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Optimization of Cellulase Production by a Novel Endophytic Fungus *Penicillium oxalicum* R4 Isolated from *Taxus cuspidata*

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Abstract: Endophytic fungi inside a plant can degrade a portion of plant lignin and cellulose. Endophytic Penicillium is one of the industrial microorganisms with the advantage of producing enzymes with a complete enzyme system that can be secreted into the extracellular space. The natural evolution of ancient tree species from special natural geographic environments to screen out cellulase-producing strains with excellent characteristics provides a promising direction for future industrial enzymes. The present study successfully isolated and screened a novel fungal endophyte, *Penicillium oxalicum* R4, with higher cellulase activity from *Taxus cuspidata*. Under the optimized culture conditions obtained by a Box–Behnken design (BBD) and an artificial neural network–genetic algorithm (ANN–GA), yields of Filter Paperase (FPase), Carboxymethyl Cellulase (CMCase) and β-glucosidase (βGLase) produced by *P. oxalicum* R4 were 1.45, 5.27 and 6.35 U/mL, which were approximately 1.60-fold, 1.59-fold and 2.16-fold higher than those of the non-optimized culture, respectively. The discovery of cellulase-producing strains of endophytic fungi located in special natural geographic environments, such as *Taxus cuspidata*, which is known as a living plant fossil, provides new research directions for future industrial enzymes.

Keywords: bioproducts; *Penicillium oxalicum*; biodegradation; cellulases

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1. Introduction

With the rapid economic development and the rapid increase in population, the human demand for energy is also increasing [1,2]. Fossil energy, as one of the main sources of energy consumed by human beings, is irreducible and finite, and the pollutants released during the combustion process can cause serious pollution to the environment [3–5]. For the sake of sustainable economic progress and human survival, people have to gradually focus on the use of renewable energy [6,7]. Biomass, the most abundant and cheapest renewable resource on Earth, is usually disposed of in a simple and crude way, such as burning or burying straw on site, causing serious air pollution and even haze. Cellulose is a promising renewable fuel and chemical raw material. After hydrolysis of cellulose, it has important applications in textile, food, pharmaceutical, biofuel and other fields [8,9].

Cellulase hydrolysis is more acceptable and feasible, and is generally considered more environmentally friendly as well as economically justified [10]. There are three main cellulolytic enzymes namely exo-β-glucanase (Pfase), endo-β-glucanase (CMCase) and β-glucosidase (βGLase), which are capable of efficiently hydrolyzing the glycosidic bonds of cellulose. Thus far, the screening of cellulase-producing strains and the modification of genes has achieved certain results, but they are still far from satisfying the demand. The ability to obtain cellulase strains with high yield and high enzyme activity is still an issue and is a problem that needs to be solved in the future [8,11]. Most of the commercially

available cellulases are from fungi. Compared with bacteria, fungi have cellulase potency and are easy to extract due to their extracellular secretory enzyme characteristics [12,13]. In recent years, more and more scholars have started to study endophytic fungal enzymes as a novel source of cellulase.

Endophytic fungi refers to a type of fungi that widely exists in healthy plant tissues and are an important part of the plant microecosystem [14]. Plants infected by endophytic fungi do not show obvious symptoms [15]. Endophytic fungi inside the plant are able to degrade a portion of plant lignin and cellulose. The discovery that fungal endophytes can produce such cellulases as *Trichoderma*, *Penicillium*, *Aspergillus* and *Chrysosporium* have been reported [16–18]. Since endophytic fungi and plants develop a long-term mutually beneficial symbiotic relationship, endophytic fungi obtain nutrients from plants for growth and reproduction, while the cellulase produced by the endophytic fungi helps host plants to protect themselves against invasive pathogens [19]. The endophytic *Penicillium oxalicum* is one of the microorganisms with the advantage of enzyme production, which has a complete enzyme system that can be secreted into the extracellular space [20]. The natural evolution of cellulase-producing endophytic fungi from ancient tree species, in a special natural geographical environment, provides a new research direction for future industrial enzymes [21]. *Taxus cuspidata* is recognized as a living fossil plant with a history of 2.5 million years on Earth and has abundant endophytic fungi.

In this study, we compared response surface methodology (RSM) with an artificial neural network–genetic algorithm (ANN–GA) to obtain parameters for optimizing the enzyme activity production process based on a single-factor test of the effect of culture conditions on the cellulase production activity of endophytic fungi of *T. cuspidata*. The optimization of the cellulose enzyme activity process parameters is a nonlinear fitting process. Cellulases with high βGLase/Pfase ratio as an index were generated to evaluate the potential of enzyme-producing endophytic fungi for biotechnological applications. It provides economically viable cellulase strains with a high yield and high enzymatic activity suitable for producing industrial enzymes in the future.

2. Materials and Methods

2.1. Chemicals and Microorganisms

All chemicals were purchased from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). The endophyte fungi were isolated from *Taxus cuspidata* and cultivated at 30 °C for 3–5 days and preserved at 4 °C on a potato dextrose agar medium (PDA) for further use. The *Hibiseu manihot* L. waste stems (HWS) were provided by the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University. The dried HWS was passed through 40 mesh sieves. Cellulose, hemicellulose and lignin in the dried HWS was 43.6 ± 0.2%, 27.7 ± 0.5%, and 12.8 ± 0.1%, respectively, and were prepared for subsequent treatment.

2.2. Screening for Cellulase-Producing Fungi

Each fungal isolate (5 mm agar disc) was grown on a cellulose congo red medium for 4 days at 30 ± 2 °C. This was achieved by pouring all Petri dishes with Congo red solution (1% *w*/*v*), placing at room temperature for 15 min, then pouring off the solution before adding in 1 mol/L NaCl for a while [14].

