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Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment

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ABSTRACT

Three corn feedstocks (fibers, cobs and stover) available for sustainable second generation bioethanol production were subjected to pretreatments with the aim of preventing formation of yeast-inhibiting sugar-degradation products. After pretreatment, monosaccharides, soluble oligosaccharides and residual sugars were quantified. The size of the soluble xylans was estimated by size exclusion chromatography. The pretreatments resulted in relatively low monosaccharide release, but conditions were reached to obtain most of the xylan-structures in the soluble part. A state of the art commercial enzyme preparation, Cellic CTec2, was tested in hydrolyzing these dilute acid-pretreated feedstocks. The xylose and glucose liberated were fermented by a recombinant *Saccharomyces cerevisiae* strain. In the simultaneous enzymatic saccharification and fermentation system employed, a concentration of more than 5% (v/v) (0.2 g per g of dry matter) of ethanol was reached.

1. Introduction

World-wide, a strong drive is manifest towards increasing the production of bioethanol from biomass. Leading countries are Brazil and the USA, where bioethanol is produced from sucrose (sugar cane) and starch (corn), respectively. The general concern with these 'first generation' processes is that crops for biofuels will put high pressure on the availability of agricultural crop land that is needed for food (Marris, 2006). Alternative 'second generation' lignocellulosic feedstocks have to be used if bioethanol as a fuel is to considerably grow in a sustainable way (Lynd, 1996). Preferably, the feedstocks are byproducts of existing industries that will not put an extra pressure on land use. In the USA, the average productivity of corn grains is 7 MT ha^{-1} annually (Somerville et al., 2010), and about 8% of the grain is fiber. This amount of corn results in 3 MT ha^{-1} $year^{-1}$ stover, and about 20% of the stover are cobs.

These three corn wastes, fibers, cobs and stover, are mainly composed of cellulose, hemicellulose and lignin. Cellulose is a crystalline linear polymer of β-(1,4)-linked glucose moieties. The hemicelluloses present in the cell walls of grasses and cereals largely comprise xylan, a polymer of linear chains of β-(1,4)-linked D-xylopyranosyl residues. On this backbone, depending on the feedstock, various substitutions can be present, like α-L-arabinofuranosyl, (4-*O*-methyl)-α-D-glucuronic acids and *O*-acetylesters (Huisman et al., 2000). These substitutions can be organized as short chains. Mono- or dimeric ferulic acid ester-linked to the arabinose moieties, tend to link the different polymers within plant cell walls (Allerdings et al., 2006; Carpita et al., 2001; Saulnier et al., 1995a).

In order to convert second generation feedstocks into bioethanol, the cellulose and hemicellulose need to be released as monosaccharides. Hereto, either thermochemical approaches (usually referred to as pretreatment), enzymatic approaches or a combination of the two methodologies (Gírio et al., 2010; Sassner et al., 2008; Taherzadeh and Karimi, 2007) are proposed. A pretreatment can serve to either completely liberate the sugars, or to make the polymeric compounds more accessible to subsequent enzymatic attack. Different types of pretreatment include liquid hot water, steam explosion, acid pretreatment, alkali pretreatment, e.g., AFEX, and ionic liquid pretreatments (Alvira et al., 2010). However, pretreatments should not lead to the formation of inhibitory compounds for the further enzymatic hydrolysis and fermentation (Chundawat et al., 2010). Inhibitory compounds in lignocellulose hydrolysates comprise aliphatic acids, furaldehydes, aromatic compounds and extractives (Martın and Jönsson, 2003). Furaldehydes, such as furfural and 5-hydroxymethylfurfural (HMF), and some aliphatic acids, such as formic and levulinic acid, can be formed during pretreatment as degradation products from carbohydrates. Other compounds, like acetic acid, from hemicellulose, and phenolics, from the partial breakdown of lignin, can not be avoided since they are intrinsic parts of the cell wall structure.

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In the last decade concentrated efforts have been put in creating cheaper enzyme cocktails for the degradation of cellulose and hemicelluloses. This includes the commercial preparations, Cellic CTec2 (http://www.bioenergy.novozymes.com) and Accelerase (http://www.genencor.com). These cocktails were mainly developed for improved cellulase activity, although they contain also some hemicellulase activity.

To ferment the sugars and to produce ethanol, the yeast *Saccharomyces cerevisiae* is the organism of choice in the ethanol industry because of its high efficiency and conversion rate and its robustness in industrial environments. However, this yeast is incapable of fermenting xylose. This has been overcome by insertion of a *Piromyces* xylose isomerase gene in *S. cerevisiae* and upregulation of the pentose phosphate pathway (Kuyper et al., 2005). Alternatives are insertion of a xylose reductase and a xylitol dehydrogenase or using bacteria that can already grow on xylose, but these are not as efficient in producing ethanol as *S. cerevisiae* with xylose isomerase because of high xylitol formation, non-robustness and a low ethanol efficiency.

In this paper, it is demonstrated that corn feedstocks (fibers, cobs and stover) can be used in second generation bioethanol production. In the overall process, it is important to prevent the formation of yeast-inhibiting sugar-degradation products. Therefore, upon pretreatment, monosaccharides, soluble oligosaccharides and insoluble sugars were quantified along with the yeast-inhibiting compounds furfural and hydroxymethylfurfural. A commercial enzyme preparation was employed in hydrolyzing the mild pretreated feedstocks into fermentable sugars. Furthermore, a xylose-fermenting *S. cerevisiae* strain was put to the test in producing ethanol. It produced more than the economically relevant 5% (v/v) (Gnansounou and Dauriat, 2010) of ethanol from the 'real life' pretreated corn feedstocks.

