BIODIVERSITAS
Volume 24, Number 10, October 2023
Pages: 5653-5663

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241047

Isolation, phylogenetic analysis and bioprospection of myxobacteria from Vietnam

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Manuscript received: 16 September 2023. Revision accepted: 26 October 2023.

Abstract. *Yen NTN, Chung DD, Hong NTK, Cham NP, Nhan VT, Linh DTL, Ngoc NLB, Thai NM, Nga ND, Anh NT. 2023. Isolation, phylogenetic analysis and bioprospection of myxobacteria from Vietnam. Biodiversitas 24: 5653-5663.* Myxobacteria have been considered microbial factories for producing secondary metabolites that have a variety of potential biological actions for discovering and isolating new biological molecules. Myxobacteria were isolated from soil samples collected in some provinces/cities in Vietnam. The purified isolates were identified based on morphology, biochemical test and phylogenetic analysis inferred from 16S rRNA gene. High-throughput screening assays including 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DDPH) for antioxidant properties and microdilution for antimicrobial activity were performed with myxobacterial extracts. Compounds from potential strain were predicted using liquid chromatography coupled with mass spectrometry. Forty-three myxobacterial strains were isolated and classified into seven genera of *Angiococcus*, *Archangium*, *Chondromyces*, *Corallococcus*, *Cystobacter*, *Melittangium*, and *Myxococcus*. The extract from CT21 strain had the highest total antioxidant activity (IC_{50} = 52.34 ± 1.47 and 30.28 ± 0.74 µg/mL for the DPPH and ABTS, respectively). It is worth noting that all strains isolated myxobacterial strains show inhibitory activity against at least one of the tested microorganisms. The most potent antimicrobial strain was *Myxococcus stipitatus* GL41, which inhibited all tested microorganisms, and the minimal inhibitory concentration (MIC) values were 1 µg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Streptococcus faecalis*, *Candida albicans*, and *Aspergillus niger*. Mass spectrometry analysis revealed the presence of althiomycin-the polyketide antibiotic from ethyl acetate fraction. In the present study, myxobacteria were isolated from soil sample collected from Vietnam, analyzed phylogenetically, and screened for biological activities.

Keywords: Antimicrobial, antioxidant, mass spectrometry, myxobacteria, phylogeny, soil

INTRODUCTION

Myxobacteria is a group of Gram-negative bacteria, rod shaped organisms that are widespread in natural habitats such as soil, water, bark, herbivore dung, desert, marine areas, and saline-alkaline soil (Wang et al. 2021). Two stages make up their elaborate life cycle: the vegetative stage, during which myxobacteria, rod-shaped cells, swarm on solid surfaces and feed on other bacteria or break down cellulose (Cao et al. 2015; Thakur et al. 2018). The cells generate fruiting bodies that contain heat-stable myxospores in unfavorable conditions by going through morphogenesis and displaying a structured social behavior (Shimkets et al. 2006). It has been theorized that the exceptional ability to produce novel bioactive compounds in myxobacteria has been facilitated for their survival to thrive in the harsh and competitive environment (Bader et al. 2020). Myxobacteria have been documented as a promising microorganism with broad-ranging bioactivities including antioxidant, antivirus, antimicrobial, antimalarial, and cytotoxic activities (Shrivastava and Sharma 2021). Traditional and high-throughput screening techniques have allowed scientists to discover a series of Myxococales-represented unique compounds from naturally isolated species. Hundreds of compounds have been found through decades of research, among which the novel compound known as epothilone, whose derivative, has been commercialized as a medication for human breast cancer treatment (Saggu et al. 2023). More than 100 metabolites and 600 derivatives related to myxobacteria have been found (Saadatpour and Mohammadipanah 2020). Myxobacteria were considered outstanding secondary metabolite producers for drug development because they are able to produce novel compounds that have useful bioactivities, after Actinobacteria, they are the second most prolific makers of these compounds (Thakur et al. 2018). They can be classified as predators and cellulose-degraders based on their dietary requirements. Myxobacteria are extremely interesting due to their high potential to produce a wide variety of bioactive secondary metabolites, in addition to their interesting lifestyle as demonstrated by the development of frequently colorful fruiting bodies and dry resistant myxospores (Shrivastava and Sharma 2021).

There are many myxobacteria in the natural habitat. Less than 10% of all wild species have, however, so far been isolated (Cortina et al. 2012). Therefore, increasing ability to identify new myxobacteria would have significant potential economic value and give more strains for pharmaceutical testing and development (Shimkets et al. 2006). Indeed, many notable compounds have been discovered in recent years, including archazolids isolated from *Archangium gephyra*, ajudazols extracted from *Chondromyces crocatus*, cruentaren from *Byssovorax cruenta*, bithiazole derived from *Myxococcus fulvus*, myxoprincomide extracted from *Myxococcus xanthus*, indiacens A and B isolated from *Sandaracinus amylolyticus*, salimyxin B and enhygrolide A from *Enhygromyxa salina*, and pyxipyrrolones derived from *Pyxidicoccus* (Kjaerulff et al. 2017; Shrivastava and Sharma 2021). The secondary metabolites produced by myxobacteria are promising agents. The secondary metabolites of myxobacteria are uncommon hybrids of polyketides and nonribosomal generated peptides with high pharmacological value, the chemical structures of their metabolome are also exceptional both in diversity and biological activities (Kjaerulff et al. 2017).

By 2000, although only 40 myxobacterial species had been described due to challenges in isolating myxobacterial strains from natural habitats, a large number of strains producing various metabolites and structural variants had been isolated (Gaspari et al. 2005). It showed that myxobacterial isolation from unexploited natural areas is still an effective strategy in the search for novel products. Indeed, their biodiversity and metabolite products may depend on their different habitats (Wrótniak Drzewiecka et al. 2016). According to Jingjing Wang's report summarizing research on the diversity of myxobacterial distribution worldwide (Wang et al. 2021), the map of myxobacterial research spreads across continents and seas, but not for Vietnam. Our country is believed to possess a diverse geographical area and a typical tropical climate rich in natural microbiomes. Therefore, in this study, myxobacteria were isolated and identified from the soil collected from provinces/cities in Vietnam and screened for antioxidant and antimicrobial activities from their raw extracts. This research is the first project that aims at providing novel information on the isolation, phylogenetic relationships and bioactivities of myxobacterial strains in Vietnam.

