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BIOMASS AND BIOENERGY XXX (2010) 1–11

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ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form
23 June 2010
Accepted 1 July 2010
Available online xxx

Keywords:
Energy crops
Cannabis sativa
Lignocellulose
Enzymatic hydrolysis
SSF
Saccharomyces cerevisiae

ABSTRACT

Biomass can be converted into liquid and gaseous biofuels with good efficiency. In this study, the conversion of industrial hemp (*Cannabis sativa* L.), a biomass source that can be cultivated with a high biomass yield per hectare, was used. Steam pretreatment of dry and ensiled hemp was investigated prior to ethanol production. The pretreatment efficiency was evaluated in terms of sugar recovery and polysaccharide conversion in the enzymatic hydrolysis step. For both materials, impregnation with 2% SO_2 followed by steam pretreatment at 210 °C for 5 min were found to be the optimal conditions leading to the highest overall yield of glucose. Simultaneous saccharification and fermentation experiments carried out with optimised pretreatment conditions resulted in ethanol yields of 163 g kg^{-1} ensiled hemp (dry matter) (71% of the theoretical maximum) and 171 g kg^{-1} dry hemp (74%), which corresponds to 206–216 l Mg^{-1} ethanol based on initial dry material.

1. Introduction

Interest in biomass-based renewable fuels has increased, and ethanol produced from lignocellulosic feedstock is a promising candidate. The carbohydrate portion of lignocellulose biomass (containing cellulose and hemicellulose) is suitable for ethanol production but difficult to access when cellulose, hemicellulose and lignin are associated. Several pretreatment methods have been developed to increase the accessibility of cellulose [1], and a wide variety of lignocellulosic substrates have already been proven to be suitable raw materials, including wood materials (spruce [2], willow [3]), agricultural by-products (such as corn stover [4], wheat straw [5]), sugar production by-products (sugar cane bagasse [6], sweet sorghum bagasse [7]), reeds [8] or switchgrass [9].

The target plant of this study was industrial hemp (*Cannabis sativa* L.), which is an annual plant mostly cultivated for its strong fibres. Hemp has not, to our knowledge, previously been investigated for ethanol production, and has several features that make it an interesting alternative biomass. The plant is

Abbreviations: SPH, steam pretreated hemp; SPHS, steam pretreated hemp silage; WIS, water insoluble solids.

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doi:10.1016/j.biombioe.2010.07.003

rather drought tolerant, and can reach high biomass yields per hectare [10]. The need for herbicides can be reduced because hemp is able to overgrow weeds. Thus, it is advantageous to use hemp in a crop rotation, especially in organic farming. Hemp fibre already has many industrial applications, for example in the textile or pulp and paper industries [11]; it can be used in fibre-reinforced composites [12] or as a construction material [13]. Hemp has already been reported to be feasible solid fuel for combustion [14].

Hemp can be cultivated in various climates. In warmer areas, hemp can be dried in the field and stored dry. In other areas where rain is common during the harvest period in the autumn, ensiling can be a more suitable storage method. In addition, during ensiling acids are formed that could later act as catalysts in the physico-chemical pretreatment, which might decrease the need for addition of extra chemicals to the process. When using hemp as a biomass source for fuel production rather than as a fibre crop [15], harvesting should be postponed for 1-2 months to achieve the highest biomass yield.

The cellulose content of the hemp stem is quite high (about 44%) compared to other agricultural lignocellulosic materials, e.g., corn stover [16] or wheat straw [5], both with 37% (dry matter) cellulose. The high cellulose content and high biomass yield make hemp a good potential crop for bioethanol production. The hemp stem consists of bast fibres and a woody core. The bast fibre is rich in cellulose and has rather low lignin content, while the woody core has significantly higher lignin content [17].

Steam pretreatment of hemp fibres has previously been studied in order to separate the fibres from the other components [17]. Treatment with alkali impregnation followed by steam pretreatment at 200 °C with a residence time of 90 s was found to be optimal for this purpose. In another study, steam pretreatment of hemp fibres at 185 °C for 2 min increased the cellulose content from 60% to 74%, whereas enzyme (pectinase) assisted retting followed by steam pretreatment resulted in a 78% cellulose content of the remaining solid material [18]. Enhancing the enzymatic breakdown of hemp via electron beam irradiation was previously tested [19], where the improvement in enzymatic hydrolysis was more evident in the hydrolysis of xylan than in that of cellulose.

The aim of the present study was to optimise steam pretreatment parameters for hemp in order to achieve the highest glucose yield in enzymatic hydrolysis for conversion to ethanol. Steam pretreatment was performed both on dry hemp stems and hemp silage (stem and leaves together) under different conditions with and without SO_2 impregnation. The efficiency of the pretreatment was evaluated by enzymatic hydrolysis of the whole slurry. Mass balance calculations were performed on the pretreatment and the enzymatic hydrolysis to estimate the overall glucose yield, the efficiency of hemicellulose solubilisation and the sugar degradation. Simultaneous saccharification and fermentation (SSF) was performed on samples at optimised conditions. SSF of both the whole pretreatment slurry and of the separated solid fraction of the pretreated material was performed. Fig. 1 shows the schematic representation of the experiments.

2. Materials and methods

2.1. Raw material

Industrial hemp (*C. sativa* L.) of the variety Futura 75 was cultivated at Nöbbelöv, close to Lund, Sweden (N55°43′, E13°08′). The hemp was sown on 4 April 2007 and harvested on 3 and 4 September. Stems were cut a few centimetres above ground. The average dry matter (DM) yield was 16 Mg ha^{-1}. The hemp was air-dried indoors in open air at 10–20 °C after harvest to a DM content of 91.7%. There were no visible signs of microbial degradation during drying. Stems and leaves (including fine stems) were separated manually after drying and weighed. Dry stems were comminuted with a garden shredder (AXT 2500 HT, Robert Bosch GmbH, Germany) to a length of 2–3 cm. For the steam pretreatment equal amounts of air-dried hemp from three different fertilization levels (115, 150 and 200 kg N ha^{-1}) were mixed due to shortage of substrate.

