

Enhancing Tobacco Stem Utilization via Response Surface-Optimized Cellulase Hydrolysis for Cigarette Production

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Abstract

This study aims to enhance the valorization of tobacco stems in cigarette manufacturing by optimizing the cellulase hydrolysis process. A sequential statistical approach was employed, integrating single-factor experiments, Plackett-Burman design (PBD), steepest ascent method, and response surface methodology (RSM). Initially, Cellulase N was selected as the most effective enzyme via single-factor analysis. PBD identified temperature, enzyme concentration, and time as the most significant factors. The optimal response region was then approximated using the steepest ascent method and precisely refined by RSM. The established optimal parameters were: enzyme concentration, 508 U/mL; temperature, 49.1°C; time, 5.36 h; rotation speed, 120 rpm; pH, 5.0; and solid-liquid ratio, 1:40. Under these conditions, the process achieved a reducing sugar yield of 36.2% and a cellulose degradation rate of 70.7%. Structural analysis using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) confirmed the significant degradation and depolymerization of the tobacco stem's fibrous structure by Cellulase N. Furthermore, sensory evaluation demonstrated a marked improvement in the treated samples' quality over the control, especially in mitigating ligneous taste. This work establishes a robust parameter system for Cellulase N-mediated hydrolysis and provides a theoretical and practical basis for developing advanced enzyme cocktails. The findings are crucial for increasing tobacco stem inclusion in cigarette blends and advancing the sustainability of the tobacco industry.

Introduction

Tobacco stem, a coarse-veined byproduct of tobacco threshing and redrying, represents a significant biomass resource, with cell wall polymers (cellulose, hemicellulose, lignin, and pectin) comprising up to 43% of its mass [1–3]. With global annual production exceeding 2 million tons, improper disposal of this biomass poses environmental risks while squandering natural resources [1, 4]. The primary valorization strategy involves converting tobacco stems into cut stems or reconstituted tobacco for incorporation into cigarette blends [5, 6]. Leveraging its high filling value, cut stem reduces raw material costs and dilutes harmful smoke constituents like nicotine, tar, and CO [6–8]. Nevertheless, the high lignocellulosic content limits its inclusion ratio. The pyrolysis of these components during combustion generates undesirable and toxic byproducts, including low-molecular-weight aldehydes (formaldehyde, formic acid), phenols, and carcinogenic polycyclic aromatic hydrocarbons (PAHs), while also imparting woody odors and irritation that degrade sensory quality and smoking safety [9–11]. Cellulose, the dominant polymer in tobacco stems, is a glucose polymer linked by β -1,4-glycosidic bonds. Its pyrolysis exhibits a dual nature: it yields desirable caramel-like furfurals in the 300–500°C range but forms confirmed carcinogens like naphthalene and anthracene at temperatures above 600°C [11, 12]. Consequently, the efficient and selective degradation of cellulose to modulate its pyrolysis pathway is critical for unlocking the industrial potential of tobacco stems.

Conventional chemical treatments (acid/alkali) are plagued by equipment corrosion, environmental hazards, and uncontrolled side reactions [11, 13]. Conversely, enzymatic hydrolysis has emerged as a promising, eco-friendly alternative due to its mild conditions and high substrate specificity [14, 15]. While studies such as Li et al. have shown that cellulase treatment reduces cellulose and enhances sugars and aroma in tobacco leaves, research addressing the unique lignocellulosic matrix of tobacco stems remains scarce [12]. A major bottleneck is the suboptimal and unstable degradation efficiency (typically < 50% cellulose degradation) resulting from a lack of systematic process optimization, which fails to meet industrial demands for efficiency and stability. Moreover, the selectivity and adaptability of cellulase cocktails for the dense structure of tobacco stems require urgent investigation. Therefore, developing a tailored enzyme system and overcoming efficiency limitations through multi-parameter optimization are pivotal for advancing tobacco stem bioprocessing.

This study aims to systematically optimize the enzymatic hydrolysis of tobacco stems using commercial cellulase. A sequential statistical approach was employed, integrating single-factor experiments, Plackett-Burman design (PBD), steepest ascent method, and response surface methodology (RSM) to optimize critical parameters: temperature, pH, time, enzyme concentration, and solid-liquid ratio. The objective was to establish a robust and efficient hydrolysis system. This research provides a direct technical foundation for improving the sensory quality of cut stems and reducing cigarette harmfulness, while also pioneering a new pathway for the green and high-value utilization of tobacco stem biomass.

Materials and Methods

Materials and Reagents

The cut tobacco stem used in this study was provided by China Tobacco Hunan Industry Co., Ltd. The stems were sourced from Longshan, Hunan Province (2024 crop year) and processed into cut stems (0.17 mm width) strictly in accordance with the Cut Stem Process Specification of China Tobacco Hunan Industry Co., Ltd.. The preparation process included washing, conditioning, cutting, and drying. The cellulase enzymes used in this study were supplied by Sunson Industry Group, Qingdao Vland Biotechnology Incorporated, Luoyang Ouke Baike Biotechnology Co., Ltd., Wuhan Sunhy Biology Co., Ltd., Henan New Yangshao Enzymes Co., Ltd., and Novozymes Biotechnology Co., Ltd., respectively. Chemical reagents, including glucose, xylose, and arabinose standards, as well as sodium carboxymethyl cellulose (CMC-

Na), potassium sodium tartrate tetrahydrate, 3,5-dinitrosalicylic acid (DNS), sulfuric acid, citric acid, and sodium citrate, were all purchased from Beijing BioDee Biotechnology Co., Ltd.

Tobacco Stem Sample Pretreatment

Cut tobacco stem samples (10.0 g, accurately weighed, same source as described in Section 2.1) were placed in a 2 L stoppered conical flask. A stepwise extraction pretreatment was employed: initially, 1 L of preheated distilled water ($80 \pm 0.5^{\circ}\text{C}$) was added, and the mixture was subjected to constant-temperature oscillation at 120 rpm for 1 h. Subsequently, an additional 1 L of distilled water (preheated to the same temperature) was added, and the mixture was transferred to a constant-temperature water bath oscillator ($80 \pm 0.5^{\circ}\text{C}$) for a further 30 min of extraction. After completion of the reaction, the solid and liquid phases were separated using a vacuum filtration apparatus. The filtrate was discarded, and the solid residue was transferred to an electric blast drying oven. The residue was dried at 105°C until a constant weight was achieved. The resulting pretreated tobacco stem matrix was stored in a desiccator for subsequent use.

Optimization of Cellulase Hydrolysis Conditions

Single-factor experiments

A 0.4 g sample of pretreated cut tobacco stem was accurately weighed and placed in a 50 mL conical flask. Different types of cellulase solutions at a concentration of 100 U/mL were added separately, and the mixture was made up to a total volume of 15 mL with citric acid-sodium citrate buffer (pH 6.0). After sealing, the flask was placed in a constant-temperature water bath shaker at 50°C and shaken at 120 rpm for 2 h. The reaction was immediately filtered upon completion. For analysis, 1 mL of the filtrate was boiled for 15 min to inactivate the enzyme and used for reducing sugar content determination. The residue was thoroughly washed with distilled water and dried in an oven at 105°C to a constant weight for cellulose content analysis. Based on the identification of the optimal cellulase type, single-factor optimization of the hydrolysis conditions was conducted sequentially. The factor levels were as follows: enzyme concentration (5, 25, 50, 100, 150, 200, 250, 300, 350 U/mL), solid-liquid ratio (1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50), pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0), rotation speed (0, 60, 90, 120, 150, 180 rpm), temperature (30, 40, 50, 60, 70°C), and time (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 h). Except for the factor under investigation, all other conditions were maintained according to the basic reaction system described above. A blank control group (without enzyme addition) was also established to eliminate interference from residual reducing sugars in the substrate, following the same procedure. The yield of reducing sugar was calculated according to the following equation:

$$\text{Reducing sugar yield} = (C \times V \times F) / (M_0 \times 1000) \times 100\% \quad (1)$$

Where: C = Reducing sugar content (mg/mL); V = Total volume of the enzymatic hydrolysate (15 mL); F = Dilution factor of the enzymatic hydrolysate; M_0 = Oven-dried mass of the hydrolyzed sample (0.4 g); 1000 = Unit conversion factor.

PBD

Based on the results of the single-factor experiments, six key factors—enzyme concentration, solid-liquid ratio, temperature, pH, rotation speed, and time—were selected. A PBD incorporating three center points ($N = 15$) was employed to screen for significant factors. The reducing sugar yield was used as the response value. Each factor was set at two levels: high (+1) and low (-1). The detailed settings of the factors and levels are presented in Table 1.