MATERIALS AND METHODS

Soil samples collection

Soil samples were collected from 20 provinces in Vietnam at a depth of 10 cm and stored in sterile falcons or bags (Charousova et al. 2017). The samples were air-dried to reduce the natural moisture and inhibit the development of fungi and worms (Shimkets et al. 2006).

Isolation and purification of myxobacteria

Myxobacteria were isolated from ground soil using three typical methods including (i) WCX (water cycloheximide) medium (w/v, agar 1.5%, $CaCl_2$ 0.07%, cycloheximide 0.01%) with *Escherichia coli* streaks (*E. coli* was cultivated in Luria-Bertani broth) for bacteriolytic strains (W), (ii) ST21CX medium (w/v, agar 1%, cycloheximide 0.01%, $CaCl_2.2H_2O$ 0.1%, $FeCl_3$ 0.02%, K_2HPO_4 0.1%, KNO_3 0.1%, $MgSO_4.7H_2O$ 0.1%, $MnSO_4.7H_2O$ 0.01%, yeast extract 0.002%) with filter paper for cellulolytic decomposers (F), and (iii) the wild rabbit dung method that contain natural substrate suitable for *Myxococcus* and *Corallococcus* isolation (R) (Shimkets et al. 2006).

Dried soil was heated in a water bath at 80℃ for 30 min before being used to fill a half of Petri dish. Cycloheximide solution (100 µg/mL) and amphotericin B (10 µg/mL) were added to control the growth of fungi. The Petri dishes were wrapped with paraffin before being incubated at 30℃ for 3-30 days and checked daily by stereoscopy to recognize fruiting bodies' appearances. Myxobacterial isolates were purified by transferring the swarm edge and fruiting bodies many times on VY2 dishes (w/v, agar 1.5%, Baker's yeast 0.5%, $CaCl_2.H_2O$ 0.14%, cyanocobalamine 0.00005%, cycloheximide 0.01%, and the final pH 7.2). The purity of isolates was confirmed by inoculation in the enzymatic hydrolysate of casein (CEH) medium (w/v, casein hydrolysate 1.0%, K_2HPO_4 0.025%, $MnSO_4.7H_2O$ 0.1%) for 24 h (Shimkets et al. 2006).

Bacterial identification

Morphological identification

Isolated bacteria were observed in their swarms and fruiting bodies. The size and shape of vegetative cells and myxospores after Gram staining were identified and the type of fruiting bodies observed were compared to the published descriptions (Rosenberg et al. 2014).

DNA isolation and 16S rRNA gene amplification

DNA extraction was performed using TopPure Genomic DNA Extraction Kit, (ABT, Vietnam) following the manufacturer's instructions. The quantity and quality of the eluted DNA was determined using gel electrophoresis and the Nanodrop Spectrophotometer, respectively. The samples that had a concentration lower than 20 ng/µL were used for PCR (polymerase chain reaction). The 16S rRNA gene was amplified using the 27F and 1492R primers (Meliah and Lisdiyanti 2018). The final volume of PCR was 25 µL, including 5 µL of DNA, 1X reaction buffer (Tris KCl-$MgCl_2$), 5 mmol/L $MgCl_2$, 1 mmol/L dNTP, 0.4 µmol/L of each primer, and MyTaq HS DNA Polymerase (5 U/µL). The PCR program was initial denaturation at 95℃ for 5 min and 30 cycles (denaturation at 95℃ for 80 sec, annealing at 57℃ for 30 sec, and extending at 72℃ for 80 sec) (Kumar et al. 2017). The amplicons were visualized using a 1.0% agarose gel and the correctly sized products were purified using the HiYieldTM Genomic DNA Mini Kit (ABT, Vietnam) and sent for sequencing at Axil Scientific Pte Ltd, Singapore.

Phylogenetic analysis

The raw sequencing data was compiled using the Lasergene tool following quality verification. The newly

discovered myxobacteria's 16S rRNA genes were deposited in GenBank. The assembled 16S rRNA sequence data were compared to those from other myxobacteria using the NCBI's Nucleotide BLAST program, which uses the Basic Local Alignment Search Tool (BLAST). The data matrix includes myxobacterial sequences in this study and reference sequences retrieved from the NCBI databases for phylogenetic analysis. The selected reference strains were representatives of the main genera belonging to the three suborders Cystobacterineae, Sorangiineae, and Nannocystineae, order Myxococcales. Nucleotide sequences were aligned using the MUSCLE program. MEGA software version 11 was used to reconstruct the phylogeny using the maximum likelihood method with Jukes-Cantor model and 1000 bootstrap replicates (Kumar et al. 2017). *Desulfovibrio vulgaris* DSM 644, which belongs to the delta-proteobacteria group, was used as outgroup taxa (Meliah and Lisdiyanti 2018).

Preparation of myxobacterial crude extracts

The purified isolates were inoculated in 100 mL P media (w/v, baking yeast 0.4%, $CaCl_2$ 0.1%, ferric EDTA 0.0008%, Hepes 2.38%, $MgSO_4$ 0.1%, peptone 0.2%, soluble starch 0.8%, yeast extract 0.2%, pH 7.5) and shaken at 180 rounds per min at room temperature for ten days. On the 4th day of cultivation, 1-2% Amberlite XAD-16N adsorbent resin was added. After fermentation, the resin was collected by filtering and then extracted with 80 mL acetone and 80 mL methanol for 2 h each, respectively. The organic solvents were evaporated in a vacuum device to a final volume of 1 mL crude extracts and stored at 4-8°C (Charousova et al. 2017).

Evaluation of antioxidant activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate, Sigma, USA) stock solution (1 mmol/L) was diluted with a solvent to achieve an absorbance of 0.68 (± 0.02) at 517 nm. The mixture was prepared by mixing 180 µL DPPH solution and 20 µL myxobacterial extracts. The absorbance at 517 nm was recorded (Grajeda et al. 2016).

ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity

ABTS radical cation ($ABTS^{\bullet+}$) solution was made by mixing ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, Sigma) stock solution (7 mmol/L) with 2.45 mmol potassium persulfate ($K_2S_2O_8$) solution. The final volume included 20 µL crude extract, 100 µL $ABTS^{\bullet+}$ solution (A_{734} nm = 0.70 ± 0.02), and 80 µL of the methanol and distilled water mixture (1:1, v/v). The absorbance was measured at 734 nm. The well containing 100 µL $ABTS^{\bullet+}$ solution, 50 µL methanol, and 50 µL distilled water, was used as the negative control (Gaber et al. 2021).

The IC_{50} values (half-maximal inhibitory concentration) or the extract concentrations that requires scavenging of 50% free radicals (µg/mL) were predicted from the regression model and used to express the antioxidative activities. Ascorbic acid and Trolox were used as the standards for establishing the ratio of $IC_{50\ (sample)}$ to $IC_{50\ (standard)}$ in the DPPH and ABTS methods, respectively (Wang et al. 2019). All measurements were conducted in triplicate and averaged as Mean ± SD used Excel 2013.

Evaluation of antimicrobial activity

The test microorganisms obtained from the American Type Culture Collection (ATCC) included: Methicillin-susceptible *Staphylococcus aureus* or MSSA (ATCC 25923), methicillin-resistant *Staphylococcus aureus* or MRSA (ATCC 43300), *Streptococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404), *Penicillium* sp. (Pe), *Mucor* sp. (Mu), and *Rhizopus* sp. (Rh) were supplied by the University of Medicine and Pharmacy at Ho Chi Minh City.

Microdilution method

Muller Hinton broth and RPMI-1640 medium were used for bacterial and filamentous fungi strains, respectively, and the supplement of 2% glucose was used for yeast *C. albicans*. Minimum inhibitory concentrations (MICs) of extracts were determined by the microdilution method using 96-well microtiter plates. The extracts were diluted in DMSO (dimethyl sulfoxide) with the highest concentration (512 µg/mL) in well one, followed by a serial dilution of ½. Fifty microliters of the test microorganism inoculate and 30 µL resazurin (0.015 mg/mL) as an indicator for cell viability were added and the plates were incubated at 35°C for 16-48 h (Zargaran et al. 2017). The MIC endpoints were observed visually when viable cells changed their color from purple to pink (Markantonatou et al. 2020). Amikacin, fluconazole, and amphotericin B (Sigma) were used as positive controls for antibacterial, anti-Candia, and antifungal activities, respectively (Cioch et al. 2017; Manandhar et al. 2019; Rodrigues et al. 2019).

Analysis of metabolites with liquid chromatography coupled mass spectrometry

GL41 crude extract was fractionated by liquid-liquid with solvents that has increasing polarity (n-hexan, chloroform, and ethyl acetate). The antimicrobial activities of fragments were assessed by well diffusion assay with test microorganisms. The high bioactive fragments were purified on semi-chromatography column (column Luster C18 250×10 mm, 10 µm, Dikmatech, Büchi Reveleris X2 system) and the purities of each fragments were checked on HPLC system with PDA detector.

The partially purified fraction was performed on an HPLC-hrMS (Agilent 1900, USA) using C18 XTerra column (4.6×50 mm, 2.5 µm). The solvents were consisted of methanol (solvent A) and deionized water with 0.1% formic acid (solvent B). Initial gradient elution was 90% A in 10 min, increased to 100% B within 5 min, and maintained with 90% A. Flow rate was 0.35 mL/min, sample injection volume was 5 µL, and column oven temperature was at 30°C. Mass information was analyzed by high-resolution mass spectrometry (Series 6500 Q-TOP, Agilent, USA). MS parameters were under the following

conditions: ionization voltage was 3.5 kV; capillary voltage ± 24 V; sheath gas and auxiliary gas were 35 and 15 psi, respectively; vaporizer temperature and capillary temperature were 300°C. Data were acquired in electrospray (ESI) positive scans ranging from 50-1000 m/z (Hoffmann et al. 2018). Identification of bacterial compounds was performed with the filter to appearance in myxobacterial extract and in none of the medium blank extracts. Data evaluation was performed with Masshunter Qualitative Analysis B.07.00 (Agilent) and published documents.

RESULTS AND DISCUSSION

Myxobacterial isolation

Soil samples were mainly loam, peat, and silt soils that were collected in rice fields or near the roots of large trees in fruit gardens in Vietnam that gives the best yields (Shimkets et al. 2006). Forty-three myxobacterial strains were isolated using the rabbit dung pellets method, WCX method, filter paper method, and soil extract method from 20 soil samples (Table 1). Pellets made from rabbit dung are a natural growing medium for myxobacteria. Sterilized rabbit dung pellets can induce fruiting bodies in as little as two to three days, and the majority of these fruiting bodies are *Myxococcus*, which are simple to purify. Natural rabbit dung pellets, on the other hand, are rich in organic materials and are susceptible to mold contamination during the induction process, which covers the fruiting bodies of myxobacteria. Only 15 (34.9%), 16 (37.2%), and 12 (27.9%) strains of myxobacteria were isolated using the various isolation techniques, using the rabbit dung, filter paper, and *E. coli* bait/WCX procedures, respectively.

Morphological characteristics and 16S rRNA gene sequences

All of the soil-isolated strains were successfully inoculated in VY2 medium. The morphology of fruiting bodies, myxospores, and vegetative cells were observed under a stereoscopic microscope. Based on morphological characteristics and 16S ribosomal RNA gene sequences, the 43 strains belonged to Myxococcales. The morphology of the colonial edge, the opaque and the radial pattern; the color and shape of fruiting bodies, aggregate or solitary structure; and the size of vegetative cells were observed on the dissecting and optical microscopes. Vegetative cells were generally characterized by slender rods and rounded ends, about 3-12 µm in length and 0.5-1 µm in width (Figures 1 and 2).

Nucleotide BLAST revealed that the 43 isolates had a high sequence similarity (99.65-100%) to Myxococcales. These strains were assigned to seven specific genera: *Angiococcus* (1 strain), *Archangium* (3 strains), *Chondromyces* (2 strains), *Corallococcus* (15 strains), *Cystobacter* (1 strain), *Melittangium* (1 strain), and *Myxococcus* (20 strains), belonging to three families (Myxococcaceae, Archangiaceae, and Polyangiaceae), and two suborders (Cystobacterineae and Sorangiineae). Among these 43 isolated strains, 24 were identified to the species level, while 19 were assigned to the genus level (Figure 3; Table 1).

