

Research Article

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Secondary metabolites of Antarctic fungi antagonistic to aquatic pathogenic bacteria

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Abstract: Polar microbial derived antibiotics have potential as alternatives to traditional antibiotics in treating fish against pathogenic bacteria. In this paper, 23 strains of polar fungi were fermented to detect bacteriostatic products on three aquatic pathogenic bacteria, subsequently the active fungus was identified. It was indicated that secondary metabolites of 23 strains were distinct; of these, the extract of strain B-7 (belonging to *Bjerkandera* according to molecular identification) demonstrated a strong antibacterial activity to *Streptococcus agalactiae*, *Vibrio anguillarum* and *Aeromonas hydrophila* ATCC7966 by Kirby-Bauer paper strip method. During one fermentation cycle, the pH curve of the fermentation liquor became lowest (4.0) on the 4th day and rose back to 7.6 finally after 5 days, The residual sugar curve was decreased before stabilising on the 6th day. It is presumed that a large amount of alkaline secondary metabolites might have been produced during fermentation. This study focuses on antagonism between aquatic pathogenic bacteria and fermentation metabolites

from Antarctic fungi for the first time, which may provide data on research of antibiotics against aquatic pathogenic bacteria.

Keywords: fungus; *Streptococcus agalactiae*; *Vibrio anguillarum*; *Aeromonas hydrophila*; secondary metabolites

1 Introduction

China is the largest country of fish production, consumption, processing and exporting, supplying one-third of fish stocks in the world. Aquaculture yield accounts for 72% of domestic and 60% of worldwide fishery output, occupying half of global gross production [1]. However, excessive development of fish farming and the spread of fish diseases affects sustainable development. Common fish pathogens include *Aeromonas hydrophila*, *Vibrio anguillarum* and *Streptococcus agalactiae*.

As a typical human-animal-fish common pathogen, *A. hydrophila* is widely distributed in a variety of natural waters [2]. It often causes furunculosis, fish hemorrhagic and human intestinal diseases after infection [3], it is listed as one of candidate pollutants and indicators for water pollution by the US Environmental Protection Agency [4]. *V. anguillarum* is usually found in cultured or wild fishes, seawater bivalve and crustacean animals. It can cause fatal bleeding disease—vibriosis. The disease has caused global breeding disasters and serious economic losses [5]. *Streptococcus agalactiae* is generally parasitic in the gastrointestinal and genital tracts of organism. It is the main pathogen to cause neonatal bacteremia, pneumonia and meningitis with the mortality rate of 5% to 20% [6] in addition to fish death. At present, the threats to children and adults are primarily through severe sepsis, accompanied by deep abscess and even endocarditis [7].

At present, antibiotics are still the main method to kill these pathogens, but antibiotic resistance has been a global public health challenge [8]. Statistically, almost all types of pathogens exposed to antibiotics have generated

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resistance from mutation over 75 years [9]. Researchers have already found variant of *A. hydrophila* (tolerant to chloramphenicol, streptomycin, tetracycline and oxytetracycline) [10-14], *V. anguillarum* (tolerant to chloramphenicol, sulfonamides, streptomycin, ampicillin, trimethoprim and tetracycline) [15-17] and *S. agalactiae* (tolerant to telithromycin and tigecycline) [18,19].

The development of active substances from polar microbes is in the ascendant. Since 2001, scholars have found 219 new compounds of microbes, lichens and marine organisms from the North and South Poles [20]. More than 1,000 Antarctic fungi have been isolated for identification since 1847 [21]. According to the number and species analysis, Antarctic fungi may be the species with widest biodiversity in the region [22]. Fungi usually secrete compounds to adapt to the extreme living environment, such compounds may also possess anti-cancer, anti-bacterial and anti-oxidative properties [23-25]. The antimicrobial activity of polar microbes can act through its secondary metabolites including terpenoids, phenols and nitrides. These secondary metabolites are synthesized by taking primary metabolites such as carbohydrates as the precursor during a certain period of growth. Without an, as yet, identified function for life activities, these substances are therefore new drug sources full of potential [26].

The experiment focused on the antagonistic effects of 23 Antarctic fungi fermentation metabolites to 3 aquatic pathogens as well as preliminary investigation of the antibacterial and fermentation characteristics of the screened active fungi.

2 Materials and methods

2.1 Experimental materials and instruments

Antarctic fungi were donated by the China Polar Center, and preserved in potato dextrose agar plate culture medium. *A. hydrophila* ATCC7966, *V. anguillarum*, and *S. agalactiae* were obtained from National Aquatic Pathogen Database of Shanghai Ocean University.

Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Mueller-Hinton Agar (MHA) and antimicrobial drug sensitive slips were purchased from Hangzhou Microbiology Reagent Co., Ltd.; the tryptone and yeast extract from Oxoid Corporation, Basingstoke, UK; the nutrient broth (NB) from Haibo Biotech Co., Ltd, Qingdao High-tech Industrial Park; the Ezup Column Fungi Genome DNA Extraction Kit and SanPrep Column DNA Glue Recovery Kit from Tiangen Biotech Co., Ltd; the

qualitative filter paper from Hangzhou Specialty Paper Co., Ltd; the remaining drugs from Sinopharm Group. The above reagents were analytical pure.

PDA, PDB, NB and MHA mediums were prepared according to the instructions.

Glucose Peptone Yeast (GPY) medium was prepared as follows. 10 g glucose, 1.5 g tryptone, 3.5 g yeast extract, 0.5 g potassium dihydrogen phosphate, 0.25 g magnesium sulfate heptahydrate, 2.5 g sodium chloride and 1 g calcium carbonate were mixed and dissolved in 500 mL distilled water for high temperature sterilization.

2.2 Fungal fermentation and product extraction

A small amount of cells were scraped from the plate which preserved 23 strains of polar fungi. After that, these cells were put in PDB medium at 24°C for 180 rpm shaking cultivation for 2 days, followed by fermentation in GPY medium of 2L flask at 20°C for 10-day. The fermentation liquor was filtered by four layer gauze, and then routinely extracted with isometric butyl acetate at 1:1 (v: v). The extraction was repeated twice. Extract mass, cell dry mass (pretreating at 80°C for drying to constant weight) and supernatant volume were determined. Each strain was conducted with three parallel tests.

2.3 Bacteriostatic test of fermentation products

The above extract was dissolved in methanol to final concentration of 5 g/L, from which 10 µL solution was dropped onto a 6 mm diameter sheet. *A. hydrophila* ATCC7966, *V. anguillarum* and *S. agalactiae* activated with bacterial nutrient broth mediums were coating MHA medium plate respectively. Then the experimental sheet and antimicrobial drug sensitive slips were pasted on the plate for 30°C static cultivation. After 24h, the diameter of the inhibition zone was observed and recorded. Here are 12 kinds of positive control drug sensitive sheet standards: erythromycin (S1), penicillin (S2), cefotaxime (S3), chloramphenicol (S4), sulfamethoxazole (S5), butylamine (S6), doxycycline (S7), gentamicin (S8), florfenicol (S9), enrofloxacin (S10), streptomycin (S11) and cefradine (S12). The methanol solution was taken as blank control.

The extract with antibacterial effect was selected to repeat the above antibacterial experiments.

The antibacterial effect was judged according to the following standard.

If inhibition zone diameter ≤ 8 mm, then the effect will be insensitivity.

If $8 \text{ mm} < \text{inhibition zone diameter} \leq 13$ mm, then the effect will be low sensitivity.

If $13 \text{ mm} < \text{inhibition zone diameter} \leq 19$ mm, then the effect will be medium sensitivity.

If inhibition zone diameter > 19 mm, then the effect will be high sensitivity.

2.4 Morphological and molecular identification of active fungi

The morphological characteristics of strain B-7 were observed under inverted microscope.

The genomic DNA of strain B-7 was extracted according to Ezup column fungus genomic DNA extraction kit. After that, sequence 18S rDNA was amplified by PCR. The primer was as follows.

NS1 :5'GTAGTCATATGCTTGTCTC3'

NS6:5'GCATCACAGACCTGTTATTGCCTC3'

PCR reaction system: Template DNA (50ng/ μl) 0.5 μl ; 10 \times Buffer 2.5 μl ; 2.5mM dNTP 1 μl ; Taq ferment 0.2 μl ; NS1 (10uM) 0.5 μl ; NS6 (10uM) 0.5 μl ; DDW 25 μl .

PCR loop condition: Initial denaturation: at 94°C for 4 min; 30 cycle: at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min; repair extension: at 72°C for 10 min; cessation reaction: at 4°C.

PCR products were conducted with electrophoresis by 1% agarose at 150V and 100mA for 20min. Electrophoresis strip was cut to obtain the desired DNA bands. The target bands were purified by the SanPrep column DNA gel recovery kit, and sequenced directly using PCR primers. The fungi were three parallel fermentation samples. The obtained 18S rDNA sequence was compared with the known sequence in GenBank to identify species.