2.3. Identification of the Selected Fungal Strain

The potential cellulase-producing fungi was identified based on its morphological observation and molecular methods. The morphological observation was examined using a Nikon microscope (Eclipse TE2000-U, Tokyo Japan) after 3 days of growth on PDA plates. The strain was grown on potato dextrose agar medium (PDA) at 28 °C for 7 days, and then cultivated in 150 mL conical flask contained 50 mL potato dextrose broth medium (PDB). The fungus was shaken at 160 rpm/min at 30 °C for 4 days, and the total genomic DNA was extracted using CTAB protocol [15]. The ITS region of

endophytic fungi was used by general sequence ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). After amplification, the identification of strain was finished by comparing with the data available in NCBI using BLAST (http://www.ncbi.nlm.nih.Gov/BLAST/accessed accessed on 25 January 2021).

2.4. Culture Conditions for Cellulase Production through Single-Factor Test

The endophyte fungi R4 was cultivated at 30 °C for 7 days on PDA. A diameter of 5 mm good-well endophytic fungus R4 block was cultivated in 100 mL conical flask contained 30 mL of basal salt medium, as well as 2.0 g sterilized HWS powder. The components of the basal salt medium were as follows: 1 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.1 g/L NaCl, 0.2 g/L yeast extract and ammonium sulfate. The initial pH of the mixture was adjusted to 5.0. The flask was cultivated at 30 °C for 4 days at 120 rpm. The samples were centrifuged at 12,000 rpm for 10 min, the supernatant for determination. Experiments were carried out with nitrogen source, pH, incubation temperature and incubation time as the main influencing factors to investigate the effects of nitrogen source (peptone, $(NH_4)_2SO_4$, $NaNO_3$, beef extracts and yeast extracts), initial pH (3.0–8.0), incubation temperature (25–50 °C) and incubation time (1–5 d) on cellulase activity.

2.5. Response Surface Methodology

Based on optimization of the experimental results single factor, Box–Behnken (BBD) experimental design provided fermentation temperature (A), fermentation time (B) and initial fermentation pH(C), three factors as independent variables for level three factors and response surface analysis experiments in BBD, where in response, Fpase activity value is Y_1, Y_2 is CMCase activity, and Y_3 is βGLase activity. Three enzymes dynamic second-order multiple regression equation is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \ (k = 3) \quad (1)$$

where Y is the response variable, x_i, x_j are independent variables, and β_0, β_j, β_{jj}, β_{jj} are constant terms. Factor level experimental design is shown in Table S1, and each experiment was repeated three times. Analysis of variance (ANOVA) was carried out, data processing was done with statistical software Design-Expert 8.0.6 (State Ease, Inc, Minneapolis, MN, USA), and three-dimensional response surface plot using Statistica 7.0 software (State Ease, Inc., Address: 1300 Godward St NE, Suite 6400, Minneapolis, MN, USA).

2.6. Artificial Neural Network and Genetic Algorithm

To compare the fit of neural networks and response surface methods, simulations and predictions were performed based on the BBD experiments described above. The three-layer ANN model (input layer, hidden layer, and output layer) was used to build an optimization model for the test factors. Genetic algorithm (GA) was performed to study the optimal culture conditions based on including training (70%), testing (15%), and validation (15%). Three factors (temperature, time and initial pH) were selected as input layer nodes of the network and normalized to the range [0, 1], and three neurons in the output layer named cellulase activity of Fpase, CMCase and βGLase. The program was written through MATLAB R2016b software (The Mathworks, Inc., Natick, MA, USA) and its accompanying genetic algorithm toolbox [22].

The normalization method was calculated according to the following equation:

$$T_i = T_{imin} + (T_i' - a) \times \frac{T_{imax} - T_{imin}}{b - a} \quad (2)$$

where T_i is the ith input of training sample data; T_i' is the normalized data of T_i, $T_i \in [a, b]$; T_{imax}, T_{imin} are the maximum and minimum values of the ith input of training sample data, respectively.

2.7. Determination of Cellulases Activity

Filter paper activity, according to the method Filter paper activity (Fpase) was described by Hankin [23] and estimated by using Whatman No. 1 filter paper (FP, 50 mg, 1.0 × 6.0 cm) in 100 mM, pH 5.0 of citrate buffer 1.0 mL, containing 0.5 mL of suitably diluted by the crude enzyme solution (DCES) for 60 min at 50 °C, and the released reducing sugar yield was examined by DNS method. The activity of one unit of filter paper (FPU) was defined as the amount of one milliliter of crude enzyme released per minute and one μmol of glucose.

Carboxymethyl cellulase activity was described in Miller [24]. In order to determine the ability of fungal isolates to produce cellulose, each strain was cultivated in 150 mL conical flask contained 50 mL of basal salt medium amended with HWS (10 g/L) as the sole carbon source. In short, the reaction system included 0.2 mL of crude enzyme solution and 1.8 mL of 1% CMC in 100 mM sodium phosphate buffer at pH 5.5, 50 °C in water bath for 25 min. Then, the reaction system was stopped by adding DNS reagent and placing it in a water bath at 100 °C for 15 min. The OD was measured at 520 nm, and the reducing sugar concentration was measured.

B-glucosidase (βGLase) was mentioned by Leite [25], the reaction system was contained 0.5 mL of crude enzyme and 1.0 mL of 0.4% cellobiose dissolved in 0.05 M citrate-phosphate buffer at pH 4.8, 50 °C for 30 min. The DNS method was used to measure the amount of glucose released.

2.8. Statistical Analysis

All data were expressed as the mean ± standard deviation (SD) ($n = 3$). The obvious differences were calculated by ANOVA. The $p < 0.05$ were considered to be significant.

