ARTICLE

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Do New Cellulolytic Enzyme Preparations Affect the Industrial Strategies for High Solids Lignocellulosic Ethanol Production?

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ABSTRACT: Production of ethanol from lignocellulosic materials has a promising market potential, but the process is still only at pilot/demonstration scale due to the technical and economical difficulties of the process. Operating the process at very high solids concentrations (above 20% dry matter—DM) has proven essential for economic feasibility at industrial scale. Historically, simultaneous saccharification and fermentation (SSF) was found to give better ethanol yields compared to separate hydrolysis and fermentation (SHF), but data in literature are typically based on operating the process at low dry matter conditions. In this work the impact of selected enzyme preparation and processing strategy (SHF, presaccharification and simultaneous saccharification and fermentation—PSSF, and SSF) on final ethanol yield and overall performance was investigated with pretreated wheat straw up to 30% DM. The experiments revealed that an SSF strategy was indeed better than SHF when applying an older generation enzyme cocktail (Celluclast-Novozym 188). In case of the newer product Cellic CTec 2, SHF resulted in 20% higher final ethanol yield compared to SSF. It was possible to close the mass balance around cellulose to around 94%, revealing that the most relevant products could be accounted for. One observation was the presence of oxidized sugar (gluconic acid) upon enzymatic hydrolysis with the latest enzyme preparation. Experiments showed gluconic acid formation by recently discovered enzymatic class of lytic polysaccharides monoxygenases (LPMO's) to be depending on the processing strategy. The lowest concentration was achieved in SSF, which could be correlated with less available oxygen due to simultaneous oxygen consumption by the yeast. Quantity of glycerol and cell mass was also depending on the selected processing strategy.

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KEYWORDS: high dry matter; bioethanol; SSF; SHF; LPMO; GH61

Introduction

Nowadays only a few pilot/demo plants are converting lignocellulosic biomass into bioethanol, nevertheless contributing greatly to build up the necessary knowledge and experience to understand how industrial bioconversion of lignocellulose should be done. Examples of these plants are: Inbicon in Denmark, Sekab in Sweden, Iogen in Canada and US, Abengoa in Spain, and Chemtex's plant in Italy (Larsen et al., 2012). Even though different biomass pose different challenges when it comes to efficient conversion to ethanol, some similarities for "industrial relevant conditions" has been identified. From an industrial point of view operating at high solids conditions is essential to make feasible bioethanol production (Di Risio et al., 2011; Larsen et al., 2008; Modenbach and Nokes, 2012), mostly by lowering the minimum ethanol selling price (MESP) (Macrelli et al., 2012). There are numerous benefits by operating the process at high solids concentrations of which the final high ethanol concentration and consequently lower cost for distillation is the most obvious. Also capital and production costs are more beneficial due to reduced size of equipment and reduced energy consumption for processing (Katzen et al., 1999; Wingren et al., 2003).

Processing of lignocellulosic materials at high solids concentrations (above 20% dry matter) has previously been proven technically difficult (Kargi et al., 1985; Mohagheghi et al., 1992). One technical challenge is the fibrous structure of the lignocellulosic materials causing initial high viscosity and poor mixing, which impair performance of the enzymes (Kristensen et al., 2009). This is especially the case when conventional industrial pretreatment technologies such as hydrothermal, diluted acid, or alkali pretreament are applied. In these cases the lignin and fibrous structure are maintained, even if severily swollen. In contrast, a method such as ionic liquids cause a more complete dissolution of the cellulose and lignin structure (Lee et al., 2009), that likely facilitates easy handling even at high solids concentration. Despite this, the latter technology is still considered far from applicable at industrial scale due to high operational costs. The lignocellulosic biomass used in this work was hydrothermally pretreated wheat straw with a final

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dry matter content of about 30% prior to the enzymatic hydrolysis and subsequent fermentation.

Lab scale equipment commercially available on the market capable of mixing at more than 20% dry matter (DM) is rather limited, and there are only few examples capable of handling 25% DM at industrial scale (Di Risio et al., 2011; Larsen et al., 2012). Recently, a simple lab scale technology was proposed capable of mixing material up to 30% DM solids. The mixing principle is based on horizontal rotation of the whole reactor, thus the name roller bottle reactor (RBR), but has been, to the best of our knowledge, only successfully applied to enzymatic hydrolysis (Roche et al., 2009). In this study, we have assembled a system following the same principle as the RBR, but capable of supporting many flasks to be used as reactors for not only enzymatic hydrolysis but also fermentation.

Historically, two major strategies have been applied to pursue the enzymatic hydrolysis and fermentation of lignocellulosic biomass: (1) separate hydrolysis and fermentation (SHF) and (2) simultaneous saccharification and fermentation (SSF) (Tomas-Pejo et al., 2008; Wingren et al., 2003). In SHF, enzymatic hydrolysis and fermentation are carried out in separate steps, whereas in SSF both are combined into one step. From an application point of view there are several aspects to consider when selecting which strategy to implement. The main difference is that in SHF the hydrolysis and fermentation are both operated at their optimal conditions, especially regarding the temperature. In SSF the temperature is mandated by the microorganism (typically no more than 35°C), which implies that the enzymatic hydrolysis is at suboptimal condition. Even though the enzymatic hydrolysis is operated at the optimal temperature in SHF (typically 45–50°C), the hydrolysis yield is then affected by end-product inhibition, which does not occur in SSF due to the simultaneous removal of glucose by the fermenting organism. Using older generations of cellulase enzyme preparations, higher yields of ethanol production was typically found pursuing the SSF strategy. This has been well documented for a number of different biomasses and pretreatments and especially at conditions involving higher solids concentrations (Erdei et al., 2010; Mohagheghi et al., 1992; Olofsson et al., 2008; Tomas-Pejo et al., 2009). Recently, new cellulases preparations have been developed and made commercially available. The characteristics of these are first of all higher β-glucosidase activity and the presence of a new class of enzymes termed lytic polysaccharides monoxygenases, LPMO's (previously known as GH61) (Cannella et al., 2012; Harris et al., 2010; Horn et al., 2012; Lo Leggio et al., 2012; Westereng et al., 2011). Additional improvements may involve replacement of individual cellulases by better and more stable counterparts (Brienzo et al., 2008). Altogether, these new characteristics have generally increased the performance of newer generations of cellulase preparations (Rosgaard et al., 2006). The relation between choice of enzyme preparation, process strategy at industrial relevant conditions and the overall process performance, that is, final ethanol yield, therefore needs to be investigated.