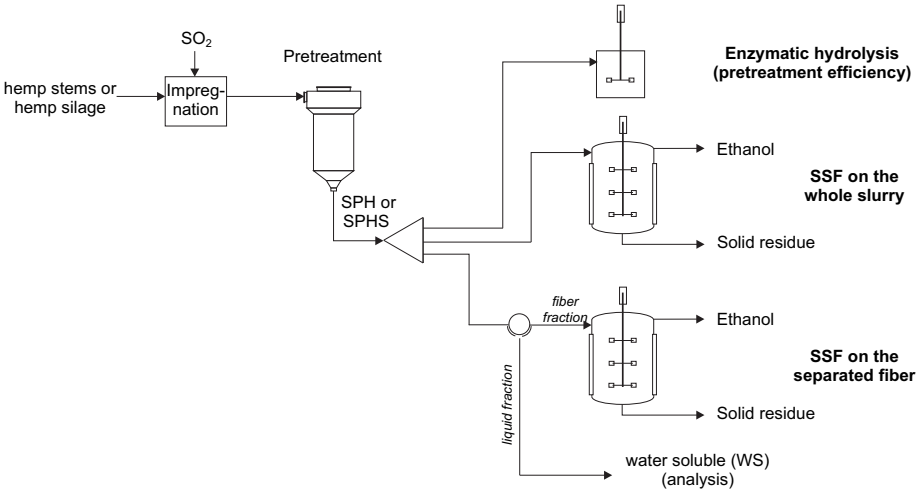

Fig. 1 – Schematic representation of the experimental process (Abbreviations: SPH: steam pretreated hemp, SPHS: steam pretreated hemp silage, SSF: simultaneous saccharification and fermentation).

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The mixture is referred to as dry hemp in the paper. The leaves were not pretreated since they easily fell apart into smaller pieces not suitable for the pretreatment unit used. The stems were sprayed with deionised water to a moisture content of 50%, mixed and stored at room temperature for two days prior to steam pretreatment.

Silage was prepared from hemp (full plant including leaves) fertilized with nitrogen at 200 kg ha^{-1}, which was harvested and shredded to ~2 cm pieces using a Claas Jaguar maize forager equipped with a traditional maize header. The hemp was ensiled without additives in round bales made by an Orkel MP 2000 stationary baler. The round bales where wrapped in silage plastic and stored for eight months, from September 2007 to May 2008, when samples were taken from the ensiled hemp. Ensiled hemp samples were stored frozen from May 2008 until pretreatment started (September, 2008). Ensiled hemp (stems and leaves) had oven dry matter content of 25%, therefore no spraying was needed before steam pretreatment.

2.2. Enzyme preparations

The enzymes used in the hydrolysis were Celluclast 1.5L and Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark). Filter paper activity of Celluclast 1.5L was 60.9 FPU ml^{-1} and β-glucosidase activities were 32.8 IU ml^{-1} and 502.3 IU ml^{-1} for Celluclast 1.5L and Novozym 188, respectively. FPA and β-glucosidase activities were measured [20,21] prior to experiments.

2.3. Chemicals

Nutrients for fermentations (KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and $(NH_4)_2HPO_4$) were purchased from Merck (Hochenbrun, Germany). Yeast extract was purchased from Applichem (Gatersleben, Germany). 72% sulphuric acid for analysis of structural carbohydrates was purchased from Fluka (St. Louis, MO, USA). Sugar standards for HPLC analysis were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.4. Steam pretreatment

Pretreatment conditions for dry hemp and ensiled hemp were tested at different temperatures and SO_2 impregnation was used when indicated. In the case of dry hemp, three different temperatures (205 °C, 210 °C and 215 °C) were investigated with 2% SO_2 impregnation and a 5-min residence time. Ensiled hemp was pretreated at four temperatures without SO_2 impregnation (190 °C, 200 °C, 210 °C and 220 °C) and at two temperatures (200 °C and 210 °C) with 2% SO_2 impregnation.

Impregnation was performed in batches of 300 g dry matter (DM) by injecting SO_2 (2% based on water content) into plastic bags. After 20 min of impregnation, the bags were ventilated before the material was steam pretreated. Pretreatment was performed in a 10 l batch reactor, described earlier [22]. After steam pretreatment, the slurry was collected from the flash cyclone and stored at +5 °C a few days. There were no visible signs of microbial degradation during storage. Samples for analysis were washed with distilled water to remove water soluble from water-insoluble solids (WIS). A part of the pretreatment slurry was separated into solid and liquid fractions using a manual hydraulic press (Sixten Torne AB, Malmö, Sweden). The solid fraction was used for the SSF without additional washing step in the case, when only the solid fraction was used, and the liquid was used for the yeast cultivation (as described in Section 2.5).

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed to evaluate the effect of different steam pretreatment conditions. A low substrate concentration (2% water-insoluble solids (WIS) using the whole pretreatment slurry) was used to minimise product inhibition. Enzyme loading for hydrolysis was 15 FPU g^{-1} WIS Celluclast 1.5L and 23 IU g^{-1} WIS Novozym 188. The hydrolysis was performed with 500 g total mass in 1 l bottles immersed in a water bath at 40 °C. Agitation with a frequency of 5 Hz was ensured by overhead stirring. The pH was set to 4.8 with a 0.05 mol l^{-1} sodium acetate buffer. Samples were taken after 0, 2, 4, 8, 24, 48, 72 and 96 h and analysed for monomer sugar content by high-performance liquid chromatography (HPLC). All hydrolysis experiments were run in duplicate.

2.6. Yeast cultivation

Yeast cultivation was performed in three steps (propagation, batch and fed-batch cultivation) [5]. The strain of *Saccharomyces cerevisiae* used was purified from commercial yeast (Jästbolaget AB, Rotebro, Sweden). Cells were added to a 300 ml Erlenmeyer flask with 70 ml of a water solution containing 23.8 g l^{-1} glucose, 10.8 g l^{-1} $(NH_4)_2SO_4$, 5.0 g l^{-1} KH_2PO_4 and 1.1 g l^{-1} $MgSO_4 \cdot 7H_2O$. The water solution also contained 14.4 ml l^{-1} of a trace-metal solution and 1.4 ml l^{-1} of a vitamin solution [23]. The pH was adjusted to pH 5 with 0.25 mol l^{-1} NaOH. The Erlenmeyer flask was closed with a cotton plug and incubated at 30 °C for 24 h on an incubator shaker using an agitation frequency of 2.5 Hz.

Batch cultivation was then performed in a 2 l fermenter (Infors AG, Bottmingen, Switzerland) with a working volume of 250 ml, similarly to the procedure described earlier [24] with some modifications. Cultivation was started by adding a 60 ml inoculum to a medium containing 40.0 g l^{-1} glucose, 22.5 g l^{-1} $(NH_4)_2SO_4$, 10.5 g l^{-1} KH_2PO_4, 2.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 60.0 ml l^{-1} trace-metal solution and 6.0 ml l^{-1} vitamin solution [23]. The pH was continuously adjusted to pH 5 with 10% NaOH solution. The stirring frequency was 8.3 Hz and the aeration rate was 0.25 l min^{-1} corresponding to a space velocity of 1 min^{-1}. The dissolved oxygen concentration was continuously measured throughout batch cultivation with an oxygen sensor. Batch cultivation was changed to fed-batch cultivation when a rapid increase in oxygen concentration was observed.

