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Screening, Isolation and Identification of High Antioxidant Strains from 16 Kinds of Mushrooms

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To promote the rational exploration of the mushroom resources, this study evaluated the *in vitro* antioxidant activity of 16 kinds of mushrooms collected from the Sichuan province in China and completed the identification of the optimal active strains.
Place and Duration of Study: School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan province, China. April 2019 to May 2020 (13 Months).
Methodology: 16 kinds of mushrooms were collected in the wild. These strains were isolated and cultivated. The extract's antioxidant activities of fermentation broth and mycelium were assessed using the DPPH radical-scavenging method. The higher activity strains were also evaluated by Trolox equivalent antioxidant capacity (TEAC) and the Ferric reducing antioxidant power (FRAP) assay. Meanwhile, the contents of total flavonoids and total phenols were determined. Finally, The highly active strains were identified by internal transcribed spacer (ITS) Identification.
Results: All extract of fermentation broth and mycelium from 16 kinds of mushrooms had scavenging effects on DPPH. Among them, strain No. 6 and No. 7 exhibited obvious antioxidant capacity, and the scavenging rates of their fermentation broths on DPPH were 89.47% and 84.27%, respectively. And the results of TEAC and FRAP were consistent with those of DPPH. Besides, these two kinds of mushrooms were all rich in phenolic and flavonoid compounds, and the content in the fermentation broth was significantly higher than that in the mycelium, indicating that phenolic

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and flavonoid substances may be the material basis of their antioxidant effect. The strain No. 6 and No. 7 were confirmed to be *Bjerkandera* and *Nigrospora sp,* respectively by 16S nrDNA sequence identification.

Conclusion: The results showed that *Bjerkandera* and *Nigrospora sp.* possessed the highest antioxidant capacities with rich total phenolic and total flavonoids. They could be potential sources of natural antioxidants for further exploration and application.

Keywords: Mushroom; antioxidant activity; total phenolic; total flavonoids; ITS.

1. INTRODUCTION

Most biological systems are constantly exposed to active oxidants from endogenous and exogenous sources [1]. Reactive oxygen species (ROS) include superoxide free radical, hydrogen peroxide, hydroxyl free radical, and singlet oxygen [2]. Free radical-induced oxidative damage is involved in the pathogenesis of many chronic and degenerative diseases, such as cardiovascular disease, cancer, diabetes, neurodegenerative disease, and aging [3,4]. Antioxidants are substances capable of scavenging ROS and protecting from oxidative damage [5]. With the development of free radical research, the development of natural antioxidants has become a research hot spot.

Fungi are considered to be one of the most diverse, ecologically significant, and economically significant organisms on the planet [6]. More and more researchers are looking for natural antioxidants targeting fungi [7,8]. Among them, Macrofungi, also known as mushroom, has played an important role in Traditional Chinese Medicine for centuries. Its dried extracts from fruit bodies have occupied a lucrative market in the herbal medicine market in western countries [9-10]. In recent years, a host of mushrooms that possess antioxidant activity have gradually become a hot spot in the development of nutritious food and functional food ingredients [11]. In this study, sixteen species of macrofungus (mushrooms) collected in the wild were used as experimental materials to study their antioxidant capacity, determine the total phenolic and flavonoid content. Then, highly active strains were identified by 16s nrDNA sequence analysis. These above works provided a theoretical basis for further development and utilization of these mushroom resources.

2. MATERIALS AND METHODS

2.1 Materials

Sixteen kinds of mushrooms were collected in Longshan Experimental Base of Southwest University of Science and Technology, Mianyang, Sichuan province, China. (Fig. 1). The voucher specimens were kept in the Microbiology Laboratory of the Southwest University of Science and Technology.

1,1-diphenyl-2-bitracinium free radical (DPPH), 2, 2'-azinobis(3-ethylbenothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2, 4, 6-Tri(2-pyridyl)-s-taiazine (TPTZ) were purchased from TCI (Shanghai) Chemical Industry Development Co., Ltd. Folin-Ciocalteu was purchased from Nanjing Oddfoni Biological Technology Co., Ltd. BHA (Butyl hydroxyanisole) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Sodium carbonate and other reagents were purchased from Chengdu Kelon Chemical Reagent Factory. All chemicals used in the experiment were of analytical grade.

