INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–079/2019/22–6–1311–1319
DOI: 10.17957/IJAB/15.1203
http://www.fspublishers.org

*Full Length Article*

# Identification and Tracking Activity of Fungus from the Antarctic Pole on Antagonistic of Aquatic Pathogenic Bacteria

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## Abstract

To seek the lead compound with the activity of antagonistic aquatic pathogenic bacteria in Antarctica fungi, the work identified species of a previously collected fungus with high sensitivity to *Aeromonas hydrophila* ATCC7966 and *Streptococcus agalactiae*. Potential active compounds were separated from fermentation broth by activity tracking and identified in structure by spectrum. The results showed that this fungus had common characteristics as Basidiomycota in morphology. According to 18S rDNA and internal transcribed space (ITS) DNA sequencing, this fungus was identified as *Bjerkandera adusta* in family Meruliaceae. Two active compounds *viz*., veratric acid and erythro-1-(3, 5-dichlone-4-methoxyphenyl)-1, 2-propylene glycol were identified by nuclear magnetic resonance spectrum and mass spectrum. Veratric acid was separated for the first time from any fungus, while erythro-1-(3, 5-dichlone-4-methoxyphenyl)-1, 2-propylene glycol was once reported in *Bjerkandera*. In addition, 3-chlone-4-methoxybenzoi acid and 4-chlone-2-methoxybenzoic acid were identified by GC-MS. In the non-active compounds, the metabolic intermediate, veratraldehyde was identified with spectrum. The results concluded that there were various antibacterial active compounds in the extractum of fermentation liquor, which provides reference for developing new preventive drug against aquatic pathological bacteria. © 2019 Friends Science Publishers

**Keywords:** Antarctica fungus; Purification; Secondary metabolites; Structural identification

## Introduction

China supplies one-third of global fish production, in which aquiculture yield occupies the half of global gross production (Cao *et al.*, 2015). However, sustainable development is often affected by fish diseases spreading caused by aquatic pathogenic bacteria such as *Streptococcus agalactiae* and *Aeromonas hydrophila*. Both of them result in fish death (Money and Dobson, 2004), especially for *A. hydrophila*, which is the main reason for furunculosis and fish hemorrhagic diseases after infection (Cutuli *et al.*, 2015). At present, the use of antibiotic is the main method to withstand these pathogens. Antarctic fungi might be the most diverse species in Antarctic fungi (Bridge and Spooner, 2012), which have more than 1,000 species identified (Bridge *et al.*, 2008). Antarctica microbes present antimicrobial activity with their secondary metabolites, such as terpenoids, phenols and nitrides, which might become new drug sources (Giudice *et al.*, 2007).

In early study, the fermentation products of 23 polar fungi were extracted to detect antibacterial activity by kirby-bauer paper strip method (Esser and Elefson. 1970). A fungus with strong antibacterial activity was screened. This fungus was highly sensitive to *A. hydrophila* ATCC7966 and *S. agalactiae*. During 10-day's fermentation, pH curve tended to increase after decreased, while the curve of sugar residual tended to decrease (Zhao *et al.*, 2018). It was initially speculated that lots of alkaline secondary metabolites had generated. The strain was roughly inferred to belong to *Bjerkandera* based on 18S rDNA. The work further identified this strain via morphological characteristics of every growth stages, more detailed molecular identification and characteristic compound even. Furthermore, potential active compounds were separated by activity tracking from fermentation broth to identify structures by spectrum and GC-MS.

## Material and Methods

### Morphology Identification of Strain B-7

Antarctic fungi were from Polar Research Institute of

**To cite this paper:** Cai, C., H. Yu, H. Zhao, X. Liu, B. Jiao and B. Chen, 201x. Identification and tracking activity of fungus from the Antarctic pole on antagonistic of aquatic pathogenic bacteria. *Intl. J. Agric. Biol*., 22: 1311–1319

China, while *A. hydrophila* ATCC7966 and *S. agalactiae* were from National Aquatic Pathogen Database of Shanghai Ocean University and preserved under -80°C in 60% glycerol. Potato Dextrose Broth (PDB), Mueller-Hinton Agar, nutrient broth and Glucose Peptone Yeast medium were all prepared according to Zhao's method (Zhao *et al.*, 2018). Potato Dextrose Agar slant medium was prepared as follows: mix 6 g PDB and 4 g agar and dissolved in 200 mL distilled water to make slant medium in tube. Fungus strain B-7 was preserved in Potato Dextrose Agar slant medium. In resuscitation, some thalluses was scraped and placed in 5 mL PDB medium for 2-day excitation. The fungus was activated at 24°C and 180 rpm in incubator for shaking culture. After confirming the increase of bacterium concentration by microscopic examination, fungus strain B-7 was inoculated on Potato Dextrose Agar slant with sterilized bamboo stick for static culture at 37°C. After hypha overgrowed on the slant, clear it with sterilized distilled water and then transfer to a 75 mL PDB medium as seed solution. All the hypha was cultured by shaking at 28°C and 180 rpm. After the seed solution was cultured to an appropriate density, shake evenly and pour it to 500 mL Glucose Peptone Yeast medium for fermentation at 20°C with continuous oscillation frequency of 180 rpm. After fermentation for 10 days, every bottle of fermentation liquor was filtered with four layers of gauze. Butyl acetate were used to extract the filter liquor in 1:1 (v:v) twice and dried by rotary evaporation.