Fed-batch cultivation was performed on the liquid fraction of the pretreatment slurry by continuous addition of 858 ml of liquid fraction supplemented with glucose and salt solutions to a total volume of 1000 ml. The glucose concentration in the pretreatment liquid solution was adjusted to 80 g l^{-1}. Salts were added to the solution to concentrations of 11.3 g l^{-1} $(NH_4)_2SO_4$, 5.3 g l^{-1} KH_2PO_4 and 1.1 g l^{-1} $MgSO_4 \cdot 7H_2O$. The final concentration of the diluted liquid fraction was equivalent to that obtained when the slurry from pretreatment had been diluted to 7.5% WIS. The diluted and adjusted liquid fraction

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was added to the fermenter at constant flow rate for 14–16 h. The pH was continuously adjusted to 5 with 10% NaOH solution. The stirring frequency was 13.3 Hz and the aeration rate was 1.875 l min^{-1} at the end of the fed-batch cultivation, corresponding to 1.5 min^{-1} space velocity. Cells were harvested by centrifugation of the broth at 150 Hz for 5 min and were washed two times with deionised water.

2.7. SSF

SSF experiments were performed in 2 l laboratory fermenters (Infors AG, Bottmingen, Switzerland) with 1.4 kg of working mass using 7.5% WIS substrate concentration. As nutrients 0.5 g l^{-1} $(NH_4)_2HPO_4$, 0.025 g l^{-1} $MgSO_4$ and 1 g l^{-1} yeast extract were added. The fermenter with the substrate and the nutrients in separate bottles were sterilised at 121 °C for 20 min. Cultivated yeast was added at a concentration of 5 g l^{-1}. The experiments were performed at 37 °C and pH 5, maintained using a 10% NaOH solution. The experiments were run for 72 h with 5.8 Hz agitation. Enzyme loading was 20 FPU g^{-1} glucan Celluclast 1.5L and 23 IU g^{-1} glucan Novozym 188. SSF experiment samples were analysed by HPLC for sugars, lactic acid, acetic acid and ethanol content.

2.8. Analysis

Extractive contents of the dry hemp samples were determined according to the NREL protocol [25], with the modification that samples were dried at 105 °C before and after extraction and the extractives were calculated as loss in weight by the samples. Analysis of structural carbohydrates and lignin (based on DM) were performed on the extracted dry hemp stem samples as well as on the solid fraction of the silage and on the solid fractions after pretreatment. The analyses were performed according to the NREL protocol [26]. Silage and solid fractions were washed with distilled water prior to analysis. The content of various sugars in the liquid fractions after steam pretreatment and in the liquid fraction of the untreated silage, obtained by filtration, was analysed according to the NREL protocol [27]. The difference in monomer sugar concentrations with and without dilute acid hydrolysis of the liquid samples was determined as oligomer sugars. Each sample was analysed in duplicate.

2.9. HPLC

The carbohydrate and inhibitor content in the liquid samples were analysed with an HPLC system (Shimadzu, Japan) equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was used for the separation and determination of cellobiose, glucose, mannose, arabinose, lactic acid, glycerol, acetic acid, ethanol, HMF and furfural using 5 mmol l^{-1} H_2SO_4 as the eluent at a flow rate of 0.5 ml min^{-1} and a column temperature of 65 °C. An Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) was used for the separation of cellobiose, glucose, xylose, galactose, arabinose and mannose with Millipore quality water as the eluent at a 0.6 ml min^{-1} flow rate and a column temperature of 85 °C. Calibration of the equipment for each compound was performed with 6 level calibration standards at a range of 0.15–10.0 mg ml^{-1}.

2.10. Mass balance calculations

During the experiments, process streams were quantified and analysed as described above.

The "volatile/further degraded" fraction in the mass balances was calculated on the basis of the difference in total solids loaded to the reactor and collected from the cyclone. The mass balance calculation includes all measurement errors from the process.

2.11. Statistical evaluation

The effect of fertilization and ensiling on the composition of hemp stems and solid fraction of hemp silage was investigated using Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA) One-way ANOVA followed by Tukey's HSD test was used for multiple comparison of the fibre characteristics between treatments.

Table 1 – Composition of dry hemp grown at different fertilization levels and solid fraction of hemp silage as percentage of dry weight. Mean values of duplicates are presented. Relative standard deviations in percentage are indicated in parenthesis.

	Dry hemp (115 kg ha^{-1} fertilizer)	Dry hemp (150 kg ha^{-1} fertilizer)	Dry hemp (200 kg ha^{-1} fertilizer)	Solid fraction of hemp silage
Glucan	44.1 (3.6%)	43.7 (1.9%)	43.0 (2.7%)	45.2 (0.4%)
Mannan	2.0 (3.4%)	2.1 (1.0%)	1.8 (1.7%)	2.6 (0.2%)
Xylan	10.1 (4.5%)	11.0 (1.5%)	10.3 (2.8%)	10.1 (3.4%)
Galactan	2.1 (4.4%)	2.0 (0.4%)	2.1 (1.7%)	1.7 (0.2%)
Arabinan	0.7 (4.8%)	0.6 (2.5%)	0.7 (1.4%)	b.d.l.
Acid-soluble lignin	6.5 (0.3%)	6.7 (0.1%)	6.7 (7.2%)	4.7 (3.7%)
Acid-insoluble lignin	14.5 (0.2%)	15.0 (1.3%)	15.3 (0.4%)	18.7 (2.1%)
Water extractives	13.5	11.9	11.0	n.d.
Ethanol extractives	2.7	1.4	0.8	n.d.
Total determined compounds	96.1	94.3	91.6	83.0

n.d. – not determined.
b.d.l. – below detection limit.

