Appl Biochem Biotechnol (2008) 148:23–33
DOI 10.1007/s12010-008-8134-2

Pretreatment of Whole-Crop Harvested, Ensiled Maize for Ethanol Production

M. H. Thomsen · J. B. Holm-Nielsen · P. Oleskowicz-Popiel · A. B. Thomsen

Received: 25 April 2007 / Accepted: 3 January 2008 /
Published online: 22 February 2008

Abstract To have all-year-round available feedstock, whole-crop maize is harvested premature, when it still contains enough moisture for the anaerobic ensiling process. Silage preparation is a well-known procedure for preserving plant material. At first, this method was applied to obtain high-quality animal feed. However, it was found that such ensiled crops are very suitable for bioenergy production. Maize silage, which consists of hardly degradable lignocellulosic material, hemicellulosic material, and starch, was evaluated for its potential as a feedstock in the production of bioethanol. It was pretreated at low severity (185 °C, 15 min) giving very high glucan (~100%) and hemicellulose recoveries (<80%)—as well as very high ethanol yield in simultaneous saccharification and fermentation experiments (98% of the theoretical production based on available glucan in the medium). The theoretical ethanol production of maize silage pretreated at 185 °C for 15 min without oxygen or catalyst was 392 kg ethanol per ton of dry maize silage.

Keywords Maize silage · Bioethanol · Lignocellulose · Pretreatment · Simultaneous saccharification and fermentation

Introduction

Bioethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future [1]. Brazil and the United States are the largest producers of ethanol for transport, accounting for about 90% of world production. Both countries currently produce about 16 billion liters per year with a displacement of 40% of gasoline use in Brazil but only 3% in the United States with

M. H. Thomsen (✉) · P. Oleskowicz-Popiel · A. B. Thomsen
Bioenergy and Biomass Programme, Biosystems Department, Risø National Laboratory,
Technical University of Denmark, Building BIO-301, Frederiksborgvej 399,
P.O. Box 49, 4000 Roskilde, Denmark
e-mail: mette.hedegaard.thomsen@risoe.dk

J. B. Holm-Nielsen
ACABS Laboratories, Aalborg University Esbjerg, Niels Bohrs vej 8, Esbjerg, Denmark

sugarcane (*Saccharum* L.) and corn (*Zea mays* L.) as the primary feedstock, respectively [2]. In 2005, Europe produced only about 2.6% of the world's bioethanol production, but with a bioethanol sector growing with 70.5% between 2004 and 2005 primarily in Germany and Spain but with new producer countries like Hungary and Lithuania coming up [3]. The EU countries have an indicative target year 2010 at 5.75% biofuels in the transportation sector.

Recently, a 10% binding minimum target was decided to be achieved by all EU member states for the share of biofuels in overall EU transport petrol and diesel consumption by 2020 [4].

In Denmark, industrial production of bioenergy mainly exists in the form of incineration of straw, wood chips, and wood pellets in CHP plants, but extensive research is carried out to develop second-generation bioethanol production from lignocellulosic materials and successful pilot scale facilities have been developed, such as the Integrated Biomass Utilization System (IBUS) plant [5] with maximum capacity to pretreat 1 ton of straw per hour. While the production of ethanol from sugars and starch is more straightforward (first-generation bioethanol), production from lignocellulose creates additional technical challenges, such as a need for pretreatment. Lignocellulosic materials contain cellulose and hemicellulose that are bound together by lignin. Cellulose and hemicellulose are both polymers built up by long chains of sugar monomers which first after pretreatment and hydrolysis can be converted into ethanol by microbial fermentation. The aim of the pretreatment is to open up the lignocellulosic structure to enable enzymatic hydrolysis. In the enzymatic hydrolysis, the monomeric sugars bound in cellulose and hemicellulose are released and becomes available for conversion into ethanol. The mostly used microorganism for ethanol production is the ordinary baker's yeast, *Saccharomyces cerevisiae*. In the pretreatment process, some inhibitors are formed [6] and *S. cerevisiae* is one of the most inhibitor-tolerant microorganisms used for the conversion [7]. However, it can only convert the hexoses, such as glucose and mannose, and not the pentoses such as xylose and arabinose that are found in the hemicellulose part of the straw. Inhibitor formation and pentose fermentation are the main challenges in second-generation bioethanol.

