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Title:

Isolation and characterization of diesel-degrading bacteria from hydrocarbon-contaminated sites, flower farms and soda lakes

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Abstract

Background: Hydrocarbon-derived pollutants are becoming one of the most concerning ecological issues. Thus, there is a need to investigate and develop innovative, low-cost, eco-friendly, and fast techniques to reduce and/or eliminate pollutants using biological agents. The current study is conducted to isolate, characterize, and identify potential diesel-degrading bacteria.

Results: Samples were collected from flower farms, lakeshores, old aged garages, asphalt, and bitumen soils and spread on selective medium (Bushnell Hass Mineral Salts Agar) containing diesel as the growth substrate. The isolates were characterized based on their growth patterns using OD measurement, biochemical testing and gravimetric analysis and identified using the Biolog database, and 16S rRNA gene sequencing techniques. Subsequently, six diesel degraders

were identified and belong to *Pseudomonas*, *Providencia*, *Roseomonas*, *Stenotrophomonas*, *Achromobacter*, and *Bacillus*. Among these, based on gravimetric analysis, the three potent isolates AAUW23, AAUG11 and AAUG36 achieved 84%, 83.4%, and 83% diesel degradation efficiency, respectively, in 15 days. Consequently, the partial 16S rRNA gene sequences revealed that the two most potent bacterial strains (AAUW23 and AAUG11) were *Pseudomonas aeruginosa*, while AAUG36 was *Bacillus subtilis*.

Conclusion: This study demonstrated that bacterial species isolated from hydrocarbon-contaminated and/or uncontaminated environments could be optimized to be used as potential bioremediation agents for diesel removal.

Keywords: Biodegradation; BioLog; Gravimetric analysis; Hydrocarbon-degradation; 16S rRNA gene

Background

Hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs), benzene, kerosene, and diesel are important organic pollutants and inputs for different industries, vehicles, and household activities as a source of energy [2, 8, 14, 29, 35, 36, 40, 47]. Among these, diesel is known to be used massively for engine fuel and industrial applications. It is one of the products of petroleum compounds formed during fractional distillation (between 25 °C and 36 °C boiling point) and composed of a mixture of carbon chains between 9 and 25 carbon atoms that may include both aromatic and aliphatic hydrocarbon components [11, 29, 36]. These hydrocarbon components can be discharged into environments (groundwater, soil, and air) from different sources (point and non-point) such as garages, gas station services, chemical, and petrochemical industries, agricultural waste, automobile exhaust spillage of petroleum, run-off asphalt pavements, vehicular emission and combustion of fossil fuel [43, 44, 49]. This phenomenon may happen intentionally or accidentally mainly from anthropogenic activities as a result of urbanization, industrialization and civilization [5, 9, 14, 19, 43, 44] and, to some extent, by natural disaster [26]. As a result, the hydrocarbon-derived pollutants are immuno-toxicant, mutagenic and carcinogenic to humans and animals, and affect natural ecosystem functioning in many ways [8, 9, 14, 19, 29, 34, 36, 38- 40, 45, 46].

There are different methods of mitigating hydrocarbon pollutions. These include mechanical, chemical, and biological approaches. The first two aforementioned means of mitigation of pollutants need high operational costs and are prone to secondary pollution that necessitates integrated pollution management to reduce and/or remove the toxic pollutants from the environment [5, 24]. On the other hand, the biological method (bioremediation) is another promising technology that is prominent, eco-friendly, cost-effective, efficient and easily

applicable for the treatment of hydrocarbon-contaminated environments [1, 10, 14, 19, 26, 28, 39, 44, 47] but it possibly requires a long period of time for complete degrading of pollutants [18]. This approach mainly relies on two main techniques viz. bioaugmentation and biostimulation [24]. The bioaugmentation involves an introduction of selected hydrocarbon-degrading microbial strains or consortia to the polluted environment to boost the already existing potential microbial communities for the biodegradation process [1, 24, 27, 38, 44]. Biostimulation, however, engages the amendment of macro- and micronutrients, sustains physical parameters (pH, temperature and aeration) and supplies surface-active substances (surfactants) in contaminated sites to optimize soil conditions and enhance biodegradation by increasing the growth rate of inhabitant hydrocarbon-degrading microorganisms [16, 24, 29, 34, 36, 38, 44]. This technique can be applied in situ and ex situ [27] to enhance biodegradation by increasing the bioavailability of the pollutants and the growth rate of inhabitant (indigenous, autochthonous) hydrocarbon-degrading microorganisms [1]. Thus, potential microbes use hydrocarbon-derived pollutants as a source of carbon and energy [18, 37] and/or co-metabolite, finally leading to the complete mineralization of contaminants to carbon dioxide, water, mineral salts and biomass [5, 8, 13, 14, 28, 39, 44].

Many studies showed that diverse microorganisms or microbial communities, namely, bacteria, fungi, yeasts, protozoa, and algae, play a great role in the biodegradation of hydrocarbon pollutants and, among those, bacteria are the dominant and active degraders [9, 13, 14, 19, 39, 46]. The hydrocarbon-degrading bacteria are ubiquitous [36] of which the most known genera are: *Achromobacter, Marinobacter, Actinobacter, Alcaligenes, Mycobacterium, Arthrobacter, Bacillus, Rhodococcus, Corynebacterium, Micrococcus, Flavobacter, Nocardia, Bravibacterium, Streptococcus, Bacillus, Stenotrophomonas, Methylobacterium, Enterobacter;*

and *Pseudomonas* [13, 21, 29, 35, 41, 48]. Their effectiveness for biodegradation and detoxification of hydrocarbon pollutant is because of their diverse enzymatic activities including hydrolases, oxygenase, demethylase, dehalogenases, transferases, and oxidoreductases that can catalyze different degradation routes aerobically or anaerobically [10, 14, 19, 29, 31, 37, 41, 44], as well as their effective reproduction potential [26]. The fast and absolute degradation of hydrocarbon or other organic pollutants is brought via aerobic conditions [18, 31, 32, 37, 46].

This process mostly utilizes oxygenase enzymes (monooxygenases and dioxygenases) [49] for oxidative attack of alkyl side chains and the hydroxylation of aromatic rings (benzene, toluene, xylene, and naphthalene). However, the anaerobic degradation is catalysed by anaerobic or facultative bacteria using different final electron acceptors such as sulfate, nitrate, iron, manganese and CO_2 [6, 37]. The first step in oxidative biodegradation pathways is the activation of the ring for cleavage (*meta* or *ortho* cleavage) by hydroxylation using oxygenase enzymes [31, 49]. Both short- and long-chain hydrocarbons are oxidized to the corresponding alcohol that is later converted into aldehyde by an alcohol dehydrogenase, and the aldehyde oxidized into the acid by an aldehyde dehydrogenase. Consequently, the resulting fatty acids go through the ß-oxidation system to acetate (even-chain alkanes) and propionate (odd-chain alkanes). The hydrocarbon products are then subsequently oxidized into the Krebs cycle intermediates and eventually mineralized to CO_2 and water [12, 49].

The microbial enzymes for biodegradtion of hydrocarbon are encoded by genes located on chromosomal or plasmid DNA [31, 37]. Some of the well studied genes are: *alkB* (encoding alkane monooxygenase), *ndo/nah (*encoding naphthalene dioxygenase), *xyl* (encoding xylene dioxygenase), *bssA* (encoding for benzylesuccinate synthase A), *assA* (encoding alkaylsuccinate synthase A) [25, 30, 37], *almA* and *ladA* (encoding for long chain alkane monooxygenase), and

many of the gene-encoding transcriptional regulators of alkane biodegradtion, such as *merR, araC2 alkS* [48]. Therefore, indigenous microorganisms can degrade hydrocarbon-derived pollutants through natural remediation or attenuation into less or non-toxic forms in the environment [14, 26, 43, 44, 49]. However, natural attenuation is often limited when there is a lack of proper nutrient availability, high capability of microbial communities, and necessary catabolic genes for complete hydrocarbon degradation [22]. In addition, individual bacteria can metabolize only a limited range of hydrocarbon substrates such as alkanes, and others that are paraffinic and aromatic, but a bacterial consortium with broad enzymatic capacities is required to synergistically degrade complex mixtures of pollutants [13, 26, 49]. Studies also showed that for successful biodegradation, the number of hydrocarbon-degrading bacteria should be in the range of 10^4 to 10^7 CFU per gram of soil [5] and considerably higher in hydrocarbon-contaminated sites [32].

