Screening and performance of L-14, a novel, highly efficient and low temperature-resistant cellulose-degrading strain

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Abstract: In view of the low bioconversion efficiency of agricultural biomass waste in low-temperature environments in winter, a low-temperature-resistant cellulose-degrading strain, L-14, was successfully screened by restrictive cultures from humus-rich soil in the Daqing Zhalong wetland region. According to morphological observations and 18S rDNA sequence analysis, the cellulose-degrading strain L-14 was identified as a *Neurospora* sp, belonging to fungus. Different parameters, such as temperature, initial pH, carbon, nitrogen and lecithin, were optimized using a single-factor experiment and a response surface methodology (RSM). When the temperature was 16 ℃, the optimal conditions for enzyme production were an initial pH 8.20, 10.45 g/L of bran, 5.28 g/L of yeast powder, and 4.25 g/L of lecithin. The carboxymethyl cellulase (CMCase) activity of strain L-14 was 63.598 IU/mL. Strain L-14 had a high level of cellulose degradation activity, excellent resistance to low temperatures and environmental adaptability, indicating its good application prospects in substrates pretreatment of biogas engineering.

Keywords: low-temperature-resistant, *Neurospora* sp., 18S rDNA sequence, carboxymethyl cellulase, response surface methodology

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1 Introduction

There is much agricultural and forestry matter rich in fiber resources in northern China each year, and disposal of incineration products, storage as landfill or stacking of limbs results in a waste of resources and severe environmental pollution[1,2]. The average temperature is relatively low in Northeast China, most of the mesophilic and thermophilic cellulose degradation strains currently studied have common problems in practical applications, such as low enzyme activity, low-temperature resistance, and poor genetic stability[3,4]. Low-temperature cellulase-producing bacteria usually grow in areas with an average annual temperature ≤5 ℃. Coping with the degradation of physiological, biochemical and other properties caused by low temperature, these organisms synthesize enzymes that perform high-quality catalysis at low temperatures, but their thermal stability is very low[5]. To date, research on cellulose-degrading bacteria has been most concentrated on deep-sea psychrophile and cold-adapted bacteria. Few studies have focused on psychrotrophic cellulose-degrading bacteria[6,7]. However, there are few studies on the screening and isolation of low temperature-tolerant strains in soil and wetlands. In the northeast of China, the low-temperature season lasts about six months. Therefore, studies based on cryophile cellulose-degrading bacteria in the soil of the Daqing wetland and forest area in Heilongjiang Province of China have provided us with crucial information to enrich and expand various resources and apply low-temperature cellulose-degrading bacteria.

The objective of this study was to isolate a novel psychrotrophic cellulose-degrading strain, named L-14, by restriction culture screening, and to determine in detail the morphological observations, the 18S rDNA sequences and the CMC enzyme production conditions, respectively. The cellulose-degrading strain L-14 was identified as a *Neurospora* sp, which belongs to fungus. Results laid a theoretical foundation for the application of low-temperature cellulose-degrading bacteria.

2 Materials and methods

2.1 Preparation of microorganism and media

Potato Dexrose Agar (PDA) medium: 300 g of freshly peeled diced potatoes were taken, and then 1000 mL distilled water was added. The potatoes were boiled for 30 min. The residue was filtered, and the leaching solution was cold to room temperature. Then, 25 g of glucose and 15 g of agar were added to 1000 mL of distilled water at normal pH. Finally, the solution was sterilized at 121 ℃ and 101 kPa for 25 min.

Enrichment medium: 5 g peptone, 5 g filter paper, 0.5 g NaCl, 2 g yeast powder, 2 g $CaCO_3$, and 1000 mL distilled water. The solution was sterilized at 121 ℃ and 101 kPa for 25 min.

Screening medium: 15 g sodium carboxymethyl cellulose, 2 g $MgSO_4$, 2 g KH_2PO_4, 1 g NH_4NO_3, 0.3 g NaCl, 1 g yeast powder, 15 g agar, natural pH, and 1000 mL distilled water. The solution was sterilized at 121 ℃ and 101 kPa for 25 min.

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Liquid enzyme production medium: 15 g sodium carboxymethyl cellulose, 2 g $MgSO_4$, 2 g KH_2PO_4, 1 g NH_4NO_3, 0.3 g NaCl, 2.2 g $MnSO_4$, 0.01 g $FeSO_4$ ·$7H_2O$, 1 g yeast powder, and 1000 mL distilled water. The solution was sterilized at 121 ℃ and 101 kPa for 25 min.

Cellulose medium: 10 g microcrystalline cellulose, 2 g $MgSO_4$, 2 g KH_2PO_4, 1 g NH_4NO_3, 0.3 g NaCl, 2.2 g $MnSO_4$, 0.01 g $FeSO_4$ ·$7H_2O$, 1 g yeast powder, and 1000 mL distilled water. The solution was sterilized for 25 min at 121 ℃ and 101 kPa.

