Screening and Characterization of a Low-Temperature-Resistant Cellulose-degrading Strain, *Trichoderma harzianum* L-8, from a Primitive Forest

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Low temperature is a major factor limiting the bio-sustainable and efficient conversion of cellulose-based resources in cold regions. In this study, a low-temperature resistant cellulose-degrading fungus with high cellulase production was screened from samples found in a primitive forest in Daqing by straw using the enrichment-restricted culture technique. The fungus was identified as genus *Trichoderma harzianum*, strain L-8 by morphological and molecular biological analysis. The enzyme production conditions were optimized *via* response surface methodology, and the optimal conditions for the enzyme production of *Trichoderma harzianum* L-8 were as follows: a CMC-Na addition of 10.63 g·L^{-1}, an ammonium sulfate addition of 2.22 g·L^{-1}, an initial pH of 5.29, and a lecithin addition of 5.18 g·L^{-1} when the CMCase reached 53.40 IU·mL^{-1}. The leading enzyme families of *Trichoderma harzianum* L-8 were identified *via* proteomic analysis. Proteases including glycosyl hydrolase family 3-4 and cellobiohydrolase play important roles in cellulose degradation. The strain *Trichoderma harzianum* L-8 showed a strong cellulose degradation ability under low temperatures, providing strain resources for cellulose resource biotransformation technology in cold regions.

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INTRODUCTION

As the most abundant carbohydrate in nature, cellulose is an essential class of renewable resources (Li *et al*. 2021). Its biotransformation degradation technology has become a research hotspot for both energy and the environmental sciences because of its high degradation efficiency, low energy consumption, safety, and lack of pollution (Awais *et al*. 2021). However, little research has been reported on the screening and performance of low-temperature resistant cellulose-degrading strains. After a long period of evolution, low-temperature microorganisms have a particular structure and metabolic mechanism to adapt to low-temperature environments, and most of their enzymes have low-temperature catalytic and heat-unstable properties (Yusof *et al*. 2021). Cold-adapted microorganisms regulate their metabolic activities by producing cold-active enzymes to adapt to low temperature environments (Abdellah *et al*. 2021).

Cold-active enzymes have a low optimal reaction temperature and can bind to substrates and have catalytic activity under low-temperature conditions (Wang *et al*. 2021). The screening of low-temperature tolerant cellulose-degrading strains and the search for their optimal conditions for cellulase production have great importance for the utilization of cellulose resources in cold regions (Sun *et al*. 2020). In order to solve the problem of the difficulty of the utilization of cellulose resources during the cold climate in autumn and winter in cold regions, it is necessary to find a low-temperature resistant cellulose degrading strain and optimize its enzyme production performance (Dai *et al*. 2016; Gong *et al*. 2020). In view of the cold and long winter characteristics in cold regions, the key to optimizing the biomass conversion process, reducing production costs, and resolving the waste of cellulose resources is to find strains that can adapt to the cold environment and efficiently produce cellulase and optimize their enzyme production conditions (Sun *et al*. 2018). Research on the characteristics and application conditions of cellulase can further improve the strains, which can provide valuable strain resources for subsequent practical applications.

EXPERIMENTAL

Source of Strain

The strain was isolated from a primitive forest in Daqing.

Experimental Method

Screening and identification of strains

The initial screening and re-screening of the strain were performed according to the method described in Duncan *et al*. (2008), outlined as follows:

First, 30 μL of a pure strain obtained from screening was spotted at the center of a potato dextrose agar (PDA) plate and incubated at a temperature of 17 °C for 5 to 7 d to observe the morphology and growth characteristics of the colony to determine the genus of the strain. The pure isolated strain was inoculated into PDA plates and incubated at a temperature of 15 °C for 5 d, and the spore morphology was observed *via* scanning electron microscopy. Molecular biology 18S rDNA sequence analysis was used, and PCR amplification was performed using universal fungal primers. Shanghai Biotechnology determined the sequences of the amplification products, and the results were matched with the sequences of standard strain samples on the NCBI website. The phylogenetic tree of the strain was constructed using MEGA software (7.0, Penn State University, State College, PA).

Enzyme activity assay

The enzyme activity of filter paper (FPA) was determined according to the method described in Silveira *et al*. (2012). The cellulose enzyme activity (CMCase) was determined by following the international standard method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987).

Optimization of the conditions for enzyme activity

The optimum reaction temperature, optimum reaction pH, and thermal stability of cellulase and filter paper enzymes were investigated experimentally utilizing the method of Sriariyanun *et al*. (2016) for the study of the enzymatic properties.

Using a Plackett-Burman (PB) experimental design method and analysis of variance for the results, several incubation conditions, including the carbon source, nitrogen source, initial pH, lecithin addition, metal ions added, incubation temperature, and incubation time, were examined to screen out the primary influencing factors among them. Each factor was assigned a high and a low level, expressed as "+1" and "-1", respectively.