Different pretreatment methods exist, such as wet oxidation [8, 9] which is also used in this study, as well as other methods such as acid treatment, steam explosion, and hydrothermal treatment [5, 10, 11]. Wet oxidation, a reaction involving oxygen and water at elevated temperature and pressure, was presented in the early 1980s to pretreat lignocellulose (wood) as an alternative to the well-studied steam explosion [9]. Compared to other pretreatment processes, wet oxidation has been proven to be more efficient for treating some lignocellulosic materials, because the crystalline structure of cellulose is opened during the process. Organic molecules, including lignin, decompose to CO_2, H_2O, and simpler and more oxidized organic compounds, mainly to low-molecular-weight carboxylic acids [12]. Wet oxidation appears to have the advantage of producing fewer byproducts, such as furfural and hydroxymethylfurfural [13, 14]. Under the conditions of wet oxidation, aliphatic aldehydes and saturated carbon bonds are very reactive; hence, the sugar degradation products, which are known inhibitors of microbial growth [15], are not expected to be produced at high concentration.

In Denmark, energy crop cultivation is taking place to a very limited extend, less than 5% of the cropland is utilized for oil seed rape to the European biodiesel sector, since the economical feasibility compared to other available feedstocks is considered poor until crude oil prices increase further. Danish growth conditions for maize for animal feeding purposes yields 12–15 tons dry matter (DM)/ha [16], but it has the potential of rising to 16–20 tons DM/ha in the context of biorefining [17]. Silage making is a method of moist forage preservation which is widely used all over the world, accounting for more than 200 million tons of dry matter stored annually in Western Europe and USA [18]. The aim of silage making is to preserve the crop with minimum loss of nutrients. Preservation can be

achieved either by encouraging the lactic acid bacteria to dominate the fermentation or alternatively by inhibiting microbial activity with chemical additives. In Denmark, maize is primarily harvested as whole crop and ensilaged to be stored as a "wet" animal fodder.

Maize silage consists of the whole harvested maize plant (stem, leaves, and grain), which is cut and ensiled anaerobically. Thus, the whole-crop maize silage consists of hardly degradable lignocellulosic material, hemicellulosic material, and starch. Preliminary trials of wet oxidation of maize silage, using optimum temperature, time, and pH found for corn stover and wheat straw [6, 13, 19] have shown that it is a promising raw material for bioethanol production [20], giving higher sugar and ethanol yields compared to wheat straw, which is the most abundant lignocellulosic resource in Denmark.

In the present study, pretreatment of whole-crop maize silage was studied with the aim of optimizing the bioethanol process. The influence of temperature, time, and pH on sugar recovery and yield after pretreatment and enzymatic hydrolysis was studied as well as the ethanol yield in simultaneous saccharification and fermentation (SSF) with *S. cerevisiae*.

Materials and Methods

Maize Silage

The maize silage was delivered from Aalborg University Esbjerg. It was collected from Niels Tobiasen, Dairy Farm, Farup Ribe and kept in the freezer at the laboratory (at −20 °C) until use in pretreatment experiments. The harvesting time for whole-crop maize in Denmark is October (2–3-week period for harvesting and chopping). The dry matter content of the maize at harvest is between 30% and 35% at harvest or 1.18–1.20 TS/Nordic Feeding Unit (which is equivalent to 1 kg of Danish spring barley grains). The harvested maize is chopped in the harvester, thoroughly compressed, and stored completely air tight. The process is fully anaerobic, and no additives were added. The silage was stored in a filed silo (approximately 1.5 m high, 15 m wide, 30–50 m long) and can be kept up to 12 months until next harvesting. Content of total solid of the silage was around 25%, and it was dried in a heating cabinet at 40 °C for 2–3 days and milled to a size of less than 2 mm prior to pretreatment and further analysis.

Pretreatment

Maize silage was pretreated using eight different set of process parameters (including wet oxidation) as shown in Table 1. The pretreatments were performed in a loop autoclave constructed at Risø National Laboratory using 6% dry matter [13]. After pretreatment, the material was separated by filtration into a solid filter cake (containing fibers and lignin) and a liquid fraction (containing soluble sugars and various degradation products). Pretreated liquids were stored at −20 °C until further analysis and use, and the filter cakes were dried and kept in a climate cabinet at 20 °C and 65% relative humidity.