Table 1. Identification of isolates according to 16S rRNA analysis

Genus (No. of species)	Isolation methods	Genera/species	Number of isolates	Name of samples
Angiococcus (1)	1W	*A. disciformis*	1	DN11
Archangium (3)	1R, 1W, 1F	*A. gephyra*	2	BP181, QT15
		A. disciforme	1	TV11
Chondromyces (2)	1W, 1F	*C. apiculatus*	1	HCM21
		C. pediculatus	1	BRVT1
Corallococcus (15)	5R, 8W, 2F	*C. coralloides*	4	AG11, LA43, LA44, TV5
		Corallococcus sp.	11	BDi23, BT92, BT101, DN23, GL43, PY1, QT72, ST11, TH41, TV22, VL32
Cystobacter (1)	1R	*Cystobacter* sp.	1	LA41
Melittangium (1)	1F	*M. boletus*	1	BDi22
Myxococcus (20)	9R, 4W, 7F	*Myxococcus* sp.	7	BP182, CT21, HG225, NB83, TG23, TH48, TH52
		M. fulvus	7	BP161, BP213, DN21, NB71, NB81, TG131, VL25
		M. stipitatus	5	BD2, BT43, GL41, HG223, VL21
		M. virescens	1	HCM81
Total			43	

Note: R: Rabbit dung method, W: WCX medium with *E. coli* streaks, and F: Filter paper method

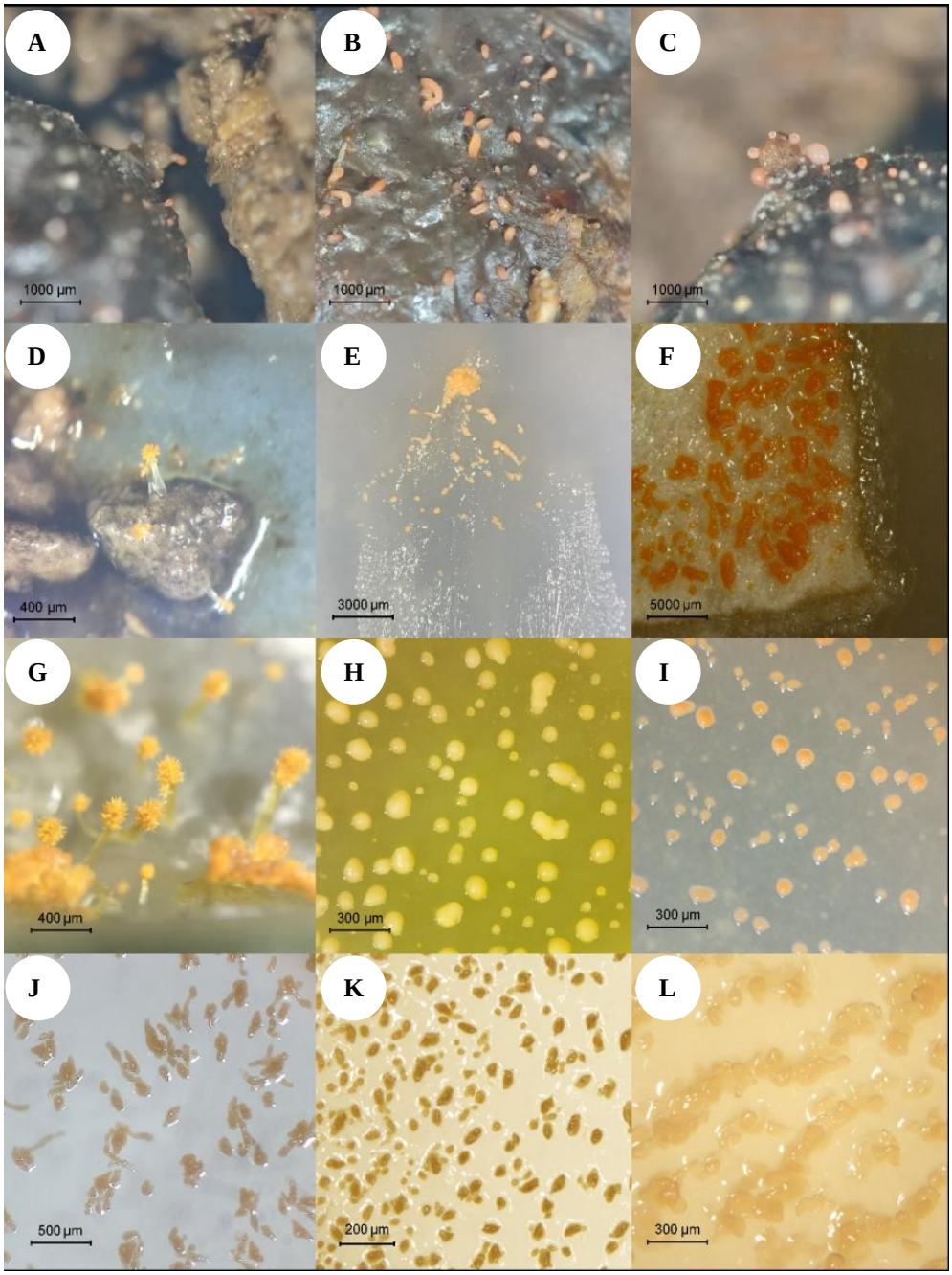

Figure 1. Myxobacterial fruiting bodies**.** Fruiting bodies on rabbit dung (a-c) and WCX agar (d), gliding movement on WCX agar (e), fruiting bodies on filter paper (f). Fruiting bodies of myxobacterial isolates on VY2 medium (g-n): (g) *Chondromyces apiculatus* (HCM21), (h) *Myxococcus* sp. (NB83), (i) *Myxococcus fulvus* (VL25), (j) *Corallococcus* sp. (ST11), (k) *Corallococcus* sp. (BT101), and (l) *Archangium gephyra* (BP181) (under a stereo microscope)

