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Fungal Community Analysis and Biodeterioration of Waterlogged Wooden Lacquerware from the Nanhai No. 1 Shipwreck

Yin Jia [1,†], Liuyu Yin [1,†], Fengyu Zhang [1], Mei Wang [1], Mingliang Sun [1], Cuiting Hu [1], Zijun Liu [1], Yue Chen [2], Jie Liu [2] and Jiao Pan [1,*]

1 Key Laboratory of Molecular Microbiology and Technology of the Ministry of Education, Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China; 117759@zju.edu.cn (Y.J.); 1510836@mail.nankai.edu.cn (L.Y.); 2120171023@mail.nankai.edu.cn (F.Z.); 1510821@mail.nankai.edu.cn (M.W.); 2120181154@mail.nankai.edu.cn (M.S.); 2120160931@mail.nankai.edu.cn (C.H.); 2120150877@mail.nankai.edu.cn (Z.L.)

2 Conservation and Restoration Institute, Chinese Academy of Cultural Heritage, Beijing 100029, China; chenyue@cach.org.cn (Y.C.); liujie@cach.org.cn (J.L.)

* Correspondence: panjiaonk@nankai.edu.cn; Tel.: +86-022-2350-5961

† The two authors have the equal contribution to this work.

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Abstract: To avoid the lacquerware of the Nanhai No. 1 shipwreck from being corroded by microorganisms and to improve the knowledge on microbial ecology of the wood lacquers, we conducted a series of tests on the two water samples storing the lacquerware and colonies on the surface of the lacquerware. The high-throughput sequencing detected dominant fungal communities. After that, the fungal strains were isolated and then identified by amplification of ITS- 18S rRNA. Then the activity of ligninolytic and cellulolytic enzymes was detected on potato dextrose agar (PDA) plates with 0.04% (*v*/*v*) guaiacol and carboxymethyl cellulose (CMC) agar plates. Finally, we tested the biocide susceptibility of these fungi. *Penicillium chrysogenum* (NK-NH3) and *Fusarium solani* (NK- NH1) were the dominant fungi in the sample collected in April 2016 and June 2017. What is more, both showed activity of ligninolytic and cellulolytic enzymes. Four biocidal products (Preventol® D7, P91, BIT 20N, and Euxyl® K100) inhibited the growth of the fungal species in vitro effectively. In further research, the microbial community and environmental parameters in the museum should be monitored to assess the changes in the community and to detect potential microbial outbreaks.

Keywords: Nanhai No.1 shipwreck; wood lacquers; fungi; high-throughput sequencing; carboxymethyl cellulose activity; biocides

1. Introduction

In traditional Chinese art, lacquerware refers to a vessel made of bamboo, wood or metal, coated with a hard resin layer on the surface. Arising in the Neolithic Age, lacquering was initially regarded as a form of waterproof protection and later developed into an important decorative art. The application of multiple layers on lacquerware can protect the core material [1]. Various types of items could be lacquered, such as furniture and many other household objects, cosmetics boxes, domestic ware, musical instruments, food-serving implements, even coffins. The cores of the lacquerware were made from various materials—for instance, the core of a lacquered tray was made of wood and fabric. Sometimes other materials such as iron, pottery, rattan, leather or the like were also used as lacquer cores. Chinese lacquerware continues to develop and has a far-reaching impact on Japanese and Korean art as well as Indian culture.

Large quantities of lacquerware have been excavated at different sites in China. For example, there were thousands of pieces of wood lacquerware found in Nanhai No.1 shipwreck in Guangdong province. Unearthed wood lacquers were usually preserved in water to prevent degradation caused by the microbes existing in the air. Soaking in the water for a long time however can reinforce the damage of wood lacquers, including the deterioration, lacquer layer loss, and microbial damage [2]. Plenty of studies have uncovered the fact that many kinds of degradation and microbial successions may occur in waterlogged wooded objects. In waterlogged sites, tunneling bacteria and erosion bacteria have been proved to be able to degrade wooden items in near anaerobic environments [3,4]. In fact, some fungi also can degrade these waterlogged lignocellulosic materials as a part of the carbon cycle [5]. Typical fungi inhabitants of waterlogged wooden objects are the species belonging to the group of Ascomycetes and Deuteromycota such as species in genus *Cladosporium*, *Acremonium*, *Fusarium*, and *Chaetomium*, among others [6,7].

