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Lignin enrichment and enzyme deactivation as the root cause of enzymatic hydrolysis slowdown of steam pretreated sugarcane bagasse

Research Paper

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The enzymatic hydrolysis (EH) rate normally decreases during the hydrolysis, leaving unhydrolyzed material as residue. This phenomenon occurs during the hydrolysis of both cellulose (avicel) and lignocellulosic material, in nature or even pretreated. The progression of EH of steam pretreated sugarcane bagasse was associated with an initial (fast), intermediate (slower) and recalcitrant (slowest) phases, at glucan to glucose conversion yields of 61.7, 81.6 and 86%, respectively. Even though the EH of avicel as a simpler material than steam pretreated sugarcane bagasse, EH slowdown was present. The less thermo-stable endo-xylanase lost 58% of initial enzyme activity, followed by β-glucosidase that lost 16%, culminating in FPase activity loss of 30% in the first 24 hours. After 72 hours of EH the total loss of FPase activity was 40% compared to the initial activity. Analysis of the solid residue from EH showed that lignin content, phenolic compounds and ash increased while glucan decreased as hydrolysis progressed. During the initial fast phase of EH, the total solid residue surface area consisted predominantly of internal surface area. Thereafter, in the intermediate and recalcitrant phases of EH, the ratio of external:internal surface area increased. The proposed fiber damage and decrease in internal surface area, probably by EH action, was visualized by scanning electron microscopy imagery. The higher lignin/glucan ratio as EH progressed and enzyme deactivation by thermo instability were the main effects observed, respectively to substrate and enzyme.

Introduction

The use of lignocellulosic agricultural residues such as sugarcane bagasse for the production of second generation biofuels (2G bioethanol) represents an important development into renewable energy, and is receiving international interest. Bioethanol can be produced from lignocellulosic biomass, such as sugarcane bagasse, by pretreatment, enzymatic hydrolysis (EH) and sugar fermentation processes. However, the recalcitrance of the lignocellulosic biomass as well as the cost of pretreatment, enzymes production and enzymatic biomass conversion still represents major process bottlenecks for industrial application of the process [1].

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Biomass recalcitrance to enzymatic hydrolysis and subsequent bioconversion is due to several physical and chemical properties of the lignocellulose. The β-1,4 orientation of the glucosidic bonds within cellulose results in the formation of intra- and intermolecular hydrogen bonds between glucose monomers, making the microfibrils highly crystalline and resistant against biological and chemical breakdown [2]. Hemicellulose and lignin also limit the cellulose accessibility to cellulases. Lignin, which is a complex cross-linked polymer of phenyl propane units, plays a major role in the recalcitrance and structural rigidity of the substrate fibers [3]. Furthermore, lignin also encrusts the carbohydrate polymer matrix of cellulose and hemicellulose by forming covalent as well as hydrogen bonding in the lignin–carbohydrate complexes (LCCs) [2], thus limiting enzyme access for hydrolysis.

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Pretreatment (chemical/mechanical/thermal) is critical to reduce the recalcitrance of heterogeneous lignocellulosic material and increase the accessibility of the substrate to EH [4]. However, the EH of pretreated lignocellulose still requires high dosages of enzyme and long incubation periods compared with other enzyme processes [5]. Depending on the pretreatment, after EH there is normally a fraction of the biomass carbohydrates that is not completely hydrolyzed [6]. The kinetic profile during EH of pretreated lignocellulose is characterized by a fast hydrolysis phase and a recalcitrant/slowdown phase. The factors that influence the overall performance during EH of lignocellulose can be placed into two categories: (1) those related to the substrate structure, which are heavily influenced by the feedstock chosen and the type of pretreatment and condition, (2) and those related to the mechanism and interactions of the cellulase enzymes with cellulose, which depend on the nature and source of the enzyme complex. There is no consensus as to which of these factors/categories have the most significant impact on the hydrolysis process. However, it is probably an interaction among the several effects related to both substrate and enzyme factors, which contribute to incomplete EH.

The incomplete cellulose to glucose conversion yield and decreased cellulose to glucose conversion rate during EH caused by lignocellulosic substrate changes (category 1) could be explained by: changes in the heterogeneous substrate composition [7] cellulose crystallinity [8], degree of polymerization [9] and surface accessibility [6]. On the other hand, the slowdown caused by the enzymes (category 2) could be explained by: thermal- and mechanical deactivation [10], unproductive binding to lignin [11], end-product inhibition [12], steric hindrances (by lignin and/or hemicellulose) and loss of synergism between the individual cellulase enzymes [8].

