

Bioremediation of Contaminated Diesel and Bioelectricity Generation Using Marine Bacterial Consortium Integrated with Microbial Fuel Cell

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Abstract

Biodegradation is a commonly used method to treat seawater polluted by petroleum. In addition to its ability to selectively degrade pollutants, it is also important to investigate the effectiveness of degradation and the benefits of restoration. This study focused on selecting a group of bacteria that can degrade diesel from marine sediment. The bacterial consortium called MB11 was found to be the most effective in removing diesel, with a removal rate of 53.77 ± 0.59 %. The consortium MB11 consists mainly of 6 types of bacteria: *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Raoutella planticola*, *Enterobacter soli* and *Oceanotoga teriensis*. The effective bacterial consortium MB11 was integrated with the floating MFC for electricity generation. The maximal open circuit voltage (OCV) and power density (PD) of 676.88 ± 5.94 mV and 0.16 ± 0.02 W/m³, respectively.

Keywords: Dehydrogenase, Electricity generation, Marine bacteria, Microbial fuel cell, Petroleum degrading bacteria

Introduction

In the past decade, the growth of the world economy has led to a significant increase in energy consumption, resulting in high demand for petroleum crude oil [1]. This crude oil is typically imported via ocean routes from Arabian regions such as UAE, Iran, Saudi Arabia and Iraq. Unfortunately, oil spills during the transfer process can harm marine life and human health due to the toxic effects of polyaromatic hydrocarbon (PAH) found in crude oil [2]. Statistical analysis indicates that between 2010 and 2015, approximately 330,000 tons of petroleum crude oil leaked [3]. Due to the properties of petroleum, such as high viscosity, low solubility and high stability, it is challenging to remove and can remain in the contaminated area for an extended period [4].

There are several methods available for the remediation of petroleum-contaminated sites, including leaching, chemical oxidation, incineration, microbial treatment and landfills. The goal of these methods is to extract, remove or transform petroleum into a safe or stable form [5]. While incineration can remove up to 99 % of petroleum, it also releases toxic substances such as furans, polychlorinated biphenyls (PCBs) and volatile heavy metals into the atmosphere [6]. Studies have shown that mercury (Hg), cadmium (Cd) and lead (Pb) are among the most harmful volatile heavy metals released during petroleum waste incineration [7]. As a result, there is a growing interest in eco-friendly methods, such as bioremediation, that can remediate petroleum-contaminated sites without releasing harmful substances.

Microbial remediation is an effective method for completely degrading petroleum pollutant into water (H₂O), carbon dioxide (CO₂), inorganic substances and cell protein [8]. Bacteria, fungi and microalgae are the most effective microbes for petroleum remediation in marine environments [9]. Previous research has identified specific hydrocarbon-degrading bacteria within genera *Pseudomonas*, *Flavobacterium*, *Enterobacter*, *Bacillus*, *Burkholderia*, *Arthrobacter*, *Alcanivorax*, *Altermonas*, *Thalassolituus*, *Oleispira* and *Cycloclasticus* [10-12]. Some enzymes like catalase, peroxidase, laccase and dehydrogenase play important role in microbial bioremediation of petroleum hydrocarbons. Among these enzyme, dehydrogenase is the most effective in bacterial degradation [13-15].

The microbial fuel cell (MFC) is a fascinating technology that employs microbial metabolism to convert the chemical energy of organic matter into electrical energy by breaking chemical bonds. The MFC has been applied to wastewater treatment and electricity generation and has shown promise in the remediation of petroleum from soil, water, wastewater and sediment. However, the current MFC model is

limited by high structural costs and is only available in small-scale units [16,17]. To address this, a study was conducted to select a petroleum-degrading bacterial consortium from marine sediment samples with dehydrogenase and laccase activities. A low-cost floating MFC was designed for petroleum treatment in seawater, and the selected bacterial consortium was applied to the anodic electrode for both petroleum degradation and electricity generation processes.

Materials and methods

Enrichment of diesel-degrading consortium

The total of 25 marine sediment samples were collected using a sterile method from the Gulf of Thailand in Surat Thani province, Thailand and immediately placed in an icebox. The samples were then transported to the Microbiology Laboratory at Thaksin University for the first round of enrichment.