However, the functioning of bacterial community structures and complete degradation is influenced by different factors such as the inherent genetic characterstics of microbial population (catabolic gene or types of enzymes), number of microbes (size), microbial mitigation or interaction (single strain or consortia), microbial diversity (bacteria, algae and fungi), microbial competition (synergistic or antagonistic), the nature and characteristics of hydrocarbon pollutants (chemical structure, concentration, bioavailability and toxicity level) and the physical environment (nutrients, temperature, water activity, pH, soil moisture and types of electron acceptors for respiration), etc. [1, 7, 22, 19, 26, 28- 30, 36, 38, 39, 42, 44, 47, 49].

The objective of this study was to isolate, characterize, and identify the potential diesel-degrading bacteria from hydrocarbon-contaminated samples from different study sites. Hence, indigenous diesel-degrading bacteria were isolated and screened for their efficacy and further

145 characterized by cultural (colony characteristics), growth pattern (OD), biochemical tests, and
146 identified using BioLog and 16S rRNA gene sequencing.

147 **Materials and Methods**

148 **Study area**

149 Soil samples were collected from sites such as old aged garages (from the Addis Ababa
150 region, namely Amanuel and Akaki), old aged asphalt (from the Addis Ababa region, Amanuel),
151 bitumen spill areas (from the Addis Ababa region, Woira Sefer) and the Gallica flower farm
152 located in Menagesha (22 km of West of Addis Ababa) that were potentially exposed to
153 hydrocarbon contamination. Sites such as Chitu soda lake (180 km from Addis Ababa and
154 located at in the Southern Rift Valley of Ethiopia) have no known potential exposure to
155 hydrocarbon contaminants. The soil samples were designated as AAUA (soil samples from
156 Akaki/Amanuel Garages), AAUAs (soil samples from Amanuel old aged asphalt sites), AAUG
157 (soil samples from Galica flower farms), AAUW (samples from Woira Sefer bitumen soils) and
158 AAUC (soil samples from Chitu soda lake).

159 **Sample size and sampling methods**

160 Approximately 10 g of humid soil samples were collected from topsoil (5–10 cm) of each
161 of the selected study sites (in triplicate) using the simple random spatial sampling method. The
162 samples were transferred into sterile polyethylene bags, labeled, kept in the icebox, and
163 transported to the Microbial Biotechnology Laboratory at Addis Ababa Science and Technology
164 University and stored in a refrigerator (EVERmed LR270W, Motteggiana (MN), Italy) at 4 °C
165 until use.

166

Enrichment of diesel-degrading bacteria

The isolation of the hydrocarbon-degrading bacteria was undertaken by using enrichment medium with the modified method of [5, 6, 22, 26, 39].The enrichment medium or modified basal salt medium (BSM) contained (g/l of distilled water): KH_2PO_4 (1.36), Na_2HPO_4 (1.39), KNO_3 (1.25), $MgSO_4$ (0.06), $CaCl_2$ (0.02), $(NH_4)_2SO_4$ (7.7), NH_4Cl (1.5), NH_4NO_3 (0.85), K_2HPO_4 (0.53), and 100 mL of a trace mineral solution containing 0.01 g of $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, H_3BO_4, $CoCl_2{\cdot}6H_2O$, $Fe_2SO_4{\cdot}2H_2O$, $CuCl_2{\cdot}2H_2O$, and $NaMoO_4{\cdot}2H_2O$. The triplicate soil samples of each site were manually homogenized and sieved using a sterile 2 mm mesh screen. Then, one gram (1 g) of each sample was weighed and mixed into 9 mL of saline solution (0.99% of NaCl) from which 1 mL of the supernatant was transferred into 50 mL of enrichment medium supplemented with 0.5% (*v/v*) of diesel. The diesel used in this experiment was obtained from a local oil filling station (Jemal Tulu Dimtu Total oil and Gas Station, Ethiopia) and it was filter-sterilized using 0.45 μm diameter of the membrane filter [14] in 100 mL capacity Erlenmeyer flasks. The flasks were incubated in an intelligent thermostatic shake cultivation cabinet (ZHP-Y2112L series, Yangzhou Sanfa Electronic Co. Ltd., Jiangsu, China) with 150 rpm at 30 °C, for 6 days [43]. Then, 10% (*v/v*) of the enriched culture was subsequently transferred into the enrichment media three times for refreshment.

Isolation and plate counting of diesel-degrading bacteria

Following enrichment, the microbial cultures were prepared to appropriate dilutions using sterile saline solution (0.99% NaCl) (10^{-1}, 10^{-3}, 10^{-5} and 10^{-7}), and from which 0.1 mL suspension was spread on Bushnell Has Mineral Salt (BHMS) agar medium containing 0.5% (*v/v*) diesel. The medium contained g/l: $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1), K_2HPO_4 (1), NH_4NO_3 (1) and 2 drops from 60% of $FeCl_3$ with pH 7.2. Two controls (negative) were used

190 i.e., BHMS media with diesel but not enriched culture and BHMS media with enriched culture
191 but not diesel supplements. The plates were incubated for 6 days at 30 °C to observe and
192 determine the colony-forming units (CFU g−1). Isolates with distinct colonies were purified by
193 streak plating onto nutrient agar medium (HiMEDIA, Bengaluru, India). Then, isolates were
194 designated as AAU (Addis Ababa University) isolates with their identification sites (A = Akaki
195 and Amanuel garage; C = Chitu Lake; G = Galica Flower farm; As = Amanuel old aged asphalt
196 and W = Woira Sefer bitumen soil) and respective identification numbers and preserved in 25%
197 *v/v* glycerol at −20 °C (IGnIS CHEST FREEZER C0450W, Milano, Italy) for a month and
198 subsequently subcultured for refreshment.

199 **Biochemical tests**

200 **Gram stain technique**

201 For the Gram's staining standard protocol, the pure isolates were allowed to grow on
202 nutrient agar for 24 h. The distinct grown colonies were transferred on a plane slide and heat
203 fixation was applied. The primary dye (methylene blue) was added for 1 min and rinsed with tap
204 water. The slide was immersed into a jar containing the mordant (Gram's Iodine) for 2 min and
205 rinsed with water. Then, the decolorizer (95% ethanol with an equal amount of acetone) was
206 added for 15–30 s. Following this, the counter stain (safranin) was added on the smear for 1 min.
207 Then, the slide was air-dried and microscopy was performed. Based on the microscopy results,
208 the isolates' morphological characteristics and Gram's results were studied.

209 **Catalase test**

210 Three drops of hydrogen peroxide (3%) were added into the overnight grown culture in
211 the test tubes and the formation of vigorous bubbles indicated catalase activity [35, 40].

212

213 **Casein hydrolysis**

214 Isolates were grown overnight in nutrient broth and inoculated onto Skim Milk Agar
215 (HiMEDIA) and incubated at 30 °C for 48 h [35, 40]. The formation of a clear zone around the
216 isolates against the white background indicated the casein hydrolysis activity of the isolates.

217 **Urease test**

218 The pure bacterial isolates were inoculated into urea broth (Difco, BD, Wokingham, UK)
219 and incubated at 30 °C for 24–48 h [40]. The change of color from yellow to pink indicated that
220 there was urease production.

221 **Starch test**

222 Isolates were grown overnight in nutrient broth and inoculated into starch agar medium
223 (Alpha Chemika, Mumbai, India) and incubated at 30 °C for 48 h [35, 40]. The plates were
224 flooded with Gram Iodine. The formation of a clear area around the isolate against the blue-black
225 background indicated starch hydrolysis.

226 **The biodegradation capacity of diesel-degrading bacteria**

227 Overnight bacterial culture [13] with a total plate count of 10^8 cells/ml [12] for each
228 isolate was inoculated into 100 BHMS broth containing 1% and 3% (*v/v*) diesel as substrate in
229 250 mL flasks and kept in a shaker incubator at 150 rpm at 30 °C [49] for 5, 10, and 15 days.
230 The growth (turbidity) was measured using a UV/Vis spectrophotometer (Mecasys, Optizen POP
231 Series, K LAB, Daejeon, Mecasys Co., Ltd, South Korea) at 600 nm (OD_{600}) in triplicate against
232 BHMS medium as blank.

