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Original Research Paper

Screening of Dominant Cellulose-Degrading Microbe in Humus and Optimisation of its Enzyme Producing Conditions and Application Optimization of Enzyme Production Conditions and Evaluation of Efficient Straw Degradation by *Penicillium oxalicum*

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Abstract: The slow degradation of returned straw adversely affects seedling survival rate. To shorten the degradation time of lignin in maize straw in warm temperate regions, a cellulolytic microbial strain that can secrete polymer with high enzymatic activity was isolated from the humus, which helped to identify an effective strain to expedite the decomposition of returned maize straw. Using the molecular biology techniques of primary screening and re-screening using selective media, the dominant cellulolytic microbes in the humus were identified as *Penicillium oxalicum* (*P. oxalicum*) (KY781806.1). Inoculum size, initial pH, fermentation time, and temperature were optimized to increase cellulase activity. The activities of sodium Carboxymethyl Cellulose (CMC) and Filter Paper (FPA) were increased by 23.04 and 25.10%, respectively. The ability of *P. oxalicum* to utilize cellulose was also demonstrated using a filter paper degradation test and maize straw utilization experiment. Finally, the results of a field test showed that the addition of *P. oxalicum* X_5 caused the returned straw to degrade completely within 30 days.

Keywords: Humus, Polymer, Cellulolytic Microbial Species, Purification, Function, Enzyme

Introduction

Crop straw contains a large amount of organic matter and nutrients. Organic matter is dominated by cellulose, a large polysaccharide composed of glucose. Cellulose is insoluble in water and organic solvents and is the main component of plant cell walls. They are typically found in hemicellulose, pectin, and lignin. It is one of the most widely distributed and abundant polysaccharides in nature, accounting for more than 50% of the carbon content in plants worldwide. Natural cellulose, on the other hand, is difficult to degrade due to its complexity, making cellulose in straw one of the world's most abundant and underutilized renewable resources. In recent years, the Government of China has actively supported and promoted the use of crop straw as a resource in various ways. These interventions have led to the primary utilization of crop straw as fertilizer, feed, and fuel and its supplementary utilization as a base and raw material (Charaya, 1999; Gong *et al*., 2020). Accordingly, approximately 60% of the straw is returned to the field as fertilizer.

With the development of agricultural mechanization, maize or wheat straw is mechanically crushed before being turned over, buried, mulched, and returned to the field. Because the mechanized crushing and return of straw to the field can improve soil water and fertilizer retention performance conveniently and quickly, it is popular with many plantation households. In fact, mechanically crushed straw accounts for 95.37% of the crop straw returned to fields (Chen *et al*., 2017).

However, in China, this practice involves returning straw directly to the field after crushing, making it difficult for lignocellulosic components of crop straw to

degrade under natural conditions. Given China's geographic location in the warm temperate zone, maize-wheat rotations are practiced in most areas and maize straw is typically returned to fields as whole plants. However, the decomposition of maize straw will not be complete until March-April of the following year. This seriously affects the normal rooting of wheat seedlings, diminishes their resistance to drought and frost, and increases the risk of drought and frost death (Fan and Wu, 2020).

To reduce the time required for straw decomposition in the field, biotechnological methods have been employed by researchers in China and elsewhere to promote the decomposition of returned straw. Among the selected putrefactive agents, high cellulase-producing strains mainly comprise fungi of the genera *Trichoderma*, *Aspergillus*, and *Penicillium* (Saratale and Oh, 2011; Cadete *et al*., 2017). However, the undesirable effects of certain factors such as straw maturation time, fertility, and biotope type have hindered the widespread use of these agents. Moreover, these strains are more commonly used in animal feed processing, as well as microbial fermented feed.

Thus, in this study, to shorten the degradation time of lignin in maize straw in warm temperate regions, a cellulolytic microbial strain that can secrete polymer with high enzymatic activity was isolated from the humus, which helped to identify an effective strain to expedite the decomposition of returned maize straw.

Materials and Methods

Sampling and Processing of Humus

The experimental material was taken from the humus layer between the annual leaf litter and the soil layer of poplar trees at the southern foot of Baiyun Mountain, song county, Henan province, China (111°24′-112°22′ E, 33°35′-34°21′ N). The temperature in this area is perennially below 25°C. After aseptic collection, humus samples (10.0 g) were diluted with 100.0 mL of sterile saline, mixed, and left to stand. The supernatant was then withdrawn and set aside.

