academicJournals

Vol. 12(15), pp. 1862-1868, 10 April, 2013
DOI: 10.5897/AJB11.2638
ISSN 1684–5315© 2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Studies on some active components and antimicrobial activities of the fermentation broth of endophytic fungi DZY16 Isolated from *Eucommia ulmoides* Oliv.

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Accepted 8 November, 2012

Research into plant-derived endophytic fungi has grown in recent decades. Endophytic fungi still have enormous potential to inspire and influence modern agriculture. In this study, the endophytic fungi DZY16 isolated from *Eucommia ulmoides* Oliv. was tested for its bioactive components and antimicrobial activities using phenol-sulfuric acid method, high performance liquid chromatography method and growth inhibition measurements. The results show that variation trend of extracellular polysaccharide content at different growth stages of the strain DZY16 and the maximum content of extracellular polysaccharide was 2.02 g/L at the sixth day. Moreover, the fermentation broth of the DZY16 contained guanosine, uridine and adenosine; the contents were 1.54 mg/g, 1.07 mg/g and 1.36 mg/g respectively. On the other hand, the strongest antimicrobial activity was exhibited by the acetylacetate extract of strain DZY16 against *Rhizoctonia solani* and *Gibberella zeae*, showing 59.84 and 70.86% respectively. The strain DZY16 was identified by internal transcribed spacer (ITS) sequence as belonging to *Nigrospora*. The results indicate that the endophytic fungi DZY16 of the plant *E. ulmoides* Oliv. is a promising source of novel bioactive compounds.

Key words: *Eucommia ulmoides* Oliv., endophytic fungi, extracellular polysaccharide, nucleotides, antimicrobial activity.

INTRODUCTION

Endophytic fungi are microorganisms that reside in living plant tissues, apparently without inflicting negative effects. They are quite ubiquitous and have been found in all plant species examined to date (Arnold et al., 2000). In recent years, many reports have showed that endophytic fungi can be capable of synthesizing active compounds produced in host plants, and the compounds are potentially useful for modern medicine and agriculture (Hung et al., 2006; Strobel, 2003). Hence, screening with activity of endophytic fungi as alternative sources of medicinal plant are crucial for conservation and utilization of fungal resources in plants.

Eucommia ulmoides Oliv. is one of the most valuable timber resources in China, and the only species both in its genus and in its family (Ma et al., 2007). This natural resource is now in short supply because of the over-collection of the wild plant. Therefore, it is important to find an alternative way to produce its active constituents to satisfy the demand. On the basis of the above studies of endophytic fungi, it is feasible to study the products of

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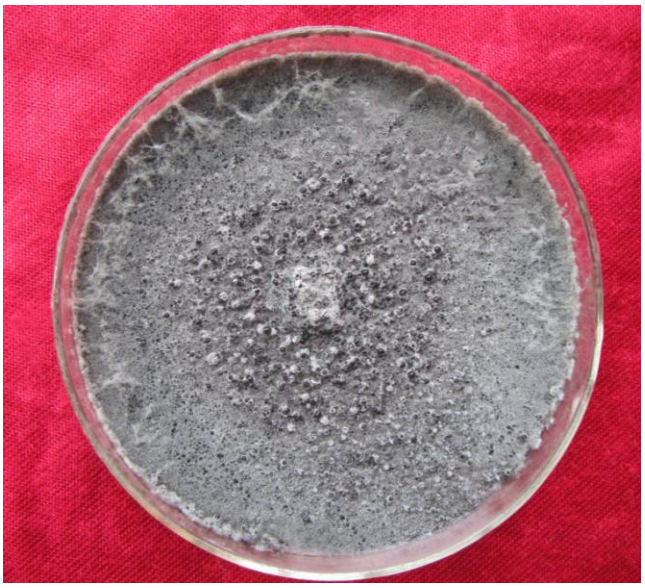

Figure 1. The colony characteristics of the strain DZY16.

secondary metabolism of the endophytic fungi isolated from *E. ulmoides* Oliv. During the initial stage of the researches on the endophytic fungi of *E. ulmoides* Oliv., a total of 78 strains were obtained from the leaves, stems and fruits of *E. ulmoides* Oliv., and the strain DZY16 isolated from the leave was found to have better antimicrobial activity using growth inhibition measurements ; the colony morphology has shown in Figure 1. In order to make an intensive study of the strain DZY16, this study was carried out to analyze the extracellular polysaccharide and nucleosides of the fermentation broth of DZY16 and to detect the anti-phytopathogen activity of the extracts of the fermentation broth. The research may contribute to the development and utilization of endophytic fungi DZY16.