2.5 Observation on the fermentation process of active fungi

Strain B-7 was fermented for 10 days. The experiment was designed as follows. There are 2 groups (A & B) of strain B-7 which were fermented. In A group, fermentation broth was sampled daily to measure pH values and residual sugar content (anthrone sulfuric acid method [27]). While group B were conducted with pH and residual sugar measurement at the end of the fermentation. The liquid loss caused in A group might affect the strain growth state. The 100 mL sterilized distilled water was added to each fermentation broth in A group for continuous cultivation

on the 6th day. The pH and residual sugar values were measured before and after adding water. In each group three parallel were taken.

2.6 Statistical analysis

The conducted research is not related to either human or animals use. All data were shown as mean \pm standard deviation. Statistical differences between the experimental groups were determined by one way ANOVA.

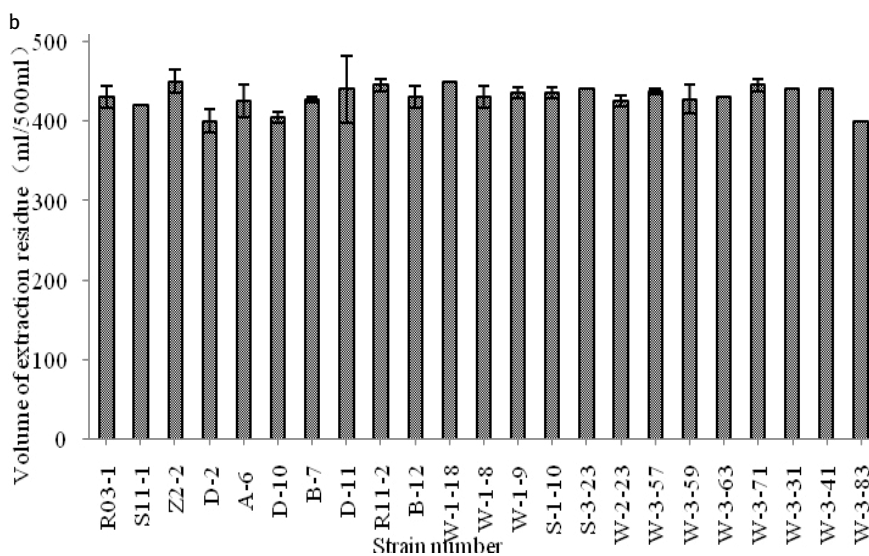
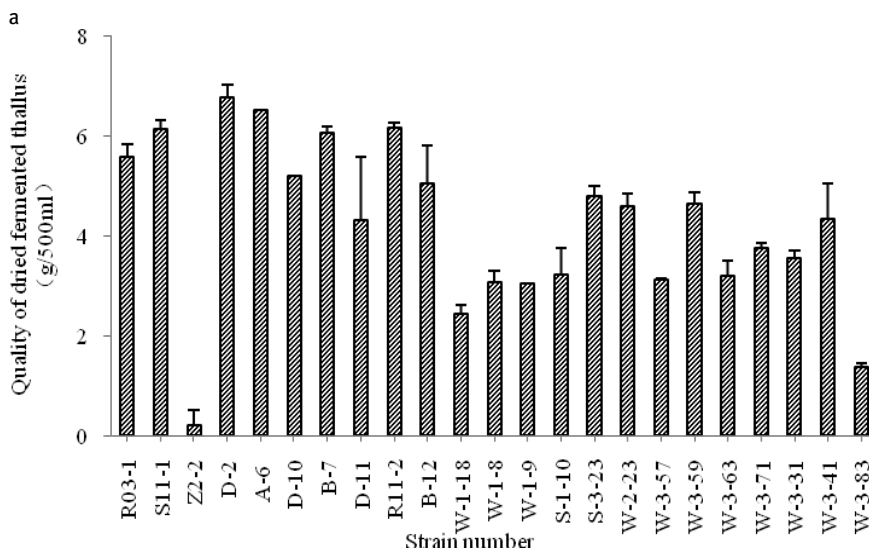
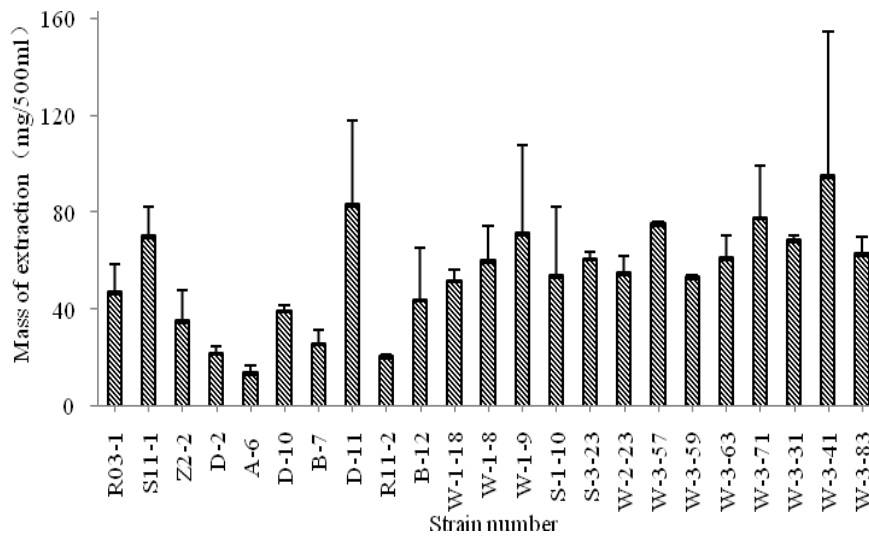
3 Results

