Bacillus cihuensis PVGOC-3 isolated from Guanabara Bay, Brazil, and its high potential for oil degradation

Rachel de M. Ferreira (rachelmoraes@hotmail.com.br)
National School of Public Health, Oswaldo Cruz Foundation
Rodrigo P. do Nascimento
Federal University of Rio de Janeiro
Danielle M. A. Stapelfeldt
Federal University of Rio de Janeiro
Lucy Seldin
Federal University of Rio de Janeiro
Maria de F. R. Moreira
National School of Public Health, Oswaldo Cruz Foundation

Research Article

Keywords: Biodegradation, Bacillus cihuensis, lubricating oil, biosurfactant

Posted Date: May 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1651008/v1

Abstract

The aim of the study was to isolate bacterial strains from an environment contaminated by hydrocarbons and investigate the degradation potential of petroleum derivatives with the use of used lubricating oil from a ship engine. Nine strains of possibly hydrocarbon-degrading bacteria were isolated from a sample of contaminated moist soil in the marginal rhizosphere at Guanabara Bay, located in the city of Rio de Janeiro, Brazil, and a triage for biosurfactant-producing bacteria was performed. Based on the results for degradation of used ship engine lubricating oil with dye 2.6 dichlorindophenol as a redox indicator, the PVGOC-3 scan was selected for growth and degradation tests. Amplification and sequencing of the rRNA 16S gene showed that the PVGOC-3 strain belongs to the species *Bacillus cihuensis* strain. The PVGOC-3 strain was able to degrade about 60.8% of the lubricating oil in 168 h at an initial concentration of 4.534 g L^{-1} according to the biodegradation analysis. According to the studies, *Bacillus cihuensis* presented a high potential in degrading used ship engine oil.

1. Introduction

The pandemic by the new Coronavirus brings with it the reaffirmation of the need for an increasingly sustainable and resilient world. Production chains, economy, health, and the environment have never been more clearly interconnected (Ivanov, 2020). We must preserve the instinct of survival and fraternity born in this pandemic. The current demand for new technologies, supplies, and energy has shown the deficiency of many countries in technological self-sufficiency. In addition to the lack of new energy technologies, such an absence transmits an idea of an urgent need to improve those existing, including oil exploration in energy generation, transportation, and production of materials such as protective equipment, among others (Lima *et al*., 2020).

It is essential to improve oil exploration technologies by using cleaning techniques for areas with oil spills and their derivatives to guarantee environmental health (Rabiei *et al*., 2013). Similarly, efficient methods for the treatment of industrial effluents must be developed (Wang *et al*., 2019; Lima *et al*., 2020).

There are several effluent treatment and remediation techniques for oily wastewater such as separation by gravity, chemical coagulation, inverse osmosis, bioremediaton, coalescers, adsorption, air flotation, membrane filtration, extraction, among others (Cunha, 2017; Kayvani *et al*., 2016; Kureel *et al*., 2017; Tian *et al*., 2018). However, such techniques are costly and often inefficient generating toxic products and producing secondary contamination from by-products (Hackbarth, 2014; Tian *et al*., 2018).

The use of microorganisms capable of degrading hydrocarbons is ordinary in both effluent treatment technologies and recovery of contaminated areas, which are necessary for the sustainability of oil exploration operations and their derivatives. Studies have shown the effectiveness of bioremediation as a biological method for cleaning oil residues and remediating hydrocarbons in many environments due to their spontaneous growth with the use of carbon sources, and the cost-benefit ratio (Rabiei *et al*., 2013; Tian *et al*., 2018).

Bioreactors are widely used for effluent treatment. The possibility of controlling and manipulating the entire degradation process makes that technique an attractive alternative due to its best cost-benefit (Banerjee and Ghoshal, 2016; Kereel *et al*., 2017).

Another exciting use of microorganisms is microbial-enhanced oil recovery (MEOR) in oil exploration. Such a technique improves the oil exploration efficiency since microorganisms and their metabolites can release the oil trapped in the rocks by reducing surface and interfacial tensions, change of wettability, changes in the flow pattern, gas production, and reduction of oil viscosity. Those microorganisms produce biosurfactants, responsible for reducing surface and interfacial tensions accumulated between oil and water (Rabiei *et al*., 2013).

Bacteria, widely studied due to their rapid development and high productivity of metabolites, are among the microorganisms capable of degrading hydrocarbons and producing biosurfactants. Different Bacillus species abundant in the environment provide extensive research on the various genera of bacteria for oil degradation. A study conducted with *Bacillus salmalaya* observed surfactant production and a 71.5% degradation of lubricating oil (2% v/v) in 20 days (Dradania et al., 2015). A study with *Bacillus subtilis* achieved the production and characterization of biosurfactants and the degradation of 60% of crude oil (2% v/v) in one day (Sakthipriya *et al*., 2016). The same species of that microorganism could degrade 65% of oil (0.3% v/v) in 5 days with high surfactant production (Wang et al., 2019), whereas *Bacillus* sp could degrade 70.3% of diesel (1% v/v) in 7 days (Lima et al., 2020). A study with *Bacillus cereus* obtained degradation of 98% in 6 days of water production of oil extraction (Banerjee & Ghoshal, 2016), and the same species degraded 98.6% of used engine oil (1% v/v) in 20 days (Bhurgri et al., 2017). On the other hand, diesel oil (3% v/v) was 50% degraded in 18 days by *Bacillus amyloliquefaciens* (Ayed et al., 2015). Therefore, biodegradation by microorganisms is a promising and versatile technique.

Another bacterium of the genus Bacillus was identified and named *Bacillus cihuensis*. In 2014, the strain was isolated from the rhizosphere of sediment in the Cihu area of Taiwan and characterized as Gram-positive Bacillus of rod-shaped morphology, with elliptical endospores formation. In addition to the characterization and the best growth conditions presented, knowledge about the possible biotechnological applications of that microorganism is scarce so far (Li *et al*., 2014).