3. Results

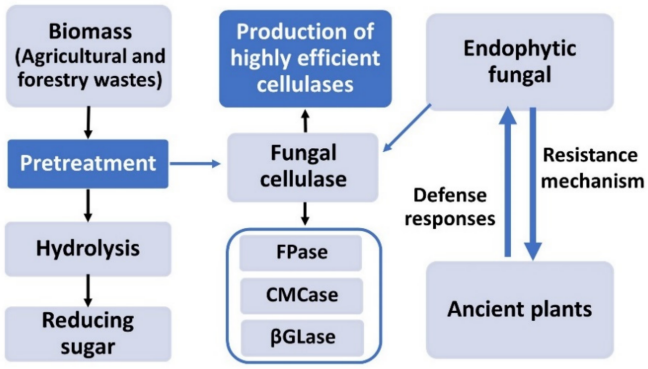
3.1. Screening and Identification of Fungal Isolate

Among 58 endophytic fungi isolated from 20-year-old *Taxus cuspidata*, congo red and cellulase, activity assays were used to screen the cellulose decomposition activity of the isolated fungi. Congo red, which could be firmly combined with cellulose, have been used in screening cellulose decomposition activity. When the macromolecular polysaccharide like cellulose degraded by cellulase, the small molecular sugars could not interact with the congo red. The endophytic fungal with cellulase production would have transparent circles around the colony. Furthermore, the larger the ratio of transparent circle diameter to colony diameter, the higher the activity of cellulase produced [24]. The results shown in Figure S1 indicated that six isolates were able to produce cellulases on Congo red plates which was estimated by clear zone diameter (cm) ranging from 0.6 to 2.8 cm. Considering that the largest clear area diameter and the highest cellulase activity were recorded by the strain R4, the strain R4 was selected as the better potential producer for identification. Similar results were demonstrated by Yao and Visser [11,26], who selected the cellulase-producing fungal dishes that showed the largest halo forming zone when all dishes were flooded with an aqueous solution of Congo red. Moreover, it graphically illustrated that all six isolates were able to produce cellulase, which ranged from 1.07 to 3.70 U/mL (Figure S1). The isolated strain R4 showed the highest cellulases activity.

3.2. Identification of the Most Potent Fungal Isolate

Endophytic fungal R4 showed the highest cellulases activity, afterward, it was selected as the most potent isolate and subjected for identification according to its morphological characteristics and molecular biology techniques. The colonies of strain R4 were nearly flat, thin felt-like, dark green in color on the front and yellow-green on the back. Conidiophores were in broom-like branches, ellipsoidal conidia grew at the tip of the pedicel in chains (Figure 1). In addition, molecular biology techniques were applied for identification combined with morphological identification. The genomic DNA was extracted using CTAB protocol. The ITS genes were amplified by PCR technology and sequenced. The length of the amplified rDNA fragment was about 564 bp. The identification was achieved by

comparing the contiguous DNA sequence with data from the reference and phylogenetic tree clustering of ITS sequences of cellulase-producing strains in GenBank isolated from *Taxus cuspidata*. The phylogenetic relationship showed that this strain was very closer to the type strains of *Penicillium* genus deposited in culture collection center of National Centre for Biotechnology Information (Figure 1). The strain R4 showed a high degree of homology (99%) with strain *Penicillium oxalicum* (accession number MH367526 and MT597864). Therefore, this strain was named as *Penicillium oxalicum* R4, which ITS1-5.8S-ITS2 sequence has been uploaded to GenBank under accession numbers MZ1899974. *Penicillium* have the ability to produce higher cellulose degrading enzymes and have certain advantages in terms of degradation performance of the enzyme system and growth rate of the strain [27], showing great potential for industrial applications.



Systematic strategies to obtain effectiveness of cellulase production.

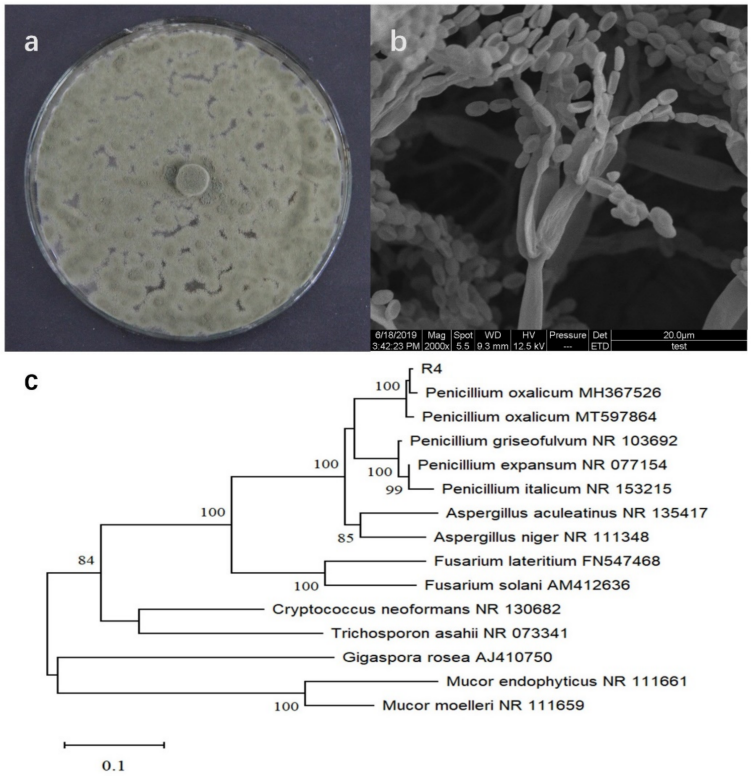


Figure 1. Phylogenetic relationship of ITS sequences of Cellulase-producing Strains in GenBank. (**a**): Colonial morphology, (**b**): The SEM of spore and hyphae, (**c**): Phylogenetic relationship of ITS sequences of R4 in GenBank).

