Wet oxidation pretreatment of rape straw for ethanol production

Efthalia Arvaniti*, Anne Belinda Bjerre1, Jens Ejbye Schmidt
Risø DTU Biosystems, P.O. Box 49, DK-4000 Roskilde, Denmark

Article info
Article history:
Received 3 October 2010
Received in revised form 13 November 2011
Accepted 21 December 2011
Available online xxx

Keywords:
Wet oxidation
Ethanol
Rape straw
Enzymatic hydrolysis

Abstract
Rape straw can be used for production of second generation bioethanol. In this paper we optimized the pretreatment of rape straw for this purpose using Wet oxidation (WO). The effect of reaction temperature, reaction time, and oxygen gas pressure was investigated for maximum ethanol yield via Simultaneous Saccharification and Fermentation (SSF). To reduce the water use and increase the energy efficiency in WO pretreatment features like recycling liquid (filtrate), presoaking of rape straw in water or recycled filtrate before WO, skip washing pretreated solids (filter cake) after WO, or use of whole slurry (Filter cake + filtrate) in SSF were also tested. Except ethanol yields, pretreatment methods were evaluated based on achieved glucose yields, amount of water used, recovery of cellulose, hemicellulose, and lignin.

The highest ethanol yield obtained was 67% after fermenting the whole slurry produced by WO at 205°C for 3 min with 12 bar of oxygen gas pressure and featured with presoaking in water. At these conditions after pre-treatment, cellulose and hemicellulose was recovered quantitatively (100%) together with 86% of the lignin. WO treatments of 2–3 min at 205–210°C with 12 bar of oxygen gas produced higher ethanol yields and cellulose, hemicelluloses, and lignin recoveries, than 15 min WO treatment at 195°C. Also, recycling filtrate and use of higher oxygen gas pressure reduced recovery of materials. The use of filtrate could be inhibitory for the yeast, but also reduced lactic acid formation in SSF.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction
Exploitation of sugars of lignocellulosic biomass resources for sustainable production of feed, energy, and fibers bespeak overcoming recalcitrance of biomass to fermenting yeasts [1]. For this purpose two strategies have been developed in the last decades. First, a pretreatment step was introduced that destroyed the coherence of biomass and improved its digestibility [2]. Secondly, hydrolytic enzymes (cellulases, hemicellulases) were introduced to hydrolyze carbohydrates of pretreated biomass to fermentable monosaccharides [3]. To support environmental sustainability of ethanol production, priority is given to use of agricultural and forest residues [4]. Rape straw is an abundant agricultural residue that has been proposed recently as feedstock for ethanol production [5]. Rape straw consists mainly of cellulose, hemicellulose, and lignin polymers, but also from other minor compounds like waxes, ashes, organic acids etc. The complete composition was been published earlier [6]. Cellulose is a highly crystalline mono-polymer of glucose, tightly packed in microfibrils, and surrounded by hemicellulose that is acetylated heteropolymers of C5 sugars (xylose, arabinose)
and C6 sugars (mannose, glucose, and galactose), and lignin that is a 3D network of phenyl-propane units [7]. The enzymes needed for complete saccharification of cellulose to glucose are endoglucanases, exoglucanases and β-glucosidases that work synergistically. The enzyme digestibility of cellulose is enhanced by loosen up the structure of cellulose and removing part of the lignin and hemicellulose coatings [1,8], to allow cellulolytic enzymes to access cellulose. Such a complicated task is carried out by pretreatment.

Numerous thermo-chemical pretreatment technologies have been developed in the last decades. Examples of pretreatment methods tested on rape straw are dilute acid [9], phosphoric acid and acetone [9], hydrothermal treatment at neutral [6], acidic [10], or alkaline pH [6], wet explosion [11], ozone [6], and wet oxidation [5]. So far, optimal pretreatment method rape straw has not been assigned. Qualifications of a pretreatment method sought for ethanol production are: maximum recovery of sugars, complete and fast digestibility of pretreated rape straw by enzymes and yeasts (high product yields and productivities), and low economy (energy, water, chemicals) [8]. Lack of information in pretreatment mechanisms is underscored by use of empirical formulas like severity factors [12]. Many times the processing cost is the limiting factor in pretreatment selection, because pretreatment methods are many times energy and chemical demanding [13]. Moreover, the severity of the process destroys sugars, and forms degradation compounds inhibitory for enzymes and yeasts [14].

Wet oxidation (WO) is an aqueous high temperature high pressure pretreatment method, that uses oxidative agents. The mechanism lies on formed hydroxyl radicals, and auto-catalyzing by formed organic acids [15]. It has been tested on various feedstocks, including a preliminarily study with rape straw [5]. However, the pretreatment conditions applied on rape straw were only estimated based on optimal results on wheat straw [16]. Given that pretreatment methods are tailor-made for a given biomass [8], optimization for rape straw is required.