Various studies have indicated the effects that changing individual parameters have on efficient EH. However, it is most probably that a combination of factors will contribute to the observed cellulose to glucose conversion rate and yield during EH. In this context, the aim of this work was to study the correlation of hydrolysis rate with changes in physicochemical properties of the steam pretreated sugarcane bagasse that occurs during EH. Un-catalyzed steam explosion was used to reduce the recalcitrance of industrial sugarcane bagasse to EH as it has been widely used and has shown to be effective for herbaceous materials without the need of adding catalyst [13]. The chemical composition, crystallinity, degree of polymerization and accessibility of the substrate were also evaluated. Complementary, the enzyme activities (FPase, CMCase, β-glucosidase and endo-xylanase) under EH conditions as well as the cellulase adsorption pattern during EH were monitored for 72 hours. Elucidating the substrate characteristics that contribute to slowdown the cellulose to glucose conversion rate and incomplete conversion could contribute to the implementation of successful enzyme and substrate feeding and enzyme recovery strategies for optimal EH procedures.

Materials and methods

Material

Sugarcane bagasse (SCB) from an industrial plant in Malelane, Mpumalanga, South Africa was supplied by TSB Sugar. The samples were packed in zipped plastic bags and stored in a temperature and moisture controlled room (20°C and 65% humidity) until used for chemical composition and pretreatment. Steam explosion pretreatment was conducted in a pilot plant located in installations of CIEMAT biomass unit (CIEMAT – Renewable Energies Department, Madrid, Spain) [14]. The material was pretreated at 210°C for 5 min. The resulting pretreated material (slurry) was hand pressed in a piston with 20 ton weight to obtain a separate liquid and solid fraction. The solid fraction was washed 10-fold (10 g water/g pretreated solid) and the resulting water insoluble solids (WIS) were used in the EH experiments.

Chemical composition analysis

Carbohydrate, lignin and ash content of the raw material and steam pretreated solid residue from EH were determined according to the National Renewable Energy Laboratory (NREL, Golden, CO, USA) methods [15].

Enzymes, enzyme activities and protein determinations

The commercial enzyme preparations used for the study were Spezyme CP and Novozym® 188, kindly donated by Genencor (Genencor International, Rochester, NY, USA) and Novozymes A/S (Bagsværd, Denmark), respectively. Spezyme CP is a cellulase produced by *Trichoderma reesei* with an activity of 60 FPU/mL, and protein concentration of 188 mg/mL. Spezyme CP also has endo-xylanase activity of 5263 IU/mL. Novozym® 188 is a β-glucosidase produced by *Aspergillus niger* with an activity of 700 IU/mL and protein concentration of 114 mg/mL.

The level of enzyme activities of the enzyme mixture used for EH was monitored during an incubation period of 24, 48 and 72 hours at 50°C with an agitation of 100 rpm. The filter paper activity and β-glucosidase activity of the enzyme preparations were determined using filter paper Whatman No. 1 and cellobiose solution as substrates, respectively, with assays conditions as previously described [16]. In the case of β-glucosidase activity, the glucose was detected using Glucose Oxidase/Peroxidase (GOPOD, Megazyme) reagent. 0.05 mL of the β-glucosidase reaction was mixed with 1.5 mL GOPOD and incubated for 20 min. The absorbance was read at 510 nm. The CMCase and xylanase activities were carried out using 0.5% CMC and 1% birchwood xylan as substrate (Sigma), respectively. The assays were conducted by adding 0.05 mL of enzyme dilution to 0.450 mL substrate and incubated for 5 min at 50°C. The reaction was stopped by adding 0.750 mL DNS and boiled for 15 min, and absorbance reading at 540 nm [17].

The protein concentration of the enzyme preparations as well as the supernatant resulting from EH was determined by Bicinchoninic acid (BCA) assay (Thermo Scientific Pierce BCA Protein Assay) using bovine serum albumin as protein standard.

Enzymatic hydrolysis of WIS

EH was performed by transferring 2 g (2%, m/v) of WIS to a 250 mL Erlenmeyer flask containing 100 mL of sodium-citrate buffer (0.05 M), pH 5. Mixing was provided by orbital shaker set at 100 rpm and temperature at 50°C. Cellulase was added to ensure an activity of 15 FPU/g WIS (44 mg/g) and β-glucosidase activity of 15 U/g WIS (2.44 mg/g). Samples were taken at 0, 3, 6, 9, 12, 18, 24, 48 and 72 hours for analysis of glucose, xylose and cellobiose by HPLC as described below and to determine changes in the