3.3. Optimization of Submerged Fermentation Conditions for Maximum Cellulases Production by P. oxalicum R4

3.3.1. Effect of Nitrogen Source

Nitrogen is an important element for the synthesis of enzymes and other microbial proteins syntheses [28]. For that, the best N source is the material initiates enzymes production. In this study, five kinds of nitrogen sources were screened for cellulases production. Ammonium sulphate as organic N was applied as a nitrogen source for production of cellulases enzymes by *P. oxalicum* R4. The results were illustrated in Figure S2a, it was clear that the optimum nitrogen source was ammonium sulphate, and high enzyme production reached 1.47, 5.27 and 6.24 U/mL for Pfase, CMCase and βGLase, respectively. Therefore, ammonium sulphate as inorganic N was applied as the nitrogen source for production of cellulases enzymes by *P. oxalicum* R4. The ammonium salt of $(NH_4)_2SO_4$ can be directly decomposed to produce ammonia and used by microorganisms, which are also known as a quick-acting nitrogen source. It has been shown that ammonium sulfate as a nitrogen source is more reasonable and balanced for *Penicillium* to produce cellulases, which is similar to the results of this study [12].

3.3.2. Optimization of Initial pH Value

The pH value of microbial growing media is regarded as one of the most important factors for microbial growing as well as enzymes production [29]. It does not mean the optimum growth pH value is considered the optimum degree for enzymes production. The pH of the growth medium plays an important role in enzyme secretion by microorganisms. In this study, six different pH values ranged between 3.0 and 8.0 were applied. Cellulase activity production was low when *P. oxalicum* R4 was cultured in a medium with an initial pH of 3.0 (Figure S2b). The cellulase production of *P. oxalicum* R4 increased when the medium pH reached 5.0. The enzyme activity decreased when the medium pH exceeded 5.0. *P. oxalicum* R4 grew on a medium with an initial pH of 5.0 and produced three cellulase enzymes yield of 1.49, 4.82, and 5.81 U/mL for Fpase, CMCase and βGLase, respectively. Thus, pH 5.0 was the optimum condition for cellulase production, and it was assumed that the cellulase-producing endophytes isolated were more suited to an acidic environment than a neutral one. Most of cellulases from *Penicillium sp.* had an optimum initial pH of 5.0–5.5 [30–32]. The next step in response surface optimization experiments are at pH 4.0, 5.0, 6.0.

3.3.3. Effect of Incubation Temperature

Incubation temperature plays an important role in the metabolic activities of all microorganisms. In this experiment, six different temperature degrees were examined and the results represented in Figure S2c, which showed that *P. oxalicum* R4 had the ability to degrade cellulose compound under a wide range of incubation temperatures (25–50 °C). The data demonstrated that the lowest CMCase and β-glucosidase activity were recorded on 25 °C while the lowest activities of Fpase was recorded at 50 °C. Generally, the activities of the three estimated enzymes gradually increased till to their maximum activity at 35 °C and then decreased. The highest activity of the three estimated enzymes were recorded when the fungal strain incubated at 35 °C with values of 1.56, 5.15 and 5.77 U/mL for Fpase, CMCase and βGLase, respectively. Many researchers have reported differences in the optimum temperature for maximum cellulase production by different fungi, such as *Penicillium*, *Trichoderma*, *Aspergillus*, white rot fungus, etc. [19,30,33]. This suggests that the optimal temperature for cellulase production also depends on the strain of microorganism.

3.3.4. Effect of Incubation Period

Clearly, the incubation period directly affects enzymes production. In this trend, the maximum yield of cellulases was tested at different incubation periods and the data was shown in Figure S2d. The results indicated that cellulase production was lowest during 0–24 h, the strain should be using the medium to maintain its own growth and development.

At 48–72 h, cellulases production increased significantly. The highest production of Fpase and CMCase was achieved at 72 h, while the highest production of βGLase was at 96 h (4d). The results indicated that 3–5 d was the optimal incubation period for cultivating cellulases [34,35].

3.4. Optimization and Experimental Design of BBD Test

Response surface methodology (RSM) is a biological process optimization statistical experimental design, which can create a surface model with continuous variables and factors to evaluate to determine the optimum level range. As the growth environment changes, the degradation function also changes. The optimal conditions for the production of the three cellulases of *P. oxalicum* R4 were determined by RSM experiments in Table 1, which had a significantly effect on the interaction between incubation temperature, pH and time. Regression model equations for these independent variables were developed. By using multivariate regression analysis, Fpase (Y_1), CMCase (Y_2) and βGLase (Y_3) was predicted according to following Equation:

$$Y_1 = 1.46 - 0.042 \times {}_1 + 0.085X_2 - 0.020X_3 - 7.5 \times 10^{-3}X_1X_2 + 0.012X_1X_3 - 0.067X_2X_3 - 0.17{X_1}^2 - 0.23{X_2}^2 - 0.16{X_3}^2 \quad (3)$$

$$Y_2 = 5.22 - 0.21X_1 + 0.33X_2 - 0.100X_3 - 0.032X_1X_2 + 0.092X_1X_3 - 0.22X_2X_3 - 0.57{X_1}^2 - 0.89{X_2}^2 - 0.53{X_3}^2 \quad (4)$$

$$Y_3 = 6.60 - 0.20X_1 + 0.40X_2 - 0.081X_3 - 0.035X_1X_2 + 0.055X_1X_3 - 0.29X_2X_3 - 0.74{X_1}^2 - 0.98{X_2}^2 - 0.66{X_3}^2 \quad (5)$$

Table 1. Experimental design with actual and predicted response value.