3.1 Characteristics of fungal fermentation products

To investigate the characteristics of fungal fermentation products, 23 strains of polar fungi were extracted after 10 d fermentation. Extracting of supernatant with isometric butyl acetate, the content per 1L fungal fermentation broth was 28 (strainA-6)-190 mg (strainW-3-31) (See Figure 1a). After the fermentation liquid was filtered, the dry mass of fungi increased from 0.4 (strain Z2-2) to 13.6 g (strain D-2) (See Figure 1b). And the volume of supernatant had little change between 800 and 900 mL (See Figure 1c), indicating similar demands of cell fermentation for water.

3.2 Bacteriostatic effect of fermentation products

The antagonism between aquatic pathogenic bacteria and Antarctic fungi were studied and compared. After screening by the Kirby-Bauer paper strip method, one-third of the 23 polar fungi fermentation products had no inhibitory effect on the three pathogens; while those that did exhibit inhibition had no obvious antibacterial effect (inhibition zone ≤ 8 mm) [28]. The only exception to these products above was that fermentation products of strain B-7 had certain inhibitory on the three bacteria (See Figure 2). In addition, three bacteria were moderately sensitive to erythromycin, amikacin, gentamicin and streptomycin, and highly sensitive to cefotaxime, chloramphenicol, doxycycline, florfenicol and enrofloxacin, and insensitive to penicillin and sulfamethoxazole according to inhibition zone of 12 antibacterial drugs sensitive paper positive controls, indicating that some broad-spectrum antibiotics had significant inhibitory effects on the three aquatic pathogens. However, the abuse of these antibiotics



c

Figure 1. Experiment of fermentation by fungi. 23 strains of polar fungi were taken for shake flask fermentation for 10 days in 20°C followed by extraction with butyl acetate. Mass of extraction product (a) and dried fermented thallus (b), and volume of extraction residue (c) were measured. Three parallel experiments were set.

