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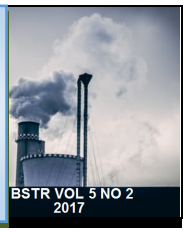


**Short communication**

# Screening of Hydrocarbon-degrading Bacterial Isolates Using the Redox Application of 2,6-DCPIP

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**ABSTRACT**

Petroleum hydrocarbons remain as the major contaminants that could be found across the world. Remediation approach through the utilisation of microbes as the bioremediation means widely recognised due to their outstanding values. As a result, scientific reports on the isolation and identification of new hydrocarbon-degrading strains were on the rise. Colourimetric-based assays are one of the fastest methods to identify the capability of hydrocarbon-degrading strains in both qualitative and quantitative assessment. In this study, the hydrocarbon-degrading potential of nine bacterial isolates was observed via 2,6-dichlorophenolindophenol (DCPIP) test. Two potent diesel-utilising isolates show a distinctive tendency to utilise aromatic (ADL15) and aliphatic (ADL36) hydrocarbons. Both isolates prove to be a good candidate for bioremediation of wide range of petroleum hydrocarbon components.

## INTRODUCTION

Petroleum hydrocarbon contamination represents a major concern around the globe. The incessant application of this noxious compounds in transportation and power generation remains as the principal source of pollution [1]. The basis of hydrocarbon compounds is from two major elements of hydrogen and carbon. Due to their repetitive and redundancy in their structure, the individual atoms have a high probability to be linked together in any number of fashions resulting in the formation of several hydrocarbon structures such as chains, circles, and other complex shapes. These hydrocarbon complexes formed from a varied structure made up a large part of the petroleum composition [2]. As the structure of different hydrocarbons may differ from one another, they can be categorised into several basic class such as alkanes (saturated hydrocarbons), alkenes (unsaturated hydrocarbons), cycloalkanes and aromatics [3,4].

The toxic effects of petroleum hydrocarbon substances are highly dependent on the structure of the hydrocarbon itself. Generally, the greater the length of the carbon chain and the number of aromatic rings, the higher the toxicity of the petroleum oil [5]. Most of the hydrocarbon compounds are toxic, mutagenic and carcinogenic. According to the United States Environmental Protection Agency (EPA)[6], these toxicants are classified as the priority environmental pollutants. Among the classes of hydrocarbon components, polycyclic aromatic hydrocarbons (PAHs) are widely considered to be the most toxic because of their recalcitrance in the environment [7,8].

Microbial flora and fauna are largely considered as one of the best pollutants remediator agents as they are naturally available in the environment and have the great versatility to strive in restricting settings [9,10]. Amongst the diverse microorganisms, bacterial species appeared to be the most common scavengers of hydrocarbon pollutants [11–13]. The mechanistic approach of the biodegradation by bacterial population is through the uptake of those noxious waste as the bacterial source of carbon and energy source. The compounds were later assimilated and sequentially degraded through a succession of enzymatic mineralisation occurred within the cell. The ultimate product of the degradation is assumed to be carbon dioxide and water [14–16]. For the foundation of hydrocarbon remediation by bacteria to start, the isolation and characterisation of hydrocarbon-degrading strain are the common approaches as to assess the isolates efficacy in the

utilisation of specific hydrocarbon before the application in contaminated sites.

Numbers of procedure have been used for the identification of hydrocarbon-degrading strains. These comprise the isolation of hydrocarbon-adapted bacteria from the liquid, and solid media supplemented with a range of different hydrocarbon substrates, turbidity measurements, oxygen consumption and carbon dioxide production. Nevertheless, these methods are time-consuming, painstaking, cost-inefficient and not reliable [17]. Colourimetric method is one of the favoured methods in the identification of hydrocarbon-degrading strains. The advantages of this practice are cost-effective, rapid detection, and microbially based approach in which natural co-substrate such as oxygen is substituted by a synthetic mediator [18]. One of the colourimetric indicators is 2,6-dichlorophenolindophenol (DCPIP). The principle of this redox indicator lies in the oxidation of the carbon source (hydrocarbon substrates) in which electrons are transferred to the electron acceptors [19]. According to Hanson et al. [20], the utilisation of substrate can be observed based on the loss of indicator's blue colour. Numbers of the study also reported using this method as the preliminary method for isolation of hydrocarbon-degrading strains [19,21,22].

In this work, the competency of nine isolated bacteria from the pristine soil of Southern Victoria Island, Antarctica in utilisation of diesel as sole carbon source was observed. The isolates that able to utilise diesel oil were assessed in utilising different hydrocarbon substrates (*n*-alkanes, aromatics, and PAHs). All isolates were adapted previously in diesel supplemented medium. The results obtained in this study will be used to further characterise the capability of the potential isolate for hydrocarbon bioremediation.

