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Hot water extraction and steam explosion as pretreatments for ethanol production from spruce bark

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HIGHLIGHTS

- Hot water extraction (80 °C) and steam explosion were studied as pretreatments.
- Steam explosion of spruce bark should be carried out without acid catalyst.
- Hot water extraction is a suitable pretreatment for spruce bark with right enzymes.
- Ethanol production from pretreated enzymatically hydrolysed barks was efficient.
- Spruce bark is a potential feedstock for the production of lignocellulosic ethanol.

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ABSTRACT

Spruce bark is a source of interesting polyphenolic compounds and also a potential but little studied feedstock for sugar route biorefinery processes. Enzymatic hydrolysis and fermentation of spruce bark sugars to ethanol were studied after three different pretreatments: steam explosion (SE), hot water extraction (HWE) at 80 °C, and sequential hot water extraction and steam explosion (HWE + SE), and the recovery of different components was determined during the pretreatments. The best steam explosion conditions were 5 min at 190 °C without acid catalyst based on the efficiency of enzymatic hydrolysis of the material. However, when pectinase was included in the enzyme mixture, the hydrolysis rate and yield of HWE bark was as good as that of SE and HWE + SE barks. Ethanol was produced efficiently with the yeast *Saccharomyces cerevisiae* from the pretreated and hydrolysed materials suggesting the suitability of spruce bark to various lignocellulosic ethanol process concepts.

1. Introduction

The utilization of biomass for the production of transport fuels, chemicals and materials is increasing because of fluctuating price and limited availability of oil, and the need to reduce greenhouse gas emissions. Upgrading of biomass to value-added products may also provide additional profits when compared to its combustion to heat and electricity.

Bark, which constitutes of ca. 12% of the total weight of a tree (Surminski, 2007), is an abundantly available biomass feedstock that is already efficiently collected at pulp, paper, and sawmill sites. With annual industrial wood consumption of 70 Mm^3 (ca. 35 Mt), approximately 3–5 Mt of industrial bark is produced per annum alone in Finland (Finnish Forest Industries Federation, 2011). Most of this bark is combusted for electricity and heat at mill site. In addition to debarking lines producing mixed bark varieties, also pure streams of e.g. spruce and birch bark are available.

Spruce bark and bark in general have been surprisingly little studied recently as a lignocellulosic feedstock for 2nd generation biorefinery processes. The composition of spruce bark is very complex and not well understood as it contains several compounds such as polyphenols and extractives which are not found in wood (Laks, 1991). Bark is not generally considered as a very good source of fermentable sugars because of the high amount of lignin and extractives in the material (Kim et al., 2005; Robinson et al., 2002; Torget et al., 1991; Vazquez et al., 1987). In fact, some non-lignin derived compounds condense and precipitate in sulphuric acid showing up as Klason lignin and making the analysis of true bark lignin difficult (Laks, 1991). Spruce bark contains ca. 19% cellulose and a varying amount of non-cellulosic sugars that are present as free sugars and bound in hemicellulose, pectin and glycosides (Laks, 1991). Most abundant non-cellulosic monosaccharides are glucose, arabinose, galacturonic acid, mannose, xylose and galactose (Le Normand et al., 2011). Additional challenges come from

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high seasonal variation in the bark composition, and the fact that industrial bark may be stored for months and wetted and pressed during debarking, which alters its composition compared to that of fresh bark.

However, some spruce bark extractives, such as lignans, stilbenes, and flavonoids, have been extensively studied because of their antioxidative and biologically active properties (Manners and Swan, 1971; Co et al., 2012). These components are all at least partly extractable with water. The total extraction yield with pressurized or unpressurized water from bark is rather high, 19–35% of bark dry matter (Co et al., 2012; David and Atarhouch, 1987), and unpressurized water extraction can be used to fractionate for example water soluble polyphenols (tannins), stilbenes, stilbene glycosides and hemicellulose from bark (Kylliäinen and Holmblom, 2004). Spruce bark tannins are interesting compounds that could find use for example in adhesives and insulating foam applications (Pizzi, 2006; Tondi and Pizzi, 2009).

In order to design a profitable biorefinery, all components of the feedstock must be efficiently utilized. In this study, the suitability of spruce bark to bioethanol production was studied as such and after extraction of tannins with hot water. Steam explosion was studied as a pretreatment for industrial bark, and the need for further pretreatment after hot water extraction to obtain efficient enzymatic hydrolysis was examined. Finally, the suitability of the materials for ethanol production was studied in industrially relevant conditions.

2. Methods

2.1. Materials

Industrial spruce bark was collected from UPM-Kymmene Oyj Jämsänkoski mill debarking unit (Finland). The dry matter content of the bark was ca. 32%. The material was stored 1–2 weeks outside (subzero temperature) before pretreatment and afterwards below −20 °C.

Enzymes Celluclast 1.5 L, Novozym 188 and Pectinex Ultra SP-L were purchased from Novozymes A/S (Bagsvaerd, Denmark). Filter paper unit (FPU) activity of Celluclast 1.5 L was 49 FPU mL^{-1} measured according to Ghose (1987), β-glucosidase activity of Novozym 188 was 5910 nkat mL^{-1} measured according Bailey and Nevalainen (1981) and pectinase activity of Pectinex Ultra SP-L was 81,900 nkat mL^{-1} measured according to Bailey and Pessa (1989). In addition the xylanase activity (Bailey et al., 1992) and mannanase activity (Stålbrand et al., 1993) of Pectinex Ultra SP-L was measured.