Estimation of diesel biodegradation efficiency by gravimetric analysis

The isolates amounted to 10^8 cells/mL and were inoculated into 100 mL BHMS supplemented with 5% diesel dispensed in 250 mL conical flasks on a rotary shaker (150 rpm), and incubated at 30 °C for 10 and 15 days. The residual concentration of diesel was assessed using the method with slight modification [7, 34, 39]. Thus, 1% 1N HCl was added into the culture to stop the bacterial activity and the residual was extracted from the whole content using petroleum ether and acetone (1:1 *v/v* ratio). Then the flask was placed on the shaker at 120 rpm for 20 min and oil-containing solvent was collected from the upper portion of the flask and poured into the pre-weighted petriplate [47]. It was repeated three times to ensure complete extraction. The extracted component was allowed to evaporate in a hot air oven at 72 °C. Then, the residual diesel was calculated as percentage of degradation using the following formula [19, 45, 47]:

$$\text{Percentage degradation } (\%) = \frac{(\,Initial\ \ concentartion\ \ \ of\ the\ diesel - Final\ \ concentartion\ \ \ of\ \ diesel\,)}{Initial\ \ concentartion\ \ \ of\ \ diesel} * 100 \qquad (1)$$

Identification of diesel-degrading bacteria using BioLog

Preliminary identification of the isolates was made using Omnilog/BioLog systems according to the manufacturer's specifications (BIOLOG Inc., Hayward, CA, USA) at Sebeta National Animal Health Diagnostic Center, Ethiopia. The 96 wells of the MicroPlates were filled with carbon sources and other nutrients and the utilization of carbon sources and/or resistance to inhibitory chemicals was colorimetrically determined using tetrazolium redox dyes. The bacterial isolates were grown on Biolog Universal Growth (BUG) agar medium using protocol "A" that used inoculation fluid A (IF A) and a default protocol to identify the vast majority of bacterial species, and then suspended in a special "gelling" inoculating fluid 3 (IF) at the 90–98% cell density. The cell suspension was then inoculated into the GEN III MicroPlate (100 µl per well)

and incubated at 91.40 °F for 16 h. After incubation, the phenotypic fingerprint of purple wells were compared to BioLog's extensive species library (databases of the microorganisms) using a microplate reader.

Genetic characterization of the isolates

PCR amplification using bacterial Specific 16S rRNA primers

The freeze–thaw technique was used to extract the genomic DNA from each pure bacterial colony for use as a template to amplify a bacterial domain-specific ca.1500 bp 16S rRNA gene. For this purpose, a colony was suspended in 50 µl sterile water and lysed by boiling for 5 min. It was then centrifuged at 12,000 × g for 10 min from which 1 µl of the supernatant of lysed cells was transferred into 20 µl of the PCR master mix. The master mix consisted of 16.2 µl PCR grade water, 2 µl of 10 × PCR buffer (Life Technologies, Carlsbad, CA, USA), 0.4 µl of 10 mM DNTP mix (Life Technologies), 0.4 µl of 20 mg/ml Bovine Serum Albumin) (BSA), 0.8 µL of 25 mM $MgCl_2$, 0.08 µl of 50 µM of each 8F (5′–AGAGTTTGATCCTGGCTCAG–3′) and 1492R (5′–GGTTACCTTGTTACGACTT–3′), and dream Taq-Polymerase (Life Technologies). DNA amplification was performed using a Thermocycler (Verticycler, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The PCR cycling program was as follows: initial denaturation at 96 °C for 10 min, 35 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 1 min and a final extension of 7 min at 72 °C. Reagent composition of the PCR reaction mixture (50 µL) contained genomic DNA extract (5µl), 10X Taq polymerase Buffer, dNTPs mixture (2.5 pmol), each primer (20 pmol), and Taq DNA polymerase (2.5 U). Finally, the 16S rRNA PCR amplicons were purified using the Illustra Exostar DNA purification kit (GE Health Care) according to the manufacturer‘s specifications. The purified PCR product then underwent partial sequencing using the 8F primer (monodirectional sequencing) at the

sequencing facility of Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Germany. The 16S rRNA amplification was performed using the bacterial universal oligonucleotide primers 8F and 1492R using the Verticycler PCR system (Applied Biosystems) as per described by [33].

Nucleotide sequencing and phylogenetic analysis

Partial 16S rRNA gene sequencing was performed using the Illumina/Solexa sequencing facility, as described by [25] and the raw DNA chromatogram sequences were viewed and edited using Sequence Alignment Editor Version 7.0.5.3 [15] and stored in FASTA format. The sequences were compiled and compared to the NCBI (http://www.ncbi.nlm.nih.gov) DNA sequence database using BLASTn to verify proximate phylogenetic positions

Data analysis

Numerical data were analyzed by analysis of variance (ANOVA) followed by a multiple comparison test (Duncan) with SAS statistics software (version 9.1.3; 2003, Cary, NC, USA), considering statistically significant differences to be those with a p-value <0.0001 of potent diesel-degrading bacteria. The phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis X (MEGA X; Pennsylvania State University, USA) with bootstrap values of 1000 replications using the maximum likelihood method [23] and Kimura-2 parameter model [21].

Results

Isolation of diesel-degrading bacteria

Nineteen diesel-degrading bacteria were isolated from the enrichment culture of different sampling sites (Table 1). The data showed that the population density of diesel-degrading bacteria was found to be 2.2×10^3 CFU for the old aged asphalt site, 1.7×10^6 CFU for the Akaki and Amanuel garage sites, 5.7×10^4 CFU for Woira sefer bitumen soil, which was

expected to have exposure to hydrocarbon contamination, and 2.7×10^3 for the Gallica flower farm, which uses different agrochemicals containing polycyclic hydrocarbons. Diesel-degrading bacteria were also recovered from Chitu soda lake that has no known history of previous hydrocarbon contamination.

Bacterial identification and characterization

Characterization of isolates based on cell morphology and biochemical test

In this study, 19 bacterial isolates were characterized using Gram's staining and biochemical tests (Table 1). Based on Gram's reaction, the majority of the bacteria (85%) were Gram-negative and rod-shaped whereas 15% were Gram-positive rods. The isolates were also characterized based on standard biochemical tests and all isolates were catalase positive, except AAUG10 (*Roseomonas cervicalis*). In addition, the majority of diesel degraders (68%) were capable of casein hydrolysis, excluding *Providencia rettgeri*, *Achromobacter xylosoxidans,* and *Stenotrophomonas maltophila*. The data also showed that 42% of the isolates were urase-positive and three Gram-positive *Bacillus spp*. were able to hydrolyze starch.

Identification of diesel-degrading species using BioLog

The GEN III MicroPlate test panel provides a standardized micro-method to profile and identify a broad range of Gram-negative and Gram-positive bacteria based on 65.5 to 99.9 % accuracy of identification of the species within genera (Table 1). Thus, the identified bacterial genera were *Pseudomonas spp., Roseomonas spp., Bacillus spp., Providencia spp., Achromobacter spp.* and *Stenotrophomonas spp*.

Table 1. Population density, morphological and physiological characteristics of diesel-degrading bacteria isolated from different sampling sites.

Isolates Code	BioLog ID	Site	CFU	Gram's	Shape	Catalase	Urase	Casein	Starch
AAUG8	*P. rettgeri*	Flower	2.7×10^3	−	Bacilli	+	+	−	−
AAUG9	*P. aeruginosa*	Flower		−	Bacilli	+	−	+	−
AAUG10	*R. cervicalis*	Flower		−	Coccobacilli	−	+	+	−
AAUG11	*P. aeruginosa*	Flower		−	Bacilli	+	−	+	-
AAUA12	*B. cereus*	Garages		+	Bacilli	+	+	+	+
AAUA13	*B. cereus*	Garages	1.7×10^6	+	Bacilli	+	+	+	+
AAUA14	*S. maltophila*	Garages		−	Bacilli	+	+	−	−
AAUA15	*S. maltophila*	Garages		−	Bacilli	+	+	−	−
AAUAs16	*A. xylosoxidans*	Asphalt	2.2×10^3	−	Bacilli	+	−	−	−
AAUAs17	*P. rettgeri*	Asphalt		−	Bacilli	+	−	−	−
AAUC18	*P. viridilivida*	Soda lake		−	Bacilli	+	+	+	-
AAUC19	*P. aeruginosa*	Soda lake	2.9×10^3	−	Bacilli	+	−	+	−
AAUC20	*S. maltophila*	Soda lake		−	Bacilli	+	−	−	−
AAUC21	*P. aeruginosa*	Soda lake		−	Bacilli	+	−	+	−
AAUW22	*P. aeruginosa*	Bitumen		−	Bacilli	+	−	+	−
AAUW23	*P. aeruginosa*	Bitumen		−	Bacilli	+	−	+	−
AAUW24	*P. aeruginosa*	Bitumen	5.7×10^4	−	Bacilli	+	−	+	−
AAUW25	*P. aeruginosa*	Bitumen		−	Bacilli	+	−	+	−
AAUG36	*B. subtilis*	Flower		+	Bacilli	+	−	+	+

Diversity of diesel-degrading bacteria

Among the isolated species, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* accounted for 42% and 16%, respectively (Table 2). Besides, *Bacillus cereus* and *Providencia rettigeri* each accounted for the third group (11% of the distribution). The isolates were recovered from different sites that were predominately contaminated with hydrocarbon components or had no history of direct contamination of hydrocarbon constituents.

