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Short Communication

Reed canary grass as a feedstock for 2nd generation bioethanol production

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HIGHLIGHTS

- Hydrolysis of reed canary grass harvested in the spring and autumn was compared.
- Additional β-glucosidase was shown to improve hydrolysis of cellulose and xylan.
- Supplementary CBHII increased the hydrolysis level of RCG by 10%.
- 82% ethanol yield was obtained on steam pretreated RCG.

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ABSTRACT

The enzymatic hydrolysis and fermentation of reed canary grass, harvested in the spring or autumn, and barley straw were studied. Steam pretreated materials were efficiently hydrolysed by commercial enzymes with a dosage of 10–20 FPU/g d.m. Reed canary grass harvested in the spring was hydrolysed more efficiently than the autumn-harvested reed canary grass. Additional β-glucosidase improved the release of glucose and xylose during the hydrolysis reaction. The hydrolysis rate and level of reed canary grass with a commercial *Trichoderma reesei* cellulase could be improved by supplementation of purified enzymes. The addition of CBH II improved the hydrolysis level by 10% in 48 hours' hydrolysis. Efficient mixing was shown to be important for hydrolysis already at 10% dry matter consistency. The highest ethanol concentration (20 g/l) and yield (82%) was obtained with reed canary grass at 10% d.m. consistency.

1. Introduction

In recent years, growing attention has been devoted to the conversion of renewable raw materials into fuel ethanol. Key drivers behind this development include the objectives of counteracting the climate change, decreasing the dependency of imported oil, and finding new earning principles for agriculture and forestry. As an example of concrete policy measures, the EU has set an aim to replace by the year 2020 up to 10 energy-% of all road transport fuels consumed within the EU with energy of renewable basis. Similar policy measures are in place in many of the leading G20 countries.

To achieve these goals production of bioethanol from materials rich in cellulose must be taken to industrial scale. Various herbaceous crops and crop harvesting residues, such as corn stover (Kumar and Wyman, 2009), and switchgrass (Martin and Grossman, 2012) have been studied as potential feedstock for production of ethanol or other biofuels. Worldwide, these materials are abundantly available. However, cultivation of many of these grasses and crops is not feasible or even possible in northern climate.

In northern Europe and America, reed canary grass (*Phalaris arundinacea* L.) has aroused interest as an energy crop for production of electricity and heat by combustion (Jasinkas et al., 2008), and for production of ethanol (Digman et al., 2010). Reed canary grass is a rhizomatous, perennial grass species that can be cultivated on the low value areas, such as bogs after peat production, and on fields, which are not needed for food production. In Finland, reed canary grass is typically harvested in the spring when the water content of the biomass has decreased to the level enabling storage without additional drying.

The aim of the present work was to study the feasibility of reed canary grass as a feedstock for production of cellulosic ethanol. Steam explosion followed by enzymatic hydrolysis was chosen as the basic process option to produce the fermentable sugar solution.

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Barley straw, which is also a material available in northern climate, was used as a reference material in the experiments.

2. Methods

2.1. Raw materials

Reed canary grass (*P. arundinacea* L.) harvested in spring (April 2005) and autumn (August 2005), as well as barley (*Hordeum vulgare* L.) straw were obtained from the experimental fields of MTT Agrifood Research Finland in Jokioinen (60°49N, 23°28E). Raw materials were pretreated by steam explosion at Lund University, Sweden at 190 °C for 5 min or 200 °C for 5 min (for barley straw only) after impregnation with 2% SO_2 (Öhgren et al., 2005). The sugar compositions of the feedstocks were analysed after total acid hydrolysis (Puls et al., 1985). The resulting monosaccharides were analysed by high performance anion exchange chromatography (HPLC) (Tenkanen and Siika-aho, 2000). The composition of the material dissolved in the steam explosion was analysed by HPLC after a secondary enzymatic hydrolysis in order to hydrolyse possible oligosaccharides to monosaccharides (Tenkanen et al., 1999).

2.2. Enzymes

Celluclast 1.5L and Novozym 188 enzymes were provided by Novozymes and Econase CE by AB Enzymes. Spezyme CP was obtained from Genencor. Filter paper activity was measured by the method of Ghose (1987) and β-glucosidase activity was measured according to Bailey and Linko (1990). Protein content was analysed by the Lowry method (Lowry et al., 1951).

Trichoderma reesei cellulases (CBH I/Cel 7A, CBH II/Cel 6A, EG I/Cel 7B, and EG II/Cel 5A) were produced and purified as described by Suurnäkki et al. (2000). Xylanase (XYL II, i.e. Family 11 xylanase with pI 9) was purified as described by Tenkanen et al. (1992), but omitting the last gel filtration step.