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3. Results and discussion

3.1. Raw material compositions

Three samples of dry hemp (only stems) from different nitrogen-fertilization levels and solid fraction of hemp silage were analysed for their composition (see Table 1). One-way ANOVA was performed to compare the fibre composition of hemp fertilized with different nitrogen levels and hemp silage. The statistical analysis has shown, that the effect of these treatments for carbohydrate content, i.e. glucan and xylan, was not significant ($p = 0.16$ and 0.48, respectively at 95% confidence limit). For acid-soluble lignin and acid-insoluble lignin, the statistical analysis has shown, that that there was a significant difference in lignin content of the materials ($p = 0.005$ and 0.0001, respectively at 95% confidence limit). Tukey's HSD test showed that the silage had a significantly different content of both acid-soluble lignin and acid-insoluble lignin while the content was not significantly different in the three samples with different fertilization level. The difference in acid-insoluble lignin content of the silage might be due to that no extractive measurement was performed on hemp silage, which may give an overestimation of this fraction. A mixture of the three dry hemp samples (equal parts of stem samples) was used for steam pretreatment due to the shortage of the feedstock. The composition used during the calculations is the average of the compositions in Table 1. The liquid fraction of hemp silage had a pH of 4.5 and contained four main components: 17.6 g l^{-1} lactic acid, 7.6 g l^{-1} acetic acid, 3.0 g l^{-1} glucose and 1.2 g l^{-1} ethanol. The dominance of lactate and acetate as fermentation products and the low pH indicate that the silage was well preserved.

3.2. Composition of WIS after pretreatment

Pretreatment of hemp stem using 2% SO_2 impregnation was performed at 205, 210 and 215 °C. Previous studies at Lund University, Department of Chemical Engineering, suggested that SO_2 impregnation and a temperature above 200 °C is needed for sufficient pretreatment of dry hemp (data not published). Table 2 shows the composition of the WIS fractions from the steam pretreated hemp stem (SPH). The solid fraction was mainly composed of glucan (65–67%) and lignin (25–30%) while hemicellulose was solubilised to a large extent. Only small differences in WIS composition were observed after steam pretreatment at 205–215 °C with SO_2 impregnation.

Table 2 – DM and WIS contents of pretreated slurry and composition of washed WIS fraction of steam pretreated (2% SO_2 impregnation) dry hemp (SPH). WIS compositions are presented as percentage of dry weight. Mean values of duplicates are presented. Relative standard deviations in percentage are indicated in parenthesis.

	205 °C	210 °C	215 °C
DM [%]	15.1	11.3	10.9
WIS [%]	12.4	8.2	8.3
Glucan	66.8 (5.3%)	66.8 (4.5%)	64.7 (3.0%)
Mannan	2.1 (0.6%)	1.8 (1.9%)	1.4 (0.4%)
Xylan	3.8 (3.0%)	3.0 (2.0%)	2.7 (1.5%)
Galactan	0.7 (4.8%)	b.d.l.	b.d.l.
Arabinan	0.2 (3.9%)	b.d.l.	b.d.l.
Acid-soluble lignin	3.8 (3.8%)	3.7 (3.1%)	3.7 (1.3%)
Acid-insoluble lignin	19.3 (5.2%)	21.2 (0.3%)	25.9 (1.4%)
Lignin ash	0.3 (3.7%)	0.4 (6.9%)	0.4 (3.5%)
Total determined compounds	96.8	96.7	98.8

b.d.l. – below detection limit.

Steam pretreatment of hemp silage was performed both with and without SO_2 impregnation. A wide range of pretreatment temperatures (190–220 °C) were tested due to the scarce available knowledge on steam pretreatment of ensiled materials. Compositions of the WIS of the steam pretreated hemp silage (SPHS) are presented in Table 3. At milder pretreatment conditions without SO_2 impregnation (190 and 200 °C), the solubilisation of the hemicellulose was not sufficient; a significant amount of xylan was present in the WIS

Table 3 – DM and WIS contents of pretreated slurry and composition of washed WIS fraction of steam pretreated hemp silage (SPHS). WIS compositions are presented as percent of dry weight. Mean values of duplicates are presented. Relative standard deviations in percentage are indicated in parenthesis.

	no SO_2				SO_2	
	190 °C	200 °C	210 °C	220 °C	200 °C	210 °C
DM [%]	8.8	10.7	10.9	10.8	12.6	11.0
WIS [%]	6.2	7.5	7.4	7.1	7.8	7.5
Glucan	57.9 (5.8%)	62.4 (1.7%)	67.1 (1.8%)	60.7 (2.3%)	68.7 (1.2%)	66.0 (0.0%)
Mannan	2.7 (9.3%)	2.5 (1.0%)	2.2 (0.0%)	1.8 (4.5%)	2.2 (4.6%)	1.8 (2.5%)
Xylan	8.4 (5.2%)	7.5 (0.1%)	1.3 (2.5%)	0.6 (4.6%)	1.3 (4.2%)	0.4 (0.2%)
Galactan	1.0 (2.8%)	0.7 (0.4%)	0.7 (5.5%)	0.5 (2.6%)	0.7 (0.7%)	0.8 (1.2%)
Arabinan	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Acid-soluble lignin	3.6 (6.4%)	3.2 (3.0%)	2.9 (3.3%)	3.3 (6.6%)	2.8 (0.3%)	2.9 (1.0%)
Acid-insoluble lignin	19.4 (8.2%)	19.1 (6.8%)	23.4 (0.5%)	26.0 (0.8%)	21.0 (0.7%)	24.7 (5.6%)
Lignin ash	2.0 (2.3%)	1.5 (2.5%)	1.3 (3.8%)	2.3 (1.7%)	1.6 (2.9%)	0.5 (3.6%)
Total determined compounds	94.9	96.8	98.9	95.2	98.0	97.1

b.d.l. – below detection limit

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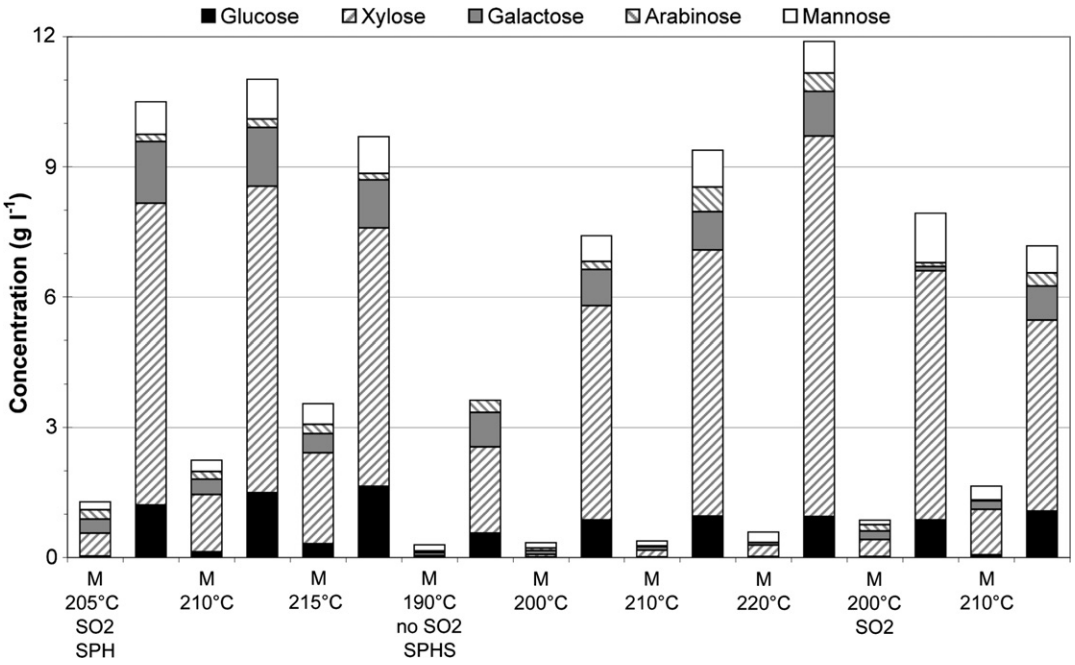

