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EXPERIMENTAL ARTICLES

Fungi in Sediments of the Sea of Japan and Their Biologically Active Metabolites

Yu. V. Khudyakova*, M. V. Pivkin*, T. A. Kuznetsova*, and V. I. Svetashev**

**Pacific Institute of Bioorganic Chemistry, Far Eastern Division, Russian Academy of Sciences, Vladivostok, 690022 Russia*

***Institute of Marine Biology, Far Eastern Division, Russian Academy of Sciences, Vladivostok, 690022 Russia*

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Abstract—The most abundant marine fungi encountered in various regions of the Sea of Japan belong to the genera *Penicillium, Aspergillus, Wardomyces, Trichoderma, Chrysosporium*, and *Chaetomium*. Facultative marine fungi of the genera *Scytalidium, Verticillium*, and *Oidiodendron* and obligate marine fungi of the genus *Dendryphiella* are much less abundant. The composition of marine sediments and the anthropogenic load on them were found to influence the abundance and species diversity of fungi, as well as the occurrence of fungal strains producing hemolytically active substances. The biodiversity of mycobiota and the abundance of hemotoxin-producing fungi in marine sediments may be used to evaluate the anthropogenic load on marine biocenoses. Hemolytic compounds were produced by 57% of the fungi isolated from marine sediments. The hemolytic activity of *Chaetomium spiculipilium* was revealed in the fraction of the culture liquid containing extracellular fatty acids and pigments. The fatty acid composition of this marine fungus was determined.

Key words: facultative and obligate marine fungi, *Chaetomium spiculipilium*, fatty acids, hemolytic activity

Marine fungi are the least studied group of marine organisms. There are only few reports concerning the investigation of mycobiota in marine sediments [1–4]. In the last decade, marine fungi became the subject of intense investigation as producers of biologically active compounds [5–7], including those which are of substantial ecological importance [4]. Until now, marine fungi of the Sea of Japan have not been virtually studied.

The aim of this work was to study the species diversity of fungi in marine sediments and to evaluate the effect of ecological factors, such as the composition of sediments and anthropogenic load on them, on the fungal diversity and the abundance of strains producing hemolytically active substances. We also attempted to isolate the hemolytically active compounds produced by the *Chaetomium spiculipilium* L.M. Ames strain and to analyze them by ^{1}H NMR, UV spectroscopy, and gas-liquid chromatography.

MATERIALS AND METHODS

Eleven samples of marine sediments of different compositions (Table 1) were withdrawn from depths of

Table 1. Effect of the type of marine sediments on the abundance and species diversity of fungi

	Parameter				
marine sediment	number of species	total number of isolates	number of isolates of the most abundant species	Shannon diversity index	mean alignment error
Mud	44	167	22	2.45 ± 0.14	0.88
Sandy mud	34	106	15	2.41 ± 3.09	1.05
Sand	22	126	28	1.88 ± 0.73	0.69
Sandy pebble	3	4	2	1.038 ± 0.03	0.95
Pebble	9	31	21	1.46 ± 0.06	0.64
Coquina	3	9	7	0.68 ± 0.07	0.62

Note: Confidence limits are given for a significance level of 0.01%; $P > 0.01$.

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Table 2. Effect of anthropogenic load on the abundance and species diversity of fungi in marine sediments

	Parameter				
area	number of species	total number of isolates	number of isolates of the most abundant species	Shannon diversity index	mean alignment error
Amur Bay	56	224	28	1.65 ± 0.95	0.79
Troitsa Bay	25	112	21	1.91 ± 0.70	0.76
Tumannyi Cape	13	48	15	0.99 ± 0.99	0.39

Note: Confidence limits are given for a significance level of 0.01%; $P > 0.01$.

Table 3. Effect of the type of marine sediments on the abundance and species diversity of hemolytically active fungi

	Parameter						
marine sediment	number of species	total number of isolates	number of isolates of the most abundant species	percent of hemolytically active strains	degree of dominance of hemolytically active strains	Shannon diversity index	mean alignment error
Mud	21	78	22	46.71	0.47	1.31 ± 1.23	0.57
Sandy mud	1	2	2	1.46	0.02	0.16 ± 0.48	0.23
Coquina	2	7	7	77.78	0.78	0.38 ± 0.02	0.54
Sandy pebble	2	2	1	50.00	0.5	0.69 ± 0.12	0.99
Sand	4	7	4	5.56	0.06	0.96 ± 0.06	0.45
Pebble	0	0	0	0	0	0	0

Note: Confidence limits are given for a significance level of 0.01%; $P > 0.01$.