The modified method of Shi *et al.* [15], was used to prepare artificial seawater, which included 5.00 g/L NaCl, 0.06 g/L Na₂HPO₄, 0.05 g/L KH₂PO₄, 0.02 g/L CaCl₂, 0.04 g/L FeSO₄ and 0.14 g/L MgSO₄. The seawater was sterilized at 121 °C for 15 min before use.

For enrichment, 10 g of marine sediment was added to 90 mL of sterilized seawater with 0.1 % (v/v) diesel, and incubated at room temperature (30 ± 2 °C) with shaking at 150 rpm for 48 h. The 10 mL of mixed culture was then transferred to 90 mL of seawater with 0.1 % (v/v) diesel and re-enriched 10 times to ensure the selected consortium can use diesel as the sole carbon sources for growth. The selected consortium was collected for further use.

Selection

The 10 mL of bacterial consortium (1.0×10⁸ cell/mL) that was 48 h old was mixed with 90 mL of artificial seawater containing 0.1 % (v/v) diesel. The mixture was incubated at room temperature for 48 h while being shaken at 150 rpm.

For diesel removal, the 10 mL of n-hexane was added and shaken for 20 min, then transferred into the separating funnel. The supernatant obtained from the separating funnel was collected for testing purposes. The concentration of the remaining diesel in the sample was determined by measuring its absorbance at 225 nm using UV-Vis spectrophotometry (Shimazu, Japan) [18].

Dehydrogenase activity

The dehydrogenase activity of the bacterial consortium was measured for 7 days using method described by Xue *et al.* [19]. The method involved adding 0.2 mL of the consortium (1.0×10⁸ cell/mL) in normal saline (0.85 % w/v NaCl) to a mixture of 2.5 mL of nutrient broth (containing 3.0 g/L beef extract and 5.0 g/L peptone) and 0.7 mL of artificial seawater containing 0.1 % (v/v) diesel. The reaction was incubated at room temperature for 60 min with shaking at 150 rpm. After that, 0.1 mL of 0.1 % (v/v) 2,3,5-triphenyltetrazolium chloride (TTC) was added, and the mixture was statically incubated at room temperature for 6 h to generate triphenylformazan (TPF). The TPF was then extracted with 4.0 mL of amyl alcohol and measured at 445 nm using UV-Vis spectrophotometry. The dehydrogenase activity was measured at regular intervals of 24 h for 7 days.

Laccase activity

The laccase activity of the bacterial consortium was measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. The supernatant was collected from a 48 h-old consortium after centrifugation at 12,000 rpm and 4 °C. A volume of 100 µL of the supernatant was mixed with 900 µL of 0.1 M ABTS solution in sodium acetate buffer (containing 5.77 g/L CH₃COONa and 1.78 g/L CH₃COOH). The solution was incubated for 5 min, and the absorbance was measured at 420 nm using a UV-Vis spectrophotometry to monitor the laccase activity.

Diesel removal

The 10 mL of 48 h old-bacterial consortium (1.0×10⁸ cell/mL) was mixed with 90 mL of artificial seawater containing 0.1 % (v/v) diesel. The reaction was incubated for 7 days under room temperature with shaking at 150 rpm. The concentration of the remaining diesel in the sample was determined by measuring its absorbance at 225 nm using UV-Vis spectrophotometry [19].

Next-generation sequencing

The bacterial consortium that can degrade diesel was cultured in a modified nutrient broth containing 3.0 g/L beef extract, 5.0 g/L peptone and 5.0 g/L NaCl with shaking at 150 rpm under room temperature for 48 h. The bacterial cell was collected by centrifugation at 12,000 rpm for 10 min.

The genomic DNA was extracted from the selected bacterial consortium using QIAamp DNA Kits manufactured by Qiagen, Germany. The DNA was then randomly fragmented using Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit to prepare the sequencing library.

The library protocol used was 16S Metagenomic Sequencing Library Preparation Part #15044223 Rev. B. Illumina SBS Technology was employed for sequencing, and the microbial community structure of selected consortium was analyzed.