Table 1
PBD matrix for evaluating factors influencing the reducing sugar yield

Run	Factors						Reducing sugar yield (%)
	Temperature (A, °C)	pH (B)	Rotation speed (C, rpm)	Time (D, h)	Solid-liquid ratio (E)	Enzyme concentration (F, U/mL)	
1	50 (+ 1)	4 (-1)	150 (+ 1)	2.5 (-1)	1:35 (-1)	200 (-1)	12.6
2	50	6 (+ 1)	90 (-1)	3.5 (+ 1)	1:35	200	15.2
3	30 (-1)	6	150	2.5	1:45 (+ 1)	200	7.5
4	50	4	150	3.5	1:35	300 (+ 1)	19.7
5	50	6	90	3.5	1:45	200	16.7
6	50	6	150	2.5	1:45	300	18.2
7	30	6	150	3.5	1:35	300	10.2
8	30	4	150	3.5	1:45	200	8.3
9	30	4	90	3.5	1:45	300	10.8
10	50	4	90	2.5	1:45	300	15.3
11	30	6	90	2.5	1:35	300	7.9
12	30	4	90	2.5	1:35	200	6.8
13	40	5	120	3	1:40	250	14.9
14	40	5	120	3	1:40	250	15.1
15	40	5	120	3	1:40	250	14.2
Steepest ascent experiment							

Following the single-factor and PBD experiments, factors significantly influencing the reducing sugar yield were subjected to a steepest ascent experiment. The step size and direction of variation for each factor were established by integrating the PBD effect estimates, single-factor trends, and preliminary data. The experiment consisted of seven distinct gradients, detailed in Table 2.

Table 2
Experimental designs and the results of steepest ascent experiment for reducing sugar yield

Run	Temperature (A, °C)	pH (B)	Rotation speed (C, rpm)	Time (D, h)	Solid-liquid ratio (E)	Enzyme concentration (F, U/mL)	Reducing sugar yield (%)
1	30	5.0	120	2.0	1:40	150	5.8
2	35			2.5		200	11.1
3	40			3.0		250	17.1
4	45			3.5		300	23.5
5	50			4.0		350	27.4
6	55			4.5		400	30.5
7	60			5.0		450	25.9

RSM

Following the PBD and steepest ascent trials, a Box-Behnken design (BBD) was employed to optimize the three most significant factors: temperature, enzyme concentration, and time. The design, comprising 15 runs for three factors at three levels with three replicates at the center point, was created using Design-Expert 11 software. The factor levels for the center point were established based on the optimal combination identified in the steepest ascent experiment, as detailed in Table 3.

Table 3
The BBD and the results for reducing sugar yield

Run	Temperature (A, °C)	Enzyme concentration (B, U/mL)	Time (C, h)	Reducing sugar yield (%)
1	40	250	4.5	21.9
2	70	250	4.5	2.6
3	40	550	4.5	28.7
4	70	550	4.5	3.9
5	40	400	3	21.7
6	70	400	3	2.4
7	40	400	6	28.7
8	70	400	6	3.9
9	55	250	3	24.0
10	55	550	3	30.0
11	55	250	6	28.3
12	55	550	6	32.9
13	55	400	4.5	33.1
14	55	400	4.5	32.9
15	55	400	4.5	32.0

Cellulase Activity Assay

The cellulase activity was measured by the 3,5-dinitrosalicylic acid (DNS) method [16]. The reaction was initiated by adding 25 μ L of enzyme solution (diluted in 50 mmol/L, pH 6.0 citrate-sodium citrate buffer) to 225 μ L of 1% CMC-Na solution (in the same buffer) pre-warmed at 50°C. After a 10-min incubation, the reaction was terminated by adding 250 μ L of DNS reagent, followed by heating in a boiling water bath for 15 min. After cooling, 250 μ L of 40% potassium sodium tartrate tetrahydrate solution was added to stabilize the color. The absorbance of the mixture was then recorded at 540 nm. The concentration of reducing sugars was determined from a glucose standard curve. Cellulase activity (U/mL) was defined as the amount of enzyme that catalyzed the production of 1 μ mol of glucose per minute under the assay conditions.

Assay for Reducing Sugars

Reducing sugar content was quantified by the DNS method [16]. To generate a standard curve, 250 μ L of glucose standards (0–0.3 mg/mL) were reacted with 250 μ L of DNS reagent in a boiling water bath for 15 min. After cooling, 250 μ L of 40% potassium sodium tartrate tetrahydrate solution was added, and the absorbance was recorded at 540 nm. The standard curve was constructed by plotting absorbance (OD_{540}) against glucose concentration. For sample analysis, the hydrolysate was centrifuged at 10,000 rpm for 5 min. The resulting supernatant was diluted with pH 6.0 citrate-sodium citrate buffer to a suitable concentration. A 250 μ L aliquot of the diluted sample was then mixed with DNS reagent and processed identically to the standards. The reducing sugar concentration was determined by interpolating the sample's absorbance at 540 nm from the glucose standard curve.

Determination of Cellulose, Hemicellulose, and Lignin Content

The contents of cellulose, hemicellulose, and lignin in tobacco stems and enzymatic hydrolysis residues were determined according to the standard method for carbohydrate composition analysis (National Renewable Energy Laboratory, NREL) [17]. The specific procedure is as follows:

A sample of 0.3 g (accurate to ± 0.01 g) of oven-dried solid (constant weight) was placed in a stoppered tube. Then, 3.00 mL (accurate to ± 0.01 mL) of 72% (w/w) sulfuric acid was added. The mixture was stirred evenly with a glass rod and incubated in a water bath at 30°C for 60 min, with stirring every 5–10 min. After hydrolysis, 84.00 mL of deionized water was added to dilute the acid concentration to 4% (w/w). The tube was sealed and autoclaved at 121°C for 1 h. After cooling to room temperature, the hydrolysate was filtered.

For sugar analysis, 10 mL of the filtrate was placed in a 50 mL conical flask. Calcium carbonate was slowly added to neutralize the pH to 7.0. After standing for precipitation, 1 mL of the supernatant was centrifuged at 10,000 rpm for 5 min. The resulting supernatant was filtered through a 0.22 µm membrane and analyzed by High-Performance Liquid Chromatography (HPLC) to determine glucose, xylose and arabinose content. Cellulose content was calculated based on glucose, while hemicellulose content was calculated based on xylose and arabinose. The HPLC conditions were as follows: Bio-Rad Aminex HPX-87H column; column temperature, 35°C; flow rate, 0.5 mL/min; refractive index detector temperature, 35°C; mobile phase, 5 mmol/L sulfuric acid solution.

The hydrolysis residue retained in the sintered glass crucible was washed with distilled water until neutral. The crucible containing the residue was dried in an oven at 105°C to a constant weight, cooled in a desiccator, and weighed (M1, accuracy 0.0001 g). Subsequently, the sample was ashed in a muffle furnace at 575°C for 4 h. After cooling, the residue was weighed again (M2). The acid-insoluble lignin content was calculated using Eq. (2):

$$\text{Acid-insoluble lignin (\%)} = (M1 - M2) / M0 \times 100\% \quad (2)$$

The acid-soluble lignin content in the hydrolysate was determined using UV-Vis spectrophotometry (TU1901, Beijing Puxi General Instrument Co., Ltd., Beijing, China). Deionized water was used as a blank control, and absorbance was measured at 320 nm. If the absorbance exceeded the linear range (0.7–1.0), the sample was appropriately diluted and re-measured. The dilution factor and absorbance value (accurate to 0.001) were recorded. All measurements were performed in duplicate and completed within 6 h after hydrolysis. The acid-soluble lignin content was calculated using Eq. (3):

$$\text{Acid-soluble lignin (\%)} = (A \times D \times V) / (30 \times M0) \times 100\% \quad (3)$$

Where: A = Absorbance of the sample; D = Dilution factor of the sample; V = Total volume of the hydrolysate (0.087 L); 30 = Absorption coefficient ($\text{L} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$); M0 = Oven-dried mass of the hydrolyzed sample (g).

Degradation Rate

The degradation rate of cellulose, hemicellulose, and lignin was calculated based on their contents in tobacco stems before and after enzymatic treatment, which were measured as described in Section 2.6. The following formula was used:

$$E_i = (C_0 - C_i) / C_0 \times 100\% \quad (4)$$

In this equation, E_i represents the degradation rate; C_0 and C_i denote the contents of the target component (cellulose, hemicellulose, or lignin) in the untreated and enzymatically hydrolyzed samples, respectively.

Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) Observation

FTIR analysis was performed on a Nicolet™ iS™ 50 spectrophotometer (Thermo Scientific™, Waltham, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector [18]. Spectra were collected over the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans per sample. For each measurement, approximately 5 mg of finely ground sample was used.

Surface morphology was examined by SEM using an EVO 18 instrument (ZEISS, Jena, Germany) at an accelerating voltage of 10 kV. Images were acquired at magnifications of 250×, 500×, 1000×, and 2000× to reveal hierarchical structural details, following the imaging protocol established in our previous work [18].

Sensory Evaluation

Cut tobacco stems, both enzymatically hydrolyzed and untreated (control), were ground to a particle size of 0.150 mm. Reconstituted tobacco sheets were prepared from these powders following the "Dense Slurry Method" as specified in the "Manufacturing Specifications for Reconstituted Tobacco by China Tobacco Hunan Industry Co., Ltd." The sheets were then cut and rolled into cigarettes of a standard weight. Sensory evaluation was performed by a panel of seven qualified professionals, with quantitative assessment conducted according to the industry standard "The evaluation methods of sensory quality for reconstituted tobacco (paper-process)" (YC/T 498–2014) [19].

Statistical Analysis

Data are presented as the mean ± standard deviation of three independent replicates. Statistical analysis was performed using SPSS 24.0 (IBM Corp., USA) to determine significant differences. Figures were plotted with Excel 2019 (Microsoft, USA) and Design-Expert 11 (Stat-Ease, Inc., USA).

Result and Discussion

Single-Factor Analysis of Cellulase Degradation of Cellulose in Cut Tobacco Stem

Effect of cellulase type

The enzymatic degradation of cellulose in tobacco stems is highly dependent on the source-specific properties of cellulases, particularly their substrate specificity [20]. To identify the optimal enzyme, six commercial cellulase preparations were evaluated for their hydrolytic efficiency. As illustrated in Fig. 1a, treatment with Cellulase N yielded the highest amount of reducing sugars, followed by Cellulase XS. In contrast, Cellulase XH and Cellulase O exhibited the lowest degradation capabilities. These results align closely with the measured cellulose degradation rates, confirming that Cellulase N is the most effective enzyme for hydrolyzing tobacco stem cellulose. The superior performance of Cellulase N can be attributed to its ability to efficiently convert cellulose into glucose and reducing oligosaccharides. We hypothesize that this efficiency stems from the unique enzymatic characteristics of Cellulase N, specifically its high substrate specificity for tobacco cellulose and its resistance to inhibition by complex matrix components [20]. This observation is consistent with findings by Liu et al., who reported significant variability in the degradation of tobacco stem pectin when using different pectinases [21]. Collectively, these results underscore that the intrinsic enzymatic properties are a decisive factor in determining the hydrolytic outcome.

Effect of enzyme concentration

Enzyme concentration is a pivotal parameter governing the efficiency of enzymatic hydrolysis [22]. While increasing enzyme dosage generally promotes substrate conversion, excessively high concentrations can trigger substrate competition among enzyme molecules and product inhibition, thereby limiting efficiency gains and escalating production costs [23–25]. Consequently, identifying the optimal enzyme loading is crucial for process optimization. As illustrated in Fig. 1b, treatment with Cellulase N significantly enhanced the reducing sugar yield compared to the untreated control. The yield increased progressively with rising enzyme concentration, peaking at 17.3% at a dosage of 250 U/mL. Beyond this threshold, no significant further increase was observed. This saturation profile was mirrored by the cellulose degradation rate, which also maximized at 250 U/mL and plateaued at higher concentrations. These findings align with established literature. For instance, Li et al. (tobacco cellulose), Wang et al. (astaxanthin extraction), and Xu et al. (flavonoid extraction) similarly reported that enzymatic efficiency improves with concentration up to a saturation point, after which it stabilizes [12, 26, 27]. This plateau effect is typically attributed to the saturation of available substrate binding sites.

Effect of solid-liquid ratio

The solid-liquid ratio is a pivotal factor affecting enzymatic hydrolysis, as it dictates substrate concentration, viscosity, and mass transfer efficiency [23, 25, 28, 29]. Figure 1c demonstrates that elevated ratios increase viscosity and reduce fluidity, limiting enzyme accessibility to tobacco stem cellulose and restricting product diffusion. This exacerbates product inhibition, resulting in suppressed reducing sugar yields and cellulose degradation. Decreasing the solid-liquid ratio mitigates these issues by lowering viscosity and enhancing fluidity, which improves enzyme-substrate contact and alleviates product inhibition. Peak hydrolysis efficiency was observed at a ratio of 1:40, yielding 11.3% reducing sugar and 36.8% cellulose degradation. Further reductions in the ratio, however, resulted in diminishing returns due to insufficient substrate concentration, which impaired enzyme-substrate binding and reduced hydrolysis rates. This trend is consistent with established literature, where optimal ratios vary by system (e.g., 1:20 for pectinase II on tobacco stems; 1:30 for cellulase on bagasse) but universally reflect a trade-off between substrate loading and mass transfer limitations [21, 23, 25, 30, 31]. The identified optimum represents the equilibrium point where these competing factors are balanced to maximize hydrolysis outcomes.

Effect of pH

pH is a pivotal factor governing enzymatic hydrolysis kinetics, as it exerts a dual influence on both the biocatalyst and the substrate [25]. It directly modulates the three-dimensional conformation, catalytic activity, and structural stability of the enzyme, while simultaneously affecting the ionization state of functional groups on the substrate surface, which in turn dictates enzyme-substrate affinity and complex formation [24, 27, 29, 32–35]. Therefore, defining the precise pH optimum is essential for maximizing process efficiency. Figure 1d demonstrates that Cellulase N exhibits markedly higher hydrolytic efficacy under acidic conditions compared to neutral or alkaline environments. Within the pH 4–6 range, the enzyme achieved peak performance, with reducing sugar yields and cellulose degradation rates remaining significantly elevated. This profile is characteristic of acid cellulases, which typically exhibit maximal activity between pH 4.0 and 5.0 due to the protonation state of key amino acid residues in the active site [36, 37]. A critical finding of this study is the strong correlation between the enzyme's optimal pH and the inherent physicochemical properties of the substrate. The natural pH of tobacco stem slurry is approximately 4.7, which falls squarely within the optimal operational window of Cellulase N. This intrinsic compatibility offers a distinct industrial advantage: it enables "self-buffered" hydrolysis without the requirement for chemical pH adjustment. From a process engineering perspective, eliminating the need

for acid/alkali additives simplifies the workflow, minimizes salt waste generation, and significantly reduces operational costs. This synergy between enzyme characteristics and substrate properties underscores the high potential of Cellulase N for scalable and cost-effective biomass conversion.

Effect of temperature

Temperature serves as a pivotal master variable in enzymatic catalysis, exerting a dual influence on the degradation of cellulose by modulating both reaction kinetics and protein conformational stability [22, 25–27, 35]. For the Cellulase N-catalyzed hydrolysis of tobacco stems, identifying the optimal temperature is crucial for maximizing catalytic turnover and ensuring process viability in industrial settings. The experimental results (Fig. 1e) reveal a typical temperature-dependent profile for enzymatic reactions. At lower temperatures (e.g., < 30°C), the reaction is kinetically limited; the insufficient thermal energy reduces the frequency of effective collisions between enzyme and substrate molecules, thereby suppressing hydrolysis efficiency. As the temperature increases to 40°C, the system gains sufficient activation energy, significantly enhancing molecular mobility and the affinity between Cellulase N and cellulose. Consequently, both the reducing sugar yield and cellulose degradation rate peak at 40°C. This optimum aligns with the classification of Cellulase N as a mesophilic enzyme, which generally exhibits maximal activity between 40°C and 50°C. Conversely, temperatures exceeding 60°C lead to a drastic decline in efficiency, with degradation rates plummeting to only 35.6% of the maximum. This sharp decrease is attributed to the thermodynamic instability of the enzyme; high thermal agitation disrupts the non-covalent interactions (e.g., hydrogen bonds and hydrophobic interactions) maintaining the enzyme's tertiary structure, leading to irreversible denaturation and active site deformation [24, 26]. Although elevated temperatures theoretically increase substrate solubility and diffusion rates, the loss of enzymatic activity outweighs these benefits once the thermal stability threshold is breached. From an industrial perspective, these findings are highly relevant to current tobacco processing protocols, which often involve hot water soaking of stems at 40–70°C for 1–2.5 hours. The identified optimal temperature (40°C) and the recommended operational window (40–50°C) align seamlessly with these existing parameters. This compatibility eliminates the need for extensive process re-engineering or specialized heating equipment. Furthermore, this temperature range is consistent with the optimal profiles of other commercial enzymes used in the tobacco industry (typically 40–70°C), validating its practicality for large-scale bio-processing [21, 38–42]. In conclusion, successful implementation of enzymatic hydrolysis in tobacco stem valorization requires a holistic optimization strategy. By selecting a temperature of 40–50°C, one can strike a critical balance: maintaining high catalytic activity while preserving enzyme stability and integrating smoothly with current thermal pretreatment workflows [22]. This strategic temperature control is fundamental to unlocking the full potential of enzymatic biomass conversion.

