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Antibacterial activities and characteristics of some marine fungi strains isolated from Co To beach, Quang Ninh province

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ABSTRACT

Resistance to pathogenic bacteria may lead to serious health problems. Scientists found that discovering novel antimicrobial compounds is possible by exploring rarely investigated environments. Therefore, this work focused on isolating and identifying some fungal strains collected from the Co To sea. We tested whether such strains can produce compounds with vital activities, including antibacterial and antifungal. The antimicrobial activity of the marine fungi crude extracts was performed by the Bioassay method in a 96-well tray. The minimum inhibitory concentration (MIC) test results showed that 22 strains of marine fungi from samples with different geographic coordinates and 20/22 strains had antibacterial activity against at least two strains of microorganisms tested. The biological evaluation revealed that strains M257 and M238 inhibited 4 to 5 tested strains with MIC values equal to or lower than positive controls. Using a BLAST analysis in the GenBank database, morphological comparisons of the two selected candidate strains with similar known species and phylogenetic analyses were conducted on the 18S rRNA gene regions, and maximum likelihood revealed that M257 belongs to *Talaromyces* genus, and M238 belongs to *Aspergillus penicillioides*. The isolates were analyzed in a phylogenetic tree based on MegaX software.

Keywords: Fungus, antimicrobial activity, bioassay, MIC, marine fungi, 18S rRNA.

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INTRODUCTION

Marine-derived fungi are essential in cycling of dissolved organic matter in marine environments. They have been found in every corner of the oceans, from open water bodies of marine macro- and microorganisms: alga, sponges, invertebrates, sediments etc. [1]. Marine fungi grow well on or inside other living things like algae, crustaceans, mollusks, corals, and sponges, even at the ocean's depths.

In the past, marine fungi received little attention. Recently, the use of culture-independent methods for studying environmental fungi and next-generation sequencing methods has revealed a diversity of fungi [2]. According to the latest report on www.marinefungi.org until 15/7/2022, there are 1,857 marine fungi species distributed in 769 genera, 226 families, 88 orders, 22 classes, and seven phyla into *Aphelidiomycota, Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Mortierellomycota, and Mucoromycota.* In 1945, Marine fungi began to attract attention when a class of β-lactam antibiotics isolated from the *Cephalosporium chrysogenum* [3]. The secondary metabolites isolated from marine fungi displayed pharmaceutically significant bioactivities, including antibiotic, antiviral, antioxidant, and anti-inflammatory. These metabolites can be used in medical, pharmaceutical, agriculture, and cosmetics [4, 5]. On the other hand, marine fungi can express some catalytically active enzymes (e.g., catalases, laccases, and peroxidases), so they also support biological treatment in the decomposition of organic compounds [6].

Although many complex and biologically active secondary compounds have been extracted from marine fungi, most remain unexplored. The functional roles of most fungi in marine ecosystems are unknown, and the discovery of new bioactive secondary metabolites from marine fungi is increasing. Fungi were the first organisms to degrade lignin and convert it into carbon dioxide and water, leading to global carbon redistribution [1]. Biological research on marine fungi has allowed the identification of several metabolites with antimicrobial activity, which are active against multidrug-resistant strains [7]. Many other bioactive compounds have continued to be isolated from marine fungi: More than 3,500 marine fungal secondary metabolites have been reported [8]. Since then, only broad-spectrum cephalosporin C can be traced as a marine fungi-derived drug. Cephalosporins were isolated from *Acremonium chrysogenum* obtained in a sample collected in sewage water from the Sardinian coast in 1940, and the only one compound: the Plinabulin (diketopiperazine class) isolated from a marine fungus (*Aspergillus* sp.) that is in phases of clinical trials by Beyond Spring Pharmaceuticals [9, 10].

Compared with the extracting medicinal compounds from plant or other macro-organisms sources, industrial microbiology is often achieved for large-scale fermentation processes to synthesize products by optimizing culture conditions. So, this work is necessary to build a sustainable ocean economy to develop alternative drugs in an eco-friendly manner.

In Vietnam, Ngo et al., (2023) reported that the fungi derived from marine samples collected in the research journey of the Akademik Oparin vessel in the North Sea of Vietnam had significant antimicrobial. The antimicrobial screening results showed that 25 strains of marine fungi exhibited inhibitory activity against at least four pathogens tested [11].