Standad	Temperature	Time	pH	Actual Value (U/mL)			Predicted Value (U/mL)			ANN–GA (U/mL)		
Order	(X_1, °C)	(X_2, h)	(X_3)	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
1	−1(30)	−1(48)	0(5)	0.99	3.59	4.56	1.01	3.61	4.64	0.99	3.59	4.56
2	1(40)	−1(48)	0(5)	0.92	3.23	4.24	0.94	3.26	4.31	0.92	3.23	4.24
3	−1(30)	1(96)	0(5)	1.21	4.36	5.58	1.19	4.33	5.51	1.21	4.36	5.58
4	1(40)	1(96)	0(5)	1.11	3.87	5.12	1.09	3.85	5.04	1.11	3.87	5.12
5	−1(30)	0(72)	−1(4)	1.23	4.63	5.67	1.2	4.53	5.53	1.23	4.63	5.67
6	1(40)	0(72)	−1(4)	1.12	4.03	5.16	1.09	3.92	5.03	1.12	4.03	5.16
7	−1(30)	0(72)	1(6)	1.11	4.04	5.13	1.14	4.15	5.26	1.11	4.04	5.13
8	1(40)	0(72)	1(6)	1.05	3.81	4.84	1.08	3.91	4.98	1.05	3.81	4.84
9	0(35)	−1(48)	−1(4)	0.93	3.28	4.29	0.94	3.36	4.35	0.93	3.28	4.29
10	0(35)	1(96)	−1(4)	1.2	4.32	5.53	1.24	4.45	5.74	1.20	4.32	5.53
11	0(35)	−1(48)	1(6)	1.08	3.72	4.98	1.03	3.59	4.77	1.08	3.72	4.98
12	0(35)	1(96)	1(6)	1.08	3.89	5.05	1.07	3.81	4.99	1.08	3.89	5.05
13	0(35)	0(72)	0(5)	1.46	5.19	6.73	1.46	5.22	6.6	1.47	5.27	6.57
14	0(35)	0(72)	0(5)	1.49	5.42	6.67	1.46	5.22	6.6	1.47	5.27	6.57
15	0(35)	0(72)	0(5)	1.45	5.23	6.26	1.46	5.22	6.6	1.47	5.27	6.57
16	0(35)	0(72)	0(5)	1.42	5.11	6.55	1.46	5.22	6.6	1.47	5.27	6.57
17	0(35)	0(72)	0(5)	1.47	5.16	6.78	1.46	5.22	6.6	1.47	5.27	6.57

The regression model was statistically validated by analysis of variance (ANOVA). Regression equation coefficients significant test results are shown in Table 2, statistical data was conducted on the experimental data through Design-Expert 8.0.6 software (State Ease, Inc., Address: 1300 Godward St NE, Suite 6400, Minneapolis, MN 55413). The larger the *F*-value and the smaller the *p*-value in the results, the higher the significance of the corresponding variable. Fpase, CMCase and βGLase activities for these three model groups were $p < 0.0001$, indicating the accuracy and applicability of these models. Meanwhile, the lack of fit ($p > 0.05$) of three model groups indicated that the models were fitted successfully. R^2 were 0.9578, 0.9592 and 0.9285, respectively, showing that the fitted empirical model can be used for prediction.

Table 2. Fpase, CMCase and βGLase yield response surface quadratic model from experimental results.

	Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value Prob > F	
Fpase activity	Model	0.59	9	0.066	41.34	<0.0001	significant
	X_1	0.014	1	0.014	9.05	0.0197	
	X_2	0.058	1	0.058	36.19	0.0005	
	X_3	3.200×10^{-3}	1	3.200×10^{-3}	2.00	0.1998	
	X_1X_2	2.250×10^{-4}	1	2.250×10^{-4}	0.14	0.7185	
	X_1X_3	6.250×10^{-4}	1	6.250×10^{-4}	0.39	0.5514	
	X_2X_3	0.018	1	0.018	11.41	0.0118	
	X_1^2	0.13	1	0.13	78.67	<0.0001	
	X_2^2	0.22	1	0.22	136.74	<0.0001	
	X_3^2	0.10	1	0.10	65.60	<0.0001	
	Residual	0.011	7	1.578			
	Lack of Fit	8.500	3	2.833	4.23	0.0987	not significant
	Pure Error	2.680	4	6.700			
	Cor Total	0.61	16				
	Source	Sum of Squares	df	Mean Square	F-value	*p*-value Prob > F	
CMCase activity	Model	8.03	9	0.89	42.85	<0.0001	significant
	X_1	0.35	1	0.35	16.94	0.0045	
	X_2	0.86	1	0.86	41.20	0.0004	
	X_3	0.080	1	0.080	3.84	0.0908	
	X_1X_2	4.225×10^{-3}	1	4.225×10^{-3}	0.20	0.6660	
	X_1X_3	0.034	1	0.034	1.64	0.2407	
	X_2X_3	0.19	1	0.19	9.09	0.0195	
	X_1^2	1.35	1	1.35	65.06	<0.0001	
	X_2^2	3.35	1	3.35	160.96	<0.0001	
	X_3^2	1.17	1	1.17	56.20	0.0001	
	Residual	0.15	7	0.021			
	Lack of Fit	0.089	3	0.030	2.10	0.2435	not significant
	Pure Error	0.057	4	0.014			
	Cor Total	8.18	16				
	Source	Sum of Squares	df	Mean Square	F-value	*p*-value Prob > F	
βGLase activity	Model	11.11	9	1.23	24.07	0.0001	significant
	X_1	0.31	1	0.31	6.09	0.0430	
	X_2	1.29	1	1.29	25.12	0.0015	
	X_3	0.053	1	0.053	1.03	0.3440	
	X_1X_2	4.900×10^{-3}	1	4.900×10^{-3}	0.096	0.7662	
	X_1X_3	0.012	1	0.012	0.24	0.6420	
	X_2X_3	0.34	1	0.34	6.67	0.0363	
	X_1^2	2.32	1	2.32	45.30	0.0003	
	X_2^2	4.05	1	4.05	78.90	<0.0001	
	X_3^2	1.81	1	1.81	35.25	0.0006	
	Residual	0.36	7	0.051			
	Lack of Fit	0.19	3	0.062	1.44	0.3550	not significant
	Pure Error	0.17	4	0.043			
	Cor Total	11.47	16				