Fig. 2 – Concentrations of monomer (M) and oligomer (O) sugars (g l^{-1}) detected in the liquid fractions of SPH and SPHS (oligomers were measured as monomers after dilute acid hydrolysis, and are presented here as concentrations of monomers).

fraction after pretreatment. At higher temperatures, the xylan content of the WIS decreased to below 1.5%. However, the lower glucan content in case of the 220 °C treatment (without SO_2 impregnation) compared to the 210 °C (with or without SO_2) suggests that using this high temperature, the cellulose component of the solid fraction was partly solubilised or degraded during the pretreatment. It should also be noted that the SPHS slurry had a strong unpleasant smell after pretreatment, in contrast to the SPH slurry.

3.3. *Composition of liquid fraction after pretreatment*

Fig. 2 shows the sugar concentrations of monomeric and oligomeric sugars in the liquid fractions of the steam pretreated materials. The oligomeric sugars were detected at significantly higher concentrations than the monomeric sugars. In case of SPH the presence of the oligomers glucose, galactose and mannose were around 1.0–1.5 g l^{-1} (expressed in monomeric concentration), while the oligomeric xylan concentration was 6.0–7.0 g l^{-1}. These data suggest that during the steam pretreatment the hemicellulose fraction of the hemp stem was solubilised, but not degraded to monomeric sugars. In case of SPHS, similar trends were observed, but generally with lower oligomeric sugar concentrations. In general terms, the harsher pretreatments yielded higher sugar concentrations in the supernatants.

Concentrations of 5-hydroxymethyl-furfural (HMF) and furfural (degradation products of C6 and C5 sugars, respectively) were below reported inhibiting levels for ethanol fermentation [28] for all samples except SPH treated at 215 °C with SO_2 (Table 4). The low concentration of these sugar degradation products suggests that degradation of

Table 4 – Concentrations of organic acids and inhibitory compounds in g l^{-1} measured in the liquid fractions of pretreated SPH, SPHS and untreated hemp silage.

		Lactic acid (g l^{-1})	Acetic acid (g l^{-1})	HMF (g l^{-1})	Furfural (g l^{-1})
SPH	205 °C SO_2	0.21	1.27	0.08	0.29
	210 °C SO_2	0.31	1.93	0.16	0.51
	215 °C SO_2	0.57	3.15	0.31	0.93
SPHS	untreated	17.6	7.6	0.18	b.d.l.
	190 °C	4.86	2.20	0.09	0.06
	200 °C	5.90	3.21	0.05	0.12
	210 °C	6.10	4.02	0.07	0.22
	220 °C	5.47	5.00	0.09	0.55
	200 °C SO_2	6.85	3.88	0.07	0.21
	210 °C SO_2	6.26	4.49	0.15	0.54

b.d.l.below detection limit.

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Table 5 – Sugar concentrations, glucan conversions in enzymatic hydrolysis (expressed as percentage of the theoretical) and the overall glucose yield (including both pretreatment and enzymatic hydrolysis) for SPH and SPHS substrates pretreated at different conditions. Mean values of duplicate experiments and standard deviations are presented.

		Glucose (g l^{-1})	Glucan conversion (%)	Overall glucose yield[a] (g kg^{-1} raw material)
SPH	205 °C SO_2	10.9 ± 2.4	72.4 ± 1.4	328.9 ± 6.4
	210 °C SO_2	12.7 ± 1.4	83.1 ± 1.0	373.3 ± 4.5
	215 °C SO_2	13.3 ± 0.9	87.6 ± 6.9	383.1 ± 30.1
SPHS	190 °C	8.4 ± 0.6	64.0 ± 5.3	254.8 ± 21.1
	200 °C	8.3 ± 0.4	58.3 ± 2.9	258.5 ± 12.8
	210 °C	10.8 ± 0.7	71.0 ± 1.4	325.8 ± 6.4
	220 °C	10.6 ± 0.3	78.6 ± 2.0	318.5 ± 8.6
	200 °C SO_2	11.7 ± 0.5	74.7 ± 3.0	341.8 ± 13.7
	210 °C SO_2	13.6 ± 0.2	89.3 ± 2.0	372.3 ± 8.3

[a]for SPHS, the base of the glucose yield was ensiled hemp.

monomeric sugars from hemicellulose was not significant during the pretreatment. The ratio of sugar degradation products based on the raw material were below 1.2 g kg^{-1} for HMF and 2.0 g kg^{-1} for furfural based on untreated material, except for 215 °C SO_2 (SPH), 220 °C and 210 °C SO_2 (SPHS), where 3.7–4.5 g kg^{-1} furfural formation was observed.

A slight lactic acid formation was observed in case of SPH, while a significantly higher amount was detected in the supernatant of SPHS, which likely originated from the ensiling process. The concentration of acetic acid released during the pretreatment of hemp stem was measured at 1.2–3.1 g l^{-1}. In case of SPHS, it was significantly higher, up to 5 g l^{-1}, which corresponds to 1.8–4.1% of the initial raw material. Acetic acid originates both from the ensiling and from the acetyl groups in the hemicellulose released during the pretreatment. Weak acids have previously been found to have an inhibitory effect on ethanol production by *S. cerevisiae* [29].