Due to the previous promising preliminary results of WO pretreatment on rape straw [5], we wanted to study this combination in more detail, with oxygen gas as sole chemical. The efficiency of the WO process was measured mainly by the ethanol yields, but also by the glucose yields, the amount of water used, the recovery of cellulose, and the recovery of hemicellulose and lignin. The examined pretreatment parameters were reaction temperature, oxygen pressure, reaction time, and also featured configurations like recycling filtrate, presoaking of rape straw before WO in water or filtrate, skip rinsing of pretreated rape straw after WO, and using whole slurry for ethanol production.

2. Materials and methods

2.1. Raw material

Oilseed rape straw (Brassica Napus, variety Carakas) was collected from fields of Hornsherred near Lyngby, Denmark in August 2007, air-dried to 90–95% humidity and stored at room temperature. Before use, the straw was reduced in 2 mm particle size by knife mill.

2.2. Pretreatment

Pretreatment experiments were carried out in a 2 L loop reactor with recirculation and stirring [17]. 60 g of dried milled rape straw were suspended in 1 L of demineralized water (6% DM). The overhead chamber of 1 L was either air at ambient pressure for hydrothermal treatment (control: experiment A), or impregnated with 12 bar of oxygen gas for Wet oxidation (WO; Experiment B).

Hydrothermal pretreatment (A) was the control of the wet oxidation (WO) pretreatment (B). After pretreatments A and B, fibers were separated from liquid via vacuum filtration with 0.1 mm mesh at 30 C, and filter cake was rinsed very rapidly with 1 L tap water. After separation, both filter cake and filtrate were stored in freezer (−20 °C) before further use.

In total, 15 pretreatment strategies were investigated as presented in Table 1. Experiments of same capital letter had same reaction time and temperature (experiments A to E); while subscript characterized other parameters tested like use of oxygen at lower pressure (BlowP), recycling filtrate for WO at two dilutions (Brecyc50, or Brecyc90), presoaking of fibers before WO (Bpresoak/recyc50), or changing rinsing strategy of filter cake (Bno-wash), or use of whole slurry downstream (Bno-wash/slurry), as well as combinations of the above parameters (e.g. BlowP/presoak/recyc50).

2.3. Liquefaction and simultaneous saccharification and fermentation

Simultaneous Saccharification and Fermentation (SSF) was preceded by a pre-hydrolysis (liquefaction) step [18]. For liquefaction, moist filter cake of pretreated rape straw (17% DM) was suspended in water and mixed with 15 FPU/g DM Cellubrix (Novozymes, Bagsvaerd Denmark). at 12.5% DM and at pH 4.8 in duplicates. In experiments Bpresoak/no-wash/slurry and Dpresoak/no-wash/slurry fibers were suspended in filtrate instead of water. The filters were sealed and shaken at 50 °C and 120 rpm for 24 h. For SSF, 20 FPU/g DM of Cellubrix L were added, together with 60 ppm urea, and 2.5 g/l dry baker’s yeast (Malteserkors Gær, Denmark). The bottles were flushed with nitrogen gas, sealed with air-tight locks and shaken at 32 °C and 100 rpm. SSF with use of whole slurry (cake – filtrate) lasted for 333 h, and SSF with use of water (no filtrate) lasted for 162 h. Ethanol production was monitored via the CO2 weight-loss method, and bottles were weighed every 0, 2, 5, 21, 25, and 29 h. After the end of SSF, spent fibers were separated from beer through decanting, were dried at 105 °C for 24 h, and milled for further analysis. The ethanol-rich beer was centrifuged at 4000 rpm for 5 min and stored at −18 °C for further analysis.

2.4. Analytical methods

2.4.1. Analysis of composition of raw material, filter cake and spent fibers

The composition of the raw material (app. 94% DM) was analyzed for lipophilic extraction: 5 g of dried milled raw
material were boiled in Soxhlet apparatus with 1000 ml ethanol for 24 h. After drying the fibers at 60 °C overnight and then for 1 h at 105 °C, the weight difference of the fibers gave the total lipophilic extractives. Raw material, filter cake from pretreatment, and spent fibers after SSF were first dried overnight at 105 °C and knife-milled to 0.5 mm particle size, and then analyzed via strong acid hydrolysis for total sugars and Klason lignin content [5].

