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Full Length Article

Optimization of Solid-state Fermentation Conditions for β-glucosidase Production by *Aspergillus fumigatus*

Xiaohua Cao[1,2,3], Qinyou Wen[4], Yaru Zhang[4], Zuxin Cheng[1,2,3], Xinying Huang[1,2,3], Jingui Zheng[1,2,3*] and Zhiwei Huang[4,5*]

[1]*Fujian Engineering Technology Research Center for Breeding and Utilization of Special Crops, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[2]*Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[3]*Key Laboratory of Crop Biotechnology (Fujian Agriculture and Forestry University), Fujian Province University, Fuzhou 350002, China*
[4]*College of Food Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[5]*Fujian Provincial Key Laboratory of Quality Science and Processing Technology in Special Starch, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[*]Corresponding author: hzwfau@163.com

Abstract

β-glucosidase is a key enzyme for the hydrolysis of lignocellulose into glucose. It plays a key role in cellulose hydrolysis and lignocellulosic ethanol production. This study aims at improving the production of β-glucosidase by solid-state fermentation of *Aspergillus fumigatus* A27. Based on single factor and response surface optimizations, the optimal conditions were as follows: ratio of straw powder to bran, 5:0; nitrogen source, peptone; nitrogen content, 0.9%; ratio of material to water, 1:2; culture temperature, 36.4°C; culture time, 6 d; initial medium pH, 3.16; and inoculum size, 10%. The activity of β-glucosidase achieved under the optimal conditions was 63.950 IU/g, which was 2.3 times higher than that obtained under non-optimal conditions. © 2019 Friends Science Publishers

Keywords: β-glucosidase; *Aspergillus fumigatus*; Solid-state fermentation; Response surface Optimization

Introduction

China is one of the main producers of crop straw. If straw is used to produce renewable and clean energy, such as ethanol, a bio-based fuel, the energy crisis and environmental pollution problems could be resolved. Currently, the production of cellulosic ethanol in China has been industrialized; however, there are problems such as high cost of cellulase, which results in high production costs and difficulty in expanding the industrial scale (Qu *et al*., 2018). Therefore, the development of low-cost, high-performance cellulase hydrolysis can be one of the key breakthrough technologies to resolve the bottleneck in industrial production of cellulosic ethanol.

β-glucosidase (β-D-glucosidase, EC3.2.1.21), also known as β-D-glucoside glucohydrolase, cellobiase, *etc*., is a class of enzyme that catalyzes the hydrolysis of glucosidic bond between glycosyl and aromatic base or hydroxy group (Esen, 1993). It belongs to cellulase enzymes, which mainly include exoglucanases, endoglucanases and β-glucosidases (Patel *et al*., 2019). In the degradation of cellulose, enzymes including endoglucanase and exoglucanase decompose cellulose into cellobiose, and β-glucosidase further hydrolyzes cellobiose, as well as other low molecular weight fiber dextrin, into glucose. The three enzymes must work together for the complete degradation of cellulose (Lynd *et al*., 2002; Sukumaran *et al*., 2005). β-glucosidase is a key enzyme for the complete hydrolysis of lignocellulose into glucose and plays a key role in cellulose hydrolysis and lignocellulosic ethanol production (Das *et al*., 2015).

To date, the cellulase production strain *Trichoderma reesei* Rut-C30 has been widely studied, and it has a wide range of applications (Peterson and Nevalainen, 2012) due to its ability to produce cellulase with high yield. However, the strain has some disadvantages, including low β-glucosidase activity, which causes the accumulation of cellobiose in the cellulose hydrolysis product, thereby affecting the hydrolysis efficiency. After cellulose is hydrolyzed, β-glucosidase activity is increased, which can significantly reduce the accumulation of cellobiose, effectively improve the enzymatic hydrolysis efficiency of cellulose, and thus can reduce the amount of cellulase used, as well as its cost (Sorensen *et al*., 2013).

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Aspergillus fumigatus is a ubiquitous saprophytic filamentous fungus. As a fungus known to have good heat tolerance (Fang and Jin, 2018), it can secrete various enzymes such as cellulase (Sharma *et al*., 2011; Ang *et al*., 2013; Cao *et al*., 2015). The fungus has also been reported to be able to produce β-glucosidase with high yield (Dodda *et al*., 2018).

