CHARACTERIZATION OF INDIGENOUS OIL-DEGRADING BACTERIA FROM OIL-POLLUTED SOIL IN CAM RANH, KHANH HOA

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ARTICLE INFO

Received: 28/6/2023
Revised: 25/7/2023
Published: 25/7/2023

KEYWORDS

Bacterial consortium
Biodegradation
Indigenous bacteria
Oil-degrading bacteria
Oil-contaminated soil

ABSTRACT

Bioremediation of oil-contaminated soils using biodegradative activities of microorganisms is a promising approach that is of interest to researchers. The study aimed to isolate oil-degrading bacterial strains from the contaminated soil of Cam Ranh, Khanh Hoa, and develop a robust indigenous bacterial consortium for bioremediation of crude oil pollution. From a total of seven oil-polluted soil samples collected in Cam Ranh, after 3-fold enrichment in liquid mineral salt medium containing 5% crude oil mixed in diesel DO (w/v), 7 bacterial isolates were achieved. Based on the antagonism of 7 strains, 3 microbial consortia were formed and developed well in the condition of liquid mineral salts with 5% crude oil added mixed in DO. In particular, the TH2 consortium revealed the best ability to develop and oil-decomposition of 90% after 13 days of incubation. By analysis of 16S rRNA sequences, four oil-degrading bacteria of TH2 were identified as *Bacillus subtilis* CR1 (OQ940649), *Bacillus siamensis* CR4 (OQ940652), *Bacillus amyloliquefaciens* CR5 (OQ940653), and *Pseudomonas citronellolis* CR7 (OQ940655), respectively. The optimal conditions for four bacterial isolates were determined at salinity 20‰, pH 7 and temperature 30-37°C. They are suitable for application in the bioremediation of oil-contaminated environments.

ĐẶC ĐIỂM VI KHUẨN BẢN ĐỊA PHÂN HỦY DẦU TRONG ĐẤT NHIỄM DẦU TẠI CAM RANH, KHÁNH HÒA

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THÔNG TIN BÀI BÁO

Ngày nhận bài: 28/6/2023
Ngày hoàn thiện: 25/7/2023
Ngày đăng: 25/7/2023

TỪ KHÓA

Tổ hợp vi khuẩn
Phân hủy sinh học
Vi khuẩn bản địa
Vi khuẩn phân hủy dầu
Đất nhiễm dầu

TÓM TẮT

Ứng dụng khả năng phân hủy sinh học của vi sinh vật để xử lý đất ô nhiễm dầu là một hướng tiếp cận đầy hứa hẹn đang được các nhà nghiên cứu quan tâm. Nghiên cứu này nhằm mục đích phân lập các chủng vi khuẩn phân hủy dầu từ đất bị ô nhiễm ở Cam Ranh, Khánh Hòa và phát triển một tổ hợp vi khuẩn bản địa có khả năng xử lý ô nhiễm dầu tại đây. Từ tổng số 7 mẫu đất nhiễm dầu được lấy tại Cam Ranh, sau khi làm giàu 3 lần trong môi trường muối khoáng lỏng chứa 5% dầu thô hòa trong diesel (DO) (w/v), đã phân lập được 7 chủng vi khuẩn. Dựa vào kết quả nghiên cứu tính đối kháng của 7 chủng, 3 tổ hợp vi sinh vật được hình thành phát triển tốt trong môi trường muối khoáng lỏng có bổ sung 5% dầu thô pha trong DO. Trong đó, tổ hợp TH2 cho thấy khả năng phát triển và hiệu quả phân hủy tốt nhất đạt 90% sau 13 ngày ủ. Bằng kỹ thuật phân tích trình tự 16S rRNA, 4 chủng vi khuẩn phân hủy dầu của tổ hợp TH2 được khảo sát và định danh lần lượt là *Bacillus subtilis* CR1 (OQ940649), *Bacillus siamensis* CR4 (OQ940652), *Bacillus amyloliquefaciens* CR5 (OQ940653) và *Pseudomonas citronellolis* CR7 (OQ940655). Chúng phát triển tối ưu ở độ mặn 20‰, pH 7 và nhiệt độ 30-37°C. Giá trị này phù hợp với điều kiện tự nhiên tại Cam Ranh, cho thấy tiềm năng ứng dụng các chủng vi khuẩn để xử lý sinh học môi trường đất nhiễm dầu.

