RESEARCH ARTICLE

Screening of cellulose-degrading fungi from spent mushroom substrate and optimization of enzyme production conditions

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Spent mushroom substrate (SMS) contains a large amount of cellulose, hemicellulose, lignin, and other components that are difficult to utilize. To improve the cellulose degradation rate and overall utilization efficiency of SMS, this study isolated a highly efficient cellulose-degrading strain from SMS using the clear-zone method and enzymatic activity assay. Through morphological observation, physiological and biochemical tests, and molecular identification, the strain was identified as *Aspergillus tubingensis* and designated X-5. The culture conditions for the strain were optimized using single factor experiments and response surface methodology (RSM) to further enhance its enzymatic activity. The results showed that the optimal conditions for producing β-endoglucanase in liquid fermentation medium were 10.92 g/L sucrose, 2.28 g/L yeast extract powder, 5.84 initial pH, and approximately 67 hours of incubation. Under these optimized conditions, the β-endoglucanase activity reached 543 U/mL, which was 16.7 times higher than it was before optimization. The optimal conditions for producing β-exoglucanase were 11.12 g/L sucrose, 1.99 g/L yeast extract powder, 5.59 initial pH, and approximately 66 hours of incubation. Under these optimized conditions, the β-exoglucanase activity reached 518.27 U/mL, which was 20.1 times higher than it was before optimization. The optimal conditions for producing β-glucosidase were 11.55 g/L sucrose, 2.29 g/L yeast extract powder, 5.76 initial pH, and approximately 66 hours of incubation. Under these optimized conditions, the β-glucosidase activity reached 533.31 U/mL, which was 31.18 times higher than it was before optimization. By optimizing the fermentation conditions for cellulase production using RSM, the X-5 strain achieved a high overall level of cellulolytic enzyme activity. This study provided a theoretical foundation for the recycling and utilization of spent mushroom substrate in the edible fungi industry.

Keywords: *Aspergillus tubingensis*; cellulase; β-endoglucanase; β-exoglucanase; β-glucosidase; single-factor experiment; response surface methodology (RSM).

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Introduction

With the flourishing development of the edible fungi industry, China has become the world's largest producer and consumer of shiitake mushrooms. While mushroom cultivation brings significant economic benefits, it also generates a staggering amount of agricultural waste, spent mushroom substrate (SMS) [1]. SMS is the residual cultivation medium after harvesting edible fungi, primarily composed of lignocellulosic residues such as sawdust, cottonseed hulls, straw, underutilized nitrogen sources like bran, mycelial metabolites, and abundant fungal protein [2]. Statistics indicate that approximately 5 kg of SMS is produced for every 1 kg of shiitake mushrooms harvested [3]. However, environmentally friendly and effective methods for managing SMS remain inadequately addressed. Traditional disposal practices such as indiscriminate stacking, discarding, or landfilling not only occupy substantial land resources but also lead to the anaerobic decomposition of organic matter in SMS, generating malodorous gases like CH_4, H_2S, and NH_3, which pollute the atmosphere and disrupt ecological balance [4]. SMS itself is an underutilized valuable bio-resource. Rich in mycelial protein, polysaccharides, vitamins, and various trace elements, it holds great potential for use as organic fertilizer, animal feed, a substrate for secondary cultivation, and raw material for biofuel production [5]. Consequently, developing efficient, environmentally sound, and value-added strategies for utilizing SMS, transforming waste into wealth, has become a critical issue for the sustainable development of the edible fungi industry and a current research hotspot in agricultural environmental protection and the circular economy.

The high-value utilization of SMS faces a core bottleneck, its complex chemical composition. The basal substrate of SMS primarily consists of a lignocellulosic complex, where lignin, cellulose, and hemicellulose are intricately cross-linked, forming an exceptionally stable structure that is difficult to decompose and utilize directly [6]. Therefore, the efficient degradation of lignocellulose is a prerequisite for unlocking the resource utilization potential of SMS. In nature, microorganisms are the primary agents for lignocellulose degradation. They secrete a series of extracellular enzymes that work synergistically to break down the lignin barrier encapsulating carbohydrates, subsequently hydrolyzing cellulose and hemicellulose into fermentable sugars [7]. However, after one or multiple mushroom production cycles, the original Lentinula edodes mycelium and its associated microbial community within the SMS tend to decline or die off, leading to a significant decrease in degradative enzyme activity, which results in the slow natural decomposition of spent substrate. Accelerating the decomposition of SMS through the application of high-efficiency microbial inoculants is considered one of the most promising biological treatment technologies. Consequently, considerable research efforts have been devoted to enhancing microbial degradation efficiency for SMS. Guo *et al*. reported that *Bacillus amyloliquefaciens* achieved lignin degradation rates of 46.7% and 42.4% for straw and SMS, respectively, within 20 days [8]. *Aspergillus awamori* has demonstrated high cellulose-degrading capability, exhibiting a filter paper activity of 420 U/mL [9]. Li *et al*. isolated three highly efficient cellulose-degrading strains from *Pleurotus eryngii* SMS, identified as *Bacillus subtilis*, *Aspergillus niger*, and *Trichoderma reesei* [10]. However, there are significant variations in enzyme systems, specific enzymatic activities, and adaptability to particular substrates among strains from different sources. Therefore, the targeted screening of native, dominant strains that exhibit high specificity, superior degradation efficiency, and strong environmental adaptability specifically to shiitake mushroom SMS is a crucial step for developing effective microbial inoculants.

The enzyme-producing capacity of a strain is not static but is influenced by culture conditions. Unoptimized conditions often fail to maximize a strain's enzymatic potential. Therefore, using SMS as the raw material and enzymatic activity as the key indicator, this study screened for strains with high cellulose-degrading efficiency. Subsequently, response surface methodology (RSM) was employed to optimize the enzyme production conditions for the selected strain, aiming to develop a fermentation process with the highest enzymatic activity. This work provided a solid theoretical basis and technical support for the future large-scale production of degrading enzyme preparations or the development of composite microbial agents.