Floating MFC design and operation

Figure 1 illustrates the floating MFC used in this experiment, which includes a microwave modified graphite plate as the anodic electrode [20], a ceramic plate as a proton exchange membrane [21], and the air-cathode made from Pt-coated carbon cloth. The polyethylene foam was used as the floating part. To immobilize the bacteria consortium, 100 mL of active consortium in modified nutrient broth was added onto the graphite plate and allow to adsorb and immobilize for 30 h under room temperature [22]. The immobilized bacterial consortium on graphite surface was monitored by scanning electron microscopy as showed in **Figure 2**.

During operation, the floating MFC with immobilized bacterial consortium was placed in a grass chamber filled with 1,000 mL of diesel-contaminated seawater at a concentration of 1 % (v/v). The open circuit voltage (OCV) was recorded every 60 min over 24 h period. The polarization curve was plotted by measuring the close circuit voltage (CCV) at 300 - 5,000 Ω . The electrochemical properties were calculated using Ohm's law.

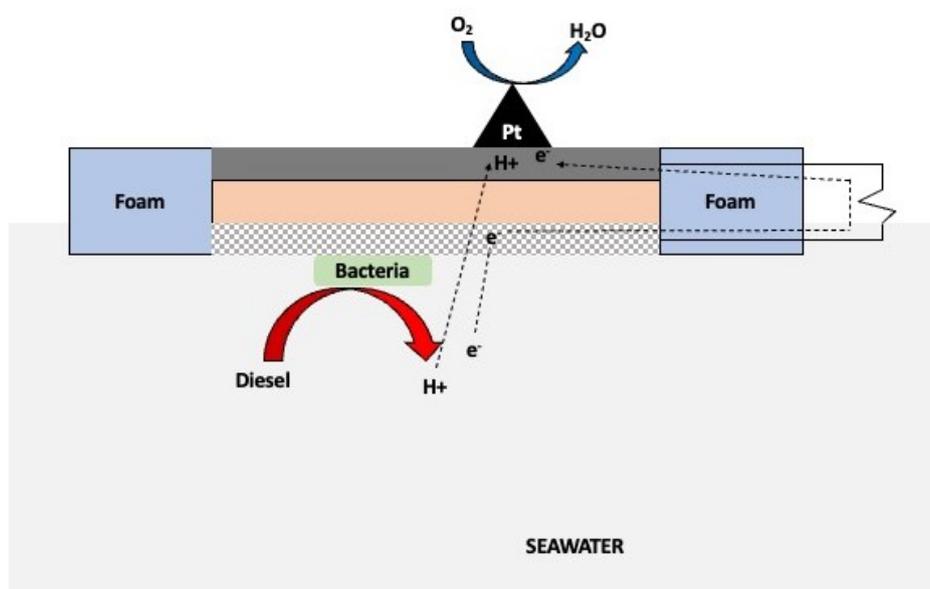


Figure 1 Diagram of floating MFC used in this experiment.

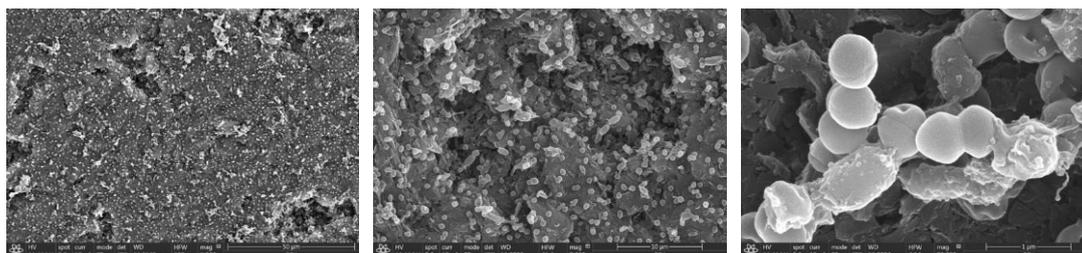


Figure 2 The bacterial consortium - immobilizing graphite electrode under scanning electron microscope.

Results and discussion

Selection

In order to gain a bacterial consortium capable of using diesel as sole carbon, marine sediments were inoculated in seawater that had been contaminated with diesel. The bacterial consortium that showed the highest efficiency in removing diesel was MB11, which was able to remove 21.30 ± 1.04 % of the diesel after incubated at room temperature for 48 h without any additional substances added. The next most efficient bacterial consortium was MB15, which able to remove 18.19 ± 0.06 % of the diesel (**Figure 3**).