In one of our studies, *A. fumigatus* A27, was screened from 49 cellulose-degrading bacteria preserved in our laboratory. Its β-glucosidase activity was determined to be 8.990 IU/g, which was 2.15 times higher than the activity obtained from the control strain *Trichoderma viride* GIM3.141 (data to be published). Thus far, there are only a few reports on the process conditions for the production of β-glucosidase by solid-state fermentation of *A. fumigatus*. In this study, we applied single factor test and response surface analysis to optimize the process conditions for β-glucosidase production by *A. fumigatus* A27 and to increase the yield and activity of β-glucosidase. The study can lay a foundation for the development of low-cost and high-hydrolysis cellulose.

Materials and Methods

Materials

Fungal strain. *A. fumigatus* A27, which was stored in our laboratory, was used.

Rice straw powder. Rice (variety 2B948) was cultivated in the laboratory. Straw was harvested and crushed with a pulverizer, and thereafter was screened through a 40 mesh sieve.

Preparation of Media

Solid enzyme-producing medium. Five grams of straw powder was mixed with 15 mL of inorganic salt culture solution (16 g/L $(NH_4)_2 SO_4$, 4 g/L $KH_2 PO_4$, 1 g/L $MgSO_4$).

Solid-state fermentation enzyme-producing medium. Straw powder and bran, nitrogen source, water were mixed at different proportions. The mixtures were then sterilized at 121°C for 20 min.

Preparation of Reagents

DNS reagent. About 600 mL of water was added into a beaker, which was then preheated in a 50°C water bath. Ten grams each of 3,5-dinitrosalicylic acid and sodium hydroxide were then added and stirred until dissolved. After that, 200 g of sodium potassium tartrate, 2 g of phenol (re-distilled) and 5 g of anhydrous sodium sulfite were sequentially added. After all components were dissolved (as indicated by a clear solution), the solution was removed from the water bath and then cooled down to room temperature, and its final volume was adjusted to 1000 mL with water. The obtained solution was stored in a brown reagent bottle and stored in the dark for 7–10 days before use.

Sodium citrate buffer (0.05 mol/L, pH 4.8): 4.83 g of citric acid monohydrate and 7.94 g of trisodium citrate were weighed and then dissolve in about 750 mL of water. The solution pH was adjusted to 4.8; after that, its final volume was adjusted to 1000 mL with water. The solution was stored until subsequent use.

For 1% salicin solution, 1 g D(-)-salicin was weighed (with a precision of up to 0.1 mg), dissolved in 100 mL of sodium citrate buffer and then stored in a refrigerator until subsequent use.

Inoculation

Preparation of fungus. The *A. fumigatus* A27 strain was removed from a 4°C refrigerator, transferred to a fresh Potato Dextrose Agar (PDA) medium slant and then incubated at 30°C for 5–6 days.

Preparation of fungal suspension. *A. fumigatus* A27 was scraped off the slants using an inoculating loop into a 250 mL flask containing 50 mL of sterile physiological saline. The flask was shaken at 150 rpm at 30°C for 10–12 h to obtain a spore suspension with a concentration of about 1 x 10^7 cells/mL.

To produce the enzyme, the prepared fungal suspension was added to the enzyme-producing medium, and solid-state fermentation was carried out.

Preparation of Crude Enzyme

One hundred milliliters of distilled water was mixed evenly with the cultured solid enzyme and then incubated at 30°C for 1 h. After that, it was subjected to centrifugation at 12000x at 10°C for 10 min. The supernatant, which contains the crude enzyme, was collected.