Based on the primary influencing factors screened by the Plackett-Burman test, a 4-factor, 5-level response surface analysis was conducted for the conditions of cellulase production using a central composite design (CCD). The final equation model between the factors and the corresponding indicators is shown in Eq. 1,

$$Y = a_0 + \sum_{i=1}^{k} a_i X_i + \sum_{i=1}^{j-1} \sum_{j=1}^{k} a_{ij} X_i X_j + \sum_{i=1}^{k} a_{ij} {X_i}^2 \quad (1)$$

where Y is the enzyme activity, a_0 is the offset, a_i is the linear offset, a_{ij} is the second-order offset, and X_i is the value of each factor.

Proteomic analysis

Shanghai Biotech performed proteomic analysis on the total protein samples *via* liquid chromatography-mass spectrometry (LC-MS).

RESULTS AND DISCUSSION

Isolation and Morphological Identification of the Low Temperature Resistant Cellulose Degrading Strains

By comparing the size of the hydrolysis circle, observing the degradation of microcrystalline cellulose, and determining the enzyme activity, eight strains with good enzyme production were obtained. The results of the colony diameter, hydrolysis ring size, microcrystalline cellulose degradation, and CMCase are shown in Table 1. After comparing the strains, L-8 was selected as the experimental strain.

Table 1. Hydrolytic Circle and Colony Diameter

Strain	Hydrolytic Circle Diameter (cm)	Colony Diameter (cm)	Degradation of Microcrystalline Cellulose	CMCase ($IU \cdot mL^{-1}$)
L-1	2.75	1.40	+	6.253
L-2	6.20	2.50	+++	23.102
L-3	4.05	2.30	++	11.291
L-4	5.40	2.00	+++	18.102
L-5	3.00	1.00	+	3.621
L-6	6.70	6.55	+++	23.704
L-7	5.58	3.15	++++	26.067
L-8	8.60	8.40	++++	33.825

Note: “+” indicates slight degradation signs ; “++” indicates slight degradation; “+++” represents moderate degradation; and “++++” indicates good degradation effect

The Congo red staining, colony morphology, and spore morphology of strain L-8 are shown in Fig. 1. The colony was round, with an average diameter of approximately 8 cm, annular outward diffusion, edge folds, dense clumps, white flocculent at the early growth stage, and produced dark green spores. Under the scanning electron microscope, one or more conidial peduncles were observed on the lateral branches of the mycelium, which grew in an upright position. There were branches at the end of the arbuscular, and the top of the arbuscular was tightly non-dispersed. The spore masses were formed on the lateral branches, and the spore masses were spherical with smooth surfaces. The colony was preliminarily identified as a *Trichoderma* sp. strain.

Fig. 1. Morphological characters of L-8: (A) characteristics of the hydrolysis circle of the Congo red staining of the strain; (B) the growth morphology of the strains attached to the screening medium; and (C) the hyphae and spores observed under a scanning electron microscopy

Molecular Biological Identification of the Low Temperature Resistant Cellulose Degrading Strains

The strain DNA was used as a template to amplify the 18S rDNA of the strain, and the sequence of 1050 bp was sequenced after linkage with the T vector. The genes were compared with the sequences in the GenBank database by BLAST, and the phylogenetic tree was constructed using MEGA 7.0 software. The evolutionary status of *Trichoderma harzianum* L-8 is shown in Fig. 2. *Trichoderma harzianum* is one of the most common "aggregate species" of *Trichoderma* (Chaverri *et al*. 2015). Cellulase, hemicellulase, xylanase, chitinase and protease produced by fermentation are usually widely used in agriculture, feed and environmental protection.

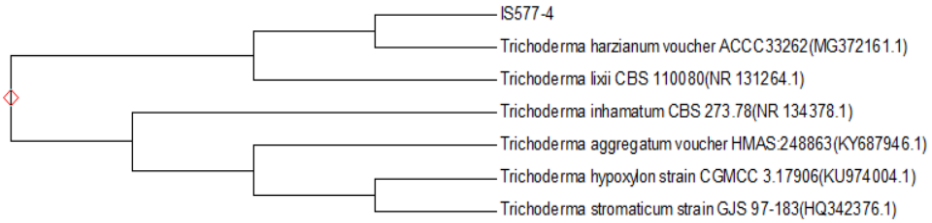

Fig. 2. Phylogenetic tree with strain L-8

Enzymatic Properties of the Strains

The effects of the reaction temperature on the cellulase and filter paper enzymes activities produced by *Trichoderma harzianum* L-8 are shown in Fig. 3. The cellulase and filter paper enzymes had the highest enzymatic activities under 30 °C reactions. When the

reaction temperature exceeded 35 °C, the FPA and CMCase activity significantly decreased (p-value less than 0.05). Other reports on cellulases also reflected similar properties (Zhang *et al*. 2009; Li *et al*. 2020). During the fermentation process, the influence of temperature primarily manifested in terms of microbial growth and reproduction, metabolic synthesis, and physicochemical properties of the fermentation broth. Low-temperature cellulase activity was the strongest at the optimal temperature range, and the enzymatic reaction rate was the largest. However, the enzymatic reaction was affected beyond the optimal temperature, thereby inhibiting the synthesis of metabolites. The cellulase and filter paper enzymes produced by *Trichoderma harzianum* L-8 had good activity when reacting under low-temperature conditions. They had good adaptability to low-temperature, which could be adapted to the autumn temperature in the cold region of northeast China.

