A MYXOBACTERIUM STRAIN ISOLATED IN VIETNAM PRODUCES EREMOPHILENE-LIKE SESQUITERPENE

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ABSTRACT

Sesquiterpenes, a class of terpenes, consist of three isoprene units, constituting a highly diverse class of natural bioactive compounds that are found principally in plants but also in fungi and some invertebrates and myxobacteria as well. In this study, by using different approaches for isolation, analysis of morphology and bio-physio-chemical properties and comparison of the sequences of 16S ribosomal RNA genes, three strains belonging to the group of myxobacteria were isolated from soil samples collected from some areas in Vietnam. The sesquiterpene biosynthesis in the isolated strains was investigated. The data showed that the isolated strain DL1 was able to form fruiting body and sysnthesize an eremophilene-like sesquiterpene.

Keywords: 16S rRNA, eremophilene, Myxobacteria, sesquiterpene, secondary metabolites.

Phân lập và phát hiện chủng vi khuẩn Myxobacteria ở Việt Nam có khả năng tổng hợp Sesquiterpene tương tự Eremophilene

TÓM TẮT

Sesquiterpene được tìm thấy chủ yếu ở thực vật, nấm và một số động vật không xương sống. Gần đây, sinh tổng tổng hợp sesquiterpene ở một số vi khuẩn thuộc nhóm myxobacteria đã được phát hiện. Myxobacteria là một nhóm vi khuẩn tiềm năng được nhiều nhóm nghiên cứu trên thế giới quan tâm nhằm khai thác các hợp chất thứ cấp mới ứng dụng trong y dược. Tuy nhiên cho đến nay ở Việt Nam hầu như chưa có nghiên cứu về phân lập và xác định đặc điểm của các vi khuẩn này. Trong nghiên cứu này, bằng cách sử dụng các kỹ thuật phân lập, quan sát mô tả hình thái, đặc điểm sinh lý hóa và so sánh trình tự 16S rRNA, 3 chủng vi khuẩn thuộc nhóm myxobacteria đã được phân lập từ các mẫu đất thu thập ở nhiều địa phương khác nhau ở Việt Nam. Khảo sát khả năng tổng hợp sesquiterpene từ các mẫu vi khuẩn phân lập cho thấy chủng DL1 có khả năng sinh thể quả và tổng hợp một sesquiterpene giống như eremophilene.

Từ khóa: 16S rRNA, Eremophilene, hợp chất tự nhiên, Myxobacteria, Sesquiterpene.

1. INTRODUCTION

Sesquiterpenes constitute a highly diverse class of natural bioactive compounds for medicine, flavors and fragrance ingredients, food additives, and agrochemicals (Bártíková *et al.*, 2014). So far, sesquiterpenes have been reported as secondary metabolites synthesized mainly in higher plants, fungi, and some invertebrates. Recently, some research groups have found that the biosynthesis of sesquiterpenes occurs in some bacteria, notably, myxobacteria. These bacteria have been reported as an excellent natural sources for the discovery of new polyketides for wide-range application (Wenzel and Müller, 2007).

Myxobacteria are large groups of bacteria living predominantly in the soil. These bacteria do not have flagella and move on solid surfaces by gliding (Schneiker *et al.,* 2007). Of proteobacteria, only myxobacteria belonging to delta protobacteria (δ-proteobacteria) are able to develop fruiting body under unfavorable conditions including pH, temperature or lack of nutrient. The spores (myxospores) are separated or grouped together to form fruiting body that can appear on the surface of the medium (observed in the genera *Melittangium and Stigmatella*) or in the medium (observed in the genera *Angiococcus, Polyangium, Cystobacter, and Sorangium)* (Shimkets *et al.*, 2006). The fruiting bodies of myxobacteria vary from species to species with different size, shape and colour (Shimkets *et al.*, 2006). The life cycle of myxobacteria is quite complicated because of the change of morphology in different growth stages including vegetative stage, myxospore and fruiting body. The ability to form fruiting body is one of the easiest sign to be recognized and it is also one of the interesting characteristics of myxobacteria.

