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Article

Effects of lignocellulolytic enzymes activities under different culture conditions from *Wolfiporia cocos*

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Abstract: Objective: In order to explore the degradation mechanism of *Wolfiporia cocos* fungus lignocellulose, reveal the main enzymes of poria cocos lignocellulose system and their relationship with culture methods, and explore the production and application of lignocellulose-related enzyme resources. **Method:** Poria cocos strain YX2 collected in the field was observed microscopically to understand its culture characteristics. Its DNA was extracted by fungal kit and then amplified by PCR. After the obtained products were compared by BLAST, the phylogenetic tree of brown rot fungus was constructed by using biological analysis software ClustalX and building Phylogenetic Trees from Neighbor-Joining with MEGA. The activities of cellulase, hemicellulase and lignin-degrading enzymes were determined by MicroplateReader, and the magnitude of the nine enzyme activities were calculated. The maximum secretion of exo-β-glucanase, endo-β-glucanase and β-glucosidase in the cellulase group was 16-17 U/mL, 32-35 U/mL and 36-37 U/mL, respectively, and the maximum secretion of xylanase, mannanase and α-glucosidase in the hemicellulase group was 28-38 U/mL, 280-342 U/mL and 9-11 U/mL, respectively, under the conditions of treatment with or without the addition of pine wood chips. 280-342U/mL, and 9-11U/mL, respectively.The maximum secretion of MnP, Laccase and LiP, which are lignin degrading enzymes, in four different culture solutions: A. without Mn^{2+} were 0.015U/mL, 0.031U/mL and 0.017U/mL, respectively; B. with Mn^{2+} were 0.081U/mL, 0.032U/mL and 0.109U/mL, respectively; C. with The highest secretion of wood chips was 0.026U/mL, 0.025U/mL, 0.105U/mL, respectively; D. The highest secretion of 2,6DMP was 0.025U/mL, 0.029U/mL, 0.067U/mL, respectively. **Conclusion:** Through the combination of morphological and molecular biological identification of Poria cocos, the taxonomic status of Poria YX2 was clarified, and the brown rot fungus in There is both a connection and a genetic gap in the affinity. The size of the enzymatic activity in lignocellulase in the order of mannanase > xylanase > β -glucosidase > endo-β-glucanase > exo-β-glucanase > α-glucosidase > LiP > MnP > Laccase, and to provide a basic enzymatic reference for the study of the mechanism of action of the lignocellulase system produced by Porphyromonas.

Keywords: *Wolfiporia cocos*; ITS sequences; ligninolytic enzymes; manganese peroxidase; lignocellulase

Introduction

The structure of wood cell walls is complex, mainly composed of cellulose, hemicellulose, lignin, pectin polymers and glycoproteins and other macromolecules, which make the cell wall intricately arranged, especially coniferous wood lignans [1]. It is dominated by guaiac-based structural units, with very few syringyl and p-hydroxyphenyl structural units, and they are connected by ether bonds (-C-O-C-) and carbon-carbon bonds (-C-C-), making wood The structure is very hard, and it is

difficult for some microorganisms to degrade wood at first, but as the wood declines year by year, the enzymes that decompose lignocellulose become active [2]. Therefore, it is one of the hotspots to study the enzymatic ability of lignocellulose-degrading enzymes. Poria cocos (Wolfiporia cocos) is a wood-decaying fungus, and it is also a traditional Chinese medicinal material used for both medicine and food in my country. Its pharmacological activity is widely recognized. Only with excellent strain and degradability can the sclerotium production capacity and the transformation efficiency of catalytic degrading enzymes be improved. At present, many studies have been carried out on the chemical components and pharmacological effects of Poria cocos, genomics [3], polysaccharide anticancer [4], germplasm resources and cultivation techniques [5], and the degradation ability of Poria lignocellulase has been carried out. There is little research.

Poria (W. cocos) is a high-temperature aerobic saprophytic fungus, also known as Yuling, Fuling, Wanlinggui, Poria, etc., mostly parasitic on the roots of tree species such as Pinus massoniana or P. densiflora. Most of them grow in the field and form irregular sclerotia, so it is difficult to excavate wild Poria cocos [6]. The lignocellulase of brown rot fungus is a multi-component complex enzyme system, including hydrolases related to the degradation of cellulose and hemicellulose, etc. Cellulase (Cels) is a kind of β - A general term for multi-component enzymes that generate D-glycosidic bonds and generate glucose, they can secrete a series of endo-β -glucanases (endo-β -glucanase/CBH), exo-β -glucanases (exo-β -glucanase/EG) and β -glucosidase (β -glucosidase/BG) to degrade cellulase and xylanase activity in Trichoderma[7]) is a complex enzyme system, including a variety of enzymes such as xylanase, mannanase, and xylosidase [8]; oxidases related to lignin degradation, mainly Including manganese peroxidase (Manganese peroxidase/MnP), lignin peroxidase (Lignin peroxidase/LiP), laccase (Laccase/Lac), etc. [9]. Study [10] found that the brown rot fungus Fomitopsis palustris produces three major cellulases (endo-β -glucanase, exo-β-glucanase and β-glucosidase) simultaneously and degrades crystalline cellulose. Study [11] reported that Pleurotus sp.2 and Trametes gallica were able to produce strong lignocellulose degrading enzyme activity in liquid medium with faster rate of enzyme production. Study [12] reported that the activities of carboxymethyl cellulase, xylanase and laccase were significantly affected by different carbon and nitrogen source culture conditions in P. pombe. Gloeophyllum saepiarium can secrete more than 20 kinds of amylase, cellulase, hemicellulase, ligninase, pectinase, protease, lipase, maltase, mucilase and urease Enzymes, the decomposition of these complex organic matter requires the participation of a series of complex enzymes, in order to show a strong synergistic effect, to decompose the extremely complex plant cell wall materials into simple carbohydrates [13].

Study [14] isolated and purified MnP from Poria cocos (W. cocos) which showed that Poria cocos had at least two enzymes, MnPI and MnPII, but had no effect on its secreted cellulase, hemicellulase and lignin degrading enzymes. Other enzymes are used for purification and optimization of culture conditions. The differences of the main enzyme systems and the laws of enzyme activities in the lignocellulose-degrading enzyme systems need to be explored. Therefore, this paper attempts to further analyze the degradation mechanism of lignocellulose-related enzymes from the

enzymatic level, explore the optimal culture conditions for degrading enzymes and the influence of the induction process of degrading enzymes, and reveal the main enzymes of the lignocellulase system of Poria cocos The relationship between medium components and induction substrates and other conditions can provide a theoretical basis for the production and application of lignocellulose-related enzymes in the future [15].

1. Materials and methods

1.1. Source of bacteria

Wild Poria cocos strain (Wolfiporia cocos) was picked in the wild in February 2020 from the forest area of Chingzhai Village, Zhongguan Town, Yuexi County, Anqing City, Anhui Province (30°85′ N, 116°35′ E), Poria cocos under a decaying pine stump The sclerotia, which have been isolated and identified, are now stored in a 4 ℃ refrigerator in the Animal and Plant Quarantine Laboratory of Anqing Normal University, and the liquid Poria cocos mycelium YX2 is preserved in the China Center for Type Culture Collection, with the preservation number CCTCC NO: M 2021434; buy common microorganisms in China Culture Collection and Management Center CGMCC Poria 5.78 [16].

1.2. Culture medium

Solid PDA medium: peeled potatoes 200 g, glucose 20 g, agar powder 17 g, $KH_2PO_4 3$ g，$MgSO_4 1.5$ g, dilute to 1000 mL, put them into conical flasks, sterilize by autoclaving at 121 °C for 30 min, and set aside.