## MATERIALS AND METHODS

### Chemicals and media

Diesel oil was purchased from PETRONAS gas station located in Serdang, Selangor. *n*-hexane, *n*-heptane, *n*-tetradecane, *n*-hexadecane, xylene, and toluene were obtained from Sigma Aldrich, Germany. Phenol was bought from Nacalai Tesque, Inc. Japan. Bushnell-Haas (BH) media was used as the standard media for the experiment. The BH media contents were as follows: 0.2 g/L $MgSO_4$, 0.02 g/L $CaCl_2$, 1.0 g/L $KH_2PO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $NH_4NO_3$, 0.05 g/L $FeCl_3$. The solution was stirred evenly, with the pH adjusted to pH 7.0 ± 0.2 at 25°C [23].

### Preparation of bacterial inoculum/cell suspension

For cell suspension preparation, isolates were initially grown in 100 ml (250 ml flask) of standard nutrient broth for 48 hours before cultivation. The isolates were incubated in a rotary shaker with a temperature of 20°C with an agitation speed of 150 rpm. The grown culture was then centrifuged at 5000×g for 30 min. The pelleted cells were then washed with 1X phosphate-buffered saline (PBS) solution, and the cell density was adjusted to $OD_{600}$ nm = 1.0 [20].

### Screening test for hydrocarbon-utilising bacteria

The screening experiment was based on the screening experiment done by Hanson et al. [20] with several modifications. The isolates were initially assessed based on the capability to utilise 0.1% (v/v) diesel within 24 h. Isolates that showed positive results were subsequently tested in 0.5% (v/v) of diesel for the same period (24 h). The utilisation of the potent diesel-degrading isolates on different hydrocarbons (*n*-hexane, *n*-heptane, *n*-tetradecane, *n*-hexadecane, xylene, toluene, and phenol) as sole carbon source was verified using three $\mu$l of cell suspension, 2 $\mu$l of hydrocarbon substrate, 150 $\mu$l of BH media and 45 $\mu$l of 1.0 g/L DCPIP. Utilisation of selective substrate was detected from the changes of DCPIP blue colour (oxidised) to colourless (reduced). Plates were incubated at 25°C for 24, 48 and 72 h. Two set of controls were also prepared to assess the interactions of the assay components and the redox indicator. Control 1 (C1) consisted of only DCPIP, BH medium, and substrate (different control for each different substrate). Control 2 (C2) contained DCPIP, BH medium, and cell suspension. The function of the latter control was to determine the influence of inoculum to DCPIP over time. The discolouration of DCPIP was observed qualitatively after 24, 48 and 72 h. All DCPIP assay were done in 96-well microtitre plates (Corning®).

## RESULTS

### Screening test for hydrocarbon-utilising bacteria

**Table 1** shows the utilisation of 0.1% (v/v) and 0.5% (v/v) diesel oil by nine isolates within 24 h. From the results, isolate ADL15, ADL36, ADL37, and ADL41 showed fast utilisation of 0.1% (v/v) within 24 h. When diesel concentration was increased to 0.5% (v/v), only two potent isolates was adept to utilise the elevated substrate within 24 h.

**Table 1.** The qualitative observation of primary and secondary screening using DCPIP indicator after 24 h

| Isolate | Decolourisation | |
|---|---|---|
| | **0**.1% (v/v) diesel | **0.5% (v/v) diesel** |
| ADL11 | no | no |
| ADL13 | no | no |
| ADL14 | no | no |
| ADL15 | yes | yes |
| ADL16 | no | no |
| ADL32 | no | no |
| ADL36 | yes | yes |
| ADL37 | yes | no |
| ADL41 | yes | no |

The secondary screening of both isolate (ADL15 and ADL36) in several *n*-alkanes, simple aromatics, and PAHs reveals distinctive utilisation of major hydrocarbon substrates between the isolates. Diesel oil was also tested with an elevated concentration (1.0% (v/v)) to observe the best diesel degraders from the two isolates. **Table 2** shows the utilisation of different hydrocarbon compounds by isolate ADL15 and ADL36 within 24 h.

