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Identification of hydrocarbon-degrading bacterial consortium isolated from the oil-contaminated muddy soil in Hanoi, Vietnam

D. T. Tuyen, N. T. K. Thanh, N. X. B. Khoa, N. C. Cuong ✉

Joint Russia–Vietnam Tropical Science and Technology Research Center
63 Nguyen Van Huyen St., Cau Giay, Ha Noi, Vietnam

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Abstract. Bioremediation is a promising approach for treating oil-contaminated environments. The main objective of this study was to isolate bacteria capable of degrading hydrocarbons for application in oily wastewater treatment from oil-contaminated sites in Hanoi, Vietnam. The bacterial consortium studied was obtained from an oil-contaminated muddy soil sample enriched with crude oil mixed with diesel oil as a carbon source. The reconstituted consortium was able to degrade 93% of the oil content after 7 days of testing. A total of five pure bacterial strains were isolated on TSA agar from the complex microbial communities and were selected as potential candidates for oily sludge biodegradation processes. These isolates were identified based on their morphological and biochemical characteristics. By using molecular biology techniques, five hydrocarbons degrading bacteria were investigated and identified as *Pseudomonas mendocina* strain MD1 (OL687411.1), *Pseudomonas hydrolytica* strain MD2 (OL771695.1), *Brucella intermedia* strain MD3 (OL687412.1), *Pseudomonas stutzeri* strain MD4 (OL687413.1), and *Stenotrophomonas nitritireducens* strain MD5 (OL687414.1). The morphological and biochemical characterization of these bacteria showed that five of them were Gram-negative, rod-shaped, catalase positive, the ideal pH was neutral, and the optimum growth temperature was 30ºC in a culture medium with a salinity of 0.5%. These strains are capable of producing extracellular enzymes, such as lipase, amylase, cellulase, and protease.
Keywords: bacterial consortium, biodegradation, hydrocarbons-degrading bacteria, oily wastewater, treatment

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✉ *Corresponding author*. Biotechnology Sub-Institute, Joint Russia–Vietnam Tropical Science and Technology Research Center, Vietnam.

ORCID and e-mail addresses: Do Thi Tuyen: https://orcid.org/0000-0003-0070-5425, tuyendodhkh@gmail.com; Nguyen Thi Kim Thanh: https://orcid.org/0000-0001-9840-7527, kimthanhnt2301@gmail.com; Nghiem Xuan Bach Khoa: https://orcid.org/0000-0002-7046-1369, khoa.bach01@gmail.com; Ngo Cao Cuong: https://orcid.org/0000-0002-9286-0571, cuongnc@vrtc.org.vn.

INTRODUCTION

Up to now, petroleum hydrocarbons products are still an indispensable source of raw materials and fuel in an industrial society, effectively serving socio-economic development. However, the exploitation, processing, and oil transportation often lead to the release of oil into the environment. Use of petroleum products also cause many harms, especially many types of untreated petroleum are discharged polluting on both soil and water environment (Nandy et al., 2020). Due to its hazardous effects, it is necessary to treat oily appropriately (Khusnuryani et al., 2016).

Oil-polluted effluents come from many different sources. Oily wastewater is a mixture of polyaromatic hydrocarbons, petroleum hydrocarbons, phenols …, which are numerous toxins and difficult to degrade (Tian et al., 2018). The process of the mineralization of organic chemicals depends on dehydrogenase activity of oil-degrading microorganisms. The result of biological breakdown of hydrocarbons is to the formation of CO_2, H_2O, and microorganism biomass. Therefore, crude-oil biodegradation in the environment by mixed microbial populations and aerobic hydrocarbon-degrading (ex-situ treatments) is a popular approach for bioremediation of regions contaminated because of ability to utilize diesel oil as sole source of carbon of many naturally occurring microorganisms (Welz et al., 2021).

Although several studies have reported for the degradation of multiple hydrocarbons by pure culture bacteria, such as *Mycobacterium* sp., *Stenotrophomonas* sp., and *Sphingomonas* sp. (Mariano et al., 2007; Patowary et al., 2016; Kumari et al., 2018). However, some studies of using bacterial consortium in waste treatment have been conducted. The results indicated that the efficiency of degradation process was enhanced by a bacterial consortium rather than a single strain because their enzymatic and metabolic function was stimulated by the communities involved as a consortium (Sathishkumar et al., 2008). Bento showed that the inoculum of pre-selected indigenous strains of microorganisms from their own environment is the best simple approach of cleaning in situ bioremediation of diesel-contaminated soils (Bento et al., 2003). Kumari proposed that the consortium of bacteria has significant potential in remediation of multiple polycyclic aromatic hydrocarbons compounds in crude oil (Kumari et al., 2018).