Congo red primary screening medium: 2 g sodium carboxymethyl cellulose, $MgSO_4, 7H_2O$ 1.88g，$KH_2PO_4 0.5g$, 2 g peptone, 15 g agar powder, dilute to 1000 mL, autoclave at 121 ℃ for 30 min, and set aside.

Cellulase seed medium: 20 g peptone, 10 g sodium carboxymethyl cellulose, NaC15g，$KH_2PO_4 1g$, and 130 mL in a 300 mL conical flask, respectively, sealed and autoclaved at 121 °C for 30 min, and set aside.

Cellulase enzyme production medium: peptone 10 g, yeast extract 15 g, sodium carboxymethyl cellulose 10 g, NaC15g，$KH_2PO_4 1g$, pH = 7, dilute to 1000 mL, and put 130 in a 300 mL conical flask. mL, sealed, autoclaved at 121 °C for 30 min, and set aside.

LNAS medium: the preparation of liquid medium, and the corresponding mineral solution is selected according to the needs of different groups.

1.3. ITS sequence amplification and phylogenetic analysis

The total genomic DNA of Poria cocos was obtained, about 20 mg of fresh mycelia were scraped with a sterilized knife, and the powder was fully ground with a cell breaker until the cell walls were broken. PCR uses 3 fungal universal primer pairs, 5.8S rRNA uses ITS1: (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4: (5’-TCCGCTTATTGATATGC-3’), ribosomal large subunit (LSU) uses NL1: (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL4: (5’-GGTCCGTGTTTCAAGACGG-3’), transcription elongation factor (EF-a) using

EF1-688F: (5'-CGGTCACTTGATCTACAAGTGC-3') and EF1-1251R: (5'-CCTCGAACTCACCAGTACCG-3'). The PCR amplification system is: template DNA 0.50 μL, primers 1.75 μL each, 2 × Rapid Taq Master Mix 25.00 μL, sterilized ddH_2O to make up to 50.00 μL, the above three PCR amplification reaction conditions are the same, and the products are stored in −20 °C refrigerator.

1.4. Qualitative culture of cellulase produced by Poria cocos

Under sterile conditions, the mycelia were inoculated on the sterilized Congo red primary screening medium, cultured at 28 °C for 5 d, first immersed with 2 g/L Congo red dye for 30 min, and then 1 mol/ L of NaCl did not cover the medium, evenly covered and decolorized for 30 min, the diameter of the hydrolysis circle/colony diameter was measured and the relative activity A value of the enzyme was calculated.

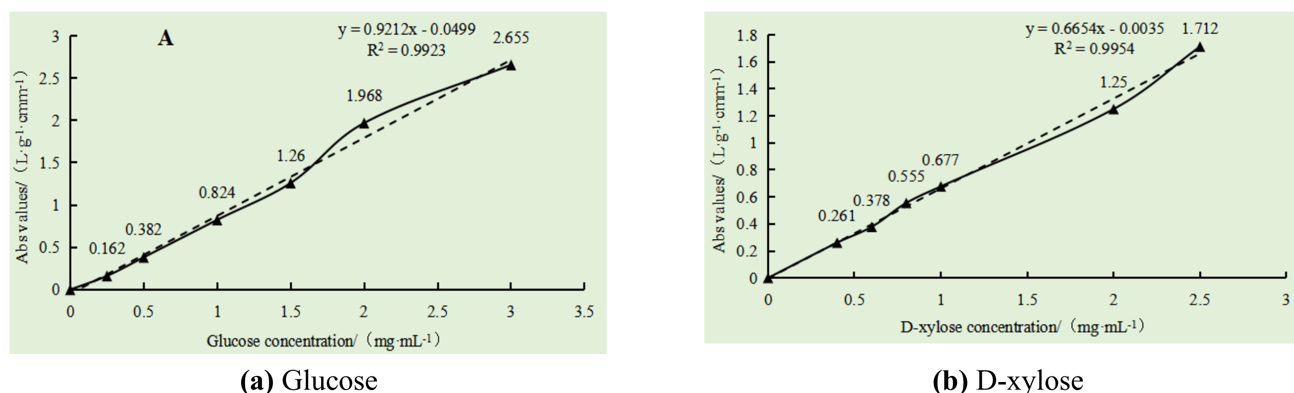
1.5. Enzyme production culture method

The lignocellulose-degrading enzyme system all adopts the solid PDA medium to cultivate, when the mycelium is about to cover the plate, use a puncher to punch, and each bottle of seed medium contains 5 apertures and is a 1cm bacterium cake, 28 °C, cultivated in a constant temperature shaking incubator at 150 rpm, and the cellulose and hemicellulose-degrading enzymes were inoculated into the enzyme-producing medium with a pipette after the mycelium in the seed medium grew and matured, and the inoculation volume was 0.5 mL per bottle, LNAS medium with four different substrates was used for the detection of lignin-degrading enzymes, A. Mineral solution without adding; B. Mineral solution with; C. Mineral solution with 2g pine chips ; D. Add 2,6-dimethoxyphenol (2,6-DMP) mineral element solution. The culture solution, namely extracellular enzyme solution, was extracted in the ultra-clean workbench. The cellulase system continued to draw for 12 days, and the ligninase system continued to draw for 21 days. Each time, 1.3 mL of the culture solution was quantitatively and quantitatively drawn with a pipette gun each time. The extracted crude enzyme solution was centrifuged at 13200 rpm for 5min, and the enzyme activity was measured.

1.6. Enzyme activity determination method and preparation of standard curve

Using a microplate reader (Multiskan GO full-wavelength microplate reader) to measure the change of the optical density value under a certain light wave as the basis for the enzyme activity. Preparation of glucose standard curve: Dilute 10 mg •mL^{-1} glucose standard solution with distilled water to 3.0, 2.0, 1.5, 1.0, 0.5, 0.25 mg •mL^{-1}, which is the standard dilution solution. Preparation of D-xylose standard curve: Dilute 33 D-xylose standard solution with distilled water to 2.5, 2, 1, 0.8, 0.6, 0.4 mg•mL^{-1}, which is the standard dilution solution. The microplate reader was preheated for more than 30 min, adjusted to 540 nm, and zeroed with distilled water. Taking the standard diluent as the abscissa (x) and its corresponding ΔA standard as the ordinate (y), draw the standard curve to obtain the linear regression equation $y = kx + b$, and bring the ΔA measurement into the formula to obtain x (mg•mL^{-1}). Draw the standard curve of glucose, the regression equation is y = 0.9344, x = 0.0763, the correlation

coefficient R^2 = 0.991, as shown in **Figure 1a**; draw the D-xylose standard curve, the regression equation is y = 0.6666, x = 0.0056, the correlation coefficient R^2= 0.9937, **Figure 1b**.



(a) Glucose **(b)** D-xylose

Figure 1. Glucose and D-xylose standard curve of Wolfiporia cocos. **(a)** Glucose; **(b)** D-xylose.

