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Short communication

High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations

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ABSTRACT

The hydrolysis of hydrothermally treated sugarcane bagasse at dry matter (DM) content of 5%, 15%, and 20% using a laboratory-made blend of *Trichoderma reesei* Rut C-30 and *Aspergillus awamori* (TrAa) supernatants was compared with that of commercial enzymes: Celluclast-Novozym 188 (Cel188), Cellic CTec2 (CT2), and CT2 blended with HTec2 (CTH2). Although the glucose yields (80–86%) achieved with all the enzymes were similar at 5% DM, CTH2 and CT2 performed better than Cel188 and TrAa at 20% DM, with yields of 72%, 69%, 45%, and 57%, respectively. Noticeably, TrAa promoted faster liquefaction in the early stages of hydrolysis. The superior CT2's performance at high DM contents might be associated with its improved resistance to end-product inhibition, as this preparation was the least affected in experiments containing initial exogenous glucose. However, the performance of all the enzymes decreased in the presence of exogenous glucose in comparison to experiments without exogenous glucose.

1. Introduction

In biorefineries, lignocellulosic biomass can be processed to produce fermentable sugars, which can then be used as platform molecules for the integrated synthesis of different chemical feedstocks and fuels [1]. Biomass processing by enzymatic hydrolysis has long been accepted as the most appropriate method to depolymerize biomass into sugars that will be further converted through microbial processes into ethanol or other bioproducts. However, to make biomass enzymatic conversion economically feasible, it is necessary to conduct enzymatic hydrolysis at high-solids content [2].

High-solids enzymatic hydrolysis is defined as a process in which very little or no free water is present in the slurry (i.e., with a total dry matter (DM) content of ≥20%) [3]. Working at high DM content is advantageous in enzymatic hydrolysis of lignocellulosic biomass, as it increases the final product concentration, thus improving the plant output while lowering energy and water inputs. However, enzymatic hydrolysis of large amounts of biomass leads to a decrease in yields, especially because of the initial high viscosity of fibrous materials, resulting in poor mixing and impaired enzyme performance [4].

Although cellobiohydrolases are essential for the depolymerization of crystalline cellulose, research has shown that endoglucanases are the most efficient enzymes in rapidly reducing the viscosity of the biomass suspension [5]. Therefore, preparations containing efficient enzymes that enable partial hydrolysis in the liquefaction stage can improve poor mass transfer in high solids by rapidly converting it into a more flowable fluid [6].

Another important factor affecting high-solids enzymatic hydrolysis is end-product inhibition by glucose and cellobiose of cellobiohydrolases, endoglucanases and β-glucosidases [7]. In high-solids enzymatic hydrolysis, inhibition by sugars can have a more prominent effect on the abovementioned enzymes, as the high-solids loading limits the diffusion of products away from the enzyme's catalytic site [8]. Under these conditions, it is important to use enzymes with a high tolerance to end-product inhibition.

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This study evaluated the hydrolytic efficiency of a laboratory-made cellulase blend in comparison to that of three commercial preparations in hydrolysis assays with low and high DM contents. Hydrothermally pretreated sugarcane bagasse was used as the substrate. The biomass liquefaction ability of the preparations in the first hours of hydrolysis, final conversion yields, and performance in the presence of a high initial concentration of the final hydrolysis product were then compared.

2. Materials and methods

2.1. Source of materials and determination of the chemical composition of the biomass

Hydrothermally pretreated sugarcane bagasse (190 °C, 12 min) was kindly supplied by Inbicon A/S (Denmark). The analyses of the chemical composition of the pretreated samples were performed according to reported procedures [9]. The enzyme mixtures tested were the commercial preparations Celluclast 1.5 L and Novozym 188 (Novozymes A/S Bagæverd, Denmark), Cellic CTec2 and Cellic HTec2 (Novozymes A/S Bagæverd, Denmark), and concentrated supernatants of *Trichoderma reesei* Rut C-30 and a selected *Aspergillus awamori* strain. Cellic CTec2 was also tested on its own.

Celluclast 1.5L-Novozym 188 and *T. reesei-A. awamori* are based on blends of enzymes from *Trichoderma* and *Aspergillus*, which produce high amounts of cellulases and β-glucosidases, respectively. Cellic CTec2 is a cellulase preparation with a high β-glucosidase content, and HTec2 is rich in endoxylanases, with a cellulase background. The blend of Cellic CTec2 and Cellic HTec2 was evaluated to investigate the influence of hemicellulases on cellulose hydrolysis, as earlier reported [11].

Supernatants of *T. reesei* Rut C-30 and *A. awamori* fungi were obtained as follows. *T. reesei* Rut C-30 was cultivated in a 20-L instrumented fermenter containing 15 L of growth medium consisting of (g/L): 0.30 urea, 1.40 $(NH_4)_2SO_4$, 2.00 KH_2PO_4, 0.30 $CaCl_2$, 0.30 $MgSO_4{\cdot}7H_2O$, 6.0 yeast extract (Merck, Darmstadt, Germany), 6.0 corn steep liquor (Sigma-Aldrich,USA), 30.0 lactose (Merck, Darmstadt, Germany) as the carbon source; and trace elements (mg/L): 5.0 $FeSO_4{\cdot}7H_2O$, 20.0 $CoCl_2{\cdot}6H_2O$, 16.0 $MnSO_4{\cdot}4H_2O$, 14·0 $ZnSO_4{\cdot}7H_2O$ (Merck, Darmstadt, Germany). A 1.5-L inoculum was prepared from a spore suspension and cultivated for 2 days at 30 °C and 200 rpm before being used for starting the 15-L fermentation. The same growth medium and culture conditions were used for the inoculum preparation and the 15-L fermentation.