Effect of rotation speed

Rotation speed is a pivotal operational variable that governs the hydrodynamic conditions of the reaction system, directly impacting mixing efficiency, mass transfer coefficients, and the frequency of enzyme-substrate collisions [22, 25]. While increased agitation generally alleviates external mass transfer limitations by reducing the boundary layer thickness around substrate particles, excessive rotational speeds introduce hydrodynamic shear stress, which poses a risk of enzyme denaturation [43]. Thus, optimizing rotation speed represents a trade-off between maximizing kinetic rates and preserving biocatalyst integrity. The experimental results (Fig. 1f) reveal a non-linear relationship between rotation speed and hydrolytic performance. As the speed increased from low values to 120 rpm, the system benefited from enhanced turbulence and improved solid-liquid contact, resulting in a significant surge in both reducing sugar yield and cellulose degradation rate. This phase represents the "mass-transfer-limited" regime, where increased agitation directly correlates with higher reaction rates. However, beyond the threshold of 120 rpm, a marked decline in efficiency was observed. This inflection point suggests a transition to a "shear-induced inactivation" regime. High shear forces likely disrupt the non-covalent interactions (e.g., hydrogen bonds and hydrophobic interactions) stabilizing the enzyme's tertiary structure, leading to active site deformation and a consequent loss of catalytic function [43]. These findings have profound implications for industrial scale-up. In large-scale bioreactors, power input per unit volume is a key economic driver. Operating above the optimal speed not only fails to improve yields due to enzyme inactivation but also escalates energy costs disproportionately, undermining the process's economic feasibility. Furthermore, in viscous lignocellulosic slurries (common in tobacco stem processing), excessive shear can alter the rheological properties of the substrate, potentially complicating downstream solid-liquid separation. Consequently, a rotation speed of 120 rpm is identified as the optimal operational window for this system. It ensures sufficient mass transfer to overcome diffusion limitations while remaining below the critical shear threshold for Cellulase N. This parameter setting aligns well with the requirements for cost-effective industrial enzymolysis, offering a practical guideline for the design and operation of bioreactors treating tobacco stems. The robust performance of Cellulase N under these moderate shear conditions highlights its suitability for large-scale biomass conversion applications.

Effect of time

Enzymatic hydrolysis time is a critical determinant of reaction kinetics, influencing substrate conversion, enzyme activity retention, product inhibition, and overall process economic feasibility [25]. As illustrated in Fig. 1h, the reaction profile exhibited distinct phases. During the initial

stage (0–1.5 h), despite the high activity of Cellulase N, abundant substrate availability, and minimal product inhibition, the reducing sugar yield and cellulose degradation rate remained relatively low. This limitation is attributed to the insufficient reaction time required for effective enzyme-substrate interactions and catalytic turnover [25]. As the reaction progressed (2–3.0 h), the hydrolysis rate decelerated due to the combined effects of substrate depletion, gradual enzyme deactivation, and the onset of product inhibition [25]. Nevertheless, the cumulative conversion continued to increase, with both the reducing sugar yield and cellulose degradation rate reaching their maximum values at 3.0 h. Extending the hydrolysis time beyond 3.0 h did not result in a further increase in reducing sugar yield; notably, the cellulose degradation rate exhibited a slight decline. While a plateau in hydrolysis efficiency is typically expected as the reaction approaches equilibrium, the marginal decrease in degradation rate observed in this study may be attributable to analytical variability or minor systematic errors inherent in the experimental protocol [35, 44]. From an industrial application perspective, a 3.0 h hydrolysis period, while slightly longer than the conventional hot water soaking time for tobacco stems (1–2.5 h), is significantly more efficient compared to other enzymatic treatments reported in the literature. For instance, protocols utilizing Pectinase II require up to 8 h, while laccase treatments often exceed 24 h [21, 45]. Therefore, process design must balance hydrolysis efficiency against time costs and production continuity. The optimal duration should be calibrated based on specific production constraints to maximize throughput while maintaining cost-effectiveness.

PBD

Based on the results of the single-factor experiments, the central points and high/low levels for six critical factors—temperature (A), pH (B), rotation speed (C), time (D), liquid-solid ratio (E) and enzyme concentration (F) —were established for the PBD. Given that the reducing sugar yield and cellulose degradation rate exhibited consistent trends in the single-factor tests, the reducing sugar yield was selected as the sole response variable for the PBD and subsequent optimization steps to streamline the experimental workflow. The PBD matrix was generated using Design Expert 11 software, and the experimental results are presented in Table 1. The reducing sugar yield varied significantly across the different combinations, ranging from a minimum of 6.8% (Run 12) to a maximum of 19.7% (Run 4). A first-order polynomial regression equation was derived to describe the relationship between the factors and the response:

$$Y = - 17.69 + 0.3861A + 0.173B + 0.0105C + 2.100D + 0.388E + 0.02498F \text{ (5)}$$

Analysis of Variance (ANOVA) (Table 4) confirmed that the model was statistically significant ($P < 0.05$) with a coefficient of determination (R^2) of 0.9622, indicating a strong correlation between the predicted and experimental values and validating the model's predictive capability for reducing sugar yield.

The Pareto analysis and effect estimates revealed the order of significance for the factors influencing reducing sugar yield as follows: temperature > enzyme concentration > time > liquid-solid ratio > rotation speed > pH. Notably, temperature, enzyme concentration, and time exhibited significant positive effects on the yield ($P < 0.05$). Consequently, the non-significant factors—liquid-solid ratio, rotation speed, and pH —were fixed at 1:40, 120 r/min, and 5.0, respectively. The subsequent optimization phase focused exclusively on the three significant variables (temperature, enzyme concentration, and time) using RSM to determine the optimal conditions for maximizing reducing sugar yield.

Table 4
Statistical analysis in PBD for assessing the reducing sugar yield

Source	Degree of freedom	Adjusted sum of squares	Adjusted mean squares	F-Value	P-Value	Rank	Significance
Model	7	226.556	32.365	25.45	0.000	-	***
Linearity	6	214.165	35.694	28.07	0.000	-	***
Temperature	1	178.846	178.846	140.64	0.000	1	**
pH	1	0.361	0.361	0.28	0.611	6	
Rotation speed	1	1.195	1.195	0.94	0.365	5	
Time	1	13.232	13.232	10.41	0.015	3	*
Solid-liquid ratio	1	1.806	1.806	1.42	0.272	4	
Enzyme concentration	1	18.724	18.724	14.72	0.006	2	**
Bending	1	12.391	12.391	9.74	0.017	-	
Error	7	8.902	1.272				
Lack of fit	5	8.469	1.694	7.82	0.117	-	
Pure error	2	0.433	0.217				
Total	14	235.458					
$R^2=0.9622$, $R^2_{adj}=0.9244$							

* $P < 0.05$, significant at 5% level; ** $P < 0.01$, significant at 1% level; *** $P < 0.001$, significant at 1‰ level.

“–”, no data.

Steepest Ascent Method

The results of the steepest ascent experiments (Table 2) revealed a distinct "rise-then-fall" trend for the reducing sugar yield in response to variations in temperature, enzyme concentration, and time. As the factor levels were incrementally increased from Run 1 (30°C, 150 U/mL, 2.0 h; yield: 5.8%) to Run 6 (55°C, 400 U/mL, 4.5 h; yield: 30.5%), the reducing sugar yield exhibited a consistent upward trajectory. However, further intensification of the reaction conditions beyond Run 6 resulted in a decline in yield, indicating that the optimal region had been surpassed, likely due to factors such as enzyme denaturation or severe product inhibition. Consequently, the parameter combination of Run 6 (55°C, 400 U/mL, 4.5 h) was designated as the center point for the subsequent RSM design. Notably, under the synergistic action of these multiple factors, the reducing sugar yield was significantly enhanced from the maximum value obtained in the single-factor experiments (17.3%) to 30.5%, representing a substantial increase of 76.3%. This improvement was achieved despite the fact that the enzymatic conditions (higher enzyme loading, extended duration, and elevated temperature) were significantly more rigorous than those in the single-factor tests. This result not only validates the necessity of multi-parameter joint optimization but also underscores the robust catalytic activity of the cellulase complex at elevated temperatures (55°C). Such thermal stability suggests that this enzyme preparation holds considerable potential for application in intensified enzymatic hydrolysis processes, where high-temperature operation is often desirable to increase reaction rates and reduce microbial contamination risks.