MATERIALS AND METHODS

Sample collection and processing

The marine samples were collected by SCUBA at Co To beach, Quang Ninh province, in Northeastern Vietnam. The specimens were collected in cleaned, sanitized, and autoclaved bottles with 30% glycerol in an icebox during transported to the laboratory of Institute of Marine Biochemistry for further analysis. In the Lab., the samples were stored at 4^{o}C until used (not longer than 3 weeks).

Fungal isolation

The culture media used for the isolation and cultivation of marine microorganisms were

SWA, ISP2, PDA, MEA, and Czapek. The pH of the culture medium was adjusted to 7.0. Samples (0.5 g weight or smear pattern) was crushed and diluted ten times in sterile water, homogenized by vortexing for 5 minutes. Aliquots of 50 µL were spread on isolation media. The petri plates were incubated at 25^{o}C for 7 days. All these isolated strains were observed for morphology during culture on agar plates, such as pigmentation and texture. Colony morphology is a commonly used tool for initial classification. Fungi emerge as textured colonies that are powdery or fuzzy. Fungi hyphae often grow over the substratum, penetrate surface, or produce aerial mycelium. Colonies from the isolation plates were purified through several rounds of transfer to Czapek culture media [9, 10].

Fermentation, production of crude extracts and antibacterial bioassay

Isolates were inoculated in 1,000 mL flasks containing 500 mL Czapek medium (Instant ocean: 30 g/L; Sucrose: 30 g/L; Dipotassium phosphate: 1 g/L; Magnesium sulfate: 0.5 g/L; Potassium chloride: 0.5 g/L; Ferrous sulfate: 0.01 g/L; Agar: 15 g/L; Final pH (at 25^{o}C) 7.3 ± 0 .2)), and incubated for 7 days at 25^{o}C, and 150 rpm. The culture was filtered and extracted with ethyl acetate (5 times). These ethyl acetate extracts were purified by column chromatography on silica gel, eluted with an n-hexane/acetone gradient, and washed with CH_2Cl_2/MeOH to give fractions. The extracts were evaporated under reduced pressure to yield crude extracts. The antibacterial activity of those crude extracts was evaluated against Gram-negative bacteria (*Escherichia coli* ATCC25922 (E.C), *Pseudomonas aeruginosa* ATCC27853 (P.A), *Salmonella enterica* ATCC13076 (S.A)), and three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212 (E.F), *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579 (B.C)) and one fungus strain *Candida albicans* ATCC10231 (C.A)).

The test bacteria were cultivated for 12 hours at 37^{o}C and diluted according to the McFarland standard 0.5 scales. The antibacterial bioassay was carried out in flat-bottom 96-well microliter plates. This dilution yielded a starting inoculum of approximately 2×105 CFU/mL. The stock extracts were diluted in DMSO (Dimethyl sulfoxide) at a decreasing concentration range: 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, and 2 µg/mL with the number of experiments. Streptomycin (Sigma) and cycloheximide (Merck) were positive control for bacteria and fungi.

Antimicrobial activities are often reported as MIC (Minimum Inhibition Concentration) values, which typically denote the lowest concentration of test compound, which inhibits growth (97–100%), reproducible and yielding values expressed in µg/mL. MIC denotes the lowest test compound concentration that completely prevents the visible growth of a microorganism. MIC values were determined accurately based on BioTeck spectrophotometer and Raw Data Software turbidity measurements. The MIC of extracts against the pathogens was performed as described by [12, 13].

Taxonomic characterization of pure isolated strains

The identification and classification of a strain is based on a combination of genotypic and phenotypic data. Morphological characterization of pure isolated strains was observed according to spore-producing, diffusible pigments, melanin pigment formation, and hyphae and conidia in culture media. Sequences of 18S rRNA were used for taxonomical identification of the fungal strains. The PCR mixture (50 µL) contained 2.0 µL fungi DNA, 25 µL Master Mix 2X, 19 µL of H_2O, 2.0 µL of 10 pmol/µL for each primer NS3F (5'-GCAAGTCTGGTGCCAGCAGC C-3') and NS8R (5'-TCCGCAGGTTCACCTA CGGA-3'). The mixtures were amplified using a protocol of 94^{o}C for 3 minutes, 30 cycles at 94^{o}C for 1 minute, primer annealing at 58^{o}C for 1 minute, and 72^{o}C for 1 min 20 secs, extension at 72^{o}C for 8 minutes, eventually, the reaction mixture was cooled to 4^{o}C in an automated MJ Thermal cycler (Bio-Rad). An aliquot of 5 µL of each amplified product was electrophoresed in 1.0% agarose gel. The gel was stained with

ethidium bromide for 10 minutes and photographed under UV light illumination [12, 13].