So far, more than 100 different basic compounds and approximately 500 structural variants have been isolated from myxobacteria (Reichenbach, 1999; Gerth *et al.*, 2003). About 7.500 different myxobacteria have been isolated and analyzed chemically (Gerth *et al.*, 2003; Wenzel and Müller, 2007). In the large group of myxobacteria, the genus *Sorangium* produces nearly 50% of total metabolites isolated from myxobacteria (Gerth *et al.*, 2003; Schneiker *et al.*, 2007). With a high potential of application, isolation and characterization of new strains of myxobacteria are of significance.

In this study, soil samples collected from different areas of Vietnam were used for isolation of myxobacteria. Analysis of morphology, characterization of bio-physio-chemical properties and comparison of the sequences of the 16S ribosomal RNA gene from isolated strains were carried out. Sesquiterpenes released from culture media of these strains were also analyzed.

2. MATERIALS AND METHODS

2.1. Collecting soil samples

Soil samples collected from different areas in Vietnam, i.e. Da Lat, Bac Giang, Gia Lam, Kon Tum, Ninh Binh, Soc Son, Son Tay, Thai Nguyen, Tuyen Quang, Vinh Phuc, Yen Bai were used to isolate myxobacteria. Collected soil samples were dried and stored in closed plastic bags at 25 to 30°C and moisture from 75 to 85%.

2.2. Isolation media and antibiotics

For isolation of myxobacteria, several media ST21, ST21CX, VY/2, WAT, EBS and LB were used (Reichenbach and Dworkin, 2001). The components of the ST21 medium included macro elements (g/L): K_2HPO_4 1, yeast extract 0.02, KNO_3 1, $MgSO_4.7H_2O$ 1, $CaCl_2.2H_2O$ 1, $FeCl_3$ 1, $MnSO_4.7H_2O$ 0.1, agar 10, and microelements (mg/L): $MnCl_2.4H_2O$ 0.1, $CoCl_2$ 0.02, CuSO4 0.01, $Na_2MoO_4.2H_2O$ 0.01, $ZnCl_2$ 0.02, LiCl 0.005, $SnCl_2.2H_2O$ 0.005, H_3BO_3 0.01, KBr 0.02, EDTA Na-Fe^{3+} 0.008. The ST21CX medium was modified from ST21 by adding 25 µg/ml cycloheximide. The medium VY/2 included (g/L): Baker's yeast 5, $CaCl_2.2H_2O$ 1, cyanocobalamin 0.5,agar 15**.**The WAT medium included: $CaCl_2.2H_2O$ 1.1% (w/v), agar 1.5% (w/v). The medium EBS included (w/v in percent): peptone from casein 0.5, proteosepeptone 0.5, peptone from meat 0.1, yeast extract 0.1, pH 7.0. The LB medium included (w/v in percent): peptone 1, yeast extract 0.5, NaCl 1, pH 7.5. Several antibiotics including cyclohexamide (CX), nystatin (NYS), kanamycin (KA), gentamicin (GEN) were used at 2.5 mg/100 mL.

2.3. Isolation of myxobacteria

The collected soil samples were treated with a combination of two antibiotics CX and KA before isolation. A combination strategy using different methods were applied to isolate myxobacteria including trapping method by rabit dung, filter paper based ST21CX medium, YV/2 medium for the formation of spore and fruiting body, andWAT-*E.coli* based medium for

development of fruiting body (Reichenbach and Dworkin, 2001; Shimkets *et al.*, 2006; Hyun *et al.*, 2008; Li *et al.*, 2013).

2.3.1. Enrichment of myxobacteria by trapping method

The dry soil samples were prepared as a layer with 10 - 15 mm thick on a petri dish and kept moist by sterile water during the incubation time. Dried rabit dung pellets (about 0.5 cm) were sterilized by autoclaving and put on the dry soil samples and incubated at 30°C (Figure 1). After one week, the dung pellets were transferred to the filter paper based ST21CX medium.

2.3.2. Isolation on the filter paper based ST21CX medium

The filter paper based ST21CX medium used for isolation myxobacterium was previously described by Shimkets *et al.* (2006), Hyun *et al.* (2008), Li *et al.* (2013). Soil samples were spread on a sterile filter paper and plated on the ST21CX medium and incubated at 30°C for 20 to 30 days. The formation of bacterial colonies and fruiting bodies were observed continuously during the incubation period.