1.6.1. Determination of cellulase activity

The activities of exo-β -glucanase, endo-β -glucanase and β -glucosidase were measured in a 150 µL reaction system. First, the amount of crude enzyme solution was 25 µL, 50 µL, and 50 µL; secondly, different volumes of substrate solutions were taken, and the substrate solution used for the detection of exo-β -glucanase was 2% microcrystalline Cellulose solution 50 µL, the substrate solution used for detection of endo-β -glucanase was 1% sodium carboxymethylcellulose solution 25 µL, and the substrate solution used for detection of β -glucosidase was 1% water 25 µL of salicin solution; put it into a 50 ℃ constant temperature water bath for water bath reaction, the reaction time is 2 h, 30 min, 30 min in turn; again, add DNS color developing solution to 75 µL; finally, after mixing, the boiling water bath time is long Both were 5 min, and the absorbance at 540 nm was measured after cooling. Units of enzymatic activity are defined as the amount of enzyme required to break down a specific substrate to release 1 µmol of reducing sugars per minute. No sawdust and pine sawdust (P. massoniana) were used as control treatments, and the enzyme activities were detected on the 2nd, 4th, 6th, 8th, 10th, and 12th d, respectively, with three replicates each.

1.6.2. Determination of hemicellulase activity

The activity determination of xylanase, mannanase, and α -glucosidase were all set to a 200 µL reaction system. First, the amount of crude enzyme solution was 67 µL, 67 µL, and 6.7 µL; Volume of substrate solution, the substrate solution used for the detection of xylanase is 33 µL of 0.8% xylan solution, and the substrate solution used for the detection of mannanase is 33 µL of 0.8% mannan solution, and the detection of α—The substrate solution used for glucosidase was 60 µL of 0.4 % pNPG solution; the water bath reaction time in a constant temperature water bath at 50 °C was 30 min, 30 min, and 60 min in turn; again, the reaction was terminated, xylanase, mannan Carbohydrases were all terminated with DNS chromogenic solution, and 100 µL of DNS reagent was added. Finally, the mixture was mixed in a boiling water bath for 5 min. After cooling, the absorbance at a wavelength of 540 nm was measured. The α—

glucosidase was added with 66.7 μL of 1 mo1 · $L^{-1}Na_2CO_3$ solution to terminate the reaction, and the absorbance value at the wavelength of 410 nm was measured after shaking and mixing. The unit of enzymatic activity is defined as the amount of enzyme required to break down the substrate to release 1 μmol of reducing sugar per minute. No sawdust and pine sawdust were used as control treatments, and the enzyme activities were detected on the 2nd, 4th, 6th, 8th, 10th, and 12th d, respectively, with three replicates each.

1.6.3. Determination of lignin-degrading enzyme activity

The activities of manganese peroxidase (MnP), laccase (Laccase), and lignin peroxidase (LiP) were measured using 2,6-DMP, veratrol (VA), and ABTS as substrates, respectively. The 100 μL reaction system was referred to [17], and the changes of absorbance at 470 nm, 420 nm, and 310 nm within 3 min were measured respectively. Enzyme activity unit (U): The amount of enzyme required to catalyze 1 μmol of 2,6-DMP, ABTS or VA per minute under the above conditions [18]. In the calculation, $\varepsilon 470$ = 49,600 (mol · $L^{-1}cm)^{-1}$, ε 420 = 36 000 (mol · $L^{-1}cm)^{-1}$, ε 310 = 9300 (mo1 · $L^{-1}cm)^{-1}$.

1.6.4. Data processing and statistical analysis

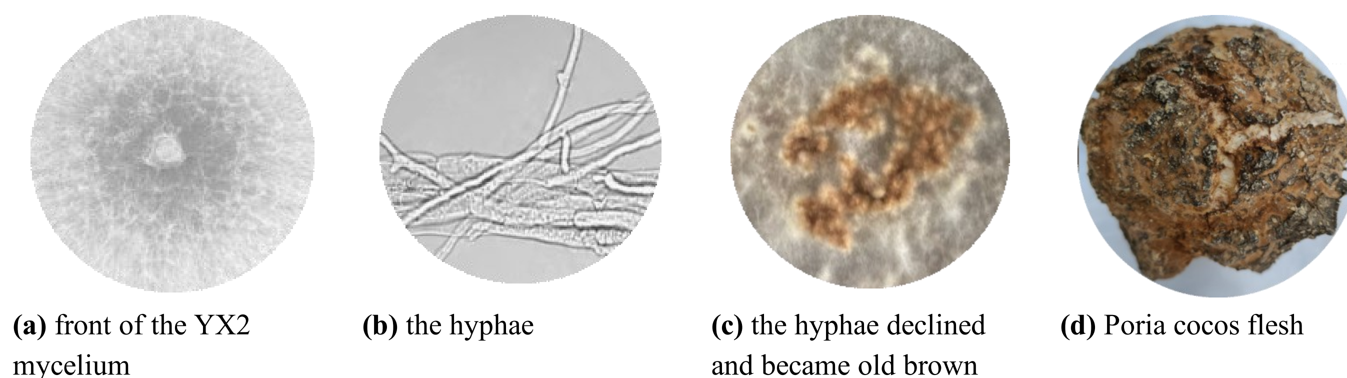
The phylogenetic tree was constructed by MEGA6.1, the data was processed and analyzed by Microsoft Excel software, the variance analysis was performed on the data by SPSS 26.0 and the significance of differences was evaluated, and Origin 2018 was used for graph drawing [19,20].

2. Results and analysis

2.1. Biological properties of Poria YX2

The obtained Poria cocos (W. cocos) YX2 was inoculated in PDA medium and cultured for 4–5 days, and then placed on an optical microscope to observe the macro and micro growth characteristics such as the formation, color, structure and separation of the Poria mycelium. The front of the YX2 mycelium, as shown in **Figure 2a**, the Poria cocos mycelium is composed of many branched hyphae, the hyphae are crisscrossed, densely running through the substrate or spreading on the surface of the substrate, the mycelium is white the villous occasional lock-like association phenomenon (10 × 20 times) is shown in **Figure 2b**. There is a diaphragm in the hyphae that divides the hyphae into multiple linear cells. The width of the hyphae is 2 to 5 μm. Concentric ring-shaped colonies are common when cultured in petri dishes, and radial hyphae are common when cultured in bacterial bags, which proves that the hyphae have strong vitality. After 5 to 7 days, the plates were covered with plates, and then transferred to a light incubator, where light and dark were alternately cultured. After 28 days, the hyphae declined and became old brown, and the fruit bodies were honeycomb-shaped, as shown in **Figure 2c**. Poria YX2 has homogeneous, spherical, ellipsoid sclerotia measuring 140 by 180 mm. It is an aggregation of latent mycelium. When it is fresh, it is easily torn, supple, and has a moisture level of no more than 30%. Once dried, it is difficult and challenging to the overall moisture content is less than 18% when the sclerotium is burst open; when the sclerotium is covered in dirt, the surface is rough, mostly showing tumor-like shrinking, and a layer of skin that

resembles a shell is present. Poria cocos flesh was white, dark brown, as **Figure 2d** illustrates.



(a) front of the YX2 mycelium **(b)** the hyphae **(c)** the hyphae declined and became old brown **(d)** Poria cocos flesh

Figure 2. Microstructure and biological culture characteristics of W.cocos YX2. **(a)** front of the YX2 mycelium; **(b)** the hyphae; **(c)** the hyphae declined and became old brown; **(d)** Poria cocos flesh.