2.4.2. Analysis of composition of pretreated filtrate and beer
The filtrate collected after pretreatment was analyzed: for total soluble sugars via weak acid hydrolysis method [19]; for free sugars (glucose, xylose, arabinose); for organic acids (formic acid, acetic acid, lactic acid, succinic acid, glycolic acid); and for furans (2-furfuraldehyde, 5-hydroxy-methyl-furfuraldehyde, and 2-furoic acid) in HPLC (see below). Moreover, filtrate was analyzed on total phenolics through Prussian blue method. The beer after SSF was analyzed for free sugars, and ethanol in HPLC.

2.4.3. Enzyme assay of pretreated rape straw
The cellulase activity of Cellubrix L measured as volumetric Filter-paper activity (FPA) was 89 FPU/ml, and volumetric activity of β-glucosidase (βG) was 28 IU/ml. FPA was measured by Gose method [20], and βG activity by Berghem method [21].

For testing enzyme digestibility of pretreated rape straw moist filter cake was suspended at 2% DM in demineralized water, and mixed with 30 FPU/g DM Cellubrix L in a total working volume of 8 ml in triplicates. Finally, in enzyme assays of experiments Bno-wash/slurry, B presoak/no-wash/slurry and D presoak/no-wash/slurry, fibers were suspended in filtrate instead of water, and filtrate corresponded to 80%v/v of the solution. The vials were sealed and shaken rotary at 50 °C for 24 h together with enzyme blank. After the end of the assay, supernatants were measured for free sugars (glucose, xylose, and arabinose) in HPLC.

2.4.4. HPLC analysis
A Shimadzu Corp HPLC (Kyoto, Japan) system equipped with BioRad HPX-87H column (Amminex) at 63 °C, using 4 mM H2SO4 as eluent at 0.6 ml/min flow rate for detecting the ORGANIC ACIDSs, furans, ethanol, and sugars. The detector for furans was a Diode array SPD-M10AVP (Shimadzu Corp, Kyoto, Japan) and for the other compounds was a RID-10A RI-detector (Shimadzu Corp, Kyoto, Japan).

2.5. Calculations
Cellulose recovery (glucose) and hemicellulose recovery (xylose, arabinose) after pretreatment were calculated as a percentage of the raw material sugars added in the pretreatment reactor. The recoveries were calculated for both soluble and insoluble fractions (cake, filtrate):

\[\text{Recovery} = \frac{g \text{ sugar in pretreated material}}{g \text{ sugar in untreated material}} \times 100\%\]

where \(C_{\text{sugar}}\) (g sugar/g pretreated solids), is the concentration of glucose, xylose and arabinose; \(g_{\text{r}}\) (g Pretreated solids/g untreated material) is the recovery of insoluble solids after pretreatment; \(C_{\text{RH}}\) (g Carbohydrate/g Raw material) is the concentration of carbohydrates (e.g. cellulose, hemicellulose) in the raw material; and \(H_f\) is the Hydration factor of each sugar (g sugar/g Carbohydrate); for glucose \(H_f\) is 1.1, and for xylose and arabinose \(H_f\) is 1.136.

Glucose yield% in enzyme assays was calculated in a percentage basis of the glucose potential of added pretreated biomass (100%):

\[Y_{\text{gl}} = \frac{g \text{ released glucose after enzyme hydrolysis}}{g \text{ glucose potential of added in enzyme hydrolysis}} \times 100\%\]

where \(Y_{\text{gl}}\) is the released glucose in enzyme hydrolysis experiments (g/l), \(C_{\text{gl}}\) is the concentration of cellulose added in the enzyme assays (g/l), and \(H_f\) is the hydration factor of glucose (g glucose/g cellulose) equal to 1.1.

Since the same principle, cellulbiose yield% and xylose yield% was calculated, as the percentage of the released cellulbiose and xylose after enzyme hydrolysis of the cellulbiose and xylose potential of cellulose and hemicellulose respectively (100%). In these two cases, \(H_f\) is 1.05, and 1.136 respectively.

Ethanol yield after SSF was calculated in a percentage basis on the percentage of the theoretical ethanol potential of cellulose of raw material:

\[Y_{\text{EtOH}} = \frac{\text{g ethanol produced}}{\text{g theoretical ethanol potential of added cellulose in SSF}} \times 100\%\]

where \(Y_{\text{EtOH}}\) measured (g/l), \(Y_s\) is the yield of solid biomass from pretreatment step (g Total Solids/g Raw material), \(C_s\) is the DM of biomass in SSF (g Total Solids/l), \(C_{\text{cel}}\) is the cellulose content of raw material (g cellulose/g RM), \(H_f\) is the hydration factor of glucose (g glucose/g cellulose) which is 1.1, and \(Y_{\text{EtOH}}\) is the theoretical ethanol yield of glucose(g ethanol/g glucose) 0.51.