Materials and methods

Enrichment and screening of strains

Spent mushroom substrate (SMS), second flush, was obtained from Nanshan Biotechnology Co., Ltd., Zhumadian, Henan, China. 3 g of SMS was mixed with 300 mL of sterile water and stir on a magnetic stirrer for 15 minutes to ensure thorough blending before centrifuging the mixture at 5,000 rpm for 5 minutes and collecting the supernatant. The supernatant was used to perform a three-zone streak inoculation on a Potato Dextrose Agar (PDA) solid medium (Hangzhou Baisi Biotechnology Co., Ltd., Hangzhou, Zhejiang, China) composed of 200 g/L potato infusion powder, 20 g/L dextrose, and 15 g/L agar. The inoculated medium was incubated at 28°C for 36 hours. The dominant colonies were transferred onto Congo red medium (Hangzhou Baisi Biotechnology Co., Ltd., Hangzhou, Zhejiang, China) containing 1.0 g $NaNO_3$, 1.2 g Na_2HPO_4, 0.9 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.5 g yeast extract, 0.5 g acid-hydrolyzed casein, 0.2 g Congo Red, 5.0 g cellulose powder, and 15.0 g agar in one liter volume and incubated at 28℃ for 48 hours. The strains with cellulose degradation ability were screened by measuring the size of the hydrolyzed transparent zone of Congo red [11].

Morphological observation and biological identification of strains

The target strains were inoculated onto PDA plate and cultured for 5 days. Observations were made on the growth characteristics of the colonies including colony size, shape, edge, luster, texture, color, and transparency. A slide covered with mycelium was prepared and observed under a microscope. The target strains were sent to Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) for 18S rDNA gene sequencing followed by sequence alignment analysis using the Blast tool in the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed by using the neighbor-joining method in MEGA5.0 software (https://www.megasoftware.net). Based on the results, the species of the strains were determined [12].

Determination of activity

The crude enzyme fermentation broth was prepared by inoculating target strain into 200 mL of PDA liquid medium and culturing at 35°C, 150 rpm, for 48 hours. The resulting seed culture was then transferred at an inoculum size of 2.5% (v/v) into a liquid enzyme-production medium and incubated at 35°C, 150 rpm, for 72 h before centrifugation at 5,000 rpm for 10 minutes to collect the supernatant as the crude enzyme fermentation broth [13]. The β-endoglucanase activity was determined *via* the 3,5-dinitrosalicylic acid (DNS) method with 1% Carboxymethyl Cellulose Sodium (CMC-Na) (Damao Chemical Reagent Factory, Tianjin, China) as substrate [14]. 1 mL of CMC-Na solution was mixed with 0.50 mL crude enzyme fermentation broth at 50°C for 30 min. Reactions were terminated with 1 mL DNS reagent and heated at 100°C for 10 min. The absorbance at 540 nm was measured [15]. The reducing sugar (glucose equivalents) was calculated by using a standard curve of 0 - 1 mg/mL glucose. One unit (U) was defined as the amount of enzyme releasing 1 µmol glucose equivalent per minute. The activity of β-exoglucanase was determined by using 1 mL of a 1% microcrystalline cellulose solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as the substrate, while the activity of β-glucosidase was determined by using 1 mL of a 1% salicin solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as the substrate. The enzymatic activity (E) was calculated as follows.

$$E\ (U/mL) = 1{,}000 \times C \times V \times D / (t \times v \times 186.16)$$

where C was the concentration of product from the standard curve (mg/mL). V was the total reaction volume (2.5 mL). D was the dilution factor. T was the reaction time (30 min). v was the enzyme volume used (0.5 mL). 180.16 represented the molecular weight of glucose (g/mol).

Single factor optimization of enzyme production conditions

The effects of four single factors including nitrogen source, carbon source, cultivation time, and initial pH were examined on the cellulase production capability of the strain. Effect of different carbon sources on enzyme production was explored by using different liquid enzyme-production media prepared using CMC-Na, sucrose, glucose, or starch as the sole carbon source at a concentration of 1%. The media were inoculated at 2.5% (v/v) and cultured at 35°C, 180 rpm, for 72 h. Enzyme activity was determined according to the standard assay method [16]. After identifying the optimal carbon source, its concentration was varied at 4, 6, 8, 10, and 12 g/L to prepare different media. Enzyme activity was measured under the aforementioned conditions to determine the optimal concentration. Effect of different nitrogen sources on enzyme production was determined by using different liquid enzyme-production media prepared using peptone, ammonium chloride, ammonium sulfate, or yeast extract powder as the sole nitrogen source at a concentration of 1%. The media were inoculated at 2.5% (v/v) and cultured at 35°C, 180 rpm, for 72 hours. Enzyme activity was determined according to the standard assay method [17]. After identifying the optimal nitrogen source, its concentration was varied at 0.8, 1.6, 2.4, 3.2, and 4.0 g/L to prepare different media. Enzyme activity was measured under the aforementioned conditions to determine the optimal nitrogen source concentration. Effect of cultivation time on enzyme production was then measured. Under the inoculation volume of 2.5%, the liquid enzyme-production medium was cultured at 35°C, 180 rpm, for 1, 2, 3, 4, and 5 days, respectively. Enzyme activity was measured after each interval to determine the optimal cultivation time for enzyme production by the strain. Effect of initial pH of the medium on enzyme production was identified with an inoculation volume of 2.5%, the various initial pH of the liquid enzyme-production medium at 5.0, 6.0, 7.0, 8.0, and 9.0 using a citrate buffer. The cultures were then incubated at 35°C, 180 rpm, for 72 hours before the enzyme activity was measured to determine the optimal initial pH for enzyme production by the strain.

Optimization of enzyme production conditions using RSM

Based on the results of single factor experiments, a Box-Behnken experimental design (BBD) was implemented by using Design-Expert 13 software (https://www.statease.com/software/design-expert/). With enzyme activity as the response value, a four-factor, three-level design was established to identify the optimal parameters for response surface analysis. This study aimed to optimize the enzyme production conditions of the cellulase-producing strain. The coded factors and levels for the response surface experiments were presented in Table 1.

