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A Multistage Process to Enhance Cellobiose Production from Cellulosic Materials

Caroline Vanderghem · Pascal Boquel · Christophe Blecker · Michel Paquot

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Abstract Cellobiose, a disaccharide, is a valuable product that can be obtained from cellulose hydrolysis. In this study, a simple methodology is presented to enhance the production and improve the selectivity of cellobiose during enzymatic hydrolysis of cellulose. The approach consisted of a multistage removal of filtrate via vacuum filtration and resuspension of the retentate. By this process, the remaining solid was further hydrolyzed without additional enzyme loading. Compared to the continuous hydrolysis process, the production of cellobiose increased by 45%. Increased selectivity of cellobiose is due to the loss of β-glucosidases in the filtrate, while enhanced productivity is likely due to mitigated product inhibition.

Keywords Cellobiose · Cellulose hydrolysis · Multistage process · Cellulase binding · β-glucosidases

Introduction

Biomass represents the most abundant renewable carbon source on earth for the production of bioenergy [1]; biomaterials; high-value chemicals such as fragrances, flavoring agents, food-related products, and nutraceuticals [2]; and biosurfactants [3].

Cellulose is part of the biomass and is composed of D-glucose subunits jointed together in long chains by β-1,4-glycosidic bonds. By cellulose saccharification, glucose can be obtained and readily fermented to produce fuel ethanol [4], organic acids [5], and other chemicals [6]. Another cellulose hydrolysis product is the disaccharide cellobiose that has gained attention, for example, in the form of cellobiitol, a low-calorie bulking agent suitable for incorporation into formulated foods [7]. In an acylated form, it is suited to thickening or structuring a water-immiscible liquid in a cosmetic formulation [8].

C. Vanderghem · P. Boquel · C. Blecker · M. Paquot (✉)
Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium
e-mail: paquot.m@fsagx.ac.be

For polysaccharide catalysis, enzymatic hydrolysis is preferred over acid-catalyzed hydrolysis. The former produces better yields [9], and the latter has the disadvantages that concentrated acids are toxic, hazardous, require expensive corrosion-resistant reactors, and have major waste disposal problems [10, 11]. However, cellulose enzymatic hydrolysis encounters three main problems: (1) the high cost of cellulase enzymes often hinders large-scale application [12]; (2) the enzymatic hydrolysis of cellulose is limited by reaction products such as glucose and cellobiose [13, 14], resulting in incomplete utilization of the cellulosic material; and (3) lignified material must be pre-treated in order to favor cellulase accessibility to the substrate.

Efficient cellulosic bioconversion is a complex process and requires the synergistic action of three kinds of enzymes: endoglucanases, exoglucanases, and β-glucosidases [15–17]. Endoglucanases act randomly at internal amorphous sites in the cellulose polysaccharide chain, producing oligosaccharides of various lengths and consequently new sites for the exoglucanases. These ones act in a processive manner on the reducing or non-reducing ends of cellulose, liberating cellobiose as major product. β-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose [18]. Thus, for high production of cellobiose, the presence of β-glucosidases is undesirable.

A method widely studied to recycle cellulase during saccharification is ultrafiltration. However, Knutsen and Davis [19] investigated another method of recovering and reusing cellulase enzyme bound in the solid phase. The method consisted of simple solid/liquid separation using a large-pore vacuum filtration unit for recovering active cellulose enzymes bound to the solid substrate and reuse of these enzymes.

In this study, a multistage cellulose hydrolysis process was investigated in order to upgrade the production of cellobiose. The approach consisted of a multistage removal of filtrate via vacuum filtration and resuspension of the retentate without any enzyme addition. By this process, it is expected to increase the production of cellobiose by removing inhibitory reaction products such as glucose and cellobiose, while retaining solid particles and bound enzymes. In an additional experiment, the vacuum filter cake was washed with buffer in order to eliminate residual glucose and cellobiose.

Finally, experiments were performed in order to investigate which cellulases were eliminated during the filtration step.

Materials and Methods

Cellulose and Cellulase Enzymes

Cellulose fibers (Alba-Fibre; C-200) were acquired from Mikro-Technik GmbH & Co., KG, Germany (98% purity according to the producer). The cellulase mixture from *Trichoderma reesei* ATCC 26921 (Celluclast® 1.5 L) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The filter paper activity of cellulase was determined with the official International Union of Pure and Applied Chemistry method with filter paper strips (Whatman no. 1) [20, 21]. Values were expressed as filter paper units (FPU)/milliliter. The activity of the commercial enzyme concentrate was 17 FPU/mL.