The aim of the study was to isolate bacterial strains from an environment contaminated by hydrocarbons and investigate the degradation potential of petroleum derivatives with the use of used lubricating oil from a ship engine.

2. Materials And Methods

2.1 Isolation of bacterial strains and sediment contamination

The strains of bacteria were isolated from a sample of the moist and sandy soil of the rhizosphere (10 to 20 cm), marginal to Guanabara Bay, located at S 22”50’023 and W 43”14’465, in the municipality of Rio de Janeiro. Another batch of bacterial strains was isolated from an aliquot of 25g of the sediment

contaminated with 250µL of lubricating oil (Lubrax 25W50) in a 125mL Erlenmeyer and incubated for 15 days at 35ºC (Dantas, 2016). For isolation, both samples were suspended in 0.8% sterile saline solution, diluted in series, spread in nutrient agar (NA), and incubated at 35°C for 48 h (Atlas, 1993).

2.2 Triage of biosurfactant-producing bacteria

2.2.1 Emulsification test

The strains were tested for emulsification capacity to verify the production of biosurfactants. The microbial cells were stirred in a nutrient broth (10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 1 g L^{-1} NaCl) at 150 rpm, pH 7 and 35ºC for 48 h. After centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant were removed, added to 2 mL of lubricating oil and stirred for 2 min in a vortex. Then, the system was kept resting for 6 hours to verify the stability of the foam formed (Cooper and Goldenberg; 1987).

2.2.2 Oil displacement test

The strains were previously inoculated in 5 g L^{-1} glucose for 24 h at 35ºC and 150 rpm. A volume equal to 15mL of distilled water was added to Petri dishes of 5 cm diameter. To produce an apolar layer, 250 µL of lubricating oil were slowly added to the formation of the oil film. Then, 10 µL of the supernatant, from the inoculum of the microorganism in glucose were placed on the film formed. Oil spreading and formation of a halo was considered a positive and negative result for the drop dissolved in water, respectively. The negative control used was distilled water, whereas a 5% Extran solution, the positive one (Kreischer & Silva, 2017).

2.3 Biodegradability test using DCPIP redox indicator

Bacterial cells were inoculated in a nutrient broth at 150 rpm and 35ºC for 48 hours (Peixoto & Vieira, 2005). The standardization of the microorganisms concentration was determined for tube No. 3 using the Mac Farland scale. In 20 mL penicillin vials, the system consisted of 1.5mL of Bushnell-Hass medium (1 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4NO_3, 200 mg L^{-1} $MgSO_4.7H_2O$, 50 mg L^{-1} $FeCl_3$, 20 mg L^{-1} $CaCl_2.2\ H_2O$) at pH 7, 480uL of inoculum and 20uL of used ship engine oil, under agitation at 150 rpm and 35 °C for 168 h. The negative control was also performed without adding the inoculum. After that period of time, the addition of 1.5 µL of 1 g L^{-1} 2.6 dichlorindophenol (DCPIP) indicated the presence or absence of oxidation of the medium, and systems remaining colorless after the DCPIP were considered positive (Hanson et al., 1993).

2.4 Characterization of ship oil by Infrared Spectroscopy (IR)

Shimadzu's infrared spectrophotometer equipment, RAffimty-1, produced the absorption spectra in the infrared region between 400 and 4000 cm^{-1} for used ship engine oil.

2.5 Determination of bacterial growth in the presence of hydrocarbons

Microorganisms grown in a nutrient broth for 48h and 35°C were centrifuged, suspended in an 0.8% saline solution, and standardized at an absorbance equal to 1,000 in a UV-Visible spectrophotometer (Bisector sp-22 manual). In Erlenmeyers, 2.5 mL standardized inoculum in a 22.25 mL of Bushnell Haas medium (BH) contaminated 250 µL of used ship engine oil (Mobil Delvac SAE 40) and applied a rotation of 150 rpm for seven days and 35°C. After the incubation period, taken a 2 mL aliquot was taken and measured at 600 nm to verify the growth of microorganisms. (Das & Mukherjee, 2007).

2.6 Hydrocarbon degradation in solid and liquid medium

Ship engine oil used as a carbon source evaluated the ability of microorganisms to access a carbon source in a solid medium (Arulazhagan, P. & Vasudevan, 2011; Almeida *et al*., 2017). The strain was inoculated by the technique of surface spreading in Petri dish with BH medium, agar 15 g L^{-1}, at pH 7, supplemented with 1 % oil. Negative controls containing only BH and agar medium at pH seven were inoculated with the same microorganism and incubated for seven days at 35ºC.

The strain previously grown in nutrient broth for 48h, 35ºC, 150 rpm, and standardized to OD equal to 1,000 was inoculated in 20mL penicillin vials, containing 1,780 mL BH medium, 200uL inoculum and 20uL of ship engine oil, kept under 150 rpm and 35°C for 7 days. The negative control was also performed without adding the inoculum.

After that period, 5mL of chloroform were added to each vial, left under agitation for 15 min, and the supernatant removed. Then, the organic solution was analyzed by UV-Visible spectroscopy (UV-2600 SHIMADZU). Next, the quantification of the oil occurred at 239 nm following a scanning between 200 and 800 nm (APHA 1985; Henderson *et al*., 1999). The difference between oil concentration in negative controls and samples inoculated with bacterial strains determined the oil degradation.

Equation 1 calculates the oil biodegradation percentage as follows:

$$\%\,\boldsymbol{biodegradation} = \left(\frac{C_{CN} - C_f}{C_{CN}}\right) \times 100\% \qquad \text{Equation 1}$$

Where,

C_{CN}: Initial oil concentration, performed by the negative control.

C_f : Final oil concentration.

2.7 Bacterial growth time

The bacterial growth time test contributes to the optimization of the biodegradation process, in which it is possible to monitor the viability time of microbial cells still alive, as well the time of each generation (Kereel *et al*., 2017).