2.2 Isolation and identification of cellulase-producing microorganisms

2.2.1 Isolation and screening of strains

Cultured substrate containing leaching solution was coated and then cultured in an incubator (17±1) ℃ for 7-10 d. Colonies with good growth and different shapes were selected and scored to a single colony. The plate was cultured in an incubator at 5 ℃ for 5-15 d, and well-grown colonies were selected. These plates were stained with Congo red staining and rinsed with NaCl solution. The size of the cellulose degradation hydrolysis zones of colonies was measured, and strains with good degradation ability were selected[8]. Two loops of pure bacteria were inoculated into enzyme production medium and cultured at 17 ℃ in a 120 r/min shake flask for 7 d to determine the CMCase activities. The cellulose culture solution was treated in the same manner as the enzyme-producing medium, and the strains degraded microcrystalline cellulose. Based on the results of the above two experiments, the strains with high cellulase activity and good pure cellulose degradation were selected as the strains to be further investigated.

2.2.2 Identification of strains

The low-temperature cellulose-degrading bacteria were inoculated in beef extract peptone medium by the plate scribing method, and the cold-adapted cellulose-degrading bacteria were cultured at a constant temperature for 48 h. Then, the colony morphology was observed. Physiological and biochemical identification was performed by referring to the Fungal Identification Manual and the Flora Fungorum Sinicorum[9]. In addition, 18S rDNA sequence analysis method of molecular biology was used, and the universal fungal primers were synthesized by the Shanghai Shenggong Co., Ltd. The specific reaction system and conditions were previously reported[10]. The 18S rRNA gene sequences were compared with other 18S rRNA gene sequences available in the GenBank database, and a phylogenetic tree was constructed using MEGA7.0 software[11].

2.3 Methods of enzyme assay

Filter paper enzyme activity (FPA) determination method: take 1 mL of crude enzyme solution diluted 5 times into a 25 mL stoppered test tube, add 2.5 mL of citric acid-sodium citrate buffer (pH 4.8), and use 50 mg of filter paper as the substrate to be hydrolyzed. After 30 min of incubation in a water bath at 30 ℃, the reaction was terminated by adding 3,5-dinitrosalicylic acid (DNS) to 3 mL of DNS coloring solution. After boiling for 10 min in a boiling water bath, the test tube was cooled to room temperature by cold water. The absorbance was measured at 540 nm by an ultraviolet spectrophotometer, and the reducing sugar content calculated based on a glucose standard curve was used to calculate the FPA (Table 1). The amount of enzyme required to produce 1 μg of glucose (hydrolyzed product) per min is defined as one unit of enzyme activity, an IU.

CMCase activity assay: Cellulase activity was determined using international standard methods recommended by the International Association of Theoretical and Applied Chemistry (IUPAC)[12].

Enzyme activity calculation method:

$$X=1000\times A\times n/180/T \quad (1)$$

where, X is the units of enzyme activity, IU/Ml; A is the glucose content (mg/mL) calculated by experimental determination of OD values and a standard curve; n is the dilution factor; and T is the reaction time, min.

Table 1 Standard factory glucose solution preparation

Tube number	1	2	3	4	5	6
Glucose standard solution/mL	0	0.2	0.4	0.6	0.8	1
Distilled water/mL	1	0.8	0.6	0.4	0.2	0
DNS reagent/mL	3	3	3	3	3	3
Glucose concentration/mg mL^{-1}	0	0.2	0.4	0.6	0.8	1.0

Note: $y=7.2497x-0.2765$, $R^2=0.9991$.

2.4 Optimization of conditions for enzyme activity

2.4.1 Single-factor experiment optimization

pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were used in the experiment groups. pH was used as a single variable in the experiment groups. Bran, corn flour, cellulose, filter paper and carboxymethyl cellulose-Na were used as the sole carbon source in the experimental groups. Ammonium sulfate, peptone, yeast powder, soy flour and urea were used as nitrogen sources in the experimental groups. All experiments were performed in triplicate, and after 9 d of culture at (17±1) ℃, the cellulase activity was measured.

2.4.2 Interactions among carbon

Based on the results of single-factor experiments, a Plackett-Burman (PB) approach was used to design the experiments, analyze the experimental data and investigate the multifactor interaction of carbon source, nitrogen source, initial pH, lecithin addition, culture temperature and culture time. When the number of factors in the test design is less than 19, an appropriate number of empty items are set in the test design, and each factor has two levels, high and low, which are respectively denoted "+1" and "–1".

2.4.3 Central Composite Design (CCD) test design

The independent variables were the amounts of bran, yeast powder, and lecithin added and pH, which were mainly used by the PB test as independent variables. The CCD method was used to optimize the enzyme production conditions, and CMCase activity was the only response variable and was used to verify whether the model was reliable. The experimental design factors are shown in Table 2. Experimental data from the CCD test were analyzed and fitted according to the second-order polynomial in Equation (1), which included direct effects and interaction effects for each variable:

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

where, Y is the enzyme activity; a_0 is the offset; a_i is the linear offset; a_{ij} is the second-order offset; X_i is the value of each factor[13].

