Cellulase Production by *Penicillium Oxalicum* Ti-11 with Traditional Chinese Medicine Residue as Substrate

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Abstract

Purpose

The study aims to search for efficient cellulase producer and explore the possibility of traditional Chinese medicine residue as a substrate for cellulase production, so as to realize the waste utilization of traditional Chinese medicine residue.

Methods

The cellulase-producing strain was identified through morphological and molecular biological methods. Scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR) were used to characterize the structure of traditional Chinese medicine residues before and after fermentation. The enzyme activity was determined by DNS method, and the enzyme production conditions were optimized by single factor and response surface methodology.

Result

The strain grew well in forsythia leaf residue, and the highest FPA could reach 2.06 IU/mL. In addition, the structural characteristics of traditional Chinese medicine residue that before and after enzymatic hydrolysis were characterized by SEM and FTIR. The results showed that the structure of the residue was destroyed after enzymatic hydrolysis, the damage of forsythia leaf residue was the most serious, and enzymatic hydrolysis promoted the dissolution of cellulose, lignin and hemicellulose. The enzyme production conditions of the strain were optimized by Plackett-Burman design and response surface analysis. The FPA could reach 2.79 IU/mL under the optimal conditions of FLR concentration 24.84 g/L, (NH₄)₂SO₄ concentration 2 g/L, temperature 34.44°C, pH 6.20, rotational speed 200rpm, inoculum 6%, which was 35.44% higher than that before optimization.

Conclusions

The results showed that traditional Chinese medicine residue could be used as the induced substrate for fungal cellulase production. This study provides an idea for the low-cost production of fungal cellulase and the waste utilization of traditional Chinese medicine residue.

1. Introduction

The huge consumption of fossil fuels and other non-renewable resources causes the energy crisis become the most serious issue facing mankind in the future (Pietrosemoli and Rodríguez-Monroy 2019; Höök and Tang 2013). Exploring green, cheap and easily available renewable resources with considerable output may be the only solution (Elavarasan 2019; Bahrampour et al. 2020).

Lignocellulosic feedstocks were saccharified to produce ethanol, butanol and hydrogen is considered the best choice as a future Fossil fuel substitutes due to their economic, environmental friendly and

sustainable characteristics (Kumar et al. 2020). The key step to produce bioethanol in industry is the conversion of cellulose to glucose, however the high production costs and low yields of cellulase become a significant limiting factor for its development (Srivastava et al. 2018). As an inducible enzyme, cellulase often requires inducers to produce the enzyme (Suto and Tomita 2001). Studies have shown that cellulose can be used as inducers for the production of fungal cellulase (Luo et al. 2020), and sophorose is the most efficient inducer as is known currently (Xia et al. 2018; Mandels and Reese 1960), but the high cost of production contributes to it cannot be widely used in practice production. Cow dung (Yan et al. 2018), waste paper (Dong et al. 2021) and domestic sewage (Libardi et al. 2017) have been reported to be used as substrates for microorganisms to produce cellulase, they have the common characteristics that rich in cellulose. Seeking for more low-cost but efficient substrate is the future development direction.

Traditional Chinese medicine residue (TCMR) is the residue left after the boiling process of traditional Chinese medicine, however not been fully utilized to date (Chassagne et al. 2019). Traditional Chinese medicine has been proved to have a positive therapeutic effect on COVID-19 (Zhao et al. 2021; Luo et al. 2020), hence the development and promotion of traditional Chinese medicine are widely valued, resulting in the accumulation of a large amount of TCMR. According to statistics, the annual discharge of TCMR in China reaches 35 million tons (Tao et al. 2021). TCMR is rich in lignin, cellulose, hemicellulose and abundant nutrients such as polysaccharides, crude protein, crude fat and amino acids (Lu and Li 2021). It is likely to be used as a low-cost substrate for the production of microbial cellulase. After the first extraction of traditional Chinese medicine, about 30% of its active components remain in the residue due to the low utilization efficiency of traditional Chinese medicine, including bioactive substances such as alkaloids, terpenoids, flavonoids and quinones remain (Huang et al. 2021). Stacking, incineration and landfill are the main treatment methods of TCMR in traditional Chinese medicine production enterprises in China (Wang et al. 2021; Zhan et al. 2017), not only causing serious harm to the ecological environment, but also leading to a great waste of resources. Therefore, it is of significant to develop an environmentally friendly treatment method for TCMR, consequently solving the problem of environmental pollution and realizing the efficient utilization of resources.