**Molecular Identification of Strain B-7**

18S rDNA and ITS DNA from genomic DNA of strain B-7 were amplified by PCR using primers as follows. 18S rDNA amplification primer: NS1: 5'GTAGTCATATGCTTGTCTC3'; NS6: 5'GCATCACAGACCTGTTATTGCCTC3'. ITS DNA amplification primer: ITS1: 5' TCCGTAGGTGAACCTGCGG 3', ITS4: 5' TCCTCCGCTTATTGATATGC 3'. PCR products were identified by electrophoresis and purified by the SanPrep column DNA gel recovery kit to sequence by Applied Biosystems 3730XL sequencer. The sequencing results were analyzed. For each sample, 100 most similar sequences from genbank were obtained according to their matching degree and score, followed by comparison using DNAMAN software. After comparison, the Maximum likelihood phylogenetic tree was built with MEGA6.06 (Tamura *et al.*, 2013) in Kimura 2 - parameter model.

**Overall Flow of Separating Fermented Extractum**

Fungus strain B-7 was fermented, and a total of 80L fermentation liquor was extracted with butyl acetate twice. The bacteriostatic activity of two pathological bacteria was detected with paper strip for rough extractum (Zhao *et al.*, 2018). Active components were particularly separated by activity tracking. Moreover, other components were also separated or analyzed by liquid chromatography mass spectrometry technology (GC-MS) for identification (Fig. 1) to understand the overall components in fungus B-7 fermented extractum. The sample for GC-MS analysis was pretreated by 4% vitriol-methyl alcohol heating reflux esterification method (Pang *et al.*, 2013) with loading quantity of 1 $\mu$L. HP-5 capillary column (30 m × 0.25 mm) was used. For heating procedure, keep at 50°C for 1 min and then rise the temperature to 200°C by 25°C $min^{-1}$ and to 230°C by 3°C $min^{-1}$. Finally, keep at 230°C for 18 min. The split ratio was 50:1. The flow rates of nitrogen, hydrogen and air were 30, 450 and 40 mL $min^{-1}$, respectively.

**Steps for Separation of Fermentation Broth**

Rough extractum was dissolved with dichloromethane in 1:1 (g $mL^{-1}$) and mixed with silica gel in 1:1 (m:m) followed by grinding and blowing dry. The sample was loaded on preequilibrated silica gel column [9 cm (d) × 6 cm (h)] and eluted with different liquors (mixture of petroleum ether and ethyl acetate at ratio of 50:1, 30:1, 15:1, 8:1 or 3:1, ethyl acetate and methyl alcohol). Every part of eluent was detected by thin-layer chromatography and concentrated, followed by detection of antibacterial activity of two pathological bacteria.

**Purification of Components**

Eluted components (the eluent petroleum ether: ethyl acetate=3:1, No. 1-6) dissolved in methyl alcohol were conducted by thin-layer chromatography. The developing solvents were as follows: pure petroleum ether, petroleum ether and ethyl acetate with ratio of 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 4:1, 3:1, 2:1, and 1:1. The components were detected with 254 nm ultraviolet and dyed with vanillin. Component No. 1-6 was taken for liquid chromatogram with chromatographic column, YMC. 20–100% methyl alcohol was used for 1h linear elution. Components 1-6 was separated by chromatography on ODS column using eluent of methyl alcohol solution in different concentrations (45, 55, 65, 75, 85 and 100%) followed by antibacterial activity detection.

After thin-layer chromatography detection, the eluted components (tube 1–8 with 45% methyl alcohol, No. 1-6-1) was separated by silica gel using eluent of petroleum ether, dichloromethane, ethyl acetate and methyl alcohol in ratio of 5:5:1:1. The separated components were loaded on ODS-HG-5 [5 $\mu$m, 10 mm (i.d.) × 25 cm] column for HPLC detection using methyl alcohol as mobile phase. The eluted component (No. 1-6-1-2) was dissolved with deuterated chloroform for $^{1}H$, $^{13}C$ and DEPT spectrum.

After comparative thin-layer chromatography detection with developing solvents of petroleum ether and acetone in ratio of 3:1, petroleum ether and ethyl acetate in ratio of 3:1, and dichloromethane and methyl alcohol in ratio of 9:1, the

eluted components (tube 19–24 of 45% methyl alcohol, No. 1-6-4) was separated by silica gel using eluent of dichloromethane, and the mixture of dichloromethane and methyl alcohol at ratio of 99:1, 95:5, and 9:1, respectively. Then one of eluted components (eluent was composed of dichloromethane and methyl alcohol in ratio of 95:5, No. 1-6-4-3) was purified by LH20 column [1cm (d) × 56 cm (h)] using eluent composed of dichloromethane and methyl alcohol in ratio of 1:1. Likewise, one of eluted components (No. 1-6-4-3-3), after HPLC detection, was taken for $^{1}H$, $^{13}C$, DEPT and Mass spectrum.

The eluted component (petroleum and ethyl acetate in ratio of 8:1, No. 1-5) was separated by LH20 column. Then one of eluted components (mixture of tube 21-24, No. 1-5-2), was purified by LH20 column again after thin-layer chromatography detection. In the same way, one of eluted components (tube 19, No. 1-5-2-2) was taken for $^{1}H$, $^{13}C$ and DEPT spectrum after HPLC detection.