Microorganisms grown in nutrient broth for 24 h at 35°C were centrifuged, suspended in a 0.8% (m/v) saline solution, and standardized at 600 nm to 1.000 nm optical density (OD) in a spectrophotometer UV-Visible. In Erlenmeyer flasks (500 mL), 10 mL of a standardized inoculum were inoculated in 89 mL of BH medium and 1 g of ship engine oil, rotated at 110 rpm, and 35°C for ten days, all in triplicate. The serial dilution technique counted the colony-forming units (CFU) taken from aliquots of 1 mL every 24 h (Das; Mukherjee, 2007; Kereel *et al.*, 2017).

The total aliquots volume removed was less than 20% from the entire system. According to statistical sampling methods, the value found for the difference between initial and end system concentrations is not statistically significant when the variation in volume is less than ± 20% (Skoog, 2020).

The growth rate (μ) calculation for the bacterial strain was calculated according to equation 2 for each microorganism specific stationary phase (Schmidell et al., 2001).

$$lnN - lnN_0 = \mu \times (t - t_0) \qquad \text{Equation 2}$$

Where,

N: number of microorganism cells at the end of the stationary phase.

N_0: number of microorganism cells at the beginning of the experiment.

t: time at the end of the stationary phase (h).

t_0: initial time (h).

The generation number (n) was calculated according to equation 3 (Schmidell *et al.*, 2001).

$$n = \frac{lnN - lnN_0}{ln2} \qquad \text{Equation 3}$$

The generation time (g) given by equation 4 (Schmidell *et al.*, 2001).

$$g = \frac{ln2}{\mu} \qquad \text{Equation 4}$$

2.8 Reinoculation test

The microorganisms were grown in nutrient broth for 24 hours at 35°C, centrifuged, resuspended in a 0.8% (m/v) saline solution, and standardized to an OD equal to 1.000 of absorbance at 600 nm in a UV-

Visible spectrophotometer. In 20 mL penicillin vials, the system consisted of 2.67 mL of BH medium, 300 µL of standardized inoculum, and 30 µL of used ship engine oil, under agitation at 150 rpm for 10 days at 35° C (Hanson et al., 1993). The composition of the system was the same for negative controls but with no addition of inoculum.

The UV-visible spectrophotometry performed the oil quantification using extraction in chloroform in six replicas. From three of them, 2 ml aliquots were taken and added to 20 ml penicillin vials containing 2 ml of BH medium and 40 µl oil, agitated (150 rpm) at 35ºC for another ten days. Then, the resulting oil underwent extraction and analysis. Negative controls with no inoculum were also prepared in triplicate. The percentage of oil biodegradation was calculated by equation 1.

2.9 Phenotypic and molecular characterization

The strain was inoculated in Petri dishes containing nutrient Agar medium for 20h at 35ºC. After observing the macroscopic characteristics of the colonies formed, the Gram staining allowed us to verify the microscopic structural features.

For sequencing of the gene encoding 16S rRNA and phylogenetic analysis, DNA was extracted and purified according to the ZR Fungal/Bacterial DNA MiniPrep system (Zymo Research, Irvine, CA, USA) commercial kit. PCR amplified the sequence of the gene encoding 16S rRNA with the primers pA (5' AGAGTTTGATCCTGGCTCAG 3'- forward) and pH (5' AAGGAGGTGATCCAGCCGCA 3'- reverse) (Massol-Deya et al., 1995). The PCR products were purified with the "Wizard™ Rapid PCR Purification System" (Promega®) commercial kit and sequenced by the DNA Sequencing Platform - RPT01A – Fiocruz. The resulting gene sequence encoding 16S rRNA was compared to reference strains with validly published names, using the GenBank database and the BLAST-N tool (www.ncbi.Nlm.nih.gov/blast) to confirm the identity of the PVGOC-3 strain. Then, the sequences were aligned in the BioEdit Sequence Alignment Editor and imported for the construction of a phylogenetic tree by the Neighbor-Joining method in the MEGA X program (Tamura *et al.*, 2013).

3. Results And Discussion

3.1 Isolation of bacterial strains and sediment contamination

Nine distinct bacterial strains were isolated from the contaminated sediment. Such contamination with lubricating oil limited mineral nutrients to simulate a common problem in marine environments contaminated with oil due to an unbalanced relationship among carbon, phosphorus, and nitrogen. Microorganisms degrading hydrocarbons tend to adapt to limitations by using their genetic products. Those adaptation processes may produce a sudden increase in the cell mass after a temporary decrease in the overall number of microorganisms. Thus, the genetic ability of the microbiota is improved, resulting

in more excellent enzymatic capability for the biodegradation of pollutants, which use those compounds as a source of carbon and energy (Abbasian et al., 2016; Maier & Gentry, 2015).

3.2 Emulsification test

The nine isolated strains gave positive results for emulsification, indicating the formation of a biosurfactant since they showed permanent foam after 6 hours. The sparkling action is one of the characteristics of biosurfactants. These compounds help in the promotion of biodegradation since they reduce interfacial and superficial stresses caused by the contact of polar compounds with apolar, as well as contribute to reducing the critical concentration of micelles (Dadrasnia & Ismail, 2015). In the oil industry, they can be applied in enhancing oil recovery, cleaning oil spills, oil contaminated ship cleaning, viscosity control, oil emulsification, and crude oil removal from sludge (Montagnolli et al., 2015).

The different microbial biosurfactants produced have unique structures and many potential applications, from biotechnology to environmental cleaning. Therefore, they may be more appropriate than synthetic and traditional chemical surfactants. Their properties make them suitable for numerous industrial uses, involving detergency, emulsification, lubrication, foaming capacity, wetting capacity, solubilization, and phase dispersion.