The inoculation of an enriched mixed microbial consortium into water waste is a strategy for ex-situ treatments biodegradation water waste contaminated with diesel oil. In this paper, the bacterial strains were isolated from an enriched bacterial consortium capable of degrading crude oil mixed in diesel oil (DO). And the objective of this study was also to identify the bioremediation diesel oil microbial consortium from enriched samples.

MATERIAL AND METHODS

Samples, media and culture. Nine oil-contaminated muddy soil samples for the isolation of bacteria capable of degrading crude oil were collected from oil contaminated sites in Hanoi, Vietnam. Surface sediment samples were collected at some wastewater discharge outlets by using a sterile stainless steel spoon, packed in sterile plastic bags for transport to the laboratory and stored at 4ºC (Mariano et al., 2007).

Mineral salt medium (GOST 9023-74) contained the following: KNO_3 – 4 g; KH_2PO_4 – 0.5 g; Na_2HPO_4 – 1.4 g; $MgSO_4$ – 0.8 g, distilled water 1L, addition 5% crude oil mixed in DO (at a ratio of 5:95). The DO fuel used for enrichment and the petroleum products used for biodegradation experiments were collected from petroleum company (Petrolimex, Vietnam) (Hien et al., 2013). For solid medium, GOST 9023-74 broth was supplemented with 20 g/L agar. All the media were autoclaved at 121ºC for 30 min. The cultures were grown at 30ºC in an incubator or a rotary shaker at 180 rpm for liquid media (Patowary et al., 2016).

Isolation and selection of crude hydrocarbons-degrading bacteria. Oil-contaminated muddy soil samples were used as a natural source for oil degrading bacteria. Enrichment culture technique using GOST 9023-74 medium was applied to isolate bacteria. Enrichment of microbial culture was carried out in 1000 mL Erlenmeyer flasks containing 300 mL of GOST 9023-74 liquid medium supplemented with 5% crude oil mixed in DO at a ratio of 5:95 (w/v). Soaking a total of 10 g oil-contaminated muddy soil samples in 100 mL sterile water contained in 250 mL conical flask. The flask was adequate shaken and blended at 180 rpm for 30 min, after that standing for 20 min. 30 mL of the supernatant was extracted and added into 300 mL GOST 9023-74 liquid medium containing 5% crude oil mixed in DO (w/v) as the only energy and carbon source (Patowary et al., 2016). These flasks were incubated on a rotary shaker of 180 rpm at 30ºC for 7 days. At weekly intervals during the initial enrichment, 30 mL of the enrichment culture was extracted and transferred into the same fresh medium. After three consecutive cultures, total bacteria were isolated and numbered by using the pour dish technique on plate count agar (Sathishkumar et al., 2008). The liquid culture (1 mL) of each sample was added to 9 mL of physiological saline solution and agitated for 1 min. The liquid culture was performed tenfold serial dilution to a concentration of 10-8, 10-9, 10-10. Spreading 100 µL of each dilution aliquots aseptically onto a range of culture Tryptic soy agar (TSA) media (containing peptone from casein 15 g/L, peptone from soymeal 5 g/L, NaCl 5 g/L, agar 15 g/L) in Petri dishes and incubated for 24 – 48 h at 30ºC. The observation and counting of colony-forming units (CFU) was performed on the plates appearing 25 – 250 colonies. The single isolates with phenotypic differences were streaked onto similar medium to obtain pure strains of oil-degrading bacteria. Then the single colonies were transferred into TSA media and stored in fridge at -20ºC in 20% glycerol for further studies (Tian et al., 2018).

Characterization of isolates. Morphological and biochemical characterization of bacterial strains were compared to the Bergey manual. The isolated colonies were incubated on TSA agar for 24 – 48 h and examined the morphological properties by optical microscopy at 400× and 1000× magnification. The morphological properties was examined by an Axio Image 2 (Imager.Z2) microscope display with integrated Zeiss Axiocam 503 Color Camera Unit at 100X objective, incorporating Zen 3.3 software (blue version) (Phuong et al., 2021).