Conditions for Hydrolysis

Cellulose (50 g/L) was suspended in citrate buffer (0.05 M, pH4.8) at 50°C. Hydrolysis took place in 500-mL glass bottles incubated in a water bath maintained at 50°C equipped

with a submersible magnetic stirrer (2mag MIXdrive 6, Munich, Germany). Stirring was continuously performed with a stir bar at 400 rpm. Prior to enzyme loading, a sample was taken to measure background glucose and cellobiose concentrations. Samples were treated with cellulase at a concentration of 0.4 FPU/g cellulose. The control experiment consisted of a continuous hydrolysis performed over a 24-h period. During the multistage experiment (four times, 6 h), hydrolysis was performed continuously for 6 h, and then the hydrolyzate was filtered with a vacuum filter funnel (500 mL, 95 mm diameter) assembled with a fritted disk (40–100 μm pore size; Robu Glasfilter-Geräte GmbH). Vacuum was run until filtrate was no longer being collected (about 10 min). Following filtration, retentates were resuspended in fresh buffer at the same concentration as in the first stage (50 g dry matter/L), at 50°C under stirring, to continue the hydrolysis for further 6 h. Steps were repeated four times in order to complete the 24-h period. The second multistage experiment was identical to the first one, but in addition, the vacuum filter cake was washed twice with buffer (1:1; cake: buffer) to remove any enzymes not bound to the solids and remaining cellobiose and glucose. In order to perform hydrolysis kinetics, samples of hydrolyzates were removed at determined times. Samples were submerged for 10 min at 90°C in order to inactivate the enzymes before gas chromatographic (GC) analysis. Each experiment was performed in duplicate.

Investigation of the Cellulases Eliminated in the Filtrate

Experiments were performed in order to investigate which cellulases were eliminated during the filtration step. For the multistage experiment, after the first 6 h of continuous hydrolysis, the filtrate was recuperated after vacuum filtration and divided into two parts. The first part was incubated over 6 h. In the second part, fresh cellulose was added and also incubated over 6 h. The two parts were assessed for glucose and cellobiose production.

Determination of Reactions Products

GC analysis was performed in order to quantify the concentration of glucose and cellobiose [22, 23]. A sample of withdrawn hydrolyzate was filtered (0.45 μm) in order to separate insoluble material. Before GC analysis, samples were subjected to derivatization of the carbohydrates in order to increase volatility. Of the filtered hydrolyzate, 750 μL was added to 250 μL 2-deoxyglucose (3 mg/mL; internal standard). Of each solution, 400 μL were added to 2 ml of sodium borohydride in a solution of dimethyl sulfoxide (20 g/L), and the mixture was heated at 40°C for 90 min. Subsequently, the samples were cooled at room temperature; and 600 μL of acetic acid, 400 μL of 1-methylimidazole, and 4 mL of anhydride acetic were added. The mixture was heated at 93°C for 2 h, in order to acetylate all the alcohol functions. After cooling the reaction mixture to 5°C, 10 mL of distilled water and 3 mL of dichloromethane were added, vortexed, and decanted for 2 h. The organic inferior phase was recuperated and injected in the GC.

GC analyses were carried out with a Hewlett-Packard (HP 6890) gas chromatograph equipped with a flame ionization detector. The components were separated using a high performance capillary column, HP1-methylsiloxane (30 m×320 μm, 0.25 μm, Scientific Glass Engineering, S.G.E. Pty. Ltd., Melbourne, Australia). The airflow was 400 mL/min. The initial temperature was 140°C, followed by 240°C and 290°C. Detection temperature was performed at 300°C. Data were analyzed using ChemHP software.

2-Deoxyglucose (internal standard), glucose, and cellobiose solutions of known concentrations were used as standards.