With regard to enzyme performance, several articles have also studied the beneficial effect upon hydrolysis by addition of poly(ethylene glycol) (Börjesson et al., 2007; Li et al., 2012; Kristensen et al., 2007). Generally, the understanding is that PEG lowers the interaction between lignin and enzyme, thereby reducing the non-productive adsorption of enzyme. Higher conversion is therefore obtained at same enzyme loading or addition of PEG could be used to reduce the enzyme loading without compromising the yield. Most of these studies have been conducted at low solids concentrations. The economic feasibility is still questionable due to the price of PEG, but due to the limited data available using PEG in hydrolysis and fermentation performed at high solids concentrations it is not possible to assess the benefit under industrial relevant conditions.

In the present study, the effect upon overall performance of two different cellulases preparations applied in three different processing strategies has been investigated. In addition, a mass balance around cellulose has been used to study how different process conditions (strategy and enzyme) affect the distribution of the most important products (ethanol, glycerol cell mass, and gluconic acid). Especially the role of the LPMO's in the various strategies has been investigated.

Materials and Methods

Enzymes

Two commercial cellulase preparations Celluclast 1.5L and Cellic CTec2 and the β-glucosidase preparation Novozym 188 were used, all from Novozymes A/S (Bagsværd, Denmark). Filter paper activity was determined according to Ghose (1987) and β-glucosidase activity was measured using 5 mM *p*-nitrophenyl-β-D-glucopyranoside as substrate (Saha and Bothast, 1996). Protein content was measured using the Ninhydrin assay with BSA as protein standard (Starcher, 2001). The results for Cellic CTec2 were: 120 FPU/g, 2,731 U/g (β-glucosidase activity), and 161 mg protein/g; for Celluclast: 62 FPU/g, 15 U/g (β-glucosidase activity), and 127 mg protein/g; for Novozym 188: 231 U/g (β-glucosidase activity) and 220 mg protein/g.

Biomass Pretreatment and Composition Analysis

Wheat straw (*Triticum aestivum* L.) was pretreated at the Inbicon A/S pilot plant in Skærbæk, Denmark. The hydrothermal pretreatment was conducted at 195°C with a residence time of 18.5 min without addition of chemicals. A washing and pressing step was applied after the pretreatment, which reduced the content of soluble molecules generated during the pretreatment, that is, pentose sugars and toxicants such as furfural, HMF, and acetic acid (Larsen et al., 2008, 2012). The final dry matter content was 32.5%. The material was stored frozen until use.

The composition of the solid material was analyzed by strong acid hydrolysis using a modified version of the TAPPI standard procedure (1998), the modification being that the

standard curve was treated similarly to the samples to correct for sugar degradation. Before analysis the material was washed with water to remove soluble sugars by repeated centrifugation and suspending in demineralized water. The solids were then dried at 60°C over night. The monosaccharides D-glucose, D-xylose, L-arabinose, D-mannose, and D-galactose were measured on a Dionex ICS5000-system equipped with a CarboPac-PA1 column and using PAD-detection (Dionex, Sunnyvale, CA). The composition of the pretreated material was: glucan (cellulose) 53.7%, xylan 3.5%, klason lignin 34%, and ash 6.1%. The toxicant concentrations in the final material when adjusted to the dry matter content of 30% (after washing and pressing) were: acetic acid 2.53 g/kg, hydroxymethyl furfural 0.32 g/kg, and furfural 0.58 g/kg.

Roller Bottle Reactor Assembling

The RBR consisted of 100 mL blue cap glass bottles. Sampling and gas outlet was done through two ports made in the cap. The gas outlet was connected to a water trap via a silicon tube. The setup enabled anaerobic conditions and sampling. Twelve reactors so assembled were placed on a roller bottle holder with four segment cradles connected to a horizontal rotator capable of a rotation at a speed up to 60 RPM (Stuart 4/4, Bibby Scientific, Stone, UK). The rotator unit assembled with the drum and bottles was placed in an incubator oven. The whole unit was positioned with an incline of about 30° and the bottles were oriented having the outlets in the higher position. The rotation speed was 10 RPM for all the experiments.

Enzymatic Hydrolysis and Fermentation Strategies

The study included 33 different scenarios of enzymatic hydrolysis and fermentation. This involved testing scenarios at two dry matter concentrations (20% and 30% DM), two different enzyme preparations (Cellic CTec 2 and a 5:1 (weight) mix of Celluclast and Novozym 188) at two enzyme loadings (5 and 7.5 FPU/g DM) using three different process strategies, with and without a surfactant PEG3000 (all the conditions are summarized in Table I). In the following Cellic CTec2 referred as CT2 and the mixture of Cellulcast and Novozym 188 as CN. The enzymatic dosage was design to have a high and low dosage (respectively 7.5 and 5 FPU per gram of dry matter) for both enzyme preps, which corresponded to 22.8 and 15 mg protein per gram of cellulose for CT2, and 30 and 22.8 mg per gram of cellulose for CN. Three different hydrolysis and fermentation strategies were applied:

SHF was conducted as following: the pretreated biomass was enzymatically hydrolyzed for 72 h at 50°C, cooled to 34°C, inoculated with yeast and fermented for 96 h. Separation of solids and liquid was not performed.
PSSF (Alvira et al., 2013), where the biomass was partially hydrolyzed for 24 h at 50°C, cooled to 34°C, inoculated with yeast and fermented for 120 h. No solids separation occurred among hydrolysis and fermentation steps as described above.
SSF, where the enzymes and yeast were added to the biomass at the same time and kept at 34°C for 168 h. Moreover, poly(ethylene glycol) with molecular weight of 3000 (PEG3000) was added at a concentration of 0.01 g PEG/g DM to another set of experiments with 30% DM. Each scenario was tested in triplicate, using a biological triplicate approach for the fermentations.

