Enzymatic Preparation of Cello-oligosaccharides Using Bamboo Materials

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Cello-oligosaccharides (COS) are products of the preliminary hydrolysis of cellulose. They have been the subject of significant research and application potential across various fields, including food, feed, and biotechnology. This study explored an eco-friendly, efficient process for producing COS from bamboo biomass. Subsequently, the optimal hydrolysis conditions using microcrystalline cellulose as the substrate were determined to establish the best process for converting bamboo cellulose into COS. The resulting hydrolyzate was analyzed, with cellobiose content (mg/L) serving as the response variable to identify the optimal conditions for pH, hydrolysis temperature, enzyme addition amount, substrate addition amount, reaction time, and inhibitor addition amount. Finally, various bamboo pretreatment technologies and cellulose hydrolysis methods were integrated to determine the most suitable hydrolysis technology for bamboo cellulose. The results of this study demonstrate that enzymatic hydrolysis can be employed as a production method to convert bamboo cellulose to COS.

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INTRODUCTION

Cello-oligosaccharides (COS) are low molecular weight polymers with a degree of polymerization ranging from 2 to 10. They consist of glucose molecules linked by beta-1,4-glycosidic bonds. The polysaccharide has water solubility and cannot be digested by human intestines; it is derived from biomass rich in cellulose (Ávila *et al*. 2021). COS has anti-oxidation, anti-tumor, lipid-lowering, and immune-regulating effects, with promising applications in the food and pharmaceutical industries (Saini *et al.* 2022). COS can also prevent cavity-causing bacteria from forming a matrix, thereby preventing tooth decay (Kim and Rajapakse 2005); wholly or partially esterified cellobiose to prepare a powerful thickener in cosmetics (Chen *et al.* 2021). Furthermore, studies have demonstrated that cellobiose exhibits a superior prebiotic index (PI) compared to the currently popular fructooligosaccharides, positioning it as a more promising source of oligosaccharide probiotics (Sanz *et al.* 2005). If COS can be produced economically in large quantities, it will bring tremendous opportunities to various industries (Siccama *et al.* 2022; Ahmed and Goyal 2024). China is the country with the most abundant bamboo resources in the world (Clark *et al.* 2022). Bamboo proliferates and has high cellulose content, making it a superior biomass raw material with cellulose content ranging from 45% to 55% (Batalha

et al. 2011; Wu *et al.* 2024). In addition, various bamboo processing factories generate a large amount of residual waste annually, all of which can be used as raw materials for COS production. Suppose the basic conditions for establishing a COS production plant can be met, such as convenient transportation, low-cost water and electricity, and affordable land. In that case, bamboo could be an excellent raw material for COS production. The preparation methods of COS mainly include enzymatic hydrolysis (Liu *et al.* 2024), acid-base hydrolysis (Xiong *et al.* 2023), hydrothermal liquefaction hydrolysis (Yang *et al.* 2023), and microbial method (Han *et al.* 2019). Enzymatic hydrolysis is the most promising method for producing oligosaccharides, with higher product yield, more substantial substrate specificity, and more environmentally friendly production conditions than other methods (Vanderghem *et al.* 2010).

Cellulase is a collective term for enzymes that degrade cellulose to generate glucose. It is not a single enzyme, but rather a multi-component system that works synergistically. It mainly consists of endoglucanase (EG), exoglucanase (CBH), and beta-glucosidase (BG). During the hydrolysis of cellulose, endoglucanase (EG), and cellobiohydrolase (CBH) work cooperatively to release cellobiose, which is subsequently hydrolyzed by β-glucosidase (BG) to produce glucose. Inhibiting BG activity allows for the accumulation of cellobiose. Numerous studies have explored methods to inhibit BG activity in cellulase, such as cultivating *Trichoderma reesei* in the presence of a specific concentration of $CaCO_3$, which results in cellulase with reduced BG enzyme activity (Wu 2003). Additionally, BG becomes irreversibly inactive under alkaline conditions (Xu *et al.* 2012). Chitosan has the ability to bind to EG and CBH within cellulase solutions, but it does not interact with BG; thus, BG can be separated through chitosan affinity precipitation (Homma *et al.* 1993). Furthermore, cellulase exhibits adsorption behavior on cellulose surfaces, allowing BG removal by centrifuging the upper liquid layer (Ooshima *et al.* 1983). Moreover, gluconolactone (GDL), glucose oxidase, and gluconic acid demonstrate selective inhibitory effects on BG (Kim and Day 2010).

Before enzymatic hydrolysis, biomass materials typically require pretreatment, which is essential for the effective hydrolysis of biomass raw materials. The primary aims of pretreatment are to remove lignin from the raw material, disrupt the compact structure of cellulose, eliminate inhibitory compounds, enhance hydrolysis efficiency, and increase product yield. Common pretreatment technologies include physical methods (Fei *et al.* 2020), chemical methods (Hafid *et al.* 2021), physical-chemical combination methods (Yu *et al.* 2020), and biological methods (Machado and Ferraz 2017). For instance, acid pretreatment employs hydrochloric acid, sulfuric acid, and succinic acid, which can reduce the length and size of fibers, although the cellulose content remains low (Gao *et al.* 2023). In the alkali treatment (NaOH) process, catalysts such as H_2O_2 or sodium hypochlorite ($NaClO_2$) are introduced, followed by dilute acid treatment. This acid-alkali composite treatment method maximizes the removal of lignin and hemicellulose from wood fibers, thereby enhancing cellulose's content and crystallinity and improving cellulose's susceptibility to hydrolysis (Hafid *et al.* 2021). Adopting new green solvents to replace traditional organic solvents for biomass pretreatment has emerged as a promising research direction. Deep eutectic solvents (DESs) are created by mixing two green organic solvents (Ling *et al.* 2022), such as deep eutectic solvents formed by combining choline chloride (ChCl) and lactic acid (LC) for the pretreatment of wood fibers. This approach can achieve a lignin removal percentage of 94.4% and a cellulose recovery rate of 91% (Liu *et al.* 2019).

This study explored an eco-friendly, efficient process for producing COS from bamboo biomass. Methods for inhibiting the activity of the BG enzyme were screened. Subsequently, the optimal hydrolysis conditions using microcrystalline cellulose as the substrate were determined. Finally, various bamboo pretreatment technologies and cellulose hydrolysis methods were integrated to identify the most suitable hydrolysis technology for bamboo fiber. This study provides new insights for utilizing bamboo raw materials and producing COS.

EXPERIMENTAL

Materials

Bamboo species, including *Phyllostachys pubescens*, *Dendrocalamus latiflorus*, *Dendrocalamus farinosus*, and *Bambusa beecheyana*, were collected from the bamboo research base at Southwest University of Science and Technology. Healthy, pest-free two-year-old bamboo specimens were selected, and their surface dirt was removed with distilled water. The bamboo was dried to a constant weight at 60 °C, ground into a powder, and sieved through a 100-mesh sieve. The sieved bamboo powder was ground for another 10 h in a planetary ball mill, which operated with a forward rotation for 10 min at a speed of 500 rpm.

