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ISOLATION, SCREENING ANTIMICROBIAL ACTIVITY AND IDENTIFICATION OF FUNGI FROM MARINE SEDIMENTS OF THE AREA THANH LAN, CO TO, VIETNAM

Le Thi Hong Minh✉, Nguyen Mai Anh, Vu Thi Quyen, Vu Thi Thu Huyen, Doan Thi Mai Huong, Pham Van Cuong, Chau Van Minh

Institute of Marine Biochemitry, Vietnam Academy of Science and Technology

✉ To whom correspondence should be addressed. E-mail: lhminhbk@gmail.com

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SUMMARY

Marine environment is rich in natural product resources, including marine microorganisms, especially fungi which are not only seen as a potential source of highly applicable bioactive substances but also can provide for science new chemical structures. The objective of this study is to isolate and screen fungal strains with antibacterial activity from the marine environment. Twenty five strains of fungi were isolated from marine sediments of Thanh Lan, Co To island and assessed on antibiotic activity against 7 tested microbial strains, including three Gram-negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212, *Stapphylococus aureus* ATCC25923, *Bacillus cereus* ATCC 13245), and the yeast *Candida albicans* ATCC10231. The minimum inhibitory concentration (MIC) against the tested microorganisms was determined for the crude extracts obtained from the culture broths after ethyl acetate extraction and vacuum rotary evaporation. Three strains with the highest antimicrobial activity M26, M30 and M45 were capable of inhibiting 4 - 5 of the 7 tested microorganisms with MIC values from 64 to 256 µg/ml, depending on each tested strain. Morphological and phylogenetic investigations based on 18S rRNA gene sequences of the three selected strains showed that strains M26 and M30 belonged to the genus *Penicillium*, whereas strain M45 belonged to the genus *Neurospora*. The sequences of 18S rRNA gene of three strains M26, M30 and M45 were registered on GenBank database with accession numbers: MH673730, MH673731, MH673732, respectively. Research results showed that marine environment has a great potential in isolation of fungal strains for the search for antibacterial substances as well as other biologically active compounds.

Keywords: *antimicrobial activity, marine sediments, MIC, Fungi, 18S rRNA gene sequences*

INTRODUCTION

Fungi produce a large amount of secondary metabolites, some of those are highly valuable products with pharmaceutical applications such as *penicillins*, a group of structurally related ß-lactam antibiotics isolated from *Penicillium chrysogenum,* griseofulvin from *Penicillium griseofulvum* has been used for human diseases treatment (Khan *et al*., 2014).

The increasing needs for drugs to control new diseases or to fight with drug-resistant strains of microorganisms have been stimulating researchers to search for unconventional new sources of natural bioactive products. Advanced approaches of target-based discovery using bacterial genomics, combinatorial chemistry are time consuming and so far did not lead to an approvable bioactive compound. The traditional, culturing-based approach based on isolation and screening seems to be still very effective (Busti *et al*., 2006).

The ocean which occupies approximately 70% of the Earth's surface is a particularly extreme living environment because of its poor nutrient content and high salinity. As marine microorganisms have been able to adapt to these harsh environmental conditions, they might have opened prospects of detecting new biological activity compounds including anti-tumor, antibacterial, antiviral, antifungal, anti-inflammatory, anti-cancer activity, and enzyme inhibitory (Prakash *et al*., 2005). After 40 years of intensive research, chemistry of marine natural products has become a mature field (Zhang *et*

al., 2010). Many promising compounds with new and complicated structures have been isolated from the oceans and some have been identified as leading preclinical anticancer compounds. Marine derived fungi are rich sources of structurally novel and biologically active secondary metabolites, which have become attractive as important resources for new chemicals in drug discovery (Rejeev *et al*., 2004; Molinski *et al*., 2009).

Herein, we reported on the isolation, taxonomic characterization and antimicrobial activity of the fungus strains isolated from sediment samples collected at Co To island, Thanh Lan of Vietnam.

MATERIALS AND METHODS

Chemicals

Genomic DNA isolation kit was purchased from Promega (Madison, WI, USA). PCR master mix was purchased from Bioneer. Glucose and all other chemicals (for media) were obtained from Himedia (India), Duc Giang (Vietnam) and Sigma-Aldrich (St. Louis, MO, USA).

Test microorganisms

Microorganisms used for antibacterial test were from ATCC including three Gram negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), three Gram positive bacteria (*Enterococcus faecalis* ATCC29212, *Stapphylococus aureus* ATCC25923, *Bacillus cereus* ATCC 13245), one yeast strain *Candida albicans* ATCC10231.