2.2. ITS sequence and phylogenetic analysis of Poria cocos YX2

The nucleotide sequences of 1652 bp, 660 bp and 545 bp were obtained by sequencing the PCR products of Poria cocos (W.cocos) YX2. The sequences were submitted to GenBank (NCBI accession numbers are: ON129554, ON129553, ON155840, respectively. The identification results show that: Poria cocos The ITS region sequence of YX2 (ON129554) is 1652 bp: 1-5 bp is 18S rRNA sequence, 6-1005 bp is ITS1 sequence, 1006-1167 bp is 5.8S rRNA sequence, 1168-1626 bp is ITS2 sequence, 347-508 bp is 28S sequence rRNA sequence; Poria YX2 (ON129553) ribosomal large subunit (LSU) sequence is 1-660 bp; Poria YX2 (ON155840) CDS coding sequence of transcription elongation factor EF1 alpha (translation elongation factor EF1 alpha) has a total of 545 bp and is 1-129 bp, respectively , 179–320 bp, 380–545 bp sequences, according to the three Poria cocos YX2 sequences, the brown rot fungus phylogenetic tree was constructed by MEGA 6.1 software, and the NJ clustering method was used to analyze it. It can be seen from **Figure 3** that the phylogenetic tree is divided into internal transcription intervals Region (ITS), ribosomal large subunit (LSU), transcription elongation factor (TEF) three-part sequence, Poria cocos (Wolfiporia cocos) YX2 and 22 Poria strains are relatively close and clustered together, while Pachyma hoelen, Wolfiporia cocos cocos, Poria cocos and Macrohyporia cocos are synonymous strains of Poria cocos. The genetic distance between Poria cocos species is the closest. Four synonymous Poria cocos ITS, LSU and EF sequences are clustered together, and the other three brown strains are clustered together. The Pt-2, 277, and 12388 strains of the rot fungus Piptoporus betulinus are the outgroup strains as the reference, which have both homology and differences with the interval sequences of Poria cocos ITS, LSU, and TEF, respectively. The same in NCBI The origin is more than 90%, and the ITS sequence, LSU sequence, and TEF sequence of the Poria YX2 strain are genetically linked in the phylogenetic tree. Therefore, it is clear that the wild Poria cocos in Yuexi (W. cocos) YX2 is a Poria strain under the genus Poria.

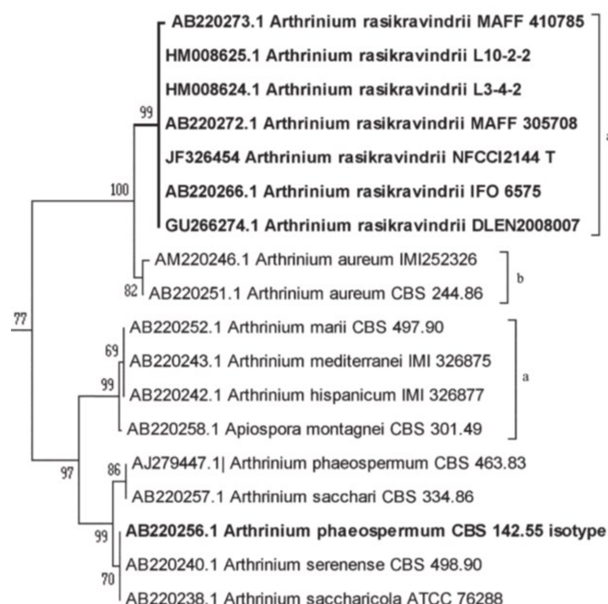
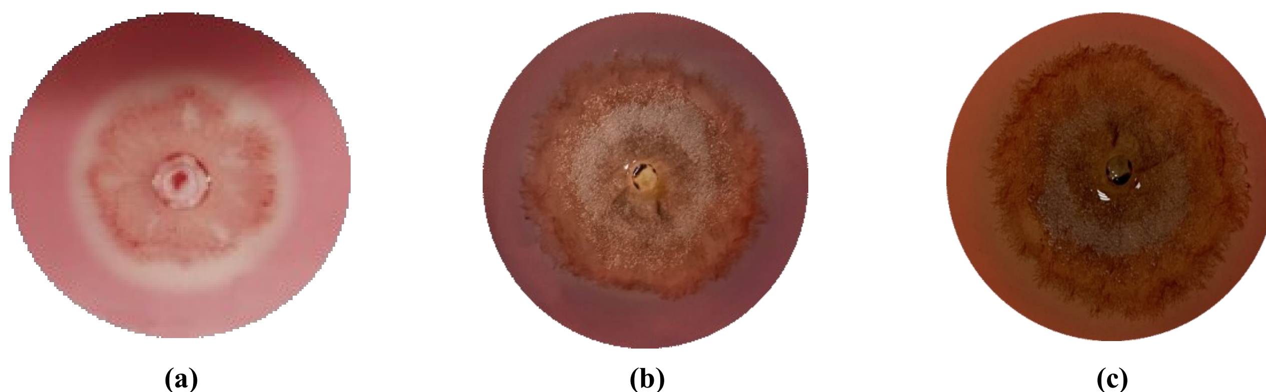


Figure 3. Phylogenetic Trees of Neighbor-Joining from W. cocos YX2.

2.3. Qualitative culture of lignocellulase

Poria hyphae were inoculated on Congo red qualitative medium, cultured at 28 ℃ for 5d, first immersed with 2g/L Congo red dye for 30min, and then decolorized with an appropriate amount of 1mol/L NaCl for 30min, measuring the maximum transparency of YX2 strain. The diameter of the circle is 14mm, as shown in **Figure 4a**. It was observed that the tested strains had obvious transparent circles, as shown in **Figure 4b,c** comparison, indicating that Poria YX2 has a certain cellulase activity.



(a) **(b)** **(c)**

Figure 4. Qualitative culture of lignocellulase from W. cocos YX2.

2.4. Enzyme activity assay of Poria lignocellulase in different culture methods

2.4.1. Cellulase activity test results of Poria cocos

The cellulase activity of Poria cocos is in the medium without pine sawdust substrate. During the 12-day culture, the experimental group Poria YX2 and the control group Poria 5.78 exo-β -glucanase, endo-β -glucan Changes in enzyme and β -glucosidase activities are shown in **Figure 5**. It can be seen from the figure that the exo-β -glucanase activities of the control group and the experimental group were

15.87U/mL and 28.644U/mL respectively on the 2nd day under the condition of no pine sawdust, and the maximum value of 16.818U/mL was reached on the 4th day without YX2 sawdust. mL, began to gradually decrease on the 6th day, while the control group reached a maximum value of 33.781U/mL on the 8th day of 5.78, showing a fluctuating decrease as a whole; the endo-β-glucanase in the control group and the experimental group were both decreased under the condition of no pine sawdust. It reached the maximum value on the 12th day, which were 32.245U/mL and 66.385U/mL respectively, showing an overall upward trend; β-glucosidase reached the maximum value on the 12th day in both the control group and the experimental group under the condition of no pine sawdust. They were 36.704U/mL and 78.871U/mL respectively, showing a steady upward trend overall. Under the condition of containing pine sawdust, the exo-β -glucanase activity of the experimental group reached a maximum of 16.711U/mL on the 4th day, and then gradually decreased, while the control group reached a maximum of 37.741U/mL on the 8th day. The volatility decreased; the endo-β-glucanase reached a maximum value of 34.706U/m on the 8th day in the experimental group under the condition of containing pine sawdust, and the overall curve changed, and the control group reached a maximum value of 106.267U/m on the 12th day. mL, showed a steady upward trend overall. On the whole, it showed an upward trend; β-glucosidase reached a maximum value of 36.811 U/mL on the 8th day in the experimental group under the condition of containing pine sawdust, and the overall curve changed, and the control group reached a maximum value of 112.689 on the 12th day, showing a steady overall Upward trend.