MATERIALS AND METHODS

The test microorganisms were obtained from the School of Plant Protection, Anhui Agricultural University (31°86′ N and 117°25′ E), AnHui Province, Southeast China.

Culture of the shaking flask seeding

The endophytic fungi DZY16 taken from the slope were added to potato dextrose liquid medium in Erlenmeyer flasks and incubated at 28°C and 160 rpm with normal daily light and dark periods for six days, and the shaking flask seeding of DZY16 was obtained.

Preparation of the crude extracellular polysaccharides

The shaking flask seeding culture of DZY16 were added to 60 mL potato dextrose liquid medium with 10% (V/V) inoculation quantity in Erlenmeyer flasks and incubated at 28°C and 160 rpm with normal daily light and dark periods for ten days. Then, the flasks was sampled at special time every day during incubation period of DZY16, and biomass was separated by filtering with Whatman filter paper to be dried in a freeze-drying system (FreeZone12, Labconco Ltd., U.S.), so that the supernatant (the fermentation broth) was obtained.

The crude extracellular polysaccharides were precipitated from the supernatant for 12 h at 4°C by a three fold volume of 95% ethanol alcohol, after the precipitation, the crude extracellular polysaccharides were washed three times with 100% ethanol, acetone, ethyl ether successively, and finally, they were dissolved in water and freeze-dried.

Determination of extracellular polysaccharide

The contents of extracellular polysaccharides consisting in the crude extracellular polysaccharides were determined by phenol-sulfuric acid method.

Fermentation and treatment of the fermentation broth of DZY16

The strain DZY16 was added to 200 mL potato dextrose liquid medium in Erlenmeyer flasks and incubated at 28°C and 160 rpm with normal daily light and dark periods for ten days. The mycelia of the DZY16 were separated by centrifugation (5000 r. min^{-1}, 10 min), so that the fermentation broth of DZY16 was obtained, and finally the fermentation broth of DZY16 was freeze-dried in freeze-drying system.

Preparation of the nucleotides of the fermentation broth of DZY16

The freeze-dried fermentation broth of DZY16 (1.0 g) was dissolved in 70% ethyl alcohol in 5 mL volumetric flask to the mark, ultrasonic-tion (40 KHZ, 400 W) was performed at room temperature under optimized conditions: 70% ethyl alcohol solvent; 5 mL volume of solvent, and 30 min extract time. After centrifugation (10000 r. min^{-1}, 5 min), the supernatant was filtered with 0.22 μm Millipore filters before HPLC analysis.

High-performance liquid chromatography (HPLC) analysis

High performance liquid chromatography method was used for the determination of the contents of nucleoside in the samples (Acme9000HPLC, YOUNGLIN Ltd., Korea); detection was performed on Waters Spherisorb ODS_2 (4.6×250mm, 5μm) column by UV730D detector at 254 nm with methanol and KH_2PO_4 (0.01mol·L^{-1}) as the mobile phases at a flow rate of 1.0 ml/min, and the volume ratio of the solution methanol and KH_2PO_4 was 10:90.

Calibration curves

Adenosine (0.2500 g), uridine (0.2455 g), guanine (0.2480 g) and inosine (0.2550 g) were dissolved in 70% ethyl alcohol in 50 mL volumetric flask to the mark, to get four standard solutions, then the stock solution of standards was prepared and diluted with 70% ethyl alcohol to appropriate concentrations for the establishment of calibration curves. At least five concentrations of the four standards mixture were injected in triplicate, respectively and then the calibration curves were constructed by plotting the peak areas versus the concentration of each standard.