A large percentage of the world's cultural heritage artifacts have been severely and irreversibly damaged and degraded by microorganisms [8,9]. In July 2012 to August 2013, the Chengdu Institute of Cultural Relics and the Jingzhou Cultural Relics Protection Center formed a joint archaeological team who excavated four pits in the wooden tombs of the Western Han Dynasty in Chengdu, and uncovered hundreds of precious bamboo and wood lacquers, including some bamboo cases. In order to avoid the damage caused by microbial degradations during the period of water conservation, the research team collected the microbial samples from the unearthed bamboo cases. *Penicillium* sp., *Aspergillus* sp., and *Trichoderma* sp. were identified by isolation and purification, DNA-ITS, and other biological methods [10]. Therefore, the protection against mildew should be carried out in time [11]. These studies on microbial degradations of cultural heritage artifacts revealed the occurrence of fungi. Moreover, the Nanhai No.1 shipwreck is suffering an outbreak of a white filamentous fungus. To this end, the detection and identification of biodegradative microorganisms are an essential means for the control and prevention of microbial biodeterioration, since only if we understand the biological and physiological properties of organisms can we have the capacity to control the potential harm they cause [12].

The aims of this study were (1): to isolate and identify fungi colonizing the surfaces of the wood lacquers excavated from Nanhai No.1 shipwreck; (2) to detect fungi dwelling in the water storing the lacquerware; (3) to assess the ability of the fungi recovered from the surfaces of the wood lacquers to degrade cellulose and lignin; (4) to evaluate the susceptibility of the fungi recovered from the surfaces of the wood lacquers to four different biocides.

2. Materials and Methods

2.1. Sample Collection

This research was carried out in June 2017 in the Marine Silk Road Museum of Guangdong. This museum is the world's first underwater archaeology theme museum that shows the excavation, protection, display, and research of Nanhai No.1 shipwreck. It displays dynamic demonstrations of the excavation of the underwater archeology site. The museum is divided into two theme areas: exhibit area of Nanhai No.1 and exhibit area of Maritime Silk Road. Nanhai No.1 shipwreck was wholly salvaged from the seabed more than 20 m deep and moved to the museum in 2007 [13]. In November 2013, the excavation of the Nanhai No.1 shipwreck began and a number of archaeological objects including lacquerware, ornaments, bronze mirrors, wood combs, and even seeds of plants were extracted from the wreck. The map of Nanhai No.1 can be seen in our former study about the microbial community of waterlogged archaeological wood from Nanhai No.1 shipwreck. In this research, the overall microbiological analyses were to identify the fungi present on the wood lacquers extracted from Nanhai No.1 and to reveal fungal communities thriving in water samples of the wood lacquers. So far, the wood lacquers have been stored in a 4 °C fridge and contained with moist gauze to preserve moisture with deionized water as the storage water of the lacquerware.

Lacquerware surface sample collection using non-invasive sampling with PDA medium (Figure 1). The lacquerware surfaces were sampled with sterile swabs and then inoculated in petri dishes with PDA medium in a sterile operation. Three distinct fungal plaque areas on each lacquerware surface were selected for inoculation. Afterwards, these petri dishes were incubated in the laboratory and pure colonies were isolated for identification and investigation. In addition, 50 mL sterile centrifuge tubes were used to collect water samples with one water sample for each lacquerware. Sample NHI935 was collected from lacquerware 2016NHIT0501⑩935 and sample NHI58 was collected from lacquerware 2015NHIT0202③:58. The two water samples were conducted with high-throughput sequencing and purification.

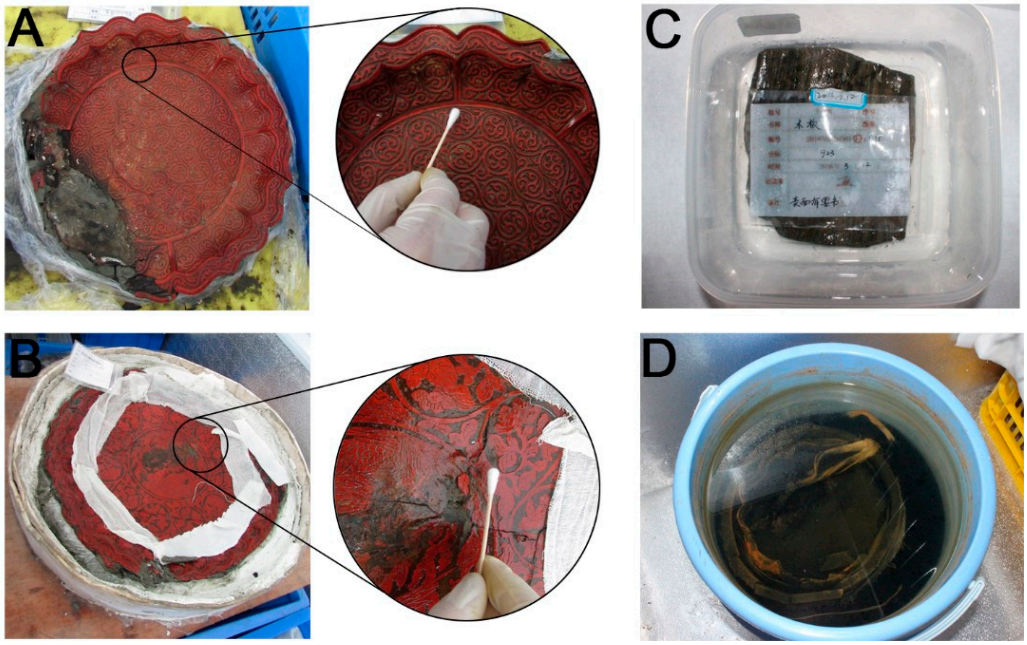

Figure 1. Sampling areas for microbiological analyses on the wood lacquers surfaces: (**A**) lacquer NHI566; (**B**) lacquer NHI883; (**C**) water sample NHI935; (**D**) water sample NHI58.