Saccharomyces cerevisiae strain VTT-B-08014 was used in the fermentation experiments. The strain was pre-cultivated aerobically overnight in YP medium supplemented with 2% glucose at 30 °C, and overnight in YP medium supplemented with 5% glucose at 30 °C before inoculation. Yeast cells were washed with 0.1 M sodium phosphate solution pH 7 and diluted to Yeast Nitrogen Base before inoculation.

All used chemicals were of analytical grade.

2.2. Analysis of the substrate composition

Neutral monosaccharide composition of the materials was determined with high-performance anion exchange chromatography with pulsed amperometric detection after a two-step hydrolysis with sulphuric acid according to the NREL Laboratory Analytical Procedure. The analysis was run on Dionex ICS-3000 liquid chromatograph (Dionex Corp., Sunnyvale, CA) according to Tenkanen and Siika-aho (2000) with minor modifications (equilibration with 15 mM NaOH, isocratic elution with water). Acidic monosaccharides galacturonic acid, 4-*O*-methyl glucuronic acid and glucuronic acid were analysed from solid samples after metanolysis and silylation by gas chromatography of the silylated sugar monomers according to Sundberg et al. (1996).

Extractives content was determined gravimetrically after 5 h extraction with boiling heptane in soxhlet. The apparent lignin content was determined after the removal of extractives according to the NREL Laboratory Analytical Procedure with slight modification as previously described by Varnai et al. (2010). Lignin content was calculated as the sum of the Klason- and acid-soluble lignin which was determined at 203 nm with reference absorptivity of 128 g L^{-1} cm^{-1}. The ash content of the samples was measured gravimetrically after combustion in a furnace at 550 °C.

2.3. Pretreatments

Steam explosion (SE) was conducted with and without an acid catalyst. Acid catalyst was added by impregnating the bark prior to steam explosion in 0.5% H_2SO_4 solution for 30 min after which the bark was filtered on a 6 μm wire to ca. 18% dry matter content. Steam explosion of the acid-impregnated and untreated bark was carried out in a 10 L pressurized vessel. The vessel was heated with steam to the desired temperature (190 °C/11.6 bar or 205 °C/16.3 bar) and maintained there for 5 min before releasing the pressure to a collection vessel. In the steam explosion of the hot water extracted material the bark was contained in a 5 L metal wire cage inside the vessel to prevent solid material losses to the collection vessel and to decrease material wetting. The steam condensate was vented out of the reactor during heating. After steam explosion of untreated bark and acid-impregnated bark the dry matter content was from 12% to 17%, and after steam explosion of hot water extracted bark it was 27%. Steam exploded materials were washed four times with hot tap water to remove soluble sugars before hydrolysis experiments.

Hot water extraction (HWE) of bark was carried out in a 250 L horizontal Drais reactor with ploughshare mixing (Dreiswerke, Germany). Consistency during the extraction was 8% and mixing speed 100 rpm. The extraction was carried out in two steps including first a 120 min cold water extraction at 20 °C, after which the extract was let out and replaced with hot water. Hot water extraction was conducted at 80 °C for 120 min. After extraction the bark was pressed to 21% dry matter content. Part of this bark was steam exploded without acid catalyst at 190 °C producing sequentially extracted and steam exploded (HWE + SE) bark.

Another batch of hot water extracted bark was made for fermentation experiments with only 120 min hot water extraction at 80 °C. Experimental data from a large extraction series (data not shown) showed that the amount and composition of the extract as well as the carbohydrate composition of the extracted bark coming from sequential cold and hot water extraction of spruce bark and single 120 min hot water extraction were very similar. Thus in this study both batches of hot water extracted bark can be considered comparable in their composition and behaviour in hydrolysis and fermentation.

2.4. Hydrolysis experiments

Enzymatic hydrolysis experiments were carried out with combinations of different dosages of commercial cellulase, β-glucosidase and pectinase products. Cellulase Celluclast 1.5 L was dosed 10 FPU g^{-1} (low dosage) or 25 FPU g^{-1} (high dosage), β-glucosidase Novozym 188 was dosed 200 nkat g^{-1} (low dosage) or 500 nkat g^{-1} (high dosage) and pectinase Pectinex Ultra SP-L was dosed 0 or 5000 nkat g^{-1} (high dosage). High dosage of Celluclast 1.5 L brought additional 220 nkat g^{-1} and low dosage additional 90 nkat g^{-1} β-glucosidase activity to the mixture on top of the β-glucosidase activity obtained from Novozym 188. The β-glucosi-

dase activity of Pectinex Ultra SP-L and the pectinase activities of Celluclast 1.5 L and Novozym 188 were insignificant (<5%) compared to the dosed activities. High dosage of Pectinex Ultra SP-L brought 115 nkat g^{-1} xylanase activity and 1140 nkat g^{-1} mannanase activity to the enzyme mixture.

Hydrolysis experiments were performed in test tubes in 3 mL reaction volume at 1% dry matter content in 50 mM sodium acetate buffer pH 5, with 0.02% sodium azide to control microbial action. The tubes were placed in a water bath at 45 °C and magnetic mixing was applied at 300 rpm. The materials were not dried after pretreatment but used as such. Hydrolysis was followed by terminating the reaction by boiling the tubes for 10 min after 4, 24 and 48 h from the start. Hydrolysis yield was analysed by measuring reducing sugars with 3,5-dinitrosalicylic acid (DNS) method according to Bernfeld (1955) and by analysing neutral monosaccharides with HPAEC-PAD as described above. Solubilised acidic monosaccharides were analysed similarly as neutral monosaccharides but with a modified gradient (Tenkanen and Siika-aho, 2000). Triplicate tubes were prepared for each sampling point.

