PRODUCTION OF 2ND GENERATION BIOETHANOL FROM LUCERNE – OPTIMIZATION OF HYDROTHERMAL PRETREATMENT

Sune T. Thomsen,* Morten Jensen, and Jens E. Schmidt

Lucerne (*Medicago sativa*) has many qualities associated with sustainable agriculture such as nitrogen fixation and high biomass yield. Therefore, there is interest in whether lucerne is a suitable biomass substrate for bioethanol production, and if hydrothermal pretreatment (HTT) of lucerne improves enzymatic convertibility, providing sufficient enzymatic conversion of carbohydrate to simple sugars for ethanol production. The HTT process was optimised for lucerne hay, and the pretreated biomass was assessed by carbohydrate analysis, inhibitor characterisation of liquid phases, and by simultaneous saccharification and fermentation (SSF) of the whole slurry with Cellubrix enzymes and *Saccharomyces cerevisiae* yeast. The optimal HTT conditions were 205°C for 5 minutes, resulting in pentose recovery of 81%, and an enzymatic convertibility of glucan to monomeric glucose of 74%, facilitating a conversion of 6.2% w/w of untreated material into bioethanol in SSF, which is equivalent to 1,100 litre ethanol per hectare per year.

**Keywords:** Bioethanol; Biomass; Chemical composition; Lucerne; Alfalfa; Pretreatment

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**INTRODUCTION**

A combination of a sustainable agricultural production system, economic feasibility, and effective production will ultimately strengthen the argument for producing 2nd generation bioethanol, i.e ethanol that is derived from non-food biomass. While industries first and foremost will focus on the economic gain, it is the responsibility of other stakeholders to elaborate on the sustainable production all the way from agriculture to end products.

Lucerne (*Medicago Sativa*, also known as alfalfa) is the highest yielding leguminous forage plant cultivated today, with high biomass yield per hectare and capability of growth under diverse climatic conditions (Lamb et al. 2007; Samac et al. 2006; Sheafer et al. 1992). Furthermore, lucerne has many other qualities in sustainable agriculture. Because lucerne is a perennial crop with a deep nitrogen fixating root system it has several positive traits, e.g. substitution for synthetic fertiliser, high drought resistance, prevention of nutrient leaching, and improvement of soil carbon content; as a consequence of less ploughing, green house gas emissions are reduced (Bauchan 2009; Bowman et al. 2002; Pereyra et al. 2008; Samac et al. 2006; Testa et al. 2011). Thus, lucerne should not be regarded as a dedicated energy crop, but as a natural fertiliser in low input cropping systems that have the additional benefit of being a high-yielding
lignocellulosic energy producer (Blume 2008). Lucerne is cultivated across the world with average national dry matter yields of up to 40 tons per hectare (The Czech Republic 2010) (eurostat.ec.europa.eu). In Denmark the average yield is 14 tons per hectare (Blume 2008; eurostat.ec.europa.eu).

In order to reach bioethanol yields of economic interest it is essential to apply the most suited pretreatment of the lignocellulosic material (Eggeman and Elander 2005; Felby et al. 2003; Sarkar et al. 2012). The term pretreatment is presently used for the processing of the lignocellulosic biomass to increase the availability of carbohydrates for lignocellulytic enzymes (Martin et al. 2006). Pretreatment methods such as wet oxidation (WO) (McGinnis et al. 1983), steam explosion (Sun and Cheng 2002), ammonia fiber explosion (Krishnan et al. 2010), liquid hot water (Ingram et al. 2009; Rogalinski et al. 2008), and wet explosion (Sorensen et al. 2008) are all pretreatment methods used in 2nd generation bioethanol research. Hydrothermal treatment (HTT) can be performed without addition of chemicals, oxygen, or additional pressure, which is advantageous when designing pretreatment solutions for large quantities of lignocellulosic biomass (Oleskowicz-Popiel et al. 2011; Thomsen et al. 2008a,b; Thomsen and Hauggaard-Nielsen 2008). HTT is currently moving close to commercial reality through the Danish company Inbicon, which is running a demonstration plant in Denmark based on this technology (Larsen et al. 2008; www.inbicon.com). Using HTT, Inbicon can produce more than 16 g bioethanol per 100 gram DM untreated wheat straw, and with an average biomass yield under Danish conditions of 3.9 tons per hectare per year, over 810 L bioethanol per hectare can be produced annually (estimated from (Blume 2008; Larsen et al. 2008)).