Limits of detection and quantification

The stock solutions containing reference compounds were diluted with 70% ethyl alcohol to appropriate concentrations, and an aliquot of the diluted solutions was injected into the HPLC for analysis. Limits of detection (LOD) and quantification (LOQ) for each standard were determined at a signal to noise ratio (S/N)of about 3 and 10 respectively.

Accuracy

The recovery was determined by adding a known amount of individual standards to the freeze-dried fermentation broth of DZY16 (1.0 g). The mixture was extracted and analyzed using the methods mentioned above. The quantity of each analyte was subsequently obtained from the corresponding calibration curve**.**

Preparation of three extracts of the fermentation broth of DZY16

With a solid to liquid ratio of 1:15 (w/V), the freeze-dried fermentation broth of DZY16 was extracted two times for 5 h by petroleum ether, ethyl acetate and methanol in sequence at 45°C. Three extracts were evaporated by vacuum concentration, and were dissolved in sterilized water at a concentration of 1 mg/ mL, then the solutions were sterilized by filtration with 0.22 μm Millipore filters.

Growth inhibition measurements

The antifungal activity of three extracts of the fermentation broth was determined in two pathogenic fungi: *Rhizoctonia solani* and *Gibberella zeae*. Prior to testing, indicator organisms were cultured in PDA medium at 28°C

0.5 mL samples were poured into sterile Petri dishes containing 15 mL PDA, followed by adequate mixing. A negative control was prepared using 0.5 mL sterilized water. For the positive controls, 100 mg/L carbendazim was used for two pathogenic fungi.

A 5 mm diameter plug of the actively growing mycelium of the pathogenic fungi was placed in the center of the each plate. The plates were incubated at 28°C in the dark (3 plates per treatment). The diameters of the inhibition zones were measured by vernier caliper. According to the growth rate of each fungus, colony diameter data taken after 2 days (*R. solani*) and 5 days (*G. zeae*) were used. The inhibitory activity of each treatment was carried out using the following formula, where DC = diameter of control, and DT = diameter of fungal colony with treatment. The experiments were repeated twice and the data presented here are the averages of two experiments.

$$\text{Growth inhibition (\%)} = \left[\frac{DC—DT}{DC}\right] \times 100\%$$

DNA extraction, PCR amplification, and sequencing

The strain DZY16 was identified using molecular tools. Genomic DNA was extracted from ground mycelium (Lee et al, 1988). Primers ITS5 5′ TCCGTAGGTGAACCTGCGC 3′ and ITS4 5′ TCCTCCGCTTATTGATATGC 3′ were used to amplify the 5.8S and flanking ITS regions of the orphospecies. The DNA fragment was amplified and sequenced according to previously described methods (Guo et al, 2000). The sequence data from this study have been submitted to GenBank under accession No. JQ359020.

RESULTS

Determination of extracellular polysaccharide at different growth stages of the strain DZY16

The variation trend of content of extracellular polysaccharide at different growth stages of the strain DZY16 is shown in Figure 2. Firstly, the content of extracellular polysaccharide of DZY16 was slowly increased at lag phase and logarithmic phase of the strain DZY16, then the content of extracellular polysaccharides had a trend of rapid growth at stationary phase of the strain DZY16, and the maximum content of extracellular polysaccharide was 2.02 g/L at the sixth day. Subsequently, the content of extracellular polysaccharide significantly decreased, until the eighth day; downtrend of the content of extracellular polysaccharides tended stable. On the other hand, the trend of the biomass of the strain DZY16 slowly increased and eventually was stable; at the eighth day the maximum biomass reached 14.30 g/L.

Identification and quantification of the investigated nucleosides in the fermentation broth of DZY16

Linearity, regression, and the linear ranges of the four analytes were determined using the HPLC method. The correlation coefficient values indicated appropriate correlations between the investigated compound concentrations and their peak areas within the test ranges. The limits of detection and quantification were less than 1.0 and 2.0 ng respectively (Table 1) and the method had good accuracy with the overall recovery of 94.6 to 101.4% for the analytes (Table 2). The results indicated that this HPLC method was accurate and sensitive for quantitative determination of the four compounds.