For the A. *awamori* enzymes production, the fungus was cultivated in a 5-L fermenter containing 3 L of growth medium consisting of (g/L): 1.2 $NaNO_3$, 3.0 KH_2PO_4, 6.0 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.05 $CaCl_2$, 12.0 yeast extract (Merck, Darmstadt, Germany) and 30.0 wheat bran. A 300-mL inoculum was prepared from a spore suspension and cultivated for 2 days at 30 °C and 200 rpm before being used for starting the 3-L fermentation. The same growth medium and culture conditions were used for the inoculum preparation and the 3-L fermentation. After 7 days of cultivation for both fungi, the cultures were filtered in diatomaceous earth and the resulting supernatants were concentrated 10-fold by ultrafiltration using a 10-kDa membrane.

2.2. Measurements of enzymatic activity and protein concentrations

The total cellulolytic activities of the enzymes evaluated in this study (filter paper activity [FPase]) were determined according to Adney and Baker [12]. One unit of FPase (FPU) corresponds to the release of 1 micromole of glucose per minute, following the assay proposed by Ghose [13] using an enzyme dilution providing 2 mg of glucose after 60 min assay reaction. β-glucosidase activities were determined according to Ghose [13]. The concentration of liberated glucose was measured using a glucose assay kit (GAHK20; Sigma, MO, USA). One unit of β-glucosidase (BGU) was defined as the amount of enzyme that converted 1 μmol of cellobiose into glucose in 1 min at 50 °C.

Prior to measuring the protein concentration, the samples were pretreated with the Compat-Able Protein Assay Preparation Reagent Set (Pierce, IL USA) to precipitate the proteins in the samples and eliminate possible interference by other substances. The protein pellet was easily solubilized in ultrapure water, and the protein concentration was measured using a BCA Protein Assay Kit (Pierce, IL USA), with bovine serum albumin as the standard protein. The results for protein concentration and enzymatic activities measurements are shown in Table 1.

2.3. Enzymatic saccharification

Enzymatic saccharification assays were conducted in triplicate at 50 °C for 72 h using 5%, 15%, or 20% (w/w) pretreated bagasse and an enzyme dosage of 20 FPU per gram of glucan in all the assays. The quantification of the FPU dosage was done taking into account the total cellulase activity for all enzyme preparations including the overall activity of the enzyme blends. The total DM percentages calculated in this study are based on water insoluble solids (WIS) alone, as we have used water washed pretreated samples. Blends of Celluclast 1.5L-Novozym 188 and *T. reesei-A. awamori* were prepared to reach an FPU:BGU ratio of 1:3. A mixture of Cellic CTec2 and HTec2 was prepared in a ratio of 9:1 (v/v). The biomass (2.5 g, 7.5 g, or 10.0 g dry basis, depending on aimed initial solids content of 5%, 15% or 20%, respectively) and the enzyme cocktail were added to sodium acetate buffer (50 mM, pH 5.0) in 250 mL capped flasks to a total reaction mass of 50 g and the assays were incubated at 50 °C in a rotatory shaker (150 rpm). The samples were withdrawn at 0, 6, 24, 48, and 72 h, and the sugar content was determined by HPLC, as described below. STATISTICA (v.7.0, StatSoft, Inc., 2004) software was used to compare the hydrolysis yields using Fisher's LSD test ($p < 0.05$). Glucose yields were calculated according to the following equation:

$$\text{Glucose yield} = \frac{\left(C_{glucose} - C_{glucose_0}\right)}{1.111\left(\frac{W_t}{V_{h0}}\right)F_{ins0}F_{glucan}} \times 100 \quad (1)$$

where $C_{glucose}$ is the glucose concentration in the hydrolysates (g/L); $C_{glucose_0}$ is the initial glucose concentration in the hydrolysis assay; W_t is the total weight of the hydrolysis assay (g); V_{h0} is the initial volume of liquid (L); F_{ins0} is the initial mass fraction of insoluble solids in the total hydrolysis assay; F_{glucan} is the initial mass fraction of glucan in insoluble solids. V_{h0} is the volume that corresponded to the initial mass (g) of liquid added to the hydrolysis assay ($W_t - W_{ins0}$).

In order to evaluate the early liquefaction effect of enzymatic preparations, the total insoluble solids were determined after a 6-h hydrolysis using 20% (w/w) of pretreated bagasse with *T. reesei-A. awamori* blend, Cellic CTec2, *T. reesei* or *A. awamori* supernatants (used individually). The reactions were stopped after 6 h by boiling all the assays and the whole hydrolysis content were filtered through glass fiber filters. The solids recovered were washed exhaustively until no glucose was detected in the filtrate, which was monitored using the YSI 2700 Select Biochemistry Analyzer. The total insoluble solids were determined by drying the material overnight in an oven at 105 °C until constant weight. Control experiments containing 20% (w/w) of pretreated bagasse with buffer were also run in parallel.