2.2. Microbial Isolation, Cultivation, and Identification

According to the common cultural laboratory practices, the PDA medium plates containing lacquerware surface samples were incubated at 28 °C for 5–30 days. Fungi were isolated and pure cultures obtained after subsampling. The DNA of the pure strains isolated from the surface of lacquerwares were extracted by the hexadecyltrimethylammonium bromide (CTAB) method [14,15]. Two primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′), were used to amplify the fungal ITS region [16]. The PCR reaction consisted of 50 µL mixtures, including genomic DNA (2 µL), 10× Reaction Buffer (5 µL), 2.5 mM deoxy-ribonucleoside triphosphate (dNTP) mix (4 µL), 10 µM forward primer (2 µL), 10 µM reverse primer (2 µL), 5 U/µL Transtaq-T DNA polymerase (0.5 µL) (TransGen Biotech, China), and dd H_2O to 50 µL. The thermocycling program was set as follows: 95 °C for 3 min, followed by 95 °C for 32 cycles of 30 s, 54 °C for 30 s, 72 °C for 20 s, 72 °C for 5 min as a final extension [17]. After detecting the PCR products with 1% agarose gel electrophoresis, an AxyPrep PCR Clean Up Kit (Axygen, California, USA) was used for purification.

The purified PCR products were sequenced by GENEWIZ (Beijing, China). The sequences were used as queries in BLAST searches [18]. The sequences retrieved were deposited in the NCBI GenBank under the accession number SRP219493 for fungi.

2.3. High-Throughput Sequencing Analysis

Water samples of lacquers were collected using 50 mL centrifuge tubes. Total DNA of the water samples was detected by the MoBio PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA).

Fungal communities were studied by amplifying internal transcribed spacer 1 (ITS1) fragments using primers ITS5-1737F (5′-GGAAGTAAAAGTCGTAACAAGG-3′)/ITS2-2043R (5′-GCTGCGTTCTT CATCGATGC-3′) combined with adapter sequences and barcode sequences. Amplifications were carried out in a 50 µL mixture including 25 µL of Master Mix (2X), a 0.5 µM final concentration of the forward and reverse primers, 10 ng of template DNA and nuclease-free water to 50 µL. The PCR conditions were 98 °C for 1 min, followed by 30 cycles of 10 s at 98 °C, 30 s at 55 °C for ITS region amplification, and 30 s at 72 °C, with a final extension of 5 min at 72 °C.

After extraction, the total DNA was operated in Novogene (Beijing, China) including amplification by amplicons to analyze the fungal community. The construction of a library used the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) [13]. The library was sequenced on an Illumina Hiseq2500 PE250 platform. The sequences were processed and analyzed by Novogene (Beijing, China).

2.4. Activity of Carboxymethyl Cellulose (CMCase) Production by Fungi

Fungal spores of pure isolates strains illustrated in Section 2.2 at a concentration of 2×10^7 CFU/mL were added to the liquid fermentation medium. The liquid fermentation medium formulation consisted of 1.0% CMC-Na, 1.0% peptone, 0.5% beef extract, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$ and 1 L of tap water. Four single variables were set for time, temperature, pH, and $FeCl_3$ concentration. Fungi were cultivated under single variable conditions as above.

For measuring enzyme activity, 2 mL of fermentation brothwas centrifuged at 12,000 r/min, 4 °C for 5 min. Then 0.5 mL of the supernatant was incubated with 0.5 mL of 1% CMC-Na prepared with 0.05 mol/L citric acid buffer (pH 4.8) at 50 °C for 30 min. The reaction was terminated by addition of 3 mL of 3,5-dinitrosalicylic acid reagent. All of the assay tubes were placed in boiling water for 5 min. The absorbance of the contents in the tubes was measured at 530 nm. To the blank control group was added 0.5 mL of the fermentation supernatant after boiling to make sure it would not react with the reagents.

The unit of CMCase (IU) was defined as the amount of enzyme required to produce 1 µmol of glucose per minute and per mL of crude enzyme solution under reaction conditions. The activity of CMCase calculation formula is $X = (A \times V_T \times 1000)/(M \times V \times T)$. X is the CMCase activity ($IU \cdot mL^{-1}$) in the sample; A is the glucose content ($mg \cdot mL^{-1}$) calculated according to the standard curve; V_T is the total volume of the reaction solution (mL); M is the molar mass of glucose (180.2 $g \cdot mol^{-1}$); V is the volume of the crude enzyme solution (mL) added in the reaction; T is the reaction time (min).

