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Enzymatic Hydrolysis and Ethanol Fermentation of High Dry Matter Wet-Exploded Wheat Straw at Low Enzyme Loading

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Abstract Wheat straw was pretreated by wet explosion using three different oxidizing agents (H_2O_2, O_2, and air). The effect of the pretreatment was evaluated based on glucose and xylose liberated during enzymatic hydrolysis. The results showed that pretreatment with the use of O_2 as oxidizing agent was the most efficient in enhancing overall convertibility of the raw material to sugars and minimizing generation of furfural as a by-product. For scale-up of the process, high dry matter (DM) concentrations of 15–20% will be necessary. However, high DM hydrolysis and fermentation are limited by high viscosity of the material, higher inhibition of the enzymes, and fermenting microorganism. The wet-explosion pretreatment method enabled relatively high yields from both enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) to be obtained when performed on unwashed slurry with 14% DM and a low enzyme loading of 10 FPU/g cellulose in an industrial acceptable time frame of 96 h. Cellulose and hemicellulose conversion from enzymatic hydrolysis were 70 and 68%, respectively, and an overall ethanol yield from SSF was 68%.

Keywords Saccharomyces cerevisiae · Simultaneous saccharification and fermentation · Wet explosion · Pretreatment · Wheat straw · High dry matter · Ethanol

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Introduction

The demand for fuel ethanol has increased tremendously because of environmental, economic, and national energy security concerns. This has increased the necessity of using lignocellulosic wastes from agriculture and forestry as alternative raw materials in addition to corn, grain, and sugarcane, which are nowadays the primary feedstocks used for production of fuel ethanol. Wheat straw is the most abundant agricultural waste in Europe and the second largest agricultural residue in the world [1]. The global annual production of bioethanol from wheat straw has been estimated to 104 million m^3 [1].

The nature of lignocellulose, such as straw, makes it rather resistant to enzymatic hydrolysis, and pretreatment is, therefore, required to open the lignocellulose structure to facilitate further hydrolysis of the carbohydrates to monomeric sugars. To obtain high overall ethanol yields, the pretreatment should maximize enzymatic convertibility, minimize sugar decomposition or loss, not require addition of chemicals toxic to the enzymes or the fermenting microorganisms, be effective for treatment of materials at high DM content, and be scalable to industrial scale [2]. Several pretreatment technologies such as wet oxidation [3], steam explosion [4], dilute acid [5], and hydrothermal [6] have been used for pretreatment of wheat straw. Recently, a new pretreatment technology, wet explosion, has been developed at The Technical University of Denmark (DTU) [7]. The method is a combination of thermal hydrolysis, wet explosion, and wet oxidation, enabling (1) operating with high biomass concentrations up to 50%, (2) handling of big particle sizes, thereby, avoiding initial energy intensive mechanical milling, (3) an easy controllable process, (4) low total energy consumption, and (5) minor generation of inhibiting compounds [7]. Since 2006, the MaxiFuels pilot plant for production of ethanol from lignocelluloses has been operating in Denmark. The pilot plant tests wheat straw as the primary feedstock.

Most studies dealing with enzymatic hydrolysis or simultaneous saccharification and fermentation (SSF) at laboratory scale are performed at low substrate concentrations, usually concentrations below 10% dry matter (DM). However, to improve process economics, operating at higher substrate concentrations would be advantageous. This is because of increased final ethanol concentration, reduced reactor volume, lower operating cost, lower downstream processing cost, and reduced wastewater [8]. It has also been stated that ethanol concentration should be above 5% (*v*/*v*) in the fermentation broth for an economically viable lignocellulose-based ethanol process. For most types of lignocellulosic materials, this requires operating enzymatic hydrolysis or SSF processes at solid concentrations above 15% DM [9]. However, operating at initial substrate concentrations above 10% DM is limited by (1) inefficient mixing because of high initial viscosity of the material, (2) higher inhibitor concentrations that could reduce the performance of cellulases and hemicellulases, (3) higher concentrations of compounds inhibitory to fermenting organism, and (4) higher enzyme loadings needed for efficient hydrolysis within an industrial acceptable time frame [9]. Many studies have shown that washing the pretreated material results in improved enzymatic hydrolysis of the cellulose fraction and more efficient fermentation of glucose to ethanol because of removal of some of the inhibitors, both for enzymes and the microorganisms [8, 10, 11]. However, in large scale, including a washing step will not be advantageous because of the generation of a large waste stream. Thus, the whole slurry after pretreatment should be further hydrolyzed and fermented.