To visualize the effects of various factors and their interactions on the cellulase yield of *P. oxalicum* R4, see the three-dimensional regression equation simulation diagram in Figure 2 and Figure S4. The interaction of pH (4–6) and temperature (30–40 °C) on the activity of Fpase, CMCase and βGLase produced by *P. oxalicum* R4 was shown in Figure 2a. When temperature is 35 °C and pH is 5, the mean values of highest activity are: Fpase (1.47 U/mL), CMCase (5.06 U/mL) and βGLase (6.28 U/mL). The initial pH value of the medium was from 4 to 4.9, yield increased gradually, while the increasing pH value, the enzyme activity of cellulase decreased and extreme temperature was not beneficial to the growth of mycelium. It also proofen that inappropriate pH could change the osmotic

pressure and affected fungal growth, inhibition of cellulose synthesis and metabolism. From Figure 2b, incubation time had a marked effect on CMCase production. When incubation time was below 24 h, cellulase activity were very low because of physiological processes for self-growth. Cellulase yield increased with time, but a certain cultivation time and temperature were exceeded, and the cellulase activity decreased. The nutrients in the medium were gradually depleted, which further affected the growth of the strain and reduced enzyme secretion. The interaction between fermentation times and pH was shown in Figure 2c, when the initial pH (<5) and time (>96 h) increased, βGLase yield also increased. It showed that the proper time and pH were conducive to the growth and metabolism of *P. oxalicum* R4, promoted the production of metabolites and increased cellulase yield. Combined with the BBD experiment and response surface analysis, the optimal culture conditions for the cellulases yield of endophytic fungi R4 from *Taxus cuspidata* can be simulated under temperature 34 °C, time 77 h, and the initial pH 5, the predicted response value of Fpase, CMCase and βGLase activity were 1.47 U/mL, 5.28 U/mL and 6.66 U/mL, respectively.