## Results

### Morphology of Strain B-7

On the slant, strain B-7 fungus dispersed and grew in cluster from white to light yellow. After rushing the hypha on slant to medium with sterile purified water, the microscopic examination showed that there were many hypha studs which had not grown into branches (See Fig. 2). Taking every segment of hypha as a chamber, there were many cells without interval. All the cells consisted of basidium, while basidiospore, which was young ascus, would form on the outside of basidium. Microscopy showed that there were multiple spores, showing that basidiospores with sexual propagation. This was the common feature for fungi in *Basidiomycota* (Tamura *et al.*, 2013).

After strain B-7 revived and was inoculated with PDB medium, fungus grew slowly and developed in filiform in stationary state. The medium consumed a little, while the background was deep in microscopic examination. The thallus was hollow with many branches in vegetative growth. After strain B-7 fungus grew in PDB medium for 2 days, hypha showed significant increase with more branches. At this time, the density reached the requirements for fermentation. After multiple cultures, we found that this fungus seed liquid had two states. One was that both thallus and medium were dark yellow with dense hypha in branch. There was no obvious spore or sporangium. This state was speculated as vegetative growth or sexual propagation. The other one was that both thallus and medium are nearly white. Calabash-like sporangium was adhered to some hypha in medium. In general, sporangium generates at the top of vegetative hypha, and the protoplasm in sporangium was separated into a large amount of small pieces which developed into sporangiospore. Some hypha was connected with botryoid conidium generated by a certain method from the tip of specific hypha (conidiophore) with solitary,

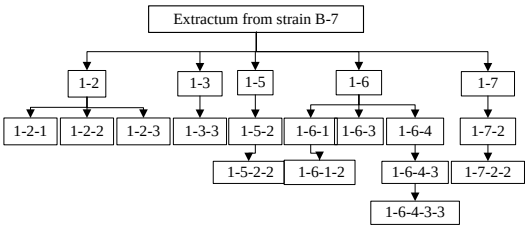


**Fig. 1:** General flow of separation of fungus strain B-7 fermentation extract

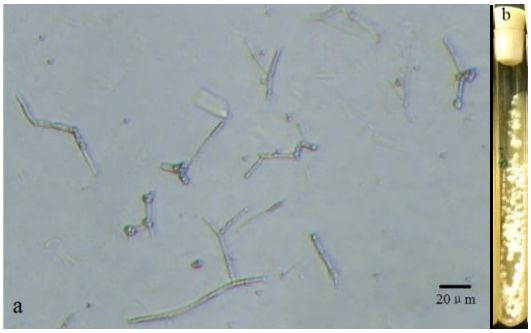


**Fig. 2:** Microscopy and morphology of fungus strain B-7 on slant culture-medium
**a**. cell morphology on slant culture-medium; **b**. morphology of fungus on slant culture-medium

scattered, cluster or fascicular peduncle (See Fig. 3). Sporangiospore and conidium were two typical types of fungi asexual reproduction. At the beginning, PDB medium was yellow, and then became shallow after fermentation, so it was speculated that fungi in vegetative propagation consumed nutrition faster. To maintain growth, strain B-7 fungi seed liquid was yellow in most cases of sexual propagation and vegetative growth. The subsequent studies showed that the extractum obtained in fermentation of fungi in both states had antibacterial activity. After 45-day growth, the medium was covered by a thick layer of white hypha under which all the thallus linked together and developed in mushroom. Some individual thalluses were in sphere or cylinder with thick density.

After 10 days of fermentation, the hypha grew significantly and dispersed in branch. The quantity of thallus was large, and some thallus got together to form white globule. At this time, both medium and moisture had been used up, while the secondary metabolite substantially produced to make fermentation liquor pasty (See Fig. 4).

### Molecular Identification of Strain B-7

There were a great variety of fungi with great variation, so two sequences, 18S rDNA and ITS DNA, were used as molecular marker for species identification of strain B-7. By comparison, these sequences had a large amount of similar sequence matching in NCBI. The similarity of the first 100 sequences was up to 99%. 18S rDNA is a target gene for the identification of eukarya molecule and for phylogeny

analysis. After removing sequences of uncertain species, ninety 18S rDNA sequences were distributed in 34 categories, including *Phanerochaete* (13), *Phlebia* (12), *Bjerkandera* (6), *Trametes* (6), *Rhizochaete* (5), *Hyphodermella* (5), *Ceriporia* (5), *Agaricomycetes* (4), *Antrodia* (3), *Ceriporiopsis* (3), *Hexagonia* (3), *Antrodia* (3), *Diplomitoporus* (2), *Tyromyces* (2), *Lopharia* (1), *Sistotrema* (1), *Porostereum* (1), *Oxyporus* (1), *Phlebiopsis* (1), *Climacodon* (1), *Hapalopilus* (1), *Coriolopsis* (1), *Byssomerulius* (1), *Abortiporus* (1), *Earliella* (1), *Fomitopsis* (1), *Pulcherricium* (1), *Ganoderma* (1), *Amauroderma* (1), *Albatrellus* (1), *Oligoporus* (1), *Gloeoporus* (1), *Cerrena* (1) and *Trichaptum* (1). The 18S rDNA marker reflected the phylogenetic relation between different species that significantly clustered in the evolutionary tree. Here, according to 18S sequence (Genbank accession number: MG669556), strain B-7 had close relationship with *Bjerkandera*, *Lopharia*, *Antrodia*, *Oxyporus* among *Trichaptum* in different families and *Agaricomycetes*. The first three genuses belonged to *Polyporales*, while the last two belonged to *Hymenochaetales*. Thus, strain B-7 was likely belonged to *Bjerkandera* according to the number of similar 18S rDNA sequences by contrast.