PDA medium (potato 200 g, glucose 20 g, agar 17 g, $MgSO_4$ 1.5 g, water 1 000 mL) was used for the isolation and expansion of the strain. PD medium (potato 200 g, glucose 20 g, $MgSO_4$ 1.5 g, water l 000 mL) was used for liquid culture.

2.2 Methods

2.2.1 Strain isolation and culture

Strain isolation and culture referred to Shin TY's method [12]. The pileus of the fresh mushroom was cut off with a scissor and placed on a filter paper, covered with a beaker for 24h, and the spores were collected.

The spores were inoculated and cultured on PDA medium at 25℃. The strains were isolated and purified by the streak plate method. At last, 16 pure strains of mushrooms were obtained through many times streaking and preserved in glycerin agar at -20℃ before use.

The purified strains were activated on the PDA medium for 5-7 days using the streak plate method. Then, the single colony was inoculated in a 500 mL triangle flask containing 300 mL PD

Fig. 1. The collection samples of 16 kinds of mushrooms

medium under aseptic operation in a constant temperature oscillating incubator at 25℃ for at least 30 days. The fermentation state was observed and recorded every 5 days. When the mycelium was no longer growing, the culture was complete

2.2.2 Sample extraction

After the cultivation was completed, the myceliu m and the fermentation broth were obtained by fil tration. The mycelium was extracted twice by ethyl acetate (mycelium/ethyl acetate=1:5 mL) as Yadav M's description [13]. The extraction and filtration operation was repeated several times until the extract solution was close to colorless. Similarly, ethyl acetate solution (ethyl acetate/H_2O= 1:1) was added to the fermentation broth and extracted liquid-liquid for several times until the extraction solution was nearly colorless. These extracts were condensed into solid using a rotary evaporator, then sealed in a glass bottle filled with N_2 for standby use.

2.2.3 Antioxidant assays

2.2.3.1 DPPH radical-scavenging activity

DPPH solution (0.03 mg·mL^{-1}) was prepared with anhydrous ethanol, which was stored in the dark until used. Antioxidant activity of the extract was measured using Pyrzynska's method [14] with slight modification. 0.2 mL supernatant of various extracts were added into 4 mL of DPPH solution separately. The mixture was vigorously shaken and incubated for 30min at room temperature in darkness. Then, the supernatant was transferred to the cuvette and the absorbance of the sample

was measured at a wavelength of 517 nm. The decrease in the absorbance indicated radical-scavenging activity. The antioxidant capacity of the sample can be expressed by the scavenging rate (SR %) and calculated using the following formula:

$$SR\ \% = (1-(Ai - Aj)/A0) \times 100\%$$

Ai: Absorbance of 0.2 mL test solution mixed with 4 mL DPPH solution;
Aj: Absorbance of 0.2 mL test solution mixed with 4 mL anhydrous ethanol solvent;
A0: Absorbance of 0.2 mL of the solvent used in preparing the test solution after mixing with 4 mL of DPPH solution.

2.2.3.2 Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay was carried out to determine the free radical scavenging activity using the ABTS radical cation according to the method of Koh et al. [15] with a slight modification. Briefly, 7 mM ethanolic ABTS solution was mixed with 2.6 mM potassium persulfate in volume 1:1 for 16 h in the dark at room temperature. Then, this mixture was combined with 0.2 mL of the sample solution in a tube. After storing the mixture in the dark for 10 min, its absorbance at 650 nm was measured. Ascorbic acid (Vitamin C) and dibutyl hydroxytoluene (BHT) were used as positive controls at a concentration of 0.1-1.0 mg/mL. Standard solution (240 µL) and ethanol (60 µL) were regarded as negative controls. The calculation method is the same as the DPPH method in section 2.2.3.1.

2.2.3.3 Ferric-reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out according to Uysal [16] with some modifications. Briefly, the FRAP reagent was prepared from sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution (40 mM HCl as solvent), and 20 mM iron (III) chloride solution in a volume ratio of 10:1:1, respectively. The FRAP reagent was prepared fresh daily and warmed to 37°C in a water bath before use. A mixture containing 3 mL of freshly prepared and prewarmed (at 37°C) FRAP reagent and an aliquot of sample extract was incubated at 37°C for 30 min and the absorbance was then recorded at 593 nm. The preparation and calculation methods of the samples and positive controls were the same as the ABTS method in item 2.2.3.2.