Progress of ethanol fermentation is calculated from equivalents of CO2 vent during fermentation or SSF. Equivalent for each gram of CO2 vented is 1045. From this amount of ethanol, ethanol yields are calculated as described above.

Intensity of pretreatment method was assessed by the empirical Severity factor \(R_0\) (Eq. (4)), that translates pretreatment temperature and reaction time into impact factors on deconstruction and alteration of lignocellulosic biomass [22]:

\[R_0 = \exp \left( \frac{T - 100}{14.75} \right) \times t\]

where \(T\) is reaction temperature and \(t\) is reaction time.
Standard deviation was used for analyzing the dispersion of replicate experiments, and stems all graphic illustrations in figures. The standard deviation was calculated using STDEVA formula of Excel software.

One-way analysis of variance (ANOVA) analyzed data for 5% and 10% significance level by grouping pretreatment parameters (treatments) at different conditions (levels). In the case that group contained more than two levels, and results were significantly different, Newman and Keuls post-hoc statistical analysis tool was applied for 5% or 10% significance level, to identify in which levels of that treatment were significantly different. Newman and Keuls was calculated with DSAATSTAT macro v. 1.101 (Perugia, Italy) in Excel software.

Correlation coefficient (COCO) was applied between two arrays of variables. Here is used to relate either a dependent to an independent variable, or two dependent variables, using a model from Excel software Microsoft office 2007.

3. Results and discussion

Following experiments are categorized on oxygen gas pressure, recycling of filtrate, presoaking of rape straw before WO, combinational change of temperature and reaction time, skip of rinsing, use of filtrate for ethanol production (see Table 1).

3.1. The effect of oxygen

To evaluate the effect of oxygen gas pressure on the pretreatment and the digestibility of fibers the following experimental setup was used. Low and high oxygen pressure of gas in WO were compared in experimental pairs of BlowP (8 bars) and B (12 bars, see Table 1), and BlowP/presoak/recyc50 (8 bars) and B/presoak/recyc50 (12 bars). Moreover, experiment B that is the base case of WO was compared to experiment A that is hydrothermal treatment, and to Control experiment that received no treatment.

The results of the recoveries of cellulose and lignin were significantly different for BowP and B, but not for hemi-cellulose. Cellulose recovery increased from 96% to 99% by decreasing pressure from 12 bar to 8 bar (in B to BlowP respectively, see Fig. 1A), lignin recovery increased from 58% to 68% (Fig. 1C), whereas hemi-cellulose recovery ranged 71%–77% (Fig. 1B). For the pair Bpresporecyc50 and BowP/presporecyc50 that combined recycling of the filtrate and presoaking of rape straw, cellulose and lignin recovery again significantly increased from 84% to 101 for cellulose by lowering oxygen pressure from 12 bar to 8 bar (in Bpresporecyc50 to BlowP/presporecyc50 respectively), and from 77% to 92% for lignin respectively. Hydrothermal treatment (exp. A) had comparable recoveries with WO (exp. B) with cellulose at 96% and hemi-cellulose at 89%. However, the recovery of lignin was much higher (95%) than B (see Fig. 1C).

Digestibility of cellulose of pretreated rape straw is shown in Fig. 2. The yields for increasing oxygen pressure significantly increased from 45% to 51% from B to BlowP, and decreased from 65% to 56% for Bpresporecyc50 and BowP/presporecyc50 respectively. Glucose yield of hydrothermal treatment was as low as 48%, and without rape straw cellulose without pretreatment was hydrolyzed only by 16%. In the enzymes assays except glucose (Fig. 2), also cellobiose was detected, which is a dimmer of glucose and main substrate of βG. Cellobiose accounted for 17–30% of yield and 1.6-4.0 g/l in enzyme assays in B, BlowP, Bpresporecyc50, and BowP/presporecyc50. However, in the same ranges was observed for the total of experiments. Cellulbiose accumulation is more likely caused by inhibition of βG. Back in 2004 Varga et al. (2004) had reported that glucose yield of WO

<table>
<thead>
<tr>
<th>Table 1 – Nomenclature of experiments, and description of the conditions and featured configurations of the applied pretreatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment label</strong></td>
</tr>
<tr>
<td>No pretreatment</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>BlowP</td>
</tr>
<tr>
<td>Bpresporecyc50</td>
</tr>
<tr>
<td>Brecyc50</td>
</tr>
<tr>
<td>Bpresporecyc50</td>
</tr>
<tr>
<td>BLowP/presporecyc50</td>
</tr>
<tr>
<td>Bno-wash</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>Bno-wash/slurry</td>
</tr>
<tr>
<td>Bpresporecyno-wash/slurry</td>
</tr>
<tr>
<td>Dpresporecyno-wash/slurry</td>
</tr>
</tbody>
</table>