Determination of β-glucosidase Activity

The determination of β-glucosidase activity was carried out based on the cellulase preparation (QB 2583-2003) in the light industry standard of China. Four 20 mL stoppered test tubes (one of which was used for the blank control group) were used. One and a half milliliters of 1% salicin solution was accurately pipetted and placed in the four stoppered test tubes (as a control, 2.0 mL of DNS reagent was also pipetted to one of the tubes). After the tubes were added with 0.50 mL of diluted enzyme solution, they were simultaneously placed in a 50°C water bath and then immediately removed from it after 30 min. Immediately after that, 2.0 mL of DNS reagent was added to the three sample tubes. After mixing, all four tubes were simultaneously placed in a boiling water bath for 10 min and thereafter were immediately cooled down to room temperature in ice water. Subsequently, the tubes were diluted to 20 mL using distilled water, covered with stopper and then mixed. The control sample was used as a blank to zero adjust the spectrophotometer, and the three samples

were subjected to absorbance measurement at 540 nm. The reducing sugar content was calculated by the absorbance and the linear regression equation.

Definition of β-glucosidase activity: The activity of β-glucosidase is defined as the amount of enzyme required for the hydrolysis of salicin substrate to produce 1.0 μmol of glucose in 1 min. The enzyme activity has a unit IU/g, *i.e*., the unit of enzyme activity contained in fermentation culture with 1 g dry weight. It was calculated as follows: β-glucosidase activity (IU/g) = [glucose content (mg) × 1 (min) × 1000 (μmol/mmol) × dilution factor]/[180 (mg/mmol) × 30 (min) × 0.5 (mL)].

Optimization of Conditions for Enzyme Production by Solid-state Fermentation

The effects of eight factors, including rice straw to bran ratio, nitrogen source, nitrogen content, material to water ratio, culture temperature, culture time, initial medium pH and inoculum size, on the production of β-glucosidase by solid-state fermentation of *A. fumigatus* A27 were evaluated. Each treatment was carried out in three replicates. To determine the optimal conditions, the Box-Behnken experimental design principle, which includes the three-factor and three-level response surface analysis methods, was used.

Results

Effects of a Single Factor on Activity of β-glucosidase Produced by Solid-state Fermentation

Effect of rice straw to bran ratio: In the preparation of solid enzyme-producing medium for solid-state fermentation, straw powder and bran (the carbon sources) were mixed at different ratios (5:0, 4:1, 3:2, 2:3, 1:4 and 0:5). To prepare the crude enzyme, the fungal suspension containing *A. fumigatus* A27 was inoculated at 10% (the inoculum size) and cultured at 30°C for 72 h; after that, the β-glucosidase activity was measured. The results are shown in Fig. 1. When the ratio of straw powder to bran was 5:0, the activity of β-glucosidase produced by *A. fumigatus* A27 was highest. Conversely, when the ratio of bran was increased, the enzyme activity gradually decreased. When the ratio of straw powder to bran was 0:5, the enzyme activity was lowest. The results indicate that the activity of β-glucosidase produced by *A. fumigatus* A27 was highest when straw powder was used as the sole carbon source.

Effect of nitrogen source: In the preparation of solid enzyme-producing medium used in solid-state fermentation, ammonium sulfate was replaced with different nitrogen sources, which include urea, ammonium nitrate, sodium nitrate, peptone, yeast powder and potassium nitrate. The total nitrogen content was controlled at 0.6%. The inoculation, culture and measurement of enzyme activity were carried out according to section "Effect of rice straw to bran ratio", and the results are shown in Fig. 2. The results showed that the effects of seven different nitrogen sources on β-glucosidase activity produced by A. fumigatus A27 can be ranked as followed: peptone > ammonium sulfate > potassium nitrate > ammonium nitrate > sodium nitrate > yeast powder > urea. The enzyme activity was highest when peptone was used as nitrogen source. The finding suggests that peptone should be used as the nitrogen source for the production of β-glucosidase by *A. fumigatus* A27 in solid-state fermentation.

Effect of nitrogen content in medium: The solid enzyme-producing medium used in solid-state fermentation was prepared by replacing ammonium sulfate with different amounts of peptone so that the nitrogen contents were 0.2, 0.6, 1.0, 1.4 and 1.8%. The inoculation, culture and measurement of enzyme activity were carried out according to section "Effect of rice straw to bran ratio". The results are shown in Fig. 3. When the nitrogen content was 1.0%, the activity of β-glucosidase produced by *A. fumigatus* A27 was highest. By contrast, the enzyme activity decreased with the increase or decrease of the amount of peptone added. Therefore, we may conclude that the optimum nitrogen content in the medium used for the production of β-glucosidase by *A. fumigatus* A27 using solid-state fermentation is 1.0%.