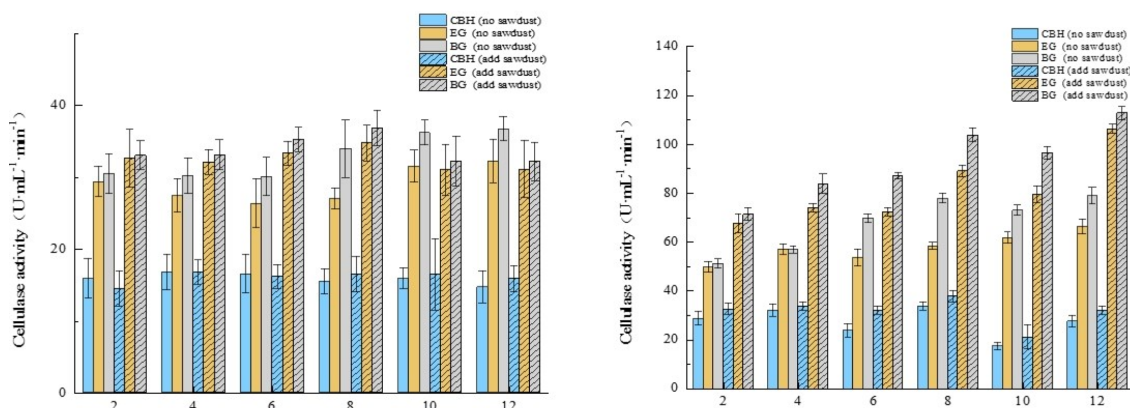


Figure 5. Cellulase activity of Wolfiporia cocos YX2and 5.78.

When Poria YX2 was cultured with or without pine sawdust, the exo- β -glucanase activities were 14.48U/mL and 15.87U/mL on the 2nd day, and the maximum enzyme activity secretion on the 4th day was 16.71U/mL, respectively. mL and 16.82U/mL, the enzyme activity decreased on the 6th day, and the overall fluctuation of the enzyme activities in the two groups was small; the enzyme activities of endo- β-glucanase were 32.6U/mL and 29.35U respectively from the 2nd day /mL, the enzyme activity showed an upward trend on the 4th to 6th day. On the 8th day, the enzyme activity with pine sawdust reached the maximum enzyme activity of 34.71U/mL, and the fluctuation decreased after the 10th day, while the enzyme activity without pine sawdust reached the 12th day. The maximum value was 32.245U/mL, and there was still an upward trend; the enzyme activities of β-glucosidase were

33.03U/mL and 30.46U/mL on the 2nd day with and without pine sawdust, respectively. On the 8th day, the enzyme activity with pine sawdust reached the maximum enzyme activity of 36.81U/mL, and then gradually decreased, while the enzyme activity without pine sawdust reached the maximum value of 36.7U/mL on the 12th day, and then the enzyme activity slowed down. Decreased, the overall enzyme activity of YX2 cellulase was compared with exo-β-glucanase < endo-β-glucanase <β-glucosidase activity.

Analysis of variance showed that the exo-β -glucanase, endo-β -glucanase and β -glucosidase activities of Poria cocos YX2 reached significant differences among different culture methods and different culture days, and the inter-subject effect was significantly different. In the test, the interspecies was 222.47*, the mean values of the three enzymes were 14.51U/mL, 30.71U/mL and 33.33U/mL, respectively, F culture method = 5.53 * and F time = 2.62 * all reached significant differences, F three enzymes = 127.53*** all reached extremely significant difference (**Table 1**).

Table 1. ANOVA results of three cellulase activities of Poria cocos with different substrates.

Strain YX2	Type III sum of squares	df	Prtial Eta square	F	P
Intercept	74033.03	1	0.99	2523.28	0.00
Species	172.47	2	0.56	222.47*	0.00
Culture methed	162.14	1	0.32	5.53 *	0.37
Three enzymes	7483.63	2	0.99	127.53 ***	0.00
Culture methed×Three enzymes	36.26	2	0.09	0.62	0.56
Culture time	38.28	5	0.70	2.62 *	0.05
Errors	352.08	12			

2.4.2. Poria hemicellulase activity detection results

Theeicellulase activity in the Poria cocos enzyme solution was detected by a microplate reader. Under the condition that there was no pine sawdust substrate in the medium, during the 12-day culture, the experimental group Poria cocos YX2 and the control group Poria 5.78 xylanase, mannan the changes of enzyme and α -glucosidase activities are shown in **Figure 6**. It can be seen from the figure that under the condition of no pine sawdust, the xylanase activities of the experimental group YX2 and the control group 5.78 were 19.244U/mL and 92.963U/mL respectively on the 2nd day, and the enzyme activity of YX2 showed an upward trend on the 4th day, and the 8th day. The experimental group reached the maximum enzyme activity of 28.786U/mL on the 10th day, and then decreased on the 10th day, while the enzyme activity peaked at 5.78 on the 12th day, and the enzyme activity reached 112.316U/mL; YX2 α mannanase was active on the 8th day. The enzyme activity reached the maximum value of 217.497U/mL, and the overall curve changed. The control group reached a maximum value of 76.195U/mL on the 12th day on 5.78, showing a steady upward trend as a whole; YX2α glucosidase reached a maximum value of 9.877 on the 8th day. U/mL; 5.78 in the control group reached a maximum of 9.699 U/mL on the 12th day, showing a steady upward trend overall. Under the condition containing pine sawdust, the enzyme activity of YX2 xylanase in the experimental group reached the maximum

value of 37.312U/mL on the 8th day, and the whole showed a curve-shaped change. The enzyme activity of the control group 5.78 reached the maximum value of 142.817U/mL on the 12th day; The enzyme activity of glycanase reached the maximum value of 341.284U/mL on the 8th day, and the overall curve changed. The enzyme activity of the control group 5.78 reached the maximum value of 97.706U/mL on the 12th day, showing a steady upward trend. The enzyme activity of glucosidase reached the maximum value of 10.662U/mL on the 8th day, and the overall change was in a curve shape. The enzyme activity of the control group 5.78 reached the maximum value of 10.413U/mL on the 12th day, showing a steady upward trend as a whole. Small.

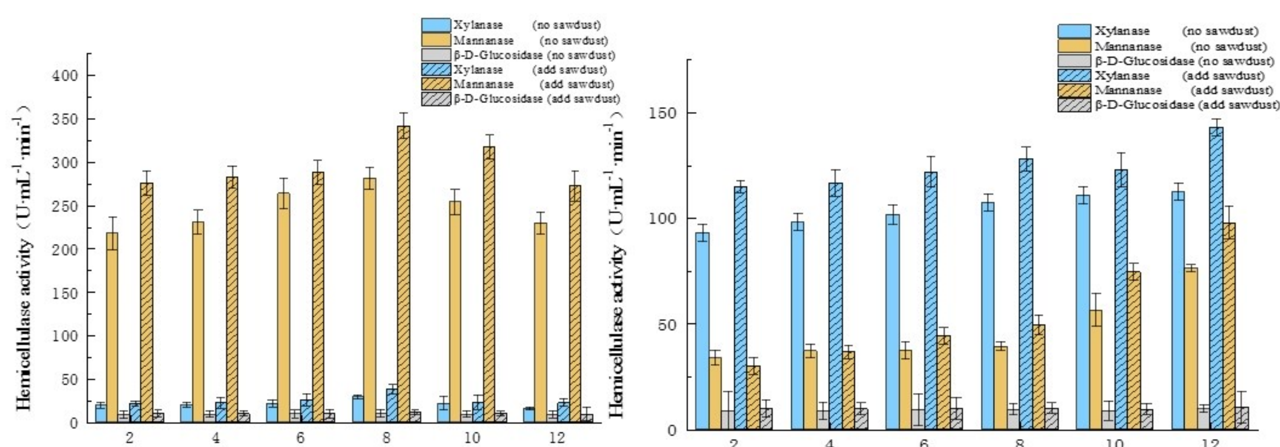


Figure 6. Hemicellulase activity of Wolfiporia cocos YX2and 5.78.