2.3.3. Isolation on VY/2 medium

The VY/2 medium used to evaluate the bio-physiological properties of isolated myxobacteria including the formation of spore and fruiting body was previously described by Shimkets *et al.* (2006) and Hyun *et al.* (2008). The development of fruiting bodies on the surface layer of the medium was visualized based on typical sign of myxobacteria. The fruiting bodies on the ST21CX medium were carefully collected and transferred to VY/2 medium to observe gliding of the bacterium and the formation of fruiting bodies. A different combination of antibiotics were applied to avoid contamination for the isolates of myxobacteria.

2.3.4. Isolation on WAT-E. coli based medium

The WAT-E. coli based medium was used for the purification of myxobacteria (Shimkets *et al.*, 2006). *E. coli* obtained from liquid media was sterilized and spread on agar WAT medium to form WAT- *E. coli* based medium. The fruiting bodies of myxobacteria on ST21CX medium were streaked on WAT- *E. coli* based medium to observe further development of the fruiting bodies.

2.3.5. Purification of myxobacteria

Myxobacteria were purified using fruiting bodies obtained from selected media. The purification steps were carried out several times to remove bacteria that may contaminate the gliding colonies. Two alternative methods based on temperature and antibiotics were applied. When the fruiting bodies or spores were formed on selected media, the temperature was increased to 58°C for 10 minutes to eliminate some bacteria (Reichenbach, 1983). In the methods using antibiotics, the fruiting bodies were transferred to EBS medium and shaked at 30°C overnight in the presence of combinations of different antibiotics (CX,KAN, GEN and NYS). This step was carried out several times and the fruiting bodies were then transferred to the VY/2 medium for the formation of the vegetative form of myxobacteria (Reichenbach, 1983).

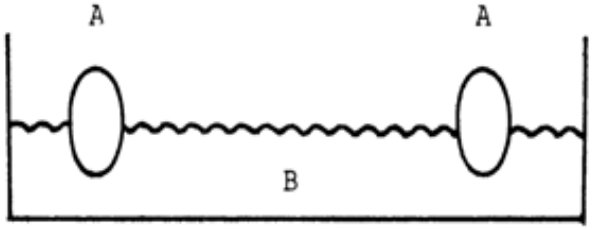

Figure 1. Draw showing the trapping method to enrich myxobacteria

Note: A: Sterilized dried rabbit dung pellets, B: soil sample

2.4. Characterization of bio-physio-chemical properties of isolated bacteria

The cellulase activity of myxobacteria, especially *Sorangium* were observed by the degradation of filter paper on the ST21CX medium in 10, 15, 20, 25, 30 days. The sterile paper was plated on the surface of the medium. The method was applied for the cells in the vegetative stage or gliding form. An agar medium containing cellulose as substrate was also applied to observe the cellulase activity (Reichenbach and Dworkin, 1986). The gram staining was carried out as described (Gram,1884).

2.5. DNA extraction, amplification and analysis of 16S ribosomal RNA

The pure isolated myxobacteria were cultured on liquid ST21CX medium for 4 days at 26ºC with shaking at 200 rpm/min. Cell pellets were collected by centrifuge at 10.000 g for 10 min. The DNA extraction was carried out according to the method described by Wilson (2001). The 16S ribosomal RNA sequence was amplified using primers W4F (GTAAGACAGAGGGTGCAAACGT) and 16SmycoR (GGGTTGCGCTCGTTGCG) (Wu *et al*., 2005; Hyesook *et al*., 2008). The PCR (25 µl) included: 0.5 µl DNA (~100 ng); 0.5 µl W4F (10 pmol), 0.5 µl 16SmycoR (10 pmol); 1.5 µl $MgCl_2$ (25 mM); 2.5 µl 10X PCR buffer; 2 µl dNTP (2.5 mM); 0.25 µl Taq DNA polymerase (5 U/µl). PCR thermal cycle: 95°C/4 min; 35 cycles (94°C/1 min; 53°C/1 min; 72°C /45 min); 72°C/10 min. PCR product was purified with QIAquick PCR Purification Kit (QIAGEN) and sequenced. The data were analyzed using Bioedit program and the sequences were compared with nucleotide database in GenBank by BLAST at NCBI (www.ncbi.nlm.nih.gov).

