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Use of Cellulase Inhibitors to Produce Cellobiose

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Abstract The economics driving biorefinery development requires high value-added products such as cellobiose for financial feasibility. This research describes a simple technology for increasing cellobiose yields during lignocellulosic hydrolysis. The yield of cellobiose produced during cellulose hydrolysis was maximized by modification of reaction conditions. The addition of an inhibitor from the group that includes glucose oxidase, gluconolactone, and gluconic acid during cellulase hydrolysis of cellulose increased the amount of cellobiose produced. The optimal conditions for cellobiose production were determined for four factors; reaction time, cellulase concentration, cellulose concentration, and inhibitor concentration using a Box-Behnken experimental design. Gluconolactone in the cellulase system resulted in the greatest production of cellobiose (31.2%) from cellulose. The yield of cellobiose was 23.7% with glucose oxidase, similar to 21.9% with gluconic acid.

Keywords Cellobiose · Cellulase · Glucose oxidase · Gluconolactone · Gluconic acid · Cellulase inhibition

Introduction

Cellobiose is a homoglucan disaccharide linked β-1,4 and is the repeating unit of cellulose. It offers enormous potential as a source of renewable products for the food, cosmetic, and pharmaceutical industries. Enzymes capable of breaking down cellobiose are absent in humans. Also, it is poorly fermented by intestinal microbes, having an estimated a caloric value of only 2 kcal/g [1]. Cellobiose has a higher prebiotic index (PI) than a currently favored prebiotic fructooligosaccharide [2]. Cellooligosaccharides containing greater than 70% cellobiose are metabolized by both Bifidobacterium and lactic acid bacteria but are not utilized by *Clostridium perfringen* and can be used for pharmaceuticals and functional foods [3]. Cellobiose fermentation by human fecal slurries produced much higher butyric acid content, which has been shown to have cancer-preventing properties [4, 5] than fructooligosaccharides

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[2]. Orally administered cellobiose reduced both neural fat in the liver and total cholesterol, indicating a potential drug use for prevention and treatment of life style-related diseases [6]. Cellobiose has also been utilized for the production of lactic acid by *Lactobacillus delbrueckii* [7]. There are some reports on oligomers containing cellobiose as a component producing specific functionalities. For example, a certain cellobio-oligosaccharides showed potential as an anti-fungal and anti-cariogenic agent [8]. A cellobiose-acarbose analogue is reported to be an inhibitor of β-glucosidase [9, 10] and fully or partially esterified cellobiose is a strong thickener in cosmetics [11]. Cellobiose can be an effective and functional carbohydrate in many applications if it can be produced economically.

There is a scarcity of information on the costs and methods for commercial production of clean cellobiose in quantity. The oldest and simplest method for producing cellobiose is by acidic hydrolysis of cellulose. This process causes numerous problems, including acid corrosion, neutralization requirements, side products and difficulty in size control. Enzymatic hydrolysis of cellulose avoids many of these problems because it utilizes defined substrates under mild conditions, i.e., pH 4–7 and temperatures between 30–50 °C [12]. When cellulase hydrolyzes cellulose, cellobiose is released synergistically by endo-β-glucanase (EC 3.2.1.4) and exo-β-glucanase (EC 3.2.1.91) in a cellulase system. It is then hydrolyzed to glucose by β-glucosidase (EC 3.2.1.21; [13]). Based on the regulatory patterns for each catalytic reaction by cellulase, blocking β-glucosidase accumulates cellobiose. Gluconolactone and gluconic acid have been reported to inhibit β-glucosidase selectively in a cellulase system [14–16]. In other words, both exo- and endo-glucanase are possible to act synergistically with uncoupled from the hydrolytic action of β-glucosidase in a cellulase system during cellulose hydrolysis in the presence of these inhibitors. Therefore, use of these inhibitors is expected to increase amounts of cellobiose being produced. In this study, cellobiose was produced from cellulose and sugarcane bagasse using cellulose reaction modification by incorporating inhibitors to the reaction mixture.