Please cite this article in press as: Arvaniti E, et al., Wet oxidation pretreatment of rape straw for ethanol production, Biomass and Bioenergy (2012), doi:10.1016/j.biombioe.2011.12.040
Fig. 1 – Recovered main components of rape straw after pretreatment: (A) recovered cellulose both soluble (dark) and insoluble fractions (light); (B) recovered C5 sugars, both insoluble (dark) and soluble (light) fractions; and (C) recovered insoluble lignin. The error bars show the standard deviation of the duplicates.
pretreated corn stover in the same assay increased only by 2% when Cellubrix was supplemented by βG [23]. Potential inhibition of Cellubrix at high sugar concentration applications needs to be studied further.

In SSF experiments where glucose is insitu consumed by yeast, cellobiose was not detected. Thereby βG was no evidence for inhibition [24], and it was decided to use Cellubrix for all SSF experiments. Ethanol yields were not significantly different for BlowP and B ranging 61–67%, or for B_presoak/recyc50 and B_lowP/presoak/recyc50 ranging 57–58% (see Fig. 3). The ethanol yield for hydrothermal treatment was 51%, and for the control was 18%. Previous reports about optimal hydrothermal treatment of rape straw for ethanol production are contradictory [25,26]. For this conditions of hydrothermal treatment were selected based on optimal of wheat straw [16]. The statistical analysis of BlowP and B, as well as of B_presoak/recyc50/B/rs and BlowP/presoak/recyc50 from ANOVA and Newman–Keuls post-hoc analysis are listed in Tables 2 and 3.

3.2. Recycling

Goal of this experiment is to reduce the amount of water used in WO pretreatment by recycling filtrate. For this, experiments B (no recycle), B_recyc50 (50% recycle of filtrate), and B_recyc90 (90% recycle of filtrate) are compared.

Cellulose recovery was not significantly different for B, B_recyc50, and B_recyc90, varying at 89–96% respectively (see Table 2 and Fig. 1A). Recovery of hemicellulose (see Fig. 1B) significantly decreased by recycling from 77% (no recycling) to 51% (90% recycle). Lignin recovery was significantly different also...
and increased by increasing recycling fraction from 58% (no recycling) to 73%, and 96% (90% recycling, see Table 3 and Fig. 1C).

The most common soluble degradation products found in the filtrates after pretreatment are presented in Fig. 4. The pH of filtrate after B, B_recyc50, and B_recyc90 pretreatment ranged 3.4 – 3.5 (data not shown). There was significant difference in concentrations of formic acid and acetic acid for the B, B_recyc50, B_recyc90 experiments (see Table 3, and Fig. 4A), where both acids increased by increasing recycling fraction of filtrate (See Fig. 5A). Other acids like glycolic acid and succinic acid were in the order of 0.3 g/l and 0.2 g/l (data not shown). Formic acid and acetic acid are expected to come primarily from hydrolysis of hemicellulose and uronic acids (pectin) [6] under low pH. For raw rape straw Alexander et al. (1987) accounted acetic acid and formic acid for 5% [6]. For furfural and soluble phenolics (see Fig. 4B) there was significant difference for B, B_recyc50, and B_recyc90 (see Tables 2 and 3). Furfural increased from 0.3 g/l to 1.1 g/l by recycling, and soluble phenolics increased from 1.8 g/l to 2.4 g/l.

Correlation coefficient (COCO) of soluble organic acids found in filtrate with insoluble lignin in B, B_recyc50 and B_recyc90 pretreatments was close to 0.93, COCO of organic acids with soluble CS sugars was ~0.96, and also COCO of soluble phenolic compounds with insoluble lignin was close to 0.96. Moreover, when the amounts of soluble organic acids and soluble phenols of B filtrate were subtracted from the resulting recycled filtrate B_recyc50 or B_recyc90, the correlations were unchanged. Finally COCO of formic acid with acetic acid was 0.98. COCOS indicate that organic acids have strong affiliation with insoluble hemicellulose and lignin, and that organic acids increased with high recovery of lignin. The latter observation of concurrent increase of soluble product and insoluble parent compound was observed also for (soluble) phenolics/insoluble lignin that had a COCO of 0.96.

Acetic acid and formic acid are not only hydrolysis products, but also degradation products of furans and phenolics that are in turn degradation products of carbohydrates and lignin [16]. The fractionation pattern of lignin during chemical pre-treatment is: Solid lignin → Soluble lignin → Phenolic derivatives → Carboxylic acids → CO₂ + H₂O [16]. We observed an increase of intermediate degradation products of lignin concurrently with the recovery lignin at low pH (recycling the filtrate) that has been reported previously [27], and could be explained by the production of “pseudolignin” by condensed phenolics and sugars.