The cellulose conversion to glucose and cellobiose were calculated by the following formulas:

$$\text{Cellulose converted to glucose (\%)} = \frac{\text{Glucose released (g)} \times 0.9}{\text{Initial cellulose (g)}} \times 100 \quad (1)$$

$$\text{Cellulose converted to cellobiose (\%)} = \frac{\text{Cellobiose released (g)} \times 0.95}{\text{Initial cellulose (g)}} \times 100 \quad (2)$$

Adsorption of Enzymes

The free enzyme was quantified by determining the protein content of the filtrates and washings by the Bradford method [24], with bovine serum albumin as the standard. The total amount of bound enzyme was calculated from the difference between initial quantity of enzyme and free enzyme quantities in the filtrates and washings.

Results and Discussion

Effect of the Multistage Hydrolysis-of-Cellulose Process on the Formation of Glucose and Cellobiose

The main purpose of these experiments was to upgrade the production of cellobiose in the hydrolyzate with a minimum of enzyme loading. For this purpose, a process was tailored based on a multistage approach. Hydrolysis was carried out by a commercial mixture from the fungi *T. reesei*, which is poor in β-glucosidases, thus restricting the conversion of cellobiose to glucose [25]. Cellulose fiber was the substrate used to assess our approach. The solid concentration was set to 50 g/L with an enzyme loading of 0.4 FPU per gram of cellulose. As the substrate is almost pure cellulose, higher loadings of enzyme are unnecessary.

Time courses of formation of glucose and cellobiose during enzymatic reaction for the multistage process (four times, 6 h), for non-washed solids, is presented in Fig. 1. During the first 6-h hydrolysis step, glucose and cellobiose reached a similar production (around 600 mg), thus presenting a glucose/cellobiose ratio about 1. Presence of glucose and cellobiose in the first stage of hydrolysis denoted that the overall enzymes were active in the commercial mixture.

After the first 6 h of hydrolysis, the hydrolyzate was filtered, and the retentate was resuspended at a concentration of 50 g cellulose (dry matter)/liter. Immediately after, a sample of medium was collected and analyzed to determine the amount of glucose and cellobiose that remained in the retentate. The remaining glucose and cellobiose were about 118 and 37 mg, respectively.

Following 6 h of further incubation at 50°C, after reduction of the residual glucose and cellobiose in the resuspended solid particles, the glucose formation drastically diminished to 138 mg in the second stage. In contrast, the cellobiose formation only slightly diminished compared to the first hydrolysis stage. The glucose/cellobiose ratio was about 0.3, denoting a less presence of β-glucosidases in the second hydrolysis stage. The third and fourth stages followed similar trends. These results clearly showed that hydrolysis continued in the three

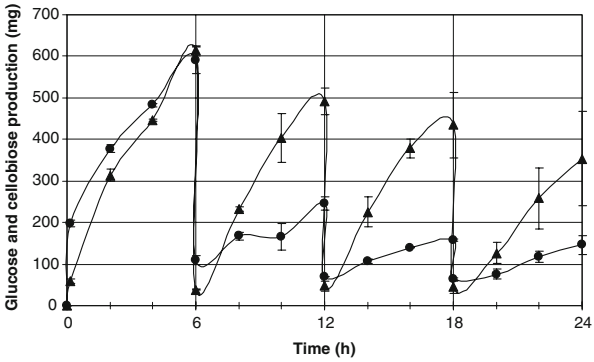

Fig. 1 Time courses of formation of glucose (*circles*) and cellobiose (*triangles*) during enzymatic reaction for the multistage process (four times, 6 h). Error bars represents standard deviation for two repeated experiments

last stages, thus supporting the theory of the adsorption of the enzymes to the cellulose. According to Tomme et al. [26] and Suurnäkki et al. [27], the extracellular enzyme system produced by *T. reesei* possesses the ability to adsorb onto cellulose through a cellulose-binding domain, except for endoglucanase III and β-glucosidases. Cellulase adsorption is a necessary step prior to hydrolysis [15].

The cellulose conversion to glucose and cellobiose for the continuous process and for the multistage process are shown in Figs. 2 and 3.