2.2.4 Total phenolic content determination

The Folin-Ciocalteu method was used to measure the total phenolic contents. This method relied on the transfer of electrons from phenolic compounds to the Folin-Ciocalteu reagent in alkaline media, the reaction product had maximum absorption at 765 nm and the absorbance value was linear with polyphenol content.

1.5 mg gallic acid was dissolved in 10 mL absolute ethanol to prepare the stock solution. And sample solutions were made by diluting the stock solution to five different concentrations including 50, 75, 100, 125, and 150µg·mL^{-1}. 0.2 mL of sample solution was mixed with 0.5 mL Folin-Ciocalteau reagent and 4.0 mL of pure water in 10 mL volumetric flasks. Then, 200 µL of 20% sodium carbonate solution was added in and the final volume was 10.0 mL with distilled water. The absorbance of the reaction mixtures was measured at 760 nm after incubation for 30 minutes at room temperature. The total phenolic content was expressed as micrograms of gallic acid equivalent per milligram of crude extract (µg GAE/mg CE).

2.2.5 Total flavonoids content determination

Total flavonoids (TF) content was estimated using the modified colorimetric method [17]. The standard control--Rutin was diluted to different concentrations. Two milliliters of standard sample solutions of different concentrations were placed into a test tube and blended with 1 mL 5% $NaNO_2$ for 6 min, and then 1 mL 10% $Al(NO_3)_3$ was added. Six minutes later, 10 mL 1 mol/L NaOH was added and kept at 25℃ for 15 min. The absorbance at 510 nm was detected and the sample's TF content was calculated using the standard curve.

2.2.6 Strains identification

The mushrooms with the optimum antioxidant activities were selected. Their DNAs were extracted using a fungal genomic DNA extraction kit, and the genomic DNA was used as the template for PCR reaction.

Fungal internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) were amplified from extracted DNA samples using the primers ITS1 and ITS4. The primer information up and down was: ITS1: CTTGGTCATTTAGAGGAAGTAA, ITS4:

TCCTCCGCTTATTGATATGC. The amplification system includes 2XTaq-PCR MIX 25 μL, ITS1 2 μL, IST4 2 μL, Genomic DNA 2 μL, and ddH_2O 19 μL. The sequence was as follows: 95℃ for 5 min, then 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, followed by 72℃ for 5 min. The original solution of PCR amplification will be sent to Beijing Hitro Biotechnology Co., LTD for identification.

2.3 Statistical Analysis

All the experiments were performed in triplicate, and the results were expressed as mean ± SD (standard deviation). Statistical analysis was performed using MS Excel 2007 and SPSS 15.0.

3. RESULTS AND DISCUSSION

3.1 Comparison of DPPH Antioxidant Activity

In this study, the antioxidant abilities of 16 strains were compared based on the scavenging of the DPPH radical. The SR% values were shown in Table 1. All strains showed DPPH free radical scavenging ability, and most of the fermentation broth was stronger than the mycelia. It was found that 7 strains with a strong DPPH clearance rate(>50%) accounted for 43.75% of the tested strains. The two strains with the highest antioxidant ability were No.6 and No.7, which were selected for further TEAC and FRAP assays, respectively.

Table 1. Results of the DPPH scavenging rate of 16 kinds of mushrooms

Number	Fermentation broth	Mycelia
1	53.21%	<30%
2	38.25%	<30%
3	<30%	<30%
4	<30%	<30%
5	<30%	<30%
6	89.47%	33.38%
7	84.27%	58.54%
8	<30%	<30%
9	58.87%	<30%
10	<30%	<30%
11	<30%	<30%
12	<30%	<30%
13-white	58.07%	<30%
13-black	57.60%	<30%
14	<30%	<30%
15	<30%	<30%
16	57.60%	<30%

3.2 Determination of TEAC

The TEAC assay is used to determine the ability to scavenge ABTS radicals. As can be seen from Fig. 2, with the increase of concentration, the ABTS free radical scavenging rates of the two strains were significantly increased. ABTS free radical scavenging abilities of different parts were also different, and the scavenging ability of fermentation broth was slightly higher than that of mycelium, which was consistent with the results of the DPPH method. When the concentration was reached 10 mg/mL, the free radical scavenging rates of these two strains were close to that of positive control BHT and Vitamin C. Strain No. 6 had a slightly higher free radical scavenging capacity than strain No. 7 because it achieved a higher free radical scavenging rate at lower concentrations.