In lab-scale hydrolysis and fermentation tests, between 10 and 35 FPU/g cellulose is commonly used as the optimum enzyme loading, depending on both substrate concentration and digestibility of the material used. However, considering both the overall cost of the ethanol process and enzyme cost that could be up to 50% of SSF process [12], more realistic in the industrial process is enzyme concentrations in the range of 10–15 FPU/g cellulose.

The overall objective of the work presented in this paper was to investigate the efficiency of high solid enzymatic hydrolysis and SSF of wet-exploded wheat straw combined with the use of low-enzyme loading. The hydrolysis and fermentation tests were carried out with pretreated wheat straw at 14% DM, and an enzyme loading of 10 FPU/g cellulose. The effect of the use of different oxidizing agents (H_2O_2, O_2, and air) in the pretreatment is also presented as a way to improve further the effectiveness of the pretreatment.

Material and Methods

Wet-Explosion Pretreatment

Wet explosion pretreatment [7] was performed batch-wise by suspending 350 g wheat straw pellets in 1.8 l of deionized water in a 3.5-l high-pressure reactor with a paddle stirrer with a maximum stirring speed of 2,000 rpm. The reactor was equipped with an injection device for injection of H_2O_2 solution, pure oxygen, or air. The reactor was heated by water jacket, which was connected to a heat exchanger controlled by an oil heater. The highest temperature in the reactor was 190 °C. The temperature and pressure inside the reactor were monitored by two temperature sensors (one in the bottom and one in the head space) and one pressure sensor. After the pretreatment, the material was flashed into a 5-l container connected to the reactor.

In this study, the temperature for pretreatment was 180–185 °C based on the previous optimization trials. After reaching the desired temperature, the required amount of oxidant [i.e., O_2 (12–18 bars), H_2O_2 solution (35%, *v*/*v*), or normal air (12–18 bar)] was injected by over-pressure air supply, and the timing of the reaction was initiated. H_2O_2 solution was only injected once, whereas three or five times of injection were conducted, respectively, when O_2 or air were used as an oxidizing agent. The reaction time was in total 15 min, and the stirrer speed was 1,000 rpm for all pretreatments presented in the paper.

Straw pellets obtained from DONG Energy pellet plant in Køge, Denmark, were pretreated by wet explosion using different oxidizing agents, i.e., H_2O_2, O_2, or air. Composition of straw pellets expressed as percent of DM is given in Table 1, and it was determined by two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory (NREL) [13]. The pretreated wheat straw out of the reactor was with a DM of 14% and consisted of hemicellulose, cellulose, and lignin.

Enzymatic Hydrolysis

For evaluation of the effect of the different oxidants (H_2O_2, O_2, or air) used in the wet-explosion pretreatment on convertibility of the material, enzymatic hydrolysis was carried out for 72 h using the different pretreated wheat straw materials (i.e., pretreated with H_2O_2, O_2, or air). The experiment was performed in 57-ml vials with unwashed slurry (20 g) with

Table 1 Composition of straw pellets expressed as percent of DM.

Cellulose	Xylan	Klason lignin	Ash
41.8	25.3	23.4	5.6

5% DM and an enzyme loading of 20 FPU/g cellulose. The DM content was adjusted to 5% by addition of a corresponding volume of 50 mM sodium acetate buffer (pH=4.8) to undiluted pretreated wheat straw (14% DM). The experiment was done in triplicate.

Enzymatic hydrolysis with the undiluted pretreated wheat straw slurry (14% DM, pretreated with O_2) was performed in duplicate in 1-l bottles containing 400 g material with pH 4.8 and an enzyme loading of 10 FPU/g cellulose. The pH of the material was increased to 4.8 using 10 M NaOH. The pH was measured at each sampling and, if necessary, adjusted to 4.8 by addition of 2 M NaOH.