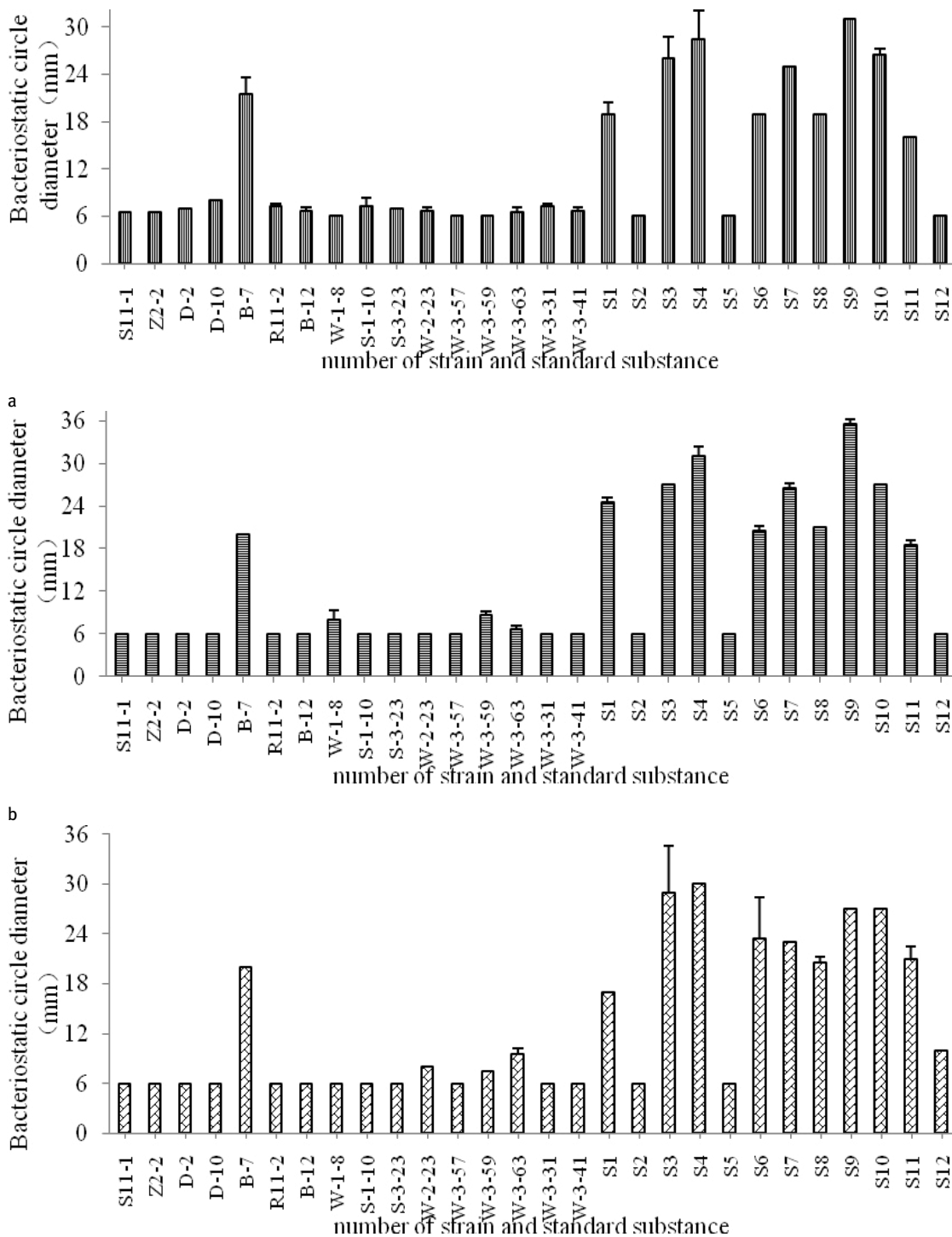


Figure 2. Bacteriostasis of fermentation product from 16 strains polar fungi. 16 strains of polar fungi were taken for shake flask fermentation for 10 days in 20°C followed by extraction with butyl acetate. Then Kirby-Bauer method was used to detect bacteriostatic circle diameter of fermentation products on *Streptococcus agalactiae* (a), *Vibrio anguillarum* (b) and *Aeromonas hydrophila* ATCC 7966 (c). Each paper was 6 mm in diameter with concrete of 100 µg. Observation time was 24 h after patch. 12 kinds of standard substance taken as positive control were as follows: erythromycin (S1), penicillin (S2), cefotaxime (S3), chloramphenicol (S4), sulfamethoxazole/trimethoprim (S5), butylamine (S6), doxycycline (S7), gentamicin (S8), florfenicol (S9), enrofloxacin (S10), streptomycin (S11), cefradine (S12). Three parallel experiments were set.

accelerates the production of super-resistant bacteria. Besides, antibiotics eventually enter human body with food chain to reduce the drug sensitivity. After doubling the extract concentration per unit paper, the three pathogenic bacteria were highly sensitive to the extract of strain B-7; the rest extracts remained inactive (See Figure 2).

3.3 Morphological and molecular identification of strain B-7

We then preliminary classified the strain B-7 by morphological and molecular identification. By microscopic examination, strain B-7 had mycelium of 5 μm in diameter and septa. There were spores at both ends of the mycelium, thus constituting the basidium (See Figure 3).

The sequencing results of three parallel samples of strain B-7 were compared in NCBI. For each sample, five most similar references were obtained according to matching degree and score. There were no RefSeq numbers in all 15 references. It was found that 18S rDNA sequence of strain B-7 had high similarity with *Bjerkandera* and *Trichaptum* genera.

All above sequences were compared by DNAMAN software. The remaining nucleotides were removed using

the Gblocks online software [29] where the parameters were set as follows: the least stringent settings, allowing smaller final blocks, gap positions within the final blocks, less strict flanking positions and many contiguous non-conserved positions. The 1,696 loci were obtained from 2,533 nucleotide sites. After comparison, the sequences were used to make UPGMA phylogenetic tree in MEGA6.06 [30], and repeated by number of differences genetic distance model and 1000 bootstrap. The phylogenetic tree showed that *Bjerkandera* genus was more stable and closer to sequence of the experimental sample. For the species of *Trichaptum* genus, 18S rDNA sequence had large range of variation (See Figure 4).

3.4 Investigation on the fermentation characteristics of strain B-7

We finally investigate on the productive process of secondary metabolites of strain B-7 by fermentation. During the fermentation process, the pH value increased after decrement. It reached the minimum (4.0) on the 4th day and 7.6 on the 10th day. The residual sugar curve decreased to become stable on the 6th day, indicating that the dextrose had been exhausted (See Figure 5).