3.3 Determination of FRAP

The FRAP method is based on the REDOX reaction. Under acidic conditions, Fe^{3+} and TPTZ form Fe^{3+} -TPTZ complex. In the presence of reducing substances in the solution, the Fe^{3+} -TPTZ complex is reduced to Fe^{2+} and shows an obvious blue color. The maximum absorption occurs at 596 nm, and the change in absorbance is proportional to the content of the reduced substance. Fig. 3 showed that both No. 6 and No. 7 strains have certain reducibility. With the increase of concentration, their absorbance increased significantly to close to the positive control BHT.

3.4 Correlation between DPPH, FRAP and TEAC Assays

The correlation between FRAP value and TEAC value adapt simple linear regression model analysis. For the fermentation broth and mycelium of the two strains, TEAC and FRAP showed a good linear relationship (R^2 were 0.996, 0.939, 0.998, and 0.993, respectively. $P<0.05$) (Fig. 4), which illustrated that the two methods were generally consistent for evaluating antioxidant capacities in these strains. This phenomenon explained the antioxidant active substances from the two strains could not only reduce oxidants (ferric ions) but also scavenge free radicals ($ABTS^{\bullet+}$).

3.5 Total Phenolic and Total Flavonoid Content

Many studies have found that the content of flavonoids and total phenols are positively

correlated with the antioxidant capacity as flavonoids and phenols are the main natural antioxidant components in many plants and mushrooms [18-20]. Higher concentrations of flavonoids and phenols generally demonstrated higher antioxidant capacity [21]. In this study, the Fulin-ciocalteu reagent was used to determine the total phenolic content of the two screened strains with gallic acid equivalent (the standard curve equation: y=0.0039x+0.0029, R^2 = 0.9903). The flavonoid content was expressed in terms of rutin equivalent (the standard curve equation: y=1.2084x-0.0027, R^2 = 0.9982). As shown in Table 3, the results showed that the content of fermentation broth was significantly higher than that of mycelia, which was consistent with the experimental results of DPPH antioxidant capacity. The higher the content of these two compounds, the stronger the antioxidant capacity is, suggesting that their high antioxidant activity may be attributed to phenolic and flavonoids components.

3.6 Strains Identification

The extracted DNA was detected by 1.5% agarose gel electrophoresis, and the DNA bands were clear and bright without obvious trailing phenomenon, indicating that the genomic DNA was complete and could be used as a template for PCR. Using DNA as template and ITS1 and ITS4 as primers, The PCR amplification of 16s nrDNA ITS segments showed that the fragment size was about 400 bp (Fig. 5), which was consistent with the expected results.