Enzymatic Hydrolysis of Solid Fraction

The enzymatic convertibility of the solid fraction after pretreatment was determined for the pretreated materials as well as for the untreated maize silage. The enzymatic hydrolysis was carried out at 50 °C, pH 4.8, with 2% DM and an enzyme load of 30 FPU/g DM. The enzyme used was Cellubrix L (Novozymes, Denmark) and the amounts of hydrolyzed sugars were determined by high-performance liquid chromatography (HPLC; see "Analysis

Table 1 Experimental conditions used for screening of pretreatment of whole-crop maize silage.

Temperature (°C)	Time (min)	Catalyst Na_2CO_3 (g/l)	Oxygen (bar)
195	15	2	12
185	15	2	12
195	15	–	12
185	15	–	12
195	15	–	–
185	15	–	–
195	10	2	12
185	10	2	12

Methods" for further details). The experiments were carried out in triplicates for each solid pretreatment fraction and for the untreated maize silage.

Simultaneous Saccharification and Fermentation

After pretreatment, 8 g DM of the solid fraction (filter cakes) was mixed with 60 ml of filtrate, and the raw sample was mixed with 60 ml of water (pH 4.8) in 250-ml fermentation flasks. All experiments were done in duplicate. Liquefaction was performed at 50 °C with an enzyme (Cellubrix L) load of 15 FPU/g DM for 24 h. After cooling to room temperature, 15 FPU/g DM enzymes (Cellubrix L), 0.2 g dry commercial yeast (Malteserkors tørgær, De Danske Spritfabrikker A/S, Denmark), and 0.2 ml urea (24%) were added. The head space in the flasks was flushed with N_2, and the flasks were equipped with yeast locks filled with glycerol. The flasks were then incubated at 32 °C and the amount of produced ethanol was determined as weight loss caused by CO_2 off gassing. The final ethanol concentration was determined by HPLC (see "Analysis Methods" for further details).

Analysis Methods

Dry Matter and Ash Content

Duplicates of 0.5 g solid material or 10 ml of liquid sample were dried at 105 °C overnight to determine the dry weight. The samples were then heated to 550 °C for 3 h to determine the ash content.

Analysis of Carbohydrates in Solid Fraction

To quantify the sugar polymers in the raw material and the solid fraction after wet oxidation, a two-step acid hydrolysis was performed. The first hydrolysis step was performed at 30 °C for 60 min with 1.5 ml of H_2SO_4 (72%) for 0.16 g DM. Then, 42 ml water was added and the second step was performed at 121 °C for 60 min. The hydrolysate was filtered and the dried filter cake subtracted for ash content is reported as Klason lignin.

Analysis of Carbohydrates in Liquid Fraction

To quantify the sugar content in the liquid fraction, a weak hydrolysis was performed at 121 °C for 10 min using 4% H_2SO_4, in duplicate. The concentrations of sugar monomers were determined by HPLC as described below.

HPLC Analysis

The amounts of released sugar monomers in the hydrolysate as well as concentration of produced ethanol were determined by HPLC (Shimadzu) using a Rezex ROA column (Phenomenex) at 63 °C and 4 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min. A refractive index detector (Shimadzu Corp., Kyoto, Japan) was used.

Calculations

Recoveries were calculated according to Eq. 1. Yields were calculated as percent of theoretical (in gram per gram original cellulose or hemicellulose in raw material; Eqs. 2 and 3). Total yields were calculated as the total yield of hemicellulose/glucose in the liquid fraction and after enzymatic hydrolysis of the solid fraction (Eq. 4). The theoretical ethanol production based on the pretreatment and hydrolysis yields was calculated according to Eq. 5. The ethanol yield in SSF experiments was calculated as percentage of theoretical based on cellulose content of the fiber fraction and glucose in the filtrates (Eq. 6). The ethanol concentration was calculated from weight loss due to CO_2 of gassing by Eq. 7 .