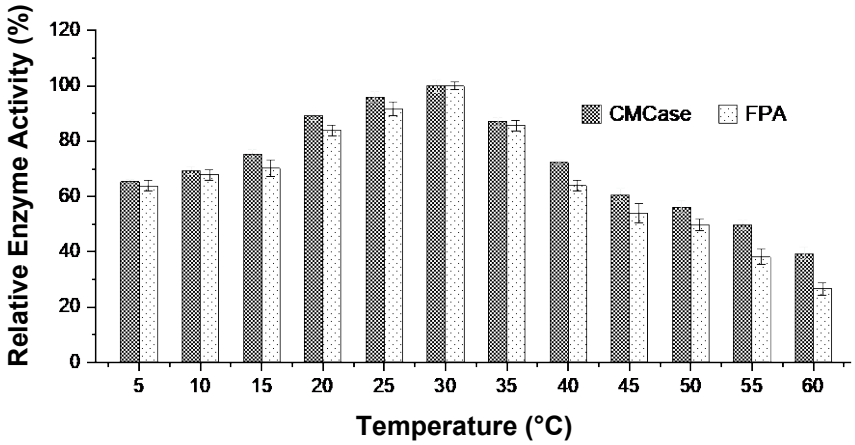

Fig. 3. Effect of the temperature on cellulase and filter paper enzyme

The thermal stability of the cellulase is shown in Fig. 4. As shown, the stability of the cellulase was high at a temperature range of 5 °C to 20 °C (p-value less than 0.05), and the relative enzyme activity was greater than 90% after holding for 2 h. The relative enzyme activity rapidly decreased after the temperature exceeded 30 °C. It could be seen that the performance of the cellulase remained stable under low-temperature conditions, which was consistent with low-temperature enzyme characteristics. This characteristic makes the strain more valuable for application, as the degradation of cellulose resources in most northeastern regions of China, where there is a considerable diurnal temperature difference and long-term cold in autumn and winter, requires enzymes with a strong ability to adapt to temperature changes.

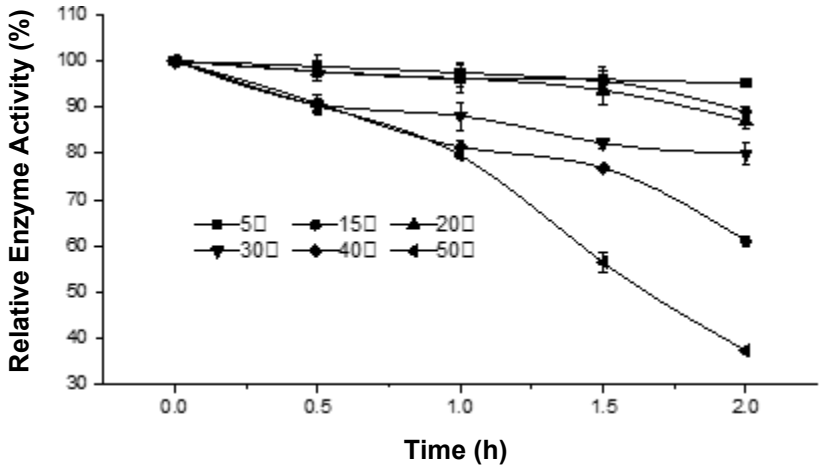

Fig. 4. Effect of the temperature on the stability of the cellulase

The effect of the pH on the enzymes produced by the strains is shown in Fig. 5. The optimal reaction pH for *Trichoderma harzianum* L-8 cellulase was 6.0. The activity conditions of the filter paper enzymes and cellulase were similar, with the relative enzyme activity reaching 100% at a pH of 5.0. The primary reason for the effect of the pH on the enzyme activity was the change in the dissociation state of the enzyme active site. The difference in the optimal reaction pHs between cellulase and the filter paper enzymes may be due to the differences between the two enzymes, which led to the deviation in the optimal activity conditions. It was seen that although there was a deviation in the optimal pHs of the two enzymes, their optimal activity conditions were the same, with both enzymes functioning at a neutral to acidic level as well as being well adapted to alkaline environments, with both enzymes being able to adapt to a wide pH range. This result was similar to Legodi *et al*. (2019) and Steiner and Margesin (2020) regarding low-temperature tolerant cellulases.