Table 2. Diversity and community structures of isolates from hydrocarbon-contaminated sites and non-polluted natural sites (Chitu soda lake).

Genus of the Isolates	Distribution (%)	Species of the Isolates	Distribution (%)	Species Distribution (%)	
				Contaminated Sites	Non-Contaminated Sites
Pseudomonas	47	*Pseudomonas aeruginosa*	42	31.5	10.5
		Pseudomonas viridilivida	5	0	5
Bacillus	16	*Bacillus cereus*	11	11	0
		Bacillus subtilis	5	5	0
Providencia	11	*Providencia rettgeri*	11	11	0
Roseomonas	5	*Roseomonas cervicalis*	5	5	0
Stenotrophomonas	16	*Stenotrophomonas maltophilia*	16	11	5
Achromobacter	5	*Achromobacter xylosoxidans*	5	5	0

Screening of isolates for effective diesel degradation

Diesel biodegradation (1%)

Bacteria utilize diesel for their growth, energy and an increase in biomass [2]. The growth or increase in biomass is indicated by turbidity in the growth medium (BHMS). In this study, the growth pattern (effective degradation) of 19 bacterial isolates was enumerated on BHMS medium supplemented with 1% diesel. The isolates showed significant growth on the 10^{th} day of incubation ranging from an OD of 0.41 ± 0.002 to 1.3 ± 0.004, indicating significant differences ($p < 0.0001$) in their ability to degrade diesel. Among the isolates, *B. cereus* (AAUA13) showed an OD value of 1.3 ± 0.004, followed by *A. xylosoxidans* (AAUAs16) with an OD value of 1.28 ± 0.002. Their degrading potential reached the peak on the 10th day of growth incubation, which was 3-fold higher than the 5th day of incubation (Fig. 1). Similarly, *P. aeruginosa* (AAUW24), *B. subtilis* (AAUG36), *P. rettgeri* (AAUAs17), *P. aeruginosa* (AAUG11, AAUC19, and AAUW25) and *S. maltophilia* (AAUA14 and AAUA15) showed no significant difference in

their growth. In this study, it was investigated that only one isolate, *P. rettgeri* (AAUG8), showed an increased growth measured in terms of OD from the 10th day of incubation (0.41 ± 0.002) to the 15th day of incubation (0.62 ± 0.002).

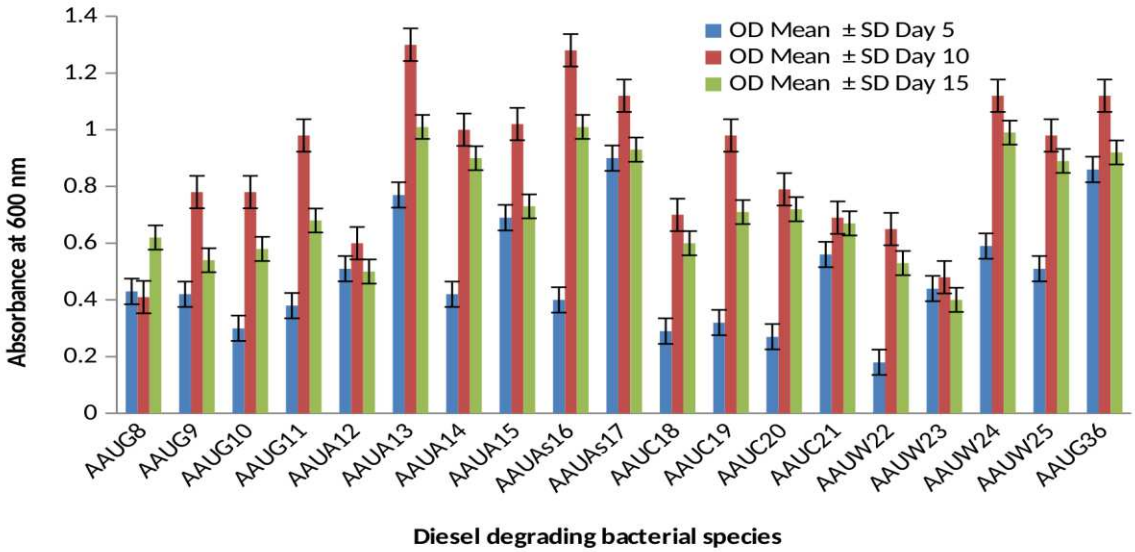

Fig. 1 Growth capacity of isolates on diesel (1% concentration at different growth periods).

Diesel biodegradation (3%)

The growth pattern of bacterial isolates for hydrocarbon degradation on the diesel medium (3%) was also studied (Fig. 2). The maximum growth was recorded on the 10th day of incubation. Isolate AAUW23 (*P. aeruginosa*) showed a significant growth capability with an OD value of 2.14 ± 0.016 ($p < 0.0001$) compared to other isolates. Earlier study also showed that *P. aeruginosa* is efficient for the degradation of high concentrations of hydrocarbon contaminants [49]. In addition, the remaining isolates identified as *P. aeruginosa* (AAUC21, AAUG11, and AAUW22), *P. viridilivida* (AAUC18), and *S. maltophilia* (AAUA14) also showed modest diesel biodegradation activities with OD values ranging from 1.43 ± 0.003 to 1.71 ± 0.022. From a previous study, *S. maltophilia* was identified as a key hydrocarbon-degrading bacterium [8]. Some isolates also showed increased activities as the incubation period continued above 10 days,

with a significance difference of OD value. Accordingly, *S. maltophilia* (AAUC20), *Providencia rettgeri* (AAUG8), *A. xylosoxidans* (AAUAs16) and *P. aeruginosa* (AAUW22) were identified as potential diesel degraders with OD values of 0.92 ± 0.075; 1.42 ± 0.047; 1.6 ± 0.022 and 1.78 ± 0.038, respectively. Therefore, *P. aeruginosa* considerably showed effective diesel degradation capacity. This could be due to the fact that *P. aeruginosa* has unique adaptive potential to survive in a range of diverse conditions including environments that harbor substantial concentrations of hydrocarbon sources such as diesel [39].

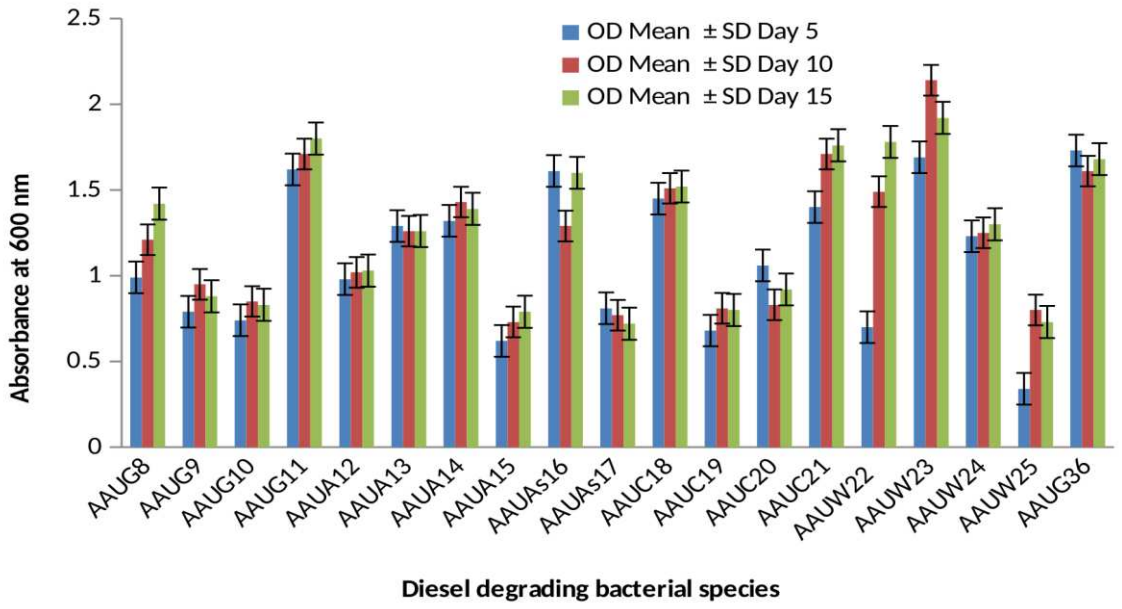

Fig. 2 Growth capacity of isolates on diesel (3% concentration at different growth periods)

Gravimetric analysis for diesel biodegradation efficacy

Seven bacteria isolates were found to grow and showed effective degradation competence in BHMS media containing 3% diesel concentration. These selected potential isolates were then provided with 5% diesel as a growth substrate and gravimetric analysis was performed on the 10th and 15th day of incubation (Fig. 3). The result showed that two isolates of *Pseudomonas spp.* (AAUW23 and AAUG11) and *B. subtilis* (AAUG36) showed 83.6%, 84%, and 83% diesel degradation efficacy, respectively, on the 15th day of incubation. The remaining isolates *P.*

viridilivida (AAUC18), *P. rettigeri* (AAUG8), and *S. maltophila* (AAUA14) showed relatively lower degradation efficiency for diesel (58%, 48%, and 31.6%, respectively) for the same day of incubation. The previous study by [34] also showed that the maximum degradation of diesel observed after 15 days of incubation was 53% when grown at 0.5% diesel concentration. Therefore, in this study, diesel was degraded at a high concentration (5%) and short exposure time.