The hydrolysis tests were performed at 50 °C in a rotary shaker at 200 rpm, and samples were taken regularly for sugar analysis. In all hydrolysis experiments including the SSF, enzymes consisted of a mixture of Celluclast 1.5 l and Novozym188 (both provided by Novozymes A/S, Bagsvaerd, Denmark) in a volume ratio of 3:1.

Simultaneous Saccharification and Fermentation

The SSF experiment was performed with unwashed and undiluted slurry of pretreated wheat straw (14% DM, pretreated with O_2) in a 2-l reactor [14] with a total working volume of 1.2 l and stirring at 250 rpm under no-aseptic conditions for 4 days. Before the material was added to the reactor, pH was adjusted to 5 using 10 M NaOH. Liquification of the material was performed at 50 °C for 24 h at an enzyme loading of 10 FPU/g cellulose. After liquification, the temperature was decreased to 35 °C, trace metals and vitamins were added, and the reactor was inoculated with 2 g/l dry yeast *Saccharomyces cerevisiae* (Ethanol Red^{2122}, Fermentis). The SSF continued for another 3 days. The pH was maintained at 5 by addition of 2 M NaOH. Samples were taken for sugar and ethanol analysis. The experiment was repeated once. The composition of the specific yeast addition was in milligrams per liter as follows: *Vitamins* Biotion, 0.050; Ca–pantothenate, 1.0; myo-inositol, 25.0; thiamin HCl, 1.0; pyridoxine HCl 1.0; para-aminobenzoic acid 2.0; trace elements ethylenediaminetetraacetic acid (EDTA) 15.0; $ZnSO_4.7H_2O$, 4.5; $MnCl_2.2H_2O$, 1.0; $CoCl_2.6H_2O$, 0.3; $CuSO_4.5H_2O$, 0.3; $Na_2MoO_4.2H_2O$, 0.4; $CaCl_2.2H_2O$, 4.5; $FeSO_4.7H_2O$, 3.0; H_3BO_3, 1.0; KI, 0.1.

Analysis

Glucose, xylose, cellobiose, ethanol furfural, and hydroxymethylfurfural (HMF) were analyzed using a high-performance liquid chromatography (HPLC) refractive index (RI) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) at 60 °C with 4 mM H_2SO_4 as eluent with a flow rate of 0.6 ml/min. Before HPLC analysis, samples were centrifuged at 10,000 rpm for 10 min, followed by filtration through a 0.45-μm membrane filter. DM content was determined according to standard methods [15]. The composition of pretreated wheat straw, e.g, cellulose, xylan, Klason lignin, and ashes was determined by a two-step acid hydrolysis according to the procedure published by the NREL [13].

Results and Discussion

Comparison of Pretreated Materials

The effect of different oxidizing agents (H_2O_2, O_2, or air) on the pretreatment of wheat straw pellets by wet explosion was evaluated. For comparison reasons, the pretreatment conditions used were the same for all oxidizing agents. These conditions were the optimum pretreatment conditions determined in a previous wet-explosion study employing H_2O_2 as oxidizing agent (unpublished data), which is the strongest of the tested oxidants. The evaluation was based on glucose and xylose liberated per 100 g raw material during enzymatic hydrolysis (Fig. 1). This approach takes into account solid recovery values in the pretreatment step and, thus, allows simple evaluation of the overall process efficiency (i.e., amount of potential sugar from the raw material) [4].

The use of H_2O_2 and O_2 as oxidizing agents resulted in rather similar glucose yields of 31–32 g per 100 g raw material after hydrolysis (Fig. 1), which corresponds to a glucose yield of 69% based on the glucose content in the raw material. These yields were on average 25% higher than that obtained from the material pretreated with air. However, material pretreated with H_2O_2 gave the lowest xylose yield of 16 g/100 g raw material (corresponding to a yield of 55% based on xylose content in the raw material), which was 22% lower than in the case of using oxygen as oxidant. On the other hand, material pretreated with H_2O_2 gave the highest amount of xylose and furfural present in the liquid fraction after pretreatment (Fig. 2). This combined with the fact that overall xylose yield was lowest after pretreatment with H_2O_2 indicated that more xylan was solubilized and hydrolyzed but that an increased fraction was further degraded to furfural or other degradation products. The low glucose and xylose yields obtained in the material pretreated with air were probably caused by low accessibility of the slurry to the enzymes. HMF was not detected during the HPLC analysis even for undiluted pretreated materials (data not shown).