$$\text{Recovery} = \frac{(\text{sugar in filtrate}(\text{g}/100\ \text{g}) + \text{sugar in solid}(\text{g}/100\ \text{g}))}{(\text{sugar in raw material}(\text{g}/100\ \text{g}))} \times 100\% \quad (1)$$

$$\text{Pretreatment yield}_{\text{sugar}} = \frac{\left(\text{mass}_{\text{sugar}}\ \text{in filtrate}\right)}{\left(\text{mass}_{\text{sugar}}\ \text{in raw material}\right)} \times 100\% \quad (2)$$

$$\text{Hydrolysis yield}_{\text{sugar}} = \frac{\left(\text{mass}_{\text{sugar}}\ \text{after enzymatic hydrolysis} \times 0.9\right)}{\left(\text{mass}_{\text{sugar}}\ \text{in raw material}\right)} \times 100\% \quad (3)$$

$$\text{Total yield}_{\text{sugar}} = \frac{\left(\text{mass}_{\text{sugar}}\ \text{in filtrate}\right) + \left(\text{mass}_{\text{sugar}}\ \text{after enzymatic hydrolysis}\right)}{\left(\text{mass}_{\text{sugar}}\ \text{in raw material}\right)} \times 100\% \quad (4)$$

$$\text{EtOH yield} = \frac{\text{EtOH}_{\text{Gravimetric/HPLC}}}{\text{glucan in solid} \times 0.51 + \text{glucose in filtrate} \times 0.51} * 100 \quad (5)$$

$$\text{Theoretical ethanol production} = \text{TSC*} \times \text{Yield in SSF**} \times 0.51 + \text{TSH***} \times \text{Recovery****} \times 0.51 \quad (6)$$

*TSC=Total sugar from cellulose
**Conversion yield obtained in SSF experiments (g/100 g raw material)

***TSH=Total sugar from hemicellulose

****Total hemicellulose recovery in solid and liquid fraction (g/100 g raw material)

$$\mathrm{EtOH(g/l)} = \text{Weight loss* of } \mathrm{CO_2}\left[\frac{\text{g total } \mathrm{CO_2} \text{ lost}}{\text{ml liquid}}\right] \cdot \frac{1}{44}\left[\frac{\text{mol } \mathrm{CO_2}}{\text{g } \mathrm{CO_2}}\right] \cdot \frac{1}{1}\left[\frac{\text{mol EtOH prod.}}{\text{mol } \mathrm{CO_2} \text{ prod.}}\right] \cdot 46\left[\frac{\text{g EtOH}}{\text{mol EtOH}}\right] \cdot 1,000\left[\frac{\text{ml liquid}}{\text{l}}\right] \quad (7)$$

*Off-gassed $\mathrm{CO_2}$

Results and Discussions

Raw Material Composition

Maize silage has a high glucan and total sugar content compared to other lignocellulosic materials that have been examined for bioethanol production by wet oxidation [5, 19, 21, 22]. Table 2 shows the composition of the maize silage used in this study; it contained approximately 52% glucan from both starch and cellulose present in this "whole-crop" harvested material. The strong acid hydrolysis used for carbohydrate analysis makes no distinction between glucan from starch and cellulose—but the average maize silage contains approximately 20% lignocellulose and 30% starch (according to official Danish fodder quality assessment). The maize silage also contains significant amounts of hemicellulose (approximately 20%). Silage fermentation is facilitated by spontaneous conversion of water-soluble carbohydrates by lactic acid bacteria. Most strains of lactic acid bacteria are able to utilize the pentose sugars, which, to some degree, could be released from the material during shredding and compression in the silo. The DM loss during ensiling is 5–10% (see "Materials and Methods" section). The lignin content is slightly lower than in traditional lignocellulosic materials such as straw (e.g., wheat and rye), but still it is sufficient for maize silage to be a promising raw material in integrated bioethanol/power production processes such as the Danish IBUS process [5], where the lignin residue from bioethanol production is used for incineration in the power plant, thereby improving the energy balance of the ethanol process.

Sugar Recovery after Pretreatment

Maize silage was pretreated by wet oxidation and hydrothermal treatment as described in Table 1. The sugar recoveries based on analysis and overall mass balance of the pretreated fractions (solid fibers and liquid) and the raw material have been calculated according to Eq. 1, and the results are shown in Fig. 1. The total recovery of glucose was very close to a 100% in all experiments (not shown), while the recovery of hemicellulose sugars depended very much on pretreatment conditions.

In maize silage pretreated at 195 °C, 25% to 50% of the total pentoses in the raw material was degraded. The lowest recovery was observed at 195 °C in the experiment

Table 2 Composition of the maize silage raw material used in this study.