Calcium carbonate ($CaCO_3$, AR), sodium hydroxide (NaOH, AR), sodium carbonate (Na_2CO_3, AR), sodium chlorite ($NaClO_2$, AR), benzene (C_6H_6, AR), ethanol (C_2H_6O, AR), and lactic acid ($C_3H_6O_3$, AR) were supplied by Chengdu Kelong Chemical Co., Ltd. (Chengdu, China). 4-Nitrophenol ($C_6H_5NO_3$, pNP; AR), 4-nitrophenyl β-D-cellobioside ($C_{18}H_{25}NO_{13}$, pNPC; AR), and 4-nitrophenyl β-D-glucopyranoside ($C_{12}H_{15}NO_8$, pNPG; AR) were obtained from Shanghai Lanji Technology Co., Ltd. (Shanghai, China). Glucono-δ-lactone ($C_6H_{10}O_6$, GDL; AR), microcrystalline cellulose (MCC, CP), and choline chloride ($C_5H_{14}ClNO$, ChCl; AR) were supplied by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Cellulase (BR) and potato dextrose agar (PDA, BR) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). *Trichoderma reesei* (strain ATCC 26921, BR) was provided by Wuhan Huana Biotechnology Co., Ltd. (Wuhan, China). Glucose, cellobiose, cellotriose, and cellotetraose (chromatographic grade) were also acquired from Shanghai Yuanye Bio-Technology Co., Ltd.

Pretreatment of bamboo

First, 5 g of the sample was added to 150 mL of an alkali extraction mixture, which was extracted at 80 °C under a condenser for 5 h using a reflux apparatus. The sample was washed, dried, and dissolved in 150 mL of an ice acetic acid solution after extraction. The mixture was extracted a second time at 80 °C under a condenser for 4 h in a reflux apparatus, after which the sample was washed until the filtrate was neutral. The sample was dried in an oven and stored (Ventura-Cruz *et al.* 2020). In the NaOH-$NaClO_2$ treatment method, 15 g of the sample was added to 150 mL of an alkali extraction liquid, which was sterilized at high pressure in a pressure cooker for 45 min. The sample was washed and dried before the addition of $NaClO_2$ extraction liquid. The mixture was treated at 120 °C in a pressure cooker for 2 h, washed, dried, and stored (Hafid *et al.* 2021). The DES treatment method (Liu *et al.* 2019) involved dissolving 15 g of bamboo powder in a mixture of benzene and ethanol, followed by a 4-h extraction at 90 °C using the Soxhlet extraction

method. After washing and drying, the sample was further processed using DES (ChCl:LC = 1:9) with a bamboo powder to DES ratio of 1:25. The mixture was treated at 120 °C for 3 h in an oil bath, after which it was washed, dried, and stored.

Cellulase treatment and enzyme activity assay methods

The activity of BG in cellulase is inhibited by various treatment methods to achieve a high yield of COS during cellulose hydrolysis. Utilizing the $CaCO_3$ medium method (Wu 2003), *Trichoderma reesei* was activated, inoculated onto PDA medium, and cultured at 28 °C for 5 to 7 days. Following Mandle's nutrient salt formulation, 20 g/kg of cellulose powder and 5 g/kg of wheat bran were added, along with 0.4 g/kg of $CaCO_3$, and the culture was maintained at 28 °C for 4 days. The culture was sterilized at 121 °C for 30 min. The mycelia of *Trichoderma reesei*, cultured on the aforementioned PDA, were transferred to a constant temperature shaker operating at 170 r/min and cultured at 28 °C for another 4 days. After this period, the solids were removed to yield a cellulase solution with low BG activity. In the inhibitor treatment method (Kim *et al.* 2010), 3.9% GDL was added to the cellulase solution (Cellulase was purchased from Shanghai Yuanye Biotechnology Co., 50 U/mg) to produce a cellulase solution exhibiting low BG activity. The renaturation method following alkali treatment (Xu *et al.* 2012) involves maintaining a constant temperature of 25 °C while adding 0.1% (w/v) NaOH to adjust the pH of the cellulase solution to 9. This mixture was allowed to stand for 30 min before the pH was adjusted to 4.8 using a 0.05 mol/L citrate-phosphate buffered saline (CPBS) solution. The alkaline-treated enzyme solution was then allowed to stand for 30 min to facilitate the renaturation of the cellulase, resulting in a cellulase solution with low BG activity. The "adsorption-separation" two-stage method (Ooshima *et al.* 1983) entails preparing a cellulose solution (The ratio of cellulose to sterile water was 1:20), adding a measured amount of cellulase, stirring thoroughly, and placing the mixture in an ice bath for 1 h. Following centrifugation, the upper liquid phase was removed, and the solid phase was retained. Sterile water was added to restore the original solution volume, and this process was repeated twice to obtain a cellulase solution with low BG activity.

The enzyme activity of cellulase components was assessed using the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Meng *et al.* 2021). Specifically, this was the p-nitrophenol (pNP) method for measuring the activities of CBH and BG enzymes. Additionally, the DNS method was employed to measure EG enzyme activity. Enzyme activity was defined as the amount of enzyme required to decompose 1 μmol of the substrate per min under specified temperature and pH conditions, with the unit expressed in IU/mL.

Mechanism of cellulase inhibitors

Following the method described by Holtzapple *et al.* (1990), 100 μL of GDL solution at concentrations of 0, 10, or 25 mg/mL was added to a microcentrifuge tube. Next, 100 μL of cellulase solution with activities of 1, 5, 10, 15, or 20 U/mL was added and mixed thoroughly. The mixture was incubated in a water bath at 50 °C for 30 min. After this incubation, 50 μL of a 15% pNPG solution, preheated to 50 °C, was introduced, and the reaction was continued in the water bath at the same temperature for 30 min. To terminate the reaction, 100 μL of 10% Na_2CO_3 was added. For the blank control group, the GDL solution was replaced with an equal volume of distilled water. Finally, the absorbance was measured at 420 nm. The reaction rate (V) was determined, and a plot was created with the reaction rate (*V*) on the ordinate and the cellulase concentration on the abscissa.

According to the aforementioned test method, the reciprocal of the reaction rate (1/*V*) was utilized as the ordinate and the reciprocal of the substrate concentration (1/*S*) as the abscissa to construct a double reciprocal graph known as the Lineweaver-Burk plot. This graph was employed to assess the competition of GDL for cellulase.

Box-Behnken Design

Single-factor experiment

Based on pre-experiment, the pH values were established at 3, 3.5, 4, 4.5, and 5. Hydrolysis temperatures were set at 30, 35, 40, 45, and 50 °C. The amounts of enzyme added were varied at 0.5, 1, 1.5, 2, and 2.5 FPU/L. Hydrolysis times were designated as 2, 4, 6, 8, and 10 h, while microcrystalline cellulose addition amounts were specified at 4, 6, 8, 10, and 12%. The resulting hydrolyzate was analyzed, with cellobiose content (mg/L) serving as the response variable to identify the optimal conditions for pH, hydrolysis temperature, enzyme loading, microcrystalline cellulose loading, reaction time, and inhibitor addition amount.

