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Research paper

Complete saccharification of cellulose through chemo-enzymatic hydrolysis

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ABSTRACT

A new method for total hydrolysis of cellulose using a chemo-enzymatic system to combine chemical depolymerization and enzymatic hydrolysis, is described in this paper. The approach described herein involves the dissolution of cellulose in an ionic liquid, depolymerization by acidic solid-catalyst, and use of an antisolvent to obtain the resulting cello-oligomers. These were subjected to chemical depolymerization, after which virtually all the soluble cello-oligomers were hydrolyzed to glucose by β-1,4-D-glucan glucohydrolase. This glucohydrolase is newly identified from a species of *Paenibacillus* (HPL-001), and is different from commercial β-1,4-D-glucosidase. Continuous recycling (99%) of ionic acid and organic solvent completely broke down the cellulose into cello-oligomers (soluble sugars) shorter than six anhydrous glucose units. The cello-oligomers of soluble sugars were easily connected to a new single-enzyme system for complete hydrolysis to glucose. The efficiency of this technology could solve the dissolution and selective deconstruction problem retarding the major production of glucose from cellulose, and could provide a crucial advance in the production of un-degraded glucose as final product. This approach provides an alternative techno-economic process to traditional, expensive, three-enzyme hydrolysis of cellulose.

1. Introduction

Biorefinery is a process utilizing integrated biological and chemical conversions to produce biofuels or platform chemicals from photosynthetic biomass. This is the only foreseeable, sustainable source of carbon fuels and materials available to humanity [1–3]. One of the most challenging issues to make possible the sustainable operation of lignocellulose-based biorefinery is to overcome the intrinsic recalcitrance of these materials. The crucial step in the production of biofuels or chemicals from biomass is the hydrolysis of cellulose to fermentable sugars. The conversion of lignocellulose typically involves a disruptive pretreatment process followed by enzyme-catalyzed hydrolysis of the cellulose to fermentable sugars [4–6]. However, examination of these cellulose deconstruction methods reveals that no pretreatment technology offers complete conversion of cellulose into fermentable sugars.

Enzymatic hydrolysis is still not feasible due to the price of enzymes, despite the high specificity of enzymes for polysaccharide substrates [7]. On the other hand, the major disadvantages of chemical biomass hydrolysis are low specificity for the target substrate and low efficiency in the selective fractionation of lignocellulose [8,9].

Thus, a different strategy has been proposed: the pretreatment of lignocellulose using ILs (ionic liquids). This methodology can effectively remove the lignin and reduce the crystallinity of the cellulose to permit enzymatic hydrolysis at high solid loadings and low enzyme concentrations. Thereby, it substantially accelerates the rate of enzymatic hydrolysis and increases the yield of fermentable sugars [10,11]. The advantage is that the ILs can be completely recovered and are therefore not present during the hydrolysis step. However, complete degradation of cellulose to its glucose monomer units or a reduction of the degree of polymerization (DP) to soluble cello-oligomers by IL treatment alone has not been reported [5]. Another strategy is the use of alternative ILs with

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acidic properties that allow the integration of several processes. An effective methodology of cellulose depolymerization using a solid catalyst in an ionic liquid has also been reported [5,6].

Apart from cellulose dissolution, the traditional hydrolysis of cellulose involves the synergistic activity of endo-β-1,4-glucanases (EC 3.2.1.4, EG), exoglucanases [including both cellobiohydrolases (EC 3.2.1.91, CBH) and sometimes glucohydrolases (EC 3.2.1.74)] and β-glucosidases (EC 3.2.1.21). All three enzyme classes must be present in this system in order to produce glucose. A model for total cellulose hydrolysis by herbivorous terrestrial crustaceans has been published and it is capable of producing glucose from crystalline cellulose without requiring a CBH. This contrasts with the traditional model for cellulose digestion, known from fungal cellulase systems [13]. Recently, we identified a new 1,4-β-D-glucan glucohydrolase (Ggh) that showed not only successive hydrolysis functions for cellobiose but also for cello-oligomers as substrates. The 1,4-β-D-glucan glucohydrolase gene (*Ggh*) which was isolated from *Paenibacillus* sp. strain HPL-001 (KCTC11365BP), has been cloned and expressed in *Escherichia coli*, and efficiently purified using affinity column chromatography. Virtually all the soluble cello-oligomers (e.g., cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose) can be hydrolyzed to glucose by this enzyme [14].

With this as background, we now are seeking to develop an alternative approach for complete cellulose saccharification to glucose, using a chemo-enzymatic technology and green chemistry. Chemical depolymerization could replace the EG and CBH roles of decreasing the DP over the course of traditional enzymatic hydrolysis for cellulosic substrates. After depolymerization of the cellulose, single-enzyme hydrolysis could then complete the total saccharification of the cello-oligomers without other by-products. Furthermore, the key point for using IL technology in cellulose saccharification is complete and economical separation of sugars from the reaction mixture. The question then, is how to combine chemical depolymerization with enzymatic hydrolysis.