Therefore, in this study, *Semen ziziphi spinosae* residue (SZSR), *Astragalus* residue (AR) and *Forsythia* leaf residue (FLR) were used to the production of fungal cellulase. The ability of TCMR as an inducer of cellulase production by *Penicillium oxalate* was studied, and the culture conditions of cellulase production were optimized by Plackett-Burman design (PBD) and response surface methodology (RSM). The possibility of using TCMR as fungal cellulase inducer was explored to realize the comprehensive utilization of TCMR.

2. Materials And Methods

2.1 Isolation and identification of cellulase producing fungi

The strain was obtained from rotten straw collected from suburban farmland in Zhuzhou using dilution coating method and plate streaking method. The isolation medium contained 10 g/L CMC-Na, 5 g/L

peptone, 1 g/L Congo red, 1 g/L NaCl, 1 g/L MgSO₄, 1 g/L K₂HPO₄, 1 g/L KH₂PO₄, 20 g/L agar, pH 7. The plates were incubated at 37°C for 2-3 d, and then decolorization with 1 M NaCl solution. The ratio of the clear zone diameter to colony diameter indicating the hydrolysis capacity (Rawway et al. 2018). The strain with high ratio were selected and preserved at -80°C for subsequent experiments. The potential cellulase-producing fungi was identified based on morphological observation. Further confirmation was done by 18S rDNA sequencing using universal primer ITS1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS4(5'-TCCTCCGCTTATTGATATGC-3'). After sequencing the PCR products, the obtained DNA sequences were compared with the NCBI GenBank database BLAST sequence homology analysis was performed to determine the taxonomic status of the strain.

2.2 Acquisition and preparation of TCMR

TCMR were obtained from People's pharmacy in Tianyuan District Zhuzhou city. The obtained TCMR were oven dried to constant weight for removing moisture, afterward the samples were powdered using a mill mixer, the powder was passed through a 80 mesh sieve and set aside.

2.3 Determination of cellulase activity

Filter paper activity (FPA) was measured according to the standard method described by Ghose (Ghose. 1987). Incubating 0.5 mL of the supernatant with 0.05 M(pH 4.8) citrate buffer (1.5 mL) and 50 mg Whatman No.1 filter paper. for 60 min at 50°C. The reducing sugar released was estimated using DNS method, one unit of enzyme activity was defined as the amount of enzyme required that released 1 µmol of reducing sugar in 1 min under assay conditions.

PDA medium: potato 200 g, glucose 20 g, H₂O 1000 mL, pH natural

Fermentation medium: TCMR 20 g, NaCl 5 g, (NH₄)₂SO₄ 3 g, MgSO₄ 7H₂O 3 g H₂O 1000 mL, pH natural

2.4 Statistical optimization of cellulase production

2.4.1 Screening of significant variables by Plackett-Burman

The Plackett-Burman design is a reliable technique used to select factors that significantly influenced the cellulase production with minimal trials (Vanaja and Shobha Rani 2007). The variables chosen for the present study were of TCMR concentration(X_1), (NH₄)₂SO₄ concentration(X_2), temperature(X_3), Inoculum(X_4), pH(X_5) and rotation speed(X_6). All the runs were carried out in triplicate, and the average was taken as a response.

2.4.2 Process optimization by Box–Behnken

After the selection of significant factors using PBD, Box–Behnken experimental design (BBD) provided FLR concentration(A), temperature (B) and pH(C), three factors as independent variables for level three factors and response surface analysis experiments in BBD. Analysis of variance (ANOVA) was carried out, data processing was done with statistical software Design-Expert 10.0.7.