2.5. Fermentation

The unwashed pretreated spruce bark was mixed with 0.1 M sodium citrate buffer at pH 5 before moving it to the fermenter. Before starting the fermentations, pH of spruce bark was adjusted to 5 with 10 M NaOH and during the fermentations it was adjusted with 5 M KOH and 5 M H_3PO_4.

The experiments were carried out in a Biostat CT-DCU fermenter by B. Braun (Germany) as a simultaneous saccharification and fermentation (SSF) combined with a short prehydrolysis. Fermenter was equipped with a powerful marine propeller for mixing and a special samples collector device, which enabled sampling during high consistency fermentations. Working volume of 1.5 L was used. Redox balance, dissolved oxygen and pH were measured in situ. Oxygen, carbon dioxide and ethanol were analysed from the exhaust gases with photoacoustic measurement (1309 Multipoint sampler, 1313 Fermentation monitor and LumaSoft Gas Multi Point 7850 software) manufactured by Innova Air Tech Instruments A/S (Denmark). The fermenter was sterilized before the experiment.

A 6 h prehydrolysis in 15% dry matter content was conducted at 45 °C, with 1 L min^{-1} aeration (air) and 100–150 rpm mixing with 10 FPU g^{-1} Celluclast 1.5 L, 500 nkat g^{-1} Novozym 188 and 5000 nkat g^{-1} Pectinex Ultra SP-L.

The consequent simultaneous saccharification and fermentation was carried out at 35 °C under 1 L min^{-1} nitrogen gas flow. Cell density in pre-cultivated yeast was calculated from absorbance at 660 nm (OD_{660}) and the fermenter was inoculated with 3.0 g L^{-1} yeast (OD 10). Samples were withdrawn from the reactor and dry matter content, main metabolic products and monosaccharides were analysed from the supernatant of centrifuged samples using Dionex ICS-3000 liquid chromatograph. Fast Acid Analysis Column (Bio-Rad Laboratories, USA) and Aminex HPX-87H column (Bio-Rad Laboratories, USA) were used with 0.3 mL min^{-1} flow of 5 mM H_2SO_4 as an eluent. Impurities were removed by Cation-H Refill Cartridges (Bio-Rad Laboratories, USA) as a pre-column. Waters 2487 dual λ absorbance detector (wavelength 210 nm) and Waters 2414 refractive index (RI) detector were used.

3. Results and discussion

3.1. Pretreatment of spruce bark

Three types of pretreatments were carried out for spruce bark. The composition of the starting material and the solid fractions after treatments were analysed and component yields compared in order to evaluate how the treatments affected the feedstock composition. First the effects of acid catalyst and temperature conditions during steam explosion were shortly investigated. Mass and component yields in the steam explosion of spruce bark for 5 min at 190 °C (11.6 bar) or 205 °C (16.3 bar) with or without acid catalyst are presented in Table 1. Yields were calculated as total mass of the solid residue or component mass in the solid residue after treatment divided by the original mass or component mass before treatment. Total mass yields are rather low due to material losses during steam explosion and the following washing step.

According to the results, steam explosion dissolved or degraded hemicelluloses and increased the relative share of glucan in the solid residue compared to untreated bark. Steam explosion with acid catalyst resulted in more complete hemicellulose degradation. The relative amount of lipophilic extractives increased slightly during steam explosion whereas some ash was lost especially when using acid catalyst. More severe treatment conditions (impregnation in dilute acid, higher temperature) resulted in an increase of compounds analysed as Klason lignin in the solid residue. It has been suggested that water-soluble bark phenolics such as tannins are rendered insoluble in dilute acid treatment and show up as Klason lignin in the insoluble residue (Torget et al., 1991). High temperature may promote this phenomenon and result in increased Klason lignin content in the more severely pretreated bark samples. Robinson et al. (2002) conducted SO_2 catalysed steam explosions with mixed Douglas fir wood and bark and they observed an increase in mass yield with increasing share of bark in the feedstock mix. This could support the hypothesis that otherwise water-soluble bark components become insoluble by acid pretreatment and remain in the solid residue.

As presented later in Section 3.2, those steam explosion conditions that provided the best hydrolysability (190 °C, no acid catalyst) were used in further experiments where steam explosion and hot water extraction were compared as pretreatment methods. The hypothesis was that hot water extraction at 80 °C would not be a sufficient pretreatment to obtain high enzyme hydrolysis yields. Therefore, a sequential hot water extraction and steam explosion treatment was also carried out for spruce bark. According to the results presented in Table 2 hot water extraction dissolved and degraded less polysaccharides than steam explosion. Especially acidic polysaccharides consisting mostly of galacturonic acid were removed during steam explosion. Apparent lignin content in hot water extracted bark was lower than in untreated and steam exploded bark suggesting partial removal of water soluble phenolics during hot water extraction. Ash was mostly preserved in the treatments, but more extractives were removed in hot water extraction than in steam explosion.