Screening of the Dominant Cellulolytic Microbial Species in the Samples

Next, 200 μL of the supernatant was evenly spread onto a nutrient agar medium. This process was repeated three times. The nutrient agar medium was then incubated for 48 h at a constant temperature and humidity. Individual colonies were then streaked on the primary screening medium (Ulrich *et al*., 2008) and incubated for 48 h in a constant temperature and humidity incubator. The plates were removed and the colonies were carefully scraped off with a scalpel. Care was taken during this process to ensure that the medium remained intact. Then, 1 g L^{-1} Congo red solution was added for staining and left for 10 min before being poured off. In the next step, 1 mol L^{-1} of sodium chloride solution was added for decolorization for 10-15 min. Observations were made to check the appearance of transparent rings around the colonies. Strains with transparent rings around the colonies were selected.

Selected plants were inoculated using the spot inoculation method on a secondary screening medium (Ulrich *et al*., 2008) and incubated in a constant temperature and humidity incubator. After 3, 5, and 7 days of incubation (Saratale and Oh, 2011), the diameter of each individual colony (D) and the diameter of the surrounding Congo red-stained ring (d) were measured. The relative activity of the enzyme, ϕ (= d/D) was calculated. Colonies showing high relative enzyme activity were selected.

Colony Characteristics and Cell Morphology of the Dominant Cellulolytic Microbial Species

After 24 h of incubation, the colony characteristics of the strains with a higher relative enzyme activity on nutrient agar medium were observed and the respective colonies were photographed. Gram staining was performed and cell morphologies of the selected strains were observed under a compound light microscope and photographed.

Identification of the Dominant Cellulolytic Microbial Species Using Gene Amplification and Sequencing

The cetyltrimethylammonium bromide method was performed using the Internal Transcribed Spacer (ITS) universal fungal primers ITS_1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS_4 (5'-TCCTCCGCTTATTGATATG C-3') (Wang *et al*., 2019). Genomic DNA extracted using the above method was used as a template for Polymerase Chain Reaction (PCR) amplification. Here, 25 μL of the reaction system consisted of 2.5 μL of 10× Ex Taq buffer, 2.0 μL of dNTP mixture, 1.0 μL of each primer, 1.0 μL of genomic DNA, and 0.25 μL of Ex Taq enzyme. Water was added to a final volume of 25 μL and the components were mixed well. The PCR conditions were as follows: Pre-denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 1 min; and extension at 72°C for 10 min. The PCR products were purified by 1% agarose gel electrophoresis, ligated with the pMD19-T vector, and transformed into $DH_{5\alpha}$ competent cells. Positive clones were selected and sent to Shanghai Bioengineering Co. for further analysis. Sequence homology analysis was performed using Basic Local Alignment Search Tool (BLASTn). The sequences with high homology were selected for multiple comparisons using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA 6.0) and a phylogenetic tree was constructed by the neighbor-joining method to determine the phylogenetic relationships and taxonomic statuses of the selected strains.