Chromatograms of the mix-standard and ultrasonic 70% ethyl alcohol extract from the fermentation broth of DZY16 are shown in Figures 3 and 4. The peaks were identified by two means: (1) comparing the retention times of the unknown peaks with those of the standards eluted under the same conditions and (2) spiking the sample with stock standard solutions of analytes. By the calibration curve of each investigated compound, the contents of the four analytes in the fermentation broth of DZY16 were determined. The data is summarized in Table 3. In brief, the amounts of the inosine was undetectable, furthermore, the content of guanosine was higher than those of uridine and adenosine.

The antifungal activities of the fermentation broth of DZY16

The antifungal activities of the three extracts of the fermentation broth of DZY16 were detected in two pathogenic fungi, and the results show that the antifungal activities

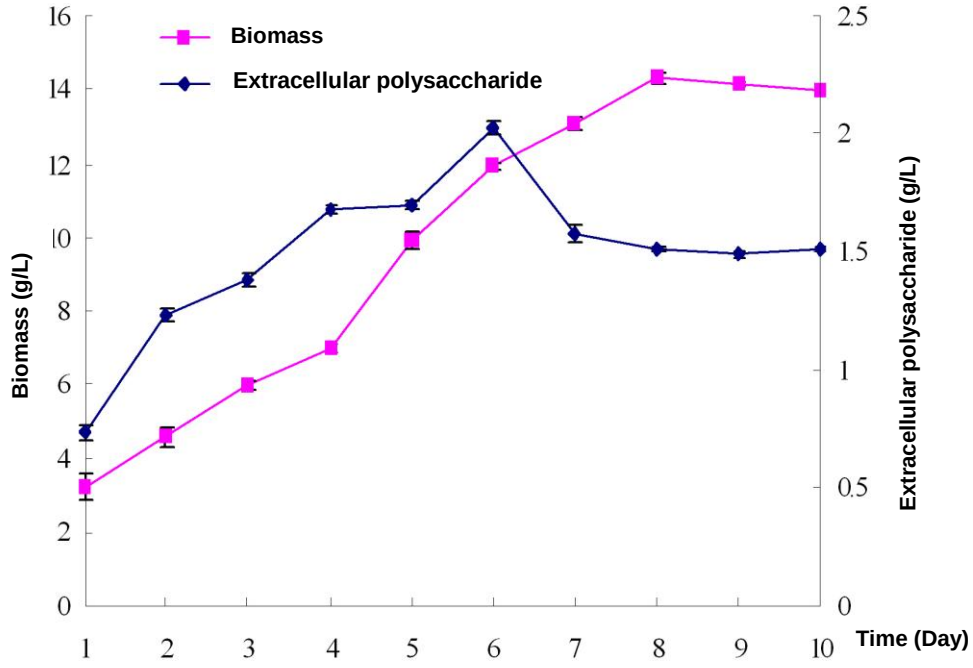

Figure 2. Time profiles of extracellular polysaccharide and biomass.

Table 1. Calibration data, LOD and LOQ of the four analytes.

Analyte	Linear regression data			LOD/ng	LOQ/ng
	Regressive equation	R^2	Test range/ mg/ml		
Uridine	Y=3227.2X-170.57	0.9975	0.0491-0.2500	0.8	1.1
Inosine	Y=3399.3X-137.15	0.9976	0.0510-0.2550	1.0	2.0
Guanosine	Y=2520.1X-125.41	0.9985	0.0496-0.2480	0.8	1.1
Adenosine	Y=5696.5X-315.05	0.9965	0.0500-0.2500	0.6	1.0

Table 2. Recoveries for the assay of the investigated components in the fermentation broth of DZY16.