Effect of material to water ratio: Different volumes of inorganic salt culture solution were added to the solid enzyme-producing medium used in solid-state fermentation at different material to water ratios of 1:1, 2:3, 1:2, 2:5, 1:3, 2:7 and 1:4. The inoculation, culture, and measurement of enzyme activity were performed based on section "Effect of rice straw to bran ratio", and the results are shown in Fig. 4. When the material to water ratio was 1:2, the activity of β-glucosidase produced by *A. fumigatus* A27 was highest. With increasing or decreasing material to water ratio, the enzyme activity gradually decreased. Thus, the optimum material to water ratio in the medium used for *A. fumigatus* A27-produced β-glucosidase is 1:2.

Effect of culture temperature: In this experiment, the production of enzyme using solid-state fermentation was conducted at various temperatures, including 25, 30, 33, 35, 37, 39, 40 and 45°C. The enzyme-producing medium was used, and the inoculation, culture and measurement of enzyme activity were carried out based on section "Effect of rice straw to bran ratio". The results shown in Fig. 5 indicate that the activity of β-glucosidase was highest when the culture temperature was 37°C; on the other hand, the enzyme activity decreased when the culture temperature were lower or higher. When the culture temperature was too low (*e.g*., at 25°C) or too high (*e.g*., at 45°C), the enzyme activity significantly decreased. This finding indicates that the optimum culture temperature for the production of β-glucosidase by *A. fumigatus* A27 is 37°C.

Effect of culture time: The solid enzyme-producing medium was used and the inoculation was carried out according to section "Effect of rice straw to bran ratio". The

culture time was varied from 1 to 10 d. The culture medium was sampled every 24 h, from which the crude enzyme was prepared and β-glucosidase activity was measured. As shown in Fig. 6, the activity of β-glucosidase produced by *A. fumigatus* A27 was highest when the culture time was 6 d, at which it remained stable until the end of the fermentation. Therefore, the optimal culture time for the production of β-glucosidase by *A. fumigatus* A27 is 6 d.

Effect of initial medium pH: In this experiment, inorganic salt culture solutions with different initial pH (2.0, 3.0, 4.0, 5.0, 6.0 and 7.0) were employed to prepare the solid enzyme-producing medium used in solid-state fermentation. The inoculation, cultivation and enzyme activity measurement were performed according to section "Effect of rice straw to bran ratio". The results illustrated in Fig. 7 show that when the initial medium pH was 3.0, the activity of β-glucosidase was highest, and when such pH was 2.0 or higher than 3.0, the enzyme activity was significantly decreased. According to the results, we may conclude that the optimum initial medium pH for the production of β-glucosidase by *A. fumigatus* A27 using solid-state fermentation is 3.0.

Effect of inoculum size: The enzyme-producing medium was used, and the inoculation, culture, and enzyme activity measurement were conducted according to section "Effect of rice straw to bran ratio". The inoculum size was varied as follows: 1, 5, 10, 15, 20 and 25%. As shown in Fig. 8, the inoculum size had a significant effect on the production of β-glucosidase, as indicated by its activity: too large and too small inoculum sizes were unfavorable for enzyme production. Based in the results, the optimum inoculum size is 10%.

Optimization of conditions for the production of β-glucosidase by *A. fumigatus* A27 using response surface

Box-behnken design and analysis of results: In this optimization, the three factors from the single factor experiment including culture temperature, initial medium pH and nitrogen content were optimized using the response surface, and the β-glucosidase activity was the response value. Other conditions were the experimental optimal values derived from the single factor optimization. The test factors and values are shown in Table 1. Seventeen experimental groups were designed based on the Box-Behnken central combination experiment principle using Design-Expert.8.0.5b software, and the results are shown in Table 2.