The production and characterization of biosurfactants produced and their biotechnological potential for biodegradation were verified for *Bacillus licheniformis, Bacillus amyloliquefaciens*, and *Bacillus subtilis* (Mukherjee et al., 2016; Ayed et al., 2015; De Oliveira *et al.*, 2017).

3.3 Oil displacement test

Of the nine isolated strains, only PVGOC-3 and PVGOC-5 could promote the dispersion of the oil film over water and produce compost with a possible capacity to reduce surface oil/water retention. Future studies on the characteristics of possible biosurfactants produced and their biotechnological applications are necessary. The strains of Bacillus *subtilis* and *Pseudomonas aeruginosa* also could produce the surface displacement of oil over water with the production of biosurfactant (Alvarez et. al., 2015).

Biosurfactant-producing bacteria can be found in various environments. However, studies show a greater tendency for the development of those microorganisms in sites contaminated by hydrocarbons (Soltanighias et al., 2019; Alvarez et al., 2015). The interest in biosurfactant-producing bacteria occurs due to their broad applicability in many industry sectors since most commercially available surfactants can be synthesized from petroleum derivatives. Moreover, biosurfactants are more effective than chemical surfactants (Alvarez et. al., 2015; Alvarez et. al., 2018).

Besides using biosurfactants in the oil sector, decontamination of areas, and MEOR, they also have great potential in the pharmaceutical and food industries. Another characteristic of interest of this compound is the production of bioemulsifiers, very important due to their high emulsification activity and ability to reduce surface and interfacial tension (Alavarez *et al.*, 2015). Those bioemulsifiers are already used in commercial food formulations as texturizers, viscosifiers, emulsifiers, and syneresis reducing agents.

However, studies on the characterization of new bioemulsifiers are scarce due to the difficulty in distinguishing them from biosurfactants (Alvarez et. al., 2018).

3.4 Biodegradability test using DCPIP redox indicator

The biodegradability test using the DCPIP redox indicator is elementary and low-cost. Several authors verified its efficiency and emphasized the vital role of that indicator in the detection of microorganisms with the ability to degrade hydrocarbons, such as bacteria (Montagnolli et al., 2015; Habib et al., 2017; Ime et al., 2018; Peixoto et al., 2018).

Only the PVGOC-3 strain could completely discolor the DCPIP dye in the biodegradability test. Therefore, subsequent tests for biodegradation and 16S rRNA gene sequencing solely utilized that strain. Oil biodegradation using DCPIP redox indicator was also successful with the *Bacillus toyonensis* (Peixoto et al., 2018). Similarly, studies with *Baccillus subtilis* (Cruz et al., 2013) and *Pseudomonas aeruginosa*in (Lopes, 2014) were victorious for the degradation of oil, diesel, and biodiesel and used automotive lubricating oil, respectively.

3.5 Characterization of ship oil by Infrared Spectroscopy (IR)

Infrared spectroscopy performed a qualitative analysis of the oil's chemical composition to verify its main chemical groups. It is necessary to investigate if the used oil has kept its main chemical characteristics after use. Figure 1 shows a typical spectrum of lubricating oil with four regions, characteristic of that kind of substance (Santos Junior, 2011). The spectrum region (a) was considered as the “fingerprint” of the material, because it refers to the axial deformation of the C-O bond of esters (Silverstein and Webster, 1998). The region (b) presents two intense bands, characteristics of angular deformation vibrations of C-H bonds in methyls and methylenes. The low-intensity bands of the region (c) are attributes of the axial deformation vibration of the C = O bond of carbonyl groups present in aliphatic esters. Finally, region (d) shows typical intense bands of the axial deformation of C-H bonds of methyl and methylene groups (Silverstein and Webster, 1998).

According to the spectrum obtained, especially in the digital printing region, it is possible to identify some constituent groups of lubricating oil (Santos Junior, 2011), as shown in Table 1. The oil used still has the main functional groups, characteristic of lubricating oil.

Table 1
Identification of chemical groups constituting used ship engine oil, their respective bands, and specifications for lubricating oils according to Santos Junior (2011).

Group	Specifications:	Bands
Alkyl zinc dithiophosphate	Anti-wear and antioxidant additive	665 and 975 cm^{-1}
Hydrocarbons	Typical constituents of lubricating oils	772, 815, 887, 1660, 2854, 2924 and 2954 cm^{-1}
Succinimida	Dispersant additive	1230 and 1705 cm^{-1}
Polymethacrylae	Typical chemical component of fluidity point lowering additives and viscosity modifiers	1154, 1169 and 1745 cm^{-1}

Our study employed used lubricating oil for ship engines, formed by unchanged molecules of basic oil, degradation products of the basic oil, inorganic contaminants, water from the combustion chamber, light hydrocarbons (unburned fuel), carbon particles produced by cracking of fuels, and the lubricant. In addition, they also generate compounds such as dioxins, organic acids, ketones, and polycyclic aromatic hydrocarbons, derived from the original formula and/or absorbed from the engine (Teramae *et al.*, 2012). The complex matrix with several constituents from the lubricating oil employed allows us to raise the hypothesis that the *Bacillus cihuensis* also has high resistance to additives, which are part of that type of oil, as well as highly toxic by-products and high potential of degradation.

3.6 Determination of bacterial growth in the presence of hydrocarbons

Inoculum OD measured the growth response of the PVGOC-3 strain after seven days of incubation in the presence of hydrocarbons. The 0.222 absorbance obtained represented a 123% increase in microbial cell density. *Bacillus subtilis* increased 6 times the number of cells in 120h and *Pseudomonas aeruginosa* increased the cells 8 times in the same period, both in crude oil as a carbon source (Das & Mukherjee, 2007). However, the presence of a large population of total heterotrophic microorganisms does not necessarily present a direct correlation with biodegradation (Townsend et al., 2007). Other hydrocarbon quantification tests are necessary to measure the real ability of the microorganism to degrade organic substances of interest.