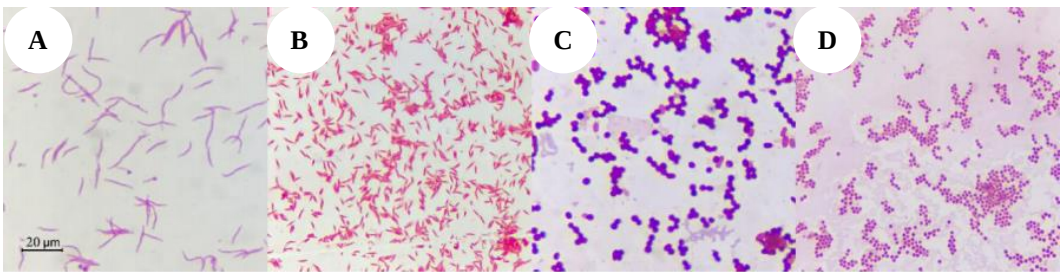

Figure 2. Morphology of Gram-negative vegetative cells and myxospores**.** (a-b) Vegetative cells of *Myxococcus stipitatus* GL41 (a) and *Myxococcus* sp. TG23 (b); (c-d) Myxospores of *Myxococcus virescens* HCM81 (c) and *Myxococcus fulvus* NB81 (d) from VY2 medium (at 1000X magnification)

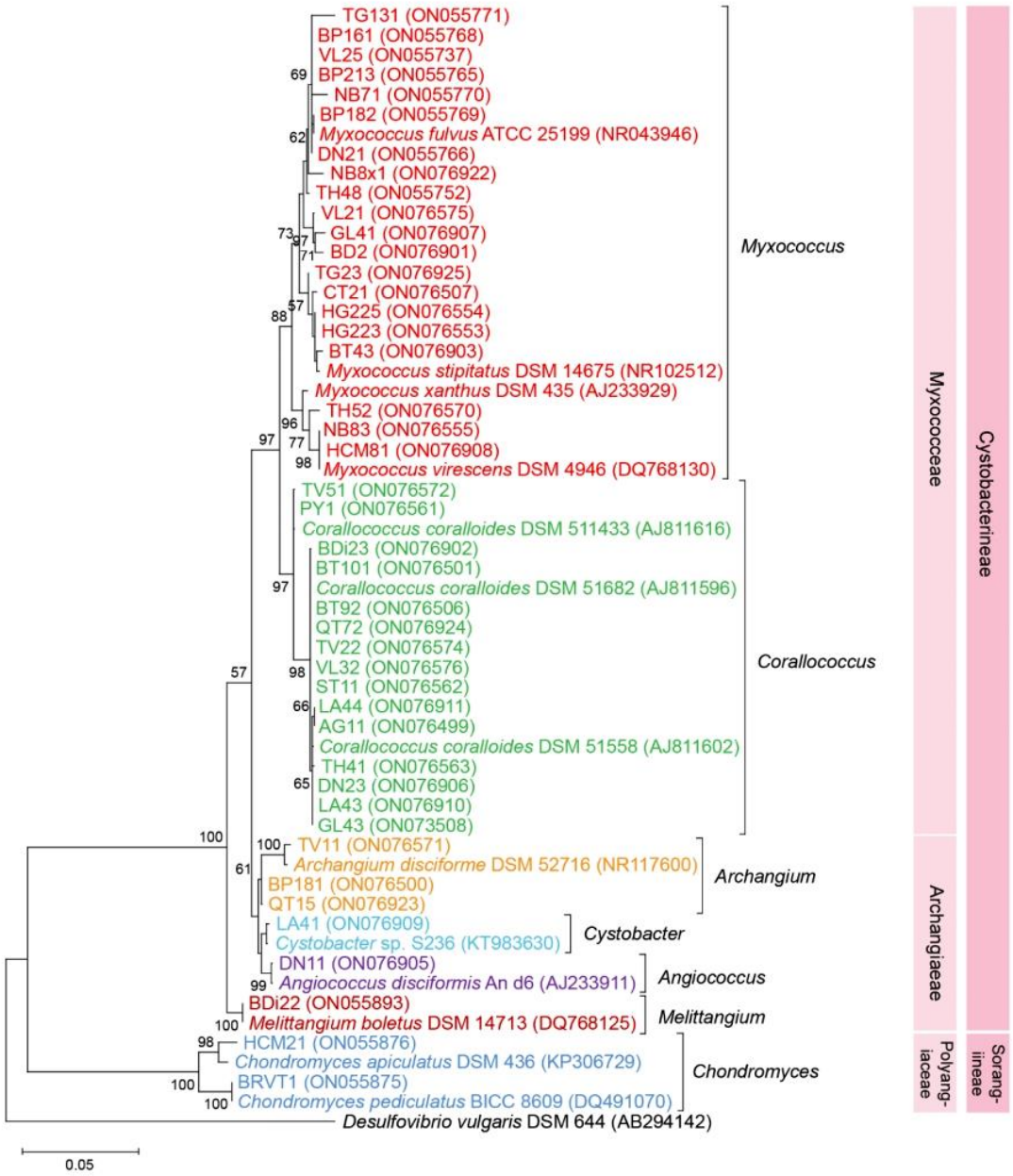

Figure 3. The maximum likelihood tree based on 57 16S rRNA gene sequences. The tree consisted of 43 myxobacterial sequences in this study and 14 reference strains retrieved from the NCBI databases. The right bars indicate family and order. The numbers in parathesis show the accession numbers of the sequences. The numbers at nodes indicate bootstrap values. The bar scale represents a number of nucleotide substitutions per site

Forty-three myxobacterial 16S rRNA genes were deposited in GenBank with accession numbers ON055737-ON055893 and ON076499-ON076924. The phylogenetic analysis was established based on the data from fifty-seven 16S rRNA sequences, consisting of 43 sequences in this study and 14 reference strains [i.e., *Myxococcus fulvus* (NR043946), *Myxococcus stipitatus* (NR102512), *Myxococcus xanthus* (AJ233929), *Myxococcus virescens* (DQ768130), *Corallococcus coralloides* (AJ811616, AJ811596, AJ811602), *Archangium disciforme* (NR117600), *Cystobacter* sp. (KT983630), *Angiococcus disciformis* (AJ233911), *Melittangium boletus* (DQ768125), *Chondromyces apiculatus* (KP306729), and *Chondromyces pediculatus* (DQ491070)]. The evolutionary tree of the 43 isolated strains revealed that they were divided into seven genera namely, *Myxococcus*, *Corallococcus*, *Archangium*, *Cystobacter*, *Angiococcus*, *Melittangium*, and *Chrondromyces*. Most of the isolates belonged to the Myxococceae and Archangiaceae families of the Cystobacterineae suborder. Only two strains of *Chondromyces* in the Polyangiaceae family of the Sorangiineae suborder were identified. Additionally, the most abundant of isolates in different locations belonged to *Myxococcus* and *Corallococcus*. In contrast, the strains of *Angiococcus*, *Archangium*, *Chondromyces*, *Cystobacter* and *Melittangium* were restricted in some areas (Figure 3; Table 1).