Table 1. Factors and levels for the response surface methodology in optimizing enzyme production conditions by strain X-5.

Level	A	B	C	D
	Sucrose concentration	Yeast extract concentration	Time	Initial pH
-1	8 g/L	1.6 g/L	2 d	5
0	10 g/L	2.4 g/L	3 d	6
1	12 g/L	3.2 g/L	4 d	7

Data analysis

All experiments were performed in triplicate. Origin 9.6 software (https://www.originlab.com) was used for data analysis of the single factor experiment. Design-Expert 13 software was employed for response surface design, fitting quadratic regression models, variance analysis, significance testing, and calculation of the coefficient of determination. Analysis of variance (ANOVA) was conducted on the regression equation. *P* value less than 0.01 was defined as highly significant effect, while *P* value less than 0.05 was defined as significant effect. By comparing the model-predicted values with the actual values from independent validation experiments, the relative error was calculated to verify the model's predictive ability. A relative error less than 5% indicated excellent predictive

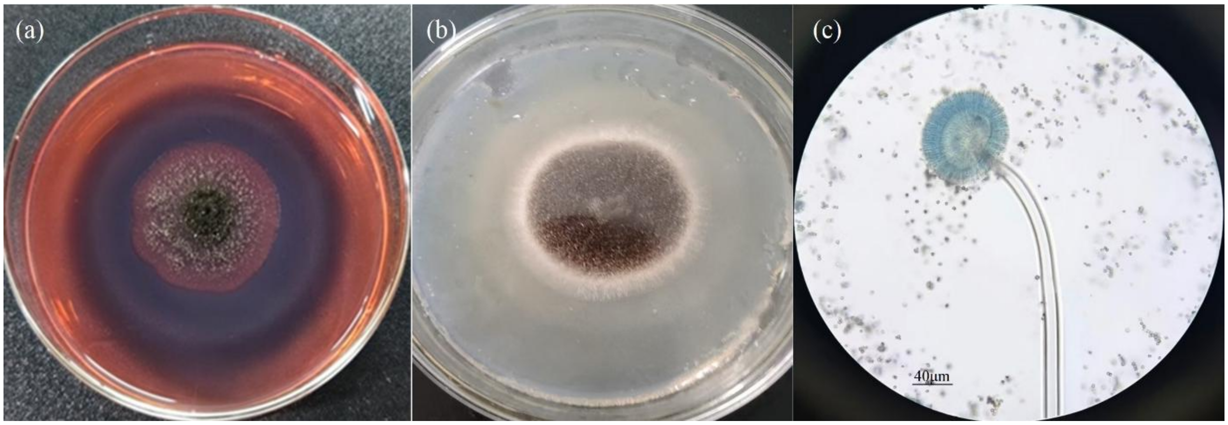

Figure 1. Transparent circle (**a**), observations of colony (**b**), and electron microscopy (**c**) of X-5 strain.

ability of the model, and a relative error between 5% and 10% indicated good predictive ability.

Results

Screening of cellulose-degrading strains

Isolated single colonies were inoculated onto Congo red selective medium and cultured for 36 hours. The size of the hydrolysis zone (transparent circle) around each colony was observed (Figure 1a). The ratio of the hydrolysis zone diameter (R) to the colony diameter (r) was used as the basis for strain screening. The results showed that strain X-5 had the highest R/r value, reaching 2.77. Enzyme activity assays indicated that X-5 exhibited the highest enzyme activity with β-endoglucanase, β-exoglucanase, and β-glucosidase activities of 31.85, 25.24, and 16.47 U/mL, respectively. Therefore, X-5 strain was selected as the target strain for subsequent studies.

Morphological and molecular identification of the cellulose-degrading strain

Upon maturation, strain X-5 abundantly produced conidial structures on PDA medium. The center of the colony turned jet-black, presenting a velvety black appearance. The colony exhibited a regular shape, being circular or elliptical, flat with slight radial furrows, and an entire, smooth margin. The mycelium was dense and thick with the central area appearing slightly floccose and the periphery uniformly velvety (Figure 1b). Microscopic observation revealed that the fungal structures possessed colorless and smooth conidiophores. The apices of the conidiophores swelled into large, spherical vesicles. The surface of these vesicles bore brown, two-layered sporogenous structures (comprising brown metulae and phialides), which ultimately produced chains of spherical, black conidia with rough ornamentation (Figure 1c). The BLAST analysis results of 18S rDNA sequence indicated that strain X-5 shared the closest phylogenetic relationship with *Aspergillus tubingensis* with a BLAST similarity of 98% (Figure 2). Based on the combined morphological characteristics and molecular identification results, strain X-5 was identified as *Aspergillus tubingensis*.

Results of single factor experiments

The results demonstrated that, when sucrose was used as the carbon source, the cellulase activity reached its highest level with β-endoglucanase, β-exoglucanase, and β-glucosidase activities measuring 92.47 U/mL, 87.38 U/mL, and 99.24 U/mL, respectively. Therefore, it was determined that sucrose was the optimal carbon source for enzyme production by X-5 fermentation (Figure 3a). When yeast extract powder was used as the nitrogen source, the cellulase activity reached its