3.7 Hydrocarbon degradation in solid and liquid media

Agar in 1% of used ship oil evaluated the ability of the PVGOC-3 strain to access carbon sources in the solid medium. That capability was based on the formation of colonies visible only after 48 hours of incubation. The microorganisms *Bacillus methylotrophicus* and *Pseudomonas sihuiensis* also resulted in a solid medium supplemented with 0.2% Bazu oil as the only source of carbon and energy (Pereira, 2018).

The quantification of residual oil concentration allowed us to assess the performance of the PVGOC-3 for biodegrading the used ship oil in the aqueous medium for seven days. The total oil concentration decreased 60.8%, from 4.535 to 1.779 g L^{-1}. Figure 2 shows such a decrease and the negative control (CN) as the initial oil concentration. The microorganism presented a curve with a significant change in concentration and wavelength between 200 and 290nm. Those changes may represent a breakdown of the long hydrocarbon chains that constitute the oil. However, future tests using other hydrocarbon quantification methodologies will help evaluate the meaning of those modifications.

3.8 Bacterial growth time

According to Fig. 3, the bacterial strain presented a latency phase (lag) of up to 72 h, followed by an exponential phase of 168 h. Then, it entered the stationary phase for 216 h, and the death phase lasted 240 h.

The presence or absence of a lag phase is an essential indication of how metabolic pathways are induced after the exposure of microorganisms to different energy sources. The existence of a lag phase indicates that a metabolic pathway needs to be activated, that there is a need for specific enzymes, revealing that the biodegradation process will not be immediate (Oliveira, 2017).

In a study on microbial growth for media with BTEX, the absence of a lag phase at concentrations below 50 mg L^{-1}, a more extended lag phase at higher concentrations, and an increase in cell biomass were observed. This study also suggests the desirability of additional time for the production of enzymes to oxidize a large amount of the compound (Deng *et al*., 2017). In a stationary phase on the seventh day, the same behavior was observed for the species *Bacillus* sp., *Pseudomonas* sp., and *Sphingomonas* sp., in a medium with biodiesel and diesel. All those species were isolated from soil contaminated by hydrocarbons (Schultz, 2010).

The growth of *B. methylotrophicus* and *P. sihuiensis* species in BH medium with 2% Bazu oil showed a peak growth in the 10th day, with viable cells in the medium for up to 46 days (Pereira, 2018). Such results show the variability of this microorganism species' growth. However, it is essential to highlight that, according to the literature, each microorganism has specific degradation genes for each hydrocarbon group; being necessary to evaluate not only the growth rate but also the degradation percentage (Maciel, 2013, Pereira, 2018).

The specific growth rates (μ), number of generations (n) and generation time (g) were calculated, as shown in Table 2. The growth rate achieved was reduced due to limited access to carbon sources in the medium. It is worth noting that the number of generations obtained was 11.9, indicating that the microorganism of this study possibly has a fast metabolic pathway to access oil's hydrocarbons.

Table 2
Specific microbial growth rates, number of generations and time of each generation.

	μ (h^{-1})	n	g (h)
B. cihuensis	0,081	5,615	11,9

The BTEX biodegradation with the microorganisms *Burk holderiacepacia* and *Enterobacter* sp showed 0.3 and 0.5 d^{-1} growth rates, respectively, for a 10 mg L^{-1} BTEX initial concentration (Oliveira, 2017). Such results are comparable with those achieved for species in this work after concentration unit conversion.

3.9 Reinoculation test

The reinoculation test aimed to verify the oil components ability to induce microorganism's metabolic pathways (Nam and Alexander, 2001; Oliveira, 2017). According to Fig. 4, there was no statistically significant difference between oil biodegradation after reinoculation for PVGOC-3, which obtained 65.9% in the first and 63.2% in the second generation. According to statistical analysis, the biodegradation values for the first and second microorganisms generations are not different.

According to the literature, the reinoculation process may not be significant for some strains as in BTEX biodegradation by *Enterobacter* sp and *Burkholderia cepacia* (Oliveira, 2017). Besides, the difference can only be noticed after a longer degradation period, as DDT (1,2,3,4,10,10-hexachlor-1,4,4a,5,8,8a-hexahydro-exo-1,4, -endo-5,8-dimethanonaphthalene) biodegradation in soil by a Gram-negative bacillus-shaped bacterium, which biodegradation process lasted 180 days (Nam and Alexander, 2001).

The biodegradation values after re-inoculation led to a hypothesis. Although the difference after re-inoculation was not significant for the activation of specific genes to degrade oil, it was possible to observe the ability of the species to degrade oil, as no variation appeared between the first and second inoculation in the final degradation. Perhaps, in the first inoculation, the biomass could have an energy reserve coming from the medium in which it was found since the initial culture medium was rich in nutrients that favored its growth. This energy could be enough for the biomass to start the oil degradation. On the other hand, in the second inoculation, that energy reserve might not exist. Thus, biodegradation would be more negligible or not even occur. This fact was not verified since the oil degradation was proportional to the first generation. However, this may indicate that it is possible to reuse the biomass of these microorganisms for new cycles of degradation.

Table 3 shows promising results for biodegradation using PVGOC-3 strain since it presents high biodegradation capacity in a moderate time range. It might degrade 2.756 g L^{-1} of used ship engine lubricating oil in 7 days.