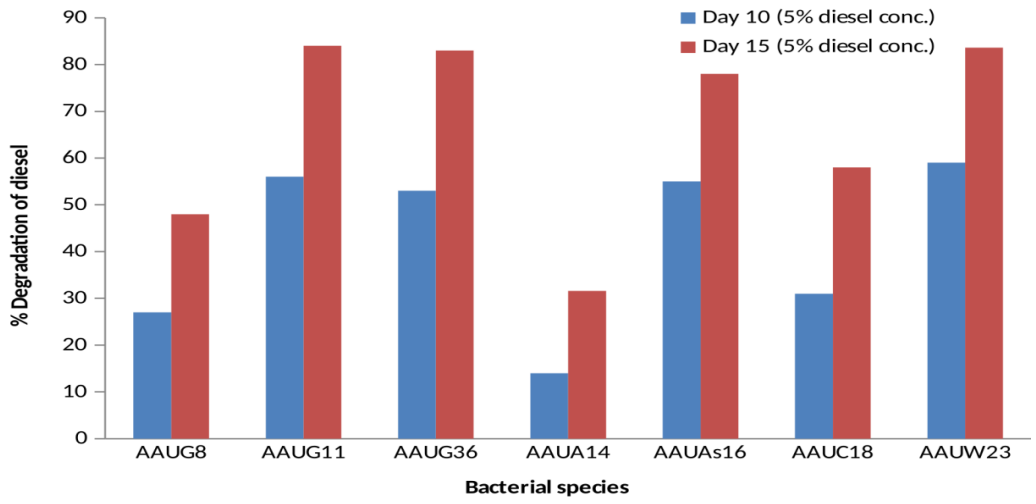

Fig. 3 Gravimetric analysis for diesel degradation

16S rRNA sequences and phylogenetic analysis of selected isolates

Three of the most efficient bacterial isolates (designated as AAUW23, AAUG36, and AAUG11) that showed maximum diesel-degrading capability were selected upon the gravimetric analysis method and their 16S rRNA were sequenced. The partial 16S rRNA sequences of the three bacterial isolates were submitted to the NCBI and their accession numbers were obtained as MT669825 for AAUW23, MT669830 for AAUG36 and MT669831 for AAUG11. The 16S rRNA sequencing and phylogenetic data analysis of these three bacteria isolates using BLAST searches confirmed that the isolates are closely related to some of 16S rRNA sequences of the cultured bacterial taxon in the Genbank database. Consequently, two isolates, AAUW23 and

AAUG11, belonged to gamma subdivisions of *Proteobacteria*, while AAUG36 belonged to *Firmicutes* (Table 3).

Table 3. Phylogenetic affiliation of 16S rRNA partial sequences of three bacterial isolates

Isolate Code	Accession Number	Top-hit Taxon	GenBank Accession	Identity (%)	Taxonomy
AAUG11	MT669831	*P. aeruginosa*	MT646431.1 MT636685.1 MT598024.1 MT626658.1 MT598019.1	99.69%, 99.69%, 99.69% 99.69% 99.69%	Bacteria;Proteobacteria; Gamma-proteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
AAUG36	MT669830	*B. subtilis*	MT677931.1 MT645308.1 MT704510.1 MT706001.1 MT733943.1	99.43%, 99.43%, 99.43% 99.43% 99.43%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
AAUW23	MT669825	*P. aeruginosa*	MT598024.1 KM216848.1 MK875780.1 MT626658.1 MT633047.1	99.23% 99.34% 93.34%, 99.23% 99.23%	Bacteria;Proteobacteria; Gamma-proteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.

The phylogenetic tree was constructed in MEGA X using the maximum likelihood method, and it depicted that bacterial isolates AAUW23 and AAUG11 could well cluster with *P. aeruginosa* while isolate AAUG36 could relate with *Bacillus spp.* (Fig. 4).

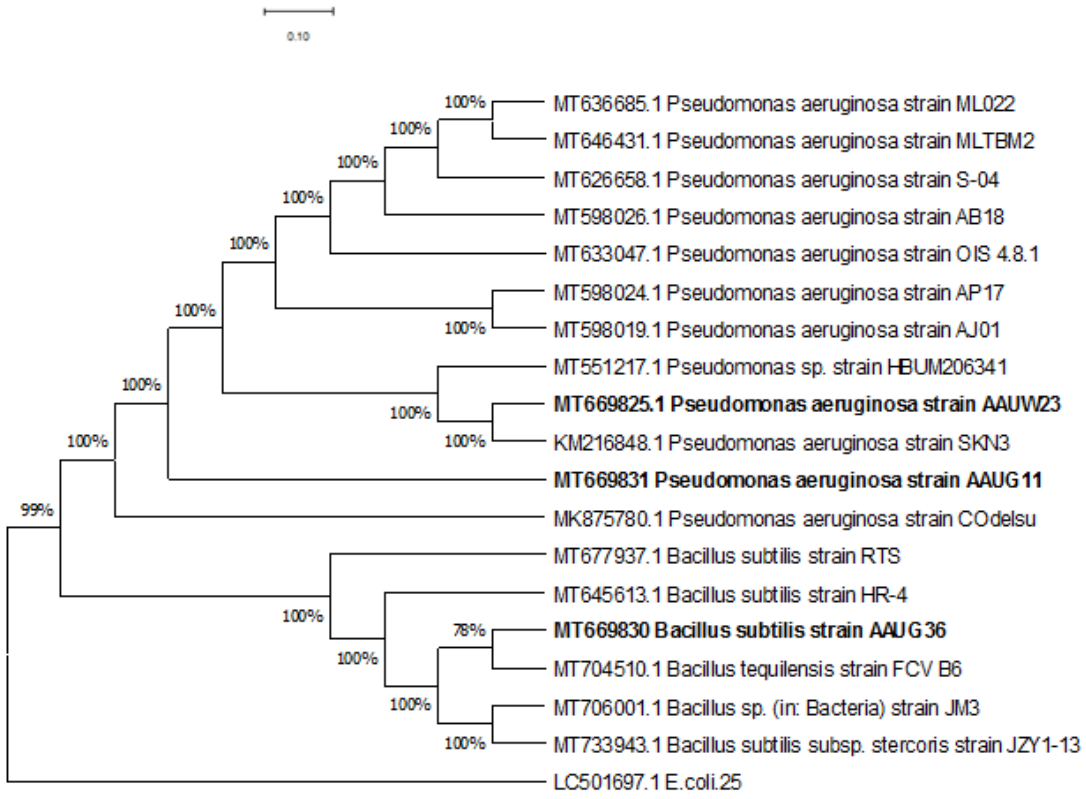

Fig. 4 Phylogenetic tree based on partial bacterial sequences of the 16S rRNA region for the two *Pseudomonas isolates* and one *Bacillus subtilis* (bold and coded with the initials "AAU"), and accession numbers of the 16S rRNA are followed by species names. Numbers at nodes indicated bootstrap values for each node out of 1000 bootstrap resembling. The phylogenetic tree was constructed in MEGA X using the maximum likelihood method [23] and Kimura-2 parameter model [21]. The *E. coli* plasmid partial sequence was used as an out-group

The constructed phylogenetic tree depicted that AAUG11 (MT669831) and AAUW23 (MT669825) shared 98% nucleotide identity with each other and ≥99% similarity with other existing bacterial 16S rRNA sequences retrieved from the database. The isolate AAUG36 (MT669831) isolated from the Gallica flower farm soil sample was affiliated to *P. aeruginosa* strain SKN3 (MK216848.1) and strain COdelsu (MK875780.1) with a similarity of 99% previously isolated from plant phyllosphre and crude oil samples, respectively. The other isolate

AAUW23 (MT669825) isolated from bitumen soil sample was also closely related to *Pseudomonas spp.* strain SKN3 (MK216848.1) and strain HBUM206341 (MT551217.1) previously described from environmental samples. In addition, the isolate AAUG36 (MT669830) was isolated from the Gallica flower farm and formed a common lineage with strain *Bacillus tequilensis* FCV B6 (MT704510.1) with 99.34% similarity (bootstrap value of 78%) previously isolated from disinfectant-contaminated biofilm sample. From this study, therefore, *Pseudomonas* and *Bacillus* are the prevailing diesel-degrading bacterial genera detected in hydrocarbon-contaminated areas such as bitumen soil and flower farms. The present study clearly revealed that bacteria inhabiting various hydrocarbon-contaminated soils/sediments could rapidly degrade diesel.