ITS DNA is the regional segment between gene 18S and gene 28S, mainly including ITS1, 5.8S rDNA and ITS2. Compared with genomic sequences of 18S, 5.8S and 28S in rDNA, ITS1 and ITS 2 which are non-coding spaces bear greater evolvement and selection pressure. Therefore, these two spaces can be taken as good markers of molecular genetics for identification of species and even species levels. The online comparison among ITS DNA sequences of strain B-7 showed that after excluding the sequences in uncertain species, 89 ITS DNA sequences were distributed in 11 species. The category was significantly more collective than that blasted by 18S sequence and species in *Bjerkandera* accounting for 60%. In the evolutionary tree of ITS DNA sequences, B-7 sequence was close to *Bjerkandera* spp. (HQ637322) cluster, followed by *Bjerkandera adusta* (KC176332) and *Thanatephorus cucumeris* (KM979725 and FJ426396) (See Fig. 5). Known as *Rhizoctonia solani* at present, *Thanatephorus cucumeris*, the widespread pathogenic fungi, belongs to *Cantharellales* of *Agaricomycetes*. *T. cucumeris* did not produce conidium, but the sporidium of sexual propagation might occasionally appear in the infected plant, which was inconsistent with the morphological characteristics of strain B-7. Therefore, it was believed that strain B-7 fungus most likely belonged to *Bjerkandera* spp. of *Meruliaceae*.

Only two species (*B. adusta* and *Bjerkandera fumosa*) had been identified as *Bjerkandera*, Both of which were distribute in high latitude area and cold-resistant (Zmitrovich *et al.*, 2016). By blast, *B. fumosa* had only one ITS DNA sequence, while that of *B. adusta* accounted for 83% among 53 sequences. Therefore, it was inferred by probability that strain B-7 was more likely belong to *B. adusta*.

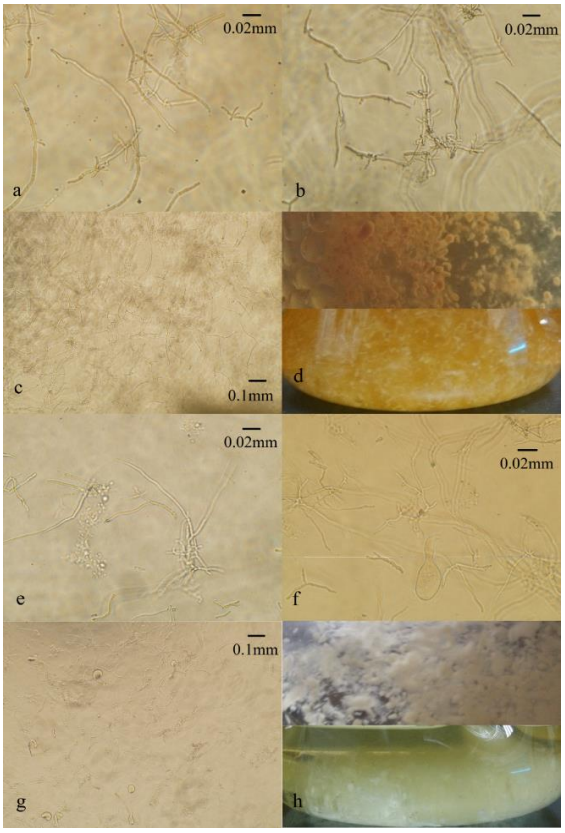


**Fig. 3:** Fungus strain B-7 seeds in different growth states
**a-c**: microscopy of yellow seed, **d**. morphology of yellow seed, **e-g**. microscopy of white seed, **h**. morphology of white seed

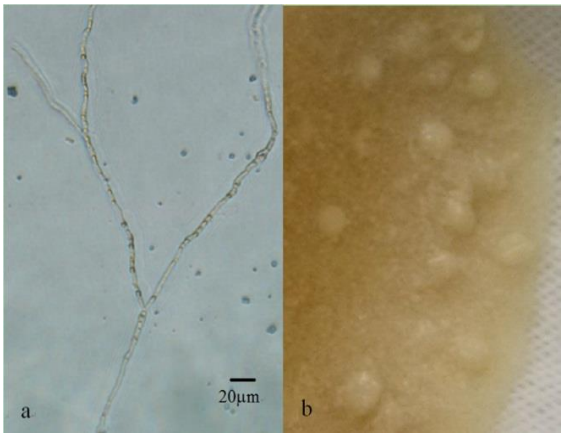


**Fig. 4:** Morphology of fungus strain B-7 after fermentation
*a*. microscopy of fermentation liquid, *b*. morphology of fermentation liquid

**First Separation of Rude Extractum**

20 g extractum was collected from a total of 80L liquor by strain B-7 fermentation. The detection by kirby-bauer paper strip method showed that *A. hydrophila* ATCC 7966 and *S. agalactiae* were highly sensitive to extractum.