Table 3
Comparison of the result with the literature

Microorganism	Degradation (%)	Time (days)	Initial concentration ($g\ L^{-1}$)	Reference
PVGOC-3	60.8	7	4.534	Our study
Bacillus sp	100	78*	1.8	Ke et al., 2018
Bacillus cereus strain	100	40*	0.5	Das et al., 2015
Bacillus cereus	95	120*	2.5	Banerjee & Ghoshal, 2016
Bacillus amyloliquefaciens	50	18*	1	Ayed et al., 2015
Bacillus salmalaya	72.6	1	0.02	Dadrasnia & Ismail, 2015
Bacillus cereus	98	20	2.71	Bhurgri et al., 2017
Bacillus subtilis	76	5	3	Wang et al., 2019
Bacillus sp	70.3	7	0.01	Lima *et al*.
Bacillus subtilis	100%	10	1	Sakthipriya et al., 2018

* hours;

Our results showed no proportional relationship between growth and degradation with the PVGOC-3 strain, identified as *Bacillus cihuensis*, since the bacterium showed low growth and a high rate of oil degradation, about 2.756 $g\ L^{-1}$ in 7 days. The test of oil degradation in a solid medium pointed to the selectivity of the microorganism in degrading hydrocarbon chains since the solid medium used as a support for oil degradation also has hydrocarbon chains. Therefore, the characteristics of degraded chains and formed by-products should be further studied.

3.10 Phenotypic and molecular characterization

The phylogenetic tree with sequences based on the 16S rRNA gene showed a higher relationship between the PVGOC-3 strain and the *Bacillus cihuensis* strain, as shown in Fig. 5. According to the GenBank database, the PVGOC-3 strain sequence obtained a 93.70% identity with *Bacillus cihuensis* strain, access number NR_148280.1. The morphological characteristics of the strain were Gram-positive in the rod format. After submission, the PVGOC-3 strain received an access number MT903350 in the GenBank.

Future studies on process optimization, characterization, and the toxicity of the products formed must be developed. Likewise, the characterization of the biosurfactant produced during fermentation for a possible biotechnological use must also be researched.

4. Conclusion

The PVGOC-3 strain, isolated from the sediment rhizosphere in Guanabara Bay, and identified as *Bacillus cihuensis* showed promising results in the biodegradation of used ship engine lubricating oil. The degradation of the oil was possible with these hydrocarbons as the only carbon source.

The qualitative triage of the isolated bacteria led to the selection of 2 potential isolates for the production of biosurfactant, of which all isolates belong to the genus Bacillus. The PVGOC-3 strain, identified as *Bacillus cihuensis*, presented the highest oil degradation potential in the biodegradation tests. These results indicate their potential for the production of biosurfactants and possibly bioemulsifiers, which directs future studies for the characterization of these possible compounds and their industrial application. Especially in pathogenicity studies, which will allow their direct applications even in products for the food and pharmaceutical industries.

Declarations

Acknowledgment

Our thanks for the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro for granting the doctoral scholarship note ten.

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Figures

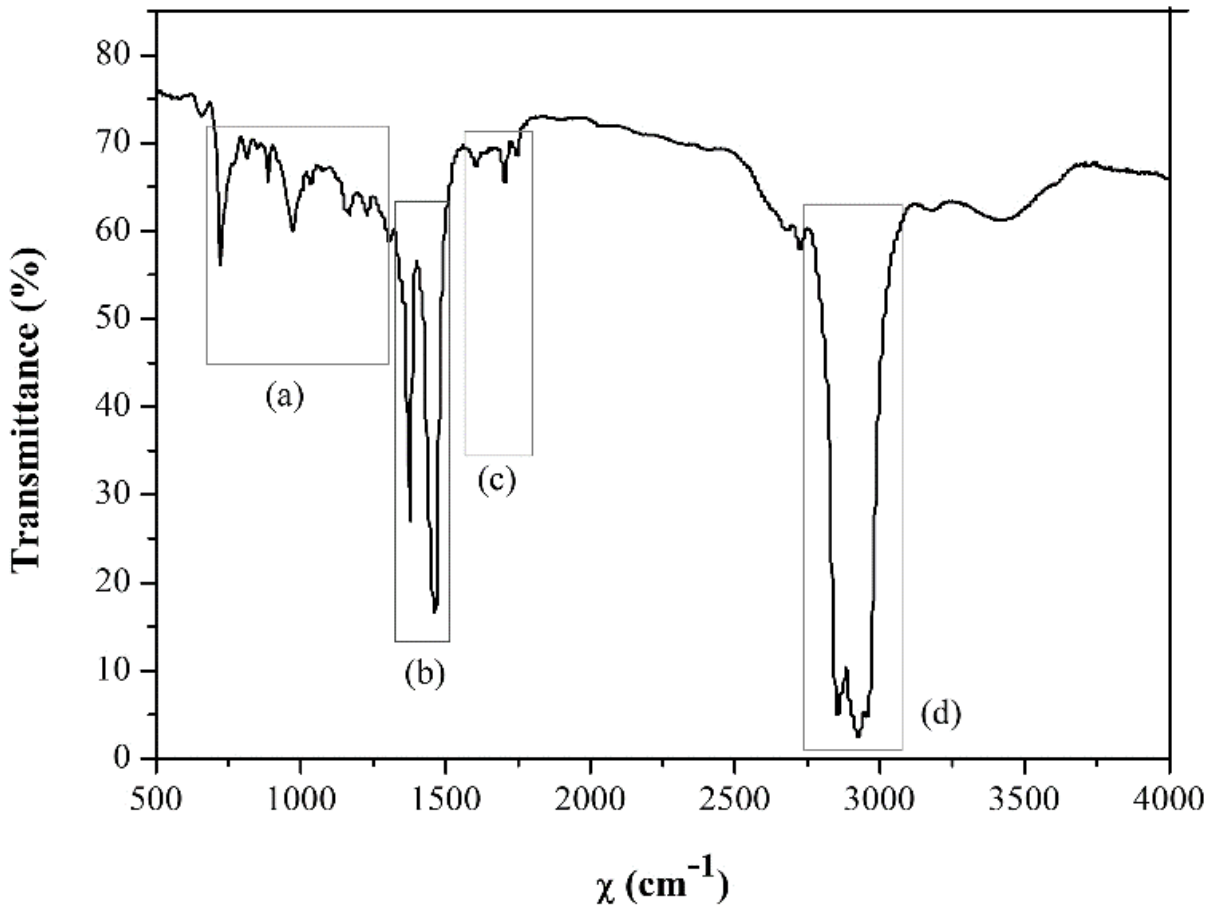

Figure 1

Analysis of used ship engine oil in the IR. Wavelength from 400 to 4000 cm^{-1}

Figure 2

UV-visible spectrophotometer scan from 200 to 800nm for ship oil quantification. Scans from 200 to 800nm and 200 to 300nm compare PVGOC-3 strain and NC.