During pretreatment it is important to avoid degradation of hemicellulose as well as lignin, to prevent the formation of inhibitory by-products, since ethanol fermentations are highly influenced by the level of inhibitors in the substrate feed (Klinke et al. 2001; Martin et al. 2007; Thomsen et al. 2009).

Phenols, carboxylic acids (acetic acid, formic acid etc.), and furans (furfural (2-furaldehyde), HMF (5-hydroxymethyl-2-furaldehyde), and 2-furoic acid) are the most problematic inhibitors of ethanol fermentations and are derived from degradation of lignin, cleavage of hemicellulose side-chains, and dehydration of sugars under thermal and acidic conditions (Martin et al. 2007, 2008). The production of the inhibitors is correlated with reaction conditions during the pretreatment (Thomsen et al. 2009). Furthermore, carboxylic acids can be thermally and oxidatively degraded into a wide spectrum of degradation products that might cause additional inhibition (Bjerre and Sørensen 1990; Thomsen et al. 2009).

The accessibility of cellulose increases with increasing severity conditions of the pretreatment. Therefore, outcomes such as high cellulose convertibility and high hemicellulose recovery often contradict each other. Work by Klinke et al. (2002), showed that the best cellulose convertibility (60% cellulose converted per amount cellulose in pretreated wheat straw) was obtained with considerable loss of hemicellulose when using wet oxidation as pretreatment (Klinke et al. 2002). Xu et al. (2010) and Redding et al. (2011) found similar results. Pretreatment of lucerne for 2nd generation bioethanol has only been evaluated on few occasions, and always it has been only on the fibrous stem fraction and not on the whole crop (Boateng et al. 2008; Dien et al. 2006; Sreenath et al. 2001).
At a laboratory scale, pretreatment and SSF are often undertaken with dry matter (DM) contents of less than 10%. However, a commercial process using these large flows of water is not economically feasible due to the energy required for heating (Petersen et al. 2009). The implication of this is not only that the dry matter of the fermentable biomass will increase, but also that the levels of fermentation inhibitors formed during pretreatment will rise. DM content of novel pilot scale facilities have been reported to be more than 35% (Maas et al. 2008).

In this study, it was investigated whether lucerne is a suitable substrate for 2nd generation bioethanol production, and if HTT of lucerne improves enzymatic convertibility providing a suitable level of free sugar (glucose) for ethanol production.

EXPERIMENTAL

Raw Material
Lucerne hay was collected from Vemmetofte farm at Zealand, Denmark (55°25'N, 12°22'E), with the harvest taking place in 2008. The lucerne hay was dried with hot air, 50°C, in a drying cylinder and milled to ≤ 1 mm.

Pretreatment of Lucerne Hay
The pretreatment of lucerne hay was performed in a 2 litre loop autoclave at Biosystems Division, Risø DTU. The loop autoclave was operated in a batch setup and was fed with 1 L tap water and 60 g DM of biomass, which was submitted to continuous circulation and stirring. The design of the loop autoclave as well as heating and cooling intervals has been described previously (Bjerre and Sørensen 1990; Bjerre et al. 1996). The pretreatments were performed at different temperatures (175 to 215°C) and at different holding times (5 to 10 minutes). The HTTs were made without any addition of chemicals. One wet oxidation pretreatment (WO) were made with the addition of 12 bar oxygen (purity 99.99%) to the 1 litre headspace of the loopreactor.

After the heat treatment the liquid and solid fractions were separated by vacuum filtration with a 100 µm nylon filter. The liquid filtrate was stored at -21°C, while the solid fraction was washed with 2 L tap water and dried at 20 to 40°C to a dry matter content of approximately 95%.