In this study, we suggest a new concept for total hydrolysis of cellulose using a chemo-enzymatic system of a solid catalyst in an ionic acid, and a single enzyme (Ggh), instead of the traditional three-enzyme system, for cellulose hydrolysis.

2. Experimental

2.1. Materials

Cellulose was purchased from Sigma-Aldrich. BMIMCl (1-n-butyl-3-methylimidazolium chloride) was purchased from BASF (Ludwigshafen, Germany) and used without further purification. Cellulase (CTEC 2) was obtained from Novozyme. All other reagents were of analytical grade and used without further purification.

2.2. Enzymatic conversion from cellulose to glucose with or without IL-pretreatment

A weighed amount of IL (1-butyl-3-methylimidazolium chloride) was added to clear glass vials with magnetic stirrers and placed into a heating water bath at 95–105 °C. A 5% (w/w) cellulose solution was prepared by combining 50 mg of cellulose with 950 mg BMIMCl in a 5 mL autoclave vial. The mixture was stirred for dissolution until the solution was nearly transparent. The solution of cellulose in IL was sucked out in a pipette and blown into water to form a solidified thread. The solid phase cellulose was thereby obtained in the form of regenerated cellulose and dried in an oven at 60 °C for 24 h before being used as a starting material. In this way, the regeneration of cellulose was obtained at room temperature. Untreated or regenerated cellulose was hydrolyzed using the same cellulase (6%, CTEC 2) in 2 mL vials at a constant temperature of 50 °C, stirred at 200 r/min in a shaking incubator, to provide a time-based reaction. The untreated cellulose controls were run concurrently with all regenerated cellulose hydrolysis experiments to eliminate potential differences in temperature history or enzyme loading. The glucose released from untreated cellulose (reagent) and IL-treated cellulose, regenerated by an antisolvent after dissolution in an IL [BMIMCl], was determined using an HPLC (Younglin Instrument Acme 9000, Korea). The total reaction period was 366 h, and samples were taken at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 216, 288, and 366 h after reaction. All experiments were carried out in triplicate. Each value recorded is therefore the arithmetic mean of three experimental data sets (data shown as mean value ± standard deviation).

2.3. Catalytic cellulose depolymerization and soluble sugar precipitation

The catalytic material Amberlyst 15DRY (Aldrich) was stirred twice into methanol (99.9%, Aldrich) for 15 min, and then dried overnight at 100 °C. The solution of cellulose in 1-butyl-3-methylimidazolium chloride (BMIMCl) was prepared by dissolving 5.00 g of Sigmacell cellulose in 100 g of BMIMCl at 100 °C with mechanical stirring. Subsequently, 1.0 g of Amberlyst 15DRY (AMB) was added to the mixture containing cellulose. The depolymerization of cellulose was carried out at 100 °C for 2 h. Residual cellulose and soluble cello-oligomers from the reaction mixture were precipitated by the addition of chloroform (25 mL) and separated by centrifugation or filtration. The solid portion of the depolymerized-cellulose was dissolved in distilled water and the insoluble cello-oligomers were separated by filtration. The aqueous solution of cello-oligomers was collected and stored at −20 °C for a posterior DNS assay and HPLC analysis. The total amount of reduced sugars was determined by reaction with 3,5-dinitrosalicylic acid (DNS) at 100 °C. The color formation was monitored at 550 nm using a StellarNet, Inc., EPP 2000 UV–Vis spectrometer. The amount of total reducing sugar (TRS) was determined using a standard curve obtained with glucose.

Soluble sugar precipitation was confirmed with standard sugars by the same method. The solution of glucose and other sugars in 1-butyl-3-methylimidazolium chloride (BMIMCl) was prepared by dissolving 1.00 g of glucose, xylan, arabinose, mannose, dextrose, galactose, fructose, and xylose in 100 g of BMIMCl at 100 °C, with mechanical stirring at 100 °C. Subsequently, 100 mL of chloroform was added to a flask containing one sugar in a BMIMCl solution. Glucose and other precipitated sugars were separated from the aqueous solution by centrifugation or filtration at freezing temperature. Glucose and other sugar precipitates were dried overnight at 100 °C overnight, and held for HPLC analysis. The amount of sugar precipitated was determined by optical density (OD) using a spectrophotometer at 600 nm.

2.4. Enzymatic glucose production

The cellulose oligomers, depolymerized by a solid catalyst in an ionic liquid, were precipitated by the addition of chloroform to the cellulose/BMIMCl solution. Among the precipitated cello-oligomers, the soluble portion was separated by dissolving in water. This aqueous solution of cello-oligomers was hydrolyzed using 50 μg of purified glucohydrolase in 2-mL vials at a constant temperature of 40 °C, with a stirring rate of 200 r/min, in a shaking incubator, to provide a time-based reaction (0, 10, 20, 30, 60, 120, 180, or 360 min). Each portion of 10-μl sample was analyzed after clean-up with boiling water for 5 min and filtering with a 0.2 μm syringe filter. An HPLC (Younglin Instrument Acme 9000, Korea) analysis was conducted as mentioned above. The conversion of