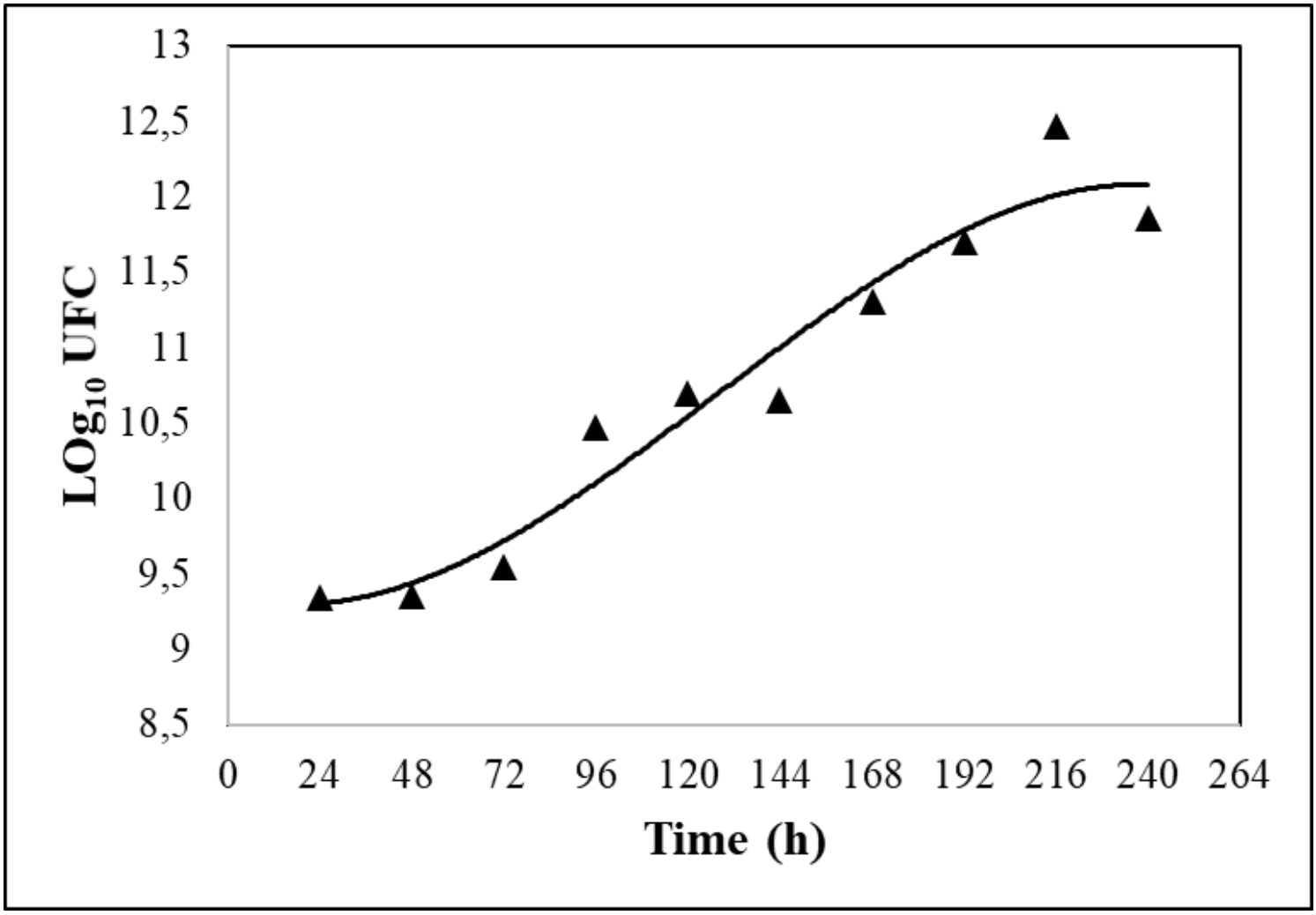

Figure 3

Growth curve. Experimental conditions: 100 mL of BH medium and 1% used ship oil, 150 rpm at 35ºC. (SD 0.92 to 2.34).