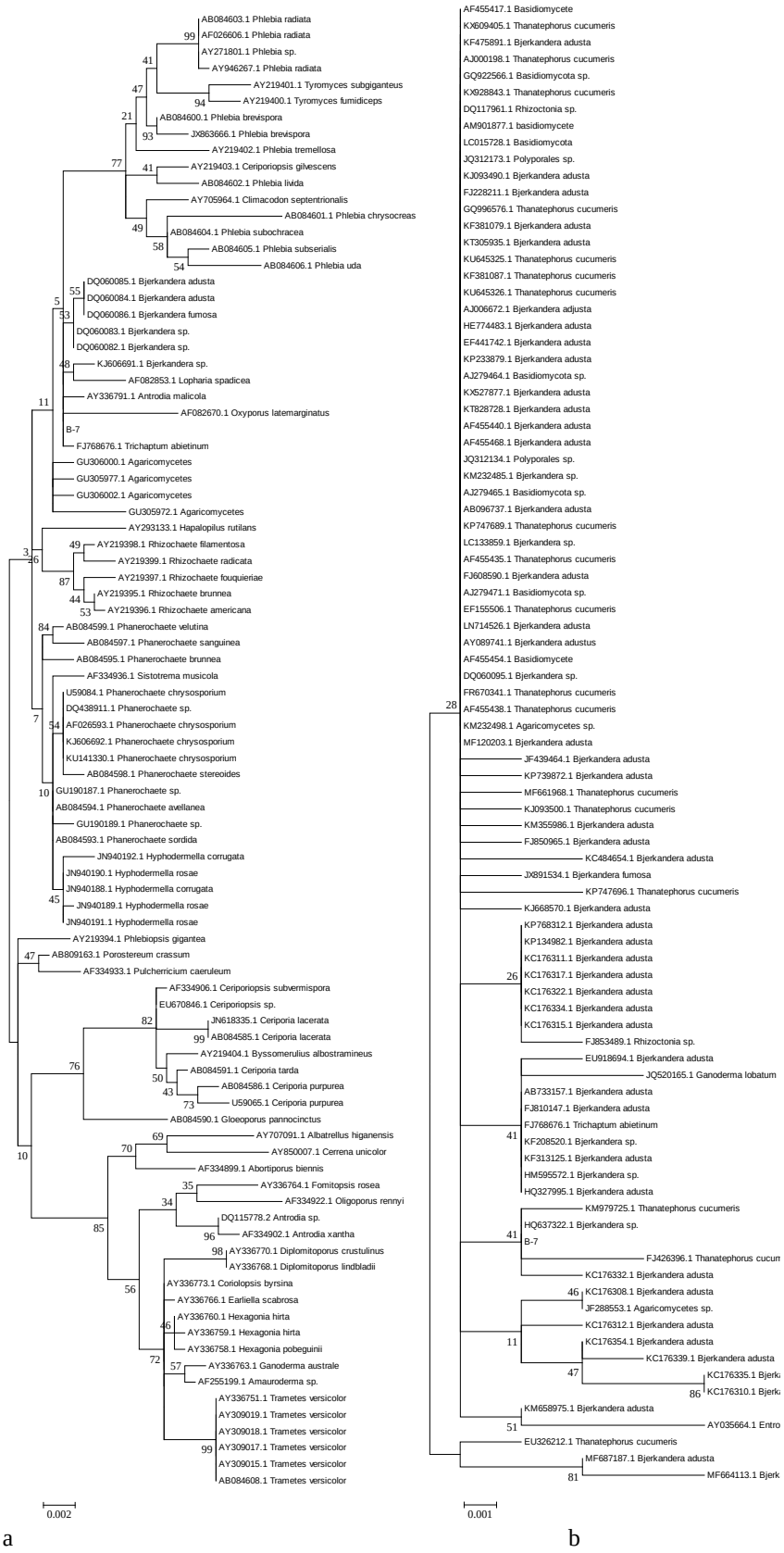


**Fig. 5:** Evolutionary tree of fungus strain B-7 and related species by 18S rDNA (**a**) and ITS (**b**) sequence

After separation by silicagel column, the elution fractions colors included white, yellow and brown, showing that components were various, wherein the most content was the eluted component of ethyl acetate (3.8 g), while that of the least content was the eluted component of petroleum ether and ethyl acetate in ratio of 3:1 (1.1 g) (Table 1). However, *S. agalactiae* and *A. hydrophila* ATCC7966 were only moderately sensitive to component No. 1-6 among 8 components.

**Separation of Component No. 1-6**

Thin layer chromatography showed that developing solvent of petroleum ether and ethyl acetate mixture could not completely separate the component No. 1-6. Therefore, the inversed phase chromatograph such as ODS was used for elution, during which the elution fractions eluted by 45–100% methyl alcohol had more contents. Taken 45% methyl alcohol as the initial concentration, component No. 1-6 was eluted with increasing methanol concentration on ODS column, followed by detection and combination. Thirteen components were obtained, among which the maximum mass was 70 mg (No. 1-6-1) and two components (No. 1-6-1 and 1-6-4) had activity of inhibiting *S. agalactiae* and *A. hydrophila* ATCC7966 (Table 2).