Plackett-Burman experiment factorial and level design

The Plackett-Burman design was developed using Design-Expert 13 software based on a single-factor experiment. The design factor levels and coding values are presented in Table 1. The experiment consisted of N=12 trials, investigating six factors: pH (A), temperature (B, °C), enzyme addition amount (C, FPU/L), microcrystalline cellulose addition amount (D, %), reaction time (E, h), and inhibitor addition amount (F, %). Additionally, a dummy factor was included to assess experimental error. Cellobiose production (mg/L) served as the response variable. Each experimental condition was replicated three times to evaluate the influence of each factor on the hydrolyzate content, allowing for the identification of significant factors. The results of the Plackett-Burman experiment indicated that the optimal hydrolysis conditions were determined by the three most significant factors: enzyme addition amount, microcrystalline cellulose addition amount, and inhibitor addition amount. Subsequently, the positive and negative effects of these factors were utilized to establish the climbing direction, with the step size determined by the response values of each factor. For the other insignificant factors, the high-level value was taken for the positive effect, while the low-level value was used for the negative effect, with cellobiose content (mg/L) remaining the response variable. Each experiment was conducted in triplicate to examine the impact of various factors on hydrolyzate content and to identify the optimal response region.

Table 1. Plackett-Burman Experiment Factorial and Level Design

Levels	B	C	D	E	F	Dummy Variable
Low (-1)	50	0.5	8	2	3	（G，H，I，J，K）
High (+1)	60	1.5	12	6	5	

Box-Behnken experimental design

Based on the results of the steepest climbing experiment, a Box-Behnken experiment was conducted with treatment 5 serving as the center point. The independent variables selected were the amounts of enzyme addition, microcrystalline cellulose addition, and inhibitor addition. Three factors were established according to the Box-Behnken design. In the horizontal experiment, cellobiose content was utilized as the

response variable to identify the optimal hydrolysis process. The design factors and their respective levels are presented in Table 2.

Table 2. Box-Behnken Experimental Design

Factors	Levels		
	-1	0	1
Cellulase (FPU/L)	2	2.5	3
Avicel (%)	12	13	14
Gluconolactone (%)	2	2.5	3

Preparation of COS by hydrolysis of bamboo with different pretreatment methods

Using bamboo cellulose treated with various pretreatment methods as raw material, COS was produced through cellulase hydrolysis. Based on the optimal hydrolysis conditions established *via* the Box-Behnken design, the pH of the reaction system was maintained at 4.5, the temperature at 55 °C, the enzyme concentration at 2 FPU/L, the microcrystalline cellulose concentration at 14%, and the hydrolysis reaction duration at 4 h, with an inhibitor concentration of 2%. Under these conditions, different types of bamboo cellulose were hydrolyzed, and the concentrations of glucose, cellobiose, cellotriose, and cellotetraose in the hydrolyzate were measured.

Measurement method of reaction products

The reaction solutions, subjected to various hydrolysis conditions, were placed in a boiling water bath for 10 min post-reaction to inactivate the cellulase. Subsequently, they were filtered using a water-based filter (PES polyethersulfone, 0.22 μm). Following moderate dilution, an ion chromatograph (Metrohm, Switzerland; model IC-881) was employed to analyze the hydrolysates. The chromatographic column used was the RCX-30 (79877), maintained at a temperature of 50 °C. An amperometric detector (896 Professional Detector 1) was utilized, with 0.1 M NaOH and 50 mM sodium acetate serving as eluents. The sampling volume was set at 20 mL, and a 0.7 mL/min flow rate was maintained. Standards were employed as references to detect and quantify glucose, cellobiose, cellotriose, and cellotetraose.

Hydrolyzate conversion rate

The conversion rate of cellulose into soluble compounds was defined as the hydrolyzate conversion rate and was calculated as follows (Siccama *et al.* 2022),

$$\text{convertion rate (g/100g cellulose)} = \frac{\text{C1}+\text{C2}\times 1.05+\text{C3}\times 1.07+\text{C4}\times 1.085}{C\times M\times 1.111}\times 100 \quad (1)$$

where C1, C2, C3, and C4 represent the concentrations of glucose, cellobiose, cellotriose, and cellotetraose in the hydrolyzate, respectively, with units expressed in grams per liter (g/L). Additionally, C denotes the initial concentration of bamboo cellulose in the sample (g/L), while M indicates the mass fraction of cellulose in bamboo. COS was quantified as 'free glucose'; thus, 1.05, 1.07, and 1.085 correction factors were applied to account for the water molecules introduced during hydrolysis. Given that cellulose possesses a degree of polymerization exceeding 500, a correction factor of 1.111 was utilized. In an ideal scenario, the maximum amount of cellulose converted into glucose was considered to be 100 g of glucose per 100 g of cellulose.

Data processing and analysis

SPSS software was utilized to analyze the data, and the significance of the means was compared using a t-test. The significance level was set at $P < 0.05$. GraphPad Prism 10 was employed to create illustrations. The Box-Behnken design was implemented using Design-Expert 13 statistical software for experimental design and data analysis, and the response surface charts were exported from Design-Expert 13.

RESULTS AND DISCUSSION

Changes in Cellulase Activity under Different Treatment Conditions

Both the inhibitor treatment method and the "adsorption-separation" method gave rise to the most significant inhibitory effects on BG enzyme activity. In contrast, the $CaCO_3$ medium method and the renaturation method following alkali treatment did not demonstrate any significant inhibitory effects. Under the inhibitor treatment method, BG enzyme activity decreased from 5.87 to 0.48 IU/mL, while CBH enzyme activity decreased from 461 to 283 IU/mL, and EG enzyme activity decreased from 71.9 to 51.4 IU/mL.