Multivariate quadratic regression fitting analysis of the test data was performed on Design-Expert.8.0.5b software, and the quadratic polynomial equation was obtained as follows:

$$\beta\text{-glucosidase activity (IU/g)} = 64.25 - 2.13A + 0.92B - 1.65C + 0.26AB - 0.75AC + 0.70BC - 3.18A^2 - 2.04B^2 - 2.59C^2$$

According to the analysis of variance of the regression model (Table 3), the linear variables A, B and C and the quadratic variables A^2, B^2 and C^2 of the regression equation have significant effects on β-glucosidase activity (P<0.0001). In addition, the effect of the interacting

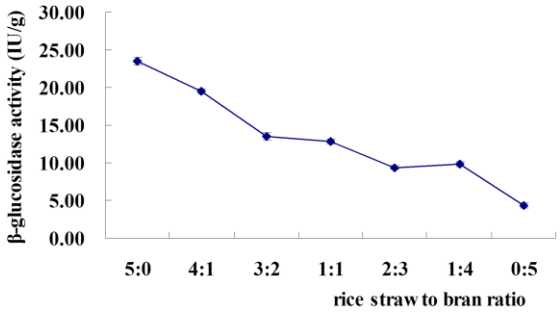

Fig. 1: Effect of straw powder and bran ratio on β-glucosidase activity

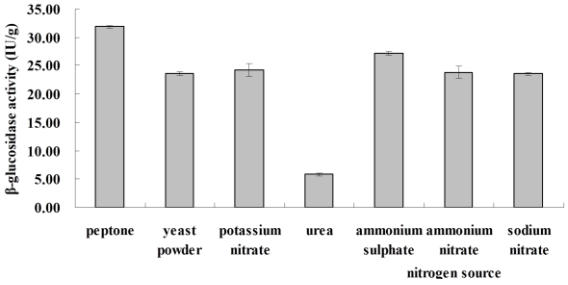

Fig. 2: Effect of different nitrogen sources on β-glucosidase activity

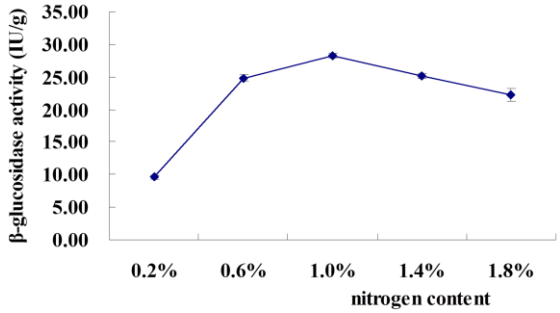

Fig. 3: Effect of nitrogen content in culture medium on β-glucosidase activity

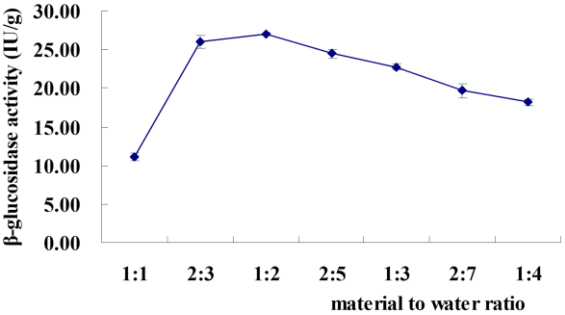

Fig. 4: Effect of material to water ratio on β-glucosidase activity

Table 1: Factors and values used in the Box-Behnken design

Number	Factors	Levels		
		-1	0	1
A	culture temperature / ℃	35	37	39
B	initial medium pH	2	3	4
C	nitrogen content in medium / %	0.6	1.0	1.4

Table 2: Arrangement and results of the Box-Behnken design

Group	Factors			β-glucosidase activity (IU/g)
	A	B	C	
1	1	0	1	53.656
2	0	1	-1	61.398
3	0	0	0	64.598
4	-1	0	-1	61.811
5	1	0	-1	58.714
6	0	0	0	63.875
7	1	1	0	58.198
8	0	1	1	59.746
9	0	-1	-1	60.882
10	0	0	0	64.392
11	-1	1	0	61.605
12	1	-1	0	55.927
13	-1	0	1	59.746
14	-1	-1	0	60.366
15	0	0	0	63.979
16	0	-1	1	56.443
17	0	0	0	64.392

variable AB on β-glucosidase activity is not significant, while that of the interacting variable AC or BC is significant. The response surface and contour maps are shown in Figs. 9 to 11. The quadratic regression equation model is significant ($P < 0.0001$), and the number of lack of fit item is not significant (P=0.3856). This indicates that the model is suitable. The determination coefficient R^2 of 0.9956 indicates that the regression equation is well-fitted. The correction decision coefficient R^2_{Adj} of 0.9899 indicates that the model can explain variables with the 98.99% response values. Based on the above analysis, the model well-fits with the experimental data and can be used to analyze and predict the activity of β-glucosidase produced by *A. fumigatus* A27 in solid-state fermentation.