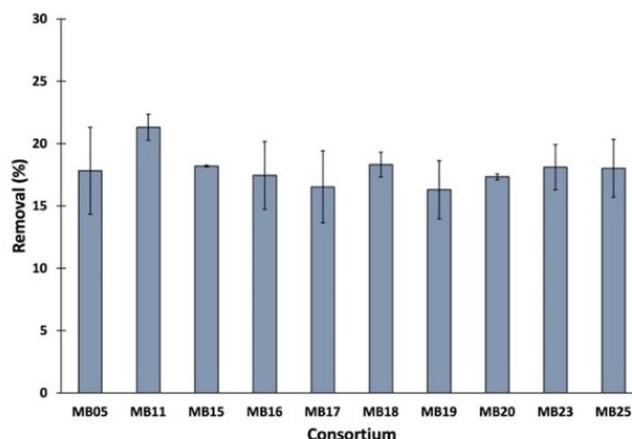


Figure 3 The diesel removal (%) of the enriched bacterial consortium.

On the other hand, the diesel-degrading bacterium *Pseudomonas aeruginosa* IU5 was isolated from the oil contaminated soil near a gas station. The 1 % (v/v) of diesel was used for bacterial screening. The diesel removal was found where the bacterium was inoculated into diesel-adding culture medium for 13 days [23]. Moreover, the diesel-degrading bacteria has been found in the fresh water sediment collected from the water reserve. The diesel removal of isolated bacterium was found when it was incubated in the minimal medium supplemented with 5 % (v/v) diesel for 21 days [24]. In Bekele *et al.* [25], the diesel-degrading bacteria was isolated from old aged garages soil. The diesel removal efficiency of isolated bacteria was found when it was cultured in the basal salt medium supplemented with mineral solution.

Enzyme activities and diesel removal

The selected bacterial consortium MB11 has been shown the maximal dehydrogenase activity of 0.005 ± 0.001 $\mu\text{g}/\text{mL}$ where it was cultured in the diesel-contaminated seawater at day-5 and day-6. In term of laccase activity, the MB11 showed the maximal laccase activity of 0.50 ± 0.01 U/mL at day-7.

The effectiveness of the bacterial consortium MB11 in removing diesel from the seawater was evaluated and compared with its enzyme activities. The highest diesel removal achieved by the MB11 was 53.77 ± 0.59 %, and this was observed after 7 days of growth in the diesel-contaminated seawater.

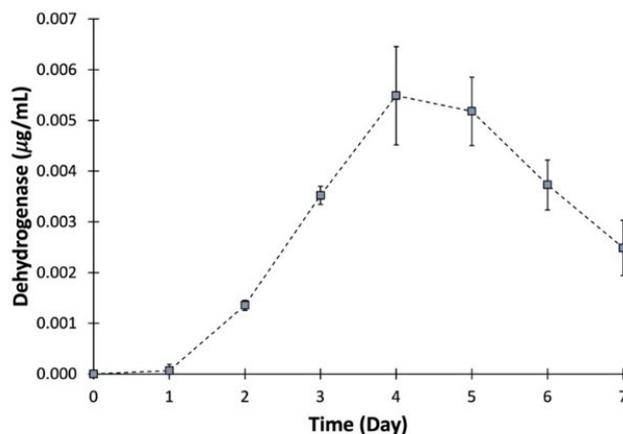


Figure 4 The dehydrogenase activity of the selected bacterial consortium MB11.

Studies conducted by Panda *et al.* [26], Zhang *et al.* [27], have shown that certain microorganisms have the ability to break down the hydrocarbon in diesel. Bacteria like *Pseudomonas* sp., *Bacillus* sp. and *Acinetobacter* sp. that are isolated from contaminated environments are proved to be effective in diesel degradation due to their ability to adapt, resist, tolerate and survive when exposed to the pollution. These bacteria have unique metabolic pathways that have evolved to prevent their death by changing or bypassing certain metabolic and enzymatic reactions [28-30].