Evaluation of antioxidant activities

Data on the 43 myxobacterial extract concentrations that neutralize 50% DPPH• ranged from 27.39 ± 1.74 to 249.43 ± 6.17 µg/mL. The strain with the highest activity was TH41 (IC_{50} = 27.39 ± 1.74 µg/mL, ratio = 2.51), followed by TG131, VL32, CT21, and GL41 with the 50% scavenger concentration of 40.28 ± 1.13 µg/mL (ratio = 3.70), 50.87 ± 1.33 µg/mL (ratio = 4.67), 52.34 ± 1.47 µg/mL (ratio = 4.80), and 57.24 ± 1.52 µg/mL (ratio = 5.25), respectively (Table 2).

All samples showed a high capacity to scavenge $ABTS^{\bullet+}$ radicals cation. The IC_{50} values from the 43 extracts differed significantly between myxobacterial extracts, varying from 30.28 ± 0.74 to 197.36 ± 2.22 µg/mL (data not shown). The CT21 extract exhibits the highest activity (equivalent 30.28 ± 0.74 µg/mL, ratio = 3.95), followed by GL41, TG131, VL32, and BDi22 extracts for the values of 42.76 ± 0.50 µg/mL (ratio = 5.57), 48.35 ± 0.58 µg/mL (ratio = 6.30), 57.22 ± 0.69 µg/mL (ratio = 7.46), and 65.25 ± 0.86 µg/mL (ratio = 8.51), respectively (Table 2).

The crude extract from CT21 displayed the highest antioxidant activity in both assays (IC_{50} = 52.34 ± 1.47 µg/mL, ratio = 4.80 for DPPH assay, and 30.28 ± 0.74 µg/mL, ratio = 3.95 for ABTS assay), proving the scavenging capacity of antioxidants towards DPPH• and $ABTS^{\bullet+}$. For TG131, the measured IC50 values were 40.28 ± 1.13 µg/mL (ratio = 3.70) and 48.35 ± 0.58 µg/mL (ratio = 6.30) for the DPPH and ABTS assay, respectively. The GL41 demonstrated a low half-maximal inhibitory concentration of 57.24 ± 1.52 µg/mL (ratio = 5.25) and 42.76 ± 0.50 µg/mL (ratio = 5.57) in the DPPH and ABTS assays, respectively.

Evaluation of antimicrobial activities

The results of MIC of myxobacterial extracts were summarized (Table 3). It showed that all the extracts had antimicrobial activity on at least one of ten test microorganisms, with broadly varied concentrations ranging from 1 to 512 µg/mL. In this study, 40, 23, and 18 strains produced bioactive metabolites against MRSA, MSSA and *S. faecalis*, respectively. However, there are only 16 strains against *P. aeruginosa*, and only one strain was found that inhibited *E. coli*. All strains show antifungal effects, in which the highest was 86% of extracts against *Mucor* sp., and the lowest was 55.8% against *C. albicans*. The susceptibility of extracts on Gram-positive bacteria and mold is better than on Gram-negative bacteria and yeast, respectively (Table 3).

Table 2. High antioxidant activities of some myxobacterial strains

Samples	DPPH assay		ABTS assay	
	IC_{50} (µg/mL)	IC_{50} ratio	IC_{50} (µg/mL)	IC_{50} ratio
BDi22	102.45 ± 2.75	9.40	65.25 ± 0.86	8.51
BT43	87.32 ± 2.35	8.01	85.02 ± 1.12	10.08
BT92	96.35 ± 2.51	8.84	118.67 ± 1.67	15.47
CT21	52.34 ± 1.47	4.80	30.28 ± 0.74	3.95
GL41	57.24 ± 1.52	5.25	42.76 ± 0.50	5.57
HCM81	122.87 ± 3.35	11.28	67.14 ± 0.85	8.75
HG223	121.30 ± 3.22	11.13	75.59 ± 0.94	9.85
LA44	81.38 ± 2.28	7.47	110.75 ± 1.35	14.44
TG131	40.28 ± 1.13	3.70	48.35 ± 0.58	6.30
TH41	27.39 ± 1.74	2.51	116.70 ± 1.43	15.29
TV51	112.13 ± 2.81	10.29	70.97 ± 0.86	9.25
VL32	50.87 ± 1.33	4.67	57.22 ± 0.69	7.46
Trolox	-	-	7.67 ± 0.82	-
Ascorbic acid	10.90 ± 0.89	-	-	-

Table 3. Antimicrobial activity of isolated genera (µg/mL)

Genera	Test microorganisms									
	MR	MS	Sf	Ec	Pa	Ca	Mu	Rh	Pe	An
Angiococcus (n = 1)	1	-	-	-	-	-	1	1	-	-
Archangium (n = 3)	3	3	2	-	1	2	2	2	-	1
Chondromyces (n = 2)	1	1	1	-	1	-	1	2	2	2
Corallococcus (n = 15)	15	7	4	-	5	7	10	13	12	15
Cystobacter (n = 1)	1	-	-	-	-	-	-	-	1	1
Melittangium (n = 1)	1	1	-	-	-	1	-	1	1	1
Myxococcus (n = 20)	18	11	11	1	9	14	17	18	14	16
Total (n = 43) (%)	40	23	18	1	16	24	31	37	30	36
	93.0	53.5	41.9	2.3	37.2	55.8	72.1	86.0	69.8	83.7