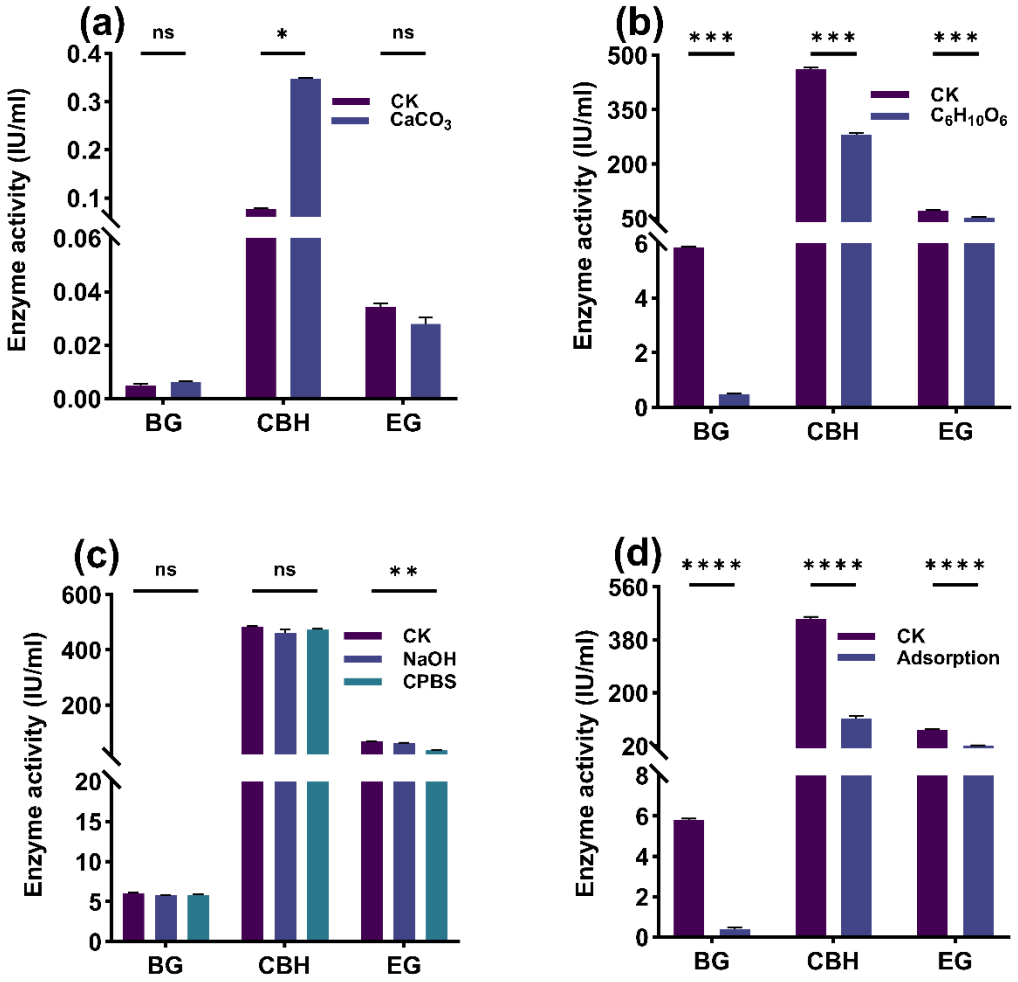


Fig. 1. Changes in cellulase activity under different treatment methods: (a) Enzyme activity in $CaCO_3$ medium method; (b) Enzyme activity in inhibitor treatment method; (c) Enzyme activity in renaturation method after alkali treatment; (d) Enzyme activity of "adsorption-separation" two-stage method. Error bars represent the standard deviation of triplicate experiments.

Similarly, under the conditions of the "adsorption-separation" method, BG enzyme activity decreased from 5.80 to 0.32 IU/mL; CBH enzyme activity decreased from 450 to 113 IU/mL, and EG enzyme activity decreased from 74.9 to 20.7 IU/mL. Although BG enzyme activity was significantly reduced, CBH and EG enzyme activity retention was notably lower compared to the inhibitor treatment method. Therefore, the inhibitor treatment method was selected for subsequent studies as the cellulase treatment approach to enhance COS production.

Research on the Inhibition Mechanism of Cellulase by Inhibitors

Figure 2 illustrates that adding inhibitors did not alter V_{max}, with the relationship $K''_m > K'_m > K_m$ observed. Notably, K'_m in the presence of inhibitors exceeded K_m in their absence and increased with higher inhibitor concentrations. The straight line of the double reciprocal plot intersects the vertical axis, which indicates competitive inhibition. This suggests that GDL exhibits competitive inhibition of BG within the cellulase component. The inhibitory effect arises from the competition between the inhibitor and the substrate for the binding site at the enzyme's active center, thereby disrupting the normal interaction between the substrate and the enzyme.

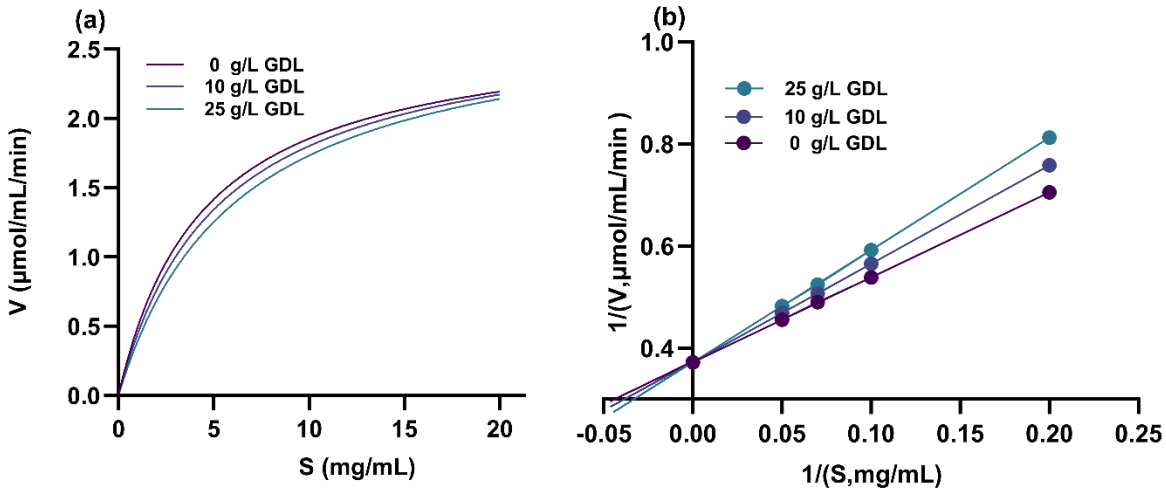


Fig. 2. The inhibitory effect of GDL on BG: (a) Competitive inhibition curve; (b) Lineweaver-Burk plot

Results of Single Factor Experiment

The single-factor experiment results of hydrolyzing microcrystalline cellulose to prepare cellobiose are shown in Fig. 3. As depicted in Fig. 3(a), cellobiose production initially increased and then decreased with increased enzyme added. The maximum cellobiose production (918 mg/L) was observed at an enzyme addition of 1 FPU/L; cellobiose production began to decline beyond this concentration. Figure 3(b) illustrates that, as pH increased, cellobiose production also followed a trend of first increasing and then decreasing. The optimal pH for cellulase activity was 4.5, at which point cellobiose production was maximized (874 mg/L). Deviations from this optimal pH, whether too high or too low, resulted in reduced enzyme activity. Figure 3(c) shows that, with increasing concentrations of the inhibitor, cellobiose production again exhibited a trend of increasing and then decreasing, with the highest production (680 mg/L) occurring at an inhibitor concentration of 4%. Figure 3(d) shows that when the substrate microcrystalline cellulose

was added at a concentration of 10%, cellobiose production reached its peak (1176 mg/L). This may be attributed to the saturation of the enzyme's active sites at higher substrate concentrations, thereby preventing further hydrolysis rate increases. Figure 3(e) indicates that the maximum cellobiose production (924 mg/L) was achieved at a reaction time of 4 h. Beyond this duration, hydrolysis efficiency gradually declined, which was likely due to the continued, albeit minimal, decomposition of cellobiose into glucose by non-deactivated BG enzyme, establishing 4 h as the optimal hydrolysis time. Lastly, as shown in Fig. 3(f), the highest cellobiose production (733 mg/L) occurred at a reaction temperature of 35 °C. Extreme high or low temperatures adversely affect enzyme activity and reduce cellobiose production.