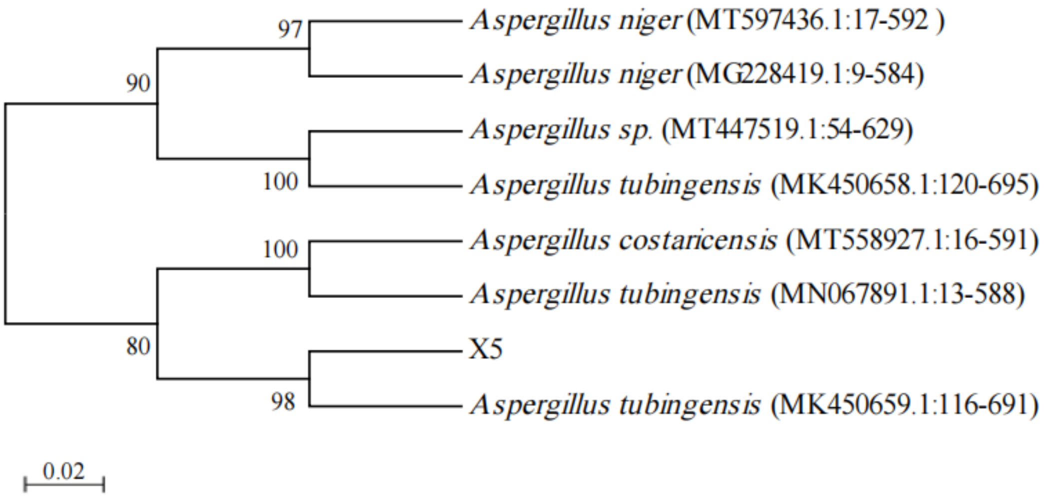

Figure 2. Phylogenetic tree based on the 18S rDNA sequences of strain X-5.

highest level with β-endoglucanase, β-exoglucanase, and β-glucosidase activities measuring 29.5 U/mL, 19.86 U/mL, and 18.01 U/mL, respectively. Thus, yeast extract powder was identified as the optimal nitrogen source for cellulase production by this strain (Figure 3b). When the sucrose concentration was 10 g/L, the strain exhibited the highest cellulase production capacity. The activities of β-endoglucanase, β-exoglucanase, and β-glucosidase reached peak values of 487.26 U/mL, 498.34 U/mL, and 504.7 U/mL, respectively. The optimal carbon source concentration for cellulase production by this strain was determined to be 10 g/L (Figure 3c). When the yeast extract powder concentration was 2.4 g/L, the strain achieved the highest cellulase production capacity. The activities of β-endoglucanase, β-exoglucanase, and β-glucosidase were 41.11 U/mL, 37.42 U/mL, and 43.06 U/mL, respectively. The optimal nitrogen source concentration for cellulase production by this strain was determined to be 2.4 g/L (Figure 3d). When the pH of the medium was 6, the β-endoglucanase and β-glucosidase activity reached its highest level of 70.03 U/mL and 71.06 U/mL, respectively. When the pH of the medium was 7, the β-exoglucanase activity reached its highest level of 71.07 U/mL. The optimal pH for cellulase production by this strain was determined to be 6 (Figure 3e). When the cultivation time was 3 days, the cellulase activity reached its highest level with β-endoglucanase, β-exoglucanase, and β-glucosidase activities measuring 125.31 U/mL, 112.8 U/mL, and 129.62 U/mL, respectively. The optimal cultivation time for cellulase production by this strain was determined to be 3 days (Figure 3f).

Response surface methodology design, development of regression model, and analysis of variance for β-endoglucanase

The response surface results were analyzed. The quadratic fitting of the influence of each factor was carried out, and the quadratic regression model was established. The quadratic multiple regression equation of β-endoglucanase activity and its influencing factors was obtained as follows.

$$Y = 500.01 + 121.97A + 3.62B - 38.3C - 27.26D - 14.31AB - 11.56AC + 2.36AD + 25.54BC + 3.64BD + 14.59CD - 77.53A^2 - 76.54B^2 - 186.62C^2 - 70.68D^2$$

where Y was the response value of enzyme activity (U/mL). A was the sucrose concentration (g/L). B was the yeast extract concentration (g/L). C was the time (h). D was the initial pH. The regression model showed $P < 0.0001$, indicating an extremely significant level. The lack-of-fit term had a P value of 0.6671, indicating that the lack-of-fit term was not significant, which proved the

