Isolation of a Heat-Resistant Aspergillus Fumigatus and Identification of its Cellulase

Ming Sui¹, Peng Qu², Yusong Zhu², Silei Zhao¹ and Ruixiao Tao¹

¹Department of Wine and Food Engineering, Sichuan Technology & Business College, Dujiangyan, Sichuan Province, China
²Xinjiang Oilfield Company, China

Abstract. Cellulose-degrading fungi have the characteristics of large enzyme production, high enzyme activity, comprehensive enzyme composition, and most of them are secreted and extracted extracellularly. Therefore, studies on cellulase-producing bacteria at home and abroad have focused on fungi. Due to its thermal stability, the high temperature resistant cellulase can meet the high temperature conversion conditions in production, which is beneficial to the improvement of cellulose degradation efficiency. This chapter mainly carried out the separation screening and identification of heat-resistant cellulase-producing fungi, and the analysis of cellulase protein components and concentrations in fermentation products by Nano LC-ESI-MS/MS.

1. Introduction
Microorganisms that degrade lignocellulose are widely distributed in nature. Fungi, bacteria, and actinomycetes are among the most studied cellulose degradation groups. The reason is that fungi have more complete and complex fibers than bacteria. The enzyme-degrading enzyme system can degrade various components in plant biomass by continuously secreting a large amount of extracellular hydrolase, and finally degrade into soluble sugar which can be absorbed by microorganisms. Moreover, the fungal hyphae can enter the cell through the secondary wall when degrading the plant cellulose substrate, secreting cellulase in the interior, and fully hydrating the cellulose structure.

2. Determination of enzyme activity
The target strain obtained by the initial screening was eluted with distilled water to prepare a bosom suspension, and inoculated into a 250 mL flask containing 50 mL of the rescreening medium, and cultured for 72 hours, and then the supernatant was centrifuged at 4000 rpm to obtain a crude enzyme solution.

(a). Drawing of the glucose standard curve
Take a 20 mL graduated tube, and then use a 1 mg/mL glucose standard solution and distilled water to prepare a glucose solution at the concentration shown in the table below.

Table 1. Drawing of the glucose standard curve

| Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--------|---|---|---|---|---|---|---|---|---|
| Glucose (mL) | 0.0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 |
| Distilled water (mL) | 2.0 | 1.8 | 1.6 | 1.4 | 1.2 | 1.0 | 0.8 | 0.6 | 0.4 |
Add 3.0 mL of DNS to the test tube, and boil water for Smin. After the reaction, remove it and cool it to room temperature with distilled water and dilute to volume. Shake well after ODsa. The tube 1 was taken as CK, and the absorbance of each of the other tubes was measured and recorded. The results were based on the glucose content as the independent variable and the corresponding absorbance as the dependent variable.

(b). Enzyme activity assay
The enzyme activity of each component of the cellulase was determined by the DNS method. The procedure is to add 0.5 mL of the crude enzyme solution to a 1.5 mL graduated tube containing 1% acetic acid buffer solution of pH 4.8, and then react in a 50 °C water bath for 30 min according to the standard curve measurement step, and then calculate the amount of reducing sugar it produces.

(c). Enzyme activity representation
The enzyme activity is expressed in international units, that is, the amount of enzyme capable of converting 1 mol of substrate in 1 min or the amount of enzyme of 1 mol of the relevant group in the substrate under specific conditions, and the calculation formula is as follows:

\[
\text{Enzyme activity (IU/mL) one (glucose content (mg) \times \text{dilution factor} \times (5.56) / (the amount of enzyme added in the reaction solution X time (min))}
\]

3. Biological identification of high temperature fungi, plate morphology and mycelial growth rate at different temperatures
(a). The total DNA of the strain was extracted using the Omega fungus genomic DNA extraction kit; then the ITS1 and ITS4 primers were used for PCR amplification to obtain the ITS sequence. Sequencing was done by Meiji. Sequence alignments were performed in the NCBI database.

(b). The plate was inoculated with a puncher (diameter 8 mm) on a CMC-Na plate, and cultured at 45°C, and the colony morphology after inoculation was photographed.

(c). Determination of mycelial growth rate: The CMC-Na medium was divided into two groups of solid medium, one group was added with glucose in an amount of 2. Sg/mL, and the other group was not added with glucose. The cells were observed at 15, 25, 35, 45, and 55°C for 72 hours, and the hyphae diameter was recorded every day to indicate the mycelial growth rate.