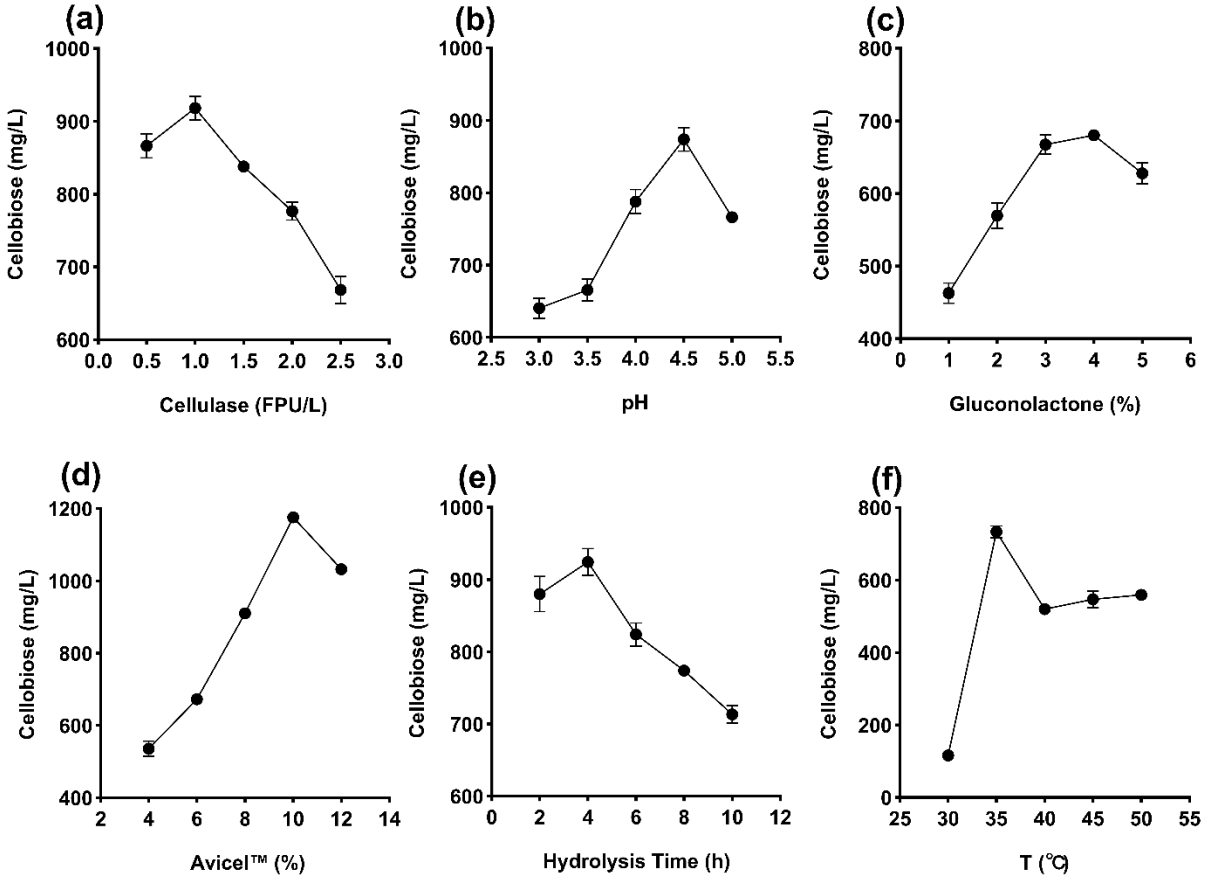


Fig. 3. Single factor test results of cellulose hydrolysis to prepare cellobiose: (a) enzyme addition amount; (b) pH; (c) inhibitor addition amount; (d) substrate addition amount; (e) reaction time; (f) reaction temperature

Results of Plackett-Burman Experimental Design

Based on the previous single-factor experiment results, an experimental design comprising 12 experiments (N = 12) was selected, with cellobiose production serving as the response variable. The experimental design and results are presented in Table 3. Using Design-Expert software, a correlation analysis was conducted on the Plackett-Burman data, deriving the half-normal probability effect diagram and the Pareto diagram of the standardized effects of six factors.

As illustrated in Fig. 4(a), the standardized effect points for factors C, D, and F are notably distant from the fitting line, while the standardized effect points for the remaining factors are closer. Thus, the amounts of enzyme, substrate, and inhibitor added are significant influencing factors ($P < 0.05$). Furthermore, Fig. 4(b) provides additional insights into the magnitude and importance of these factor effects, with factors C, D, and F exceeding the t value, thus confirming their significance.

Table 3. Plackett-Burman Experimental Design and Results

Run	A	B	C	D	E	F	Cellobiose (mg/L)
1	+1	+1	-1	+1	+1	+1	1102
2	-1	+1	+1	-1	+1	+1	901
3	+1	-1	+1	+1	-1	+1	1454
4	-1	+1	-1	+1	+1	-1	1298
5	-1	-1	+1	-1	+1	+1	1330
6	-1	-1	-1	+1	-1	+1	824
7	+1	-1	-1	-1	+1	-1	901
8	+1	+1	-1	-1	-1	+1	734
9	+1	+1	+1	-1	-1	-1	1501
10	-1	+1	+1	+1	-1	-1	1755
11	+1	-1	+1	+1	+1	-1	1669
12	+1	-1	-1	-1	-1	-1	846

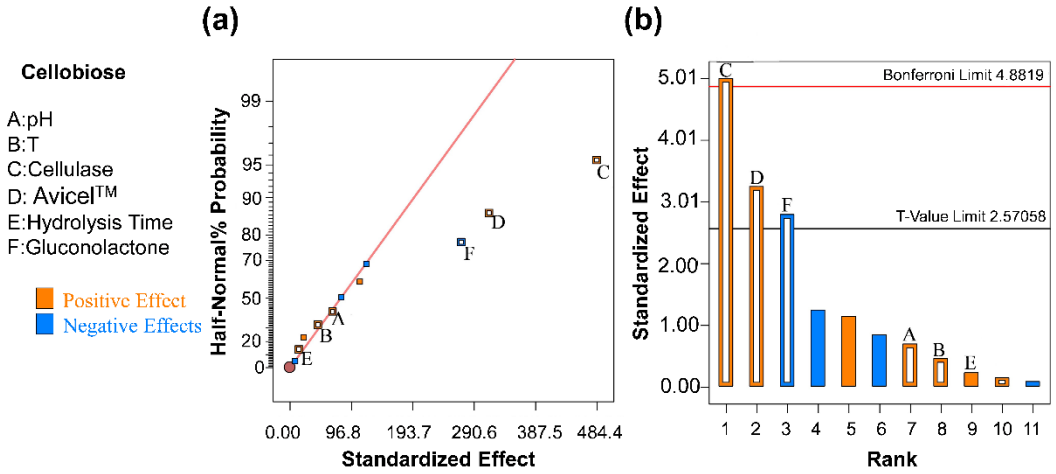


Fig. 4. (a) Half-normal probability effect diagram of standardized effect; (b) Pareto chart of standardized effect

Variance analysis was conducted on the experimental data, and the results from the Plackett-Burman design were evaluated using the Lenth method to identify significant effects, resulting in Table 4. The *P* value of the model was 0.0222, which was less than 0.05, indicating that the model was significant and reliable. The model demonstrated a good fit across the entire regression area, with a coefficient of determination of $R^2 = 0.8986$, suggesting a strong correlation in the experiment. The adjusted coefficient of determination $R^2_{adj} = 0.7770$ indicates that this regression model can explain 77.7% of the variability in the experimental data. Furthermore, the coefficient of variation (*CV*) is 14.03%; generally,

a lower *CV* signifies greater credibility and accuracy of the experiment. Adeq Precision is the ratio of the effective signal to noise, with a value of 8.1343, which exceeds the threshold of 4.0, indicating that the precision of this experiment is credible.