As illustrated in Fig. 9, the contour line has circular shape, indicating that the effect of the interaction between the culture temperature and the initial medium pH on the β-glucosidase activity is not significant. As can be seen in Fig. 10 and 11, the contour lines have an elliptical shape, indicating that the effect of the interaction between the culture temperature and the nitrogen content, and that between the initial medium pH and the nitrogen content on the activity of β- glucosidase are significant.

Validation of the quadratic regression equation model: The optimal conditions for the enzyme-producing fermentation of *A. fumigatus* A27 derived from the quadratic regression equation were as follows: the culture temperature, 36.4°C; the initial medium pH, 3.16; the nitrogen content, 0.9%; and the maximum β-glucosidase activity, 64.849 IU/g. The validation experiment was carried out under the obtained conditions in three replicates. The experimental β-glucosidase activity was 63.950 ± 0.057 IU/g, which highly resembles the predicted value (64.898 IU/g). This indicates that the process parameters

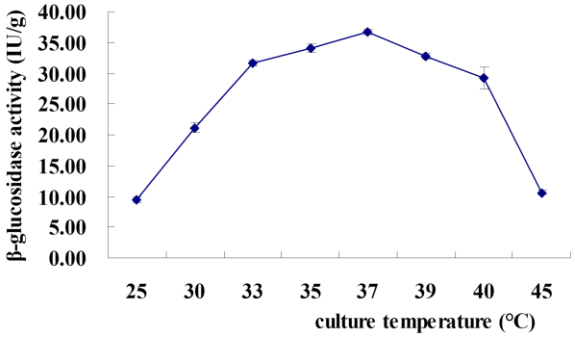

Fig. 5: Effect of culture temperature on β-glucosidase activity

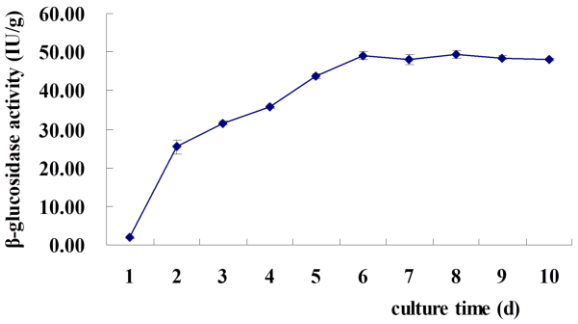