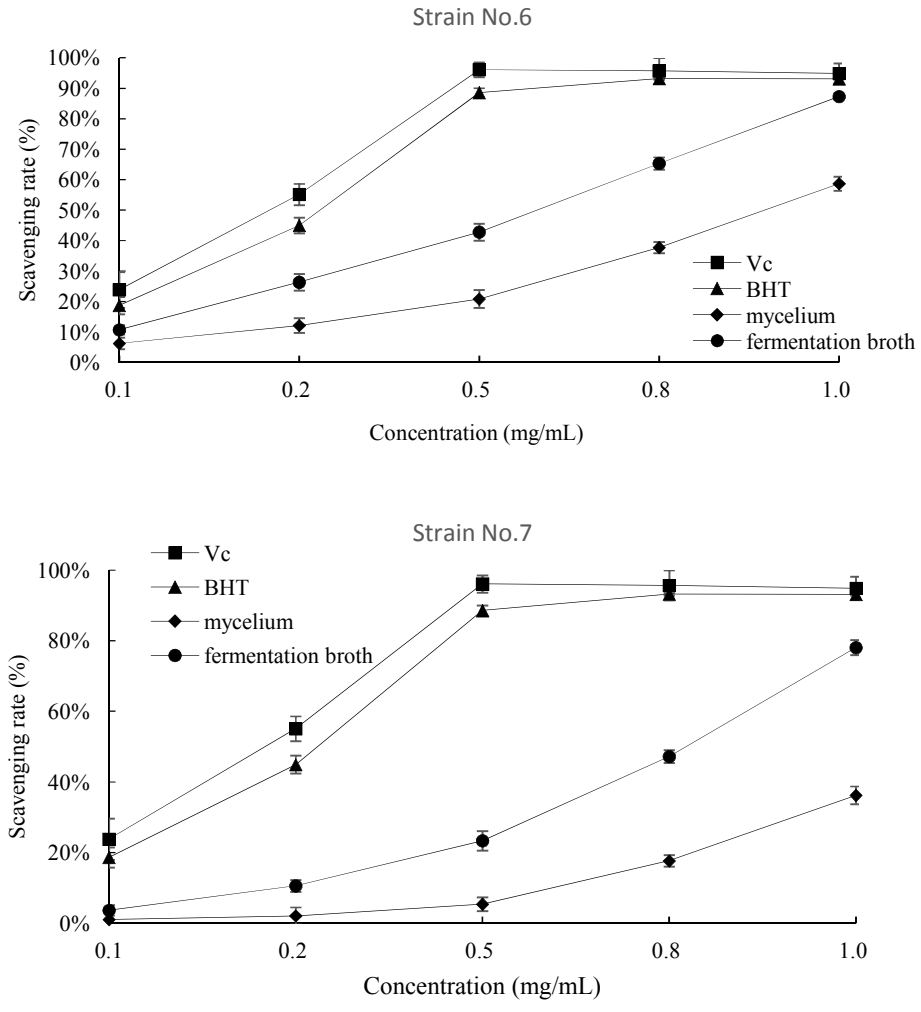


Fig. 2. ABTS free radical scavenging rate of two strains

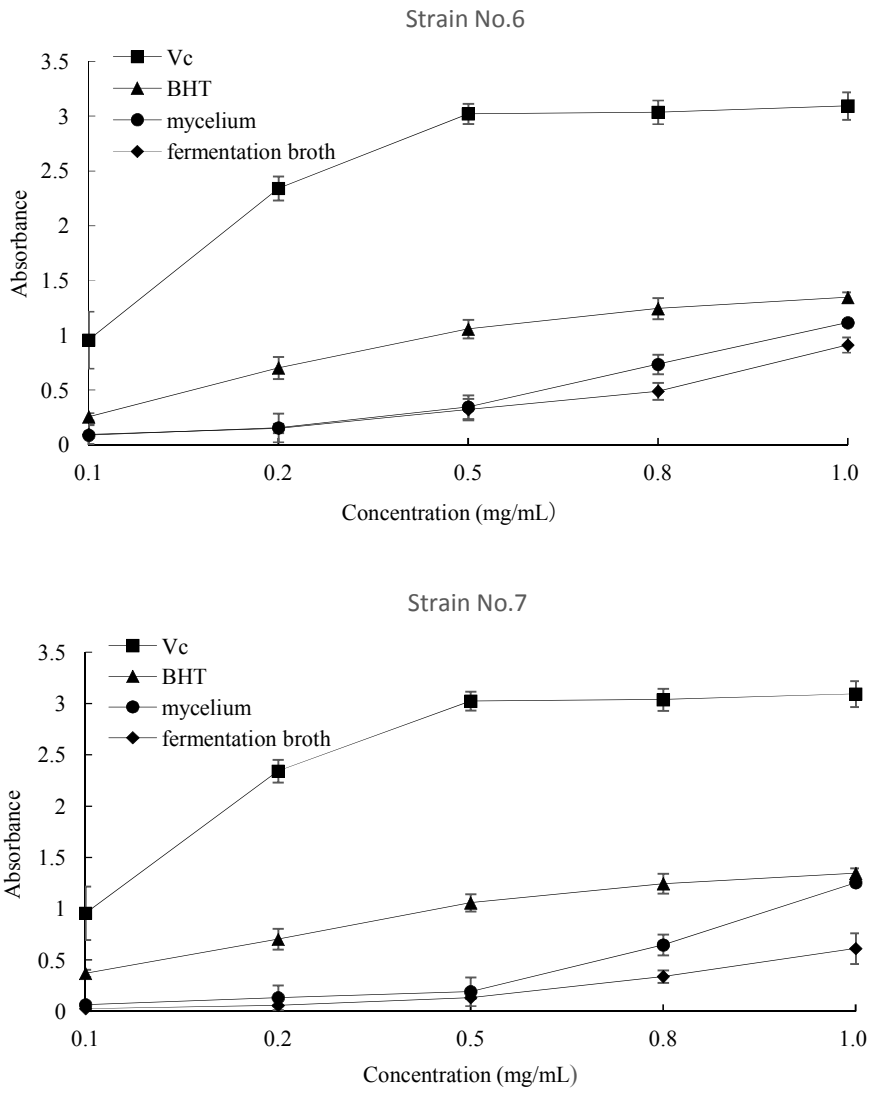


Fig. 3. Reducing power of the two strains

Table 2. IC_{50} of DPPH, ABTS and positive control

Method	Strain No. 6		Strain No. 7		Positive control	
	Mycelium	Fermentation broth	Mycelium	Fermentation broth	BHT	Vc
DPPH	2.310±0.0234	0.403±0.0067	1.141±0.0103	0.601±0.0110	0.221±0.0053	0.188±0.0098
ABTS	1.050±0.0580	0.445±0.0163	1.774±0.0337	0.716±0.0112	0.215±0.0075	0.175±0.0078

Furthermore, as can be seen from Table 2, the IC_{50} value of DPPH scavenging activity was successively Vc>BHT>fermentation broth of No.6 > fermentation broth of N0.7 > Mycelium of No.7 > Mycelium of No.6. The results were consistent with the ABTS. Above all, These data demonstrated that DPPH, FRAP and TEAC assays were consistent