Materials and Methods

Substrates, Enzymes, and Chemicals

Avicel™ Type PH 101 (FMC, Philadelphia, PA) was used as a model for "pure" cellulose. Ammonia-treated sugarcane bagasse (58.2% glucan) was donated by Dr. G. DeQueiroz (Audubon Sugar Institute, LSU Ag Center). The cellulosic substrates, Avicel™ and ammonia-treated sugarcane bagasse were soaked ten times with 20 mM sodium citrate buffer, pH 5.2 prior to hydrolysis. Cellulase utilized was a commercial mixture enzyme of exo- and endo-glucanase and β-glucosidase produced from *Trichoderma viride*. Cellulase activity was determined as a filter paper unit (FPU) according to a NREL procedure [17]. Glucose oxidase, gluconic acid, and gluconolactone were tested as inhibitors. The unit definition of glucose oxidase followed that of the manufacturer where a unit is the amount that oxidizes 1.0 μmole of β-D-glucose to D-gluconolactone and H_2O_2 in one min at pH 5.1 and 37 °C. All enzymes and chemicals except for Avicel™ were obtained from Sigma-Aldrich (St. Louis, MO).

Cellulose Hydrolysis

In the inhibition studies, 1% cellulose was used as a substrate for 0.46 FPU/ml cellulase in the presence of various concentrations of inhibitors, 0.1–10% gluconolactone, 0.5–10% gluconic acid, or 0.51–51 U/ml of glucose oxidase. Several experimental groups were

tested for optimization of cellobiose production. All studies were performed at 37 °C and 150 rpm in sodium citrate buffer at pH 5.2.

Quantification of Sugars

The concentrations of cellobiose and glucose were measured using a high-performance liquid chromatography equipped with a refractive index detector. An Aminex-HPX-87 K column (Bio-Rad Lab., Hercules, CA) was used with 0.01 M K_2HPO_4 as the mobile phase. It was run at a constant flow rate of 0.6 ml/min at 85 °C.

Box-Behnken Experimental Design

A four-factor and three-level Box-Behnken experimental design in Design Expert 7.13 software (Stat-Ease, Inc) was used to optimize cellobiose production. Several factors—the amount of cellulose, cellulase, each inhibitor and reaction time—were used to determine each of the 29 formulations given in Tables 1 and 2. The high, medium, and low levels were selected based on preliminary experiments. Optimization was performed using a desirability function to obtain the levels of X_1, X_2, X_3, and X_4.

The behavior of the system is explained by the following quadratic model equation [18];

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

where Y is predicted response, β_0 intercept, β_1, β_2, β_3, and β_4 linear coefficient, β_{11}, β_{22}, β_{33}, and β_{44} squared coefficients and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} interaction coefficients. Total 29 experiments were necessary to determine the 15 coefficients for the model (Table 3).

Results and Discussion

Inhibition Study on a Cellulase System

Gluconic acid and gluconolactone act as mixed inhibitors as shown by inhibition plots (Fig. 1). Mixed-type inhibition is sometimes called noncompetitive inhibition, indicating that the action of the inhibitor on the enzyme changes the dissociation constant for substrate from K_s to αK_s [19]. The Ki values were 2.35 mg/ml at a 5% concentration of gluconic acid and

Table 1 Independent variables used in the Box-Behnken experimental design.

Symbol	Variable	Levels		
		−1	0	1
X_1	Cellulose (%)	1	3	5
X_2	Cellulase (FPU)	0.2	5.1	10.0
X_3	Glucose oxidase (U/ml)	0.51	5.1	51
	Gluconic acid (%)	0.5	5.25	10
	Gluconolactone (%)	0.10	2.55	5.00
X_4	Reaction time (h)	12	24	36

Table 2 Formulations used in the Box-Behnken design.

Run	X_1	X_2	X_3	X_4
1	–1	–1	0	0
2	–1	0	–1	0
3	–1	0	0	–1
4	–1	0	0	1
5	–1	0	1	0
6	–1	1	0	0
7	0	–1	–1	0
8	0	–1	0	1
9	0	–1	0	–1
10	0	–1	1	0
11	0	0	–1	–1
12	0	0	–1	1
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17	0	0	0	0
18	0	0	1	–1
19	0	0	1	1
20	0	1	–1	0
21	0	1	0	–1
22	0	1	0	1
23	0	1	1	0
24	1	–1	0	0
25	1	0	–1	0
26	1	0	0	1
27	1	0	0	–1
28	1	0	1	0
29	1	1	0	0

3.5 mg/ml at 1% gluconolactone concentration. Glucose analogues such as gluconolactone and gluconic acid have been reported to inhibit cellulase, showing a mixed inhibition pattern [14, 20]. However, the role of sugars and their analogues in most kinetic studies of cellulase enzymes has been debated because the mechanism of inhibition is largely dependent on the source and concentration of the enzymes, the conditions of hydrolysis, and even the methods of analysis for the products [21–23].