Table 2 CCD factor and level table for strain L-14

Number	Factor	Level				
		–1.414	–1	0	1	1.414
X_1	Wheat bran	6.3	7.5	10	12.5	13.8
X_2	Yeast Powder	2.5	3.0	4	5.1	5.5
X_3	pH	6.5	7.1	8	9.1	9.5
X_4	Lecithin	2.5	3.0	4	5.0	5.5

3 Results and analysis

3.1 Separation and screening of low-temperature-resistant cellulose-degrading bacteria

The temperature was controlled at (17±1)℃. After 7-9 separations and purifications, Congo red staining was compared with the size of hydrolysis zones, the degradation of microcrystalline cellulose and the enzyme activity of liquid culture. A total of 10 low-temperature-resistant strains were screened. The strains with high enzyme activity are shown in Table 3. The CMCase activity of L-8, L-11 and L-14 were highest. A strain with the most CMCase activity (35.457 IU/mL), designated L-14, was selected for characterization and further analysis. The degradation of microcrystalline cellulose was obvious, and the diameter of the hydrolysis zone was 8.6 cm. Considering the three screening results, the selected strain L-14 was identified as the subject for further research.

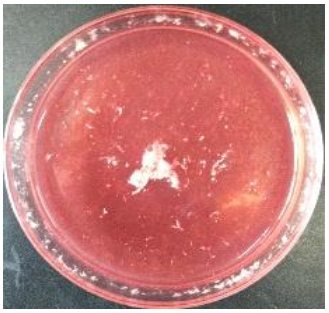
Table 3 Strain screening results

Strain	Transparent circle diameter/cm	Colony diameter /cm	Degradation of microcrystalline cellulose	CMCase /IU mL^{-1}
L-1	2.75	1.4	+	6.253
L-2	6.2	2.5	+++	23.102
L-3	4.05	2.3	++	11.291
L-4	5.4	2.0	+++	18.102
L-5	3	1	+	3.621
L-6	6.7	6.55	+++	23.704
L-7	5.58	3.15	++++	26.067
L-8	8.6	8.4	++++	33.825
L-9	4.45	2.6	++	11.898
L-10	4.75	2.9	++	12.765
L-11	7.68	2.45	++++	39.266
L-12	3.75	2.5	++	9.033
L-13	3.3	1.85	+	9.245
L-14	8.6	8.0	++++	35.457

Note: "+" indicates degradation of microcrystalline cellulose, "+" indicates the growth of the strain and that microcrystalline cellulose showed signs of degradation; "++" indicates weak degradation; "+++" indicates moderate degradation; and "++++" means that a good level of degradation occurs.

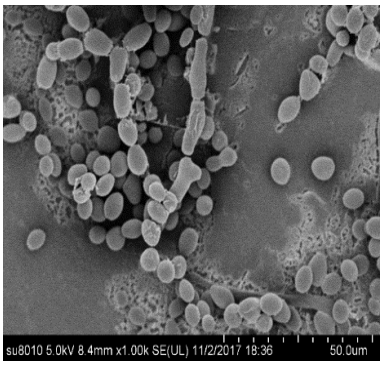
3.2 Morphology of low-temperature-resistant cellulose-degrading bacteria L-14

The hydrolysis circle in the L-14 Congo red staining medium was large (Figure 1a), and there were loose hyphae that were pale pink, separated and branched on the screening medium. The colonies were net-like with no obvious edges, the hyphae were unstable, and the spores were highly diffusive, as shown in Figure 1b. As shown by the microscope in Figure 1c, the conidia were connected in a chain shape and grew directly from the hyphae. The spores were easily separated from each other after maturity, and the individual spores were dispersed in a free state on the medium surface. The spores were oval and had a smooth surface. According to colony morphology, the colony was preliminarily identified as a *Neurospora* sp. strain.



a. The picture shows the characteristics of the strain's Congo red-stained hydrolysis circle

b. The growth morphology of the strain was connected to the screening medium



c. The picture shows the hyphae and spores observed under scanning electron microscopy

Figure 1 Morphological characteristics of strain L-14

3.3 Phylogenetic analysis based on 18S rDNA gene sequence

Molecular biological identification of L-14 strain: The strain's 18S rDNA was amplified, and a T vector was ligated and sequenced to obtain a 1050 bp sequence. The 18S rDNA gene sequences were compared with 18S rDNA gene sequences available in GenBank, and a phylogenetic tree was constructed by applying the neighbor-joining method using the MAGA 7.0 program. The L-14 strain was assigned to *Neurospora* sp. according to 98% similarity in their 18S rDNA gene sequences. The phylogenetic tree was constructed based on the 18S rDNA sequences (Figure 2).