2.5 Structural characterization of TCMR

The Microstructure morphology of TCMR that before and after enzymatic hydrolysis was analyzed by scanning electron microscopy (SEM). Fourier transform infrared spectroscopy (FTIR) was used to study the knot of TCMR before and after enzymatic hydrolysis. The structural groups were measured in the range of 400-4000 wave numbers in the infrared spectrum.

2.6 Statistical Analysis

The statistical analysis were performed using the Origin 9.0 and IBM SPSS Statistics 25. All experiments were carried out three replicates and the data were presented as means $\pm$ standard deviations (SD). The average value and standard deviation are calculated based on the data obtained from three independent experiments. Statistical differences at $p < 0.05$ were considered as significant.

3. Results And Discussion

3.1. Isolation and identification of cellulase-producing fungi

Penicillium oxalate strain was inoculated on PDA medium at 37 °C for 3 days. The morphological characteristics of Penicillium oxalate were as shown in Figure 1-A. the colony was flat, the texture was velvety, the surrounding hyphae were white, the central hyphae were dark green, and the conidia were numerous and easy to fall off. The conidium structure under the microscope is shown in Figure 1-B. the conidiophores occur in the matrix, the walls are smooth, the broom branches are usually biwhorled, occasionally tricycle, and the conidia are oval.

Rotting straw samples from farmland soil were suspended in sterile water, after gradient dilution, they were coated on Congo red medium for the isolation of cellulase-producing fungi. Cellulose can bind to congo red firmly; however, when the cellulase produced by fungi degrades cellulose to polysaccharides, the polysaccharides cannot bind to congo red, after eluted by NaCl solution, the unbound congo red is eluted, resulting in a transparent circle around the colony (Figure 1-C). Generally speaking, the greater the ratio of the diameter of the transparent circle to the diameter of the colony, the higher the cellulase activity (Sui et al. 2021). Among the 28 strains screened, Ti-11 with the highest ratio was selected as a candidate for further identification. Clustering analysis of 18S rDNA sequences of the target bacteria and model strains was carried out with MEGA 7.0 software, and the strain was classified as Penicillium oxalicum (GenBank accession number: SUB10745084 Ti-11 OL687558) as shown in Figure 2.

3.2. Fungi growth and enzyme production under three kinds of residues

Normal growth of strain is the premise of producing enzyme. The residue contains plentiful nutrients which can be used as the natural culture medium for fungi growth, some antibacterial ingredients are also existing, including alkaloids, quinones, organic acids and other compounds (Huang et al. 2021). The strain must have certain drug resistance if residues are used as enzyme production medium.

As shown in figure 3, the strain reached the logarithmic growth phase 1-6 days after inoculation, then reached the growth plateau and lasted for about 4 days. In the culture medium of three different types of drug residue, the growth cycle of the strain is almost the same, indicating that the strain has a certain resistance to drugs. The strain grew well in the drug residue medium, and there was basically no lag in the early stage, indicating that the drug residue was rich in nutrients and can be used for the growth of the strain effectively. As shown in figure 4, the enzyme production of the strain reached its peak at 6-7 days, so fermentation culture for 6 days was selected in the following experiment to study the optimization of enzyme production conditions. Under the fermentation conditions of adding residue and without residue, the enzyme activity of the strain was quite different, which verified that cellulase was a kind of induced enzyme (Havukainen et al. 2020). The medium for the production of cellulase usually contains cellulose-rich substrates as carbon sources. when using FLR as substrate, the activity of cellulase is the highest, and the other two residues also showed strong induction. FLR was selected as the induction substrate for cellulase production in the later optimization experiment.

3.3 Structural characterization of three kinds of residues

The surface morphology of the residue before and after enzymatic hydrolysis were characterized by scanning electron microscope (SEM). Fig. 5-a, 5-c and 5-e represent the SEM of SZSR, AR and FLR respectively before enzymatic hydrolysis, and fig. 5-b, 5-d and 5-f represent the SEM of SZSR, AR and FLR respectively after enzymatic hydrolysis. Results showed that the surface of the residue without enzymatic hydrolysis was dense and smooth, with slight erosion marks, which may be caused by mechanical damage in the grinding process. However, the surface of the residue after enzymatic hydrolysis showed jagged erosion marks, and the exfoliation degree was different. In addition, the erosion degree of FLR after enzymatic hydrolysis was the most serious. After the degradation of the strain, the surface of cellulose became rough from a relatively complete, and a large number of fracture traces appeared on the surface of the large fiber. Gully-like traces were observed on the messy surface in some areas, it's may be the traces left by the attachment of fungi, which is more conducive to the further enzymatic hydrolysis process of the strain (Yang et al. 2021).