Note: MR: MRSA, MS: MSSA, Sf: *S. faecalis*, Ec: *E. coli*, Pa: *P. aeruginosa*, Ca: *C. albicans*, Mu: *Mucor* sp., Rh: *Rhizopus* sp., Pe: *Penicillium* sp., and An: *A. niger*

Table 4. MIC values of isolates showing highly antimicrobial properties (μg/mL)

Samples	MR	MS	Sf	Ec	Pa	Ca	Rh	Mu	Pe	An
BD2	4	4	32	-	512	8	4	8	2	1
BDi22	128	512	-	-	-	16	32	64	1	1
BP161	4	1	16	-	512	128	64	128	-	-
BP181	1	1	4	-	512	64	128	256	-	-
CT21	128	64	512	-	-	8	16	-	2	1
GL41	1	1	1	64	128	1	16	16	8	1
HG225	16	64	64	-	512	32	16	16	1	1
NB71	4	1	16	-	512	16	32	16	1	1
VL32	64	256	-	-	-	512	128	64	1	4
Amikacin	4	2	4	1	0.25					
Fluconazole						4				
Amphotericin B							1	1	0.5	1

Note: MR: MRSA, MS: MSSA, Sf: *S. faecalis*, Ec: *E. coli*, Pa: *P. aeruginosa*, Ca: *C. albicans*, Mu: *Mucor* sp., Rh: *Rhizopus* sp., Pe: *Penicillium* sp., and An: *A. niger*

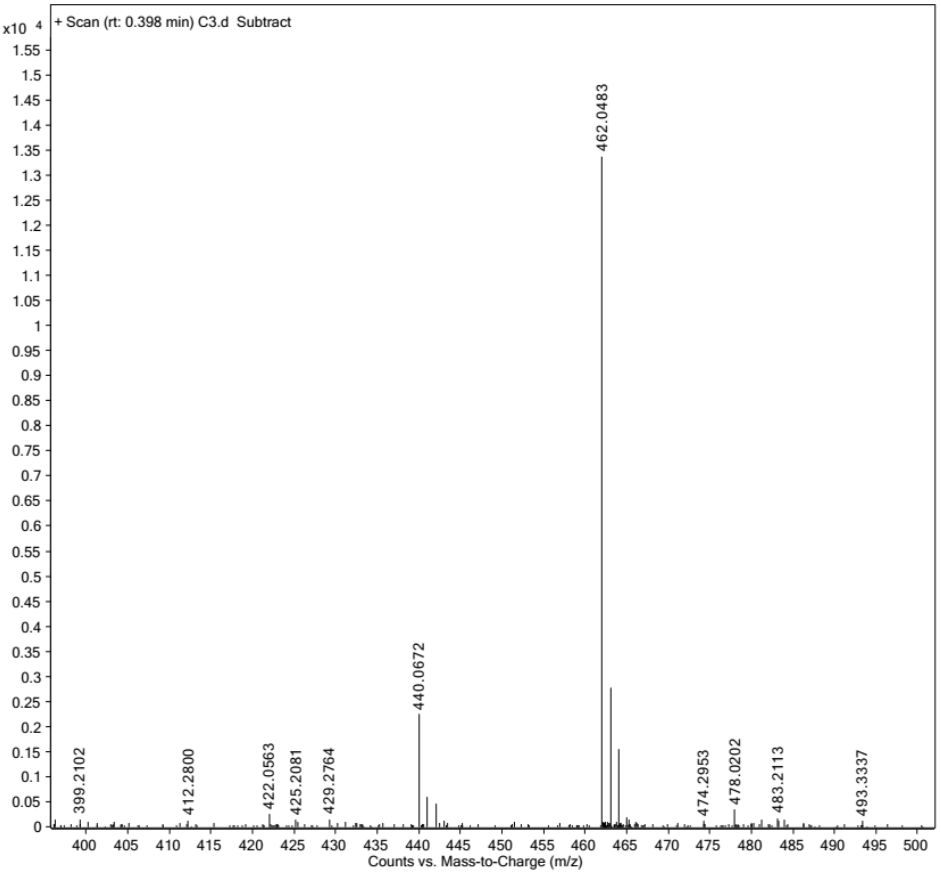

Figure 4. Mass spectrum of the fragment from strain GL41 that has only partially been purified. The presence of althiomycin can be determined thanks to the protonated ion [M+H]+ at 440.0672 *m/z*, [M+Na]+ at 462.0483 *m/z* and [M+H-H_2O] at 422.0563 *m/z*

Significantly active strains (6/9 strains) belonged to the *Myxococcus* genus, these are BD2, BP161, CT21, GL41, HG225, and NB71. Strain GL41 was found to have the highest inhibitory effect on MRSA, MSSA, *S. faecalis*, *C. albicans*, and *A. niger* (MIC = 1 μg/mL). All of the test microorganisms were indicated to be susceptible to GL41. This is also the only extract that showed inhibitory capable to *E. coli*. GL41 extract showed MIC values that were lower than the positive controls, especially on MRSA, MSSA, *C. albicans*, and *A. niger* (Table 4).

Analysis of metabolites with liquid chromatography coupled mass spectrometry

The LC-MS analysis of partially purified fragments from GL41 crude extract identified 01 secondary metabolite peaks as known structures - althiomycin. Mass spectrum determined the protonated ion at *m/z* [M+H]+ 440.0672, [M+Na]+ 462.0483 and [M+H-H_2O]+ 422.0563 (Figure 4).

Discussion

Myxobacterial isolation from natural habitat is still a strategy to uncover prospective chemicals and achieved particular effects since the ability to produce secondary compounds from myxobacteria depends upon species rather than genera (Gaspari et al. 2005). The isolation techniques have also been constantly improved since the first report of Thaxter (1892), which used minimal substrates to limit the growth of contaminants while still providing a specific, selective organic source (living *E. coli* cells, filter paper, and rabbit dung), thereby stimulating vegetative cell growth and fruiting body formation (Mohr 2018). Despite some drawbacks for not being a priority choice, such as (i) long incubation time (2-4 weeks), (ii) a large amount of soil is proportionate to a high density of soil contaminants, (iii) wild rabbit dung availability, the results showed that rabbit dung method gave the highest isolation efficiency (37.2%). In this finding, the heat pre-treatment approach by water-bathing was performed in combination with moisturization with an antifungal solution which delayed the overgrowth of molds 5-10 days later. Then, fruiting bodies were facilitated to appear in contrast to the dung pellet dark substrate for easy recognition (Shimkets et al. 2006), so that the isolation ratio differ from previous report in Xinjiang, China (3.4%) (Zhang et al. 2013). In addition, the filter paper method was selectively designed for the cellulolytic strains, but many non-cellulolytic strains were detected on filter paper e.g., *Chondromyces apiculatus* HCM21, *Myxococcus* sp., *Corallococcus* sp. and *Archangium* sp.