RSM

In the enzymatic hydrolysis experiments conducted using the BBD, the reducing sugar yield exhibited significant variation across different combinations of process parameters (Table 3). The yield ranged from a minimum of 2.4% (Run 6) to a maximum of 33.1% (Run 13). This wide variation confirms that the selected ranges for the independent variables were appropriate and that the factors exerted substantial effects on the response, justifying the use of RSM for further optimization. The experimental data were fitted using Design-Expert 11 software to establish a quadratic polynomial regression model describing the relationship between the reducing sugar yield (Y) and the independent variables: temperature (A), enzyme concentration (B), and time (C). The resulting equation is as follows:

$$Y = -223.81 + 7.854A + 0.1229B + 13.37C - 0.07331A^2 - 0.000084B^2 - 0.891C^2 - 0.000607AB - 0.0629AC - 0.00148BC \quad (6)$$

Analysis of Variance (ANOVA) (Table 5) revealed that the regression model was highly significant ($P < 0.01$), indicating that the model effectively described the experimental data. The coefficient of determination (R^2) was 0.9992, demonstrating exceptional explanatory power and predictive reliability. Furthermore, the lack-of-fit was not significant ($P > 0.05$), confirming the adequacy of the model and the absence of systematic errors, thereby validating its use for analyzing and optimizing the enzymatic hydrolysis process. Analysis of the model coefficients showed that the linear terms A (temperature), B (enzyme concentration), and C (time) all had significant effects on the reducing sugar yield ($P < 0.05$). The order of influence was consistent with the PBD results: temperature > enzyme concentration > time. Additionally, the quadratic terms A^2 , B^2 , and C^2 were significant ($P < 0.05$), suggesting distinct non-linear relationships between the factors and the response. Regarding interaction effects, the cross-terms AB (temperature $\times$ enzyme concentration) and AC (temperature $\times$ time) were significant, whereas the interaction between enzyme concentration and time (BC) was not.

Table 5
Regression coefficients and their significance for the reducing suger yield derived from the results of the BBD

Source	Degree of freedom	Adjusted sum of squares	Adjusted mean squares	F-Value	P-Value	Significant
Model	9	2070.88	230.10	681.98	0.000	***
Linearity	3	1047.26	349.09	1034.64	0.000	***
Temperature	1	973.07	973.07	2884.04	0.000	***
Enzyme concentration	1	43.72	43.72	129.57	0.000	***
Time	1	30.48	30.48	90.32	0.000	***
Square	3	1007.70	335.90	995.56	0.000	***
Temperature*Temperature	1	1004.47	1004.47	2977.10	0.000	***
Enzyme concentration*Enzyme concentration	1	13.22	13.22	39.17	0.002	**
Time*Time	1	14.85	14.85	44.01	0.001	**
Two-factor interaction	3	15.92	5.31	15.72	0.006	**
Temperature*Enzyme concentration	1	7.46	7.46	22.12	0.005	**
Temperature*Time	1	8.01	8.01	23.74	0.005	**
Enzyme concentration*Time	1	0.44	0.44	1.31	0.304	
Error	5	1.69	0.34			
Lack of fit	3	0.97	0.32	0.90	0.563	
Pure error	2	0.72	0.36			
Total	14	2072.57				
$R^2=0.9992, R^2_{adj}=0.9977$						

* $P < 0.05$, significant at 5% level; ** $P < 0.01$, significant at 1% level; *** $P < 0.001$, significant at 1‰ level.

The three-dimensional response surface plots (Fig. 2) visually illustrate the effects of the variables. The reducing sugar yield exhibited a parabolic trend with increasing temperature, rising to a peak before declining, which indicates the existence of an optimal temperature. In contrast, the yield increases associated with extending hydrolysis time or increasing enzyme concentration were less pronounced within the tested range. Notably, low enzyme concentrations combined with short reaction times resulted in minimal sugar yields. However, a significant enhancement was observed when both enzyme concentration and time were moderately increased. These trends align well with enzymatic reaction kinetics, where substrate saturation and enzyme stability limit the reaction rate. Consequently, optimizing the process requires careful consideration of factor synergy, with strict control over reaction temperature being paramount.

Using the optimization module of Design-Expert, the predicted optimal parameters were determined as follows: enzyme concentration of 508 U/mL, temperature of 49.1°C, and time of 5.36 h. Under these conditions, the predicted reducing sugar yield was 36.2%. Verification experiments yielded an actual value of 36.4%, with a relative error of only 0.55% compared to the predicted value. This high degree of agreement confirms the precision and reliability of the established model. Compared to the maximum yield obtained in the single-factor experiments (17.3%), the optimized conditions resulted in an approximately 110% increase in reducing sugar yield. Furthermore, under these

optimal conditions, the cellulose degradation rate reached 70.7% (Table 6). This value is superior to results reported in similar studies, such as those by Hou et al., which may be attributed to differences in enzymatic hydrolysis methods or the specific enzyme preparations used [11]. The degradation rate also significantly exceeded the values reported by Zhang et al. and Li et al. in their studies on tobacco cellulose degradation [12, 46]. Additionally, partial degradation of hemicellulose (20.5%) and lignin (10.0%) was observed. This co-degradation phenomenon is likely due to the disruption of the lignocellulosic matrix following extensive cellulose hydrolysis. As the cellulose backbone is depolymerized, the physical barrier protecting hemicellulose and lignin is compromised, facilitating their release and partial degradation [47]. These results suggest that the optimized enzymatic process not only maximizes sugar recovery but also effectively modifies the overall chemical structure of the tobacco stem biomass.

Table 6
Cellulose, hemicellulose, and lignin contents in cut tobacco stems subjected to different treatments

Untreated cut tobacco stem	Cellulose content (%)	Hemicellulose content (%)	Acid soluble lignin content (%)	Acid insoluble lignin content (%)
	26.6	8.5	3.1	2.4
Hot water-pretreated cut tobacco stem	25.5	10.7	3.3	2.5
Enzymatic hydrolysis blank control (soaked without enzyme)	22.9	8.3	3.3	2.7
Enzymatically hydrolyzed cut tobacco stem	6.7	6.6	2.8	2.6