DOI: https://doi.org/10.34238/tnu-jst.8235

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1. Introduction

Currently, crude oil and petroleum products are being exploited and used increasingly, leading to release into the environment and harming surrounding ecosystems. Especially in coastal areas, oil extraction and transportation activities easily cause oil leakage and spill incidents, dispersing oil into the soil environment, which is a severe reason for soil pollution.

Cam Ranh bay is an important strategic area in the socio-economic development and security - defense of Vietnam. According to the actual assessment, this area is seriously polluted with diesel (DO), total petroleum hydrocarbons (TPHs) concentrations up to 4052 mg/kg of soil, higher than the standard value of QCVN 03:2023/BTNMT, 500 mg/kg. Oil seeps into groundwater, negatively affects soil quality, loses soil's ability to metabolize, kills microorganisms, and damages ecosystems. Petroleum may damage plants, animals, and people's health since it includes dangerous and persistent compounds such as benzene, toluene, ethyl benzene, xylene, and naphthalene [1].

Bioremediation is recognized as an efficient, economical, and versatile alternative to physicochemical treatment of oil-contaminants. In recent years, using hydrocarbon degrading bacteria to clean petroleum contaminated soils has become a prevalent, efficient, and economical technique that converts toxic wastes to non-toxic end products [2]. Stimulating the growth of indigenous microorganisms, and the addition of oil-degrading bacteria to the contaminated soil, is a promising method to accelerate decomposition activities in contaminated sites without impacting ecosystems [3], [4]. Previous studies have shown that bacteria in soil could completely mineralize organic pollutants into carbon dioxide, water, inorganic compounds or simple organic compounds [4], [5]. Some studies show that indigenous bacteria from contaminated sites, such as *Arthrobacter, Bacillus, Staphylococcus, Rhodococcus*,… can degrade petroleum compounds to satisfy their cell growth and energy needs [6]. Using indigenous microbial consortium for bioremediation is more effective than individual bacterial cultures. The research of Rahman (2002) showed that the mixed bacterial consortium degraded up to 78% of crude oil while this number was only 41-66% with the individual strain [2]. The diversity of oil-degrading bacteria in the soil and their ability to degrade oil are at different rates [7]. At the same time, some components of crude oil are difficult to degrade, such as shorter and longer chain alkanes ($<C_{10}$ and C_{20}-C_{40}) and polycyclic aromatic hydrocarbons (PAHs) [8]. Therefore, it is necessary to isolate and evaluate the biodegradation capability of indigenous bacteria in the soil under saline conditions in Cam Ranh to propose effective methods of treating oil pollution. Research on bacteria capable of decomposing oil in Cam Ranh is scanty. In this paper, bacterial strains capable of degrading crude oil mixed in diesel were isolated from oil-contaminated soil samples collected at Cam Ranh. The present study aimed to identify the bioremediation indigenous microbial consortium in oil-polluted soils and evaluate the ability to degrade DO and crude oil from the enriched samples.

2. Methodology

2.1. Materials

Oil-contaminated soil samples: seven oil-contaminated soil samples collected from the depth of 0 to 120 cm surface in Cam Ranh Bay, Khanh Hoa Province, Vietnam in June 2022. The NaCl salt content in the collected soil samples was between 1.5 - 1.6%, pH 5.5 - 8.4, and TPHs concentrations from 58.1-4052 mg/kg. Entire experiments were carried out in the laboratory of the Department of Biotechnology, Joint Vietnam - Russia Tropical Science and Technology Research Center laboratory.

2.2. Methods

2.2.1. Enrichments and isolation of oil-degrading bacterial strains

Soil samples were incubated on a rotary shaker at 30 ^{o}C and 150 rpm for 7 days in mineral salt medium (GOST 9023-74) containing: 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 15‰ NaCl and 5% crude oil mixed in DO (at a ratio of 5:95) as the only energy and carbon source [9].

After 7 days of incubation, the enriched culture was extracted and transferred to the same fresh medium at a ratio of 10% (w/v). After three cycles of enrichment, total bacteria were isolated and numbered using the plate counting method on MPA modified medium (containing peptone 10 g/L, meat extract 5 g/L, NaCl 15 g/L, agar 20 g/L) and incubated at 30°C for 24h. The pure colonies with phenotypic differences (colony shapes, sizes, colors, margins, texture, etc. and cell shapes and sizes) were sub-cultured, purified and maintained for further study [10].

2.2.2. Morphological observation of isolated strains

The isolated colonies were incubated on MPA modified for 24h and examined for morphological properties. The cell morphology of the isolated bacteria was observed under a light microscope (Zeiss Axiocam 503 Color Camera Unit) with a magnification of 1000x after Gram staining.