The general setup was to add pretreated wheat straw corresponding to 30 g at 32.5% DM in 100 mL blue cap bottles. Water was added to give a final DM content of 20% or 30% (final working mass of 49 and 33 g, respectively). The pH was adjusted to 5.0 by addition of 0.2 mL of 13 M NaOH solution. In experiments with PEG, 0.2 g of PEG3000 was added. The biomass (prewarmed at 50°C) was manually mixed for few minutes after the addition of the enzymes.

Table I. Experimental conditions for all the scenarios.

	Strategies											
Parameters	A	B	C	D	E	F	G	H	I	J	K	L
Strategy	SHF	PSSF	SSF	SHF	PSSF	SSF	SHF	PSSF	SSF	SHF	PSSF	SSF
Enzyme preparation	CT2	CT2	CT2	CT2	CT2	CT2	CN	CN	CN	CN	CN	CN
Enzyme dosage (mg/g cellulose)	22.8	22.8	22.8	15.0	15.0	15.0	30.0	30.0	30.0	22.8	22.8	22.8
Hydrolysis 50°C, time (h)	72	24	0	72	24	0	72	24	0	72	24	0
Fermentation 34°C, time (h)	96	144	168	96	144	168	96	144	168	96	144	168
Process strategies	Results											
	A	B	C	D	E	F	G	H	I	J	K	L
Ethanol yield 30% DM (%)	62.8	61.76	45.84	49.15	50.72	37.67	28.82	38.36	38.21	27.32	30.60	30.04
Ethanol yield 30% DM—PEG (%)	88.77	89.07	73.99	61.05	60.15	45.96	45.94	48.51	54.14	—	—	—
Ethanol yield 20% DM (%)	73.09	84.09	86.32	59.05	70.30	73.74	54.61	65.15	66.34	42.30	57.02	59.13

The final ethanol yield at the end of each process strategy is given as percentage of the maximum theoretical ethanol fermentable from the initial amount of cellulose loaded.

After the hydrolysis or presaccharification (72 or 24 h), pH of the slurries were measured (typically pH 4.7) and adjusted to 5.0 using 13 M NaOH solution, rapidly cooled to 34°C and then inoculated with 0.5 mL of concentrated yeasts suspension (15 g/L) resulting in inoculums size of dry cells 6 g/kg of dry biomass. The pH after fermentation for both SHF and PSSF strategies was 4.9. The SSF experiments were carried out similarly as listed above with the only difference that the yeast cells were added simultaneously with the enzymes, and the pH was adjusted at 5.0 from the beginning using the 13 M NaOH solution (final pH after 168 h was 4.7). No nutrients were added during the fermentation of wheat straw.

Yeast Growth and Viability Analysis

The *Saccharomyces cerevisiae* strain Thermosacc® Dry (Lallemand, Inc., Montreal, Canada) was used in all fermentation experiments. Thermosacc® was precultured in CBS medium (Verduyn et al., 1992) containing 20 g/L of glucose for 48 h prior each inoculation. The cells were then harvested and the pellet was resuspended in 0.9% NaCl solution giving a final concentration of 15 g/L of dry cells. No growth was achieved when Thermosacc® was incubated for 96 h with CBS medium containing gluconic acid as sole carbon source. The fermentation yield was also tested in defined medium containing glucose and gluconic acid, and no ethanol was detected with the gluconic acid (168 h of incubation).

During the fermentation of wheat straw hydrolyzates, samples were plated on YPD plates for viability analysis. For each time point, the sample was plated at three different dilutions in duplicate, incubated for 48 h at 35°C after which the number of colonies were counted. The YPD plates contained 1% yeast extract, 2% peptone, and 2% glucose.

HPLC Analysis

The quantification of D-glucose, D-cellobiose, D-xylose, lactate, acetate, glycerol, and ethanol was done using an Ultimate 3000 HPLC (Dionex, Germering, Germany) equipped with refractive index detector (Shodex, Tokyo, Japan) and UV detector at 210 nm (Dionex). The separation was performed in a Phenomenex Rezex ROA column at 80°C with 5 mM H_2SO_4 as eluent at a flow rate of 0.8 mL/min.

HPAEC Analysis

Oxidized glucose (gluconic acid, Sigma Aldrich, St. Louis, MO) was quantified using ICS5000 HPAEC system, equipped with a PAD detector (both from Dionex, Sunnyvale). The separation was performed using a Dionex CarboPac PA1 2 mm × 50 mm guard column and 2 mm × 250 mm, analytical column. The column was operated at a flow of 0.25 mL/min and maintained at 30°C. Peak separation for oxidized monosaccharides was obtained by applying the following elution gradient: 0.1 M NaOH for 5 min, then a linear increase from 0.1 M NaOH to 0.1 M NaOH with 0.3 M NaOAc in 35 min, then to 0.1 M NaOH/1 M NaOAc in 5 min. The column was reconditioned with 0.1 M NaOH for 5 min before injection of next sample. The gradient used was also suitable to observe potential oligosaccharides of aldonic acids (Westereng et al., 2013).