Cellulose, hemicellulose and lignin in TCMR were characterized by Fourier transform infrared spectroscopy. Fig. 6, 7 and 8 represent the FTIR of SZSR, AR and FLR respectively before and after enzymatic hydrolysis. It had the characteristic peak of cellulose, the broad and strong absorption peak in 3400cm^{-1} was O-H stretching vibration absorption peak, the weaker absorption peak in 2900cm^{-1} was the antisymmetric stretching vibration peak of $\text{-CH}_2\text{-}$, and the absorption peaks in 1430cm^{-1} and 1057cm^{-1} belong to the bending vibration of C-H and the telescopic skeleton vibration absorption peak of cellulose glycoside bond C-O-C glucopyranose ring, respectively. The peak between 760 and 868cm^{-1} corresponds to the stretching vibration of aromatic C-H, indicating the existence of adjacent aromatic hydrogen in TCMR. $1900\text{-}1465\text{cm}^{-1}$ was the characteristic absorption peak of aromatic ring, carboxyl and carbonyl of lignin and hemicellulose (Le et al. 2017; Fan et al. 2012). The FTIR spectra were basically the same before and after enzymatic hydrolysis. However, after enzymatic hydrolysis, the absorption intensity of

some characteristic peaks changed, and some of the characteristic peaks even disappeared completely. The band intensity of the residue decreased after enzymatic hydrolysis, indicating that enzymatic hydrolysis promoted the dissolution of cellulose, lignin and hemicellulose.

3.4 Optimization of cellulase production conditions

3.4.1 Plackett-Burman

The purpose of Plackett-Burman design is to screen out the most important factors affecting cellulase production by *Penicillium oxalate* Ti-11 quickly and effectively. Six factors including FLR concentration(X_1), $(NH_4)_2SO_4$ concentration(X_2), temperature(X_3), Inoculum(X_4), pH(X_5) and rotation speed(X_6) were investigated. Each factor took 2 levels of high (+) and low (-), with cellulase activity (Y) as the response value. The experimental design and results are shown in Table 1. The first-order linear model was obtained by Minitab18 software, as follows:

$$Y=3.519+0.02237X_1+0.0107X_2-0.02549X_3-0.0308X_4-0.2201X_5+0.00105X_6$$

To test the significance of the development model, ANOVA was performed, and the results are shown in Table 2. The P value of the model was 0.004, and the decision coefficient R^2 was 94.82%, indicating that the linear model fitted with the test data in a high degree, and the error was small after it was applied to the actual prediction. The statistical analysis of Plackett-Burman test showed that X_3 , X_4 and X_5 had a negative effect, whereas X_1 , X_2 and X_6 showed a positive effect on cellulase production. X_5 (pH), X_3 (temperature) and X_1 (FLR concentration) were significant factors affecting cellulase production by *Penicillium oxalicum* Ti-11. The variables of X_2 , X_4 and X_6 were eliminated as they were insignificant towards cellulase production. It has been reported that pH and temperature had a negative effect on cellulase (Ahmed and Bibi 2018), similar statistical result was observed in our study. Substrate concentration was influential on cellulase production, it is wise to further optimize FLR concentration (Irfan et al 2017). Therefore, FLR concentration, pH and temperature were selected as the research objects, and the response surface was used to explore the best conditions for enzyme production.

Table 1. Plackett–Burman experimental design and results.