Steam explosion after hot water extraction dissolved and possibly degraded neutral and acidic hemicelluloses resulting in a very similar total yield compared to steam explosion alone except with less extractives and ash. Thus, the composition of the hot water extracted and steam exploded bark was very similar to steam exploded bark with relatively high glucan content and less acidic polysaccharides compared to the starting material (Table 2). Hot water extracted bark on the other hand was similar to the untreated spruce bark with lower glucan and higher hemicellulose content. The neutral hemicellulosic polysaccharides in the untreated bark were composed of 36% arabinan, 20% xylan, 26% mannan, 16% galactan and 3% rhamnan, and acidic hemicelluloses were composed of 89% galacturonic acid, 6% 4-*O*-methyl glucuronic acid and 5% glucuronic acid. Steam exploded bark contained less arabinan (12%) and more xylan (40%) than the hot water extracted and steam exploded material (25% and 29%, respectively). On the basis of the comparison of metanolysis and acid hydrolysis products, 79% of the glucan in the untreated material was cellulose (β-1,4-glucan) and the rest was part of hemicellulose or attached

Table 1
Composition (*C*) of spruce bark as % of dry matter and component and total mass yields (*Y*) as % of theoretical after steam explosion for 5 min at 190 °C or 205 °C with or without acid catalyst.

Component	Untreated bark	Steam exploded samples							
		190 °C, no acid		190 °C, acid		205 °C, no acid		205 °C, acid	
	C	*C*	*Y*	*C*	*Y*	*C*	*Y*	*C*	*Y*
Glucan	28.1	38.8	85	41	81	40.3	91	37.4	83
Other polysaccharides	13.6	9.1	41	3.6	15	6.7	31	3.0	14
Lignin	35.8	33.6	63	37.9	64	38.3	74	44.6	85
Extractives	4.5	6.6	91	6.7	83	6.2	88	6.6	92
Ash	3.6	3.6	62	2.4	37	3.2	56	2.4	41
Mass yield			62		55		63		62

Table 2
Composition (*C*) of spruce bark as % of dry matter, and component and total mass yields (*Y*) after different pretreatments (SE = steam explosion, HWE = hot water extraction, HWE + SE = sequential hot water extraction and steam explosion).

Component	Untreated	SE		HWE		SE after HWE	HWE + SE	
	C	*C*	*Y*	*C*	*Y*	*Y*	*C*	*Y*
Glucan	28.1	38.8	85	33.3	88	99	39.5	87
Other neutral polysaccharides	13.6	9.1	41	13.7	74	57	9.3	42
Acid polysaccharides	7.3	3.0	25	5.9	60	47	3.3	28
Lignin	32.8	33.6	63	28.2	64	98	32.9	62
Extractives	4.5	6.6	91	3.5	59	104	4.4	61
Ash	3.6	3.6	62	3.3	69	72	2.8	49
Total mass yield			62		74	84		62

as glycosides for example to stilbenes. Similar ratios were obtained for the pretreated materials (69–86%).

3.2. Effect of steam explosion conditions on the hydrolysability of spruce bark

Steam explosion was performed on industrial spruce bark at two temperatures (190 or 205 °C) with or without pre-impregnation in 0.5% sulphuric acid, and the hydrolysability of the materials was assayed with commercial hydrolytic enzymes. High or low dosages of cellulase (25 or 10 FPU g^{-1}) and β-glucosidase (500 or 200 nkat g^{-1}) were used. Experiments with and without pectinase (5000 nkat g^{-1}) were also carried out. Fig. 1 presents the results from the hydrolysis experiments. The yields based on reducing sugar assay are calculated as percentage of polysaccharides released as neutral monosaccharides in total acid hydrolysis.

The best conditions for steam explosion of spruce bark can be distinguished from the results. Highest yields and faster hydrolysis was obtained with bark treated at 190 °C without acid catalyst. Hydrolysis yields from 75% to 84% obtained in our studies are comparable to published hydrolysis results obtained for steam pretreated softwood (Monavari et al., 2009; Söderström et al., 2003). The second best steam explosion parameters appeared to be 205 °C without acid catalyst, whereas the acid catalysed materials produced lower hydrolysis rates and yields especially with lower enzyme dosages. Worst hydrolysis results were obtained with untreated material, especially with lower enzyme dosages. However, compared to the hydrolysis of native wood the hydrolysis yield of untreated bark with high enzyme dosages was surprisingly high, 68%.

According to the results, a more severe pretreatment, i.e. the use of acid or higher temperature, decreased the hydrolysis rate and yield. This result is quite the opposite to what is reported for other woody biomasses. Higher pretreatment temperature increases the hydrolysability of for example poplar wood (Schütt et al., 2011), Douglas Fir (Nakagame et al., 2011) and spruce wood (Fang et al., 2011). Possible reason for the finding could be that water-soluble phenolic compounds in the bark, such as tannins, are condensed to insoluble material analysed as Klason lignin by the action of acid and high temperatures so that they cover cellulose surfaces and reduce their accessibility to hydrolytic enzymes. This suggestion is supported by the findings of David and Atarhouch (1987) who obtained lower hydrolysis yields from spruce bark refluxed with dilute H_2SO_4 compared to bark extracted with boiling water.

High cellulase and β-glucosidase dosages improved the hydrolysis rate and final hydrolysis yields, especially when harsh pretreatment conditions were used or the materials were not pretreated at all. The addition of pectinase to the enzyme mixture produced a similar although a smaller effect.

Overall, according to the results presented here steam explosion at 190 °C without additional acid was an effective pretreatment for spruce bark as it more than doubled the hydrolysis yield of the material with low dosages of cellulase and β-glucosidase.