High-Throughput Reduced Scale Compositional Analysis

This analysis was used to monitor the cellulose content in the residual solids from hydrolysis and fermentation samples taken during all the experiments, which therefore required a high throughput method. The compositional analysis (TAPPI method) is traditionally done in 100 mL glass bottle and 300 mg of biomass is analyzed, this imposes an extensive use of equipment, chemicals, and timework when applied at hundreds of samples. In this work, we reduced the scale 100-fold following the method of DeMartini et al. (2011). In details, the procedure was: 3 mg of dry, milled, and washed sample was incubate for 2 h with 60 μL of 72% sulfuric acid at room temperature, then 840 μL of milliQ water was added; each analysis was done in five replicates. The samples were autoclaved at 121°C for 1 h. The pH was adjusted to around pH 5 (with a suspension of $CaCO_3$, 50%, w/w, of water) and filtered. The procedure was done in 1 mL glass vials (flat bottom) inserted into a custom-made aluminum plate with a standard 96-well plate format. The vials were sealed tightly by a rubber plate inserted between the glass and an aluminum top plate, which was secured by a press lock. After autoclavation, all plates were weighted to check any leakage. Then the liquids were transferred to a filtering 96-well plate, and filtered directly in a second plate. The monomeric sugars (mainly glucose) were analyzed by HPLC (see above). Ash and lignin were not quantified. As control the contribution of glucose from the yeast cells was evaluated by adding a known amount of cells (6 g/kg of dry biomass) to biomass. Only a negligible increase of glucans was measured (0.2% more glucans relative to without yeast addition). In accordance with a similar method published (DeMartini et al., 2011), the reproducibility was acceptable: standard deviation of less than 2% was achieved for 90% of the samples.

Results

The study aimed at investigating the processing strategies at conditions closely related those expected in a commercial process. All results were obtained operating at very high dry matter content (20% and 30% DM), which is higher than most of the previous work present in literature. The substrate for this work was supplied from an industrial partner and involved a hydrothermally pretreatment of wheat straw followed by a liquid–solid separation including a washing step (Larsen et al., 2012). The resulting material was about 30% dry matter and contained negligible amounts of free sugars (mostly xylose) and the concentration of inhibitory compounds (mostly acetic acid, furfural, and HMF) was also maintained low due to the washing step. Moreover, the surfactant PEG3000 was added to a further set of experiments

at 30% DM, in order to investigate the possible beneficial effect upon final yields when applied at industrial relevant condition.

The experimental plan was designed to compare industrial relevant processing strategies SHF, PSSF, and SSF (all in batch configuration), when applying a new and old generation of cellulolytic enzymes. Two different dosages for each enzyme preparation were tested. In total the screening generated 33 different experiments (tested in triplicate). The details for the experiments are provided in Table I. The hydrolysis and fermentation strategies had the same overall duration of 168 h, and the main difference was the time at elevated temperature for hydrolysis (50°C). For SHF the hydrolysis time was 72 h, for PSSF 24 h and for SSF the temperature was throughout the whole experiment constant at 34°C. Such representative screening is not possible operating in shake flask systems or with standard vertical bench reactors, mostly due to the inefficient mixing caused by operating at very high dry matter. Thus RBR technology was applied and proved efficient for both hydrolysis and fermentation. The relative large amount of samples generated need as well high throughput analytical methods, especially for the quantification of residual cellulose in hydrolysis/fermentation samples. For this goal a reduced scale compositional analysis was applied (DeMartini et al., 2011).

Ethanol Fermentation Using Different Strategies at 30% DM

A direct comparison of glucose and ethanol profiles for the two tested enzymes CT2 and CN at the highest enzyme loading revealed a clear difference in hydrolysis and fermentation performance among the three strategies (Fig. 1 and Table II). In general the newer enzyme preparation CT2 performed better than the old reference enzyme mix CN, as shown in Table II and Supplementary Table I where glucose and ethanol concentration for all the sampling time points are summarized (every 24 h). During 24 h hydrolysis CT2 released almost 2.5 times more glucose compared to CN despite loaded at the same filter paper activity (7.5 FPU/g DM), but lower protein loading (22.8 vs 30 mg EP/g cellulose) as also observed in previous work (Cannella et al., 2012). The final yields of ethanol were also higher for CT2. More interestingly, the best choice of strategy with respect to final ethanol yield was different between the two enzyme preparations. SHF and PSSF resulted in 16–17% higher ethanol yield compared to SSF using CT2. On the contrary, the ethanol yield was 10% higher using SSF or PSSF compared to SHF when CN was applied. The same trends were obtained among all the different scenarios tested at high solids conditions (30% DM) (Fig. 2). The reasons for this difference between CT2 to CN could be many, however most likely it is associated with the higher specific activity of some enzymes, not at least β-glucosidase, in CT2. Generally, SSF has previously been preferred over SHF because the first resulted in less end-product inhibition by glucose and cellobiose (due to a continuous conversion of glucose to ethanol). Even though the temperature in SSF (usually