Molecular identification of crude hydrocarbons-degrading bacteria. The isolated strains were identified based on partial sequence analysis of the 16S rRNA gene. Cells grown on TSA agar for 24 h were used to extract and purify genomic DNA by using a Kit ZR Fungal/Bacterial DNA MiniPrepTM (Zymo Research, UK). The 16S rRNA gene

fragments were amplified in the Thermal Cycler for DNA amplification (GeneAmp™ PCR System 9700, Life technologies applied biosystems, Singapore). By using 16S rDNA universal primer pair 27F (5′-AGAGTTTGATCATGGCTCAG-3′, forward primer) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′, reverse primer). Programmed PCR was performed as follows: initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, primer annealing at 52°C for 1 min, and extension at 72°C for 1.5 min, finally, extension incubated at 72°C for 5 min. Amplified PCR product was resolved by electrophoresis on 1% agarose gel and sent for directly automated sequencing at 1st Base Laboratories Sdn. Bhd., Malaysia. The 16S rRNA sequencing data were compared with GenBank sequences by the BLAST tool on GenBankk (http://www.ncbi.nlm.nih.gov/) and constructed a neighbor-Joining phylogenetic tree (Kumar et al., 2016).

Screening of extracellular hydrolytic enzymes production. The method of agar plug diffusion was used to determine the ability to produce extracellular hydrolytic enzymes of microbial strains on agar medium. The bacteria strains were analyzed for their ability to produce lipase, amylase, cellulase and protease. Mineral salt medium (GOST 9023-74) was used as basal medium with the addition of 1% substrate (w/v, tween 80, starch, gelatin, cellulose) as the only energy and carbon source (Patowary et al., 2016). The isolates were incubated on substrate agar plates for 24 h at 30ºC. The agar plugs of bacteria culture media with 9 mm diameter were placed on substrate agar medium (1%, w/v), respectively for the determination of lipase, amylase, protease and cellulase activity. Place the dishes in a 4ºC fridge for 4 – 6 hours for the diffusion of enzymes in the plug into the agar medium. The petri dishes were then incubated at 30±2°C and visible clear zone around the plug after Lugol drops on agar (de Veras et al., 2018; Do et al., 2021). All tests were repeated in triplicate.

Data analysis. A Neighbor-Joining phylogenetic tree was constructed by using a MEGA7 software based on 16S rRNA sequences (Kumar et al., 2016). The results were expressed as mean ± standard deviation (*SD*) of at least three independent experiments. Statistical data processing was performed using the software package MS Excel 2000 (Microsoft Corp.).

RESULTS AND DISCUSSION

Isolation and morphological, biochemical, physiological characterization of oil-degrading bacteria. Oil-degrading bacterial isolates were obtained from oil contaminated sites in Hanoi, Vietnam. Using the enrichment technique in liquid mineral medium, 5% crude oil mixed in DO adding to GOST medium was used as the only energy and carbon source to grow of oil-degrading strains. After a series of three further subcultures, microbial populations were determined by using the pour plate technique on plate count agar on GOST plates containing 5% crude oil mixed in DO (w/v), and count the colony-forming units, CFU/g. The results showed that the best performance in diesel oil degradation was the third enriched samples times. In this time, there is the increase of a specific microbial community and nutrient addition, diesel oil 5%, related to the dehydrogenase activity than the number of different microorganisms species. Indigenous microbial in initial muddy soil samples are well adjusted to their own environment after enrich-

ment. Thus, increasing immediately the population density of indigenous microorganisms pre-selected could ensure rapid degradation of diesel oil.

Isolation of oil-degrading bacteria were performed under similar conditions with morphological differences strains. From nine oil-contaminated muddy soil samples, after enrichment culture, a combination of microorganisms capable of degrading oil was selected for isolation and further study. The population for bacterial species after a series of three further subcultures reached a density of 9.3×10^{10} to 1.1×10^{12} CFU/mL. This indicates a rapid growth of oil-degrading bacteria populations. A total of five pure bacterial strains were isolated on the TSA agar from the complex of microorganisms and were selected as potential candidates to oily sludge biodegradation processes. Five strains were labeled as MD1, MD2, MD3, MD4, MD5. The morphological and biochemical characterization of bacteria revealed that five of them were Gram-negative and rod shape (Fig. 1**)**. All bacteria were catalase positive, optimal pH was neutral. They were cultivated in a medium with a salt concentration of 0.5% and optimal growth temperature for bacteria averaged 30ºC. Microbial consortium consisted of five strains isolated from an effectively enriched oil-contaminated muddy soil samples. This is very suitable when using a combination of these strains in microbial inoculation to treat oily wastewater in natural ecological conditions.