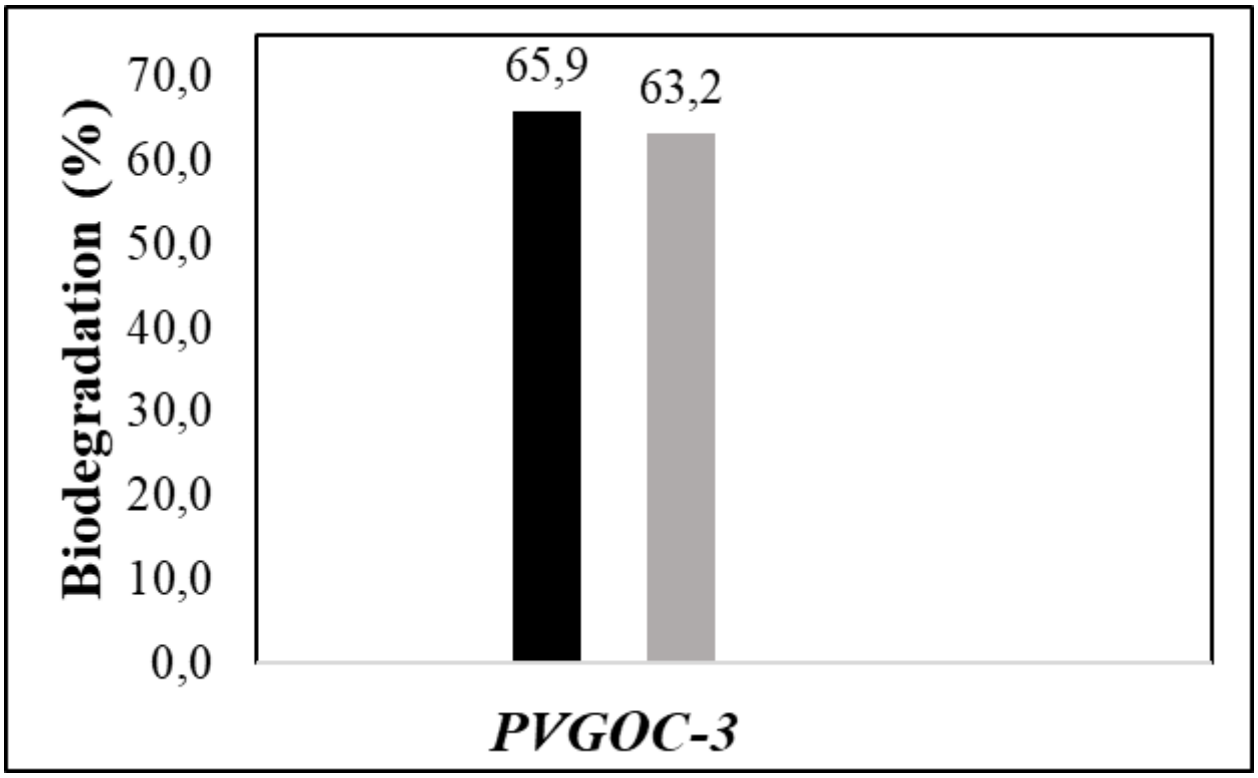

Figure 4

Comparison of biodegradation between first (■) and second (■) generation bacterial strains. Experimental conditions: 4 mL of BH medium and 1% used ship oil, 150 rpm at 35ºC for 10 days (dp 1,77 a 2,01).

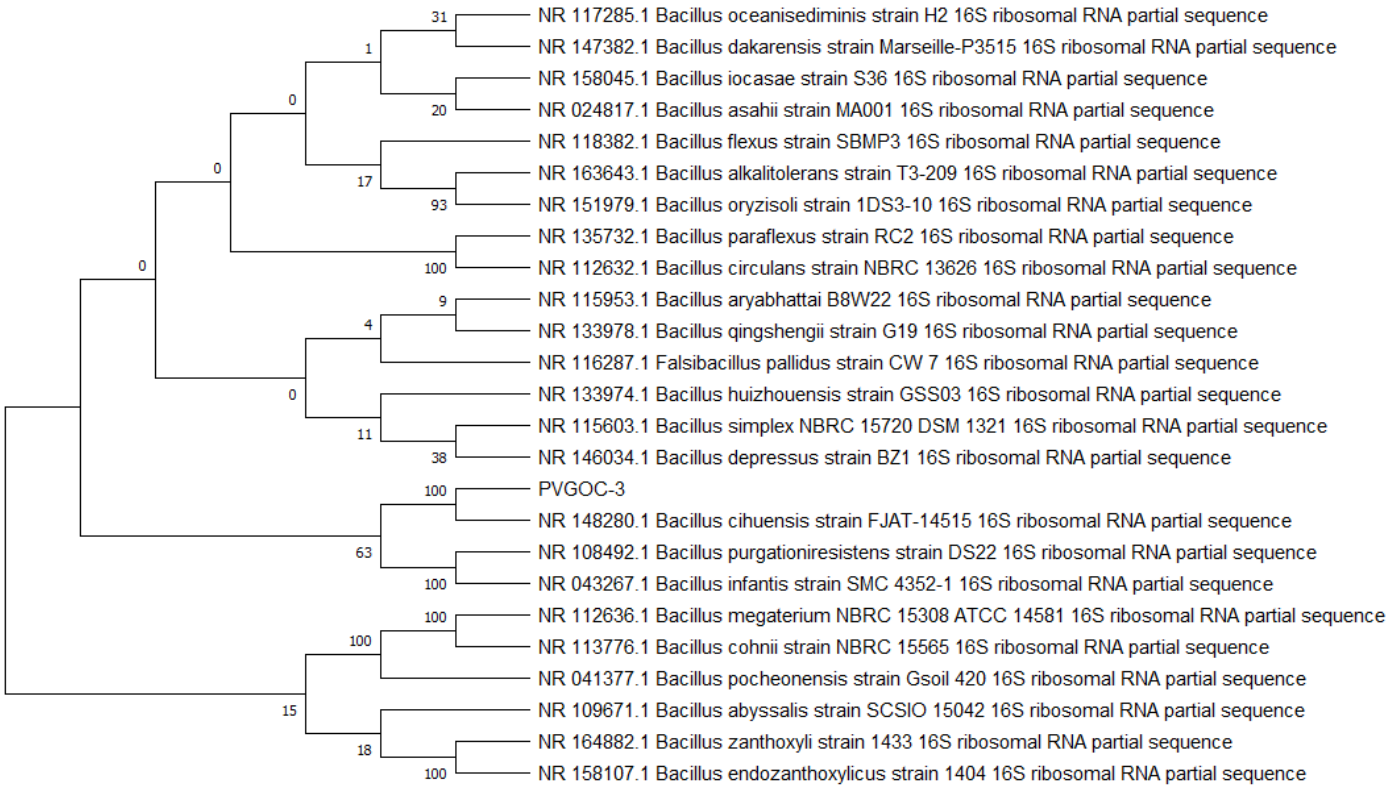

Figure 5

Phylogenetic tree of sequences of the 16S rRNA gene (approximately 1000 bp) showing the relationship between the PVGOC-3 strain and the type strains of different Species of Ensifer. The evolutionary history was inferred using the Neighbor-Joining method. Bootstrap analyses were performed with 1,000 repetitions. The GenBank access number of each species is in parentheses.