Table 3. Total polyphenols and total flavonoid contents of 2 kinds of mushroom

Strain	Total polyphenols		Total flavonoid	
	Fermentation broth	Mycelium	Fermentation broth	Mycelium
No. 6	2.48%	0.82%	7.30%	4.80%
No. 7	2.63%	2.30%	5.00%	4.62%

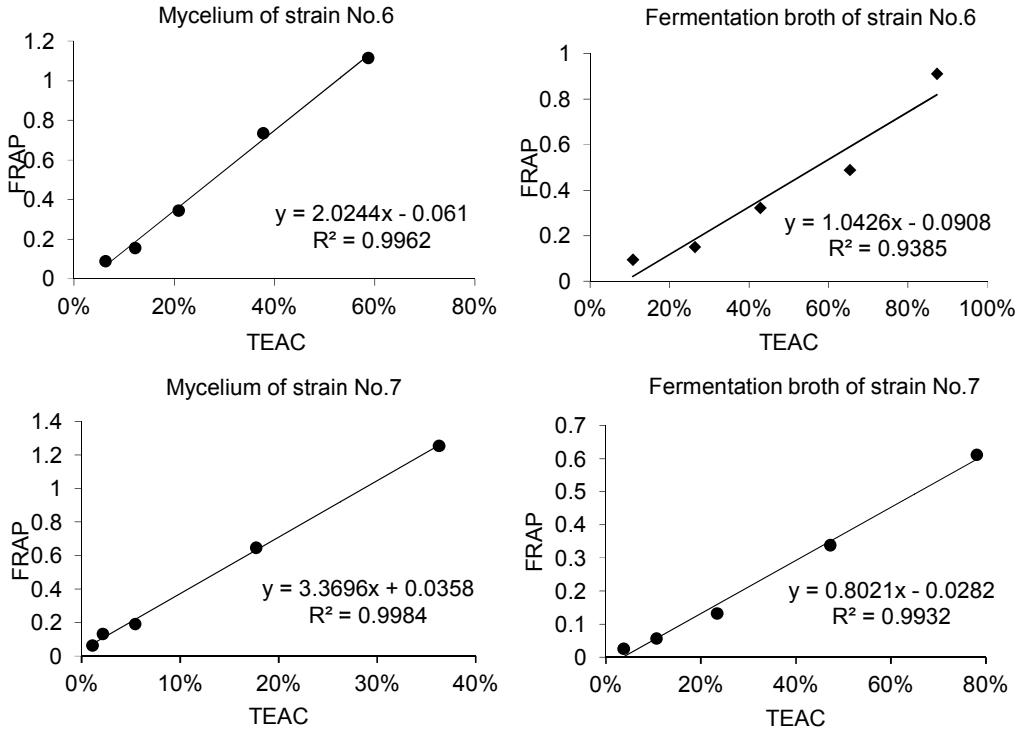


Fig. 4. Correlation between the antioxidant capacity measured by the FRAP and TEAC

The PCR amplification solution was sent to Beijing Hitro Biotechnology Co., Ltd. for identification. The phylogenetic tree was constructed by homology comparison results, as shown in Fig. 6. Strain No.6 and *Bjerkandera adusta* were clustered in one branch with the closest genetic relationship. Therefore, strain No. 6 was identified as *Bjerkandera adusta*. Similarly, strain No. 7 was identified as *Nigrospora sp*.