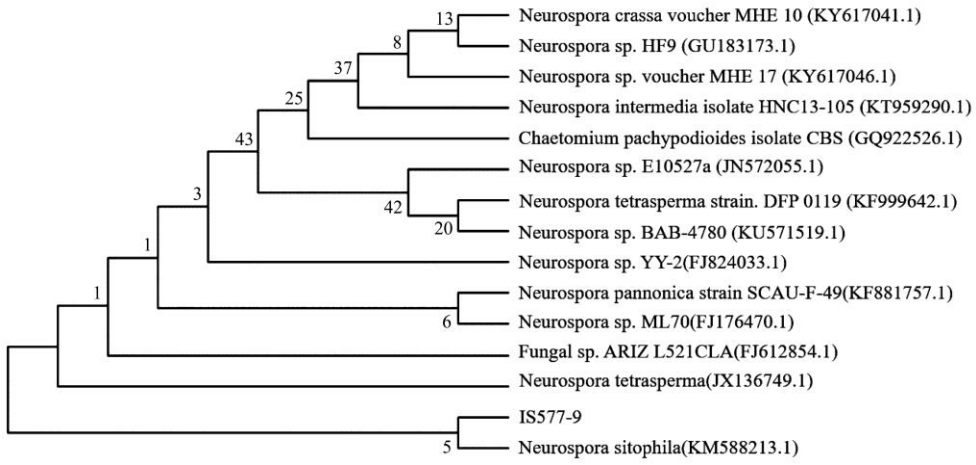


Figure 2 Phylogenetic tree with strain L-14

3.4 Enzymatic properties of strain L-14

3.4.1 Optimum temperature and thermal stability

The effect of temperature on the activities of cellulases and filter paper enzymes produced by strain L-14 is shown in Figure 3. Both CMCs and filter paper enzymes produced by strain L-14 have their highest enzyme activity at 40 ℃. When the temperature was between 20 ℃-50 ℃, the relative enzyme activity of cellulases and filter paper enzymes was maintained above 70%; the relative enzyme activity was also maintained above 50% at 5 ℃. CMCase activity increased with increasing temperature between 5 and 40 ℃ until an optimum was reached. In contrast, a further increase in temperature above 40 ℃ caused CMCase activity to gradually decrease. The cellulase and filter paper enzymes produced by strain L-14 showed high enzyme activity between 25 ℃-50 ℃. Both enzymes can adapt to low and high temperatures.

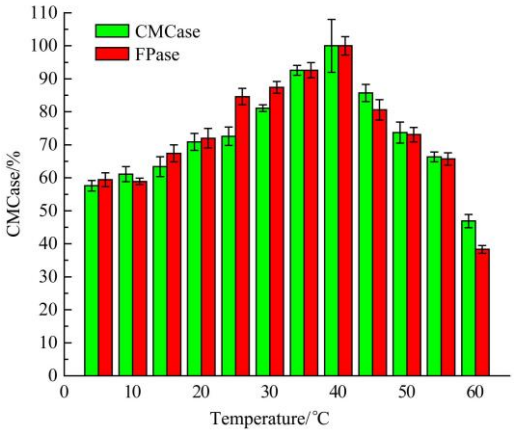


Figure 3 Effect of temperature on the enzyme activity of strain L-14

The relative stability of the cellulase activity of strain L-14 at different temperatures for 0.5-2 h is shown in Figure 4. After 2 h of storage at 5 ℃, more than 90% of the CMCase activity was retained, and the cellulase stability was the highest in this sample. The cellulase relative activity was maintained above 80% at the temperature of 5 ℃-20 ℃. The CMCase activity decreased as the temperature increased beyond above 30 ℃. The relative enzyme activity was 44% after 2 h of incubation at 30 ℃ and almost completely lost after 2 h at 50 ℃. This result was consistent with the low-temperature enzyme properties.

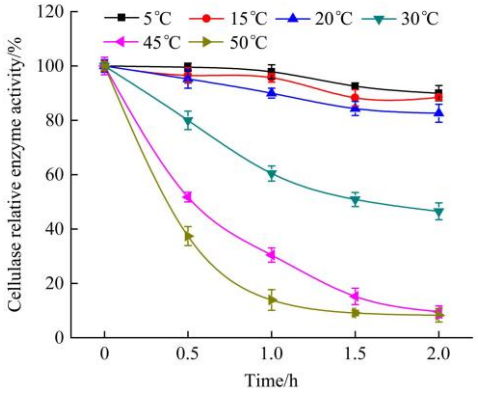


Figure 4 Effect of storage temperature on the stability of L-14 cellulase relative enzyme activity

3.4.2 Effect of pH on enzyme activity

Figure 5 presents the CMCase activity of the L-14 fermentation cultures at different initial pH values. CMCase from strain L-14 had the highest activity at pH 5.0. The enzyme was approximately 70% of its maximum activity at pH 4.0-9.0. In addition, more than 80% of the relative activity was maintained at pH 5.0-8.0. The pH-based trends in CMCase activity and FPA were basically the same for strain L-14. These two types of enzymes of strain L-14 prefer to act in a neutral alkaline environment.