Table 4. Effect of anthropogenic load on the biodiversity and abundance of hemolytically active strains

	Parameter						
marine sediment	number of species	total number of isolates	number of isolates of the most abundant species	percent of hemolytically active strains	degree of dominance of hemolytically active strains	Shannon diversity index	mean alignment error
Amur Bay	24	86	22	38.39	0.38	0.72 ± 1.84	0.38
Troitsa Ba	2	5	4	4.46	0.05	0.48 ± 0.48	0.44
Tumannyi Cape	2	3	2	6.25	0.06	0.32 ± 0.32	0.46

Note: Confidence limits are given for a significance level of 0.01%; $P > 0.01$.

4 to 79 m in three regions of the Sea of Japan in Russian terrestrial and adjacent waters: (1) Amur Bay, where the highest anthropogenic impact on marine ecosystems from the nearby city of Vladivostok was observed; (2) Troitsa Bay, on which Zarubino and Andreevka settlements are situated, where anthropogenic impact was considerably less than in Amur Bay; (3) the shelf area near Tumannyi Cape, 132 km away from populated areas, where anthropogenic load was the least.

Marine sediment samples were withdrawn by dragging or by divers. Fungi were isolated from plates inoculated with either undiluted or appropriately diluted samples [8]. The species composition of the fungi isolated in pure cultures [9] was determined with the use of the Shannon diversity index H′ [10]. The results were subjected to the variance analysis [11]. Hemolytically active fungal strains were detected on blood agar with human erythrocytes [12]. The hemolytic activity of chloroform–methanol (2 : 1) extracts or of individual compounds isolated from them was determined using the nonheparinized blood of nonbreeding white mice as described by Kalinin *et al.* [12]. The hemolytic coeffi-

Table 5. Content of fatty acids (%) in the hemolytically active fractions and in the mycelium of *C. spiculipilium*

Fatty acid	Fraction 1	Fraction 2	Mycelium
12 : 0	1.1	1.8	0.9
13 : 0	0.4	0.4	0.4
14 : 0-*i*	0.4	0.4	0.3
14 : 0	6.6	6.4	3.8
14 : 1	0.9	1.3	0.9
15 : 0-*i*	0.3	0.3	0.3
15 : 0-*a*	1.3	1.2	0.5
15 : 0	3.2	2.9	1.6
15 : 1	0.4	0.5	0.4
16 : 0-*i*	0.6	0.5	0.3
16 : 0	60.6	54.9	24.2
16 : 1 (*n*-9)	0.0	3.1	0.0
16 : 1 (*n*-7)	2.7	2.0	5.5
17 : 0-*i*	0.2	0.3	0.8
17 : 0-*a*	0.4	0.5	0.6
Phytanic-*i*	0.1	0.3	0.3
17 : 0	1.4	1.2	1.2
17 : 1	0.2	0.4	0.4
18 : 0-*i*	0.1	0.1	0.5
18 : 0	12.6	11.6	17.2
18 : 1 (*n*-9)	0.5	3.0	12.2
18 : 1 (*n*-7)	0.3	0.4	2.8
19 : 0-*i*	0.0	0.2	0.0
18 : 2 (*n*-6)	0.1	0.6	13.5
19 : 0	0.4	0.4	0.6
19 : 1	0.1	0.2	0.9
18 : 3 (*n*-3)	0.0	0.0	0.4
20 : 0	1.0	1.0	0.7
20 : 1 (*n*-11)	0.0	0.1	0.8
20 : 1 (*n*-9)	0.0	0.0	0.4
20 : 1 (*n*-7)	0.0	0.0	0.6
20 : 2 (*n*-6)	0.7	1.3	4.5
21 : 0	0.2	0.2	0.0
20 : 4 (*n*-6)	0.0	0.0	1.6
20 : 5 (*n*-3)	0.0	0.0	0.5
22 : 0	1.1	0.8	0.0
24 : 0	1.4	1.1	0.0
24 : 1	0.6	0.6	0.0

cient, HC_{50}, was defined as the minimal concentration of a substance inducing the 50% hemolysis of erythrocytes in 60 min at 37°C.

In order to obtain chloroform–methanol extracts for subsequent analysis, fungi were grown in a modified SUC-1 medium containing (g/l seawater) sucrose, 80; yeast extract, 1; and maize meal, 50 [13].Cultivation was performed in a UVMT-12-250 temperature-controlled setup on a shaker (170 rpm) at 20°C for 7 days. The biomass and the culture liquid were extracted with a chloroform–methanol (2 : 1) mixture according to the method of Bligh and Dyer [14].