Sample collection

Marine sediment samples were collected from three different locations at depth of 4 - 14 m (Table 1), seawater temperature at the sampling sites was 26 - 29^{o}C. The sediment samples were put into 15 ml or 50 ml sterile Falcon tubes, preserved in ice-box and processed within 24 h.

Table 1. Detail of the samples collected from three different locations at Co To Island, Thanh Lan.

No	Geographic coordinates	Water depth (m)	Name of sample
		Nam Cap Island	
1	21^{o}5'11"-107^{o}50'57"	14	30a
2	21^{o}5'11"-107^{o}50'57"	14	30b
		Van Trai	
3	20^{o}59'33"-107^{o}46'33"	4	31c
4	20^{o}59'33"-107^{o}46'33"	5	31d
		Hon Mon Southeast	
5	21^{o}0'14"-107^{o}46'22"	4	34d
6	21^{o}0'14"-107^{o}46'22"	6	34e

Isolation of fungi

An amount of 0.5 g of sample was suspended in 4.5 ml of sterile distilled water, homogenized by vortexing for 1 min, and the suspension was treated at 60^{o}C for 6 min. Next, 0.5 ml of the heat-treated suspension was used for serial dilution in sterile distilled water to 10^{-3}. At the final dilution step, aliquots of 50 µl were spread on four different solid media, including: A1 (10 g/l soluble starch, 4g/l yeast extract, 2g/l peptone, 30g/l instant ocean, 15g/l agar) MEA - malt extract agar (5g/l malt extract, 1g/l peptone, 30g/l instant ocean, 15g/l agar), PDA - potato dextrose agar (30g/l potato extract, 20g/l dextrose 5g/l soluble starch, 30g/l instant ocean, 15g/l agar), NZSG (20g/l soluble starch, 5g/l yeast extract, 10g/l glucose 5g/l NZ amine A, 30g/l instant ocean, 15g/l agar). Plates were incubated at 28°C for 7-15 days. Single colonies of fungi were transferred onto new petri dishes of PDA medium for further purification steps.

Preparation of crude extracts of culture broth

Fungal strains were cultivated at 28°C in sterile 1000 ml flasks containing 500 ml PDA broth medium, pH 7.0, shaked at 200 rpm and 27^{o}C. After 7 day cultivation, the culture broths were filtered by filter paper (thickness 0.35-0.5 mm, particle retention 3 µm) and then extracted with 300 ml ethyl

acetate (5 times × 15 minutes). Extracts were then evaporated under reduced pressure (250 mbar, heating bath at 45°C) to yield crude extracts (Cédric *et al.,* 2013).

Screening for antimicrobial activity of extracts from fungi

Crude extracts were diluted in DMSO at 1% concentration (10 mg/1ml DMSO) and used in screening experiments for antagonistic properties against the test microorganisms. Thus, the test microbes were grown in 96 well plates containing LB broth –supplemented with the crude extracts at different concentrations. Streptomycin was used as a positive control for bacteria and cycloheximide for the yeast *C. albicans* ATCC10231. Quantitative assay was performed by dilution series on 96 well plates for determination of MIC values of extracts against the test bacteria. The UV absorption of each sample was measured at 610 nm and compared with the UV absorption of the media as negative control. A MIC value was determined in well containing the extract at the lowest concentration completely inhibited growth of the test microorganisms after 24 hours of incubation and was correctly calculated based on the turbidity measurement on spectrophotometer Biotek (Hadacek *et al.,* 2000).

Identification of fungi

Fungal strains were grown for 7 days at 27ºC on MEA plates to observe colony morphology and conidiophore characteristics under microscope (1000 ×).

Genomic DNA of three potential isolates was extracted by Wizard® Genomic DNA Purification Kit was purchased from Promega (USA). Sequences of 18S rRNA was used for taxonomical identification of the fungal strains. Gene amplifications were performed in a 25.0 µl mixture containing 16.3 µl of sdH_2O, 2.5 µl of 10× PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP's, 0.2 µl of Taq polymerase, 1.0 µl of 0.05 mM for both primers NS3F (5'-GCAAGTCTGGTGCCAGCAGCC-3') and NS8R (5'-TCCGCAGGTTCACCTACGGA-3') and 2.0 µl of genomic DNA. The thermocycling was performed on MJ Thermal cycler (Bio - Rad), with a preheating step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 30s and extension at 72°C for 45s before a final extension of 72°C for 10 min. The PCR product size was about 1300 bp. PCR products were purified by DNA purification kit (Invitrogen) and sequenced by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were handled by BioEdit v.2.7.5. and compared with fungal 18S rDNA sequences available in GenBank database by using NBCI Blast program. The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic neighbor-joining tree by using the MEGA program version 4.1.