2.3. Enzymatic hydrolysis and fermentation

After the pretreatments the hydrolysis of washed solid fraction was carried out using commercial cellulases at a dosage of 10 FPU/g dry matter and β-glucosidase (Novozym 188) 100 nkat/g d.m. The hydrolysis experiments were carried out as triplicates in 50 mM sodium acetate buffer (pH 5) in magnetically stirred test tubes at 1% (w/w) d.m. consistency. The temperature was controlled at 45 °C. The reducing sugars in enzyme hydrolysates were monitored using the DNS method (Bernfeld, 1955) and the monosaccharides by HPLC (Tenkanen and Siika-aho, 2000).

Effect of supplementation of commercial mixture (Econase CE) with the purified major cellulases of *T. reesei* was studied using the washed solid fraction of steam exploded reed canary grass (spring harvest). The dosages of the commercial cellulase mixture and β-glucosidase (Novozyme 188) were 10 FPU/g cellulose and 100 nkat/g cellulose, respectively. This mixture was supplemented with the purified enzymes with a dosage of 5 mg/g cellulose. The cellulases studied were CBH I (Cel7A), CBH II (Cel6A), EG I (Cel7B), EG II/(Cel5A) and xylanase (XYL II). Hydrolysis was carried out as triplicates in test tubes in 1% (w/w) cellulose content.

The unwashed steam exploded raw materials were hydrolysed in Erlenmeyer flasks (liquid volume 60 ml) shaken at 100 rpm, or in stirred tank reactors (liquid volume 700 ml) equipped with a marine impeller enabling mixing at 400–1000 rpm at 10% d.m. consistency. Cellulase dosage was 20 FPU/g d.m. and β-glucosidase (Novozym 188) 200 nkat/g d.m. The experiments were carried out at 45 °C and pH 5. Hydrolyses in Erlenmeyer flasks were done as triplicates whereas hydrolyses in stirred tank reactor as single experiments.

Fermentation of pretreated unwashed reed canary grass (spring harvest,190 °C, 5 min) and barley straw (200 °C, 5 min) were carried out in Erlenmeyer flasks equipped with oil locks at 50 ml working volume and 10% dry matter consistency. Autoclaved yeast nitrogen base (Difco, dissolved in 5 ml volume) was used as a nutrient in fermentation. The material was prehydrolysed for 24 h by using enzymes Celluclast (10 FPU/g dry matter) and β-glucosidase Novozym 188 (100 nkat/g) at 45 °C, pH 5 and then inoculated with 3.5 g/l yeast (yeast strain VTT-B-03339). For fermentation phase the temperature was reduced to 30 °C. Fermentation of barley straw was continued 12 days and reed canary grass for 15 days. Fermentation was followed by measuring the mass loss due to formation of CO_2 and analysing ethanol from the broth by HPLC (Heinonen et al., 2012).

3. Results and discussion

3.1. Raw materials

All raw materials consisted mainly of glucose, xylose and arabinose, the monosaccharides present in cellulose and arabinoxylan (Table 1). Reed canary grass harvested in spring and in autumn had slightly different compositions. Generally, the proportion of stem and cellulosic fibre is higher in the spring than in the autumn (Pahkala and Pihala, 2000). Consistent with this, the content of cellulose in reed canary grass harvested in spring was significantly higher than in the autumn-harvested crop (Table 1). Expectedly, reed canary grass harvested in autumn contained fructose originating from water soluble fructan polysaccharides or from sucrose.

Table 1
The sugar composition (% of dry matter) of untreated raw materials and steam exploded (190 °C, 5 min, 2% SO_2) washed solids.

Sample	Reed canary grass (autumn)		Reed canary grass (spring)		Barley straw	
	Untreated raw material	Steam exploded washed solids	Untreated raw material	Steam exploded washed solids	Untreated raw material	Steam exploded washed solids
Rhamnose	<0.1	<0.1	0.2	<0.1	<0.1	<0.1
Arabinose	3.2	0.6	2.3	0.4	2.6	0.7
Galactose	1.3	0.3	1.3	0.2	1.0	0.3
Glucose	36.0	63.3	48.5	66.9	48.6	63.5
Xylose	17.6	8.9	19.1	5.7	23.6	12.1
Mannose	0.2	<0.1	0.5	0.2	<0.1	<0.1
Fructose	3.8	<0.1	n.d.	<0.1	n.d.	<0.1
Monosaccharides tot.	62	73	72	73	76	77
As polysaccharides	56	66	64	66	68	69

n.d. = not determined.