Analyte[a]	Original (μg)	Spiked (μg)	Found (μg)	RSD (%)	Recovery[b] (%)
Uridine	10.7	50.0	61.4	0.6	101.4
Guanosine	15.4	50.0	62.7	1.2	94.6
Adenosine	13.6	50.0	63.1	1.3	99.0

[a] the inosine was absence in the fermentation broth of DZY16, so the recovery of the inosine was not analyzed . [b] Recovery(%) = 100 × amount found - original amount)/amount spiked.

of the three extracts were significantly different. With *R. solani* and *G. zeae*, the inhibition rate of the acetyl acetate extract was significantly higher than those of petroleum ether extract and methanol extract (Table 4). With *R. Solani,* the inhibition rate of the acetyl acetate extract on the growth of *R. solani* was 59.84%, which was lower compared to that of the fungicide carbendazim on *R. solani*, whereas the inhibition rates of the petroleum ether extract and methanol extract were only 15.44 and 48.00% respectively (Table 4). The acetyl acetate extract showed higher growth inhibitory effect on the growth of *G. zeae,* where the acetyl acetate extract at 1 mg/mL caused 70.86% inhibition, compared to 63.94% for 100 mg/L carbendazim, used as the positive control, whereas the inhibition rates of the petroleum ether extract and methanol extract were 16.19 and 55.40% respectively (Table 4).

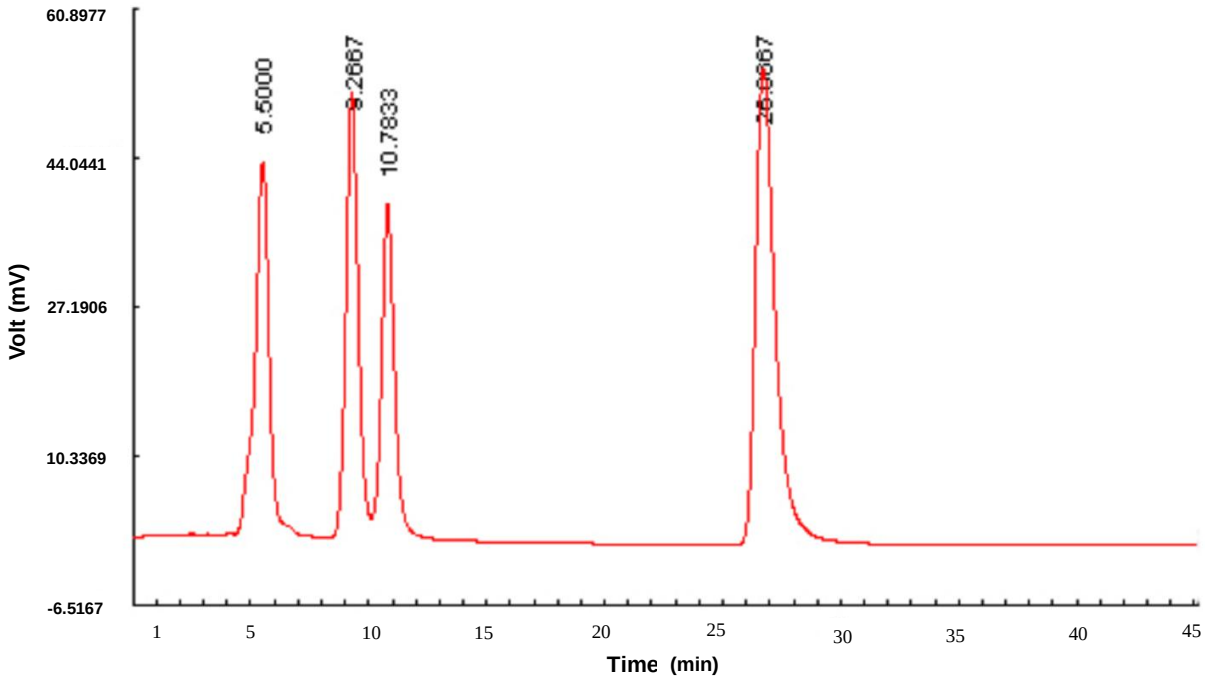

Figure 3. HPLC Diagram of mixture of nucleoside standard sample (i) uridine (ii) inosine (iii) guanosine (iv) adenosine.