2.6. Solid phase micro-extraction- gas chromatography-massspectroscopy (SPME-GC-MS) analysis of volatiles

The isolated strain D1 was grown in ST21CX liquid medium in a headspace flask sealed with septum and aluminium cap. Carboxen-polydimethylsiloxane (CAR/PDMS, 75 µm) fibre with manual holder purchased from Sigma-Aldrich was used for the extraction of volatiles. The volatiles were collected for 24h, and then analyzed by GC/MS as previously described (Ly *et al*, 2017). Volatiles were extracted by exposing the solid phase microextraction (SPME) fibre to the headspace of the culture flask that was maintained at 40°C for 30 min. For thermal desorption, the SPME fibre was quickly inserted into the GC injector. A desorption time of 3 min at 250°C was used in split mode (1:10).

3. RESULTS AND DICUSSION

3.1. Isolation of myxobacteria

As described by Reichenbach (1983), dung of many herbivores such as sheep, goat and rabit is natural substrate sources as effective baits for the isolation of myxobacteria. Many strains of myxobacteria, such as *M. fulvus, M. virescens, Sorangium cellulosum* were also often found in dung of herbivores (Reichenbach,1999). In the present study, rabit dung pellets were utilized to trap myxobacteria (Figure 2).

After 1 week of incubation, rabit dung pellets were transferred to filter paper based ST21CX medium. Some colonies were formed on the filter paper but this process took a long time, up to several weeks (data not shown). Although this method was efficient as reported elsewhere, in this study, however, the amount of fruiting bodies appeared very low and hardly to observe. Therefore, an alternative method was carried out by placing soil samples directly on the surface of the filter paper based ST21X medium (Figure 3A, B, C). Athough various combinations of antibiotics and fungicide such as cyclohexamide, nystatin, kanamycin, and gentamycin were used in the isolation and purification steps, cycloheximide treated soil samples were most efficient because this antibiotic inhibited the protein production of eukaryotic cells including fungi. The data of antibiotic selection and inducing for fruiting bodies are summarized in Table 1. Results

showed that the duration for growth and degradation of filter paper was about 10 days. In some samples, molds were observed after the 10th day of incubation. Due to the ability to degrade filter paper and form fruiting bodies, colonies of myxobacteria are quite easy to select. Several cycles of purification were carried out by a combining formation of fruiting bodies and germination of myxospores from collected fruiting bodies (Figure 3B, D, E).

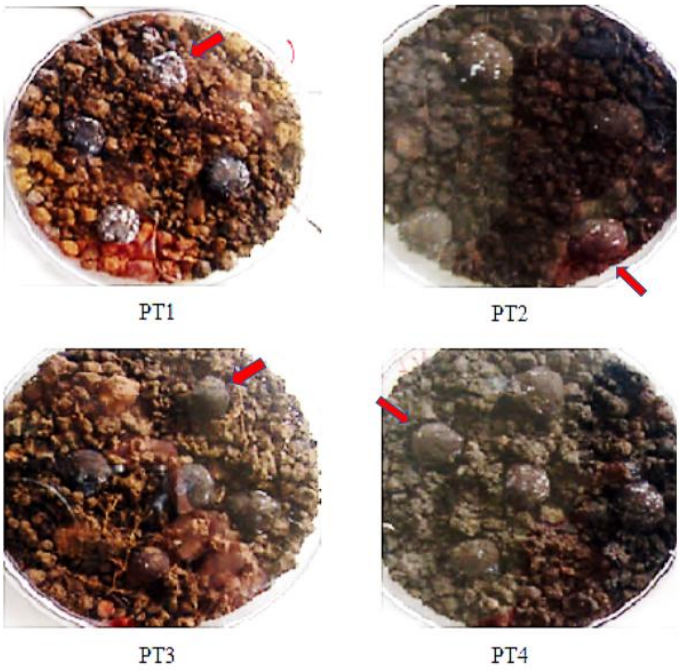

Figure 2. Trapping of myxobacteria by using rabit dung pellets

Note: PT1-2 and PT3-4 are soil samples collected from Gia Lam and Ba Vi, respectively. Arrows indicate the rabit dung