RESULTS AND DISCUSSION

Isolation and screening for antimicrobial activity of marine fungi

From the marine sediments randomly collected, serial dilution and plating on different media were carried out for fungal isolation. After two weeks of incubation, 25 fungal strains were isolated and screened for antibacterial activity. These fungi were cultivated in PDA broth medium. The culture broths were extracted 5 times with ethyl acetate then the extracts were evaporated under reduced pressure to yield crude extracts. Crude extracts were tested against 7 reference strains.

The result of screening showed that most of the isolates were active against both Gram positive and Gram negative bacteria. Most notably, 3 strains (M26, M30, M45) were chosen for the highest biological activity. Strains M30, M45 inhibited 5 of the 7 strains of microorganisms tested with MIC values equal or lower than the positive control. Specifically, strain M30 inhibited *P. aeruginosa* and strain M45 inhibited all three Gram-positive strains at MIC values of 128 µg/ml which is lower than the MIC value 256 µg/ml of streptomycin control. Strain M26 also showed an inhibitory effect on 4 of the 7 tested strains with MIC values in the range from 64 to 256 µg/ml, depending on the tested microorganisms. In addition, the two strains M30 and M26 had a good inhibitory effect on *C. albicans* ATCC10231 with MIC values of 64 and 256 µg/ml, respectively (Table 2).

Research by Kustiariyah *et al.,* 2011, showed that ethyl acetate extract of 10 fungi strains isolated from marine environment in Indonesia had inhibitory activity against two Gram-positive bacteria (*B. subtilis, S. aureus*) with inhibition zone diameters ranging from 24 to 34 mm while only 3 of 10 strains inhibited *P. aeruginosa* and 6 of 10 strains inhibited *E. coli* with inhibition zone diameters ranging from 8 to 13 mm.

Screening results also indicated that the crude extracts of the isolates were active against Gram positive bacteria better than gram negative ones. The reason for different sensitivity toward Gram positive and Gram negative bacteria could be explained by differences of cell envelope in these microorganisms. The outer membrane of Gram negative carrying the structural lipopolysaccharide components, making the cell envelop impermeable to lipophilic solutes. In contrast, the Gram positive bacteria should be more susceptible by having only an outer peptidoglycan layer which is not an effective permeable barrier.

Table 2. Antimicrobial activity of crude ethyl acetate extracts from 3 fungal strains.

No	Isolates	Gram-positive			Gram-negative			Yeast
		E. faecalis **ATCC29212**	***S. aureus*** **ATCC25923**	***B. cereus*** **ATCC13245**	***E. coli*** **ATCC25922**	***P. aerugin osa*** **ATCC27853**	***S. enterica*** **ATCC13076**	***C. albicans*** **ATCC10231**
	Unit	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**
1	*M26*	*256*	*-*	*128*	*-*	*256*	*-*	*256*
2	*M30*	*256*	*256*	*256*	*-*	*128*	*-*	*64*
3	*M45*	*128*	*128*	*128*	*64*	*-*	*256*	*-*
Streptomycin		*256*	*256*	*128*	*32*	*256*	*128*	*-*
Cycloheximide		*-*	*-*	*-*	*-*	*-*	*-*	*32*