3.3. Comparison of the effect of pretreatments on the hydrolysability of spruce bark

Steam explosion (SE) is not and ideal pretreatment process due to high investment and running costs and possible formation of inhibitors at high temperature during the treatment. It is also not suitable for fractionation and isolation of water soluble polyphenolic components from spruce bark, because the components are not extracted efficiently into the small amount of condensate and the high temperature may cause degradation of the desired compounds (Gaugler and Grigsby, 2009). Unpressurized hot water extraction (HWE) on the other hand allows separation of water-soluble tannins from bark material with less degradation (Kaijaluoto et al., 2010). We investigated whether hot water extraction is a sufficient pretreatment for spruce bark prior to enzyme hydrolysis and fermentation to ethanol or whether additional steam explosion is required. The enzymatic hydrolysis rate and yield of steam exploded (SE), hot water extracted (HWE) and sequentially hot water extracted and steam exploded (HWE + SE) spruce bark were

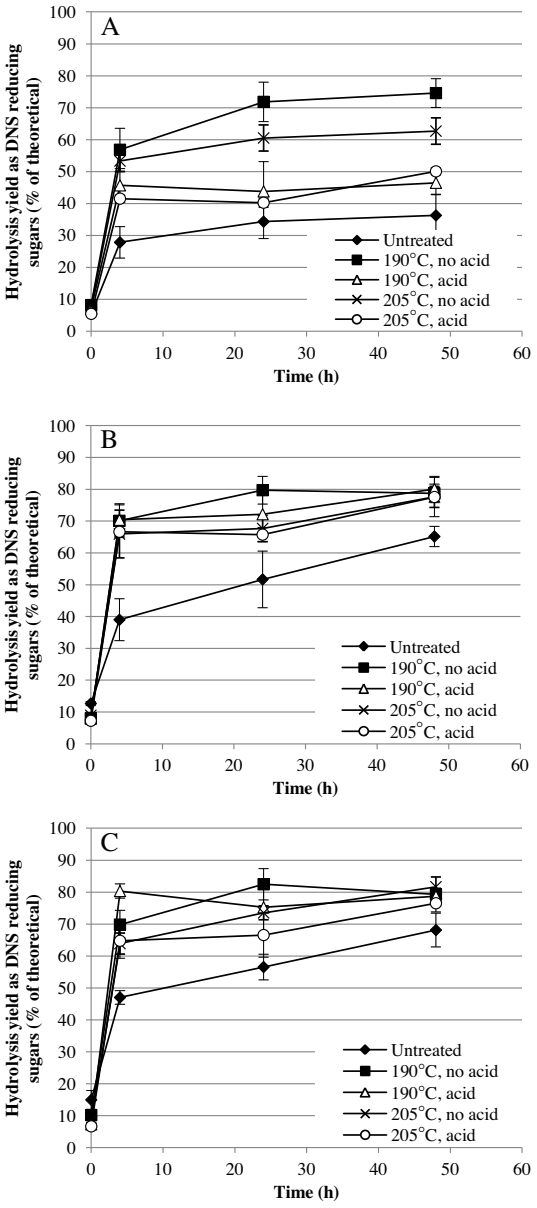

Fig. 1. Hydrolysis yield of untreated and steam exploded spruce bark in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. (A) Low dosage of cellulase and β-glucosidase. (B) High dosage of cellulase and β-glucosidase. (C) High dosage of cellulase, β-glucosidase and pectinase.

compared (Fig. 2). The yields based on reducing sugar assay were calculated as percentage of the sum of monosaccharides released in acid hydrolysis (neutral monosaccharides) and methanolysis (acidic monosaccharides) from the solid materials.

SE and HWE + SE barks behaved similarly in hydrolysis although SE bark appeared to hydrolyse somewhat faster with high enzyme dosage. This similar behaviour was expected for these two bark materials as their chemical composition was also similar (Chapter 3.1). HWE bark differed from these two as it hydrolysed slower and the final yield was 22% lower with low enzyme dosages. High cellulase and β-glucosidase dosages improved the hydrolysis of HWE bark but only the addition of pectinase significantly improved its hydrolysis to a comparable level with the two other pretreated materials.

When 500 nkat g^{-1} β-glucosidase and 5000 nkat g^{-1} pectinase but no cellulase was used, the hydrolysis yield was the highest for HWE bark (33% or carbohydrates) and less for SE (28%) and HWE + SE bark (27%) supporting the hydrolysis results with cellulase, β-glucosidase and pectinase (data not shown).

The positive effect of pectinase on the hydrolysis of HWE spruce bark was evident also when low cellulase and high β-glucosidase dosage was used (Fig. 3). Additional pectinase improved the hydrolysis of HWE bark to a comparable level with SE bark. The improvement in the hydrolysis with pectinase was 57% after 4 h and 24% after 48 h. The pectinase activity corresponds only to additional 4% in the protein mixture, which suggests that use of additional pectinase may be inexpensive compared to the total cost of the enzyme treatment. Pectinex Ultra SP-L contains, as well as Novozym 188 to a lesser extent, several accessory activities in addition to pectinase or β-glucosidase activities, which may contribute to the improved hydrolysis of pretreated barks.