**Separation of Component No. 1-6-1**

After many trials, component No. 1-6-1 was separated by silica gel column with eluent composed of petroleum ether, dichloromethane, ethyl acetate and methyl alcohol in ratio of 5:5:1:1. One of the components (1-6-1-2), after HPLC detection, was conducted for spectrum analysis. Main spectrum data of this component were as follows:

H-NMR: $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.77 (dd, *J* = 8.4, 1.7 Hz, 4H), 7.60 (d, *J* = 1.6 Hz, 4H), 7.27 (s, 1H), 6.92 (d, *J* = 8.5 Hz, 4H), 3.95 (d, *J* = 5.7 Hz, 23H), 1.62 (d, *J* = 7.1 Hz, 2H), 1.26 (s, 6H).

C-NMR: $^{13}$C NMR (101 MHz, $CDCl_3$) δ 217.36 (s), 198.96 (s), 180.55 (s), 143.74 (d, *J* = 0.4 Hz), 125.07 – 124.30 (m), 112.81 – 112.00 (m), 110.65 – 110.03 (m), 106.93 (s), 88.63 – 88.42 (m), 56.47 – 55.61 (m), 33.52 – 33.11 (m), 16.66 – 16.11 (m), 11.22 (s), -3.50 (s), -21.80 – -22.00 (m).

Dept spectrum: $^{13}$C NMR (101 MHz, $CDCL_3$) δ 171.35 (s), 153.59 (s), 148.59 (s), 124.46 (s), 121.70 (s), 112.26 (s), 110.24 (s), 80.28 – 77.68 (m), 77.38 (d, *J* = 29.8 Hz), 76.94 (s), 76.75 (d, *J* = 32.1 Hz), 55.93 (d, *J* = 5.7 Hz).

The component No. 1-6-1-2, whose spectrum data was consistent with references (Yang *et al.*, 2010; Zhang *et al.*, 2013; Xiong *et al.*, 2016), was identified as 3,4-dimethoxy benzoic acid (CAS No.: 93-07-2).

**Separation of Component No. 1-6-4**

In thin-layer chromatography detection, component No. 1-6-4 showed sapphirine points after dying with vanillin. The points had still existed after the following chromatography by silicagel column (No. 1-6-4-3) and LH20 column (No. 1-6-4-3-3). No. 1-6-4-3-3 was conducted for spectrum analysis and main spectrum data were as follows:

H-NMR: $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, *J* = 17.7 Hz, 4H), 7.24 (d, *J* = 17.7 Hz, 4H), 4.58 (d, *J* = 4.0 Hz, 1H), 4.58 (d, *J* = 4.0 Hz, 1H), 4.04 – 3.80 (m, 6H), 4.14 – 3.81 (m, 6H), 1.21 (s, 1H), 1.21 (s, 1H), 1.03 (d, *J* = 6.4 Hz, 4H), 1.03 (d, *J* = 6.4 Hz, 4H), -0.05 (s, 1H), -0.05 (s, 1H).

C-NMR: $^{13}$C NMR (101 MHz, $CDCl_3$) δ 127.09 (s), 76.00 (s), 70.97 (s), 60.73 (s), 17.19 (s).

Dept spectrum: $^{13}$C NMR (101 MHz, $CDCl_3$) δ 137.79 (s), 129.18 (s), 126.98 (s), 77.54 – 76.20 (m), 70.87 (s), 60.62 (s), 17.09 (s).

ESIMS mass spectrum of this compound showed mass spectra peaks of M, M+2 and M+4 in abundance ratio of 9:6:1, showing that there were two chlorine atoms in molecule. There was a $[M+COOH]^-$ peak at 295.0158 m/z in HRESIMS spectrum. Combined with $^{13}$C NMR, it was known that the molecular formula of this compound was $C_{10}H_{12}Cl_2O_3$. There were two overlapping hydrogen signals at $\delta_H$7.24 in $^{1}$H NMR spectrum, indicating that there was a tetra-substitutional benzene ring in molecule. In addition, there was a methoxy structural fragment (δ3.85, 3H, S) and two methine signals, $\delta_H$ 4.57 (1H, d, 4.0 Hz) and $\delta_H$ 3.95 (1H, m) in $^{1}$H NMR. The molecular formula showed that there was a hydroxy between two methine signals. By comparing reference (Swarts *et al.*, 1998), this compound was erythro-1-(3,5-dichloro-4-methoxy phenyl)-1,2-propanediol.

**Separation of Component No. 1-5**

Component No. 1-5 was separated by LH20 column twice and the spectrum data of the main eluant component, No. 1-5-2-1, were as follows:

H-NMR: $^{1}$H NMR (500 MHz, $CDCl_3$) δ 9.86 (s, 1H), 7.53 – 7.39 (m, 2H), 7.26 (s, 3H), 6.98 (d, *J* = 8.2 Hz, 1H), 5.29 (s, 2H), 3.95 (t, *J* = 9.1 Hz, 7H), 3.48 (s, 15H), 1.59 (s, 7H), 1.25 (s, 5H), 0.07 (s, 1H).