Table 1. Influence of antibiotic treatment on soil samples

Soil sample	Antibiotic treatment				
	CX+ NYS	CX+ KAN	CX+GEN	CX+KAN+NYS	CX+KAN+GEN
DL1	+	+	C	-	-
BG4.1	+	+	-	-	*
BG6.1	+	-	-	-	*
BG6.2	C	C	C	+	-
BG10	-	+	-	*	
GL1	+	+	-	-	-
KT1	-	-	+	*	*
NB5	C	C	C	-	+
SS1	+	C	-	*	*
ST1	C	C	C	-	+
TN1	+	-	-	*	*
TQ7	+	+	-	*	*
VP1	C	C	-	C	+
YB2	+	+	-	*	*

Note: DL, BG, GL, KT, NB, SS, ST, TN, TQ, VP, and YB were soil samples collected from Da Lat, Bac Giang, Gia Lam, Kon Tum, Ninh Binh, Soc Son, Son Tay, Thai Nguyen, Tuyen Quang, Vinh Phuc, Yen Bai, respectively. The number in dicates the number of collected samples. C: contamination, (-) no colony; (+): colony forms fruiting body; (*): colony without forming fruiting body

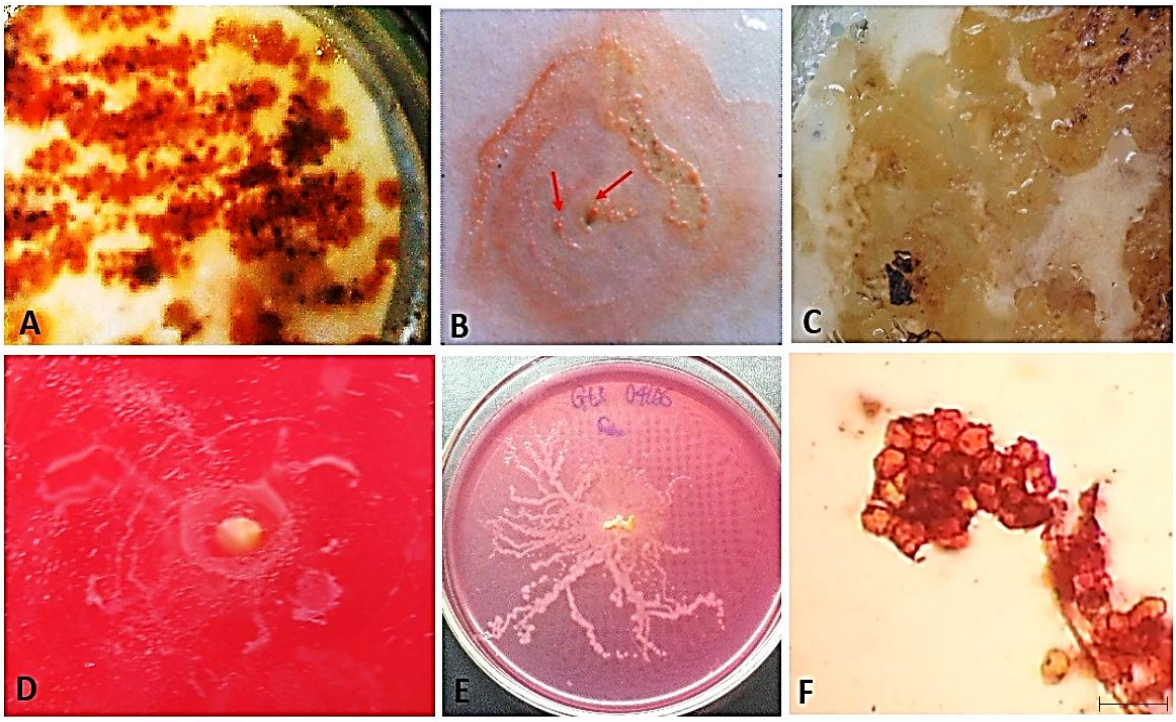

Figure 3. Morphology and formation of fruiting bodies of isolated bacteria

Note: A: Growth and gliding of vegetative form on filter paper based ST21CX medium; B: formation of fruiting bodies, red arrows indicate the fruiting bodies; C: gliding of bacteria on filter paper based ST21CX medium (vegetative stage); gliding of bacteria on VY/2 medium; F: an opened fruiting body containing myxospores observed under microscope (gram negative), scale bar indicates 10 µm.