The cellulose conversion to glucose (9%) was higher in the continuous hydrolysis than for the multistage process (6.5%). In contrast, a higher conversion of cellulose to cellobiose was observed for the multistage process (14.3%), compared to the continuous hydrolysis (9.9%). These results could be explained by two factors: (1) a possibly fewer presence of β-glucosidase, since the second hydrolysis stage may lead to a less hydrolysis of cellobiose; and (2) during the continuous hydrolysis, the concentration of glucose and cellobiose probably reached inhibitory levels, thus inhibiting cellulases and reducing the rate of hydrolysis. According to Andrić et al. [28], glucose directly inhibits β-glucosidase, which results in accumulation of cellobiose. The latter is a strong inhibitor of cellobiohydrolases [15]. Cellobiose inhibition study was reported on the commercial enzyme preparation Celluclast® 1.5 L acting on *para*-nitrophenyl β-D-cellobioside as substrate [29]. The authors showed that with a concentration as low as 5.9 mmol/L of cellobiose in the reaction mixture, a 12.8% inhibition of cellobiohydrolases was reached. It is quite difficult to compare this data with our results due to different substrate type. However, in the present

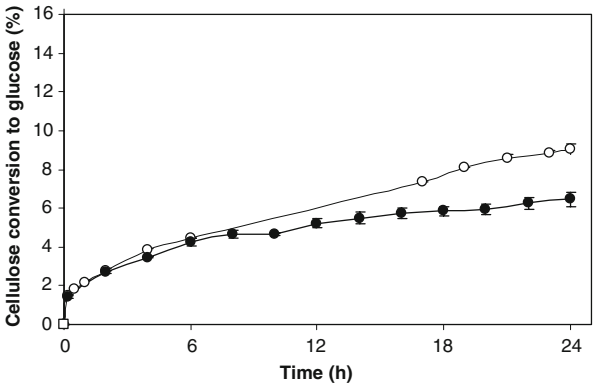

Fig. 2 Cellulose conversion to glucose (percent) for the multistage process (*closed circles*) and the continuous process (*open circles*) over a 24-h period. Error bars represents standard deviation for two repeated experiments

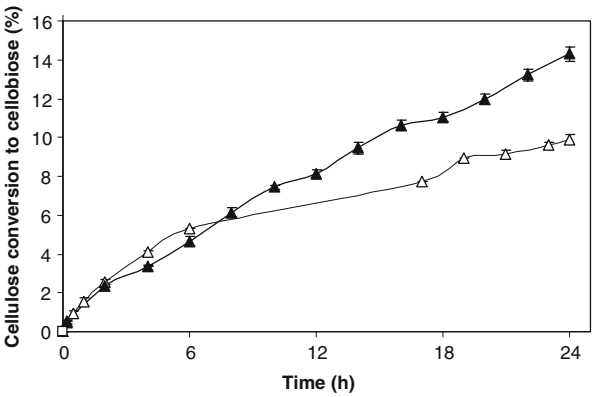

Fig. 3 Cellulose conversion to cellobiose (percent) for the multistage process (*closed triangles*) and the continuous process (*open triangles*) over a 24-h period. Error bars represents standard deviation for two repeated experiments

work, after 6 h of continuous hydrolysis, the cellobiose concentration reached 8.7 mmol/L, which could be an inhibitory concentration for cellobiohydrolases.

The initial cellulose concentration (50 g/L) used in this study is very low, and this will implicate high costs associated with the separation of dilute cellobiose from large quantities of water. However, a higher concentration of cellulose could increase the concentration of the inhibitory reaction products.

A second multistage experiment was performed, but this time, the vacuum filter cake was washed twice with buffer (1:1; cake: buffer) to remove any enzymes not bound to the solids and remaining cellobiose and glucose. This additional step of washing did not result in appreciably enhanced cellobiose production (results not shown). In fact, the concentrations of cellobiose and glucose that remained in the retentate after the filtration step were very small and probably were not enough to induce inhibition.

Investigation of the Cellulases Eliminated in the Filtrate

After 6 h of continuous hydrolysis, the filtrate was recuperated after vacuum filtration, divided into two parts, and subjected to two independent experiments. The first experiment consisted of incubating the filtrate over 6 h. The second experiment consisted of adding fresh crystalline cellulose at a concentration of 50 g/L to the filtrate and incubating the mix. Both experiments were assessed for glucose and cellobiose production. The results in Fig. 4a showed that in the filtrate without addition of cellulose, glucose concentration increased from 2.5 to 3 mg/mL, after 6 h of incubation. In parallel, cellobiose concentration diminished from 2.6 to 2.2 mg/mL (Fig. 4b). A mass balance reinforces the glucose and cellobiose measurements, as approximately 0.5 mg/mL glucose was formed, while 0.4 mg/mL of cellobiose was reacted. The increase in glucose is probably due to hydrolysis of the cellobiose contained in the filtrate by β-glucosidases.