C-NMR: $^{13}$C NMR (101 MHz, $CDCl_3$) δ 190.95 (s), 126.92 (s), 109.67 (d, *J* = 145.5 Hz), 77.08 (dd, *J* = 42.1, 21.8 Hz), 56.13 (d, *J* = 16.9 Hz), 50.91 (s).

Dept spectrum: $^{13}$C NMR (101 MHz, $CDCl_3$) δ 190.95 (s), 126.93 (s), 110.40 (s), 108.95 (s), 77.23 (s), 56.13 (d, *J* = 17.0 Hz), 53.44 (s), 50.91 (s), 29.73 (s).

By comparing reference (Dohi *et al.*, 2012), component No. 1-5-2-1 was veratraldehyde (3,4-dimethoxy benzaldehyde).

**Analysis on other Components**

Not all the components from extractum of strain B-7 were identified, and some of them were conducted for GC-MS identification (Table 3). The non-active components of extractum (No. 1-2, 1-3, and 1-7) contained a large amount of fatty acid, while in active component (No. 1-6), the work

**Table 1:** Components of fungus strain B-7 fermentation extract after first isolation

| Number | Flow phase | Column volume (V) of elution | Component mass (g) |
|---|---|---|---|
| 1-1 | petroleum ether | 3 | 1.189 |
| 1-2 | petroleum ether: ethyl acetate =50:1 | 6 | 3.464 |
| 1-3 | petroleum ether: ethyl acetate =30:1 | 6 | 2.805 |
| 1-4 | petroleum ether: ethyl acetate =15:1 | 6 | 1.994 |
| 1-5 | petroleum ether: ethyl acetate =8:1 | 6 | 2.142 |
| 1-6 | petroleum ether: ethyl acetate =3:1 | 6 | 1.078 |
| 1-7 | acetate | 4.5 | 3.792 |
| 1-8 | methanol | 3 | 2.703 |

**Table 2:** Elution composition of components No. 1-6 purified by ODS column

| Number | Mobile phase | Elution volume (L) | Number of collection pipe | Component mass (mg) |
|---|---|---|---|---|
| 1-6-1 | 45% methanol | 3 | 1-8 | 70 |
| 1-6-2 | | | 9-13 | 26 |
| 1-6-3 | | | 14-18 | 12 |
| 1-6-4 | | | 19-24 | 33 |
| 1-6-5 | | | 25-31 | 34 |
| 1-6-6 | | | 32-42 | 6 |
| 1-6-7 | 55% methanol | 2.7 | all | 25 |
| 1-6-8 | 65% methanol | 2 | all | 57 |
| 1-6-9 | 75% methanol | 2 | all | 10 |
| 1-6-10 | 85% methanol | 2 | 1-10 | 6 |
| 1-6-11 | | | 11-20 | 28 |
| 1-6-12 | | | 21-25 | 2 |
| 1-6-13 | 100% methanol | 2 | all | 32 |

**Table 3:** GC-MS identification of some components from fungus strain B-7 fermentation extract

| Component | Name | Probability (%) |
|---|---|---|
| 1-2 | palmitate | 85.1 |
| 1-3-3 | palmitate | 89.4 |
| 1-6-3 | 4-chloro-2-methoxybenzoic acid | 82.0 |
| 1-6-3 | 3-chloro-4-methoxybenzoic acid | 92.7 |
| 1-7-2-2 | stearic acid | 78.0 |
| 1-7-2-2 | palmitate | 89.4 |
| 1-7-2-2 | palmitate | 97.4 |

detected another two chlorine-containing methoxybenzoic acids with more than 80% reliability. This further proved the identification of strain B-7 of *Bjerkandera* species and predicted the development potential of chlorine-containing aromatic compound in inhibiting aquatic pathogenic bacteria.

## Discussion

In the work, *S. agalactiae* and *A. hydrophila* ATCC7966 were moderately sensitive to component No. 1-6. In previous reports, Guo (*et al.*, 2016) extracted 3 pure compounds from fermentation product of *Aspergillus tubingensis* SZX-6, including methyl 6-acetyl-5,7,8-trihydroxy-4-methoxy-2-naphthoate, with moderate inhibitory activity against *Vibrio anguillarum*. Moreover, Yue *et al.* (2014) obtained 5 strains of epiphytic fungi by separating atiniae, the fermentation product of which had inhibitory activity against 6 bacterial strains. For *Emericella* spp., after static fermentation and component separation, the maximum inhibition zone against *Staphylococcus aureus* was 21.3 mm (highly inhibitory), while that against *Vibrio parahaeolyticus* was 10.2 mm (low inhibitory).

The 3,4-dimethoxy benzoic acid, also named veratric acid, was obtained from fungus for the first time in this experiment. It was also separated from radix rannculi ternate (Xiong *et al.*, 2016), crofton weed (Zhang *et al.*, 2013) and illicium verum (Yang *et al.*, 2010). The 3,4-dimethoxy benzoic acid had antidrug resistant TB activity (Xiong *et al.*, 2016), antifungal effect and preventive role against platelet aggregation (Yu *et al.*, 1998). At present, there has been a mature chemical synthetic method. Meanwhile, it is an important intermediate of synthetic drug, itopride, and can be used to prepare benzamide compound. As a common pharmacophore skeleton, benzamide compound had activity of antineoplastic (Wang *et al.*, 2015), antipsychotic (Seeman, 2013), sterilization, *etc*. and inhibitory effects against Gram negative and positive bacteria (Kumar *et al.*, 2007). Moreover, it could be further modified as oxazole benzamide to inhibit staphylococcus aureus by interdicting cell-division protein FtsZ (Stokes *et al.*, 2014). Phthalic diethyl ester and phthalic dibutyl ester that were aromatic compounds could inhibit *A. hydrophila* from infecting golden fish with survival rate of 60–80% (Anusha *et al.*, 2014). Moreover, the fermentation product of *Aspergillus tubingensis* SZX-6, methyl 6-acetyl-5,7,8-hydroxy-4-methoxy-2-formate (Guo *et al.*, 2016) had moderate inhibitory activity against *V. anguillarum*. It seems that the study on the inhibitory effects of organic and natural product on aquatic pathogenic bacteria has drawn more attention in recent years.