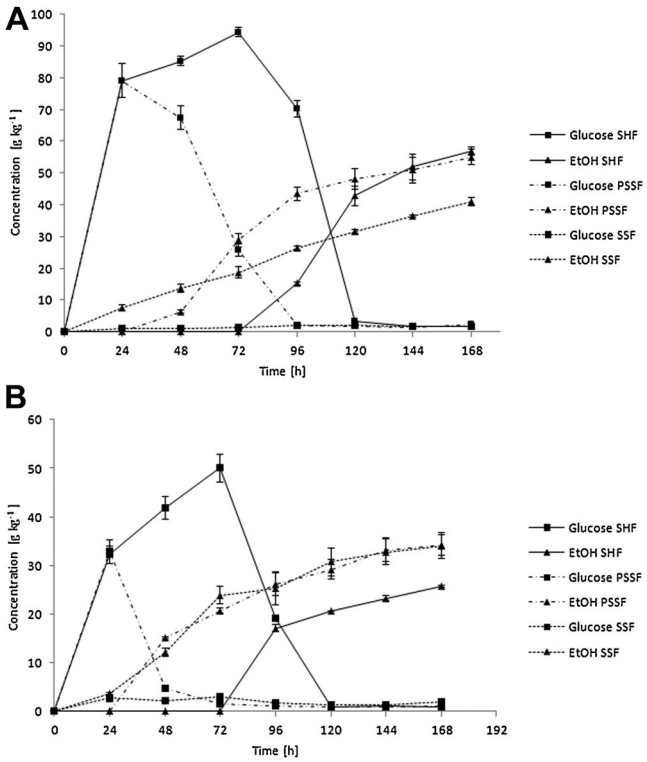

Figure 1. Profile of glucose (squares) and ethanol (triangle) during SHF (solid lines), PSSF (dash-dotted lines), and SSF (dotted lines) at 30%DM. Yeast was inoculated at 72, 24, and 0 h, respectively. The enzymes applied were CT2 (**Panel A**: scenarios A–C) and CN (**Panel B**: scenarios G–I), all at the high enzyme loading.

∼35°C) is far from the optimal for hydrolysis (50°C), this was found to be the most advantageous strategy with enzyme preparations such as CN (Mohagheghi et al., 1992). Despite 50°C being the optimum temperature for hydrolysis, the high temperature for extended periods of time could result in deactivation of enzyme. This would also favor SSF over SHF in case of less stable enzyme preparations. In case of CT2 end-product inhibition affected less severely due to the higher β-glucosidase activity in this preparation, and maybe also a

Table II. Final ethanol yield after 168 h.

Conditions	SHF	PSSF	SSF
Overall ethanol yield with CT2 enzyme			
30% DM	62.80	61.76	45.84
30% DM + PEG3000	88.77	89.07	73.99
20% DM	73.09	84.09	86.32
Overall ethanol yield with CN enzyme			
30% DM	28.82	38.36	38.21
30% DM + PEG3000	45.94	48.81	54.14
20% DM	54.61	65.15	66.34

The data are calculated in % of the maximum ethanol yield obtainable at the DM loading indicated. The experiments were done at the highest enzyme dosage 22.8 and 30 mg protein/g cellulose for CT2 and CN mixtures, respectively (7.5 FPU/g dry biomass).

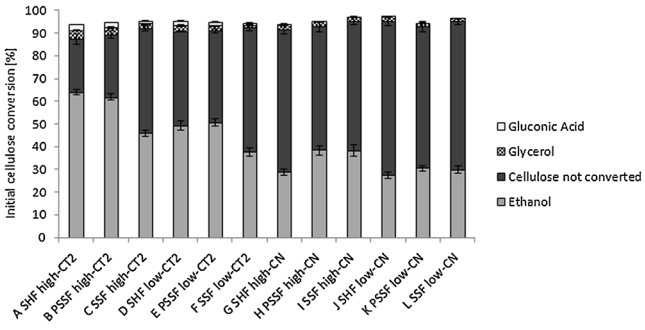

Figure 2. Final products yield as percentage of initial cellulose for all scenarios at 30%DM, after 168 h. Each bar represents the percentage of initial cellulose converted into the indicated products. Cellulose is the amount of fibers not hydrolyzed during the strategies as directly measured by compositional analysis. Gluconic acid, glycerol, and ethanol was measured and stoichiometric converted back to corresponding amount of cellulose. Ethanol includes the carbon dioxide.

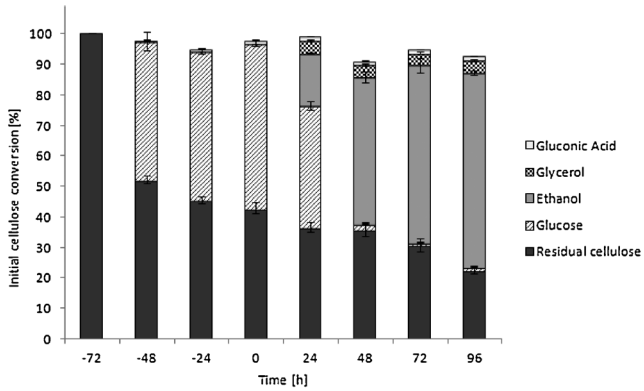

Figure 3. Product profiles as percentage of initial cellulose for scenario A, SHF with CT2 at high enzyme loading, and 30% DM. Time -72, -48, and -24 h represent the hydrolysis step at 50°C. Time 0 h represents onset of fermentation, that is, yeast addition and temperature change to the fermentation temperature of 34°C.

less glucose sensitive β-glucosidases. This higher tolerance made it possible to work at optimal temperature (50°C) despite high glucose concentrations (maximum in this study was ~150 g/kg, scenario A with PEG3000). Data presented show that PSSF and SHF led to higher ethanol yields, or achieving the same ethanol yields as SSF with 30% less enzymes (by comparing scenarios C with respect to D and E, Table I), when applying latest cellulolytic preparation CT2. Furthermore, the presence of LPMO enzymes plays a prominent role in increasing the overall activity of the enzymatic cocktail, especially at high dry matter content as will be discussed in details below.