4. Nano LC-ESI-MS/MS Analysis
A. fumigatus LY1 was cultured for 120 h under a suitable carbon and nitrogen source, and the fermentation broth was centrifuged at 8000 rpm for centrifugation. The supernatant was filtered through a membrane and concentrated with a 10 kDa ultrafiltration tube to obtain a protein sample.

The protein sample was concentrated to a certain volume, and then SDS buffer was added for reduction acetamidine reaction, and then digested with sequencing standard trypsin, and the enzyme-cut buffer was 100 mM ammonium hydrogen carbonate. The skin section obtained by digestion was extracted through acetonitrile and completely dried by a freeze dryer. The dried sample is redissolved in the sample solution. The samples were then tested via nanoscale liquid phase-electrospray-tandem mass spectrometry.

The sample was passed through a high pressure liquid phase into a C18-based reversed-phase column that was 8 cm long and 75 um in internal diameter. The filled C18 matrix was 3 um in size and contained a pore size of 300 people. The sample was injected into the column for 20 min liquid phase solution A (97.5% water, 2% acetonitrile and 0.5% formic acid); solution B (9.5% water, 90% acetonitrile and 0.5% formic acid). The total gradient time was 45 min, the mobile phase was gradually increased from 2% B to 90% B, and the last 5 min of the column was rinsed. The column flow rate is approximately 800 um/min and the injected sample volume is approximately 3 um.
Table 2. Mass spectrometry parameter

| Parameter name            | Parameter value                                      |
|---------------------------|------------------------------------------------------|
| Ion mode                  | Positive ion mode                                    |
| Primary scan range        | 350-1600Da                                           |
| Secondary scan range      | Depending on the primary parent ion mass-to-charge ratio |
| Secondary collision energy| 33%                                                  |
| Capillary temperature     | 100°                                                 |
| Ion source voltage        | 2000 V                                               |
| Broken mode               | CID                                                  |

The liquid phase is coupled in-line with the mass spectrometer, and the sample eluted from the liquid chromatography column is directly ionized into the mass spectrometer via electrospray. Ion source voltage: between 1.2KV and 1.5KV; capillary temperature: 110°C. The mass spectrometer is set up in a data-dependent mode to obtain tandem mass spectrometry data by CID (collision induced dissociation), which includes a full mass range and three microscans. The dynamic exclusion setting, the repeat count within 0.3 min and the exclusion interval of 0.4 min, the excluded mass range is 4Da.

5. Isolation and screening of high temperature cellulase-producing fungi

The calculation of the relative content of the protein is based on the method published by Griffin et al., in which the scoring formula is based on mass spectrometry abundance in mass spectrometry and tandem mass spectrometry data. The normalized SIN is calculated by the following formula. The relative abundance of the final protein is the ratio of SIN of each protein to the SIN of all proteins in one sample.

\[
SI_N = \frac{\left[ \sum_{k=1}^{m} \left( \sum_{j=1}^{a} i_j \right) / \sum_{j=1}^{a} SI_j \right]}{L}
\]

Using a dilution plate method, a strain of high temperature resistant fungus capable of growing CMC-Na as a sole carbon source and producing a stalk at 45 °C was isolated from 15 soil samples and named LY1. The strain LY1 was cultured in the rescreening medium for 72 hours, and the fermentation broth was centrifuged at 4000 r/min for 10 min to obtain the crude enzyme solution. The cellulase activity was measured as shown in Table 3:

Table 3. Results of enzyme activity assay of LY1 cellulase

| Strain | β-glucosidase (IU/mL) | Endoglucanase (IU/mL) | Exoglucanase (IU/mL) |
|--------|----------------------|------------------------|----------------------|
| LY1    | 1.05±0.1             | 0.32±0.04              | 0.33±0.09            |

It can be seen from Table 3 that the susceptibility fungus LY1 can produce three cellulases such as glucosylase, endoglucanase and exoglucanase, among which glucagonase has the highest activity, reaching 1.05 addition and subtraction. 0.1 IU/mL. Thus, LY1 is a fungus that produces cellulose complex enzymes with the ability to degrade intact cellulose into glucose.

6. Strains LY1 classification identification results

Figure 1 is a diagram showing the hyphal and colony morphology of the LY1 strain. It can be observed that the hyphae of the strain LY1 is branched and branched, and the bud-like branches and long or short branches are visible on the main branch, the stalks belong to the branching stalks, and the top end is expanded into a rounded sac. It can be seen from the morphology of the colony on the plate containing only CMC as the sole carbon source. The mycelial growth state of LY1 is good at 45°C, and it is in a culture environment containing only CMC as the sole carbon source, indicating that the strain has better bacteria. Cellulose utilization capacity. After 24 hours of culture, the hyphae were white. After 72 hours of culture, the hyphae had a green stalk at the edge of the mycelium. After 72 hours of cultivation, the
mycelium was covered with a 3/4 plate and the surface was covered with green stalks. Further cultivation of the hyphae will spread the entire plate, and the stalk will slowly change from green to smoky gray. From the results of hyphal microscopy and plate colony morphology observation, it can be preliminarily judged that LY1 is a genus Aspergillus.