2.2.3. Screening of antagonistic interactions between bacteria isolated

Selected strains of bacteria were screened for antagonistic interactions by the cross-streak method [11]. They were streaked in horizontal and vertical rows that crossed each other on the same plate of MPA agar and incubated at 30°C. The antagonistic effect was indicated by the failure of the target strain to grow in the confluence area.

2.2.4. Oil degradation ability of the bacterial consortium

Bacterial consortia were grown in liquid mineral salt medium containing 5% crude oil mixed in DO (w/v) on a rotator shaker at 180 rpm, 30°C for 13 days. Non-inocula was used as the control. The estimation of bacterial growth was carried out by measuring OD600 nm in a UV–visible spectrophotometer.

Crude oil remaining from the culture broth after 13 days of testing was extracted using solvent extraction method with n-hexane. After the evaporation of the solvent, the amount of residual oil was determined by gravimetric methods. Uninoculated flasks served as control. The percentage of oil degradation was determined by the following formula [12]: Percentage of degradation = $((m_{control} - m_{residual})/m_{control})\times 100$, where $m_{control}$ is weight of the oil in control flasks after treatment (g), $m_{residual}$ is weight of the residual oil in testing flasks after treatment (g).

2.2.5. Effect of environmental conditions on growth of bacteria isolated

The bacterial species were cultured in MPA broth with different conditions including: Incubation temperatures of 10, 20, 30, 37 and 45°C; autoclaved medium initial pH of 3, 5, 6 7, 9 using 1M HCl or 1M NaOH; the salinity with NaCl, concentrations of 5, 10, 20, 30 and 40‰. After incubation on a rotary shaker at 150 rpm for 24 hours, the cell density was measured the optical density (OD) at 600 nm in a UV–visible spectrophotometer.

2.2.6. Molecular identification of isolates by 16S rRNA sequencing

Extraction of genomic DNA from the bacterial isolates was done using Kit ZR Fungal/Bacterial DNA MiniPrep^{TM} (Zymo Research, UK) according to the manufacturer's instructions. 16S rRNA genes were amplified by PCR using the universal primer pairs 27F and 1492R. The product of PCR was sequenced by 1st Base Laboratories Sdn. Bhd., Malaysia. The

16S rRNA sequencing data were compared with the corresponding sequences of the strains registered on GenBank using the BLAST tool on NCBI (http://www.ncbi.nlm.nih.gov/). The Neighbor-Joining phylogenetic tree was constructed using MEGA X software based on 16S rRNA sequences.

2.2.7. Data analysis

All experiments were performed in triplicate. The mean and standard deviations of the three experiments were calculated using Microsoft Excel 2010.

3. Results and discussions

3.1. Isolation and morphology of oil-degrading bacteria

After three times of enrichment in a mineral salt medium with 15‰ NaCl and 5% crude oil mixed in DO at a ratio of 5:95 (w/v) used as the sole source of carbon, the bacterial populations reached a density of 1.4×10^{10} to 1.05×10^{11} CFU/mL. This demonstrated that the oil-degrading bacterial populations grew rapidly. The isolated colonies with different morphologies were selected to form a microbial consortium capable of degrading oil and for further study. A total of 7 bacterial strains were isolated on the MPA agar from seven oil-contaminated soil samples in Cam Ranh (Table 1). 5/7 strains were found to be Gram-positive spore-forming rods labeled as CR1, CR2, CR3, CR4, CR5, and the remaining isolates CR6, and CR7 were Gram-negative rods. The number of isolates is not much, indicating that the enrichment process has initially screened bacterial strains that are able to adapt to the carbon source in crude oil.

Table 1. *Morphological characteristics of seven oil-degrading bacterial isolates cultured on MPA medium*

Isolates	Morphology of colony				Gram stain	Shape of cell
	Form	Color	Elevation	Margin		
CR1	Irregular	White creamy	Umbonate	Smooth	+	Rods
CR2	Irregular	White creamy	Convex	Undulated	+	Rods
CR3	Circular	White creamy	Flat	Smooth	+	Rods
CR4	Irregular	White creamy	Raised	Smooth	+	Rods
CR5	Irregular	White	Raised	Smooth	+	Rods
CR6	Irregular	Yellow green	Raised	Undulated	-	Rods
CR7	Circular	White creamy	Convex	Smooth	-	Rods

3.2. Antagonistic interactions between bacteria isolated

According to some studies, using a consortium of indigenous microorganisms to treat environmental pollution showed that it is more effective than single strains [5]. Therefore, in order to improve the oil degradation efficiency, it is necessary to select a consortium of microorganisms that do not have antagonistic interactions, grow stably, and decompose oil quickly [7].