The total amount of protein in the supernatant was measured by the Enhanced Lowry Assay Kit (Leagene, Beijing). The specific activity of CMCase is calculated and defined as the unit of enzyme activity per mg of protein (IU/mg) [19].

2.5. Biocide Susceptibility of Fungal Strains

The biocide susceptibility test used the disk diffusion method [15]. Four biocidal products were evaluated, Preventol® D 7, P 91, BIT 20 N and Euxyl® K100 [13]. In brief, the tested fungi were dipped with a sterile cotton swab and painted on PDA medium plates. Afterwards five paper discs (radius of 3 mm) were put on the medium and then four were loaded with 30 µL of the corresponding 0.5% biocides, one was soaked with sterile water as a control. After incubating at 28 °C for 4 days, the inhibition zones around the discs were measured and statistical analysis of these measures was conducted.

3. Results

3.1. Fungi Communities Analyzed by Classical Cultivation Methods and High-Throughput Sequencing

Fungal strains from the surface of two lacquers were isolated. As shown in Table 1, the cultivable fungal strains were identified by molecular identification. Two isolates were obtained from the surface of lacquer NHI556, and three isolates from the surface of lacquer NHI883. These isolates were most closely related to *P. chrysogenum* and *F. solani*.

Table 1. Molecular identification of pure strains isolated from lacquerware surfaces.

Taxonomy	Closet Strain	Similarity (%)	Accession Number (GenBank Database)
NHI566			
Isolate 1	*Penicillium chrysogenum*	99%	KF986423
Isolate 2	*Fusarium solani*	99%	KX349467
NHI883			
Isolate 3	*Penicillium chrysogenum*	99%	KF986423
Isolate 4	*Fusarium solani*	99%	KX349467
Isolate 5	*Colletotrichum dematium*	99%	JN998109

The fungal community composition of two water samples (NHI935 and NHI58) was analyzed. High-quality reads numbering 171,814 were obtained after filtering. There were five fungal phyla in the two samples (Figure 2A). Ascomycota was the most abundant phylum, accounting for 97.50% in sample NHI935 and 95.33% in sample NHI58. Four additional fungal phyla including Glomeromycota, Basidiomycota, Zygomycota, and Chytridiomycota accounted for a small proportion. In the genus level, high-throughput sequencing revealed the presence of a total of 48 genera in the two water samples. The main fungal genera are summarized in Figure 2B, with "Other" consistently being the largest category. *Fusarium* belongs to the phylum Ascomycota and was the most dominant genus in the samples, where it accounted for 34.4% and 7.6% of the community. *Cadophora* was the second largest genus in NHI935, accounting for 16.7%, but only 0.8% in NHI58. *Rhizopus*, *Pestalotiopsis*, *Cryptococcus*, *Alternaria*, *Aspergillus*, *Talaromyces*, and *Exophiala* were detected in all two samples with average abundance 0.13%, 1.23%, 0.58%, 1.21%, 0.05%, 0.71%, and 0.69%, respectively.

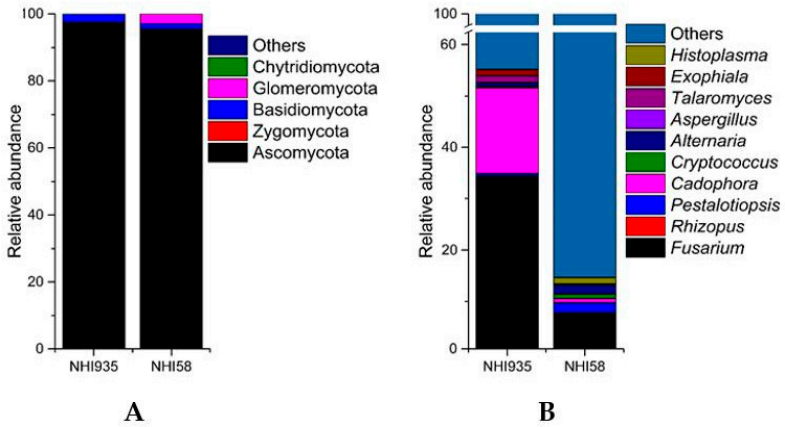

Figure 2. Fungal community at phyla (**A**) and genera (**B**) level detected in the two water samples.

3.2. Isolation of Dominant Fungi

The plate pictures and photomicrographs of five fungi isolated from the surface of the lacquerware are presented in Figure 3. Meanwhile, as was shown in Table 1, the strain 'isolate 1' from the sample NHI566 and the 'isolate 3' from the sample NHI883 were the same species and the strain *P. chrysogenum* was named NK-NH3(MH392741). The strain 'isolate 2' from the sample NHI566 and the 'isolate 4' from the sample NHI883 were the same species and the same as *F. solani* NK- NH1(KY410238) in the former study on Nanhai No.1 shipwreck in 2015 [13].