YX2 hemicellulase was active in the medium with or without pine sawdust as the substrate. During the 12-day culture, the changes in the activities of xylanase, mannanase and α -glucosidase were shown in **Figure 6**. It can be seen from the figure that the xylanase activities were 20.89U/mL and 19.24U/mL on the 2nd day under the two culture methods with and without pine sawdust, and the enzyme activities started to increase from the 4th day, and the enzyme activity on the 8th day. The maximum enzyme activity reached 37.31U/mL and 28.79U/mL, respectively, and the enzyme activity decreased to 21.9U/mL and 15.37U/mL after 10 days. The enzyme activities were 275U/mL and 217.49U/mL on the 2nd day, the enzyme activity showed an upward trend on the 4th day, and the maximum enzyme activities were 341.28U/mL and 280.74U/mL on the 8th day, respectively. Gradually decreased to 272.04U/mL and 229.09U/mL respectively; α -glucosidase enzyme activities were 9.23U/mL and 8.84U/mL respectively from 2 days onwards in the presence or absence of pine sawdust; on the 4th day at the beginning, the enzyme activities showed an upward trend. The maximum enzyme activities were 10.66U/mL and 9.88U/mL on the 8th day, and the enzyme activities decreased to 8.84U/mL and 8.77U/mL after 10 days. Overall enzyme activity compared to α -glucosidase < xylanase < mannanase.

Analysis of variance showed that the exo-xylanase, mannanase and α-glucosidase activities of Poria YX2 were significantly different between different culture methods and different culture days. *, the mean values of the three enzymes were 22.94U/mL, 270.7U/mL and 9.29U/mL, respectively, F culture method = 5.53 * and F time = 2.62 * all reached significant differences, F three enzymes = 127.53*** all reached very

significant difference (**Table 2**).

Table 2. ANOVA results of three kinds of hemicellulase activities of Poria cocos with different substrates.

Strain YX2	Type III sum of squares	fd	Partial Eta square	F	P
Intercept	1101149.2	1	0.99	2523.28	0.00
Species	276.42	2	0.56	262.86*	0.00
Culture methed	8831.29	1	0.51	12.576*	0.004
Three enzymes	1558901.87	2	0.99	1109.983***	0.00
Culture methed×Three enzymes	13730.31	2	0.09	0.62	0.003
Culture time	9037.53	5	0.45	9.65***	0.00
Errors	8426.63	12			

YX2 hemicellulase was active in the medium with or without pine sawdust as substrate. During the 12-day culture, the changes in the activities of xylanase, mannanase and α -glucosidase were shown in **Figure 5**. It can be seen from the figure that the xylanase activities were 20.89U/mL and 19.24U/mL on the 2nd day under the two culture methods with and without pine sawdust, and the enzyme activities were on the rise from the 4th day, and the enzyme activity on the 8th day. The maximum enzyme activity reached 37.31U/mL and 28.79U/mL, respectively, and the enzyme activity decreased to 21.9U/mL and 15.37U/mL after 10 days; The enzyme activities were 275U/mL and 217.49U/mL on the 2nd day, the enzyme activity showed an upward trend on the 4th day, and the maximum enzyme activities were 341.28U/mL and 280.74U/mL on the 8th day, respectively. Gradually decreased to 272.04U/mL and 229.09U/mL, respectively; α -glucosidase enzyme activities were 9.23U/mL and 8.84U/mL respectively from 2 days onwards under the two culture methods with and without pine sawdust; on the 4th day at the beginning, the enzyme activities all showed an upward trend. The maximum enzyme activities were 10.66U/mL and 9.88U/mL on the 8th day, and the enzyme activities decreased to 8.84U/mL and 8.77U/mL after 10 days. Overall enzyme activity comparison α glucosidase < xylanase < mannanase.

2.4.3. The detection results of Lignin-degrading enzyme activity of Poria cocos in 4 different culture solutions.

The MnP enzyme activity of 100μl of the Poria cocos experimental group YX2 and the control group 5.78 were detected by a microplate reader under the condition of the culture medium containing 4 different substrates during the 21d culture process. It can be seen from the figure that the culture methods withoutMn^{2+} all reached the maximum value of 0.0159 U/mL and 0.0028 U/mL on the 15th day, respectively; the culture methods containing Mn^{2+}reached the maximum value of 0.0806U on the 15th day, respectively. /mL and 0.0018U/mL, these two culture methods show that the enzyme activity of the control group 5.78 is less than that of the experimental group YX2. Comparing the results of the two mediums without Mn^{2+} and containingMn^{2+}shows that Mn^{2+} is the induction of MnP production by Poria cocos One of the factors; the maximum enzyme activity was 0.0269 U/mL and 0.0037 U/mL

on the 15th day in the culture method with pine sawdust as the substrate, and the enzyme activity of the control group was 5.78 less than that of the experimental group YX2. All three of the culture methods' enzyme activity had a generally curvilinear variation. Though the enzyme activity was not high and the secretion peak might not occur, all of the culture techniques utilizing 2,6-DMP as substrate attained their maximum values of enzyme activity within 21 days, which were 0.0251 U/mL and 0.0005 U/mL, respectively. The findings demonstrated that the variations in enzyme activity in the experimental group YX2 and control group 5.78 were in line with the four culture techniques. The MnP production of Poria cocos at 21d using the four culture techniques is depicted in **Figure 7**.

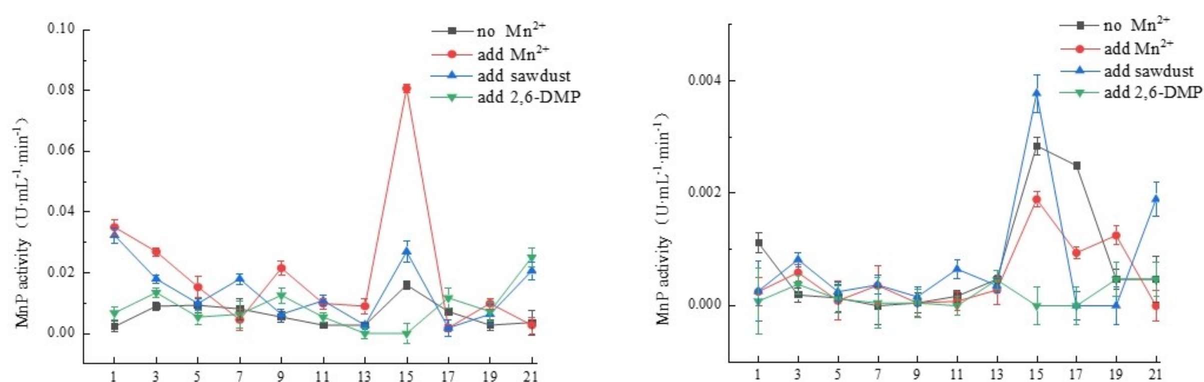


Figure 7. MnP activity of Wolfiporia cocos YX2and 5.78.