By performing multiple regression fitting on the data, the regression equation was obtained in Eq. 2.

$$Y = -101.23 + 67.7A + 4.46B + 484.36C + 78.72D + 3.6E - 135.46F \quad (2)$$

The regression equation reveals that the partial regression coefficient for factor C was 484.37, indicating a positive influence of factor C on the content of cellobiose. Specifically, as factor C increases, the content of cellobiose exhibits an upward trend. Additionally, factor D demonstrates a positive effect, while factor F has a negative effect.

Table 4. Factor Levels and Significance Analysis of Plackett-Burman Experiment

Source	*df*	Sum of squares	Mean square	*F* value	*P* value	significance
Model	6	1240000	207000	7.39	0.0222	*
A	1	13747	13747	0.4907	0.5149	n.s
B	1	5977	5977	0.2134	0.6635	n.s
C	1	704000	704000	25.12	0.0041	**
D	1	298000	298000	10.62	0.0225	*
E	1	623	623	0.0223	0.8872	n.s
F	1	22000	2.2000	7.87	0.0378	*
Residual	5	140000	28017			
Correlation total	11	1380000				
CV (%)	14.03					
R^2	0.8986			Pred R^2	0.4161	
R^2_{adj}	0.7770			Adeq Precision	8.1343	

The steepest climbing experiment utilizes the gradient direction of the response value change as the climbing direction, determining the step length based on the effect values of various significant factors. This approach allows for a rapid and cost-effective convergence towards the maximum response area. Following the Plackett-Burman experiment, the most significant factors identified were C, D, and F, with factor C being the most prominent. Consequently, factor C is employed as the climbing unit.

$$C(climbing\ unit) = \frac{484.36}{484.36} \times \frac{(1.5-0.5)}{2} = 0.5\ FPU/L \quad (3)$$

$$D(climbing\ unit) = \frac{78.72}{484.36} \times \frac{(12-8)}{2} = 0.32\% \quad (4)$$

$$F(climbing\ unit) = \frac{135.46}{484.36} \times \frac{(5-3)}{2} = 0.28\% \quad (5)$$

The step sizes of factors C, D, and F were, respectively, 0.5FPU/L, 1%, and 0.5% according to the actual situation.

The climbing experiment comprehensively considers various factors, including cost and workload. Factors A, B, and E demonstrated positive effects, as illustrated in Fig. 4, with each positive effect quantified at +1. The pH was maintained at 4.5, reflecting the actual conditions. Additionally, the temperature was set at 55 °C, and the reaction time was

established at 4 h. The experimental design and results are detailed in Table 5. Notably, the experimental findings indicate that the cellobiose content in treatment 5 reached its maximum value, leading to the selection of treatment 5 as the central point for the response surface experiment.

Table 5. Experimental Design and Results of the Steepest Climb

Run	Step size	A Positive Effects	B Positive Effects	C Positive Effects	D Positive Effects	E Positive Effects	F Negative Effects	Cellobiose (mg/L)
1	0	4.5	55	0.5	9	4	4.5	833
2	0+1△	4.5	55	1	10	4	4	1115
3	0+2△	4.5	55	1.5	11	4	3.5	1139
4	0+3△	4.5	55	2	12	4	3	1381
5	0+4△	4.5	55	2.5	13	4	2.5	1871
6	0+5△	4.5	55	3	14	4	2	1580

Box-Behnken Experiment

The results of the steepest climbing experiment indicate that, with treatment 5 as the central point, the amounts of enzyme added (A, FPU/L), substrate added (B, %), and inhibitor added (C, %) serve as independent variables. Design-Expert 13 software was utilized to design a Box-Behnken three-factor, three-level experiment, with the cellobiose content in the hydrolysis reaction as the response variable. The experimental design and results are presented in Table 6.

Table 6. Box-Behnken Experimental Design and Results

Run	A	B	C	Cellobiose (mg/L)
1	-1	-1	0	1749
2	-1	-1	0	1833
3	-1	-1	0	1875
4	-1	-1	0	1894
5	-1	0	-1	1896
6	-1	0	-1	1633
7	-1	0	-1	1465
8	-1	0	-1	1871
9	0	-1	-1	1761
10	0	-1	-1	2022
11	0	-1	-1	1792
12	0	-1	-1	1747
13	0	0	0	1498
14	0	0	0	1510
15	0	0	0	1490
16	0	0	0	1497
17	0	0	0	1508

The Box-Behnken experimental design and its results were processed and analyzed using Design-Expert 13. The variance analysis of the quadratic model is presented in Table 7. In this table, the model significance test was found to be highly significant ($P < 0.0001$), confirming the statistical relevance of the model. Specifically, the linear factors A, B, and C significantly influenced cellobiose, while the interaction terms AC and BC had an extremely significant effect. Additionally, the squared terms A^2, B^2, and C^2 also

significantly impacted cellobiose. These findings indicate the degree of influence ranked as follows: C > B > A, corresponding to inhibitor > substrate loading > enzyme loading.

The lack of fit term was utilized to assess the degree of fit between the employed model and the laboratory data. In this experiment, the P value for the lack of fit term is 0.1568 ($P > 0.05$), suggesting that the lack of fit is not significant, which is advantageous for the model and indicates no loss of fit. Therefore, the regression equation can be used to analyze the experimental results. The model's coefficient of determination, $R^2 = 0.9983$, and the adjusted correlation coefficient, R^2 (adj) = 0.9961, both exceeding 0.80, demonstrate that the model exhibited a firm fit. A quadratic polynomial equation is derived by applying quadratic multiple regression to the data.

$$Y = 42601.87 - 3418.98A - 1872.37B - 32.67AB + 668.83AC - 153.42BC + 446.56A^2 + 225.48B^2 + 417.05C^2 \quad (5)$$

Table 7. Box-Behnken Experimental Design Quadratic Model Variance Analysis

Source	*df*	Sum of squares	Mean square	F value	*P* value	significance
Model	531000	9	58965	458.24	< 0.0001	***
A	7492	1	7492	58.23	0.0001	**
B	20284	1	20284	157.63	< 0.0001	***
C	23943	1	23943	186.07	< 0.0001	***
AB	1067	1	1067	8.29	0.0237	*
AC	112000	1	112000	869.09	< 0.0001	***
BC	23536	1	23536	182.91	< 0.0001	***
A^2	52478	1	52478	407.83	< 0.0001	***
B^2	214000	1	214000	1663.53	< 0.0001	***
C^2	45772	1	45772	355.71	< 0.0001	***
Residual	900.75	7	128.68			
Residual	624.78	3	208.26	3.02	0.1568	n.s
CV（%）	0.66		R^2	0.9983		
			R^2_{adj}	0.9961		
			Pred R^2	0.9804		
			Adeq Precision	63.0011		

The regression model was employed to predict cellobiose production under various hydrolysis conditions. To facilitate the observation of the model's prediction results, both the residual standard distribution diagram and the model prediction scatter diagram were generated (Fig. 5).