Comparison of the total yields of the different pretreated materials combined with the generation of furfural in the liquid fraction shows that the pretreatment with O_2 was the most efficient in enhancing overall convertibility of the raw material to sugars. The total

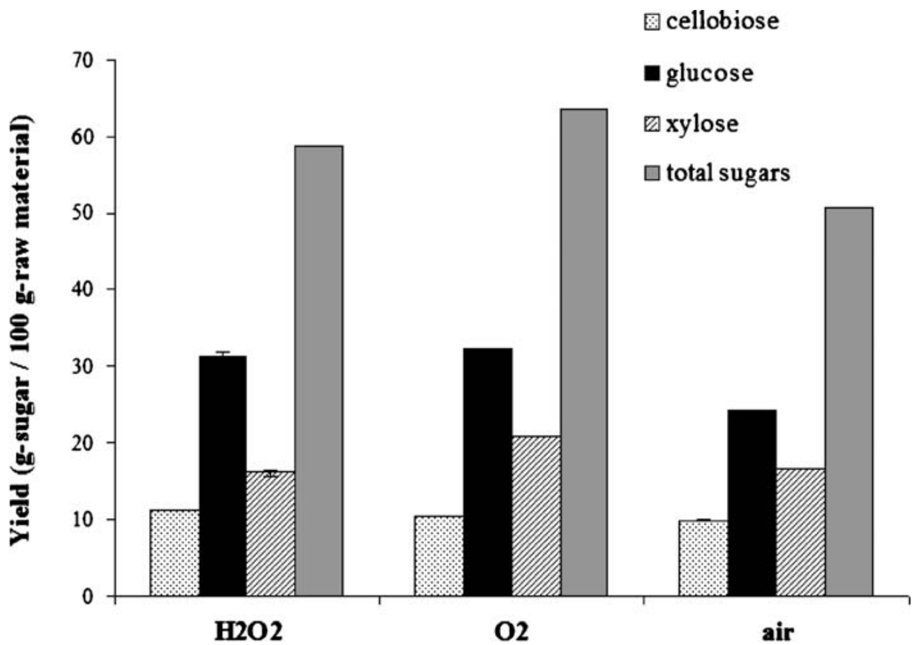

Fig. 1 Glucose, xylose, and cellobiose yields after 72 h enzymatic hydrolysis of pretreated wheat straw by wet explosion using different oxidizing agents (H_2O_2, O_2, and air). The yields are given as sugar liberated during the hydrolysis per 100 g raw wheat straw. Results are average of triplicates

overall yield of glucose, xylose, and cellobiose of this pretreatment was 63 g/g raw material, corresponding to 93% cellulose and 72% hemicellulose conversion based on the glucose and xylose content in the raw material. High recovery yield of the sugars, no production of HMF, and rather low production of furfural (Figs. 1 and 2) show that wet-explosion pretreatment using O_2 as oxidizing agent is an efficient method for fractionation of wheat straw. Moreover, replacement of H_2O_2 with oxygen could also be beneficial by reducing the cost of the pretreatment step. Based on the results, material pretreated with O_2 was used for further hydrolysis and SSF tests.

Enzymatic Hydrolysis

In this study, both enzymatic hydrolysis and SSF were investigated, because we have previously shown that the thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 could effectively co-ferment glucose and xylose present in undetoxified wet-exploded wheat straw hydrolysate [16]. However, this organism can only be used as an ethanol producer if the process is carried out as separate hydrolysis and fermentation (SHF) because of differences in both temperature and pH optimum between this microorganism and the enzymes. Another possibility is to perform, first, SSF using *S. cerevisiae* followed by xylose fermentation by *Thermoanaerobacter* BG1L1. It is, therefore, important to know which of the two scenarios (SHF or SSF) would be the most efficient in converting the pretreated material into fermentable sugars.