A microplate reader was used to detect the changes of Laccase enzyme activity of 100 $\mu 1$ of Poria cocos experimental group YX2 and control group 5.78 in culture medium containing 4 different substrates during the 21 d culture. It can be seen from the figure that the culture methods without Mn^{2+} reached the maximum value of 0.0315 U/mL and 0.0167 U/mL on the 7th day, respectively; the culture methods containing Mn^{2+} reached the maximum value of 0.0324U on the 7th day, respectively. /mL and 0.0309U/mL, these two culture methods show that the Laccase enzyme activity of the control group 5.78 is less than that of the experimental group YX2, and the results of the two culture solutions without $Mn^{2+}$and containing Mn^{2+} show that Mn^{2+} is the production of Laccase by Poria cocos. One of the induction factors, but the enzyme activity of Laccase did not change significantly; in the culture mode with pine sawdust as the substrate, the maximum enzyme activity reached 0.0253 U/mL and 0.0679 U/mL on the 9th day, respectively, and the control group was 5.78 higher than the experimental one. The enzymatic activity of group YX2 showed a curvilinear change in the three culture methods as a whole. However, the culture methods adding 2,6-DMP as the substrate all reached the maximum enzyme activity of 0.0296 U/mL and 0.0247 U/mL in 21 days, respectively; the secretion peak may not appear, and there is still a tendency to secrete Laccase enzyme activity. The results showed that the enzyme activities of the control group 5.78 and the experimental group YX2 in the four culture methods were consistent, but the overall Laccase enzyme activity was not high and the range of changes was small. **Figure 8** shows the production of Laccase by Poria cocos in 4 kinds of culture medium at 21d.

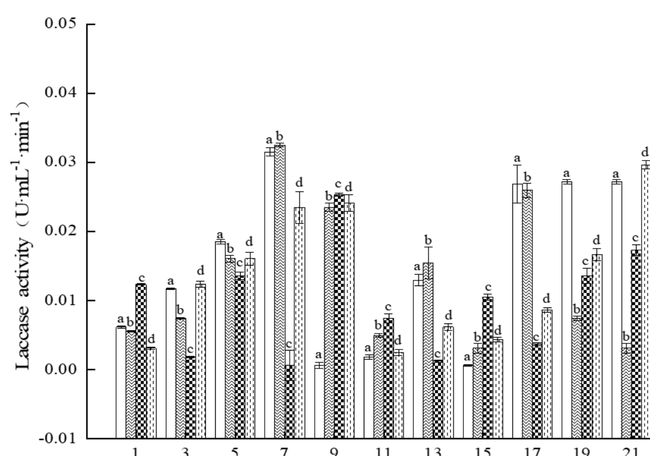


Figure 8. Laccase activity of Wolfiporia cocos YX2and 5.78.

A microplate reader was used to detect the changes of Laccase enzyme activity in 100 $\mu 1$ of Poria cocos experimental group YX2 and control group 5.78 LiP enzymes in the culture medium containing 4 different substrates during the 21d culture. It can be seen from the figure that the culture methods without Mn^{2+} all reached the maximum value of 0.0176 U/mL and 0.0347 U/mL on the 17th day, respectively; the culture methods containing Mn^{2+} reached the maximum value of 0.109U on the 11th day, respectively. /mL and 0.0391 U/mL, these two culture methods showed that YX2 in the experimental group containing Mn^{2+} secreted LiP enzyme activity earlier than the control group 5.78 without Mn^{2+}; in the culture method adding pine sawdust as the substrate, the activity reached the 11th day. The maximum enzymatic activities were 0.1046 U/mL and 0.0256 U/mL, respectively, and the enzymatic activity of the control group was 5.78 higher than that of the experimental group YX2; while the culture methods adding 2,6-DMP as the substrate reached the maximum enzymatic activity in 21 days, respectively 0.0671U/mL and 0.0738U/mL; the peak of secretion may not appear, and there is still a tendency to secrete LiP enzyme activity. The results showed that the changes of the enzyme activities of the control group 5.78 and the experimental group YX2 in the four culture methods were consistent, but the overall LiP enzyme activity was not high and the change range was small. **Figure 9** shows the production of LiP by Poria cocos in the four cultures on 21d.

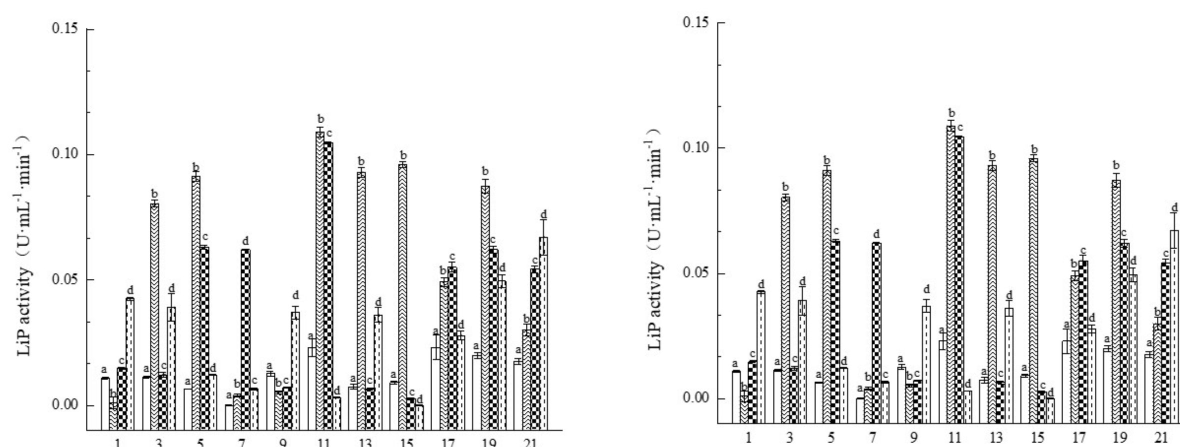


Figure 9. LiP activity of W.cocos YX2and 5.78.

3. Discussion

3.1. Identification of wild Poria

This study to wild Poria YX1 as the object of study, from the agglomerated mycelium culture, mycelium with septa, occasionally see the phenomenon of locking joint, Poria nuclei are also a dormant body for Poria mycelium group. Fresh mycelium kernel cut Poria meat is white, which can also be used as the parent species or cultivated species of Poria cocos meat citation. Combined with the identification of molecular biology, selected ITS, LSU, TEF sequence amplification, analysis of PCR products, YX1 and Birch Stripping Tubers Pt-2, 277, 12388 strains are the same as the brown rot bacteria, in the kinship of both the connection and genetic gap [21]. Poria cocos (Pachyma hoelen), Wolfiporia cocos, Poria cocos and Macrohyporia cocos have four synonyms, and they are closely related to each other in the phylogeny, according to the Dictionary of Mycology, 10th edition, 2008 and with reference to the latest compilation of the eight-world system of According to the Dictionary of Mushrooms, 10th edition and referring to the latest compilation of the eight-boundary system of mycological taxonomy, Poria cocos YX1 (Wolfiporia cocos) belongs to the family of Stramenophora (Basidiomycota), the subphylum of mushrooms (Agaricomycotina), the class of mushrooms (Agaricomycetes), the order of polypore mushrooms (Polyporales), and the family of Fomitopsidaceae (Fomitopsidaceae) [22].