Several types of colonies with colours were observed on filter paper including white, yellow, orange, pink and brown that spread on the filter papers adjacent to the position of soil samples (Figure 3A, C). This phenomenon was very common in a number of reports (Shimkets *et al.*, 2006). After 20 days of incubation, isolated bacteria from 26 soil samples formed fruiting bodies. Although it has been reported that the fruiting bodies are formed under unfavorable conditions, in this study, the formation of fruiting bodies was observed when there was a lack of nutrients or the surface of the filter paper became dry. Fruiting bodies appeared in some samples from the 20th days of incubation and existed in a short time, from 2 to 3 days. By using gram staining method, the myxospores from the fruiting body of the strain DL1 were observed (Figure 3F). The morphology of the myxospores from strain DL1 was similar to those of *Sorangium cellulosum* (Gerth *et al.*, 2003).

3.2. Sequence comparison and analysis

The isolated DNA samples from purified strains GL2, YB2, DL1, NA2 and TQ7 (Figure 4, left) were used as template for PCR to amplify the 16S ribosomal RNA gene (16S rDNA). Data showed that the size of 16S rDNA was about 600 bp (Figure 4, right). The sequences of the 16S rDNA were obtained by service of 1st BASE sequecining Company (data not shown). Local alignment search tool (BLAST) with sequences of 16S rDNA as queries revealed that the 16S rDNA sequence of the strain GL2 showed the highest similarity (99%) to *Sporocytophaga myxococcoides* DSM 11118 (NR025463) (Figure 5A) and the 16S rDNA sequence of the strain YB2 showed 98% similarity to uncultured bacterium KMS200711-118 (EU881332.1) and 97% similarity to *Myxococcales bacterium* (FJ435064) (Figure 5B). The 16S rDNA sequence of DL1 showed similarity of 99% to *Polyangium* sp. (KC862608) and 98% to *Sorangium cellulosum*

strain X3T8 (HQ623117) (Figure 5C). The 16S rDNA sequences of the strains NA2 and TQ7 were highly similar to those of *Pseudomonas* sp. (data not shown). In general, by the sequence comparison, the isolated bacteria with 97-98% similarity of 16S rRNA were classified into an operational taxonomic unit. All isolated strains belonged to delta-protobacteria. Based on the morphology, bio-physio-chemical properties, the bacterial strains GL2 and YB2 and DL1 samples were classified into myxobacteria and belonged to *Sporocytophaga myxococcoides* GL2, *Myxococcales bacterium* YB2 and *Polyangium* sp. DL1, respectively.

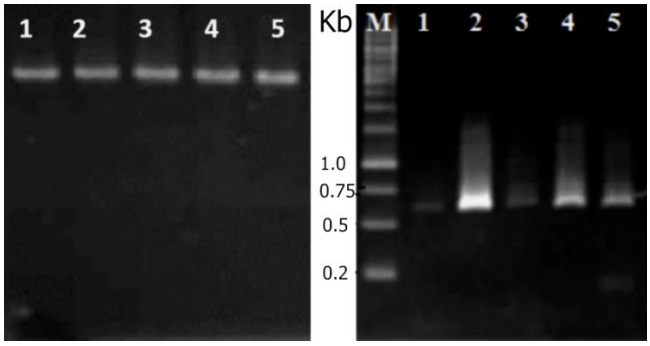

Figure 4. DNA extraction and PCR amplification of 16S ribosomal RNA

Note: Agarose gel electrophoresis of total DNA of samples GL2, YB2, DL1, NA2 and TQ7 (left) and PCR products of 16S ribosomal RNA (right), respectively. M: DNA ladder FluoroBand 1KB (0,25-10kb). The PCR products have size of about 600 bp (0.6 Kb)