To evaluate the material after wet explosion with O_2 under realistic conditions, both enzymatic hydrolysis and SSF were performed on unwashed material at high DM (14% DM) and with low enzyme loading of 10 FPU/g cellulose. Cellulose and hemicellulose conversion of 60 and 68%, respectively, were obtained in 96 h (Fig. 3). The initial

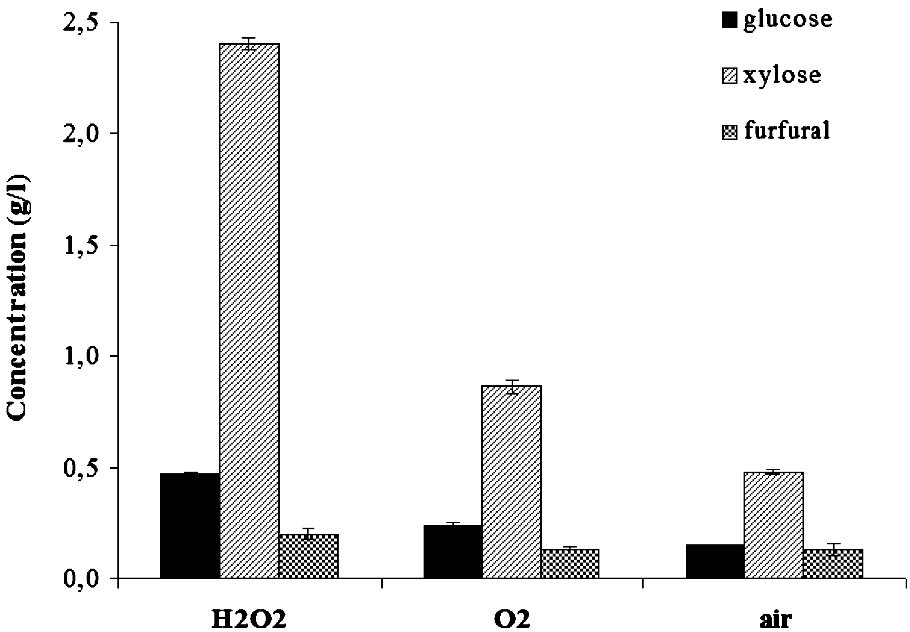

Fig. 2 Glucose, xylose, and furfural present in the liquid fraction in wet-exploded wheat straw (5% DM) pretreated with the use of H_2O_2, O_2, or air as oxidizing agents. Results are average of triplicates