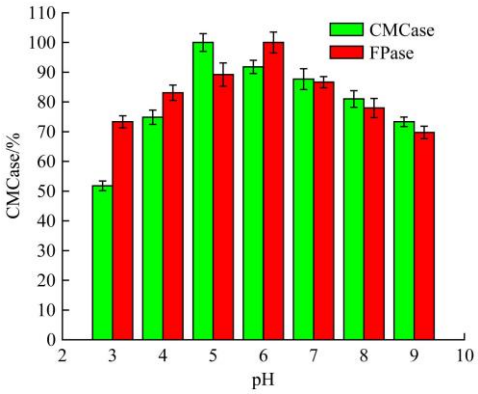


Figure 5 Effect of pH on the stability of L-14 enzyme activity

3.4.3 Effect of metal ions on cellulase enzyme activity

The effect of adding metal ions to the crude enzyme solution of strain L-14 is shown in Figure 6. Ca^{2+}, Na^{+}, Co^{2+} and a small amount of Fe^{2+} promoted the cellulase activity of strain L-14. The enzyme activity was obvious when the concentration of Co^{2+} was low. When 0.6 mg/L Co^{2+} was added, the relative activity of cellulase was the highest, and the highest value was 135%. When the Co^{2+} concentration was 0.3 mg/L, the relative activity of cellulase was 117%. The CMCase activity decreased rapidly as the amount of Co^{2+} increased beyond 0.6 mg/L, indicating that the activity of the enzyme was obvious at low concentrations. The promotion by Na^{+} and Ca^{2+} ions was relatively stable. When the concentration of Na^{+} was 0.3 mg/L, the relative cellulase activity was 127%; when the Ca^{2+} concentration was 0.6 mg/L, the relative cellulase activity was 121%. The relative enzyme activity was 97% when 0.3 mg/L Fe^{2+} was added. When the Fe^{2+} concentration was increased to 0.6 mg/L, the relative enzyme activity increased to 117%, followed by a downward trend with further increasing concentration. Mg^{2+} and Mn^{2+} ions had a negative effect on the enzyme activity that was enhanced with further addition of Mg^{2+} and Mn^{2+}. When the Mg^{2+} and Mn^{2+} concentration reached 1.2 mg/L, the corresponding relative enzyme activities were 62% and 48%, respectively. CMCase activity in the fermentation cultures with various metal ions was in order of Co^{2+}>Na^{+}>Ca^{2+}>Fe^{2+}>Mg^{2+}>Mn^{2+}.

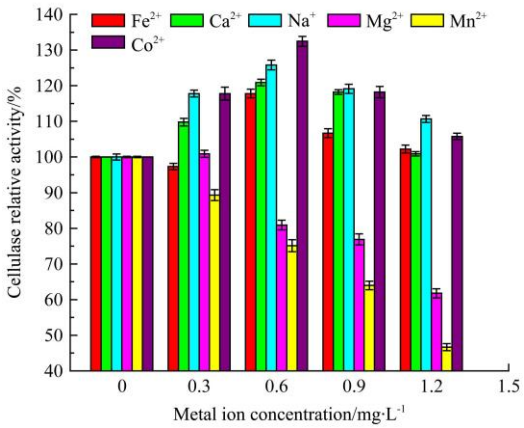


Figure 6 Effect of metal ions on strain L-14 CMCase

3.4.4 Optimization of enzyme production

The optimum operating temperature of the CMCase was

assessed from 10 ℃ to 20 ℃ with an interval of 2 ℃. The results are shown in Figure 7. The strain L-14 CMCase activity first increased and then showed a downward trend. The curve decreased slightly between 16 ℃-18 ℃, and with increasing temperature, the activity of CMCase decreased significantly. The cultures grown at 16 ℃ exhibited the greatest CMCase activity (33.504 IU/mL) and the best growth, indicating that strain L-14 produced the most cellulase when cultured at 16 ℃-18 ℃. As the pH increased, the CMCase activity of strain L-14 gradually increased (Figure 8), and reached a maximum of 41.241 IU/mL at pH 8.0. When the pH value was between 8.0 and 9.0, the CMCase activity decreased gently, indicating that L-14 could grow in an alkaline environment and that the enzyme produced was adapted to a wide range of initial medium pH values. Therefore, the initial pH value suitable for the enzyme is between 7.0 and 9.0.

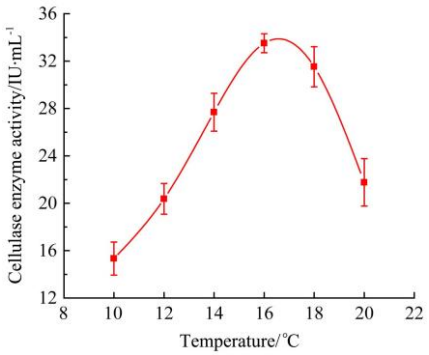


Figure 7 Effect of culture temperature on the cellulase activity of strain L-14

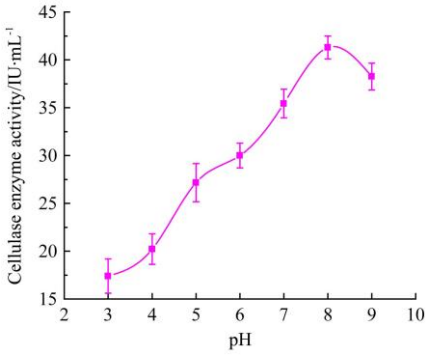


Figure 8 Effect of initial pH value on the cellulase enzyme activity of strain L-14

The effects of carbon sources on L-14 enzyme activity are shown in Table 4. The result showed that L-14 has high CMCase activity cultured in fermentation medium with sodium carboxymethyl cellulose and reached its highest CMCase activity when 7.5-12.5 g/L bran was added. The optimal concentration of bran in the medium was 10 g/L, where the CMCase activity reached 43.445 IU/mL.