FTIR Spectroscopy Analysis

To elucidate the structural modifications induced by different treatments, FTIR analysis was performed on four samples: untreated cut tobacco stem, hot water-pretreated cut tobacco stem, enzymatic hydrolysis blank control (soaked without enzyme), and enzymatically hydrolyzed cut tobacco stem. As shown in Fig. 3, the overall spectral profiles of all samples were similar, indicating the preservation of the basic lignocellulosic framework. However, distinct variations in the intensity and shape of characteristic absorption bands were observed, reflecting differences in chemical composition and supramolecular structure. A broad absorption band centered at approximately 3350 cm⁻¹, attributed to O–H stretching vibrations involved in intermolecular and intramolecular hydrogen bonding within cellulose and hemicellulose, was present in all samples [48]. Compared to the raw cut tobacco stem, the hot water-pretreated sample exhibited a significantly enhanced intensity at this wavenumber. This increase is likely due to the swelling of the fibrous structure induced by hot water, which exposes more hydroxyl groups and strengthens hydrogen bonding networks, thereby facilitating the subsequent enzymatic deconstruction of the cellulose and hemicellulose matrices [22]. The blank control sample showed a slight reduction in intensity compared to the hot water-pretreated sample, suggesting that prolonged soaking in warm water alone causes partial structural disruption. Notably, the enzymatically hydrolyzed sample displayed the lowest absorbance intensity at 3350 cm⁻¹, indicating that Cellulase N effectively cleaved the glycosidic bonds in cellulose, leading to a significant reduction in the ordered hydrogen-bonding network. The absorption peak at approximately 2903 cm⁻¹ corresponds to C–H stretching vibrations in methyl and methylene groups, primarily originating from cellulose, hemicellulose, and lignin [49]. The variation trend at this wavenumber mirrored that observed at 3350 cm⁻¹, further corroborating that hot water pretreatment, prolonged soaking, and enzymatic hydrolysis all impact the structural integrity of the lignocellulosic components, with the most pronounced degradation of cellulose induced by Cellulase N. The band near 1728 cm⁻¹ is assigned to the stretching vibration of C = O groups in acetyl and uronic ester linkages of hemicellulose, as well as ferulic and p-coumaric acid esters in lignin [48, 50]. A significant increase in the intensity of this peak was observed in the hot water-pretreated and blank control samples, which may be attributed to the "unmasking" effect caused by structural relaxation and increased hydrogen bonding accessibility. Conversely, the intensity of this peak decreased after enzymatic hydrolysis, suggesting that in addition to degrading cellulose, the enzyme cocktail also hydrolyzed easily degradable hemicellulose fractions. The absorption bands at 1612 cm⁻¹ and 1516 cm⁻¹ are characteristic of aromatic skeletal vibrations and acetyl group vibrations in lignin, respectively [48, 50, 51]. The trend at 1612 cm⁻¹ was consistent with the aforementioned peaks; the significantly lower intensity in the enzymatically hydrolyzed sample compared to the raw material indicates that the degradation of the cellulose matrix was accompanied by the partial degradation or solubilization of lignin. Interestingly, at 1516 cm⁻¹, the absorbance intensity of the enzymatically hydrolyzed sample was lower than that of the hot water-pretreated and blank control samples but remained higher than that of the raw sample. This phenomenon can be explained by the "exposure effect": as the cellulose matrix is enzymatically removed, the lignin aromatic skeleton becomes more exposed, resulting in a relative increase in absorbance compared to the raw state, despite some lignin degradation. In the range of 1453–1250 cm⁻¹, several minor bands were observed. Specifically, peaks at 1421 cm⁻¹ and 1318 cm⁻¹ are associated with C–H bending and C–O stretching in cellulose and hemicellulose, while the peak at 1254 cm⁻¹ corresponds to aryl–O stretching in lignin [18, 48, 52]. The intensity variations at 1421 cm⁻¹ and 1318 cm⁻¹ followed the trend of the 2903 cm⁻¹ peak, further confirming the consistent impact of different treatments on cellulose structure. The variation at 1254 cm⁻¹ was similar to that at 1516 cm⁻¹, likely sharing the same underlying mechanism of lignin exposure. Notably, the peak at 1384 cm⁻¹, attributed to C–H deformation in cellulose, exhibited the highest intensity and sharpest profile in the

raw sample, which diminished upon treatment, indicating a loss of cellulose crystallinity [52]. Finally, the peaks at 1160 cm^{-1} , 1054 cm^{-1} , and 896 cm^{-1} correspond to the asymmetric stretching of C–O–C glycosidic bonds, C–O stretching in cellulose/xylan, and the characteristic vibration of β -glycosidic linkages, respectively [48, 52, 53]. The changes in intensity at these positions were consistent with the trends observed at 2903 cm^{-1} , reinforcing the conclusion that the treatments effectively disrupted the fibrous structure of the cut tobacco stem. In summary, the FTIR spectral analysis provides compelling evidence that enzymatic hydrolysis with Cellulase N significantly alters the chemical structure of cut tobacco stem. The reduction in characteristic cellulose and hemicellulose peaks, coupled with the relative exposure of lignin structures, corroborates the quantitative results regarding component degradation (cellulose, hemicellulose, and lignin) discussed in previous sections.

SEM Analysis

To visually elucidate the impact of different treatment conditions on the microstructure of cut tobacco stem, SEM was employed to observe samples under four distinct conditions: untreated cut tobacco stem, hot water-pretreated cut tobacco stem, enzymatic hydrolysis blank control (soaked without enzyme), and enzymatically hydrolyzed cut tobacco stem (Fig. 4). Observations were conducted at magnifications of $250\times$, $500\times$, $1000\times$, and $2000\times$ to comprehensively characterize surface morphological variations. As depicted in Fig. 4, the untreated cut tobacco stem exhibited typical structural features of a dense lignocellulosic matrix. The surface was smooth and flat, with a uniformly arranged fiber matrix that was tightly interconnected. No significant structural defects, cracks, or fractures were observed, demonstrating the high density and structural integrity characteristic of natural biomass materials. In contrast, the morphology of the residue following hot water pretreatment underwent initial alterations. While the overall structure remained relatively compact, the surface displayed a degree of looseness and minor damage. This phenomenon is primarily attributed to the mild nature of hot water pretreatment, which mainly removes soluble sugars, small molecular impurities, and partial extractives from the cut tobacco stem. The treatment intensity is relatively low; although it weakens the inter-fiber binding forces, it is insufficient to completely disrupt the overall lignocellulosic skeleton. Nevertheless, this slight surface roughening and increase in porosity theoretically facilitate the penetration of subsequent chemical reagents or enzyme molecules into the substrate. The surface morphology of the enzymatic hydrolysis blank control (soaked without enzyme) showed distinct differences compared to the raw material and the pretreated samples. This group exhibited a fluffy surface texture accompanied by distinct wrinkles and curling. These observations suggest that during prolonged soaking in warm water, despite the absence of enzymatic catalysis, physical swelling effects and the continuous action of hot water still induced partial disruption to the fiber structure, leading to the separation and reorganization of fiber bundles. Compared to the three aforementioned groups, the enzymatically hydrolyzed sample exhibited the most dramatic microstructural changes. The surface was no longer smooth; instead, it was characterized by numerous voids and pits of varying sizes, and the degree of fiber tearing was extremely severe. More significantly, a large number of fragmented lamellar structures were observed peeling off and scattering across the surface. These morphological features provide compelling evidence that Cellulase N efficiently targeted the cellulose components within the cut tobacco stem, particularly performing deep hydrolysis and exfoliation of the cellulose skeleton. This enzymatic degradation not only led to the collapse and laceration of the cell wall but also fully exposed the internally encapsulated components, resulting in a porous residue with a high specific surface area. The severe microstructural destruction observed herein is highly consistent with the significant increase in reducing sugar yield and cellulose degradation rate reported in the previous sections. This strong correlation validates the high catalytic efficiency of Cellulase N from a structural perspective and confirms that the enhanced enzymatic accessibility resulting from physical pretreatment and structural disruption is the key driver for the improved conversion efficiency.

Sensory Evaluation

Sensory evaluation was conducted on the control and enzymatically hydrolyzed cut tobacco stem according to the methodology described in Section 2.9. The results are summarized in Table 7. Statistical analysis revealed that the comprehensive sensory score of the cut tobacco stem treated with cellulase was approximately 5 points higher than that of the control sample. Regarding specific sensory attributes, the lignes was significantly attenuated, showing a reduction of 40.27%. Additionally, the throat irritation was notably alleviated, with an improvement rate of 10.4%. The overall enhancement in sensory quality is primarily attributed to the specific degradation of lignocellulosic components induced by enzymatic hydrolysis. Specifically, cellulase facilitates the directional hydrolysis of cellulose into reducing sugars, thereby optimizing the sugar-alkaloid ratio within the cut tobacco stem—a critical factor influencing combustion quality and smoke harshness [54]. Simultaneously, the degradation of certain lignin precursors suppresses the generation of ligneous aroma compounds. These experimental findings demonstrate that this biological treatment process can effectively reduce irritating components and ligneous taste by modulating the carbohydrate composition of tobacco stems. Consequently, this study provides a viable biotechnological approach for improving the sensory quality of cigarettes, offering a promising strategy to enhance the utilization value of tobacco by-products.

Table 7
Sensory evaluation results of reconstituted tobacco leaves made from cut stems before and after treatment

Sample	Aroma		Smoke	Offensive taste			Irritancy		Smoking taste		Total score
	Quality of aroma	Quantity of aroma	Density of smoke	Smooth	Ligneous	Other offensive taste	Irritation	Burning	Clarity	Comfort	
Enzymatic hydrolysis blank control (soaked without enzyme)	5.36	5.64	5.43	6.07	5.14	7.85	6.07	6.07	6.07	5.79	59.49
Enzymatically hydrolyzed cut tobacco stem	5.79	5.86	5.79	6.14	7.21	8.00	6.93	6.14	6.36	6.21	64.43
Note: Sensory evaluation was scored on a 100-point scale (0–10 points per attribute).											