Figure 3. Morphological identification of strain B-7. (a) Strain B-7 morphology in seed liquid medium; (b) Strain B-7 colony morphology on cant medium.

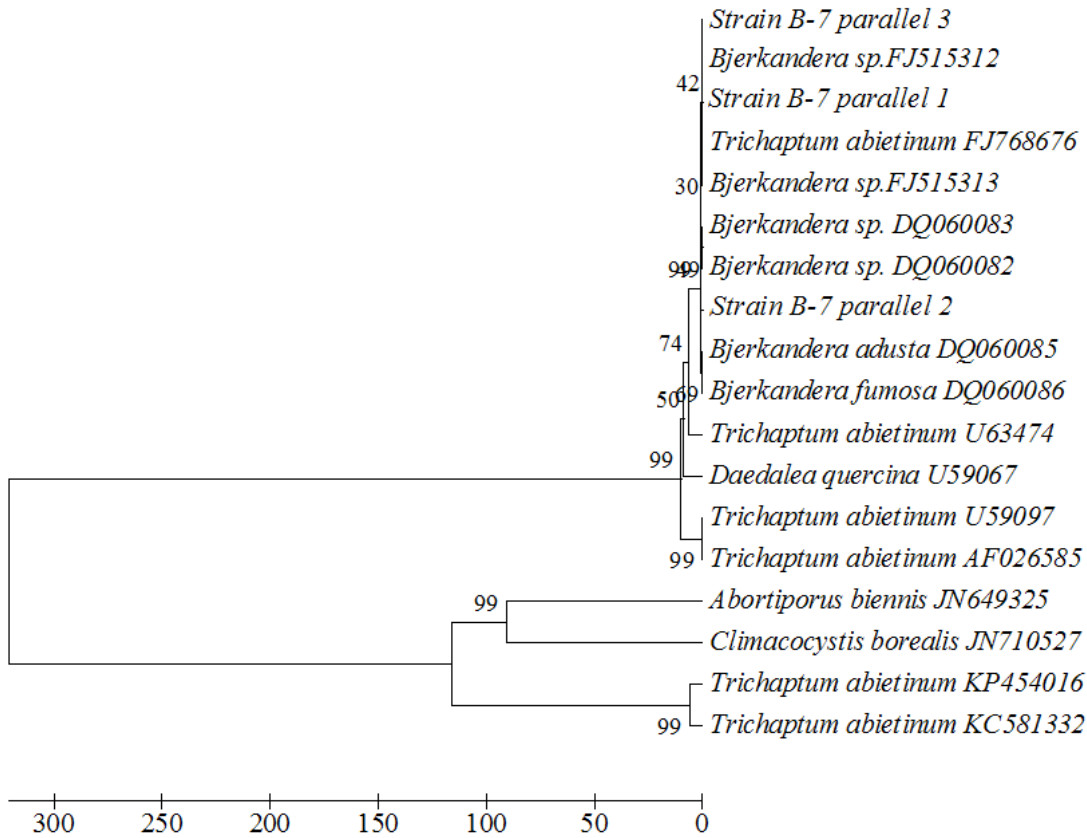


Figure 4. Molecular phylogenetic analysis of strain B-7 and 15 species similar in 18S rDNA. All the sequences are compared by DNAMAN software. The remaining nucleotides are removed using the Gblocks online software. The 1,696 loci are obtained from 2,533 nucleotide sites. After comparison, the sequences are used to make UPGMA phylogenetic tree in MEGA6.06, and repeated by number of differences genetic distance model and 1000 bootstrap.