Table 2. *Antagonistic interactions among bacteria isolated from oil-contaminated soil*

Strains	CR1	CR2	CR3	CR4	CR5	CR6	CR7
CR1	N	-	-	-	-	+	-
CR2	-	N	-	+	+	+	-
CR3	-	-	N	+	+	+	-
CR4	-	+	+	N	-	+	-
CR5	-	+	+	-	N	+	-
CR6	+	+	+	+	+	N	-
CR7	-	-	-	-	-	-	N

Notes: - no antagonistic interactions; + antagonistic interactions; N no test for antagonism

From seven isolated bacterial strains, the antagonism among them was investigated to build the consortia of strains applied to decompose oil. The results were shown in Table 2. CR6 strain had antagonistic interactions with almost all strains CR1 to CR5. In contrast, CR7 strain did not show antagonism against all other strains. Screening for biological antagonists among seven isolated strains was a simplistic approach but it should yield useful information for conducting oil-degrading bacterial consortia.

Bacterial isolates that did not inhibit each other in pairs and simultaneously grown under the same conditions of nutrient medium were grouped into a consortium to study the oil degrading efficiency. Three bacterial consortia were formed from 7 isolated strains and designated as Consortium TH1 (CR1, CR2, CR3, CR7), TH2 (CR1, CR4, CR5, CR7), TH3 (CR6, CR7).

3.3. Biodegradation of crude oil by a bacterial consortium

Three bacterial consortia TH1, TH2, TH3 were studied for their ability to decompose oil. The disappearance of DO and crude oil from the culture medium and the change in color of the medium showed that the consortia were able to emulsify oil effectively. After 13 days, the levels of microbial growth and oil biodegradation were analyzed using spectrometry-based and gravimetric methods (Figure 1). The results in Figure 1a showed that the OD600 values of all the consortia increased slightly and peaked at 7-9 days of incubation. The maximum OD600 was 1.67 ± 0.03 at TH2, followed by TH3 and TH1 with 0.76 ± 0.02 and 0.48 ± 0.01, respectively, suggesting that the strains could degrade crude oil. After 9 days, the curve stayed stable, which indicated that the growth and death of the bacterial cells had stabilized. The increase in cell mass in terms of turbidity directly indicates the utilization of DO and crude oil as the sole source of carbon.

The OD600 value and gravimetric analysis in figure 1b showed that Consortium TH2 produced the highest crude oil degradation of 90 ± 3.1% compared to Consortium TH1 and TH3 with 79.4 ± 2.1% and 87.7 ± 1.5%, respectively. This value is not too different from some other studies in the world. Zhang (2010) showed the result that during a 7-day incubation, about 95.8% of TPHs of diesel oil were degraded by a bacterial consortium containing seven strains from oil-contaminated soils [12]. The consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate *Rhodococcus erythropolis* from oil-polluted soil was able to degrade hydrocacbons by 90% in 6 weeks in liquid culture [4]. There was no biomass and significant degradation of diesel oil and crude oil in the microbial-free control. The biomass of the bacterial consortium isolated was proportional to the degradation of oil. TH2 showed the best oil degradation ability, therefore four 4 strains CR1, CR4, CR5, CR7 were further studied for treatment applications.