Raw material	Glucan (g/100 g DM)	Hemicellulose (g/100 g DM)	Lignin (g/100 g DM)
Maize silage	51.7	19.5	16.6

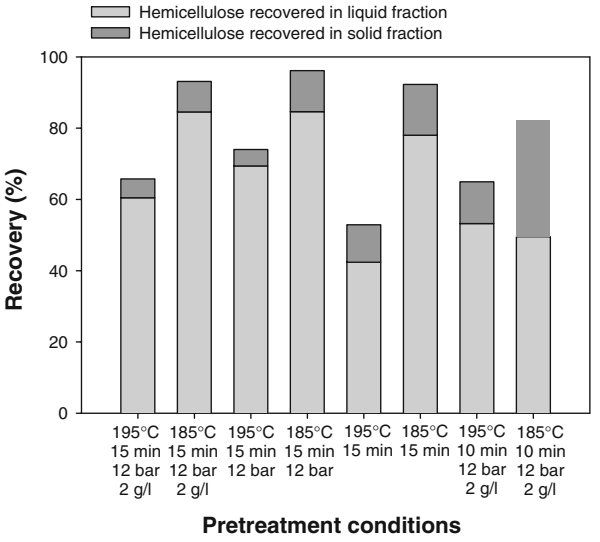

Fig. 1 Recovery of hemicellulose sugars (pentoses) in pretreatment of maize silage using different pretreatment parameters (see Table 1)

where no oxygen or alkaline catalyst was used. This is coherent with wet oxidation studies on wheat straw, which shows that oxygen and Na_2CO_3 have a positive effect on hemicellulose recovery [13]. Decreasing residence time from 15 to 10 min does not improve hemicellulose recovery in this study. However, decreasing the temperature to 185 °C has a significant effect on the recovery, and in these experiments, recoveries of 80–96% are achieved. The three experiments at 185 °C with a residence time of 15 min gave very similar results, with the wet oxidation experiment (O_2 addition) giving a slightly higher recovery.

Sugar Yield after Pretreatment and Enzymatic Hydrolysis

To evaluate the effect of the different pretreatments on the enzymatic convertibility of the materials, this was tested both on the raw maize silage and on the solid fractions of the pretreated material. Figure 2 shows the fraction of glucan (solid bars) and hemicellulose sugars (hatched bars) liberated during pretreatment (black) and enzymatic hydrolysis (gray). During pretreatment of maize silage, a significant part of both glucan and hemicellulose sugars was extracted into the liquid fraction (pretreatment yield). In "pure" lignocellulosic material such as wheat straw, which has not been ensilaged, only very small amounts of glucose are released during pretreatment (2–7%) [5]. In these experiments, 60% to 98% of the glucan and almost all the recovered hemicellulose are released during pretreatment, probably because a significant part of the glucan in this material is starch. During enzymatic hydrolysis, even more sugars were liberated (mainly glucan), giving very high total sugar yield in the best experiments (~100% of glucan). The highest glucan (~100%) and hemicellulose yields (78–89%) were obtained in the three experiments performed at 185 °C with a residence time of 15 min (enz. hydr. for exp. 185 °C, 15 min is missing—Fig. 2). The enzymatic convertibility of the raw maize silage gave a very low yield, proving that pretreatment is necessary to achieve high ethanol yields.

SSF of Pretreated Maize Silage

Simultaneous saccharification and fermentation was performed on the raw maize silage and on pretreated fibers suspended in the filtrates to a dry matter content of approximately 13%.

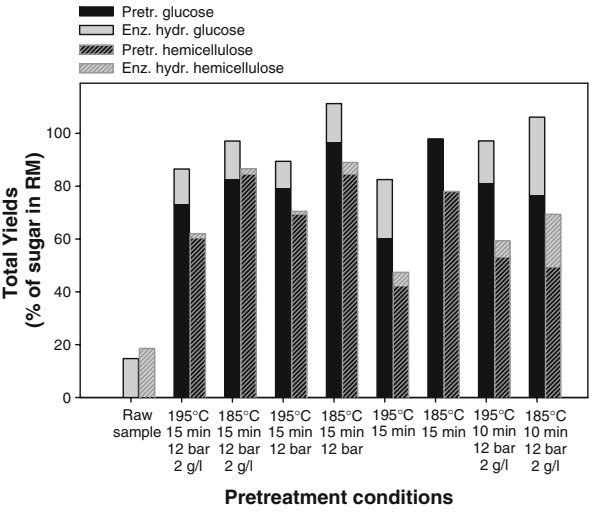

Fig. 2 Total yield of sugar after pretreatment and subsequent enzymatic hydrolysis of maize silage using different pretreatment parameters (see Table 1)