2. Methods

2.1. Feedstock material

Corn fiber (87% dry matter (DM)), corn cobs (95% DM) and corn stover (90% DM) were generously supplied by ADM (Decatur, IL, USA). These raw materials were milled with a Retsch ZM200 mill (Retsch, Leeds, UK), equipped with a 1 mm screen, and stored in the freezer until use. High dry matter corn stover hydrolysate pretreated by NREL (Aden, 2008) was kindly provided by Novozymes (Bagsvaerd, Denmark) and stored at −20 °C (20% DM). In the NREL pretreatment a temperature of 190 °C with a residence time of 105 s and reactor acid concentration of 1.64% (wt/wt) was used. The solids loading to the reactor was 30% total solids.

2.2. Pretreatment setup

Dilute acid thermal pretreatments were performed with the milled raw feedstocks. Concentrated H_2SO_4 (97%) was added to the feedstock slurry in a range of 0–2 g per 100 g of feedstock dry matter before thermal treatment. For pretreatments a 1L Parr (Moline, IL, USA) bench-scale high-pressure reactor (Hastelloy) was used. To increase the heating rate, a spiral coil (Parr, Moline, IL) was installed in the reactor vessel. During the heating phase, steam at 20 bar was sent through the coil. During the treatment, temperature was controlled electrically (PID controller). After the desired treatment time the feedstock was rapidly cooled by sending water (15 °C) through the coil. Typical maximum heating and cooling rates were 87 and 60 °C min^{-1}, respectively. During pretreatment the feedstock was continuously stirred with a turbine type impeller (Parr, Moline, IL) at 300 rpm.

For fermentation, pretreated material with 20% DM was used. At this high DM content both corn fibers and stover were too viscous to treat in the PARR bench scale reactor. To overcome the viscosity issue, corn fibers were pretreated (20% DM, 140 °C, 10 min, 2% H_2SO_4) in closed 500 mL glass bottles (Schott, Mainz, Germany) in an HST 4 × 5 × 6 autoclave (Zirbus, Bad Grund, Germany). For corn stover, the NREL pretreated stover was used (see Section 2.1). The corn cobs were pretreated (20% DM, 140 °C, 10 min, 2% H_2SO_4) as described above.

2.3. Analysis of the feedstocks, and pretreated materials

Pretreated samples were centrifuged using a Labofuse400e (Heraeus, Buckinghamshire, UK) centrifuge (15 min, 25 °C, 1500*g*). The supernatants were collected. For pretreated fiber this was 33 ml, for cobs 38 ml and for stover 30 ml. Residues were washed three times with distilled water (15 ml), and the washing water was added to the supernatants. Washed residues are referred to as 'Solid'. The combined supernatants and washing water are referred to as 'Liquid'. Both the Solid and Liquid were freeze dried in a Christ Alpha 1–4 LD (Christ, Osterode am Harz, Germany).

2.3.1. Carbohydrate analysis

The neutral sugar composition of the raw material and the freeze dried pretreated Liquids and Solids was determined by gas chromatography as described by Englyst and Cummings (1984), using inositol as an internal standard. The samples were treated with 72% w/w H_2SO_4 (1 h, 30 °C), followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, and the constituent sugars released were analyzed as their alditol acetates by using GC. To determine the starch content a well defined amount of feedstock was incubated with amyloglucosidase (Spirizyme Plus, Novozymes, Bagsvaerd, Denmark) (0.5% protein on DM) and the amount of glucose released was determined by free sugar analysis. The analyses were performed in duplicate.

2.3.2. Free sugars

Monosaccharides (glucose, xylose, arabinose, galactose, mannose, fructose) of the pretreated material, the enzymatically saccharified material, and the fermented material were analyzed by using high-performance anion-exchange chromatography (HPAEC). HPAEC was performed on a Dionex (Sunnyvale, USA) ICS3000 system equipped with a Dionex CarboPac PA-1 column (4 mm ID × 250 mm) in combination with a Dionex CarboPac PA guard column (4 mm × 50 mm) and an ED50-detector (Dionex). Isocratic elution (20 °C, 1 mL min^{-1}) of 23 min was carried out with water. Each elution was followed by a washing (2 min 0.15 M NaOH, 3 min 0.15 M NaOH + 1 M NaOAc, 1 min 0.15 M NaOH) and an equilibration step (1 min water). Analyses were performed in duplicate.

2.3.3. Lignin

Feedstocks (untreated) were analyzed for acid insoluble lignin. To each sample of 300 mg (dry weight) 3 mL of 72% H_2SO_4 was added and samples were hydrolyzed (1 h 30 °C). After this prehydrolysis, 37 mL of distilled water was added to each sample and samples were put in a boiling water bath for 3 h. Each half hour samples were shaken. Samples were filtered over G4 glass filters. The residual part was washed until it was free of acid and dried overnight at 105 °C. The weight of the dried residual part is a measure for the acid insoluble lignin content. Analyses were performed in duplicate.