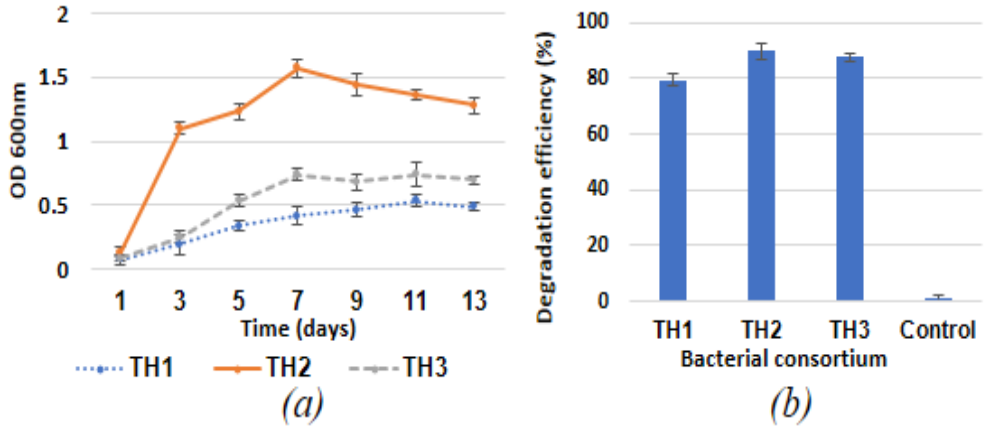

Figure 1. *The oil biodegrading ability of bacterial consortium (a) Bacterial growth curve in liquid mineral salt medium with crude oil mixed in DO as a sole carbon source (b) Percentage of crude oil removal measured by gravimetric after a 13-day incubation period.*

3.4. Effect of environmental conditions on the growth of bacteria isolated

Environmental conditions such as salinities, initial pH values and temperature affect the growth and decomposition of oil by microorganisms.

Four strains CR1, CR4, CR5, CR7 of TH2 were studied for optimal growth conditions. As shown in Figure 2a, the highest cell concentration of isolated strains was achieved with 20 g/L of NaCl. Isolated strains had a wide salinity range of 5 – 40‰ NaCl, suitable for application in normal and saline conditions. The soil in Cam Ranh Bay is moderately salty, NaCl content is about 15-20 g/L. Therefore, the indigenous microbial strains isolated here had shown good adaptability to the soil conditions for oil-polluted treatment.

Studies on the effect of the pH showed that the initial pH significantly affected the growth of the bacteria (Figure 2b). When the initial pH was 3, there was no sign of microbial growth. Bacterial density increased rapidly with increasing pH and reached its highest OD value in the range of 1.26 -1.43 at neutral pH 6-7. Then, with an increase in the initial pH of the medium, the cell concentration decreased slightly but bacteria could still grow at a pH of 9. The strain showed weaker resistance to acid environments but exhibited superior tolerance to alkaline environments. Overall, neutral media was favorable for all the bacterial isolates. A neutral pH of 7 has been reported to be optimal for growth and oil degradation rate and extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons [13].

As shown in Figure 2c, 30°C was the optimal growth temperature for most strains, except for strain CR1 showed maximum growth at 37°C (OD600 = 1.57 ± 0.02). When the temperature was lower than 30°C or higher than 40°C, the number of bacteria significantly decreased. This indicates that isolated strains were less active at temperatures outside the optimal region. This result was consistent with studies on suitable temperature conditions for bacterial growth [13].

Salinities, pH values and temperature affected the physical properties, chemical composition of the oil. Thus, it was affecting the rate at which microorganisms degrade hydrocarbons and the composition of the microbial community [2]. The study of B. Liu (2016) showed that the salinity of 30 g/L, pH of 7.5 and temperatures of 30°C was considered optimal for maximum microbial growth and biodegradation [13].

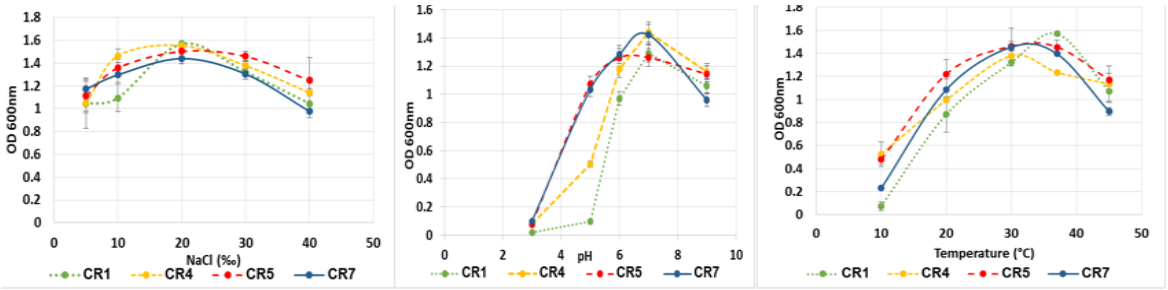

Figure 2. *Optimization of diffirent growth conditions of the bacterial isolates (a) NaCl, (b) pH, (c) Temperature*