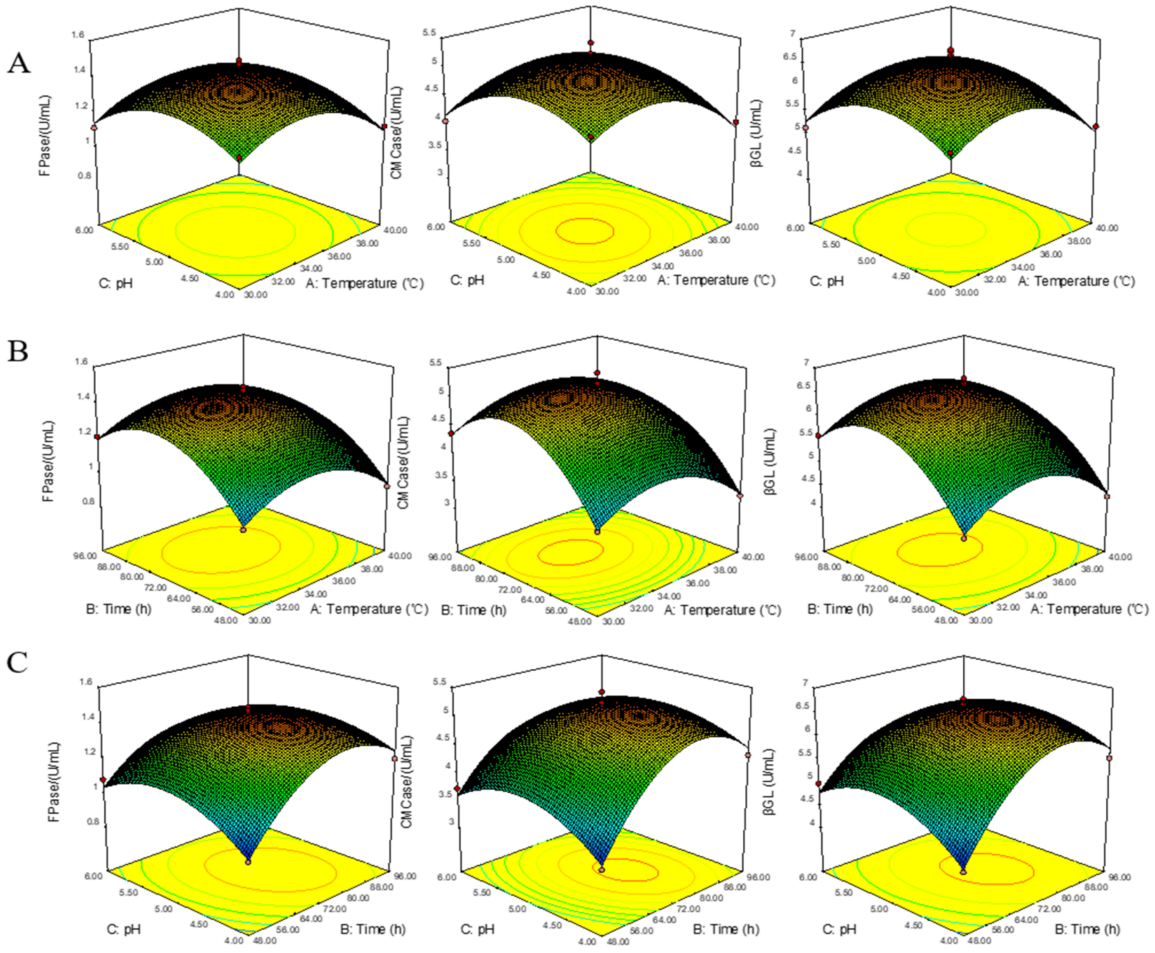


Figure 2. Three-dimensional regression equation simulation diagram of cellulase production activity of *P. oxalicum* R4 showing (**A**) varying time and temperature, (**B**) varying pH and temperature and (**C**) varying pH and time.

3.5. Artificial Neural Network and Genetic Algorithm

To build the optimal ANN model for predicting the response, it was confirmed by repeatedly training of the network to obtain the minimum mean square error (MSE) and the maximum R^2. The same data used to construct the RSM model were used to build the

ANN model for predicting cellulase production. The regression model was obtained from fitting the experimental group data, and the optimal value was calculated. The optimal structures of the neural network models trained by the Levenberg–Marquardt algorithm were three neurons in the input layer, ten neurons in the hidden layer, and three neurons in the output layer. According to the minimum MSE and maximum R-value for training, validation and testing of the network, 10 neurons in the hidden layer of the network were selected. From Figure S3, it can be concluded that the network learns well as the number of periods increases and obtains its best performance at the 6th epoch, where the MSE value of 0.076. As shown in Figure 3, the R-values of 0.99948 and 0.99178 for the training and validation datasets, respectively, confirm the good correlation between the experimental data used for model development and the predicted data. For the test dataset, the R-value was 0.99706, confirming the good predictive power of the ANN model for the unseen data. Overall, the R-value of 0.99723 for all datasets indicated that the predicted data from the 17 experiments were in great agreement with the experimental results (Table 1). The artificial neural network model was combined with a genetic algorithm to obtain optimal values of independent process parameters to obtain optimal cellulase yield. The optimal fermentation conditions predicted by the ANN model were temperature 35 °C, time 78 h, and the initial pH 5, the predicted response value of Fpase, CMCase and βGLase activity were 1.45 U/mL, 5.27 U/mL and 6.35 U/mL, respectively.

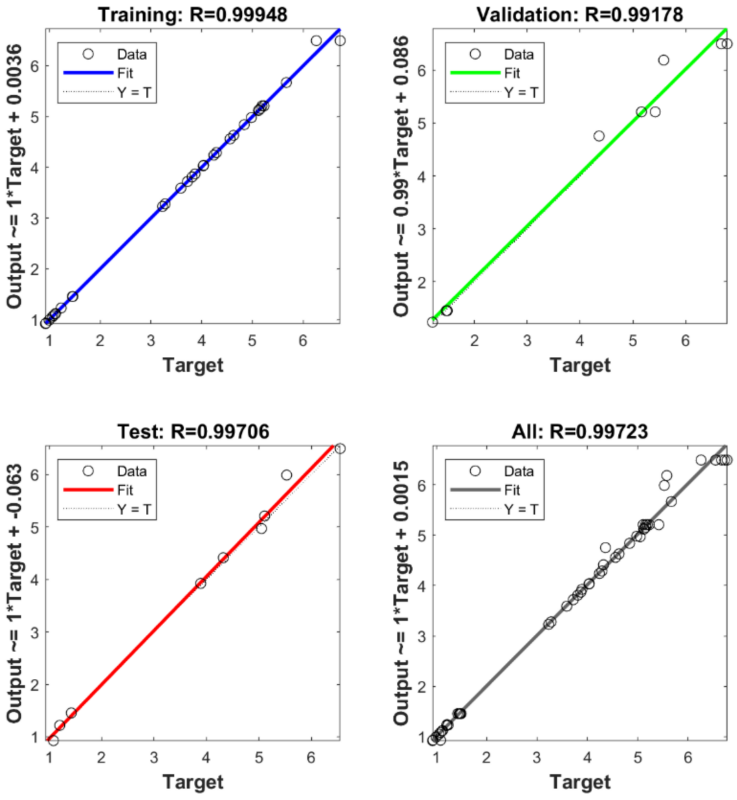


Figure 3. Regression analysis plot of the generated ANN model for training, testing, validation, and all data sets.

3.6. Verification and Comparison of Models

The optimized cellulase yields predicted by the RSM and ANN models, the corresponding actual values were shown in Table 1. It can be seen that the results predicted by the ANN models were closer to the best simulation curve, indicating that the ANN model fit the data better than the RSM model, and these results were also consistent with other related studies [36,37].

Validation tests were performed with the optimal conditions predicted by the two methods (Table 3), and the measured values were the average of three parallel experiments. The results showed that the Pfase, CMCase and βGLase optimized by ANN–GA were 1.45 U/mL, 5.27 U/mL and 6.35 U/mL, respectively, and the relative errors with the measured values were less than 0.5%, and the predicted PFase, CMCase and βGLase obtained by the RSM were 1.47 U/mL, 5.28 U/mL and 6.66 U/mL, respectively, the relative errors were in the range of 0.76–5.0%. Comparing the two methods, there were some deviations in the prediction of the optimal value by RSM. The predicted maximum cellulase yield was not the real maximum value. ANN–GA had a higher fit between the measured results and the predicted results in terms of the accuracy of the prediction, and the obtained optimal value were closer to the limit value. Based on the above results it could be concluded that both RSM and ANN–GA were feasible in the optimization of cellulase production by the endophytic fungus R4 of *Taxus cuspidata*, but the latter was superior to the former.