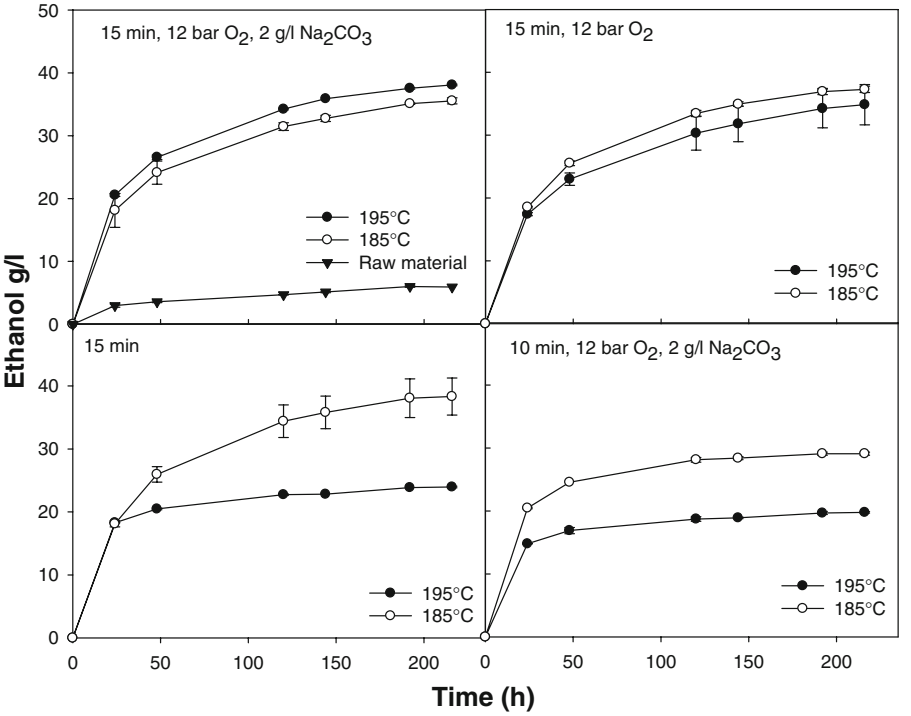

Fig. 3 Simultaneous saccharification and fermentation of raw maize silage and maize silage pretreated at different parameters settings (see Table 1). Ethanol production was followed as weight loss due to CO_2 off gassing and calculated by Eq. 7

Thirteen percent dry matter is the upper limit of what can be handled in shake flask experiments. Since *S. cerevisiae* were used in this SSF, only glucose were converted to ethanol, and yields are based only on the conversion of glucan. The ethanol production curves from SSF of the different pretreated materials are shown in Fig. 3, and the final ethanol concentrations measured by HPLC are shown in Table 3. The ethanol yield and the productivity on the raw material was low, but was improved significantly by pretreatment of the materials. This is also due to the low enzymatic convertibility of the raw material (Fig. 2). Fermentation of all pretreated materials started without a lag phase, indicating that the level of inhibitors produced in the pretreatments was low enough for the yeast to start producing ethanol. In the fermentation of toxic materials, the yeast will detoxify the medium by converting the toxic aldehydes to less toxic alcohols, giving a lag phase where no ethanol is produced [23, 24]. The best results were obtained when using maize silage pretreated with a residence time of 15 min. SSF of material pretreated for 15 min gave very similar ethanol yields of 91–98% of the theoretical production (based on available glucan in the medium determined by HPLC—Table 3), except for the experiment at 195 °C using no oxygen and no catalyst; in this experiment the yield was only approximately 61%, probably due to the low hemicellulose recovery (Fig. 1). This shows that the protective effect of hemicellulose sugars under alkaline wet oxidation conditions becomes more pronounced at higher temperatures, which is an advantage when pretreating materials such as wheat straw [13] and corn stover [19] where high temperatures are needed for high cellulose convertibility. However, these results show that in pretreatment of maize silage, 185 °C is sufficient to achieve high ethanol yields, and the addition of oxygen and catalyst can be avoided, meaning that energy can be saved and the cost of oxygen and catalyst eliminated. The ethanol yield in SSF of material pretreated at 185 °C for 15 min without oxygen or catalyst was 98% of the theoretical production (based on available glucan in the medium) when measured by HPLC (Table 3).