3.5. Identification of the bacterial isolates

The 16S rRNA gene sequences of seven strains were identified and submitted into NCBI databases under the GenBank accession numbers shown in Table 3. The isolates were grouped into two main clusters related to the genera of *Bacillus* and *Pseudomonas* with the identity of more than 99% homology. This result was in line with studies suggesting spore-forming bacteria were crucial in oil biodegradation. Species belonging to this genus such as *Bacillus subtilis, Bacillus siamensis,* and *Bacillus amyloliquefaciens* were known to be able to produce biosurfactants regarding hydrocarbon degradation capacity and degrade oil in soil and water both

under normal and saline conditions [14]-[16]. The strain *Pseudomonas citronellolis* was the predominant strain among the isolates from oil-contaminated soil samples in different geoclimatic sites in India. They could degrade a wide range of organic pollutants including polycyclic aromatic hydrocarbons, halogenated derivatives, and recalcitrant organic residues [17]. The previous study by Xiumei Tian suggested that bacterial consortium made from *Bacillus* sp. and *Pseudomonas* sp. isolated from heavy oil-contaminated soil in Bohai Bay, China could degrade 80.64% of crude oil, 76.30% of crude oil alkanes [18]. The results of Rahman showed that the mixed bacterial consortium could carry out up to 78% of the degradation after 20 days of incubation, which was more efficient than the crude oil degradation of the individual cultures, the ratio degraded by *Pseudomonas* sp. and *Bacillus* sp. was 66%, 59%, respectively [2].

Table 3. *Identification of bacterial isolates using 16S rRNA gene sequences*

Isolates	Accession number (NCBI)	Identification	Identity (%)
CR1	OQ940649	*Bacillus subtilis* CR1	99.69
CR4	OQ940652	*Bacillus siamensis* CR4	99.79
CR5	OQ940653	*Bacillus amyloliquefaciens* CR5	100
CR7	OQ940655	*Pseudomonas citronellolis* CR7	99.81

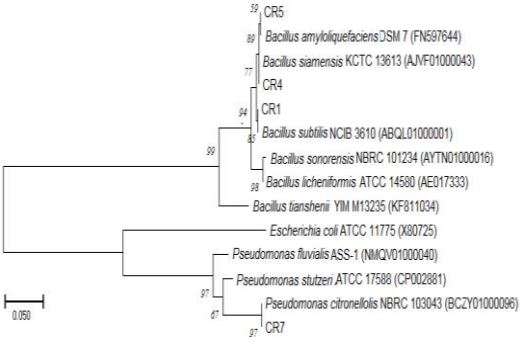

Figure 3. *Phylogenetic tree (1000 bootstrap replications) of the isolated strains based on 16S rRNA sequences using neighbour-joining method by MEGA X*

This high rate of crude oil degradation may be caused by the strains' complementing impacts on metabolic pathways, resulting in better degradation of petroleum hydrocarbons and a higher capacity to adapt to mineral salt media with crude oil mixed in DO. Through symbiosis and constructive collaboration between several strains, the complex microbial consortium was produced and showed significant efficiency in oil degradation. Besides, a combination of bacterial consortia containing various enzymes with strong abilities to degrade complex hydrocarbons is necessary for the effective remediation of oil-polluted soil [7].

Based on 16S rRNA sequences of seven isolates, a phylogenetic tree was constructed by the Neighbor-joining method. In the phylogenetic tree, CR1, CR4, and CR5 strains of the genus *Bacillus* form a separate branch different from strains of the genus *Pseudomonas* CR7 (Figure 3).

4. Conclusions

In the present study, seven bacterial strains were isolated from the oil-contaminated soil in Cam Ranh, Khanh Hoa. In this study, 3 microbial consortia formed from 7 bacterial strains isolated showed the ability to degrade oil from 79.4-90%, in which, bacterial strains in consortium TH2 showed the highest survival and oil-degrading activity. Four bacterial strains of consortium TH2 developed an ideal pH value of neutral. The optimum growth temperature was 30 ºC in a culture medium with a salinity of 20‰. The 16S rRNA gene sequences were submitted in the NCBI-GenBank databases under the accession numbers: *Bacillus subtilis* CR1 (OQ940649), *Bacillus siamensis* CR4 (OQ940652), *Bacillus amyloliquefaciens* CR5 (OQ940653), *Pseudomonas citronellolis* CR7 (OQ940655). In summary, the isolated indigenous bacterial strains have potential applications in the remediation of oil-contaminated soil in Cam Ranh, Khanh Hoa.

Acknowledgments

This work is studied in the biotechnology laboratory and financially supported by *Joint Vietnam - Russia Tropical Science and Technology Research Center*.

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