Discussion

Microorganisms play a vital role in biodegradation (bioremediation) of hydrocarbon pollutants from polluted milieu [4]. In this study, the bacterial isolates were recovered from the soil samples of known or unknown hydrocarbon-contaminated environments using BHMS medium supplemented with 0.5% of diesel as a carbon source to enrich their growth pattern and determine their degrading potential. The population density enumerated from all sampling sites is within the recommended number of 10^4 to 10^7 CFU per gram of soil for successful hydrocarbon biodegradation [44]. However, there were differences in the number of bacterial isolates in the sampling sites. The fact that different numbers of colonies were obtained from these sites might be associated with the diversity of bacteria capable of degrading hydrocarbons and their derivatives [39]. In addition, a greater number of diesel-degrading bacteria could be recovered from garage sample sites and other various petroleum compound contaminated sites [13, 14, 29, 35, 45, 49]. This could be associated with the potential of bacterial survival on

different types of hydrocarbon components such as aliphatic (diesel) and aromatic (monocyclic or polycyclic) hydrocarbons [14]. However, indigenous microorganisms that can degrade these pollutants through natural attenuation are very low [26, 44]. In addition, the current work demonstrated that hydrocarbon-degrading bacteria also isolated from non-hydrocarbon-contaminated sites such as soda lake (Chitu). In agreement with this, studies also showed that several hundred strains of hydrocarbon-degrading bacteria have also been isolated from the environments with no known hydrocarbon contamination [20, 48] This could be due to the existence of hydrocarbons from natural and anthropological origin or produced by the degradation and synthesis processes of some microorganisms [3, 17] and such natural environments are expected to contain highly reduced forms of hydrocarbon that are important to support microbial communities as good sources of carbon and energy [3, 9, 16, 32].

The majorities of isolated diesel-degrading bacteria were Gram negative, mainly belonging to five genera viz. *Pseudomonas spp.*, *Stenotrophomonas spp.*, *Providencia spp.*, *Roseomonas spp.*, *and Achromobacter spp*., and found to be 47, 16, 11, 5 and 5% of the total isolates, respectively. Other studies also showed that the Gram negative species of the genus *Pseudomonas spp.* (38.94%) and *Achromobacter spp.* (7.96%) were characterized as diesel degraders [45]. In addition, among the total isolates identified in this study as diesel degraders, 16% were found to be Gram positive isolates, which belong to species of the genus *Bacillus*. The diesel-degrading bacterial species were also characterized using some standard biochemical tests based on their catalytic activities. Some isolates showed positive results for degradation of hydrogen peroxide, casein, starch, and urea, but others did not. This could be a preliminary indication that the isolates have diverse enzymes for catalyzing the degradation of various and/or specific substrates. They were also identified using BioLog data of which the majority of isolates

were represented by *Pseudomonas species* with 84.9 to 98.1% accuracy. The next dominant diesel-degrading species was *S. maltophilia* (78.7–97.5%), followed by *Bacillus spp*. (65.5–87.5%), *P. rettigeri* (85.6–99.9%), *R. cervicalis* (70%) and *A. xylosoxidans* (95.7%). The *P. aeruginosa, B. cereus, S. maltophilia, A. xylosoxidans* and *P. rettgeri* were recovered from garages, old aged asphalts and bitumen soil environments which are contaminated with hydrocarbon components by anthropological activities. Other related studies also confirmed that *P. aeruginosa, S. maltophilia* and *B. cereus/subtilis* were also isolated from wide variety of aliphatic and aromatic hydrocarbon-contaminated soils [39, 49]. In addition, *P. rettgeri, P. aeruginosa,* and *R. cervicalis* were isolated from flower farms while *P. aeruginosa, P. viridilivida and S. maltophilia* were also recovered from the Chitu soda lake site, which has no direct contact with hydrocarbon components. From the current and other previous studies, it could be recognized that *P. aeruginosa* is potentially obtained from various soil environments, mainly due to its ubiquity in terms of its diverse metabolic capability for diesel degradation.

The growth capacity of the isolates was then detected at different diesel concentrations (1%, 3%, and 5%). At 1% diesel concentration, two bacterial isolates, *B. cereus* (AAUA13) and *P. aeruginosa* (AAUAs16), showed significant growth patterns on the tenth day of incubation. This indicates that *Bacillus spp.* and *Pseudomonas spp.* displayed superb diesel degradation potential [39, 42]. In addition, *P. rettgeri and S. maltophilia* were identified as potential diesel degraders [2, 10]. Notably, the current study demonstrated that species of the genus *Pseudomonas, Achromobacter, Providencia,* and *Stenotrophomonas* were identified as potential candidates in diesel degradation/utilization compared to the other isolates for the relatively longer culture time (15 days) and a higher concentration of the substrate (3% diesel). In addition, the study also showed that isolates performed better activity on the degradation of 3% diesel

concentration than 1% concentration on the same day of incubation. This is due to the fact that an increase in OD with an increase in diesel concentration in the growth medium indicates an increase in hydrocarbon-degrading bacterial population as they use it as a carbon and energy source [9].

P. aeruginosa (AAUW23 and AAUG11) were the most efficient biodegraders of diesel. Many studies also confirmed that *Pseudomonas sp*. shows superb diesel degradation efficacy [2, 7, 9, 19, 39, 45, 46, 49]. This is because it is an oleophilic microorganism [26] and has metabolic versatility, or it may be symbiotically associated in soils [47], or it produces biosurfactant, which effectively makes the diesel more available for utilization [11, 7, 29, 38]. In addition, other studies also showed that such bacterial species possess enzyme systems to degrade and utilize diesel oil as a source of carbon and energy [9, 39]. In addition, *B. subtilis* (AAUG36) was also identified as a potent bacterial species for the degradation of diesel. The study also showed that this species is able to be found in different environments due to its ability to produce endospore to pass harsh environments [5, 24] and surface-active substances (biosurfactants) to decrease surface and inter-surface tension and increase bioavailability of contaminants for efficient biodegradation [24]. This biological characteristic is important to augment the bioavailability of poorly accessible diesel and to enhance the biodegradation rates. Therefore, this study indicated that *P. aeruginosa* and *B. subtilis* showed maximum degradation with a higher concentration of diesel (5%) and without using any synthetic surfactants.

The 16S rRNA gene sequence alignments using BLASTn search in NCBI as well as the BioLog identification system for the three species of potential diesel-degrading bacterial isolates (AAUW23, AAUG36 and AAUG11) revealed that the isolates were found to exhibit 99% and above identity for existing cultured bacteria in the database. The 16s RNA gene partial

sequencing (Table 3) identified two different bacterial genera viz., *Pseudomonas spp*., and *Bacillus spp.* and that both isolates, AAUW23 and AAUG11, were recognized as *P. aeruginosa* and isolate AAUG36 as *B. subtilis.* Interestingly, the sequences of these isolates were aligned with the analogous sequences of several other known hydrocarbon-degrading organisms, and the resulting phylogenetic tree indicated that these isolates were grouped into phyla *Proteobacteria* (AAUW23 and AAUG11) and *Firmicutes* (AAUG36), with Gamma-proteobacteria being the most represented class (Fig. 4). The report from earlier studies also described that the phylum *Proteobacteria*, in most of the cases, has characteristics that are closely associated with aliphatic and aromatic hydrocarbon-degrading organisms [45, 49].