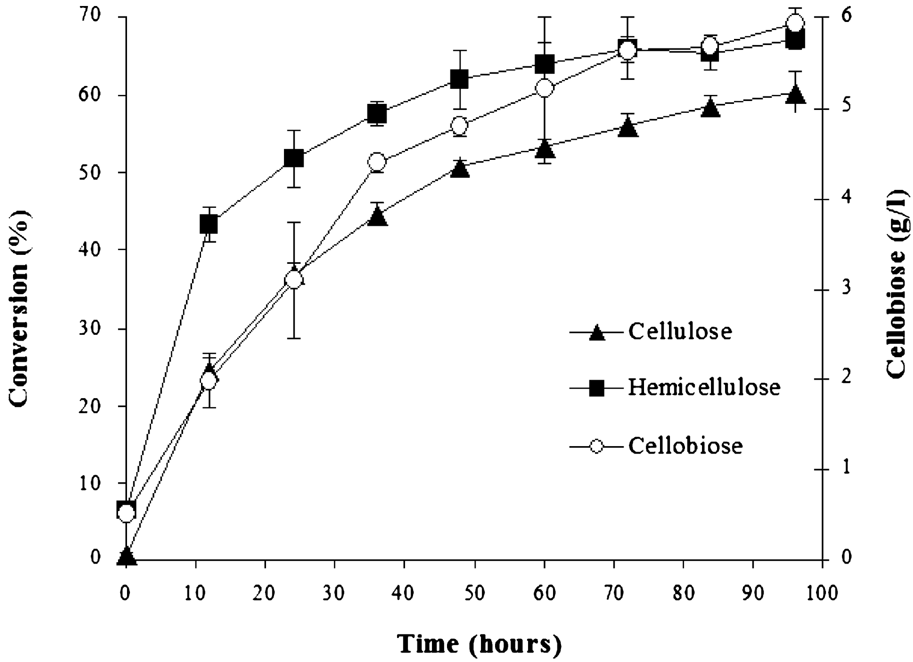

Fig. 3 Time course of cellulose and hemicellulose conversion and cellobiose production during enzymatic hydrolysis of wet-exploded wheat straw at 14% DM and an enzyme loading of 10 FPU/g cellulose. Degree of cellulose and hemicellulose conversion was calculated as amount of glucose and xylose, respectively, released during the hydrolysis relative to the maximum theoretical. Results are average of duplicates

hemicellulose hydrolysis rate appeared to be faster than the cellulose hydrolysis rate, which was probably caused by the partial solubilization of hemicelluloses during the pretreatment. These oligomers were fast hydrolyzed to xylose by the enzymes. Cellulose was hydrolyzed more slowly and a significant accumulation of cellobiose was also observed (Fig. 3). This relative large amount of cellobiose could result in pronounced inhibition of the cellulases. If the production cellobiose was taken into account, the cellulose conversion would increase by 17%, thereby, resulting in a final cellulose conversion of 70% in 96 h.

Reduced cellulose conversion in the range of 10–20% with an increase in substrate concentration from 2 to 10% DM has been reported for various substrates such as pretreated wheat straw [9] and softwood [17, 18], as well as for pure cellulose [19]. To our knowledge, only one other study has investigated enzymatic hydrolysis of pretreated wheat straw at solid concentrations higher than 10% DM [9]. In that study, cellulose conversion of approximately 65% was obtained with 12 FPU/g cellulose, which is comparable to the cellulose conversion reported herein. However, the hemicellulose conversion was 10% lower compared to that obtained in our study. This difference is most likely because of the removal of some of the easy degradable hemicelluloses in the washing step in the pretreatment in that study, whereas all hemicelluloses remain in the pretreated material used in this study. The reduced efficiency of enzymatic hydrolysis of cellulose with increased substrate concentration is primary caused by increased product inhibition (glucose) of cellulases. In this study, a significant accumulation of cellobiose was observed. Furthermore, some inhibition of the cellulases by hemicellulose-derived sugars [20] and degradation compounds formed during the pretreatment [21] have been also reported.

Simultaneous Saccharification and Fermentation

SSF is considered the most promising process for converting cellulose into ethanol. This technology enables immediate conversion of released glucose into ethanol, thus, preventing glucose accumulation in the fermentation broth and, therefore, no product (glucose) inhibition. The potential of performing the process as an SSF process was studied using *S. cerevisiae*. This fermenting organism was chosen, as it is the most efficient organism for fermentation of glucose and proven to be rather robust to the toxicity of various pretreated materials or hydrolysates [9, 22–24]. The pretreated wheat straw was liquefied for 24 h to decrease the viscosity of the material and, thus, to avoid mass transfer limitations. Glucose was consumed rapidly, and already after 10 h of SSF, the glucose liberated during the liquification step was utilized (Fig. 4). The ethanol concentration reached 12.3 g/l, corresponding to an ethanol productivity of 1.23 g/l per h (Fig. 4). These results indicate that unwashed wet-exploded wheat straw with high DM content of 14% is not toxic to the yeast and, thus, could be effectively fermented. High fermentability of the slurry at high DM is important, as detoxification or washing of the material is not needed.

After the initial fast fermentation in the first 10 h, a phase with minor increase in ethanol concentration was seen, indicating that glucose was still being released from the cellulose fraction, although at a very slow rate. The ethanol concentration reached 22.2 g/l after 72 h of SSF. Assuming a theoretical ethanol yield of 0.51 g ethanol/g consumed glucose, the cellulose conversion could be estimated to at least 68%. This result shows that, under the condition tested, the amount of cellulose hydrolyzed in the SSF was increased by at least 14% compared to that obtained in enzymatic hydrolysis (60%). It is also notable that, in the SSF, the cellobiose concentration was constantly low, thereby, avoiding the inhibition of cellulases by cellobiose. Although, that unwashed and high solids pretreated material was used for SSF process, operating the SSF in fed-batch [24], using a shorter liquification step [25] or minor increase in enzyme loading could enhance cellulose and hemicellulose conversion, thus, resulting in shorter process time and/or higher ethanol yield.