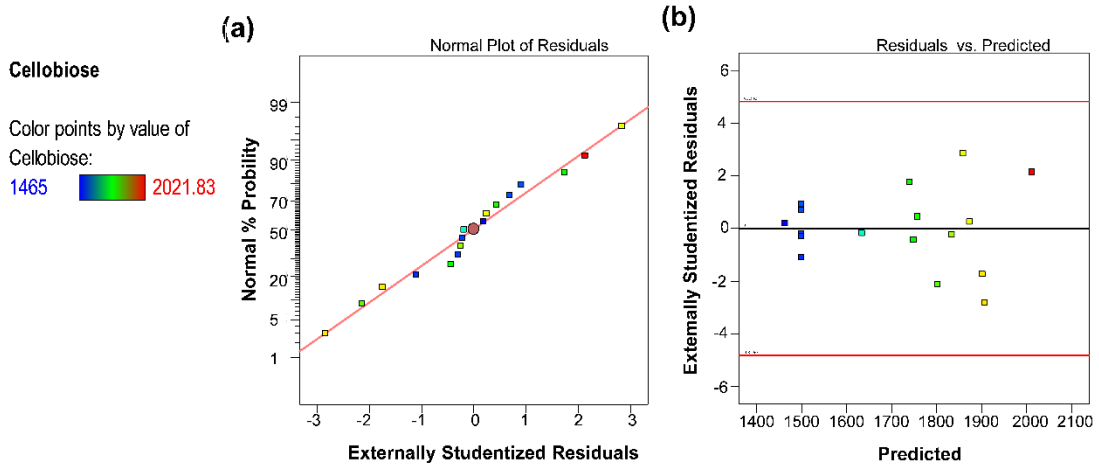
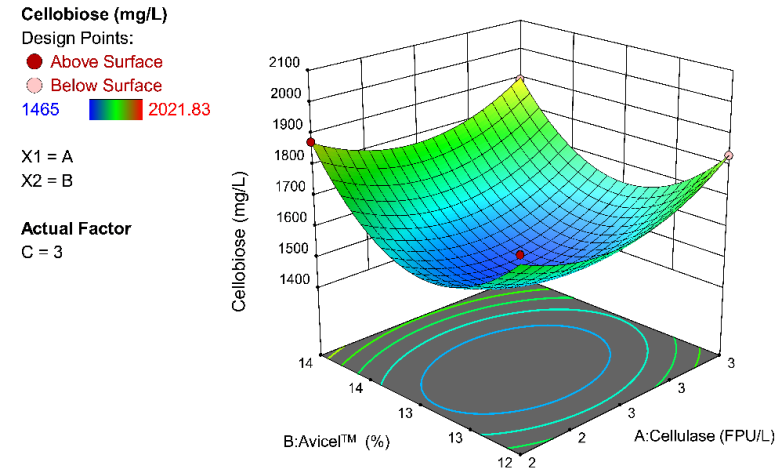
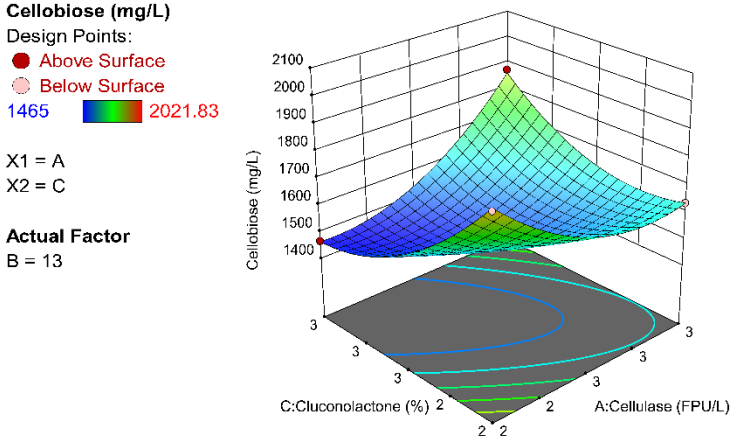


Fig. 5. (a) Residual normal distribution diagram; (b) residual error versus model prediction scatter diagram





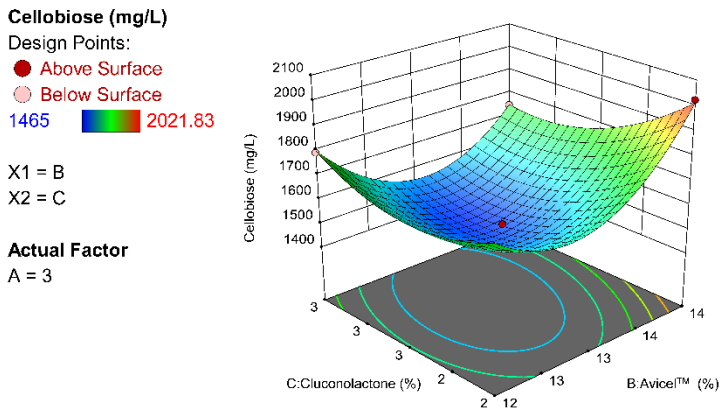


Fig. 6. Response surface contour plot of the interaction between factors

As shown in Fig. 5(a), the sample points were clustered closely around the 45° diagonal, indicating a strong correlation between the model's predictions of cellobiose yield and the experimental values, with minimal discrepancies. Typically, a robust model necessitates that the prediction error for the majority of samples does not exceed ±2SD. Figure 5(b) reveals that all sample points fell within the ±2SD range of the model, with no outliers present. This finding suggests the model exhibited high prediction accuracy and was suitable for process analysis in cellobiose preparation.

The response surface analysis diagram generated by Design-Expert is illustrated in Fig. 6, depicting the impact of three factors-enzyme addition, inhibitor addition, and substrate addition-on cellobiose production and the interrelationships among these factors and cellobiose yield. The interaction of yield is evident in the 3D response surface diagram, where the maximum yield value is identified. Following the central composite experimental design principle of Box-Benhken, three significant factors influencing cellobiose production were selected: enzyme addition amount (A, FPU/L), substrate addition amount (B, %), and inhibitor addition amount (C, %). A response surface analysis experiment involved these three factors at three levels. The optimal process conditions identified were: pH 4.5, temperature 55 °C, enzyme addition of 2 FPU/L, substrate addition of 14%, reaction time of 4 h, and inhibitor addition of 2%. Under these conditions, the theoretical yield of cellobiose was calculated to be 2277 mg/L, with a cellulose conversion rate to cellobiose of 15.5%. Verification experiments under the established conditions yielded an experimental cellobiose production value of 2231 mg/L, which approximates the theoretical prediction. This indicates that the model effectively predicts the hydrolysis process, thereby demonstrating the feasibility of optimizing cellobiose yield using response surface methodology.