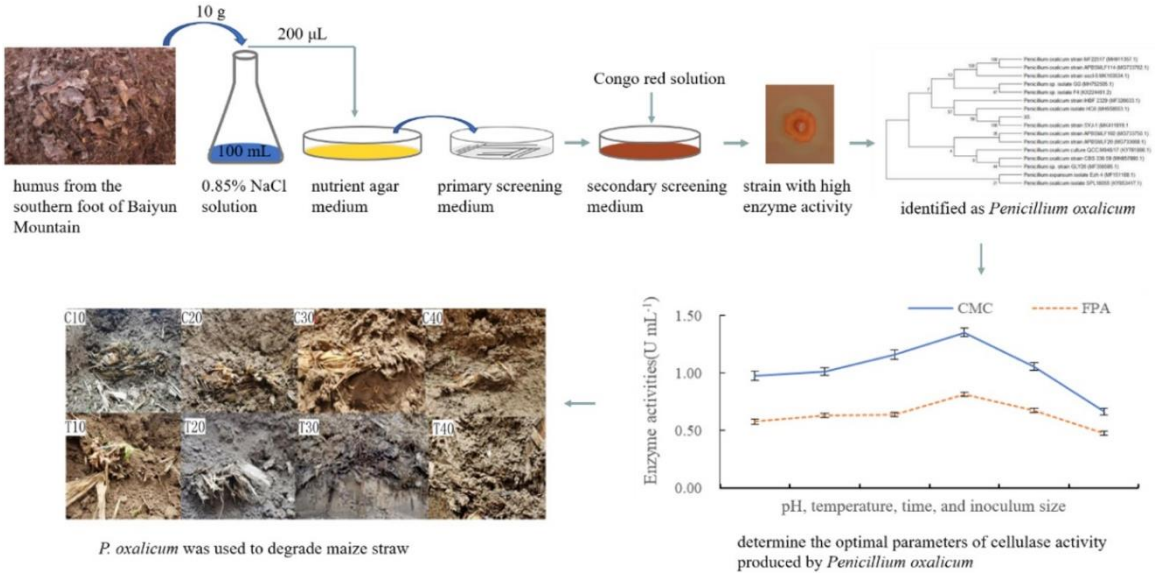

Fig. 1: Test design flow chart

Optimization of the Enzyme Production Conditions of the Dominant Cellulolytic Microbial Species

The effects of inoculum size (1, 2, 3, 4, 5, and 6%), initial pH (3, 4, 5, 6, 7, 8, and 9), fermentation time (12, 24, 36, 48, 60, 72, 84 and 96 h) and fermentation temperature (25, 28, 31, 34, 37 and 40°C) on cellulase activity (assessed via sodium carboxymethyl cellulose hydrolysis and filter paper degradation) of the selected isolates were investigated under existing fermentation conditions (inoculum size: 1%, Initial pH 5.6, fermentation time of 24 h and fermentation temperature of 28°C).

Filter Paper Activity (FPA) was determined using the 3,5-dinitro salicylic acid method (Ulrich *et al*., 2008). The enzymatic hydrolysis of sodium carboxymethyl cellulose was determined using the reducing sugar method (Mašková and Kunc, 1988).

Filter Paper Degradation Experiment

The screened isolates were incubated in a nutrient broth for 24 h. After the screening, 1.5 mL of the microbial suspension was extracted and added to a 250 mL conical flask containing 150 mL of filter paper degradation liquid medium (Kovács *et al*., 2022). Next, a piece of Whatman® double-ring qualitative filter paper (diameter: 7.0 cm) was placed in the flask. The experiment was performed in triplicate and three control groups without the addition of the culture suspension were established. The mixture was incubated in a constant-temperature shaker at 37°C and 140 rpm. Degradation of the filter paper was observed on days 3, 4, 5, and photographs were taken.

Inoculation of Maize Straw with the Dominant Cellulolytic Microbial Species

Briefly, 1% of the culture suspension was inoculated daily in a sterilized straw decomposition liquid medium (Ulrich *et al*., 2008), with incubation at 37°C and 120 rpm. Five replicates were performed for each experimental and control group. The straw was dehydrated, dried at 105°C for 6 h, and placed in the medium. The extent of decomposition was regularly examined. The experiment was conducted for 240 h (Chu *et al*., 2021).

The straw was filtered through 12 layers of gauze, soaked in distilled water for 30 min, and filtered through 12 layers of gauze again. This step was repeated several times until the filtrate was neutral. Maize straw was then dehydrated and dried at 105°C for 6 h, after which its weight was recorded (Kovács *et al*., 2022).

Weight loss = (mass of straw before incubation-mass of straw after incubation)/mass of straw before incubation (× 100%).

Observation of Straw Decomposition Carried out by the Dominant Cellulolytic Microbial Strain

A review of the local agricultural tillage habits indicated a soil layer thickness of 40 cm, a corn straw thickness of 20 cm, and a straw humidity of 50%. A microbial culture suspension (2.5×10^6 CFU mL^{-1}) at a volume equivalent to 10% of the corn straw volume was used for fermentation for 24 h. The test design flow chart was shown in Fig. 1. The temperature of the soil layer at a depth of 40 cm during the experiment was shown in Fig. 2.

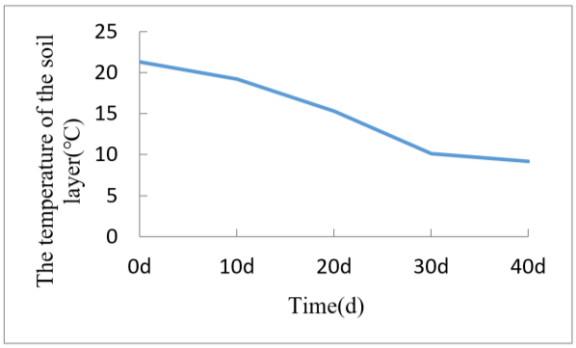

Fig. 2: Changes in the temperature of the soil layer during returning straw experiment

Results

Screening and Identification of the Dominant Cellulose-Degrading Microbes in the Humus

A total of 23 strains were obtained after isolation and purification. After Congo red staining, the three strains exhibiting the largest diameters of transparent rings around the colonies were labeled X_4, X_5, and X_6. The relative enzyme activities of the three strains were measured after 3, 5, and 7 d of culture. The results are presented in Table 1. It can be observed that strain X_5 secreted the cellulase with the strongest relative activity.