Theoretical Ethanol Production from Pretreated Maize Silage

A theoretical ethanol production based on the achieved yields was calculated for each wet oxidation experiment. For glucan sugars, the yield obtained in SSF was used, and for the hemicellulose sugars (which were not converted by baker's yeast in the SSF) it was assumed that all recovered sugars could be converted to ethanol at a yield of 0.51 g ethanol/g sugar (Eq. 6). These results are shown in Fig. 4 and compared to the maximum ethanol

Table 3 Final ethanol production in SSF experiments measured by HPLC.

Temperature (°C)	Time (min)	Catalyst Na_2CO_3 (g/l)	O_2 (bar)	Ethanol (g/l) HPLC	Ethanol (% of theoretical) HPLC
Raw	–	–	–	5.9	15
195	15	2	12	38.1	97
185	15	2	12	35.6	90
195	15	–	12	34.9	89
185	15	–	12	37.3	95
195	15	–	–	24.0	61
185	15	–	–	38.3	98
195	10	2	12	19.8	51
185	10	2	12	29.1	74

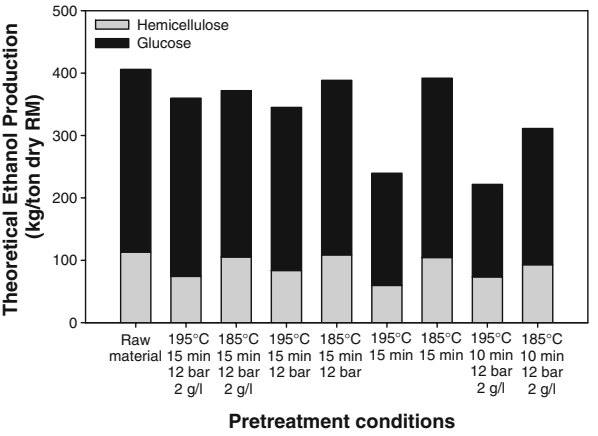

Fig. 4 Theoretical ethanol production based on sugar yield after pretreatment and enzymatic hydrolysis of maize silage pretreated at different parameters settings (see Table 1). The results are compared to the maximum production based on total sugar in the raw maize silage (*bar 1*)

production from the available sugars in the raw maize silage. Four hundred kilograms of ethanol can be produced from 1 ton of dry maize silage (corresponding to 100 kg/ton wet silage—25% DM) based on the sugar content of the material (bar 1—Fig. 4). Two experiments gave similar theoretical ethanol production of 388 kg/ton dry maize silage and 392 kg/ton dry maize silage, namely the two experiments performed at 185 °C for 15 min with and without oxygen, respectively. The experiment performed without oxygen is the best choice, because oxygen increases the cost of the bioethanol process and makes scale-up of the process more complex.

Conclusions

From the results found in this study on bioenergy potential of maize silage, it can be concluded that maize silage is a very promising raw material for bioethanol production.

Maize silage has a high glucan and total sugar content compared to other lignocellulosic materials that have been examined for bioethanol production by wet oxidation, and the result presented in this paper shows that it can be pretreated at low severity (185 °C, 15 min) giving very high glucan (~100%) and hemicellulose recoveries (89%)—as well as very high ethanol yield in SSF experiments (98% of the theoretical production based on available glucan in the medium). In contrast to other lignocellulosic materials, most of the sugars (both glucan and hemicellulose) are extracted during pretreatment, probably meaning that enzyme loadings can be reduced significantly. Furthermore, addition of oxygen and catalyst can be eliminated, which further reduces the price of ethanol production. The theoretical ethanol production of maize silage pretreated at 185 °C for 15 min without oxygen or catalyst was 392 kg ethanol per ton of dry maize silage.

The results presented in this paper were only a very preliminary study of pretreatment of maize silage. Trials should be made at lower temperatures to examine if more energy could be saved in the process. It would also be interesting to determine the content of starch and cellulose separately by enzymatic hydrolysis, instead of total glucan as is the case in this study. Also, enzymatic hydrolysis and SSF using low enzyme loadings (of both cellulases and amylases) should be made to fully see the potential of this promising raw material for bioethanol production.