Table 2
Sugar yields (% of original carbohydrates) in washed solid fraction and soluble fraction after steam explosion (190 °C, 5 min, 2% SO_2).

Sample	Reed canary grass, autumn		Reed canary grass, spring		Barley straw	
	Solid fraction	Soluble fraction	Solid fraction	Soluble fraction	Solid fraction	Soluble fraction
Rhamnose	0	0	0	0	0	0
Arabinose	13	75	13	72	19	82
Galactose	14	86	9	77	20	80
Glucose	94	6	97	3	92	4
Xylose	30	70	21	71	36	64
Mannose	0	99	19	81	0	100
Fructose	0	81	0	100	0	0
Total monosaccharides	66	32	72	25	71	27

Barley straw resembled reed canary grass in sugar compositions but had slightly higher xylose content. A relatively high proportion, 56–68% of the d.m. of reed canary grasses and barley straw were carbohydrates.

3.2. Effect of steam explosion

During the pretreatment by steam explosion, a major part of arabinose, galactose, xylose, mannose, and fructose was solubilised, whereas glucose was enriched in the solid fraction (Tables 1 and 2). Thus, most cellulose remained in the solid fraction and the hemicellulosic material, mainly arabinoxylan, was efficiently solubilised. Water soluble carbohydrates, e.g. glucans, fructans, and sucrose, can explain the concentrations of glucose and fructose in the soluble fractions, especially in the autumn-harvested reed canary grass. The pretreatment caused, however, some losses of arabinose, xylose, and fructose, which are the sugars most susceptible for degradation. The low amount of rhamnose present in raw materials could not be detected after steam explosion.

3.3. Enzymatic hydrolysis with commercial enzymes

Nearly theoretical amounts of reducing sugars were produced in small scale enzymatic hydrolysis with Celluclast 1.5L supplemented by -glucosidase in 72 h hydrolysis (Table 3). The degree of hydrolysis of reed canary grass harvested in the spring was higher than that of the autumn-harvested crop. This might be because a higher amount of xylan was removed by the pretreatment from the spring-harvested reed canary grass. Ageing during the winter in the field might also have brought about chemical and physical changes improving the efficiency of the steam explosion.

The samples after the enzymatic hydrolysis were analysed by HPLC. In contrast to reducing sugar assay, release of glucose was lowest with Celluclast 1.5L and highest with Econase in hydrolysis of reed canary grasses, and with Spezyme in hydrolysis of barley straw. Based on HPLC analysis, the highest glucose yields obtained in the 72 h hydrolysis were 82%, 89% and 86% for reed canary grass harvested in autumn and in spring, and for barley straw, respectively. The total glucose yields after pretreatment and enzymatic hydrolysis were similar for spring harvested reed canary grass (89%) and barley straw (90%) but clearly lower for autumn-harvested reed canary grass (82%).

The amount of xylose released from reed canary grasses was similar with all commercial enzymes. However, clearly the highest amount of xylose was formed from barley straw by Econase, supplemented with β-glucosidase. Presumably, the higher xylanase activity in Econase enhanced the xylose release most significantly in the material having the highest xylan content. The total xylose yields were higher than the glucose yields: 94%, 87%, 97% for reed canary grass harvested in autumn, and in spring, and for barley straw, respectively. Part of the sugars might have remained as oligosaccharides after enzymatic hydrolysis especially with Celluclast, thus explaining the differences in the results of reducing sugars and HPLC assays.

Addition of β-glucosidase improved the formation of glucose in hydrolysis of all materials, but the most significant improvement was found on reed canary grass (spring harvest) with Econase and with barley straw with all commercial enzymes (Table 3). The yield of xylose was also slightly enhanced by additional β-glucosidase. It has been observed that the increased hydrolysis of cellulose synergistically improves the hydrolysis of xylan in the

Table 3
Reducing sugars, and glucose and xylose yields by HPLC after 72 h hydrolysis (1% d.m., 45 °C, pH 5) of steam exploded washed solid fraction of reed canary grass and barley straw with different commercial cellulases with or without β-glucosidase (Novozym 188).

