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Synergy between cellulases and pectinases in the hydrolysis of hemp

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HIGHLIGHTS

- The synergy between cellulases, pectinases and xylanase in the hydrolysis of hemp was evaluated.
- Xylan did not limit the access of pectinase to the pectin in hemp.
- Pectinase was more efficient than xylanase in the hydrolysis of fresh and ensiled hemp.
- Clear synergy between cellulases and pectinase was observed in the hydrolysis of ensiled hemp.

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ABSTRACT

The impact of pectinases in the hydrolysis of fresh, steam-exploded and ensiled hemp was investigated and the synergy between cellulases, pectinases and xylanase in the hydrolysis was evaluated. About half; 59.3% and 46.1% of pectin in the steam-exploded and ensiled hemp, respectively, could be removed by a low dosage of pectinases used. Pectinases were more efficient than xylanase in the hydrolysis of fresh and ensiled hemp whereas xylanase showed higher hydrolytic efficiency than the pectinase preparation used in the hydrolysis of steam-exploded hemp. Clear synergistic action between cellulases and xylanase could be observed in the hydrolysis of steam-exploded hemp. Supplementation of pectinase resulted in clear synergism with cellulases in the hydrolysis of all hemp substrates. Highest hydrolysis yield of steam-exploded hemp was obtained in the hydrolysis with cellulases and xylanase. In the hydrolysis of ensiled hemp, the synergistic action between cellulases and pectinases was more obvious for efficient hydrolysis.

1. Introduction

Hemp (*Cannabis sativa* L.) is an annual herbaceous plant of the Cannabaceae family and is one of the fastest growing plant species known able to produce up to 25 tons of dry matter per hectare in southern Europe (Struik et al., 2000). Today, it is cultivated mostly in the EU, Central Asia, Philippines, and China (Shahzad, 2011). Cultivation of hemp only requires low amounts of pesticides and herbicides. Varieties deficient in tetrahydrocannabinol have been developed and they have several potential industrial uses in various sectors including paper, textiles, composite materials and fuel industries.

Hemp fibers are composed mainly of cellulose, hemicellulose, lignin and pectin (Sipos et al., 2010; Shahzad, 2011; Pakarinen et al., 2012). The polysaccharides; cellulose, hemicellulose and pectin are potential sources of fermentable platform sugars for the production of chemicals or biofuels (Sipos et al., 2010; Prade et al., 2011; Kreuger et al., 2011). In order to efficiently hydrolyze cellulose, synergistic action of the major cellulases; *i.e.* endoglucanases, cellobiohydrolases and β-glucosidase is required. Complete hydrolysis of xylan, the main hemicellulose in hemp, needs the enzymes hydrolyzing the main chain into monosaccharides (endoxylanases and β-xylosidases) as well as the side group cleaving enzymes (α-L-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases, and feruloyl esterases).

Pectins are high molecular weight, negatively charged, acidic, complex polysaccharides present in the primary cell wall and comprise, along with lignin, the major components of the middle lamellae (Jayani et al., 2005; Pedrolli et al., 2009). Pectins are composed of a backbone of galacturonic acid units partially esterified by methyl groups (Thibault et al., 1993). Some of hydroxyl groups at C-2 and C-3 of the galacturonic acid residues may be acetylated (Pilnik and Voragen, 1970). In addition, galacturonic acid residues may carry side chains of neutral sugars, including galactose,

Abbreviations: βG, glucosidase; CBH, cellobiohydrolases; CEL, cellulase; DM, dry matter; EG, endoglucanase; HPAEC-PAD, high-performance anion exchange chromatography coupled with pulsed amperometric detection; PEC, pectinase; SE hemp, steam-exploded hemp; XYL, xylanase.

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arabinose and xylose. Pectins are readily hydrolyzed to sugars by pectinases, which comprise an enzyme group which hydrolyzes methylated and non-methylated pectins through depolymerization and de-esterification reactions (Pedrolli et al., 2009). In addition, pectins are degraded by pectin and pectate lyases.

Today, pectinases are of great significance in the food and textile industries (Bhat, 2000; Kashyap et al., 2001; Ortega et al., 2004), saccharification of food residues (Spagnuolo et al., 1997; Wilkins et al., 2007; Dien et al., 2008; Zhang et al., 2010), as well as in the pulp and paper industry (Viikari et al., 2001; Beg et al., 2001; Reid and Ricard, 2004). In the hydrolysis of grapefruit peel waste, the pectinase preparation used was found to be more effective than the cellulase preparation, obviously due to the wider spectrum of activities in the pectinase preparation (Wilkins et al., 2007). Pectinases also played an important role in the hydrolysis of sugar beet pulp, by increasing the access of cellulases to cellulose. Clear synergism between this commercial cellulases and commercial pectinases was observed (Spagnuolo et al., 1997). Supplementation of cellulases with pectinases resulted in clear improvement of cellulose hydrolysis of dilute acid-pretreated corn stover. Addition of pectinases was more beneficial than the other two enzyme supplements, β-glucosidase and xylanase studied (Berlin et al., 2007). Addition of pectinase to the hydrolysis of ammonia-pretreated corncob had a more significant effect on the glucose and xylose yields than that of xylanase (Zhang et al., 2010). In addition to pectinases, other activities, especially accessory hemicellulases in the pectinase preparations, releasing arabinose and xylose, have contributed to increased hydrolysis yields (Dien et al., 2008; Zhong et al., 2009). Previously, the hydrolysis yields of untreated, pretreated, and ensiled hemp by commercial cellulases was found to increase by addition of pectinases, which efficiently removed pectin in the substrates (Pakarinen et al., 2012).