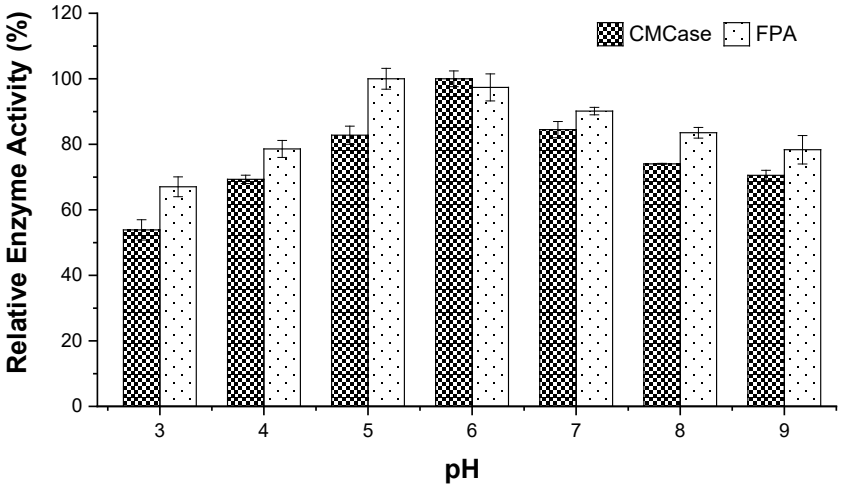

Fig. 5. Effect of the pH on the cellulase and filter paper enzyme

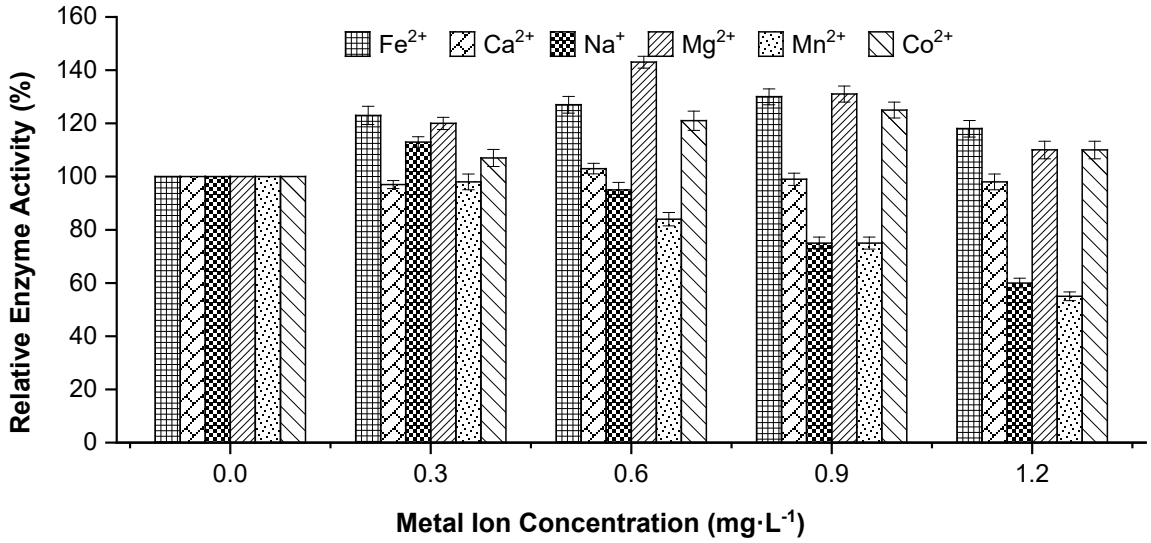

Fig. 6. Effect of metal ions on cellulase

The effect of each metal ion on the enzyme reaction is shown in Fig. 6. As shown in Fig. 6, it was found that Mg^{2+}, Fe^{2+}, Co^{2+}, and low concentrations of Na^{+} have a facilitative effect on the cellulase produced by *Trichoderma harzianum* L-8. The addition of Mn^{2+} was detrimental to the catalytic action of cellulase produced by *Trichoderma harzianum* L-8. This was consistent with the conclusion of Picart *et al*. (2008), who found that the addition of Mn^{2+} inhibited the catalytic action of cellulase. However, the results of Zhang *et al*. (2016) showed that the Mn^{2+} in metal ions had a considerable activating effect on cellulase activity. Further studies on the mechanism of the effect of metal ions on cellulase are needed.

Screening of the Significant Factors

After the single-factor test, the best enzyme production temperature for strain *Trichoderma harzianum* L-8 was 16 °C. The best enzyme production effect was achieved when the initial pH was 4.0 to 5.0. For different carbon and nitrogen sources, the best carbon source for strain *Trichoderma harzianum* L-8 was CMC-Na, and the enzyme production effect was better when the addition amount was 7.5 $g \cdot L^{-1}$ to 15.0 $g \cdot L^{-1}$. The enzyme production effect was better when the addition amount of ammonium sulfate was 2 $g \cdot L^{-1}$ to 4 $g \cdot L^{-1}$ (as the nitrogen source), and lecithin affected the enzyme production effect of the strain. Based on this, the effect of each factor, metal ion, and lecithin addition on the enzyme production of the strain was determined *via* a single-factor test, and a PB test was used to screen the significant factors comprehensively.

The PB test was conducted with a shaking bed culture with CMCase as the response value, and the factors affecting the cellulase production ability of the strain were screened. The factor levels are shown in Table 2. The experimental design and results are shown in Table 3. CMCase was used as the response value *Y* for the significant factor analysis, and the significance level of each factor is shown in Table 4.