In order to clarify the taxonomic status of the strain, molecular biology identification was carried out on the basis of morphology. The total DNA of the strain was extracted using the Omega fungus genomic DNA extraction kit; PCR amplification was performed using ITS1 and ITS4 primers to obtain the ITS sequence. BLAST comparison was performed in NCBI, and phylogenetic trees were obtained by phylogenetic analysis of strains with higher homology.

The ITS1 and ITS4 primers were used for PCR amplification to obtain the ITS sequence, and the phylogenetic tree of strain LY1 and related strains was prepared, as shown in Figure 2:

The results in Figure 2 indicate that the 18S rDNA sequence of strain LY1 is 100% homologous to Aspergillus fumigates ITCC 6915 in the GeneBank database. Therefore, LY1 can be identified as a strain of A. fumigates based on 18S rDNA sequencing results, and was initially named as Aspergillus fumigatus LY1o.

7. Mycelial growth rate at different culture temperatures
Aspergillus fumigatus LY1 was cultured at different temperatures, and the growth diameter of the colony on the plate was recorded to examine the effect of the culture temperature on the growth of LY1 hyphae. The results are shown in Table 4. From the results, it was found that the growth rate of LY1
strain was significantly higher than that of the plate without glucose on the plate with glucose added, which indicated that the addition of fast-acting carbon source glucose promoted mycelial growth. When the culture temperature is 15°C and 55°C, the hyphae can hardly grow, can still grow under the suitable growth temperature at 15°C, and can not grow under the condition of returning to room temperature at 55°C. It is preliminarily judged that the mycelium has died. Therefore, the strain grew well between 25°C and 45°C in the test range, and the maximum growth rate was obtained at 35°C.

| Temperature | 24h         | 48h         | 72h         | Survival state                  |
|-------------|-------------|-------------|-------------|---------------------------------|
| 15 (+glucose) | 0.80±0.06  | 0.98±0.08  | 1.00±0.10  | Recover to room temperature to grow |
| 15 (+glucose) | 22.58±0.67  | 35.31±0.75  | 56.76±0.84  | Growing vigorously               |
| 25 (+glucose) | 26.32±1.02  | 42.29±0.93  | 60±1.34     | Growing vigorously               |
| 35 (+glucose) | 32.17±0.98  | 53.35±1.24  | 78±1.00     | Growing vigorously               |
| 45 (+glucose) | 28.86±2.00  | 44.12±1.47  | 64.06±2.44  | Growing vigorously               |
| 55 (+glucose) | 0           | 0           | 0           | death                           |

8. Conclusion

In this study, a strain of fungus LY1 was isolated and screened from soil, and it was finally identified as Aspergillus fumigatus by morphological and molecular biological identification. The strain grows well under the culture condition of 45 °C, and it can produce a high temperature resistant and diverse cellulose degrading enzyme system, and has the potential to completely degrade cellulose into glucose. Many studies at home and abroad have also screened a number of Aspergillus fumigatus in the same environmental conditions, showing its widespread existence. The wild strain Aspergillus fumigatus LY1 crude enzyme solution is similar to the enzyme production level of wild strains such as Aspergillus fumigatus E4 and Aspergillus fumigatus ZS found in China.

Although the Aspergillus fumigatus strain can efficiently degrade lignocellulose, its conditional pathogenicity causes immunodeficiency patients to infect aspergillosis, making it rarely used in industrial enzyme production or as a model strain for studying the mechanism of lignocellulose degradation. Nonetheless, due to the efficient cellulose degradation ability of Aspergillus fumigatus, there are still many studies on the production of cellulase by Aspergillus fumigatus. Moreover, with the rise of bioinformatics research and mass spectrometry, multiple Aspergillus fumigatus have been genome-wide sequencing and transcriptome analysis. The results of genomic sequence and transcriptome analysis indicated that the Aspergillus fumigatus genome is rich in CAZymes gene, various types of lignocellulolytic enzymes, and large extracellular protein secretion can promote the transformation into a mature lignocellulolytic enzyme producing strain.

References

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