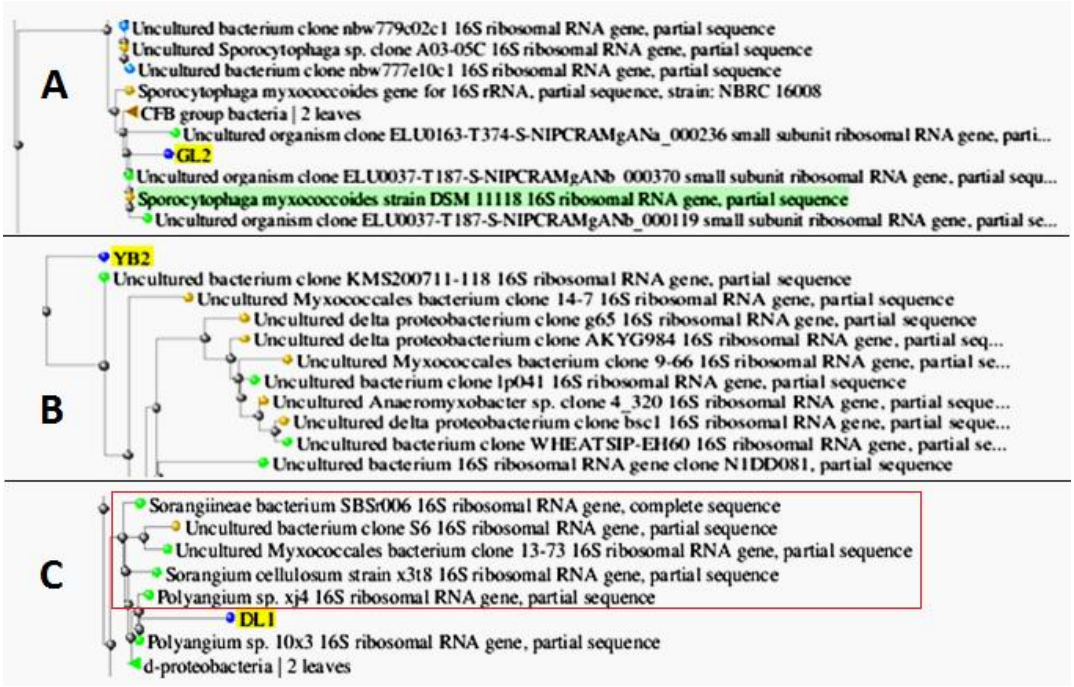

Figure 5. Phylogenetic analysis of 16S rDNA of isolated bacteria

Note: A: 16S rDNA of GL2 shows 99% similarity to Sporocytophaga myxococcoides DSM 11118 (NR025463); B: 16S rDNA of YB2 shows 97% similarity to Myxococcales bacterium (GenBank accession number: FJ435064); C: 16S rDNA of DL1 shows 99% similarity to Polyangium sp. (KC862608) and 98% to Sorangium cellulosum strain X3T8 (HQ623117). All isolated strains belong to delta-protobacteria. The phylogenetic trees (A, B, C) were plot directed from the online BLAST at NCBI. No bootstrap values were displayed

3.3. Sesquiterpene detection from volatile by SPME-GC/MS analysis

A solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC/MS) was set up to detect volatile compounds released from the culture. Analyzing the entrapped volatile compounds from the cultures of three isolated strains revealed that in the metabolites of the strain DL1, sesquiterpene was detected with a relatively high content (Figure 6). The relative ion abundances of this compound were similar to those of eremophilene produced by myxobacterium *Sorangium cellulosum Soce56* (Schifrin *et al.*, 2015) for ion fragments: 53, 67, 79, 90, 105, 118, 133,1 47, 161, 189, 204 m/z ions (Figure 7). This suggested that the detected sesquiterpene was likely eremophilene. In addition, GC-MS analysis also revealed several types of volatile compounds including ketones, alcohols, esters, and aromatic compounds (Figure 6A). Several reports showed that eremophilene could be also oxidized to form multiple derivatives which may have cytotoxic, anti-inflammatory and antiviral properties (Schifrin *et al.*, 2015). In terms of ecological function, the sesquiterpene eremophilene may help the myxobacteria to "communicate" or showing social activity to facilite the complex lifestyle such as gliding and forming of fruiting bodies upon starvation or stress condition.