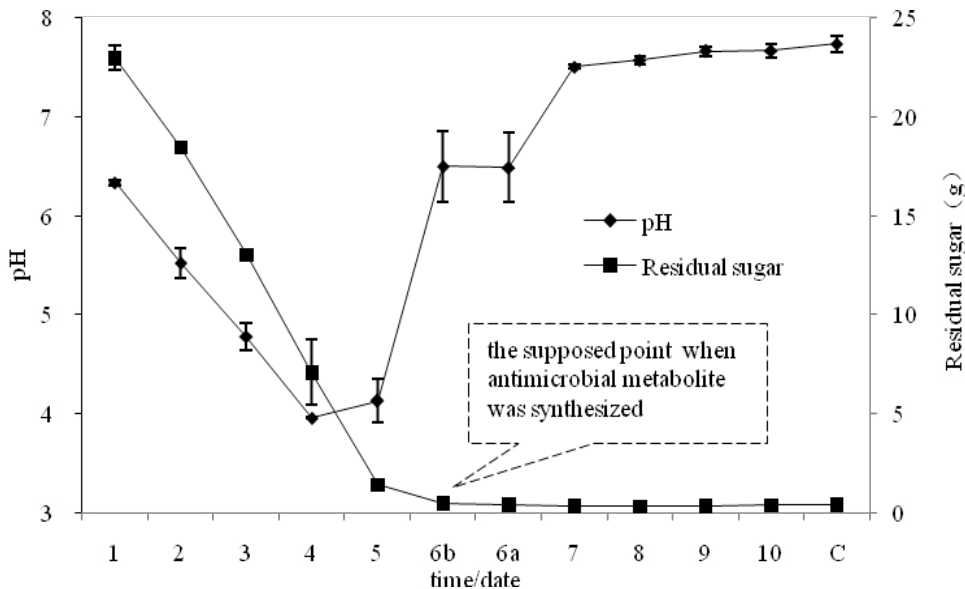


Figure 5. Observation of strain B-7 fermentation. Strain B-7 was fermented for 10 days under 180 RPM at 20°C using GPY fermentation medium. pH value (a) and residual sugar (b) of fermentation liquor were detected every day during fermentation, wherein 100 ml of sterilized distilled water were added in 6th days and the parameters were also detected before (6b) and after(6a) water adding. For control group(C), the parameters were only detected at the end of the fermentation. Three parallel experiments were set.

4 Discussion

Antarctic fungi may be the species with widest biodiversity in the region. In this experiment, Antarctic fungi (at 20°C) have a lower fermentation temperature than general fungi (at 25°C) [31], which may be caused by its crymophilia. In the fermentation process, different strains show various forms and colors. Fermented extract content, cell morphology and dry mass have large difference (See Figure 1). This is consistent with the variety of Antarctic fungi [22], thus providing more possibility for product variety.

There are still few reports of aquatic pathogens inhibited by Antarctic fungi which have been discussed for more than one century. The fungi used in this experiment are randomly selected from China Polar Center. From the results of the inhibition zone, the fungi with high activity (inhibition zone diameter > 1.9 cm) account for only 4%. However, the fermentation product of fungal strain B-7 has inhibition effect to the three pathogens, thus indicating certain broad spectrum. Henriquez *et al.* proposed that 3 and 1 strains of 101 polar sac fungi have significant inhibition effects to *Staphylococcus aureus* ATCC25922 and *Xanthomonas campestris* 833, respectively (inhibition zone diameter > 1.0 cm) [25]. In addition, there is no strain that has a significant inhibition effect on *Pseudomonas aeruginosa* and *Clavibacter michiganensis* 807. It is found that even the most abundant species in the South Pole has low probability to screen the strains with apparent antimicrobial activity. Positive drug sensitive sheets are tested to derive that the broad-spectrum antibiotics (such as cefotaxime, chloramphenicol and florfenicol) have generally better antimicrobial effect. The drugs inhibiting the three bacteria are initially selected. Tetracyclic (doxycycline) and chloramphenicol (florfenicol) antibiotics can be the first choice, which is followed by moderately inhibited aminoglycosides (amikacin, gentamicin and streptomycin) in this experiment. Sulfonamides antibiotics (sulfamethoxazole) are inappropriate. In the β -lactam antibiotics, penicillin has poor effect on three bacteria, while the effect of cefotaxime is very significant.

Fungus B-7 might be a kind of Basidiomycota. The obtained Fungus B-7 consists of multicellular hyphae with septa. Basidia are formed by hyphae and spores. This is the common feature of the Basidiomycota fungi [32]. Basidiomycota, as the highest division in fungi, has wide distribution, large quantity and various kinds. There are more than 20,000 kinds of edible or medicinal species [33]. The 18S rDNA molecular identification shows that the fungus belongs to *Bjerkandera*, Meruliaceae (or

Trichaptum, Polyporaceae), Polyporales, Agaricomycetes, Basidiomycota. The fungi of *Trichaptum* were found in 1904, widely distributed in the world [34]. There are 34 species recorded in documents. In the genus *Bjerkandera*, there are only two species (*B. adusta* and *B. fumosa*) distributed in the high latitudes, belonging to hardy species [35]. In evolutionary tree, strain B-7 is closer to the genus *Bjerkandera*, so it is preliminarily classified as *Bjerkandera*.