From strain B-7, we purified erythro-1-(3,5-dichloro-4-methoxy phenyl)-1,2-propanediol, which was firstly

obtained from living body by Henk who separated it from *Bjerkandera* spp. BOS55. Moreover, single chlorine substitutional product, tremetol (Swarts *et al.*, 1998), was also obtained. Components containing 3,5-dichloro-4-methoxyphenyl perssad with main characteristics of helium atom and benzene ring was very common in *Bjerkandera* category, *e.g*., cam aldehyde and cam alcohol (Jong *et al.*, 1992; Mercantini *et al.*, 1993; De *et al.*, 1994; Spinnler *et al.*, 1994; Swarts *et al.*, 1997a), chloro 4-p-hydroxybenzoic acid ester derivatives (Swarts *et al.*, 1996), chloro-benzenediol derivative (Spinnler *et al.*, 1994; Teunissen *et al.*, 1997), 1-(3-chlorine-4-methoxy phenyl)-3-hydroxy-1-acetone (Swarts *et al.*, 1998), and chloro veratryl alcohol (Swarts *et al.*, 1997b). The last one was analogue of veratric acid. All of these products, in turn, might corroborate the identification results of the aforementioned strain B-7 species. The previous studies showed that dichloro aromatic alcohol and aldehydes material had antibacterial activity (Becker *et al.*, 1994) and inhibitory effect against chitin synthetase. For example, taking *Paecilomyces variotii* and *Mucor miehei* as reference, the inhibition rate of 3,5-dichloro-4-methoxybenzyl alcohol against chitin synthetase was up to 40%, but this effect could be eliminated by lecithin (Pfefferle *et al.*, 1990). This was different from the common bactericides by breath inhibition, including acrylics and oudemansin in the market.

Veratraldehyde which was soluble in ethyl alcohol and diethyl ether existed in the eluted component of petroleum ether and ethyl acetate in ratio of 8:1. Veratraldehyde is sensitive to air and is oxidized as 3,4-dimethoxybenzoic acid in the light, which means they are front and back midbodies in metabolic pathway. 3,4-dimethoxybenzoic acid existed in the eluted fraction of petroleum and ethyl acetate in ratio of 3:1 in this experiment. As a commonly known compound, veratraldehyde (CAS No.: 120-14-9) was synthesized with veratrole, 3,4-dimethoxy toluene (Xu, 1998) or 3,4-dihydroxybenzaldehyde (Liu *et al.*, 2013), *etc*. Besides, veratraldehyde was used as an organic compound substrate to produce the veterinary drug, trimethoprim diaveridine. The latter was added to the feed by 0.02% to increase the efficacy of iodide, thus preventing and controlling poultry bacterial infections. Meanwhile, veratraldehyde was used as medical intermediate when synthesizing antibiotic drugs, including vasodilator pyrazolazine hydrochloride, epinephrine β- receptor blocker Bevantolol, and preventive drug for allergic asthma and allergic rhinitis Rizaben (Xu, 1998). Veratraldehyde was also food-grade spice according to national standard.

## Conclusion

Strain B-7, likely belonged to *Bjerkandera adusta* in *Meruliaceae*, had the common characteristics of *Basidiomycota* and showed sexual propagation of basidiospore on slant, vegetative growth or sexual propagation in yellow seed liquid, and vegetative propagation of sporangiospore and conidium in white seed liquid. From 20 g extractum via fermentation, veratric acid and erythro-1-(3,5-dichlone-4-methoxy phenyl)-1,2-propylene glycol were identified by nuclear magnetic resonance spectrum and mass spectrum. The former was firstly separated from fungus, while the latter was firstly separated from *Bjerkandera* spp. previously. By GC-MS comparison, 3-chlorine-4-methoxy benzoic acid and 4-chlorine-2-2-methoxy benzoic acid were obtained. The metabolic intermediate, veratraldehyde, was identified by spectrum in non-active component.

## Acknowledgement

This research work was financially supported by National Key R&D Program of China (Grant No. 2018YFC1406705), General Financial Grant from the China Postdoctoral Science Foundation (Grant No. 2015M572743), Ocean Public Welfare Scientific Research Project, Guangdong Oceanic Administration (Grant No. GD2012-D01-001), and Key Laboratory of Integrated Marine Monitoring and Applied Technologies for Harmful Algal Blooms, S.O.A. (Grant No. MATHAB201817). The funders did not play a role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

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**[Received10 May 2019; Accepted 28 May 2019; Published (online) 22 Dec 2019]**