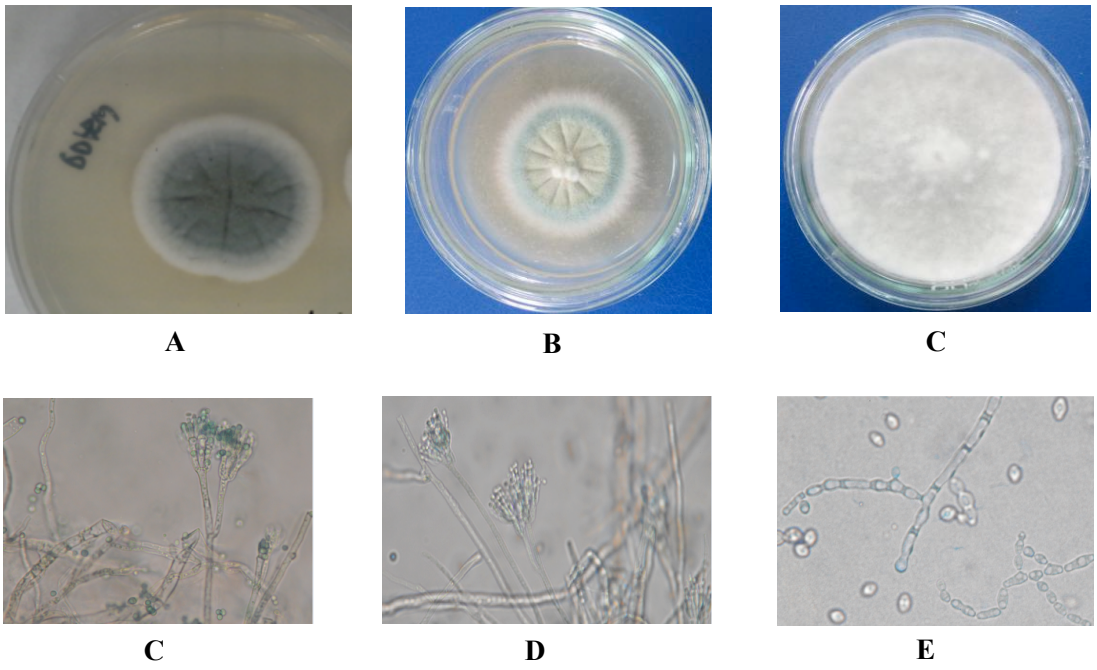

Figure 1. Colony morphological characteristics of the strains M26 (A), M30 (B) and M45 (C) grown on MEA medium for 1 weeks at 27°C; and the conidiophore observed under microscope (1000 X) of M26 (D), M30 (E) and M45 (F).

Identification of the fungi

The morphological and conidiophore characteristics are considered as one of the important characteristics in the taxonomical identification of fungi (Figure 1). Well grown colonies of strains M26 and M30 on the MEA medium had diameter from 4.5 – 5.0 cm after 7 days at 27°C. The colonies were

velvety and orange-green in colour with thin white margin. Reverse sides of the colonies were bright yellow to olive brown. Mature colonies were deeply radiantly wrinkled. Spores were abundant with grey-green shades. Colonies did not produce odour and exudates. Conidiophores were approximately 70-80 × 2 µm in size and had smooth walls. Phyloides were strictly monoverticillate, consisting of small verticels. Five to eight or ten parallel sterigmata were present on verticels. Sterigmata were 10-12 x 0.2-2.5 µm in length. Spores were arranged in chains. Conidial chains were up to 100 µm long (Raper *et al.,* 1968; Domsch *et al*., 1980).

Strain M45 grew rapidly on the MEA medium, producing colonies of 2.5 cm in diameter in one day at 27°C, fully covered the agar surface in petri dish. The mature colonies were white and turned yellow when formed spores. The spores were arranged in chains like hyphae break, then quickly separated to form a group of powder in the dry conditions, the shape and size of the anomaly 10-15 µm x 5-10 µm (Robert *et al*., 1984).

The three potential isolates were subjected to identification by 18S rRNA gene sequencing. The 18S rRNA genes were amplified by PCR by using specific primers NS3F and NS8R, giving products of 1300 bp (Figure 2). Comparative analyses of 18S rRNA gene sequences of these three isolates showed that strains M26 and M30 exhibited the highest similarity (99%) to *Penicillium chrysogenium*; whereas strain M45 showed the highest similarity (99%) to *Neurospora crassa* (Figure 3). The sequences of 18S rRNA gene of M26, M30 and M45 isolates were registered in GenBank database with the accession numbers MH673730, MH673731, MH673732, respectively.

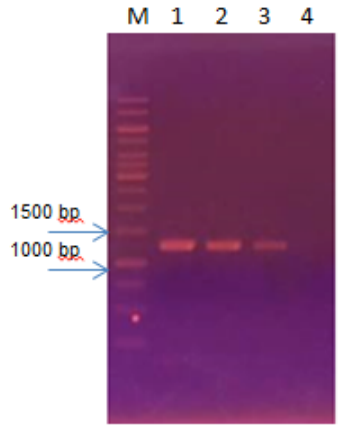

Figure 2. Electrophores is image of PCR products18S rRNA gene of isolates. Lane M: Represents the 1Kb DNA ladder of Fisher Scientific. Lanes 1-3: PCR products of M26, M30, and M45 isolates. Lane 4: PCR product control without DNA template.