When fresh cellulose was added to the filtrate, cellobiose concentration did not increase after 6 h of incubation (Fig. 4b). This result strongly supported the fact that endocellulases and cellobiohydrolases were not significantly present in the filtrate.

As previously shown, when the retentates were resuspended in fresh buffer at a concentration of 50 g of cellulose (dry matter)/liter, the concentration of cellobiose had an increase of 2.3 mg/mL after 6 h of incubation. This result denoted the activity of the endocellulases and cellobiohydrolases in the resuspended solid particles (Fig. 5). In contrast, when fresh cellulose was added to the filtrate, cellobiose concentration diminished about 0.5 mg/mL, after 6 h of incubation.

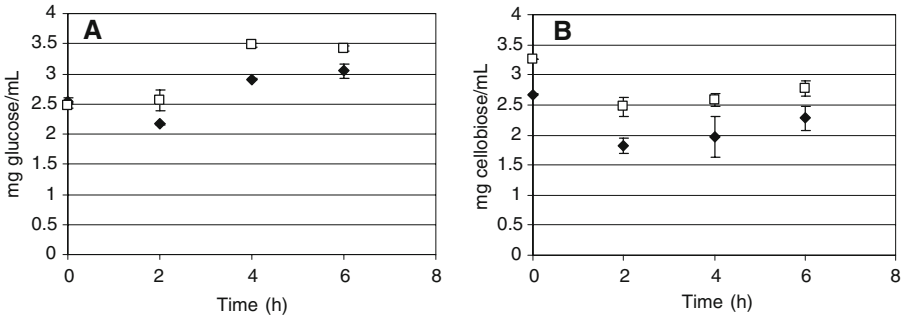

Fig. 4 Time courses of formation of glucose (**a**) and cellobiose (**b**) on the filtrate with added cellulose 50 g/L (*squares*) and non-added cellulose (*diamonds*). Error bars represents standard deviation for two repeated experiments

To further confirm the hypothesis of enzyme adsorption, free enzyme was quantified by determining the protein content of the filtrates and washings. The total quantity of bound enzyme was calculated from the difference between initial enzyme loading and free enzyme concentration in the filtrate and washings. The initial enzyme load was about 14.8±0.1 mg, and after the multistage processes, where solids were not washed, the final protein content in the cellulosic material was 10.8±0.1 and about 10.1±0.1 mg where the solids were washed. As shown, after the four cycles, the majority of the protein remains bound to the solid part after filtration. Only a small amount of enzyme is lost after our washings conditions, but this loss did not have a significant impact in the hydrolysis rate and in the final concentrations of glucose and cellobiose compared to that of the multistage process without washings.

Conclusion

In this study, cellulose enzymatic hydrolysis was investigated in a multistage scheme in order to enhance cellobiose production. The capability of some cellulases to bind their substrate was used in this process. By our approach, the cellobiose yield was 1.45 times higher than with a continuous hydrolysis process without the addition of further enzyme. A washing step of the retentate was shown not to significantly further enhance the total

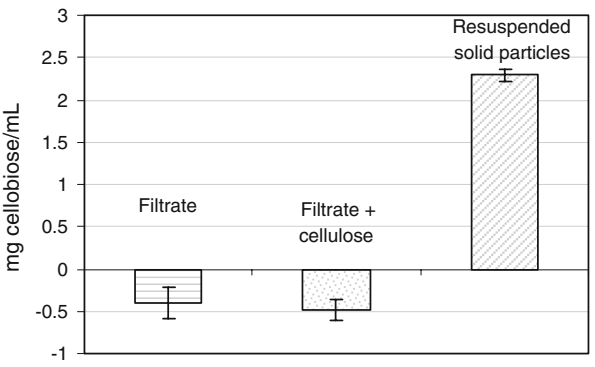

Fig. 5 Decrease or increase of cellobiose concentration after 6 h of incubation: in the filtrate, filtrate with cellulose addition, and resuspended retentate. Error bars represents standard deviation for two repeated experiments

cellobiose formation. We confirmed that mostly cellulases (endoglucanases and cellobiohydrolases) remained bound to the substrate and that β-glucosidases were eliminated in the filtrate during the first separation step. This approach is expected to be useful to upgrade the production of cellobiose and other oligosaccharides by enzyme hydrolysis of cellulosic biomass.

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