Effects of Inhibitors on Hydrolysis of Cellulose

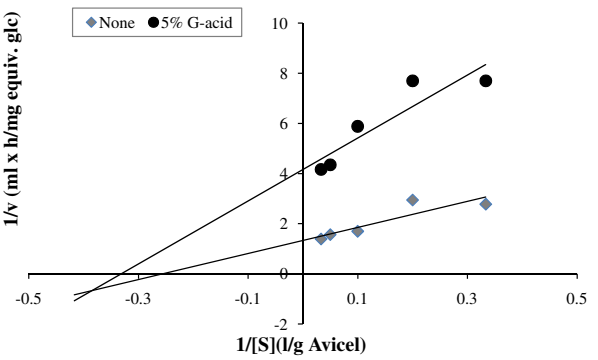
Cellobiose concentrations were monitored over a time course of 38 h during the hydrolysis of cellulose at 37 °C in the presence of glucose oxidase, gluconic acid, or gluconolactone. A significant increase in cellobiose concentrations in reaction mixtures was observed when the loading of glucose oxidase was 0.51 U/ml or higher (Fig. 2). The concentration of cellobiose in the reaction mixture increased with increasing glucose oxidase loading up to 12 h. Saturation of the reaction with glucose oxidase appears to be around 51 U/ml of

Table 3 Analysis of variance for the cellobiose production model.

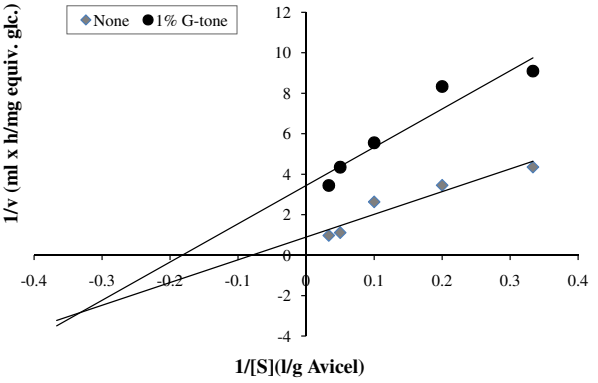
Source	Sum of squares	*df*	Mean square	*F* value	Probability >$F^{a,b}$
Glucose oxidase					
Model	19,390.89	14	1385.06	10.44	<0.0001
Residual	1,857.44	14	132.67		
Correlation total	21,248.32	28			
Gluconic acid					
Model	11,242.33	14	803.02	7.38	0.0003
Residual	1,523.63	14	108.83		
Correlation total	12,765.96	28			
Gluconolactone					
Model	6,957.93	14	497.00	4.40	0.0045
Residual	1,580.11	14	112.87		
Correlation total	8,538.05	28			

[a] Probability >*F*, level of significance

[b] Values of "probability >*F*" less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant



(a) Gluconic acid



(b) Gluconolactone

Fig. 1 Lineweaver-Burk plots for cellulase in the presence of gluconic acid (**a**) and gluconolactone (**b**)

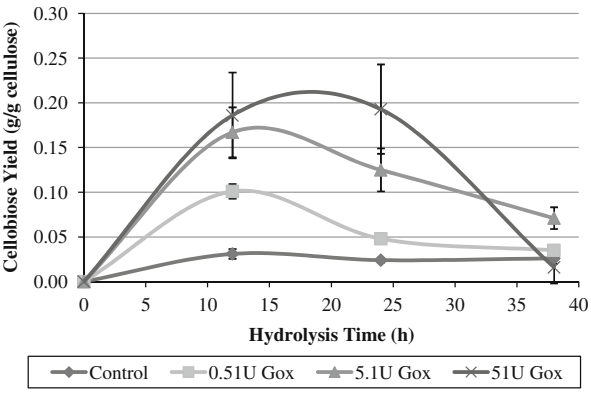


Fig. 2 Impact of various glucose oxidase loading on the production of cellobiose during Avicel™ hydrolysis