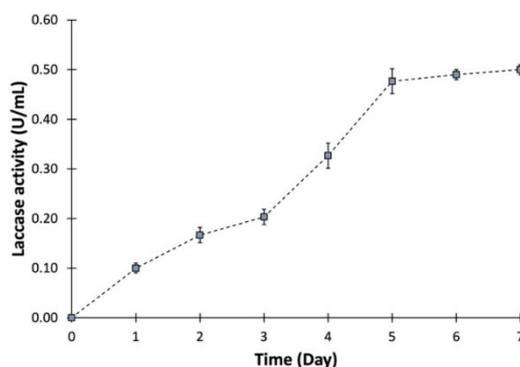


Figure 5 The laccase activity of the selected bacterial consortium MB11.

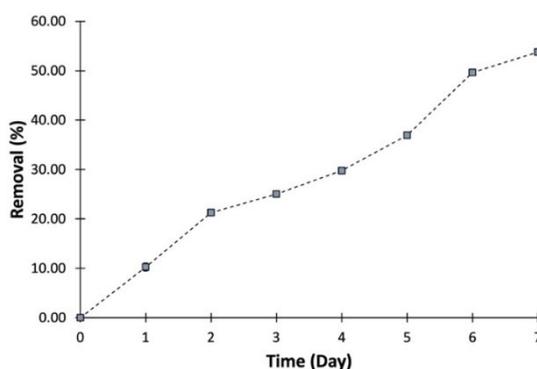


Figure 6 The diesel removal (%) of the selected bacterial consortium MB11.

The study conducted by Fu *et al.* [31], involved using a marine bacterium *Pseudomonas* sp. YT-11 that was obtained from seawater. The bacterium was immobilized on the cinnamon shell and used it to remove diesel from the contaminated samples. After 5-days of incubation, the results showed that about 60 % of diesel has been removed owing to the biodegradation and bio-adsorption processes. On the other hand, the diesel-degrading bacteria with dehydrogenase activity were isolated from the seawater sample and enriched in the mineral salt medium supplemented with yeast extract and mineral solution. The free cell biodegradation efficiency of 44.79 % has been gained [32]. Moreover, the diesel-degrading bacterium *Pseudomonas* sp. J4AJ has been used for diesel bioremediation by synergic degradation with plant *Scirpus triqueter*. The maximal diesel removal of 54.51 % was achieved after 60 days operation [33]. Dai *et al.* [34] utilized an engineered laccase producing-bacterial consortium to remediate diesel-contaminated soil. After a period of 100 days, the maximal amount of diesel removed was found to be 66.5 %.

Microbial structure

The community's richness and diversity were displayed in **Table 1**. The phylum distribution of 50.1 % is similar to the phylum Proteobacteria, followed by 41.4 % of the phylum Firmicutes, 4.2 % of the Thermotogae, 2.8 % of the phylum Synergistetes and 1.4 % of the Bacteroidetes, respectively.

The class distribution of 50.1 % is similar to class Gammaproteobacteria, followed by 36.4 % of the class Bacilli, 4.2 % of the class Thermotogae, 4.0 % of the Bacilli, 2.8 % of the Synergistia, 1.4 % of the Bacteroidia and 1.0 % of the Tissierellia, respectively.

The order distribution of 38.3 % is similar to order Enterobacterales, followed by 36.3 % of the order Lactobacillales, 11.8 % of the order Pseudomonadales, 4.2 % of the order Petrotogales, 4.0 % of the order

Eubacteriales, 2.8 % of the order Synergistales, 1.4 % of the order Bacteroidales, 1.0 % of the order Tissierellales and 0.1 % of the order Bacillales, respectively.

The Family distribution of 36.3 % is similar to the family Enterococcaceae, followed by the 20.5 % of the family Enterobacteriaceae, 17.8 % of the family Morganellaceae, 11.8 % of the family Pseudomonadaceae, 4.2 % of the family Petrotogaceae, 3.5 % of the family Clostridaceae, 2.8 % of the family Synergistaceae, 1.4 % of the family Tannerellaceae, 1.0 % of the family Tissierellaceae and 0.1 % of families Paenibacillaceae and Peptostreptococcaceae, respectively.

The Genus distribution of 36.3 % is similar to genus *Enterococcus*, followed by 17.8 % of the genus *Proteus*, 11.8 % of the genus *Pseudomonas*, 10.8 % of the genus *Raoutella*, 9.7 % of the *Enterobacter*, 4.2 % of the genus *Oceanotoga*, 2.9 % of the genus *Paraclostridium*, 2.7 % of the genus *Aminobacterium*, 1.4 % of the genus *Parabacteroides*, 1.0 % of the genus *Tissierella*, 0.6 % of the genus *Clostridium*, 0.3 % of the genus *Anaerotignum* and 0.1 % of the genus *Paenibacillus*, *Natranaerovirga* and *Acetoanaerobium*, respectively.