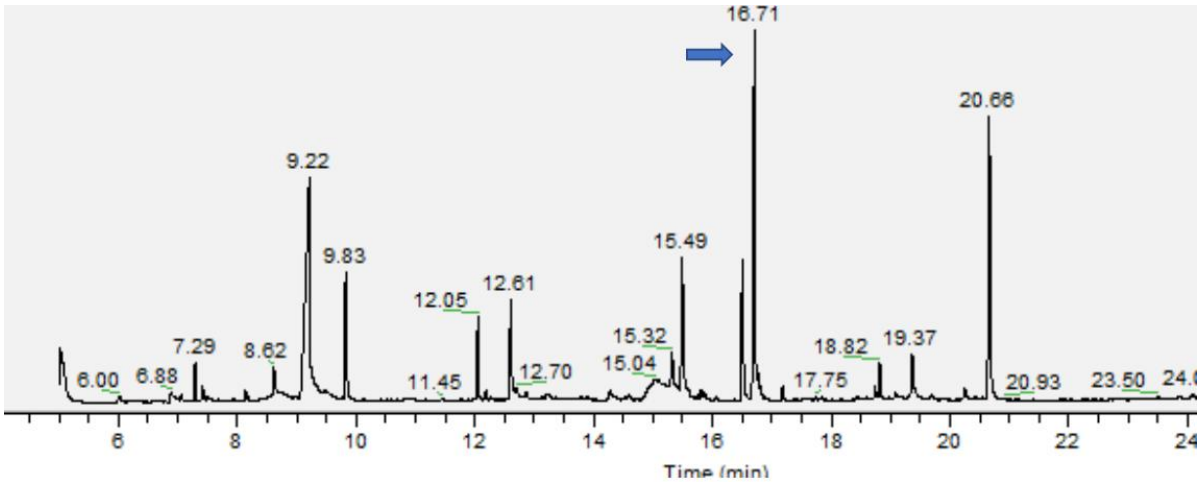

Figure 6. Total ion chromatograms of volatile metabolites

Note: Volatile metabolites collected from the culture medium of the isolatedstrain DL1. The arrow represented the peak with highest content of a sesquiterpene

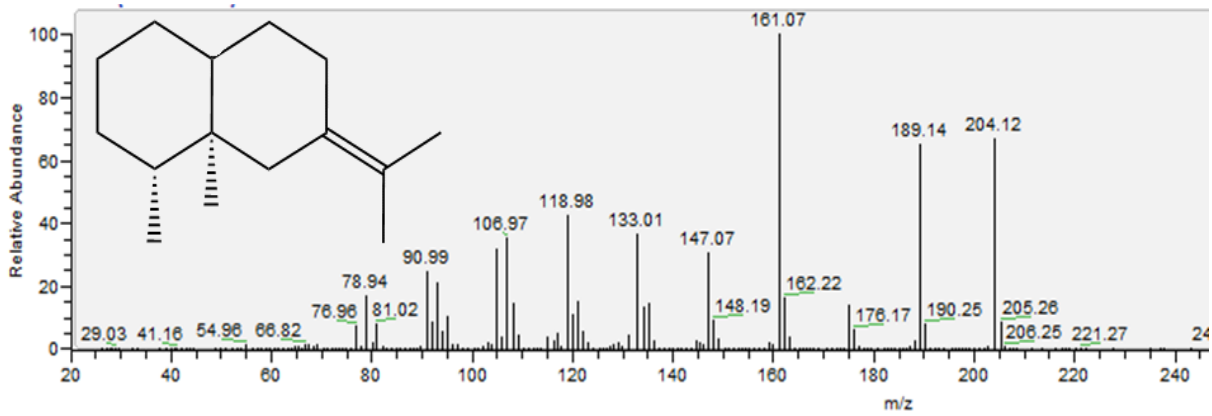

Figure 7. Mass spectra of the detected sesquiterpene

4. CONCLUSION

Study on morphology, bio-physio-chemical properties and phylogenetics of isolated soil bacteria showed that three bacterial strains GL2, YB2, DL1 were likely classified into the group of myxobacteria. These bacterial strains were able to glide and form fruiting bodies on the filter paper based ST21CX medium. The strain DL1 showed 99% similarity to *Polyangium* sp. and 98% to *Sorangium cellulosum* strain X3T8 and was able to produce a sesquiterpene-like eremophilene. With a high potential of application, isolation and characterization of new strains of myxobacteria, especially in Vietnam, might contribute to the exploitation of novel secondary metabolites for pharmaceuticals, fragrances and agrochemicals.

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