glucose oxidase. The highest concentration of cellobiose (0.18 g/g cellulose) was achieved after 24 h of hydrolysis in the presence of 51 U/ml of glucose oxidase and was eight times higher than in the absence of glucose oxidase. A decrease in cellobiose concentration was observed after it reached a maximum concentration. A large excess of glucose oxidase (above 51 U/ml) suppresses the production of cellobiose (data not shown) as it interferes with the cellulase hydrolysis, physically obstructing the approach of the cellulase to cellulose [16]. Without glucose oxidase, only 0.02 g cellobiose (per gram cellulose) was produced. Gluconic acid in the reaction mixtures produced an increase in cellobiose during cellulose hydrolysis (Fig. 3). The addition of 5% or 10% gluconic acid produced similar patterns of increased cellobiose. The highest yield of cellobiose was 0.3 g/g cellulose in the presence of 5% gluconic acid over a 38 h hydrolysis. This was ten times greater than the control. There were large differences in cellobiose concentration when gluconolactone was added to reaction mixtures containing 1% Avicel™ and 0.46 FPU/ml cellulase (Fig. 4). The highest yield of cellobiose (0.31 g/g cellulose) was achieved after 24 h of cellulose hydrolysis by supplementing with 1% gluconolactone. Excess gluconolactone (>1%) inhibited both cellobiose (Fig. 4) and glucose production (data not shown). The addition of 0.1% gluconolactone was insufficient for consistent production of cellobiose, since cellobiose concentration decreased rapidly with time.

Optimization of Cellobiose Production

On the basis of the cellulase-inhibitor relationship studies, 1–5% cellulose, 0.2–10 FPU of cellulase, 12–36 h reaction time, and each inhibitor; 0.5–51U of glucose oxidase, 0.5–10% gluconic acid, and 0.1–5% gluconolactone were selected as the ranges to optimize cellobiose production, employing a Box-Behnken design. A response surface methodology (RSM) was used to study the effect of interactions between independent variables on the production of cellobiose. The estimative response model equations for estimation of the cellobiose production were as follow:

$$Y = -77.01 + 45.32X_1 - 0.07X_2 + 4.64X_3 + 3.19X_4 + 1.18X_1X_2 - 0.18X_1X_3 - 0.22X_1X_4 - 0.05X_2X_3 - 0.0007X_2X_4 - 0.02X_3X_4 - 4.67X_1^2 - 0.22X_2^2 - 0.05X_3^2 - 0.05X_4^2 \text{ for a glucose oxidase model;}$$

$$Y = -10.43 + 2.63X_1 + 16.76X_2 + 8.16X_3 + 2.41X_4 + 0.86X_1X_2 - 0.77X_1X_3 + 0.38X_1X_4 + 0.75X_2X_3 - 0.06X_2X_4 - 0.12X_3X_4 - 1.56X_1^2 - 1.95X_2^2 - 0.38X_3^2 - 0.06X_4^2 \text{ for a gluconic acid model;}$$

$$Y = -25.12 + 20.43X_1 + 4.05X_2 + 11.03X_3 + 4.48X_4 + 0.59X_1X_2 + 0.25X_1X_3 + 0.05X_1X_4 + 0.97X_2X_3 + 0.04X_2X_4 - 0.07X_3X_4 - 3.66X_1^2 - 0.76X_2^2 - 2.22X_3^2 - 0.10X_4^2 \text{ for a gluconolactone model}$$

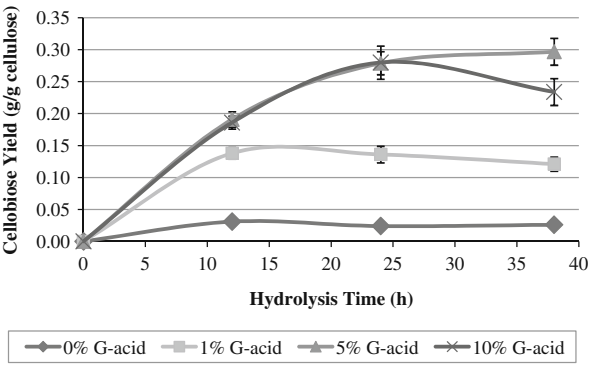


Fig. 3 Impact of gluconic acid concentration on the production of cellobiose during Avicel™ hydrolysis

where Y is the response factor, relative cellobiose yields (%), X_1, X_2, X_3, and X_4 are the real values of the independent factors; cellulose concentrations (%), cellulase concentration (FPU), inhibitor (U/ml for glucose oxidase and % for others), and reaction time (h). The fit qualities of the models were confirmed by analysis of variance (ANOVA). All three models are highly significant as evidenced from F_{model} values (10.44 at $p<0.0001$ in glucose oxidase; 7.38 at $p=0.0003$ in gluconic acid; 4.40 at $p=0.0045$ in gluconolactone) in the ANOVA.