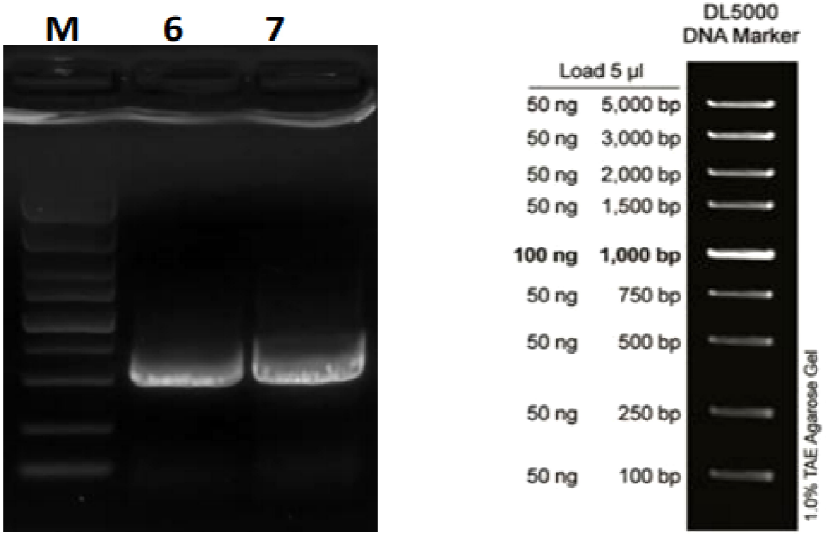
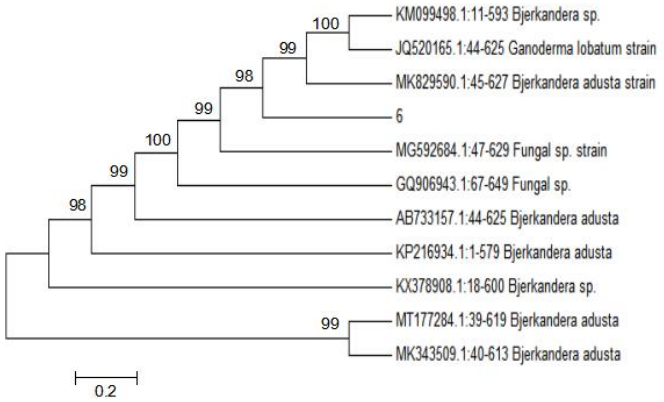
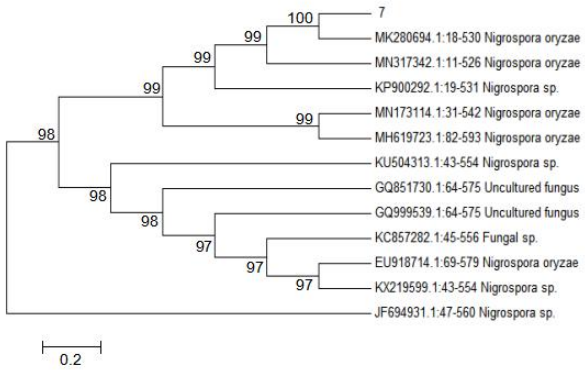


Fig. 5. DNA and PCR amplification electrophoresis of strains No. 6 and No. 7



a



b

Fig. 6. Phylogentic tree of two strains (a: No.6, b: No.7) based on 16S nrDNA sequence

4. CONCLUSION

In this study, the DPPH assay was used to evaluate the antioxidant activity of 16 strains of mushrooms. The DPPH free radical scavenging rate in the fermentation broth of strains No. 6 and No. 7 were over 80%. Their antioxidant capacities were furtherly confirmed using the TEAC and FRAP methods, respectively. A significant correlation between the TEAC and FRAP values suggested that antioxidants in these strains were capable of reducing oxidants and scavenging free radicals. There are rich in phenolic and flavonoids in these strains also high compared to other literature [22,23], indicated that the compounds could be the main contributors to the antioxidant capacities of these strains.

At last, strains No. 6 and No. 7 were identified as *Bjerkandera* and *Nigrospora SP* by 16S nrDNA sequence analysis. The results of this study indicated that these two mushrooms may be a potential source of natural effective antioxidant used in the food and pharmaceutical industries. In the future, the compounds with high antioxidant capacities would be isolated, purified, and identified from these two mushrooms.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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