The 18S rRNA gene sequencing products were analyzed by capillary electrophoresis on DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were analyzed by BioEdit version 7.2 and compared with known 18S rRNA gene sequences in the GenBank database by multiple sequence alignment using the CLUSTAL W program. Evolutionary analysis was performed using the Maximum Likelihood method, whereas the evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model [14].

RESULTS

The research team commits that using samples in this study complies with international guidelines and considers the conservation of marine resources.

Sample collection and processing

Marine samples were collected with SCUBA diving at a depth of 4–12 m under sea level, and the water temperature ranged from 26–30^{o}C in different geographic coordinates in Co To beach (Table 1).

Marine fungi isolation

Observations of colony feature such as color, size, shape, hyphae, conidia, conidiophores, and arrangement of spores visible by the naked eye were used classically to identify fungi. Colonies showed powder consistency and stuck firmly to agar surface from the isolation plates were purified through several rounds of transfer to suitable culture media. A total of 22 strains were isolated from the marine samples. The numbers of samples and isolates in each sample are presented in Table 1.

All potential colonies were purified and stored in glycerol stock in -80^{o}C.

Table 1. Isolated strains from Co To sea

No.	Samples	Organisms	Sampling geographic coordinates/Water depth	Isolated agar medium
1	M226	Sponge	21^{o}0’14”N-107^{o}46’22”E/4 m	SWA
2	M228	Mollusca	21^{o}5’11”N-107^{o}50’57”E/4 m	SWA
3	M229	Sponge	21^{o}0’14”N-107^{o}46’22”E/4 m	ISP2
4	M231	Sponge	21^{o}5’11”N-107^{o}50’57”E/4 m	ISP2
5	M232	Mollusca	21^{o}5’11”N-107^{o}50’57”E/4 m	ISP2
6	M233	Seaweed	20^{o}59’33”N-107^{o}46’33”E/12 m	CZ
7	M235	Sponge	21^{o}0’14”N-107^{o}46’22”E/4 m	PDA
8	M236	Soft coral	21^{o}0’36”N-107^{o}40’50”E/12 m	CZ
9	M237	Sponge	20^{o}59’33”N-107^{o}46’33”E/12 m	MEA
10	M238	Soft coral	20^{o}59’691”N-107^{o}46’936”E/5.5 m	ISP2
11	M239	Mollusca	2^{o}5’11”N-107^{o}50’57”E/4 m	PDA
12	M240	Sponge	21^{o}5’11”N-107^{o}50’57”E/4 m	CZ
13	M241	Animal	20^{o}59’33”N-107^{o}46’33”E/12 m	MEA
14	M242	Sponge	20^{o}59’33”N-107^{o}46’33”E/12 m	PDA
15	M244	Seaweed	2^{o}0’36”N-107^{o}40’50”E/12 m	ISP2
16	M245	Scallop	21^{o}5’11”N-107^{o}50’57”E/4 m	ISP2
17	M246	Animal	20^{o}59’691”N-107^{o}46’936”E/5.5 m	MEA
18	M249b	Shiny Red Scallop	21^{o}0’830”N-107^{o}48’512”E/6 m	PDA
19	M257	Scallop	21^{o}2’387”N-107^{o}45’661”E/4.5 m	SWA
20	M259	Crustacea	21^{o}2’387”N-107^{o}45’661”E/4.5 m	SWA
21	M262	Seawater	21^{o}1’904”N-107^{o}46’440”E/4.5 m	MEA
22	M265	Polychaete Worms	21^{o}1’904’N-107^{o}46’440”E/4.5 m	PDA

Antibacterial bioassay

96-well microtiter plates were covered using parafilm and incubated at 37°C in ambient air. The final MIC was read after 24 hours. The turbidity value was measured to determine the MIC. Experiments were performed in triplicate and repeated three times with similar results.