Analysis of Carbohydrates in Solid Fractions
The dry matter (DM) content was determined according to the method of Clesceri et al. (1998). The carbohydrate and klason lignin content of (pretreated) biomasses was determined by strong acid hydrolysis, where (pretreated) biomass samples were subjected to strong sulphuric acid (72w/w%) at 30°C for one hour, and thereafter the remaining polymers were hydrolysed by autoclaving in weak sulphuric acid (4w/w%) for 60 minutes at 121°C. Sugar degradation was compensated including sugar spiked samples to the setup. Derived D-glucose, D-xylose, and L-arabinose were analysed by High Pressure Liquid Chromatography (HPLC) (Biorad Aminex HPX-87H column 87H (Hercules, CA; USA), 4 mM sulphuric acid as eluent, sugar standards were run in parallel, RI detector).
Prior to the strong acid hydrolysis a lipophilic extraction with hot 96% ethanol was performed for 24 hours in a soxhlet setup with reflux condenser.

**Analysis of Carbohydrates in Liquid Fractions**

The glucan (mainly cellulose) and pentosan (xylan and arabinan) polymers and oligomers in the liquid fractions were hydrolysed with 4 w/w % H₂SO₄ by autoclaving for 10 minutes at 121°C. Derived D-glucose, D-xylose, and L-arabinose, were analysed on HPLC (under the same system and run conditions as described before, and with appropriate standards).

**Analysis of Phenols, Carboxylic Acids, and Furans in Liquid Fractions**

The total phenol content was determined according to the method described by Graham (1992) by means of a colour forming reaction between FeCl₃ and phenols.

Malic acid, succinic acid, glycolic acid (2-Hydroxyethanoic acid), formic acid, acetic acid, lactic acid, propionic acid, and ethanol were determined by HPLC (under the same system and run conditions as described before, and with appropriate standards).

5-hydroxy-2-methylfurfural (HMF), 2-furfural (furfural), and 2-fuoric acid were analyzed on HPLC (under the same system and run conditions as described before, however with appropriate standards and with a UV detector).

**Enzymatic Convertibility of Solid Fraction**

To determine the amount of glucan that could be converted with cellulase enzymes to glucose, 30 Filter Paper Unit (FPU) Cellubrix® per g DM was added to a 2% DM dissolution of (pretreated) biomass sample in acetate buffer (pH 4.8). The samples were incubated at 50°C for 72 hours with continuous vertical rotation, whereafter the hydrolysate was analysed on HPLC. The enzyme activity of the Cellubrix® was 70 FPU/mL, determined according to the method of Ghose (1987). The Cellubrix® was generously donated by Novozymes, Bagsvaerd, Denmark.

**Simultaneous Saccharification and Fermentation (SSF)**

Fermentations were carried out as simultaneous saccharification and fermentation (SSF), with a preceding liquefaction step. The SSFs were carried out as pairs and were made with 10% g DM of solid fraction and with a liquid phase consisting of either liquid fraction from the pretreatment or in case of the untreated biomass, water with pH adjusted to 4.8 using NaOH or H₂SO₄. The SSFs where carried out in 250 mL blue cap flasks with yeast locks in a shaker/incubator set to 100 rpm. The cellulytic enzyme mixture used in this study was the commercial product Cellubrix®. A liquefaction step (prehydrolysis) was carried out at 50°C for 24 hours with an enzyme load of 15 FPU/g DM. After the liquefaction the bottles were cooled to room temperature and an additional 20 FPU’s of Cellubrix® were added as well as 0.020 g yeast/g DM. The head space of the bottles was flushed with N₂/CO₂ gas and sealed with yeast locks filled with glycerol. Hereafter, the SSFs were carried out at 32°C and were monitored gravimetric by CO₂ weight loss. After 144 hours the SFFs were terminated, and the exact content of ethanol, lactose, glucose, xylose, xylitol, lactic acid, formic acid, acetic acid, and propionic acid.
were evaluated by HPLC (under the same system and run conditions as described before, and with appropriate standards).

The yeast used was a purified species of *Saccharomyces cerevisiae* obtained from commercial dry yeast (Malteserkors tørgær, De Danske Spritfabrikker A/S, Denmark, purchased in Denmark, produced in Canada).