For this study, it was crucial to measure the residual cellulose after hydrolysis and fermentation in order to establish a mass balance for cellulose. Determining the composition of lignocellulosic materials is usually a cumbersome procedure, but for this work a high throughput method was applied, which enabled us to measure residual cellulose during the process and in the final fermentation broth. Together with measurement of the most common fermentation products (ethanol, glycerol, and acetic acid) and products of the hydrolysis (monosaccharides and gluconic acid) a simplified mass balances around cellulose could therefore be made. In Figure 3, all these parameters are shown following the time course of scenario A. For these calculations it was assumed that the mentioned fermentation products could only originate from cellulose derived glucose. Besides the mentioned products, some glucose will be used by the yeast for cell growth. Despite yeast viability (CFU) was measured it was not possible to make a precise estimation of the amount of glucose that would be used for cell growth. This contribution was therefore omitted in the mass balance. On average the cellulose mass balance closed around 94% for all the strategies applied (Figs. 2 and 3). We therefore conclude that the mass balance describes the system rather well and that the most important products (except cell growth) have been accounted for.

Ethanol Yield at Low Dry Matter (20%)

Operating at high dry matter conditions results in higher final ethanol concentrations but have proven to result in lower conversion and ethanol yield compared to similar experiments performed at lower dry matter (Jørgensen et al., 2007). For comparison, the different scenarios were also tested at lower dry matter conditions (20% DM). It should be noticed that even 20% DM were considered high dry matter until recently, but reports on industrial processing of lignocellulosic materials consider conditions above 20% as most realistic (Larsen et al., 2012). Tables I and II shows the percentage of cellulose converted to ethanol for all scenarios at both dry matter conditions. As expected, lower yields were obtained when passing from low to high dry matter and this could be observed for both enzyme preparations. Around 40–50% less ethanol was produced in all strategies with CN enzyme. Instead, for CT2, the effect of increasing dry matter was very much depending on the strategy applied. For SHF the yield only decreased by 14% but for SSF by almost 50%. Remarkably, at 20% DM SSF conditions therefore resulted in higher ethanol yield than SHF (Fig. 4), which was opposite the trend observed at 30% DM conditions. The glucose and ethanol concentration measurements during all the experiments run at 20%DM are summarized in Supplementary Table III.

We speculate that the better SSF performance at 20% DM is due to faster liquefaction. For efficient mixing and distribution of enzymes and yeast the materials needs to be liquefied. At 20% DM this will happen rather quickly even at the lower fermentation temperature. At 30% DM the material stays very solid-like for a much longer period, especially if the temperature is 34°C. The water is mostly constrained in the fibrous structure, which negatively affects the water activity, and thereafter enzymes and yeasts performances.

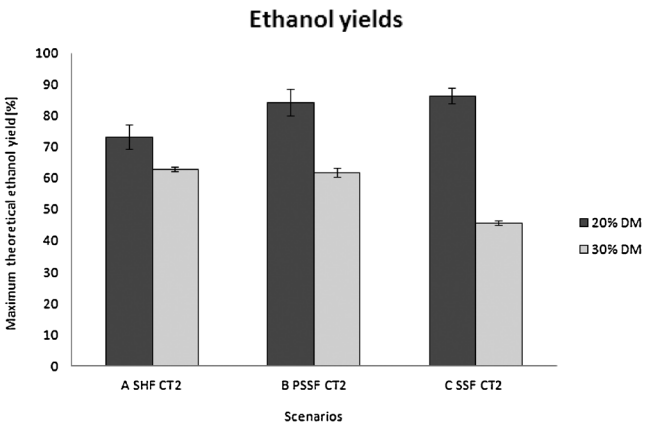

Figure 4. Comparison of final ethanol yield among scenarios A–C, at 20% and 30% dry matter.

Yeast Viability and Glycerol Production

Yeast growth detection is challenging during fermentation of lignocellulosic hydrolyzates. This is mainly due to presence of insoluble solids and soluble lignin derivatives that makes it impossible to use any colorimetric and UV absorption technique or even simple dry weight measurements. Instead the classic plating technique for measuring colony-forming units (CFU) was applied to monitor the yeast growth, moreover this technique will only account for living cell and not fully caputre the total cell mass increase during the time course of a fermentation. The cell population increased during the first 48 h, then kept constant for the next 2 days after which the yeast started reducing in number (Fig. 5). Almost no yeast growth was observed between 120 and 168 h. Among the three different strategies, yeasts growth was clearly more pronounced in SSF compared to the other strategies. As observed previously, pretreated wheat straw as substrate can sustain yeast growth without addition of nutrients (Jørgensen, 2009).

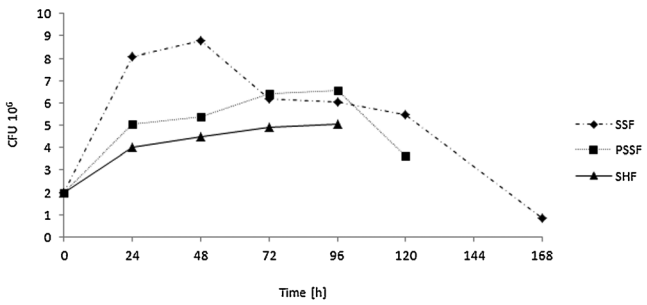

Figure 5. Cell viability in scenarios A–C, with CT2 at high enzyme loading, and 30% DM. Time 0 h represent the initial concentration of yeasts given by the inoculum.