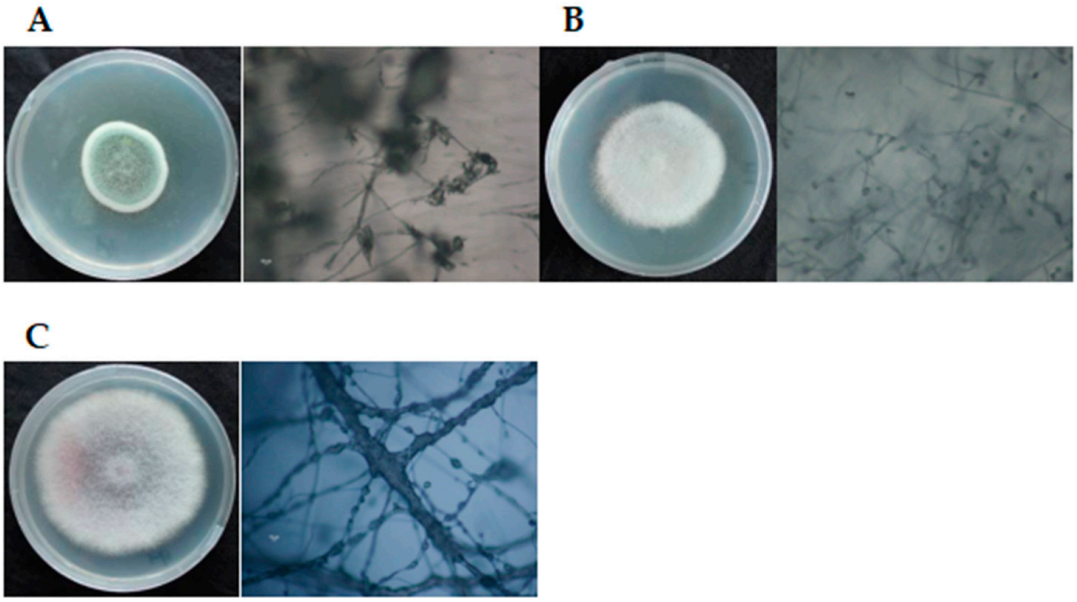

Figure 3. The plate pictures (left) and photomicrographs (right) of five fungi isolated and purified from the surface of the lacquerware. (**A**) Isolate 1 and 3: *P. chrysogenum*, the scale bar is 10 μm; (**B**) Isolate 2 and 4: *F. solani*, the scale bar is 5 μm; (**C**) Isolate 5: *C. dematium*, the scale bar is 10 μm.

3.3. Activity of CMCase Production by F. Solani NK-NH1 and P. Chrysogenum NK-NH3

During the protection process, the pH value and iron ion concentration of the lacquerware from ocean change, resulting in changes in the activity of CMCase. In the CMCase activity test, *P. chrysogenum* NK-NH3 reached a peak (0.5272 IU/mL and 0.3481 IU/mg, respectively) when the fermentation medium pH was 3.0, the $FeCl_3$ concentration was 0.2 g/L, cultured under 32 °C for 5d. While, *F. solani* NK-NH1 reached the highest CMCase activity and specific enzyme activity (0.11 IU/mL and 0.096 IU/mg, respectively) when the pH of fermentation medium was 5.4, and cultured under 32 °C for 5d. The effect of Fe^{3+} ion concentration on the CMCase activity is not significant compared with when no ferric ion is added. The results are illustrated in Figure 4 and three replicates were conducted per conditions for each activity test. As is shown in Figure 4, CMCase presents different effects on the two strains. The difference in the enzymatic properties of the two strains is related to the strain and its genetic characteristics—we will conduct further study. If the CMCase of the two fungi are separated and purified, the optimal pH value of these enzymes and other physiological and biochemical characteristics can be further determined.