Hydrolysis of Bamboo Cellulose and Analysis of COS Products

In recent years, numerous studies have sought to extract COS from various biomass raw materials, such as corn cobs (Chu *et al.* 2014), asparagus (Siccama *et al.* 2022), sugarcane straw (Barbosa *et al.* 2020), and miscanthus (Kendrick *et al.* 2022). Using pretreated bamboo as the raw material, bamboo cellulose was hydrolyzed under optimal conditions determine using the Box-Behnken designed experiment, and the cellobiose content in the hydrolyzate was measured. The results are presented in Fig. 7. The yield of cellobiose obtained from the DES-treated *Bambusa beecheyana* and *Dendrocalamus latiflorus* was

the highest. The cellulose hydrolysis reaction products of the four DES-treated bamboos were analyzed, and the conversion percentage of the cellulose hydrolyzate was calculated. As indicated in Table 8, the total yield of COS in the *Bambusa beecheyana* was the highest at 7.8 g/100 g cellulose, with the cellobiose yield also being the highest at 5.48 g/100 g cellulose. Additionally, the yield of cellotriose hydrolyzed from *Dendrocalamus latiflorus* was the highest at 1.09 g/100 g cellulose, while the yield of cellotetraose hydrolyzed from *Phyllostachys pubescens* was the highest at 1.43 g/100 g cellulose. Comparing the conclusions of this study with previous research, it was found that the cellobiose yield of 1.6 g/100 g cellulose, obtained by hydrolyzing microcrystalline cellulose according to the optimal response surface process, was significantly higher than that reported by Vanderghem *et al.* (2010), which was 74 mg/100 g cellulose. Furthermore, under DES treatment, the total yield of COS from *Bambusa beecheyana* cellulose was also higher compared to the conversion rate of 36 mg/100 g cellulose achieved by Siccama *et al.* (2022) through enzymatic conversion of white asparagus waste into COS.

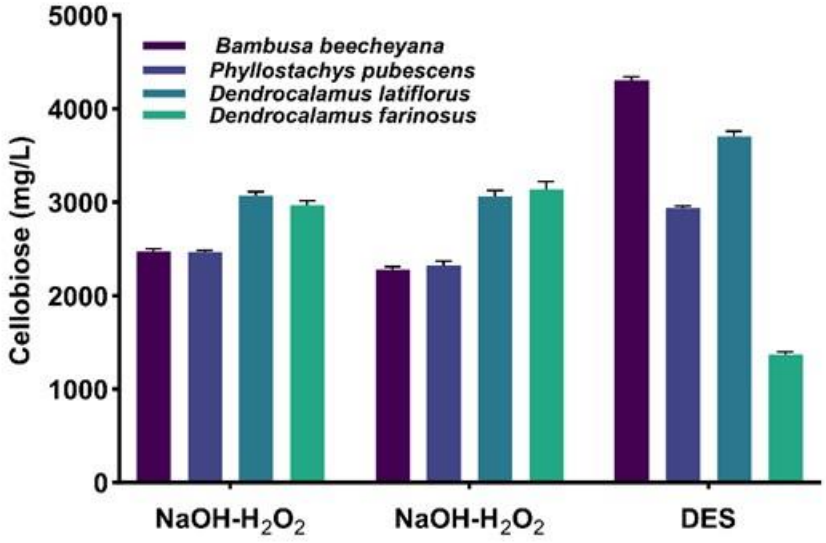


Fig. 7. Effects of different pretreatment methods on bamboo cellobiose yield

Table 8. Hydrolyzate Conversion Extents of Different Bamboos under DES Pretreatment

Bamboos	Total (g/ 100 g cellulose)	Cellobiose (g/ 100 g cellulose)	Cellotriose (g/ 100 g cellulose)	Cellotertrose (g/ 100 g cellulose)
Bambusa beecheyana	7.8±0.9	5.48±0.9	1.03±0.2	1.29±1.4
Phyllostachys Pubescens	7.01±0.3	4.51±0.2	1.07±0.9	1.43±0.9
Dendrocalamus latiflorus	7.28±0.4	4.82±0.3	1.09±0.8	1.39±0.1
Dendrocalamus farinosus	3.23±0.7	1.61±0.8	0.85±0.3	0.76±0.4

Additionally, Kim *et al.* (2010) reported a cellobiose yield of 21.9% using cellulase inhibitors in the hydrolysis of sugarcane bagasse. The results of this study indicate that while bamboo can serve as a raw material for producing a certain quantity of COS, the

conversion efficiency remains insufficient for large-scale production. This limitation primarily arises from cellobiose's significant inhibitory effect on the cellulase components, specifically CBH and EG. To enhance hydrolysis efficiency in future research, the following approaches are recommended: (1) Conducting an in-depth investigation into the interaction mechanisms between cellobiose and the CBH and EG components of cellulase, alongside the development of engineered cellulases that exhibit improved tolerance to cellobiose. This may involve modifications to the spatial structure of the active site to alter the binding affinity between cellobiose and the enzyme, thus minimizing the inhibitory effects of cellobiose and preserving the enzyme's efficacy in cellulose hydrolysis. (2) Optimizing the process by timely removal of cellobiose from the reaction system, such as utilizing membrane bioreactors (MBRs) to mitigate the product's inhibitory effects on the enzyme while streamlining the steps required for product separation and purification.

CONCLUSIONS

1. This study initially identified suitable β-glucosidase activity inhibition techniques and bamboo pretreatment methods, integrating them with an optimized hydrolysis process to regulate the formation of cello-oligosaccharides (COS) during the hydrolysis of bamboo cellulose. The introduction of gluconolactone (GDL) effectively inhibited β-glucosidase (BG) enzyme activity, decreasing from 5.87 to 0.48 IU/mL, while the activities of endoglucanase (EG) and cellobiohydrolase (CBH) remained largely unaffected.
2. Analysis of the inhibition curve and the Lineweaver-Burk plot for GDL's inhibitory effect on BG revealed that the inhibition was of the competitive type, characterized by competition between the inhibitor and substrate for the enzyme's active site, thereby disrupting the regular interaction between the substrate and the enzyme.
3. The pretreatment process enhanced biomass hydrolysis efficiency, with the DES pretreatment method demonstrating the most pronounced effect. This method not only enhanced the lignin removal but also improved the retention percentage of cellulose while partially disrupting the tight structure of cellulose.
4. The variety of bamboo was shown to significantly influence the conversion of hydrolysis products. The highest cellobiose yield was obtained from the DES-treated *Bambusa beecheyana* and *Dendrocalamus lactiferous*, with *Bambusa beecheyana* exhibiting the highest COS yield.

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