The responses of surface plots (Fig. 5) represent the effect of four factors-the concentration of cellulose, cellulase, reaction time, and inhibitor and their reciprocal interactions with cellobiose yield on holding two of the factors constant at a middle level. As shown in Fig. 5a, the greatest cellobiose production was achieved with glucose oxidase loadings between 25.7 and 38.4 U/ml with 3% cellulose and a 24-h reaction time. The effects of cellulase concentrations and reaction times on cellobiose production are shown in Fig. 5b. The cellobiose production was sensitive to both cellulase concentration and reaction time. The optimal yield was obtained at 5.1 FPU of cellulase and 24-h reaction time. In the gluconic acid model, the best results are shown with cellulase loading between 2.6 and 7.5 FPU, whereas an increase in cellulose concentration increased cellobiose yields at 5.25% gluconic acid and 24-h reaction time (Fig. 5c). Figure 5d represents the relationship between concentrations of gluconic acid and cellulase on cellobiose production

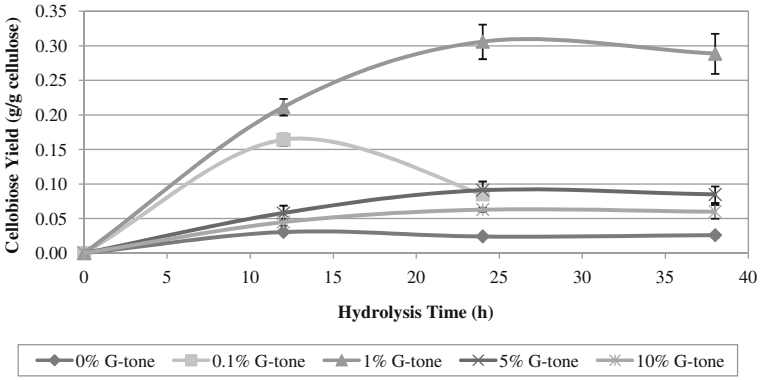


Fig. 4 Impact of gluconolactone concentration on the production of cellobiose during Avicel™ hydrolysis

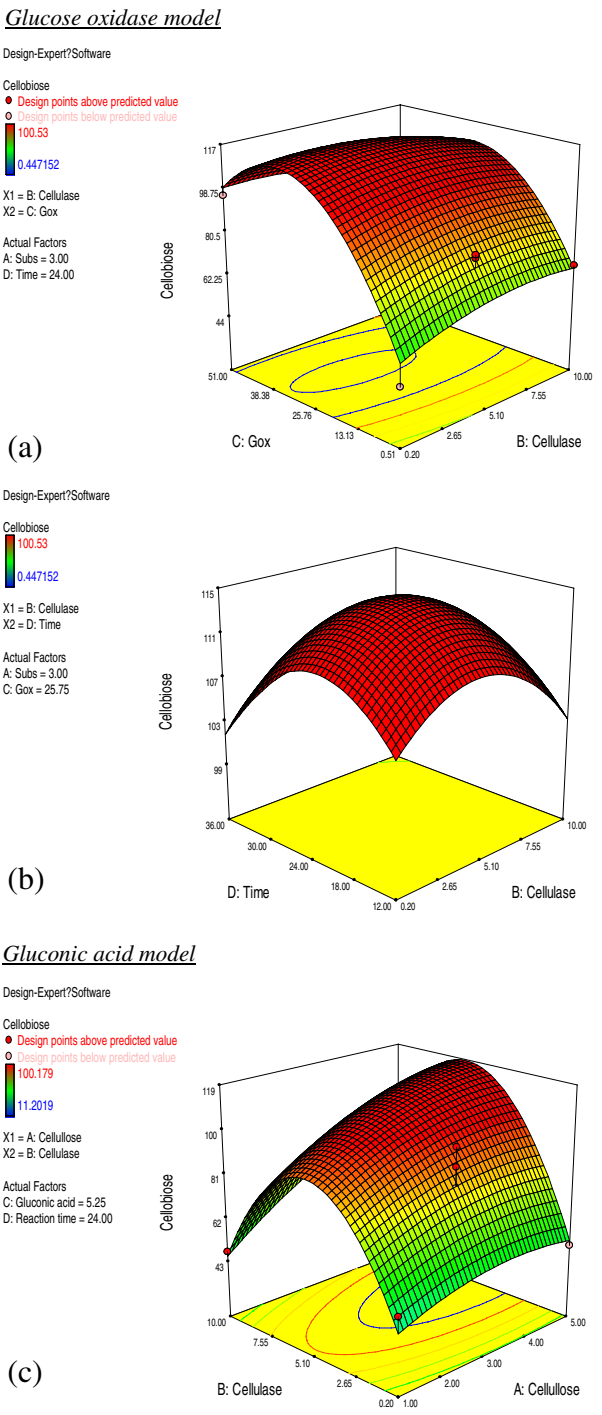
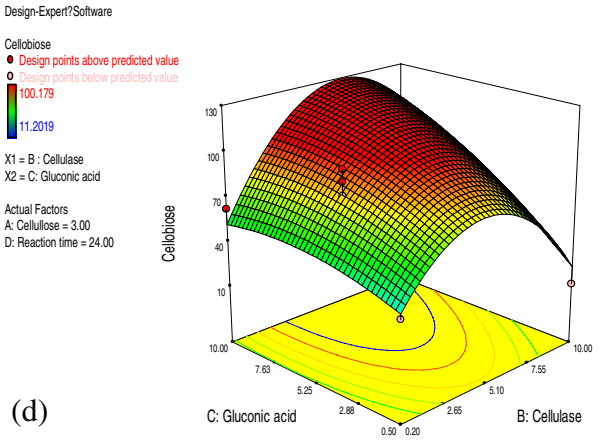