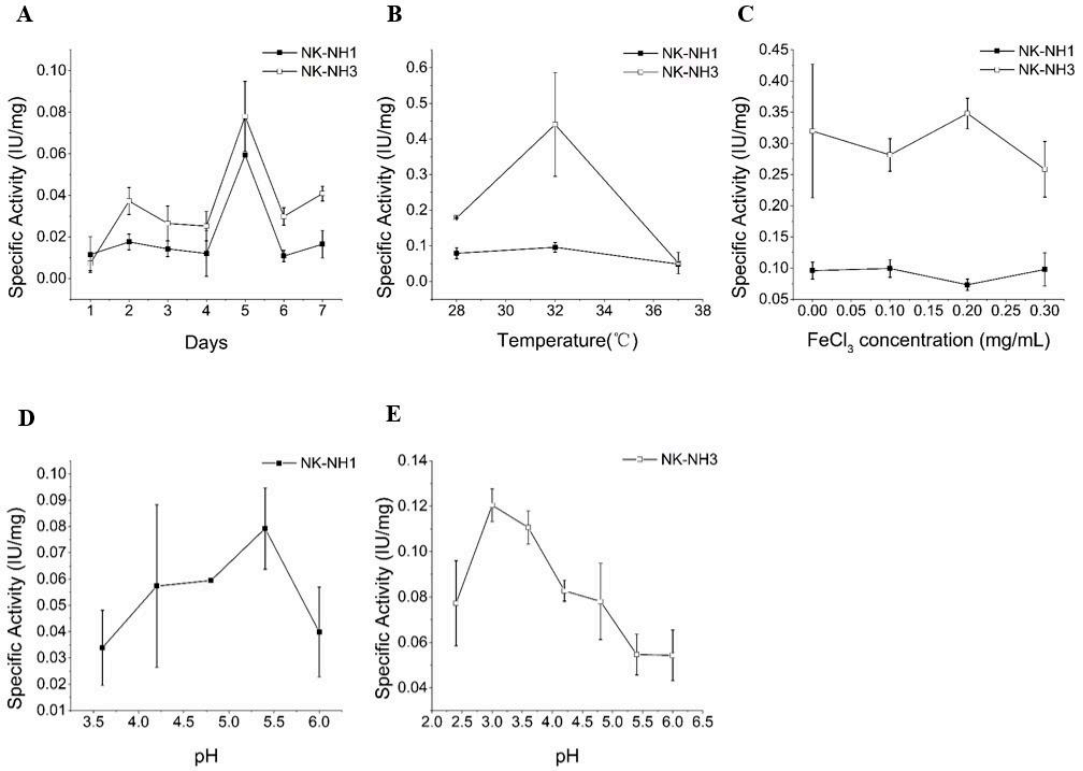

Figure 4. Effects of time, temperature, ferric chloride concentration, and pH on the CMCase activity of *F. solani* NK-NH1 and *P. chrysogenum* NK-NH3. (**A**) Trend of CMCase activity of two strains cultured in PDA liquid medium for at 28 °C 1–5 days. (**B**) Trend of CMCase activity of two strains cultured in PDA liquid medium at 28, 32, and 37 °C for 5 days. (**C**) Trend of CMCase activity of two strains cultured in PDA liquid medium (*F. solani* NK-NH1 at pH 5.4 and *P. chrysogenum* NK-NH3 at pH 3.0) containing 0.1, 0.2, and 0.3 mg/mL $FeCl_3$ for five days. (**D**) Trend of CMCase activity of *F. solani* NK-NH1 cultured in PDA liquid medium with pH 3.6–6.0 at 28 °C for five days. (**E**) Trend of CMCase activity of *P. chrysogenum* NK-NH3 cultured in PDA liquid medium at pH 2.4–6.0 at 28 °C for five days.

3.4. Efficiency of Biocide Products against Targeted Strains

Four biocides used in the protection of cultural relics, K100, Preventol® D7, P91, and BIT 20N, were selected to test the susceptibility of strains. *F. solani* NK-NH1 and *P. chrysogenum* NK-NH3 were used for the tests. Four biocides were able to inhibit the fungal growth when applied at 0.5% concentration, which is much lower than the concentration (2%) recommended by manufacturers. In this study, the most effective agent was Preventol® D7, based on isothiazolinone, followed by BIT 20N (isothiazolinone derivative). Biocide products P91 combining bronopol and isothiazolinone had similar efficacy when compared with BIT 20N. Another product K100 based on the combination of benzyl alcohol and isothiazolinone derivative had the lowest efficacy against fungal strains.

Under the treatment of 0.1% and 0.5% concentration bacteriostatic agents, obvious inhibition zones appeared around the filter paper with the bacteriostatic agent added, indicating that all the four bacteriostatic agents had biocide effects on the strains. Under the treatment of 0.1% concentration of bacteriostatic agent, the treatment effects of P91 and K100 were poor, and the treatment differences of the other bacteriostatic agents were not obvious. For strains *F. solani* NK-NH1 and *P. chrysogenum* NK-NH3, under the treatment of 0.5% concentration of bacteriostatic agent, the biocide efficacy of the four bacteriostatic agents could be clearly seen, D7 had the strongest effect, P91 was second, 20N was poor, and K100 was the weakest. The results are shown in Figure 5 and statistical analysis is presented in Figure S1.