Run	Variables						FPA (IU/mL)
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	
1	130	12	145	-15	19	1200	1.05
2	-120	-11	-130	17	1	1	1.03
3	1	1	-1	1	-16	-1160	2.09
4	-1	1	-1	-1	-1	1	2.01
5	1	-1	1	-1	-1	-1	1.57
6	-1	1	1	1	-1	1	1.42
7	1	1	-1	1	1	-1	1.34
8	-1	-1	1	1	1	-1	0.90
9	-1	1	1	-1	1	-1	0.88
10	1	-1	-1	-1	1	1	1.58
11	1	-1	1	1	-1	1	1.79
12	-1	-1	-1	-1	-1	-1	1.85

Table 2. ANOVA for Plackett-Burman model.

Source	Effect	SS	MS	F value	P value
Model		1.91378	0.31896	15.24	0.004
X ₁	0.2237	0.15008	0.15008	7.17	0.044
X ₂	0.0107	0.00034	0.00034	0.02	0.903
X ₃	-0.3823	0.43854	0.43854	20.95	0.006
X ₄	-0.0617	0.01141	0.01141	0.55	0.494
X ₅	-0.6603	1.30812	1.30812	62.50	0.001
X ₆	0.0420	0.00529	0.00529	0.25	0.636

3.4.2 Response surface analysis

BBD is one of the Response Surface Methodology (RSM) designs, and supposed to more influential and easier to adjust and interpret the experiments (Selvam et al. 2014). In the study, FLR concentration, temperature and pH were selected as three influencing factors, which are regarded as independent variables A, B, C, and -1, 0, 1 represent the level of each variable, and the independent variables were

encoded, as shown in Table 3. The FPA (Y) was used as the response value to design the BBD, and results are shown in Table 4.

Table 3. Box-Benhnken experimental design.

Variables	Levels		
	-1	0	1
A	20	25	30
B	30	35	40
C	5	6	7

Table 4. Box-Benhnken experimental results.

Run	A	B	C	FPA
1	30	5	25	1.84
2	35	6	25	2.71
3	30	6	20	2.12
4	35	5	30	1.99
5	35	5	20	1.73
6	40	7	25	2.05
7	40	5	25	1.69
8	35	6	25	2.62
9	35	6	25	2.80
10	40	6	20	2.06
11	30	7	25	2.24
12	35	6	25	2.69
13	30	6	30	2.15
14	35	7	30	2.02
15	35	7	20	2.21
16	40	6	30	1.95
17	35	6	25	2.69

The quadratic polynomial regression model of Y (FPA) to A (FLR concentration), B (temperature) and C (pH) was obtained by quadratic multiple regression fitting of Table 4 data using the software Design

Expert 10.0.7:

$$Y=2.70-0.073A+0.16B-8.750 \times 10^{-4}C-0.011AB-0.038AC-0.12BC-0.33A^2-0.41B^2-0.30C^2$$

In order to verify the feasibility of the model, significance test and variance analysis were carried out as shown in Table 5. According to the ANOVA results, F-value of the model was 54.06, which implied the model was significant. Besides, the “Lack of Fit F-value” of 0.80 indicate that the lack of fit was not significant relative to the pure error. The predicated R^2 (0.9009) and adjusted R^2 (0.9676) values for the cellulase production were in reasonable agreement with the value of R^2 (0.9858), which is closer to 1.0. It shows that the better fitness of the model in the experimental data and the experiments are very reliable. Values of prob > F less than 0.05 indicate model terms are significant (Al Azkawi et al. 2018). The statistical analysis indicates that A, B, BC, A^2 , B^2 , C^2 are significant model terms for cellulase production.

Table 5. Analysis of variance of response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	Prob>F
Model	2.04	9	0.23	54.06	<0.0001
A	0.043	1	0.043	10.29	0.0149
B	0.20	1	0.20	47.58	0.0002
C	6.125×10^{-6}	1	6.125×10^{-6}	1.464×10^{-3}	0.9705
AB	5.062×10^{-4}	1	5.062×10^{-4}	0.12	0.7382
AC	5.776×10^{-3}	1	5.776×10^{-3}	1.38	0.2784
BC	0.053	1	0.053	12.70	0.0092
A^2	0.46	1	0.46	110.72	<0.0001
B^2	0.72	1	0.72	172.02	<0.0001
C^2	0.37	1	0.37	89.19	<0.0001
Residual	0.029	7	4.184×10^{-3}		
Lack of Fit	0.011	3	3.667×10^{-3}	0.80	0.5542
Pure Error	0.018	4	4.572×10^{-3}		
Cor Total	2.07	16			