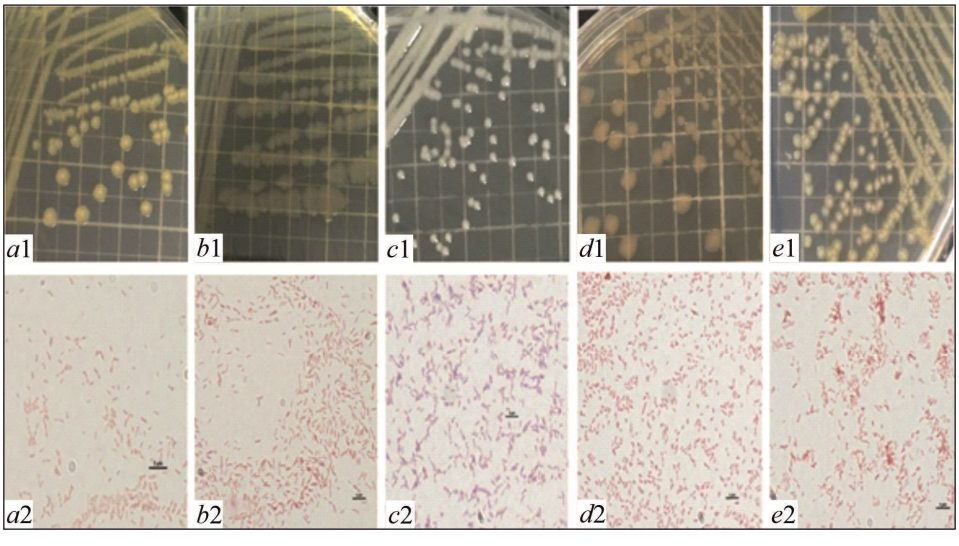


Fig. 1. Morphology characteristics of the five strains. The colony morphologies of MD1 (*a*1), MD2 (*b*1), MD3 (*c*1), MD4 (*d*1), MD5 (*e*1) strains on TSA agar and the Gram-stained MD1 (*a*2), MD2 (*b*2), MD3 (*c*2), MD4 (*d*2), MD5 (*e*2) cells observed under 1000× light microscope, respectively

Identification of oil-degrading bacteria. A total of five trains were cultured onto appropriate media to obtain pure growth and the partial sequencing of the 16S rDNA gene were identified. The 16S rRNA gene sequences analysis led to identification three

different genera represented among the strains: *Pseudomonas* sp. (MD1, MD2 and MD4), *Brucella* sp. (MD3), and *Stenotrophomonas* sp. (MD5). The 16S rDNA gene sequences of these strains were submitted into NCBI databases under the following GenBank accession numbers: MD1 (OL687411.1), MD2 (OL771695), MD3 (OL687412.1), MD4 (OL687413.1) and MD5 (OL687414.1), respectively (Table 1). Phylogenetic tree of the five strains based on 16S rDNA sequences was constructed by the neighbour-joining method using MEGA 7 software. In the phylogenetic tree, MD1 and MD2 strains form a separate branch along with MD4 strain (Fig. 2).

Table 1. Identification of five strains based on 16S rDNA sequences

Isolate	Accession number / NCBI	Identification
MD1	OL687411.1	*Pseudomonas mendocina* MD1
MD2	OL771695.1	*Pseudomonas hydrolytica* MD2
MD3	OL687412.1	*Brucella intermedia* MD3
MD4	OL687413.1	*Pseudomonas stutzeri* MD4
MD5	OL687414.1	*Stenotrophomonas nitritireducens* MD5