The chloroform–methanol extract (276 mg) was dried under a vacuum and separated by Flash-chromatography on a KSK Silica Gel in 100 ml of the following solvent systems: benzene; benzene–chloroform (1 : 1); chloroform; chloroform–ethanol (1 : 1); ethanol. Preparative thin-layer chromatography was performed in a petroleum ether–diethyl ether–glacial acetic acid (9 : 1 : 0.1) system. Adsorption column chromatography was carried out in the following solvent systems: benzene–chloroform (1 : 1); chloroform–ethanol (1 : 1); ethanol. Chromatography on Sephadex LH-20 was performed in the chloroform–ethanol (1 : 1) system. Thin-layer chromatography (TLC) was carried out in chloroform–methanol (2 : 1) and chloroform–benzene (5 : 1) systems. The absorption spectra of the ethanol solutions of pigments were recorded on a UV-VIS M 40 Specord spectrophotometer (Germany). Fatty acid methyl esters were analyzed on a GC-9A gas-liquid chromatograph equipped with a flame ionization detector and a Supelcowax 10 (Supelco) capillary column (30 m × 0.25 mm). The carrier gas was helium. The chromatograph was interfaced to a C-R 317 Chromatopak integrator (Shimadzu).

^1H NMR spectra were recorded on a WM-250 Bruker spectrometer (250 MHz) with tetramethylsilane (TMS) as the internal standard.

RESULTS AND DISCUSSION

The 442 fungal strains isolated from 11 samples of marine sediments taken from different regions of the Sea of Japan were found to belong to 79 species of 24 genera. The majority of strains (98%) were facultative marine fungi of the genera *Penicillium, Aspergillus, Wardomyces, Trichoderma, Chrysosporium*, and *Chaetomium.* Some fungal isolates belonged to the genera *Scytalidium, Verticillium*, and *Oidiodendron.* About 2% of the isolates were obligate marine fungi of the genus *Dendryphiella.* The maximum level of fungal diversity was revealed in the marine sediment samples taken from Amur Bay, which suffers from the highest anthropogenic load (Table 2). One of the isolates obtained from these samples, *Aspergillus fumigatus* Fresen. var. *ellipticus* Raper et Fennell, is a human pathogen [15]. Marine sediments from the Tumannyi Cape region, where anthropogenic load was minimum, were characterized by the lowest level of fungal diversity (Table 2).

About 57% of the fungal strains isolated were found to be able to synthesize hemolytic compounds. The percentage of hemolytically active isolates was maximum among representatives of the genera *Chaetomium, Pen-*

icillium, Aspergillus, and *Trichoderma* (Tables 3 and 4). Marine sediments with intense anthropogenic load were dominated by hemolytically active strains, while hemotoxin-producing strains in ecologically sound regions (Troitsa Bay and Tumannyi Cape) were much less abundant.

Species diversity and the abundance of hemolytically active strains were also dependent on the composition of marine sediments. In particular, fungal strains isolated from pebble and coquina were not numerous, and no hemolytically active strains were found on pebble (Tables 1 and 3).

The fungus *C. spiculipilium*, exhibiting the maximum production of hemotoxins, was chosen for further studies. Hemolytically active substances were detected in a crude preparation obtained from the chloroform–methanol extract of the culture liquid of this strain by flash-chromatography on silica gel in a chloroform–ethanol (1 : 1) solvent system. This preparation was fractionated by preparative TLC into two fractions, which were further purified by chromatography on silica gel and Sephadex LH-20. One of these fractions (fraction 1) contained lipophilic, chloroform-soluble, substances, and the other (fraction 2) contained polar, methanol-soluble, substances. Both fractions taken at a concentration of 0.01 mg/ml showed similar hemolytic activities. Their analysis by ^{1}H NMR and gas-liquid chromatography (GLC) showed that they both mainly consisted of fatty acids with a small amount of pigments (less than 10%). The absorption spectra of these fractions in ethanol had maxima at 390, 406, and 432 nm. The fractions differed in their fatty acid profiles (Table 5): the content of 12 : 0, 16 : 1 (*n*-9), 18 : 1 (*n*-9), 18 : 2 (*n*-6), 19 : 1, and 20 : 2 (*n*-6) acids in fraction 2 was, respectively, 1.8, 3.0, 6.0, 6.0, 2.0, and 1.8 times higher than in fraction 1. The fatty acids of both fractions were dominated by 16 : 0 and 18 : 0 acids, whereas the amount of each of the other acids (13 : 0, 15 : 1, 17 : 1, 19 : 0, 19 : 1, 21 : 0, and 24 : 1) did not exceed 1% of the total. The comparison of the fatty acid compositions of the mycelium and the two fractions showed that 16 : 1 (*n*-9), 21 : 0, 22 : 0, 24 : 0, and 24 : 1 acids were not accumulated in the mycelium but were completely excreted into the medium (Table 5). This suggests that these fatty acids are of the most ecological importance.

Thus, the abundance and species diversity of fungi in marine sediments depend on their type and the anthropogenic load on them. The species composition of marine mycobiota, particularly the proportion of hemolytically active fungal species, may serve as an index of anthropogenic load on marine ecosystems. The hemolytic activity of the fungus *C. spiculipilium* was found to be associated with the production of extracellular fatty acids.

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