From the ANOVA shown results in Tables 2 through 4, the experimental design model of *Trichoderma harzianum* L-8 PB was highly significant (the p-value equals 0.009, which is less than 0.01).

Table 2. Plackett-Burman Design Factor and Level Table for *Trichoderma harzianum* L-8

Serial Number	Factors	Levels	
		Low (-1)	High (1)
X_a	Addition of bran ($g \cdot L^{-1}$)	10	15
X_b	Addition of CMC-Na ($g \cdot L^{-1}$)	7.5	12.5
X_c	Addition of corn flour ($g \cdot L^{-1}$)	10	15
X_d	Additional amount of ammonium sulfate ($g \cdot L^{-1}$)	2	4
X_e	Additional amount of yeast powder ($g \cdot L^{-1}$)	1	3
X_f	Addition of urea ($g \cdot L^{-1}$)	1	3
X_g	Void item		
X_h	pH	4	6
X_i	Void item		
X_j	Addition of potassium dihydrogen phosphate ($g \cdot L^{-1}$)	0.2	0.4
X_k	The addition amount of ferrous sulfate ($g \cdot L^{-1}$)	0.3	0.5
X_l	Addition of sodium chloride ($g \cdot L^{-1}$)	0.5	0.6
X_m	Addition of manganese sulfate ($g \cdot L^{-1}$)	0.3	0.5
X_n	Addition of magnesium sulfate ($g \cdot L^{-1}$)	0.2	0.4
X_o	Addition of calcium chloride ($g \cdot L^{-1}$)	0.1	0.3
X_p	The addition amount of lecithin ($g \cdot L^{-1}$)[1]	3	5
X_q	Cultural temperature (°C)	15	20
X_r	Incubation time (d)	7	11
X_s	Void item ($g \cdot L^{-1}$)		

Table 3. Plackett-Burman Design and Results of *Trichoderma harzianum* L-8

No.	X_a	X_b	X_c	X_d	X_e	X_f	X_g	X_h	X_i	X_j	X_k	X_l	X_m	X_n	X_o	X_p	X_q	X_r	X_s	Y
1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	32.700
2	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	24.319
3	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	21.523
4	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	35.797
5	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	21.859
6	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	38.406
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	6.996
8	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	12.909
9	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	38.184
10	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	23.745
11	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	16.511
12	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	27.745
13	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	16.915
14	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	12.219
15	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	19.823
16	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	24.508
17	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	21.440
18	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	28.771
19	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	46.778
20	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	7.712

Note: The X_a to X_s in the table represents different factors, the 1 to 20 represents the conditions given by 20 sets of experimental designs, and Y indicates the CMCase activity ($IU \cdot mL^{-1}$)

The four factors that had the most significant effect on cellulase in order were X_b (CMC-Na addition), was greater than X_d (ammonium sulfate addition), which was greater than X_h (initial pH), which was greater than X_p (lecithin addition), and the p-values of X_b, X_d, X_h, and X_p were less than 0.01, which indicated that these factors had significant effects on the cellulase and filter paper enzyme yields. Therefore, these four factors were selected as the primary factors for the following optimization experiments.

The significant factors identified by the PB experiment on the enzyme production of strain *Trichoderma harzianum* L-8 were set as variables. The four significant factors and their levels are tabulated in Table 5. The results of the CCD experimental design are shown in Table 6. In the experiment, except for the primary influencing factors identified in the PB experiment, the values of the other factors were taken as the best values in the single-factor experiment, and the corresponding CMCase (Y) values were obtained experimentally.

Table 4. Plackett-Burman ANOVA Results of *Trichoderma harzianum* L-8

Factors	Sum of Square	Degrees of Freedom	Mean Square	F-value	p-value
X_a	52.893	1	52.893	2975.069	0.012
X_b	740.974	1	740.974	41677.671	0.003
X_c	0.297	1	0.297	16.704	0.153
X_d	386.149	1	386.149	21719.780	0.004
X_f	0.368	1	0.368	20.686	0.138
X_g	18.163	1	18.163	1021.629	0.200
X_h	479.476	1	479.476	26969.152	0.004
X_i	3.948	1	3.948	222.071	0.043
X_j	0.397	1	0.397	22.344	0.133
X_k	27.716	1	27.716	1558.932	0.016
X_l	31.595	1	31.595	1777.111	0.015
X_m	0.122	1	0.122	6.849	0.232
X_n	0.170	1	0.170	9.589	0.199
X_o	18.873	1	18.873	1061.549	0.020
X_p	595.954	1	595.954	33520.679	0.004
X_q	7.064	1	7.064	397.328	0.032
X_r	1.507	1	1.507	84.793	0.069
X_s	0.075	1	0.075	4.195	0.289
Model	2365.741	1	2365.741	7392.563	0.009
Residual value	0.018	1	0.018		
All items	2365.759	1			

Note: The X_a to X_s in the table represents different factors, a p-value less than 0.05 indicates the factor or model is significant, and a p-value less than 0.01 indicates the factor or model is extremely significant

Table 5. Central Composite Design Factor and Level Table for *Trichoderma harzianum* L-8