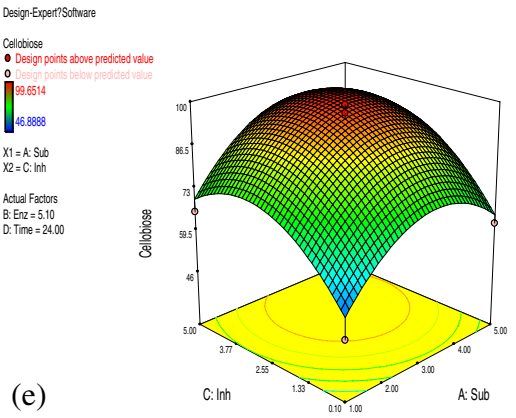
Fig. 5 Response surface plots illustrating the effect of varying concentrations of Avicel™, cellulase, and inhibitor, and reaction time on their reciprocal interactions with cellobiose production. Other factors are held constant at 3% Avicel™ and 24-h reaction time (**a**) in the glucose oxidase model; 3% Avicel™ and 25.75 U glucose oxidase in the glucose oxidase model (**b**); 5.25% gluconic acid and 24-h reaction time in the gluconic acid model (**c**); 3% Avicel™ and 24-h reaction time in the gluconic acid model (**d**); 5.10 FPU cellulase and 24-h reaction time in the gluconolactone model (**e**); 3% Avicel™ and 24-h reaction time in the gluconolactone model (**f**)

Fig. 5 (continued)

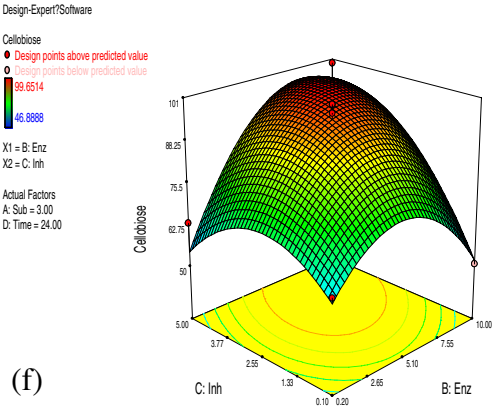


(d)

Gluconolactone model



(e)