Conclusion

Bioremediation is one of the current approaches in environmental microbiology or environmental biotechnology that has been exercised for the reduction and/or removal of hydrocarbon pollutants. Microorganisms, typically bacteria that have particular metabolic capacities, are essential for the biodegradation of hydrocarbon pollutants. The present study provides a scientific investigation on diesel-degrading bacteria obtained from different soil environments based on culture-dependent techniques. It was found that potential bacteria that could degrade diesel would readily be isolated from hydrocarbon-contaminated soil samples or other natural environments that have no direct contamination with hydrocarbon residuals. This could be a ground work indication for a possible search of potential bacterial isolates for the bioremediation of hydrocarbon-contaminated environments. The 16S rRNA gene sequencing and phylogenetic tree construction inferred that the potential bacterial isolates AAUW23 (MT669825) and AAUG11 (MT669831), closely affiliated to species of the genus *Pseudomonas*, and AAUG36 *(*MT669830), which is affiliated to *Bacillus*, might able to predominately survive

and thrive in 1%, 3%, and 5% (*v/v*) diesel. The isolates also exhibited maximum diesel degradation efficiency using the gravimetric analysis method. Therefore, this study attests that bacterial species inhabiting different habitats are considered to be potential biological agents for the efficient biodegradation of diesel. This study also adds to the existing body of knowledge contributing to further improvements in the study towards minimizing environmental pollution contaminated with hydrocarbon components such as diesel.

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Author Contributions

Mr. Gessesse kebede contributed to investigation and Writing—original preparation of the manuscript. Dr. Eshetu Mekonen performed molecular techniques, sequencing while Dr. Adugna Abdi worked on bioinformatics analysis. Dr. Tekile Tafesse contributed for conceptualization of the project and editing the manuscript. Dr. Fassil Assefa and Dr. Mesfin Tafesse were project advisors. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The partial 16S rRNA sequences of bacterial isolates were submitted to the NCBI and their accession numbers were obtained as MT669825, MT669830 and MT669831.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest and the funders had no role in the decision to publish the results.

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Figures

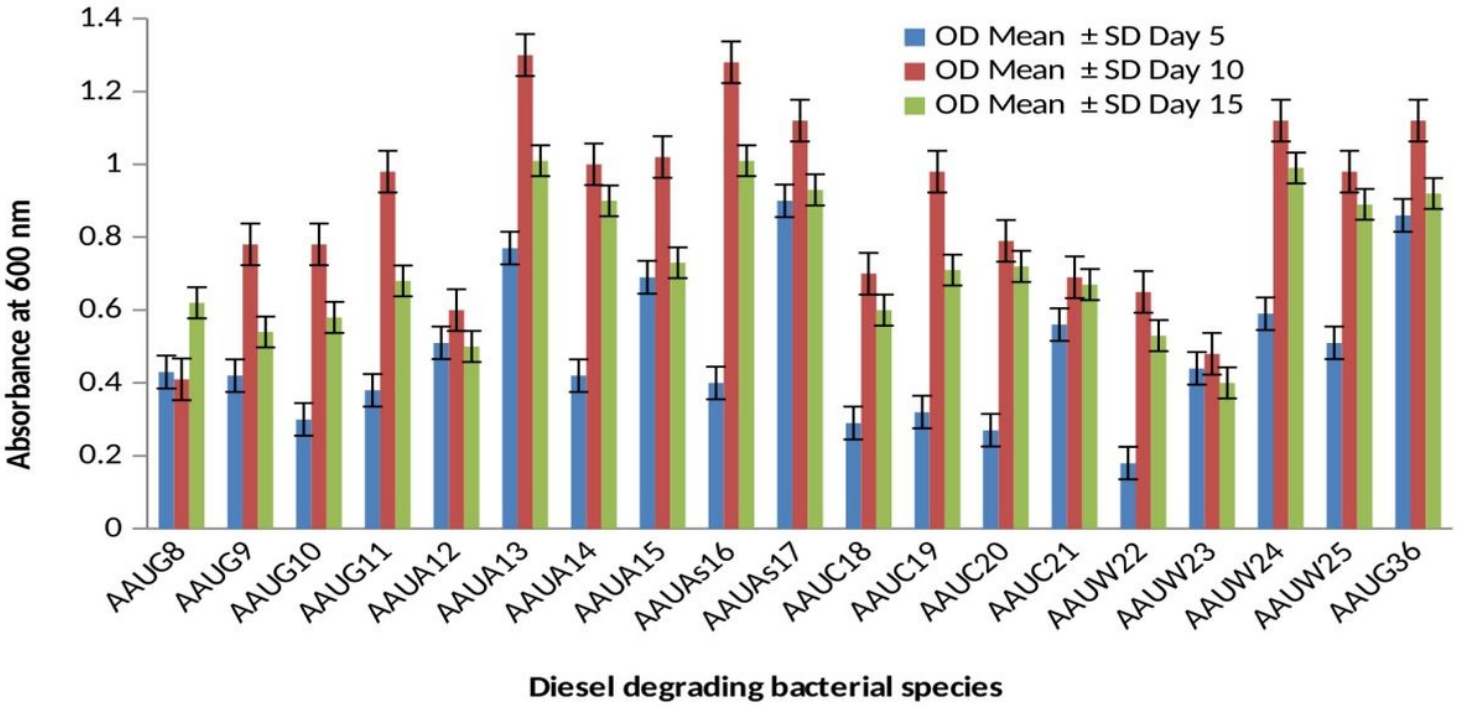

Figure 1

Growth capacity of isolates on diesel (1% concentration at different growth periods).

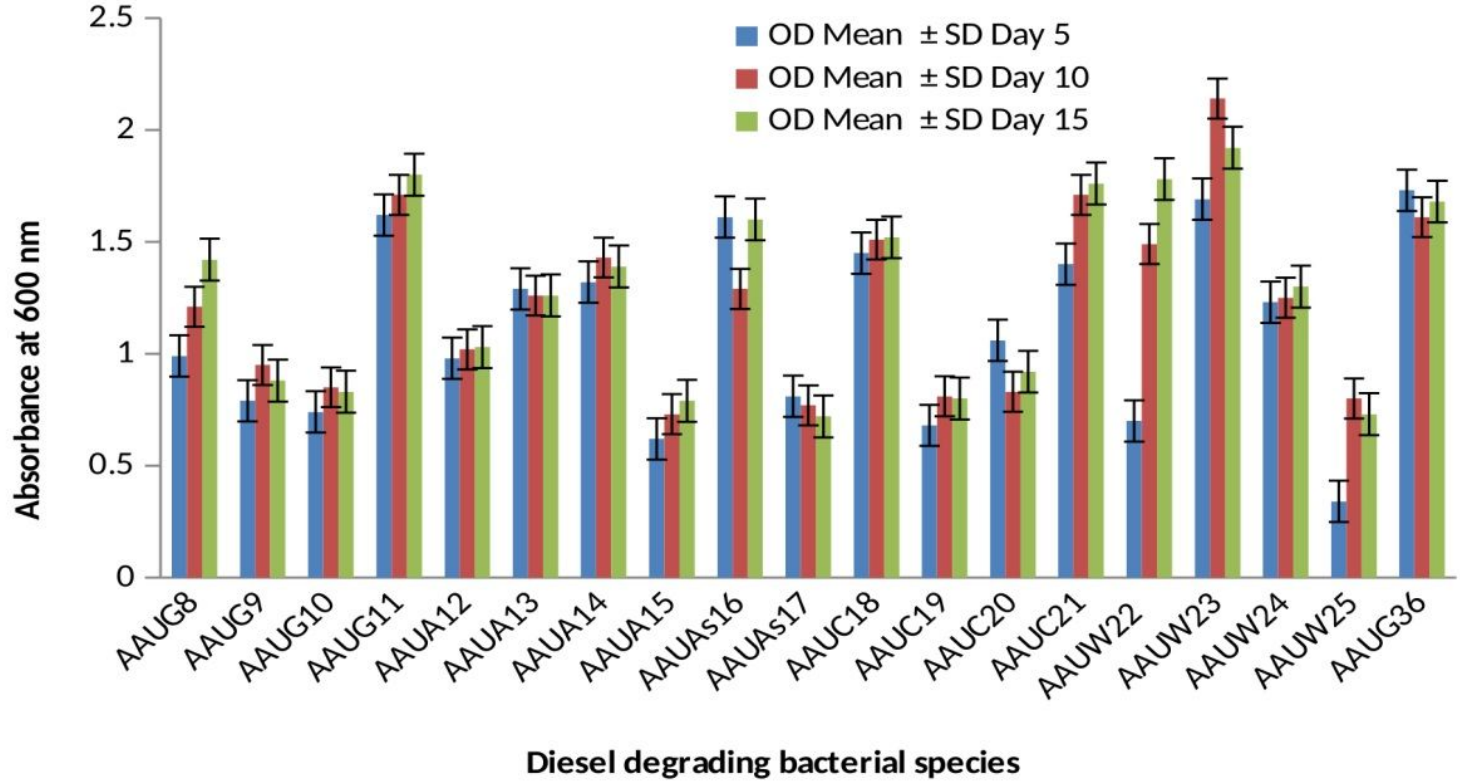

Figure 2

Growth capacity of isolates on diesel (3% concentration at different growth periods)