Table 3. kinds and contents of nucleosides in the fermentation broth of DZY16 [a]Undetectable.

Sample	Adenosine (mg/g)	Uridine (mg/g)	Guanosine (mg/g)	Inosine[a] (mg/g)
The fermentation broth of DZY16	1.36±0.09	1.07±0.07	1.54±0.02	

Table 4 Antifungi activities at different extracting solvents of DZY16.

Sample	Growth inhibition (%)	
	Gibberella zeae	***Rhizoctonia solani***
Petroleum ether extract	16.19 ± 0.29[d]	15.44 ± 0.72[d]
Acetylacetate extract	70.86 ± 0.55[a]	59.84 ± 0.72[b]
Methanol extract	55.40 ± 0.63[c]	48.00 ± 0.62[c]
Positive control	63.94 ± 0.25[b]	71.19 ± 0.67[a]

The different lowercase show significant difference among treatments at 95% confidence level using the Duncan multiple comparisons.

Molecular phylogenetics

ITS of DZY16 strains were sequenced, the sequence long was 776 bp, including 18 S, 5.8 S, 28 S and interval region. The sequences were aligned by ClustalW first, and then the alignment sequences were subjected to the construction of the phylogenetic trees based on the Bootstrap neighbor-joining (NJ) method with Kimura 2-parameter model by using MEGA version 3.1 (Figure 5). The sequence similarity can also be demonstrated in tree graphs. DZY16 and *Nigrospora* had high homology; one of the largest homology was up to 98%, so DZY16 strain is theoretically a member of *Nigrospora*.

DISCUSSION

During this studies, the fermentation broth of DZY16 was found to contain extracellular polysaccharide, and the maximum content of extracellular polysaccharide reached 2.02 g/L. Qualitative HPLC analysis showed that the fermentation broth of the strain DZY16 had uridine, guanosine and adenosine; the type of nucleoside was more