Serial Number	Factors	Levels				
		-1.414	-1	0	1	1.414
X_1	CMC-Na	6.25	7.5	10	12.5	13.75
X_2	Ammonium sulfate	0.5	1	2	3	3.5
X_3	pH	3.5	4	5	6	6.5
X_4	Lecithin	1.5	2	3	4	4.5

Using Design-Expert 8.0 software (Stat-Ease Inc., Minneapolis, MN), a response surface regression analysis was performed on the experimental results, and a multivariate quadratic equation was obtained, as shown in Eq. 2,

$$Y = 51.679 + 3.653X_1 + 3.523X_2 + 4.101X_3 + 3.141X_4 + 1.666X_1X_2 + 3.539X_1X_3 + 1.213X_1X_4 + 1.602X_2X_3 + 0.148X_2X_4 + 1.338X_3X_4 + 10.493X_1^2 + 10.051X_2^2 + 8.757X_3^2 + 8.489X_4^2 \quad (2)$$

The results of the fitted model ANOVA are presented in Table 7, which shows that the model used for *Trichoderma harzianum* L-8 in this experiment was highly significant (a p-value less than 0.001), which indicated a significant regression relationship between CMCase and the factors.

Table 6. Central Composite Design and Results for *Trichoderma harzianum* L-8

No.	X_1	X_2	X_3	X_4	Y
1	0	0	0	0	52.338
2	0	1.414	0	0	36.915
3	1	1	1	1	30.721
4	-1	-1	-1	-1	10.427
5	0	0	0	0	50.024
6	1	1	1	1	35.197
7	-1	1	-1	-1	7.006
8	0	0	0	0	53.028
9	1	-1	-1	1	12.085
10	0	0	0	0	53.148
11	1	1	-1	1	19.583
12	1	-1	1	1	20.574
13	-1	1	1	1	12.354
14	0	-1.414	0	0	25.095
15	0	0	0	0	53.148
16	0	0	0	0	50.001
17	-1		1	-1	13.663
18	0	0	0	-1.414	28.476
19	0	0	0	0	49.084
20	-1.414	0	0	0	25.711
21	1	-1	-1	-1	3.214
22	1	-1	1	-1	14.908
23	0	0	0	0	53.014
24	1.414	0	0	0	34.532
25	0	0	1.414	0	39.249
26	-1	-1	1	1	10.326
27	0	0	0	0	52.116
28	-1	-1	-1	-1	6.612
29	0	0	0	1.414	39.783
30	1	1	-1	-1	5.474
31	-1	-1	1	1	6.445
32	0	0	-1.414	0	27.539
33	-1	1	-1	1	14.330
34	0	0	0	0	51.642
35	0	0	0	0	52.687
36	0	0	0	0	52.210

The effect of a single factor on the response values was highly significant. Only the two-two interaction between the independent variables in the model X_2X_4 (ammonium sulfate and lecithin) was not significant (the p-value equals 0.7410, which is greater than 0.05), presumably because lecithin had the effect of dispersing insoluble substances. In contrast, ammonium sulfate, as a source of inorganic nitrogen, was easily soluble in the medium, so the interaction between the addition of lecithin and ammonium sulfate was not significant. The interaction between the remaining factors were ranked from largest to smallest as follows: X_1X_3 (CMC-Na addition *vs.* pH), was greater than X_1X_2 (CMC-Na addition *vs.* ammonium sulfate addition), which was greater than X_2X_3 (ammonium sulfate addition *vs.* pH), which was greater than X_3X_4 (pH *vs.* lecithin addition), which was greater than X_1X_4 (CMC-Na addition vs. lecithin addition); all the p-values were less than 0.05. The coefficient of determination of the fitted equation for cellulase R^2 was 99.04% and the predicted coefficient of determination Pred R^2 was 97.20%, which indicated that the model

explained the variance in the analysis and the regression equation fit well. The out-of-fit term, with a non-significant p-value (the p-value equals 0.1026, which was greater than 0.05), indicated that there was not much data out of the fitted equation, and the data was reliable.

Table 7. ANOVA of *Trichoderma harzianum* L-8

Source	Sum of Square	Degrees of Freedom	Mean Square	F-value	p-value
X_1	266.953	1	266.953	85.440	< 0.0001
X_2	248.171	1	248.171	79.429	< 0.0001
X_3	336.355	1	336.355	107.652	< 0.0001
X_4	197.328	1	197.328	63.156	< 0.0001
X_1X_2	44.389	1	44.389	14.207	0.0011
X_1X_3	200.446	1	200.446	64.154	< 0.0001
X_1X_4	23.551	1	23.551	7.537	0.0121
X_2X_3	41.051	1	41.051	13.139	0.0016
X_2X_4	0.351	1	0.351	0.112	0.7410
X_3X_4	28.639	1	28.639	9.116	0.0064
X_1^2	1070.788	1	1070.788	342.712	< 0.0001
X_2^2	982.572	1	982.572	314.478	< 0.0001
X_3^2	762.858	1	762.858	244.157	< 0.0001
X_4^2	700.862	1	700.862	224.315	< 0.0001
Model	11299.044	14	800.075	258.309	< 0.0001
Salvage value	65.614	21	3.124		
Missing item	43.920	10	4.392	2.227	0.1026
Pure error	21.694	11	1.792		
All items	11364.658	35			
Note: R^2 = 99.04%; and Pred R^2 = 97.20%					