Colony and Cell Morphological Characteristics of the Dominant Cellulolytic Microbial Species

Figure 3A shows that the colonies formed by the X_5 strain were round, approximately 8 mm in diameter, loose, dry, rough, opaque, lustreless, and pink. They showed a raised center and a powdery edge.

Figure 3B, Gram staining of strain X_5 showed cells with a blue-purple color and the spores were oval-shaped with mycelia. Given the morphology of the colonies and microscopic characteristics of the strain, it was tentatively identified as a fungus.

Identification of the Dominant Cellulolytic Microbial Species

Using molecular biology methods, the DNA of the dominant cellulose-degrading strain was extracted and the ITS region was amplified by PCR. A 1500 bp target band was amplified using 1.5% agarose gel electrophoresis, in Fig. 4.

The band was recovered by gel cutting and sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The sequencing results were compared with sequences of existing strains in the national center for biotechnology information Gene database. Strain X_5 was identified as *Penicillium oxalicum* (KY781806.1; 100% homology) by homology analysis. A phylogenetic tree was constructed using the neighbor-joining method (Fig. 5).

Table 1: The relative enzyme activity(ϕ) of the three strains

Time/d	X_4	X_5	X_6
3	0.094±0.0024[a]	0.25±0.0085[b]	0.13±0.0019[c]
5	0.076±0.011[a]	0.28±0.0058[b]	0.12±0.0046[c]
7	0.069±0.0024[a]	0.26±0.0088[b]	0.12±0.0014[c]

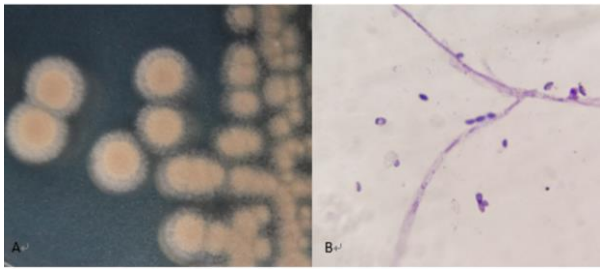

Fig. 3: X_5 bacterial colony and Cell morphology (100×)

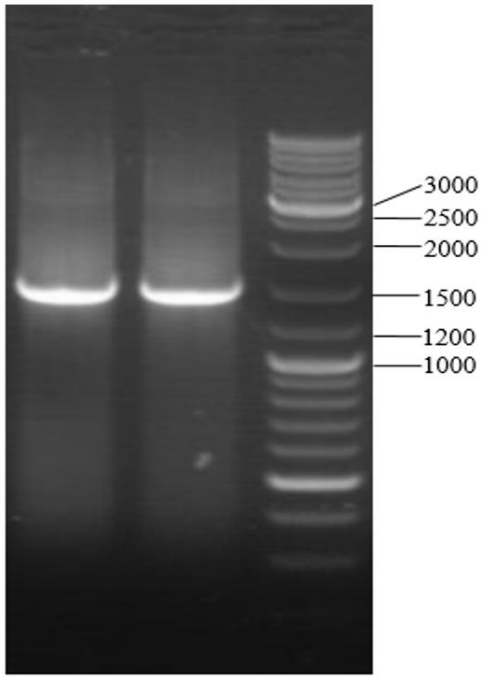

Fig. 4: PCR amplification of X_5 strain DNA

Influence of P. oxalicum X_5 of Inoculum Size on Enzyme Production

The effect of the inoculum size (1, 2, 3, 4, 5, and 6%) on the enzymatic activity of the fungal cellulase is shown in Fig. 6 and Table 2. For inoculum sizes of 1-2%, cellulase activity increased with inoculum size. When the inoculum size was 2%, cellulase activity, in terms of sodium carboxymethyl cellulose hydrolysis and FPA, was the highest (1.050 and 0.546 U mL^{-1}, respectively). The cellulase activity gradually decreased when the inoculum size exceeded 2%. Therefore, the optimum inoculum size was 2%.

