L-arabinofuranosidase, $Cu(NO_3)_2$, and ascorbic acid) resulted in a small but significant ($P<0.05$: one-way ANOVA) improvement of 13% to 19% more glucose (both g/L and achieved % theoretical yields, compared with the use of a non-fed-batch variant of the aerated HTM method without any supplements) at 15% and 25% (w/v) solids loadings tested, respectively (**Fig. 10B**). As previously observed with the aerated HTM system (in comparison with the standard SI method), the degree of improvement for both response parameters (g/L and achieved % theoretical glucose yields) increased as solids loading increased but in this case with the greatest improvement observed at 15% w/v solids loading (19% improvement in glucose yields). Increasing solids loading to 25% w/v then reduced the relative degree of improvement in achieved glucose yields to only 15% more compared with the aerated HTM system alone. This suggested that 15% solids loading was a 'sweet spot' in terms of optimal conversion efficiency (optimal achieved % theoretical yields). This ever smaller relative increase in glucose yields even with the inclusion of all the optimisations suggested the point of diminishing returns had likely been reached. It suggested that simultaneous inclusion of all the additives (supplementary enzymes and enzymatic cofactors) alongside the main cellulolytic enzyme cocktail (Cellic® CTec2) would not likely be economically viable in terms of their incorporation into any large scale enzymatic saccharification process as use of any one supplementary additive alone would likely do the job effectively and facilitate a good degree of enhancement of saccharification. The choice of which particular additive (if any) would likely depend solely on what was