The species distribution was showed in **Figure 7**, the result indicated that 36.3 % is similar to *Enterococcus faecalis*, followed by 17.8 % of the *Proteus mirabillis*, 11.8 of the *Pseudomonas aeruginosa*, 10.8 % of the *Raoutella planticola*, 9.7 % of the *Enterobacter soli*, 4.2 % of the *Oceanotoga teriensis*, 2.9 % of the *Paraclostridium benzoelyticum*, 2.7 % of the *Aminobacterium colombiense*, 1.4 % of the *Parabacteroides chartae*, 1.0 % of the *Tissierella praeacuta* and 0.1 % *Paenibacillus aquistagni*, *Natranaerovirga hydrolytica* and *Acetoanaerobium sticklandii*, respectively.

Bhuvanewar *et al.* [35], has reported that the bacteria such as *Pseudomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Bacillus* sp., *Flavobacterium* sp., *Achromobacter* sp., *Klebsiella* sp., *Actinomycetes* sp., *Acetobacter* sp. and *Rhodococcus* sp. isolated from the contaminated environment can break down the hydrocarbon in diesel and utilize it as a sole carbon source [35]. **Table 2** displays diesel degradation efficiency by isolated bacteria.

Table 1 The community richness and diversity of the MB11 consortium.

Consortium	OTUs	Chao1	Shannon	Gini-simpson
MB11	48	48	2.82	0.80

Table 2 Diesel degradation efficiency by isolated bacteria.

Bacteria	Culture time (Day)	Medium	Diesel removal (%)	Reference
<i>Enterococcus faecalis</i> <i>Proteus mirabillis</i> <i>Pseudomonas aeruginosa</i> <i>Raoutella planticola</i> <i>Enterobacter soli</i> <i>Oceanotoga teriensis</i>	7	Artificial seawater	53.77 ± 0.59	This study
<i>Vibrio alginolyticus</i>	14	Mineral salt medium	94.22	[36]
<i>Pseudomonas aeruginosa</i> <i>Sphingomonas</i> sp.	21	Mineral salt medium	35.00	[37]
<i>Bacillus aquimaris</i> <i>Bacillus anthracis</i> <i>Bacillus cereus</i>	63	Sand medium	18.00	[38]
<i>Rhodococcus</i> sp. <i>Pseudomonas</i> sp. <i>Achromobacter piechaudii</i> <i>Rhodococcus qingshengii</i> <i>Sphingomonas</i> sp. <i>Bacillus mycoides</i>	49	Soil medium	90.00	[39]
<i>Pseudomonas stutzeri</i> GQ-4 <i>Pseudomonas</i> sp. SZ-2 <i>Bacillus</i> sp. SQ-2	56	Soil medium	48.00	[40]