In fungus fermentation process, organics such as sugar and fat decompose to produce acidic substances, decreasing the pH value of the culture medium. E.g., after solid state fermentation by *Mucor* and *Aspergillus oryzae* for 2 days, the pH of surimi fermentation medium decreases from 6.9 to 6.2 [36]. In this experiment, the fungi are conducted with liquid state fermentation. During the first half of fermentation, the acid metabolites increase to cause the decrease of pH with the consumption of dextrose. In this process, the fermentation broth is clear. During the second half, the dextrose exhausts to make cloudy fungus liquid, thus increasing the pH. The amount of fermented extract rises after extraction. This is probably because massive secondary metabolites of alkaline lipids are produced (See Figure 4). The alkaline agent in secondary metabolites is derived from a nitrogen-containing ring, such as the alkaloid [37]. It is known that there are two ways to synthesize nitrogenous secondary products. First, dextrose is used to produce aliphatic amino acids by glycolysis and tricarboxylic acid cycle. After derivatization, the nitrogenous secondary product is synthesized. Secondly, dextrose degrades to produce phosphoenolpyruvate or erythrose-4-phosphate. After that, aromatic amino acids are produced by shikimic acid pathway metabolism to finally synthesize nitrogenous secondary product [1, 38]. Further research is required to determine whether the extreme weather in Antarctic region indicates new metabolic pathway for the fungus.

Partial plant polysaccharides have synergistic effects of inhibition of, and immunity to water pathogens. Kamilya *et al.* demonstrated that the survival rate of *Catla catla* infected by *A. hydrophila* rises from 15% to 65% after being fed on SC Glucan. If SC Glucan is taken as a vaccine adjuvant simultaneously, the survival rate will increase by 15% again [39]. Wu *et al.* proposed that the survival rate of *Carassius auratus* infected by *A. hydrophila* rises from 24.4% to 68.9% after being fed on *Coriolus versicolor* polysaccharide with 1 gram per kilogram body weight for 2 weeks [40]. If the *C. auratus* is fed after immunization, the survival rate after 5 weeks rises from 50% to 85% [41]. Synergistic reaction of *Ganoderma lucidum* and *Astragalus* (0.5%) has immunoprotectiveness on *Cyprinus carpio*,

thus increasing survival rate from 40% to 60% [42]. In general, the antibacterial activity of the polysaccharide is mainly reflected in improving host immunity. It should be mixed with the feed for promotion and application. The polysaccharide is easily soluble in water and harmless.

In contrast, the secondary metabolites of fungi are small molecules that are insoluble in water [25]. Once active molecules are screened, the structure will be easily determined, and prepared by chemical synthesis. However, the yeast is the main fungus used to inhibit these aquatic pathogens at present. For example, *Debaryomyces hansenii* strain CBS 8339 is applied for *Mycteroperca rosacea* [43]; *Saccharomyces cerevisiae* for *Oreochromis niloticus* [44-46] and *Labeorohita* [47]. Besides, the extract of yeast can strengthen the resistance to the inflection of *A. hydrophila* by improving host immunity. For example, commercial yeast extract (Himedia Company from Mumbai, India) is applied for *Labeorohita* [48]; *Saccharomyces cerevisiae* β -glucan for *Cyprinus carpio* [49-51].

Lokesh *et al.* [52] found that the yeast manno oligosaccharides or commercial glucan enhance the ability to resist *V. anguillarum* by promoting the expressions of *Gadus morhua* proinflammatory (il1b, il8 and ifng) and anti-inflammatory factors (il10). Similarly, *Oreochromis niloticus* is fed by the feed containing 0.3% *Saccharomyces cerevisiae* to improve the ability to resist *S. agalactiae* [53]. It can be seen that the fungi or their extracts antagonize aquatic pathogens by improving host immunity, which is consistent with the above plant polysaccharides.

In the paper, secondary metabolites of fungi B-7 fermentation are directly used to inhibit bacteria. Yueyang *et al.* [54] used similar method to isolate 5 epiphytic fungi from the anemone, and the ethyl acetate extracts of fermentation product have inhibition effects on 6 strains of bacteria. Wherein, the fungus of the genus *Emericella* sp. has the best inhibition effect, wherein the maximum diameters of inhibition zones are 21.3 mm (high inhibition) and 10.2 mm (low inhibition) for *Staphylococcus aureus* and *Vibrio parahaemolyticus*, respectively.

In subsequent experiments, we will study the antimicrobial components in strain B-7 through activity tracking. The structure of active compound will be identified for activity verification.

5 Conclusions

The antibacterial activity of 23 polar fungi were screened, wherein strain B-7 has strong antibacterial activity to *S. agalactiae*, *V. anguillarum* and *A. hydrophila* ATCC7966

by the Kirby-Bauer paper strip method. According to 18S rDNA identification, the fungus belonged to genus *Bjerkandera*. During 10 days fermentation process, the pH curve of the fermentation liquor became lowest (4.0) on the 4th day and rose back to 7.6 finally after 5 days, and the residual sugar curve showed a downward trend. It is presumed that massive alkaline secondary metabolites are produced since the 6th day.

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