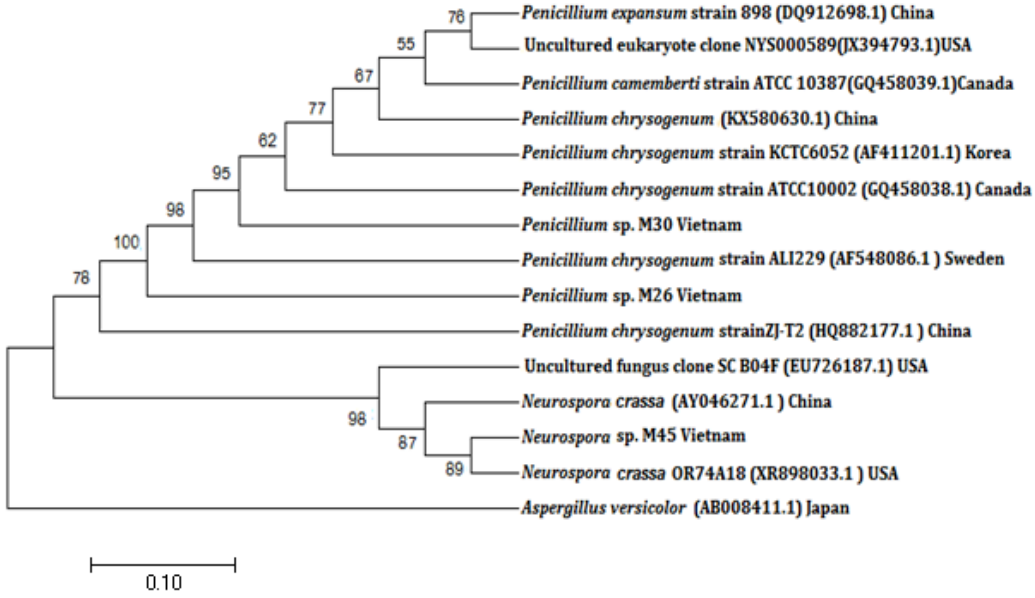

Figure 3. Neighbor-joining tree based on 18S rRNA gene sequences showing relationships between the strains in groups and representative members of the genera *Penicillium* and *Neurospora*. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; bootstrap values below 50% were eliminated.

The genus *Penicillium* consists of more than 354 species, of which several are of industrial importance. Well known are the industrial penicillin producer *Penicillium. chrysogenum* and other *Penicillia* used for the production of many pharmaceutically important secondary metabolites and enzymes such as cellulases, protease, amylase and in food production (Jens *et al*., 2017). Marine environment is a habitat of a wide range of distinct *Penicillium,* some of those have been reported for antibiotic and enzyme production. Thus, the marine environment is a potential source for novel bioactive compounds that need to be explored.

Essential antibacterial compounds, such as xanthocillin X, and 14 other known compounds including three steroids, two ceramides, six aromatic compounds, and three alkaloids were isolated from *Penicillium commune* SD-118 (Shang *et al*., 2012). Several other members of conidiogenone were isolated from culture extracts of *Penicillium chrysogenum* QEN-24S derived from an unidentified marine red algal species of the genus *Laurencia*. This compound has shown potential cytotoxic effect to the human leukemia cell line (HL-60) (Gao *et al*., 2011). Arumugam *et al.* 2015 successfully isolated a piezotolerant fungus *Nigrospora* sp. NIOT from deep sea environment and cultured it under submerged fermentation. Secondary metabolites produced from this organism showed potent antimicrobial and anticancer activities with immediate application to cosmetics and pharmaceutical industries.

Recently, in research of fungal diversity in coastal marine ecosystems, Babu *et al.* (2010) isolated strains of *Neurospora crassa* in both sea areas of Poombugar and Nagapattinam in Southeastern, India. Strain *Neurospora crassa* F7 which can provide enzyme lipase has also been isolated from palm oil wastewater of Pedavegi palm oil extracting plant in India (Suseela *et al.,* 2014). In another study, Kumar *et al.* (2015) isolated *Neurospora crassa* from marine samples at Machilipatnam coast of Andhra Pradesh, India. Crude extract of this *Neurospora* sp. dissolved in DMSO showed inhibitory effects against four tested microorganisms *Escherichia coli, Bacillus* sp*., Salmonella* sp*., Streptococci* sp. at concentrations of 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml, respectively, with inhibition zone diameters ranging from 10 to 22 mm, depending on each tested strain.