Table 4 Effect of carbon sources on cellulase produced by strain L-14

Addition /g Ł$^{-1}$	Variety carbon sources				
	Wheat bran	Corn meal	Cellulose	Filter paper	CMC-Na
	CMCase/IU mL^{-1}				
5	20.372	10.066	10.916	13.873	26.371
7.5	32.012	12.693	16.530	15.950	37.074
10	43.445	16.131	21.041	14.515	29.741
12.5	34.950	9.619	17.182	9.253	22.802
15	17.564	4.226	14.1895	8.108	19.445

The effects of nitrogen sources on L-14 enzyme activity are shown in Table 5. The organic nitrogen is more beneficial to enzyme production by strain L-14 than the inorganic nitrogen sources ammonium sulfate and urea. When the organic nitrogen sources of peptone, yeast powder and soybean powder were present at 4-5 g/L, the CMCase activity of strain L-14 reached its corresponding maximum values of 16.602 IU/mL, 49.790 IU/mL and 37.885 IU/mL, respectively, demonstrating that the enzyme-producing effect of strain L-14 was preferable when the yeast powder concentration was 3-6 g/L or the soybean powder concentration was 4-6 g/L.

Table 5 Effect of nitrogen sources on cellulase produced by strain L-14

Addition /g Ł$^{-1}$	Variety carbon sources				
	Peptone	Yeast powder	Ammonium sulfate	Soybean powder	Urea
	CMCase/IU mL^{-1}				
1	5.694	8.324	18.467	17.010	7.241
2	13.148	25.126	22.935	17.780	8.204
3	15.784	34.484	14.773	15.025	6.167
4	16.602	47.320	13.596	37.885	3.870
5	12.086	49.791	10.122	32.368	3.056
6	7.482	40.826	9.621	19.054	1.889

3.5 Screening for significant factors

3.5.1 Plackett-Burman design

A PB design was used to optimize key factors influencing enzyme-producing effect of strain L-14 with CMCase activity as the response value. Variance analysis of the PB test showed that the four factors most influential to strain L-14 CMCase activity were ranked as X_8 (initial pH) > X_2 (bran added) = X_4 (yeast powder added) > X_{16} (lecithin added). As shown in Table 6, the p values were all less than 0.01, indicating that these four factors had a significant impact on CMCase activity, and were used as the main factors for CCD optimization experiments.

Table 6 PB ANOVA results for strain L-14

Factors	Sum of square	Degrees of freedom	Mean square	*F* value	*p* value
X_1	5.200	1	5.200	450.848	0.030
X_2	278.693	1	278.693	24161.897	0.004
X_3	32.378	1	32.378	2807.102	0.012
X_4	345.150	1	345.150	29923.525	0.004
X_5	3.105	1	3.105	269.164	0.039
X_6	0.214	1	0.214	18.555	0.145
X_7	8.763	1	8.763	759.764	0.023
X_8	412.246	1	412.246	35740.520	0.003
X_9	6.787	1	6.787	588.449	0.026
X_{10}	11.625	1	11.625	1007.821	0.020
X_{11}	0.388	1	0.388	33.654	0.109
X_{12}	15.870	1	15.870	1375.880	0.017
X_{13}	21.930	1	21.930	1901.254	0.015
X_{14}	0.575	1	0.575	49.884	0.090
X_{15}	7.729	1	7.729	670.048	0.025
X_{16}	210.997	1	210.997	18292.858	0.005
X_{18}	1.099	1	1.099	95.273	0.065
X_{19}	4.676	1	4.676	405.356	0.032
Model	1367.425	18	75.968	6586.214	0.009
Residue value	0.012	1	0.012		
All items	1367.437	19			

Note: X_1-X_{19} in the table indicate different factors, p<0.05 indicates that the factor or model is significant, and p<0.01 indicates that the factor or model is extremely significant.

3.5.2 Optimization of the response surface experiment

The response surface experimental design and its results are shown in Table 7. Design-Expert 8.0 software was used to carry out a response surface regression analysis of the experimental results to obtain optimal enzyme production conditions for X_2 (bran addition), X_4 (yeast powder addition), X_8 (initial pH), and X_{16} (lecithin addition). The multivariate quadratic equation was as follows:

$$Y = 60.028 + 3.057X_1 + 3.458X_2 + 2.854X_3 + 4.173X_4 + 2.333X_1X_2 + 1.333X_1X_3 - 1.709X_1X_4 + 1.126X_2X_3 + 1.258X_2X_4 + 1.618X_3X_4 - 9.852X_1^2 - 8.002X_2^2 - 9.322X_3^2 - 9.012X_4^2$$

Table 7 CCD and results for strain L-14

Test number	X_1	X_2	X_3	X_4	Y
1	0	1.414	0	0	46.310
2	–1	–1	–1	–1	14.997
3	1.414	0	0	0	41.926
4	–1.414	0	0	0	36.054
5	–1	1	1	1	34.369
6	0	0	0	0	57.590
7	–1	1	1	–1	15.597
8	1	–1	1	1	26.591
9	0	0	0	0	61.594
10	0	0	0	–1.414	34.795
11	0	0	0	0	63.215
12	1	1	–1	1	28.671
13	–1	–1	1	–1	14.911
14	1	1	1	–1	34.449
15	0	0	1.414	0	40.250
16	1	–1	–1	1	18.877
17	1	1	1	1	45.637
18	0	0	–1.414	0	39.850
19	0	–1.414	0	0	39.071
20	0	0	0	1.414	46.545
21	0	0	0	0	60.384
22	–1	1	–1	1	24.483
23	–1	1	–1	–1	14.431
24	0	0	0	0	59.625
25	0	0	0	0	60.304
26	0	0	0	0	62.005
27	0	0	0	0	59.326
28	1	1	–1	–1	25.203
29	0	0	0	0	62.590
30	1	–1	1	–1	22.683
31	1	–1	–1	–1	17.689
32	–1	–1	1	1	27.403
33	0	0	0	0	61.974
34	0	0	0	0	58.473
35	0	0	0	0	58.576
36	–1	–1	–1	1	20.769

Note: The numerals 1-30 represent 30 different experimental conditions, and Y represents carboxymethyl cellulose activity (IU/mL).