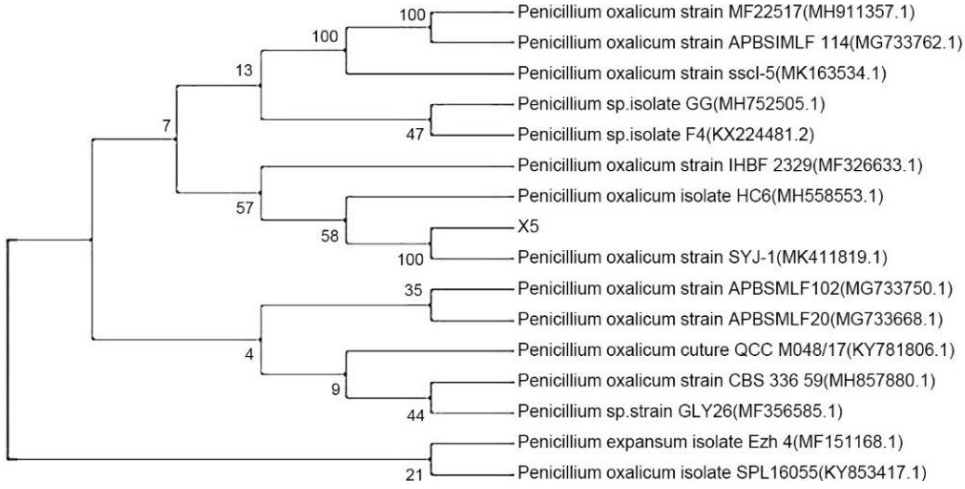

Fig. 5: Phylogenetic tree of strain X_5 based on its full sequence analysis

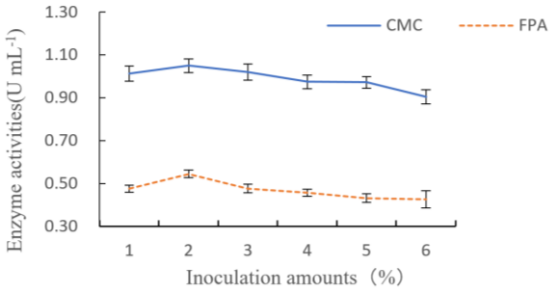

Fig. 6: Effects on CMC and FPA enzyme activities of different inoculation amounts

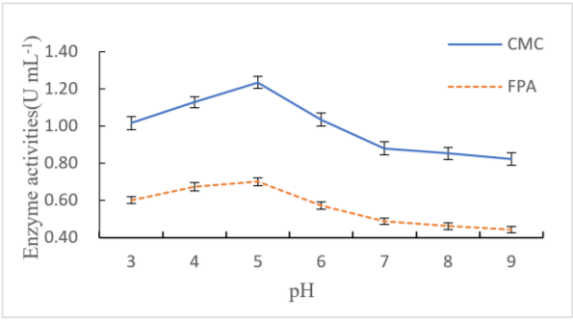

Fig. 7: Effects on CMC and FPA enzyme activities at different initial pH

Table 2: Effects on CMC and FPA enzyme activities of different inoculation amounts

	Enzyme activities(U/mL)	
Inoculation amounts (%)	CMC	FPA
1	1.013	0.476
2	1.050	0.546
3	1.020	0.477
4	0.975	0.458
5	0.972	0.432
6	0.906	0.427

Table 3: Effects on CMC and FPA enzyme activities at different initial pH

	Enzyme activities(U/mL)	
pH	CMC	FPA
3	1.216	0.672
4	1.229	0.684
5	1.235	0.701
6	1.035	0.572
7	0.880	0.488
8	0.853	0.463
9	0.822	0.443

Influence of the Initial pH of P. oxalicum X_5 on Enzyme Production

Figure 7 illustrates the effect of different initial pH values (3, 4, 5, 6, 7, 8, and 9) on the enzymatic activity of cellulase. Figure 7, cellulase activity increased with an increase in initial pH from 3-5. When the initial pH was 5, cellulase activity in terms of sodium carboxymethyl cellulose hydrolysis and FPA, reached maximum values of 1.235 and 0.701 U mL^{-1}, respectively. When the initial pH exceeded 5, cellulase activity displayed a decreasing trend (Table 3). Therefore, the optimal initial pH was determined to be 5.