**Table 2.** Biodegradability experiment of hydrocarbon substrates using DCPIP indicator at 24, 48 and 72 h.

| Hydrocarbon substrates 1.0% (v/v) | Isolate | | | | | |
|---|---|---|---|---|---|---|
| | ADL15 | | | ADL36 | | |
| | 24 | 48 | 72 | 24 | 48 | 72 |
| *n*-hexane | no | no | no | no | no | no |
| *n*-heptane | no | no | no | no | no | no |
| *n*-tetradecane | no | no | no | yes | - | - |
| *n*-hexadecane | no | no | no | yes | - | - |
| diesel | yes | - | - | yes | - | - |
| xylene | yes | - | - | no | no | no |
| toluene | yes | - | - | no | no | no |
| phenol | yes | - | - | no | no | yes |

The results show that ADL15 has higher tendency to utilise aromatic hydrocarbons while ADL36 has greater affinity for aliphatic hydrocarbons. Although ADL36 proved to be a better consumer of aliphatics, the isolate cannot utilise short-chain *n*-alkanes even after 72 h. However, isolate ADL36 showed a promising potential to utilise diesel at a higher concentration as the decolourisation of DCPIP occurred less than 24 h (data not shown).

## DISCUSSIONS

Colourimetric assays such as DCPIP redox assay are valuable due to their fast detection of microbial metabolism occurrence in both aerobic and anaerobic studies, low resource output and cheap. DCPIP is an enzyme-catalysed redox electron acceptor that is blue colour in oxidised state and colourless in its reduced form. According to Yoshida et al. [24], loss of DCPIP colour is observed at its peak wavelength (600 nm). Some studies reviewed by Konidari et al. [25] have reported that DCPIP is likely unstable in the dark or when long exposure towards the light source. Besides, the colour of the DCPIP indicator is more practically stable in the presence of light and can be used consistently from time to time [26].

The regulation of metabolic pathways regulated within microbial cells is particularly sensitive to the cell's need. Common redox reactions and specific redox couples occur consistently in all of the major metabolic pathways in cells. The concentrations of the redox couples assist in the flow regulation of metabolites through these pathways. According to Buchanan [27], these intracellular concentrations of redox couples might also be responsive to receptor agonists, as well as cell nutrients. Conferring to this important fact, using chemical analysis, such alterations in the concentration can be estimated. Hydrocarbon oxidation processes by microbes involve redox reactions, in which electrons are transferred to electron acceptors, such as $O_2$, nitrates, and sulfate [28]. Therefore, it is possible to determine the ability of a microorganism to utilise a hydrocarbon substrate with the incorporation of DCPIP as the terminal electron acceptor into the experiment by simply monitoring the colour change from blue (oxidised) to colourless (reduced).

The principle of the discoloration can be described by the molecular conformation of the DCPIP indicator. As bacterial cells utilise the hydrocarbon substrates, electrons are liberated to the environment. Molecular conformation of the indicator will then takes place and reflects the light in a different angle, turning its colouration from blue to colorless [29].

During experimenting, the intensity of the DCPIP concentration must be considered. 1.0 g/L is selected as the concentration in regards to previous studies [22,29,30]. A high concentration may produce a dark blue colour thus any reduction may not easily detect and observed [31]. In contrast, a lower DCPIP concentration may produce an insufficient indication for any noticeable decreases. A near neutral pH (7.25) was chosen as the reasonable pH for DCPIP since the indicator is not adequately stable in higher pH values. In regards to the effect of temperature, the study was done at 25°C as the established maximum response for DCPIP ranges from 297 to 303 K (~24°C to 30°C) [32].

The affinity of isolate ADL15 to utilise aromatic substrate and PAHs reveals a promising potential for bioremediation of the highly toxic and recalcitrant compounds. Several bacterial genera that commonly associated with degradation of aromatic hydrocarbons and PAHs are *Pseudomonas* [33–37], *Mycobacterium* [38–41] and *Sphingomonas* [42–45]. On the other hand, the ability of isolate ADL36 to degrade middle-chain aliphatic hydrocarbons shows a similarity towards the genus *Rhodococcus* [46–50]. In brief, although both isolates showed a different range of catabolic metabolism, both isolates exhibited a degradation potential for diesel oil. The ability of isolate ADL36 to utilise diesel oil rapidly might occur due to the composition of the diesel oil itself, which tends to have a higher percentage of aliphatic components [51,52].

## CONCLUSION

Microbes are excellent petroleum hydrocarbon degraders that widely available in many environmental niches. Therefore, the isolation and identification of these minute organisms are fundamental in proposing for a bioremediation tool. Colourimetric assessment proved to be the quickest method for the detection of microbial metabolism from carbon sources such as hydrocarbons. Although the detection of microbial ability to degrade hydrocarbon can be observed by this remarkable colourimetric approach, a full characterisation and identification based on the physiological, biochemical and genetic adaptability must be considered to completely comprehend the significance of the isolated bacteria in the big picture of microbial remediation.

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