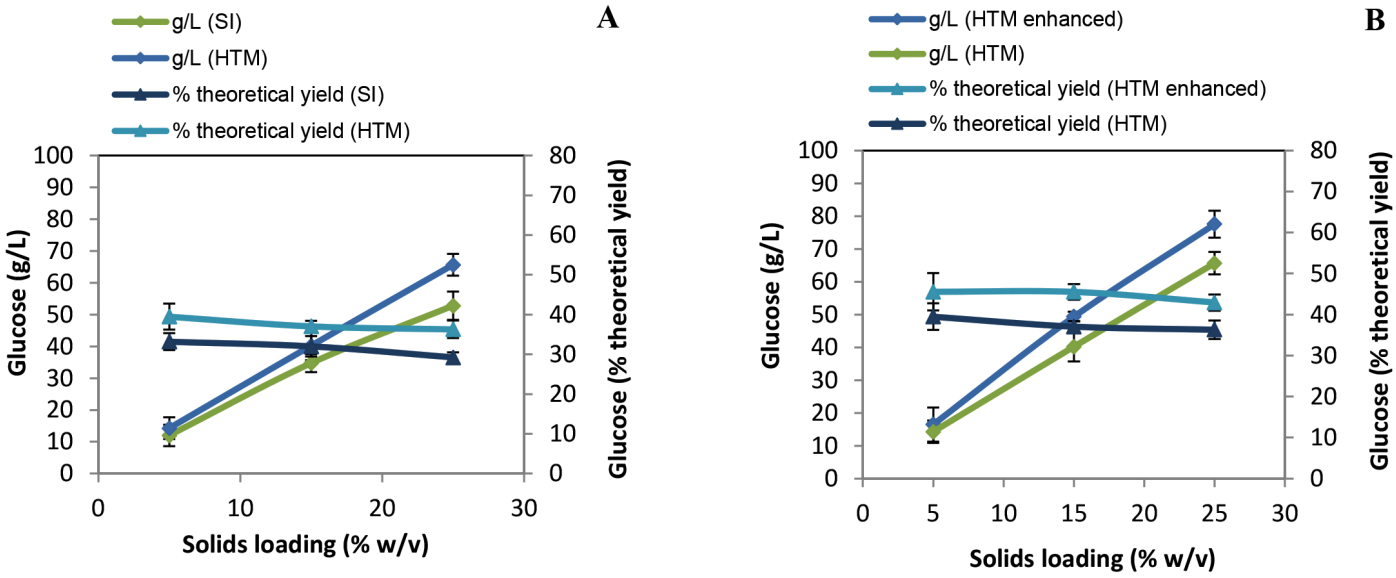

Fig.10. Comparison of the effect of variation of solids loading on post enzymatic saccharification glucose concentrations achieved using different methods. **A:** shaking incubator (SI) method compared with the aerated high-torque mixing (HTM) method. **B:** aerated HTM method alone compared with the consolidated method (aerated HTM with additional enzymes and cofactors). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10 FPU/g biomass and incubated at 50°C for 72 h) on pretreated BSG (1% HCl 121°C 30 min, 25% w/w solids loading) over a 5%-25% solids loading range. **A:** Standard shaking incubator (SI) system (150 rpm) compared with aerated high-torque mixing (HTM) system (150 rpm). **B:** HTM aerated method (non-fed-batch with no additives) compared with a HTM aerated method fed-batch (biomass split into 2 × aliquots; second added after 24 h) with additional enzymes: FAE; ferulic acid esterase, AXE; acetyl xylan esterase, AFD; α-L-arabinofuranosidase, HTec2; Cellic® HTec2 xylanases (all at 100 μL doses), and $Cu(NO_3)_2$ and ascorbic acid (both at 10 mM). Data are the mean ± SD of 3

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the most economically viable and readily available in the quantities required for large scale saccharification. Overall, even with all the suggested optimisation strategies employed simultaneously, only relatively low % theoretical glucose yields were achieved (43% when using 25% w/v solids loading). This could be due to a number of possible factors. Some degree of product inhibition (catabolite repression) is a possibility as cellobiose has been shown to feedback inhibit various endocellulases (Xiao et al., 2004). However, the inclusion of high concentrations of β-glucosidase enzymes within the Novozymes Cellic® CTec2 preparation should in theory combat this issue to a significant degree through cleaving any cellobiose molecules into their constituent monomeric glucose sub-units. As such, this was considered unlikely to be the cause of the low yields. In addition, HPLC analysis of all post enzymatic saccharification supernatants (the hydrolysates produced from all experiments, etc.) did not show any significant cellobiose concentrations to be present that would have possibly been an indicator of feedback inhibition. Alternatively, inhibition of the cellulolytic enzymes from deleterious product formation that may have occurred during the upstream pre-treatment process (such as liberation of lignin based hydroxycinnamic acids or furan based hemicellulose or cellulose degradation products) can be discounted in this case as all experiments were conducted using thoroughly washed biomass prior to the enzymatic hydrolysis reactions. In addition, HPLC analysis was conducted to screen for these inhibitory compounds and concluded negligible quantities were present. Another possible factor is that adsorption of various cellulases to the cellulose substrate has been experimentally shown to decrease with increasing solids loading (Kristensen et al., 2009). This could possibly be due to mass transfer limitations due to the insoluble nature of the slurries used when attempting high solids loading enzyme saccharification (the low free water content and thus low 'mobile phase' in which the enzymes can freely move throughout the media). This is particularly applicable to pretreated lignocellulosic substrates such as BSG as the upstream processing (the thermo-chemical pre-treatment step in particular) generates a wide range of sizes of fragments of the material (some of which are very small; < 106 μm) that saturates the liquid or 'mobile phase'.

Additionally, the build-up of hydrolysis end products such as high concentrations of glucose and cellobiose have been shown to physically impede the adsorption of endocellulases to cellulose (Stutzenberger and Lintz, 1986). The general observed trend of a linear increase in liberated glucose concentrations (g/L) as solids' loading was increased (for much of the experimental data presented here) would suggest that when very high solids loadings were used (>25% w/v), sufficient glucose concentrations may be present to impede any further successful adsorption (or mobility of) the CBMs. This could assist in explaining the sub-optimal achieved % theoretical glucose yields. It is the CBMs that facilitate the adsorption and subsequent activity and mobility of the critical endocellulases that initially cleave internal β-1,4 glycosidic linkages thus generating more free reducing ends that other cellulolytic enzymes can attack (Arantes and Saddler, 2010). The modification of endocellulase CBMs (including their substitution for higher binding affinity variants) in order to enhance both their activity and their resistance to high solid to liquid ratio environments thus improving their function at high solids loading has also been considered (Taylor et al., 2012).