Conclusion

This study systematically investigated the optimal process conditions for the degradation of tobacco stem cellulose using Cellulase N by integrating single-factor experiments with a multivariate optimization strategy. The results demonstrated that Cellulase N exhibited superior performance in both reducing sugar yield and cellulose degradation rate, attributable to its excellent substrate specificity and high catalytic efficiency. Based on single-factor tests, a PBD was employed to identify enzyme concentration, temperature, and time as the critical influencing factors. Subsequent optimization using the steepest ascent method and RSM established a predictive model with high goodness-of-fit ($R^2 = 0.9992$). The optimal enzymatic hydrolysis conditions were determined as follows: enzyme concentration of 508 U/mL, temperature of 49.1°C, and time of 5.36 h. Under these conditions, the reducing sugar yield reached 36.4% and the cellulose degradation rate achieved 70.7%, representing a significant enhancement compared to pre-optimization levels (17.3%). Furthermore, structural characterization via FTIR and SEM revealed that the disruption of the cellulose framework facilitated the dissociation of partial hemicellulose and lignin, suggesting new possibilities for the comprehensive utilization of tobacco stem resources. Sensory evaluation indicated that the optimized enzymatically hydrolyzed samples exhibited significantly reduced ligneous aroma and throat irritation, alongside improved smoke aroma quality and comfort, without introducing any adverse odors. In summary, this research successfully established an efficient process system for the degradation of tobacco stem cellulose using Cellulase N. These findings not only provide a reliable technical pathway for the high-value utilization of tobacco stems in cigarette formulations but also offer a theoretical basis and practical reference for promoting resource conservation and sustainable development within the tobacco industry.

Declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors approved the consent for publishing the manuscript to *Applied Biochemistry and Biotechnology*.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

Lin Dai and Tianjiao Wang: Project administration. Saihua Zhu: Methodology, Conceptualization. Yu Zhao and Jinghao Ma: Data curation, software. Guangsen Fan: Writing – original draft, Visualization. Yong Jin: Review and suggestions. Xiaoyan Liu: Edited the language. Dafeng Yin & Guangsen Fan: Supervision and guidance, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data Availability

Data will be made available on request.

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Figures

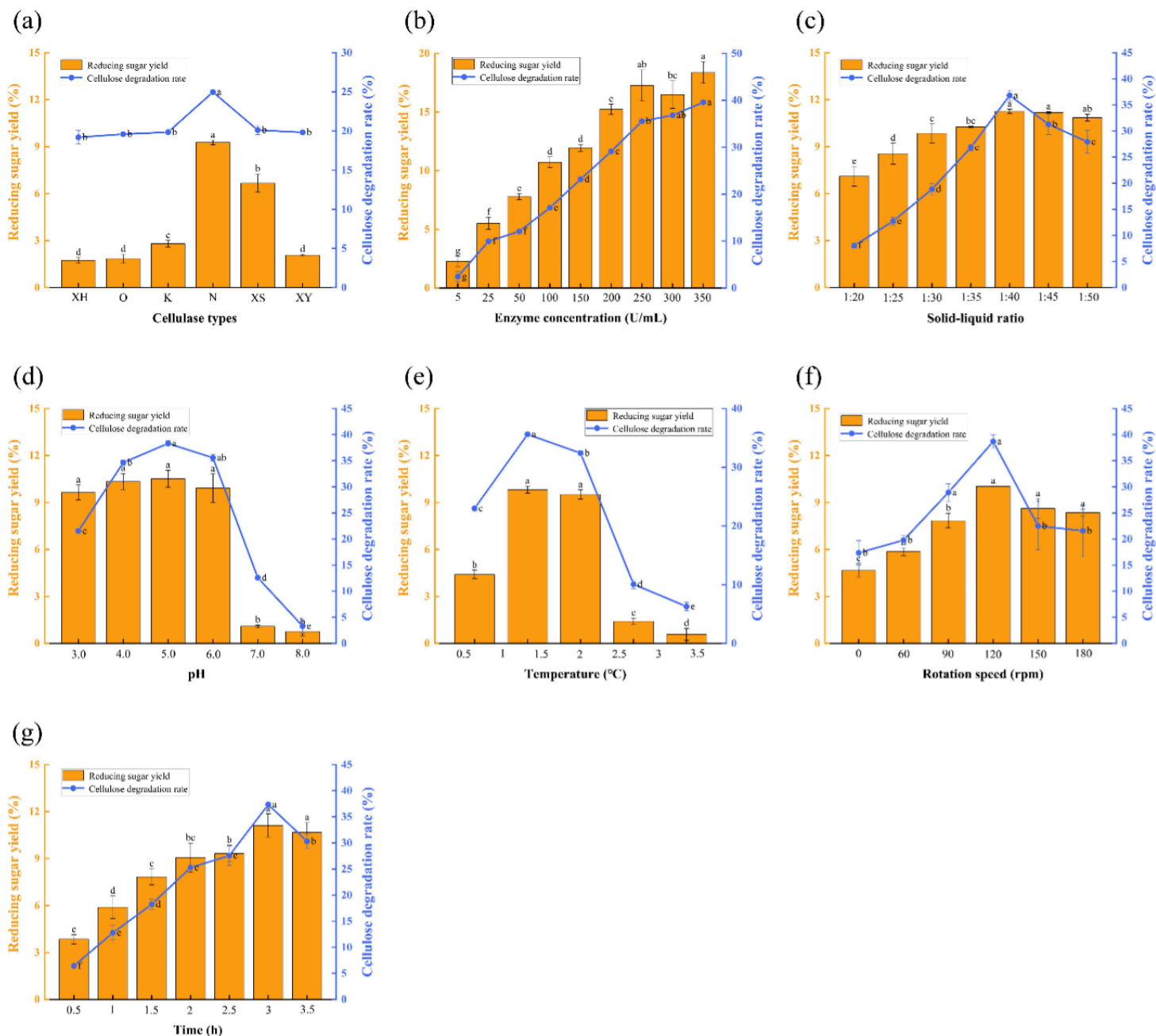


Figure 1

Effects of various factors on reducing sugar yield and cellulose degradation rate of cut tobacco stem. (a) Cellulase types (XH, O, K, N, XS, and XY); (b) enzyme concentration (5, 25, 50, 100, 150, 200, 250, 300, 350 U/mL); (c) solid-liquid ratio (1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50); (d) pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0); (e) temperature (30, 40, 50, 60, 70 °C); (f) rotation speed (0, 60, 90, 120, 150, 180 rpm); (g) time (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 h). Vertical bars and line represent the mean $\pm$ standard deviation. Bars and lines labeled with different letters indicate statistically significant differences based on the Tukey test ($P < 0.05$).

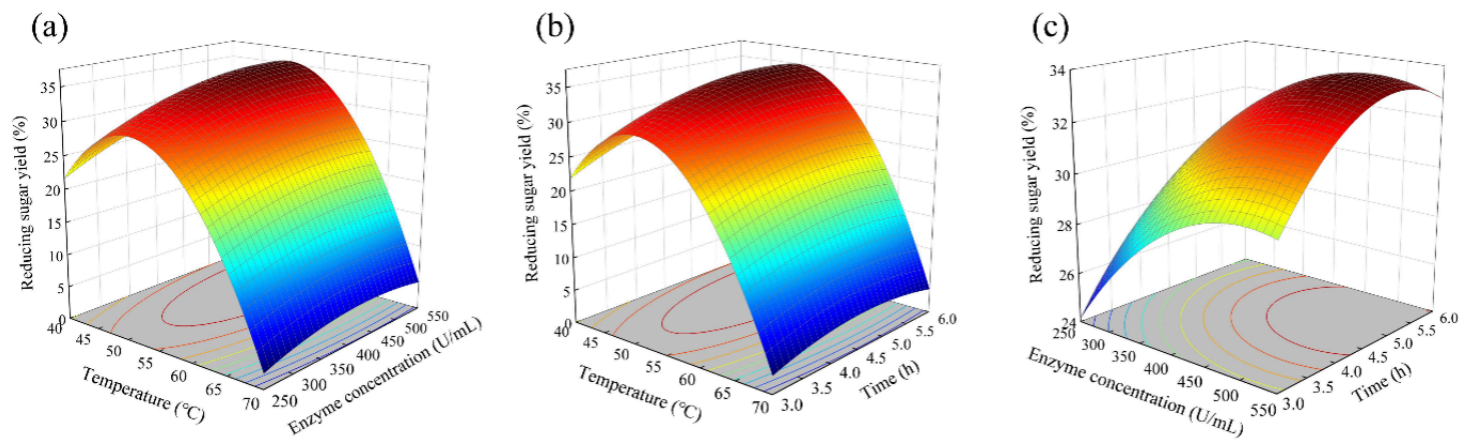


Figure 2

Three-dimensional surface maps illustrating the effects of pairwise interactions among various factors on the reducing sugar yield. a, Interaction between temperature and enzyme concentration; b, interaction between time and temperature; c, interaction between enzyme concentration and time.