**RESULTS**

**Pretreatment**

The composition of solid fractions of untreated and pretreated lucerne is presented in Table 1. When comparing pretreated lucerne with untreated Lucerne, it is clear that the glucan content was increased, while the level of lipophilic extractives and residuals was decreased after pretreatment. Addition of oxygen (wet oxidation, WO) increased this effect compared to HTT. It can also be seen that with increasing pretreatment temperature the amount of glucan and lipophilic extractives were increased, while the amount of pentosan and residuals were decreased. During the pretreatment a mass loss of the solid phase was observed due to solubilisation of biomass constituents, and this was also correlated with the increase in temperature.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glucan</th>
<th>Pentosan†</th>
<th>Klason lignin</th>
<th>Ash</th>
<th>Extractives Residuals‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT, 175°C, 10min</td>
<td>31.7</td>
<td>12.1</td>
<td>22.9</td>
<td>8.3</td>
<td>11.1</td>
</tr>
<tr>
<td>HTT, 185°C, 10min</td>
<td>31.5</td>
<td>10.3</td>
<td>23.6</td>
<td>12.3</td>
<td>13.3</td>
</tr>
<tr>
<td>HTT, 195°C, 10min</td>
<td>35.8</td>
<td>9.8</td>
<td>26.6</td>
<td>9.1</td>
<td>13.3</td>
</tr>
<tr>
<td>HTT, 205°C, 5min</td>
<td>35.7</td>
<td>8.2</td>
<td>25.6</td>
<td>9.1</td>
<td>15.3</td>
</tr>
<tr>
<td>HTT, 215°C, 5min</td>
<td>36.1</td>
<td>5.0</td>
<td>25.7</td>
<td>10.4</td>
<td>18.0</td>
</tr>
<tr>
<td>WO, 12barO₂, 195°C, 10min</td>
<td>43.6</td>
<td>5.2</td>
<td>25.9</td>
<td>12.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Lucerne whole crop</td>
<td>19.4</td>
<td>12.1</td>
<td>11.9</td>
<td>11.8</td>
<td>22.1</td>
</tr>
</tbody>
</table>

† Xylan and arabinan, ‡ Residuals is the share not otherwise accounted for.

The carbohydrates released into the liquid phase during pretreatments were analyzed. On that basis it was possible to calculate the recovery of carbohydrates after the pretreatments. In Fig. 1 it can be seen that there are no significant loss of glucose during the pretreatments. Figure 2, on the other hand, shows a loss of pentoses, which is correlated with the increased temperature. Addition of molecular oxygen decreased pentosan recovery.
**Inhibitor Studies**

The liquid phases after pretreatment were analyzed for phenols, carboxylic acids, and furans. Phenolic compounds were only produced in low amounts, which did not vary with temperature (results not shown).

Table 2 provides an overview of the produced carboxylic acids from the different pretreatments. The concentration of malic acid decreased with increase in temperature, while the concentration of glycolic-, formic-, acetic-, and lactic acid increased with
increasing temperature. WO caused a lower malic acid concentration in the liquid fraction but an overall increase in carboxylic acid production compared to the HTT. This is correlated with the WO liquid fraction having the lowest pH. Since malic acid concentration decreased with increasing temperature of the pretreatments, it follows that malic acid originated from the lucerne, while the other carboxylic acids appear to be degradation products from the thermal decomposition of the biomass.

Table 2. Carboxylic Acids and pH in Liquid Fraction after Pretreatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Malic acid [g/L]</th>
<th>Succinic acid n.d.</th>
<th>Glycolic acid 0.11</th>
<th>Formic acid 0.52</th>
<th>Acetic acid 0.84</th>
<th>Lactic acid 0.13</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT, 175°C, 10min</td>
<td>1.53</td>
<td>n.d.</td>
<td>0.11</td>
<td>0.52</td>
<td>0.84</td>
<td>0.13</td>
<td>5.38</td>
</tr>
<tr>
<td>HTT, 185°C, 10min</td>
<td>1.51</td>
<td>n.d.</td>
<td>0.15</td>
<td>0.63</td>
<td>1.07</td>
<td>0.16</td>
<td>5.17</td>
</tr>
<tr>
<td>HTT, 195°C, 10min</td>
<td>1.37</td>
<td>n.d.</td>
<td>0.15</td>
<td>0.67</td>
<td>1.22</td>
<td>0.16</td>
<td>5.08</td>
</tr>
<tr>
<td>HTT, 205°C, 5min</td>
<td>1.40</td>
<td>n.d.</td>
<td>0.13</td>
<td>0.67</td>
<td>1.25</td>
<td>0.14</td>
<td>5.03</td>
</tr>
<tr>
<td>HTT, 215°C, 5min</td>
<td>1.26</td>
<td>n.d.</td>
<td>0.22</td>
<td>0.78</td>
<td>1.37</td>
<td>0.18</td>
<td>4.89</td>
</tr>
<tr>
<td>WO, 12bar O₂, 195°C, 10min</td>
<td>0.68</td>
<td>0.37</td>
<td>0.41</td>
<td>1.34</td>
<td>2.06</td>
<td>n.d.</td>
<td>4.38</td>
</tr>
</tbody>
</table>