3.4. *Enzymatic hydrolysis*

Enzymatic hydrolysis of the pretreated slurry was performed to evaluate the accessibility of the cellulose and thus the efficiency of the pretreatments. Enzymatic hydrolysis was performed on the pretreated slurries of SPH and SPHS. Table 5 shows the final glucose concentrations and the glucan conversions in the enzymatic hydrolysis as well as the overall glucose yield including both pretreatment and enzymatic hydrolysis. For SPH pretreatment at 215 °C resulted in the highest glucan conversion and overall glucose yield, but it should be kept in mind that this material contained high concentrations of furfural and HMF and thus the ethanol fermentation might be significantly inhibited. Pretreatment at 210 °C gave nearly as high glucan conversion and glucose yield but lower levels of inhibitors. For SPHS pretreatment at 210 °C for 5 min with SO_2 addition gave the highest overall glucose yield among the conditions investigated. Lower temperatures and pretreatments without catalyst resulted insufficient glucan conversion, therefore lower glucan yields. For both SPH and SPHS pretreatment at 210 °C using SO_2 catalyst resulted in a considerable increase in glucan breakdown, resulting in a glucose yield of 373 and 372 g kg^{-1}, respectively.

Although hydrolysis was performed using equal substrate concentrations (2% WIS), higher glucose concentration does not necessarily mean higher glucan conversion, as the compositions of the WIS fractions differ (Tables 2 and 3). The availability of the glucan varied in different samples, for instance in the case of SPHS pretreated without SO_2 at 210 °C, where the glucan content of the WIS was as high as for the SPH treated with SO_2 at the same temperature (data in Tables 2 and 3), and the conversion was significantly lower for SPHS (Table 5).

Fig. 3 shows conversion values reached in enzymatic hydrolysis as a function of pretreatment temperature. When SO_2 impregnation was applied (closed symbols), the increase of the temperature resulted in more remarkable increased glucan conversion compared to pretreatments without the acid catalyst.

3.5. *Mass balance analysis*

Mass balance analyses were performed for both the pretreatment alone and in combination with enzymatic hydrolysis. Fig. 4A and B show the carbohydrate recoveries after steam pretreatment for hemp stem and hemp silage, respectively. In the calculations for the pretreatment, the amount of ensiled hemp was taken as 100%, i.e., possible loss during ensiling was not taken into consideration. The exact

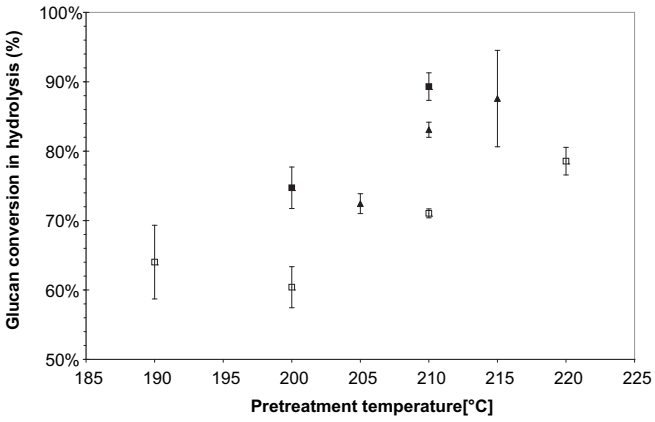

Fig. 3 – Glucan conversion in enzymatic hydrolysis of steam pretreated hemp and hemp silage as a function of pretreatment temperature. Mean values of duplicate experiments and standard deviations are presented (steam pretreated hemp with SO_2 (▲); steam pretreated hemp silage with (■) and without (□) SO_2).

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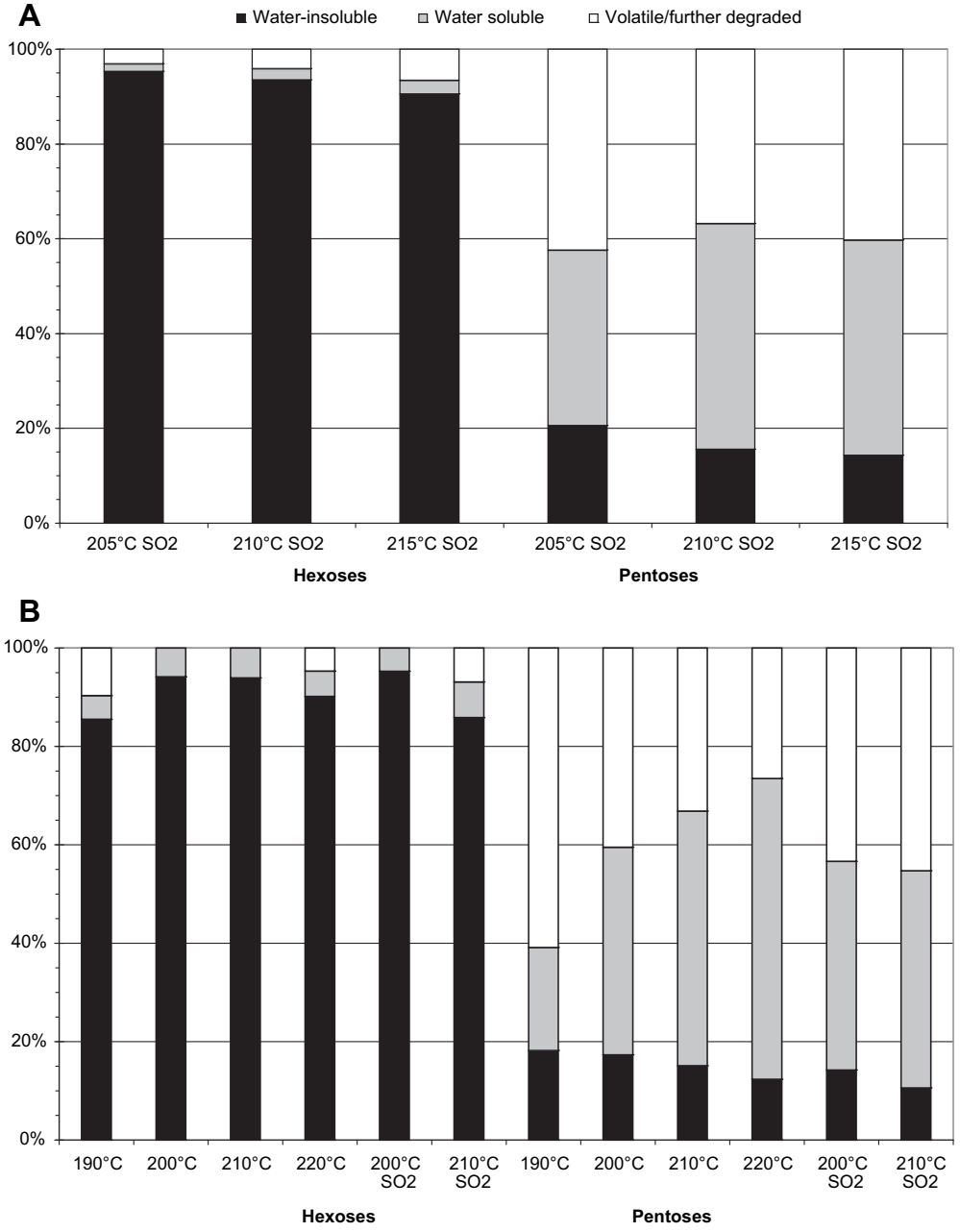