Influence of P. oxalicum X_5 of Fermentation Time on Enzyme Production

The effect of fermentation time (12, 24, 36, 48, 60, 72, 84, and 96 h) on the enzymatic activity of the fungal cellulase is shown in Fig. 8 and Table 4. The highest enzyme activity in terms of sodium carboxymethyl cellulose hydrolysis and FPA was 1.293 and 0.796 U mL^{-1}, respectively, and was observed after 12 h of fermentation. After the fermentation time exceeded 12 h, cellulase enzyme activity gradually decreased. Therefore, the optimal fermentation time was 12 h.

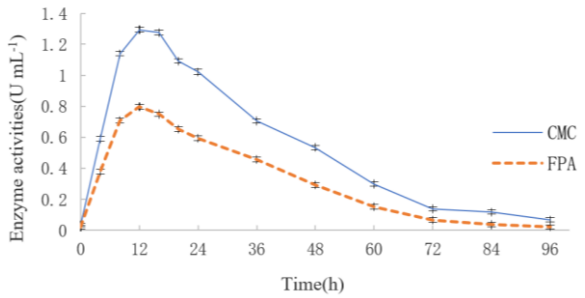

Fig. 8: Effects on CMC and FPA enzyme activities of different fermentation times

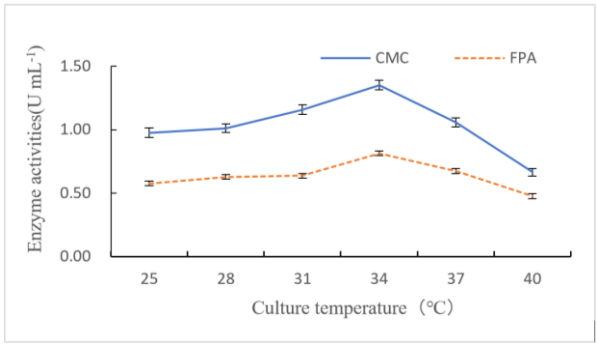

Fig. 9: Effects on CMC and FPA enzyme activities of different culture temperatures

Table 4: Effects on CMC and FPA enzyme activities of different fermentation times

	Enzyme activities (U/mL)	
Time(h)	CMC	FPA
0	0.000	0.000
4	0.588	0.379
8	1.138	0.707
12	1.293	0.796
16	1.276	0.748
20	1.091	0.653
24	1.021	0.594
36	0.705	0.456
48	0.534	0.292
60	0.297	0.015
72	0.137	0.065
84	0.116	0.035
96	0.065	0.021

Table 5: Effects on CMC and FPA enzyme activities of different culture temperatures

	Enzyme activities(U/mL)	
Culture temperature (°C)	CMC	FPA
25	0.977	0.576
28	1.011	0.628
31	1.158	0.638
34	1.352	0.813
37	1.057	0.674
40	0.664	0.476

Table 6: Changes in CMC and FPA enzyme activities before and after optimization (U mL^{-1})

	CMC	FPA	Measurement time
Before optimization	1.344±0.0292[a]	0.715±0.0382[a]	24 h
After optimization	1.653±0.0271[b]	0.894±0.0327[b]	12 h

* The letters on the right side of the numbers in each group are different, which means that the difference is significant ($p< 0.05$)

Influence of P. oxalicum X_5 of Fermentation Temperature on Enzyme Production

The effect of fermentation temperature (25, 28, 30, 34, 37, and 40°C) on the enzymatic activity of the fungal cellulase is shown in Fig. 9 and Table 5. Cellulase activity gradually increased as the fermentation temperature increased from 25-34°C. The highest fight enzyme activity, in terms of sodium carboxymethyl cellulose hydrolysis and FPA, was observed at a fermentation temperature of 34°C and was 1.352 and 0.813 U mL^{-1}, respectively. After the fermentation temperature exceeded 34°C, cellulase activity showed a decreasing trend. Therefore, the optimum fermentation temperature was determined to be 34°C.

Optimal Enzyme Production Conditions for P. oxalicum X_5

The optimum conditions for the cellulase production of *P. oxalicum* X_5 were as follows: Inoculum size 2%, initial pH 5, fermentation time 12 h, and fermentation temperature 34°C.

Variations in Enzyme Activity after Optimisation of the Enzyme Production Conditions for P. oxalicum X_5

Table 6 illustrates that the optimal cellulase activity in terms of sodium carboxymethyl cellulose hydrolysis and FPA was 1.653 and 0.894 U mL^{-1}, respectively. These values are higher than those obtained before optimization by 23.04 and 25.10%, respectively.