Fig. 6: Effect of culture time on β-glucosidase activity

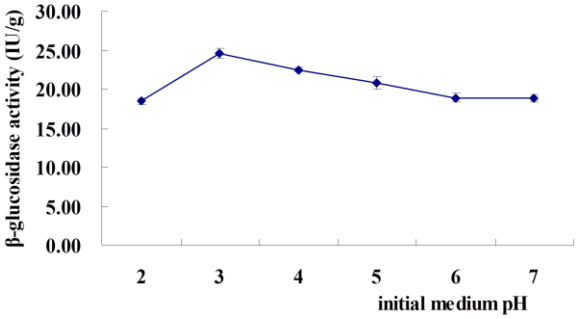

Fig. 7: Effect of initial medium pH on β-glucosidase activity

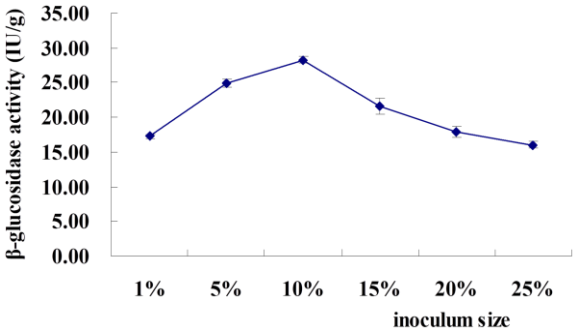

Fig. 8: Effect of inoculum size on β-glucosidase activity

Table 3: Analysis of variance for the quadratic regression model

Origin	Square sum	Degree of Freedom	Mean square	F	*P*	
model	167.48	9	18.61	174.77	< 0.0001	significant
A	36.26	1	36.26	340.56	< 0.0001	
B	6.71	1	6.71	63.06	< 0.0001	
C	21.82	1	21.82	204.95	< 0.0001	
AB	0.27	1	0.27	2.50	0.1577	
AC	2.24	1	2.24	21.04	0.0025	
BC	1.94	1	1.94	18.24	0.0037	
A^2	42.56	1	42.56	399.71	< 0.0001	
B^2	17.59	1	17.59	165.19	< 0.0001	
C^2	28.15	1	28.15	264.40	< 0.0001	
residual quantity	0.75	7	0.11			
misstated item	0.37	3	0.12	1.32	0.3856	not significant
net error	0.38	4	0.094			
total	168.23	16				

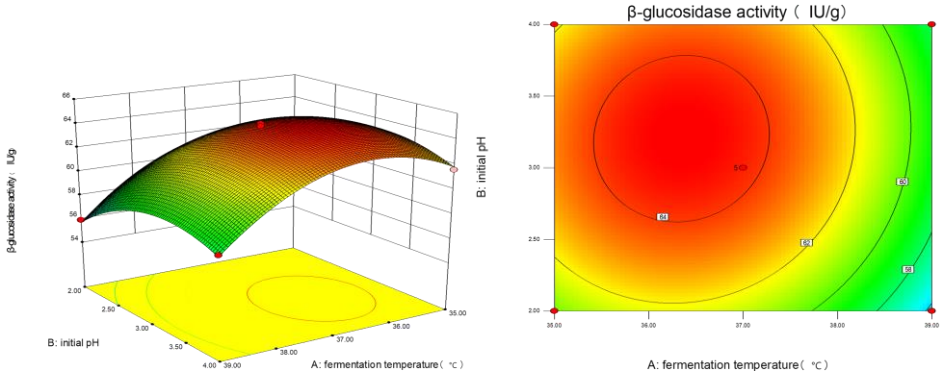

Fig. 9: Response surface and contour map showing the effect of f (A, B) on β-glucosidase activity

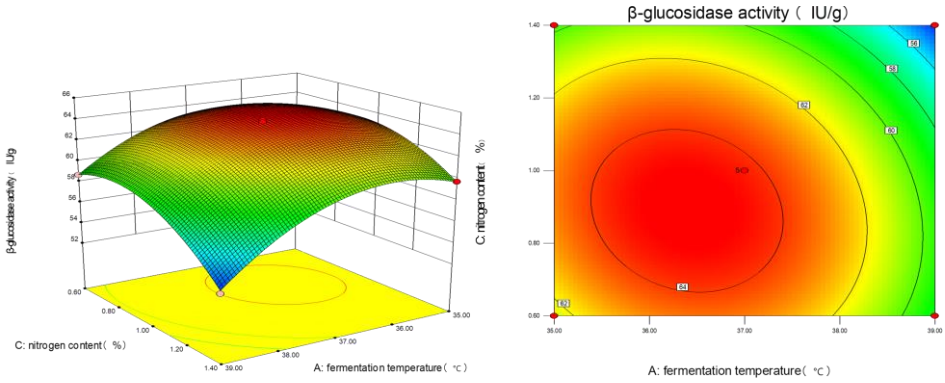

Fig. 10: Response surface and contour map showing the effect of f (A, C) on β-glucosidase activity

obtained by the response surface optimization for the production of β-glucosidase by *A. fumigatus* A27 fermentation are accurate and reliable.