Raw material	Enzymes	Glucose (% of theoretical)	Xylose (% of theoretical)	Reducing sugars (% of theoretical)
Reed canary grass, autumn	Celluclast	63	63	69
	Celluclast + Novozym	76	72	91
	Econase	70	63	77
	Econase + Novozym	82	71	84
	Spezyme	72	64	74
	Spezyme + Novozym	80	68	79
Reed canary grass, spring	Celluclast	66	74	75
	Celluclast + Novozym	78	76	103
	Econase	68	67	82
	Econase + Novozym	89	74	97
	Spezyme	78	72	76
	Spezyme + Novozym	88	77	89
Barley straw	Celluclast	61	68	80
	Celluclast + Novozym	78	69	92
	Econase	63	73	83
	Econase + Novozym	80	81	93
	Spezyme	70	74	87
	Spezyme + Novozym	87	72	93

Table 4
Hydrolysis of washed solid fraction of steam exploded reed canary grass (spring) by supplementing commercial cellulase mixture (Econase CE: 10 FPU/g cellulose + Novozym 188 -glucosidase: 100 nkat/g cellulose) with purified enzymes; CBH I, CBH II, EG I, EG II, or XYL II (5 mg/g cellulose). Hydrolysis conditions were 1% cellulose content, 45 °C, pH 5. Hydrolysis products analysed as reducing sugars.

Enzyme mixture	Supplementation	Hydrolysis products (% of theoretical)			
		0 h	6 h	24 h	48 h
Econase + Novozym	–	1.6	32	61	78
Econase + Novozym	CBH I	1.6	37	68	83
Econase + Novozym	CBH II	1.6	41	69	88
Econase + Novozym	EG I	1.6	34	64	87
Econase + Novozym	EG II	1.5	33	57	81
Econase + Novozym	XYLII	1.5	37	63	85
Reference	–	1.1	1.1	1.2	1.1

complex lignocellulose material (Varnai et al., 2011). The other side activities of the β-glucosidase preparation might also have enhanced the hydrolysis of side groups of xylan and mixed xylo-oligomers. Synergy between xylanases and cellulases in hydrolysis of pretreated corn stover has been frequently observed (Hu et al., 2011; Kumar and Wyman, 2009).

The possibility to enhance the hydrolytic performance of a commercial *T. reesei* cellulase mixture (Econase CE) by addition of potential rate-limiting enzymes was studied by overdosing the mixture by the major cellulases and xylanase (Table 4). CBH II improved the hydrolysis of the washed solid fraction of reed canary grass most significantly, by 13% after 48 h hydrolysis. The hydrolysis yield was also enhanced by EGI, probably due to its strong hemicellulolytic side activities (xylanase and xyloglucanase; Benko et al., 2008) and only slightly less by CBH I and xylanase. EGII had practically no effect in the hydrolysis and thus the amount of EGII was not considered to limit the hydrolysis rate or degree.

3.4. Enzymatic hydrolysis and fermentation at 10% dry matter consistency

The hydrolysis yield, 75%, obtained in 10% consistency with unwashed steam exploded reed canary grass (spring harvest) was clearly lower than with washed materials at 1% consistency (results not shown). One major reason for low hydrolysis results at higher dry matter content presumably is the inefficient mixing by shaking, resulting in insufficient diffusion of enzymes and reaction products. By more efficient mixing in stirred tank reactor significantly higher hydrolysis yield of reducing sugars, 96%, and faster hydrolysis was obtained. It thus seems that improved mass transfer by efficient mixing increased the hydrolysis results. In addition to improved diffusion and access of enzymes to the substrate, the more even glucose and cellobiose concentrations obtained by efficient mixing probably decreased the end product inhibition of the enzymes. The HPLC analysis of the hydrolysates showed that both reed canary grass and barley straw were hydrolysed equally well with 95% hydrolysis yield.

Both reed canary grass and barley straw were hydrolysed and fermented at 10% dry matter consistency. Neither of the materials was toxic to the yeast even at 10% dry matter consistency, and the formed sugars could be efficiently fermented to ethanol without a lag phase. The ethanol concentration obtained from reed canary grass was 20.4 g/l, and from barley straw 17.1 g/l. The ethanol yields on the two raw materials were 82% and 74% from the theoretical yield from glucose, and 59% and 50% from both glucose and xylose, for reed canary grass and barley straw, respectively. Fermentation results showed that reed canary grass is a promising raw material for ethanol production. For this, improved hydrolysis techniques for high solids consistencies should be developed and the composition of enzymes should further be optimised to make the process more efficient.

4. Conclusions

The enzymatic hydrolysis and fermentation of reed canary grass, harvested in the spring and autumn, and barley straw were studied. As expected, additional β-glucosidase improved the hydrolysis of cellulose and xylan in pretreated raw materials by the commercial enzymes studied. Supplementation of a commercial *Trichoderma* cellulase mixture with CBH II resulted in the highest improvement of hydrolysis, when steam exploded reed canary grass was studied. High 95% hydrolysis yield was obtained with both the raw materials at 10% consistency. In fermentation the highest ethanol yield, 82% of theoretical based on glucose, was obtained on reed canary grass.

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