The complete monitoring of secondary metabolites during fermentation is outside the scope of this work. However, the production of glycerol is a particular phenomenon of yeast metabolism as response to the osmotic stress given by the presence of high amount of soluble compounds (Maris et al., 2006). This becomes even more relevant during lignocellulosic ethanol fermentation, and at high dry matter conditions (Petersson and Liden, 2007). Figure 2 shows the fraction of cellulose converted into glycerol. There are no significant differences that seem to be related to the type of enzymatic cocktail applied. But, in the early phase of fermentation in SHF and PSSF the yeast was exposed to high concentrations of glucose and consequently experienced a high osmotic stress that drove the production of glycerol up to 6.8 and 5.6 g/kg, respectively. By applying an SSF strategy, where glucose never reached concentrations above 1–2 g/kg, the glycerol production was significantly lower at 3.5 g/kg. This trend was observed within all enzyme cocktails and dosage scenarios, confirming that the glycerol production was a response to osmotic stress caused by the presence of soluble molecules, mainly glucose. Especially at high dry matter conditions, high concentration of products (e.g., glucose above 100 g/kg in PSSF and SHF) is a key factor, and the osmotic stress will thus become a serious problem for yeasts when inoculated. As a response the yeast will start to produce glycerol to balance the osmotic gradient. To be noted, the highest amount of glycerol produced was observed when the highest concentration of glucose was present at time of inoculation (Fig. 6). This also corresponded with the lowest yeast growth, and generally the amount of glycerol was directly correlated with initial amount of glucose. On the contrary, the highest yeast growth was detected when glycerol production was lower (SSF strategies). After complete fermentation of glucose, the glycerol produced was not metabolized by the yeast.

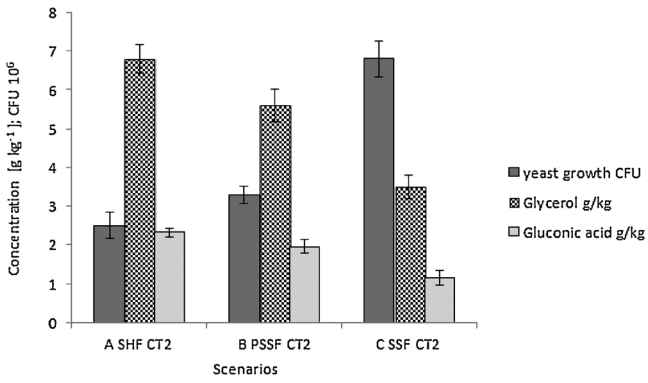

Figure 6. Final amount of gluconic acid, glycerol and yeast growth of scenarios A–C at 30%DM without PEG3000. Yeast growth represents the highest CFU-value during the process subtracted the CFU value in the inoculum.

Oxidized Sugars and Role of O_2

As previously found in our group, new cellulase cocktails contain carbohydrate oxidizing activities ascribable to the LPMO enzyme class (previously called GH61). It was also suggested that high dry matter conditions and mainly lignin has a boosting effect on LPMO's activity (Cannella et al., 2012). Results in Figure 2 reveal that a significant percentage of cellulose (up to 2.5% of released glucose) was converted into oxidized products (exclusively gluconic acid) using CT2, whereas none of this was observed using CN. As previously suggested, temperature and oxygen play a role in LPMO's activity during hydrolysis and fermentation (Cannella et al., 2012; Podkaminer et al., 2012). It is known that oxygen is a substrate in the oxidative reaction, and its removal leads to a strong inhibition of LPMO's (Phillips et al., 2011; Vaaje-Kolstad et al., 2010). In the initial phase of fermentation, yeast will use any available oxygen for respiration, but otherwise the fermentation will be anaerobic. In case of an SSF configuration, yeasts and enzymes are inoculated simultaneously, thus lead to a competition for oxygen between yeast and LPMO's. Theoretically, SSF conditions should therefore result in less oxidized carbohydrates being formed compared to the other two tested strategies. This was indeed confirmed in this study. In SSF, the amount of gluconic acid detected was lower than the other strategies where enzymes are introduced before yeast (Fig. 6). In SHF, the enzymes were let to hydrolyze the material for 72 h before inoculation with yeasts, and in such conditions 2.3 g/kg of gluconic acid was detected, whereas only 1.1 g/kg was produced in SSF. These concentrations correspond to 2.5% and 1.2% of total glucose released in the two scenarios. Likewise, these data might also partially explain the lower cell growth detected in SHF and PSSF compared to SSF (Fig. 5), given that LPMO's consume part of the oxygen during the hydrolysis phase, and then less oxygen is present at time of inoculation of the yeast. This would result in a shorter transition phase where the yeast can grow aerobically. Nevertheless, as already suggested above, the conspicuous amount of glucose at the onset of fermentation could also partially inhibit cell growth due to osmotic shock. As noticed in Figure 3, during fermentation yeast metabolize glucose to ethanol and glycerol, but not change in gluconic acid concentration was observed. Control experiments on defined medium also confirmed the inability of *S. cerevisiae* to metabolize gluconic acid. The action of LPMO's is therefore a delicate balance between on one site their ability to improve cellulose hydrolysis and on the other site the production of gluconic acid and consequently loss of potential ethanol.

From the results obtained, the anoxic environment generated during SSF is inhibitory for LPMO's, instead the conditions are more favorable during SHF and PSSF were the enzymes and yeast do not compete for dissolved oxygen. Thus a new model of enzymatic hydrolysis and fermentation of lignocellulose can be drawn. In presence of an electron donor, the oxygen rich environment favor LPMO's activity generating oxidative cuts on the lignocellulose surface, which represent new entry sites for exocellulases, ultimately increasing the overall amount of products released.