* n.d. = not detected.

The formation of furans during the pretreatment is shown in Table 3. 5-HMF, a degradation product of C6 sugars, was only detected in the WO. Furfural and 2-furoic acid, degradation products of C5 sugars, increased with temperature in the HTT even though there was no apparent difference from HTT 195°C 10 minutes to HTT 205°C 5 minutes. The amount of furfural produced in the WO was 5-fold higher than the amount produced in the corresponding HTT (HTT 195°C 10 minutes).

Table 3. Furans in Liquid Fraction after Pretreatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>5-HMF [mg/L]</th>
<th>Furfural [mg/L]</th>
<th>2-furoic acid [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT, 175°C, 10min</td>
<td>n.d.</td>
<td>4.27</td>
<td>18.50</td>
</tr>
<tr>
<td>HTT, 185°C, 10min</td>
<td>n.d.</td>
<td>8.66</td>
<td>24.53</td>
</tr>
<tr>
<td>HTT, 195°C, 10min</td>
<td>n.d.</td>
<td>10.41</td>
<td>32.28</td>
</tr>
<tr>
<td>HTT, 205°C, 5min</td>
<td>n.d.</td>
<td>10.45</td>
<td>31.34</td>
</tr>
<tr>
<td>HTT, 215°C, 5min</td>
<td>n.d.</td>
<td>25.97</td>
<td>38.44</td>
</tr>
<tr>
<td>WO, 12bar O₂, 195°C, 10min</td>
<td>15.61</td>
<td>51.01</td>
<td>35.52</td>
</tr>
</tbody>
</table>

* n.d. = not detected.

**Enzymatic Convertibility Test and SSF**

The HTTs as well as the WO had a positive effect on the enzymatic convertibility of the remaining glucan in the solid fractions after pretreatment, which can be seen in Table 4. For the HTT solid fractions the enzymatic convertibility increased with increasing temperature. In case of the WO the addition of the oxygen increased the enzymatic convertibility compared to HTT 195°C 10 minutes.
The ethanol conversion efficiency based on the glucan content of the pretreated materials can also be seen in Table 4, where HTT 215°C 5 minutes and WO 195°C 10 minutes gave the highest yields.

When taking the DM loss during pretreatment into account and basing the ethanol yields on untreated material (g ethanol per 100 g DM untreated material), the WO gave the highest yield. 205°C 5 minutes and 215°C 5 minutes gave the highest yields among HTTs. For HTT at 205°C and 5 minutes the ethanol yield corresponds to an annual yield 1,100 litres of bioethanol per hectare, assuming the average growth of lucerne in Denmark of 14 tons per hectare (Blume 2008; eurostat.ec.europa.eu).