Figure 10 and Table 7, when the optimized conditions were used to culture *P. oxalicum*, the highest cellulase activity in terms of sodium carboxymethyl cellulose hydrolysis and FPA was 1.602 and 0.874 U mL^{-1}, respectively, for a fermentation time of 12 h. After the fermentation time exceeded 12 h, cellulase enzyme activity gradually decreased. When the fermentation time reached 108 h, cellulase activity, in terms of both sodium carboxymethyl cellulose hydrolysis and FPA, was 0 U mL^{-1}.

Results of the Filter Paper Degradation Test of P. oxalicum X_5

The experimental and control groups were observed and photographed on days three, four, and five. The results are shown in Fig. 11. The filter paper in the experimental group (to which the culture suspension was added) exhibited

degradation. In addition, the degradation of the filter paper became more evident with time.

Degradation Test Results of Maize Straw

Figure 12 and Table 8, the weight loss rate of the straw continued to increase over the 240 h straw decomposition experiment, with all experimental groups showing significantly higher decomposition than that of the control group ($p<0.05$).

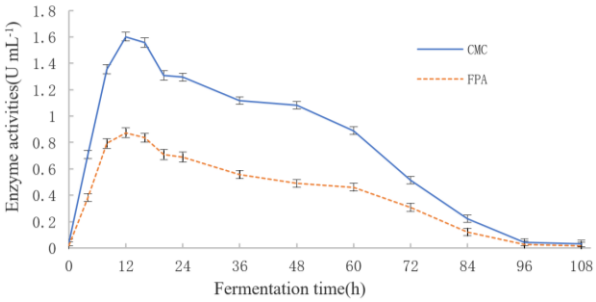

Fig. 10: After optimization, the enzyme activities of CMC and FPA changed with fermentation time

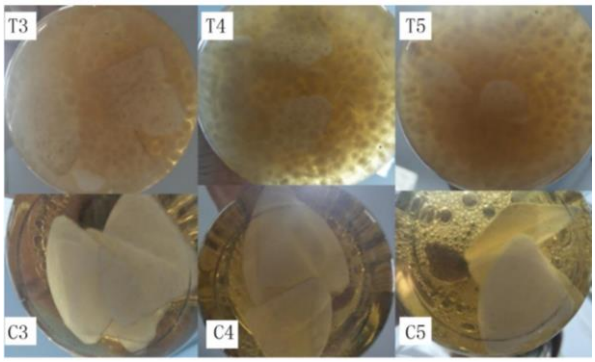

Fig. 11: Results of the filter paper degradation test

Table 7: After optimization, the enzyme activities of CMC and FPA changed with fermentation time

Fermentation time(h)	Enzyme activities(U/mL)	
	CMC	FPA
0	0.000	0.000
4	0.708	0.383
8	1.356	0.793
12	1.602	0.874
16	1.558	0.836
20	1.310	0.709
24	1.296	0.688
36	1.117	0.557
48	1.083	0.489
60	0.889	0.461
72	0.515	0.309
84	0.223	0.121
96	0.044	0.025
108	0.032	0.017

Table 8: Effect of *Penicillium oxalate* X_5 on weight loss rate of maize straw

Time(h)	Weight loss of maize straw (%)	
	Control groups	Test groups
24	1.41	2.800
48	1.54	2.910
72	1.98	3.540
96	2.37	4.240
120	2.51	5.430
144	2.87	7.510
168	3.11	8.610
192	3.21	11.08
216	3.34	12.56
240	3.34	12.73

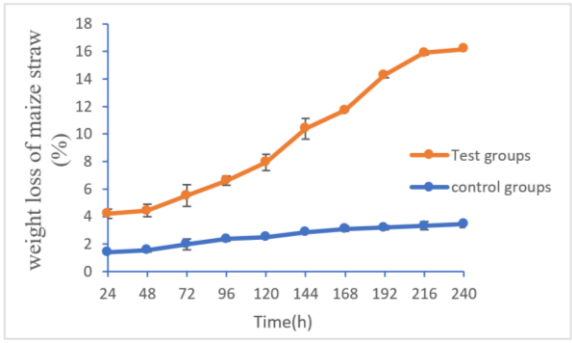

Fig. 12: Effect of *Penicillium oxalate* X_5 on weight loss rate of maize straw

Fig. 13: Changes of decomposing maize straw of *P. oxalate* X_5; *Cross sections of the control group (C) and experimental group (T) at 10, 20, 30, and 40 days, respectively. Owing to the different light intensity and humidity when taking photos, a color difference in the picture was observed

Effectiveness of Returning Straw to the Field after the Decomposition of P. oxalicum X_5

Figure 13, by day 30, the straw in the experimental group (the group to which *P. oxalicum* had been added) had decomposed fully, and demarcation with the soil layer was no longer obvious. By day 40, the straw had already been mixed with the soil layer. The straw in the control group, on the other hand, retained its original structure on day 40 and no decomposition occurred.