Table 3. Comparison of optimization of cellulase production by different methods.

Method	Temperature (°C)	Time (h)	pH	Predictive Value			Actual Value		
				Fpase	CMCase	βGLase	Fpase	CMCase	βGLase
RAW							0.87	3.31	2.83
RSM	34.22	77.09	4.87	1.47	5.28	6.66	1.45	5.24	6.34
ANN–GA	34.83	78.11	4.98	1.45	5.27	6.35	1.45	5.27	6.35

Under the optimized culture conditions obtained by the Box–Behnken design (BBD) and the artificial neural network–genetic algorithm (ANN–GA), yields of Filter Paperase (FPase), Carboxymethyl Cellulase (CMCase) and β-glucosidase (βGLase) produced by *P. oxalicum* R4 were approximately 1.60-fold, 1.59-fold and 2.16-fold higher than those of the non-optimized culture, respectively.

4. Discussion

Scholars all over the world are concerned with searching and discovering novel strains of cellulase enzymes with high efficiency in nature [38], improving strains to obtain maximum cellulase production, optimizing fermentation processes to improve production efficiency, designing inexpensive cellulase bioprocesses to further improve their saccharification efficiency, and making cellulase production costs economically feasible [30,39]. Endophytic fungi are a special kind of fungi that parasitize in the tissues of healthy living plants and have no obvious symptoms to the host. They have co-evolved with the host plant for a long period of time, forming a mutually beneficial symbiosis relationship [19,40]. Endophytic fungi inside the plant also have the ability to degrade a portion of plant lignin and cellulose. Recently, endophytic fungal enzymes have received increasing attention as a novel source of cellulase [41]. Studies have shown that endophytic fungi invade and colonize plant tissues and obtain nutrients, and the highly active cellulases produced to help the host plant defend themselves against invading pathogens [8,19]. However, most endophytic fungi are rarely reported as a source of enzymes for biotechnological applications. Although there are many reports on cellulase-producing strains, obtaining cellulase strains with high yield and enzymatic activity is still a key issue to be solved [13,38].

The natural evolution of ancient tree species from special natural geographic environments to screen out cellulase-producing strains with excellent characteristics provides a promising direction for future industrial enzymes [21]. *Taxus cuspidata* is recognized as an endangered and rare natural anti-cancer plant in the world, with a history of 2.5 million years on earth, and is a living plant fossil. An important criterion for the evaluation of cellulase activity is the βGLase/FPase ratio. It has been reported that the lower the βGLase/PFase ratio, the higher the accumulation of cellobiose, leading to the occurrence

of a feedback inhibition mechanism, which inhibits PFase and CMCase activities [3]. The strain R4 showed the highest cellulases activity from *Taxus cuspidata*.

In recent years, research on cellulase components, production conditions, fermentation methods and expansion of production has led to great progress in cellulase production and application. The research on liquid fermentation conditions of cellulase is of practical significance and suitable for large-scale production, because the liquid fermentation method has the characteristics of high utilization of raw materials, easy to control the culture conditions, high yield and stable quality, etc. In terms of liquid submerged fermentation (SmF) culture, cellulase-producing strains need a good growth condition to meet the nutrients for their own growth in order to produce cellulase efficiently. Hu et al. used *Aspergillus niger* as the test material and optimized its medium and fermentation conditions by SmF, which increased the the activity of FPase, CMCase and βGLase by 120.55–141.52%, respectively, compared with the unoptimized conditions [42]. Han et al. used UV mutagenic strain UVIII of *Trichoderma pseudokoningii* TH as the study object, the activities of FPase, CMCase and βGLase were increased 0.60–2.34 times, respectively, after the optimal medium and fermentation conditions [43]. We examined the production profile of cellulolytic enzymes by *P. oxalicum* R4 in experiments using 5% HWS as the substrate. Both RSM and ANN–GA were applied in this experiment to fit the data based on the experimental design to obtain the regression models, and then the optimal values of the model were calculated. The response surface method is widely used in general optimization, and its fitting is mainly based on a quadratic nonlinear regression equation, which can reflect the influence of each factor on the response value [44]. However, the artificial neural network applies a global nonlinear fitting approach, and the prediction of extreme values using genetic algorithms for the model has a high accuracy [22,36].

5. Conclusions

In this study, an endophytic *Penicillium oxalicum* R4 was isolated from *Taxus cuspidata*, which was known as a plant fossil, with high cellulose utilization ability. The cellulase system produced by R4 was relatively complete, and the yield of the three cellulases were more reasonable and balanced. Optimizing cultivation conditions through RSM and ANN–GA, cellulases with high βGLase/PFase ratio were generated to evaluate the potential of enzyme-producing endophytic fungi for subsequent bioethanol production from forestry waste.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13116006/s1, Figure S1: Screening of fungal isolates for production of cellulases enzymes using Congo red assay (a) and cellulase activity assay (b); Figure S1: Single factor optimization of cellulase activity of endophytic fungus R4 from *Taxus cuspidata*. Nitrogen source (a), pH (b), Temperature (c), Time (d); Figure S3: Network training curves with Epochs number for trained subsets for cellulases yield.

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Abbreviations

BBD	Box–Behnken design
ANN–GA	Artificial neural network–genetic algorithm
FPase	Filter Paperase
CMCase	Carboxymethyl Cellulase
βGLase	β-glucosidase
RSM	Response surface methodology

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