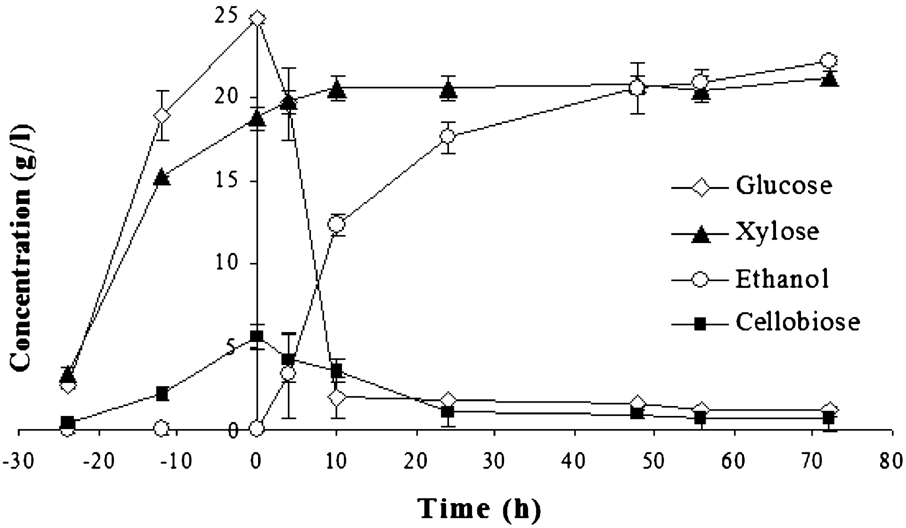

Fig. 4 Time course of ethanol, glucose, xylose and cellobiose concentrations during SSF of wet-exploded wheat straw at 14% DM and enzyme loading of 10 FPU/g-cellulose. Results are average of duplicates

Previous studies investigating the SSF of pretreated wheat straw have mostly been conducted with washed material, substrate concentrations less than 10% DM, and higher enzyme loading [6, 11, 26, 27]. Nevertheless, the overall ethanol yields obtained in those studies (60–70%) are in the same range as the results reported herein. In an SSF study with wheat straw with rather similar substrate concentration (16% DM), a cellulose conversion of 70% was obtained [8]. However, this yield was obtained with washed pretreated material, which could be due to the potential removal of inhibitors for both enzymes and the yeast.

Xylose fermentation was not tested, but with our thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 (16), ethanol production could theoretically be increased by more than 39% by including the xylose. Based on the results from the current study, 285 l ethanol per ton DM could be possible from unwashed or undetoxified wet-exploded wheat straw. Pilot scale studies are now carried out at the Maxifuels pilot plant (DTU, Denmark) to optimize the pretreatment method at higher DM up to 30%.

Conclusions

The pretreatment of wheat straw pellets by wet explosion using three different oxidizing agents (H_2O_2, O_2, and air) under the same conditions showed that the pretreatment with O_2 was the most efficient in enhancing overall convertibility of the raw material to sugars and minimizing generation of furfural as a by-product. The wet-explosion pretreatment with O_2 as oxidizing agent enabled relatively high yields from both enzymatic hydrolysis and SSF to be obtained even on unwashed slurry with 14% DM and a low enzyme loading of 10 FPU/g cellulose in an industrial acceptable time frame of 96 h. From the enzymatic hydrolysis experiment, cellulose and hemicellulose conversion of 70 and 68%, respectively, was achieved, and the overall ethanol yield from SSF was 68%. Overall, the experimental data from the enzymatic hydrolysis and SSF reported in this paper seem rather encouraging in view of the feasibility of the wet-exploded pretreatment method as an efficient method for pretreatment of wheat straw.

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