PEG Affects the Conversion Yield

In an attempt to improve the cellulose conversion, poly(-ethylene glycol) (PEG3000) at a dosage of 0.01 g/g DM was added. Previous works have shown that addition of PEG increase the enzymatic saccharification of lignocellulosic biomass (Börjesson et al., 2007; Kristensen et al., 2007; Li et al., 2012). The suggested mechanism behind the positive effect of PEG is the decrease in non-productive binding of enzymes to lignin present in the biomass. With the addition of PEG, the cellulose hydrolysis rate was 45% higher within the first 24 h with subsequent higher ethanol fermentation (45% more ethanol in PSSF containing PEG compared to the PSSF without PEG) (Fig. 7). In accordance with other reports, the addition of PEG resulted in a significant increase in ethanol production for all scenarios, and for both enzyme cocktails (Supplementary Table II). The tendency discussed above on which strategy gives better conversion with different cocktails was not affected by addition of PEG. To be remarked is the yields obtained with the SHF or PSSF strategy using CT2 in presence of PEG (strategies A and B). In this case an almost complete hydrolysis of cellulose was obtained (up to 89% cellulose to ethanol conversion was achieved), thereby resulting in almost 80 g/kg of ethanol in the fermentation broth. Particular importance then has to be attributed to a dedicated step for the enzymatic hydrolysis of the biomass at its optimal temperature of 50°C, and to the extent of it. Based on the higher enzymatic loading for the CT2 enzymes tested in this work, 24 h of presaccharification resulted in a better choice accounting for the cellulose hydrolysis yield and enzyme stability due to the detrimental effects of temperature. Shorter presaccharification length of 8 and 16 h resulted in lower final ethanol yield: 76.5% and 80.8%, respectively (data not shown). When using CT2 at low dosage (scenarios

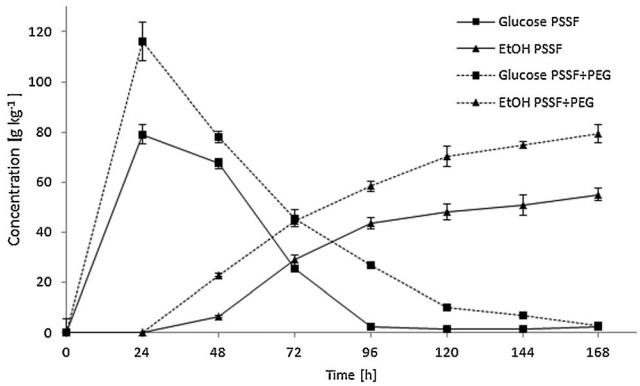

Figure 7. Concentration of glucose (squares) and ethanol (triangle) for scenario B at 30% DM comparing with PEG3000 (dashed lines) and without (solid lines). Time 24 h represent the yeast addition and temperature switch from 50 to 34°C.

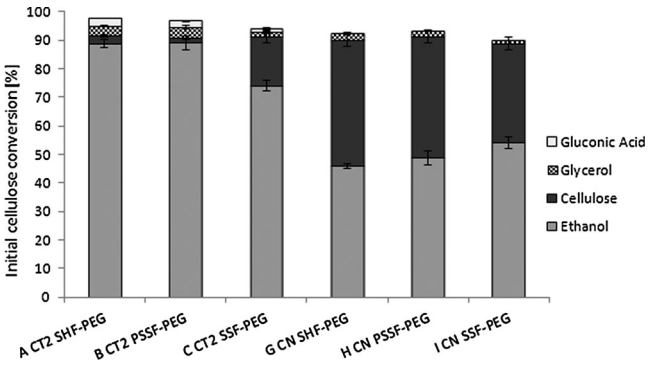

Figure 8. Final products yield as percentage of initial cellulose for scenarios A–C and G–I containing 1% of PEG3000. Scenarios A, B, and C represent SHF, PSSF, and SSF at 30% DM, respectively, applying CT2 at high enzyme loading. Scenarios G, H, and I represent SHF, PSSF, and SSF at 30% DM, respectively, applying CN at high enzyme loading.

D–F) in presence of PEG, yields close to the higher dosage without PEG (scenarios A–C) was achieved (Fig. 8), this indicates that a direct saving of 30% of enzymes when applying the polymer could be possible (Table I and Table II). An industrial application of PEG could be considered in order to replace part of the enzymes since these stands as major impact on cellulosic ethanol selling price (Macrelli et al., 2012), and maximize the cellulase activity since PEG seems to be beneficial to the enzyme stability for future recycling purposes (Chylenski et al., 2012). Nevertheless, the industrial applications will mostly depend on the selling price of PEG relative to the enzymes price.

Conclusions

The main achievement of this work is the observation that process strategies affect the overall ethanol yield and enzyme usage for production of bioethanol at very high solids loading. Moreover, the new advanced cellulolytic enzyme preparation has been found to alter the common perception of which production strategies to apply: at 30% DM, PSSF and SHF strategies performed significantly better than SSF, which is contrary to many previous studies conducted with ancestor enzymatic preparations. This change in the historical paradigm is due to incorporation of highly active β-glucosidases and the new class of oxidative enzymes LPMO's. The oxygen requirement of LPMO's adds a new degree of complexity to the application of these enzymes during production of cellulosic bioethanol. The absence of yeasts during the enzymatic hydrolysis was clearly beneficial for LPMO's activity, thus avoiding any competition for dissolved oxygen and generating higher amount of oxidative cuts on the cellulose surface as observed by increased formation of gluconic acid. The extras entry sites created by the oxidative activity seems to have a determining effect during a dedicated aerobic hydrolysis step, e.g. an "SHF-like" strategy, and is definitively of high importance to any high dry matter cellulose hydrolysis strategy. However, the drawback of excessive LPMO's activity is the significant amounts of gluconic acid, which cannot be metabolized by the yeast and in other words represents a loss in potential ethanol. Thus a delicate balance of the oxidative activity together with classic hydrolyzing enzymes is of importance for next generations of cellulolytic mixtures. Moreover, the application of PEG3000 surfactant (together with a low dosage of enzyme) could be used as a mean to replace part of the enzymes (in our case 30%) and still achieve same final yield at any strategy tested in this work. Lastly, other metabolites such as glycerol and cell mass were also depending on the selected strategy: SHF led to higher glycerol and lower cell mass, whereas the trend was inverted for SSF.

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