In this work, the solubilization of pectin from fresh, ensiled and steam-exploded hemp by pectinases was investigated. The synergistic actions between cellulases, xylanase, and pectinases in the enzymatic hydrolysis of different hemp substrates were evaluated using purified and commercial enzyme preparations. The aim of the work was to investigate the role of pectin, restricting the enzymatic hydrolysis of pectin-containing hemp substrates in order to improve the hydrolysis of hemp fibers to fermentable sugars.

2. Methods

2.1. Materials

Hemp was harvested from Southern Finland in 2008. Fresh hemp, steam-exploded (SE hemp) and ensiled hemp (3.4% formic acid at 5–10 °C for 8 months) were prepared as described by Pakarinen et al. (2012). Fresh hemp was steam exploded at KCL (Finnish Pulp and Paper Research Institute) in a batch reactor under a pressure of 14.5 bar at 200 °C for 5 min. The contents of cellulose, xylan, polygalacturonic acid and lignin in fresh, SE and ensiled hemp are shown in Table 1. All other chemicals used were of analytical grade and purchased from Sigma or Merck.

2.2. Enzymes

Pectinase (PEC, pectinex Ultra SP-L) was purchased from Novo Nordisk A/S (Bagsvaerd Denmark). The main enzymes in Pectinex are polygalacturonase, pectate lyase and pectinesterase, and it contains low amounts of hemicellulases and cellulases as side activities. Cellobiohydrolases (CBH I) from *Thermoascus aurantiacus*, fused with the *Trichoderma reesei* CBHI cellulose-binding domain, endoglucanase (EG II) originating from *T. aurantiacus*, β-glucosidase (βG) from *Acremonium thermophilum* and xylanase preparation (XYL) originating from *T. aurantiacus*, were produced in a genetically modified *T. reesei* strain where the genes *cbh1, cbh2, egl1* and *egl2*, encoding for CBH I, CBH II, EG I and EG II, respectively, had been deleted as described elsewhere (Suominen et al., 1993; Leskinen et al., 2005; Vehmaanperä et al., 2007). The thermostable enzyme preparations were kindly provided by Roal Oy (Rajamäki, Finland). All thermostable enzyme preparations were adjusted to pH 6.0 and treated at 60 °C for 2 h to inactivate the background *T. reesei* enzymes. Protein was quantified by the Lowry method, using bovine serum albumin (Sigma Chemical Co., USA) as standard (Lowry et al., 1951).

Table 1
Chemical composition (% of dry matter) of the hemp substrates (Pakarinen et al., 2012).

	Cellulose	Xylan	Polygalacturonic acid	Lignin
Fresh hemp	46.1	9.5	5.9	18.0
SE hemp	69.6	5.5	1.3	16.0
Ensiled hemp	46.4	9.0	6.1	15.3

2.3. Enzymatic hydrolysis

The hydrolysis of the hemp substrates by PEC and XYL was carried out in tubes with a working volume of 3 ml in 50 mM sodium citrate buffer (pH 5.0) containing 0.02% NaN_3 at 50 °C. The DM content of substrate was 2% and enzyme preparations were dosed based on the amount (mg) of protein in the enzyme preparation per gram DM of hemp. Samples were withdrawn at 48 h and boiled for 10 min to stop the enzymatic hydrolysis. After cooling, the samples were centrifuged and the supernatants were analyzed for reducing sugars with xylose as standard. Monosaccharides and galacturonic acid were analyzed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Two replicate tests were carried out in all hydrolysis experiments and average values were presented.

The synergy between cellulases, pectinases and xylanases in the hydrolysis of the hemp substrates was investigated as described above. Enzyme loading was expressed as protein amount (mg) of enzyme preparation per gram DM of substrate. The cellulase preparation (CEL) contained: CBHI (8 mg), EGII (2 mg) and βG (1 mg) per gram DM of substrate. Samples were withdrawn at 6, 24 and 48 h and boiled for 10 min to stop the enzymatic hydrolysis. After cooling, the samples were centrifuged and the supernatants were analyzed for reducing sugars (glucose as standard), as well as for monosaccharides by HPAEC-PAD.

2.4. Analysis of carbohydrates

The amount of reducing compounds liberated was determined using the dinitrosalicylic acid method with xylose or glucose as standard (Miller, 1959). Monosaccharides in the hydrolysates were analyzed using HPAEC-PAD system equipped with a Waters 2707 autosampler, three Waters 515 HPLC pumps, Waters 2465 pulsed amperometric detector using Empower 2 software for instrument control and data analysis (Waters Corporation, Milford, MA, USA). The analytical CarboPac PA-1 column (4.0 mm ID × 250 mm) in combination with the CarboPac PA-1 guard column (4.0 mm × 50 mm) (Dionex Sunnyvale, USA) was used. The eluents were H_2O and 0.2 M NaOH with a total flow rate 1 ml/min with post column addition of 300 mM NaOH at a flow rate of 0.3 ml/min. D-Glucose, D-galactose, D-xylose, D-arabinose, and D-mannose (Merck, Darmstadt, Germany) were used as external standards (Pakarinen et al., 2011).

The analytical CarboPac PA-100 column (250 × 4 mm) and the guard column PA-100 (50 × 4 mm) (Dionex, Sunnyvale, CA, USA)