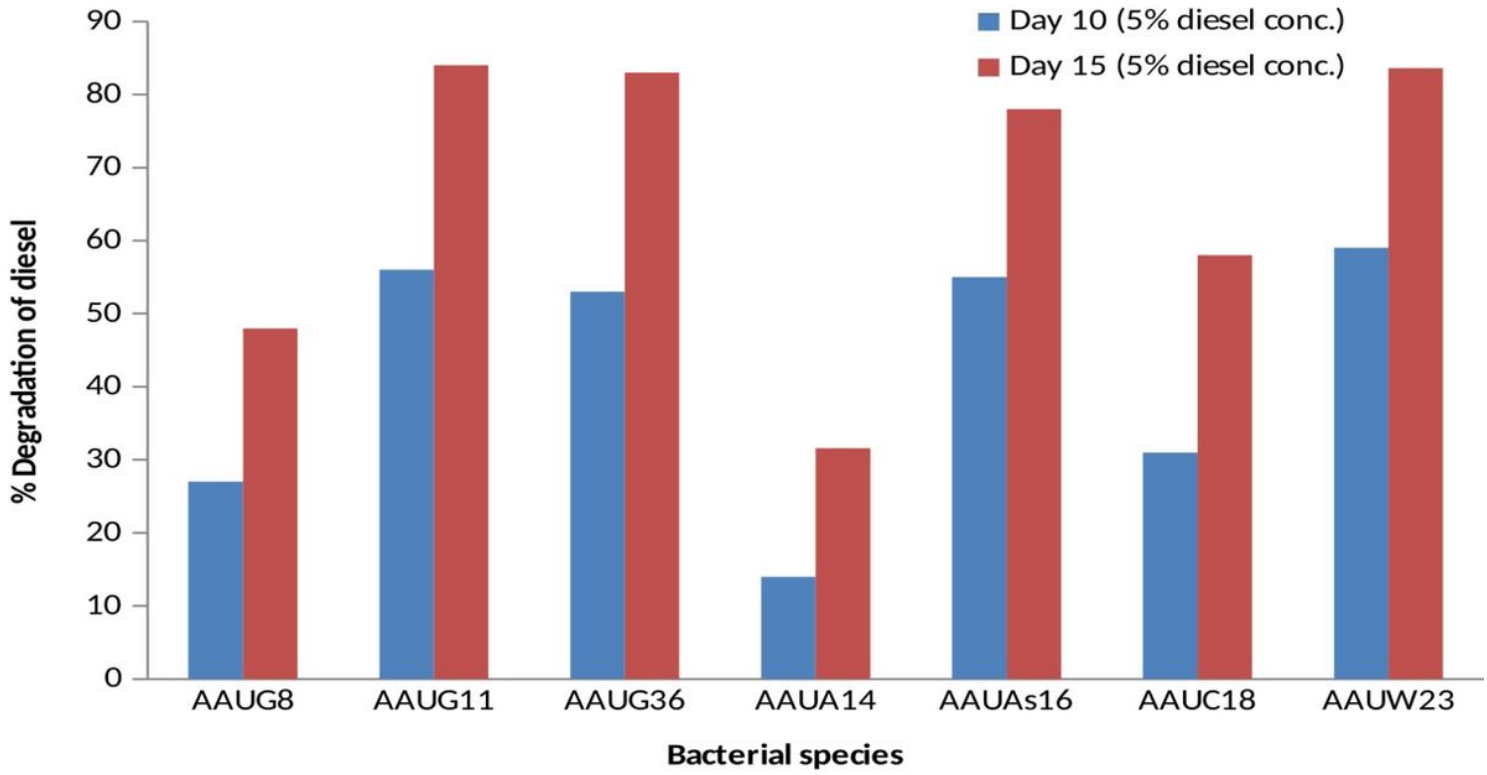

Figure 3

Gravimetric analysis for diesel degradation

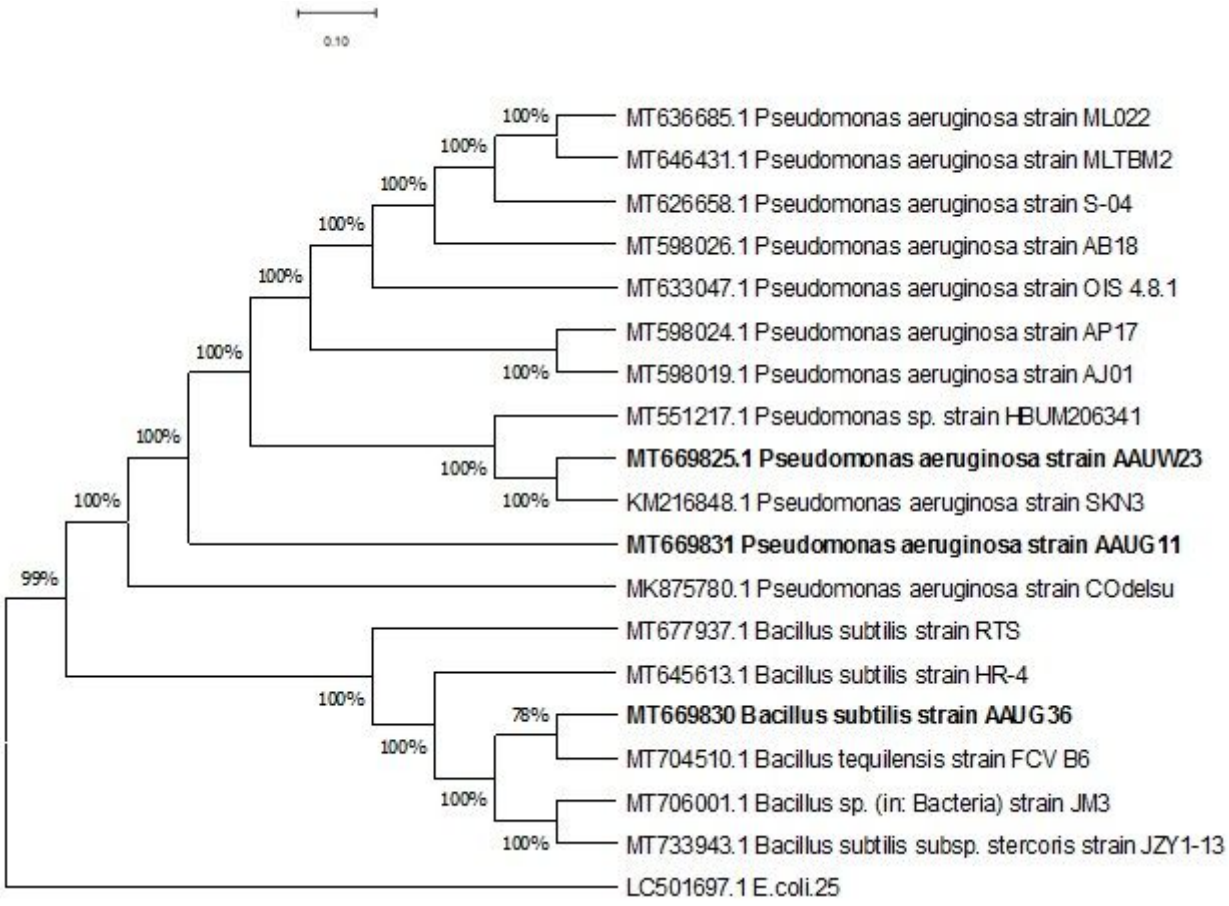

Figure 4

Phylogenetic tree based on partial bacterial sequences of the 16S rRNA region for the two Pseudomonas isolates and one Bacillus subtilis (bold and coded with the initials —AAU□), and accession numbers of the 16S rRNA are followed by species names. Numbers at nodes indicated bootstrap values for each node out of 1000 bootstrap resembling. The phylogenetic tree was constructed in MEGA X using the maximum likelihood method [23] and Kimura-2 parameter model [21]. The E. coli plasmid partial sequence was used as an out-group