The response surface plots (using Design Expert 8.0 software) for the effect of the four significant factors on the enzyme production of *Trichoderma harzianum* L-8 are shown in Fig. 7. The response surface plots showed the interaction between the four significant factors and the range of optimal enzyme production culture conditions. Further analysis *via* the Design Expert 8.0 software yielded the optimal fermentation conditions for *Trichoderma harzianum* L-8, listed as follows: a CMC-Na addition of 10.63 $g \cdot L^{-1}$, an ammonium sulfate addition of 2.22 $g \cdot L^{-1}$, a pH of 5.29, and lecithin addition of 5.18 $g \cdot L^{-1}$; under these conditions, the CMCase could reach 53.401 $IU \cdot mL^{-1}$. To validate the response model to verify the reliability of the predicted enzyme production capacity of the strain, the strain was verified by three sets of parallel tests under optimal conditions. The obtained CMCase value of *Trichoderma harzianum* L-8 was 53.4 $IU \cdot mL^{-1}$, which was close to the predicted value; this indicated that the model constructed *via* the response surface method had a good and stable fitting performance with accurate and reliable data.

The present results can be compared to other studies. For example, Sharma *et al*. (2020) who studied the optimal conditions of strain EF2 cellulase screened from *Eisenia fetida* and obtained enzyme activity up to 35.307 $IU \cdot mL^{-1}$. Li *et al*. (2020) selected a high yielding strain HQ of low-temperature cellulase with enzyme activity up to 32.5 $IU \cdot mL^{-1}$ after the optimization of the fermentation conditions. In comparison, the strain *Trichoderma harzianum* L-8 in this study had a high cellulase production capacity (enzyme activity up to 53.4 $IU \cdot mL^{-1}$), which is worthy of more in-depth research for cellulase production applications.