Fig. 4 – Hexose and pentose recoveries after steam pretreatment of dry hemp (A) and hemp silage (B) as percentage of the theoretical. Values are based on carbohydrates in the raw material.

change in DM and energy content during ensiling is difficult to determine if the mass and composition of the material is not determined before and after ensiling [30]. The mass was not determined in the farm scale ensiling used. Based on the amount of solubilised sugars and fermentation products (i.e. lactic acid, acetic acid, glucose and ethanol) measured in the liquid of the silage, a rough estimation of the material balance of ensiling can be performed, which shows that during the ensiling, approximately 8% of the raw material was turned into these products.

SPH hexoses (mainly glucan) remained in the solid fraction (90–95%), and only 1.5–4.0% were solubilised (Fig. 4A). Further degradation of hexoses was not significant. In the case of pentoses (mainly xylan) only 15–20% of the initial amount remained in the solid fraction. Only 36–48% of the xylan was transferred into the liquid fraction, and the amount of the further-degraded or not-determined material was rather high, 36–42%. Similar mass balances have been achieved for other lignocellulosic materials (corn stover, salix, spruce) [3]. The lowest amount of further-degraded/not-determined compounds and the highest solubilisation of pentoses were obtained with pretreatment at 210 °C for 5 min. For SPHS, similar trends were observed concerning the recovery after pretreatment (Fig. 4B). Hexoses remained in the solid fraction (86–96%), and only a minor part was solubilised (5–7%) or further degraded (0–10%). A minor part of the initial xylan

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(10–18%) from SPHS remained in the solid fraction, while 20–75% was solubilised, and a large amount (26–60%) was further degraded or not determined. The reason for the high amount of these compounds for pretreatment at 190 °C is probably that this was the least severe condition which has led to poor pretreatment and rather heterogenous material. The samples taken for analysis could have been non-representative and add an error for mass balance calculation. In this comparison it should be kept in mind that only stems were used from dry hemp while hemp silage contained both stems and leaves.

Fig. 5 shows the mass balances both in the pretreatment and the enzymatic hydrolysis for the different experimental setups. The fractions are represented as percentage of the initial dry raw material and are defined as: i) the water-insoluble fraction (mainly lignin), which can be further utilised as solid fuel; ii) glucan remained in the solid residue after hydrolysis; iii) glucan solubilised during the enzymatic hydrolysis; iv) sugars solubilised in the steam pretreatment; and v) volatile and further degraded compounds, which were not accounted for. The goal of the experiments was to maximise yield of solubilised glucose in the enzymatic hydrolysis of the steam pretreated material (grey part of the bars in the figure).

In the case of dry hemp (Fig. 5 and Table 5) the maximal glucose yields were similar for pretreatment at 210 °C and 215 °C with SO_2 impregnation (373 and 383 g kg^{-1}, respectively), but there was a significant difference in the amount of solubilised hemicellulose sugars (249 and 199 g kg^{-1}). Both pretreatment conditions were found to be efficient for improving cellulose hydrolysis, but the lower temperature resulted in less inhibitor formation. As the process economy is strongly affected by the utilisation of the hemicellulose fraction [31], pretreatment at 210 °C for 5 min after SO_2 impregnation was found to be the best condition for hemp stem. The optimal pretreatment condition (210/5 min/2% SO2) for hemp and hemp silage is similar to pretreatment conditions obtained for agricultural lignocellulosics (200 °C/10 min/2% SO_2 for corn stover [16], 190 °C/10 min/0.2% H_2SO_4 for wheat straw [5]; or for woods (210 °C/5 min/2.5%SO_2 for softwood [32], 205 °C/4 min/2% SO_2 for hardwood [33].

For hemp silage (Fig. 5 and Table 5), the highest glucose yield (372 g kg^{-1}) was obtained by pretreatment at 210 °C for 5 min with SO_2 impregnation, followed by the pretreatment at 200 °C with SO_2 impregnation (342 g kg^{-1}). The highest yield of sugars solubilised during the pretreatment was also obtained in the case of 210 °C with SO_2 impregnation, therefore this was chosen as the best pretreatment condition. Thus, based on glucose yields in enzymatic hydrolysis, steam pretreatment at 210 °C for 5 min with SO_2 impregnation was chosen as the optimal pretreatment conditions both for dry hemp and hemp silage.

3.6. *Results of SSF of the whole slurry and of the separated fibre*

SSF of SPH and SPHS was performed using either the whole slurry or the separated solid fraction of the materials pretreated using the selected optimal pretreatment conditions: 2% SO_2 impregnation followed by 210 °C/5 min treatment. Fig. 6 shows the glucose, xylose and ethanol concentrations during the 72 h of the SSF. Decrease of xylose concentration is an indicator for microbial contamination of the fermentation, as *S. cerevisiae* can only consume C6 sugars. Neither decrease of xylose concentration nor lactic acid production (data not shown) was observed during the SSF experiments. With SPH, the separated fibre resulted in a slightly higher ethanol concentration compared to the whole slurry (Fig. 6A). Final ethanol concentrations in the case of whole slurry and the separated fibre were determined to 18.4 g l^{-1} and 21.3 g l^{-1},