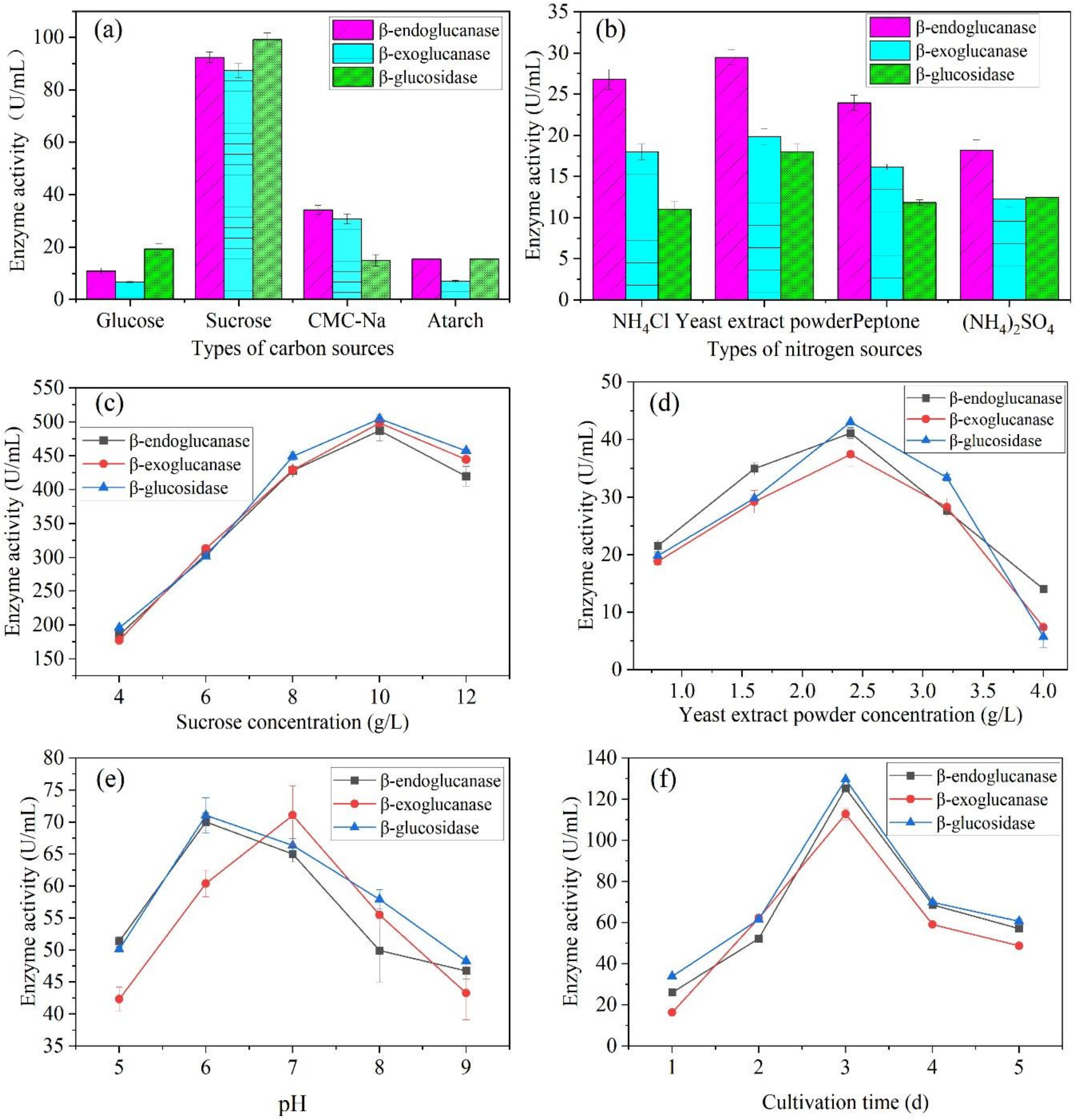

Figure 3. The influence of four factors on the enzyme activity of X-5 strain.

reliability of the model. $R^2 = 0.9859$, indicating a good fit of the equation to the experimental data and showing that the model could explain 98.59% of the variation in β-endoglucanase activity, making it highly suitable for practical applications. The adjusted coefficient of determination R^2_{Adj} was 0.9659, indicating that the model could explain approximately 96.59% of the variation in the response values. The variance analysis of the regression equation terms showed that, among the four influencing factors A, B, C, and D, factors A, C, D, A^2, B^2, C^2, and D^2 had an extremely significant impact on β-endoglucanase activity ($P < 0.01$), while factor BC and CD had a significant impact ($P < 0.05$). Factors B, AB, AC, AD, and BD had no significant impact on β-endoglucanase activity ($P > 0.05$). The order of influence of the factors on β-endoglucanase activity was A > C > D > B. First-order partial derivatives were taken for each of the four

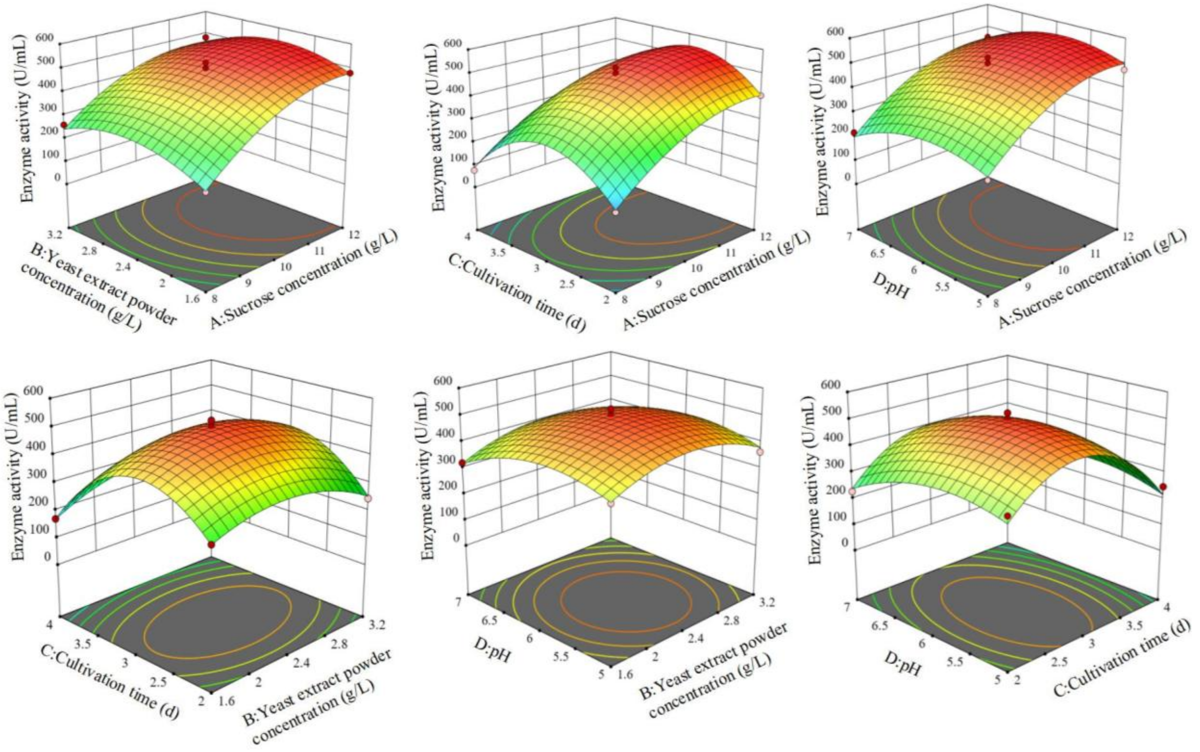

Figure 4. Response surface and contour plots depicting the interaction effects of single factors on β-endoglucanase activity of X-5 strain.