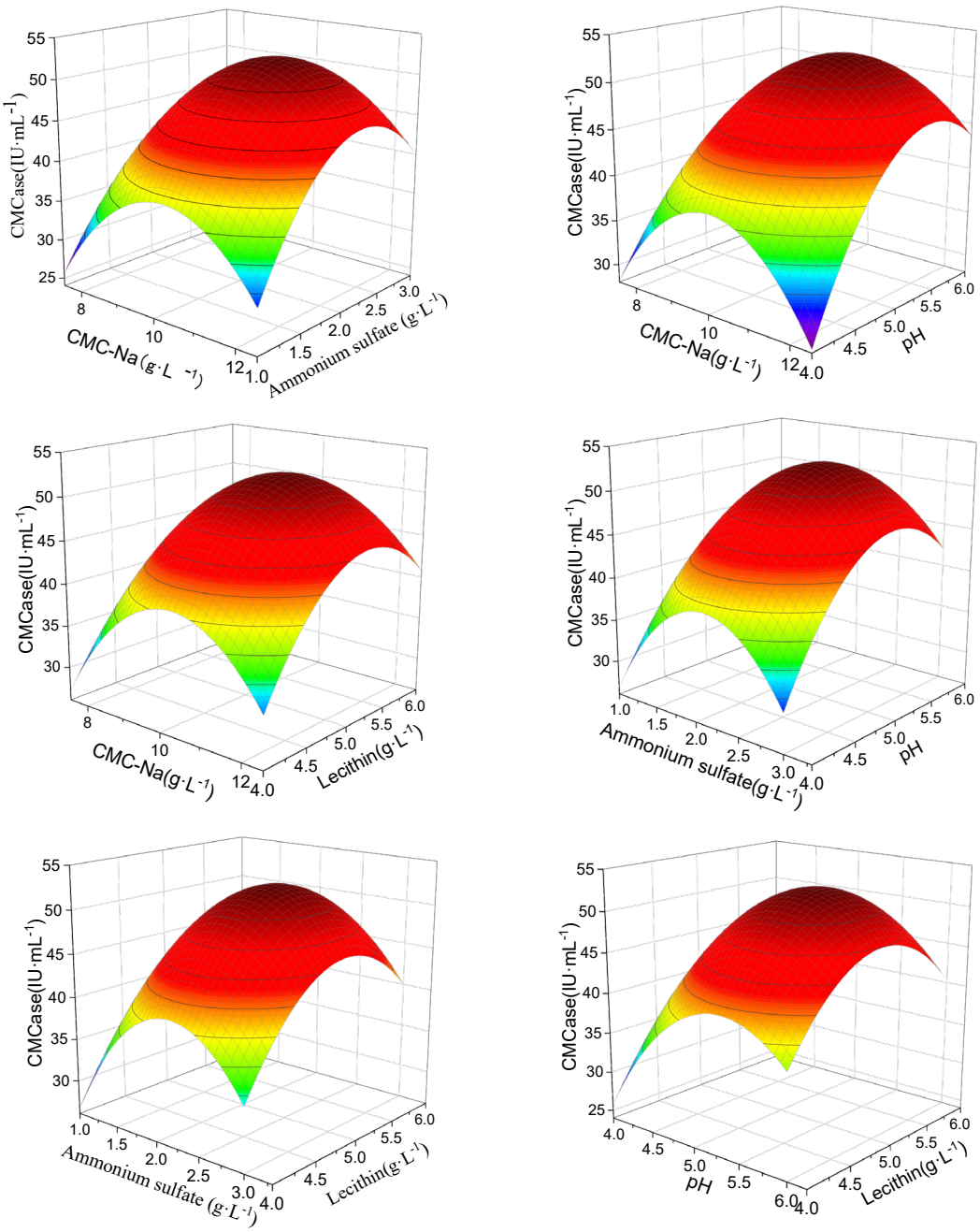

Fig. 7. Effect of the interaction of the two factors on CMCase

Proteomic Analysis

Some of the proteins identified by the proteomic analysis are shown in Table 8, including the cellulose-degrading enzyme proteins, other proteins related to cellular life activities, and some hypothetical proteins. Among them, glycosyl hydrolase family 3-4 and cellobiohydrolase were the enzyme classes involved in cellulose degradation. In cellulose degradation, cellobiose hydrolase primarily acts on the ends of the cellulose chain, which was an important component of the cellulase family and played a leading role in the cellulose degradation process. It had directional hydrolysis of the cellulose chain and could

hydrolyze the β-1,4-glycosidic bond from the non-reducing end or reducing end of the cellulose molecular chain to release cellobiose molecules and destroy the crystalline region of cellulose (Hamid *et al*. 2015). Glycosyl hydrolase family 3-4 belongs to GH3. As β-glucosidase, it participates in cellulose degradation in coordination with exoglucanase and endoglucanase. In addition, while breaking down cellulose, the strain secretes glycoside hydrolase family 92 protein and alpha-galactosidase, which degrades hemicellulose (Fan *et al*. 2021). Isoamyl alcohol oxidase is an enzyme required to degrade lignins (Aulitto *et al*. 2018).

In addition, some non-lignocellulosic enzyme proteins, *e.g.*, aminopeptidase 2 and glucoamylase, were identified. Aminopeptidase 2 is a key enzyme involved in protein processing. Glucoamylase hydrolyzes alpha-1,4 and alpha-1,6 glycosidic bonds in liquid starch and belongs to the group of proteins related to energy and substance. However, it is a protein related to energy and substance metabolism (Xian and Feng 2018). In addition, some proteins were found to be hypothetical proteins *via* mass spectrometry analysis, and their protein functions are still unclear, which may be newly discovered proteins or proteins that have just been entered into the database. The specific functions of these unknown proteins need to be further explored and studied. These proteases were directly or indirectly involved in the synthesis and catabolism of substances related to the degradation of substrates by the low-temperature tolerant strain *Trichoderma harzianum* L-8, which provides a theoretical basis for the further exploration of the mechanism of cellulose degradation by the low-temperature tolerant strains.

Table 8. Identification of the Polypeptides in the Cellulose Bound Fraction of the Crude Enzyme from L-8

No.	GI Number	Score	Mass	Search Result
1	gi|1373354180	146	89553	Glycoside hydrolase family 92 protein
2	gi|818167677	107	77436	Glycosyl hydrolase family 3-4
3	gi|818157837	84	57977	Hypothetical protein THAR02_09247
4	gi|818166643	93	48671	α-Galactosidase
5	gi|818162266	60	62466	Isoamyl alcohol oxidase
6	gi|818158875	62	116597	Aminopeptidase 2
7	gi|818159680	97	92958	Hypothetical protein THAR02_07812
8	gi|818161098	136	70380	Glucoamylase
9	gi|818166915	76	68426	Hypothetical protein THAR02_01558
10	gi|379322974	92	52662	Cellobiohydrolase

CONCLUSIONS

1. In this study, a low-temperature resistant cellulose degrading fungus with high cellulase production was screened from a primitive forest in Daqing using an enrichment-restricted culture technique with straw. This fungus was identified *via* morphological analysis and molecular biology as *Trichoderma harzianum* strain L-8.
2. *Trichoderma harzianum* L-8 had a good activity when reacting under low temperature conditions, had good adaptability to low temperatures, and had good activity at low temperatures.

3. The optimal conditions for enzyme production, determined *via* the response surface method, were as follows: a CMC-Na addition of 10.63 $g \cdot L^{-1}$, an ammonium sulfate addition of 2.22 $g \cdot L^{-1}$, a pH of 5.29, and a lecithin addition of 5.18 $g \cdot L^{-1}$; under these conditions, the CMCase could reach 53.4 $IU \cdot mL^{-1}$
4. The leading enzyme families of *Trichoderma harzianum* L-8 were identified by proteomic analysis. Proteases including glycosyl hydrolase family 3-4 and cellobiohydrolase play important roles in cellulose degradation.

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