3.2. Cellulase and hemicellulase production by Poria cocos

In this study, the preliminary qualitative culture of Poria cocos-producing cellulase was done, and there was an obvious hyaline circle phenomenon, and YX1 was screened for its ability to degrade lignocellulose, and as a quantitative culture of the research strain YX1 was used for the determination of lignocellulase activity. It was found that cellulase and hemicellulase had a convergent pattern of change under the condition of having pine wood chips and no pine wood chips as substrate. Compared with the control group without pine wood chips and the experimental group with pine wood chips as the substrate in the culture mode, except for CBH, EG and BGL were higher in the experimental group with pine wood chips than in the control group without pine wood chips, and from the comparison of the cellulase activity, CBH < EG < BGL in cellulase, and the mannanase, xylanase, and α -glucosidase were higher in the experimental group with pine wood chips than in the control group without pine wood chips, and the activity of cellulase from hemicellulose was higher in the experimental group with pine wood chips than in the control group without pine wood chips, while the activity of cellulase from hemicellulose was higher in the control group without pine wood chips. Wood chips control group, from the hemicellulase activity between the comparison, hemicellulase in mannanase < xylanase < α-glucosidase [23]. Among the three cellulases in the experimental group of Poria cocos, CBH had the highest enzyme activity at the early stage of incubation, which is consistent with the results of the study [24]. Both EG and BGL enzyme activities among cellulases reached the highest value of secretion on day 8, BGL was slightly higher than the enzyme activity of EG, and both enzyme activities were more than two times higher than CBH. The mannanase of the three hemicellulases in the

experimental group of Poria cocos was 9-32 times higher than xylanase activity and α -glucosidase, which is consistent with the results of this study [25]. The study [26] reported the cellulase and hemicellulase activities of three species of brown rot fungi compared to the present study of Poria, Poria YX1 were higher than the enzyme-producing capacity of these three species of red-rimmed laminarial fungi (Fomitopsisspinicola), birch stripping tubular fungi (Piptoporus betulinus) and yellow umbrella (Pholiota adiposa).

3.3. Lignin-degrading enzymes produced by Poria

Poria cocos is a brown rot fungus that mainly produces cellulase and hemicellulase, but also produces weak lignin-degrading enzyme activity [27,28]. In Poria cocos lignin degrading enzymes, except Laccase, MnP and LiP showed that the experimental group with pine shavings was higher than the control group without pine shavings, which indicated that the addition of Mn^{2+} and pine shavings as the substrate for culture could play an inducing role. Study [29] reported the MnP enzyme activity of fungi such as Ceriporiopsissubvermispora (Ceriporiops), Pleurotus ostreatus (Pleurotus ostreatus) and Physisporinus rivulosus (Physisporinus rivulosus) under co-culture conditions, and the highest MnP activity was detected after 7 d, and the highest MnP activity was observed after 7 d, and the highest MnP activity was observed after 7 d. The MnP enzyme activity obtained in several different culture methods ranged from 25 to 250 $U \cdot mL^{-1}$, compared with the results of previous studies, Poria cocos MnP enzyme activity was relatively weak, the highest enzyme activity of the four culture methods was only 0.081 $U \cdot mL^{-1}$. Poria cocos Laccase culture mode A and B relative to each other, culture mode B reached the secretion peak in advance, but was not much affected by Mn^{2+}; culture mode C and D, the same day 9 to reach the secretion peak, compared with culture mode A without Mn^{2+}, not affected by pine wood chips [30,31]. The study [32] reported that the four cultures of red flat mushroom Laccase enzyme activity varied from 36 to 205 $U \cdot L^{-1}$, the enzyme activity secretion under the conditions of containing Mn^{2+} and no Mn^{2+} , which was basically consistent with the results of this study, indicating that the secretion pattern of Laccase enzyme activity was not obvious, and the overall fluctuating changes, in comparison with the Poria cocos Laccase enzyme activity was weak, only 0.025-0.025 to 0.025, which was the same as that in the culture mode A, which was not affected by pine wood chips. Only 0.025-0.032 $U \cdot mL^{-1}$. LiP culture mode A and B compared, LiP is affected by Mn^{2+} , and reached the peak of secretion in advance; culture mode C, the peak of secretion was reached on the 7th day, compared with the culture mode A, the results showed that the pine wood chips as a substrate is one of the conditions for the induction of LiP; culture mode D, the 19th day of LiP enzyme activity reached the peak of secretion. Study [30] reported that LiP enzyme activity of Nigella sativa, Ganoderma lucidum, Ashwagandha and J201 fungi all reached the highest on the 9th day, which were 121.6, 143.5, 111.6 and 145.2 $U \cdot mL^{-1}$, respectively, and the pattern of enzyme production was in the form of a gradual increase and then a gradual decrease, and the enzyme producing ability was still present after reaching the peak, which was similar to the secretion pattern of the culture methods of this study that produced LiP containing Mn^{2+} and pine wood shavings. This is similar to the present

study of LiP production with Mn^{2+} and secretion in culture with pine wood chips, but the difference is that the maximum enzyme production time is reached, and the enzyme activity of LiP produced by Poria cocos is relatively weak, and the enzyme activity is only 0.017～0.109 U · mL^{-1}. From the comparison between the lignin degrading enzyme activities of Poria, the lignin degrading enzyme LiP > MnP > Laccase, due to the weak lignin degrading enzyme produced by Poria, the results of the three enzyme activities of MnP, Laccase and LiP were not analysed by further analysis of variance (ANOVA). From the comparison between lignocellulase activities, lignin degrading enzyme < cellulase < hemicellulase activity. Overall, the above results showed that Poria cocos has the ability to secrete lignocellulase, and Poria cocos as a brown-rot fungus only has weak ligninase activity, and there is a significant difference between the multiple enzymes ($P < 0.05$).

In summary, the combination of morphological characteristics of wild Poria cocos and molecular biological identification clarified the taxonomic status of Poria cocos, revealed the main enzyme families of its lignocellulase system and the connection with the culture conditions and so on, therefore, this strain can be a candidate for in-depth study. The enzyme activity of CBH in cellulase reaches the maximum amount at the early stage of culture, the enzyme activity of α -glucosidase in hemicellulase reaches the maximum amount at the early stage of culture, and the lignin degrading enzymes MnP and LiP reach the maximum amount of secretion at the late stage of culture [33,34]. Poria cocos bacteria constantly secreted more cellulase and hemicellulase enzymes, which played a dominant factor in the pre-composition process, while lignin degrading enzymes were a secondary factor. The study [35] found that Poria isolated and purified two MnPI and MnP II isoenzymes, and the optimised MnP enzyme activity was as high as 1249.7 U . mL^{-1}. Therefore, further optimisation of the enzyme-producing culture conditions is still needed to improve the enzyme production efficiency of lignocellulase from Poria cocos at a later stage. In addition, the results of this study showed that the difference in lignocellulose degrading enzyme activity might be related to the conditions of strain characteristics, cultivation methods and inducing factors, and the overall enzyme activity of lignocellulose degrading enzyme was lower, which might be related to the use of nitrogen-limiting medium, the sensitivity of enzyme labelling instrumentation detection, as well as the growth characteristics of Poria cocos itself and its nucleus formation cycle. The results of this study also showed that the enzyme activities of lignocellulase-producing enzymes in Poria were in the order of mannanase > xylanase > BGL > EG > CBH > α-glucosidase > LiP > MnP > Laccase.In terms of the structure of the decomposed lignocellulose composition, in addition to lignocellulase, Poria cocos bacteria may also contain a wealth of other auxiliary enzymes, which participate in the degradation mechanism of lignocellulose in synergistic manner with a variety of enzymes, catalysing the degradation of lignocellulose. cellulose degradation mechanism, catalysing the Poria mycelium to continuously aggregate, absorbing intra- and extra-basal nutrients, and ultimately forming agglomerated nuclei, all of which need to be further investigated.

Author contributions: Conceptualization, LD and FZ; methodology, FZ; software, FZ; validation, FZ, YY and WF; formal analysis, LD; investigation, FZ; resources, WF;

data curation, WF; writing—original draft preparation, LD; writing—review and editing, WF; visualization, LD; supervision, WF; project administration, WF; funding acquisition, FZ. All authors have read and agreed to the published version of the manuscript.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Huaihua University (granted No. HHUNSWLL_202309432, granted time.20230908). All participants signed the informed consent in this study.

Conflict of interest: The authors declare no conflict of interest.

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