variables in the regression equation, forming a system of equations, which was then solved. The results showed that the optimal fermentation conditions for strain X-5 to produce β-endoglucanase were A = 10.92, B = 2.28, C = 2.79, D = 5.84 with a theoretically predicted β-endoglucanase activity of 543 U/mL. Based on practical operational conditions, the adjusted optimal fermentation conditions for β-endoglucanase production were 10.9 g/L sucrose, 2.3 g/L yeast extract powder, 2.8 days (approximately 67 hours) cultivation time, and initial pH 5.8. Under these conditions, three parallel validation experiments obtained an actual average β-endoglucanase activity of 531.83 U/mL, which was 16.7 times higher than it was before optimization. The relative error compared to the theoretical value was 2.1%, indicating a high consistency between the actual and predicted values, which demonstrated that the constructed model had excellent and stable fitting performance, and the regression equation was reliable. After analyzing the response surface experimental results, response surface plots illustrating the interactions among the four factors were generated (Figure 4). These plots visually reflected the significant impact of the interactions among these factors on the response value (Y). The results showed that all response surfaces were smooth and curve downward, indicating that the maximum activity of β-endoglucanase could be achieved within the designed range of factor levels. The significance of the influence of the factors on the response value (Y) could be determined based on the shape of the contour lines. Elliptical contour lines indicated significant effects, whereas non-elliptical shapes suggested otherwise. In the contour plot of the BC and CD interaction, clear interactivity was observable, indicating that the effects of factors B, C, and D on β-endoglucanase activity were not independent. This finding was consistent with the results obtained from the analysis of variance of the quadratic regression model, demonstrating the reliability of the analysis.

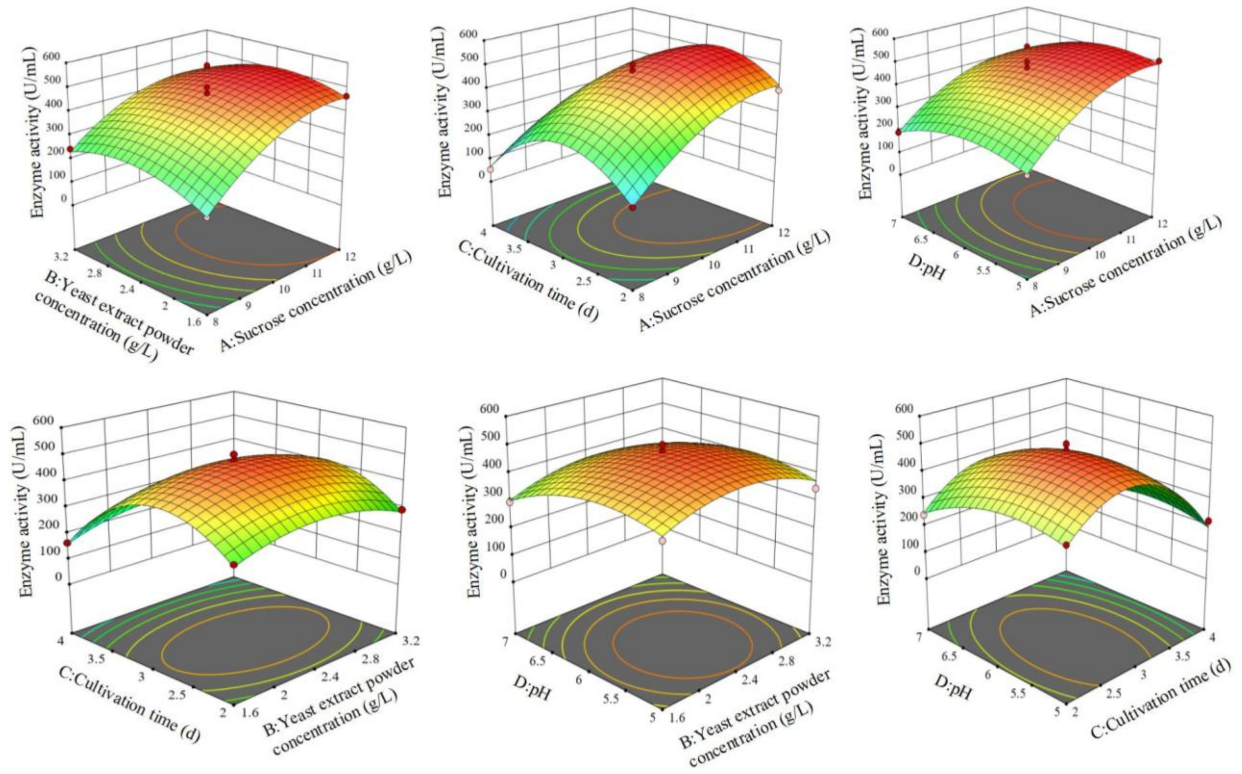

Figure 5. Response surface and contour plots depicting the interaction effects of single factors on β-exoglucanase activity of strain X-5.

Response surface methodology design, development of regression model, and analysis of variance for β-exoglucanase

The quadratic multiple regression equation of β-exoglucanase activity and its influencing factors was obtained as follows.

$$Y = 3.991E5A + 1.594E5B + 21.88C + 44{,}388.87C + 14{,}599.66D + 1{,}738.25AB + 2{,}795.43AC + 733.16AD + 142.77BC + 115.95BD + 385.77CD + 36{,}138.92A^2 + 25{,}304.38B^2 + 1.717E5C^2 + 22{,}176.59D^2$$