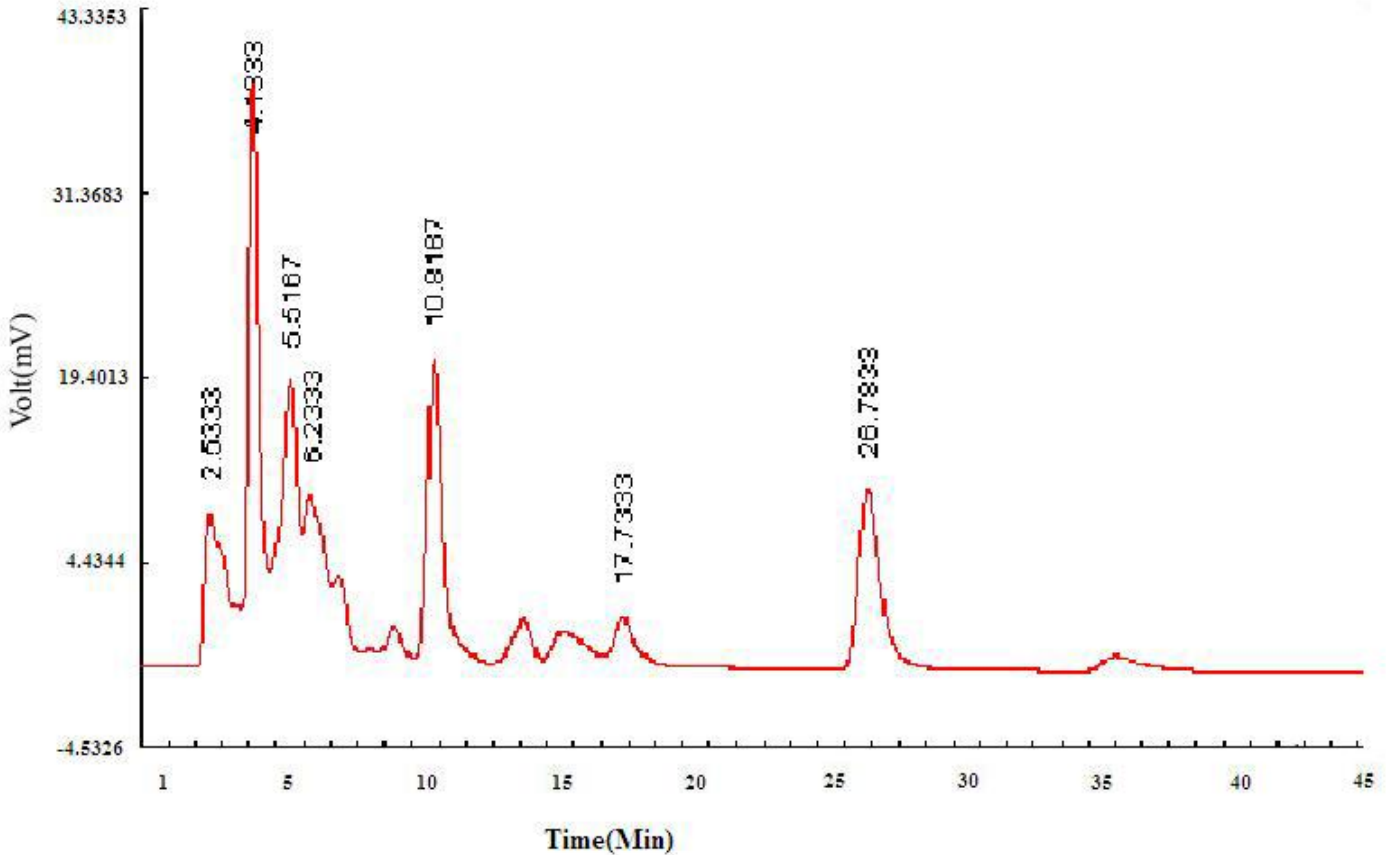

Figure 4. HPLC digram of the fermentation broth of DZY16

than that of the reported fungi *Phomopsis sp.* from *Azadirachta indica* A. Juss (Wu et al., 2008), at the same time, quantitative HPLC analysis showed the contents of uridine, guanosine and adenosine were 1.07 mg/g, 1.54 mg/g and 1.36 mg/g respectively. Thus, the above results indicated the strain DZY16 could be used as a source of extracellular polysaccharide and nucleosides, and these biological substances could be obtained by liquid fermentation easily without influence of external conditions.

Antifungal activities of plant endophytic fungi have been reported by a few groups (Liu et al., 2001; Li et al., 2005). In the present study, three extracts of the fermentation broths of endophytic fungi DZY16 were screened for antifungal activities in two pathogenic fungi, as an indication of its capability to produce secondary metabolites of potential therapeutic interest. The results show that the effect of the three extracts on the growth of *R. solani* was lower compared to the effect on *G. zeae*. Among the three extracts , the acetyl acetate extract exhibited strong antifungal activity to two pathogenic fungi; the antifungal activity of methanol extract was secondary, and the antifungal activity of petroleum ether extract was poor (Table 4). Moreover, the inhibition rate of DZY16 to *G. zeae* was 70.86% and significantly higher than that of the fungicide carbendazim (100 mg/L); it could be another potential source of bioactive antifungal agents, and their active metabolites are worth further research.

In recently years, many investigations of the *E. ulmoides Oliver.* indicated that the principal components of *E. ulmoides* Oliver. were geniposidic acid and chlorogenic acid, moreover, chlorogenic acid was widely used for its antimicrobial, anti-inxammatory, antioxidant, anti-cancer, and anti-hepatitis B virus activities (Riitta et al., 2005; Wu et al., 2007). Therefore, the next goal is to isolate and purify the acetyl acetate extract in order to confirm whether chlorogenic acid exists in the acetyl acetate extract or obtain other compounds with antifungal activities. In this way, we hope to lay a stable foundation for a new method for producing these important bioactive compounds.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (Grant No.31000871) and Projects of University Science Foundation for Outstanding Young