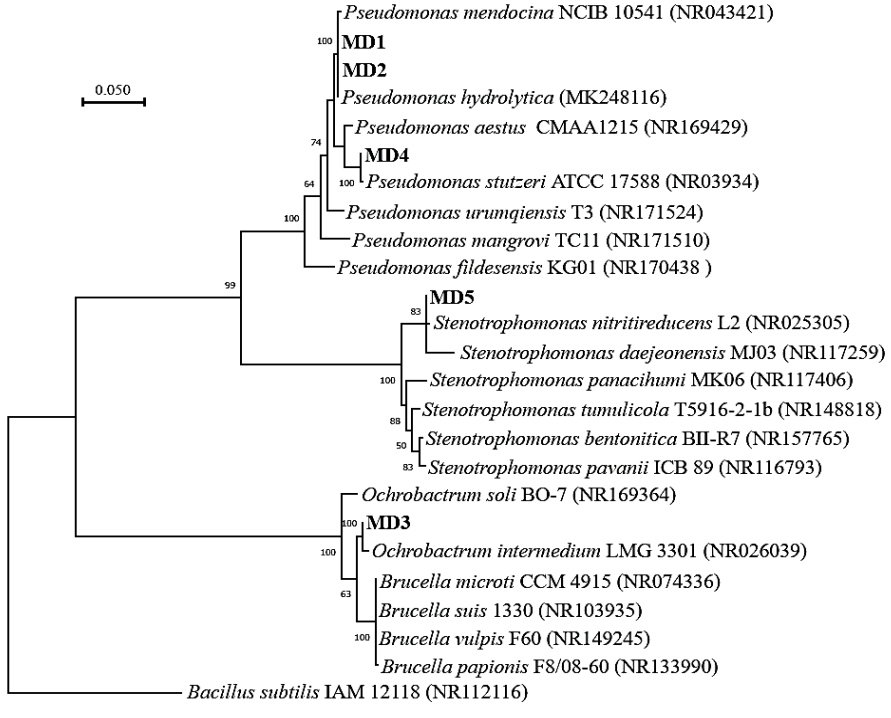


Fig. 2. Phylogenetic tree of the five strains based on 16S rDNA sequences using neighbour-joining method by MEGA 7 (The scale bar represents to a phylogenetic distance of 0.050 nucleotide substitutions per position. Bootstrap numbers 1000 replicates)

The isolation of bacteria directly from oil contaminated sites as oily sludge and soil was reported in the references. From hydrocarbons contaminated environments, many strains of the genera *Psedudomonas*, *Brucella*, *Stenotrophomonas* indicated the possibility of growth and degradation hydrocarbons (Muthukumar et al., 2003; Kumari et al., 2018; Poyraz, 2021). Pseudomonas mendocina was reported about degradation of phenanthrene, benzo[b]fluoranthene, citronellol, citronellal and citronellyl acetate (Kumari et al., 2018; Tian et al., 2002; Tozoni et al., 2010). In studies shown by Kumar, Pseudomonas aeruginosa was able to produce biosurfactants regarding hydrocarbon degradation capacity and this strain has been isolated from hydrocarbon-contaminated soil. Stenotrophomonas acidaminiphila was isolated from sludge and used to treat petrochemical effluents (Kumar et al., 2015). However, Pseudomonas hydrolytica is the first time reported about ability degradation diesel oil (Patowary et al., 2016).

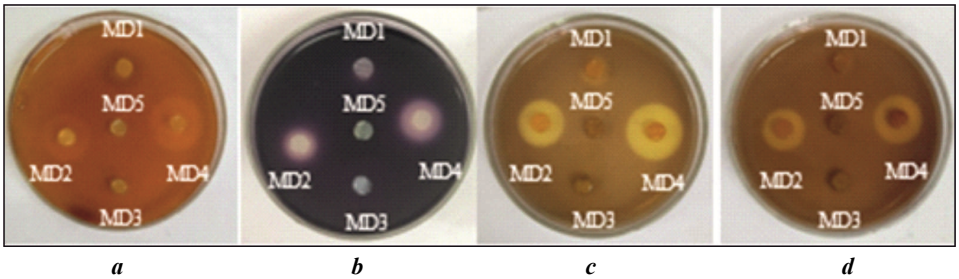
Searching for microorganisms that are native to the contaminated environment and have possibility degradation potential to treat contaminated areas is very important. The enrichment technique with 5% crude oil mixed in DO allowed the isolation of bacteria tolerant to this residue. These strains selected have greater adaptability with environmental stress, greater resistance to changes in environmental conditions.

Enzyme activity of isolated bacteria strains. There are various methods used to characterize the populations of hydrocarbon-degrading bacteria in environmental samples, such as microbial counts investigations and their extracellular enzyme capacity. Therefore, lipase activity of strains was used to monitor biodegradation of petroleum hydrocarbons, such as diesel oil, in contaminated environment (Margesin et al., 2007). In this study, the agar plug diffusion method was used to confirm extracellular hydrolytic enzymes producing ability of microbial strains on agar medium. The bacteria strains were analyzed for their ability to produce as lipase, amylase, protease and cellulase by the method of agar plug diffusion (Table 2 and Fig. 3). The result showed that all the five bacterial isolates showed li-

Table 2. Enzyme activity of bacteria isolated from oil contaminated sites

Strains	Enzyme activity (halo zone, mm)			
	Lipase	*Amylase*	*Cellulase*	*Protease*
MD1	+	–	5±0.5	+
MD2	15±0.5	7±0.5	12±0.5	9±0.5
MD3	+	–	–	–
MD4	18±0.5	9±0.5	16±0.5	13±0.5
MD5	+	+	–	–