(f)

at a fixed reaction time (24 h) and cellulose concentration (3%). Changing gluconic acid concentration did not impact the production of cellobiose, while the 5.1 U of cellulase achieved the maximal cellobiose production. Figure 5e, f show the relationships between the independent and dependent variables in the presence of gluconolactone using contour and response surface plots. The maximum production of cellobiose was reached with Avicel™ concentration between 3% and 4%, and gluconolactone concentration between 2.5 and 3.8 (Fig. 5e). The relationship between gluconolactone and cellulase shows the narrowest range of values and generates the maximum zone of the cellobiose production in the range of 1.3–3.8% gluconolactone and 2.6–7.5 FPU of cellulase. Optimal values elicited from RSM are 4% cellulose, 8.2 FPU of cellulase, 17.3 U/ml of glucose oxidase, and 16 h for a glucose oxidase model; 4% cellulose, 2.5 FPU of cellulase, 9.5% gluconic acid, and 22 h for a gluconic acid model; 4% cellulose, 7.6 FPU of cellulase, 3.9% gluconolactone, and 25 h for a gluconolactone model.

Cellobiose Production from Cellulose and Lignocellulosic Biomass

Optimal conditions from RSM were applied to the production of cellobiose from Avicel™ and ammonia-treated sugarcane bagasse as a source of lignocellulosic biomass. The concentration of ammonia-treated sugarcane bagasse had the same cellulose content as Avicel in the reaction mixture. Cellobiose production from Avicel™ was 9.48 mg/ml in the presence of glucose oxidase, 8.76 mg/ml in gluconic acid, and 12.5 mg/ml whereas the reaction mixture without inhibitor produced 1.3 mg/ml of cellobiose (Fig. 6). Ammonia-treated sugarcane bagasse did not achieve the maximum cellobiose yield which was produced from Avicel™ on the basis of yield (%). The addition of glucose oxidase yielded 23.7% cellobiose from Avicel™, but 11.48% cellobiose from bagasse. The addition of gluconic acid yielded 21.9% cellobiose from Avicel™, but 9.2% cellobiose from bagasse, and the addition of gluconolactone produced 31.2% cellobiose from Avicel™ and 11.0% cellobiose from bagasse. Lignocellulosic biomass-like sugarcane bagasse consists of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicelluloses. Even though it was pretreated, it is still complex, and many components such as lignin remnants, hemicelluloses and phenolic compounds remain, which may interfere with this system. Studies on the production of cellobiose have been conducted by several researchers [12, 24, 25]. Tanaka [25] produced 3–3.5 g of cellobiose from 50 g of microcrystalline cellulose by cellulase during 6.8–24 days. Homma et al. [12] reported that 0.9 g of cellobiose was

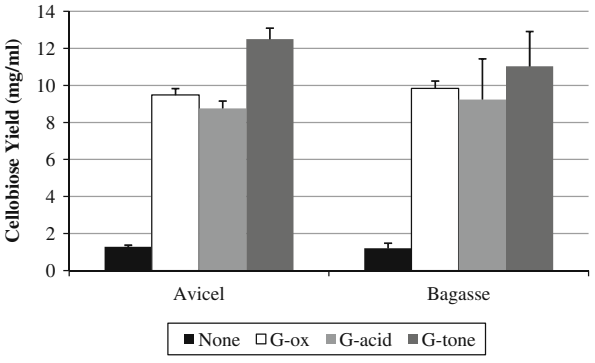


Fig. 6 Cellobiose production from Avicel™ and sugarcane bagasse using cellulase modification system

produced from 46.6 g of cellulose by cellulase, which β-glucosidase was removed, in the repeated batch reactions for 60 h. Tanaka and Oi [24] polymerized 21 mg of cellobiose from 30 g of glucose by β-glucosidase in 3 day incubation. In this study, conversion from cellulose to cellobiose by adding the appropriate inhibitor to the cellulase hydrolysis system was performed in a short time and in high amounts.

Conclusion

This study demonstrated a simple technology for increasing cellobiose yields from cellulose by adding cellulase inhibitors such as glucose oxidase, gluconic acid, and gluconolactone to an enzymatic cellulose hydrolysis system. The addition of any of the test inhibitors resulted in the production of between 7.4–10.4 times more cellobiose than in the absence of the inhibitor(s). Gluconolactone was the most effective cellulase inhibitor, yielding cellobiose as 31.2% of the theoretical conversion of cellulose. The yield of cellobiose was 23.7% with glucose oxidase, similar to 21.9% with an oxidized product of glucose, gluconic acid. Cellobiose yields using cellulase inhibition was significantly higher than has been previously reported. Significant quantities could be produced by this method as an additional product from a biorefinery.

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