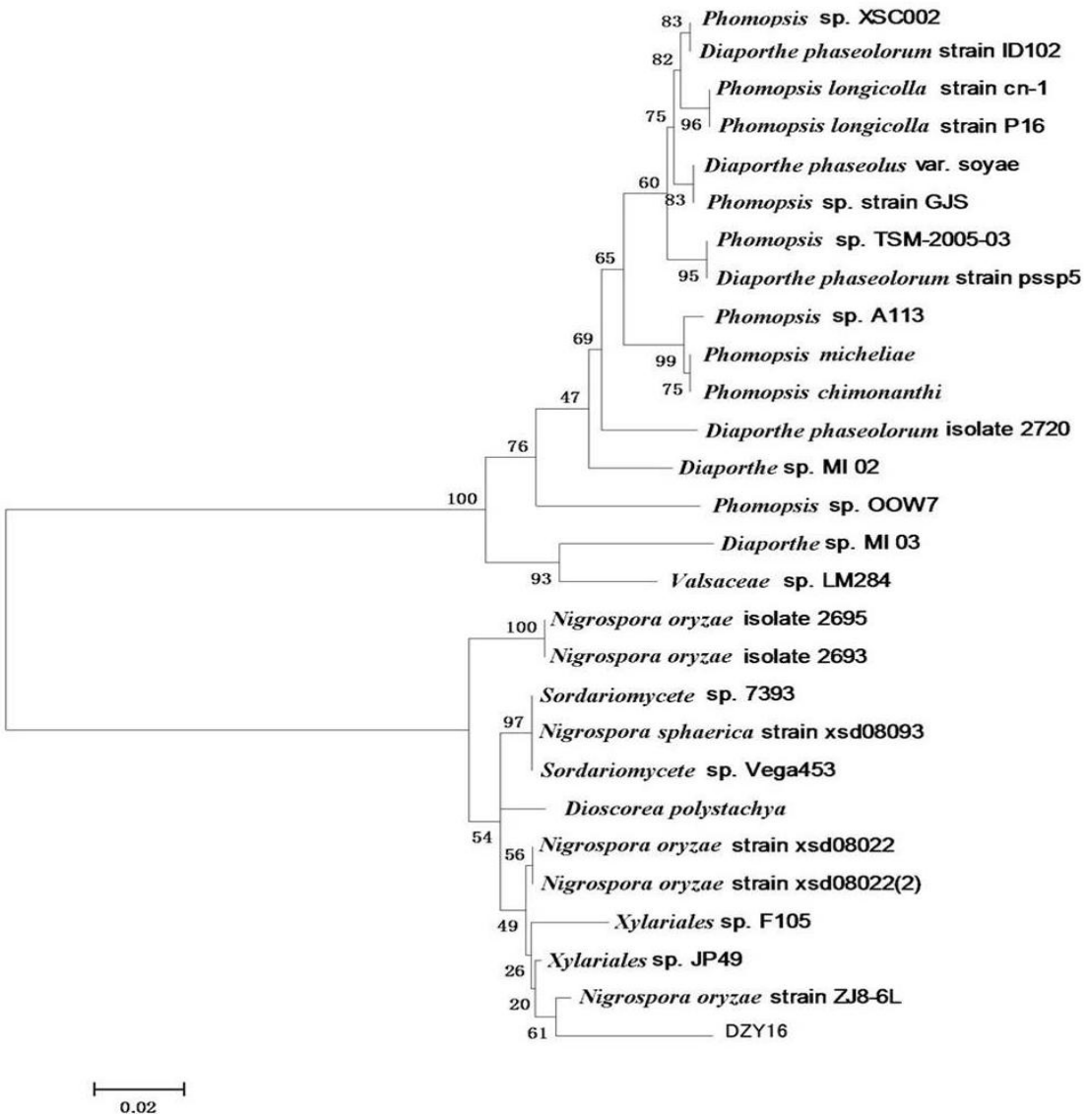

Figure 5. Strain DZY16 ITS sequence blast analysis.

Scholars of Anhui Province (Grant No. 2010SQRL063).

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