Glucose yields when filtrate was recycled in WO were found significantly highest (see Table 2) only for B_recyc50 54% (Fig. 2). The ethanol yields were found not significantly different ranging 57–61% (Fig. 3). Previous studies have shown that delignification improves glucose saccharification [6,28]. In hereby study such correlation was not observed. The glucose yields increased significantly by 50% filtrate recycling, while the lignin increased significantly from 59% (no recycling) to 73% (50% recycling).

3.3. Presoaking of rape straw before WO

The goal of this experiment is to improve the efficiency of energy use by presoaking rape straw before WO in water (neutral pH) or in filtrate (pH 3.5). For this (see Table 1), (a) B_no-wash/slurry (no presoaking) is compared to B_presoak/no-wash/slurry (presoaking in water); (b) B_recyc50 (no presoaking but WO in recycled filtrate) is compared to B_presoak/recyc50 (presoaking and WO in recycled filtrate).

Recovery of cellulose significantly decreased with use of presoaking in recycled filtrate (B_presoak/recyc50) compared to no presoaking WO (B_recyc50) from 92% to 84% (see Fig. 1A), whereas hemicellulose, and lignin, were not significantly altered by presoaking. Also furfural was significantly higher by presoaking (0.71 g/l, see Fig. 4). But these results were not in line with the known high reactivity of hemicellulose (the parent compound) and furfural (its degradation product) under low pH [29]. Also in previous results presoaking at acidic conditions hydrolized more hemicelluloses than control [30]. Finally, glucose yields were significantly higher with presoaking 65% (presoaking in filtrate). Finally, the ethanol yields were 57% for both cases.

Table 2 – Results from ANOVA analysis. Compared groups (independent variables) were Pressure, Recycling, Presoaking, Filtrate content in the insoluble solids, and combinations of temperature and time. Dependent variables are the total recovery (soluble and insoluble) of pretreated rape straw cellulose, hemicellulose, the recovery of insoluble hemicellulose and lignin, the glucose yield, the ethanol yield, and concentration of degradation products found in the filtrate of pretreated rape straw. ** = significantly different values from ANOVA by 5% significance, * = different significant values, 10% significance, ns = Not significant difference, NA = Not analyzed.

<table>
<thead>
<tr>
<th>Analyzed parameters</th>
<th>Oxygen gas pressure</th>
<th>Recycling filtrate</th>
<th>Presoaking rape straw</th>
<th>Temperature/Time combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment groups</td>
<td>B + B_LowP</td>
<td>B_presoak/recyc50</td>
<td>B_recyc50</td>
<td>B + B_recyc50</td>
</tr>
<tr>
<td>Cellulose recovery%</td>
<td>*</td>
<td>**</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Hemi-cellulose recovery%</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Insoluble Lignin recovery%</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Glucose yield%</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Ethanol yield%</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>VFA concentration (g/l)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Furfural concentration (g/l)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Phenolics (g/l)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 3  Summarizing table containing significantly different data (ANOVA) after being post-hoc analyzed by Newman–Keuls statistical tool, as well as raw data (italic font), all presented in descending order of magnitude a to d, with a the highest and d the lowest value. ns = not significant according to ANOVA (Table 2).

<table>
<thead>
<tr>
<th>Analyzed parameters</th>
<th>Oxygen gas pressure</th>
<th>Recycling filtrate</th>
<th>Presoaking rape straw</th>
<th>Filtrate in pretreated solids</th>
<th>Temperature/Time combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyzed grouped treatments</td>
<td>B</td>
<td>B&lt;sub&gt;LowP&lt;/sub&gt;</td>
<td>B&lt;sub&gt;presoak/recyc50&lt;/sub&gt;</td>
<td>B</td>
<td>B&lt;sub&gt;recyc50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Cellulose recovery%</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>ns</td>
</tr>
<tr>
<td>Hemicellulose recovery%</td>
<td>ns</td>
<td>ns</td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>Insoluble Lignin recovery%</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>c</td>
</tr>
<tr>
<td>Glucose yield%</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>ns</td>
</tr>
<tr>
<td>Ethanol yield%</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>a</td>
</tr>
<tr>
<td>VFA concentration (g/l)</td>
<td>ns</td>
<td>ns</td>
<td>c</td>
<td>b</td>
<td>a</td>
</tr>
<tr>
<td>Furfural concentration (g/l)</td>
<td>a</td>
<td>b</td>
<td>ns</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>Phenolics (g/l)</td>
<td>ns</td>
<td>ns</td>
<td>c</td>
<td>b</td>
<td>a</td>
</tr>
</tbody>
</table>
Bno-wash/slurry and Bpresoak/no-wash/slurry was not replicated; thereby statistical analysis was limited. By featuring presoaking in water the recovery of cellulose decreased from 98% to 89%, recovery of hemicellulose increased considerably from 82% to 92%, and of lignin increased slightly from 60% to 66% respectively. Glucose yield increased by presoaking in water from 45% to 52%, and ethanol yield was 3% and 1% respectively. The low ethanol yields are presumably the result of the inhibitory effect of filtrate in yeast during SSF.