Hydrolysates after 48 h enzyme hydrolysis were analysed by HPLC and the specific yield of each monosaccharide was determined (Table 3). The total hydrolysis yields as monosaccharides analysed by HPLC were on average 76% of the hydrolysis yield obtained by reducing sugars assay (standard deviation 5.2). The DNS reagent used in the reducing sugar assay gives response also to the reducing ends of oligosaccharides, and fructose which is found in spruce bark but was not analysed by HPLC. In addition, DNS reagent may degrade some oligosaccharides during the analysis generating more reducing ends. According to the reducing sugar assay, 35% of the soluble dry matter of the hot water extract was reducing compounds and according to HPLC only 1% was monosaccharides (data not shown). This leads to the suggestion that there are some other at least partly soluble components in industrial and also pretreated spruce bark that give response in reducing sugar assay.

Despite the differences in the yield levels, the conclusions from HPLC analysis were similar as those drawn from reducing sugar assay. HPLC analysis showed total hydrolysis yield of 63% for both HWE and HWE + SE bark with low dosage of cellulase and high dosage of β-glucosidase and pectinase. Pectinase improved hydrolysis to monosaccharides, in particular to galacturonic acid, which underlines the importance of the pectinolytic activity for hydrolysis of HWE bark. As spruce bark is reported to contain pectin (Laks, 1991; Le Normand et al., 2011), it can be assumed that at least the majority of the released galacturonic acid came from pectic substances, although it is possible that some activities in Pectinex Ultra SP-L released galacturonic acid from hemicellulose as well. The very low amount of galactose, 4-*O*-methyl glucuronic acid and glucuronic acid in spruce bark caused errors in the calculations and resulted in some cases in yields of over 100%.

A few hydrolysis experiments were carried out with using purified β-glucosidase (Sipos et al., 2009) instead of commercial Novozym 188 enzyme. The yields with purified β-glucosidase were generally slightly lower than with Novozym 188 (results not shown) suggesting that other components in the commercial enzyme preparate play a role in the hydrolysis.

Softwood barks have been found fairly resistant to hydrolytic enzymes in earlier studies (David and Atarhouch, 1987; Vazquez et al., 1987). Tannins contained in the untreated and partly in the pretreated bark have been suggested to bind and precipitate proteins and therefore inactivate cellulase enzymes (Walch et al., 1992). Thus, it was surprising to obtain such high hydrolysis yields (up to 68%) from untreated bark in this study. On the other hand,

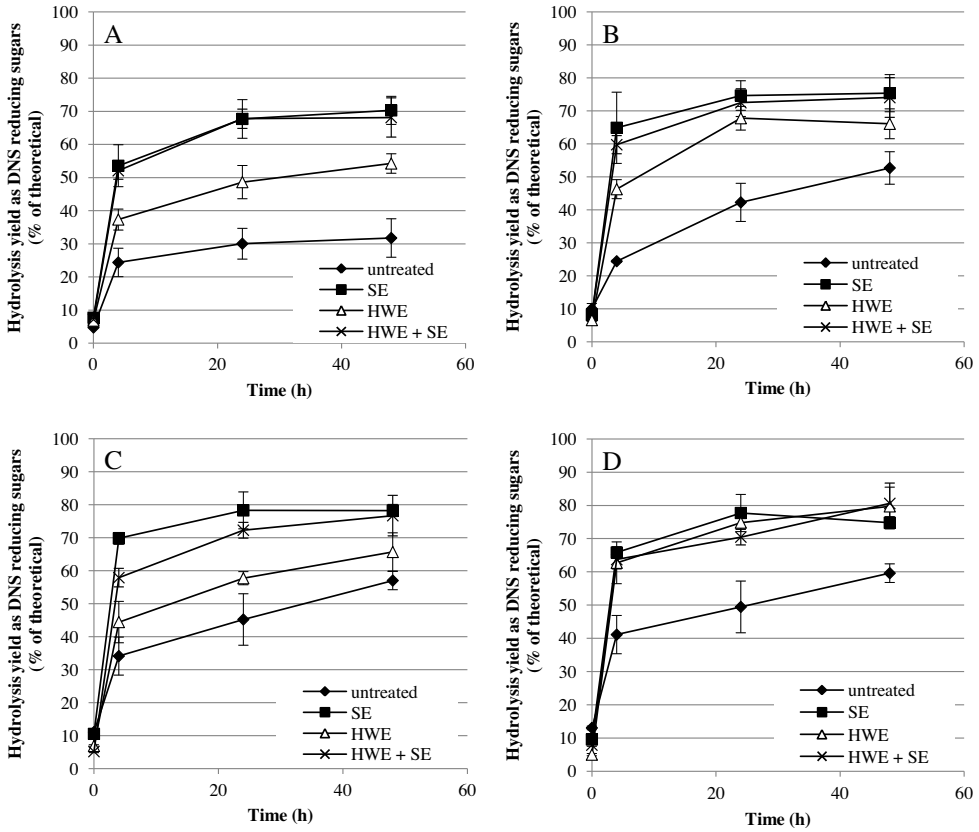

Fig. 2. Hydrolysis of pretreated spruce bark in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. Pretreatments used were hot water extraction (HWE), steam explosion (SE) and combination of the two (HWE + SE). Hydrolysis with. (A) Low dosage of cellulase and β-glucosidase, (B) low dosage of cellulase and β-glucosidase, high dosage of pectinase, (C) high dosage of cellulase and β-glucosidase, (D) high dosage of cellulase, β-glucosidase and pectinase.