The two-dimensional contour plots and three-dimensional response surface graphs (Figure 9) were plotted to illustrate the interaction of two variables and the optimum level of each variable for maximum cellulase activities. The contours can reflect the interaction among the factors, and the oval shows that

the interaction between the two factors was significant, while the circle was on the contrary (Han et al. 2017). Figure 9a and Figure 9c displayed circularity, indicating that the interaction AB and AC were insignificant, while Figure 9e tended to be elliptical, indicating that the interaction between B and C was significant. Figure 9b showed the response surface was convex indicate that the FPA increased with A and B until a certain point and then decreased. The same trend was observed in Figure 9d and Figure 9f. The results showed that A, B and C had maximum improving effects on cellulase production at optimal concentrations. Temperature that are either too high or too low could inhibit the growth of the strain and product formation (Blomqvist et al. 2010). In particular, higher temperature eventually denatures cellulase, resulting in reduced enzyme activity (Saini et al. 2017). Fungi grow over a relatively wide pH range but are sensitive to the ambient pH of cellulase processes (He et al. 2014). Substrate concentration had significant effect on cellulase production by fungi (Irfan et al. 2017; Varotkar et al. 2016), adding excessive FLR would have compressed the spatial environment of fungi growth and accumulated fungistatic substances like phenolic compounds and flavonoids, resulting in a negative effect on the growth of microorganisms.

According to the standard analysis, the predicted results of the model show that the maximum cellulase production can be achieved when pH, temperature and FLR concentration are 6.20, 34.44 °C and 24.84g/L, respectively. The maximum predicted value of cellulase yield was 2.72 IU/mL. In order to confirm the optimization results, the proposed medium components and conditions were carried out in triplicate. Under these suggested conditions, the average yield of cellulase was 2.79 IU/mL, which was consistent with the predicted value. This optimization strategy led to the enhancement of cellulase from 2.06 IU/mL to 2.79 IU/mL. Obviously, the developed model is considered to be accurate and reliable for predicting cellulase production of *Penicillium oxalicum* Ti-11.

4. Conclusion

In this study, the cellulase activity was further enhanced the application of BBD statistical optimization method. The most suitable process to produce cellulase was FLR concentration 24.84 g/L, $(\text{NH}_4)_2\text{SO}_4$ concentration 2 g/L, temperature 34.44°C, pH 6.20, rotational speed 200 rpm, inoculum 6%, the new strategy significantly increased the FPA from 2.06 IU/mL to 2.79 IU/mL. These results has revealed the ability of *penicillium oxalicum* Ti-11 to produce cellulase utilizing TCMR as carbon source.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All of the authors consent to the publication of this manuscript in *Annals of Microbiology*.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare no competing interests in publishing this manuscript.

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Authors' contributions

YKZ performed all the experimental research work included in this paper and primarily drafted the manuscript. XXZ designed and supervised this work and also perfected the manuscript. LLC, SR and CZ did enzyme activity determination experiment and data analysis. LM helped to finalize the manuscript. The authors read and approved the final manuscript.

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Figures

Figure 1

The morphological, conidium structure and congo red staining observation of strain.

Figure 2

Phylogenetic tree of *Penicillium oxalicum* Ti-11.

Figure 3

Growth curves of strain.

Figure 4

Enzyme production curve of strain.

(a)

(b)

(c)

(d)

(e)

(f)

Figure 5

SEM analysis of TCMR. (a, b) before and after enzymolysis Semen ziziphi spinosae residue; (c, d) before and after enzymolysis Astragalus residue; (e, f) before and after enzymolysis Forsythia leaf residue.

Figure 6

FTIR spectra of SZSR.

Figure 7

FTIR spectra of AR.

Figure 8

FTIR spectra of FLR.

(a)

(b)

(c)

(d)

(e)

(f)

Figure 9

Two-dimensional contour plots and three-dimensional response surface graphs showing the interaction effects of two variables on cellulase production.