Presoaking either in water or filtrate reduced recovery of cellulose, and increased glucose yields. However presoaking is not attractive if cellulose is degraded. Low recovery of cellulose is accounted for low recovery of glucose. This glucose however might be part of hemicellulose structure instead [31]. Alexander et al. has pointed out that hemicellulose origin of glucose might account for as high as 10% of total glucose of rape straw [6].

### 3.4. Filtrate concentration in pretreated rape straw

The goal is to improve efficiency of water used after pretreatment by blending pretreated cake with filtrate. For this control experiment B (rinsed cake) is compared to non-rinsed pretreated cake (Bno-wash), and Bno-wash/slurry where the whole slurry (cake + filtrate) is used.

Experiments Bno-wash and Bno-wash/slurry are not replicated, and therefore statistical analysis is limited. The degradation products in filtrate were similar since pretreatment conditions were kept the same. However, the dilution factor of filtrate in the assays was different. Glucose yields of Bno-wash that skip washing was higher (60%, see Fig. 2) than that of control (B) and Bno-wash/slurry (45% both). The enzyme assay of Bno-wash/slurry that used filtrate contained at time zero 0.9 g/l cellol-oligomers, 0.2 g/l glucose, 4.6 g/l xylooligomers, 1.2 g/l xylose, 1.0 g/l arabinooligomers, 0.2 g/l arabinose, 1.6 g/l formic acid, 1.9 g/l formic acid.

---

**Fig. 4** – Concentration of (A) formic acid (gray) and acetic acid (black), and (B) furfural (gray) and phenolic compounds (black) found in filtrate after pretreatment experiments. Filtrates from B were recycled to experiments Brecyc50, Brecyc90, Bpresoak/recyc50 and BLowP/presoak/recyc50. Filtrates from Bno-wash/slurry, Bpresoak/no-wash/slurry, and Dpresoak/no-wash/slurry were used together with filter cakes in enzyme assays and SSF experiments. Error bars show the standard deviation of duplicates.
acetic acid, 0.4 g/l furfural, and 1.3 g/l soluble phenolics (data not shown). All these components are known inhibitors for cellulolytic enzymes [30,32–35], and is believed that inhibited enzymes of B_{no-wash/slurry} enzymes and caused reduction of the glucose yield.

Skip of rinsing did not affect ethanol yields. However, use of filtrate in SSF (B_{no-wash/slurry}) considerably reduced ethanol yield (3%) compared to control B and unrinsed cake (B_{no-wash}) that ranged 61–62%. The low ethanol yield is attributed to enzyme and yeast inhibition from filtrate. After SSF, the pH was around 3.9 and the concentration of formic acid was 1.5 g/l, and of acetic acid 3.3 g/l. It has been reported that Saccharomyces cerevisiae can ferment glucose at pH below 3, but presence of ORGANIC ACIDS radically decreases yeast tolerance at low pH [36]. Moreover, filtrate contained 0.4 g/l furfural before SSF, and Delgenes et al. (1996) [37] reported that furfural at 0.5 g/l reduced 53% of growth of S. cerevisiae and 57% of ethanol production.

Finally, after SSF lactic acid was detected at 6.3, 0.2, and 0 g/l for B, Bw/1 and B_{no-wash/slurry} experiments. It appears that components present in SSF of unrinsed cake (B_{no-wash}) or whole slurry (B_{no-wash/slurry}) inhibited production of lactic acid, and presumably lactic acid bacteria (LAB). Presence of LAB was not proved, but they are common contamination in dry baker’s yeast formulations [38]. Lactic acid production was detected in all SSF flasks that used rinsed pretreated rape straw (like for example B), in amounts around 6–8 g/l (data not shown) that was estimated to account for 10% of added total glucose. Lactic acid however, was not detected in enzyme assays indicating that LAB were inoculated together with the yeast in SSF.

Summarizing, the use of non rinsed pretreated rape straw in SSF represented the best case for ethanol production, since allowed ethanol fermentation, but restricted spreading contamination by LAB. With this setup about 55 L of water were saved per liter of ethanol (data not shown).