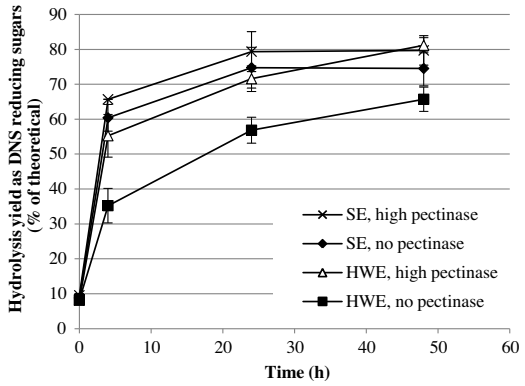

Fig. 3. Hydrolysis of pretreated spruce bark with low cellulase dosage, high β-glucosidase dosage and with or without pectinase in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. Pretreatments used were hot water extraction (HWE) and steam explosion (SE).

the specific activity and efficiency of commercial enzymes towards lignocellulosics has greatly developed since 1980's and as mentioned the reducing sugar method used in this study also appears to give response to some other dissolving components in spruce bark in addition to mono- and oligosaccharides.

When comparing our composition and hydrolysis data to literature, it must be noted that industrial bark processed at the debarking unit which we have used in our study is not the same as fresh bark obtained from a recently felled tree, which has been used in many published studied on bark utilisation (Robinson et al., 2002; Torget et al., 1991; David and Atarhouch, 1987). Very large differences have also been found in the hydrolysability of inner and outer bark. David and Atarhouch (1987) obtained 58% hydrolysis yield for spruce inner bark glucan after extraction with boiling water whereas the hydrolysis yield of outer bark was only 18%. Hydrolysability of whole bark was in between (51%), and spruce bark was determined to contain 40% inner bark and 60% outer bark. Altogether, the hydrolysis yields remained lower than those obtained in this study except when spruce bark was delignified with NaOH or NaClO after extraction with boiling water (glucan hydrolysis yields 67–99%). Pretreatment with NaOH has been found to increase the hydrolysability of pine bark only slightly (from 3% to 6%) and delignification with sodium hypochlorite was needed to achieve higher yields (>67%) in 24 h (Vazquez et al., 1987). Eucalyptus bark and hardwood bark in general appear to be easier to hydrolyse enzymatically as 60% cellulose hydrolysis yield has been obtained with eucalyptus bark pretreated hydrothermally with CO_2 (Matsushita et al., 2010). The yields obtained

Table 3
Yields of monosaccharides (% of the amount of the monosaccharides in the starting material) after 48 h enzyme hydrolysis of hot water extracted (HWE) and sequentially hot water extracted and steam exploded (HWE + SE) spruce bark with low (L) or high (H) dosage of cellulase, high dosage of β-glucosidase and with or without pectinase.

Material	Cellulase	Pectinase	Glc	Xyl	Ara	Man	Gal	Rha	GalA	4-*O*-MeGlcA	GlcA
HWE	L	No	64	33	33	20	23	0	7	11	58
	L	Yes	68	58	60	46	58	14	60	8	23
	H	No	58	32	31	25	23	0	5	35	56
	H	Yes	70	54	59	49	56	10	45	23	38
HWE + SE	L	No	57	42	115	28	47	0	19	37	167
	L	Yes	66	62	69	50	118	0	26	63	35
	H	No	68	47	45	36	118	0	6	125	59
	H	Yes	70	62	72	56	120	0	62	30	82

in this study appear to be higher than what has been reported for pretreated bark in the literature.

The results of our experiments point towards a hypothesis that in acid catalysed pretreatment of spruce bark some water-soluble phenolic compounds are condensed and increase the Klason lignin content of the material. This reaction in turn reduces the hydrolysability of the biomass by possibly reducing the accessibility of the enzymes to cellulose. On the other hand, most of these compounds are removed during hot water extraction and further acid catalysed steam explosion may not reduce the hydrolysability of bark as in the case of untreated bark. However, this hypothesis was not verified in this work since acid catalysed steam explosion was not studied on hot water extracted bark.

Altogether, the results suggest that using a right mixture of enzymes including a pectinase product in hydrolysis may render hot water extraction in 80 °C as an adequate pretreatment for industrial spruce bark and remove the need for conventional capital intensive pretreatment technologies such as steam explosion. Steam explosion can also be used to improve the hydrolysability of spruce bark but additional acid catalyst and very high temperature may reduce the efficiency of the pretreatment. It appears that the solid residue from a biorefinery producing tannin from industrial spruce bark by hot water extraction is a good feedstock for 2nd generation bioethanol production or other sugar-based production processes because of its good hydrolysability as such with right enzymes. However, if it is used as an additional feedstock in a process containing a steam explosion unit using acid catalyst, it should be fed to the process after steam explosion. These results suggest that spruce bark can be reconsidered as a feedstock for sugar biorefineries, especially in such concepts in which tannins or other water extractable components are first extracted for use as biochemicals or intermediates.

3.4. High consistency fermentation of pretreated spruce bark

The fermentability of HWE + SE and HWE barks were compared in high consistency (150 g L^{-1} substrate concentration) simultaneous saccharification and fermentation (SSF) experiments to confirm the results from hydrolysis studies and to assess the effect of possible inhibitors to the fermenting yeast *S. cerevisiae*. A new batch of HWE bark was made for this experiment and the materials were used unwashed. HWE + SE and HWE barks contained 49.8% and 48.0% total carbohydrates with 41.4% and 38.4% hexose polysaccharides, expressed as monosaccharides after acid hydrolysis.