The regression model showed $P < 0.0001$, indicating an extremely significant level. The lack-of-fit term had a P value of 0.7181, indicating that the lack-of-fit term was not significant, which proved the reliability of the model. $R^2 = 0.9843$, indicating a good fit of the equation to the experimental data and showing that the model could explain 98.43% of the variation in β-exoglucanase activity, making it highly suitable for practical applications. The adjusted coefficient of determination R^2_{Adj} was 0.9661, indicating that the model could explain approximately 96.61% of the variation in the response values. The variance analysis of the regression equation terms showed that, among the four influencing factors, A, C, D, A^2, B^2, C^2, and D^2 had an extremely significant impact on β-exoglucanase activity ($P < 0.01$), while factor AC and BC had a significant impact ($P < 0.05$). B, AB, AD, BD, and CD had no significant impact on β-exoglucanase activity ($P > 0.05$). The order of influence of the factors on β-exoglucanase activity was A > C > D > B. First-order partial derivatives were taken for each of the four variables in the regression equation, forming a system of equations, which was then solved. The results showed that the optimal fermentation conditions for strain X-5 to produce β-exoglucanase were A = 11.12， B = 1.99， C = 2.76， D = 5.59 with a theoretically predicted β-exoglucanase activity of 518.27 U/mL. Based on practical operational conditions, the adjusted optimal fermentation conditions for β-exoglucanase production were 11.1 g/L sucrose, 2.0 g/L yeast extract powder, 2.8 days (approximately 67 hours) cultivation time, and initial pH 5.6. Under these conditions, three parallel validation experiments yielded an actual average β-exoglucanase activity of 507.4 U/mL, which was 20.1 times higher than it was before optimization. The relative error compared to the theoretical value was 2.1%, indicating a high consistency between the actual and predicted values, which demonstrated that the constructed model had excellent and stable fitting performance, and the regression equation was reliable. The response surface plots showed smooth and curve downward, indicating that the maximum activity of β-exoglucanase could be achieved within the designed range of factor levels (Figure 5). In the contour plot of the AC and BC interaction, clear interactivity was observed, indicating that the effects of factors A, B and C on β-exoglucanase activity were not independent. This finding was consistent with the results obtained from the analysis of variance of the quadratic regression model, demonstrating the reliability of the analysis.

Response surface methodology design, development of regression model, and analysis of variance for β-glucosidase

The quadratic multiple regression equation of β-glucosidase activity and its influencing factors was obtained as follows.

$$Y = 3.968E5 + 1.762E5A + 390B – 43{,}039.41C - 19{,}364.45D - 270.98AB - 2{,}428.72AC + 1{,}292.31AD + 36BC + 444.24BD + 2{,}393.47CD - 27{,}746.94A^2 – 21{,}793.2B^2 - 1.491E5C^2 - 21{,}644.85D^2$$

The regression model showed $P < 0.0001$, indicating an extremely significant level. The lack-of-fit term had a *P*-value of 0.3814, indicating that the lack-of-fit term was not significant, which proved the reliability of the model. $R^2 = 0.9789$, indicating a good fit of the equation to the experimental data and showing that the model could explain 97.89% of the variation in β-glucosidase activity, making it highly suitable for practical applications. The adjusted coefficient of determination R^2_{Adj} was 0.9542, indicating that the model could explain approximately 95.42% of the variation in the response values. The variance analysis of the regression equation terms showed that, among the four influencing factors, A, C, D, A^2, B^2, C^2, and D^2 had an extremely significant impact on β-glucosidase activity ($P < 0.01$), while AC and CD had a significant impact ($P < 0.05$). B, AB, BC, AD, and BD had no significant impact on β-glucosidase activity ($P > 0.05$). The order of influence of the factors on β-glucosidase activity was A > C > D > B. The optimal fermentation conditions for strain X-5 to produce β-glucosidase were A = 11.55, B = 2.29, C = 2.75, D = 5.76 with a theoretically predicted β-glucosidase activity of 553.31 U/mL. Based on practical operational conditions, the adjusted optimal fermentation conditions for β-glucosidase production were 11.6 g/L sucrose, 2.3 g/L yeast extract powder, 2.8 days (approximately 67 hours) cultivation time, and initial pH 5.8. Under these conditions, three parallel validation experiments yielded an actual average β-glucosidase activity of 513.48 U/mL, which was 31.18 times higher than it was before optimization. The relative error compared to the theoretical value was 7.2%, indicating a consistency between the actual and predicted values, which demonstrated that the constructed model had good and stable fitting performance, and the regression equation was reliable. The response surface plots showed that all response surfaces were smooth and curved downward, indicating that the maximum activity of β-glucosidase could be achieved within the designed range of factor levels (Figure 6). In the contour plot of the AC and CD interaction, clear interactivity was observed, indicating that the effects of factors A, C and D on β-glucosidase activity were not independent. This finding was consistent with the results obtained from the analysis of variance of the quadratic regression model, demonstrating the reliability of the analysis.