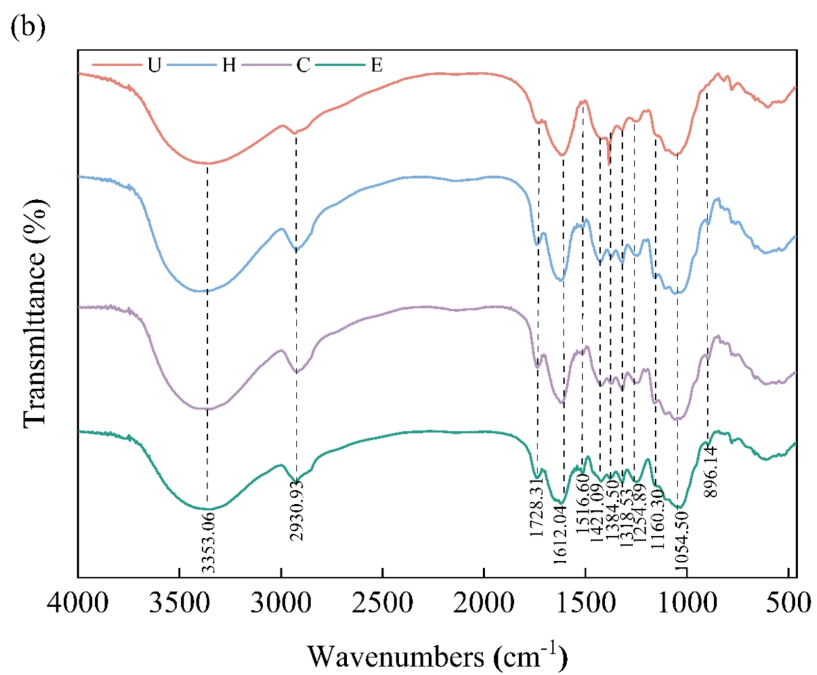
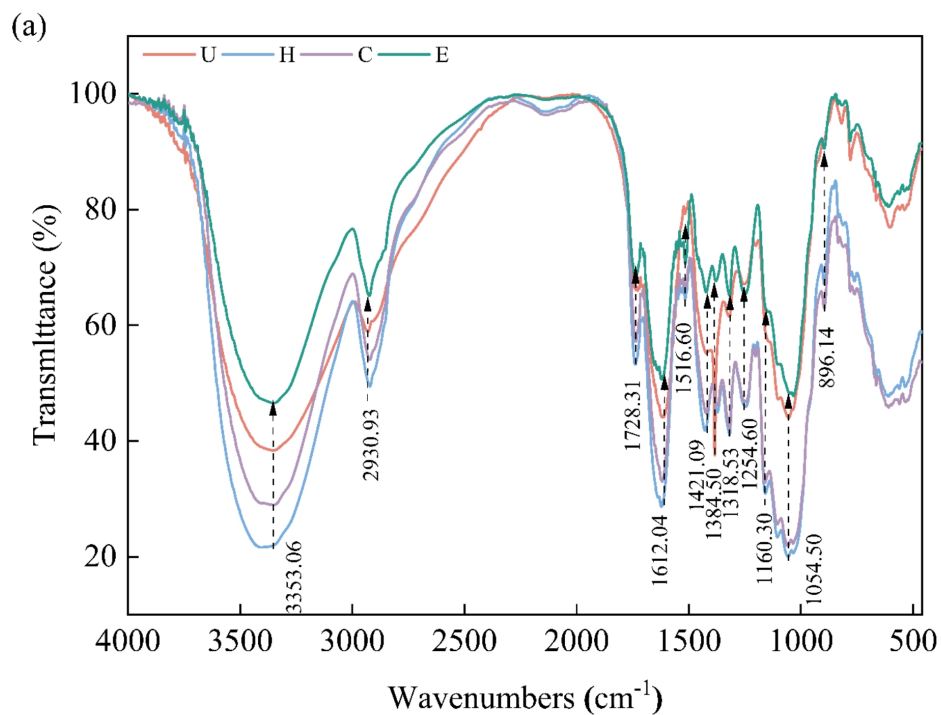


Figure 3

FTIR spectra of untreated cut tobacco stem (U), hot water-pretreated cut tobacco stem (H), enzymatic hydrolysis blank control (soaked without enzyme) (C) and enzymatically hydrolyzed cut tobacco stem (E).

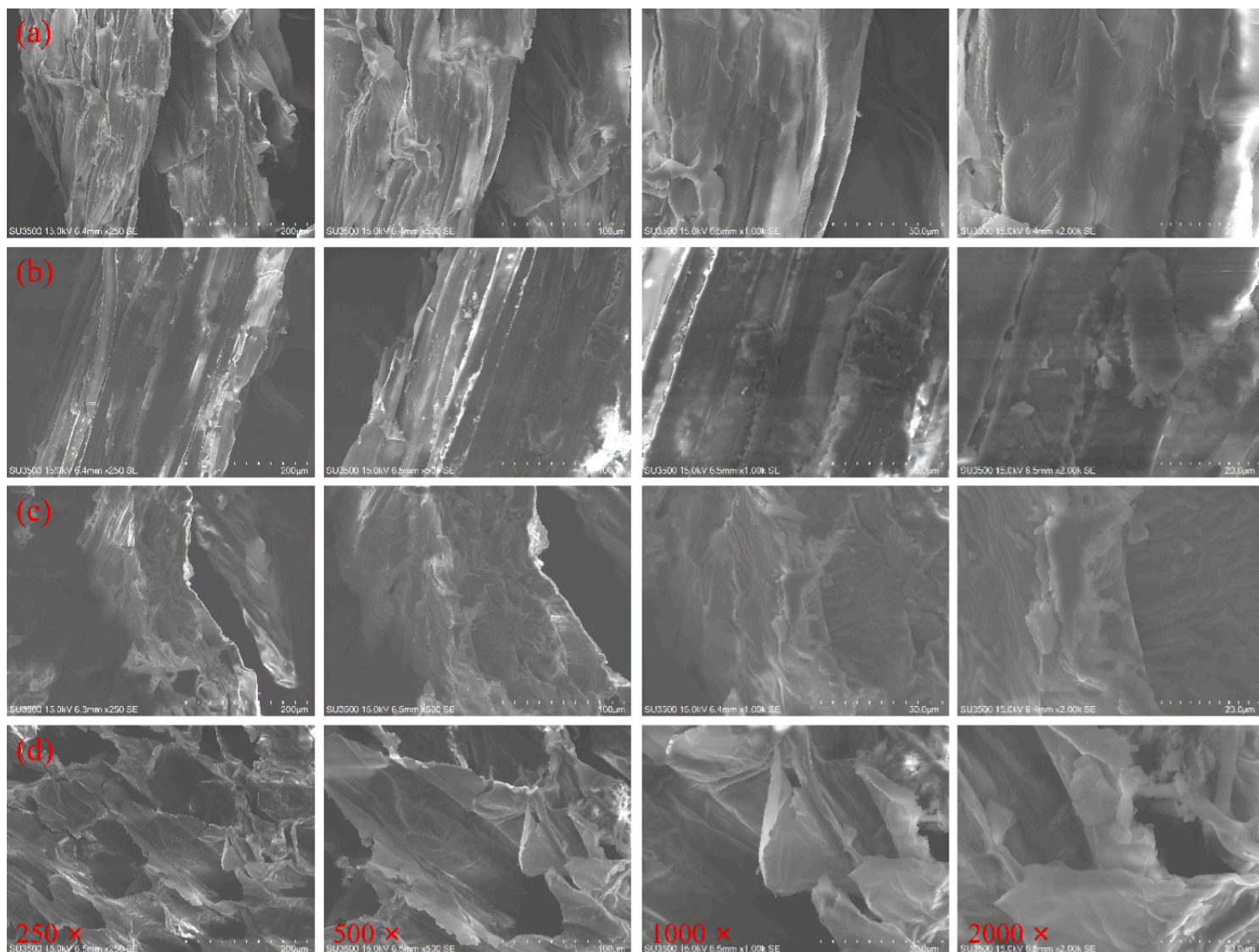


Figure 4

SEM image of untreated cut tobacco stem (a), hot water-pretreated cut tobacco stem (b), enzymatic hydrolysis blank control (soaked without enzyme) (c) and enzymatically hydrolyzed cut tobacco stem (d) at different magnifications.

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