Acknowledgments We thank Tomas Fernqvist from Risø National Laboratory. Dairy Farm, Farup Ribe is thanked for supplying good quality maize silage. Risø National Laboratory, Technical University of Denmark and Højteknologifonden, Denmark are thanked for financial support given to this study.

References

1. Thomsen, A. B., Medina, C., & Ahring, B. K. (2003). In H. Larsen, J. Kossmann, & L. S. Petersen (Eds.), *Risø energy report 2* (pp. 40–44).
2. Hazell, P., & Pachauri, R. K. (2006). 2020 Focus No. 14, November. http://www.ifpri.org/2020/focus/focus14.asp#dl.
3. Eurobserver (2006). Biofuels barometer May, pp. 57–66.
4. EU (2007). Brussels European Council 8–9 March—Presidency Conclusions (http://mediacontent.ig.publicus.com/PDF/IG35268339.PDF)
5. Thomsen, M. H., Thygesen, A., Christensen, B. H., Larsen, J., Jørgensen, H., & Thomsen, A. B. (2006). *Applied Biochemistry and Biotechnology*, *129–132*, 448–460.
6. Klinke, H. B., Olsson, L., Thomsen, A. B., & Ahring, B. K. (2003). *Biotechnology and Bioengineering*, *3*(81), 738–747.
7. Olsson, L., & Hahn-Hagerdal, B. (1993). *Process Biochemistry*, *28*, 249–257.
8. Schmidt, A. S., & Thomsen, A. B. (1989). *Bioresource Technology*, *64*, 139–151.
9. McGinnis, G. D. (1983a). WWWMCE. *Industrial & Engineering Chemistry Product Research and Development*, *22*, 352–357.
10. Lee, Y. V., Iyer, P., & Torget, R. W. (1999). *Advances in Biochemical Engineering, Biotechnology*, *65*, 93–115.
11. Galbe, M., & Zacchi, G. (2002). *Applied Microbiology and Biotechnology*, *59*, 618–628.
12. McGinnis, G. D., Wilson, W. W., Prince, S. E., & Chen, C. C. (1983b). *Industrial & Engineering Chemistry Product Research and Development*, *22*, 633–636.
13. Bjerre, A. B., Olesen, A. B., Fernqvist, T., Plöger, A., & Schmidt, A. S. (1996). *Biotechnology and Bioengineering*, *49*, 568–577.
14. Ahring, B. K., Licht, D., Schmidt, A. S., Sommer, P., & Thomsen, A. B. (1999). *Bioresource Technology*, *68*, 3–9.
15. Palmqvist, E., Hahn-Hägerdal, B., Szengyel, Z., Zacchi, G., & Reczey, K. (1997). *Enzyme and Microbial Technology*, *20*, 286–293.
16. Pedersen, C. Aa. (2006). Oversigt over Landsforsøgene 2006, Dansk Landbrugsrådgivning, Landscenteret-Planteavl (Danish).
17. Holm-Nielsen, J. B., Madsen, M., & Popiel, P. O. (2006). Proceedings: World Bioenergy 2006, Conference for Biomass for Energy, 05/2006, Jönköbing Sweden.
18. Weinberg, Z. G., & Munc, R. E. (1996). *FEMS Microbiology Reviews*, *19*, 53–68.
19. Varga, E., Schmidt, A. S., Reczey, K., & Thomsen, A. B. (2003). *Applied Biochemistry and Biotechnology*, *104*, 37–50.
20. Thomsen, M. H., Popiel, P. O., Lisiecki, P., Varga, E., Thomsen, A. B., Esbensen, K. H., et al (2005). Proceedings: 14th European Biomass Conference and Exhibitons, 17–21 October 2005, Paris, France.
21. Petersson, A., Thomsen, M. H., Haugaard-Nielsen, H., & Thomsen, A. B. (2007). *Biomass & Bioenergy*, *31*, 812–819.
22. Martín, C., Klinke, H., & Thomsen, A. B. (2007). *Enzyme and Microbial Technology*, *40*(3), 426–432.
23. Taherzadeh, M. J., Gustafsson, L., Niklasson, C., & Lidén, G. (1999). *Journal of Bioscience and Bioengineering*, *87*, 169–174.
24. Taherzadeh, M. J., Gustafsson, L., Niklasson, C., & Lidén, G. (2000). *Applied Microbiology and Biotechnology*, *53*, 701–708.