A total of 22 fungi isolates were tested for activity against 7 pathogenic microorganisms. Among all the marine fungi crude extracts tested, MIC was determined for 22 fungi isolates against E. faecalis. 20/22 isolates showed antibacterial activities against at least 2 strains of tested microorganisms. In which, isolates M238 and M257 were highly active. Isolate M238 exhibited antagonistic activity against 4 tested microorganisms, especially the ability to resist the group of microorganisms that cause intestinal diseases with MIC values equal to or lower than the positive control. M257 inhibited 5 tested microorganisms, including gram-positive, gram-negative and yeast. The detail results are presented in Table 2.

Table 2. MIC values of marine fungi crude extracts against tested microorganisms

No.	Samples	Antimicrobial activity of crude ethyl acetate extracts of 22 fungi strains						
		Gram-positive			Gram-negative			Yeast
		E.f	*S.a*	*B.c*	*E.c*	*P.a*	*S.e*	*C.a*
		MIC (µg/mL)						
1	M226	64	-	-	-	-	-	128
2	M228	32	-	-	-	-	-	256
3	M229	16	-	-	-	-	256	128
4	M231	128	-	-	-	-	-	16
5	M232	64	-	-	-	-	-	32
6	M233	32	-	-	-	256	-	256
7	M235	64	-	-	-	-	-	32
8	M236	16	-	-	-	-	-	32
9	M237	128	-	256	-	-	-	16
10	M238	32	32	8	-	-	-	32
11	M239	16	-	-	-	-	-	8
12	M240	32	-	-	-	-	-	256
13	M241	16	-	-	-	-	256	256
14	M242	32	-	-	-	-	-	-
15	M244	32	-	-	-	-	-	32
16	M245	128	-	-	-	-	-	-
17	M246	64	-	-	-	-	-	32
18	M249	32	-	-	-	-	-	256
19	M257	256	-	256	-	256	256	128
20	M259	64	-	-	-	-	-	256
21	M262	128	-	-	-	-	-	16
22	M265	64	-	-	-	-	-	32
Streptomycin		256	256	128	32	256	128	-
Cyclohexamide		-	-	-	-	-	-	32

Notes: Positive control: Streptomycin, Cycloheximide; (-): Inactive.

From the results shown in Table 2, two strains, M257 and M238, combined with the largest broad-spectrum with good control efficacy against tested organisms, were selected for further characterization and analysis.

Taxonomic characterization of pure isolated strains

The two mentioned strains grew slowly on the Czapek medium after 3–10 incubation days at 28°C to observe colony morphology, mycelium's color, and substrate mycelium's color. The shape and size of mycelium, spore-generating organs, conidia, and sporangia were observed using a Japanese Nikon ECLIPSE 80i optical microscope.

Figure 1. Colony morphological characteristics of the strains M257. (A) Colonies grown on Czapek medium for 7 days at 25°C; (B) Conidiophores; (C) Conidia, electron microscope magnified 1,000 times

M257 colonies moderately slow growing on Czapek medium at 25°C. Colonies nearly circular, protuberant in centers; margins wide 1.8–2.2 cm after 10 days, light yellow to yellowish green color, soluble pigments and exudates absent. Its reverse side is orange-white to light orange.

The sporangiophore is produced from the surface mycelium or the basal mycelium, thin-walled, smooth, measures 80–330 µm × 2.5–3.5 µm; metulae divergent, 4–8 per stipe/branch, unequal in length, vesiculate, 10–15 × 2.2–3.2 µm; spores globose to ellipsoid or lemon seed, 3.0–4.0 × 2.5–3.0 µm, smooth, forming short chains; conidia forming chains, gray-white to pale green, globose to sub-globose, somewhat ellipsoidal, 9–11 × 8–10 µm; sporangium usually ellipsoid, light yellow color, 3.8–5.0 × 2.2–3.0 µm (Fig. 1).