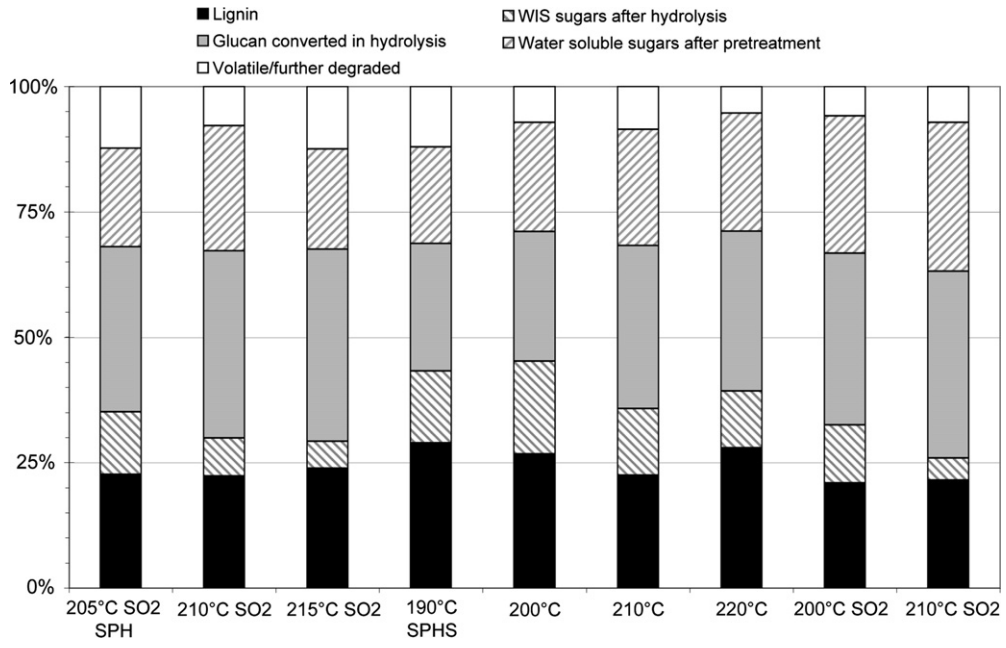

Fig. 5 – Lignin and carbohydrate fractions after steam pretreatment and enzymatic hydrolysis of dry hemp (SPH) and hemp silage (SPHS), as percentage of the dry weight of raw material.

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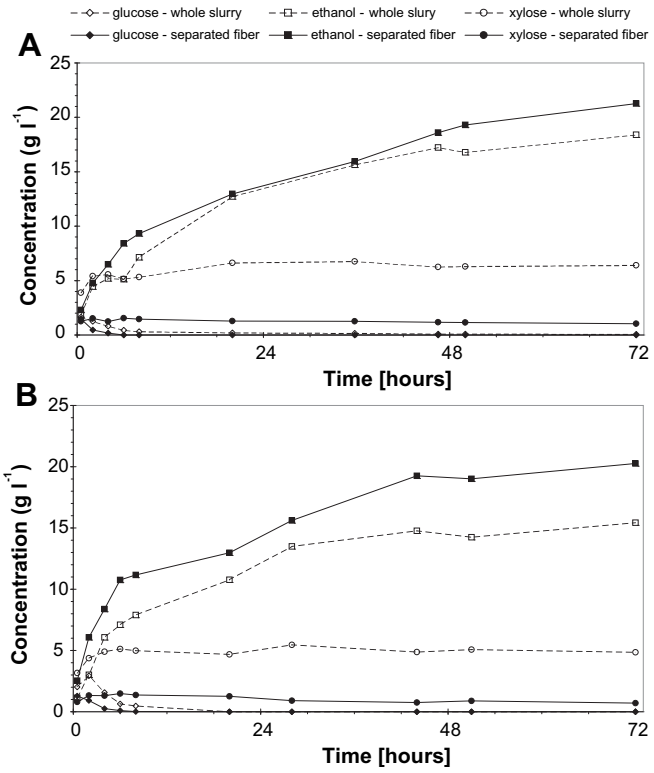

Fig. 6 – Concentrations of glucose, xylose and ethanol in SSF of SPH (A) and SPHS (B). Substrates were pretreated at 210 °C for 5 min using 2% SO_2 impregnation (open symbols and dashed lines – whole slurry, closed symbols and continuous lines– separated fibre).

respectively. This corresponds to a total process yield of 148 and 171 g kg^{-1} ethanol based on raw dry hemp DM, respectively. The overall ethanol yields were 62.4% and 74.1% of the theoretical maximum based on the glucan content in the raw material.

With SPHS, the difference between the performance of the whole slurry and the separated fibre was more pronounced (Fig. 6B). The final ethanol concentrations were 15.4 and 20.3 g l^{-1}, respectively. The total ethanol yields were 125 and 163 g kg^{-1} based on hemp silage DM, respectively, corresponding to 53.4% and 71.2% of the theoretical. The significant difference might be caused by the inhibitory effect of the organic acids present in the whole slurry [28,29]. At the beginning of the fermentation of the whole slurry, 6.0 g l^{-1} lactic acid and 6.8 g l^{-1} acetic acid were present and the concentrations of these compounds were constant during the process. The presence of acetic acid is rather important, as its pK_a value is rather close to the pH of the SSF. The inhibitory effect of the organic acids is connected with the protonated form; because it can diffuse across the plasma membrane [28] (36% of the acetic acid and 7% of the lactic acid is in protonated form at pH 5). The concentration of protonated acetic acid was calculated to be 40.8 mmol l^{-1}.

Maize silage has previously been tested for ethanol production in SSF [34]. The yield for wet-oxidised (WO) maize silage was found to be 83% (of the theoretical maximum), which corresponds to 308 g kg^{-1} ethanol based on DM WO maize silage (82% of the theoretical maximum), which is slightly less than what has been found for WO corn stover [35]. It should be noted, that during WO, beside hemicellulose, a part of the lignin also degrades, which results in a pretreated material rich in cellulose. In the case of wheat straw, 132 g kg^{-1} ethanol based on dry wheat straw SSF yield was achieved [5], while in case of *Salix*, 201 g kg^{-1} ethanol based on dry wood yield was achieved, and the ethanol yield in SSF was higher compared to the theoretical maximum than in the present study [36].

The results obtained both with SPH and SPHS show that separation of fibre and liquid fraction prior to SSF is advantageous. In the case of SPHS, the effect was more pronounced compared to SPH. Separation is beneficial not only because of the removal of inhibitory compounds with the liquid fraction, but also a new fraction arises containing mainly C5 sugars (mono- and oligomers) some C6 sugars and other organic compounds like acetic acid, furfural and HMF, which can be utilised separately, e.g., for biogas production.

4. Conclusions

Steam pretreatment with an SO_2 catalyst was shown to be an efficient pretreatment method prior to ethanol production from both dry hemp and hemp silage. In both cases impregnation with 2% SO_2 followed by steam pretreatment at 210 °C for 5 min were found to be the most suitable pretreatment conditions within the investigated intervals. No significant effect of the ensiling process was detected at the optimal conditions for conversion of hemp to ethanol. In further experiments, utilisation of the liquid fraction and SSF residue for biogas production to increase the energy recovery will be investigated.

Acknowledgements

The Environmental Fund of Region Scania, the Hungarian National Research Fund (OTKA – K 72710) and The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning are kindly acknowledged for their financial support. Enzymes were kindly donated by Novozymes A/S (Bagsværd, Denmark). Thanks to Thomas Prade at the Swedish University of Agricultural Sciences for providing dry hemp.

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