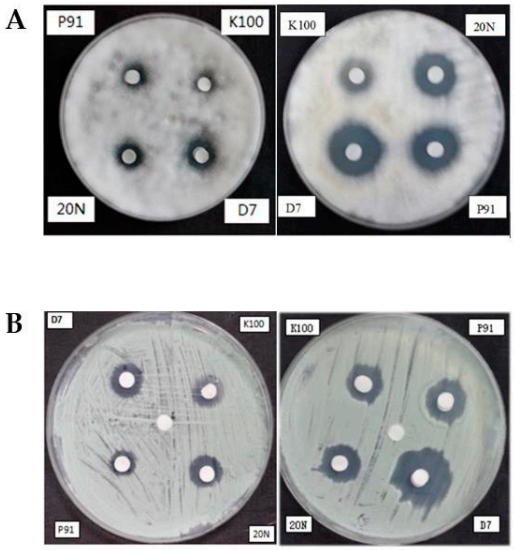

Figure 5. The inhibition of fungal growth by four biocides. (**A**) *F. solani* NK-NH1 under the treatment of 0.1%(left) and 0.5%(right) concentration biocides and cultured on PDA plates at 28 °C for 3 days. (**B**) *P. chrysogenum* NK-NH3 under the treatment of 0.1% (left) and 0.5% (right) concentration biocides and cultured on PDA plates at 28 °C for 3 days.

4. Discussion

4.1. Fungal Communities

Our previously unpublished results from 2016 showed that *Penicillium* was the main fungus genus in the water sample of lacquer, while results from 2017 in the present article showed that *Fusarium* was the dominant one (Supplementary Tables S1–S3). Moreover, fungi of these two genera, namely *F. solani* NK-NH1 and *P. chrysogenum* NK-NH3, were successfully isolated from the lacquerware surfaces.

This is the first time that *Fusarium* species have been reported to lead a putative microbial deterioration of wooden lacquerware. Another famous case concerning *Fusarium* in the biodeterioration of cultural heritage is the Paleolithic painting of the Lascaux Cave in France [20]. The ground and walls were covered by the dense white hyphae of *F. solani*. They not only destroyed the aesthetics of the paintings, but also changed the material structure of them [21]. In addition, *Fusarium* sp. was isolated from documentary heritage [22]. *Fusarium* species are mainly plant pathogens that cause root and stem rots. They live in the soil after the plant has been harvested or decayed [20]. Some members of the genus produce mycotoxins that can affect human and animal health andare often found in wounds [23]. The genus also can be found in food and indoor environments [24]. Thus in lacquerware surfaces and water *Fusarium* might have been brought from the outside environment by visitors or conservation-restorers.

It is worthy of mention that besides the *Fusarium*, the genus *Cadophora* should not be ignored, which accounts for 16.7% in sample NHI935. Most *Cadophora* members were primarily isolated from plants and soil [25]. For example, isolates related to *C. malorum* cause wood decay of kiwifruit [26]. *Cadophora* species, such as *C. malorum*, *C. luteo-olivacea*, and *C. fastigiata* have also been isolated in extreme environments in Antarctica, causing soft decay of wood huts [25,27,28]. Thus this genus may also be responsible for the deterioration of wooden lacquerware.

Another fungus, *P. chrysogenum*, is known to be a deteriorative agent and was both isolated from ancient documents and the air of indoor environments [12,29,30]. In addition, sequences of the cellulase and hemicellulase producing strain, *P. chrysogenum*, were detected. Therefore, this fungus is a threat to the wooden lacquerware.

4.2. Degradation of Lignocellulose by Fungi

Lignocellulose consists of lignin, carbohydrates, pectin, proteins, ash, salts, and minerals. Lignocellulose is highly difficult to degrade by microorganisms. Fungi are mainly responsible for lignocellulose degradation because they have two types of efficient enzymatic systems [31]. A hydrolytic system to degrade polysaccharide by producing hydrolases and an oxidative and extracellular ligninolytic system to open phenyl rings and degrade lignin.

Fusarium is an important type of parasitic pathogenic fungi. Cell wall degrading enzymes (CWDEs) are one of the main pathogenic ways by which *Fusarium* invades and infects the host [32]. According to J.Chen, CWDEs secreted by *Fusarium* mainly include pectinase, cellulase, hemicellulase, protease, amylase, and phospholipase [33]. *Penicillium* is known to be able to secrete lignocellulolytic enzymes, such as hemicellulase, cellulases, manganese peroxidase, lignin peroxidase, and laccase [34]. Therefore, *Fusarium* and *Penicillium* may be responsible for the microbial degradation of lacquerware.

According to the CMCase activity test, notably, the activity of CMCase increased to some extent under acidity and iron ions-containing conditions. Studies have shown that marine effluent wood relics usually deposit ferro-iron compounds, which are easily oxidized in effluent, causing degradation of organic matter [35]. Since the wood lacquers were effluent in the ocean, strains isolated from their surface may be adaptable to this environment. Therefore, attention must be paid to control the adverse effects of iron sulfide on wood lacquers for subsequent protection. Considering the further protection of the lacquer, it should undergo de-ironing, desalination, reinforcement, and other treatments. Lacquer serves in marine waterlogged cultural relics, it has a great degree of a problem with acidification. Therefore, during the protection process, the pH value and iron ion concentration in the wood will change. We need to examine the enzyme activity of CMCase at different pH values and iron ion concentrations of these two main fungi. In addition, the storage temperature of the lacquer is also an important factor to be considered in further protection. Therefore, we tested the enzyme activity of CMCase of two fungi at different temperatures.