During the fermentations it was noticed that a significant amount of ethanol evaporated from the reactor. Evaporation rates were 0.03–0.15 g L^{-1} h^{-1}, and the rate was related to the concentration of ethanol in the reactor. Evaporation rates as high as these have not been observed earlier in the same vessel, and it was concluded that the phenomenon was probably caused by increased gas/liquid surface area in the reactor related to the porous nature of the pretreated bark. Thus the ethanol production was calculated based on CO_2 production assuming that all CO_2 was produced from ethanol fermentation in strictly anaerobic conditions. Ethanol production was also calculated by analysing ethanol from exhaust gas and adding the amount to ethanol concentration analysed by HPLC from samples taken from the reactor. The results supported the CO_2-based calculations.

Fig. 4 presents the release and consumption of glucose and the production of ethanol during the fermentations. Glucose was quickly consumed by the yeast after inoculation at 6 h and 94% of all ethanol was produced during first 50 h in both fermentations. During HWE + SE fermentation 1.7 g L^{-1} acetate and 4.6 g L^{-1} glycerol was produced. The corresponding concentrations for HWE bark were 1.4 g L^{-1} and 4.3 g L^{-1}. The hydrolysis yield of glucan analysed by HPLC after 6 h prehydrolysis was 68.2% for HWE + SE bark and 66.0% for HWE bark showing similar and fast hydrolysability of the materials. Fermentation in 15% consistency produced 21.0 g L^{-1} ethanol from HWE + SE bark and 18.3 g L^{-1} from HWE bark. These concentrations correspond to 66.4% and 62.3% total yields from hexose polysaccharides to ethanol assuming 0.51 g g^{-1} as the theoretical maximum of ethanol from hexoses. Yield of ethanol from 1 kg of pretreated bark was 178 and 155 L correspondingly. Yield of ethanol from untreated bark was 110 and 114 L which could be increased by reducing vessel losses during pretreatment. If a total hydrolysis yield of 70–75% is expected from hexose polysaccharides according to the hydrolysis results, the fermentation yield from hexoses to ethanol was 83–95%, which can be considered as efficient ethanol production.

According to the results, pretreated spruce bark in this consistency did not contain any inhibitors that would have seriously reduced fermentation yield but instead the materials were fermented relatively efficiently. This behaviour is very different compared to spruce wood which is often severely inhibitory after steam pretreatment (Alriksson et al., 2011). Also Robinson et al. (2002) found that up to 30% addition of bark in Douglas fir whitewood pretreated with SO_2-catalyzed steam explosion had a negligible impact on the fermentation of the pretreatment hydrolysate to ethanol by yeast. Hydroxymethylfurfural and furfural production from bark during steam explosion was found to be significantly lower than from wood probably at least partly because of lower carbohydrate content of bark compared to wood. In our case it could also be speculated that hot water extraction removed some water-soluble phenolic compounds that could otherwise be inhibitory to yeast after steam explosion.

Based on this finding it appears that softwood bark produces less inhibitors during steam explosion than softwood in general. Thus it appears that industrial spruce bark either directly after hot water extraction or after pretreatment by steam explosion is a good feedstock for the production of ethanol. Alternatively, it could be used together with other 2nd generation biomass materials as a source of sugars for fermentation. The hydrolysis yield could probably be improved by using the most developed new commercial cellulase products and by optimising the enzyme mixture more carefully, which would lead to higher total ethanol yield.

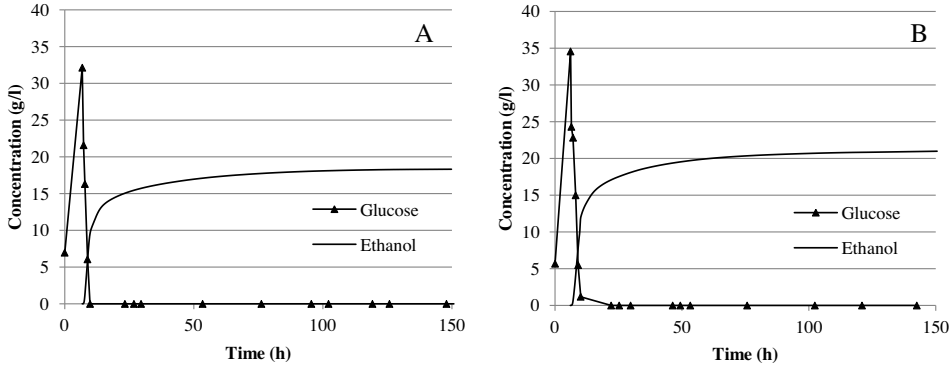

Fig. 4. Glucose release and consumption and ethanol production during the prehydrolysis (6 h) and fermentation of sequentially hot water extracted and steam exploded (A) and hot water extracted (B) spruce bark in 15% consistency.

4. Conclusions

The composition and component yields of industrial spruce bark during two types of pretreatment and their combination were studied and the suitability of the pretreated materials was assessed for 2nd generation bioethanol production. Steam explosion without acid catalyst was found to be an efficient pretreatment improving the hydrolysability of spruce bark. However, hot water extraction was also found to be a sufficient pretreatment for spruce bark when an enzyme mixture containing pectinase is used. Ethanol was efficiently produced from hot water extracted bark suggesting the suitability of hot water extraction as an only pretreatment for spruce bark.

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