Discussion

Penicillium oxalicum can be isolated from various environments, including tropical commodities, food, air, and soil (Gupta *et al*., 2020; Tian *et al*., 2018). In recent years, *P. oxalicum* has been increasingly studied as a degrading hazardous microorganism (Tian *et al*., 2018; Wang *et al*., 2021; Ren *et al*., 2021) and researchers have been interested in it primarily as a popular cellulase-producing mold and polymers such as rhamnose, xylose, fructose, and trehalose were found in the mycelium and culture medium of *P. oxalicum* (Reis *et al*., 2015). Studies on cellulase production by *P. oxalicum* fall under three main categories. The first category involves isolating *P. oxalicum* strains from different environments to screen for wild strains with high cellulolytic capacities (Santi *et al*., 2021). In this study, a cellulase-producing strain, *P. oxalicum* X_5, was isolated from woodland humus in a temperate region. The second category concerns research on cellulase production-related genes in wild-type strains (Xiong *et al*., 2018). The last step involved the creation of engineered strains to improve the ability of *P. oxalicum* to decompose cellulose, thus providing efficiently engineered *P. oxalicum* for cellulase production (Li *et al*., 2020). These three studies provide useful information to better exploit the advantages of *P. oxalicum* and further promote the use of microbial resources.

In this study, the fungus was isolated from woodland humus using a selective medium. After optimizing the fermentation conditions, the sodium carboxymethyl cellulose activity (hydrolysis) and FPA reached 1.602 and 0.874 U mL^{-1}, respectively. The enzyme activity of this strain was higher than that of most wild strains (Gupta *et al*., 2012), but lower than that of the engineered fungal strains and there are shortcomings in the research on the genes and mechanisms of cellulose degradation by *Penicillium oxalicum*. However, satisfactory results were achieved when *P. oxalicum* X_5 was used in large-scale field trials.

In China, the ground temperature is below 25°C when the straw is returned to the fields, which are located in the warm temperate zone north of 32°N latitude (Liu *et al*., 2021). To meet this temperature requirement, this study screened a strain of *P. oxalicum* (KY781806.1) from the humus layer between the annual leaf litter and soil layer in poplar wood, where the perennial temperature is below 25°C. Under these conditions, the straw lost 12.73% of its weight within 240 h. At an initial ground temperature of 21.3°C and a final temperature of 10.1°C over 30 days, the fungal strain isolated in this study can help the returned maize straw to completely decompose without affecting the normal rooting of the wheat seedlings, thus ensuring that the survival rate of the crop is not affected.

Conclusion

In this study, *P. oxalicum* X_5 was screened from humus in the soil of warm temperate woods by primary and secondary screening using selective media. Culture conditions were optimized to improve the enzyme production capacity of this strain. The ability of *P. oxalicum* X_5 to utilize cellulose was demonstrated by a filter paper degradation test and maize straw utilization experiment. Finally, *P. oxalicum* X_5 was successfully utilized to substantially decompose the returned straw in the field.

However, to allow the routine use of this strain under actual or field conditions, *P. oxalicum* should be engineered to enhance its ability to decompose returned maize straw in the field. Further research on the optimal dosage of *P. oxalicum* is required to ensure the ease of transport and preservation.

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Author's Contributions

Gailing Wang, Chendi Li, Mingcheng Wang and Linglong Xu: The experiment was carried out and analyzed the data.

Yanan Guo and Enzhong Li: Guided the designed route and provided experimental guidance for this manuscript. Revised and proofread the manuscript.

Lei Guo: Guided the designed route and provided experimental guidance for this manuscript.

Tongbiao Li: Revised and proofread the manuscript.

Availability of Data and Materials

The data used to support the findings of this study are included within the article. The datasets during and analysed

during the current study available from the corresponding author on reasonable request.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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