Discussion

In microbial fermentation to produce cellulase, raw materials containing cellulose, which has high cost, are mainly used as the carbon source. This study utilized straw powder and bran, which has low cost, as the carbon source to produce a class of cellulase enzyme, β-glucosidase, by *A. fumigatus* A27. Previous study has demonstrated that cellulose in straw powder can induce cellulase production by certain fungal strains, while bran provides additional nutrient; however, too high amount of bran can affect the permeability of the culture medium, thereby can affect the enzyme production by the fungi (Liu *et al*., 2017). As shown in Fig. 11, when the ratio of straw powder to bran was 5:0, the activity of β-glucosidase produced by *A. fumigatus* A27 was highest, which is different from the results obtained by Das *et al*. (2013). This is likely due to that the carbon

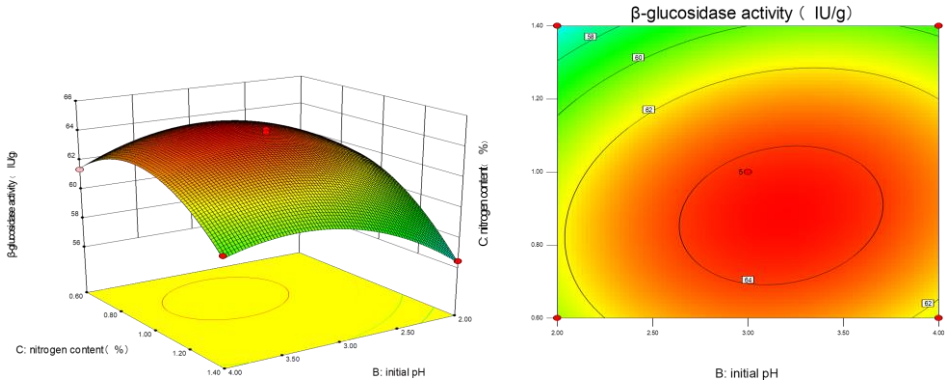

Fig. 11: Response surface and contour map showing the effect of f (B, C) on β-glucosidase activity

source and strains used in both studies are different. The results further showed that the production of enzyme by *A. fumigatus* A27 using an inexpensive raw material straw powder as the carbon source is possible and can replace other high-cost carbon sources commonly used in the industrial enzyme production (Han *et al*., 2017). In way can significantly reduce the cost of enzyme production.

The synthesis of β-glucosidase is not only related to carbon source (Liu *et al*., 2013), but also other factors, such as culture time, nitrogen source and medium pH, each of which has varying degrees of effects on enzyme synthesis (Das *et al*., 2013; Mehboob *et al*., 2014). In the optimization of culture time, the enzyme activity was first increased reaching a certain value when the culture time was prolonged. The difference between the highest and the lowest enzyme activities was 47.254 IU/g, which is higher than the highest enzyme activity obtained in the optimization of other factors. Therefore, it appears that the culture time is the most significant factor affecting the enzyme production, which is consistent with the observation by Das *et al*. (2013). The results also showed that the production of β-glucosidase using peptone as the nitrogen source was superior to that using ammonium sulfate and urea; which is consistent with the results described in Mehboob *et al*. (2014). Mold generally grows well under acidic conditions (Liu *et al*., 2017). This may explain the results obtained in this study, in which the activity of β-glucosidase was highest when the initial medium pH was 3.0.

A cellulase enzyme complex containing low amount of β-glucosidase can lead to the accumulation of cellobiose, thus causing lower efficiency of enzymatic hydrolysis. Therefore, to improve the efficiency of the enzymatic hydrolysis, it is necessary to select the target fungal strains that can produce high amount of β-glucosidase, optimize fermentation conditions, and adjust the composition of the components in the cellulose enzyme complex (Ye *et al*., 2017). In this way, a low-cost and high-performance cellulase can be developed to effectively reduce the amount and the production cost of cellulase.

Conclusions

In this study, the single factor optimization strategy and the response surface analysis were combined to determine the optimal conditions for solid-state fermentation in the production of β-glucosidase by *A. fumigatus* A27. The obtained optimal process conditions were as follows: ratio of straw powder to bran, 5:0; nitrogen source, peptone; nitrogen content, 0.9%; ratio of material to water, 1:2; culture temperature, 36.4°C; culture time, 6 d, initial medium pH, 3.16; and inoculum size, 10%. The activity of β-glucosidase obtained under the optimal conditions was 63.950 IU/g, which is 2.3 times higher than that obtained under non-optimal conditions.

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