The regression analysis of the equation is shown in Table 8. The fit of the model used for strain L-14 resulted in $p<0.01$, which suggested that the regressed fit of the equation was extremely significant; thus, there is a significant regression relationship between CMCase activity and these independent variables. The R^2, predictive R^2 and regression equation fitting values were 98.93%, 96.07% and 0.0961, respectively, which suggested that this model was of high reliability and well reflected the actual situation. A 3-D stereogram was used to display the responses to the interaction between the four major influencing factors of *Neurospora* sp. L-14 and the range of optimal values is 9.6-11.4 g /L of bran, 4.9-5.6 g/L of yeast powder, pH 7.9-8.8 and 3.9-4.5 g/L of lecithin, respectively[14,15]. According to the F value and the degree of the slope of the response surface (Figure 9), the influence of each factor on the CMCase activity was in the order X_1X_2 (the amount of bran added and the amount of yeast powder added) > X_1X_4 (the amount of bran added and the amount of lecithin added) > X_3X_4 (pH and lecithin addition amount) > X_1X_3 (bran addition amount and pH)> X_2X_4 (amount of soybean powder added and amount of lecithin added).

Table 8 Analysis of variance (ANOVA) of strain L-14

Source	Degrees of freedom	Sum of square	Mean square	F value	p value (Prob>F)
X_1	186.924	1	186.924	35.633	<0.0001
X_2	239.138	1	239.138	45.586	<0.0001
X_3	162.942	1	162.942	31.061	<0.0001
X_4	348.251	1	348.251	66.386	<0.0001
X_1X_2	87.049	1	87.049	16.594	0.0005
X_1X_3	28.409	1	28.409	5.415	0.0300
X_1X_4	46.704	1	46.704	8.903	0.0071
X_2X_3	20.268	1	20.268	3.864	0.0627
X_2X_4	25.301	1	25.301	4.823	0.0395
X_3X_4	41.861	1	41.861	7.980	0.0101
X_1^2	943.962	1	943.962	179.943	<0.0001
X_2^2	622.733	1	622.733	118.709	<0.0001
X_3^2	845.130	1	845.130	161.104	<0.0001
X_4^2	789.856	1	789.856	150.567	<0.0001
Model	10222.744	14	730.196	139.194	<0.0001
Salvage value	110.164	21	5.246		
Missing item	74.325	10	7.433	2.281	0.0961
Pure error	35.838	11	3.258		
All items	10332.908	35			

Note: R^2 = 98.93%, Pred R^2 = 96.07%.

3.5.3 Determination of optimal conditions and verification of recovery models

When the culture temperature is 16 ℃, the optimum conditions for the production of *Neurospora* sp. L-14 were as follows: 4.25 g/L lecithin, 10.45 bran g/L, 5.28 g/L yeast powder and initial pH 8.20. Under these conditions, the theoretical value of CMCase activity was 61.5978 IU/mL. The actual experimental conditions were 4 g/L lecithin, 10 g/L bran, 5 g/L yeast powder, and initial pH 8.0. Under these conditions, the L-14 CMCase activity of *Neurospora* sp. 14 was obtained: 60.590 IU/mL, which was very close to the theoretical value.

At present, many of the cellulose-degrading bacteria screened by conventional methods had optimum CMCase activity at 45 ℃-65 ℃, shown in Table 9, which have better cellulose degradation capacity. These bacterial include *Penicillium oxalicum* QSH3-3, *Streptomyces* L1 and Mutant strain CNY01. Compared with the above bacterial, strain L-14 screened by our laboratory has a higher tolerance to low temperature and better CMCase production. In our study, optimal conditions for enzyme production and low-temperature-resistant characteristics of strain L-14 were in detail investigated, and the CMCase activity reaches 35.457 IU/mL

at 15 ℃, 80% of the relative activity was maintained at pH values ranging from 6.0 to 8.0, demonstrating that strain L-14 exhibits high thermal stability and substantial pH stability and retains its activity over a wide pH range.