4.3. Biocide Discussion

The results of biocide products provide preliminary knowledge about the susceptibility of lacquerware fungi to four commercial biocides. According to these data, one may think that a biocide application based on an active compound like isothiazolinone could be a useful method to control fungal biodeterioration, and commercial biocide products based on isothiazolinone have been widely used on paper objects and stone cultural heritage monuments [36–39]. However, there are few studies on their long-term efficacy, and the potential recolonization after biocide application has not been monitored [36]. In the worst case, microbial communities may develop resistance to these biocides, and might become even more harmful to the objects [40,41]. In addition, under the selective pressure of biocides, microorganisms previously absent may replace the original microbial communities, leading to new microbial outbreaks [36]. The outbreak of the fungi *F. solani* species complex of Lascaux Cave in France is a precedent. Biocides, such as benzalkonium chloride (BC), were used to control this outbreak between 2001 and 2004 [21]. Four months after the initial application, some black stains progressively invaded the cave, marking a new outbreak. Besides, years of BC application have selected resistance populations, including a mix of *Ralstonia* and *Pseudomonas*, as well as free-living amoebae [20,21,42]. As suggested by Martin-Sanchez et al., it is necessary to weigh the pros and cons of using biocides in each specific environment. Furthermore, any biocide treatment should be carefully designed even if it may be difficult, which includes not also laboratory assays like this study, but also field assays in the real environment [21].

We believe that the choice of biocides is also very important. BC has been used as an antifungal agent against fungi isolated from cultural heritage. The biological activity of BC is ascribed to its quaternary ammonium group [43]. However, microorganisms tend to develop resistance to quaternary ammonium compounds (QACs), resulting in microorganisms colonizing surfaces treated with QACs for a long period [44]. It has been suggested that use of nitrogen-containing biocides should be avoided

because they enable several groups of micro- and macro-organisms to use it as a nitrogen source favoring recolonization [45]. The main active ingredients of the biocides we used is isothiazolinone, which is relatively environmentally friendly and efficient. A study isolated some fungi species from the stuccos of the vault of a religious building in Torino (NW-Italy), and evaluated the sensitivity of the fungi species to widely used biocidal products [46]. As a result BC, in the low range of recommended concentrations (0.25%) was demonstrated ineffective against most of the tested fungal strains, while isothiazolinone-containing biocides displayed the best effectiveness against all the strains. Based on the above considerations and our experimental results, we focused on the effect of isothiazolinone-containing biocides. However, its effects on wooden lacquerware still requires evaluation.

In the museum, the fungal contamination of lacquerware is low. To avoid breaking the relatively stable current balance, commercial biocides should not be used at the present stage. For the conservation of these wood lacquers, they should be preserved at low temperature to control fungal deterioration. Moreover, the stored water could be replaced with sterile water to avoid fungal colonization. Alternatively, continuous monitoring of the fungal community is also necessary. Once there is a significant fungal outbreak, physical, not chemical cleaning methods should be considered first, such as careful mechanical removal of fungal biomass and so on.

5. Conclusions

We studied the potential fungal biodeterioration of waterlogged wooden lacquerware from the Nanhai No. 1 shipwreck by conventional cultivation methods and modern molecular biotechnology. The high-throughput sequencing of the water samples storing the lacquerware revealed that *Fusarium* was the dominant fungus in 2017. This is the first time that *Fusarium* species have been reported to lead a putative microbial deterioration of wooden lacquerware. Considering the characteristics of *Fusarium*, we suggest that *Fusarium* might have been brought from the outside environment by visitors or conservation-restorers. Even though the corrosion and damage of the lacquerware was not very serious at a relatively stable state, the potential biodeterioration was continuous. The fungal isolates, *F. solani* NK-NH1 and *P. chrysogenum* NK-NH3, may not be directly responsible for the potential biodeterioration, while the CMCase activity test showed that they could be the potential deteriorative agents of the wood and must be regarded as a threat to the lacquerware. The results also indicated that attention must be paid to control the adverse effects of iron sulfide on wooden lacquerware in subsequent protection. The biocide susceptibility assay indicated that isothiazolinones can inhibit fungal isolate growth effectively. However, it is not recommended to use these biocides immediately without considering the actual protection of the lacquerware. In future research, the microbial community and environmental parameters in the museum should be monitored to assess the changes in the community and to detect potential microbial outbreaks at any time.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/11/3797/s1, Table S1: Fungal clone library results of NHI58 (2016.4); Table S2: High throughput sequencing results of NHI58 (2017.6); Table S3: High throughput sequencing results of NHI935 (2017.6).

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