Discussion

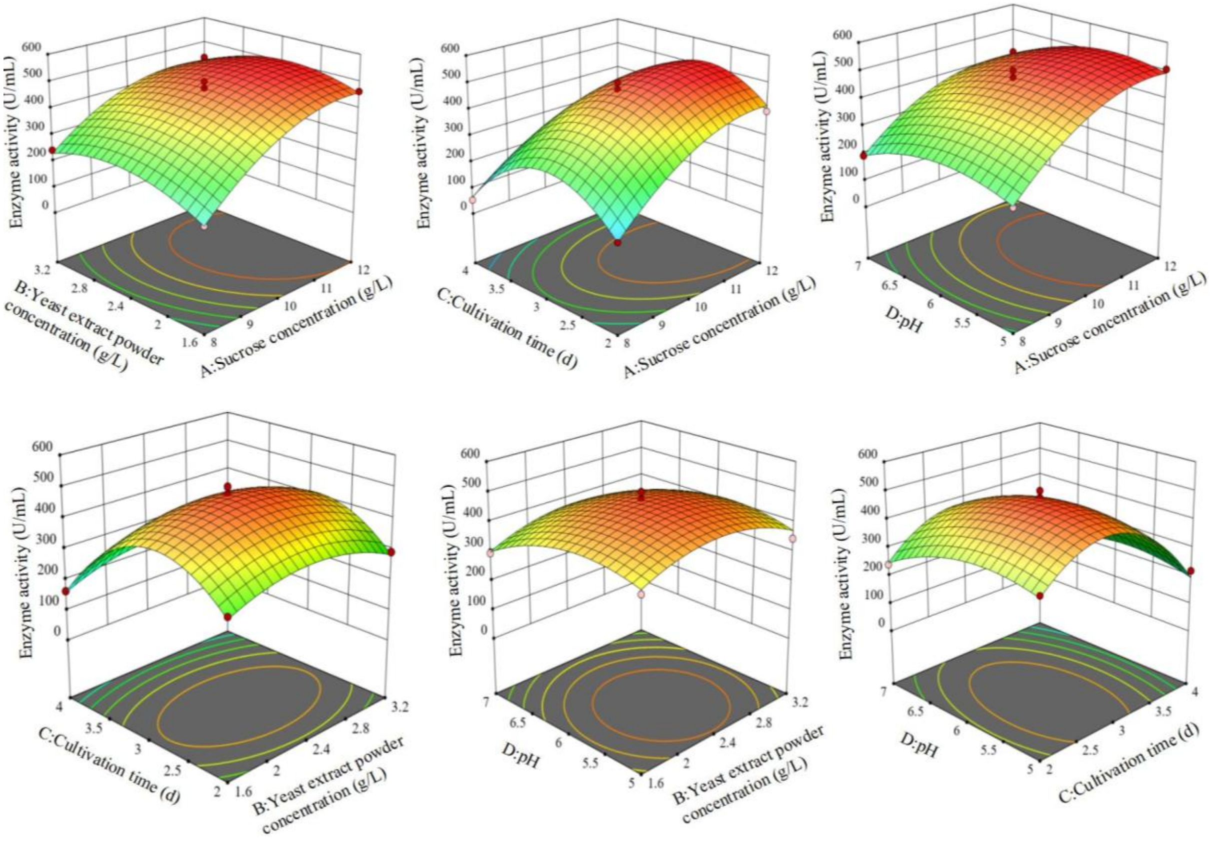

Figure 6. Response surface and contour plots depicting the interaction effects of single factors on β-glucosidase activity of strain X-5.

Screening of cellulose-decomposing microorganisms

The substrate for edible mushroom cultivation is primarily composed of agricultural waste materials such as sawdust, corn cobs, and straw. Due to its high cellulose and lignin content and low crude protein content, it is considered unsuitable for use in animal feed [18]. The cellulases and proteases secreted by edible mushrooms during growth enzymatically break down the substrate, converting hard-to-utilize macromolecules into smaller molecules that are easier for the fungi to digest, absorb, and utilize, providing essential nutrients for mushroom growth [19, 20]. Consequently, after being used for mushroom cultivation, the substrate's texture and nutritional composition improve to some extent, but its cellulose content remains high, which limits its utilization by animals [21]. Microbial fermentation, as a green and efficient method for cellulose processing, plays a significant role in improving feed quality, enhancing animal feed intake, and increasing feed utilization rates [22]. Nature hosts many microorganisms capable of secreting cellulases and utilizing cellulose as their primary carbon source. Therefore, screening microorganisms with high cellulase activity is the first step in reducing the fiber content of SMS. Currently, cellulose-degrading microbes isolated by researchers from natural environments are mainly categorized into bacteria, fungi, and actinomycetes [23]. Jia *et al*. isolated three cellulose-decomposing strains from the soil of alpine meadows in the eastern Qilian Mountains using a screening medium. Among them, strain X1-2 exhibited the highest enzyme activity and was molecularly identified as *Bacillus* sp [24]. Zhang *et al*. isolated two fungal strains from soils of different environments that efficiently degraded corn stover. Both strains showed high endoglucanase and exoglucanase activities and were molecularly identified as *Trichoderma longibrachiatum* and *Aspergillus sydowii*, respectively. Li *et al*. screened eight efficient cellulose-decomposing microorganisms from spent *Pleurotus eryngii* substrate compost including four bacterial strains (*Bacillus*), two actinomycete strains (one *Streptomyces*, one *Microbispora*), and two fungal strains (one *Seytalidium*, one *Microascus*) [26]. The screening results from this research indicated that strain X-5 had a relatively large R/r ratio and also exhibited relatively high activities of β-endoglucanase, β-exoglucanase, and β-glucosidase. However, the R/r ratio on CMC-Na solid screening medium does not always accurately reflect enzyme activity levels [27, 28]. Therefore, while the R/r ratio can to some extent indicate a strain's ability to utilize cellulose, it is subject to error. Further measurement of β-1,4-endoglucanase, β-1,4-exoglucanase, and β-glucosidase activities is necessary to comprehensively evaluate a strain's cellulose-degrading capability. This study comprehensively assessed the cellulose-degrading ability of the strains by considering the R/r ratio formed on CMC-Na solid screening medium combined with the determination of β-1,4-endoglucanase, β-1,4-exoglucanase, and β-glucosidase activities.

Optimization of enzyme production conditions for strain X-5

Cellulolytic microorganisms are widely applied in various fields such as food, papermaking, feed, energy, and agriculture due to their high efficiency in degrading cellulose. The optimization of enzyme production conditions is a crucial and fundamental step for enhancing cellulase activity and promoting industrial applications. Cellulase is a complex enzyme system comprising β-endoglucanase, β-exoglucanase, and β-glucosidase [29]. During cellulose degradation, β-1,4-endoglucanase first randomly cleaves the β-1,4-glycosidic bonds within the amorphous regions of the cellulose polysaccharide chains, generating shorter oligosaccharide chains, and creating new chain ends. Subsequently, β-1,4-exoglucanase acts on the reducing or non-reducing ends of these newly formed oligosaccharide chains, sequentially hydrolyzing β-1,4-glycosidic bonds to release glucose and cellobiose. Finally, cellobiose is hydrolyzed by β-glucosidase into glucose [30]. This process demonstrates that efficient cellulose degradation requires the synergistic action of three enzymes including β-endoglucanase, β-exoglucanase, and β-glucosidase, highlighting the importance of optimizing culture conditions for their production by microbial strains. Consequently, optimizing the conditions for the strain to produce these three enzymes becomes particularly important. In this study, the enzyme production conditions of strain X-5 were optimized through single factor experiments and response surface methodology, focusing on four key parameters of optimal carbon source and its concentration, optimal nitrogen source and its concentration, optimal pH, and optimal incubation time. These optimizations significantly enhanced the enzyme production capability of strain X-5. In future research, the strategies to effectively combine this strain with other microorganisms to create synergistic effects should be further explored to develop efficient microbial consortia for enhanced cellulose degradation and utilization of SMS.

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