**Table 4. Enzymatic Convertibility, Final Ethanol Concentration, Ethanol Conversion Efficiency and Ethanol Yield**

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Enzymatic convertibility</th>
<th>Final ethanol concentration</th>
<th>Ethanol conversion efficiency (% of max)</th>
<th>Ethanol yield (per untreated material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>175°C 10 min</td>
<td>g glucan converted per 100g glucan in pretreated material</td>
<td>g ethanol per litre in fermentation</td>
<td>g ethanol per 100 g potential ethanol from pretreated material</td>
<td>g ethanol per 100 g DM untreated material</td>
</tr>
<tr>
<td>HTT</td>
<td>67.8 (0.6)</td>
<td>8.8 (0.0)</td>
<td>41.7</td>
<td>4.9</td>
</tr>
<tr>
<td>HTT</td>
<td>68.9 (0.7)</td>
<td>9.2 (0.0)</td>
<td>43.8</td>
<td>4.9</td>
</tr>
<tr>
<td>HTT</td>
<td>72.0 (0.5)</td>
<td>11.5 (0.0)</td>
<td>55.0</td>
<td>5.7</td>
</tr>
<tr>
<td>HTT</td>
<td>73.7 (0.4)</td>
<td>12.6 (0.2)</td>
<td>59.9</td>
<td>6.2</td>
</tr>
<tr>
<td>HTT</td>
<td>91.8 (0.3)</td>
<td>13.3 (0.1)</td>
<td>62.8</td>
<td>6.2</td>
</tr>
<tr>
<td>HTT</td>
<td>81.2 (0.2)</td>
<td>15.9 (0.1)</td>
<td>62.8</td>
<td>6.2</td>
</tr>
<tr>
<td>WO 195°C 10 min</td>
<td>54.4 (0.1)</td>
<td>3.3 (0.1)</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Untreated</td>
<td>54.4 (0.1)</td>
<td>3.3 (0.1)</td>
<td>-</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Standard deviations are shown in parentheses when applicable.

**DISCUSSION**

The solid fraction after pretreatment is enriched in glucan, and the glucan is more accessible for cellulolytic enzymes with increasing severity of the pretreatment. However, recovery of pentosans decreases with greater severity. Even though solubilisation of pentosan is desirable, thermal decomposition of the derived sugars is undesirable, since inhibitors are formed, which interfere with fermentation. While the concentration of inhibitors generated in these experiments did not appear to interfere with the rate or yield of fermentation; this might not be the case when the process is scaled up and the solids loading is increased from 6%, chosen here because of mixing limitations, to 4-5 times higher, which will imply significantly higher inhibitor levels. Therefore, the inhibitor concentrations presented in Table 2 and 3 should be regarded as indications of relatively low or relatively high inhibitor levels and not as data that are directly transferable into an industrial setup. Furthermore, pentose decomposition will also affect the yields of potential co-products made from the pentose sugars, such as biochemicals, biomethane, feed, or additional bioethanol made possible by C5 fermenting microorganisms. For these
reasons it is unrealistic to use pretreatment methods that have a pentose recovery of merely 40%, such as HTT 215°C 5 minutes, even though it has a high glucan convertibility, ethanol conversion efficiency, and ethanol yield. The WO showed high glucan convertibility and ethanol yield, but again it is at the expense of a relatively low pentose recovery and the highest inhibitor levels in the liquid fraction of all the tested pretreatment methods. Furthermore, WO showed evidence of C6 sugar degradation since 5-HMF was produced; this was not the case in any of the HTT pretreatments.

The enzymatic convertibility of 59.9% for HTT 205°C 5 minutes is comparable with other biomasses and optimized pretreatment methods described in the literature (Martin and Thomsen 2007; Martin et al. 2007; Oleskowicz-Popiel et al. 2011). However, due to the relatively lower glucan content of the untreated Lucerne, the ethanol yield, on the basis of untreated material, is rather low (6.2 g bioethanol per 100 gram DM) compared to, for instance, wheat straw (16 g bioethanol per 100 gram DM untreated wheat straw). On the other hand, lucerne can give a very high biomass output per ha with very limited input from fossil sources, resulting in an annual ethanol yield per hectare of more than 1,100 litres of bioethanol, which is also comparable to other biomasses and more than wheat straw under Danish conditions (810 L bioethanol per hectare) (Blume 2008).

CONCLUSIONS

1. Hydrothermal treatment of lucerne was found to be a good method for opening the lignocellulosic structure, and glucan convertibility was increased with increasing temperature. However, the temperature 215°C decreased pentose recovery to approximately 40% (pentosan converted per pentosan in pretreated biomass). Such high temperature should therefore not be applied in a 2nd generation bioethanol concept, where pentose utilisation is required.

2. Of the tested HTT temperatures in this study, 205°C was found to be the optimal temperature, providing an ethanol yield of 60% of the potential and acceptable pentose recovery over 80%, resulting in an annual ethanol yield per hectare of more than 1,100 litres of bioethanol.

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