The average isolation rate in this research was 2.2 bacteria strains/soil sample, higher than that observed in published data of Dawid (0.9) (Dawid 2000), Israel (1.5) (Gaspari et al. 2005) and Indonesia (0.3) (Meliah and Lisdiyanti 2018). The purification ratio (0.3) was calculated on the number of purified and identified isolates compared to the number of strains observed from the initial culture plates. The low value often reflects the difficulties because of competition by fast-growing bacteria, molds, and insects, the level of contaminant agents as well as their antibiotic resistance. In addition, the explanation is also due to the fact that the same isolates occur on the plates of the indentical soil sample. Indeed, Kathrin I. Mohr (2016) reduced from over 100 original strains to obtain final 42 different strains (Mohr et al. 2016); or from the original 70 strains, Senlie Octaviana obtained 25 strains (ratio = 0.36) (Octaviana et al. 2022); (ii) The morphological similarities between other bacteria and myxobacteria result in the number of colonies suspected to be myxobacteria being higher than the actual number of strains. *Myxococcus* and *Corallococcus* are both quickly growing bacteria, giving effective isolation yield, so these two genera accounted for a high proportion (81.4%). Strains of the genus *Melittangium* or *Angiococcus* are often challenging to isolation (taken for 4.7%) because the fruiting bodies are underdeveloped, mainly vegetative cells form membranes on the medium surface. Although *Cystobacter* predominates in soil prevalence (Dawid 2000), their fruiting bodies were often submerged in slime or macerated substrate with a lot of contaminants and insects, causing the found isolates was low in present study (contributed 4.7%) (Gaspari et al. 2005). The results showed that 43 isolates assigned to 10 species belonging to 30 myxobacterial species typically isolated from soil samples in the world. In the other side, the rare strain *Chondromyces apiculatus* and the very rare ones (*Chondromyces pediculatus* and *Melittangium boletus*) were found (Dawid 2000).

The current results highlights crucial role of the phylogenetic and morphological coherence in myxobacterial identification. However, the morphological properties are not stable and depend on nutritional factors and culture time, and can be altered, degraded or lost. For instance, members of genera *Stigmatella*, *Cystobacter*, and *Melittangium*, that form *Archangium*-typed fruiting bodies (Spröer et al. 1999) can lead to identification uncertainty. In addition, Martin Dworkin also said that the data shortage on physiological and biochemical characteristics is not meet the differentiating requirements at the species level (Spröer et al. 1999). Myxobacterial identification by molecular biology techniques is an effective tool to align gene sequences with data from Genbank and construct phylogenetic trees. The results exhibited that 43 isolates belonged three main branches identified initially by 16S rRNA cataloging approach. However, small changes in 16S rRNA gene sequences between species prevented the phylogeny tool from satisfying taxonomic information in several instances (especially in high variable regions). Differentiation at the chemotaxonomic level using lipid and protein patterns and detection of species-specific gene sequences, or the use of suborder family-specific primer sequences, might contribute to a deeper comprehension (Spröer et al. 1999; Zhou et al. 2014).

Since three decades ago, myxobacteria have been identified as valuable producers of secondary metabolites with diverse biological functions. The results of this work unraveled and shed light on the scanty understanding of the free radical scavenging capacity from myxobacteria, suggest the presence of potentially endogenous compounds. Furthermore, in this study, all strains were able to inhibit growth against at least one of the 10 strains of test microorganisms, similar to the figure of 19/23 antibacterial strains (Charousova et al. 2017). The number of strains showing antifungal activity was higher than that of bacteria. Indeed, Juana Diez reported that about 54 and 29% of the bioactive compounds were antifungal and antibacterial, respectively (Diez et al. 2012). And similar to Noren and Raper's article, almost myxobacteria capable of inhibiting the growth of Gram-positive bacteria, but no effects on Gram-negative bacteria were noticed (Schäberle et al. 2014). Nearly 70 percent of 1,700 myxobacterial isolates at the Helmholtz Centre for Infection Research (Germany) were active against *S. aureus*, but only 8.1% were active against *P. aeruginosa* (Hoffmann et al. 2018). Mass spectra data of ion at *m/z* [M+H]+ 440.0672, [M+Na]+ 462.0483, and [M+H-H_2O]+ 422.0563 showed the similarity with data in the previous publication of althiomycin. As such, althiomycin, giving the molecular formula $C_{16}H_{17}N_5O_6S_2$ (molecular weight of 439), was first isolated from *Streptomyces althioticus* (1957) and later from *Cystobacter fuscus* (Kunze et al. 1982), and

biosynthetic gene cluster data encoding althiomycin was also found in *Myxococcus xanthus* DK897 (Cortina et al. 2011). It is an antibiotic that inhibits prokaryotic protein synthesis at the peptidyl transfer stage but exhibits no such effects in mammalian cells.

In conclusion, the study has described the isolation and phylogenetic relationship of myxobacteria in Vietnam. Additionally, the antioxidant and antimicrobial activities obtained from P-medium fermentation were determined. Results discovered that the strain *Myxococcus stipitatus* GL41 was determined to provide a promising total extract with impressive antioxidant and antimicrobial activities, opening up subsequent approach in determining the cytotoxic activity. Additionally, future research is encouraged by the reveal of chemical diversity through the mass spectra analysis of the other potent extracts as well as strategy of myxobacterial isolation from unusual geographic regions that may lead to the discovery of new isolates.

ACKNOWLEDGEMENTS

Yen Thi Ngoc Nguyen was funded by the Master, PhD Scholarship Programme of Vingroup Innovation Foundation (VINIF), code VINIF.2022.TS150.

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