3.5. Reaction time and temperature

The goal is to estimate the best WO reaction temperature and reaction time for rape straw among B (195 °C, 15 min), C (200 °C, 5 min), D (205 °C, 3 min), and E (210 °C, 2 min) conditions, with B as reference. The severity factors (CS) for B, C, D, and E experiments were 3.1, 0.6, 0.7, and 0.4 respectively (data not shown). Also, experiments B_{presoak/no-wash/slurry} and D_{presoak/no-wash/slurry} that are featured with presoaking and use the whole slurry (cake + filtrate) after pretreatment are compared. Experiments B_{presoak/no-wash/slurry}, D, and D_{presoak/no-wash/slurry} are not replicated in pretreatment; therefore their statistical analysis is limited.

Cellulose recovery was not significantly different (see Table 2) for B, C, D, and E experiments, ranging from 90 to 97% (see Fig. 1A). Recovery of CS sugars was significantly different only for B (see Table 3) where total hemicellulose recovery was 77% (see Fig. 1B), while for C, D, and E ranged 95–99%. Regarding recovery of insoluble hemicellulose significant maximum was for D (39%). The correlation coefficient of the insoluble hemicellulose with CS was ~0.94, in line with the predictions of the models of Overend (1987) on solubilization of xylan during hydrothermal treatments [22]. Also recovery of lignin was significantly different in all the experiments; at the lowest was 59% in experiment B and increased to 70%, 83%, and 97% for C, D, and E respectively (see Fig. 1C).

Comparing the results of WO with other studies for rape straw [10,25], all examined WO conditions (B, C, D, and E) exhibited higher glucose yields, including those for alkaline WO [5]. The presented results were only comparable to application of a two-step wet explosion pretreatment method [39]. Glucose yields for B conditions were significantly lower (45%) than C, D, and E conditions ranging from 58 to 62%. For experiment featuring presoaking the glucose yield of B_{presoak/no-wash/slurry} was 52%, compared to D_{presoak/no-wash/slurry} that was only 43%. In this study, despite the difference among the CS of B, C, D, and E experiments, the glucose yields were not significantly different. Thereby the suggested direct correlation of high CS with high projected glucose yields [40] was not verified. Moreover, as mentioned in Section 3.2, despite previous studies, removal of lignin was not also important for achieving high glucose yields. The significantly highest glucose yields were 60% and 62% and were observed for D and E WO conditions, for which the highest lignin recovery was observed also 83% and 97% respectively.

The ethanol yields for C (54%) experiment were significantly lower than B, D, and E that ranged 61–64% (see Fig. 3). For experiments featuring use of whole slurry, the ethanol yields of B_{presoak/no-wash/slurry} were 1%, compared to 67% for
**4. Conclusions**

- WO pretreatment methods of 2–3 min at 205–210 °C with 12 bar of oxygen gas pressure resulted in higher glucose and ethanol yields, and in higher recovery of cellulose, hemicellulose, and lignin compared to 15 min treatment. High recovery of sugars and lignin resulted in marginal production of degradation products in the filtrate.

- The highest ethanol yield achieved was 67% by combining a two step pretreatment process, where a soaking step at 80 °C for 20 min precedes WO at 205 °C, with 12 bar oxygen for 3 min. Under these pre-treatment conditions, cellulose and hemicellulose was recovered quantitatively together with 86% of the lignin. Therefore, for achieving high glucose yield it was not found necessary to remove large part of lignin.

- In enzyme assays β-Glucosidase of Cellubrix was inhibited by glucose evident by accumulation of cellobiose. However, such a phenomenon was not observed in SSF where glucose is readily consumed by yeast.

- Skip rinsing technique and use of whole slurry for ethanol production produced reduced production of lactic acid, and in this setup would save 55 L and 80 L of water per liter of ethanol.

- Recycling of filtrate in WO increased lignin recovery and reduced hemicellulose recovery. As a result recycling increased degradation products, and glucose yields were lower for 90% recycling glucose yields than 50% recycling. The ethanol yield however was not influenced.

- Use of 12 bar oxygen gas pressure instead of 8 bar improved ethanol yields, but decreased cellulose recovery.

**Acknowledgments**

The authors would like to thank Dr. Zsófia Kádár for providing fruitful comments; Dr. Anders Thygesen for help with the statistical analysis; Annette Eva Jensen, Ingelis Larsen, and Tomas Fernqvist for technical support. Novozymes, Bagsvaerd Denmark for kindly providing the commercial enzymes used (Cellubrix L). This work was part of the project "Biorefinery for sustainable reliable and economical fuel production from energy crops, Bio-REF", and it was funded by Danish Strategic research council EnMi 2007–2010 project no. 09-061390.

**References**