The results obtained and published studies showed that fungal strains isolated and screened from marine environments have a wide spectrum of antibacterial activity with an antibacterial concentration less than or equal to the reference substance. The study also showed that marine fungi could be a potential source for producing antibiotics based on inhibiting germs of microbial diseases. However, there is a need for research in determining the chemical structure of bioactive compounds from these fungal strains.

CONCLUSION

From six sediment samples randomly collected from Co To Island - Thanh Lan, twenty five fungal strains were isolated. The results of screening for antimicrobial activity showed that most of the isolates were active against 1 to 5 strains of microorganisms tested. Specifically, strains M30, M45 inhibited 5 of 7 strains of tested microorganisms, and strain M26 showed the inhibitory effect towards 4 of 7 strains of tested microorganisms, with MIC values ranging from 64 to 256 µg/ml, depending on the tested microbes. In addition, two strains M30 and M26 were highly active toward *C. albicans* ATCC10231 with respective MIC values from 64 to 256 µg/ml. The three strains were identified as members of the genus *Penicillium* (strains M26 and M30) and genus *Neurospora* (strain M45) based on morphological and 18S rRNA gene sequence analyzes.

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PHÂN LẬP, SÀNG LỌC HOẠT TÍNH KHÁNG KHUẨN VÀ ĐỊNH DANH CÁC CHỦNG VI NẤM ĐƯỢC PHÂN LẬP TỪ TRẦM TÍCH BIỂN CỦA VÙNG THANH LÂN, CÔ TÔ, VIỆT NAM

Lê Thị Hồng Minh, Nguyễn Mai Anh, Vũ Thị Quyên, Vũ Thi Thu Huyền, Đoàn Thị Mai Hương, Phạm Văn Cường, Châu Văn Minh

Viện Hóa sinh Biển, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Môi trường biển là nguồn cung cấp các sản phẩm tự nhiên vô cùng phong phú, trong đó các vi sinh vật biển, đặc biệt là vi nấm, được đánh giá là nguồn tiềm năng chứa các hoạt chất sinh học có giá trị ứng dụng cao, đồng thời có thể cung cấp cho khoa học các cấu trúc hóa học mới. Mục tiêu của nghiên cứu này là phân lập và sàng lọc các chủng nấm có hoạt tính kháng khuẩn từ môi trường biển. Hai mươi lăm chủng nấm đã được phân lập từ trầm tích biển đảo Cô Tô - Thanh Lân và được đánh giá hoạt tính kháng khuẩn đối với 7 chủng vi sinh vật kiểm định, gồm ba chủng vi khuẩn Gram âm (*E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. enterica* ATCC13076), ba chủng Gram dương (*E. faecalis* ATCC29212, *S. aureus* ATCC25923, *B. cereus* ATCC 13245), và nấm men *C. albicans* ATCC10231. Nồng độ ức chế tối thiểu (MIC) đối với các chủng kiểm định được xác định cho các mẫu chất chiết xuất thô thu được từ dịch nuôi cấy tế bào sau khi tách chiết bằng ethyl

acetate và làm bay hơi dung môi bằng cô quay chân không. Ba chủng có hoạt tính kháng khuẩn cao nhất là M26, M30 và M45 có khả năng ức chế 4 đến 5 trong số 7 chủng vi sinh vật kiểm định với các giá trị MIC từ 64 đến 256 µg/ml phụ thuộc vào từng chủng kiểm định, bao gồm cả *C. albicans*. So sánh đặc điểm hình thái và trình tự của gen 18S rRNA cho phép xếp hai chủng M26 và M30 vào chi *Penicillium*, và chủng M45 vào chi *Neurospora*. Các trình tự 18S rRNA của ba chủng M26, M30 và M45 đã được đăng ký trên GenBank với mã số tương ứng là MH673730, MH673731, MH673732. Kết quả nghiên cứu cho thấy môi trường biển có tiềm năng lớn để phân lập các chủng vi nấm cho mục đích tìm kiếm các chất kháng khuẩn cũng như các hoạt chất sinh học khác.

Từ khóa: *Hoạt tính kháng khuẩn, MIC, trầm tích biển, trình tự gen18S rRNA, vi nấm.*