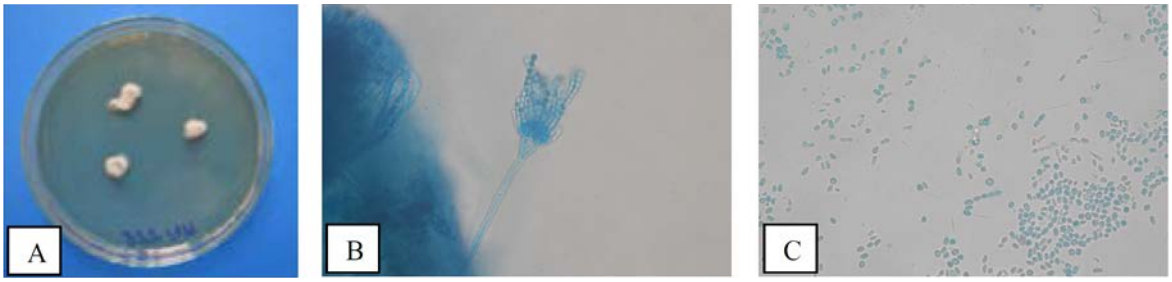

Figure 2. Colony morphological characteristics of the strains M238. (A) Colonies grown on Czapek medium for 7 days at 25°C; (B) Conidiophores; (C) Conidia, electron microscope magnified 1000 times

Colonial and microscopic morphology of M238 strain: The colonies of M238 are slow growing on Czapek medium at room temperature, filamentous, cottony in rough texture, and reach 0.5–1.0 cm in 10 days. The colonies are initially pale green to greyish green; when spores are born, they are dark yellow-green; the reverse side is colorless to dark

greenish brown. Sporangiophores are smooth, 150–500 × 3.5–9 µm; sporangium is globose to sub-globose 10–25 µm diameter, colorless or pale green; sporulation moderately dense, 6–9 × 2–3 µm. Young spores are barrel-shaped to elliptical, 3–3.5 × 4–4.5 µm; when old, they are empty to globose, rough spines (Fig. 2).

Then, these isolates will be identified by 18S rRNA gene sequences.

Agarose gel electrophoresis of PCR products from fungal genomes of the two strains showed about 1.3 kb, the approximate size of the 18S rRNA gene (Fig. 3). The 18S rRNA gene sequences of two potential strains were analyzed by BioEdit software version 7.2 and compared with known 18S rRNA gene sequences in the GenBank NCBI database by multiple sequence alignment using the ClustalW program. The result showed that an analysis of the 18S rRNA sequence of strain M238 was closely related to the genus *Aspergillus penicillioides*; this strain showed 18S rRNA gene sequence similarity with values of 99.65% for NG_063229.1 *Aspergillus penicillioides* NRRL4548, isolated by Tamura M and Sugiyama J at the Henan University of Tokyo. The M257 strain is 98.88% homologous to the 18S rRNA sequence of NG_062662.1 *Talaromyces aerugineus* CBS350.66. These identified sequences were registered on GenBank with the NCBI accession number OQ772210, *Talaromyces* sp. strains M257, and OQ772210 for M238.

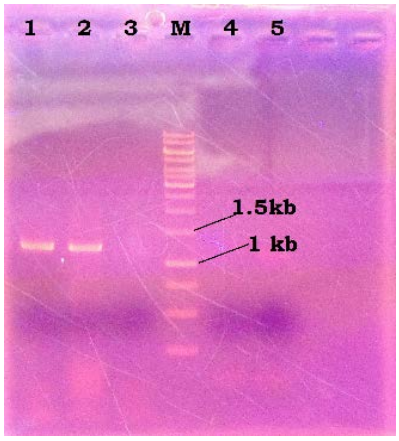

Figure 3. 1% (w/v) agarose gel electrophoresis of PCR products. Strain M238 (Lane 1); Strain M257 (Lane 2), Lane M, 1 kb plus molecular marker 1Kb DNA ladder of Fisher Scientific