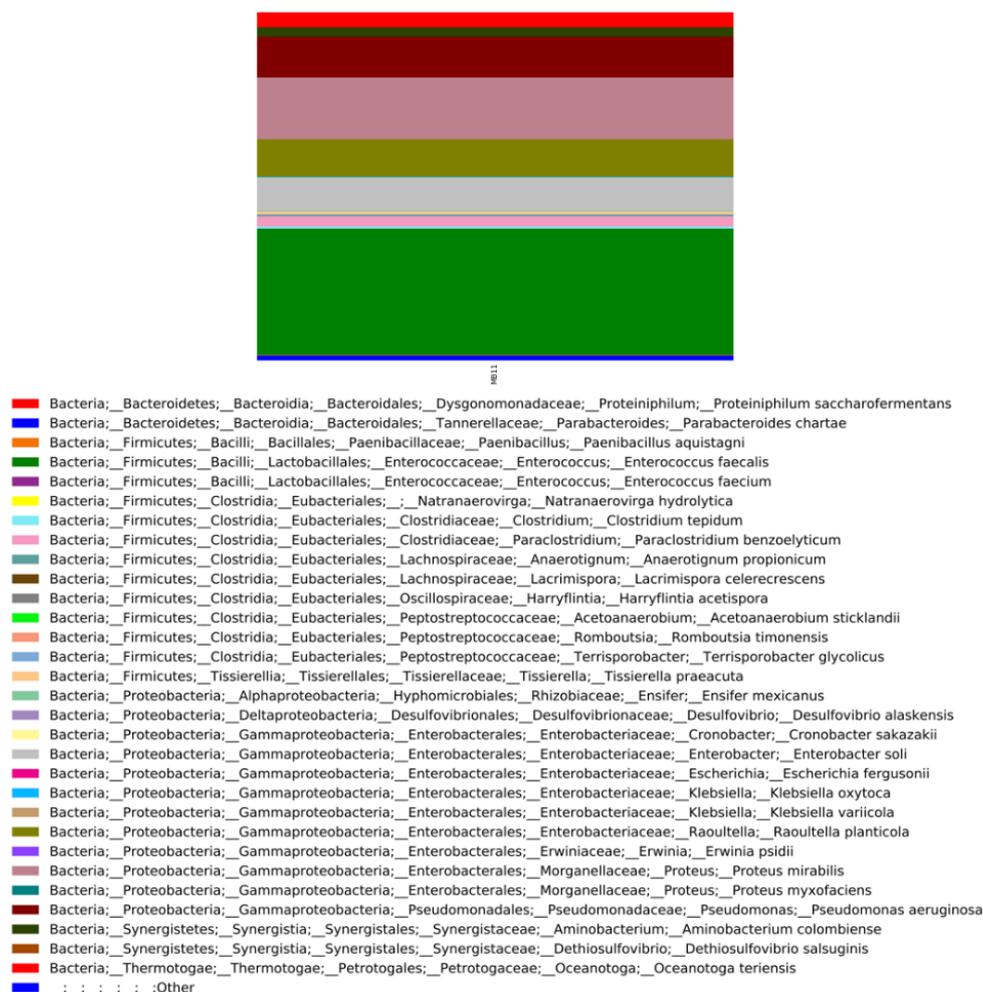


Figure 7 The species distribution of the MB11 consortium.

Electrochemical properties

In recent years, bioelectrochemical systems (BESs) have emerged as a promising alternative to conventional techniques. BESs involve the use of microbial electrochemical technologies (MET) where microorganisms act as catalysts to break down organic compounds and produce electrons that can be used to generate electricity or other useful compounds [41]. BESs encompass several applications, including microbial fuel cells (MFC), microbial electrolysis cells (MEC), microbial desalination cells (MDC) and microbial electrosynthesis cells (MES). Compared to conventional methods, BESs operate under milder and easier conditions and have been successful in treating a wide range of pollutants, including highly contaminated wastewater, soil and sediment. Additionally, BESs are cost-effective and environmentally friendly, as they do not require precious, scarce electrodes or expensive chemicals [42].

In this study, a specific bacterial consortium MB11 was immobilized on the anodic surface of a floating MFC in contaminated seawater. The highest OCV achieved was 676.88 ± 5.94 mV. The maximal power density (PD) and current density (CD) achieved based on the volume of contaminated seawater were 0.16 ± 0.02 W/m³ and 0.56 ± 0.01 A/m³, respectively.

The study conducted by Guo *et al.* [43] utilized MFC technology to degrade oil sludge and generate electricity. The experiments conducted demonstrated that the oil sludge could be utilized as a source of energy to produce electricity in the MFC, with a maximum voltage output of 299.13 mV being achieved. The wild-type bacterium, *Pseudomonas putida* strain BCRC 1059, has the ability to treat wastewater from oil refineries and generate electricity in MFC. The data obtained showed that this bacterium can produce a maximum voltage of 355 mV in MFC during the process of treating oil refinery wastewater [44]. Sarmin *et al.* [45], conducted a study where they utilized petrochemical wastewater to generate electricity using a dual-chamber microbial fuel cell (MFC) system. The results indicated that this system was capable of

producing a maximum voltage of 450 mV. **Table 3** provides a comparison of the electricity generation achieved through the use of MFC in the treatment of diesel and other petroleum hydrocarbons.