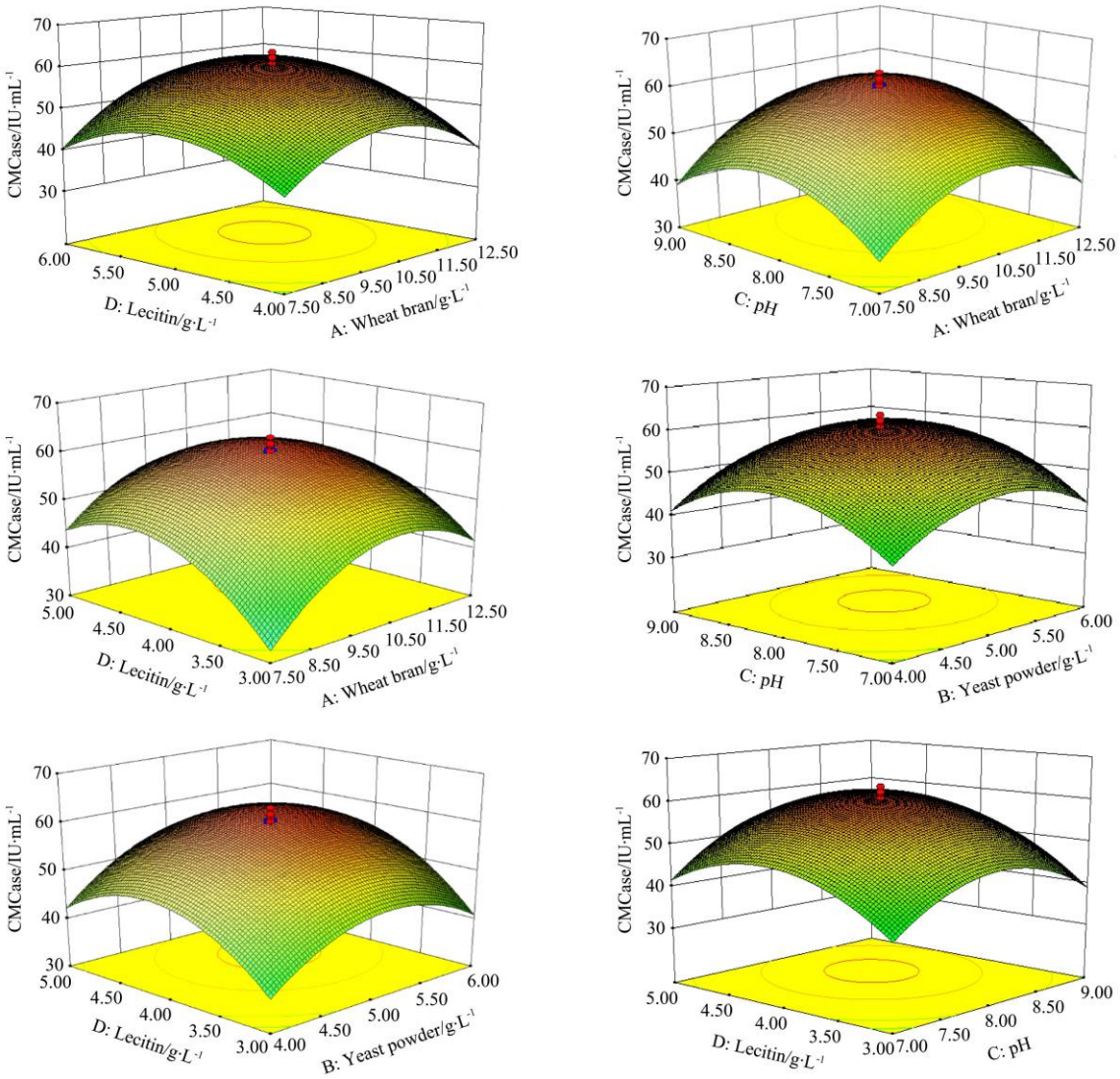


Figure 9 Response diagram for the interaction of two factors on the enzyme production of *Neurospora* sp. L-14

Table 9 Comparison of cellulose degradation characteristics

Low temperature tolerance strain	Optimum fermentation conditions	CMCase	Reference
Neurospora sp. L-14	wheat bran 10.45 g/L yeast powder 5.28 g/L pH 8.20 lecithin 4.25 g/L	61.598 IU/mL	-
Penicillium oxalicum QSH3-3	corn stalk powder 5 g/L Ammonium sulfate 2 g/L Initial pH 7.0 culture temperature 30 ℃	33.000 IU/mL	Liu et al.[16]
Streptomyces L1	-	46.59 IU/mL	Zhang et al.[17]
Mutant strain CNY01	straw powder 12 g/L wheat bran 7 g/L ammonium sulfate 5 g/L potassium dihydrogen phosphate 5.5 g/L	108.55 IU/mL	Chen et al.[18]

4 Conclusions

Low temperature is always one of the key restrictive factors confining lignocelluloses bioconversion in wastes disposal yield. So the research on low temperature-resistant bacteria has been greatly concerned in recent years. In this study, by restricting culture method, a strain L-14 was obtained from different soil samples of the Northeast cold region in China, and identified as a fungus, which shows a 98% similarity of 18S rDNA gene sequences with that of *Neurospora* sp. In this study, four significant factors including lecitin, pH, bran and yeast were screened by plackett-burman design on the basis of single factor experiment, and then the optimal fermentation conditions were determined by box-behnken central combination design experiment. The results showed that the optimal enzyme production conditions of *Neurospora* l-14 were as follows: 10.45g /L of bran, 5.28g /L of yeast, pH of 8.20, and 4.25g /L of lecithin. Under this optimization condition, CMCase reached 61.598 IU/mL, 1.74 times the previous optimization. The research provides important strain resources for exploring the lignocellulose degradation mechanism of low temperature-resistant microbials and solving the in-situ return and comprehensive utilization of crop straw in the cold region of Northeast in China.

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