The issue of this pseudo form of non-competitive inhibition by enzyme reaction products such as glucose could possibly be solved through investigation of simultaneous saccharification and fermentation (SSF) approaches. This would enable the selective uptake and metabolism of reaction products such as glucose thus reducing their deleterious feedback inhibitive effects. However, the system would need to be carefully designed as some provision for fermentation product removal would be required, especially in the case of bioethanol production as ethanol is a known inhibitor of cellulolytic enzymes although less so than the degree of feedback inhibition that occurs as a result of cellobiose build-up (Ooshima et al., 1985). The use of a thermophilic fermentative organism in conjunction with the high temperature optima (50°C) of Novozymes Cellic® CTec2 may enable some degree of evaporative recovery of any ethanol produced as an extremophile may tolerate short periods of temperature spikes which could be used to enhance recovery. Alternatively, a sealed system that artificially reduced the atmospheric pressure within the bioreactor to below the vapour pressure of ethanol could then enable lower evaporative temperatures. However, this would not solve the issue of the build-up of any other non-volatile metabolic waste product which may still be equally inhibitory towards various enzymes.

4. Conclusions

Different parameters associated with enzymatic saccharification (at high solids loading) of pretreated BSG were investigated in order to attempt to maximise the achieved glucose yields. Supplementation of the cellulolytic enzymes with additional enzymes (ferulic acid esterases, acetyl xylan esterases, and xylanases) and cofactors (ascorbate and copper) could significantly boost achieved glucose yields. Additionally, using fed-batch method and improved slurry agitation (aerated high-torque mixing) could also offer enhancements in glucose yields. From the experimental investigations, a novel, consolidated enzymatic saccharification method was developed which effectively functioned at high solids loading in an attempt to further maximise glucose yields. Glucose yields of 78 ± 4.1 g/L and ca. 43% of theoretical yield were achieved when operating at 25% w/v solids loading when using a commercially applicable low Cellic® CTec2 dose (10 FPU/g biomass). However, additional development is required in order to increase these glucose yields further when increasing the operational solids loading beyond 25% w/v.

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Supplementary Data

Table 1S.
Experimental reaction conditions for evaluation of various solids loadings (% w/v) and various enzyme doses (Novozymes Cellic® CTec2) during enzymatic saccharification of pretreated BSG (1% HCl hydrothermal method; 121°C, 30 min at 25% w/v solids loading) according to a D-optimal design space*.

Run	Factor 1 A: Enzyme dose FPU/g biomass	Factor 2 B: Solids loading (% w/v)
1	40	5
2	160	5
3	10	5
4	160	25
5	10	25
6	40	15
7	10	15
8	40	25
9	40	15
10	40	15
11	160	5
12	10	5
13	160	15
14	160	25
15	80	10
16	80	10
17	20	10
18	20	10
19	80	20
20	80	20
21	20	20
22	20	20
23	160	15
24	40	25
25	10	15
26	10	25
27	80	15
28	20	15
29	80	15
30	20	15

*D-optimal experimental design was created by DesignExpert (Stat-Ease, USA) using a collection of reaction combinations (enzyme doses and solids loadings) from which the D-optimal algorithm chose the treatment combinations to include in the design. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design.

Table 2S.
Experimental reaction conditions for evaluation of various solids loadings (% w/v) and various hydrolysis times (10-72 h) during enzymatic saccharification of pretreated BSG (1% HCl hydrothermal method; 121°C, 30 min at 25% w/v solids loading) using Novozymes Cellic® CTec2 (20 FPU/g biomass) according to a D-optimal design space*.

Run	Factor 1 A: Solids loading (% w/v)	Factor 2 B: Hydrolysis time (h)
1	25	10
2	5	72
3	25	72
4	25	48
5	5	72
6	20	48
7	15	48
8	15	48
9	5	48
10	20	72
11	15	10
12	15	24
13	25	10
14	10	24
15	25	72
16	5	10
17	5	24
18	10	24
19	25	72
20	5	10
21	10	48
22	20	24
23	15	72
24	15	24
25	5	72
26	25	10
27	10	72
28	10	10
29	20	24
30	25	24

*D-optimal experimental design was created by DesignExpert (Stat-Ease, USA) using a collection of reaction combinations (solids loadings and hydrolysis time) from which the D-optimal algorithm chose the treatment combinations to include in the design. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design.

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