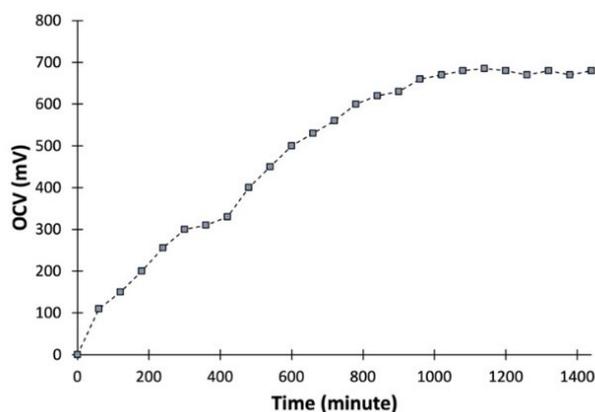


Figure 8 The OCV of the floating MFC with the bacterial consortium MB11.

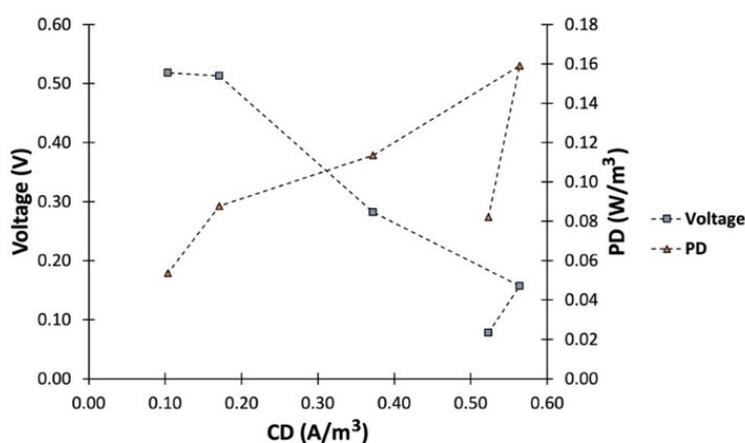


Figure 9 The polarization curve of the floating MFC with the bacterial consortium MB11.

Table 3 The electricity generation from the diesel and other petroleum hydrocarbon by MFC.

MFC type	Initial concentration (g/L)	Power output (mV)	Operating time (Day)	Predominant species	Reference
Floating MFC	Diesel 10.00	676.88 ± 5.94	1	<i>Enterococcus faecalis</i> <i>Proteus mirabilis</i> <i>Pseudomonas aeruginosa</i> <i>Raoutella planticola</i> <i>Enterobacter soli</i> <i>Oceanotoga teriensis</i>	This study
Single chamber MFC	Diesel 0.80	506.00	30	<i>Pseudomonas aeruginosa</i>	[46]
Dual chamber	Diesel 0.18	650.00	21	<i>Citrobacter</i> sp. <i>Pseudomonas</i> sp. <i>Stenotrophomonas</i> sp.	[47]
Single chamber MFC	Diesel 0.04	500.00	6	Mixed culture	[48]

MFC type	Initial concentration (g/L)	Power output (mV)	Operating time (Day)	Predominant species	Reference
Plant-MFC	Phenanthrene and Pyrene 1.00	184.90	35	<i>Geobacter</i> spp.	[49]
Single chamber MFC	Total petroleum hydrocarbon -	457.00	135	Mixed culture	[50]
Single chamber MFC	Total petroleum hydrocarbon -	282.00	135	Mixed culture	[51]
Single chamber MFC	Total petroleum hydrocarbon 16.00	190.00	66	Mixed culture	[52]

Conclusions

This study investigated the potential of biodegradation to treat seawater contaminated with diesel, and analyzed the degradation performance and bacterial community structure involved. The results showed that the biodegradation rate of diesel exceeded 53.77 ± 0.59 % compared to degradation without exogenous medium adding. The dominant bacteria in the MB11 bacterial consortium were found to be *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Raoutella planticola*, *Enterobacter soli* and *Oceanotoga teriensis*. Furthermore, the maximal voltage and power density output achieved from the floating microbial fuel cell (MFC) integrated with the bacterial consortium MB11 were 676.88 ± 5.94 mV and 0.16 ± 0.02 W/m³, respectively. These findings suggest that biodegradation, combined with MFC technology, has the potential to effectively treat diesel-contaminated seawater while generating electrical energy.

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