Notes. –, none halo zone; +, halo zone < 5 mm.



a ***b*** ***c*** ***d***

Fig. 3. Enzyme activities of isolated bacteria strains on substrate agar media, (*a*) Tween 80, (*b*) Starch, (*c*) Gelatin, (*d*) CMC

pase activity by exhibiting clearance zone around on the substrate minimal medium agar plates. Many of the bacteria isolates were able to produce extracellular hydrolytic enzymes amylase, protease and cellulase on substrate agar media. A high percentage of the bacterial strains isolated from soil contaminated with crude oil showed lipase amylase, cellulase and protease activities (Margesin et al., 2007; Ugochukwu et al., 2008).

CONCLUSIONS

A enriched bacterial consortium found to be the most effective consortium in degrading crude oil consisting of five strains was obtained from oil-contaminated muddy soil samples. The 16S rDNA gene sequences of bacterial consortium were submitted in the NCBI-GenBank databases under the accession numbers: *Pseudomonas mendocina* MD1 (OL687411.1), *Pseudomonas hydrolytica* MD2 (OL771695), *Brucella intermedia* MD3 (OL687412.1), *Pseudomonas stutzeri* MD4 (OL687413.1) and *Stenotrophomonas nitritireducens* MD5 (OL687414.1), respectively. All the five bacterial isolates were able to produce extracellular hydrolytic enzymes lipase, amylase, protease and cellulase. It was concluded that these strains are very suitable to using as a combination to treat oily wastewater in natural ecological conditions.

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Идентификация консорциума бактерий, разлагающих углеводороды, выделенных из загрязненной нефтью илистой почвы в Ханое, Вьетнам

Д. Т. Туиен, Н. Т. К. Тхань, Н. С. Б. Кхоа, Н. К. Кыонг ✉

Совместный Российско-Вьетнамский Тропический научно-исследовательский и технологический центр
Вьетнам, г. Ханой, ул. Нгуен Ван Хуен, д. 63

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Аннотация. Биоремедиация является перспективным подходом для обработки загрязненных нефтяными загрязнителями сред. Основной целью данного исследования было выделение бактерий, способных разлагать углеводороды, для применения в очистке нефтесодержащих сточных вод с нефтезагрязненных участков в Ханое, Вьетнам. Исследуемый бактериальный консорциум был получен из загрязненного нефтью илистого образца почвы, обогащенного сырой нефтью, смешанной с дизельным топливом в качестве источника углерода. Восстановленный консорциум способен разлагать 93% содержания нефти после 7 дней испытаний. В общей сложности пять чистых бактериальных штаммов были выделены на TSA-агаре из сложных микробных сообществ и были отобраны в качестве потенциальных кандидатов для процессов биодеградации нефтесодержащего осадка. Эти изоляты были идентифицированы на основе их морфологических и биохимических характеристик. С помощью методов молекулярной биологии были исследованы пять бактерий, разлагающих углеводороды, и идентифицированы как *Pseudomonas mendocina* штамм МД1 (OL687411.1), *Pseudomonas hydrolytica* штамм МД2 (OL771695.1), *Brucella intermedia* штамм МД3 (OL687412.1), *Pseudomonas stutzeri* штамм МД4 (OL687413.1) и *Stenotrophomonas nitritireducens* штамм МД5 (OL687414.1). Морфологическая и биохимическая характеристика бактерий показала, что пять из них были грамотрицательными, палочковидными, каталазоположительными, идеальное значение pH было нейтральным, оптимальная температура роста составляла 30ºC в культуральной среде с соленостью 0.5%. Эти штаммы способны вырабатывать внеклеточные ферменты, такие как липаза, амилаза, целлюлаза, протеаза.
Ключевые слова: консорциум бактерий, биодеградация, разлагающие углеводороды, нефтесодержащие сточные воды, очистка

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ORCID и *e-mail адреса*: До Тхи Туиен: https://orcid.org/0000-0003-0070-5425, tuyendodhkh@gmail.com; Нгуен Тхй Ким Тхань: https://orcid.org/0000-0001-9840-7527, kimthanhnt2301@gmail.com; Нгйем Суан Бах Кхоа: https://orcid.org/0000-0002-7046-1369, khoa.bach01@gmail.com; Нго Као Кыонг: https://orcid.org/0000-0002-9286-0571, cuongnc@vrtc.org.vn.