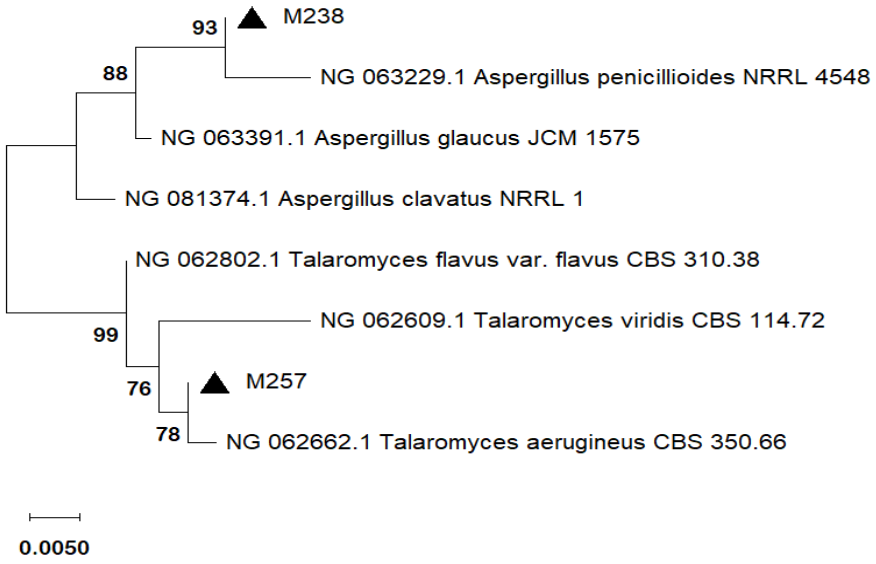

Figure 4. Molecular phylogenetic tree of M257 and M238. The evolutionary history was inferred using MEGA-X. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled datasets. National Center for Biotechnology Information (NCBI) accession numbers are provided in parentheses. The bar length represents 0.005 substitutions per nucleotide site

Evolutionary analysis was performed using the Maximum Likelihood method, whereas the evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model. This analysis involved 8 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 558 positions in the final dataset. Evolutionary analyses were conducted in MEGAX. The phylogenetic tree is shown in Fig. 4.

DISCUSSIONS

Marine fungi grow well on or inside other living things like algae, crustaceans, mollusks, corals, and sponges, even at the ocean's great depths. Positive research results on sea fungi in recent years are promising marine fungi to be a rich source for producing novel antibiotic, anti-tumor, antiviral, antifungal, and anti-yeast, and anti-inflammatory agents [15].

A new sesterterpenoid, Terretonin G, was separated from the cultivation of the marine fungus *Aspergillus* sp. OPMF00272 showed good antimicrobial activity against *S. aureus* FDA209P, *Bacillus subtillis* PCI219, and *Micrococcus luteus* ATCC9341 [16]. 5-Methoxy dihydro sterigmatocystin was obtained from *Aspergillus versicolor* MF359 isolated from the sponge Hymeniacidon perceive; this compound inhibited the growth of *S. aureus* and *B. subtilis* with relatively low MIC values [17]. The fermentation broth of *Aspergillus niger* afforded Itaconic acid derivatives nipyrones A–C, which compounds exhibited antibacterial activity against *S. aureus, B. subtilis*, and *M. tuberculosis* with MIC values in the range of 8–64 μg/mL, respectively [18]. Cottoquinazoline D was isolated from the fermentation broth of *Aspergillus versicolor* LCJ-5-4, which showed inhibitory activity against *C. albicans* with a MIC value of 22.6 μM [19]. Marine fungi *Talaromyces* sp. LF458 produced active talaromycesones A and B against *S. epidermidis* with MIC values of 3.70 and 5.48 μM, respectively [20]. The *Talaromyces* sp. ZH-154 was isolated from mangrove, afforded two small molecules 7-epiaustdiol and 8-O-methylepiaustdiol, and showed a broad spectrum of antifungal activity with MIC values ranging from 6.2 μg/mL to 50.0 μg/mL [21].

CONCLUSIONS

Studying marine fungi to find biologically active compounds is essential in drug development research. In this research, 22 isolates were isolated from the marine samples from Co To sea, Quang Ninh province. The screening results showed that 20/22 isolates inhibited 2 to 5 strains of tested microorganisms. The two potent strains, M257 and M238 (Registration number on GenBank OQ772208 and OQ772210, respectively), had suitable activities against various tested microorganisms, including gram-positive, gram-negative and yeast with MIC values equal to or lower than the positive control. Based on sequencing and phylogenetic analysis of the 18S rRNA gene, M257 belongs to the *Talaromyces* genus, and M238 belongs to *Aspergillus penicillioides*. The results of this study suggested that these isolated strains would provide bioactive compounds, which resistant to pathogenic microorganisms.

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